A Function-Based Screen for Factors that Inhibit Pluripotent Cell Differentiation

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for the degree of Doctor of Philosophy

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SUMMARY

Embryonic Stem (ES) cells are derived from the mouse blastocyst stage embryo and exhibit gene expression and developmental potential equivalent to pluripotent tissues present in the early embryo. *In vivo*, ES cells injected into blastocysts participate in development giving rise to all tissues of the adult mouse including the germ line. *In vitro*, ES cells are maintained in an undifferentiated state in the presence of Leukaemia Inhibitory Factor (LIF) or when cultured on feeders expressing LIF. The pluripotent nature of ES cells is evident by the extensive repertoire of differentiated cell types formed in embryoid bodies. ES cells therefore represent an *in vitro* system with which to study the molecular regulation of pluripotent cell maintenance and differentiation.

Molecular components important for LIF signal transduction in ES cells have been identified including Stat3, Hck, Ras and Shp-2. However, the underlying mechanism that controls ES cell pluripotence has not been approached. This work aimed to identify cellular genes important for maintenance of the undifferentiated state of ES cells in the absence of LIF signalling.

The project was designed to identify cell-autonomous factors that are capable of inhibiting ES cell differentiation. A function-based screening procedure was established taking advantage of a Polyomavirus (PyV)-based episome vector to transfect large numbers of ES cells with a cDNA expression library. Regions of the promoter for the pluripotent cell marker *Oct4* were identified that directed pluripotent cell specific gene expression. By fusing these regions of *Oct4* promoter to the neomycin resistance gene, expression of neomycin resistance was restricted to pluripotent cells, allowing selective removal of differentiated cells within a population by the addition of G418. The procedure involved supertransfection of an episome-harbouring ES cell line with a second episome containing a cDNA expression library and an *Oct4* promoter-neomycin selection cassette. ES cells were differentiated by the withdrawal of LIF and subjected to G418 selection. Cells survive only if they harbour a cDNA, the product of which acts to inhibit ES cell differentiation.

The majority of episome supertransfected ES cell colonies were shown to replicate extrachromosomal episome and express the β-galactosidase reporter gene. *Oct4*-neo

selection constructs that contained the *Oct4* Distal Enhancer (DE) were found to select efficiently against differentiated cells when supertransfected on replicating episome-based vectors. Mock screening experiments indicated that even with episomes that contain the *Oct4*-neo selection construct a small percentage of undifferentiated colonies formed in the absence of LIF and therefore represent a background in the screening procedure. Background pluripotent colony survival was circumvented by 1) trypsinising and re-seeding cells during selection and 2) harvesting episomes from surviving colonies and re-introducing these into ES cells in a second round of selection. The ability of the screen to select for cDNAs that maintain ES cells in the undifferentiated state was demonstrated by expression of *LIF* and *Stat3β* cDNAs from the base episome vector.

An episomal cDNA expression library was constructed from ES cell poly A^+ RNA and supertransfected into ES cells. The function-based screen was carried out by differentiating and selecting supertransfected ES cells in medium containing G418 but lacking LIF. Episomal DNA was extracted from pools of surviving colonies and used in a second round of screening in ES cells to enrich for functional cDNA clones. Over 7×10^5 clones from the library were screened by this procedure, and numerous pluripotent ES cell colonies were isolated. Further screening of surviving colonies by LIF titration identified several lines that required less LIF to retain the undifferentiated state. cDNAs encoding putative truncated forms of mouse Tat Binding Protein 1 (mTbp-1) and a protein related to IQ-motif containing GTPase Activating Protein 1 (IQGAP1) were isolated from selected cell lines with an altered requirement for exogenous LIF. Overexpression of the truncated *Tbp-1* cDNA in wild-type ES cells suggested a role for this protein in ES cell maintenance. Tbp-1 is a component of the proteasome and was isolated because of its ability to bind to and inhibit transactivation of HIV Tat. Given that Stat3 and other putative downstream targets of LIF signaling are degraded by ubiquitin-proteasome dependent mechanisms, the truncated Tbp-1 protein may lead to continued LIF signal transduction in the absence of LIF. Thus, these findings imply the existence of a novel mechanism that regulates pluripotent cell maintenance.

Statement of originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, it contains no material that has previously been published by any other person except where due reference is made. The author consents to the thesis being made available for photocopying and loan.

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CHAPTER 1:

INTRODUCTION

1.1 DEVELOPMENTAL BIOLOGY

Scientists have worked for centuries towards the goal of understanding mammalian embryogenesis. Historically, embryo explant and manipulation experiments were used to gain insight into the anatomical and cellular basis of mammalian embryogenesis. The application of recombinant DNA techniques such as gene targetting technology, reverse transcription polymerase chain reaction, *in situ* hybridisation and gene identification has led to rapid progress in unravelling the molecular controls of all aspects of mammalian development. As knowledge of mouse embryogenesis expands so too does our understanding of human development, predicted to lead to medical and clinical advances.

1.2 THE MOUSE AS A MODEL FOR EARLY MAMMALIAN EMBRYOGENESIS

Early development in model organisms such as *Drosophila melanogaster* is relatively well understood at the molecular level. While analogies between mammals and model organisms such as *Xenopus laevis*, *Caenorhabditis elegans* and *Drosophila* have proved instructive, especially following gastrulation, it is clear that early mammalian development is fundamentally different from that of other metazoans. Early development in the fly is directed by localised mRNAs and proteins deposited by the mother, and occurs in a syncidial blastoderm, an embryo where nuclei share a common cytoplasmic pool. In *Xenopus* maternal transcripts localised within the egg also control early developmental stages. In contrast, there is no compelling evidence for localisation of maternal transcripts in the mammalian oocyte, and maternal mRNAs and proteins in mouse embryos are rapidly degraded (Howlett and Bolton, 1985) at the 2-cell stage concurrent with transcriptional activation of the embryonic genome (Flach *et al.*, 1982).

Mammalian embryogenesis is also remarkably resilient compared to that of other vertebrates such that 80% of the mouse egg cylinder stage embryo can be ablated without disrupting embryogenesis (Snow and Tam, 1979). Likewise, a single normal embryo can be formed from aggregates of multiple pre-implantation embryos without altering its size or developmental progression (Buehr and McLaren, 1974; Lewis and Rossant, 1982; Rands, 1986).

Unique aspects of mammalian embryogenesis are best investigated in a mammalian model system, which in turn provides a better understanding of human development. However, studies of mammalian embryogenesis have been hampered by the size, fragility and experimental inaccessibility of early embryos. The mouse is the model system of choice for studying development of eutherian mammals due to its short generation time, large litter sizes and ease of husbandry. Combined with gene targetting approaches which enable generation of custom made mutant mice, these advantages make the mouse a powerful system for applying genetics to understanding the molecular controls of mammalian and therefore human development.

1.3 EARLY MOUSE DEVELOPMENT

1.3.1 Pre-implantation development

Mammalian embryogenesis begins with fertilisation of the egg by a single sperm. DNA within the male and female pronuclei undergoes replication, and during the first mitotic division fusion of the pronuclei occurs. At this point development relies on maternal transcripts and proteins synthesised during oogenesis. Half way through the 2-cell stage (27 hours post-fertilisation) embryonic gene expression begins and maternal transcripts are degraded (Hogan *et al.*, 1994). In the mouse, single blastomeres from the 2-cell or 4-cell embryo (Figure 1.1a) are totipotent, that is under the appropriate conditions they can give rise to all embryonic and extraembryonic tissues required during development to form an adult mouse. Single 8-cell blastomeres (Figure 1.1 b-c) can not form embryos in isolation. In combination with a morula single blastomeres from the 8-cell embryo can form a variety of tissues of the resulting chimaeric animal (Kelly, 1977).

Compaction of the ball of individual cells that make up the embryo at this stage results in formation of a morula in which individual cells are juxtaposed and cannot be distinguished (Figure 1.1 d-g). Polarisation of blastomeres through the formation of apical and basal membrane domains results in the formation of distinct microenvironments within the morula. The cells on the inside of the morula will become the inner cell mass (ICM) while those cells on the periphery differentiate to form trophectoderm (Hillman *et al.*, 1972; Johnson *et al.*, 1986; Sefton *et al.*, 1992; Sutherland and Calarco-Gillam, 1983). The developmental potential

Figure 1.1

Preimplantation development in the mouse.

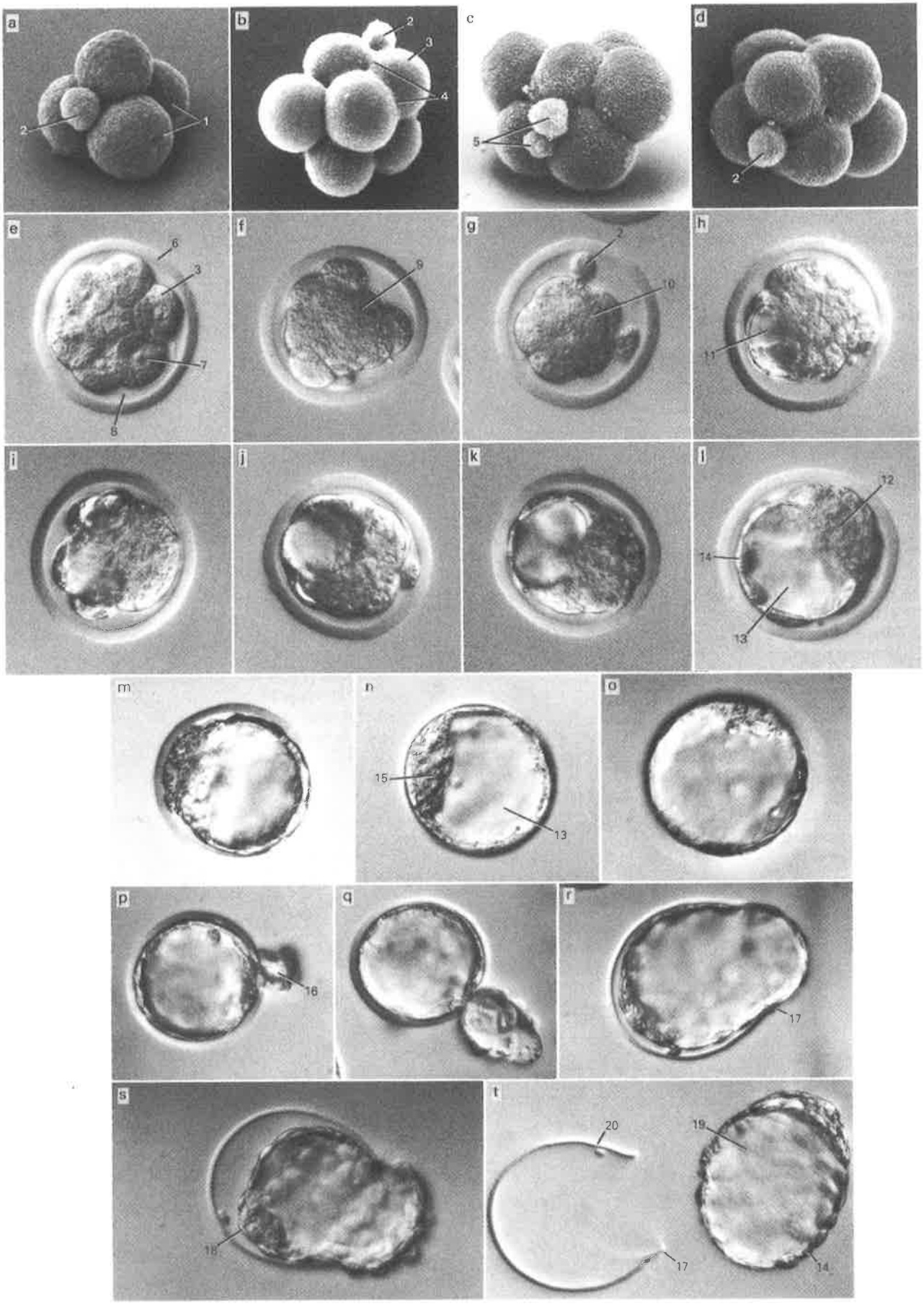
(a-d) Scanning electron micrographs of 4- and 8-cell stage embryos of pre- and early compaction stages. (a) 4-cell stage, (b) 8-cell stage, onset of compaction, (c, d) 8-cell embryo at early compaction stages.

(e-l) Progression of the 8-cell embryo to the blastocyst stage viewed by interference contrast optics. (e) 8-cell stage, (f) early morula, (g) compacted morula, (h-k) early blastulation, (l) mid blastocyst stage.

(m-t) Hatching of the blastocyst. (m-o) gradual expansion of the blastocyst and thinning of the zona pellucida, (p-s) breach of the zona pellucida and escape of the blastocyst, (t) fully expanded blastocyst.

1. blastomeres of the 4-cell embryo
2. second polar body
3. blastomere of the 8-cell embryo
4. early compaction where blastomere boundaries are no longer distinct
5. division products of the second polar body
6. zona pellucida
7. nucleus of 8-cell blastomere
8. perivitelline space
9. early morula stage embryo
10. compacted morula stage embryo
11. accumulation of blastocoelic fluid
12. inner cell mass
13. blastocoelic cavity
14. mural trophectoderm cell
15. inner cell mass
16. trophectoderm escaping through the zona pellucida
17. boundary of the breach in the zona pellucida
18. polar trophectoderm cell
19. fully expanded blastocyst
20. empty zona pellucida

Taken from Kaufman, (1992).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of cells in the morula is not yet restricted however, as blastocysts can be formed by inside or outside cells alone (Hogan *et al.*, 1994).

Passive accumulation of fluid within the embryo follows ion transport by the sodium/potassium pump, Na^+/K^+-ATPase (Watson and Kidder, 1988; Wiley, 1987). Formation of tight junction complexes between cells of the trophectoderm (Ducibella *et al.*, 1977; Enders and Schlafke, 1971; Nadijcka and Hillman, 1974) results in the gradual formation of a fluid filled cavity termed the blastocoelic cavity between the inner and outer cells. By day 3-3.5 post coitum (p.c) the outer cells have become the trophectoderm, while the inner cells form the ICM. The early blastocyst is composed of an outer layer of trophectoderm surrounding the ICM and blastocoelic cavity (Figure 1.1 h-l). The trophectoderm will contribute only to the extraembryonic ectoderm and to the trophoblast. The ICM is pluripotent, that is, it gives rise to all embryonic tissues, and in addition, to the extraembryonic endoderm. Once the blastocoelic cavity is fully expanded (Figure 1.1 o; Figure 1.2) the embryo hatches from a protective layer termed the zona pellucida, allowing the embryo to implant into the uterine wall (Bell, 1985) (Figure 1.1 p-t). Prior to implantation at day 4-4.5 p.c the pluripotent cells of the embryo undergo a second differentiation event, the formation of a layer of primitive endoderm (Figure 1.2) from ICM cells exposed to the blastcoelic cavity. Primitive endoderm goes on to form the extraembryonic tissues visceral and parietal endoderm (Figure 1.2).

1.3.2 Post-implantation development

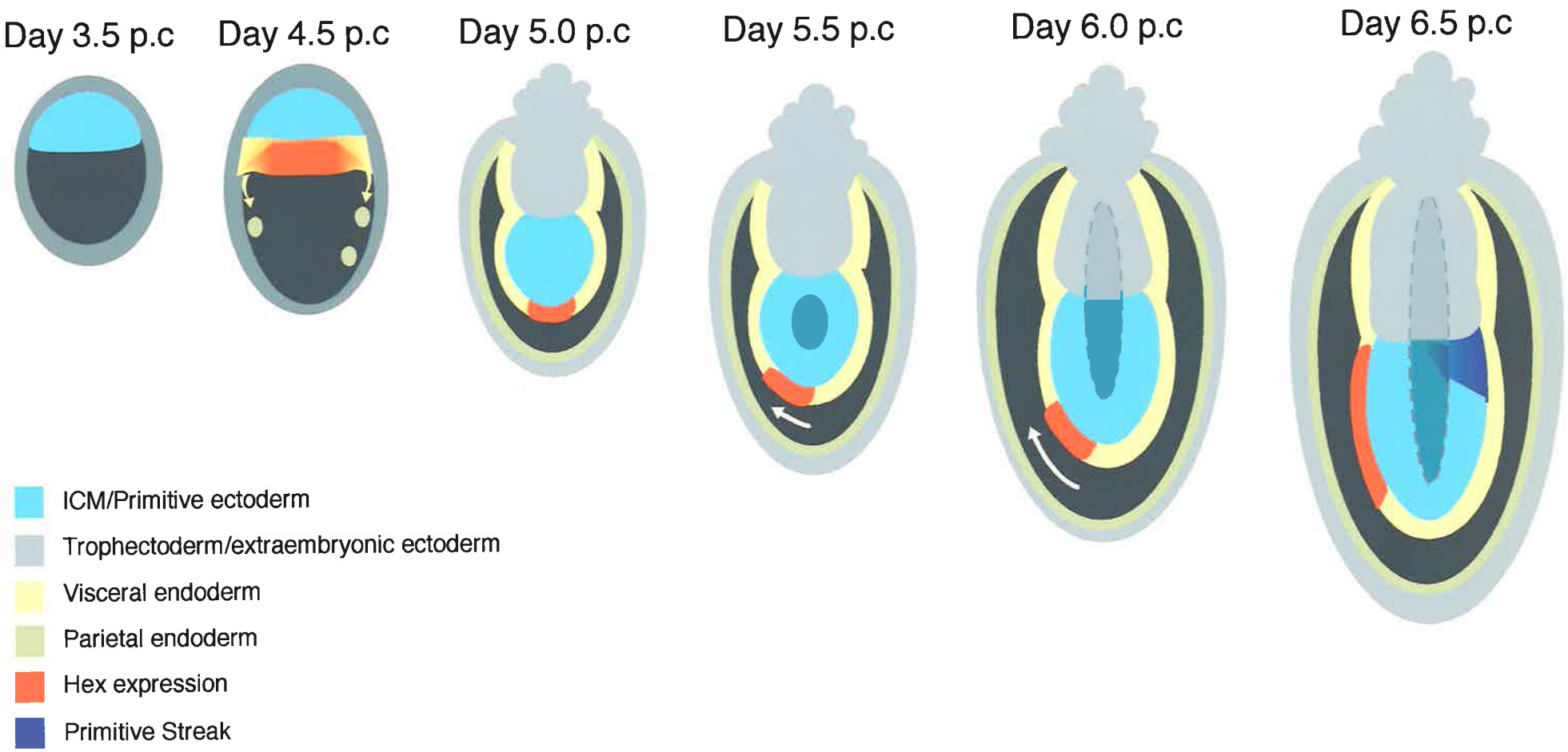
The hatched blastocyst implants into the uterine wall at day 4.5 p.c. Following implantation the primitive endoderm proliferates and migrates to encircle the blastocoelic cavity (Gardner, 1983). Extraembryonic endoderm in contact with the trophectoderm differentiates into parietal endoderm while endoderm that remains in contact with the ICM forms visceral endoderm (Figure 1.2). The ICM cells begin to divide rapidly such that in a 2 day period the number of pluripotent cells increases from 20-25 cells at day 4.5 p.c to 660 cells by day 6.5 p.c (Snow, 1977). During this time selective apoptosis of central epiblast cells results in formation of the proamniotic cavity (Coucouvanis and Martin, 1995). By day 6.5 p.c when the embryo is at the egg cylinder stage, the ICM has altered both developmental potential and morphology to form a cup-shaped pseudostratified epithelial monolayer referred to as the primitive ectoderm

Figure 1.2

Postimplantation development.

Cell movement and gene expression changes between the blastocyst, egg cylinder and early gastrulating embryo. The early blastocyst stage (day 3.5 p.c); Trophectoderm (grey) surrounds the ICM (light blue) and blastocoelic cavity (black). At day 4.5 p.c primitive endoderm forms from the ICM lining the blastocoelic cavity and begins to migrate to surround the cavity. By day 5 p.c the ICM and the extraembryonic polar trophectoderm enter a period of rapid proliferation. Primitive endoderm has formed extraembryonic tissues, the visceral endoderm (yellow, day 5 p.c) that lines the pluripotent primitive ectoderm and parietal endoderm (green) lining the blastocoelic cavity. Expression of the homeobox gene *Hex* (orange) is first seen in the distal visceral endoderm at this time and these cells give rise to progeny that migrate to line the future anterior portion of the embryo where they induce unpatterned primitive ectoderm to become anterior structures (day 5.5-6.5 p.c). The primitive streak (purple) forms marking the future posterior of the embryo.

Adapted from Beddington and Robertson, (1998).


Day 3.5 p.c
Day 4.5 p.c
Day 5.0 p.c
Day 5.5 p.c
Day 6.0 p.c
Day 6.5 p.c
ICM/Primitive ectoderm
Trophectoderm/extraembryonic ectoderm
Visceral endoderm
Parietal endoderm
Hex expression
Primitive Streak

(Figure 1.2). Primitive ectoderm cells older than day 5.5 p.c do not contribute to adult tissues following transplantation into a blastocyst and are unable to form primitive endoderm (Rossant and Ofer, 1977; Smith, 1992). The primitive ectoderm remains pluripotent however, giving rise to the three primary germ layers of the embryo, mesoderm, ectoderm, and endoderm that will form the adult (Figure 1.3).

1.3.3 Differentiation and patterning of primitive ectoderm

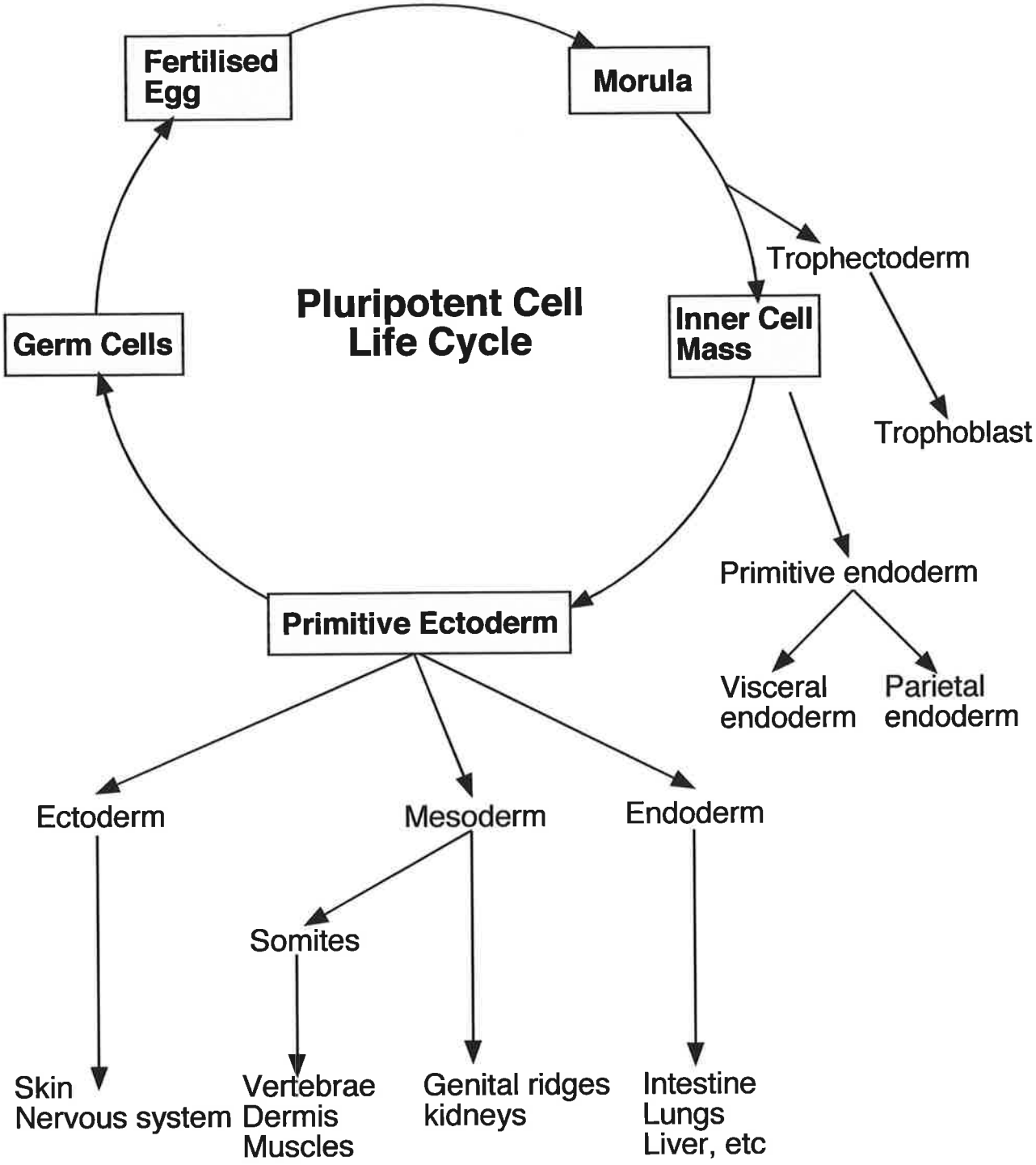
The primitive ectoderm forms a radially symmetrical cup termed the egg cylinder (Figure 1.2). Cells of the primitive ectoderm are fated to become particular embryonic tissues by virtue of their position in the layer (Tam and Beddington, 1987; Tam and Beddington, 1992; Quinlan *et al.*, 1995; Tam, 1989) but lineage tracking of individual labelled cells indicates that they can give rise to descendants in all three germ layers and the germ line (Lawson and Hage, 1994; Lawson *et al.*, 1991). Posterior and distal primitive ectoderm from day 7.5 p.c embryos, fated to form mesoderm, alters fate to that of anterior primitive ectoderm (surface ectoderm) when transplanted into anterior primitive ectoderm (Beddington, 1981; Beddington, 1982). This and other experiments (Parameswaran and Tam, 1995; Tam and Zhou, 1996) demonstrate that transplanted primitive ectoderm can take on the fate of the new area of the embryo. Cells of the primitive ectoderm are therefore pluripotent, equipotent and not committed to particular cell fates until gastrulation (Beddington, 1982; Skreb *et al.*, 1976).

Anterior-posterior patterning is first evident by expression of the homeobox gene *Hex* at the distal tip of the day 5.5 p.c embryo in the visceral endoderm (Thomas *et al.*, 1998)(Figure 1.2). Through the next day of development, progeny of these cells migrate asymmetrically to line the anterior primitive ectoderm and differentiate to form anterior visceral endoderm (Thomas *et al.*, 1998). At day 6-6.5 p.c, expression of *Hesx1*, a paired-like homeobox gene, is observed in the anterior visceral endoderm and within 24 hours expression is also seen in the adjacent primitive ectoderm implying that the anterior visceral endoderm is signalling to the adjacent primitive ectoderm (Thomas and Beddington, 1996). *Hesx1* has a role in determination of anterior ectoderm as $Hesx1^{-/-}$ embryos lack the entire hindbrain (cited in Beddington and Robertson, 1998). The importance of anterior visceral endoderm in this process is demonstrated by diminished forebrain marker expression following removal of anterior visceral

Figure 1.3

Totipotent and pluripotent cells in mouse development.

Mouse embryogenesis begins with fertilisation of the oocyte. The totipotent fertilised egg divides four times to form 16 totipotent blastomeres that comprise the morula. The first differentiation event in embryogenesis, the formation of trophectoderm, is accompanied by a gradual restriction in potential of the inner cell mass such that it can no longer form trophectoderm. Outer cells of the inner cell mass form the primitive endoderm before remaining ICM cells go through a process of rapid proliferation and cavitation to form another pluripotent cell population, the primitive ectoderm. The primitive ectoderm gives rise to the three primary germ layers of the embryo, mesoderm, endoderm and ectoderm, that will form all the tissues of the embryo proper. Germ cells also derive from the primitive ectoderm and will form sperm and oocytes for the next generation. Mesoderm gives rise to the vertebrae, dermis and muscle through formation of somites and also to the genital ridges and kidneys. Skin and the nervous system are formed from ectoderm while endoderm gives rise to internal organs such as intestines, lungs and liver.


Fertilised Egg
Morula
Trophectoderm
Pluripotent Cell Life Cycle
Germ Cells
Inner Cell Mass
Trophoblast
Primitive endoderm
Primitive Ectoderm
Visceral endoderm
Parietal endoderm
Ectoderm
Mesoderm
Endoderm
Somites
Skin
Nervous system
Vertebrae
Dermis
Muscles
Genital ridges
kidneys
Intestine
Lungs
Liver, etc

endoderm early in gastrulation (Thomas and Beddington, 1996). Juxtaposition of rabbit anterior visceral endoderm with chick primitive ectoderm results in forebrain specific marker gene expression in the primitive ectoderm implying that it takes on an anterior fate (Knoetgen *et al.*, 1999). Thus it is the anterior visceral endoderm that is responsible for specifying anterior fate to neighbouring primitive ectoderm. Pluripotent cells in the embryo are therefore responsive to specific signals that induce regionalised gene expression that in turn leads to specification and differentiation of pluripotent cells to particular cell fates.

The process of gastrulation, in which the primitive ectoderm forms multiple layers of the embryo, begins in the mouse at day 6.5 p.c. Gastrulation is marked by creation of the primitive streak, an area at the posterior of the embryo where primitive ectoderm cells delaminate from the epithelial layer and involute to form an underlying layer of mesoderm between the primitive ectoderm and visceral endoderm. As gastrulation proceeds the streak elongates and at the distal tip forms a specialised structure termed the node. Newly formed mesoderm migrates proximally into the extra-embryonic ectoderm region forming extra-embryonic mesoderm as well as laterally and anteriorly to form axial mesendoderm of the embryo. The node is responsible for formation of axial mesendoderm that will in turn form notochord and definitive gut endoderm of the embryo (Tam and Beddington, 1992). Definitive endoderm moves anteriorly from the node, displacing the visceral endoderm and gives rise to the pancreas, gut, lung, and liver (Tam and Beddington, 1992). Ectodermal tissues such as neurectoderm and epidermis are formed from primitive ectoderm cells that did not enter the streak (Quinlan *et al.*, 1995; Tam, 1989). Following gastrulation cells of the germ layers are no longer pluripotent and do not express the pluripotent cell marker Oct4 (Palmieri *et al.*, 1994)(see section 1.6.6). The germ layers are however multipotent tissues and together form all tissues of the adult.

At day 7 p.c the primordial germ cells (PGCs) have formed from the primitive ectoderm and can be observed at the posterior of the streak by their strong endogenous alkaline phosphatase activity (Ginsburg *et al.*, 1990; Lawson and Pedersen, 1992). PGCs are the only pluripotent cells present in the embryo following gastrulation. They migrate to the gonadal ridges by day 13.5 p.c giving rise to the gametes.

1.3.4 Developmental potential of the early mouse embryo

During development totipotent blastomeres of the morula undergo a pattern of cell proliferation, differentiation, apoptosis, and morphogenesis to produce all terminally differentiated cell types and tissues of the adult animal (Figure 1.3). Developmental potential becomes increasingly restricted through the time of compaction and blastocyst formation such that injected ICM cells from fully expanded blastocysts are no longer capable of forming trophectoderm in chimaeric blastocysts (Gardner, 1985). Totipotent cells have therefore progressed through the first developmental restriction to form the pluripotent ICM. The second developmental restriction occurs following formation of the primitive endoderm at day 4.0 p.c from the ICM. ICM cells go on to form the primitive ectoderm, a pluripotent cell population that progressively loses the ability to form primitive endoderm (Pedersen *et al.*, 1977). The primitive ectoderm then forms the three primary germ layers of the embryo during gastrulation. Pluripotent PGCs are segregated from this process and go on to form the gametes for the following generation. Following gastrulation somatic tissues have lost pluripotence and are restricted to one of three germ layer identities. Thus early embryogenesis is characterised by maintenance of a pluripotent cell population and concomitant restriction of cell potential to form the germ layers of the embryo. The importance of the pluripotent cell maintenance is emphasised by work that suggests that a critical cell number must be reached to allow gastrulation to commence (Power and Tam, 1993). Moreover, in response to experimentally induced cellular damage, egg cylinder stage embryos can still form normal embryos (Snow and Tam, 1979), indicating a critical role for a continuous pluripotent cell pool capable of self-renewal and differentiation during the early steps of mouse development.

1.4 PLURIPOTENT CELLS *IN VITRO*

The molecular controls of pluripotent cell maintenance and differentiation cannot be understood without the use of *in vitro* culture systems analogous to differentiation processes of early mammalian embryogenesis. Research is continuing towards the characterisation of cultured cells that retain the pluripotence of the embryonic tissues from which they were derived. *In vitro* cultured pluripotent cells can recapitulate differentiation events that occur in the

embryo and therefore study of their differentiation represents a valid approach to gaining insight into developmental cues that induce pluripotent differentiation *in vivo*.

1.4.1 Embryonal carcinoma cells

Germ cell tumours are numerous and varied but one form, teratocarcinomas, are unique because they contain both pluripotent embryonal carcinoma (EC) cells and terminally differentiated cells (Stevens, 1983). EC cells are pluripotent because single EC cells injected into isogenic mice will reform teratocarcinomas comprised of EC cells and differentiated cell types (Stevens, 1983). Teratocarcinomas arise spontaneously during spermiogenesis or oogenesis or can be formed experimentally by transplantation of early embryos or pluripotent cells into extra-uterine sites. Teratocarcinomas formed by the latter method have not gone through a primary transformation event but rather have formed due to disruption of normal pluripotent stem cell growth and differentiation cues (Stevens, 1983). Human or mouse teratocarcinomas cultured *in vitro* can give rise to EC cell lines that retain an undifferentiated phenotype (Martin and Evans, 1975b) in the presence of feeder layers (Martin and Evans, 1975b) or in medium supplemented with serum (Ruducki and McBurney, 1987). EC cells can be maintained *in vitro* in an undifferentiated state and can also be differentiated by growth at low or high densities for long periods, administration of some form of insult, or chemical induction (Ruducki and McBurney, 1987). EC cells retain molecular markers of mouse pluripotent cells such as alkaline phosphatase activity and Oct4 (Scholer *et al.*, 1989) and SSEA-1 antigen expression (Solter and Knowles, 1978).

Many EC cell lines exhibit widespread differentiation potential in that subcutaneous injection of individual EC cells into mice will give rise to teratocarcinomas containing both undifferentiated EC cells and differentiated cells derived from the three primary germ layers (Kleinsmith and Pierce, 1964). A number of EC cell lines also share many of the properties of pluripotent cells of the early embryo. For example, differentiation of EC cells by culture in suspension (Martin and Evans, 1975a; Ruducki and McBurney, 1987) or injection intraperitoneally into mice, results in the formation of cell aggregates termed embryoid bodies. Embryoid body differentiation mimics that of the early embryo (Martin and Evans, 1975b) including formation initially of at least two cell layers, an outer layer of extraembryonic

endoderm cells surrounding inner ectodermal cells (Martin and Evans, 1975a) followed by differentiation into a wide range of cell types (see section 1.5.2). Further, some EC cell lines can participate in normal development following injection into blastocyst stage embryos, giving rise to multiple tissues in chimaeric animals and in rare instances to the germ line (Martin, 1980; Rossant, 1993; Stewart and Mintz, 1981). It is clear however that few EC cell lines are truly pluripotent in that they generally do not give rise to all tissue types of the adult mouse (Papaioannou and Rossant, 1983). Unlike stem cells of the embryo, injection of some EC cell lines into blastocysts can result in formation of tumours (Papaioannou *et al.*, 1978; Smith, 1992).

EC cell lines can differentiate into a variety of differentiated cell types in the presence of different chemical inducers such as retinoic acid (RA) (Ruducki and McBurney, 1987). Nullipotent EC cell lines such as Nulli-Scc1 (Rosenstraus and Spadoro, 1981) do not differentiate in suspension culture or following intraperitoneal injection. Nullipotent EC cells presumably carry genetic alterations that render them refractory to differentiation cues unless circumvented by chemical induction.

Restricted developmental potential and tumourigenicity limit the usefulness of EC cells as an *in vitro* pluripotent stem cell model system. Selection of EC cells during tumour growth is likely to be responsible for the acquisition of mutations and an inherent genetic instability such that cells within a population are commonly aneuploid (Papaioannou *et al.*, 1978; Robertson, 1987; Smith, 1992). Chromosome instability results in accumulation of subsequent genetic aberrations that are likely to affect the ability of EC cells to contribute to the embryo when injected into blastocysts. Heterogeneity among cells in culture can also lead to complications in interpreting experimental data. In order to study cell pluripotence and early development *in vitro* there is a requirement for culture of untransformed pluripotent stem cells which faithfully mimic the population of pluripotent cells *in vivo*.

1.4.2 Embryonic stem cells

Pluripotent cell lines can also be isolated directly from the embryo, and are therefore primary cells that have not been subjected to selection pressures associated with tumour formation (Robertson, 1987). Embryonic stem (ES) cells are pluripotent cells generally derived

from cultured 129 strain mouse blastocysts (Evans and Kaufman, 1981; Martin, 1981) although ES cell lines have been isolated from other inbred mouse strains (Brook and Gardner, 1997; Kawase *et al.*, 1994; Ledermann and Burki, 1991; Roach *et al.*, 1995). ES cells can be grown *in vitro* in an undifferentiated state in the presence of feeder layers of mitotically inactivated fibroblasts, or in the presence of exogenous Leukaemia Inhibitory Factor (LIF)(Smith *et al.*, 1988; Williams *et al.*, 1988) and other IL-6-type cytokines (1.6.1) (Figure 1.4). Maintenance of pluripotence is evident from the ability of ES cells, cultured for extended periods, to contribute to adult tissues when injected into the blastocyst stage embryo (Robertson and Bradley, 1986). In the embryonic environment they routinely give rise to germ cells and undergo meiosis. ES cells are now widely used in combination with gene targetting approaches to create mice with defined genetic mutations such as loss of specific genes of interest (Thomas and Capecchi, 1987).

Derivation and growth of ES cells from species such as rat (Iannaccone *et al.*, 1994), chicken (Pain *et al.*, 1996), sheep (Handyside *et al.*, 1987; Notarianni *et al.*, 1990; Piedrahita *et al.*, 1990), rabbit (Giles *et al.*, 1993; Moreadith and Graves, 1992), mink (Sukoyan *et al.*, 1992), hamster (Doetschman *et al.*, 1988), pig (Evans *et al.*, 1990; Shim *et al.*, 1997; Wheeler, 1994), rhesus monkey (Thomson *et al.*, 1995) and marmoset monkey (Thomson *et al.*, 1996) has been reported. ES cells from pig, chicken, rabbit and rat will form chimaeric animals, although it is not currently clear if cells propagated long term in culture retain this ability. Their usefulness remains to be determined given that germ line transmission and clonal propagation have not been reported.

In the last two years there have also been reports of the derivation of human ES cells (Reubinoff *et al.*, 2000; Thomson *et al.*, 1998). Unlike ES cells from the mouse, human ES cells must be grown on a feeder layer of irradiated embryonic fibroblasts. They do not require the addition of exogenous LIF although it is not known if they will maintain pluripotence when grown on feeders which do not express LIF. Morphologically human ES cells resemble human EC cells and primate ES cells. Consistent with this, human ES cells express *Oct4* and alkaline phosphatase activity and the cell surface carbohydrate epitopes SSEA-4 and TRA-1-60 but not SSEA-1. Injection of human ES cells beneath the testis capsule of nude mice resulted in the formation of teratomas containing a wide variety of differentiated cell types (Reubinoff *et al.*,

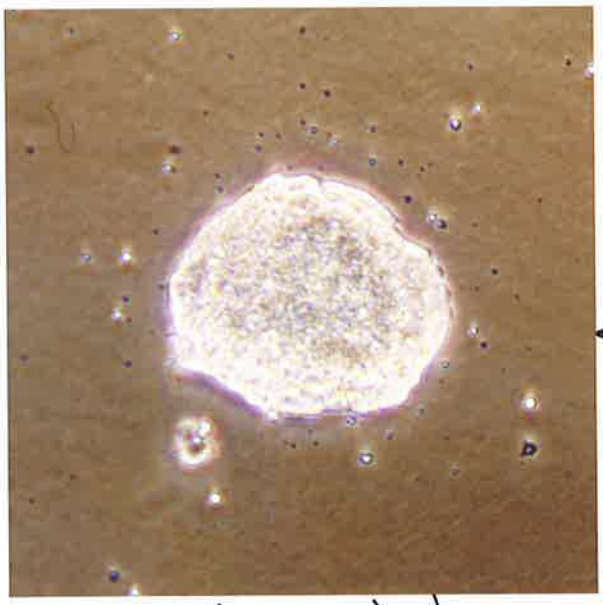
Figure 1.4

Morphology of undifferentiated and differentiated ES cells.

(A) In the presence of exogenous LIF, ES cells retain pluripotence. Morphologically undifferentiated ES cells grow as 3-dimensional colonies with high refractive index around the periphery. Individual cells cannot normally be distinguished.

(B) Differentiated ES cells. Withdrawal of LIF from ES cell cultures results in formation of a number of cell types including endoderm and mesoderm (Mummery *et al.*, 1990). ES cells differentiated by addition of retinoic acid form a more uniform population containing parietal endoderm (Mummery *et al.*, 1990) and neurons (van Inzen *et al.*, 1996).

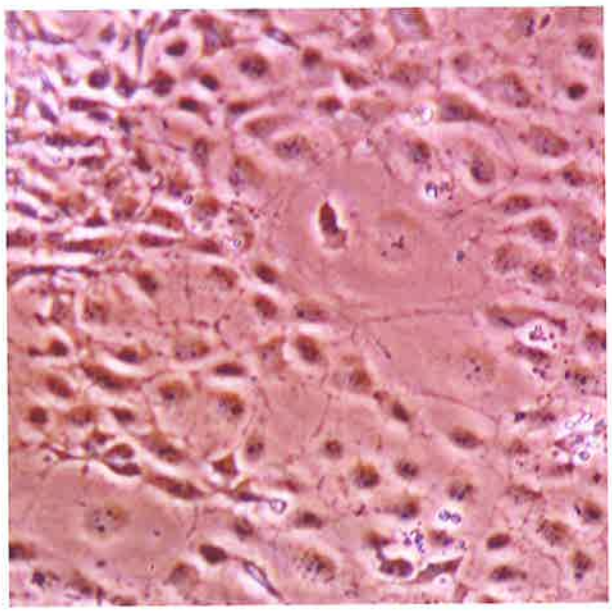
A



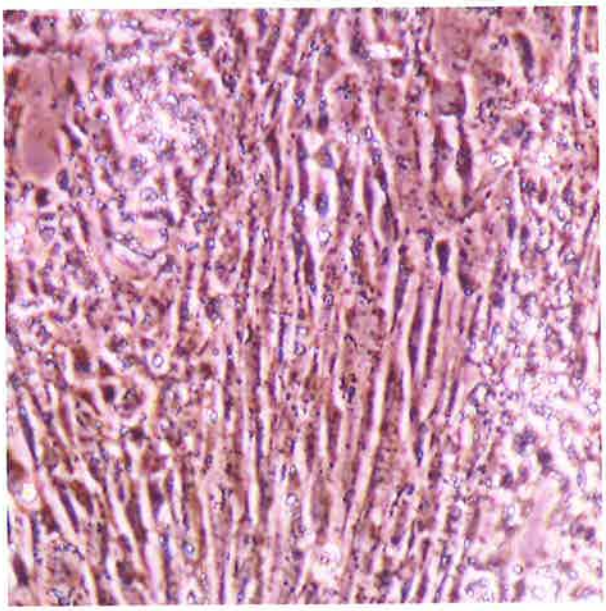
LIF

B

Withdrawal of LIF



Retinoic Acid induction



2000). The capacity of these cells to colonise the germ line is a property impossible to test experimentally for ethical reasons. This and the current technical difficulties in culturing human ES cells as a homogenous undifferentiated population means they are not the best model system for genetic and biochemical investigation.

1.4.3 Embryonic germ cells

Ectopic grafting of the genital ridge from day 11.5-12.5 p.c male 129 strain mouse embryos under the kidney capsule results in the formation of teratomas and teratocarcinomas containing EC cells (Stevens, 1983; Stevens and Mackensen, 1961). PGCs thus have the ability to form differentiated cell types and pluripotent EC cells, demonstrating their pluripotent nature. Culture of migratory PGCs or PGCs already in the genital ridge on feeder cells expressing LIF in the presence of basic Fibroblast Growth Factor (bFGF) and Stem Cell Factor (SCF) leads to the formation of Embryonic Germ (EG) cells. EG cells express pluripotent cell markers such as alkaline phosphatase, SSEA-1, and *Oct4* and give rise to teratocarcinomas upon injection into nude mice (Matsui *et al.*, 1992; Resnick *et al.*, 1992). In culture EG cells are morphologically similar to ES cells, and like ES cells can form tissues of chimaeric animals including the germ line when re-introduced into the blastocyst (Stewart *et al.*, 1994). EG cells grown long term in culture retain the ability to contribute to chimaeras and to the germ line but lose the requirement for factors other than LIF (Cheng *et al.*, 1994; Koshimizu *et al.*, 1996; Labosky *et al.*, 1994; Matsui *et al.*, 1992). Isolation of EG cells from humans (Shamblott *et al.*, 1998) and chicken (Park and Han, 2000) has also been reported.

1.4.4 The origin of ES cells

Derivation of ES cells from blastocyst stage embryos has fostered the view that ES cells are equivalent to cells of the ICM (reviewed in Gardner and Brook, 1997). Indeed ES cells and isolated ICM cells can be injected back into the blastocyst to colonise trophectoderm and extraembryonic endoderm lineages at low frequency (Beddington and Robertson, 1989) and ES cells routinely contribute to the germ line (Bradley *et al.*, 1984). Moreover ES cells retain gene expression comparable to that of the ICM (Hahnel *et al.*, 1990; Martin, 1981; Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1990). The acidic zinc finger transcription factor

Rex-1 is expressed in ES cells and in the ICM but not by the primitive ectoderm (Rogers *et al.*, 1991). In contrast, *FGF-5* is expressed in primitive ectoderm after day 5.25 p.c but not in ICM cells (Haub and Goldfarb, 1991; Hebert *et al.*, 1991) nor ES cells (Bettess, 1993). Distinct pluripotent cells derived from ES cells, termed early primitive ectoderm-like (EPL) cells express the primitive ectoderm marker *FGF-5* (Rathjen *et al.*, 1999) but not *Rex-1* which is specifically expressed in the ICM (Rogers *et al.*, 1991). These data indicate that ES cells and EPL cells are analogous to the ICM and the primitive ectoderm, respectively.

Culture of dissected ICM from early implantation embryos (day 4.5 p.c) significantly increases ES cell derivation efficiencies to 50% compared with 25% using day 3.5 p.c blastocysts (Brook and Gardner, 1997) and therefore potentially represent a more precise embryonic equivalent.

Isolated PGCs do not form chimaeras when injected into embryos (cited in Stewart *et al.*, 1994) but pluripotent EG cells isolated from PGCs and cultured *in vitro* can form chimaeric animals and give rise to the germ line. Thus it is also possible that ES cells are not a direct analogue of a cell type in the embryo and instead represent a stable pluripotent state adopted by various *in vivo* pluripotent populations under the appropriate conditions (Rossant, 1993). Injection of post-implantation primitive ectoderm into blastocysts does not yield chimaeras (Rossant, 1977; Beddington, 1983). The prediction from this scheme is that primitive ectoderm cells cultured under the correct conditions will revert to an ES-like cell and regain the capacity to form chimaeras and contribute to the germ line (Rossant, 1993).

ES cells harbour a normal number of chromosomes and only rarely form tumours upon injection into blastocysts (Hardy *et al.*, 1990) and instead efficiently contribute to adult tissues including the germ line. These characteristics and their extensive differentiation capacity *in vitro* make ES cells an excellent model for the study of cell potential and early embryogenesis without the complications of cell tumourigenicity.

1.5 ES CELL DIFFERENTIATION *IN VITRO*

1.5.1 ES cell differentiation as monolayers

ES cells cultured in monolayer can be differentiated in suspension or attached to a substratum to form a wide range of cell types. Spontaneous differentiation of ES cells attached

to a substratum is brought about by withdrawal of exogenous LIF and results in formation of extraembryonic endoderm and an unknown mesodermal cell type (Mummery *et al.*, 1990) (Figure 1.4). In the presence of chemical inducers such as RA, ES cells differentiate into parietal endoderm (Mummery *et al.*, 1990). Directed differentiation of ES cells can be achieved by co-culture with other cells or addition of conditioned medium. Co-culture of ES cells with the MCSF-deficient stromal cell line OP9 selects for ES cells that undergo haematopoiesis resulting in the formation of erythroid, myeloid, and B cell lineages (Nakano *et al.*, 1994). This system could result in the formation of haematopoietic cells as re-seeding OP9 co-cultured ES cells onto OP9 cells in presence of dexamethasone, 1α, 25-dihydroxyvitamin D_3 and M-CSF induces formation of macrophages and tartrate-resistant acid phosphatase positive cells indicative of osteoclastogenesis (Nakano *et al.*, 1994; Yamane *et al.*, 1997). Re-seeding ES cells on OP9 cells in the presence of TPO stimulates erythropoiesis while growth on MS-5 stromal cell line in HGF, TPO, bFGF results in megakaryocyte formation (Berthier *et al.*, 1997).

A more widely applicable finding is the derivation of a morphologically and developmentally distinct cell type from ES cells termed Early Primitive ectoderm Like (EPL) cells. Growth of ES cells in conditioned medium from the human hepatocellular carcinoma cell line HepG2 converts ES cells into EPL cells, a homogenous pluripotent cell type that resembles the primitive ectoderm (Rathjen *et al.*, 1999). Like primitive ectoderm, EPL cells cannot form chimaeras following blastocyst injection and express *Oct4* and *Fgf-5* but not *Rex-1* (Rathjen *et al.*, 1999). ICM cells of the blastocyst give rise to the embryo proper through formation of primitive ectoderm. EPL cells may also represent an obligatory intermediate for ES cell differentiation and therefore the study of EPL cell differentiation should allow molecular characterisation of the differentiation decisions faced by primitive ectoderm. Moreover, EPL cells could act as a starting point for the generation of specific multipotent cell types allowing further analysis of downstream differentiation events.

1.5.2 Embryoid body formation

Differentiation of ES cell aggregates in suspension causes formation of simple embryoid bodies. These spherical structures are made of extraembryonic endoderm cells at the periphery (Doetschman *et al.*, 1985; Evans and Kaufman, 1981; Martin, 1981; Robertson,

1987) surrounding, during the early steps of body formation, undifferentiated ES cells. After 4 days in culture, cystic or fluid filled embryoid bodies are formed through selective apoptosis of central cells (Coucouvanis and Martin, 1995). The surviving inner cells form an inner layer of primitive ectoderm surrounding the central cavity. The processes of cavity and layer formation in embryoid bodies are strikingly similar to the events of mouse pre-implantation development (1.3.1). Continued culture results in the formation of a plethora of differentiated structures including blood islands, visceral yolk sac and beating cardiac muscle (Doetschman *et al.*, 1985). Cell types including neurons, cardiac, smooth and skeletal muscle, chondrocytes (Doetschman *et al.*, 1985), primitive endoderm (Shen and Leder, 1992), visceral endoderm (Coucouvanis and Martin, 1995), parietal endoderm (Wilkinson *et al.*, 1988), primitive ectoderm, and mesodermal precursors (Shen and Leder, 1992; Keller *et al.*, 1993; Shen *et al.*, 1997) have been identified in cultures of embryoid bodies grown in suspension or attached to a substratum. Modification of the differentiated cell types formed in embryoid bodies can be achieved by changing embryoid body culture conditions and addition of exogenous growth factors and cytokines. By this approach haematopoietic cells such as erythrocytes (Burkert *et al.*, 1991), macrophages (Keller *et al.*, 1993; Wiles, 1993; Wiles and Keller, 1991), mast cells (Wiles and Keller, 1991; Wiles, 1993), lymphoid cells (Chen *et al.*, 1992) and cell types such as neurons (Strubing *et al.*, 1995) can be identified and expanded.

EPL cells form embryoid bodies upon suspension culture in the absence of LIF that exhibit accelerated differentiation compared with ES cell embryoid bodies (Lake *et al.*, 2000). The nascent mesoderm marker, Brachyury is expressed earlier and at much higher levels than in ES cell embryoid bodies, while expression of the ectodermal marker, *Sox-1* is not detected. The apparent differentiation into mesoderm at the expense of ectoderm is supported by the fact that EPL cell embryoid bodies form more beating muscle and macrophages but not neurons (Lake *et al.*, 2000). EPL cell embryoid bodies do not form visceral endoderm normally. Interestingly, formation of neurons in EPL cell embryoid bodies can be restored by differentiation in the presence of RA (Lake *et al.*, 2000) or visceral endoderm like signalling (Rathjen *et al.*, in press) further illustrating the importance of visceral endoderm signalling in pluripotent cell differentiation. Absence of this cell type from EPL embryoid bodies provides

an opportunity for directed differentiation of pluripotent cells in the absence of endogenous signalling (Rathjen *et al.*, in press).

1.6 MOLECULAR CONTROL OF ES CELL PLURIPOTENCE

1.6.1 IL-6-type cytokines

Maintenance of ES cells in an undifferentiated state was originally achieved by co-culture with feeder cells such as mouse embryo fibroblasts (Evans and Kaufman, 1981). A soluble source of the ES cell differentiation inhibiting activity was identified in conditioned medium from Buffalo rat liver cells (Smith and Hooper, 1987), purified and shown to identical to the cytokine Leukaemia Inhibitory Factor (LIF) (Smith *et al.*, 1988). Subsequently LIF was shown to be sufficient to maintain ES cells in the absence of feeders (Williams *et al.*, 1988; Nichols *et al.*, 1990). The differentiation inhibition activity of feeder cells results from secretion of LIF in response to paracrine signalling from pluripotent cells (Rathjen *et al.*, 1990a).

The IL-6-type cytokines LIF, Ciliary Neurotrophic Factor (CNTF), Interleukin-6 (IL-6), Cardiotrophin-1 (CT-1), Interleukin-11 (IL-11), and Oncostatin-M (OSM) interact with cell surface receptor complexes that include the ubiquitously expressed gp130 receptor subunit. IL-6 and IL-11 bind, in combination with their respective α subunits, to a homodimer of gp130 (Barton *et al.*, 2000; Hibi *et al.*, 1990; Murakami *et al.*, 1993; Yamasaki *et al.*, 1988). LIF, CNTF, and CT-1 signal through a heterodimer of gp130 and the LIF receptor (LIFR) (Davis *et al.*, 1993; Gearing *et al.*, 1991; Pennica *et al.*, 1995a). CNTF binds to gp130/LIFR heterodimers in the presence of the CNTFRα subunit (Davis *et al.*, 1991) while CT-1 binds gp130/LIFR heterodimers and a presumptive CT-1Rα subunit that is yet to be cloned (Robledo *et al.*, 1997). OSM signals through a heterodimer of gp130 and OSMR in mice (Ichihara *et al.*, 1997; Mosley *et al.*, 1996) (Figure 1.5). In accordance with the commonality of receptor subunits ES cells can be maintained in an undifferentiated state by addition of exogenous LIF, CNTF, OSM, or CT-1 (Pennica *et al.*, 1995b; Piquet Pellorce *et al.*, 1994; Rose *et al.*, 1994; Wolf *et al.*, 1994; Yoshida *et al.*, 1994). The inability of IL-6 and IL-11 to inhibit ES cell differentiation is presumably due to a lack of respective α subunit expression because IL-6 in combination with soluble IL-6Rα inhibits ES cell differentiation (Nichols *et al.*, 1994; Yoshida *et al.*, 1994).

Figure 1.5

Schematic structures of IL-6-type cytokine receptors.

IL-6-type cytokines bind to and activate receptor complexes that contain gp130 alone, or in combination with the LIFR or OSMR. Unlike the α subunits (IL-6Rα, IL-IIRα and CNTFRα), extracellular portions of gp130, LIFR, and OSMR contain fibronectin-type-III-like domains (FN III) close to the transmembrane domain. Cytokine binding modules (CBMs) comprise two fibronectin type III repeats with conserved cysteine residues (thin line) and a WSXWS motif (thick line). Domains present in gp130, LIFR and OSMR are shown.

FN III domain with four conserved cysteine residues
CBM
FN III domain with conserved WSXWS motif
Ig-like domain
FN III domain with conserved cysteine residues
CBM
FN III domain with conserved WSXWS motif
FN III domain
FN III domain
FN III domain
Transmembrane domain
Cytoplasmic domain
gp130
OSMR
OSM
Tyrosine Kinase
Tyrosine Kinase
gp130
LIFR
CNTFRα
CNTF
Tyrosine Kinase
Tyrosine Kinase
gp130
LIFR
CT-1Rα?
?
CT-1
Tyrosine Kinase
Tyrosine Kinase
gp130
LIFR
LIF, OSM
Tyrosine Kinase
Tyrosine Kinase
gp130
gp130
IL-6Rα
IL-6
Tyrosine Kinase
Tyrosine Kinase
gp130
gp130
IL-11Rα
IL-11
Tyrosine Kinase
Tyrosine Kinase

The redundant nature of IL-6-type cytokines is reflected in mice lacking these molecules. Development is unperturbed in *LIF*$^{-/-}$ mice, but homozygous adults are smaller, have decreased numbers of spleen and bone marrow stem cells (Escary *et al.*, 1993), and among others, exhibit maternal sterility (Stewart *et al.*, 1992; Escary *et al.*, 1993). The phenotype of *LIFR*$^{-/-}$ mice is more severe as neonates die exhibiting greatly reduced foetal bone volume, fewer spinal and brainstem astrocytes, and abnormal placentation (Li *et al.*, 1995; Ware *et al.*, 1995). Consistent with the observations that LIF and the LIFR are dispensable for pluripotent cell maintenance, ICM formation and growth is not inhibited in *LIF* and *LIFR* null mice. Embryos that lack gp130 die as early as day 12.5 p.c with reduced numbers of haematopoietic stem cells and progenitors and defects in the ventricular myocardium, long after formation and differentiation of the ICM and primitive ectoderm (Yoshida *et al.*, 1996), implying that either gp130 signalling has no role in ICM proliferation or that there is redundancy in receptors and/or signalling pathways capable of transducing the same signal in the embryo (Conquet and Brulet, 1990; Nichols *et al.*, 1996; Rathjen *et al.*, 1990a; Robertson *et al.*, 1993; van Eijk *et al.*, 1996).

LIFR and gp130 are expressed by pluripotent cells during early development (Nichols *et al.*, 1996; Saito *et al.*, 1992), and pluripotent cells within the early embryo are competent to respond to LIF signalling as overexpression of an extracellular matrix-associated form of the protein, LIF-M, in chimaeric embryos inhibits primitive ectoderm differentiation so that gastrulation does not commence (Conquet *et al.*, 1992)(Table 1.1). This finding is consistent with the fact that formation of primitive ectoderm in embryoid bodies is retarded by exogenous LIF (Shen and Leder, 1992). Analyses *in vitro* and *in vivo* therefore indicate a possible role for LIF signalling in pluripotent cell populations prior to primitive ectoderm differentiation at gastrulation.

1.6.2 ES cell maintenance in the absence of the LIF signalling pathway

Withdrawal of LIF results in precocious differentiation of ES cell cultures however a small percentage of 'stem cell nests', in which pluripotent cells are surrounded by differentiated cells, persist. Feedback inhibition of pluripotent cell differentiation is likely to dependent of LIF expression by surrounding differentiated cells (Mountford *et al.*, 1994; Rathjen *et al.*, 1990a).

Table 1.1 Summary of gene modifications that inhibit pluripotent cell differentiation

Gene product	Description	Modification	ES cell/Embryoid body Phenotype	Embryonic Phenotype	Reference
LIF	IL-6-type cytokine	Overexpression	No exogenous LIF requirement	With LIF-M: No primitive ectoderm diff^n	Shen and Leder 1992 Conquet *et al.*, 1992
		Null	Further suppression of ES cell renewal without LIF cf WT	No effect on development, Female sterility	Dani *et al.*, 1998 Stewart *et al.*, 1992; Escary *et al.*, 1993
Hck	Src family tyrosine kinase	Constitutively active	15-fold lower LIF requirement		Ernst *et al.*, 1994
v-Src	Src tyrosine kinase oncogene	Constitutively active	No LIF requirement; Inhibition of cell aggregation in EBs	Multiple egg cylinders in one Reichert's membrane	Boulter *et al.*, 1991
Jak1	Janus tyrosine kinase	Underexpression	Increased LIF requirement		Ernst *et al.*, 1996
Stat3	Transcription factor	Constitutively active Dominant negative	No LIF requirement Diff^n in the presence of LIF		Matsuda *et al.*, 1999;Boeuf *et al.*, 1997 Niwa *et al.*, 1998;Raz *et al.*, 1999
v-Ha-ras	GTP exchange factor	Constitutively active	8-fold lower LIF requirement		Ernst *et al.*, 1996
Shp-2	SH2 domain containing tyrosine phosphatase	Null	4-fold lower LIF requirement Impaired hematopoietic and cardiomyocyte diff^n in EBs	Defects in axial patterning and posterior development during gastrulation	Qu *et al.*, 1997;Qu and Feng 1998 Burdon *et al.*, 1999
Socs-1/3	cytokine repressors	Overexpression	Reduced cell viablity		Duval *et al.*, 2000
Pem	Paired-like homeodomain protein	Overexpression Null	Little or no EB diff^n Inhibits EB diff^n	 No effect on development	Fan *et al.*, 1999 Pitman *et al.*, 1998
RLF/L-myc	L-myc gene fusion	Overexpression	Impaired EB diff^n	Failure to develop	MacLean-Hunter *et al.*, 1994
Hsp27	Small heat shock protein	Overexpression Underexpression	No LIF: Decreased cell growth cf WT No LIF: Increased cell growth cf WT, arrested EB diff^n and apoptosis		Mehlen *et al.*, 1997 Mehlen *et al.*, 1997
AML1-d	Transcription factor	Overexpression	Poorly differentiated and apoptotic teratocarcinomas		Aziz-Aloya *et al.*, 1998
Oct4	Pou domain transcription factor	50%>WT 30-40% of WT Null	Primitive endoderm diff^n Trophectoderm diff^n *Oct4*$^{-/-}$ ES cells cannot be isolated	 Inner cells become trophoblast	Niwa *et al.*, 2000 Niwa *et al.*, 2000 Nichols *et al.*, 1998
Tbn	Taube nuss; Novel protein	Null	*Tbn*$^{-/-}$ blastocysts do not grow	ICM is not detected at day 4 p.c	Voss *et al.*, 2000
Hxt	bHLH transcription factor	Overexpression	ES cells cannot be cloned	Blastomeres fated to trophoblast	Cross *et al.*, 1995

EB, Embryoid Body; ES, Embryonic Stem (cells); WT, wild-type; cf, compared to; diff^n, differentiation.

Studies of LIF deficient ES cell lines have demonstrated that LIF is not the only factor involved in the feedback inhibition of ES cell differentiation. Feedback inhibition is not ablated in $LIF^{-/-}$ ES cells, rather, 2-3 fold fewer pluripotent colonies form. This effect is due to a soluble trypsin-sensitive factor, termed ES cell renewal factor (ESRF) that acts independently of gp130 and the Stat3 signalling pathway (Dani *et al.*, 1998). ESRF is expressed by parietal endoderm cell lines such as D7A3-PE, however conditioned medium from such lines only allows limited growth without exogenous supply of LIF (Dani *et al.*, 1998). The importance of ESRF for pluripotent cell renewal and differentiation remains to be determined.

1.6.3 gp130/LIFR receptor complex signalling in ES cells

gp130 is a widely expressed transmembrane non-tyrosine kinase receptor. It belongs to the cytokine receptor class I family by virtue of a cytokine binding module (CBM) defined by two fibronectin-type-III like domains, one containing four conserved cysteines and the other a WSXWS motif (Figure 1.5). The CBM and the Ig-like domain are important for cytokine binding (Dahmen *et al.*, 1998; Horsten *et al.*, 1997). LIF signal transduction occurs via formation of a complex between gp130 and the LIFR (Figure 1.5). LIFR contains two CBMs and like gp130 is a transmembrane non-tyrosine kinase receptor of the cytokine receptor class I family.

gp130 is a component of all receptors for IL-6-type cytokines capable of maintaining ES cells (Figure 1.5) (Heinrich *et al.*, 1998) and gp130 activation is sufficient to inhibit ES cell differentiation (Yoshida *et al.*, 1994; Ernst *et al.*, 1999). Expression of chimaeric receptors consisting of the extracellular portion of granulocyte colony stimulating factor receptor (GCSFR) and cytoplasmic portions of LIFR or gp130 demonstrate that homodimerisation of GCSF/LIFR, GCSF/gp130 or GCSFR results in maintenance of the undifferentiated state of ES cells (Ernst *et al.*, 1999). Thus, the intracellular domains of LIFR, gp130 and GCSFR are capable of transducing the differentiation inhibition signal in ES cells. ES cells differentiate in the presence of GCSF (and absence of LIF) because they do not normally express GCSFR (Ernst *et al.*, 1999). GCSFR activates the Signal Transducer and Activator of Transcription (Stat) family protein Stat3 (Tian *et al.*, 1994), leading to activation of identical signalling cascades to those of gp130/LIFR (1.6.4). This finding demonstrates that signalling via IL-6-

type cytokines is not necessary for ES cell maintenance. Cytokines other than IL-6-type cytokines could act early in embryogenesis to supply proliferation/survival signals to pluripotent cells in the absence of LIFR or gp130.

1.6.4 Signalling downstream of the gp130/LIFR complex.

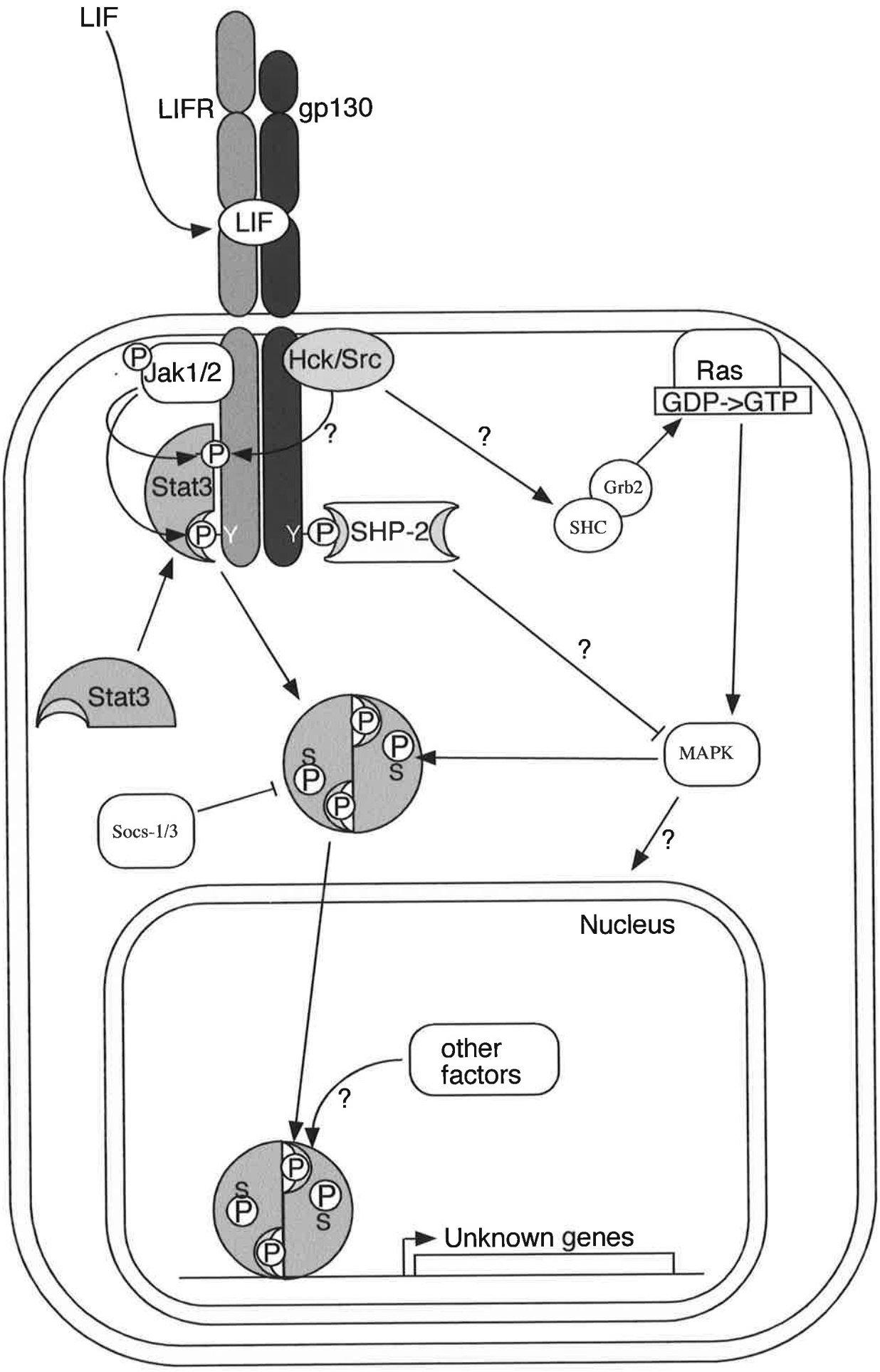
Direct interactions of gp130/LIFR with tyrosine kinases of the Jak, Tec (Matsuda *et al.*, 1995b), Fes (Matsuda *et al.*, 1995a) and Src families have been demonstrated. Three members of the Jak family, Jak1, Jak2, and Tyk2 associate constitutively with, and are activated by gp130 in a wide variety of cell lines (Lutticken *et al.*, 1994; Narazaki *et al.*, 1994; Stahl *et al.*, 1994; Guschin *et al.*, 1995). Jak kinases are activated upon receptor dimerisation in response to IL-6-type cytokines (Lutticken *et al.*, 1994; Stahl *et al.*, 1994). Dimerisation is thought to bring Jak molecules into proximity leading to cross-phosphorylation (Watowich *et al.*, 1994). Jak kinase activation leads to phosphorylation of gp130 and LIFR at specific tyrosine residues (Murakami *et al.*, 1993; Stahl *et al.*, 1994)(Figure 1.6). Stat transcription factors Stat3 and Stat1 dock with gp130 and LIFR via phosphotyrosine-SH2 domain interactions and are themselves phosphorylated (Shuai *et al.*, 1993). There is evidence that Src family as well as Jak family kinases are capable of phosphorylating Stat proteins (Chaturvedi *et al.*, 1998; Quelle *et al.*, 1995; Shuai *et al.*, 1993). Phosphorylated Stat3 and Stat1 proteins homodimerise or heterodimerise via reciprocal SH2 domain phosphotyrosine interactions (Chen *et al.*, 1998) and enter the nucleus. Stat3 activation stimulates transcription of c-fos (Hill and Treisman, 1995; Jenab and Morris, 1998), JunB (Fujitani *et al.*, 1994), CCAAT enhancer binding protein (C/EBP)δ (Yamada *et al.*, 1997), gp130 (O'Brien and Manolagas, 1997), $p27^{Kip1}$ (Kortylewski *et al.*, 1999) and acute-phase proteins such as α_2-macroglobulin, haptoglobin (Wegenka *et al.*, 1993) and lipopolysaccharide-binding protein (Schumann *et al.*, 1996).

Tyrosine phosphorylation of gp130 also leads to activation of the SH2 adaptor protein SHC and subsequently MAP kinase (Schiemann *et al.*, 1997; Stahl *et al.*, 1994). Moreover, SHC and the Raf-1 kinase bind to and are phosphorylated by activated Jak2 (Han *et al.*, 1996; VanderKuur *et al.*, 1995; Xia *et al.*, 1996). Hck, a Src family kinase, is activated in response to LIF signalling and activated Hck kinase associates with gp130 (Ernst *et al.*, 1994). Hck kinase has also been reported to activate SHC and the guanine nucleotide exchange factor $p21^{ras}$ of the

Figure 1.6

Diagrammatic representation of the LIF signalling pathway in ES cells.

LIF binds to a heterodimer of gp130 and LIFR leading to activation of Jak and Hck tyrosine kinases. Jak kinases phosphorylate gp130 and LIFR creating docking sites for Stat transcription factors. Stat3 binds to phosphotyrosine residues via an SH2 domain interaction and is itself tyrosine phosphorylated. Phosphorylated Stat3 forms homodimers or heterodimers with Stat1 (not shown), enters the nucleus, and stimulates transcription of unknown genes leading to the maintenance of ES cells. Activation of Hck kinase results in the activation of the MAP kinase (MAPK) pathway. Through an unknown mechanism that may involve serine phosphorylation of Stat3, MAP kinase activation partially inhibits ES cell differentiation. Shp-2 may act to inhibit the MAP kinase pathway in ES cells. Socs-1 and Socs-3 impair LIF signalling in ES cells presumably through inhibition of Stat3 function.


LIF
LIFR
gp130
LIF
P
Jak1/2
Hck/Src
Ras
GDP->GTP
?
?
P
Stat3
Grb2
SHC
P
Y
Y
P
SHP-2
?
Stat3
P
P
S
P
S
P
MAPK
Socs-1/3
?
Nucleus
other
factors
?
P
P
S
P
S
P
Unknown genes

Ras/Mitogen-Activated Protein (MAP) kinase pathway (Ernst *et al.*, 1996). Thus the MAP kinase pathway is activated at multiple levels following gp130/LIFR stimulation.

1.6.5 Modulation of gp130/LIFR mediated signalling

While the Jak/Stat pathway (Figure 1.6) is relatively simple, the molecular controls of this pathway are numerous and a number of factors that regulate cytokine signalling have been identified. Suppressor of cytokine signalling-1 (Socs-1, also termed Jab and SSI-1) can prevent differentiation of M1 monocyte leukaemic cells (Starr *et al.*, 1997), interact with the tyrosine kinase domain JH1 of Jak2 (Endo *et al.*, 1997) and bind to a monoclonal antibody directed against a motif within the SH2 domain of Stat3 (Naka *et al.*, 1997). Socs-1 contains a SH2 domain with a conserved motif similar to that of Stat3. The SH2 domain of Socs-1 can bind to Jak2, inhibiting its ability to phosphorylate other proteins (Suzuki *et al.*, 1998). Socs-1 antagonises LIF and IL-6 signalling in M1 cells (Starr *et al.*, 1997) and consistent with an antagonistic function, transient transfection of ES cells with Socs-1 or Socs-3, but not Socs-2, abrogates LIF dependent transcription. Stable transfection and expression of Socs-1 or Socs-3 reduced ES cell colony viability by 80 and 90%, respectively (Duval *et al.*, 2000). It is unknown if Socs gene expression has a purely cytotoxic effect or if it causes differentiation of ES cell colonies.

Members of the PIAS (protein inhibitor of activated Stat proteins) protein family interact with Stat transcription factors and inhibit their function (Liu *et al.*, 1998). PIAS3 specifically binds Stat3 but not Stat1 and inhibits Stat3 DNA binding and transactivation (Chung *et al.*, 1997). A role for PIAS3 in the maintenance of pluripotent cells has not been reported. The ubiquitously expressed tyrosine phosphatase Shp-2 is phosphorylated in response to IL-6-type cytokines in Ewing's sarcoma cell line EW-1 (Boulton *et al.*, 1994) and appears to inhibit signalling via the gp130/LIFR complex in ES cells (Qu and Feng, 1998) (Figure 1.6).

1.6.6 Oct4 is a master regulator of pluripotency

Oct4 is a member of the Pou domain transcription factor family, originally identified based on an ability to bind to the octamer motif ATGCAAAT required for tissue specific and

ubiquitous gene expression (Kemler and Schaffner, 1990; Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1989). Expression of *Oct4* is restricted to the totipotent germ line of the mammalian life cycle and to pluripotent cells *in vivo* and *in vitro*. In oocytes, maternal *Oct4* mRNA and protein are present (Palmieri *et al.*, 1994). Oct4 protein is found in 2- and 4-cell blastomeres and zygotic *Oct4* expression begins at the 4-cell stage resulting in high expression in the morula stages (Figure 1.7). During blastocyst formation Oct4 expression becomes restricted to the pluripotent cells of the ICM as Oct4 protein is rapidly lost from the newly formed trophectoderm (Palmieri *et al.*, 1994) (Figure 1.7). At day 4.5 p.c ICM cells exposed to the blastocoelic cavity differentiate into primitive endoderm. Within primitive endoderm *Oct4* mRNA expression is low but Oct4 protein levels increase transiently (Palmieri *et al.*, 1994; Scholer *et al.*, 1990). Oct4 expression is lost upon differentiation and migration of the primitive endoderm to visceral and parietal endoderm (Scholer, 1991).

Prior to gastrulation *Oct4* transcripts are observed in pluripotent primitive ectoderm cells. As gastrulation proceeds *Oct4* expression becomes restricted to PGCs. *Oct4* expression is lost from somatic tissues by day 8.5 p.c during early somitogenesis (Scholer *et al.*, 1990; Yeom *et al.*, 1996). In the adult Oct4 expression is restricted to the gonads and unfertilised oocytes (Figure 1.7) (Pesce *et al.*, 1998; Scholer *et al.*, 1989; Palmieri *et al.*, 1994). The tight expression pattern of *Oct4* therefore makes it a specific marker of the pluripotent state *in vivo*.

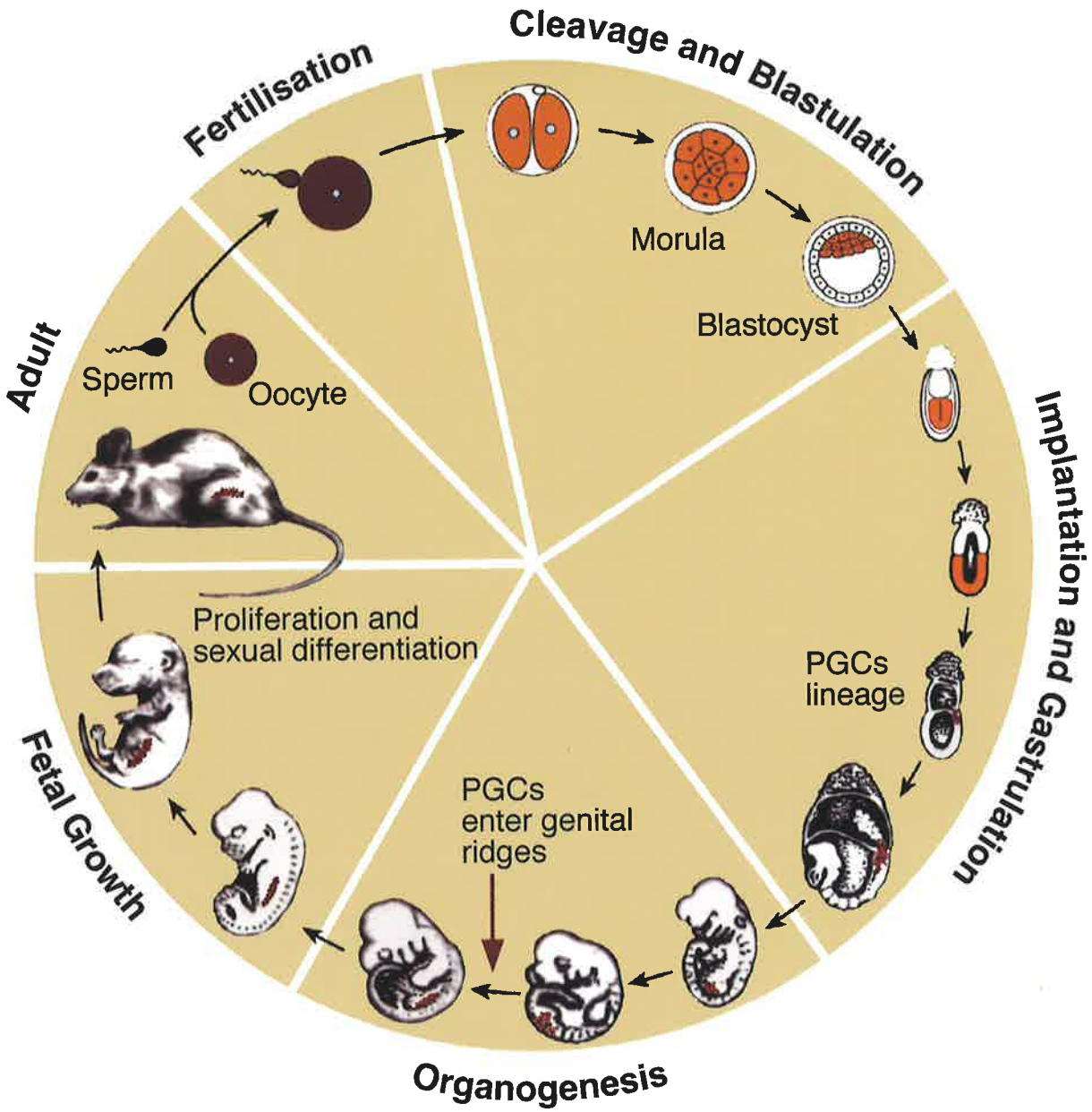
Undifferentiated ES cells and EC cells express Oct4 strongly but upon differentiation by removal of LIF or chemical induction *Oct4* mRNA and protein levels decline rapidly (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1990). Oct4 appears to have a role in ES cell differentiation and renewal. Niwa *et al*, (2000) produced ES cells carrying one endogenous *Oct4* allele ($Oct4^{+/-}$) and introduced a transgene that expressed Oct4 protein in the absence of tetracycline to a level 50% greater than wild-type undifferentiated ES cells. ES cells in which expression of the *Oct4* transgene is tetracycline repressible differentiate within 72 hours in the presence of LIF and absence of tetracycline, indicating that overexpression of Oct4 induces ES cell differentiation (Niwa *et al.*, 2000). Niwa *et al,* (2000) also removed the remaining *Oct4* allele from these ES cells creating an ES cell line in which Oct4 expression was solely dependent on transgene expression in the absence of tetracycline. Downregulation of *Oct4* transcription by addition of tetracycline, results in the formation of trophectoderm cells,

Figure 1.7

***Oct4* expression and the life cycle of the mouse.**

Maternal *Oct4* message and protein are present in unfertilised and fertilised oocytes but not in sperm. *Oct4* expression is detected at low levels in the 2- and 4- cell embryo and is upregulated in the morula stages, the ICM of the blastocyst stage and the primitive ectoderm of the egg cylinder stage. The germ cells are first observed during gastrulation in the extraembryonic mesoderm. They are passively taken into the hindgut at day 8.5 p.c and actively migrate to the gonadal anlagen at day 13.5 p.c during organogenesis. Expression of *Oct4* is indicated by red shading. PGCs are shown as dark red spots. Stages of the life cycle are indicated.

Taken from Pesce *et al*, (1998).


Fertilisation
Cleavage and Blastulation
Morula
Blastocyst
Implantation and Gastrulation
PGCs
lineage
PGCs
enter genital
ridges
Organogenesis
Fetal Growth
Proliferation and
sexual differentiation
Adult
Sperm
Oocyte

indicating that Oct4 expression is required to maintain ES cell pluripotency. Oct4 expression is not sufficient to maintain an undifferentiated state as these ES cells differentiate in the presence of tetracycline upon LIF withdrawal despite continued expression of *Oct4* transcripts. 50% higher than normal Oct4 expression also induces ES cell differentiation in the presence of LIF (Niwa *et al.*, 2000), although primitive endoderm and mesoderm are formed rather than trophectoderm, indicating that the control exerted by Oct4 is bipartite. Oct4-induced primitive endoderm formation is consistent with the transitory increase in Oct4 levels in newly formed primitive endoderm in the day 4-4.5 p.c blastocyst (Palmieri *et al.*, 1994).

Evidence for a role for Oct4 in the establishment of pluripotency has come from cell fusion experiments. Hybrid cells of pluripotent P19 EC cells and fibroblast L cells form a neuroepithelial cell type that expresses Brn-2 and nestin (Shimazaki *et al.*, 1993). These cells no longer express *Oct4*, but introduction of *Oct4* and *E1A* expression constructs can revert hybrid cells to a cell type resembling P19 parental cells that no longer express neuroepithelial markers.

A role for Oct4 in pluripotent cell maintenance *in vivo* has been demonstrated in *Oct4*$^{-/-}$ embryos. *Oct4*$^{-/-}$ blastocysts die prior to implantation and lack a true pluripotent ICM. Instead the inner cells of *Oct4*$^{-/-}$ blastocysts contain intermediate filaments, which are normally expressed in nascent trophectoderm (Brulet *et al.*, 1980), and no longer express *Sox-2* indicating that these cells have diverted to a trophoblast cell state (Nichols *et al.*, 1998). *Oct4* expression is thus not only a marker for pluripotent cells but also appears critical for the maintenance of a pluripotent ICM. It is not currently known how *Oct4* expression is established or limited to pluripotent cell populations.

1.6.7 Inhibition of ES cell differentiation by other intracellular factors

A number of cellular factors control specific differentiation events during ES cell differentiation in monolayer or during embryoid body growth (Table 1.2). While these genes are important for specific differentiation decisions they are likely to control differentiation decisions of multipotent ES cell derivatives rather than by direct inhibition of ES cell differentiation. Crucial to understanding the nature of pluripotence are cellular factors that inhibit the differentiation of ES cells (Table 1.1). Several modifications of the ES cell genome

Table 1.2 **Gene modifications that result in specific alterations to ES cell and embryoid body differentiation**

Gene product	Description	Modification	ES cell/Embryoid body Phenotype	Reference
β1 Integrin	Adhesion protein	Null	Increased neurogenic and decreased myogenic diffⁿ of EBs	Rohwedel *et al.*, 1998; Stephens *et al.*, 1995
c-Myb	Transcription factor	Overexpression	Hematopoietic commitmant and erthyromyeloid diffⁿ	Melotti and Calabretta 1996
Flk	VEGF receptor	Null	Partial block in mesoderm diffⁿ in OP9 coculture	Hidaka *et al.*, 1999
Gata-1	Transcription factor	Null	Defective in erythropoiesis	Weiss *et al.*, 1994;Blobel *et al.*, 1995
Gata-2	Transcription factor	Null	Decreased multipotential hematopoietic progenitors	Tsai and Orkin 1997
Gata-4	Transcription factor	Null	Defective vascular channel formation Impaired VE diffⁿ	Soudais *et al.*, 1995
HoxB4	Homeodomain protein	Overexpression	Enhanced hematopoietic potential	Helgason *et al.*, 1996
HNF1β	Hepatocyte nuclear factor	Null	Blocked VE diffⁿ	Coffinier *et al.*, 1999; Barbacci *et al.*, 1999
HNF3α/3β	Hepatocyte nuclear factor	Overexpression	Increased early endoderm in EBs	Levinson-Dushnik and Benvensity 1997
HNF4	Hepatocyte nuclear factor	Null	Impaired VE diffⁿ	Duncan *et al.*, 1997
Msd	Mesoderm deficiency gene	Null	Defect in mesoderm diffⁿ as EBs	Holdener *et al.*, 1994
M-twist	Basic HLH protein	Overexpression	Inhibits skeletal muscle diffⁿ	Rohwedel *et al.*, 1995
Ptpγ	Tyrosine phosphatase	Overexpression Underexpression	Increased hematopoietic diffⁿ as EBs No hematopoietic diffⁿ of EBs	Sorio *et al.*, 1997
SCL	Basic HLH protein	Null	Defect in hematopoiesis	Robb *et al.*, 1996;Porcher *et al.*, 1996
SF-1	Orphan nuclear receptor	Overexpression	Promotes steroidogenesis	Crawford *et al.*, 1997
TβRII	Type II TGFβ receptor	Dominant -ve	No mesoderm formation in monolayers	Goumans *et al.*, 1998
VE-Cadherin	Cell adhesion protein	Null	Lack of vascular structures	Vittet *et al.*, 1997
N-/P-Cadherin	Cell adhesion protein	N or N/P Null	Increased striated muscle; lack of neural tube-like structures	Moore *et al.*, 1999
MDS1/EVI1	Zinc finger nuclear factor	Overexpression	Slower cell growth; reduced numbers of differentiated hematopoietic colonies	Sitailo *et al.*, 1999
EVI1	Zinc finger nuclear factor	Overexpression	Faster growth rate; diffⁿ of greater numbers of megakaryocytic colonies	Sitailo *et al.*, 1999

EB, Embryoid body; ES, Embryonic Stem (cells); VE, Visceral Endoderm; diffⁿ, differentiation.

shown to inhibit differentiation *in vitro* in monolayer culture or as embryoid bodies are listed in table 1.1 with other genes such as *Oct4*, *Hxt* and *Tbn* which from other work (Cross *et al.*, 1995; Nichols *et al.*, 1998; Niwa *et al.*, 2000; Voss *et al.*, 2000) are known to be involved in establishment and maintenance of the pluripotent cell phenotype. Several of these, such as *LIF*, *Jak1*, *Stat3*, *Hck* and *Shp-2* have been implicated in LIF signal transduction (Table 1.1).

Jak kinases

In ES cells stimulated with IL-6/sIL-6Rα Jak2, and to a lesser degree, Jak1 are activated (Narazaki *et al.*, 1994)(1.6.3). *Jak2*$^{-/-}$ ES cells retain pluripotence state in the presence of LIF (Neubauer *et al.*, 1998) indicating that Jak2 is dispensable for LIF signalling. ES cells that express antisense *Jak1* RNA require higher concentrations of LIF to remain pluripotent, suggesting that Jak1 is necessary for LIF signal transduction in ES cells (Ernst *et al.*, 1996) (Table 1.1). These data suggest that LIF signal transduction in ES cells is primarily mediated by Jak1.

Stat transcription factors

The importance of Stat3 for ES cell maintenance is indicated by spontaneous differentiation of ES cells expressing dominant negative forms of Stat3 incapable of dimerisation (Boeuf *et al.*, 1997; Niwa *et al.*, 1998). Moreover, Stat3 activation is sufficient to inhibit ES cell differentiation, suggesting that differentiation inhibition is dependent on LIF signalling through the Stat3 transcription factor (Matsuda *et al.*, 1999).

Stat3$^{-/-}$ embryos at day 6.5 p.c are smaller than wild-type embryos, degenerate rapidly and are resorbed by day 7.5 p.c. *Stat3*$^{-/-}$ embryos undergo cavitation forming an egg cylinder although mesodermal cell types are not observed (Takeda *et al.*, 1997), indicating that the ICM forms and differentiates in the absence of Stat3. Death of the primitive ectoderm may be due to loss of Stat3 function in the visceral endoderm as *Stat3* expression is restricted to this tissue at day 6 p.c (Takeda *et al.*, 1997). These results indicate that Stat3 does not have a direct role in primitive ectoderm or ICM survival and are consistent with observations that LIF signalling is not essential for the early stages of embryogenesis. However, it is possible that loss of Stat3 function *in vivo* may be compensated by other Stat transcription factors. Blastocyst outgrowth

experiments indicate that Stat3 may function to promote cell growth in the embryo (Takeda *et al.*, 1997) rather than to inhibit cell differentiation.

ES cells express *c-fos* and *junB* in response to LIF stimulation (Duval *et al.*, 2000) presumably as a result of Stat3 activation (Fujitani *et al.*, 1994; Hill and Treisman, 1995; Jenab and Morris, 1998). *LIFR*, *ESP*, *c-jun*, and *Socs-3* are also upregulated following LIF stimulation (Duval *et al.*, 2000). Genes responsible for ES cell maintenance in response to Stat3 activation are yet to be identified (Figure 1.6).

Src family kinases

v-Src, originally isolated as an oncogene from Rous sarcoma virus, has also been identified as a chromosomal proto-oncogene, *c-Src* (Jove and Hanafusa, 1987; Stehelin *et al.*, 1976; Takeya and Hanafusa, 1983). c-Src is a non-receptor tyrosine kinase which is active in when not phosphorylated at tyrosine 527. *v-Src* has been shown to be constitutively active due to the absence of a C-terminal region encompassing tyrosine 527. Expression of *v-Src* in ES cells has been shown to abrogate their requirement for LIF (Boulter *et al.*, 1991). There are eight other members of the Src family of non-receptor tyrosine kinases, one of which, Hck kinase, has been shown to associate with gp130 in ES cells when it is constitutively activated (Ernst *et al.*, 1994)(Figure 1.6). While activated Hck does not remove LIF dependency of ES cells, the presence of activated Hck reduces the levels of LIF required for ES cell maintenance 15-fold (Ernst *et al.*, 1994). Although v-Src may mimic the role of Hck in ES cells, Hck itself is not sufficient for ES cell maintenance. The authors have suggested the existence of other factors that function in combination with Hck, perhaps Jak family kinases, which transduce a differentiation inhibition signal in ES cells (Ernst *et al.*, 1994). Other workers have demonstrated that the ability of v-Src to activate Stat3 and thus to transform fibroblast cells is dependent on Jak1 (Zhang *et al.*, 2000). It may be that v-Src expression brings about differentiation inhibition in ES cells by activating both the MAP kinase and Jak/Stat signalling pathways, while activated Hck kinase stimulates only the MAP kinase pathway. Mice deficient in Hck and Src survive until birth (Lowell *et al.*, 1996) indicating that activity of these kinases is not critical for pluripotent cell growth and differentiation *in vivo*.

The MAP kinase pathway

Modified expression or activity of proteins that comprise the MAP kinase pathway can alter the LIF dependence of ES cells (Table 1.1). Expression of the viral oncoprotein v-Ha-Ras (Ernst *et al.*, 1996), a constitutively active form of Ras, inhibits ES cell differentiation in the absence of LIF. ES cells that lack SH2 containing phosphatase (Shp-2) require 4-fold less LIF to retain pluripotence (Qu and Feng, 1998), implying that Shp-2 negatively regulates LIF signal transduction in ES cells. Differentiation of *Shp-2*$^{-/-}$ embryoid bodies is also altered so that there is reduced beating cardiac muscle, fibroblast and epithelial cell formation. This effect is likely to be caused by general inhibition of cell differentiation rather than blocking formation of particular cell lineages as trypsinisation of *Shp-2*$^{-/-}$ embryoid bodies and subsequent culture generates 5-fold more secondary embryoid bodies than normal ES cells (Qu and Feng, 1998), implying greater retention of pluripotent cells capable of forming embryoid bodies.

Mitogenic stimulation of serum starved ES cells by addition of serum and to a lesser extent LIF also leads to activation of SHC and MAP kinase phosphorylation and thus activation of the MAP kinase pathway (Ernst *et al.*, 1996). Lack of *Shp-2* also slows the growth rate of ES cells in high serum concentrations, suggesting that it is important for mitogenic signalling in response to serum (Qu and Feng, 1998). A mitogenic action of Shp-2 in other cell types has been observed (Roche *et al.*, 1996; Xiao *et al.*, 1994). Thus it seems that Shp-2 may have a dual role in ES cell maintenance, by increasing the growth rate in response to serum and/or LIF which is not characterised, and an inhibitory affect on ES cell maintenance.

Inhibition of embryoid body differentiation

Overexpression of several cellular proteins inhibits ES cell differentiation in conditions other than monolayer culture. The paired-like homeobox gene *Pem* blocks embryoid body differentiation such that Pem expressing bodies cultured in the absence of LIF are indistinguishable from normal ES cell bodies grown in the presence of LIF. Bodies as old as day 12 that overexpress Pem retain expression of the pluripotent cell marker *Oct4* and do not express visceral and parietal endoderm markers *H19* and *α-fetoprotein*, indicating that they lack the ability to differentiate in suspension (Fan *et al.*, 1999). Moreover, teratocarcinomas of normal size comprising undifferentiated cells are formed 6 weeks after subcutaneous injection

of Pem expressing ES cells. This effect is cell-autonomous because mixing populations of Pem overexpressing and normal ES cells does not stimulate differentiation of Pem expressing ES cells. Surprisingly, embryoid bodies derived from *Pem* null ($Pem^{-/Y}$) ES cells exhibit partial inhibition of embryoid body differentiation. This paradox may be due to dual roles of the Pem protein in maintenance of the undifferentiated state, and in the control of the differentiation process (Fan *et al.*, 1999). Thus Pem may act in a similar manner to Oct4 where expression levels dictate distinct differentiation outcomes (Fan *et al.*, 1999; Niwa *et al.*, 2000). The differentiation capacity of ES cells overexpressing Pem in monolayer culture has not been reported.

The acute myeloid leukaemia gene (*AML*) encodes a transcription factor containing a Runt DNA binding (RD) domain and a transactivation domain and is commonly fused to other genes in leukaemia associated chromosome translocations. Overexpression of human *AML1-d* does not perturb embryoid body differentiation *in vitro* but teratocarcinomas formed by injection of *AML1-d* expressing ES cells in syngeneic mice are poorly differentiated and contain apoptotic cells. Teratocarcinomas derived from *AML1-d* expressing ES cells consist almost entirely of morphologically undifferentiated cells and are much smaller due to extensive apoptosis (Aziz-Aloya *et al.*, 1998). The d isoform of *AML* produces a protein almost identical to chimaeric proteins associated with leukaemia and possibly represents a dominant negative isoform of the protein.

Other proteins reported to inhibit embryoid body differentiation include the RLF/L-myc gene fusion (MacLean-Hunter *et al.*, 1994) and Hsp27 (Mehlen *et al.*, 1997). The small heat shock protein (sHSP) Hsp27 and other sHSPs protect against cell necrosis and are inhibitors of apoptosis. Underexpression of *Hsp27* in ES cells increased cell growth upon differentiation and blocks embryoid body differentiation through extensive apoptosis (Mehlen *et al.*, 1997). Likewise embryoid bodies expressing high levels of *RLF/L-myc* die after 6-7 days of culture and mesoderm and ectoderm lineages were not formed in ES cell lines that expressed lower levels of *RLF/L-myc* (MacLean-Hunter *et al.*, 1994).

Many genes implicated in controlling specific cell fates in the animal also regulate the ability of ES cells to form these cell types *in vitro* (Table 1.2). Observations of this kind confirm the legitimacy of using ES cells and embryoid bodies as models for embryonic

differentiation. Discovery of genes controlling differentiation decisions made by ES cells *in vitro* can therefore provide information about molecular regulation of lineage decisions in the embryo. In this way ES cells may not only be useful as a vector for the creation of null mice strains and a means to study cellular differentiation *in vitro*, but will also contribute directly to our molecular understanding of genes involved in differentiation decisions in the embryo.

1.7 GENE DISCOVERY BASED ON GENE FUNCTION

The goal of this work was to identify genes important for pluripotent cell maintenance. Strategies of gene identification based on gene expression profile or molecular techniques can be misleading when extrapolated to putative gene function. Function-based screens can directly identify genes that exhibit a specific function of interest and thus immediately yield important information. In contrast to screening approaches based on expression or homology, genes isolated by function-based screens require less subsequent characterisation as their function has been assigned during the course of their isolation. Function-based screens involve some form of genetic perturbation, either loss of function or gain of function, followed by a specific assay for an altered cellular property and finally gene identification. As a result cellular phenotype can be correlated with the initial genetic perturbation.

1.7.1 Mutagenesis

In general, mutagenesis approaches involve application of a mutagen to increase the mutation frequency in cells to a level that allows effective screening for phenotypic alterations.

Sherman *et al*, (1981) mutagenised EC cell lines with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and then subjected them to RA or hexamethylenebisacetamide (HMBA) induced differentiation. Although almost all cells were found to differentiate, a low frequency (1 in 10^5) maintained EC cell morphology in the presence of RA (Sherman *et al.*, 1981). These colonies could be grown indefinitely in the presence or absence of inducer without detectable differentiation. Some cell lines were defective in their ability to differentiate in response to differentiation inducers other than RA, while others could still differentiate in response to HMBA.

As illustrated by this example, mutagenesis by itself yields little molecular information. It is not a simple matter to identify the underlying genetic defect responsible for alteration of the phenotype. One possible approach to this problem is gene identification via a complementation approach similar to the established procedure used in yeast. Phenotypically altered cells are transfected with a cDNA expression library to screen for genes that rescue the mutant phenotype. Isolation and identification of cDNAs responsible for the rescued phenotype defines the function of the cDNA product. Complementation is thus a two step strategy; isolation of cell lines with the desired phenotype by chemical or other mutagenesis followed by isolation of cDNAs whose product rescues this phenotype. This approach is limited because in general most mutations are recessive mutations and therefore both alleles of a gene must be disrupted. It is also dependent on high efficiency transfection of cDNAs to enable identification of low-abundance cDNAs.

Insertional mutagenesis or 'gene trapping' is a mutational approach that involves random chromosomal integration of a promoter-less selectable marker such that cells become resistant to antibiotic if the vector inserts into a gene expressed in ES cells. The expression pattern of the trapped gene can be inferred from expression of reporter genes such as *β-galactosidase* (*β-gal*) present on the trap vector (Figure 1.8a). Tagging of the locus with vector derived DNA can also provide information about the gene's chromosomal location and allows relatively simple identification of mutated genes by plasmid rescue, inverse PCR (von Melchner *et al.*, 1990) or direct sequencing (Townley *et al.*, 1997). Low abundance mRNAs can be identified by gene trap protocols because the technique does not rely on high level expression.

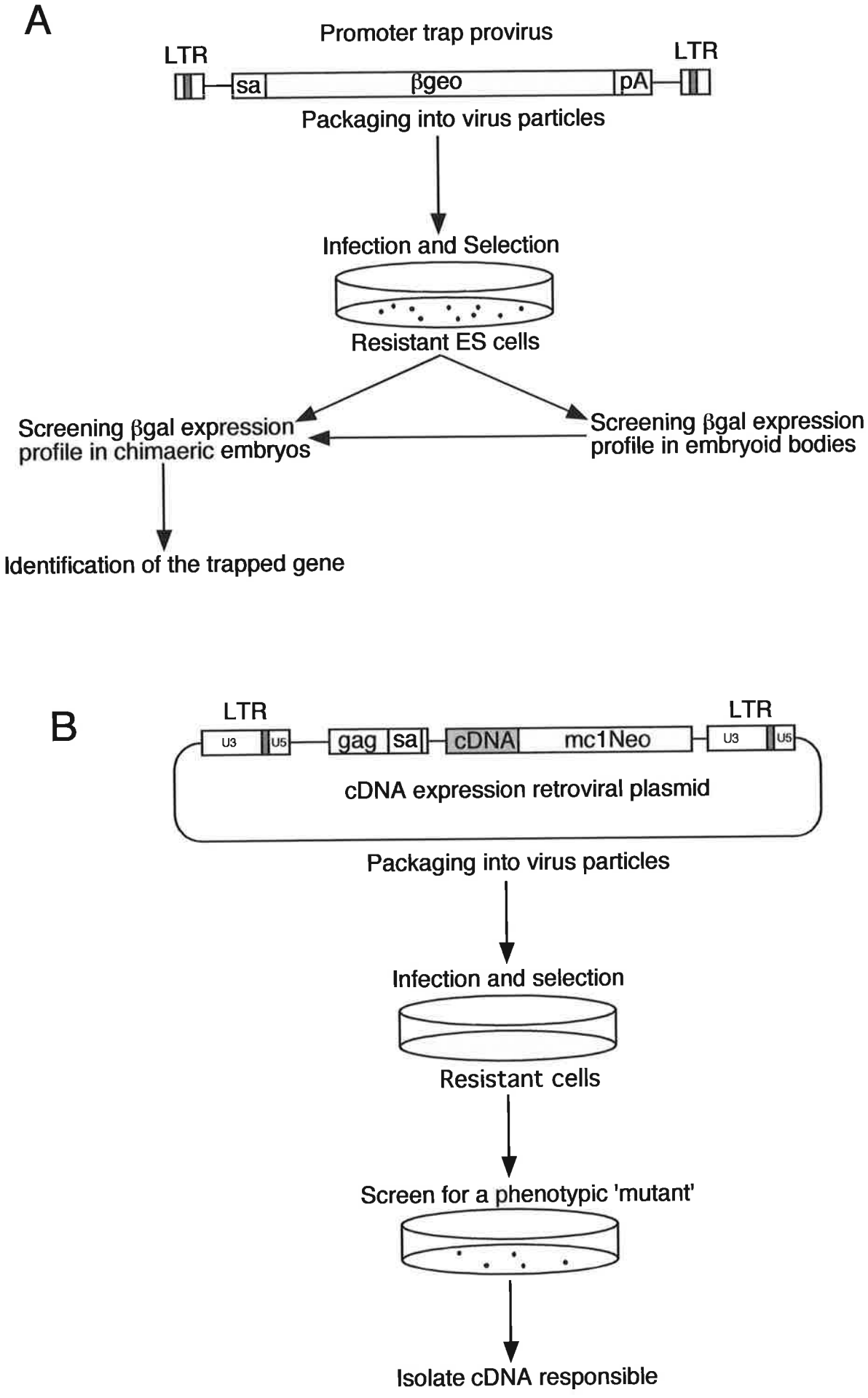
ES cells are generally used for gene trapping rather than embryos because there is a requirement for high efficiency DNA integration to allow screening of multiple targets. Gene trap methods are generally expression-based screens in that, trapped genes are assayed for interesting expression patterns in the chimaeric embryos produced by injection of gene trapped ES cells into host blastocysts (Figure 1.8a). Function is inferred initially by reporter gene expression pattern, sequence of the identified gene, or observed abnormalities in chimaeric embryos. Insertion of the gene trap vector, in the vast majority of cases, causes single hit recessive mutations that cannot be directly screened for function in diploid cells. Function can be assessed in progeny bred to homozygosity at this locus. In an attempt to isolate factors that

Figure 1.8

A depiction of gene trap and function-based screening approaches.

A. A diagrammatic representation of a generalised gene trap approach in ES cells. Recombinant retrovirus containing a splice acceptor site, βgal-neo gene fusion (βgeo) and poly A (pA) sequences but no promoter is used to infect ES cells. Retroviral vectors integrate into the genome once and ES cells are rendered G418 resistant if the βgeo ORF is transcribed, retained in mRNA species and translated. The trapped gene must therefore be expressed in ES cells. The expression pattern of the trapped gene can be inferred from β-galactosidase staining pattern in trapped ES cell embryoid bodies or in chimaeric embryos. Sequence of the trapped gene can then be recovered by inverse PCR or plasmid rescue approaches.

B. A schematic representation of a generalised function-based screen. Retroviruses capable of expressing cDNA clones that collectively comprise a cDNA library are infected into cells. Typically, G418 resistant cells are subjected to conditions that bring about phenotypic change of the whole population and colonies that do not undergo phenotypic change are isolated. Alternatively, rare phenotypic events such as transformation are sought out within the cell population. Expression of the cDNA clone should be responsible for loss or gain of the phenotypic trait of interest. The cDNA responsible is isolated by genomic PCR or plasmid rescue techniques.


A
Promoter trap provirus
LTR
sa
βgeo
pA
LTR
Packaging into virus particles
Infection and Selection
Resistant ES cells
Screening βgal expression profile in chimaeric embryos
Screening βgal expression profile in embryoid bodies
Identification of the trapped gene
B
LTR
U3
U5
gag
sa
cDNA
mc1Neo
LTR
U3
U5
cDNA expression retroviral plasmid
Packaging into virus particles
Infection and selection
Resistant cells
Screen for a phenotypic 'mutant'
Isolate cDNA responsible

control the differentiation of ES cells Gendall *et al*, (1997) devised a function-based screen designed to tag mutations that inhibit ES cell differentiation by insertional mutagenesis. One LIF independent ES cell line was isolated and found to produce higher levels of LIF protein (Gendall *et al.*, 1997).

Chemical mutagenesis and gene trap studies are significantly limited by the diploid genome of mammalian cells. Function can only be observed in animals bred to homozygosity and it is not possible to screen the genome to saturation. Further, identification of mutations with particular cellular functions may not be possible making it difficult to tackle specific biological problems.

1.7.2 Direct function screening

A more direct approach to isolation of biologically active factors is to devise a function-based screen for factors that control properties of interest in cultured cells. Function-based screens involve transfection or infection of a cDNA expression library into a cell line of interest. Cells are subjected to selection or identification so that cells with an altered phenotype of interest can be identified (Figure 1.8b). Phenotypic changes are likely to be due to expression of a cDNA product if spontaneous mutation rate for the trait is relatively low. Because the sequence of the library vector is known it is a relatively simple process to identify the cDNA responsible for the phenotypic change.

Functional screening has been used for isolation of *Socs-1* from M1 monocyte leukaemic cells which in response to IL-6-type cytokines differentiate into mature macrophages. The screen was carried out by infecting M1 cells with retroviruses carrying a cDNA expression library derived from FDC-P1 haematopoietic cells, and then inducing differentiation by addition of IL-6 (Starr *et al.*, 1997). One M1 cell line was recovered from infection of a library of 1 x 10^4 independent clones that was no longer responsive to IL-6 because it was subsequently shown to harbour a cDNA encoding *Socs-1* (1.6.5). Functional screens can be devised to screen for any phenotype that can be assayed *in vitro* including growth factor independence, cell adhesion, induction of differentiation and transformation (Rayner and Gonda, 1994).

Function-based screens rely on cDNA expression libraries and therefore require high efficiency transfection and cDNA expression. By their nature, function-based screens identify specific gain of function events, although this can result from either gain of function by cDNA expression or loss of function by overexpression of antisense transcripts or dominant negative effects (Vito *et al.*, 1996).

The efficacy of function-based screening is reliant on three factors. A cDNA expression library must be constructed from RNA most likely to contain functional cDNAs for the property under investigation. Secondly, large numbers of cDNA clones must be screened in order to identify low abundance transcripts, a process requiring techniques for efficient introduction and expression of the library in the target cells. Finally, efficient screening is dependent on a suitable assay that allows simple identification of the altered phenotype of interest.

1.8 COMPONENTS OF A SYSTEM FOR FUNCTIONAL IDENTIFICATION OF cDNAS THAT INHIBIT PLURIPOTENT CELL DIFFERENTIATION

1.8.1 ES cell differentiation as an assay for genes important for pluripotent cell maintenance

ES cells represent an *in vitro* cultured pluripotent cell type derived from the blastocyst stage embryo (1.4). Their pluripotency is demonstrated by their ability to contribute to somatic and germ line tissues in chimaeras, and to form a wide variety of cell types upon *in vitro* differentiation. Genes that function to maintain ES cells in an undifferentiated state are likely to be important for pluripotent cell maintenance in the embryo and therefore will have critical roles in the proper formation of the early embryo. ES cell differentiation should represent an ideal assay for factors that affect pluripotent cell maintenance because, in response to LIF withdrawal, they differentiate into developmentally restricted and morphologically distinct cell types. Further, markers specific for pluripotence have been identified (1.4 and 1.6.6). Therefore, identification of ES cell colonies resistant to differentiation can be relatively simply achieved using morphological criteria and confirmed by the use of marker gene expression specific to pluripotent cells.

1.8.2 A stable and efficient transfection/expression system for ES cells

The papovaviridae

The papovaviridae are small DNA tumour viruses found in various mammalian species. The monkey papovavirus Simian Virus 40 (SV40) was the first animal virus to be sequenced and characterised in detail due to its small double-stranded DNA genome and ease of propagation *in vitro*. Its 5.2 kb circular genome encodes five genes. Upon infection of permissive host cells the virus genome is released from the capsid in the nucleus, where transcription of the early genes, small-T (sT) and large-T (LT) antigens is initiated. Newly translated LT antigen initiates bidirectional replication of the virus genome at the viral origin of replication (ori), inhibits further transcription of early genes, and activates transcription of the late genes, Vp-1, Vp-2, and Vp-3, that encode the viral capsid proteins (Levine, 1982).

Replication of papovaviral genomes is strikingly similar to mammalian chromosome replication. The SV40 genome is wrapped in 24-27 nucleosomes per genome and thus is in a chromatin context (Melin *et al.*, 1985). Furthermore, semiconservative replication of the SV40 genome occurs via the use of DNA primase and Okazaki fragments on the lagging strand as does the mammalian genome (reviewed in DePamphilis, 1988). In fact replication fork elongation in papovaviral genomes is reliant purely on host replication machinery. LT antigen is, however, required for formation of the bidirectional replication fork and it is this control that allows SV40 to replicate its genome many more times per cell cycle than the host genome (reviewed in DePamphilis, 1988).

Molecular understanding of the SV40 life cycle has been the basis for its exploitation as a mammalian expression vector. There are two types of SV40 vectors, plasmid-based vectors that contain *cis* acting ori and early control regions, and viral-based vectors that contain heterologous genes in place of early or late viral transcripts. Plasmid-based vectors have an advantage in that large genes can be expressed in a range of different cell types. Most commonly, plasmids containing the SV40 ori sequences are transfected into COS monkey cell lines which contain integrated copies of SV40 virus and supply LT antigen to allow vector replication (Gluzman, 1981). Replicating plasmid vectors can be used to achieve high level transient expression of heterologous genes, or when expressing mammalian selectable markers, to stably maintain episomes in permissive host cells. Plasmid based systems are disadvantaged

however by an absolute requirement for host cell expression of SV40 LT antigen, a potent oncoprotein capable of transforming cells in culture. Non-transformed cells are therefore refractory to SV40 plasmid-based vectors due to a lack of LT expression.

Polyomavirus

Polyomavirus (PyV) is a papovavirus related to SV40 that infects most differentiated mouse cells. PyV differs from SV40 in the structure and function of the early viral genes, the sT, LT, and in PyV, but not SV40, middle-T (MT) antigen. Upon infection PyV initiates transcription of the early genes as one pre-mRNA which is differentially spliced to form three transcripts encoding the respective T antigens (Treisman *et al.*, 1981).

PyV LT antigen, like that of SV40, is necessary to recruit host factors such as DNA polymerase α to initiate replication of the chromosome (Francke and Eckhart, 1973; Fried, 1970), however PyV LT does not transform cells *in vitro*. Instead viral tumourigenicity is conferred by synergistic actions of PyV sT, MT and LT antigens (Rassoulzadegan *et al.*, 1982). Transfection of PyV MT into rat FR3T3 cells results in foci formation while transfection of PyV LT or sT has no effect (Rassoulzadegan *et al.*, 1982). PyV MT antigen is sufficient to transform established immortal cell lines but not primary cells (Rassoulzadegan *et al.*, 1982). PyV LT antigen expression decreases serum dependence in rat FR3T3 cells and renders primary rat embryo fibroblast cells immortal. Cells remain contact inhibited but can now form colonies at low density and these colonies can generate cell lines (Rassoulzadegan *et al.*, 1983). However coexpression of MT and LT antigens does not result in transformation of primary cells which appears to also require PyV sT antigen (Rassoulzadegan *et al.*, 1983).

While PyV will efficiently infect and replicate in most differentiated cells, neuroblastoma, erythroleukaemic, EC (Swartzendruber and Lehman, 1975), and ES cells (Melin *et al.*, 1991) are refractory to PyV infection due to a failure to initiate viral transcription and hence genome replication (Dandolo *et al.*, 1983; DePamphilis, 1988). Selection for PyV mutants able to grow in the refractory EC cell line F9 identified a series of mutations mapping within the region required for initiation of DNA replication. The PyF101 virus has the ability to replicate in ES cells and has two overlapping mutations (Fujimura *et al.*, 1981). The first (an A to G substitution) maps 68 bp from the centre of the core origin palindrome while the second is

a tandem duplication of 54 bp encompassing the first mutation and a region overlapping the enhancer region. The duplicated region includes a LT antigen binding site and possibly a binding site for another unknown factor (Hendrickson *et al.*, 1987a). These PyF mutations seem to affect early gene transcription or T antigen production and the replication of PyV DNA (Levine, 1982). PyV harbouring the PyF101 replication origin will infect and replicate in mouse EC and ES cells as well as normally permissive fibroblast cells.

A replicating vector for ES cells

The ability of PyV LT to support viral replication has been exploited by Gassmann and co-workers in the construction of a PyV-based vector capable of stable replication as an episome in ES cells (Gassmann *et al.*, 1995). This episome contains the early gene region encoding only the LT antigen under the control of the PyF101 mutant ori/enhancer region, and a selectable marker for use in mammalian cells (Figure 1.9a). Stable episome vector replication is thought to reflect replication of the PyV genome where recruitment of host factors to the ori by PyV LT antigen results in bidirectional replication (Hendrickson *et al.*, 1987b). Supertransfection of episome harbouring cell lines with an episome carrying the PyV ori but not the LT antigen (Figure 1.9b) elevates the transfection efficiency 100-fold, presumably due to prior LT antigen expression and consequent replication of transfected PyV-based plasmids. The advantages of increased transfection efficiency, stable transfection of ES cells, and simple isolation of the episome from ES cells make this episome system well suited for use as a library vector in ES cells.

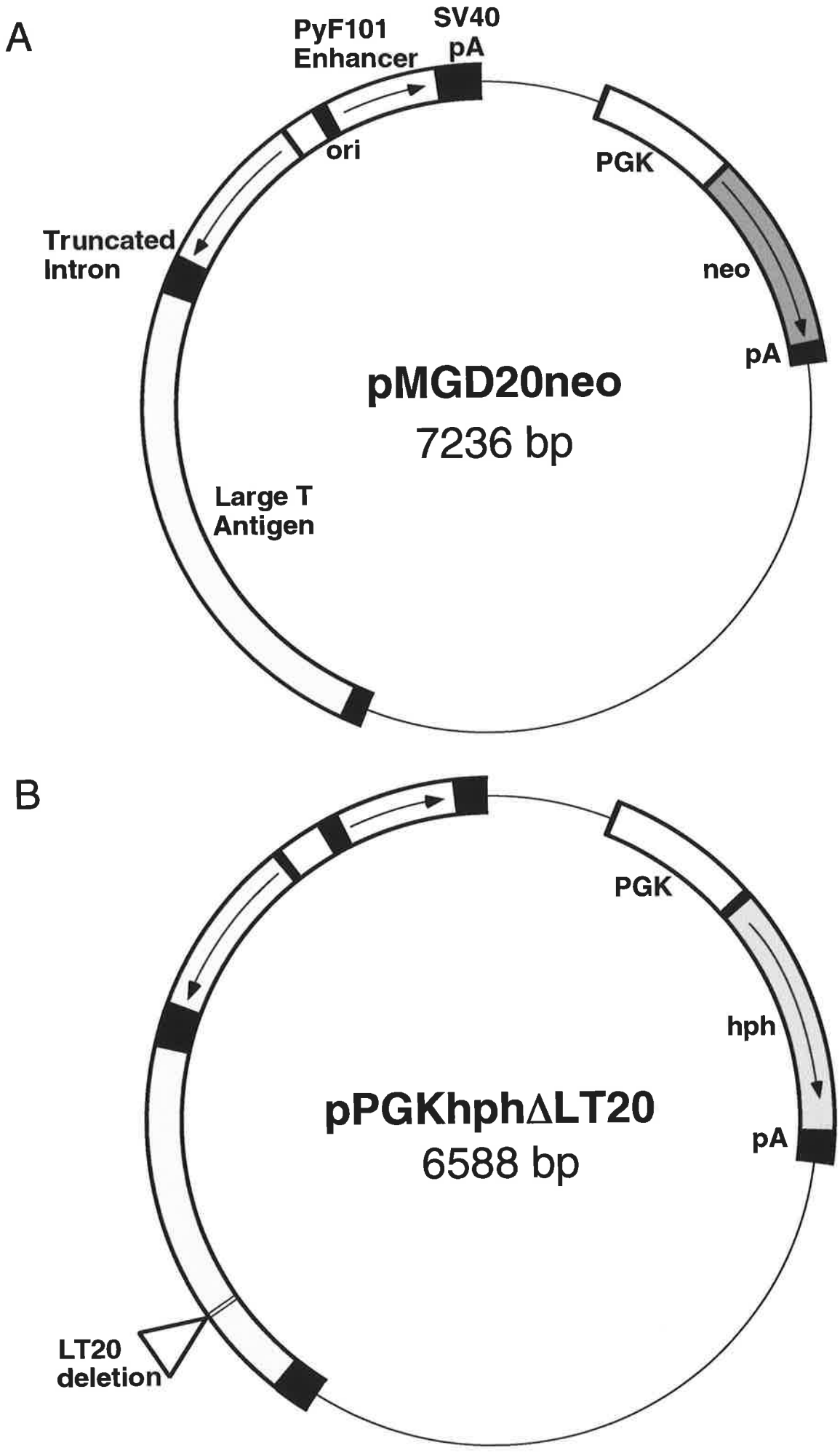
Figure 1.9

Plasmid maps of PyV-based episome vectors.

A. A representation of the pMGD20neo plasmid. pMGD20neo contains a neomycin selection cassette driven by the *PGK-1* promoter and the PyV-derived PyF101 mutant origin of replication (ori)/enhancer region and a form of the PyV early region with a truncated intron that only allows expression of the LT antigen.

B. A representation of the pPGKhphΔLT20 plasmid. pPGKhphΔLT20 contains a hygromycin selection cassette driven by the *PGK-1* promoter and the PyV-derived PyF101 mutant origin of replication (ori)/enhancer region and a form of the PyV early region carrying a truncated intron and a deletion abolishing expression of active LT protein.

Taken from Gassmann *et al*, (1995).


A
PyF101
Enhancer
SV40
pA
ori
PGK
Truncated
Intron
neo
pA
pMGD20neo
7236 bp
Large T
Antigen
B
PGK
hph
pA
pPGKhphΔLT20
6588 bp
LT20
deletion

1.9 AIMS AND APPROACH

1.9.1 Aims

Early mammalian embryogenesis is based on the maintenance, growth and programmed differentiation of pluripotent stem cells. Therefore to gain an understanding of early developmental processes that lead to formation of a three-layered embryo, it is critical to comprehend the molecular controls of pluripotent cell maintenance and differentiation. Most known factors implicated in inhibition of pluripotent cell differentiation have also been implicated in signalling via LIF/gp130 in ES cells. However there are deficiencies in our knowledge of the molecular controls of pluripotence as indicated by the fact that there has been no direct connection between these pathways and the Oct4 transcription factor which has been shown to be functionally important for pluripotence. It is therefore probable that additional components or pathways remain to be elucidated.

The work presented in this thesis was designed to gain insight into the molecular controls of pluripotent cell self-renewal and differentiation. In particular, the aim of this work was to identify using a functional assay, factors that inhibit ES cell differentiation and loss of potency in response to LIF withdrawal.

1.9.2 Approach

A function-based screening procedure was designed for the identification of cDNAs that can inhibit ES cell differentiation (Figure 1.10).

Although stable transfection of isogenic DNA into ES cells affords relatively high levels of homologous recombination (Udy *et al.*, 1997) absolute numbers of stable integrants are low compared to other cell lines. The feasibility of function-based screens in ES cells is limited by low efficiency stable integration because to identify low abundance transcripts, large numbers of independent library clones must be screened. To increase the number of transfected ES cells, a PyV-based episome vector capable of 100-fold higher stable ES cell transfection, was used as the basis of the cDNA expression library.

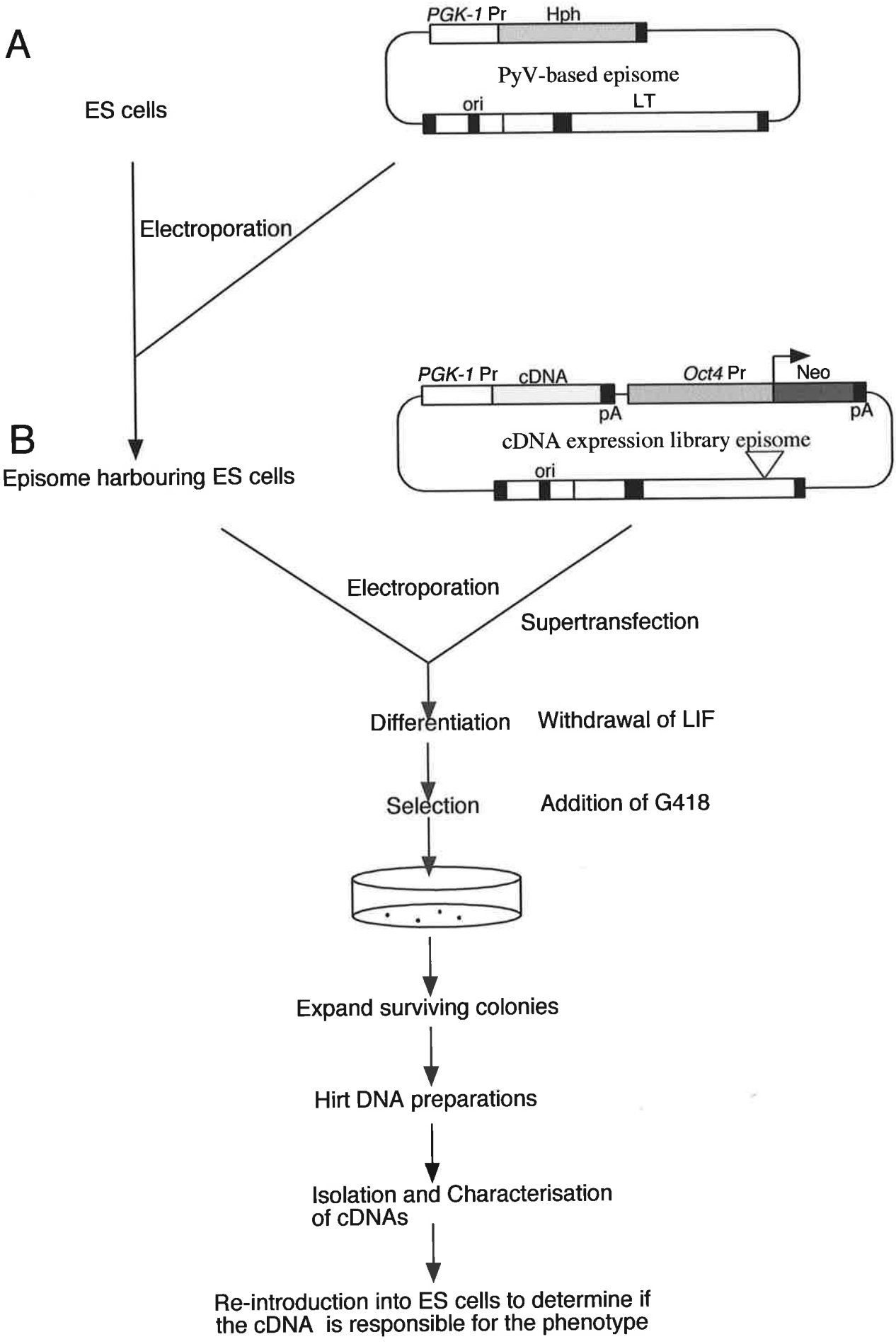
Upon ES cell differentiation, cells lose expression of the *Oct4* marker gene and upregulate LIF in a feedback loop that results in the maintenance of residual undifferentiated 'stem cell nests' (Rathjen *et al.*, 1990a). Thus LIF withdrawal may not result in complete

Figure 1.10

The screening strategy taken to isolate factors which inhibit ES cell differentiation.

A. Creation of an episome harbouring ES cell line. ES cells are electroporated with a PyV-based plasmid capable of replicating in ES cells. A line that harbours episome is identified by Southern analysis and used as a host cell line for the function-based screen. A line that already expresses LT antigen is required to achieve high efficiency stable episome transfection of the cDNA expression library.

B. An outline of the function-based screen. Episome harbouring ES cells are electroporated with a second episome that constitutes a cDNA expression library. The episomal library vector is capable of expressing cDNA clones in ES cells and contains a neomycin selection cassette capable of specifically expressing the neomycin resistance gene in undifferentiated ES cells but not differentiated derivatives. Supertransfection of episome-based plasmids is much more efficient than standard transfection efficiencies, allowing the screening of large numbers of independent cDNA library clones. Supertransfected cells are differentiated by withdrawal of LIF and selected in G418. Cells in the population differentiate, hence lose expression of *Oct4* and therefore neomycin phosphotransferase. Differentiated cells are therefore selected against. In instances where ES cells do not differentiate, retention of *Oct4* expression and neomycin resistance means that these cells survive selection in G418. Undifferentiated ES cells should have remained undifferentiated in the absence of LIF because they harbour a cDNA, the product of which is capable of inhibiting the differentiation of ES cells. Surviving colonies are picked, expanded and episomes are isolated from Hirt low molecular weight DNA preparations. cDNAs carried by episomes can be identified by colony screening, or PCR and sequencing. To confirm that isolated clones have a role in ES cell maintenance, they are re-introduced into ES cells and assayed for LIF dependency.


A
PGK-1 Pr
Hph
PyV-based episome
ori
LT
ES cells
Electroporation
B
PGK-1 Pr
cDNA
Oct4 Pr
Neo
pA
pA
cDNA expression library episome
ori
Episome harbouring ES cells
Electroporation
Supertransfection
Differentiation
Withdrawal of LIF
Selection
Addition of G418
Expand surviving colonies
Hirt DNA preparations
Isolation and Characterisation
of cDNAs
Re-introduction into ES cells to determine if
the cDNA is responsible for the phenotype

differentiation of a population of ES cells and potentially represents a background of 'false positives' that must be overcome in order to identify functional cDNAs that inhibit ES cell differentiation. One possible approach to this involves specific removal of differentiated cells from the population as they form by expressing a selectable marker gene in undifferentiated ES cells but not differentiated derivatives. Selection cassettes comprising the neomycin resistance gene driven by the *Oct4* promoter can actively select against differentiated cells leaving only undifferentiated ES cells (McWhir *et al.*, 1996; Mountford *et al.*, 1998). In the absence of differentiated cells, paracrine LIF supply to remaining ES cells will be abrogated and should allow further or complete differentiation of residual stem cells in the absence of LIF.

The screen entailed high efficiency supertransfection of ES cells that harbour a PyV-based episome will be supertransfected at high frequency with a second cDNA expression library episome that directs constitutive cDNA expression and also contains an *Oct4* promoter-neomycin selection cassette for selection against differentiated cells in the absence of LIF. The resulting transfected ES cells are differentiated by LIF withdrawal and selected using G418. *Oct4* expression and therefore neomycin resistance gene expression is lost upon ES cell differentiation and in the presence of G418 differentiated cells die. Colonies that survive this screen failed to differentiate upon LIF withdrawal and comprise only undifferentiated ES cells that retain *Oct4* and neomycin gene expression. Surviving colonies do not differentiate upon LIF withdrawal because they harbour an episome containing a cDNA whose product is capable of inhibiting ES cell differentiation (Figure 1.10). Identification of genes by this scheme will increase our knowledge of the regulatory mechanisms that inhibit ES cell differentiation

Cells may also survive under such conditions if the cDNA product activates transcription from the *Oct4* promoter. This in itself may be interesting because *Oct4* is essential for proper ICM formation and *Oct4* expression has only been observed in pluripotent cells.

Factors that maintain ES cells could influence gp130 signal transduction or could be downstream targets of Stat3 capable of regulating cell differentiation and potential. Forced expression of such factors may render ES cells unable to alter their potency. In this screen, pluripotent cell types will survive and so cells harbouring cDNAs that control differentiation potential and cell identity may be identified.

CHAPTER 2:

MATERIALS AND METHODS

2.1 ABBREVIATIONS

Ac	acetate
APS	ammonium persulphate
rATP	adenosine triphosphate
BCIG	5-bromo-4-chloro-3-indolyl-β-D-galactoside
bp	base pair
BSA	bovine serum albumin
Ci	curie
CIP	calf intestinal phosphatase
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTE	dithioerythritol
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
FCS	foetal calf serum
FLB	formamide load buffer
G418	G418 sulphate
GLB	gel loading buffer
HEPES	N-2-hydroxyethyl piperazine-N-ethane sulphonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pair
kDa	kilodalton
LB	luria broth
LIF	leukaemia inhibitory factor
LRB	long range buffer
mA	milliamperes
MBA	3-methoxybenzamide
mM	millimolar

MOPS	3-[N-morpholino]propane sulphonic acid
MQ H_2O	reverse osmosis filtered water passed through a Milli-Q™ ion-exchange matrix
NP-40	nonidet-P 40
OD_n	optical density at a wavelength of n nm
ONPG	O-nitrophenyl β-D-galacto pyranoside
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFU	plaque forming units
PSB	phage storage buffer
RA	*all-trans*-retinoic acid
RNA	ribonucleic acid
RNase	ribonuclease
RNAsin	ribonuclease inhibitor
rNTP	ribonucleotide triphosphate
rpm	revolutions per minute
SD Buffer	standard digest buffer
SDS	sodium dodecyl phosphate
SSC	salt and sodium citrate
ssDNA	sheared herring sperm DNA
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TCA	Trichloro acetic acid
TEMED	N, N, N', N'-teramethyl-ethenediamine
TFB	transformation buffer
tRNA	transfer RNA
Tween-20	polyoxyethylenesorbitan monolaurate
U	units
UV	ultra violet
V	volts
v	volume
w	weight
βME	β-mercaptoethanol

2.2 MATERIALS

2.2.1 Chemicals and reagents

All chemicals and reagents were of analytical grade and were supplied by the following.

BDH Chemicals	APS, DMF, NP-40, phenol, and PEG 6000
Sigma	agarose (Type 1), ampicillin, BSA, EtBr, EDTA, puromycin, rNTPs, MOPS, PMSF, TEMED, Tris base, Hoechst-33258, retinoic acid, gelatin, L-glutamine, bromophenol blue, and xylene cyanol
Progen	IPTG, BCIG
Roche	glycogen, herring sperm DNA, hygromycin B. DTE, DTT, dNTPs and tRNA (from brewers yeast)
Pharmacia	Sepharose CL-6B, Sephadex G50 and oligo-dT cellulose Type 7
Gibco/BRL	G418, FCS, DMEM and lipofectamine
Oxoid	Bacto-agar, Bacto-tryptone, yeast extract and Bacto Nutrient Broth
ICN	Tween-20
National Diagnostics	Sequagel 6

2.2.2 Radiochemicals

[α-^{32}P]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{33}P]dATP (1500 Ci/mmol), and [α-^{32}P]rUTP (3000 Ci/mmol) were supplied by Geneworks.

2.2.3 Kits

7-deaza-dGTP Sequencing Kit:	USB
Gigaprime labelling kit:	Geneworks
Megaprime labelling kit:	Amersham
T7 Sequenase kit:	Pharmacia
Bresaclean kit:	Geneworks
Alkaline phosphatase kit:	Sigma
Bresaspin miniprep kit:	Geneworks
Universal Riboclone cDNA synthesis kit:	Promega

2.2.4 Enzymes

Restriction endonucleases were supplied by Pharmacia and New England Biolabs. Other enzymes were obtained from the following sources:

Roche	Calf intestinal phosphatase, DNAse I, and Proteinase K RNase H and RNase A
Geneworks	*E. coli* DNA polymerase I (Klenow fragment) Taq Polymerase, and Human Placental RNase inhibitor
New England Biolabs	*E. coli* DNA polymerase I, T4 DNA polymerase

Pharmacia	T7 DNA polymerase and T4 DNA Ligase
Gibco/BRL	Superscript II
Stratagene	Pfu Turbo polymerase
Molecular Genetic Resources	AMV reverse transcriptase

2.2.5 Buffers and solutions

1 x SSC:	150 mM NaCl and 15 mM sodium citrate pH 7.4
1 x TAE:	40 mM Tris-HCl pH 8.2, 20 mM NaAc and 10 mM EDTA pH 8.2
1 x TBE:	90 mM Tris-HCl pH 8.3, 90 mM boric acid and 2.5 mM EDTA pH 8.3
1 x TE:	10 mM Tris-HCl pH 7.5 and 1 mM EDTA
10 x CIP:	500 mM Tris-HCl pH 8.5 and 1 mM EDTA
10 x GLB:	50% glycerol, 0.1% SDS, 500 μg/μl bromophenol blue and 500 μg/μl xylene cyanol.
10 x Klenow:	500 mM Tris-HCl pH 7.6 and 100 mM $MgCl_2$
10 x Ligase:	500 mM Tris-HCl pH 7.4, 100 mM $MgCl_2$, 100 mM DTT and 10 mM rATP
10 X LRB:	250 mM Tris-HCl pH 9.1 and 160 mM $(NH_4)_2SO_4$
10 x SD:	330 mM Tris-HAc pH 7.8, 625 mM KAc 100 mM MgAc (filter sterilised), 40 mM Spermidine, and 5 mM DTE.
5 x Ligase:	250 mM Tris-HCl pH 7.5, 25% PEG 6000, 50 mM $MgCl_2$, 5 mM rATP, and 5 mM DTT
50 x Denhardt's:	0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrolidone and 0.1% (w/v) BSA.
Annealing buffer:	20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 25 mM NaCl
Electroporation buffer:	20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 6 mM glucose, 0.1 mM β-mercaptoethanol and 0.001% phenol red pH 7.0
FLB:	95% (w/v) deionised formamide, 20 mM EDTA, 0.02% (w/v) bromophenol blue and 0.02% (w/v) xylene cyanol
GTE:	50 mM glucose, 25 mM Tris-HCl pH 7.6 and 10 mM EDTA.
Lysis/Solution II:	0.2 M NaOH and 1% SDS
Megadeath:	0.1 M NaOH, 0.5% SDS, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA
Na-TES:	500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 % SDS
PSB:	10 mM Tris HCl pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$

	and 0.05% (w/v) gelatin
Solution I:	50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA
Solution III:	3 M KAc and 2 M HAc
Tail Buffer:	100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS
	200 mM NaCl and 10 μg/ml RNase A
TES:	10 mM Tris-HCl pH 7.5, 1 mM EDTA and 0.1 % SDS
TFB1:	30 mM KAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$
	and 15% glycerol, pH 5.8
TFB2:	10 mM MOPS, 75 mM $CaCl_2$ and 10 mM $RbCl_2$
	and 15% glycerol, pH 6.5
TNM:	30 mM Tris-HCl pH 7.6, 150 mM NaCl, 15 mM $MgCl_2$
	and 0.4% Nonidet P 40
TUNES:	10 mM Tris-HCl pH 8.0, 7 M urea, 350 mM NaCl,
	1 mM EDTA and 2% SDS

Solutions were sterilised by autoclaving unless otherwise indicated.

2.2.6 Plasmid vectors

pBluescript II KS (Stratagene) was used for all subcloning. pBluescript II KS plasmid DNA was linearised with appropriate restriction enzyme(s) and treated with Calf intestinal Phosphatase (2.3.5) to inhibit religation of the vector alone. Vector DNA was purified as described in section 2.3.3.

Recombinant DNA plasmids were supplied by the following:

pSG513hStat3β (Caldehoven *et al.*, 1996)	Dr Rolf De Groot
pSG513hStat3α (Caldehoven *et al.*, 1996)	Dr Rolf De Groot
pBabev-srcPuro (at UC Berkeley)	Dr Steve Martin
pMGD20neo (Gassmann *et al.*, 1995)	Dr Greg Donoho
pPGKhphΔLT20 (Gassmann *et al.*, 1995)	Dr Greg Donoho
pIRESβgeo	Dr Peter Rathjen
pPGK-o-term	Dr Peter Rathjen
pEFIRESneo	Dr Murray Whitelaw
pEFIRESpuro6	Dr Dan Peet

2.2.7 Oligonucleotides

Synthetic DNA primers were synthesised by Geneworks using a 380B Applied Biosystems DNA Synthesiser.

General sequencing primers:

T7: TAATACGACTCACTATAGGGAGA

T3: ATTAACCCTCACTAAAGGGA

RSP: AAACAGCTATGACCATG

USP: GTAAAACGACGGCCAGT

Primers for library cDNA amplification

UPGK2: GCGCTGTTCTCCTCTTCCTCATCTCC

LOCT42: CCTTGAACTTCTGATCCTCTTGCCTTCC

U162: TATAAGCTTCGCCACCTTCTACTCCTC

L539: TAGGATCCATAACCAGCCACCTTGAT

Primers for RT-PCR of *mTbp-1* cDNA

Utbp96: CACGGGTGGAGAGAAGACG

Ltbp1536: CTCAGACCAGCGGACAGACT

Primers for RT-PCR of *Stat3*α and β isoforms

5′St3: TAGAATTCGATGCGACCAACATCCTG

3′St3: TAGAATTCGTCTCTGCAGCTTCTGG

Primers for genomic PCR of the mouse *Oct4* promoter

O4104U: TATAAGCTTCATGACAGAGTGGAGGAAACGGAAGATTCA

O41894L: ATGAATTCCTGGTGGAAAGACGGCTCAC

C84 sequencing primers

c84U564: CCAGGACCTCTTGCAGC

L84610: TTCATGACAGCCACACC

L84940: GTGACCACACGAGAGCA

OSM primers

mOSM1: GCCAGAGTACCAGGACCCAGTAT

mOSM4: GCCTCTGAGAGCGACATCCTGTAT

Oligonucleotides used during cDNA library construction

*Not*I-$(dT)_{18}$ Primer:

AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTT

*Eco*RI Adaptor:

5′-d[AATTCCGTTGCTGTCG]-3′

(GGCAACGACAGC)p-5′

2.2.8 Bacterial strains

DH5α strain *E. coli* were used for chemical heat shock and electroporation transformations constituting routine subcloning. Genomic library screening was performed with LE392 strain *E. coli* and DH10B strain *E. coli* were used for electroporation of cDNA library DNA.

E. coli strain genotypes were as follows:

DH5α: *supE*44 Δ*lac* U169 (phi80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1*gyrA*96 *thi*-1*relA*1

LE392: F^- e14$^-$(McrA$^-$) *hsdR514* ($r_K^- m_K^+$) *supe44 supf58 lacY1 or* Δ*(lacIZY)6 galK2 galT22 metB1 trpR55*

DH10B: F′-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) phi80d*lacZ*ΔM15 Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*) 7697 *gal*U *gal*K 1$^-$ *rps*L *nup*G

Strain stocks were stored at -80°C in 50% glycerol.

2.2.9 Bacterial growth media

Luria broth:	1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH.
LMM broth:	1% (w/v) Bacto-tryptone, 0.5% (w/v) NaCl, 0.4 % maltose, 0.2% $MgSO_4$.
Psi broth:	2% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 0.5% $MgSO_4$, adjusted to pH 7.6 with KOH.
YENB medium:	0.75% Bacto yeast extract, 0.8% Bacto Nutrient Broth.
SOC Medium:	2% Bacto-tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.
Solid Media:	Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar.
LMM agarose:	Prepared by dissolving 0.7 g agarose per 100 ml LMM broth.

Growth media were prepared in MQ water and sterilised by autoclaving.

Ampicillin (100 μg/ml) was added after the medium cooled to 55°C to maintain selective pressure for recombinant plasmids in transformed bacteria.

2.2.10 DNA markers

*Hpa*II digested pUC19 markers were purchased from Geneworks.
Band sizes (bp): 501, 489, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26.

*Eco*RI/*Hin*dIII λ DNA markers were prepared by digestion of λ DNA (NEB).
Band sizes (kb): 21.2, 5.14, 4.97, 4.26, 3.53, 2.02, 1.90, 1.58, 1.37, 0.97, 0.83, 0.56, 0.12.

2.5 kb ladder markers were purchased from Geneworks.
Band sizes from 2.5 kb to 35 kb in 2.5 kb increments

*Eco*RI digested SPP-1 bacteriophage DNA markers were purchased from Geneworks.
Band sizes (kb): 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.86, 1.51, 1.39, 1.16,
0.98, 0.72, 0.48, 0.36.

DNA fragment sizes and approximate concentrations were determined by loading agarose mini-gels with 500 ng of marker DNA.

2.2.11 Miscellaneous materials

3mm chromatography paper:	Whatman Ltd.
Biomax X-ray film:	Kodak
X-ray film:	Konica
Hybond-N^+ membrane:	Amersham
Nytran nylon:	Schleicher and Schuell
Cellulosenitrate:	Schleicher and Schuell
Tissue culture grade plates and flasks:	Falcon
Freezing vials:	Nunc Inc
100 ASA Day roll slide film:	Kodak

2.3 MOLECULAR METHODS

2.3.1 Restriction endonuclease digestion of DNA

Plasmid DNA was digested with 4 units of enzyme per 1 μg of DNA and incubated at the appropriate temperature for 1-6 hours. Genomic DNA was digested with 5 units of enzyme per microgram of DNA for 4 hours. All restriction digestions were carried out in SD buffer (33 mM Tris-HAc pH 7.8, 62.5 mM KAc, 10 mM MgAc, 4 mM Spermidine, 0.5 mM DTE). Plasmid and genomic DNA was assayed for complete digestion by TAE agarose gel electrophoresis.

2.3.2 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using horizontal mini-gels prepared by pouring 10 ml of gel solution (1% to 3% w/v agarose in 1 x TAE or 1 x TBE) onto a 7.5 cm x 5.0 cm glass microscope slide. Agarose mini-gels were submerged in 1 x TAE or 1 x TBE and samples containing 1 x GLB (5% glycerol, 0.01% SDS, 50 μg/μl bromophenol blue, 50 μg/μl xylene cyanol were typically electrophoresed at 100 mA for 30-45 minutes. DNA was visualised by EtBr staining and photographed upon exposure to medium wavelength UV light using a tracktel thermal imager.

2.3.3 Purification of linear DNA fragments

Linear DNA fragments were run on appropriate percentage TAE agarose gels and visualised under long wavelength UV light. Bands were removed from preparative gels using sterile scalpel blades and purified from agarose using the Bresaclean kit (Geneworks) according to the manufacturer's instructions.

2.3.4 Blunting of DNA fragments with overhanging 5′ and 3′ ends

Restriction digestion reactions were precipitated in the presence of glycogen, washed with 70% ethanol, air dried, and resuspended in 23 μl of MQ H_2O. DNA fragments with overhanging 5′ ends were blunted by incubation at 37°C for 30 minutes with 3 μl of 2 mM dNTPs (2 mM of each deoxynucleotide triphosphate), 3 μl of 10 x Klenow buffer (500 mM Tris-HCl pH 7.6, 100 mM $MgCl_2$) and 1 μl of DNA polymerase I Klenow fragment (6 units/μl). DNA fragments with overhanging 3′ termini were blunted by the addition of 2 μl of 10 x Klenow buffer and 6 units of DNA polymerase I Klenow fragment. The reaction was incubated at 37°C for 5 minutes. 6 μl of 2 mM dNTPs was added and the reaction was incubated for a further 15 minutes at 37°C. Blunt ended DNA fragments were purified by electrophoresis on TAE agarose gels (2.3.3).

2.3.5 Removal of 5′ phosphate groups from vector DNA fragments

To inhibit intramolecular ligation of the vector backbone 5′ phosphate groups were removed by treatment with Calf Intestinal Phosphatase prior to gel purification. Restriction digested or blunted vector DNA was precipitated in the presence of glycogen, washed in 70% ethanol, dried

and resuspended in 40 µl of MQ H_2O. The reaction was initiated by the addition of 5 µl of 10 x CIP buffer (500 mM Tris-HCl pH 8.5, 1 mM EDTA) and 1 µl (1 unit/µl) of Calf Intestinal Phosphatase. The reaction was incubated at 30 minutes at 37°C prior to purification on 1% TAE agarose gels (2.3.3).

2.3.6 Ligation reactions

Complementary end ligation reactions were carried out with 25 ng purified vector, 50 - 100 ng DNA insert in the presence of ligation buffer (50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM DTT, 1 mM rATP) and 2 units T4 DNA ligase. Reactions were incubated at room temperature for 1 hour. Sections of plasmids were removed by ligation of appropriately digested plasmid DNA in a final volume of 100 µl in ligase buffer lacking PEG 6000. Blunt end ligations were performed in ligase buffer containing PEG 6000 (50 mM Tris-HCl pH 7.5, 5% PEG 6000, 10 mM $MgCl_2$, 1 mM rATP, 1 mM DTT).

2.3.7 Preparation of $RbCl_2$ competent cells

5 mls of Psi Broth was inoculated with a single colony of DH5α strain bacteria and grown overnight at 37°C with shaking. 500 µl of overnight culture was used to inoculate 15 mls of Psi broth. The culture was grown at 37°C to an OD_{600} of 0.6. 5 mls of bacteria were subcultured in 95 mls of Psi broth and grown to an OD_{600} of 0.6 at 37°C with shaking. Cells were poured into 40 ml Oakridge tubes and chilled on ice for 5 minutes prior to centrifugation at 6000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the cell pellet was resuspended in 40 mls of TFB1 (30 mM KAc, 100 mM $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol, pH 5.8), left on ice for 5 minutes and centrifuged at 6000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet was resuspended in 4 mls of TFB2 (10 mM MOPS, 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15% glycerol, pH 6.5). After 15 minutes on ice 100 µl aliquots were snap frozen in a dry ice/ethanol bath and stored at -80°C.

2.3.8 Bacterial heat shock transformation

$RbCl_2$ competent DH5α cells were thawed on ice for 5 - 10 minutes. 50 µl aliquots were mixed with DNA (approximately 10 ng of plasmid DNA; half of a ligation reaction) and left on ice for 30 minutes. The cell/DNA mixture was heat shocked for 2 minutes at 42°C and mixed with 1 ml of LB. Cells were allowed to recover by incubation at 37°C for 45 minutes and were pelleted by brief centrifugation in a microfuge at maximum speed. The majority of the LB was removed, leaving around 100 µl, and cells were resuspended and plated on LB plates containing 100 µg/ml ampicillin. 20 µl of 50 mg/ml BCIG (dissolved in dimethyl formamide) and 50 µl of 50 mg/ml IPTG were spread onto plates for colour selection of bacteria containing recombinant plasmids, prior to plating bacteria.

2.3.9 Preparation of electrocompetent cells

An isolated DH5α strain colony was picked into 10 mls of YENB medium in a 50 ml flask and incubated overnight at 37°C with shaking. Two baffled 2 litre flasks each with 500 ml of YENB, were inoculated with 5 mls of the overnight culture. The cells were allowed to grow to an OD_{600} of 0.8 before centrifugation at 2,600 x g for 10 minutes at 4°C in a pre-cooled rotor. The supernatant was carefully removed and the cells were resuspended in 100 ml of ice-cold 10% glycerol. Following centrifugation at 2,600 x g the supernatant was removed and the pellet resuspended in 100 ml cold 10% glycerol and centrifuged again. The supernatant was removed and the cells were resuspended 2 mls of 10% glycerol. 120 µl aliquots were snap frozen in a dry ice/ethanol bath and stored at -80°C.

2.3.10 Transformation of bacteria by electroporation

Completed ligation reactions were prepared for electroporation by extraction in a 50 µl volume with an equal volume of phenol/chloroform (1:1), then chloroform alone and then precipitated in the presence of glycogen. Precipitated DNA was washed three times in 70% ethanol, air dried and resuspended in 20 µl of MQ H_2O.

Electro-competent cells were allowed to thaw on ice for 10 minutes and 40 µl aliquots were mixed with 1-2 µl of DNA (10-100 pg of supercoiled DNA; $1/10^{th}$ of ligation). The cell/DNA mix was transferred to a chilled 0.2 cm gap electroporation cuvette (BIO-RAD) and electroporated at 25 µF, 2,500 V, and 200 ohms in a BIORAD Gene Pulser. Immediately after electric shock cells were suspended in 1 ml of SOC medium and incubated at 37°C for 1 hour. The bacteria were plated onto LB plates containing 100 µg/ml ampicillin and grown overnight at 37°C.

2.3.11 Bacterial colony screening (Grunstein analysis)

Nitrocellulose filters were spotted asymmetrically with a permanent marker and placed onto bacterial transformation plates until moist. Spots were duplicated on the plate, the lift removed and transferred, bacteria side up, to 10% SDS moistened 3mm paper. A second filter was placed on the first and lightly pressed to transfer bacteria. The second filter was carefully removed from the first and placed bacteria side up on 10% SDS moistened 3mm paper. Lifts were removed after 3 minutes and placed on denaturing solution (0.5 M NaOH, 1.5 M NaCl) moistened 3mm paper for 5 minutes. Lifts were then placed on neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.4) moistened 3mm paper for 5 minutes. Finally the lifts were placed on 2 x SSC moistened 3mm paper for 5 minutes before crosslinking in a Stratalinker (Stratagene). Bacterial debris were scraped from the filters following incubation at 50°C for 30 minutes in pre-washing solution (5 x SSC, 0.5% SDS, 1mM EDTA pH 8.0).

Lifts were pre-hybridised with 10-15 ml of formamide pre-hybridisation solution (40% deionised formamide, 50 mM Tris-HCl pH 7.4, 1% SDS, 16.5% PEG 6000, 5 x Denhardt's reagent, 58.5 mg/ml NaCl, 100 µg/ml herring sperm DNA) in a petri dish at 42°C for at least 1 hour.

^{32}P-labelled DNA probes (2.3.16) were denatured for 5 minutes at 100°C and snap cooled on ice. Lifts were removed from the petri dish, ^{32}P-labelled DNA probe added, and the lifts placed back in the dish. Nylon mesh was placed on top of the lifts to prevent drying of the top lift. The petri dish was wrapped in saran wrap and placed in a 42°C oven overnight.

The following day lifts were removed from the dish and placed in 500 ml of preheated 2 x SSC/0.1% SDS and incubated at 42°C for 15 minutes. This procedure was repeated and individual lifts were checked for counts with a hand held Geiger counter. Lifts were sufficiently washed if lifts had counts of between 10 and 50 cpm. More stringent washes were performed in 2 x SSC/0.1% SDS at 65°C for 15 minutes and finally in 0.2 x SSC/0.1% SDS at 65°C for 15 minutes.

Excess moisture was removed by briefly blotting lifts onto 3mm paper. Lifts were positioned between two sheets of clear plastic in an autoradiograph cassette and exposed to fast X-ray film overnight.

2.3.12 Mini-preparation of plasmid DNA

1.5 ml of LB containing 100 μg/ml of ampicillin was inoculated with a single bacterial colony and grown overnight at 37°C in a rotating drum. Each culture was poured into a 1.5 ml Eppendorf tube and centrifuged at maximum speed for 20 seconds. The majority of the medium was removed leaving around 100 μl. Bacterial pellets were resuspended by vortexing and lysed by the addition of 300 μl of Megadeath solution (0.1 M NaOH, 0.5% SDS, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Cell debris was precipitated by mixing 155 μl of NaAc pH 5.2 with the mixture and centrifugation at maximum speed for 4 minutes. Nucleic acids were precipitated by mixing 1 ml of 100% ethanol with the supernatant. The sample was briefly vortexed and centrifuged at maximum speed for 4 minutes prior to removal of the supernatant. The nucleic acid pellet was washed by the addition of 400 μl of 70% ethanol followed by vortexing and a brief centrifugation. The remaining liquid was removed and the pellet was dried for 5 - 10 minutes at 37°C. Mini-prep DNA was resuspended in 20 μl of MQ H_2O containing 10 μg/ml RNase A.

2.3.13 Midi-preparation of plasmid DNA

50 ml of LB containing 100 μg/ml ampicillin in a 250 ml flask was inoculated with a single bacterial colony and grown overnight at 37°C with shaking. The culture was transferred to a 40 ml oakridge tube and centrifuged in a RC-5 Sorvall centrifuge and SS-34 rotor (Dupont) at 6,000 rpm for 10 minutes. The supernatant was removed and the bacterial pellet was resuspended in 3 ml of Solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA). The suspension was gently mixed with 6 ml of fresh Solution II (0.2 M NaOH, 1% SDS) to lyse the cells. Following 5 minutes incubation on ice, cell debris was precipitated by the addition of 4.5 ml of Solution III (3 M KAc, 2 M HAc). The solution was gently mixed by inversion, left on ice for 5 minutes, then mixed vigorously, left on ice for a further 15 - 20 minutes, and centrifuged at 14,000 rpm for 15 minutes at 4°C. The supernatant was mixed with

8 ml of isopropanol in a clean oakridge tube, and nucleic acids were precipitated by centrifugation at 12,000 rpm for 5 minutes at 4°C. The supernatant was aspirated and the pellet was dissolved in 400 µl of MQ H_2O. RNA was removed by incubation at 37°C for 30 minutes with 2 µl of RNase A (10 mg/ml). 8 µl of 10% SDS and 2 µl of Proteinase K (20 mg/ml) were added to the solution and incubated for a further 15 minutes at 37°C. The sample was extracted with an equal volume of phenol/chloroform and then with an equal volume of chloroform alone. The aqueous phase was precipitated by addition of 100 µl of 7 M NH_4Ac and 1 ml of 100% ethanol. DNA was precipitated for 20 minutes at -20°C then pelleted at 14,000 rpm in a bench-top centrifuge for 15 minutes. The DNA was washed in 400 µl of 70% ethanol, dried, and resuspended in 200 µl of MQ H_2O.

2.3.14 Large-scale plasmid preparation

500 ml of LB containing 100 µg/ml ampicillin was inoculated either with a single bacterial colony or 5 ml from an overnight culture, and incubated overnight at 37°C in an orbital shaker. The cells were harvested by centrifugation at 6000 rpm for 5 minutes at 4°C, and the bacterial pellets drained. Bacteria were resuspended in 6.5 mls of GTE (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) before the addition of 13 ml of fresh lysis solution (0.2 M NaOH, 1% SDS). The mixture was thoroughly mixed by inversion 20 times and placed on ice for 5 minutes. 6.5 ml of 3 M NaAc pH 4.6 was added, gently mixed by inversion and incubated on ice for 5 minutes. The mixture was mixed more vigorously and placed back on ice for a further 15 minutes. Cell debris was pelleted by centrifugation at 14,000 rpm for 15 minutes at 4°C in a SS-34 rotor and RC-5 Sorvall centrifuge (Dupont). The supernatant was transferred to a clean Oakridge tube and nucleic acid was precipitated with the addition of 15 ml of isopropanol and centrifugation at 8000 rpm for 5 minutes at 4°C. The pellet was resuspended in 7 ml of TE. 7g CsCl and 700 µl EtBr (10 mg/ml) was added and mixed immediately. EtBr/protein aggregates were removed by centrifugation at 3,500 rpm for 5 minutes at 4°C. The supernatant was transferred to a 10 ml Nalgene polycarbonate Oakridge tube and balanced with paraffin oil. A CsCl density gradient was formed by centrifugation at 45,000 rpm for 18 hours at 20°C. Plasmid DNA was visualised under long wavelength UV light and recovered using a 1 ml syringe and a 1 1/2 inch, 22 gauge needle. EtBr was removed by 5 - 7 extractions with NaCl/TE saturated isopropanol. The DNA solution was diluted 1 in 4 with MQ water in a 30 ml Corex tube before precipitation with 2.5 volumes of ethanol. Plasmid DNA was recovered by centrifugation at 9500 rpm for 20 minutes at 4°C and the pellet was resuspended in 400 µl MQ water. The DNA solution was transferred to an Eppendorf tube and precipitated again with 20 µl of NaAc pH 5.2 and 1 ml of 100% ethanol before final resuspension in MQ water. Yield and quality of plasmid DNA was determined from the absorbance of a 1 in 500 dilution at wavelengths between 210-320 nm and by electrophoresis on 1% TBE agarose gels.

2.3.15 Southern analysis

Appropriately digested DNA samples were run on 0.8-1% TAE agarose gels. The gel was stained with EtBr for 5 minutes, destained for 10-15 minutes, and photographed adjacent to a ruler for scaling purposes. A number of rinsed household sponges were placed in a shallow tray and moistened with 0.4 M NaOH. Three sheets of 3mm paper were positioned on top of the sponges, moistened, and rolled flat with a pipette. The gel was placed on the 3mm paper and lengths of parafilm were placed over each edge of the gel. A section of Hybond-N^+ membrane was cut to the exact size of the gel and moistened for 5 minutes in MQ H_2O and then in 2 x SSC. The membrane was carefully placed on top of the gel and bubbles were rolled out with a pipette. Incisions were made in the membrane to mark the position of the wells. Two pieces of 3mm paper soaked in 2 x SSC were placed on the membrane followed by a 7-10 cm stack of paper towels cut slightly smaller than the size of the gel. A glass plate was placed on top of the towels and a 500 g weight positioned on the plate. Transfers were generally allowed to proceed for 18 hours after which time the apparatus was dismantled and the blot placed DNA side up on 3mm paper soaked in 6 x SSC. The blot was then UV crosslinked in a Stratalinker (Stratagene) prior to prehybridisation.

2.3.16 Synthesis of radioactive DNA probes

DNA probes were prepared using the Gigaprime labelling kit (Geneworks) or Megaprime labelling kit (Amersham) according to the manufacturer's instructions. 20-50 ng of purified fragment DNA was oligo-labelled for 20 minutes at 37°C in a reaction containing 50 µCi of [α-^{32}P] dATP. The reaction was stopped by the addition of 10 µl of stop buffer (50% glycerol, 100 mM EDTA pH 7.0, 0.02% bromophenol blue, 0.02% SDS), MQ H_2O up to a volume of 100 µl, and incubation at 65°C for 10 minutes. Excess unincorporated label was removed from probe reactions by centrifugation at 1800 rpm for 3 minutes through a Sepharose CL-6B spin column.

2.3.17 Hybridisation of ^{32}P-labelled probes to Southern blots

A clean nylon mesh was moistened with pre-hybridisation solution (250 mM sodium phosphate buffer pH 7.2, 7% SDS, 10% PEG 6000, 1 mM EDTA, 100 ng/ml herring sperm DNA) and laid flat in a shallow tray. The Southern filter was placed on the mesh DNA side up, rolled up with the mesh, and placed in a Hybaid cylinder. 10-15 ml of warm pre-hybridisation solution was added to the cylinder, the cylinder placed in Hybaid oven, and incubated at 65°C for 1-16 hours with rotation.

^{32}P-labelled DNA probes (2.3.16) were denatured at 100°C for 5 minutes and snap cooled on ice. Following pre-hybridisation the pre-hybridisation solution was replaced and the denatured probe added. The cylinder was returned to the Hybaid oven and rotated overnight at 65°C.

2.3.18 Washing Southern filters

Hybridisation solution was replaced with 50 ml of 2 x SSC/0.1% SDS, placed in a Hybaid oven and incubated at 42°C for 15 minutes. The filter was removed from the cylinder, placed in 500 ml of prewarmed 2 x SSC/0.1% SDS and incubated at 42°C for 15 minutes. Wash solution was replaced with 500 ml of prewarmed 0.2 x SSC/0.1% SDS and incubated at 42°C for a further 15 minutes. These washes were generally sufficient to remove probe bound nonspecifically to the filter. More stringent washes were performed in 0.2 x SSC/0.1% SDS at 65°C.

2.3.19 Library screening

20 ml LMM broth was inoculated with a single LE392 bacterial colony and grown overnight at 37°C in an orbital shaker. Bacterial cultures were centrifuged at 5,000 rpm for 5 minutes at 4°C and resuspended in 10 ml of 10 mM $MgSO_4$.

Approximately 5 x 10^4 recombinant phage were added to 200 µl of plating bacteria and incubated at 37°C for 15-20 minutes. 10 ml of molten LMM agarose was transferred to the phage/bacteria mixture, briefly mixed and overlayed onto 15 cm LB plates. Plates were incubated at 37°C for 6 - 7 hours until the plaques were almost in contact. Plates were stored overnight at 4°C.

Colony/Plaque Screen hybridisation transfer membranes were spotted asymmetrically with a permanent marker and placed onto library plates for 1 minute. Spots were duplicated on the plate, the lift removed and transferred, phage side up, to 3mm paper. A second lift was placed on the plates and removed after 3 minutes. Lifts were allowed to air dry for 5 minutes. Filters were then baked at 100°C for 2 minutes, moistened with 6 x SSC, and UV crosslinked twice in a Stratalinker (Stratagene).

Lifts were pre-hybridised with 15-25 ml of formamide pre-hybridisation solution (40% deionised formamide, 50 mM Tris-HCl pH 7.4, 1% SDS, 16.5% PEG 6000, 5 x Denhardt's reagent, 1 M NaCl, 100 µg/ml herring sperm DNA) in a large petri dish at 42°C for at least 4 hours. ^{32}P-labelled DNA probes (2.3.16) were denatured at 100°C for 5 minutes and snap cooled on ice. The lifts were removed from the petri dish, pre-hybridisation solution replaced, ^{32}P-labelled DNA probe added, and the lifts placed back in the dish, one by one. Nylon mesh was placed on top of the lifts to prevent drying of the top lift. The petri dish was wrapped in saran wrap and placed in a 42°C oven overnight.

Lifts were removed from the dish and placed in 500 ml of preheated 2 x SSC/0.1% SDS and incubated at 42°C for 15 minutes. This procedure was repeated and individual lifts were checked for counts with a hand held Geiger counter. Lifts were sufficiently washed if lifts had counts of between 5 and 10 cpm. More stringent washes were performed in 2 x SSC/0.1% SDS at 65°C for 15 minutes and finally in 0.2 x SSC/0.1% SDS at 65°C for 15 minutes.

Excess moisture was removed by briefly blotting lifts onto 3mm paper. Lifts were positioned between two sheets of clear plastic in an autoradiograph cassette and exposed to fast X-ray film overnight at -80°C.

Orientation marks were copied onto autoradiographs and double positives were picked with the wide end of a Pasteur pipette into 1 ml of PSB and eluted overnight at 4°C. The eluate was placed into a clean tube and bacteria were killed by the addition of 1 drop of chloroform.

Second round screening was performed as stated above with the following changes. Dilutions of phage eluted from the first round positive plugs were used to infect 100 µl of plating bacteria and plated onto 10 cm LB plates in 4 mls of LMM agarose. Duplicate nitrocellulose lifts were taken from plates with around 300 plaques and phage were lysed by placing the lifts onto 3mm paper soaked in denaturing solution (1.5 M NaCl, 500 mM NaOH) for 5 minutes. Lifts were then placed on 3mm paper soaked in neutralisation solution (1.5 M NaCl, 500 mM Tris-HCl pH 8.0) for 5 minutes and finally onto 3mm paper soaked in 6 x SSC. DNA was crosslinked to the lifts in a Stratalinker (Stratagene). Pre-hybridisation and hybridisation was carried out in a 6 cm diameter petri dish. Isolated duplicate positive plaques were picked with the narrow end of a Pasteur pipette and phage were eluted overnight at 4°C in 400 µl of PSB.

2.3.20 High titre stock production

Isolated second round positive plaques were eluted in 400 µl of PSB overnight at 4°C. 50 µl of the eluate was incubated with 100 µl of LE392 *E. coli* before plating onto 10 cm LB plates. Plates observed to have confluent lysis were soaked in 1-2 mls of PSB for 3 hours with shaking. The PSB was transferred to Eppendorf tubes and a drop of chloroform was added to kill bacteria.

2.3.21 Phage DNA preparations

10^5 phage (50 µl of high titre stock) were mixed with 500 µl of an LE392 strain overnight culture. The mixture was incubated for 15 minutes at room temperature and added to 50 ml of LMM broth in a 250 ml flask and incubated at 37°C overnight. A few drops of chloroform were added the next day to kill bacteria. The solution was poured into 40 ml oakridge tubes and centrifuged at 5000 rpm for 5 minutes. The supernatant was poured into a new oakridge tube and treated with 10 µl RNase A (10 mg/ml) and 10 µl DNase I (100 U/µl) for 30 minutes at 37°C. 7.125mls of 4 M NaCl and 3.75 ml of 50% PEG 6000 were thoroughly mixed into the solution and the tubes chilled to 4°C for at least 2 hours. Phage particles were pelleted by centrifugation at 10,000 rpm for 10 minutes. The supernatant was drained away and the phage pellet was resuspended in 500 µl of PSB. Phage particles were lysed by the addition of 20 µl of 500 mM EDTA, 10 µl of 10% SDS, and 2.5 µl of Proteinase K (20 mg/ml). Phage proteins were digested for 60 minutes at 37°C and the protease was inactivated at 65°C for 15 minutes. The sample was phenol/chloroform extracted twice, chloroform extracted once. Phage DNA was recovered by precipitation by the addition of 1 ml of 100% ethanol and centrifuged, 70% ethanol washed, and resuspended in 100 µl of MQ H_2O.

2.3.22 Manual sequencing of plasmid DNA

15 µl of mini-prep DNA (2.3.12) was incubated at 37°C for 15 minutes with 1.5 µl RNase A (10 mg/ml) and denatured with the addition of 3.5 µl 1 M NaOH/1 mM EDTA for 15 minutes at 37°C. Denatured plasmid was purified by centrifugation at 1800 rpm for 3 minutes through a Sepharose CL-6B spin column. 1 µl of primer (4.5 ng/µl) was added to 7 µl of purified plasmid DNA in the presence of annealing buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 25 mM NaCl) in a total of 10 µl and annealed at 65°C for 2 minutes, then at 37°C for 5 minutes, and room temperature for 15 minutes, and finally kept on ice until required. Manual sequencing reactions were carried out using the T7 Sequencing kit (Pharmacia) according to the manufacturer's instructions. Briefly, 6 µl of labelling mix, 4U of modified T7 DNA polymerase, 5 µCi [α-^{33}P] dATP was added to the annealed primer/DNA mix and incubated at room temperature for 5 minutes. 3.5 µl of the mixture was mixed with 2.5 µl of each termination mix in a separate tube and incubated at 37°C for 5 minutes. Reactions were halted by the addition of 4 µl of FLB (95% (w/v) deionised formamide, 20 mM EDTA, 500 µg/µl bromophenol blue, 500 µg/µl xylene cyanol) and run on a 7M urea / 6% polyacrylamide (2:34 bis-acrylamide to acrylamide) denaturing gel. Compressions were resolved by performing reactions with Deaza-dGTP containing termination mixes.

6% polyacrylamide sequencing gels were prepared using Sequagel solution (National Diagnostics). 400 µl of 10% APS and 20 µl of TEMED were added to 40 mls of stock gel solution prior to pouring between clean glass plates (20 x 40 cm) separated by 0.4 mm spacers. After 30 minutes, the comb was removed and the well flushed with water. Gels were pre-electrophoresed for 1 hour at 2000 V/50 mA and the wells were flushed with 1 x TBE before loading samples. Gels were electrophoresed at 50 mA at a temperature of 50-55°C. After electrophoresis, the glass plates were prised apart and the gel transferred to 3mm paper. The gel was dried down at 70°C on a vacuum gel drier and exposed to fast X-ray film.

2.3.23 Automated sequencing of plasmid DNA

2 ml of LB containing 100 µg/ml of ampicillin was inoculated with a single bacterial colony and grown overnight at 37°C. Plasmid DNA was prepared from the culture using the Bresaspin miniprep Kit according to the manufacturer's instructions (Geneworks). 1 µg of miniprep DNA was subjected to cycle sequencing in the presence of 100 ng of primer and Big Dye terminator mix (PE Biosystems) in a total volume of 20 µl. The reaction was cycled through the following steps 24 times.

Step 1:	96°C for 30 seconds
Step 2:	50°C for 15 seconds
Step 2:	60°C for 4 minutes

Completed reactions were added to 80 µl of 75% isopropanol, mixed and allowed to precipitate for 15 minutes at room temperature. DNA was pelleted for 20 minutes at 14,000 rpm, washed in 250 µl of 75% isopropanol, centrifuged again for 5 minutes and air dried. Reactions were

analysed at the Institute for Medical and Veterinary Science Sequencing Centre, Adelaide, Australia and viewed on the Editview program (PE Biosystems).

2.3.24 Preparation of genomic DNA from ES cells

ES cells were grown to high density on 10 cm plates, washed once in PBS, and lysed by the addition of Tail Buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 10 μg/ml RNase A) for 15 minutes at 37°C. A further 5 ml of Tail buffer containing 200 μg/ml Proteinase K was added and the lysate was transferred to a conical tube and incubated overnight at 58°C. Genomic DNA was precipitated by gently mixing following the addition of 2.5 volumes (25 ml) of 100% ethanol. The DNA was spooled onto a glass pipette and washed twice in 5 ml of 70% ethanol. The DNA was allowed to briefly air dry and was resuspended in 1.2 ml of TE by heating to 65°C for 30 minutes with gentle agitation. Preparations of genomic DNA were left at room temperature for 2 days and then stored at 4°C.

2.3.25 Hirt extraction of episomal DNA from mammalian cells

Episomal DNA was extracted from tissue culture cells essentially as in Anant and Subramanian (1992). Briefly, cells were washed in PBS and trypsinised (2.4.4) or scraped off with a rubber policeman and centrifuged at 1,200 rpm for 4 minutes. The supernatant was removed and the cells were resuspended in 500 μl of 10 mM Tris-HCl pH 7.8, 10 mM EDTA, and vortexed. The suspension was then placed into an Eppendorf tube and mixed gently with 50 μl of 10% SDS (0.9% SDS final) for 10 minutes to lyse the cells. 140 μl of 5 M NaCl (1 M final) was mixed gently with the lysate and stored at 4°C overnight. Episomal DNA was separated from genomic DNA by centrifugation at maximum speed in a microfuge for 20 minutes. The supernatant was digested by the addition of 100 μg of Proteinase K at 50°C for 2 hours. The sample was then extracted twice with an equal volume of phenol/chloroform, once with chloroform alone, ethanol precipitated twice, and resuspended in 40 μl of H_2O. 10 μl of episomal DNA was used to transform chemically competent DH5α *E. coli*.

2.3.26 Isolation of cytoplasmic RNA from cultured cells

Cytoplasmic RNA was isolated as in Edwards *et al.*, (1985). Cells were harvested by trypsinisation and stored at -80°C until use. Cell pellets were thoroughly resuspended in 2 ml ice cold TNM (30 mM Tris-HCl pH 7.6, 150 mM NaCl, 15 mM $MgCl_2$, 0.4% Nonidet P 40) and lysed by vigorous pipetting 10 times. The suspension was placed on ice for 5 minutes, nuclei pelleted by centrifugation at 3,000 rpm for 5 minutes, and the supernatant decanted and mixed thoroughly with 2 ml TUNES (10 mM Tris-HCl pH 8.0, 7 M urea, 350 mM NaCl, 1 mM EDTA, 2% SDS). This solution was extracted twice with phenol/chloroform (1:1) and the aqueous layer was transferred to a Corex tube. RNA was precipitated by addition of $1/10^{th}$ volume NaAc pH 5.2 and 2.5 volumes 100% ethanol, and incubated at -80°C for 30 minutes. After centrifugation at 10,000 rpm for 30 minutes at 4°C, the RNA pellet was resuspended in 450 μl of MQ water, and transferred to an Eppendorf tube and precipitated again. RNA was

pelleted for 15 minutes at 14,000 rpm, resuspended in 100-200 µl of MQ water, and the concentration was determined by spectrophotometry at 260 nm. RNA samples were stored at -20°C.

2.3.27 Selection for polyadenylated RNA

Poly $(A)^+$ RNA selection was carried out essentially as in Celano *et al.*, (1993). Oligo-dT cellulose (Type 7, Pharmacia) was hydrated by three washes in Na-TES (500 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 % SDS). 1 mg of cytoplasmic RNA (2.3.26) was brought up to a final volume of 600 µl in TES (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 % SDS) and denatured at 65°C for 5 minutes and snap cooled on ice. 60 µl of 5 M NaCl was mixed with the RNA and added to 600 µl (60 mg) of hydrated oligo-dT cellulose. The tube was mixed well and incubated at 37°C for 10 minutes. Oligo-dT cellulose was pelleted by centrifugation in a benchtop centrifuge at maximum speed for 15 seconds. The supernatant was removed and the pellet was resuspended in 1 ml of Na-TES and the tube was centrifuged again. The supernatant was removed and the pellet resuspended in 1 ml of ice-cold MQ H_2O and centrifuged once more. Poly $(A)^+$ RNA was eluted by resuspending the oligo-dT cellulose in 400 µl of MQ H_2O and incubation at 55°C for 5 minutes. Following centrifugation the supernatant was placed into a siliconised Eppendorf tube. The elution was repeated twice more and eluates were again selected on oligo-dT cellulose as described above. Following the second round of selection eluted poly $(A)^+$ RNA was precipitated by the addition of 1/10th volume of 3 M NaAc pH 5.2 and 2.5 volumes of 100% ethanol. Precipitates from each tube were resuspended and pooled in 10-15 µl and the quantity and quality of the poly $(A)^+$ RNA was ascertained by spectrophotometric analysis and agarose gel electrophoresis, respectively.

2.3.28 cDNA library construction

cDNA was produced from poly $(A)^+$ ES cell RNA (2.3.27) using the Universal Riboclone cDNA Synthesis System (Promega) according to the manufacturer's instructions. Briefly, 2 µg of ES cell poly $(A)^+$ RNA was reverse transcribed with AMV reverse transcriptase (Molecular Genetic Resources) and 1 µg of *Not*I-$(dT)_{18}$ primer-adaptor (2.2.7) (Pharmacia). Second strand synthesis was performed using RNase H and *E.coli* DNA polymerase I directly after first strand synthesis. The quality and quantity of cDNA produced was determined by performing tracer reactions containing 5 µCi [α-^{32}P]dCTP and 2 µl of first or 5 µl of second strand reactions. Half of each reaction was electrophoresed on a 1% alkaline agarose gel with ^{32}P-labelled *Eco*RI digested SPP-1 phage DNA as molecular weight markers. The gel was electrophoresed in 50 mM NaOH, 10 mM EDTA for 4 hours, fixed in 10% Trichloroacetic acid (TCA) for 30 minutes, dried on a gel drier and exposed to fast film overnight. The quantity of cDNA produced was determined by TCA precipitation of the remainder of the tracer reactions according to the manufacturer's instructions.

Double stranded cDNAs were flushed by the addition of T4 DNA polymerase and extracted in phenol:chloroform:isoamyl alcohol (1:1:24) and precipitated, washed, and

resuspended in MQ H_2O. cDNA was size fractionated using Sephacryl S-400 spin columns, ligated to *Eco*RI adaptors (2.2.7), digested with *Not*I restriction enzyme, and phosphorylated using polynucleotide kinase. The cDNA was extracted in phenol:chloroform:isoamyl alcohol and size fractionated once more.

90 μg of pure pPSDEneoΔLT20 plasmid was digested with 60U of *Eco*RI and 80U *Not*I endonucleases in a volume of 300 μl for 4 hours at 37°C. A total of 18 μg of digested pPSDEneoΔLT20 was loaded onto two 0.8% TBE agarose gels and electrophoresed at 100 mA for 1.5 hours. pPSDEneoΔLT20 vector was purified away from the agarose. cDNA was ligated to gel purified *Eco*RI/*Not*I digested pPSDEneoΔLT20 vector. The ligation reaction contained approximately 200 ng of pPSDEneoΔLT20 vector and 100 U of T4 DNA ligase and was carried out overnight at 4°C. The reaction was phenol/chloroform extracted then chloroform extracted, precipitated and resuspended in 22 μl of MQ H_2O. The library was resuspended in MQ H_2O and electroporated into Electro-Max DH10B electrocompetent cells (Gibco/BRL). Bacteria were plated onto seventy 15 cm LB plates with 100 μg/ml ampicillin and grown overnight at 37°C. Plates were observed to contain 71,000 resistant colonies. Bacteria were scraped off the plates into 2 litres of LB containing 100 μg/ml ampicillin CsCl pure plasmid DNA was prepared as in section 2.3.14.

2.3.29 Genomic PCR

PCR on genomic templates was performed in a PTC-100 thermal cycler (Geneworks) as in Barnes (1994) with some modifications. Reactions were prepared in PCR tubes to a volume of 50 μl by the addition of 5 μl 10 x LRB (250 mM Tris-HCl pH 9.1, 160 mM $(NH_4)_2SO_4$, 2.5 μl 5 mM dNTPs, 1 μl of each primer (100 ng/μl), 2 μl of polymerase mix (80:1 Taq:Pfu mix diluted in Taq buffer at 1 unit Taq: 0.0125 units Pfu per μl), 100-200 ng of template DNA and H_2O up to 43 μl volume. The polymerases were activated after 90 seconds at 75°C by the addition of 7 μl of 25 mM $MgCl_2$. The final reaction contains 25 mM Tris-HCl pH 9.1, 16 mM $(NH_4)_2SO_4$, 250 μM dNTPs, 3.5 mM $MgCl_2$, 100 ng of each primer, 2 units of polymerase mix, and 100-200 ng genomic DNA. Reactions were denatured at 96°C for 1 minute and then cycled through the following steps 45 times.

Step 1: 96°C for 15 seconds

Step 2: 68°C for 110 seconds

Polymerisation was completed by a final extension at 72°C for 10 minutes.

2.3.30 cDNA synthesis

Reverse transcription was carried out with Superscript^{tm} II reverse transcriptase (Gibco/BRL) essentially following the manufacturer's instructions. Briefly, 2.5 μg of cytoplasmic RNA (section 2.3.26) and 500 ng of Oligo $(dT)_{12\text{-}18}$ (Pharmacia) were heated to 70°C for 10 minutes in a volume of 12 μl. The mixture was snap cooled on ice and incubated at 42°C for 50 minutes in a 20 μl reaction containing 500 nM dNTPs, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM

$MgCl_2$, 10 mM DTT, and 200 U Superscript^{tm} II. The reaction was diluted to 200 µl with H_2O and stored at -20°C.

2.3.31 Capillary PCR with Taq polymerase

Typically 10 ng of plasmid template or 1 µl of cDNA (2.3.30) was amplified in a reactions containing 67 mM Tris-HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 200 µg/ml gelatin, 0.45% Triton X-100, 250 µM dNTPs, 1.5 mM $MgCl_2$, 100 ng of each primer and 1 U of Taq polymerase. Reactions were performed in capillary tubes using a FTS-1 Thermal Cycler (Corbet Research) in a total volume of 20 µl. Reactions were heated to 94°C for 3 minutes and cycled through the following steps 25 times for plasmid templates or 35 times for cDNA templates.

Step 1:	94°C for 5 seconds
Step 2:	55°C for 5 seconds
Step 3:	72°C for 1 minute/kb

Reactions were incubated at 72°C for a further 5 minutes to allow extension of partial PCR products.

2.3.32 PCR with Taq polymerase

1 µl of cDNA (2.3.30) or Hirt preparation DNA (2.3.25) were amplified in 50 µl reactions containing 10 mM Tris-HCl pH 8.4, 40 mM NaCl, 0.01% Gelatin, 0.1% Triton X-100, 200 µM dNTPs, 1.5 mM $MgCl_2$, 200 ng of each primer, 1 U of Taq polymerase. Reactions were overlaid with mineral oil and heated to 94°C for 3 minutes and then cycled 35 times as follows:

Cycle 1:	94°C, 30 sec; 70°C, 1 min; 72°C, 1 min/kb
Cycle 2:	94°C, 30 sec; 67°C, 1 min; 72°C, 1 min/kb
Cycle 3:	94°C, 30 sec; 64°C, 1 min; 72°C, 1 min/kb
Cycle 4:	94°C, 30 sec; 61°C, 1 min; 72°C, 1 min/kb
Cycle 5-35:	94°C, 30 sec; 58°C, 1 min; 72°C, 1 min/kb

Reactions were incubated at 72°C for a further 5 minutes to allow extension of partial PCR products.

2.3.33 PCR with Pfu polymerase

RT-PCR reactions were carried out using Pfu Turbo (Stratagene) according to the manufacturer's instructions. Briefly, 100 µl volume PCR reactions containing 1 µl of cDNA (2.3.30), 250 ng of each primer, 200 µM dNTPs, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 100 ng/ml BSA, 0.1% Triton X-100, and 2.5 U of Pfu Turbo polymerase were overlaid with mineral oil, heated to 94°C for 3 minutes, and cycled as follows in a PTC-100 Thermal cycler.

Cycle 1:	94°C, 30 sec; 70°C, 1 min; 72°C, 1 min/kb
Cycle 2:	94°C, 30 sec; 67°C, 1 min; 72°C, 1 min/kb
Cycle 3:	94°C, 30 sec; 64°C, 1 min; 72°C, 1 min/kb
Cycle 4:	94°C, 30 sec; 61°C, 1 min; 72°C, 1 min/kb

Cycle 5-35: 94°C, 30 sec; 58°C, 1 min; 72°C, 1 min/kb

Reactions were incubated at 72°C for a further 5 minutes to allow extension of partial PCR products.

2.4 TISSUE CULTURE METHODS

2.4.1 Cell lines

Cell lines used during the course of this work were obtained from:

D3 ES cells	Dr Lindsay Williams, Ludwig Institute, Melbourne, Australia
E14 ES cells	Dr Anna Michelska, Murdock Institute, Melbourne, Australia
E14TG2a ES cells	Dr Austin Smith, CGR Edinburgh, UK.
R1 ES cells	Dr Stephen Wood, Biochemistry Department University of Adelaide, Adelaide, Australia.
COS-1 cells	ATCC
NIH3T3 cells	ATCC

2.4.2 Solutions

PBS:	136 mM NaCl, 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 pH 7.4. Sterilised by autoclaving (20 psi for 25 minutes at 140°C).
Trypsin:	0.1% trypsin (Difco) and 1 x EDTA Versene buffer solution (CSL). Sterilised by filtration through a 0.2 μM filter (Whatman).
βME/PBS:	100 mM β-mercaptoethanol (Sigma) in 14.1 ml PBS. β-mercaptoethanol/PBS solutions were not kept longer than two weeks.
L-glutamine:	100 mM L-glutamine in PBS.
LIF:	COS cell conditioned medium containing LIF prepared as described by Smith (1991) except that transfections were performed by electroporation.

2.4.3 Media

Incomplete ES cell medium:	85% DMEM medium, 15% FCS (Gibco BRL), 1% L-glutamine, 0.1 mM β-mercaptoethanol/PBS.
Complete ES cell medium:	Incomplete ES cell medium with 0.1% LIF.
Cos/NIH3T3 cell Medium:	90% DMEM medium, 10% FCS, 25 mM HEPES pH 7.5

2.4.4 Maintenance of ES cells

ES cells were maintained on gelatinised 10 cm petri dishes (Corning or Falcon) in complete ES medium at 37°C in 10% CO_2. Cells were harvested as follows: cells were washed in PBS, incubated with trypsin (1 ml) at 37°C for 1 minute and transferred to 9 ml of complete ES cell medium. The cells were centrifuged at 1,200 rpm for 4 minutes, medium aspirated, resuspended in 10 ml complete ES medium, and re-seeded at clonal density (10-80 fold dilution). Medium

was replaced with fresh medium on the second day of the passage. ES cells were re-seeded every 3 - 4 days.

2.4.5 Maintenance of NIH3T3 cells

Cells were maintained in 150 cm^2 flasks in medium containing 10% FCS at 37°C in 5% CO_2. Cells were harvested once they reached confluence as follows: cells were washed in PBS, incubated with trypsin (2.5 ml) at 37°C for 1 minute and transferred to 9 ml of medium. The cells were centrifuged at 1,200 rpm for 4 minutes, medium aspirated, resuspended in medium, and re-seeded at clonal density (5-20 fold dilution).

2.4.6 Freezing and thawing of ES cells

10 cm plates of ES cells were trypsinised (2.4.4) and centrifuged at 1,200 rpm for 4 minutes. The supernatant was aspirated and the cells resuspended in 4 ml of freezing mix (90% FCS, 10% DMSO). 1 ml was placed in each freezing vial (Nunc) and stored overnight at -80°C. Vials were placed in liquid nitrogen for long term storage.

Freezing vials were thawed in a 37°C water bath and the cells were seeded onto 10 cm plates containing 10 ml of ES cell complete medium. The next day the cells were washed in PBS and the medium replaced.

2.4.7 Spontaneous induction of ES cell differentiation

ES cells were seeded at 1,000 cells/cm^2 in incomplete ES medium. After the initial 72 hours of culture, the medium was changed daily, and the cells were cultured for a further 5 - 6 days and harvested.

2.4.8 Retinoic acid induction of ES cell differentiation

ES cells were seeded at 1 x 10^4 cells/cm^2 in incomplete ES medium containing 10 U/ml recombinant LIF (Amrad) and allowed to adhere overnight. The following day the medium was replaced with identical medium containing 10 μM retinoic acid, and the cells cultured for a further 72 hours with daily changes of medium containing 10 μM retinoic acid.

2.4.9 Cell counting

Cells were trypsinised as in section 2.4.4. 100 μl of cell suspension was mixed with 900 μl of Trypan Blue. 50 μl of this mixture placed on a haemocytometer and unstained cells were scored under light field microscopy at 20 x magnification. The number of cells per ml were determined by multiplying the number cells counted by a factor of 10^4.

2.4.10 Electroporation of ES cells

Plates of ES cells with large undifferentiated colonies were trypsinised as in section 2.4.4. The single cell suspension was added to 2 volumes of 15% complete ES cell medium (2.4.3) in a conical tube and centrifuged at 1,200 rpm for 4 minutes. The medium was aspirated and the

cells were resuspended in 10 mls of PBS. 100 µl of the suspension was taken and used to determine cell numbers using a haemocytometer (2.4.9). The suspension was centrifuged at 1,200 rpm for 4 minutes and the supernatant aspirated. The cells were resuspended in a volume of ES cell electroporation buffer (20 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, 0.1 mM β-mercaptoethanol, 0.001% phenol red pH 7.0) to form a solution that contains 1.6-5 x 10^7 cells per ml. 10-20 µg of CsCl pure plasmid DNA was added to each new 0.4 cm gap electroporation cuvette. 600 µl of cell suspension (1-3 x 10^7 cells) was added to the cuvette and mixed with the DNA using a 1 ml pipette. ES cells were electroporated once at 500 µF and 240 V. Electroporated ES cells were removed from the cuvette and plated onto 10 cm or 15 cm plates.

2.4.11 Stable ES cell selection and clone isolation

Medium was replaced one day after electroporation with ES cell medium containing 200 - 250 µg/ml G418, 110 µg/ml hygromycin B, or 1.5 µg/ml puromycin and every second day after that for 10 - 14 days. Resistant colonies were picked into 100 µl of trypsin and seeded into gelatinised wells of a 24-well tray containing ES cell medium and the appropriate selective agent. Alternatively, whole plates of resistant colonies were trypsinised as pools and passaged (2.4.4) and frozen (2.4.6).

2.4.12 Selection for ES cell survival in the absence of LIF

Electroporation was performed as described in section 2.4.10 except cells were washed in incomplete ES cell medium rather than in complete medium. Immediately following electroporation ES cell were placed in 10 cm or 15 cm plates in incomplete ES cell medium containing 200-800 µg/ml G418. Every second day the medium was aspirated and the cells were washed twice in PBS prior to addition of fresh incomplete ES cell medium containing G418. After 10 - 17 days selection in the absence of LIF the presence of pluripotent colonies was determined by histochemical staining for alkaline phosphatase activity using the Alkaline Phosphatase kit (Sigma) according to the manufacturer's instructions.

2.4.13 LIF titration assay

LIF titration assays were performed in gelatinised 24-well trays. 900 µl of incomplete medium was added to each well and 100 µl of 400 U/ml, 300 U/ml, 200 U/ml, 150 U/ml, 100 U/ml, 75 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5U/ml, and 0 U/ml recombinant LIF (ESGRO; Chemicon) in incomplete media was added to the appropriate well. Final LIF concentrations were 40 U/ml, 30 U/ml, 20 U/ml, 15 U/ml, 10 U/ml, 7.5 U/ml, 5 U/ml, 2 U/ml, 1 U/ml, 0.5 U/ml, 0 U/ml. 500 cells were added to each well and the medium was mixed to evenly disperse the cells. After 6 days the plates were stained for alkaline phosphatase activity. Plates were scanned using a Umax Powerlook II flat bed Scanner. Retention of undifferentiated colonies in cultures was quantitated by scoring undifferentiated colonies, those that lacked differentiated cells and retained AP activity, using an inverted microscope. The total number of colonies in each well

was counted and data were expressed as the percentage of the undifferentiated colonies observed versus LIF concentration.

2.4.14 Lipofection

2 x 10^5 cells were seeded in each 35 mm well 24 hours before the lipofection was carried out. 1.5 μg of sample plasmid DNA with or without 0.5 μg of CsCl purified pCH110 was diluted into 100 μl of Opti-MEM-I medium (Gibco/BRL). The DNA mixture was then mixed with 100 μl of Opti-MEM-I medium containing 5 μl (ES cells), or 8 μl (NIH3T3 cells) of Lipofectamine Reagent (Gibco/BRL) and incubated at room temperature for 15 minutes. Cells were washed in serum free medium and 800 μl of serum free medium was added to the DNA/Lipofectamine mix and carefully placed on the cells. Cells were incubated for 5 hours at 37°C at which time the medium was replaced with the respective serum containing media.

2.4.15 Harvesting cells and protein extraction

Cells were harvested 24 - 36 hours after lipofection as follows. Cells were washed three times with PBS before addition of 1 ml of TEN buffer (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl). The cells were left at room temperature for 5 minutes and scraped into an Eppendorf tube using a rubber policeman. The cells were centrifuged at 14,000 rpm for 15 seconds, the supernatant aspirated, and the cells were resuspended in 50 μl of 250 mM Tris-HCl pH 7.6. Cells were then lysed by three freeze/thaw cycles. Cell debris was pelleted by centrifugation at 14,000 rpm for 15 seconds. The supernatant was placed in a clean tube and stored at -20°C.

2.4.16 Determination of protein concentration by Bradford assay

Samples and standards were performed in triplicate and averages were used in further calculations. 2 μl of BSA standards (1-10 mg/ml) or samples were mixed 200 μl of 1 in 4 diluted Bradford Reagent (BIO-RAD) in a 96-well tray. Absorbance at 505 nm wavelength was measured in a Emax plate reader (Molecular Dynamics). Protein concentrations of samples were determined by calculation from the line of best fit of the standard curve.

2.4.17 β-galactosidase activity assay

50 μg of protein extract was mixed with 1 ml of reaction buffer (100 mM $NaPO_4$ buffer pH 7.3, 10 mM KCl, 1 mM $MgCl_2$, 50 mM β-mercaptoethanol) and incubated at 37°C for 3 - 5 minutes to activate the enzyme. 200 μl of (5 mg/ml) ONPG was added and the samples were incubated at 37°C and the time taken to reach a yellow colour was noted. The reaction was halted by the addition of 500 μl of 1 M Na_2CO_3 and the OD at 420 nm was determined in a Cary 3 Bio spectrophotometer (Varian). β-galactosidase activity was determined by multiplying the OD_{420} by 100 and by the factor required to adjust the time taken to reach a yellow colour to 1 hour.

2.4.18 Luciferase assay

Luciferase activity was determined on 25 μg of protein using the luciferase assay system (Promega) and a Berthold LB 9502 Luminometer. β-galactosidase activity in each protein extract was used to control for variation in transfection efficiencies between cell types.

2.4.19 Giemsa staining

Medium was removed from plates and the cells were washed twice in PBS. Cells were fixed in 100% methanol for 2 minutes at room temperature and then rehydrated by three washes in PBS. Cells were stained with a 1 in 10 dilution of Giemsa stain for 2 - 3 minutes. Stained cells were washed three times in PBS and allowed to dry.

2.4.20 Histochemical staining for β-galactosidase activity

The medium was aspirated and the cells were washed three times in PBS before fixing for 5 minutes in 2% gluteraldehyde/PBS. The cells were again washed three times in PBS before the addition of staining solution (0.45 mM $K_3Fe(CN)_6$, 0.45 mM $K_4Fe(CN)_6$, 1mM $MgCl_2$, 400 μg/ml BCIG). Plates were incubated for 2 - 4 hours at 37°C before blue staining colonies were counted.

2.5 PHOSPHORIMAGING, AUTORADIOGRAPHY AND DATA MANIPULATION

Filters and gels were exposed to storage Phosphor screens (Molecular Dynamics) for 1 - 4 days and analysed using a Phosphorimager (Molecular Dynamics) and the ImageQuant software package. Filters and gels were also exposed to BioMax X-ray film for 1 - 4 days. Autoradiographs and gel photographs were scanned using a Powerlook II scanner (Umax), slide scanner and Photoshop (Adobe). Images were manipulated in Canvas 6 (Deneba) and printed using a HP LaserJet 5 MP or Epson Stylus Photo 870 printer.

2.6 CONTAINMENT FACILITIES

Manipulations that involved organisms containing recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

CHAPTER 3:

ESTABLISHMENT OF A SYSTEM FOR SELECTIVE REMOVAL OF DIFFERENTIATED ES CELL PROGENY

3.1 INTRODUCTION

The proposed function-based screening approach to identify factors that inhibit ES cell differentiation (1.9.2) was designed to isolate undifferentiated ES cell colonies within a population of differentiated cells. The presence of differentiated cell types not only makes it difficult to identify genuine undifferentiated colonies within a population, but differentiated cells can also inhibit further ES cell differentiation, through the production of LIF (Rathjen *et al.*, 1990a) and ESRF (Dani *et al.*, 1998). 'Stem cell nests' that form upon differentiation of an ES cell population are surrounded by differentiated cells, express the pluripotent cell marker *Oct4* (Mountford *et al.*, 1994) and retain the capacity to differentiate (Rathjen *et al.*, 1990a). The formation of stem cell nests is reduced in *LIF* deficient ES cells (Dani *et al.*, 1998; Rathjen *et al.*, 1990a). Stem cell nests potentially provide a 'background' which would complicate the identification of colonies in which differentiation is inhibited by the transfected cDNA (1.9.2).

One method to reduce the background level of pluripotent cell maintenance would be to actively select against differentiated cells in the population. Loss of differentiated cells would be expected to reduce paracrine LIF (Rathjen *et al.*, 1990a) and ESRF (Dani *et al.*, 1998) supply to ES cells and therefore reduce the ability of undifferentiated cells to maintain pluripotency in the absence of a transfected cDNA. Differentiated cells could potentially be selected against by expressing selectable markers from an ES cell specific promoter such that differentiated cells can be eliminated in the presence of the appropriate selective agent.

3.2 ISOLATION AND CHARACTERISATION OF A PLURIPOTENT CELL-SPECIFIC PROMOTER

The pluripotent cell types of the mammalian life cycle express the Pou domain transcription factor Oct4 (Palmieri *et al.*, 1994)(Figure 1.7). *Oct4* is expressed exclusively in pluripotent cells *in vivo* and *in vitro*, and is rapidly downregulated upon differentiation of pluripotent cells (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Scholer *et al.*, 1990). This strict expression profile makes *Oct4* an excellent marker of pluripotent cells and means the *Oct4* promoter can be used to drive gene expression specifically in pluripotent cells. The promoter of the *Oct4* gene has been exploited to mark pluripotent cells with β-galactosidase (β-gal) (Dani *et al.*, 1998) or green fluorescent protein (GFP) (Yoshimizu *et al.*, 1999), and to select for

pluripotent cells. McWhir *et al,* (1996) used an *Oct4* promoter coupled neomycin resistance (*neo*) gene cassette to select against differentiated cells arising spontaneously during the derivation of ES cell lines from mouse embryos. ES cells that differentiate lose *Oct4* expression and as a consequence no longer express neomycin resistance and are therefore susceptible to G418. In this manner it is possible to remove differentiated cells from the population leaving only pluripotent ES cells. Selective removal of differentiated cells by the use of an *Oct4*-neo construct allowed the derivation of ES cell lines from normally non-permissive 87.5% CBA strain embryos (McWhir *et al.*, 1996).

Two cell type specific enhancers, mapping within 4.2 kb of the Oct4 start codon, have been identified based on reporter gene expression when fused to the Thymidine Kinase (TK) minimal promoter (Yeom *et al.*, 1996) (Figure 3.1). The Distal Enhancer (DE) drives ES cell specific expression and reporter gene expression in pre-implantation blastocysts while the Proximal Enhancer (PE) is responsible for expression in EC cells and in post-implantation primitive ectoderm (Yeom *et al.*, 1996). Expression driven by the proximal promoter (PP) in EC cells is further repressed 7.7-fold upon RA induced differentiation (Pikarsky *et al.*, 1994). Repression is due to a hormone regulatory element (HRE) 40 bp downstream of the transcription start site (Pikarsky *et al.*, 1994; Sylvester and Scholer, 1994). The HRE binds RA and retinoid X receptors and orphan nuclear hormone receptors such as COUP-TF1, ARP-1, and EAR-2. Two uncharacterised binding activities, undifferentiated cellular factor (UCF) and transiently induced factor (TRIF), bind the HRE in undifferentiated EC cells and 18-48 hrs following RA treatment, respectively (Fuhrmann *et al.*, 1999). It is not known if differentiation induced by means other than RA treatment also involves HRE nor if HRE is important to recapitulate normal *Oct4* expression *in vivo* or in ES cells and differentiated derivatives.

In order to produce a selection construct capable of selecting against differentiated cells the *Oct4* promoter was isolated. Given that the PP has been shown to play a role in *Oct4* gene expression it was chosen as the basal promoter over heterologous promoters such as the minimal TK promoter. Subsequent work by others has demonstrated that *Oct4* promoter neomycin fusions incorporating the PP region can be used to select against differentiated cells (McWhir *et al.*, 1996; Mountford *et al.*, 1998).

3.2.1 Isolation of the *Oct4* promoter by genomic PCR

The *Oct4* promoter region was isolated by amplification and cloning of a section of the *Oct4* gene from mouse E14 ES cell genomic DNA. At the time, 1,879 bp of the *Oct4* promoter including the PE had been reported (Okazawa *et al.*, 1991). Sequence of the DE region was not known and *Oct4* promoter clones could not be obtained elsewhere. O4104U and O41894L primers, incorporating *Hin*dIII and *Eco*RI sites respectively (2.2.7), were designed to amplify the region of the *Oct4* promoter from -1776 to +35 (accession No. S58422). This region contains the transcription start sites, the PP and PE regions, plus 554 bp upstream of the PE (Figure 3.1). Polymerase chain reaction (PCR) on E14 ES cell genomic DNA (2.3.29) using O4104U and O41894L primers resulted in a major species of 1,810 bp (Figure 3.2a). Restriction digestion of the PCR product with *Bam*HI produced DNA fragments of 1,300 bp and 500 bp, consistent with a product derived from the *Oct4* promoter (Figure 3.1). The PCR product was digested with *Hin*dIII and *Eco*RI, gel purified, and cloned into *Hin*dIII/*Eco*RI digested pBluescript II KS (Figure 3.2b) and sequenced by manual sequencing reactions (2.3.22) carried out with the T3 primer. A single base difference within the 200 bp sequenced was identified compared to the published *Oct4* promoter sequence (not shown) derived from P19 EC cells (Okazawa *et al.*, 1991).

3.2.2 Isolation of the *Oct4* gene from an ES cell genomic library

To obtain regions of the *Oct4* promoter upstream of the PE a λ E14TG2a genomic library (2.3.19)(Boehm *et al.*, 1991) was screened with a 515 bp *Bst*XI/*Bam*HI fragment derived from the 5′ end of the *Oct4* promoter fragment cloned by PCR (Figure 3.1 and Figure 3.2b). The library had been used successfully for isolation of genomic clones for several genes (Chapman *et al.*, 1997; Merkel *et al.*, 1999).

5 x 10^5 plaques, or 12.5% of the original library complexity, were plated on nine 15 cm plates and duplicate filter lifts were taken. Radiolabelled probes (2.3.16) were hybridised to lifts overnight and the following day lifts were washed twice in 2 x SSC/0.1% SDS for 15 minutes at 42°C then in 0.2 x SSC/0.1% SDS for 15 minutes at 42°C prior to exposure to X-ray film. Two duplicate positive clones, λ3.1 and λ4.1, were identified and subjected to a second round of screening, and a single λ3.1 positive plaque was isolated and expanded. Phage DNA

Figure 3.1

Schematic representation of the mouse *Oct4* gene.

The *Oct4* gene region present in λ3.1 as determined by comparison to Yeom *et al*, (1996) and to sequence of the mouse Major Histocompatibility Complex Class I region (accession No. AF111103). Black boxes indicate exons of the *Oct4* gene and distal enhancer (DE), proximal enhancer (PE) and proximal promoter (PP) (Yeom *et al.*, 1996) are shown. The transcription start site is indicated as an arrow. *Oct4* and *Bam*HI fragments 2 (F2) and 3 (F3) cloned into pBluescript II KS are indicated above the schematic. The genomic PCR product is shown below the schematic as a black line and 1 kb *Bgl*II and 515 bp *Bst*XI/*Bam*HI DNA probes are shown as thick black lines.

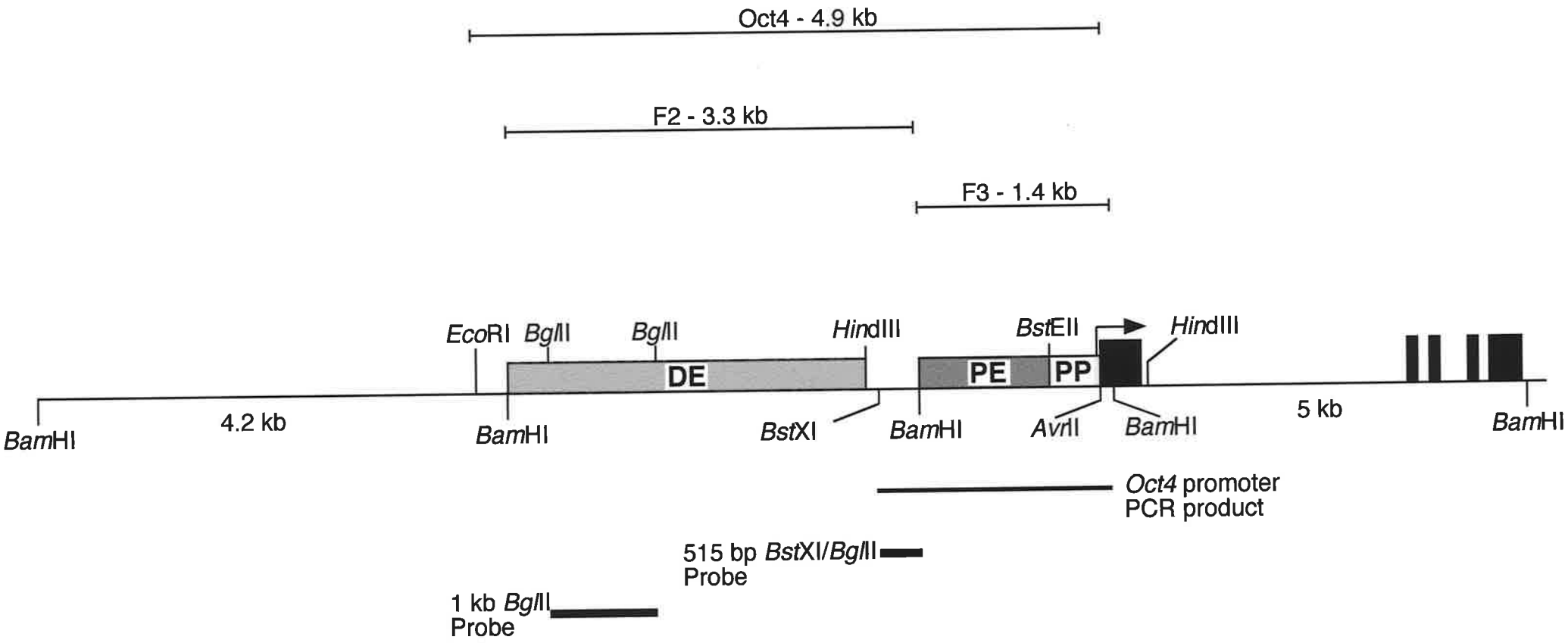
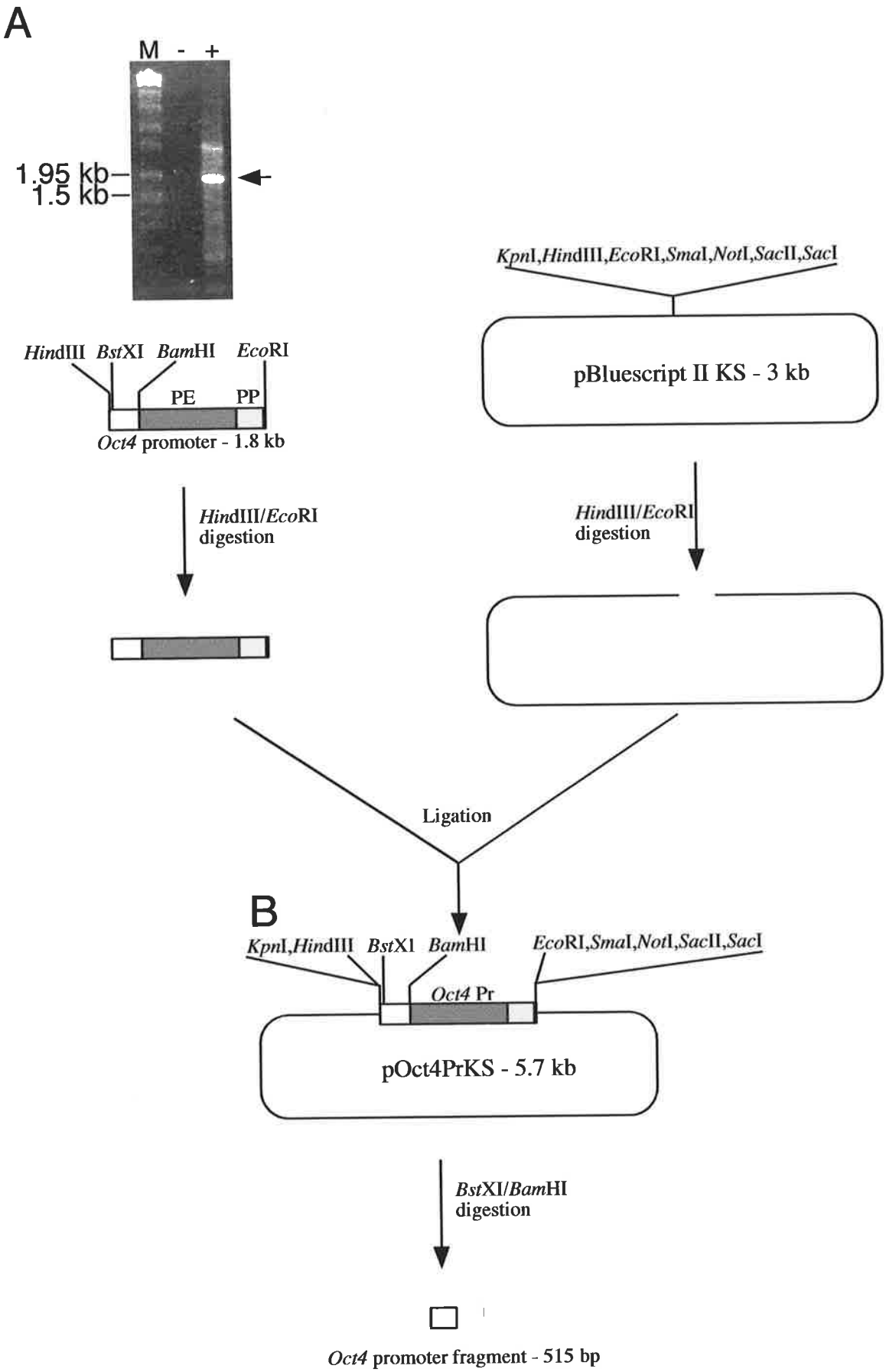

Oct4 - 4.9 kb
F2 - 3.3 kb
F3 - 1.4 kb
EcoRI
BglII
BglII
HindIII
BstEII
HindIII
DE
PE
PP
BamHI
4.2 kb
BamHI
BstXI
BamHI
AvrII
BamHI
5 kb
BamHI
Oct4 promoter
PCR product
515 bp BstXI/BglII
Probe
1 kb BglII
Probe

Figure 3.2

Genomic PCR and cloning of the proximal region of the *Oct4* promoter.

A. Genomic PCR to generate an *Oct4* fragment was performed on E14TG2a genomic DNA for 40 cycles. The amplified region (arrow) includes the proximal enhancer (PE), proximal promoter (PP), and the 5´ transcription start site. -, No DNA; +, 100 ng genomic DNA; M, *Eco*RI digested SPP-1 phage DNA molecular weight markers. 1.5 and 1.95 kb marker bands are indicated.

B. Construction of pOct4PrKS. Positions of *Bam*HI, *Bst*XI, *Eco*RI, and *Hin*dIII restriction sites are indicated. Open box, 515 bp *Bst*XI/*Bam*HI promoter fragment used as a probe; light grey box, PP region; grey box, PE region.


A
M - +
1.95 kb
1.5 kb
HindIII BstXI BamHI EcoRI
PE
PP
Oct4 promoter - 1.8 kb
HindIII/EcoRI digestion
KpnI,HindIII,EcoRI,SmaI,NotI,SacII,SacI
pBluescript II KS - 3 kb
HindIII/EcoRI digestion
Ligation
B
KpnI,HindIII BstXI BamHI
EcoRI,SmaI,NotI,SacII,SacI
Oct4 Pr
pOct4PrKS - 5.7 kb
BstXI/BamHI digestion
Oct4 promoter fragment - 515 bp

preparations (2.3.21) of λ3.1 were digested with *Bam*HI, *Hin*dIII or *Bam*HI/*Hin*dIII, run on a 1% TAE agarose gel, and Southern blotted (2.3.15). The Southern filter was probed with the 515 bp *Bst*XI/*Bam*HI *Oct4* promoter fragment used to screen the genomic library (Figure 3.1 and Figure 3.2b) and washed after overnight hybridisation to a stringency of 0.2 x SSC/0.1% SDS at 65°C.

Southern analysis confirmed that λ3.1 contained an *Oct4* promoter clone as hybridisation was observed to bands of expected sizes 3.3, 2.0, and 0.65 kb fragments with *Bam*HI, *Hin*dIII, or *Bam*HI/*Hin*dIII digestion (Figure 3.3a). The entire *Oct4* gene has been sequenced as a part of the mouse Major Histocompatibility Complex Class I genomic region (unpublished, accession No. AF111103). Restriction analysis indicated that λ3.1 contained all *Oct4* exons and at least 8.8 kb of sequence upstream of the transcriptional start sites, and encompassed the DE region (Figure 3.1 and Figure 3.3b).

3.2.3 Construction of *Oct4*-luciferase reporter plasmids

To design a promoter construct capable of driving ES cell specific expression, regions of the *Oct4* promoter including the PP were cloned in front of the luciferase reporter gene of the pGL3 basic plasmid.

pPPGL3, containing the luciferase gene under control of the PP region alone was produced by cloning *Bam*HI fragment 3 from λ3.1 into the *Bam*HI site of pBluescript II KS to form pF3KS (Figure 3.4). A 235 bp *Avr*II/*Bst*EII fragment corresponding to the PP region was then removed from pF3KS and blunt cloned into *Hin*dIII digested and blunted pGL3 vector (Figure 3.4b).

pPEPPGL3, a construct containing the PE and PP regions in front of the luciferase gene, was formed by cloning *Bam*HI fragment 3 from pF3KS into the *Bgl*II site of pGL3. This construct also contains sequence encoding the start codon and another 48 amino acids of the Oct4 open reading frame (ORF). A derivative of pPEPPGL3, pPEPPGL3HAdr was constructed to remove the Oct4 translation start site and ORF. pPEPPGL3HAdr was formed by *Hin*dIII/*Avr*II digestion, blunting and religation of pPEPPGL3 (Figure 3.4a).

pDEPPGL3, containing the DE and PP regions was formed by cloning *Bam*HI fragment 2 derived from λ3.1 into the *Bgl*II site of pPPGL3 (Figure 3.5a).

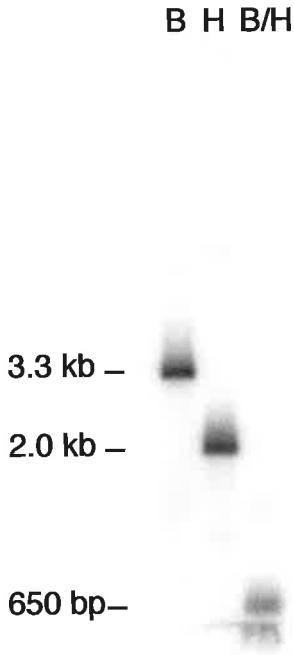
Figure 3.3

Characterisation of a λ clone containing the *Oct4* promoter.

A. Southern analysis of phage DNA preparations from *Oct4* clone λ3.1. Phage DNA was digested with *Bam*HI (B), *Hin*dIII (H), or *Bam*HI/*Hin*dIII (B/H), run on a 1% TAE agarose gel and Southern blotted. The filter was hybridised to a 515 bp *Bst*XI/*Bam*HI fragment of the *Oct4* promoter derived from pOct4PrKS (Figure 3.2) and washed at 0.2 x SSC/0.1% SDS for 20 minutes at 65°C.

B. Restriction analysis of *Oct4* clone λ3.1. λ3.1 was digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Bam*HI/*Eco*RI (B/E), *Bam*HI/*Hin*dIII (B/H), *Eco*RI/*Hin*dIII (E/H) and the digests run on a 1% TAE agarose gel. *Bam*HI fragments of sizes 4.18, 3.29, 1.39 and 1.11 kb correspond to *Bam*HI fragments 1-4 described in Yeom *et al*, (1996)(Figure 3.1). M, *Eco*RI digested SPP-1 phage DNA markers. 0.99, 1.95, 2.8, 3.6, 4.9 and 8.55 kb marker bands are indicated.

A


B H B/H
3.3 kb –
2.0 kb –
650 bp–

B

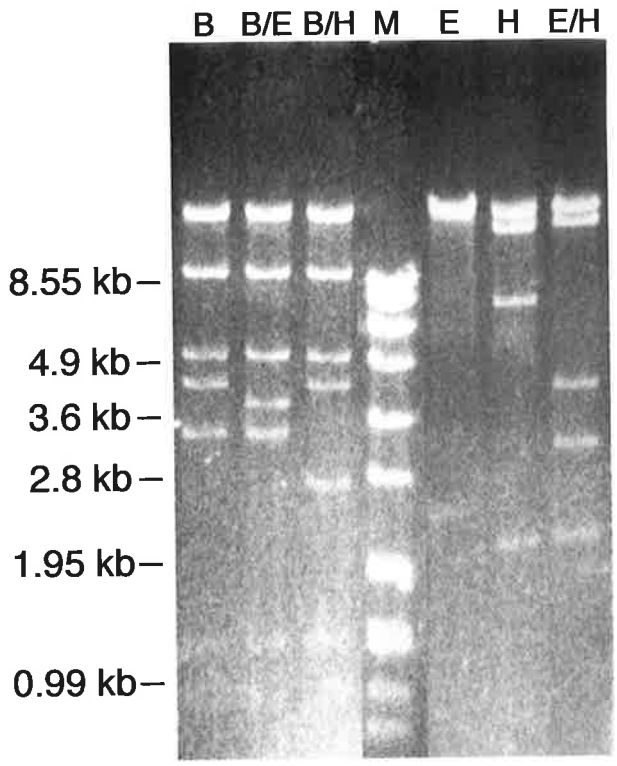

B B/E B/H M E H E/H
8.55 kb–
4.9 kb–
3.6 kb–
2.8 kb–
1.95 kb–
0.99 kb–

Figure 3.4

Construction of *Oct4*-luciferase reporter constructs pPPGL3 and pPEPPGL3HAdr.

Construction of (A) pPEPPGL3HAdr and (B) pPPGL3. pPEPPGL3 contains the luciferase gene downstream of the PE and PP regions and the initiation codon of the *Oct4* gene. The initiation codon and Oct4 coding sequence from pPEPPGL3 was removed to form pPEPPGL3HAdr in which the luciferase reporter gene is expressed from *Oct4* PP and PE regions. pPPGL3 contains the luciferase gene under the control of the *Oct4* PP region. Shading is as described in figure 3.1. Polyadenylation signals (pA) are shown as black boxes.

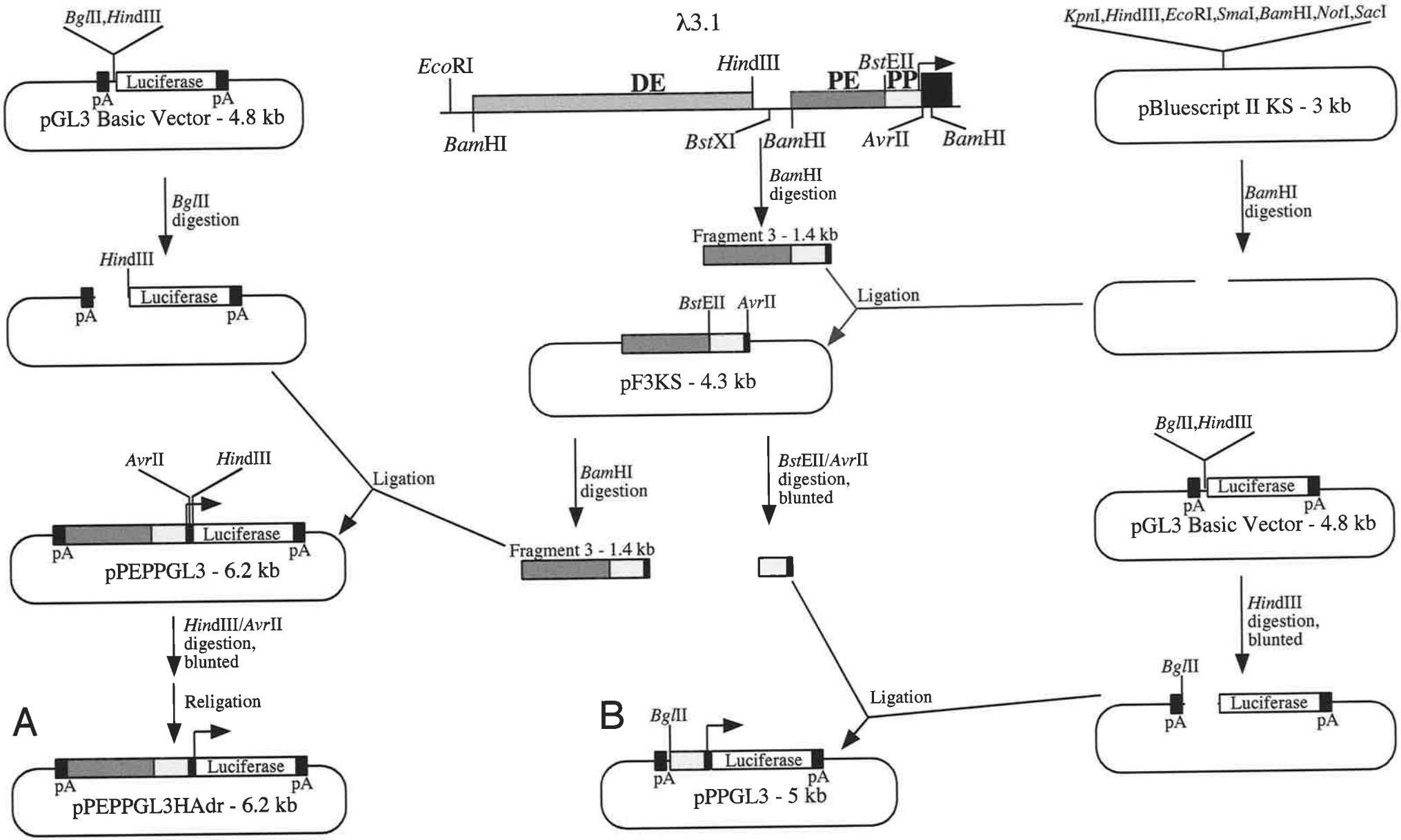
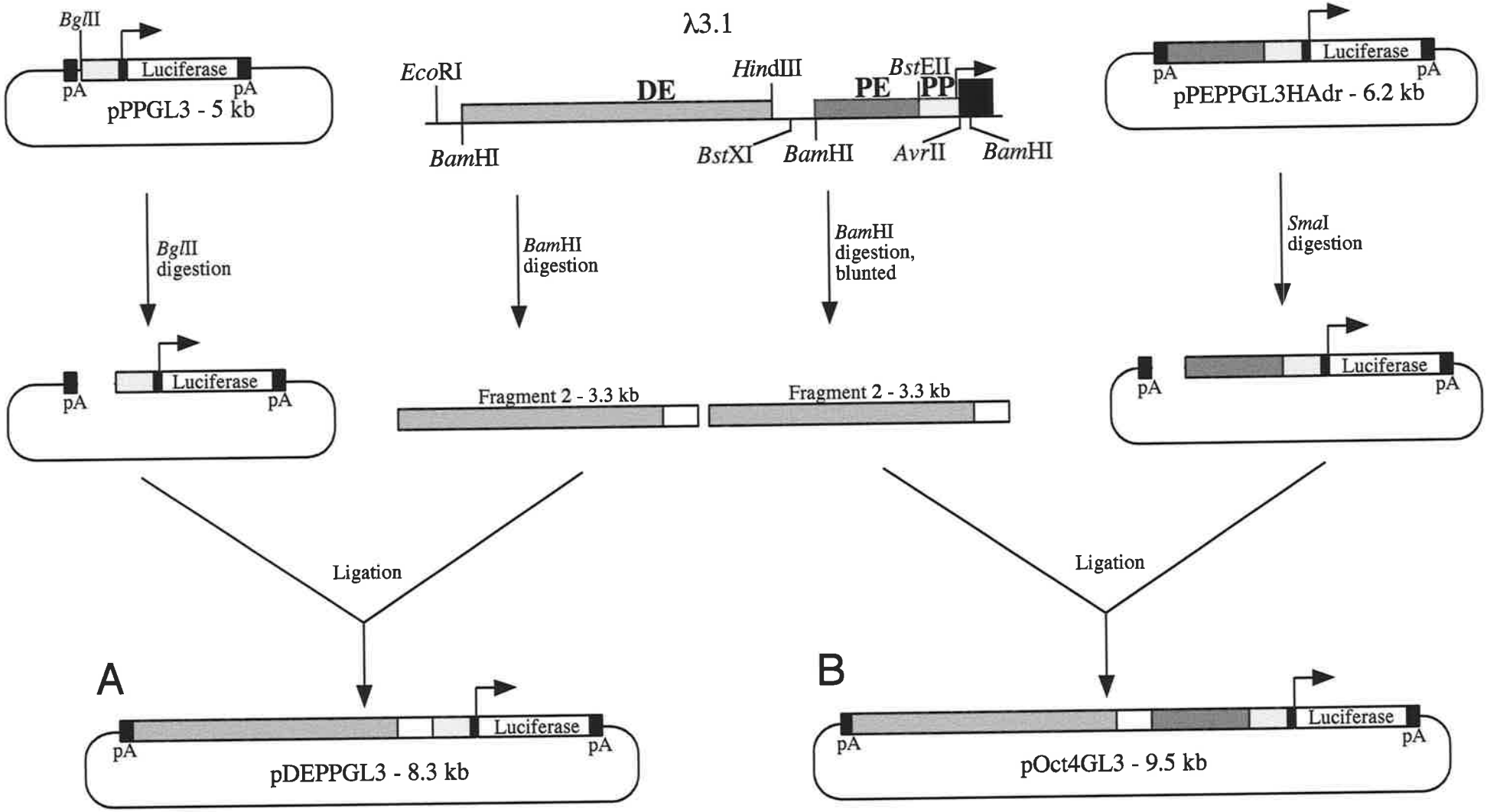

λ3.1
EcoRI
DE
HindIII
PE
BstEII
PP
BamHI
BstXI
BamHI
AvrII
BamHI
KpnI,HindIII,EcoRI,SmaI,BamHI,NotI,SacI
pBluescript II KS - 3 kb
BglII,HindIII
Luciferase
pA
pA
pGL3 Basic Vector - 4.8 kb
BglII digestion
HindIII
Luciferase
pA
pA
BamHI digestion
Fragment 3 - 1.4 kb
Ligation
BstEII AvrII
pF3KS - 4.3 kb
BamHI digestion
BstEII/AvrII digestion, blunted
AvrII
HindIII
Luciferase
pA
pA
pPEPPGL3 - 6.2 kb
Ligation
Fragment 3 - 1.4 kb
BglII,HindIII
Luciferase
pA
pA
pGL3 Basic Vector - 4.8 kb
HindIII/AvrII digestion, blunted
Religation
A
Luciferase
pA
pA
pPEPPGL3HAdr - 6.2 kb
B
BglII
Luciferase
pA
pA
pPPGL3 - 5 kb
Ligation
HindIII digestion, blunted
BglII
Luciferase
pA
pA

Figure 3.5

Construction of *Oct4*-luciferase reporter plasmids pDEPPGL3 and pOct4GL3.

Construction of (A) pDEPPGL3 and (B) pOct4GL3. Expression of the luciferase reporter gene in pDEPPGL3 is driven by the *Oct4* PP and DE regions. In pOct4GL3, the luciferase reporter gene is under the control of the *Oct4* DE, PE and PP regions. Shading is as described in figure 3.1. Polyadenylation signals (pA) are shown as black boxes.


λ3.1
BglII
Luciferase
pA
pA
pPPGL3 - 5 kb
EcoRI
DE
HindIII
PE
BstEII
PP
BamHI
BstXI
BamHI
AvrII
BamHI
Luciferase
pA
pA
pPEPPGL3HAdr - 6.2 kb
BglII
digestion
BamHI
digestion
BamHI
digestion,
blunted
SmaI
digestion
Luciferase
pA
pA
Fragment 2 - 3.3 kb
Fragment 2 - 3.3 kb
Luciferase
pA
pA
Ligation
Ligation
A
B
Luciferase
pA
pA
pDEPPGL3 - 8.3 kb
Luciferase
pA
pA
pOct4GL3 - 9.5 kb

pOct4GL3, containing the DE, PE and PP regions was formed by blunt cloning *Bam*HI fragment 2 derived from λ3.1 into the *Sma*I site of pPEPPGL3HAdr (Figure 3.5b).

3.2.4 Analysis of *Oct4* gene enhancer regions

Oct4 promoter luciferase reporter constructs and pCH110 in which expression of the *β-gal* gene is directed by the constitutive SV40 early promoter, were co-transfected (2.4.14) into undifferentiated ES cells or the fibroblast cell type NIH3T3. Fibroblasts are terminally differentiated and have been used in other studies of *Oct4* promoter activity to indicate cell type specific expression (Yeom *et al.*, 1996). Extracts were assayed for luciferase (2.4.18) and β-galactosidase activity (2.4.17). Luciferase activity was normalised against β-galactosidase activity to control for transfection efficiencies.

The PP region was sufficient to drive luciferase expression in both cell lines. Luciferase expression in NIH3T3 cells was 6.5-fold higher compared to that in ES cells (Figure 3.6), suggesting that in differentiated cells proximal promoter expression is unregulated. Addition of the DE or PE regions reduced expression in NIH3T3 cells and increased expression in ES cells. A 8.8-fold and 9.4-fold decrease in expression in NIH3T3 cells was associated with the addition of the PE or DE region to the PP respectively to levels comparable to PP expression in ES cells. The PE region increased ES cell reporter expression 2.5-fold over the PP region alone (Figure 3.6). This effect was cell type specific because pPEPPGL3HAdr expression was 3.4-fold higher in ES cells than NIH3T3 cells. The DE region drove 5.2-fold higher expression in ES cells than the PP region alone. pOct4GL3, which contains the proximal 4.8 kb of the *Oct4* promoter, including the PP, PE, and DE regions did not express luciferase counts above background in either cell line (Figure 3.6). To confirm this an identical fragment was removed from λ3.1 as a 4.8 kb *Eco*RI/*Avr*II fragment and blunt end cloned into the *Sma*I restriction site of pGL3. Significant luciferase activity was not detected with the re-cloned pOct4GL3 (not shown).

3.2.5 Discussion

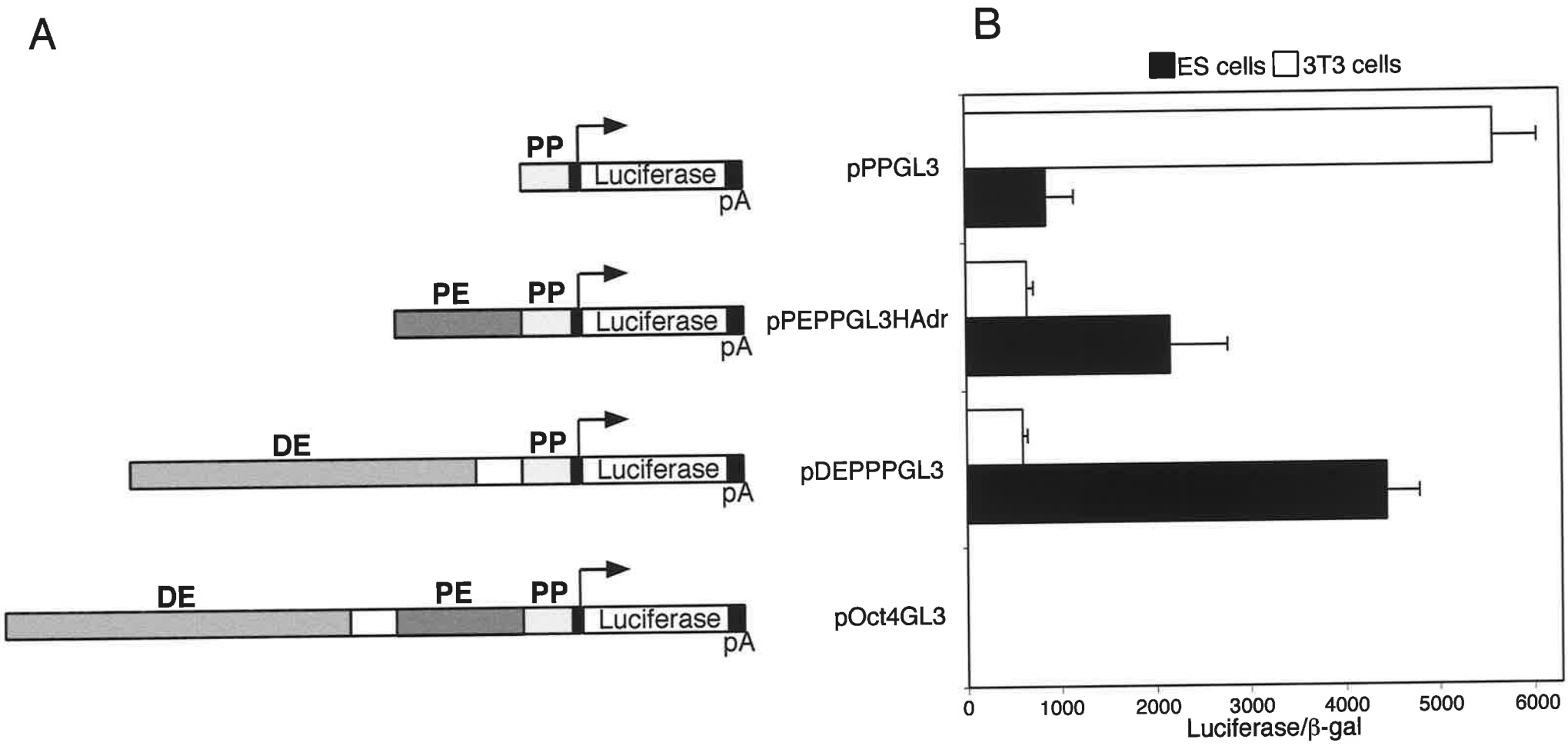
The goal of this work was to identify an expression cassette capable of driving ES cell specific expression which may function to restrict antibiotic resistance gene expression to

Figure 3.6

***Oct4*-luciferase activity in ES cells and differentiated cells.**

A. Schematic representation of the *Oct4*-luciferase constructs pPPGL3, pPEPPGL3, pDEPPGL3 and pOct4GL3.

B. Luciferase expression of *Oct4*-luciferase constructs expressed as the ratio of luciferase counts over β-galactosidase activity. 2 x 10^5 cells were seeded in 35 mm wells for 24 hours and lipofected with pCH110 and construct plasmid DNA using the Lipofectamine Reagent (Gibco/BRL). Cells were harvested 24 hours later and protein concentrations of cell extracts determined by Bradford assay (Bradford, 1976) using an Emax precision plate reader (Molecular Dynamics). Luciferase activity was determined on 25 μg of protein using the luciferase assay system (Promega) and a Berthold LB 9502 Luminometer. β-galactosidase activity in each protein extract was used to control for variation in transfection efficiencies between cell types. Transfections were performed in triplicate and standard deviations are indicated.


A
PP
Luciferase
pA
PE
PP
Luciferase
pA
DE
PP
Luciferase
pA
DE
PE
PP
Luciferase
pA
B
ES cells
3T3 cells
pPPGL3
pPEPPGL3HAdr
pDEPPPGL3
pOct4GL3
0
1000
2000
3000
4000
5000
6000
Luciferase/β-gal

pluripotent cells and thus specifically select against differentiated cells in a population. Pluripotent cell specific expression by the *Oct4* enhancers had been previously demonstrated in a heterologous reporter system by fusing DE and PE regions to the minimal TK promoter (Yeom *et al.*, 1996). Data presented here confirm that the DE region of the *Oct4* promoter directs high expression in ES cells and low expression in differentiated cells. The DE conferred 7.5-fold higher expression in ES cells than in NIH3T3 cells, compared with over 25-fold higher expression reported elsewhere (Yeom *et al.*, 1996). This difference may be attributable to inclusion of the PP in the constructs and may more accurately reflect the action of the DE in the native promoter.

ES cells transfected with pPEPPGL3HAdr expressed 2.5-fold higher luciferase activity than pPPGL3 transfected ES cells (Figure 3.6). Others have reported that the PE increases ES cell expression from the PP by less than 2-fold (Minucci *et al.*, 1996; Yeom *et al.*, 1996). The present work demonstrated that the PE region enhanced expression in ES cells 3.4-fold over NIH3T3 cells suggesting that an unrecognised ES cell specific enhancer activity is associated with the PE region. Expression directed by pPEPPGL3HAdr in ES cells is also consistent with the demonstrated use of PEPPneo fusions to select for undifferentiated ES cells in G418 (McWhir *et al.*, 1996; Mountford *et al.*, 1998) and implies that a 3.4-fold difference in expression is sufficient for selection of ES cells over differentiated cells.

Reporter constructs containing the DE, PE and PP (pOct4GL3) did not drive expression in either ES cells or NIH3T3 cells. This finding was unexpected given that constructs containing the DE, PE and the PP have been reported to drive expression in ES cells (Yeom *et al.*, 1996).

NIH3T3 cells transfected with pPPGL3 expressed high levels of luciferase reporter activity compared with basal transcription driven by TK minimal promoter. Addition of either enhancer restored reporter expression to basal levels (Figure 3.6). The DE and PE enhancers therefore act both to inhibit transcription from the PP in differentiated cells and to induce transcription in ES cells. This finding highlights the importance of the PP region and suggests that faithful reproduction of the *Oct4* expression profile may require the PP region.

PEPPneo constructs can be used to specifically remove differentiated cells (McWhir *et al.*, 1996; Mountford *et al.*, 1998). The PE and DE luciferase constructs expressed equivalent

levels of luciferase activity in differentiated cells. Given that the DE (plus the PP) provided the greatest distinction in expression level between ES cells and differentiated cells, it was the best candidate region to confer antibiotic resistance specifically to pluripotent ES cells.

3.3 ISOLATION AND CHARACTERISATION OF EPISOME-CONTAINING ES CELL LINES

The use of episome-based systems for cDNA library screening has advantages over virus-based approaches that involve retroviral infection of cells. Firstly, controlled gene expression is often not possible using integrative vectors such as retroviruses since DNA integrates into the genome with consequent effects such as disruption of endogenous genes and altered transgene expression due to positional effects. Secondly, while ES cells can be infected by retroviruses, retroviral LTRs are not functional in these cells (Teich *et al.*, 1977; Wagner *et al.*, 1985; Seliger *et al.*, 1986). A retroviral vector that is active in ES cells has been reported (Grez *et al.*, 1990) but drives variable gene expression in infected cells (Laker *et al.*, 1998). Thus it is difficult to drive cell type specific gene expression of heterologous cDNAs using retroviral gene delivery systems in ES cells.

Episomal DNA is wrapped around nucleosomes (Melin *et al.*, 1985) which may help recapitulate specific expression patterns of promoters. An episome-based system has been reported to increase transfection efficiencies to levels that make functional screening in ES cells feasible (Gassmann *et al.*, 1995). The PyV-based episome vector pMGD20neo contains a bacterial origin and ampicillin gene, *PGK*-neo cassette for selection and maintenance of the episome in mammalian cells, the PyF101 mutant enhancer/ori region that allows episome replication in ES cells and a modified form of the PyV early region capable of expressing only the LT antigen gene (Figure 1.9a). 15% of G418 resistant pMGD20neo transfected ES cells contain extrachromosomal pMGD20neo plasmid (Gassmann *et al.*, 1995). In the presence of selection pMGD20neo can replicate, without rearrangement, for at least 74 cell generations. Moreover a second PyV-based plasmid that does not express the LT antigen can be transfected and stably maintained in episome harbouring ES cells (Gassmann *et al.*, 1995). This second transfection, termed supertransfection, is remarkably efficient. High stable transfection efficiencies are not easily achieved in ES cells where the percentage of surviving cells

transiently transfected by electroporation is of the order of 0.5 % (Whyatt, 1991). Using the episome-based system the number of G418-resistant stable colonies increases by around 100-fold compared to standard stable transfection efficiencies (Gassmann *et al.*, 1995). Procedures such as function-based screening that are reliant upon the transfection of large numbers of cells (0.5-1 x 10^6 clones) therefore become feasible.

3.3.1 Construction of pMGD20hph plasmid

The screening procedure outlined in section 1.9 requires maintenance of two plasmids in ES cells using selectable markers. The plasmids pMGD20neo (Figure 1.9a) and pPGKhphΔLT20 (Figure 1.9b) were kind gifts from Dr Greg Donoho (Stanford University). pPGKhphΔLT20 contains the PyF101 enhancer/ori but does not express PyV LT antigen due to a 1,249 bp *Nco*I-*Xba*I deletion within the LT coding sequence (Gassmann *et al.*, 1995). This plasmid also contains a hygromycin B resistance gene under the control of the *PGK-1* promoter in place of the *PGK*-neo cassette in pMGD20neo.

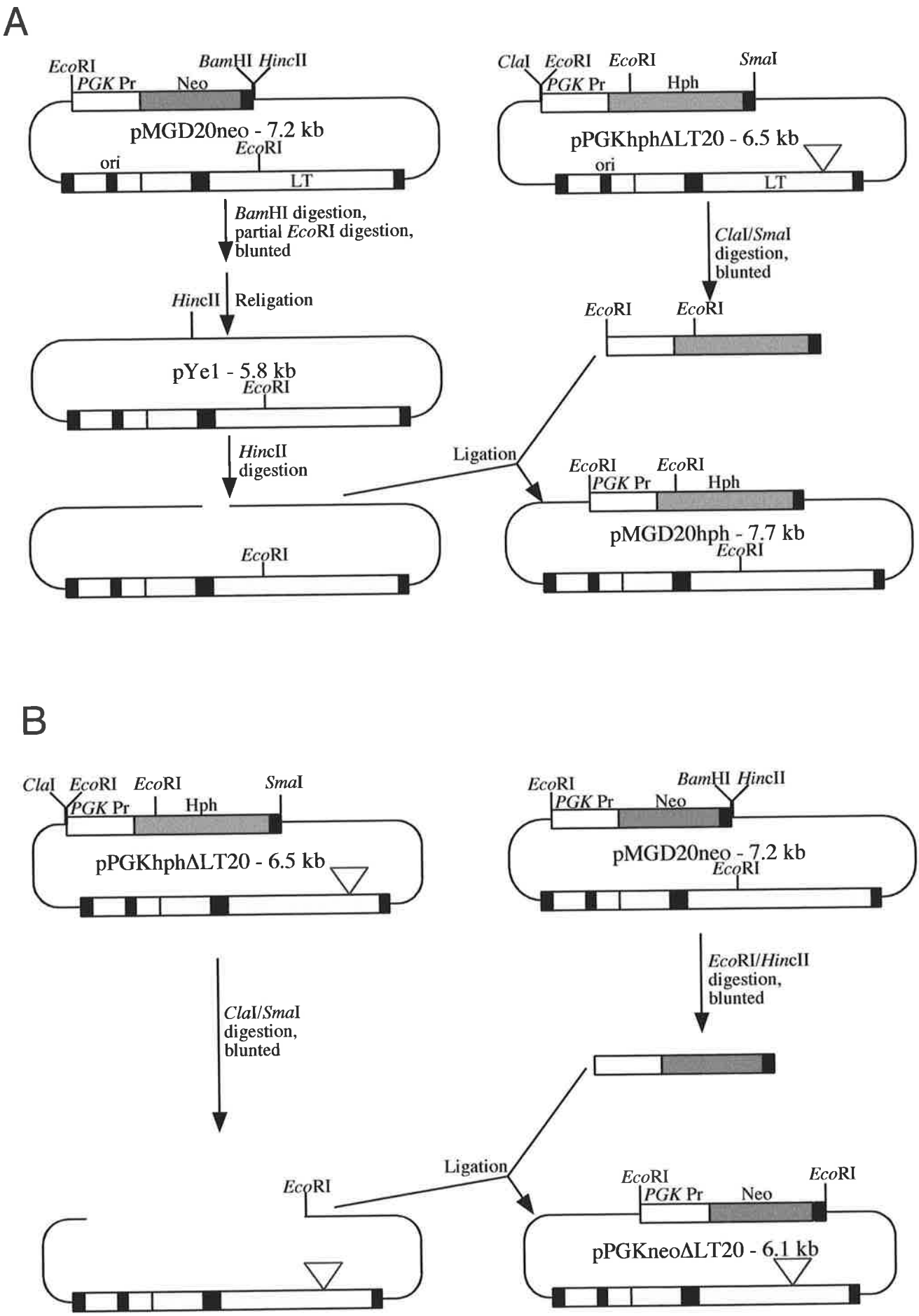
In our hands resistant ES cell colonies take as long as 14 days to visualise amongst adherent cell debris following hygromycin B selection. In contrast, G418 selection for neomycin resistant ES cell colonies is often complete within 7 days. Therefore the neomycin resistance gene was coupled with the *Oct4* promoter to produce an efficient selection cassette for use in the library plasmid. This necessitated replacement of the *neo* cassette with the *hph* selectable marker.

An episome capable of expressing PyV LT and hygromycin B resistance was generated by digestion of pMGD20neo with *Bam*HI. Partial digestion of *Bam*HI linearised pMGD20neo plasmid with 0.5 U of *Eco*RI per μg of DNA for 5-10 minutes at 37°C formed among others, a 5,850 bp fragment. This fragment could only be formed by digestion of the *Eco*RI site 3′ of the PGKneopA region and therefore lacked the PGKneopA cassette. The fragment was blunted and religated to form pPYe1, a plasmid that contains the PyF101 mutant enhancer/ori region. The PGKhphpA selection cassette derived from pPGKhphΔLT20 was cloned into the unique *Hin*cII site of pPYe1 as a 1,840 bp *Cla*I/*Sma*I fragment forming pMGD20hph (Figure 3.7a). Like pMGD20neo, pMGD20hph expresses PyV LT antigen and can be transfected into ES cells to generate episome harbouring ES cell lines.

Figure 3.7

Construction of pMGD20hph and pPGKneoΔLT20 plasmids

Construction of (A) pMGD20hph and (B) pPGKneoΔLT20. pMGD20hph contains the PGKhphpA selection cassette derived from pPGKhphΔLT20, PyF101 ori/enhancer region and LT antigen gene. pPGKneoΔLT20 contains the PGKneopA selection cassette derived from pMGD20neo, PyF101 ori/enhancer region and LT gene that harbours a deletion preventing expression of functional LT antigen. Positions of *Bam*HI, *Cla*I, *Eco*RI, *Hin*cII, *Kpn*I, and *Sma*I restriction sites are indicated. PyV-derived PyF101 ori/enhancer region and the LT antigen gene are shown at the bottom of the plasmids (see also figure 1.9, light grey shading). The 1,249 bp LT20 deletion, depicted as a triangle, abrogates expression of LT antigen. Open box, *PGK-1* promoter; dark grey box, *neo* gene; grey box, *hph* gene; black boxes, polyadenylation signals.


A
EcoRI
PGK Pr
Neo
BamHI HincII
pMGD20neo - 7.2 kb
EcoRI
ori
LT
BamHI digestion,
partial EcoRI digestion,
blunted
HincII
Religation
pYe1 - 5.8 kb
EcoRI
HincII
digestion
EcoRI
ClaI
EcoRI
PGK Pr
Hph
EcoRI
SmaI
pPGKhphΔLT20 - 6.5 kb
ori
LT
ClaI/SmaI
digestion,
blunted
EcoRI
EcoRI
Ligation
EcoRI
PGK Pr
Hph
EcoRI
pMGD20hph - 7.7 kb
EcoRI
B
ClaI
EcoRI
PGK Pr
Hph
EcoRI
SmaI
pPGKhphΔLT20 - 6.5 kb
ClaI/SmaI
digestion,
blunted
EcoRI
EcoRI
Ligation
EcoRI
EcoRI
PGK Pr
Neo
BamHI HincII
pMGD20neo - 7.2 kb
EcoRI
EcoRI/HincII
digestion,
blunted
EcoRI
PGK Pr
Neo
EcoRI
pPGKneoΔLT20 - 6.1 kb

3.3.2 Production of ES cell lines that harbour pMGD20hph episome

In order to generate ES cell lines harbouring episomally replicating pMGD20hph plasmid, 10 μg of CsCl purified (2.3.14) pMGD20hph plasmid DNA was electroporated (2.4.10) into 3 x 10^7 E14TG2a ES cells. A kill curve (not shown) established that 110 μg/ml hygromycin B was the minimum concentration required to kill untransfected cells. After 24 hours cells were subjected to hygromycin B selection for 14 days. A total of 337 colonies survived the selection procedure. Seventy two colonies were picked and expanded into ES cell complete medium containing 110 μg/ml hygromycin B (2.4.11). Forty five of these lines were screened for the presence of episomal pMGD20hph by transformation of low molecular weight Hirt preparation DNA (2.3.25)(Anant and Subramanian, 1992; Hirt, 1967) into bacteria.

Hirt preparations of two pMGD20hph stable lines, C32 and C41, were found to contain a plasmid capable of replicating in *E. coli*. This plasmid was indistinguishable from transfected pMGD20hph by *Eco*RI digestion of plasmid DNA from multiple independent *E. coli* transformants (Figure 3.8a). Hygromycin B resistant, episome containing ES cell lines formed at a lower efficiency than reported for pMGD20neo (4.4% versus 15%) (Gassmann *et al.*, 1995).

To demonstrate that pMGD20hph plasmid present in Hirt preparations had replicated extrachromosomally and was not due to contamination, Southern analysis was performed on Hirt preparation DNA from parental E14TG2a, C32, and C41 ES cells and on bacterially derived pMGD20hph plasmid DNA. DNA synthesised in mammalian cells differs from bacterially derived DNA in that it is not methylated at *Dpn*I sites, and is therefore resistant to restriction digestion by *Dpn*I endonuclease. This property can be used to determine a mammalian or bacterial origin of plasmid DNA.

Undigested and *Bam*HI, *Kpn*I and *Kpn*I/*Dpn*I digested Hirt preparation DNAs were electrophoresed on a 1% TAE agarose gel, transferred to a nylon membrane (2.3.15) and hybridised to a 544 bp *Ava*I/*Eco*RI PyV LT antigen specific cDNA probe derived from pMGD20neo (2.3.16). Hybridisation was observed to C32 and C41 Hirt DNA and bacterially derived pMGD20hph but not to Hirt DNA derived from parental E14TG2a ES cells (Figure 3.8b). Episome bands that co-migrated with bacterial pMGD20hph were present in uncut Hirt

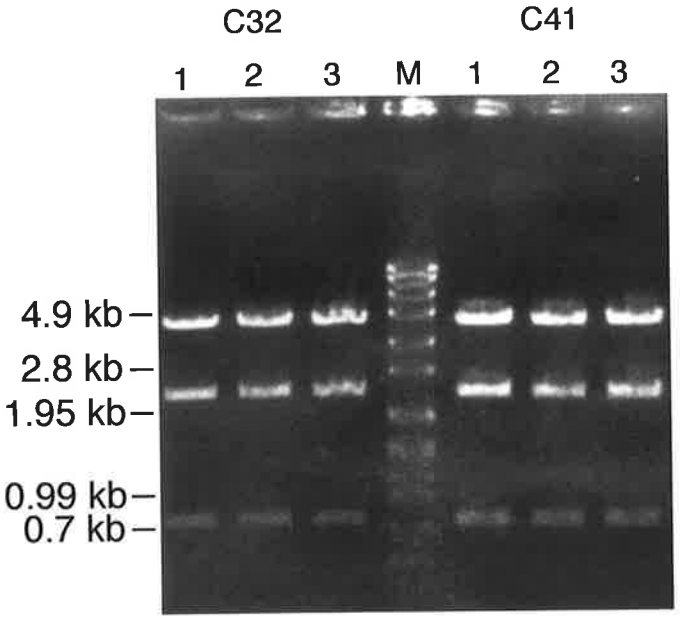
Figure 3.8

Analysis of episome harbouring ES cell lines.

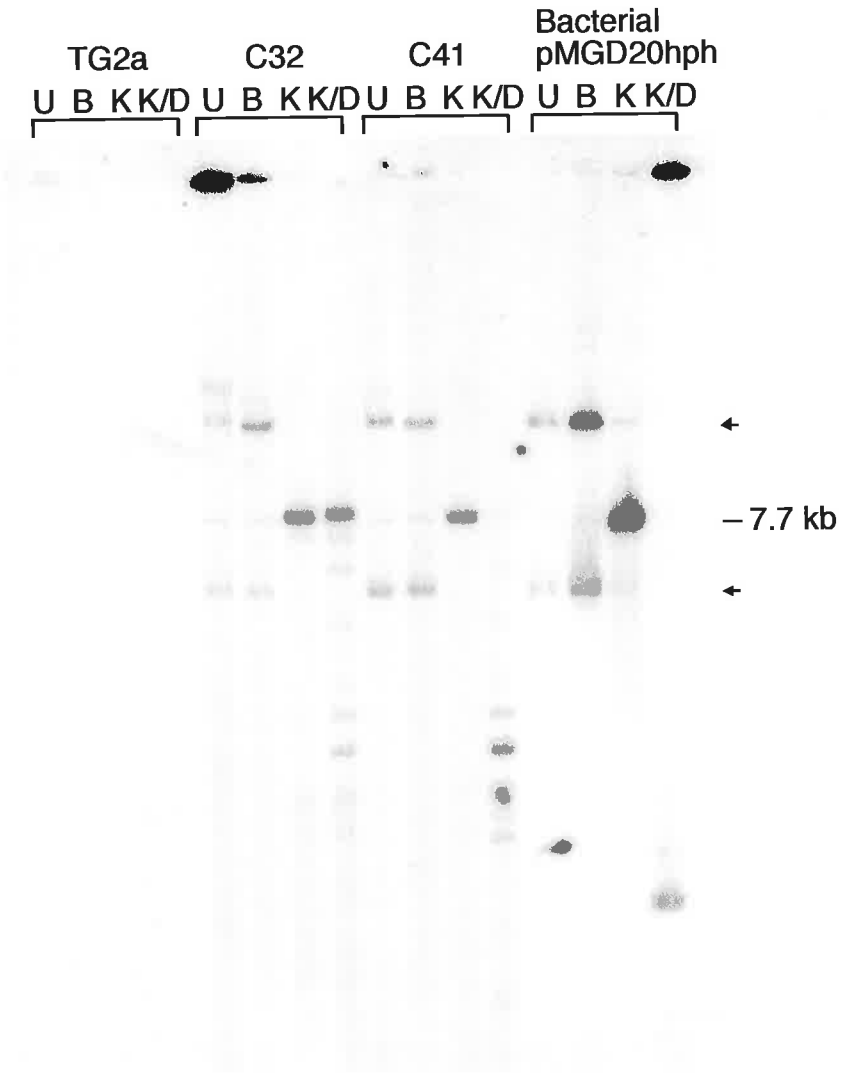
A. *Eco*RI digestion of transformed plasmids derived from C32 and C41 ES cells. Hirt preparation DNA from C32 and C41 ES cells was transformed into *E. coli*. Plasmid DNA from transformants were digested with *Eco*RI and electrophoresed on a 1% TAE agarose gel. M, *Eco*RI digested SPP-1 phage DNA markers. 0.7, 0.99, 1.95, 2.8, and 4.9 kb marker bands are indicated.

B. A Southern blot of Hirt preparation DNA from episome harbouring ES cells. Hirt preparation DNA from TG2a, C32, and C41 ES cells and 1 ng of bacterially derived pMGD20hph were digested with *Bam*HI (B), *Kpn*I (K) or *Kpn*I/*Dpn*I (K/D). Uncut Hirt preparation DNA (U) and digested DNA was electrophoresed on a 1% TAE agarose gel and Southern blotted. The filter was probed with a 544 bp *Ava*I/*Eco*RI fragment derived from pMGD20neo, specific for the PyV LT antigen gene. The blot was washed to a stringency of 0.2 x SSC/0.1% SDS at 65°C. Linearised pMGD20hph episome runs as a 7.7 kb band. Arrows designate bands observed in undigested DNA.

A


C32
C41
1
2
3
M
1
2
3
4.9 kb
2.8 kb
1.95 kb
0.99 kb
0.7 kb

B


TG2a
C32
C41
Bacterial pMGD20hph
U B K K/D U B K K/D U B K K/D U B K K/D
7.7 kb

DNA samples from C32 and C41 ES cells (Figure 3.8b, arrows) and were refractory to *Bam*HI digestion, a site not present in pMGD20hph. This indicates that at least some of the pMGD20hph derived DNA in C32 and C41 ES cells was present as extrachromosomal DNA (Figure 3.8b, lanes U and B, arrows). C32, C41 Hirt DNA and bacterially derived pMGD20hph plasmid contained a *Kpn*I fragment of 7,690 bp indicating that the plasmid in C32 and C41 cells was unrearranged pMGD20hph. This band in C32 and C41 Hirt DNA was resistant to *Dpn*I digestion (Figure 3.8b lanes K and K/D). In contrast, bacterial pMGD20hph plasmid was sensitive to *Dpn*I digestion indicating that pMGD20hph-derived DNA in the low molecular weight fraction DNA of ES cell lines C32 and C41 was unmethylated, consistent with replication in ES cells. Taken together, these data demonstrate that pMGD20hph plasmid had replicated episomally in C32 and C41 ES cells.

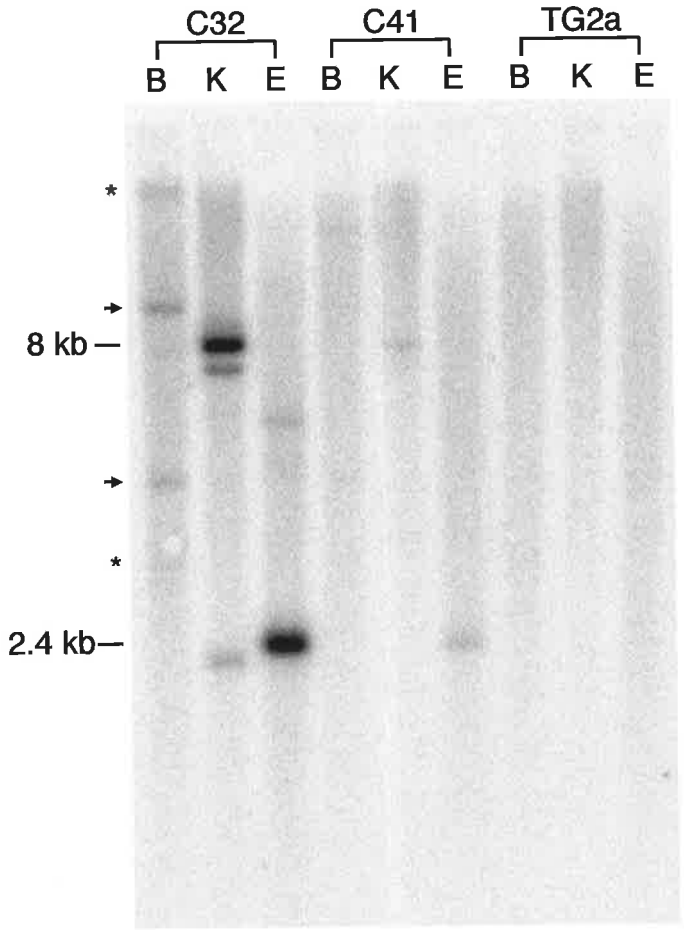
Southern blot detection of pMGD20hph episome in C32 and C41 ES cells (Figure 3.8b) demonstrated that pMGD20hph plasmid was capable of replicating extrachromosomally for as long as 18 passages or approximately 89 generations assuming a 14.5 hour cell cycle for E14 derived ES cells (Udy *et al.*, 1997). Therefore C32 and C41 ES cells stably maintained pMGD20hph episome without overt rearrangement for many passages in culture.

Southern analysis was extended to genomic DNA isolated from C32 and C41 ES cell lines to determine if pMGD20hph plasmid had also integrated into the genome. Genomic DNA isolated from E14TG2a, C32 and C41 ES cells (2.3.24) was digested with *Bam*HI, *Kpn*I, or *Eco*RI endonucleases and Southern blotted as above (2.3.15). Hybridisation was observed to bands in *Kpn*I and *Eco*RI digested C32 and C41 genomic DNA of approximately 8 kb and 2.4 kb corresponding to linearised pMGD20hph and the PyV LT antigen gene region, respectively (Figure 3.9, arrows). These bands are likely to represent episome contamination of genomic DNA because hybridisation in *Bam*HI digested genomic DNA was observed at sizes observed previously for uncut pMGD20hph plasmid (Figure 3.9, C32 lane B). However, additional secondary bands of lower intensity were also observed in digested C32 genomic DNA lanes (Figure 3.8b) and are likely to represent genomic integrated copies of pMGD20hph. C41 ES cell genomic DNA did not contain bands other than those expected from pMGD20hph episome and therefore was unlikely to contain integrated copies of pMGD20hph (Figure 3.9).

Figure 3.9

Detection of pMGD20hph derived sequence in genomic DNA.

Genomic DNA from E14TG2a, C32, and C41 ES cells was digested with *Bam*HI (B), *Kpn*I (K) or *Eco*RI (E) and analysed as in figure 3.8b. Bands of approximately 8 kb and 2.4 kb representing linearised pMGD20hph episome and the PyV LT gene region respectively are indicated. Arrows designate bands observed on previous blots (Figure 3.8b) that represent uncut pMGD20hph plasmid. Asterix mark extra bands observed in *Bam*HI digested C32 cell genomic DNA not accounted for by pMGD20hph episome contamination.


C32
C41
TG2a
B K E B K E B K E
*
8 kb
*
2.4 kb

3.3.3 Supertransfection in episome harbouring ES cell lines C32 and C41.

The PGKhphpA selection cassette from the PyV-based plasmid pPGKhphΔLT20 was removed and replaced with PGKneopA to allow selection of two episomes in the cells. pPGKneoΔLT20 was formed by cloning a 1,400 bp *Eco*RI/*Hin*cII PGKneopA fragment derived from pMGD20neo into blunted *Cla*I/*Sma*I digested pPGKhphΔLT20 (Figure 3.7b). pPGKneoΔLT20 contains a neomycin resistance selection cassette and the PyF101 mutant ori/enhancer region to allow selection and replication in mammalian cells. It also contains a form of the PyV LT gene carrying a deletion that abolishes active LT protein expression. Absence of LT expression from the second supertransfected plasmid is necessary for high efficiency supertransfection (Dr Greg Donoho, personal communication). pPGKneoΔLT20 and pMGD20hph can be selected and maintained in the same ES cells by addition of G418 and hygromycin B, respectively.

Transient transfection and stable supertransfection efficiencies were compared between parental E14TG2a and C32 ES cells. 1 x 10^7 E14TG2a and C32 ES cells were transfected with pPGKneoΔLT20 or pCH110 (2.2.6) and either selected in 200 μg/ml G418 for 10 days or stained to detect β-galactosidase activity after 24 hours, respectively. Supertransfected C32 cells formed 48-fold more G418 resistant colonies than E14TG2a cells transfected with pPGKneoΔLT20 (23,560 versus 494; Table 3.1). Two pMGD20hph harbouring lines derived from R1 ES cells could also be supertransfected at high frequency with a second episome plasmid (data not shown). The rate of supertransfection was consistent with, although lower than observations reported by Gassmann *et al,* (1995). Transient transfection rates were 2.4-fold higher in C32 ES cells than E14TG2a ES cells in this experiment. Transient transfection of C32 cells appeared less efficient than stable supertransfection with pPGKneoΔLT20. This may be due to variable expression of the *β-gal* gene in pCH110 by the SV40 early promoter and consequently underestimation of β-galactosidase positive cells or inherent differences in the ability of distinct plasmids to transfect ES cells. Finally, low level transfection of pCH110 may be below the detection limit of the β-galactosidase staining protocol while low level transfection of pPGKneoΔLT20 would result in resistant colonies as a consequence of episome replication.

To investigate whether both episome harbouring ES cell lines supported supertransfection, C32 and C41 ES cells were supertransfected with pPGKneoΔLT20 and

selected in 200 μg/ml G418 for a period of 10 days. There was a dramatic difference in the number of resistant colonies formed between lines in that C32 ES cells formed approximately 15,000 resistant colonies, 50-fold more than observed in transfected C41 plates (Table 3.1). Moreover, another ES cell line that maintained extrachromosomal pMGD20neo did not exhibit supertransfection efficiencies above normal (data not shown). Therefore the presence of extrachromosomal pMGD20hph does not necessarily confer elevated supertransfection efficiencies.

Supertransfection experiments were carried out using the pPGKβgeo as a control to compare stable transfection efficiencies of nonreplicating plasmids to supertransfection of the epsiome-based plasmid pPGKneoΔLT20. pPGKβgeo contains a β-gal-neo gene fusion is under control of the *PGK-1* promoter. This construct was formed by blunt cloning a 4.3 kb *Bst*XI/*Hin*dIII βgeo fragment from pIRES-βgeo (2.2.6) into *Eco*RI digested and blunted pPGKKS (Figure 3.10).

E14TG2a and C32 ES cells were transfected with pPGKneoΔLT20 or pPGKβgeo and either stained for β-galactosidase activity after 24 hours or selected in 200 μg/ml G418 for 17 days. As in previous experiments transient transfection of C32 ES cells was more efficient than that of E14TG2a ES cells. E14TG2a transfected ES cells formed similar numbers of resistant colonies with both plasmids (Table 3.2), comparable to efficiencies previously observed (Table 3.1). The number of G418 resistant colonies formed in pPGKneoΔLT20 supertransfected C32 ES cell cultures was 10-fold and 8.5-fold higher than transfected E14TG2a cells (Table 3.2). G418 selection for 10 days resulted in numbers of stable colonies comparable, but lower than those previously reported, while continued selection decreased the stable supertransfection efficiency to approximately 10-fold greater than standard ES cell stable transfection efficiencies.

3.3.4 Discussion

Highly efficient stable transfection is necessary to achieve effective screening of large numbers of ES cell clones and therefore cDNAs including low abundance cDNAs. Supertransfection of ES cells which contain pMGD20hph with a second episome plasmid containing the PyV ori resulted in high frequency stable transfection (Table 3.1 and Table 3.2).

Table 3.1 Transfection efficiencies of episome harbouring ES cell lines

Experiment	Plasmid	ES cells	C32	C41	C32/ES cells
No.1					
	PGKneoΔLT20	-	15136	300	-
No.2					
	pCH110(βgal stain)	4730	14,400	ND	2.4
	PGKneoΔLT20	494	23,560	ND	48

Experiments were selected in 200 μg/ml G418 for 10 days. ND, not done.

Table 3.2 The efficiency of stable transfection and supertransfection

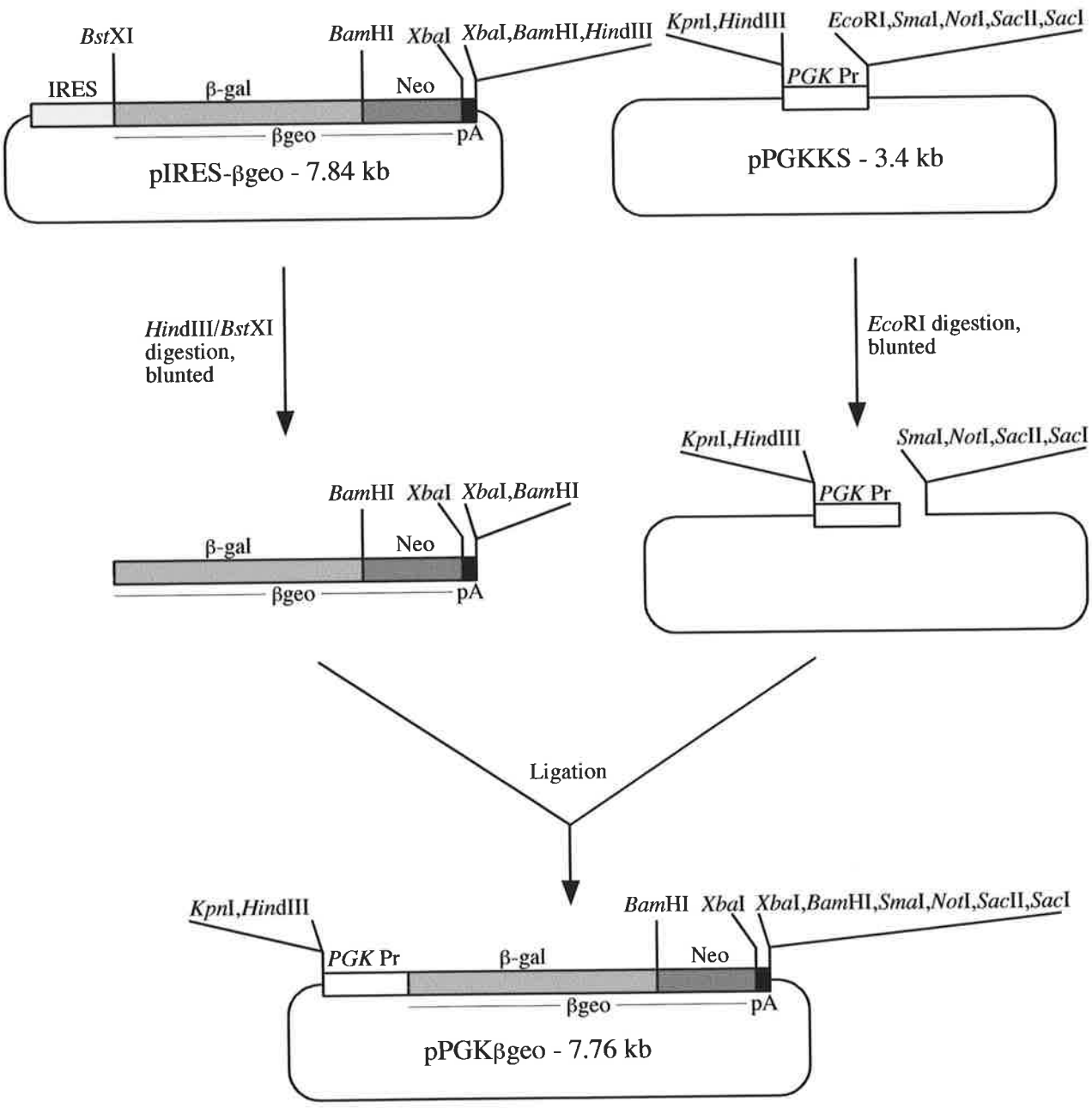
Experiment	Plasmid	ES cells	C32	C32/ES cells
No.1				
	PGKβgeo	303	1982	6.54
	PGKneoΔLT20	392	3896	9.94
No.2				
	PGKβgeo	210	348	1.66
	PGKneoΔLT20	352	3000	8.5

Experiments were selected in 200 μg/ml G418 for 17 days.

Figure 3.10

Construction of pPGKβgeo plasmid.

pPGKβgeo drives expression of the β-gal neomycin resistance gene fusion from the constitutive *PGK-1* promoter. Positions of *Bam*HI, *Bst*XI, *Eco*RI, *Hin*dIII, *Kpn*I, *Sma*I, and *Xba*I restriction sites are indicated. Open box, *PGK-1* promoter; dark grey box, *neo* gene; grey box, *β-gal* gene; black box, polyadenylation signals.


BstXI
BamHI
XbaI
XbaI,BamHI,HindIII
KpnI,HindIII
EcoRI,SmaI,NotI,SacII,SacI
IRES
β-gal
Neo
PGK Pr
βgeo
pA
pIRES-βgeo - 7.84 kb
pPGKKS - 3.4 kb
HindIII/BstXI digestion, blunted
EcoRI digestion, blunted
Ligation
pPGKβgeo - 7.76 kb

However C41 ES cells which also harbour pMGD20hph and another cell line that contains pMGD20neo formed stable colonies at rates equivalent to parental ES cells when transfected with pPGKneoΔLT20. Thus, there appears to be at least two classes of ES cell lines that contain extrachromosomal pMGD20hph plasmid: those that support supertransfection at between 10-100-fold greater than the frequency of transfection and those in which the frequency of supertransfection is not higher than that of transfection.

10-fold higher supertransfection of C32 ES cells was lower than other reports which describe supertransfection efficiencies of 100-fold (Gassmann *et al.*, 1995; Niwa *et al.*, 1998). This is unlikely to be due to cell line specific variability because episome harbouring ES cell lines derived from distinct parental ES cell lines were found to supertransfect at 10-fold higher rates also. The apparent discrepancy may be explained by variation in the manner in which supertransfection efficiency was calculated. The efficiency of supertransfection was found to decrease when selection was extended to 15 days, from an apparent frequency of 50-fold higher at 10 days to 10-fold by 15 days (3.3.3). Loss of supertransfectants following extended selection may therefore account for the lower than reported supertransfection rate given that Niwa *et al*, (1998) selected supertransfectants for only 8 days in 80 μg/ml hygromycin B. This indicates that the true rate of stable supertransfection efficiency is in the order of 10-fold greater than standard stable transfection.

While episome harbouring ES cell lines must express enough PyV LT antigen to maintain the pMGD20hph episome, it is possible that the level of LT expression is important for high supertransfection. High level resistant colony formation is not observed when episome harbouring lines are supertransfected with a second episome expressing LT antigen (Dr Greg Donoho, personal communication) suggesting that high LT expression abrogates supertransfection and replication of the second episome. While C32 and C41 cells appear to contain roughly equivalent amounts of extrachromosomal pMGD20hph plasmid (1.35-fold greater in C32 than C41, Figure 3.8b), the levels of LT antigen expressed in these cells was not measured.

ES cell lines C32 and C41 stably maintain episomes without detectable rearrangement for at least 89 cell generations. Episome stability is an essential property of this system that allows recovery and identification of functional cDNA clones from surviving ES cells.

It was estimated that approximately 125 electroporations (10 μg DNA, 1 x 10^7 cells) would screen 5 x 10^5 clones and electroporation conditions can be scaled up to reduce this number further. Thus the 10-fold increase in transfection provided by the episome system is sufficient to screen large numbers of cDNA library clones.

3.4 EPISOME-BASED SELECTION FOR PLURIPOTENT CELLS

Pluripotent cell specific expression of a selectable marker such as the neomycin resistance gene under the control of the *Oct4* promoter could selectively remove differentiated cells as they form. An *Oct4*-neo selection cassette must be constructed and tested in the context of the episomal vector for the ability to selectively remove differentiated cells as described in figure 1.10.

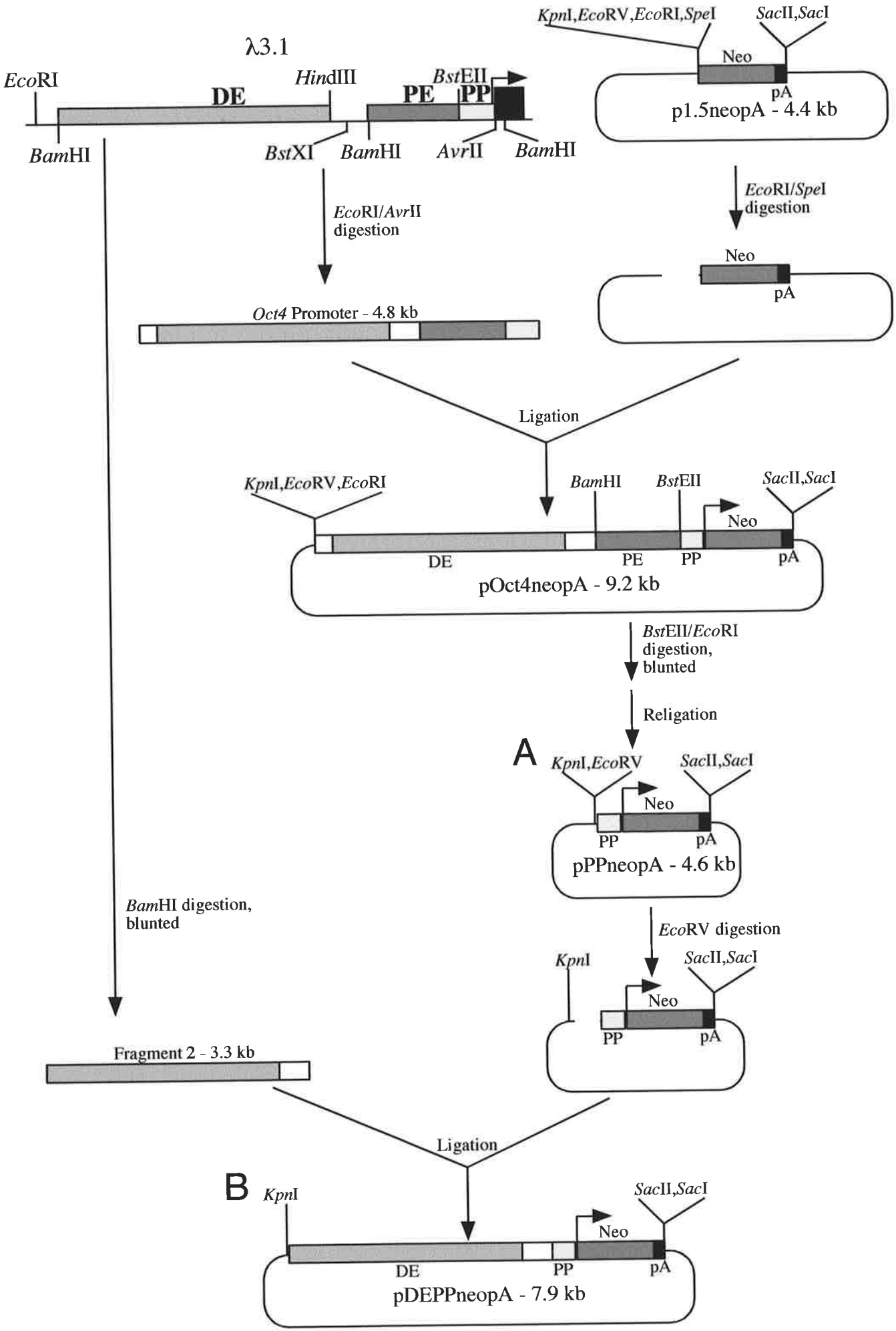
3.4.1 Construction of *Oct4*-neomycin plasmids

Oct4 promoter-neomycin plasmids were constructed to determine an appropriate region of the *Oct4* promoter for selection against differentiated cells in the context of an episome vector. A 1.5 kb neomycin-pA fragment was removed from pIRES-βgeo (Mountford *et al.*, 1994) as a *Bam*HI fragment and blunt cloned into *Not*I digested and blunted pKS to create pNeopA (not shown). Sequencing revealed that the translation initiation site of the *neo* gene in pNeopA (CGCAATATGG) conformed to the consensus for efficient translation GCC(A/G)CCAUGG (Kozak, 1991). The proximal 5 kb of the *Oct4* promoter was excised from λ3.1 as a *Eco*RI/*Avr*II fragment and directionally cloned into *Eco*RI/*Spe*I digested pNeopA. The resulting plasmid, pOct4neopA, contains the neomycin resistance gene under the control of the DE, PE, and PP regions of the *Oct4* promoter including the 5′ transcription initiation site (Figure 3.11). pPPneopA, a related construct that retains only the PP region of the *Oct4* promoter was formed by removal of a 4.7 kb *Eco*RI/*Bst*EII fragment from pOct4neopA by *Eco*RI/*Bst*EII digestion and religation (Figure 3.11a). *Bam*HI fragment 2 was excised from λ3.1 and blunt cloned into the *Eco*RV site of pPPneopA to form pDEPPneopA, a plasmid in which expression of neomycin resistance is directed by the *Oct4* promoter DE and PP regions (Figure 3.10b).

Figure 3.11

Construction of pDEPPneopA and pPPneopA.

Diagrammatic representation of (A) pPPneopA which drives neomycin expression from the PP region of the *Oct4* promoter and (B) pDEPPneopA in which neomycin expression is driven by the PP and DE regions of the *Oct4* promoter. Shading is as described in figure 3.1.


λ3.1
EcoRI
DE
HindIII
PE
BstEII
PP
BamHI
BstXI
BamHI
AvrII
BamHI
KpnI,EcoRV,EcoRI,SpeI
SacII,SacI
Neo
pA
p1.5neopA - 4.4 kb
EcoRI/AvrII digestion
EcoRI/SpeI digestion
Neo
pA
Oct4 Promoter - 4.8 kb
Ligation
KpnI,EcoRV,EcoRI
BamHI
BstEII
SacII,SacI
Neo
DE
PE
PP
pA
pOct4neopA - 9.2 kb
BstEII/EcoRI digestion, blunted
Religation
A
KpnI,EcoRV
SacII,SacI
Neo
PP
pA
pPPneopA - 4.6 kb
BamHI digestion, blunted
EcoRV digestion
KpnI
SacII,SacI
Neo
PP
pA
Fragment 2 - 3.3 kb
Ligation
B
KpnI
SacII,SacI
Neo
DE
PP
pA
pDEPPneopA - 7.9 kb

The hygromycin selection cassette was excised from pPGKhphΔLT20 by digestion with *Cla*I and *Sma*I, endfilling and religation to form pC/SdrΔLT20 (Figure 3.12). *Oct4*-neo selection cassettes were excised from pPPneopA, pDEPPneopA and pOct4neopA as *Kpn*I/*Ecl*136II fragments, blunted and cloned into *Eco*RI digested and blunted pC/SdrΔLT20. The resulting plasmids are referred to as pPPneoΔLT20 (Figure 3.12a), pDEPPneoΔLT20 (Figure 3.12b), and pOct4neoΔLT20 (Figure 3.12). pPEPPneoΔLT20 (Figure 3.12c), in which neomycin phosphotransferase expression is directed by the *Oct4* PE and PP regions was formed by removal of a 3.7 kb *Eco*RI/*Bam*HI fragment from pOct4neoΔLT20. Previous evidence (3.2.4) (Yeom *et al.*, 1996) suggests that pDEPPneoΔLT20 will confer the most efficient selection against differentiated cells.

3.4.2 Selective removal of differentiated cells and selection for undifferentiated cells using *Oct4*-neo episomes

C32 ES cells (3.3.2) were supertransfected (2.4.10) with pDEPPneoΔLT20, pPEPPneoΔLT20, pPPneoΔLT20 or pPGKneoΔLT20 that should express neomycin phosphotransferase equally well in ES cells and differentiated cells. Supertransfected cells were differentiated by LIF withdrawal and selected by culture in 800 μg/ml of G418 for 10 days (2.4.12).

ES cells supertransfected with an episome containing the *PGK*neo construct differentiated upon LIF withdrawal. Remaining undifferentiated (AP^+) ES cells and differentiated derivatives were not killed in the presence of G418 (Figure 3.13) consistent with constitutive neo expression. Neomycin phosphotransferase expression driven by the PP region alone or by PE+PP did not select against differentiated cells in the presence of G418.

In cultures supertransfected with pDEPPneoΔLT20, G418 selection resulted in a drastic reduction in the number of cells surviving selection, suggesting that these constructs can be used to select against differentiated cells (Figure 3.13). Some differentiated cells and undifferentiated AP^+ ES cell colonies were observed to survive the selection procedure. The reduced number of pluripotent colonies surviving selection in pDEPPneoΔLT20 supertransfected cultures is thought to result from a decrease in paracrine LIF supply due to reduced numbers of differentiated cells. Thus, as expected pDEPPneoΔLT20 supertransfection

Figure 3.12

Construction of *Oct4*-neo containing episomes.

A diagram of (A) pPPneoΔLT20, (B) pDEPPneoΔLT20 and (C) pPEPPneoΔLT20 episomes derived from pDEPPneopA, pPPneopA and pOct4neopA plasmids, respectively. pPPneoΔLT20 and pDEPPneoΔLT20 contain selection cassettes where *neo* gene expression is driven by the *Oct4* PP and DE plus PP regions, respectively. pPEPPneoΔLT20 was derived from pOct4neoΔLT20 and contains a selection cassette where the *Oct4* PE and PP regions are upstream of the *neo* gene. Shading is as described in figure 3.1.

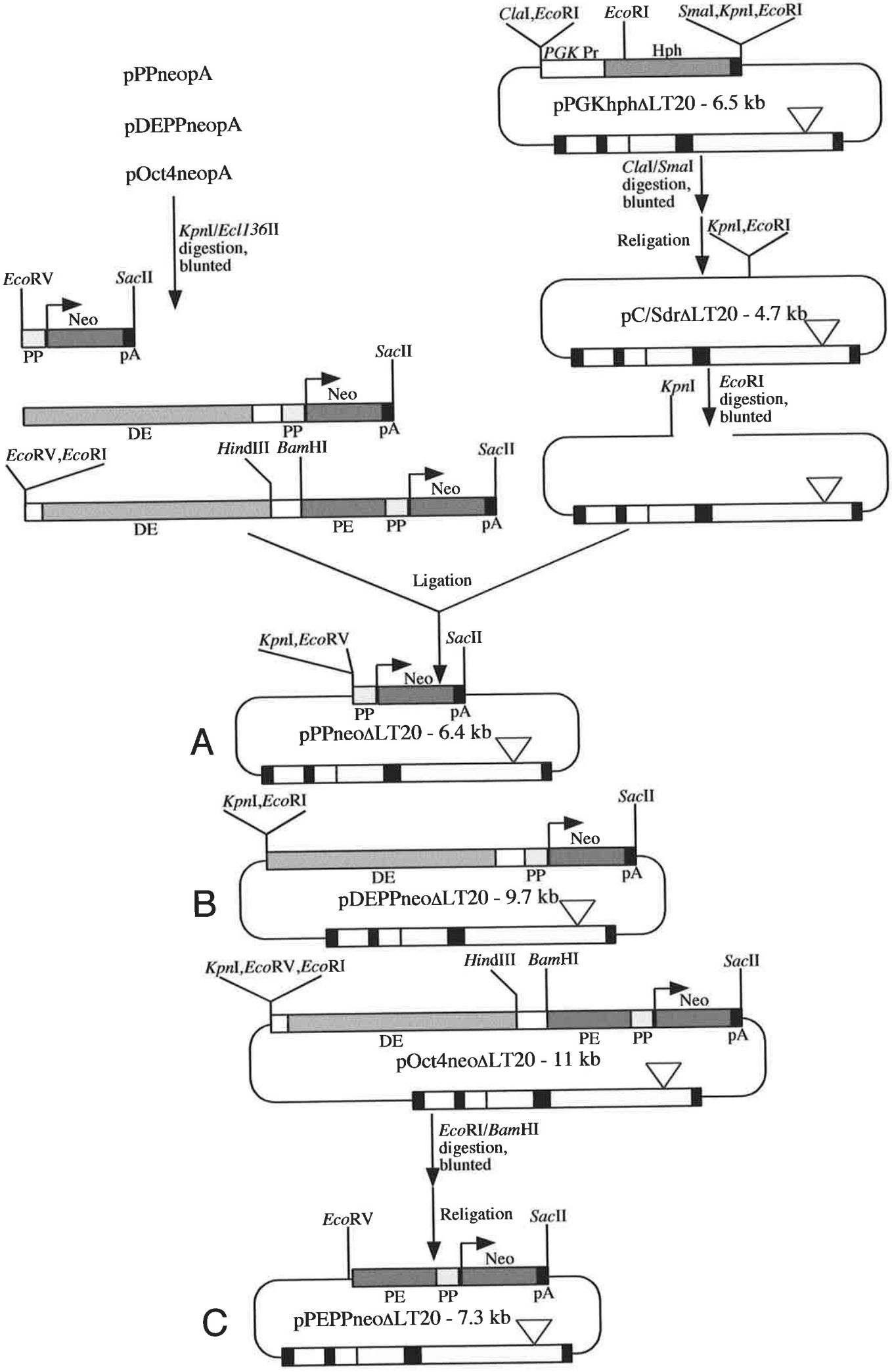
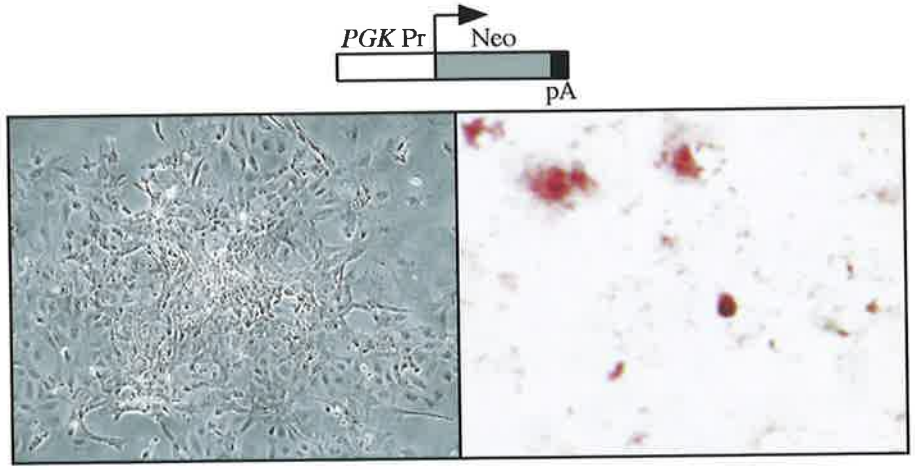

pPPneopA
pDEPPneopA
pOct4neopA
ClaI,EcoRI
EcoRI
SmaI,KpnI,EcoRI
PGK Pr
Hph
pPGKhphΔLT20 - 6.5 kb
ClaI/SmaI digestion, blunted
Religation
KpnI,EcoRI
pC/SdrΔLT20 - 4.7 kb
KpnI/Ecl136II digestion, blunted
EcoRV
SacII
Neo
PP
pA
DE
PE
KpnI
EcoRI digestion, blunted
HindIII
BamHI
EcoRV,EcoRI
Ligation
KpnI,EcoRV
A
pPPneoΔLT20 - 6.4 kb
B
pDEPPneoΔLT20 - 9.7 kb
KpnI,EcoRV,EcoRI
pOct4neoΔLT20 - 11 kb
EcoRI/BamHI digestion, blunted
C
pPEPPneoΔLT20 - 7.3 kb

Figure 3.13

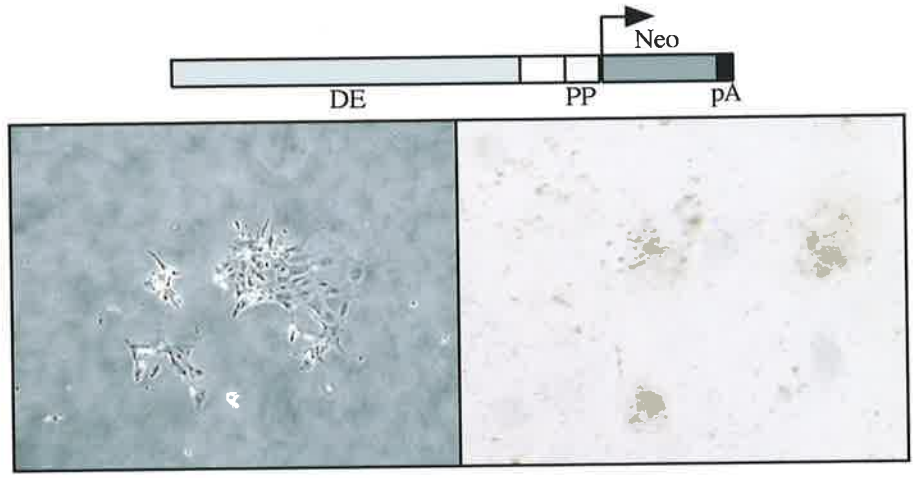
Selection against differentiated cells by expression of *Oct4*-neomycin cassettes.

C32 ES cells were supertransfected with episome-based plasmids containing *Oct4*-neo or *PGK*neo expression cassettes as indicated. Cells were differentiated in the absence of LIF and selected in 800 μg/ml G418 for a period of 10 days. Surviving cells were photographed under phase contrast microscopy at 100x magnification. Representative fields are depicted. Plates were AP stained to detect residual pluripotent cells and photographed using a dissecting microscope at a magnification of 6.3x.

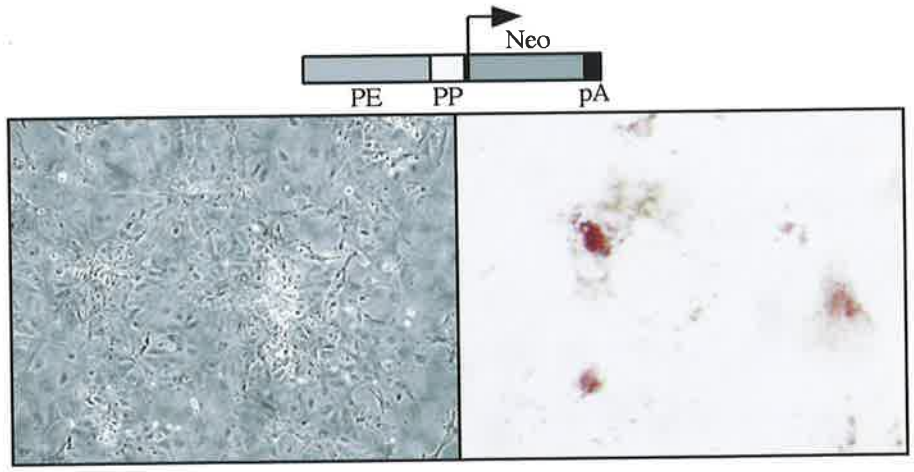
pPGKneo
ΔLT20



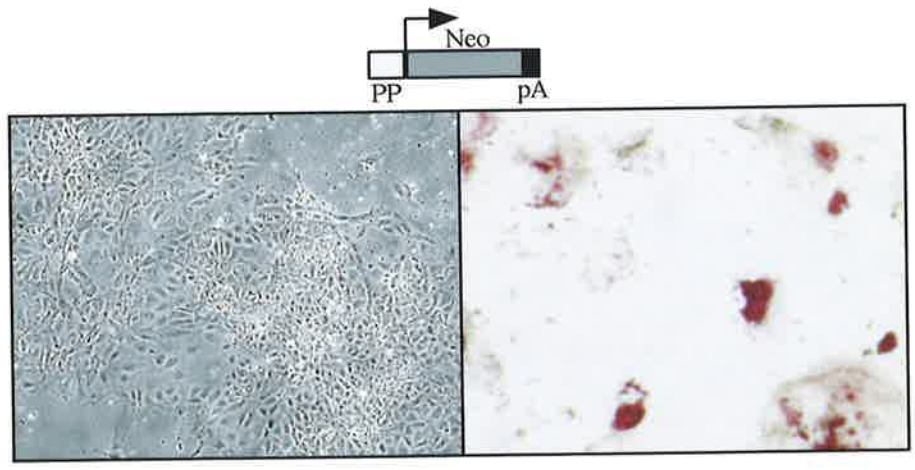
pDEPPneo
ΔLT20



pPEPPneo
ΔLT20



pPPneo
ΔLT20



resulted in selection against differentiated cells within a heterogeneous population. The selection properties of pDEPPneoΔLT20 were analysed further.

It was possible that pDEPPneoΔLT20 supertransfection selected against pluripotent and differentiated cells upon G418 addition. To confirm that pDEPPneoΔLT20 selects against differentiated cells only, and not pluripotent ES cells *per se*, pDEPPneoΔLT20 supertransfectants were differentiated in medium lacking LIF, subjected to G418 selection and examined for the presence of undifferentiated and differentiated cells.

C32 ES cells were supertransfected with pDEPPneoΔLT20 or pPGKneoΔLT20 plasmids and selected in G418 in the presence of LIF for 12 days. G418-resistant cells were pooled and passaged in the presence of LIF and G418. Pools were seeded at low density (1000 cells/cm^2) to maximise cell differentiation and selected in a low concentration of G418 (100 μg/ml) in the presence of LIF, or differentiated by LIF withdrawal. After 6 days, cells were subjected to G418 selection at 800 μg/ml for a further 5 days.

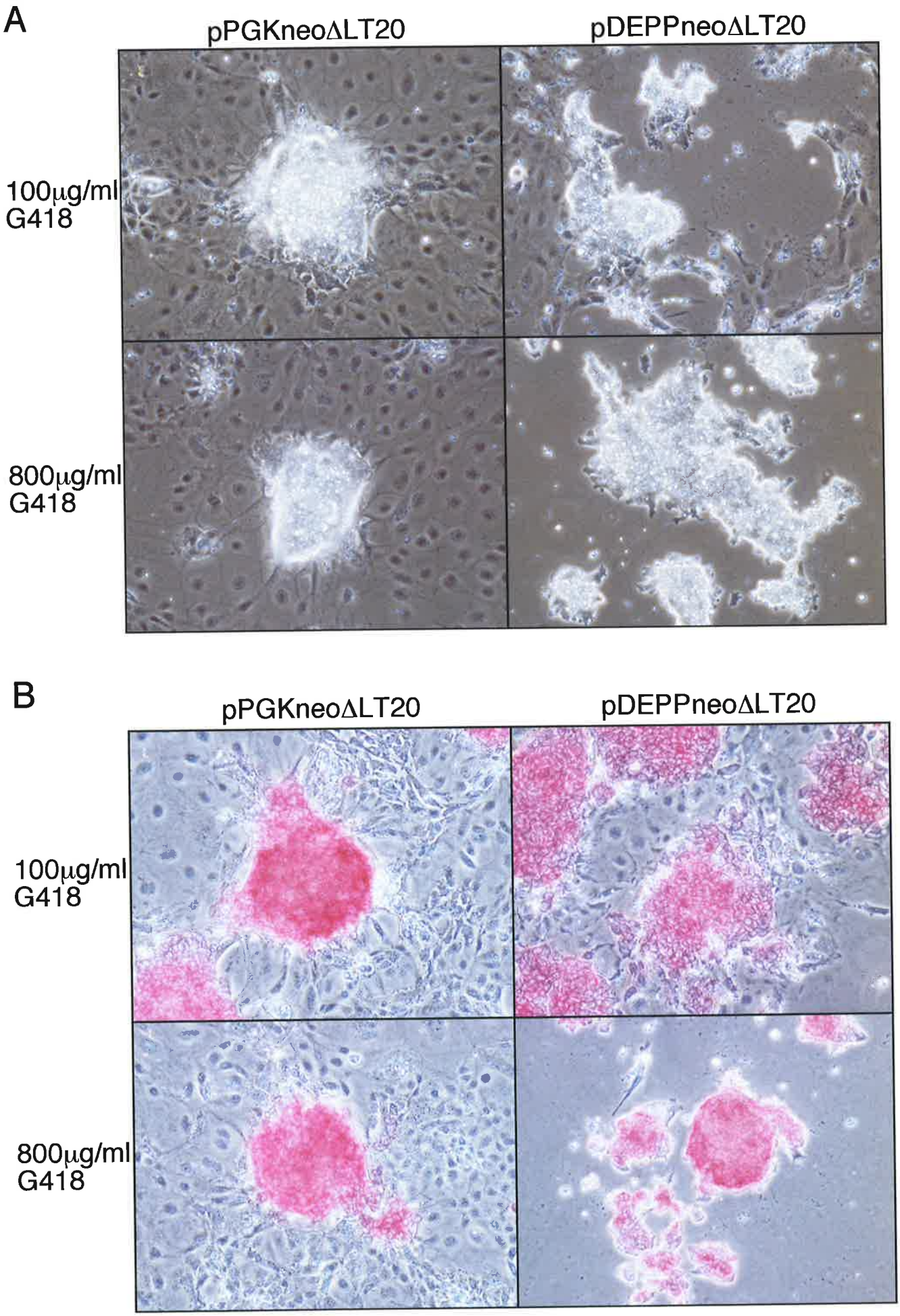
ES cells stably supertransfected with either episome formed typical ES cell colonies in the presence of LIF and G418 and retained AP expression (not shown) indicating that pluripotent pDEPPneoΔLT20 supertransfectants were resistant to high G418 selection. Although some cell death was evident in pPGKneoΔLT20 supertransfected cells, differentiated and undifferentiated cells remained viable in the culture (Figure 3.13a), consistent with constitutive expression of neomycin phosphotransferase. In pDEPPneoΔLT20 supertransfected cultures, the levels of differentiated cells was lower in 100 μg/ml G418 and at 800 μg/ml G418, differentiated cells were for the most part absent (Figure 3.14a). Surviving colonies in pDEPPneoΔLT20 supertransfected cultures grown in 800 μg/ml G418 displayed a pluripotent colony morphology and expressed AP activity (Figure 3.14b). This indicates that only pluripotent colonies survive G418 selection when supertransfected with pDEPPneoΔLT20 plasmid and demonstrates that the DEPPneopA selection cassette is capable of selecting against differentiated cells in the context of an episome vector. The observation that stem cell colonies survive selection in the absence of LIF suggests that a background of undifferentiated ES cells should be expected during function-based screening.

Figure 3.14

Specific selection against differentiated cells by supertransfection with pDEPPneoΔLT20.

A. Pooled C32 ES cell colonies stably supertransfected with pPGKneoΔLT20 or pDEPPneoΔLT20 were seeded at low density (1000 cells/cm^2) and differentiated in the absence of LIF. Cells were grown in 100 μg/ml G418 and 110 μg/ml hygromycin B for a period of 6 days. Cultures were either grown in 100 μg/ml G418 or 800 μg/ml G418 for a further 5 days. Cells were photographed under phase contrast microscopy at 200x magnification.

B. Cultures (A) were selected in either 100 μg/ml G418 or 800 μg/ml G418 for a further 2 days, AP stained and photographed under phase contrast microscopy at 200x magnification.


A
pPGKneoΔLT20
pDEPPneoΔLT20
100μg/ml G418
800μg/ml G418
B
pPGKneoΔLT20
pDEPPneoΔLT20
100μg/ml G418
800μg/ml G418

3.4.3 Discussion

Persistence of AP^+ pluripotent cells was inhibited in cells supertransfected with pDEPPneoΔLT20 (Figure 3.13). Reduction in AP^+ colony survival is probably due to a decrease in paracrine LIF supply from neighbouring differentiated cells (Rathjen *et al.*, 1990a), which do not survive G418 selection. Therefore, supertransfection with pDEPPneoΔLT20 both allows selection of ES cells from within a heterogenous population and reduces the background of pluripotent colonies that fail to differentiate.

Despite, 3.4-fold higher expression in undifferentiated ES cells (3.2.4), supertransfection with pPEPPneoΔLT20 did not result in effective selection against differentiated cells (Figure 3.13). This is in contrast to other reports from McWhir *et al*, (1996) using a neo selection construct with the PE and PP regions and approximately 650 bp of upstream *Oct4* promoter sequence and Mountford *et al*, (1998) that used the PE and PP regions to select for undifferentiated stem cells. In these reports, low G418 concentrations (50-200 μg/ml G418) selectively removed differentiated cells, presumably because stem cell-specific expression driven by the PE region is not strong (3.2.4) (Yeom *et al.*, 1996). Episome-based plasmids replicate to many copies per cell (Gassmann *et al.*, 1995) resulting in 6-10-fold higher expression of heterologous genes (Camenisch *et al.*, 1996). pPEPPneoΔLT20 dependent expression in differentiated cells, and consequent loss of the ability to select against differentiated cells may be due to increased of gene expression caused by episome replication. In support of this, G418 concentrations required to selectively remove differentiated cells in pDEPPneoΔLT20 supertransfected cultures was higher than reported by others (Figure 3.14).

3.5 COUPLED EPISOME-BASED SUPERTRANSFECTION AND *Oct4*-neo SELECTION SELECTIVELY REMOVES DIFFERENTIATED CELLS

This work has confirmed reported high efficiency supertransfection of PyV-based plasmids. The pMGD20hph episome is maintained long term in culture under selection without rearrangement, properties critical to high efficiency stable supertransfection and cDNA expression. C32 ES cells therefore exhibit the required properties for use in the proposed function-based screen (1.9.2).

Initial characterisation of the *Oct4* promoter was carried out to define upstream enhancers important for recapitulation of *Oct4* gene expression in pluripotent but not differentiated cells. In this work enhancers constructs were analysed in the presence of the PP rather than TK as previously reported. Data presented here confirm that the DE region of the *Oct4* promoter directs high expression in ES cells and low expression in differentiated cells. Supertransfected episome containing the DEPPneo cassette could be used to select against differentiated cells, generated by ES cell differentiation in the absence of LIF (Figures 3.13 and 3.14). Furthermore, remaining undifferentiated cells and ES cells grown in the presence of LIF survive this selection protocol. These experiments demonstrate the feasibility of combining the PyV-based episome system and cell type-specific antibiotic selection to achieve efficient transfection of ES cells and selection against differentiated cells. Thus the pDEPPneoΔLT20 episome exhibited the required properties for effective function-based screening for factors that inhibit ES cell differentiation (1.9.2).

CHAPTER 4:

ES CELL MAINTENANCE BY EPISOMAL EXPRESSION OF KNOWN GENES

4.1 INTRODUCTION

The function-based screen (1.9.2) requires creation of an episome-based vector that enables efficient transfection and expression of a cDNA library in ES cells, and *Oct4* promoter based selection of pluripotent cells from a heterogenous population. In this chapter, the construction and validation of pPSDEneoΔLT20 is described.

4.2 CREATION OF A EPISOME-BASED cDNA EXPRESSION VECTOR

The constitutive *PGK-1* promoter was excised from pPGK-o-Term (2.2.6) as a 500 bp *Hin*dIII/*Eco*RI fragment and cloned into *Hin*dIII/*Eco*RI digested pBluescript II KS to form pPGKKS (not shown). SV40 polyadenylation sequences were excised as a 240 bp *Xba*I fragment from pIRES-βgeo (Mountford *et al.*, 1994) and blunt cloned into the *Sac*II site of pPGKKS to form pPGKpA (Figure 4.1a). The presence of successive *Eco*RI and *Not*I restriction sites downstream of the *PGK-1* promoter allows directional cloning of cDNAs with 5′ *Eco*RI and 3′ *Not*I cohesive ends. To assist with efficient purification of *Eco*RI/*Not*I-digested vector for cDNA library construction, a stuffer DNA fragment was incorporated into pPGKpA by blunt cloning of a 720 bp *Eco*RI DNA fragment from SPP-1 bacteriophage DNA into the *Sma*I site of pPGKpA forming pPGKStufferpA (Figure 4.1b).

pDEPPneoΔLT20 was used as the base plasmid for creation of a cDNA expression vector because it enabled effective selection against differentiated cells (3.4.2). The unique *Eco*RI restriction site was removed from pDEPPneoΔLT20 by *Eco*RI digestion, endfilling, and religation, to form pDEPPneoΔLT20Edr (Figure 4.2). The PGKStufferpA expression cassette was removed from pPGKStufferpA as a 1.5 kb *Cla*I/*Ecl*136II fragment, blunted and cloned into *Kpn*I digested and blunted pDEPPneoΔLT20Edr. The resulting plasmid, pPSDEneoΔLT20 is the completed cDNA expression vector containing the PyF101 mutant ori/enhancer region, DEPPneo selection cassette and cDNA expression cassette (Figure 4.2a, Figure 1.10b). cDNAs inserted directionally into the *Eco*RI and *Not*I restriction sites of pPSDEneoΔLT20 will be expressed under the control of the constitutive *PGK-1* promoter in supertransfected cells.

Figure 4.1

Creation of a cassette that directs constitutive expression.

*Eco*RI and *Not*I restriction sites are located between the *PGK-1* promoter and the polyadenylation sequences to allow directional cloning of cDNAs under the control of the *PGK-1* promoter. Restriction sites important for this and further cloning steps are shown. White box, *PGK-1* promoter; black box, polyadenylation signals; light grey box, stuffer DNA fragment; grey box, *β-gal* gene; dark grey box, *neo* gene.

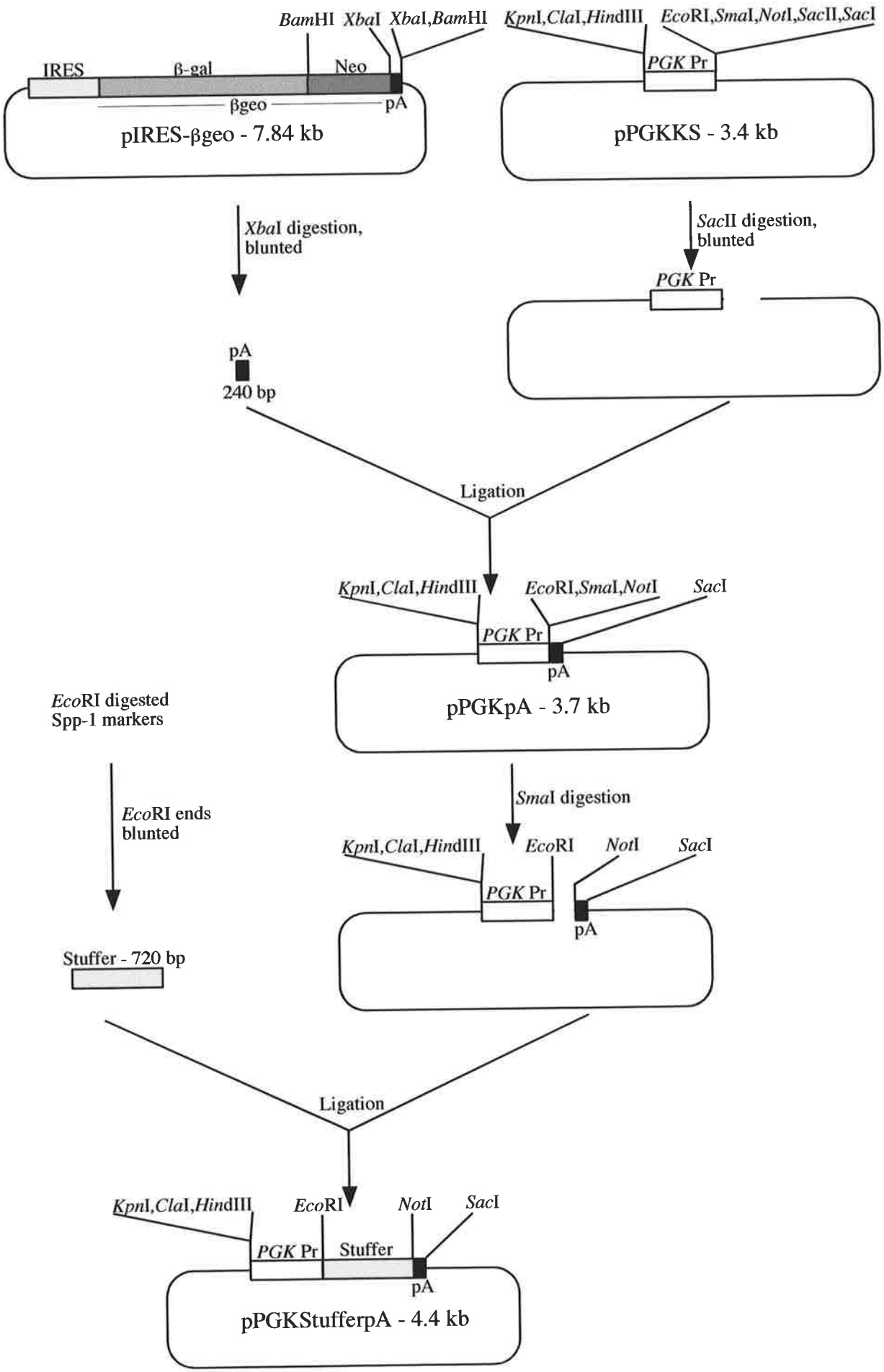
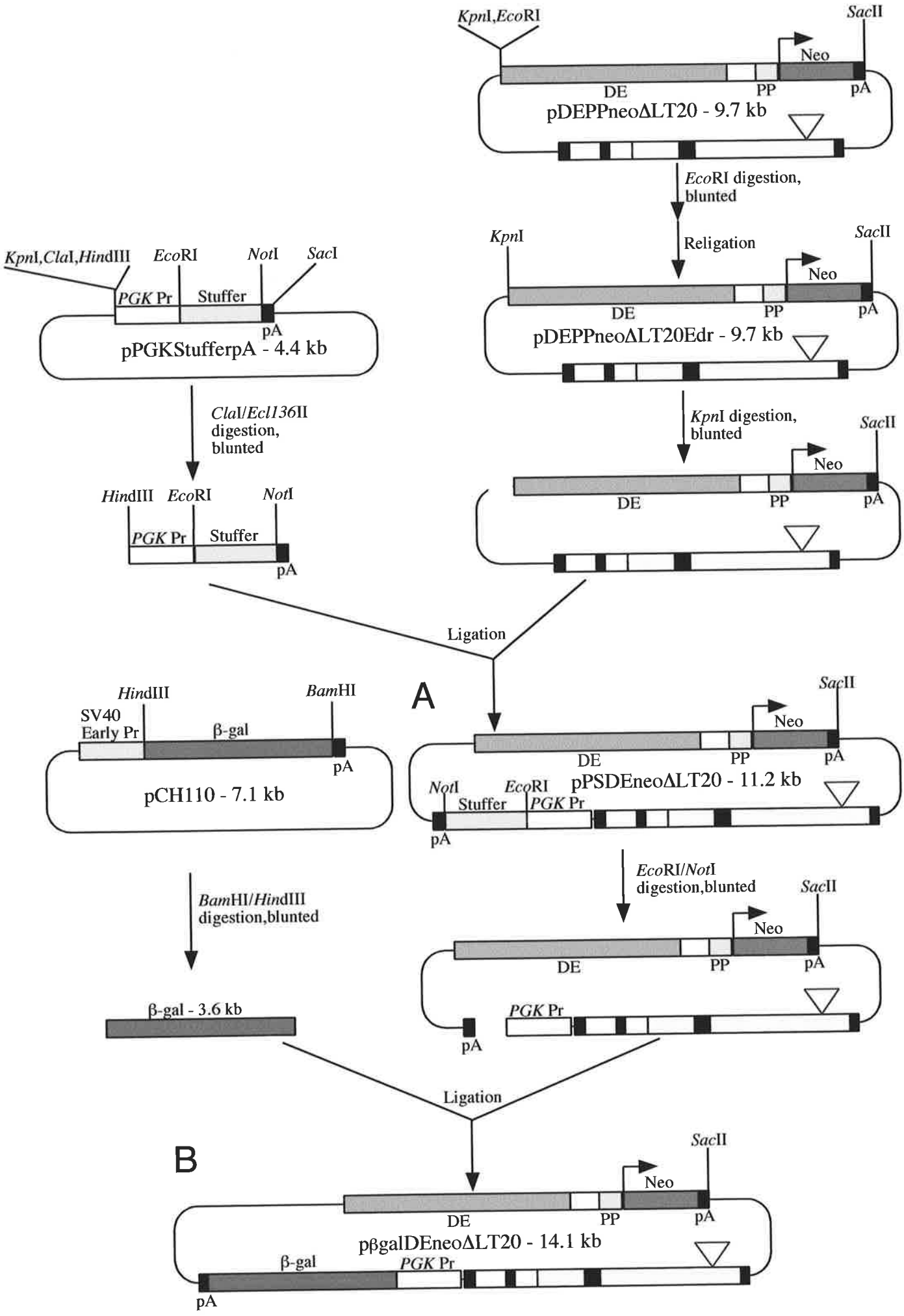

BamHI XbaI XbaI,BamHI
IRES
β-gal
Neo
βgeo
pA
pIRES-βgeo - 7.84 kb
KpnI,ClaI,HindIII EcoRI,SmaI,NotI,SacII,SacI
PGK Pr
pPGKKS - 3.4 kb
XbaI digestion, blunted
SacII digestion, blunted
PGK Pr
pA
240 bp
Ligation
KpnI,ClaI,HindIII
EcoRI,SmaI,NotI
SacI
PGK Pr
pA
pPGKpA - 3.7 kb
EcoRI digested Spp-1 markers
EcoRI ends blunted
SmaI digestion
KpnI,ClaI,HindIII
EcoRI
NotI
SacI
PGK Pr
pA
Stuffer - 720 bp
Ligation
KpnI,ClaI,HindIII
EcoRI
NotI
SacI
PGK Pr
Stuffer
pA
pPGKStufferpA - 4.4 kb

Figure 4.2

Construction of an episome-based cDNA expression library vector.

Diagram of (A) pPSDEneoΔLT20 which contains the PyV mutant ori/enhancer region, the DEPPneo selection cassette and a cDNA expression cassette. cDNAs can be directionally cloned into the *Eco*RI and *Not*I restriction sites. Construction of (B) pβgalDEneoΔLT20 that contains the *β-gal* gene under the control of the *PGK-1* promoter. White box, *PGK-1* promoter; black box, polyadenylation signals; light grey box, stuffer DNA fragment. Shading of the *Oct4* promoter is identical to that of figure 3.1.


KpnI,EcoRI
SacII
Neo
DE
PP
pA
pDEPPneoΔLT20 - 9.7 kb
EcoRI digestion, blunted
Religation
KpnI
SacII
Neo
DE
PP
pA
pDEPPneoΔLT20Edr - 9.7 kb
KpnI,ClaI,HindIII
EcoRI
NotI
SacI
PGK Pr
Stuffer
pA
pPGKStufferpA - 4.4 kb
ClaI/Ecl136II digestion, blunted
KpnI digestion, blunted
HindIII
EcoRI
NotI
PGK Pr
Stuffer
pA
Ligation
A
HindIII
BamHI
SV40 Early Pr
β-gal
pA
pCH110 - 7.1 kb
SacII
Neo
DE
PP
pA
NotI
EcoRI
Stuffer
PGK Pr
pA
pPSDEneoΔLT20 - 11.2 kb
EcoRI/NotI digestion,blunted
BamHI/HindIII digestion,blunted
β-gal - 3.6 kb
PGK Pr
pA
Ligation
B
SacII
Neo
DE
PP
pA
pβgalDEneoΔLT20 - 14.1 kb
β-gal
PGK Pr
pA

4.3 REPLICATION AND EXPRESSION OF PPSDENEOΔLT20 IN SUPERTRANSFECTED ES CELLS

Successful library screening depends on the expression of heterologous cDNAs from pPSDEneoΔLT20. Subsequent isolation of functionally relevant cDNAs responsible for an observed phenotype requires persistence of the library episome. Expression and persistence of pPSDEneoΔLT20 in supertransfected colonies was analysed using a pPSDEneoΔLT20-based plasmid modified to contain the *β-gal* reporter gene. pβgalDEneoΔLT20 was formed by blunt cloning a 3,655 bp *Bam*HI/*Hin*dIII *β-gal* gene fragment from pCH110 (Pharmacia) into the *Eco*RI/*Not*I cDNA expression site of pPSDEneoΔLT20 (Figure 4.2b).

3 x 10^7 C32 ES cells were electroporated (2.4.10) with pβgalDEneoΔLT20 and selected for 15 days in medium containing LIF, 200 μg/ml G418 and 110 μg/ml hygromycin B. 71% of surviving colonies (90 out of 127 colonies counted) stained positive for β-galactosidase activity indicating that the majority of supertransfectants express heterologous genes introduced on the pPSDEneoΔLT20 episome. G418 resistant colonies were picked from a second plate into duplicate wells and one set of duplicates was stained for β-galactosidase activity after 5 days of culture in medium containing LIF and 200 μg/ml G418. β-galactosidase activity was detected in 71% (17 out of 24) of pβgalDEneoΔLT20 supertransfected ES cell lines. Cultures of two cell lines contained β-galactosidase negative cells although these cells represented less than 50% of the total. All cell lines that expressed β-galactosidase activity remained β-galactosidase positive for a further six passages (~30 cell generations), indicating that cDNA expression from pPSDEneoΔLT20 is stably maintained in around 70% of resistant lines (Table 4.1).

Hirt preparation DNA (2.3.25) derived from each line was electroporated into electrocompetent *E. coli* (2.3.10). Lifts were taken from transformation plates and probed with a 1 kb *Bgl*II fragment specific for the DE region of the *Oct4* promoter (Figure 3.1). pβgalDEneoΔLT20 plasmid was identified in Hirt preparations from 16 of 23 ES cell lines (69.5%; Table 4.1; presence of pβgalDEneoΔLT20 could not be determined for one cell line). Moreover, 14 of the 16 (87.5%) lines that contained pβgalDEneoΔLT20 episome also expressed β-galactosidase activity. pβgalDEneoΔLT20 plasmid was generally less than 10% of the total number of transformants as determined by colony screening (not shown). This may

Table 4.1

Correlation of β-galactosidase expression and the presence of pβgalDEneoΔLT20 episome in pβgalDEneoΔLT20 stable lines.

Colony No.	βgal Positive	βgal activity after 6 passages	Presence of episome
1	+	+	+
2	-	N/D	-
3	+	+	+
4	-	N/D	+
5	-	N/D	-
6	+	+	+
7	+	+	+
8	+	+	+
9	+	+	-
10	+	+	+
11	+	+	+
12	+	+	+
13	+	+*	N/D#
14	-	N/D	-
15	+	+	+
16	-	N/D	-
17	+	+	+
18	-	N/D	-
19	+	+	-
20	+	+	+
21	-	N/D	+
22	+	+	+
23	+	+*	+
24	+	+	+
Total	17/24 (71%)	17/17 (100%)	16/23 (69.5%)

Presence of episome was determined by Grunstein analysis
* A proportion of cells do not contain β-galactosidase activity.
# Out of 30 transformants no episome was detected.
N/D, Not determined

reflect slower replication of the supertransfected, larger episome, and/or differential episome purification by the Hirt DNA extraction protocol.

In summary, approximately 70% of G418 resistant colonies resulting from pβgalDEneoΔLT20 supertransfection expressed cDNAs stably from plasmid maintained as an episome. Expression is maintained for multiple generations and persists in cells following passaging. The pPSDEneoΔLT20 episome vector therefore displays the properties required for effective function-based screening.

Genomic and Hirt DNA from pβgalDEneoΔLT20 supertransfectants were analysed for the presence of pβgalDEneoΔLT20 derived DNA by Southern blot (Figure 4.3)(2.3.15). The endogenous *Oct4* promoter was detected in genomic DNA and in some Hirt preparations as a 3.3 kb band (Figure 4.3, arrows) indicating that genomic DNA can contaminate Hirt DNA preparations. pβgalDEneoΔLT20 plasmid was detected in Hirt DNA extracts from five of the 16 lines (lines 1, 11, 17, 20 and 22) (Figure 4.3) found to contain pβgalDEneoΔLT20 by Grunstein analysis (Table 4.1) indicating that Southern analysis is less sensitive than colony screening.

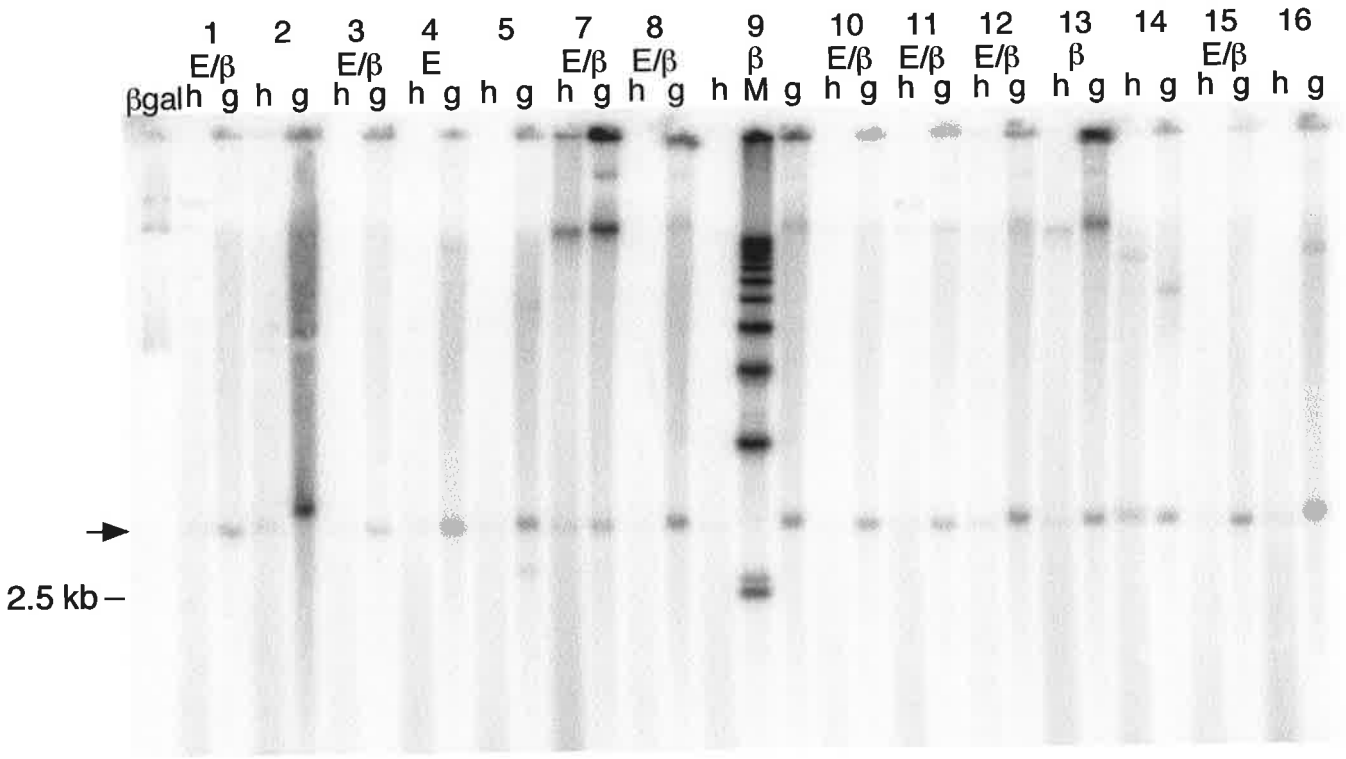
Hybridisation to *Oct4* promoter sequences was also observed to high molecular weight bands in genomic DNA from cell lines 7, 8, 9, 12, 13, 20 and 23. Hybridisation to integrated unrearranged plasmid should result in bands larger than the size of pβgalDEneoΔLT20 which does not contain *Bam*HI restriction sites. All seven lines that contained high molecular weight integrated bands expressed β-galactosidase activity (Table 4.1) and all but two contained extrachromosomal pβgalDEneoΔLT20 plasmid, indicating that genomic integration can occur in cells that maintain two episomes.

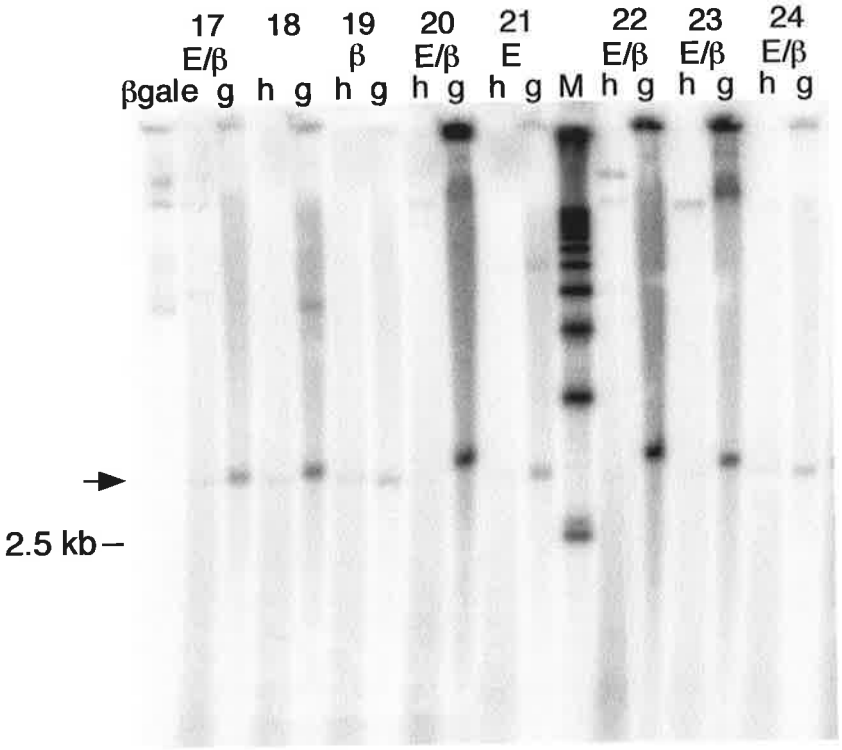
Integrated copies of pβgalDEneoΔLT20 plasmid were detected in genomic DNA in all but one (line 19) of the seven lines that did not contain episome as determined by Grunstein analysis of Hirt DNA providing an explanation for G418 resistance. Hybridisation was observed to genomic DNA bands smaller than pβgalDEneoΔLT20 plasmid in the five lines (lines 2, 5, 14, 16 and 18) that lacked episome and one (line 21) that contained episome (Figure 4.3), suggesting that the integrated episome had undergone rearrangement and providing an explanation for the lack of β-galactosidase activity (Table 4.1). A lack of cDNA expression in

Figure 4.3

Southern analysis of pβgalDEneoΔLT20 supertransfected ES cell lines.

*Bam*HI digested Hirt preparation DNA (h) and genomic DNA (g) from indicated pβgalDEneoΔLT20 supertransfected lines was electrophoresed on a 1% TAE agarose gel and Southern blotted. The filter was probed with a 1 kb *Bgl*II fragment derived from pF2KS (Figure 3.1) specific for the *Oct4* promoter region present in pβgalDEneoΔLT20. The blot was washed to a stringency of 0.2 x SSC/0.1% SDS at 65°C. The 3.3 kb fragment correspond to the endogenous *Oct4* promoter (arrowed). Those lines in which episome (E) or β-galactosidase expression (β) was detected by colony screening or β-galactosidase staining, respectively, are indicated (Table 4.1). M, 2.5 kb ladder molecular weight markers.


1 2 3 4 5 7 8 9 10 11 12 13 14 15 16
E/β E/β E E/β E/β β E/β E/β E/β β E/β
βgal h g h g h g h g h g h g h g h M g h g h g h g h g h g h g h g
2.5 kb


17 18 19 20 21 22 23 24
E/β β E/β E E/β E/β E/β
βgale g h g h g h g h g M h g h g h g
2.5 kb

selected colonies therefore results from integration of truncated episome in the majority of cases.

4.4 EPISOMAL EXPRESSION OF LIF cDNAS INHIBITS ES CELL DIFFERENTIATION

ES cell lines overexpressing LIF cDNAs do not require exogenous LIF for maintenance of the undifferentiated phenotype (Conquet *et al.*, 1992). Although overexpressed LIF is unlikely to act on ES cells in a strictly cell-autonomous manner, its inhibitory effect on ES cell differentiation is well established. Overexpression of LIF from pPSDEneoΔLT20 therefore provides a functional test for the screen (1.9.2). The diffusible and matrix-associated forms of mouse *LIF*, *mLIF-D* and *mLIF-M*, potentially provide assays for diffusible and cell-autonomous extracellular factors.

cDNAs encoding *mLIF-D* and *mLIF-M* (Rathjen *et al.*, 1990b) were cloned into pPSDEneoΔLT20. Episomal LIF expression plasmids pLIF-DDEneoΔLT20 and pLIF-MDEneoΔLT20 were created by blunt end cloning 650 bp *Eco*RI *LIF-D* and *Xho*I/*Eco*RI *LIF-M* cDNA fragments into *Eco*RI/*Not*I digested and blunted pPSDEneoΔLT20 (Figure 4.4). CsCl purified pLIF-DDEneoΔLT20, pLIF-MDEneoΔLT20 and pPSDEneoΔLT20 were supertransfected into C32 ES cells and selected for 12 days in 200 μg/ml G418 in either the presence or absence of exogenous LIF. Supertransfected C32 ES cells were cultured in the presence or absence of exogenous LIF and G418 selected for 12 days and stained for AP activity to identify pluripotent cells (Berstine *et al.*, 1973; Johnson *et al.*, 1977).

Few AP^+ colonies were observed in C32 ES cultures supertransfected with pPSDEneoΔLT20 selected in the absence of exogenous LIF (Figure 4.5a). Colonies had undergone extensive differentiation and residual pluripotent (AP^+) regions were surrounded by differentiated cells, suggesting that they were supported by feedback LIF expression (Rathjen *et al.*, 1990a). Supertransfection with pLIF-DDEneoΔLT20 and pLIF-MDEneoΔLT20 resulted in formation of morphologically normal ES cell colonies in the absence of LIF and many more AP^+ colonies were observed compared with pPSDEneoΔLT20 supertransfected cells (Figure 4.5a). Pluripotent colonies in pLIF-DDEneoΔLT20 and pLIF-MDEneoΔLT20 supertransfected C32 ES cell cultures were larger than those supertransfected with

Figure 4.4

Construction of pLIF-DDEneoΔLT20 and pLIF-MDEneoΔLT20 episomes.

Cloning of (A) the LIF-D and (B) the LIF-M coding regions into the episome-based expression vector pPSDEneoΔLT20. pLIF-DDEneoΔLT20 and pLIF-MDEneoΔLT20 plasmids can be supertransfected into episome harbouring ES cell lines to express mouse *LIF-M* and *LIF-D* transcripts from the constitutive *PGK-1* promoter. Shading is as described in figure 4.2.

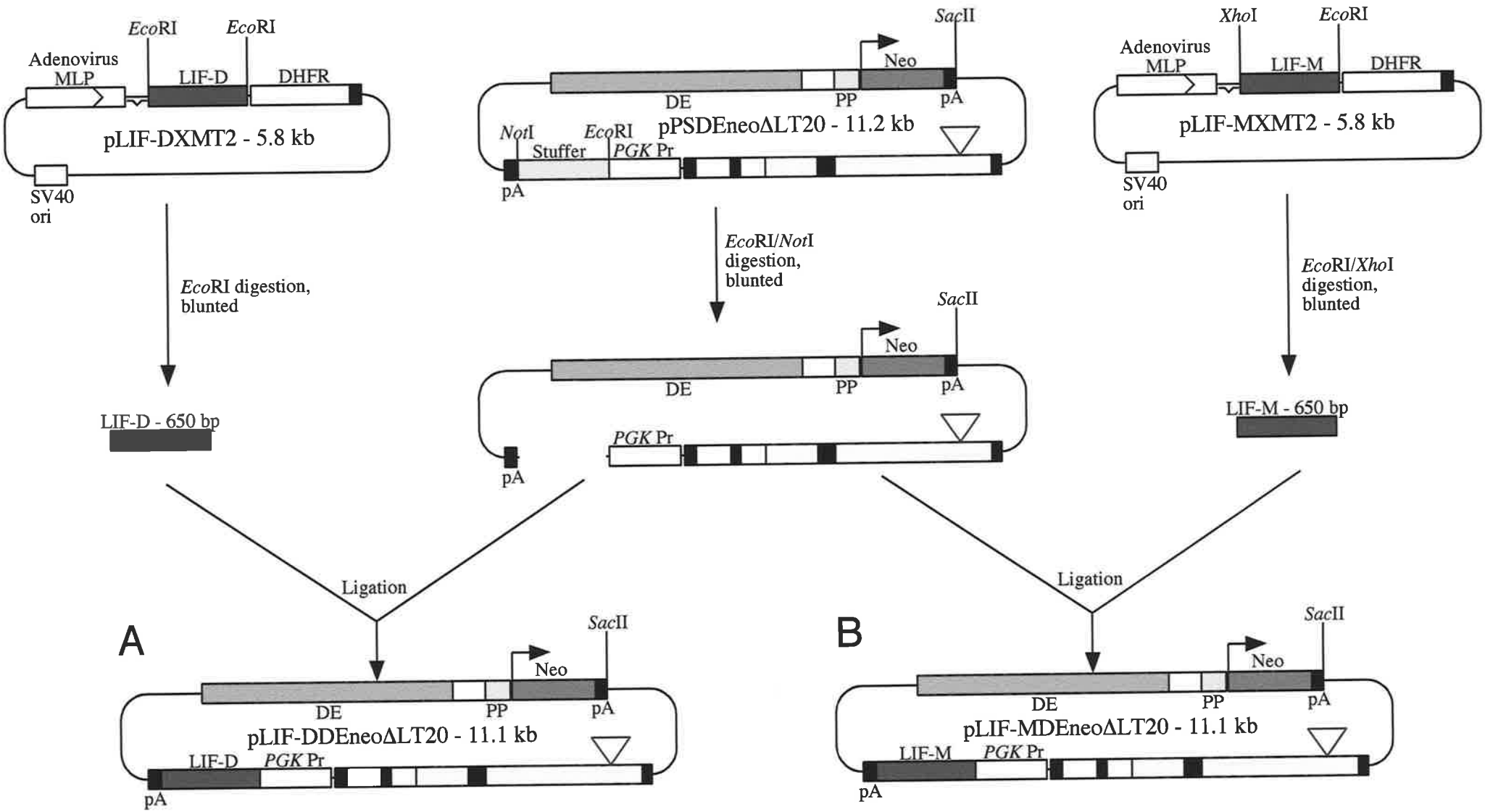

EcoRI
EcoRI
Adenovirus MLP
LIF-D
DHFR
pLIF-DXMT2 - 5.8 kb
SV40 ori
EcoRI digestion, blunted
LIF-D - 650 bp
SacII
Neo
DE
PP
pA
NotI
EcoRI
Stuffer
PGK Pr
pA
pPSDEneoΔLT20 - 11.2 kb
EcoRI/NotI digestion, blunted
SacII
Neo
DE
PP
pA
PGK Pr
pA
XhoI
EcoRI
Adenovirus MLP
LIF-M
DHFR
pLIF-MXMT2 - 5.8 kb
SV40 ori
EcoRI/XhoI digestion, blunted
LIF-M - 650 bp
Ligation
A
SacII
Neo
DE
PP
pA
pLIF-DDEneoΔLT20 - 11.1 kb
LIF-D
PGK Pr
pA
Ligation
B
SacII
Neo
DE
PP
pA
pLIF-MDEneoΔLT20 - 11.1 kb
LIF-M
PGK Pr
pA

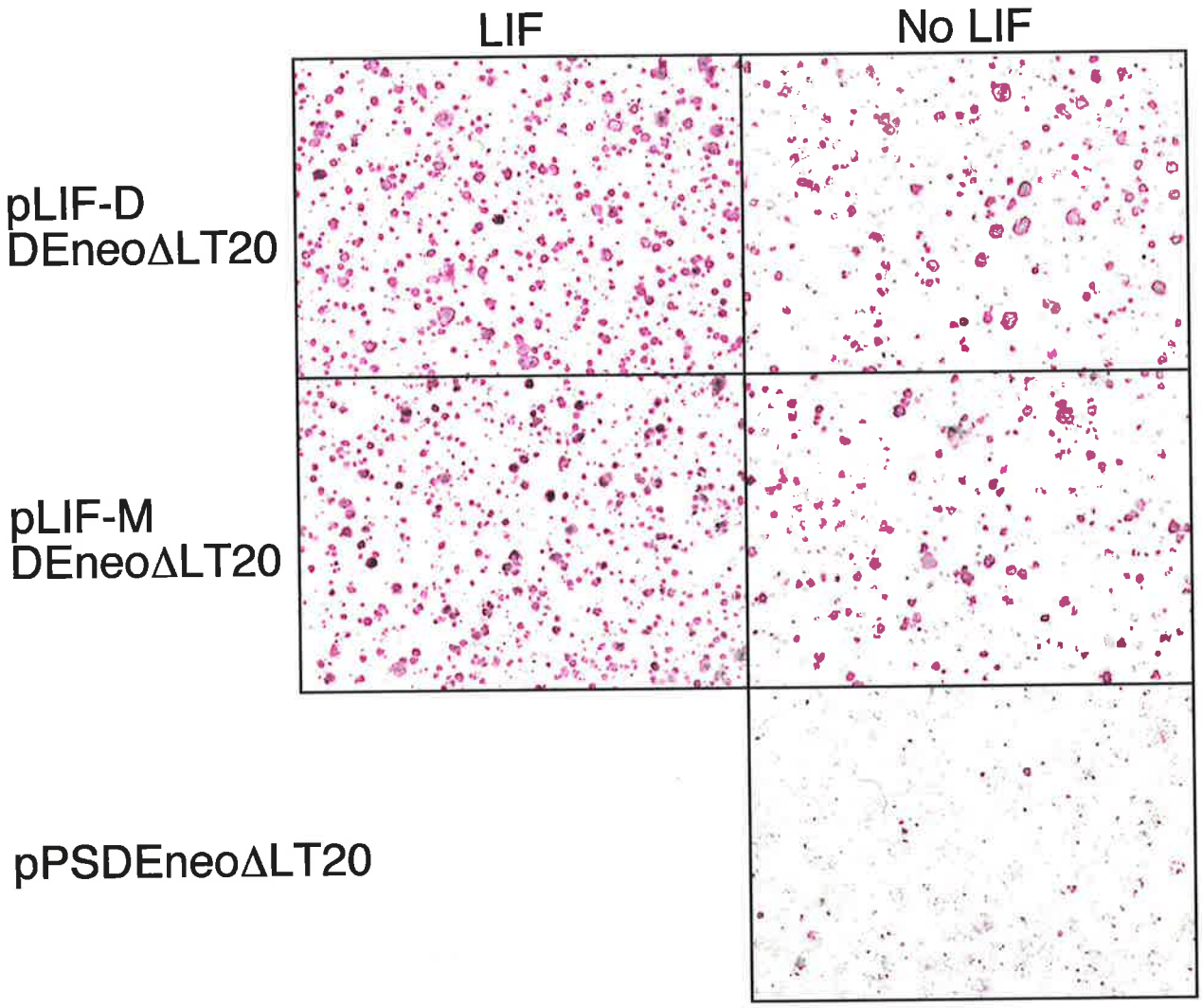
Figure 4.5

Episomal LIF expression inhibits ES cell differentiation.

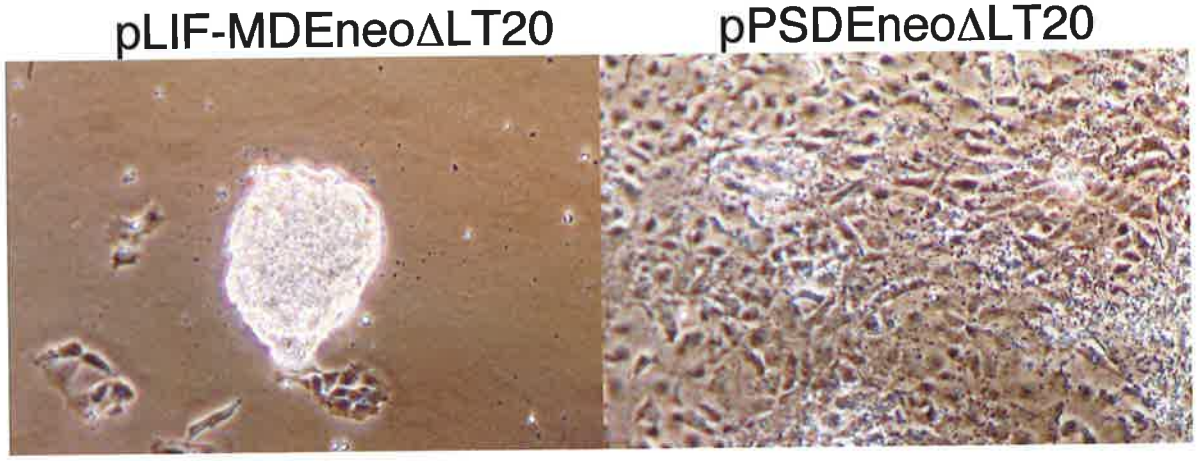
A. LIF expressing ES cells survive differentiation and selection. 3 x 10^7 C32 ES cells were supertransfected with 20 μg of CsCl purified pLIF-DDEneoΔLT20, pLIF-MDEneoΔLT20 or pPSDEneoΔLT20 plasmid DNA and 14% of the transfection was plated onto a 10 cm diameter plate. Cells were selected for 12 days in 200 μg/ml G418 in the presence or absence of exogenous LIF. Cultures were stained to detect AP activity and scanned using Umax Powerlook II scanner.

B. Stable LIF episome supertransfectants retain an undifferentiated phenotype in the absence of LIF. pLIF-MDEneoΔLT20 or pPSDEneoΔLT20 plasmid was supertransfected into C32 ES cells and selected for 10 days in 200 μg/ml G418 in the presence of exogenous LIF. Isolated colonies were cloned and expanded for three passages in 200 μg/ml G418 and exogenous LIF. Cell lines containing pPSDEneoΔLT20 or pLIF-MDEneoΔLT20 were grown at a density of 1000 cells/cm^2 for 8 days in the absence of LIF in 200 μg/ml G418 and images were taken at 200 x magnification.

A


LIF
No LIF
pLIF-D DEneoΔLT20
pLIF-M DEneoΔLT20
pPSDEneoΔLT20

B


pLIF-MDEneoΔLT20
pPSDEneoΔLT20

pPSDEneoΔLT20, showed fewer signs of differentiation and were not necessarily surrounded by differentiated cells.

While supertransfection with pLIF-DDEneoΔLT20 and pLIF-MDEneoΔLT20 episomes abrogated ES cell differentiation in the absence of LIF, the number of pluripotent colonies was higher when supertransfected cultures were selected in the presence of exogenous LIF. This indicates that rescue of the pluripotent state within the population by episomal LIF expression was not complete. The observation that not all G418-resistant pLIF-MDEneoΔLT20 supertransfected colonies retain pluripotence in the absence of exogenous LIF is consistent with a relatively cell-autonomous action of LIF within this assay. This implies that the function-based screen can be used to select for both cell-autonomous and secreted/diffusible factors that inhibit ES cell differentiation.

AP expression and ES cell morphology were retained by pooled or cloned pLIF-DDEneoΔLT20 or pLIF-MDEneoΔLT20 supertransfectants passaged in the absence of LIF for at least five passages (not shown; Figure 4.5b). This indicates that LIF cDNA expression driven by the episome vector is maintained stably, consistent with β-galactosidase expression from pβgalDEneoΔLT20 (4.3).

4.4.1 Discussion

A number of factors have been reported to impair ES cell differentiation but, with the exception of *v-Src* and *LIF* cDNAs, overexpression partially relieves the requirement for LIF instead of rendering the ES cells LIF independent.

C32 ES cells supertransfected with LIF-expressing episomes retained pluripotence in the absence of LIF. Although LIF protein expression was not directly tested in work presented here, the best interpretation is that functional LIF protein was produced by C32 ES cells supertransfected with pLIF-DDEneoΔLT20 or pLIF-MDEneoΔLT20, resulting in ES cell maintenance in the absence of exogenous LIF. The pPSDEneoΔLT20 episome is therefore likely to express functional protein at levels that inhibit ES cell differentiation.

ES cells supertransfected with library episome containing cDNA inserts that function to inhibit differentiation can therefore survive G418 selection in the absence of LIF. Further, expression is maintained stably during cell passaging, validating use of the pPSDEneoΔLT20

episome vector in this assay. This experimental approach can therefore be applied to the analysis of other factors implicated in the control of ES cell differentiation. Inhibition of ES cell differentiation in this assay is manifest as an increase in the proportion of pluripotent supertransfected colonies formed in the absence of LIF compared with pPSDEneoΔLT20 supertransfected cells. This experiment also implies that LIF was acting in a cell-autonomous manner inferring that cell-autonomous factors involved in ES cell maintenance could be isolated by the screening approach.

4.5 ES CELL CYTOTOXICITY CAUSED BY INTRODUCTION OF *v-Src*

ES cells that express v-Src have been reported to retain pluripotence in the absence of exogenous LIF (Boulter *et al.*, 1991). v-Src should act in a cell-autonomous manner given the known functions of v-Src. The v-Src cDNA therefore represented an ideal positive control to confirm that cell-autonomous factors involved in signal transduction could render ES cells resistant to the function-based screen.

To determine if *v-Src* expression inhibits ES cell differentiation in the context of the PyV-based episome, the *v-Src* cDNA was cloned into the expression cassette of pPSDEneoΔLT20 and analysed by the differentiation assay outlined previously (4.5). The pBabe v-Src puro plasmid was a gift from Dr Steve Martin (University of California at Berkeley). A 1.6 kb *Bam*HI/*Eco*RI fragment containing the *v-Src* cDNA was blunted and cloned into *Eco*RI/*Not*I digested and blunted pPSDEneoΔLT20 (Figure 4.6). Plasmid DNA preparations in which the *v-Src* cDNA was cloned into pPSDEneoΔLT20 in the 'sense' (sense pv-SrcDEneoΔLT20) and 'antisense' (antisense pv-SrcDEneoΔLT20) orientations were supertransfected (2.4.10) into C32 ES cells and photographed following 6 days of G418 selection (800 μg/ml) in the presence or absence of LIF.

G418 resistant colonies formed in the presence of LIF following supertransfection of sense pv-SrcDEneoΔLT20 into C32 ES cells were almost exclusively minute, comprising only a few cells (Figure 4.7) and did not persist with continued G418 selection. By contrast, supertransfection of C32 ES cells with antisense pv-SrcDEneoΔLT20 resulted in morphologically normal G418 resistant ES cell colonies equivalent to C32 ES cells supertransfected with pPSDEneoΔLT20 alone (Figure 4.7). Morphologically normal ES

Figure 4.6

Construction of sense and antisense pv-SrcDEneoΔLT20 plasmids.

Sense pv-SrcDEneoΔLT20 episome expresses *v-Src* from the constitutive *PGK-1* promoter in supertransfected ES cells. Antisense pv-SrcDEneoΔLT20 episome does not express *v-Src*.

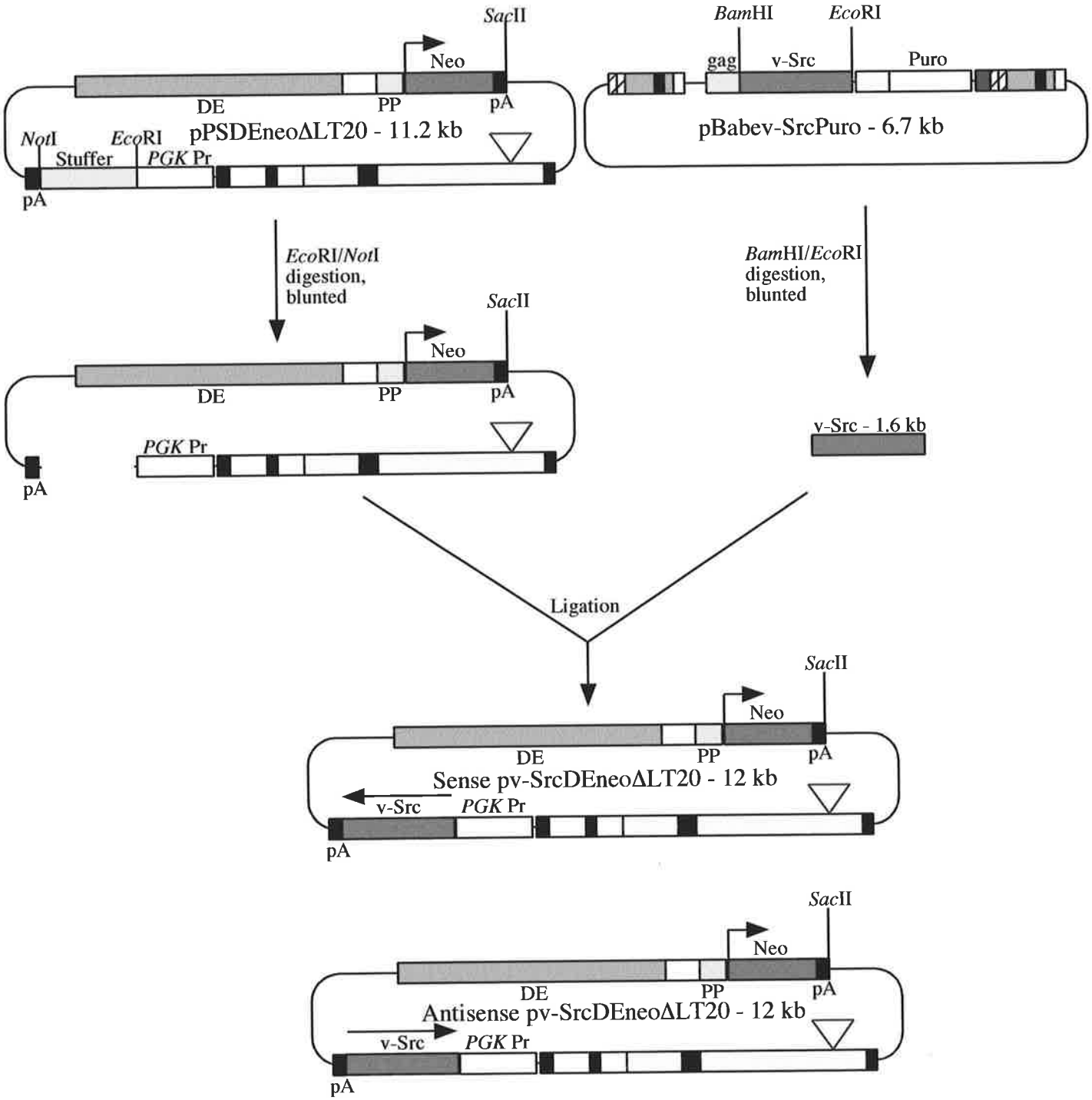
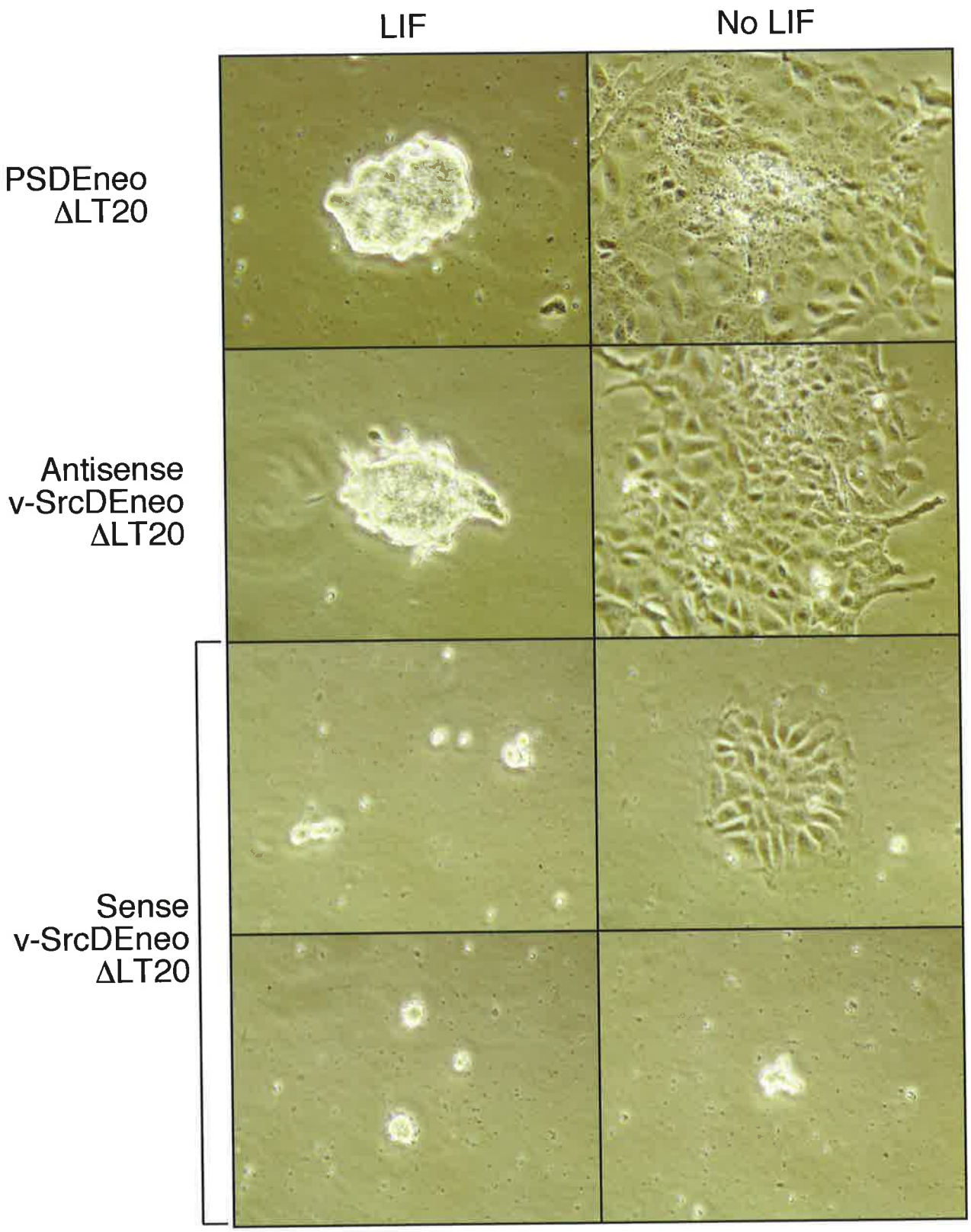

SacII
Neo
DE
PP
pA
NotI
EcoRI
Stuffer
PGK Pr
pA
pPSDEneoΔLT20 - 11.2 kb
BamHI
EcoRI
gag
v-Src
Puro
pBabev-SrcPuro - 6.7 kb
EcoRI/NotI
digestion,
blunted
BamHI/EcoRI
digestion,
blunted
v-Src - 1.6 kb
Ligation
Sense pv-SrcDEneoΔLT20 - 12 kb
v-Src
Antisense pv-SrcDEneoΔLT20 - 12 kb

Figure 4.7

Expression of the *v-Src* cDNA in ES cells using episome-based vectors.

3 x 10^7 C32 ES cells were supertransfected with 20 μg of sense pv-SrcDEneoΔLT20, antisense pv-SrcDEneoΔLT20 or pPSDEneoΔLT20. One quarter of the supertransfected cells were seeded onto 10 cm diameter plates and selected for 6 days in 800 μg/ml G418 in the presence or absence of LIF in order to kill untransfected cells. Surviving colonies were photographed under phase contrast at 200 x magnification.


LIF
No LIF
PSDEneo
ΔLT20
Antisense
v-SrcDEneo
ΔLT20
Sense
v-SrcDEneo
ΔLT20

colonies formed in sense pv-SrcDEneoΔLT20 supertransfected cultures at extremely low levels and presumably do not express significant amounts of v-Src. In the absence of LIF sense pv-SrcDEneoΔLT20 cells formed undifferentiated and differentiated minute colonies compared with antisense pv-SrcDEneoΔLT20 which were normal. The altered phenotype of sense pv-SrcDEneoΔLT20 supertransfected cells can be attributed to v-Src protein expression because supertransfection with antisense pv-SrcDEneoΔLT20 had no effect. This data suggests that v-Src expression caused cell death or growth arrest of ES cells.

v-Src is known to interact with the PyV MT antigen (Royal *et al.*, 1996). PyV MT and LT are partially homologous making it possible that the observed phenotype may result from interference with normal PyV LT function and/or episome replication by v-Src. pv-SrcDEneoΔLT20 was transfected into D3 ES cells or supertransfected into C32 ES cells to investigate this possibility. Episome-based plasmids stably transfect wild-type ES cells at conventional efficiency (3.3.3) because extrachromosomal replication will not occur due to a lack of PyV LT expression. Transfected cells were subjected to selection in 800 μg/ml G418 in the presence of LIF for 13 days, stained to detect AP activity and scored to examine the effect on colony viability. While reduced resistant clone formation may not be a rigorous measure of loss of viability, it has been taken as evidence for a cytotoxic action of Socs-1 and Socs-3 in ES cells (Duval *et al.*, 2000).

Several thousand colonies were formed following introduction of pPSDEneoΔLT20 episome into C32 ES cells, indicative of efficient supertransfection, while pv-SrcDEneoΔLT20 supertransfection resulted in only 28 morphologically normal G418 resistant colonies. 152 colonies formed when D3 ES cells were transfected with pPSDEneoΔLT20. 9-fold fewer (17 versus 152) morphologically normal G418 resistant colonies formed in D3 cultures transfected with pv-SrcDEneoΔLT20 compared with pPSDEneoΔLT20, indicating that v-Src dependent cytotoxicity was also observed in D3 ES cells that do not harbour episome. Therefore reduced colony survival in D3 and C32 ES cells can be attributed to v-Src expression and not to properties of the episome system.

AP^+ colonies that survived selection were morphologically normal (not shown) presumably because they did not express significant levels of v-Src. The number of stable

pv-SrcDEneoΔLT20 resistant colonies formed in C32 and D3 transfected cultures was roughly equivalent (17 versus 28) even given the markedly lower transfection efficiency of D3 ES cells.

4.5.1 Discussion

In contrast to other reports (Boulter *et al.*, 1991), when *v-Src* was expressed in ES cells using the episome system small resistant colonies formed that died with continued selection. Moreover, formation of resistant colonies was also impaired when pv-SrcDEneoΔLT20 was transfected into D3 ES cells, indicating that the cytotoxic effect was caused by v-Src expression alone and not through interference with episome replication. Rare G418 resistant pv-SrcDEneoΔLT20 supertransfected colonies were morphologically normal and may not express significant amounts of v-Src as such colonies were observed at comparable frequencies in D3 and C32 ES cells transfected with pv-SrcDEneoΔLT20. Formation of very low numbers of stable resistant clones may represent the frequency of pv-SrcDEneoΔLT20 integration events resulting in expression of the neomycin resistance gene but not *v-Src*, an event expected to occur at equal frequencies in D3 and C32 ES cells. Genomic integration of episomes appears to result in partial integration of the plasmid (Gassmann *et al.*, 1995), presumably due to the fact that only the neomycin resistance cassette is selected for.

While other oncogenes activate only mitogenic signals and consequently cause apoptosis, v-Src is known to activate mitogenic and survival signals via Ras, PI 3-kinase and Stat3 (Wyke *et al.*, 1993; Bromberg *et al.*, 1998), and with few exceptions (Tarpley and Temin, 1984; Wu and Hackett, 1995), does not cause apoptosis (Webb *et al.*, 2000). v-Src has recently been shown to activate an apoptotic signal when v-Src dependent survival signals are inhibited by a dominant negative form of Ras or chemical inhibitors of PI 3-kinase (Webb *et al.*, 2000). High v-Src expression has been reported to cause cytotoxicity in Chinese Hamster Ovary (CHO) cells and canine cells (Tarpley and Temin, 1984; Wu and Hackett, 1995). It is possible that unlike most cell lines expression of v-Src in ES cells may cause apoptosis.

Experiments using *v-Src* were designed to demonstrate that cell-autonomous factors are selected for in the episome-based screening approach. Episomal expression of *v-Src* instead indicated that v-Src had a cytotoxic effect. Effects on ES cell self-renewal by episomally expressed *v-Src* may however be masked by *v-Src* induced cytotoxicity. Nonetheless, the

episome-based expression system can be used to express, and functionally characterise the effect of heterologous gene expression in ES cells.

4.6 EPISOMAL EXPRESSION OF THE β ISOFORM OF Stat3 INHIBITS LOSS OF PLURIPOTENCE IN RESPONSE TO LIF WITHDRAWAL

Stat transcription factors are downstream targets of cytokine signalling via the activation of Jak family tyrosine kinases (reviewed in Heinrich *et al.*, 1998). Stat3 binds to promoters and activates transcription of acute phase response genes following cytokine stimulation. There are two isoforms of Stat3, Stat3α and Stat3β, that arise from alternative splicing. The shorter message, Stat3β, lacks 50 bp that results in a frame shift and premature termination of the polypeptide chain (Caldenhoven *et al.*, 1996; Schaefer *et al.*, 1995)(Figure 4.8). As a result the Stat3β protein is 48 amino acids shorter than Stat3α and contains seven amino acids specific to Stat3β at the C-terminus. Other Stat3 isoforms have been reported but these have not been characterised (Morton *et al.*, 1999; Nielsen *et al.*, 1997).

The properties of Stat3α and Stat3β have been extensively characterised in transfected COS-7 cells. Stat3α and Stat3β bind to the high-affinity *sis*-inducible element (hSIE) and activate transcription in response to OSM, EGF, IFNα/γ and TGF-α (Schaefer *et al.*, 1997). Unlike Stat3α, Stat3β is active in the absence of cytokine stimulation, due to constitutive phosphorylation of tyrosine 705 (Schaefer *et al.*, 1997). Stat3β is also more stable and has a greater affinity for DNA than Stat3α, but relative to DNA binding affinity, Stat3α has a greater capacity to activate transcription (Schaefer *et al.*, 1997). Maximal activation of Stat3α requires phosphorylation of both Tyr 705 and Ser 727 (Wen *et al.*, 1995). Stat3β lacks the latter residue possibly accounting for its reduced ability to activate transcription. It is of note that in HepG2 hepatoma cells Stat3α and Stat3β transmit signals from gp130 equally well (Sasse *et al.*, 1997).

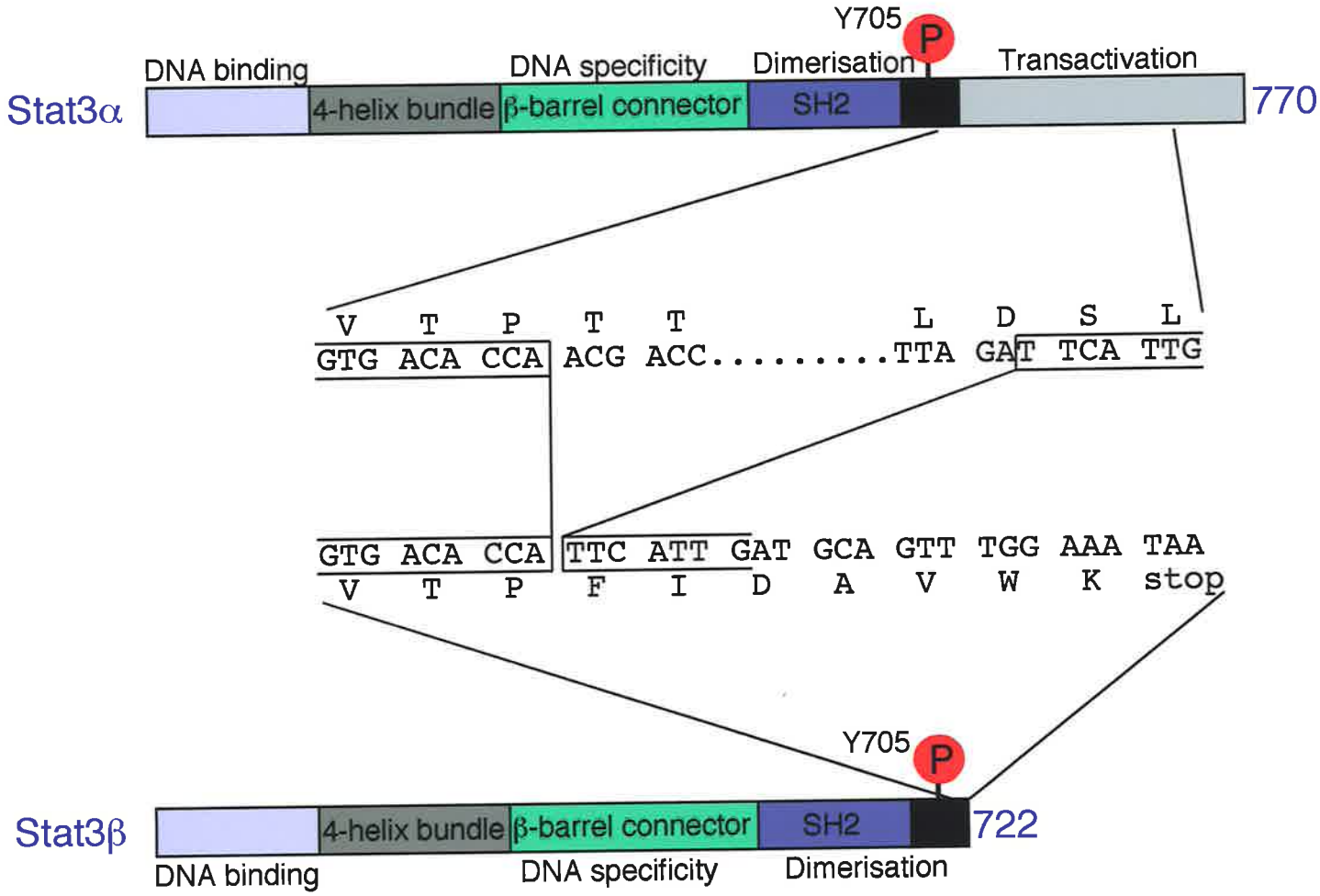
Unlike Stat3α, Stat3β can cooperate with c-Jun to activate transcription of a α2-macroglobulin-TK-CAT reporter in F9 EC cells in the absence of cytokine stimulation (Schaefer *et al.*, 1995). In COS-1 cells transfected with IL-5R, Stat3β, Stat3α and a palindromic IL-6/IFNγ response element reporter, Stat3β acts in a dominant negative fashion, inhibiting Stat3α transactivation (Caldenhoven *et al.*, 1996). $CD34^+$ bone marrow and leukaemic myeloid HL60 cell differentiation induced by G-CSF results in activation and DNA binding of Stat3β

Figure 4.8

Schematic representation of Stat3α and Stat3β transcripts and proteins.

Structural domains and regions with assigned functions are indicated. The dimerisation domain includes the SH2 domain and tyrosine 705. Tyrosine 705 is phosphorylated (red) in response to cytokine stimulation allowing reciprocal SH2 phosphotyrosine interactions to occur between monomers. Absence of a 50 bp region in the *Stat3β* message results in a shift in reading frame and hence truncation of the Stat3β protein. The altered reading frame compared to Stat3α and codons surrounding the region absent in Stat3β are depicted. The C-terminal seven amino acids are specific for Stat3β.

Adapted from Caldehoven *et al*, (1996) and Becker *et al*, (1998).


Y705
P
DNA binding
DNA specificity
Dimerisation
Transactivation
Stat3α
4-helix bundle
β-barrel connector
SH2
770
V T P T T L D S L
GTG ACA CCA ACG ACC.....TTA GAT TCA TTG
GTG ACA CCA TTC ATT GAT GCA GTT TGG AAA TAA
V T P F I D A V W K stop
Y705
P
Stat3β
4-helix bundle
β-barrel connector
SH2
722
DNA binding
DNA specificity
Dimerisation

alone, while other acute myelogenous leukaemia (AML) lines proliferate in response to G-CSF and activate Stat3α and Stat3β (Chakraborty *et al.*, 1996). Expression of Stat3β, but not Stat3α, was reported to block v-Src induced NIH 3T3 cell transformation (Turkson *et al.*, 1998), suggesting that Stat3β may inhibit Stat3α signalling in fibroblast cells. Thus Stat3β exhibits Stat3α independent biological properties and can also have positive or negative influences on the Stat3 signalling pathway. It appears likely that the observed function of Stat3β is dependent on the cell type, reporter construct and the presence of additional factors such as c-Jun.

4.6.1 Stat3β promotes ES cell survival in the absence of LIF

Stat3α activation is necessary and sufficient for ES cell self-renewal (Boeuf *et al.*, 1997; Matsuda *et al.*, 1999) while Stat3β has not been implicated in ES cell maintenance. Given that Stat3β may be a constitutively active form of Stat3 it was tested in the episome system to establish whether it had a positive effect on ES cell self-renewal in the absence of cytokine stimulation. Human *Stat3α* and *Stat3β* cDNAs were kind gifts from Dr Rolf De Groot (The University Medical Center Utrecht, The Netherlands). Human Stat3α differs by only one amino acid from mouse Stat3α. This nucleotide difference is outside the Stat3β open reading frame (Pietra *et al.*, 1998). *hStat3α* and *hStat3β* cDNAs were excised from pSG513hStat3α and pSG513hStat3β (Caldenhoven *et al.*, 1996) as 2.7 kb and 3.3 kb *Eco*RI/*Sac*I fragments, respectively. The cDNAs were blunt cloned into *Eco*RI/*Not*I digested pPSDEneoΔLT20 to form pStat3αDEneoΔLT20 and pStat3βDEneoΔLT20 plasmids (Figure 4.9a and b).

C32 ES cells were supertransfected with pStat3αDEneoΔLT20, pStat3βDEneoΔLT20 or pPSDEneoΔLT20, selected in 200 μg/ml G418 for 10 days in the presence or absence of exogenous LIF and stained for AP activity. Stable, G418-resistant ES cell colonies were formed in large numbers in cultures grown in the presence of LIF for all three plasmids (Figure 4.10a). In the absence of LIF, few pStat3αDEneoΔLT20 or pPSDEneoΔLT20 supertransfected colonies survived differentiation and selection. In contrast, pStat3βDEneoΔLT20 supertransfected cells cultured in the absence of LIF formed AP^+, G418-resistant colonies. Pluripotent colonies observed in pStat3βDEneoΔLT20 supertransfected cultures grown in the absence of LIF had not undergone extensive differentiation and were smaller than those observed in the presence of LIF (Figure 4.10a). Scoring of AP^+ colonies indicated that

Figure 4.9

Cloning of *Stat3* isoforms into the episome expression vector pPSDEneoΔLT20.

Construction of (A) pStat3αDEneoΔLT20 and (B) pStat3βDEneoΔLT20. pStat3αDEneoΔLT20 and pStat3βDEneoΔLT20 episomes can be supertransfected into episome harbouring ES cell lines to express *Stat3α* and *Stat3β* transcripts, respectively. Shading is as described in figure 4.2.

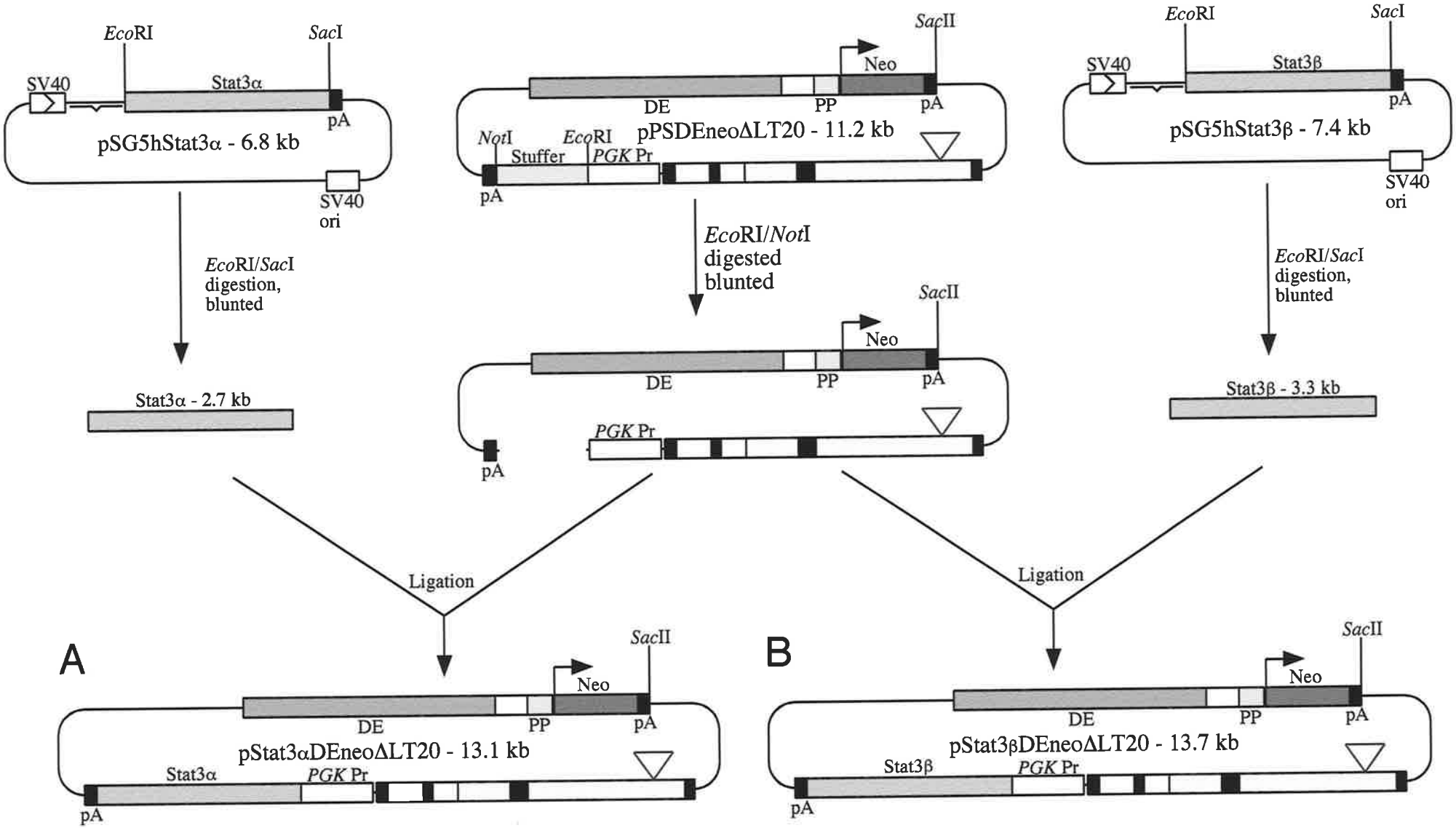

EcoRI
SacI
SV40
Stat3α
pA
pSG5hStat3α - 6.8 kb
SV40
ori
EcoRI/SacI
digestion,
blunted
Stat3α - 2.7 kb
SacII
Neo
DE
PP
pA
NotI
EcoRI
Stuffer
PGK Pr
pPSDEneoΔLT20 - 11.2 kb
pA
EcoRI/NotI
digested
blunted
SacII
Neo
DE
PP
pA
PGK Pr
pA
EcoRI
SacI
SV40
Stat3β
pA
pSG5hStat3β - 7.4 kb
SV40
ori
EcoRI/SacI
digestion,
blunted
Stat3β - 3.3 kb
Ligation
Ligation
A
SacII
Neo
DE
PP
pA
pStat3αDEneoΔLT20 - 13.1 kb
Stat3α
PGK Pr
pA
B
SacII
Neo
DE
PP
pA
pStat3βDEneoΔLT20 - 13.7 kb
Stat3β
PGK Pr
pA

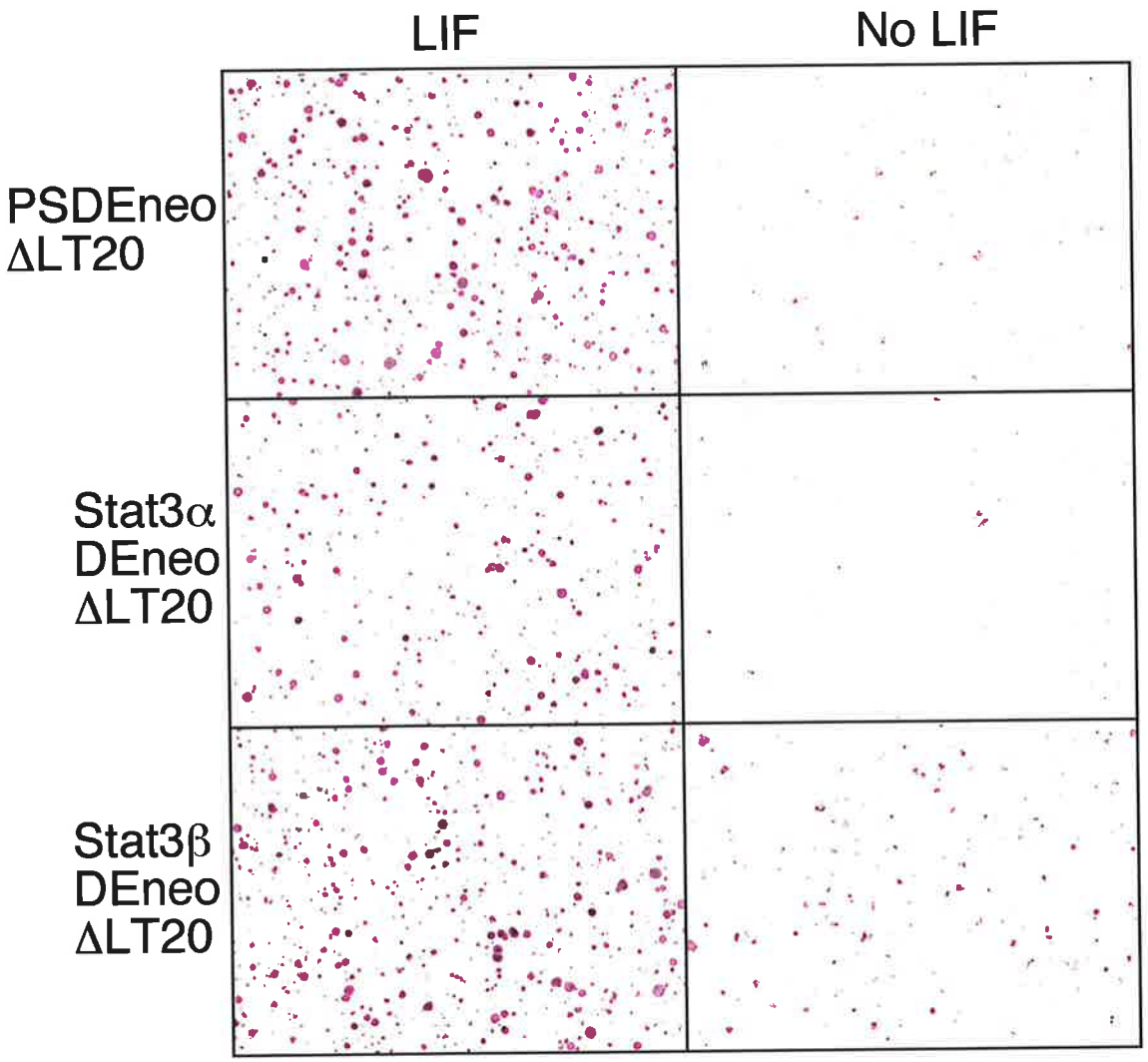
Figure 4.10

Episomal expression of Stat3β inhibits ES cell differentiation.

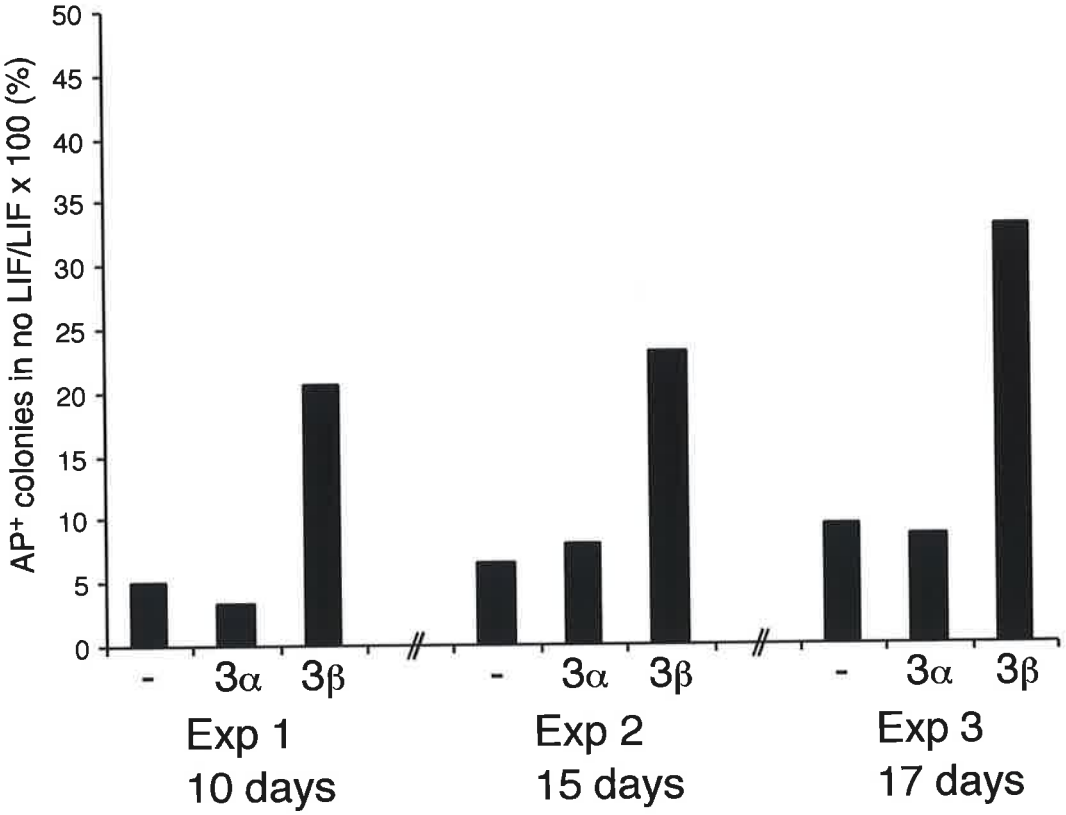
A. 3 x 10^7 C32 ES cells were electroporated with 20 μg of pStat3αDEneoΔLT20, pStat3βDEneoΔLT20 or pPSDEneoΔLT20. 7 % of the transfected cells were seeded onto 10 cm plates and selected for 10 days in 200 μg/ml G418 in the presence or absence of LIF. G418 resistant colonies were stained to detect AP activity. Plates were scanned using a Umax Powerlook II scanner.

B. A graph showing the proportion of Stat3α and Stat3β supertransfected colonies that retain AP^+ activity in the absence of LIF. AP^+ colonies were scored from plates grown with or without LIF. Data from three experiments selected for 10, 15 and 17 days in 200 μg/ml G418 respectively, are graphed separately.

A


LIF
No LIF
PSDEneo
ΔLT20
Stat3α
DEneo
ΔLT20
Stat3β
DEneo
ΔLT20

B


AP+ colonies in no LIF/LIF x 100 (%)
50
45
40
35
30
25
20
15
10
5
0
-
3α
3β
Exp 1
10 days
Exp 2
15 days
Exp 3
17 days

supertransfection with pStat3βDEneoΔLT20 elevated the number of pluripotent colonies approximately 4-fold during selection in the absence of LIF when normalised against plasmid supertransfection efficiencies (Figure 4.10b). In subsequent experiments the period of G418 selection was increased to 15 and 17 days to select episome supertransfected ES cells (3.3.3) and resistant colonies were scored by quantitation using Optimus 6.2 image analysis software. In both instances 3.5-fold more ES cell colonies survived when supertransfected with pStat3βDEneoΔLT20 compared with the pPSDEneoΔLT20 vector (Figure 4.10b). pStat3αDEneoΔLT20 supertransfected cells formed pluripotent resistant colonies in the absence of LIF at 1.29, 0.90 and 0.65-fold levels of vector control indicating that Stat3α supertransfection had no effect on the maintenance of ES cell pluripotence in this assay.

4.6.2 Discussion

Selection of pStat3βDEneoΔLT20 supertransfected ES cells in the absence of LIF resulted in a 3-4 fold increase in pluripotent colony survival compared to pPSDEneoΔLT20 or pStat3αDEneoΔLT20 supertransfected ES cells. This observation implies that Stat3β, but not Stat3α, can inhibit ES cell differentiation. Stat3β has been reported as both a constitutively active (Schaefer *et al.*, 1995; Schaefer *et al.*, 1997) and as a dominant negative form of Stat3 (Caldenhoven *et al.*, 1996). Data presented here suggest that in ES cells, Stat3β acts in a constitutively active manner given activated Stat3α maintains ES cells in an undifferentiated state (Boeuf *et al.*, 1997; Matsuda *et al.*, 1999). A role for Stat3β in pluripotent cell maintenance is consistent with constitutive phosphorylation and DNA binding activity of Stat3β in the absence of cytokine signalling (Schaefer *et al.*, 1997). Thus, Stat3β overexpression may activate the LIF pathway, resulting in increased pluripotent colony survival in the absence of LIF signal transduction.

Episomal expression demonstrated that the cell-autonomous factor, Stat3β, has a role in pluripotent cell maintenance. This confirms that the episome-based functional screen is capable of selecting for cell-autonomous factors that have an effect on the maintenance of the pluripotent state.

4.7 GENERAL DISCUSSION

Data presented in this chapter indicates the utility and versatility of the episome selection system for identification and analysis of genes that control maintenance of the pluripotent phenotype. In particular, the demonstration that both LIF, an extracellular cytokine, and Stat3β, a transcription factor, promote the survival of pluripotent cells in the absence of LIF when expressed from the episome expression vector indicate that both cell-autonomous and extracellular factors can be selected using the screening system.

Episomal expression of v-Src was observed to have a cytotoxic effect in contrast to the previous reported finding that v-Src expression inhibited ES cell differentiation in the absence of LIF (Boulter *et al.*, 1991). Thus the episome expression system can be used to demonstrate other phenotypic traits conferred by overexpressed or introduced genes in ES cells. It is conceivable that, with some modifications, the episome system could be used to conduct function-based screens in ES cells for gene induced properties such as apoptosis, cell cycle arrest, cytokine responsiveness, gene expression changes and directed differentiation to particular cell lineages.

Cellular phenotypes were observed upon episomal expression of diffusible (LIF-D), extracellular matrix-associated (LIF-M), signal transduction (v-Src) and transcription factor (Stat3) molecules. These data highlight the versatility of the episome expression system and suggest that factors of these types could be isolated by the episome-based screening approach.

CHAPTER 5:

FUNCTION-BASED SCREENING FOR FACTORS THAT INHIBIT ES CELL DIFFERENTIATION

5.1 INTRODUCTION

The functional screen was designed to identify normal cellular genes that inhibit ES cell differentiation and by analogy, are potentially involved in pluripotent cell maintenance during embryogenesis. It was reasoned that intracellular factors whose biological role is to inhibit differentiation of pluripotent ES cells will be expressed in undifferentiated ES cells. ES cells are also a source of abundant RNA, facilitating cDNA library construction. RNA isolated from mouse early embryos was also considered as starting material for cDNA library construction but was available only in limited quantities. Further, as a heterogenous cell population, the proportion of transcripts encoding proteins involved in pluripotent cell maintenance is likely to be lower in mouse embryos. The preferred source of RNA for cDNA library construction was therefore undifferentiated ES cells.

5.2 PRODUCTION AND ANALYSIS OF AN ES CELL cDNA EXPRESSION LIBRARY

5.2.1 Construction of an ES cell cDNA expression library

Double stranded cDNA was constructed from 2 μg of poly $(A)^+$ RNA using *Not*I-$(dT)_{18}$ primer-adaptor to prime first strand polymerisation. Recognition sites for *Not*I and *Eco*RI restriction endonucleases were included in the *Not*I-$(dT)_{18}$ primer-adaptor (2.2.7) to allow directional cloning of double stranded cDNA into pPSDEneoΔLT20 and subsequent subcloning of identified cDNAs via *Eco*RI digestion, respectively. First strand cDNA synthesis was performed with Avian Myeloblastosis Virus (AMV) reverse transcriptase. cDNAs reverse transcribed from poly $(A)^+$ or total RNA are not always full-length because of intrinsic RNase H activity of AMV reverse transcriptase (Gerard *et al.*, 1997). Thus library clones produced by this method may not represent full-length cDNAs.

Double stranded cDNA was produced largely by the Gubler and Hoffman method (Gubler and Hoffman, 1983) using the Riboclone cDNA synthesis system (Promega) (Figure 5.1a). Poly $(A)^+$ RNA was purified from cytoplasmic RNA through two rounds of selection on oligo-dT cellulose (Pharmacia) (2.3.27) (Celano *et al.*, 1993). A smear of RNA in the size range of 0.46 kb to 4.8 kb was isolated by this method (Figure 5.1b). The poly $(A)^+$ RNA did not contain significant quantities of 28S and 18S ribosomal RNA. Northern analysis of poly

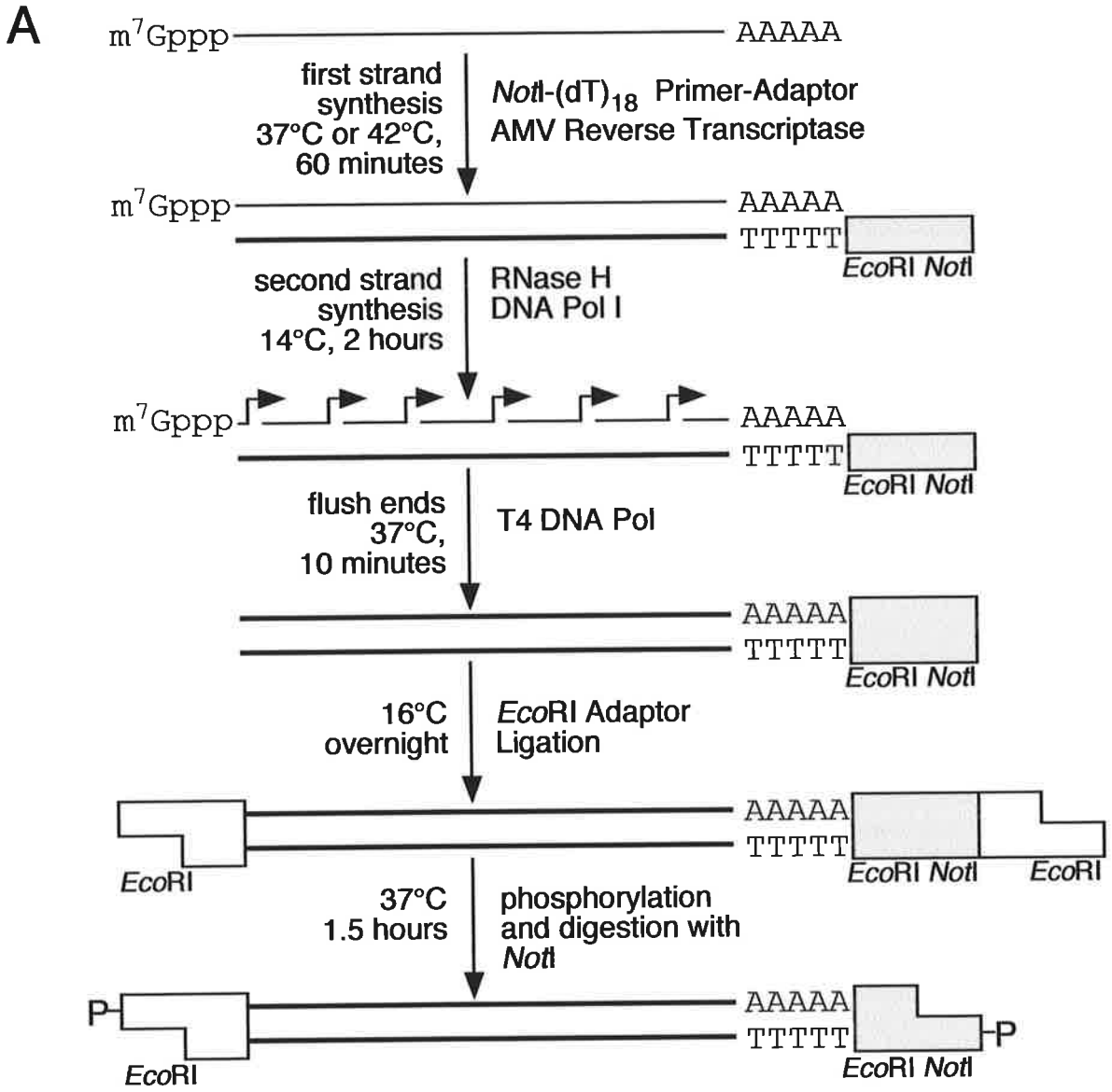
Figure 5.1

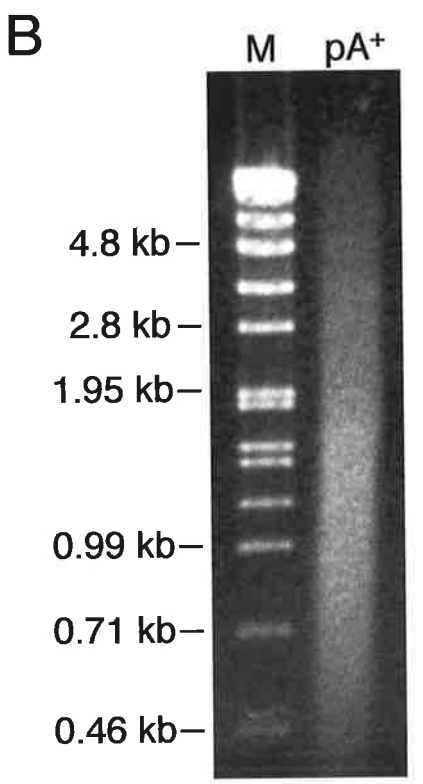
cDNA synthesis.

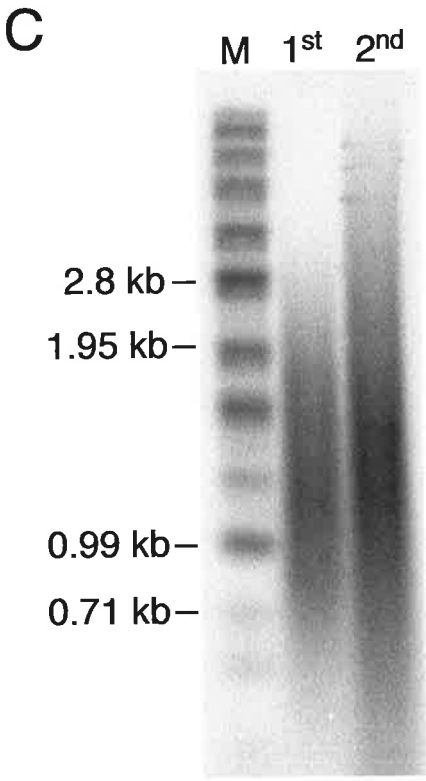
A. Schematic representation of the strategy used for cDNA synthesis and directional cloning. Poly $(A)^+$ RNA selected twice on Oligo-dT cellulose was used as a template for synthesis of the first cDNA strand by AMV reverse transcriptase. *Not*I-(dT) primer-adaptor (2.2.7) was used to incorporate a *Not*I restriction site in the 3′ end of double stranded cDNA. Second strand synthesis was continued in the same tube with addition of RNase H, *E. coli* DNA polymerase I and second strand buffer. RNase H cleaves RNA in the RNA:DNA duplex. RNA fragments act as primers for DNA polymerase I dependent DNA synthesis by nick translation. The cap structure and the remaining extreme 5′ end RNA was removed and cDNA ends were flushed by treatment with T4 DNA polymerase. cDNA longer than 400 bp was selected on Sephacryl S-400 spin columns and ligated to *Eco*RI adaptors (2.2.7) phosphorylated only on the blunt end. Ends of the cDNA were phosphorylated by T4 polynucleotide kinase and the cDNA was digested with *Not*I endonuclease. Resulting *Eco*RI and *Not*I cohesive ends at the 5′and 3′ ends of the cDNA respectively allow directional cloning into the library vector.

B. Agarose gel showing poly $(A)^+$ RNA derived from D3 ES cells used in cDNA synthesis (C). Poly $(A)^+$ RNA was selected twice on oligo-dT cellulose and heat denatured prior to loading on a 1% TBE agarose gel. pA^+, poly $(A)^+$ RNA from D3 ES cells; M, *Eco*RI digested SPP-1 phage DNA markers. 0.46, 0.71, 0.99, 1.95, 2.8 and 4.8 kb marker bands are indicated.

C. Alkaline agarose gel electrophoresis of first and second strand cDNA reactions labelled by incorporation of $[\alpha^{32}P]$-dCTP. 1^{st}, First strand reaction; 2^{nd}, Second strand reaction; M, *Eco*RI digested SPP-1 phage DNA markers. 0.71, 0.99, 1.95 and 2.8 kb marker bands are indicated.


A
m7Gppp
AAAAA
first strand synthesis 37°C or 42°C, 60 minutes
NotI-(dT)18 Primer-Adaptor
AMV Reverse Transcriptase
TTTTT
EcoRI NotI
second strand synthesis 14°C, 2 hours
RNase H
DNA Pol I
flush ends 37°C, 10 minutes
T4 DNA Pol
16°C overnight
EcoRI Adaptor Ligation
EcoRI
37°C 1.5 hours
phosphorylation and digestion with NotI
P


B
M
pA+
4.8 kb
2.8 kb
1.95 kb
0.99 kb
0.71 kb
0.46 kb


C
M
1st
2nd
2.8 kb
1.95 kb
0.99 kb
0.71 kb

$(A)^+$ RNA showed hybridisation to 7 kb and 10 kb transcripts using a probe specific for the *Icm1* gene (not shown) (Schulz, 1996) indicating that no degradation or shearing had occurred during poly $(A)^+$ RNA purification.

Tracer first and second strand reactions were carried out in the presence of 5 μCi [α-^{32}P]dCTP in order to determine the quantity and quality of cDNA produced. The second strand reaction was carried out with *E. coli* DNA polymerase I and RNase H (2.3.28). Half of each reaction was run on an alkaline agarose gel to determine the length of synthesised products. The majority of first strand products were of sizes between 710 bp and 1.95 kb (Figure 5.1c) typically observed with this methodology (Gubler and Hoffman, 1983) although longer cDNA can be obtained. A smear of products between 360 bp and 6 kb was observed following electrophoresis of the second strand reaction (Figure 5.1c), implying a continuum of sizes across this range. Increased cDNA length in the second strand reaction is probably caused by hairpin priming and polymerisation from the 5′ end of the cDNA resulting in cDNAs that run at twice the size of double stranded cDNAs formed by strand replacement. Hairpin primed cDNA generally accounts for 15% or less of the total cDNA (Gubler, 1987; Sambrook *et al.*, 1989) synthesised by the Gubler and Hoffman method. Such cDNAs will not be cloned by the method outlined in figure 5.1a because hairpin loops are not removed by nuclease treatment prior to adaptor ligation. As expected, the majority of labelled cDNAs in the second strand reaction were between 710 bp and 1.95 kb indicating that the second strand reaction reflected products of the first strand reaction (Figure 5.1c). The greatest proportion of second strand DNA was therefore produced by RNA priming and strand replacement rather than by hairpin priming.

T4 DNA polymerase was used to blunt cDNA ends and cDNAs longer than 400 bp were selected on Sephacryl S-400 spin columns (2.3.28), ligated to *Eco*RI adapters (2.2.7), phosphorylated with T4 polynucleotide kinase and restriction digested with *Not*I endonuclease (2.3.28). cDNAs were separated from excess *Eco*RI adapters through size selection on Sephacryl S-400 spin columns (2.3.28). The quantity of cDNA produced was estimated at 550 ng as determined by scintillation counting of trichloroacetic acid precipitable material from first and second strand tracer reactions (2.3.28).

The cDNA was cloned into *Eco*RI/*Not*I digested pPSDEneoΔLT20 (4.2), placing cDNAs in the sense orientation relative to the *PGK-1* promoter (Figure 5.2a). The unamplified library was electroporated into DH10B Electro-Max electrocompetent cells (Gibco/BRL) and transformants plated on ninety two 15 cm LB (plus 100 μg/ml ampicillin) plates at a density of 71,000 colonies per plate giving a total of 6.5 x 10^6 transformants.

5.2.2 Characterisation of the cDNA library

Mini-preparations (2.3.12) from 17 independent library DNA transformants indicated that all but one (94%) of the purified plasmids contained a cDNA insert. The library therefore contained over 6.1 x 10^6 independent clones. Low abundance transcripts (one transcript per cell) can generally be isolated from libraries containing several million independent clones (Sambrook *et al.*, 1989). Thus it should be possible to isolate low abundance cDNAs from this library assuming that the majority of clones express a functional protein. cDNA inserts ranged in size from 0.5 kb to 3 kb and the average insert size was determined to be 1.11 kb (Figure 5.2b). The cDNA inserts were within the size expected from this procedure (Gubler and Hoffman, 1983) but may contain a significant proportion of truncated cDNAs. Given that truncated cDNAs are likely to be present in the library, saturation screening of expressed proteins including those of low abundance or long transcripts may not be possible with this library. However, function-based screening should still be possible for short- to medium-sized transcripts and those present in more than a few copies per cell. Library clones containing truncated cDNAs could express truncated proteins with altered activity compared with wild-type proteins. In this manner dominant negative or constitutively active proteins could be identified from the screen.

In preparation for introduction into ES cells, the library was purified from bacterial transformants on fourteen 10 ml CsCl gradients (2.3.14) and a total of 8.5 mg of pure library DNA was recovered. To determine if specific cDNA sequences were present in the library, primers designed against the mouse Oncostatin M (*mOSM*) transcript (2.2.7) were used in combination with library DNA to PCR amplify *mOSM* sequences (2.3.32). PCR using reverse transcribed ES cell RNA resulted in amplification of two bands of approximately 890 bp and 760 bp representing the full-length OSM transcript and an alternate transcript that lacks exon 2,

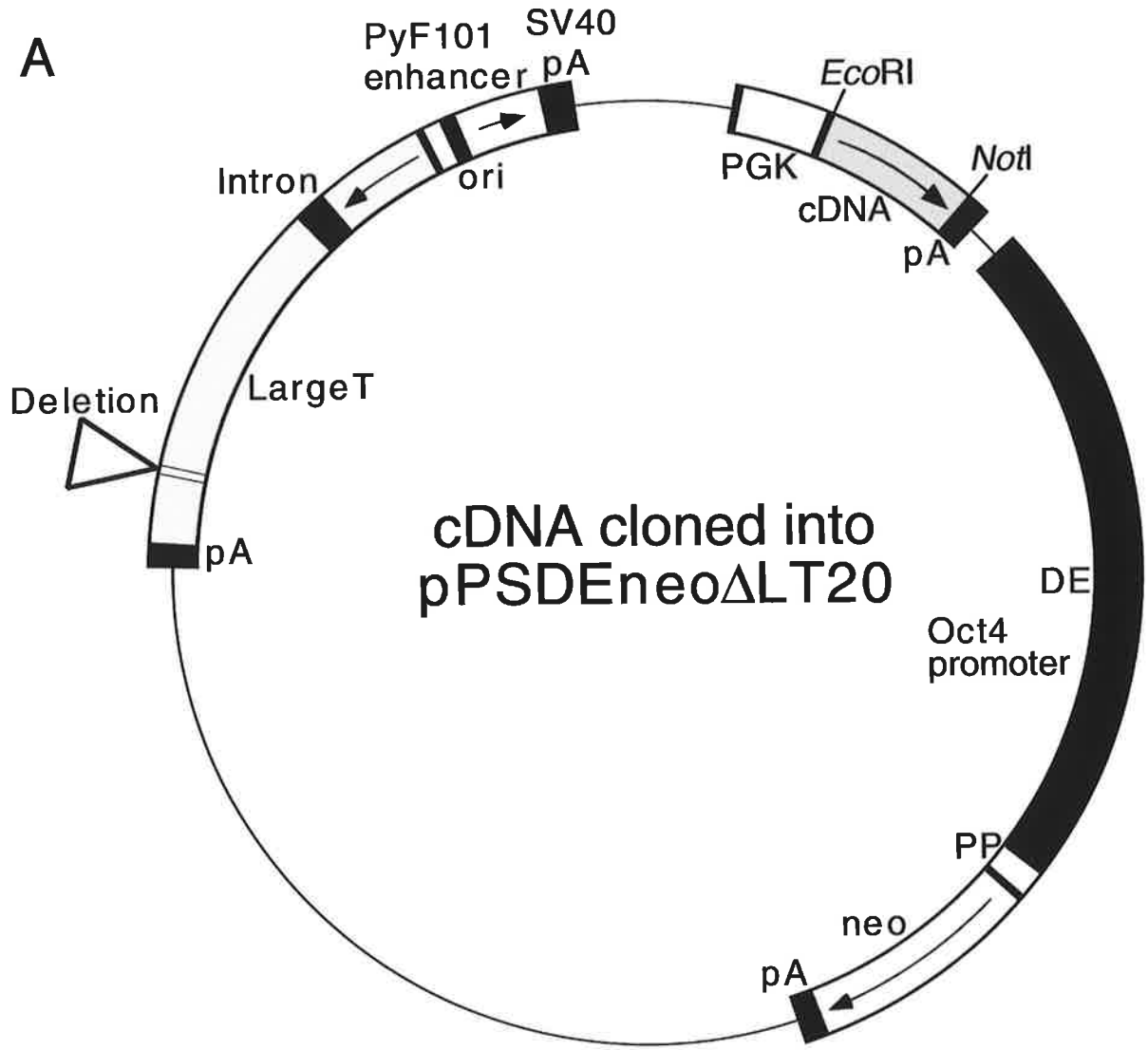
Figure 5.2

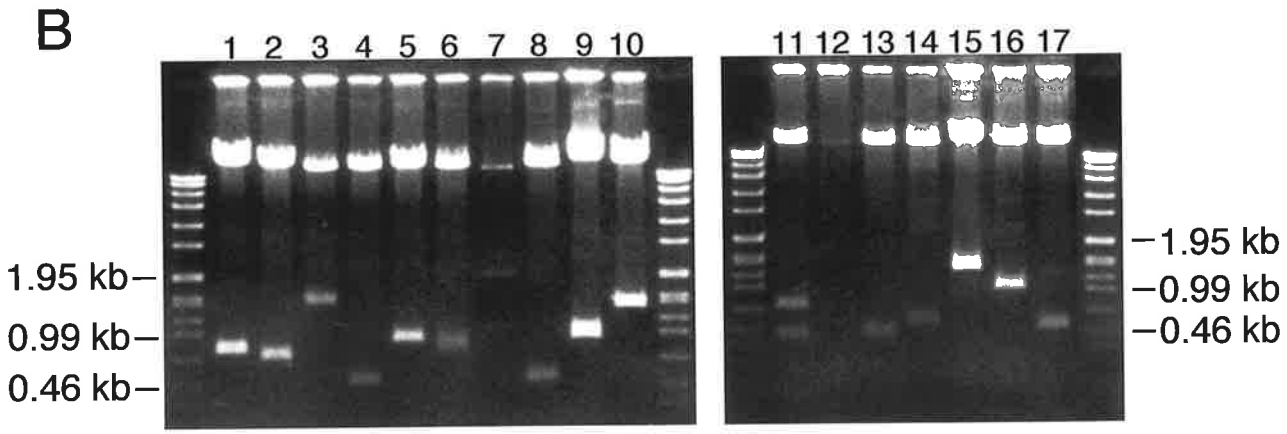
Characterisation of the cDNA library.

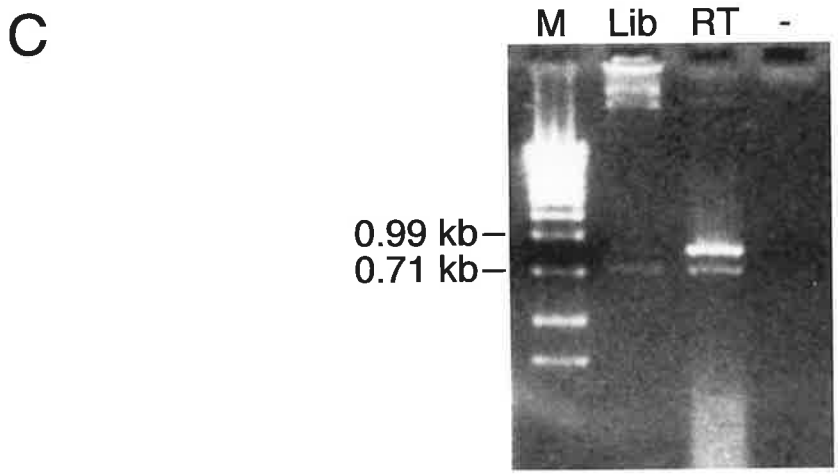
A. Diagram of the cDNA library plasmid. cDNA was directionally cloned into the *Eco*RI and *Not*I restriction sites of pPSDEneoΔLT20 placing cDNA expression under the control of the *PGK-1* promoter.

B. The size of cDNA inserts in the cDNA library. Plasmid DNA derived from primary library transformants was *Eco*RI/*Not*I digested and electrophoresed on a 1% TAE agarose gel. 1-17, transformant colonies; M, *Eco*RI digested SPP-1 phage DNA markers. 0.71, 0.99 and 1.95 kb marker bands are indicated.

C. PCR analysis of library DNA. mOSM1 and mOSM4 primers (2.2.7) were used to amplify mouse OSM specific sequences from library plasmid DNA or reverse transcribed ES cell RNA. PCR was carried out for 35 cycles at an annealing temperature of 65°C. Lib, PCR of 100 ng of library plasmid; RT, PCR of oligo-dT reverse transcribed ES cell RNA; M, *Eco*RI digested SPP-1 phage DNA markers. 0.71 and 0.99 kb marker bands are indicated.


A
PyF101
enhancer
SV40
pA
EcoRI
Intron
ori
PGK
NotI
cDNA
pA
Deletion
LargeT
pA
cDNA cloned into
pPSDEneoΔLT20
DE
Oct4
promoter
PP
neo
pA


B
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17
1.95 kb
0.99 kb
0.46 kb
1.95 kb
0.99 kb
0.46 kb


C
M Lib RT -
0.99 kb
0.71 kb

respectively (Voyle and Rathjen, 2000). *mOSM* products were also observed when library plasmid was used as a template for PCR, indicating that *mOSM* sequences were present in the library and extended 1,827 bp into the *mOSM* cDNA or to within 21 bp of the 5′ end of the full-length *mOSM* transcript (Figure 5.2c). Although the full-length transcript was amplified from library DNA it was under-represented compared with that observed upon PCR amplification of ES cell cDNA. Full-length cDNA clones corresponding to transcripts of at least 1.8 kb were therefore present in the cDNA library. Such clones should express normal cellular proteins rather than truncated protein forms, allowing functional screening of normal ES cell proteins.

5.3 OPTIMISATION OF THE FUNCTION-BASED LIBRARY SCREEN

5.3.1 Library episome replication in C32 ES cells

To determine if library DNA was maintained and replicated as an extrachromosomal plasmid in C32 ES cells, C32 ES cells were supertransfected with library DNA and selected in 200 μg/ml G418 and 110 μg/ml hygromycin in the presence of LIF. Resistant clones were formed at supertransfection efficiencies equivalent to the pPSDEneoΔLT20 plasmid (not shown) and several supertransfectants were picked, expanded and analysed for the presence of extrachromosomal library episome. Hirt DNA purified from each supertransfectant was transformed into *E. coli* and minipreparation DNA from individual transformants was analysed by *Eco*RI/*Not*I digestion and gel electrophoresis.

Plasmids of two distinct digestion patterns were observed. The majority of transformants contained 780, 2,380 and 4,550 bp *Eco*RI restriction fragments indistinguishable from *Eco*RI digested bacterial pMGD20hph plasmid. pMGD20hph therefore represented the majority of episome present in library DNA supertransfectants (Figure 5.3a), consistent with previous observations (4.3). A second plasmid was observed in Hirt DNA from three of four supertransfectants analysed. Double digestion of this plasmid with *Eco*RI and *Not*I restriction enzymes resulted in two fragments, one larger than 8.5 kb representing the pPSDEneoΔLT20 vector backbone, and a smaller fragment that was 650, 900 and 2,200 bp in lines 3, 5 and 9, respectively (Figure 5.3a). The smaller fragments represent random cDNA inserts present in individual library plasmids. The proportion of library episome found in Hirt DNA extracts was

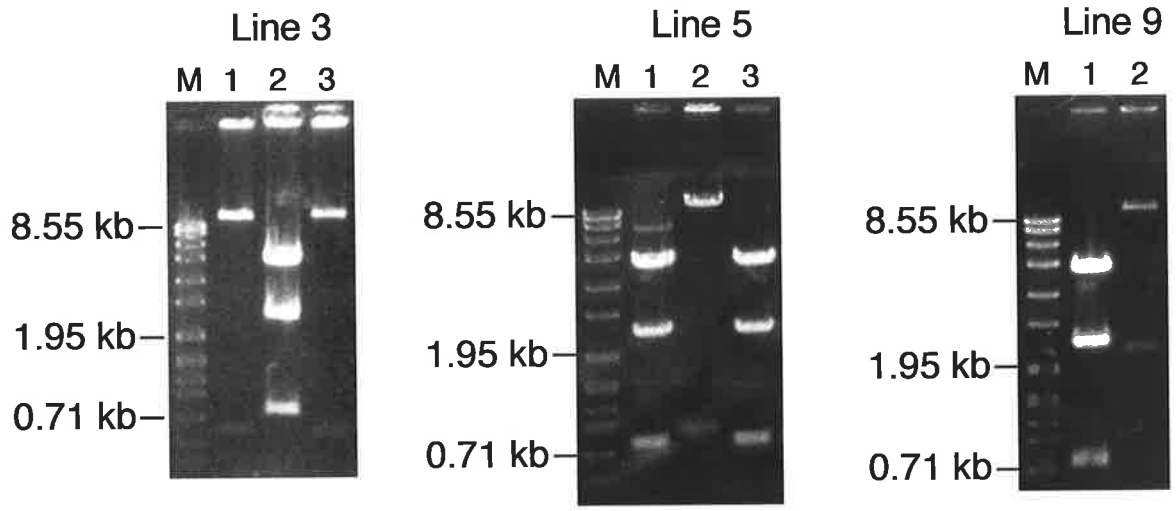
Figure 5.3

Supertransfection of episomal library DNA.

A. Electrophoretic analysis of Hirt DNA transformants derived from cell lines supertransfected with library episome. Hirt low molecular weight DNA purified from cell lines supertransfected with library episome was transformed into *E. coli.* Minipreparations from transformants were *Eco*RI/*Not*I digested and electrophoresed on a 1% TAE agarose gel (numbered 1-3). M, *Eco*RI digested SPP-1 phage DNA markers. 0.71, 1.95 and 8.55 kb marker bands are indicated.

B. Table showing the number and percentage of *Oct4* promoter-containing plasmids detected in Hirt preparations from cell lines supertransfected with episomal library DNA. Transformants that contained plasmids with *Oct4* promoter sequences were identified by Grunstein analysis of Hirt preparation transformations using a 1 kb *Bgl*II *Oct4* promoter specific radiolabelled probe (see figure 3.1).

A



B

Transformation frequency of library plasmid from supertransfectant Hirt preparations

Cell line	Total transformants	*Oct4* promoter +ve transformants	*Oct4* +ve/total x 100 (%)
1	296	29	9.8
2	56	0	0
3	103	5	4.8
4	35	2	5
5	463	29	6.3
6	182	1	0.5
7	205	4	1.9
8	456	8	1.7
9	80	5	6.2
		average:	3.8

determined by Grunstein analysis. Hirt DNA from nine supertransfectants was electroporated into *E. coli* and transformants were screened by Grunstein analysis (2.3.11) using a 1 kb *Bgl*II radiolabelled probe specific for the *Oct4* promoter (see figure 3.1). All but one (eight out of nine) supertransfectant contained plasmids with *Oct4* promoter sequence that must represent supertransfected library episome. On average, 3.8% of episomes present in these lines contained *Oct4* promoter sequences indicative of library episome (Figure 5.3b). The remainder represent pMGD20hph episome carried by the C32 ES cell line (Figure 5.3a). These data are consistent with those obtained from pβgalDEneoΔLT20 supertransfectants (4.3) and indicate that the presence of random cDNA clones had no effect on episome maintenance.

5.3.2 Optimisation of selection conditions using a pilot screen.

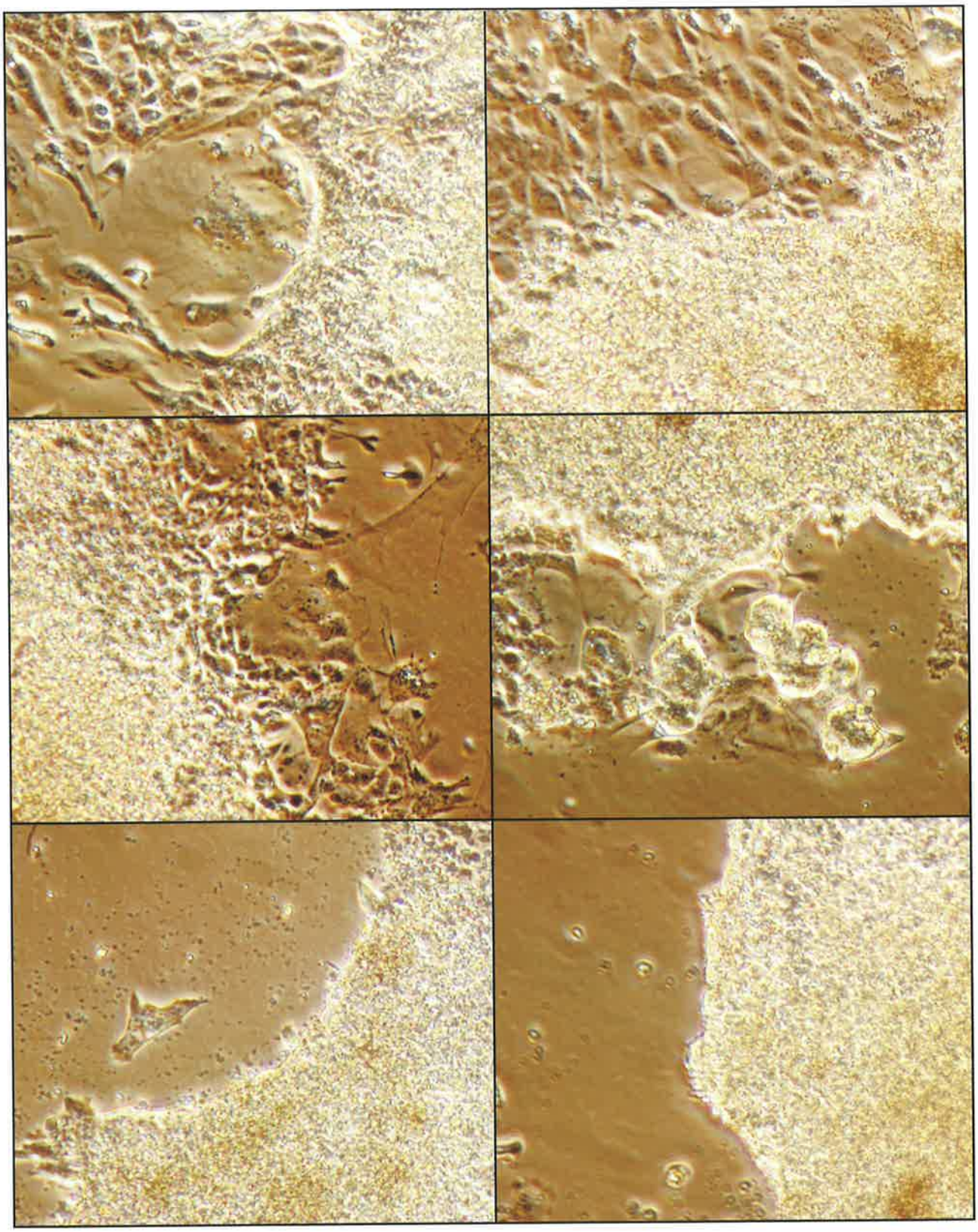
A pilot function-based screen, designed to screen 250,000 independent library clones, was performed as outlined in figure 1.10 by electroporating 160 μg of library DNA into 2.4 x 10^8 early passage (p32) C32 ES cells. Electroporated cells were plated onto twenty 15 cm diameter plates in ES cell medium lacking LIF and containing 800 μg/ml G418. To determine the number of ES cell colonies stably supertransfected, 1.3% of the cells were plated into a 6-well tray, serially diluted and selected in the presence of LIF and 800 μg/ml G418. Selective medium was replaced on screening plates each day for the first 3 days and then every second day for a further 8 days. After 2-3 days of selection surviving colonies represent G418-resistant supertransfected cells because by this time in 800 μg/ml G418, untransfected cells have died (not shown). Stable supertransfectants selected in LIF were scored after 10 days selection. A total of 50,000 independent library clones were supertransfected into C32 ES cells, indicating that fewer clones were screened than expected from previous supertransfection experiments (3.3.3) attributable to the passage of the cells used.

The pilot screen was continued to 18 days following electroporation. At this time approximately 300 colonies had survived G418 selection. Surviving colonies were large and comprised morphologically undifferentiated colonies although some differentiated cells were observed (Figure 5.4). Surviving colonies were picked, trypsinised (2.4.11) and plated into duplicate 24-well trays in the presence and absence of LIF to determine if they retained the undifferentiated phenotype in subsequent passages. Seeding densities of picked colonies could

Figure 5.4

Morphology of colonies surviving the function-based library screen.

Electroporations were plated onto three 15 cm plates in ES cell medium lacking LIF but containing 800 μg/ml G418. Selective medium was replaced on screening plates each day for the first three days and then every second day for a further 6 days. Representative G418 resistant colonies picked for further analysis are shown. Images were taken at 200 x magnification.



not be standardised, and some colonies seeded at high density in the absence of LIF. Of 100 lines tested in this way the majority underwent extensive differentiation following culture in the absence of LIF. Eight lines formed stem cell nests surrounded by differentiated cells in the absence of LIF. All colonies were observed to differentiate to some degree. Retention of stem cell nests in some lines may be in part due to high density culture because at high density ES cells form stem cell nests in the absence of LIF (Rathjen *et al.*, 1990a; Smith, 1991). These data indicated that a large proportion of colonies that survive function-based screening differentiate upon subsequent growth in the absence of LIF and therefore represent a background of 'false positives' in the procedure.

To establish if the lines isolated from the screen contained extrachromosomal library plasmid, Hirt DNA was isolated from each line and subjected to bacterial transformation and Grunstein analysis. Lifts taken from transformed Hirt DNA plates were probed with a 1 kb *Bgl*II *Oct4* promoter specific probe (see figure 3.1). The *Oct4* promoter was detected in plasmids derived from one cell line out of the eight analysed even though the total number of transformants was high, and some cases, too high to score accurately. Unlike supertransfectants selected in the presence of LIF (3.5.2 and 5.3.1), the majority of colonies that survived function-based screening did not contain extrachromosomal library. This observation is consistent with a level background of 'false positives' in the function-based screen and suggests that such colonies do not harbour episomal library DNA.

5.3.3 Reduction of background pluripotent cell survival during function-based screening.

Differentiation of ES cells as monolayers in the absence of LIF results in widespread but not complete loss of pluripotent cells from the population (Rathjen *et al.*, 1990a). A proportion of ES cells remain undifferentiated in the population and over a 6 day period these cells form colonies of pluripotent cells surrounded by differentiated derivatives, termed stem cell nests (Rathjen *et al.*, 1990a). Pluripotence is apparently maintained by LIF feedback from the surrounding cells (Rathjen *et al.*, 1990a; Mountford *et al.*, 1994). These colonies appear to remain undifferentiated in the absence of LIF by chance because cell lines derived from such colonies retain the ability to differentiate (Rathjen *et al.*, 1990a). While *Oct4*-neo selection

cassettes can be used for selection against differentiated cells (see figures 3.13 and 3.14), a residual background of pluripotent colonies survives upon supertransfection of DEPPneo-carrying episomes and G418 selection in the absence of LIF (5.3.2). These represent a background of undifferentiated colonies in the function-based screen.

Two approaches were used to overcome survival of background colonies during function-based screening: 1. disruption of paracrine and autocrine inhibition of cell differentiation by trypsinisation and re-seeding, and 2. introduction of a second round of selection.

Trypsinisation and re-seeding

It was hypothesised that the background of pluripotent colonies would be reduced if cells that survive initial selection were trypsinised, re-seeded and subjected to further selection. Following an initial period of G418 selection in the absence of LIF the total cell number is reduced dramatically. Trypsinisation at this point should allow differentiation of residual pluripotent colonies by disrupting LIF feedback inhibition due to proximity of other cells. Trypsinised cells are re-seeded at low densities, further reducing the possibility of feedback inhibition due to paracrine LIF supply.

To demonstrate that the background of pluripotent colony survival in the absence of LIF can be reduced by trypsinisation and re-seeding, C32 ES cells were supertransfected with pPSDEneoΔLT20 (3.4.3), and cultured with and without LIF in 200 μg/ml G418 for 5 days. C32 cultures initially selected in the absence of LIF were trypsinised, re-seeded and selected for a further 8 days in the presence or absence of LIF prior to AP staining. 10-fold fewer AP^+ colonies formed when C32 supertransfected cultures, initially G418 selected in the absence of LIF, were trypsinised and re-seeded in medium lacking LIF but containing G418 (87 versus 9). This reduction is attributable to selection against re-seeded pluripotent cells because 5-fold more pluripotent colonies (11800 versus 2217) formed when the selected cells were trypsinised and re-seeded into medium containing LIF and G418 (Figure 5.5a and b). These data indicate that inclusion of trypsinisation and re-seeding steps reduced survival of background pluripotent colonies in the absence of LIF. Colonies containing functional cDNAs should be amplified by this procedure, simplifying their identification.

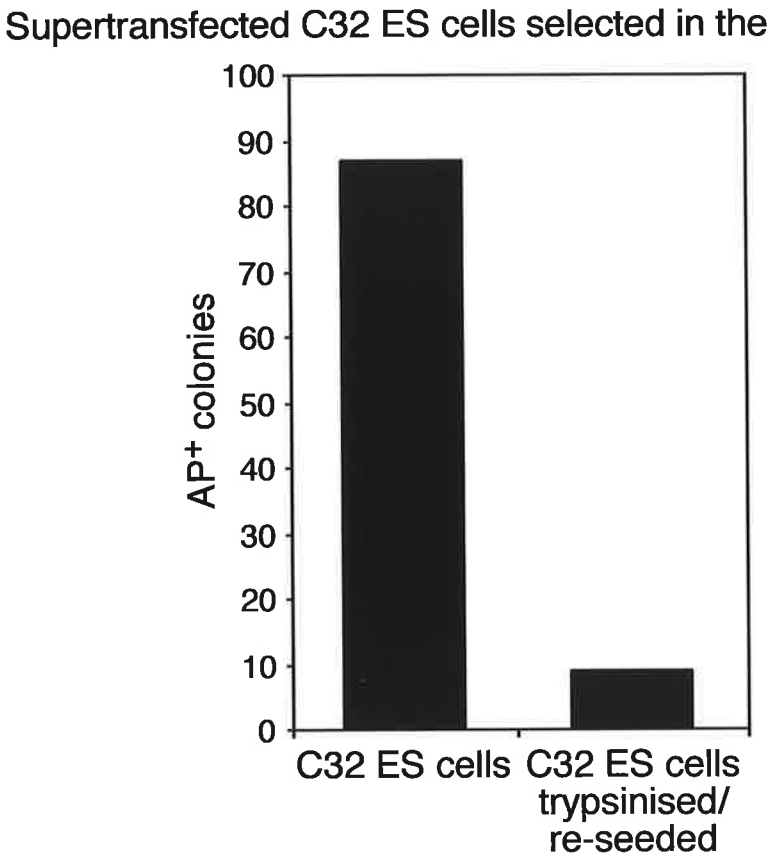
Figure 5.5

The effect of trypsinisation on pluripotent stem cell survival during G418 selection of pPSDEneoΔLT20 supertransfected C32 ES cells in the absence of LIF.

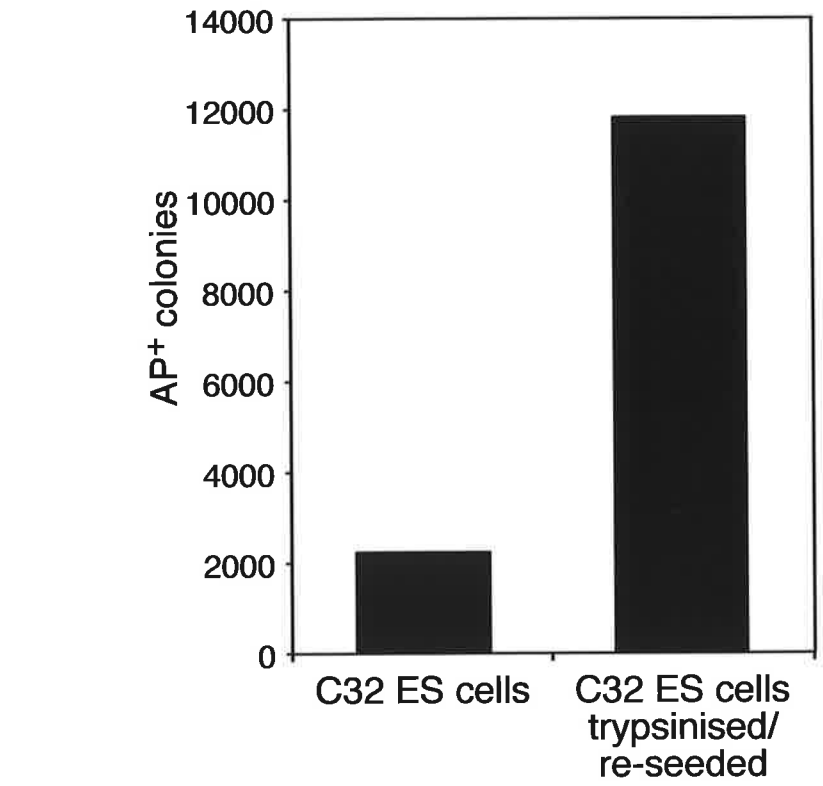
A. AP^+ colony survival in the absence of LIF with or without trypsinisation and re-seeding. 3 x 10^7 C32 ES cells were electroporated with 20 μg of CsCl purified pPSDEneoΔLT20 plasmid DNA and 1 fourteenth of the supertransfection was plated onto 10 cm diameter plates in medium containing 200 μg/ml G418 but no LIF. Cultures were G418 selected for a total of 12 days with or without trypsinisation and re-seeding after 5 days. Trypsinised cultures were selected in 200 μg/ml G418 in the absence of LIF. Plates were stained to detect AP activity. AP^+ colonies were scored and graphed for cultures selected with or without trypsinisation and re-seeding.

B. AP^+ colony survival in the presence of LIF with or without trypsinisation and re-seeding. Electroporated C32 cells (from A) were selected in 200 μg/ml G418 in the presence of LIF. Cultures were G418 selected for a total of 12 days with or without trypsinisation and re-seeding after 5 days. Trypsinised cultures were selected in 200 μg/ml G418 in the presence of LIF. Plates were stained to detect AP activity and scored. AP^+ colonies were scored and graphed for cultures selected with or without trypsinisation and re-seeding.

A


Supertransfected C32 ES cells selected in the absence of LIF
100
90
80
70
60
50
40
30
20
10
0
AP+ colonies
C32 ES cells
C32 ES cells trypsinised/ re-seeded

B


Supertransfected C32 ES cells selected in the presence of LIF
14000
12000
10000
8000
6000
4000
2000
0
AP+ colonies
C32 ES cells
C32 ES cells trypsinised/ re-seeded

Introduction of a second round of screening

Previous results suggested that formation of G418-resistant background pluripotent colonies was due to survival of cells containing integrated library episome (5.3.2). To overcome this background, a second round of screening can be incorporated into the procedure using episomes isolated from colonies that survived first round selection (Figure 5.6c). Library episome present in surviving colonies can be purified, transformed into *E. coli*, and purified. In this manner episomes containing functional cDNAs that inhibit pluripotent cell differentiation should be preferentially amplified. Supertransfected C32 ES cells can then be subjected to G418 selection in the absence of LIF to enable detection of amplified episomes that contain functional cDNAs.

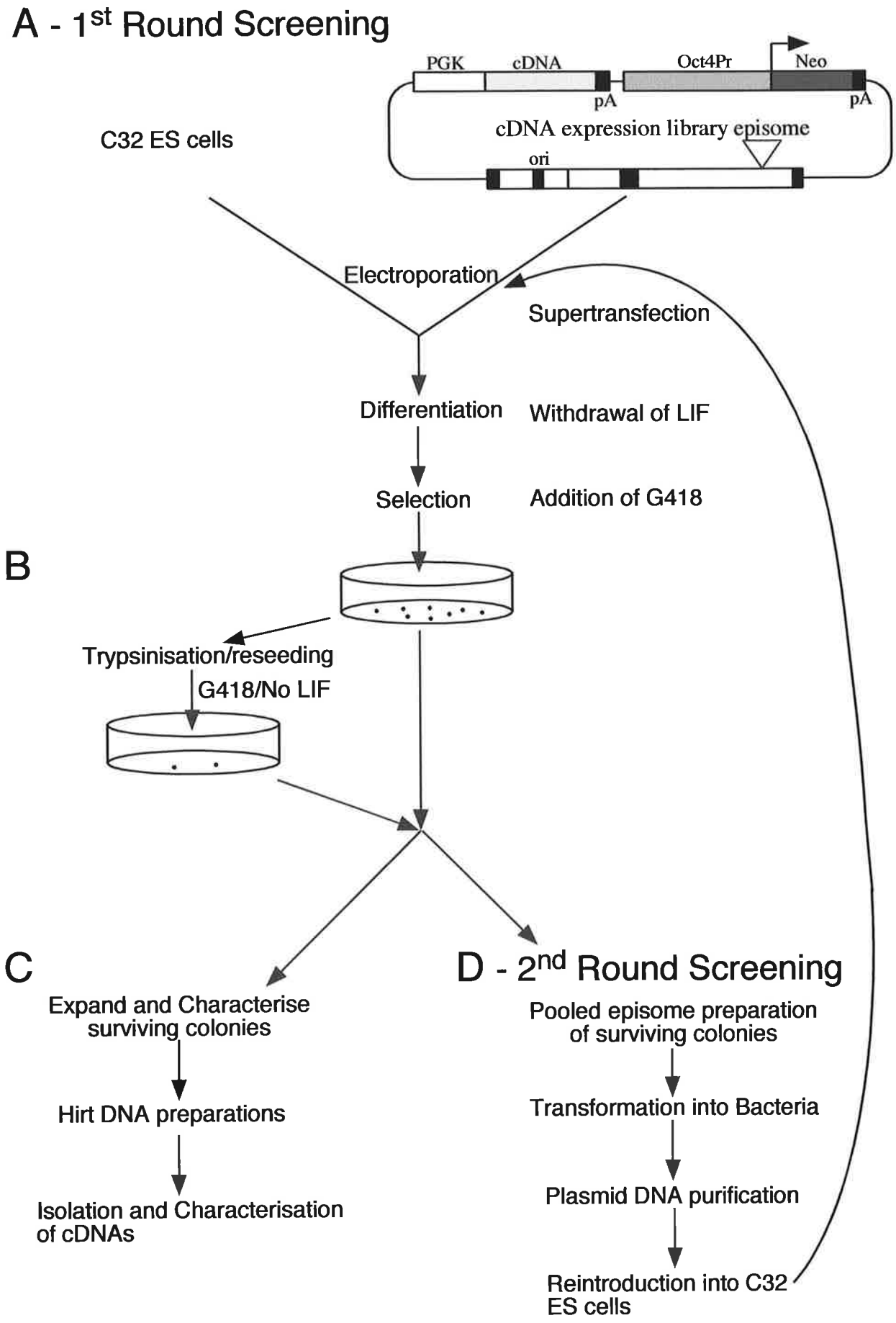
5.4 FUNCTION-BASED SCREENING

The screening protocol was designed to minimise the occurrence of false positive pluripotent colonies (Figure 5.6a-c). C32 ES cells supertransfected with episomal cDNA expression library are differentiated by LIF withdrawal and selected by addition of G418. G418-resistant colonies should comprise undifferentiated ES cells because expression of neomycin phosphotransferase, directed by the *Oct4* promoter, is restricted to pluripotent cell types. The pluripotent phenotype of surviving colonies should be supported by expression of a cDNA-encoded protein capable of maintaining pluripotence in the absence of LIF (Figure 5.6a). Background survival of pluripotent cells in the absence of LIF, due to cell-cell contact (Mountford *et al.*, 1994; Rathjen *et al.*, 1990a) will be reduced by trypsinisation and re-seeding, followed by a further period of G418 selection in the absence of LIF (Figure 5.6b). A second round screen using episomes isolated from colonies that survived first round screening can also performed (Figure 5.6d) to amplify episomes containing cDNAs that inhibit pluripotent cell differentiation.

Figure 5.6

Schematic representation of function-based screening.

First round screening (A). A cDNA expression library in pPSDEneoΔLT20 is electroporated into C32 ES cells. Supertransfected cells are differentiated by withdrawal of LIF and selected in G418. Cells in the population differentiate, hence lose expression of *Oct4* and therefore neomycin phosphotransferase. Differentiated cells are therefore selectively removed from the culture. In instances where ES cells do not differentiate, retention of Oct4 expression and neomycin resistance means that these cells survive G418 selection. Undifferentiated ES cells should have remained undifferentiated in the absence of LIF because they harbour a cDNA, the product of which is capable of inhibiting ES cell differentiation. Following selection, surviving ES cells colonies can be trypsinised and re-seeded (B) to subject cells to a second round of selection, thereby reducing background. Alternatively, following first round selection surviving colonies can be directly characterised (C) or used for Hirt DNA extraction (D) to initiate second round screening.


A - 1st Round Screening
PGK
cDNA
Oct4Pr
Neo
pA
pA
cDNA expression library episome
ori
C32 ES cells
Electroporation
Supertransfection
Differentiation
Withdrawal of LIF
Selection
Addition of G418
B
Trypsinisation/reseeding
G418/No LIF
C
Expand and Characterise surviving colonies
Hirt DNA preparations
Isolation and Characterisation of cDNAs
D - 2nd Round Screening
Pooled episome preparation of surviving colonies
Transformation into Bacteria
Plasmid DNA purification
Reintroduction into C32 ES cells

5.4.1 Identification of supertransfected cell lines resistant to differentiation in the absence of LIF

Experiment 1:

Nine electroporations (2.4.10), each with 40 μg of library episome and 6 x 10^7 C32 ES cells, were plated onto fifty 15 cm diameter plates in medium containing 200 μg/ml G418 but no LIF. G418 selection of 2% of the electroporated cells cultured in the presence of LIF (200 μg/ml G418 for 10 days) indicated that approximately 300,000 stable supertransfectants were formed. Extended selection (15 days or longer) reduces resistant colony survival (3.3.3), suggesting that this figure may be an overestimate. Medium was changed every second day and 5 days after electroporation surviving cells were trypsinised and re-seeded onto 15 cm plates in medium containing 200 μg/ml G418 but lacking LIF. Following a further 8 days of G418 selection in the absence of LIF, 100 colonies that morphologically resembled undifferentiated ES cells and contained few differentiated cells were picked (2.4.11) and seeded into duplicate 24-well trays in medium containing 200 μg/ml G418 with or without LIF supplementation. Colonies that survived selection resembled those identified from the pilot screen (5.3.2), showing little sign of morphological differentiation (not shown).

The remaining colonies, divided into 11 pools of five plates each, were harvested for Hirt DNA extraction (2.3.25). Hirt low molecular weight DNA was electroporated into *E. coli* (2.3.10), and transformants were scraped off the plates after overnight incubation. Plasmid DNA, a mix of pMGD20hph and library episome (see figure 5.3), was purified on 11 CsCl gradients. 20 μg of each plasmid preparation was electroporated into 1 x 10^7 C32 ES cells (5.3.2) which were plated onto a 15 cm plate in medium containing 200 μg/ml G418 but lacking LIF. Medium was changed every second day for 15 days and surviving colonies that retained pluripotence as assessed by morphology were picked (2.4.11) and seeded in duplicate 24-well trays in medium containing 200 μg/ml G418 with and without added LIF.

Most selected colonies differentiated extensively when cultured in the absence of LIF, indicating that pluripotence was not maintained by episome-associated cDNA expression. However, stem cell nests, defined as a group of pluripotent cells surrounded by differentiated cells, were retained in a minority of cultures in medium containing 200 μg/ml G418 but lacking LIF. All colonies formed differentiated cells in the absence of LIF indicating that selected

colonies were not incapable of differentiation. Colonies were chosen for further analysis based on retention of stem cell nests upon culture in the absence of LIF, or if the extent of differentiation in the absence of LIF was comparable to that observed in the presence of LIF. In total 11 cell lines from the first round and four cell lines from the second round (Table 5.1) fulfilled these criteria and were expanded in the presence of LIF and 200 μg/ml G418.

Experiment 2:

In a second experiment the procedure was scaled up to screen approximately 500,000 library clones based on supertransfectant survival following extended G418 selection (3.3.3). 50 electroporations were performed each with 37.5 μg library episome and 5 x 10^7 C32 ES cells. Aliquots from two electroporations were plated into medium containing 200 μg/ml G418 and LIF in order to estimate the number of colonies screened in the analysis. Following 16 days selection in G418 and LIF, G418-resistant clones were scored, indicating that approximately 390,000 colonies were screened in this analysis. The screening protocol was altered in two ways from the first large-scale screen. Cells were not trypsinised and re-seeded in order to determine the effect of performing the second round of screening in isolation. Secondly, second round supertransfected C32 ES cells were selected in 400 μg/ml G418 to improve selection against differentiated cells (3.4.2).

First round supertransfectants were selected for 10 days in 200 μg/ml G418 in the absence of LIF. 100 G418-resistant colonies that resembled undifferentiated ES cell colonies and did not contain extensive areas of differentiation (Figure 5.7) were picked and seeded in duplicate 24-well trays in medium with and without LIF in the presence of 200 μg/ml G418.

Remaining colonies were harvested and subjected to Hirt DNA extraction for second round screening. Groups of five plates were harvested as pools. *E. coli* electroporated with Hirt DNA (2.3.25) were grown overnight and recovered plasmid DNA was purified on ten CsCl gradients as described previously. 20 μg of each plasmid DNA pool was supertransfected into 3 x 10^7 C32 ES cells which were seeded onto 15 cm diameter plates in medium containing 400 μg/ml G418 but lacking LIF. Surviving colonies contained morphologically undifferentiated cells although many colonies were also surrounded by differentiated cells (Figure 5.8). Colonies were again picked on the basis of a morphological resemblance to undifferentiated ES

Table 5.1

Summary of LIF titration and Grunstein analyses performed on selected lines.

	Cell line	Altered LIF requirement	Presence of library episome
1st screen	C4	No	No
	C5	No	ND
	C6	No	No
	C8	No	ND
	C12	No	ND
	C31	No	ND
	C34	No	ND
	C46	No	ND
	C62	Yes	No
	C65	No	No
	C73	No	No
2nd Round	C84	Yes	No
	C92	No	No
	C97	No	No
	C99	No	No
2nd screen	C2.5	No	Yes
	C9.1	No	No
	C14.5	Yes	ND
	C17.1	No	ND
	C18.1	No	ND
	C18.4	No	No
	C18.6	No	Yes
	C25.1	Yes	No
	C25.2	No	No
	C27.1	No	ND
	C30.1	No	ND
	C30.2	No	ND
2nd Round	D10	Yes	No
	D12	Yes	No
	E3	No	ND
	E13	No	ND
	F11	No	ND
	2E2	No	ND
	2F1	No	ND
	2F2	No	ND
	2F3	No	ND

Grunstein analysis was used to determine if library episome was present in Hirt DNA extracts. The requirement for exogenous LIF was determined by LIF titration analysis and staining for AP activity. ND, not determined.

Figure 5.7

Morphology of colonies surviving the first round of function-based screening.

First round screening was performed as outlined in figure 5.6 a-c. Electroporations each with 37.5 μg of library DNA and 5 x 10^7 C32 ES cells were plated onto a 15 cm plate in ES cell medium lacking LIF but containing 200 μg/ml G418. Selective medium was replaced on screening plates every second day for 15 days. Representative G418 resistant colonies picked for further analysis are shown. Photographs were taken at 200 x magnification.

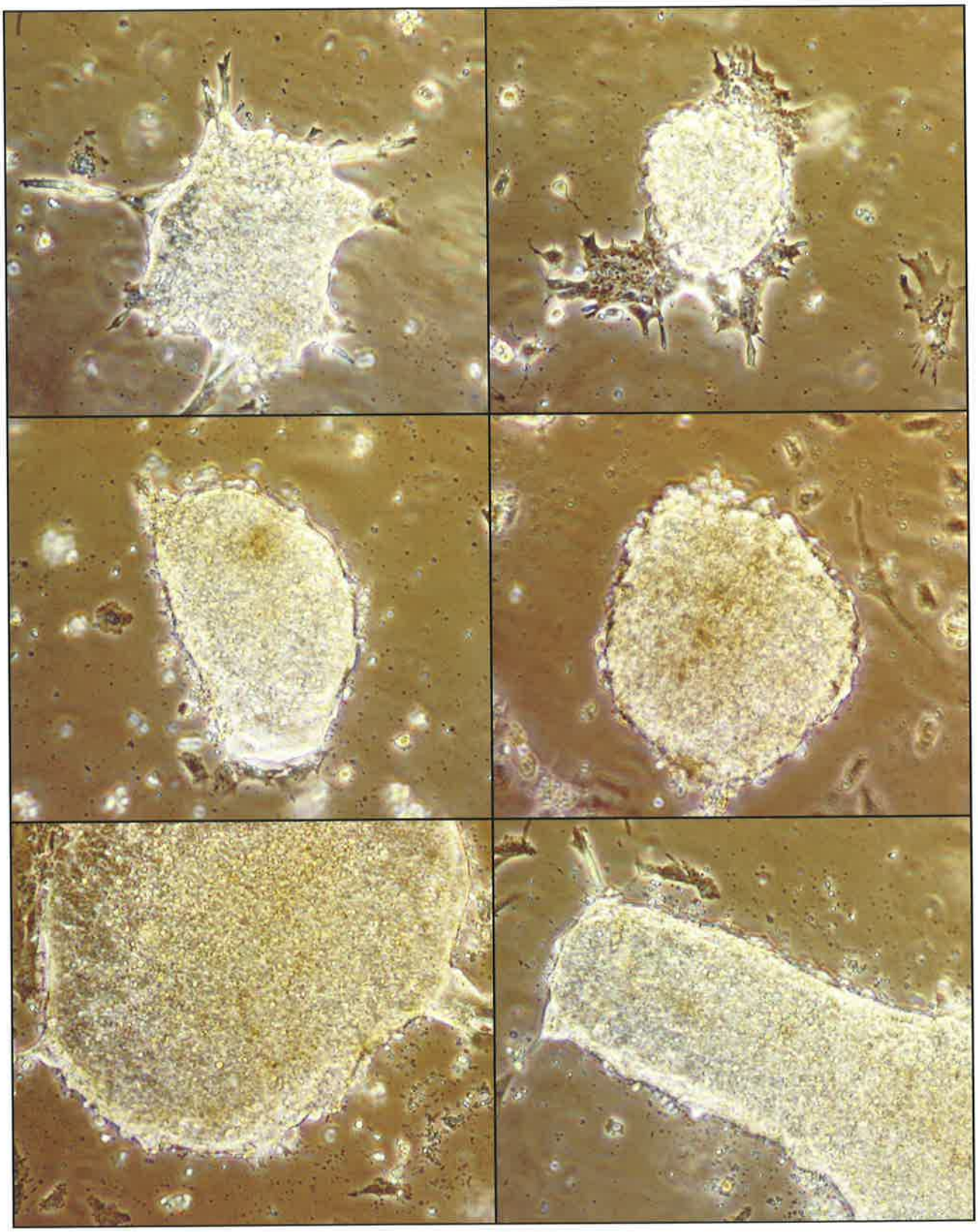
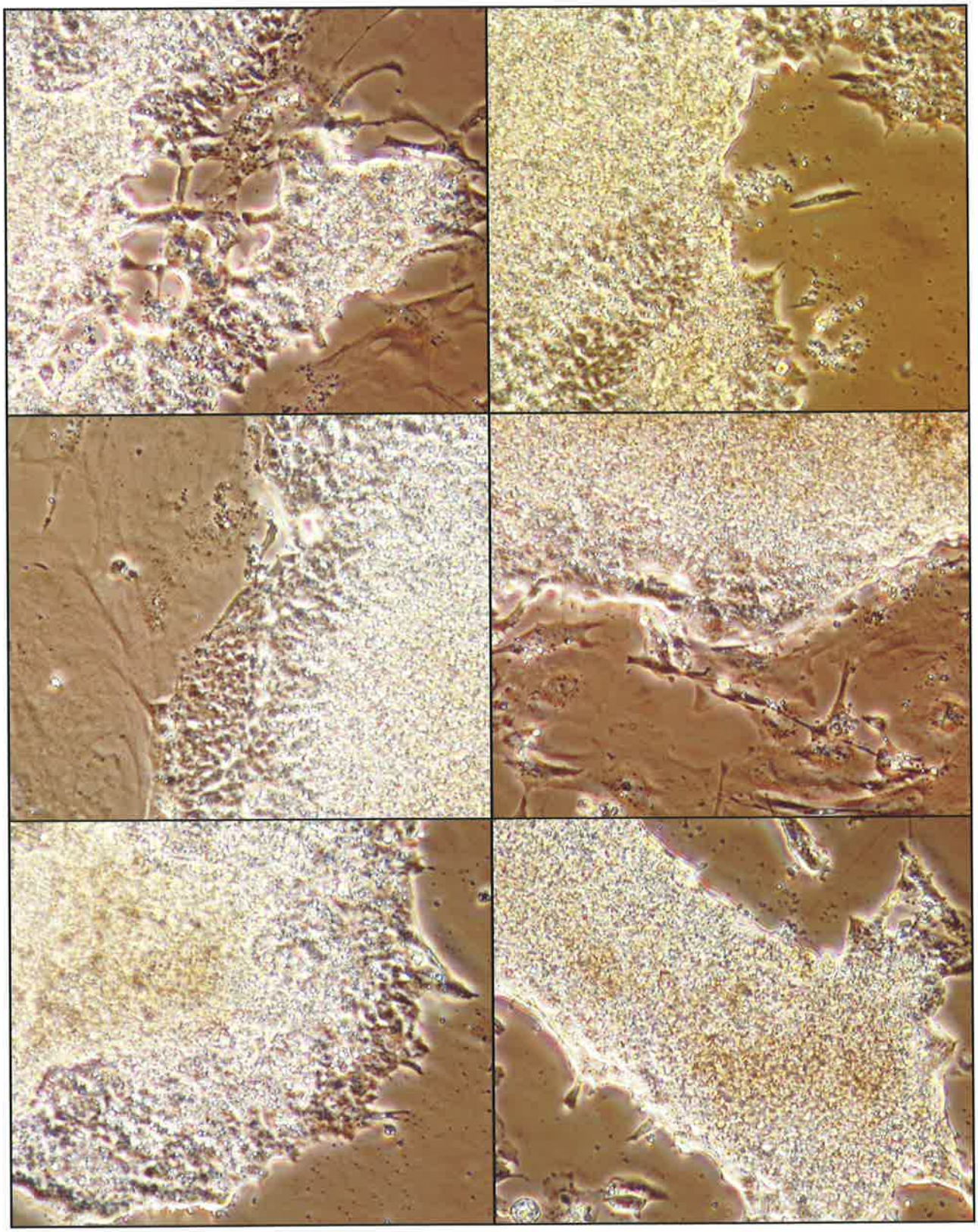


Figure 5.8

Morphology of colonies isolated from the second round function-based screening.

Screening was performed as outlined in figure 5.6d. Pooled second round episomal DNA (20 μg) from the second functional screen was electroporated into 3 x 10^7 C32 ES cells and cells were selected in the presence of 400 μg/ml G418 and absence of LIF for 11 days. Surviving colonies were photographed at 200 x magnification.



cells. 24 colonies were picked after 11 days and another 12 colonies were picked following a total of 17 days selection.

All colonies were observed to form differentiated cells in wells lacking LIF, indicating that selected colonies were not incapable of differentiation. Colonies that maintained pluripotent cells following culture in the absence of LIF were expanded in the presence of LIF for further analysis. In total, from the second large-scale screen, 12 and nine cell lines were isolated from the first and second rounds, respectively (Table 5.1).

5.5 LIF DEPENDENCE OF SELECTED CELL LINES

Cell lines that survived function-based screening and were selected for further analysis following growth in the absence of LIF (5.4.1) were expanded and analysed by LIF titration (2.4.13). 500 cells were seeded into 24-well trays at mouse recombinant LIF (ESGRO, Chemicon) concentrations between 0 U/ml and 40 U/ml. LIF concentrations higher than 40 U/ml were not assayed because only a marginally higher proportion of undifferentiated colonies was observed in the presence of 1000 U/ml LIF (see figure 5.10b). Following 6 days culture, cells were stained to detect AP activity as an indicator of pluripotence and compared to the parental C32 ES cell line. Cell lines C62 and C84 from the first screen and C14.5, C25.1, D10 and D12 from the second screen were found to retain pluripotence in lower concentrations of LIF protein.

C62:

C32 ES cells formed morphologically normal ES cell colonies that stained intensely for AP activity in concentrations of recombinant LIF greater than 30 U/ml (Figure 5.9a and b). A mixed population of undifferentiated and differentiated colonies was observed between 25 U/ml and 10 U/ml of LIF. As the LIF concentration decreased the proportion of undifferentiated colonies that retained high AP activity also decreased. The majority of colonies were spread and stained moderately for AP activity in LIF concentrations between 7.5 U/ml and 5 U/ml, indicative of differentiation (Figure 5.9a). In 1 U/ml LIF and in the absence of LIF very few colonies retained even moderate levels of AP activity, indicating that these cultures had differentiated extensively.

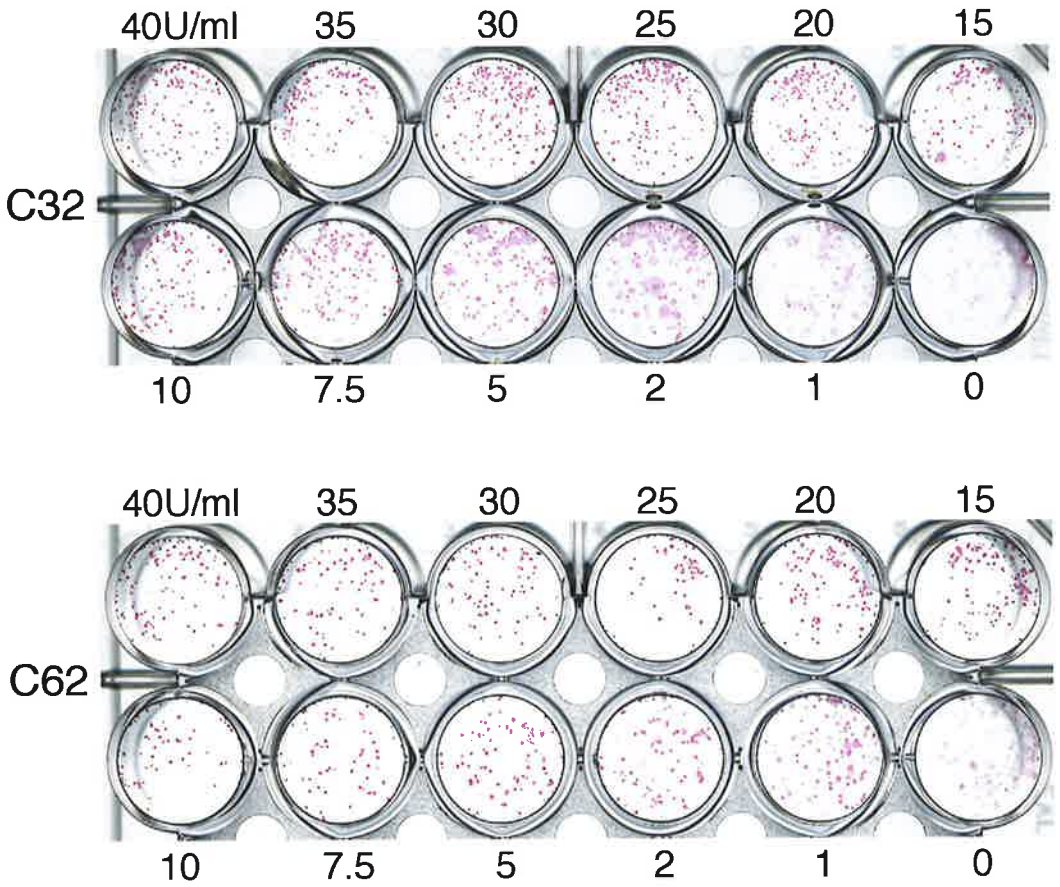
Figure 5.9

LIF dependency of cell line C62.

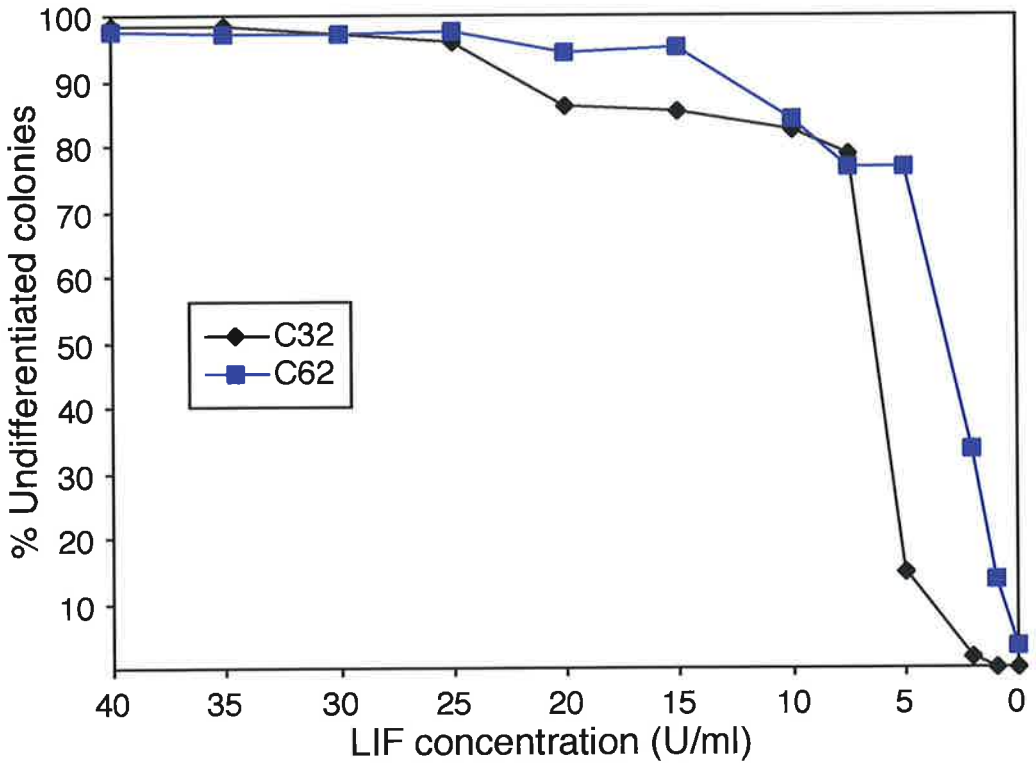
A. LIF titration assays of cell line C62 (see table 5.1) and the parental line ES cell line C32. C32 and C62 cells were seeded at 500 cells per well in ES cell medium containing 0, 1, 2, 5, 7.5, 10, 15, 20, 25, 30, 35, or 40 U/ml of recombinant LIF and allowed to differentiate for 6 days prior to AP staining. Plates were scanned using a Umax Powerlook II scanner.

B. LIF requirements of C62 cells and C32 cells. Undifferentiated colonies, defined as those that lacked differentiated cells and retained AP activity, were scored following LIF titration assay (A) and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, C32 cells; purple squares, C62 cells.

A


40U/ml
35
30
25
20
15
C32
10
7.5
5
2
1
0
40U/ml
35
30
25
20
15
C62
10
7.5
5
2
1
0

B


100
90
80
70
60
50
40
30
20
10
% Undifferentiated colonies
C32
C62
40
35
30
25
20
15
10
5
0
LIF concentration (U/ml)

In contrast, the majority of C62 colonies retained intense AP activity and morphologically resembled normal ES cell colonies in LIF concentrations as low as 10 U/ml (Figure 5.9a). Moderate AP staining was retained in the majority of colonies in wells containing as little as 1 U/ml LIF. Although colonies were more spread as the LIF concentration decreased, C62 colonies were less so compared to parallel C32 cultures (Figure 5.9a). Furthermore, AP staining colonies were still observed in the absence of LIF. The observation that the majority of C62 cells lose significant AP activity in the absence of LIF and that the colony morphology was indicative of differentiation indicates that C62 cells did not retain pluripotence in the absence of LIF and therefore remained competent to differentiate.

Scoring the number of undifferentiated colonies present in these cultures showed that C62 cultures generally contained a higher percentage of undifferentiated colonies than C32 parental cells (Figure 5.9b). The response to LIF in the two cell lines was particularly distinct at 5 U/ml LIF where 76.7% of C62 colonies remained undifferentiated compared to 14.6% of C32 colonies. Undifferentiated colonies were not observed in C32 cultures grown in 1 U/ml and 0 U/ml of LIF. In contrast, 3.4% (3 of 89) of C62 colonies cultured in the absence of LIF (0 U/ml LIF) remained undifferentiated. The LIF concentration at which 50% of the colonies were undifferentiated was 2.16-fold lower for C62 cells than C32 cells (3 U/ml versus 6.5 U/ml). Thus C62 cells appear to retain the capacity to differentiate but have a reduced requirement for LIF than C32 cells.

D10 and D12:

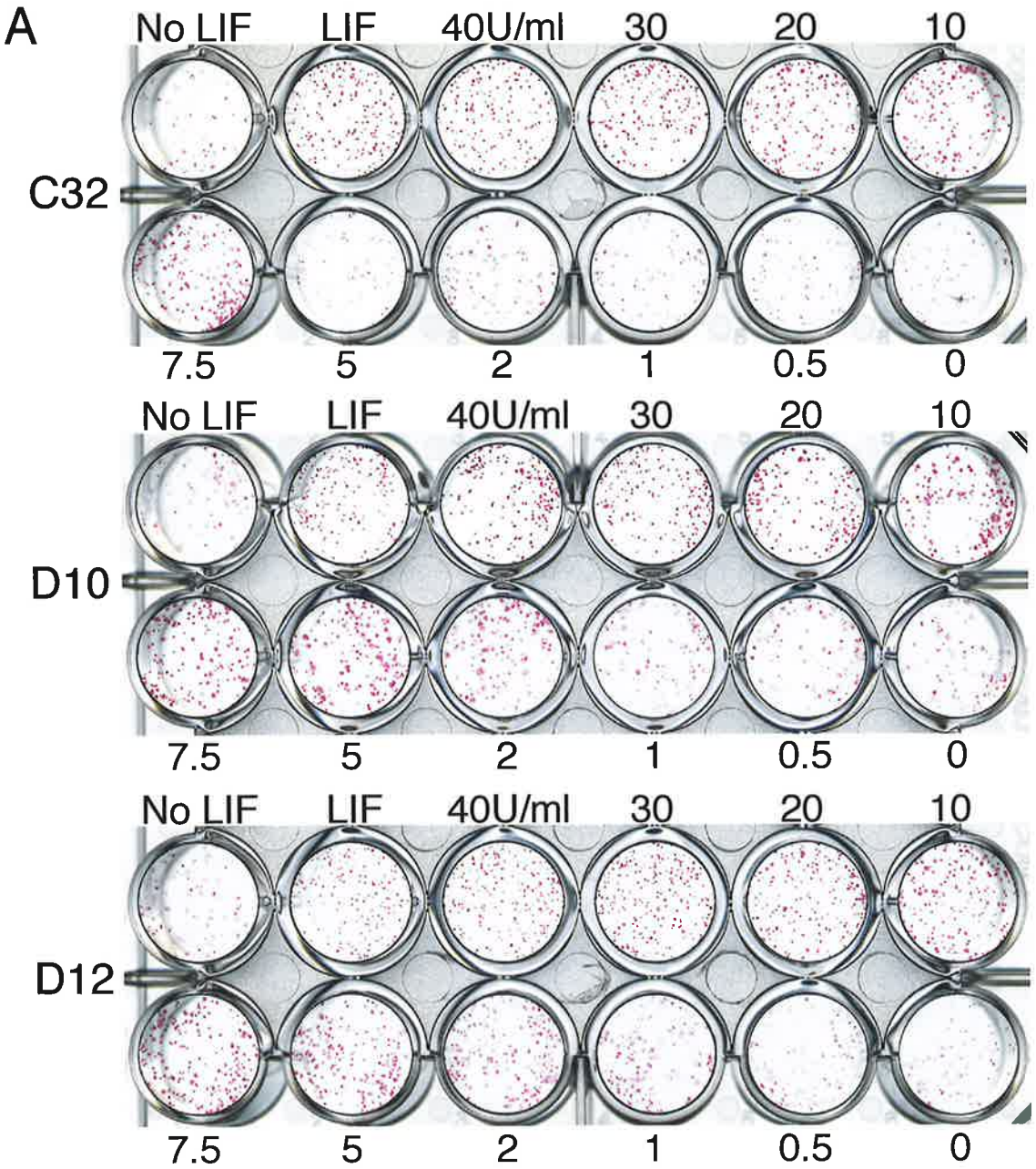
Cell lines D10 and D12 and C32 cells were analysed independently by LIF titration analysis. C32 cells in this experiment retained moderate and intense AP stained colonies in LIF concentrations as low as 7.5 U/ml LIF although at this LIF concentration colonies were spread and irregular in shape. Between 5 U/ml and 0 U/ml LIF the proportion of moderately stained colonies decreased so that in the absence of LIF only rare colonies stained positive for AP activity (Figure 5.10a). D10 and D12 retained a significant proportion of colonies staining intensely and moderately for AP activity in LIF concentrations as low as 2 U/ml. The proportion of AP^+ colonies in D10 and D12 cultures in the absence of LIF was also higher than in C32 cells (Figure 5.10a).

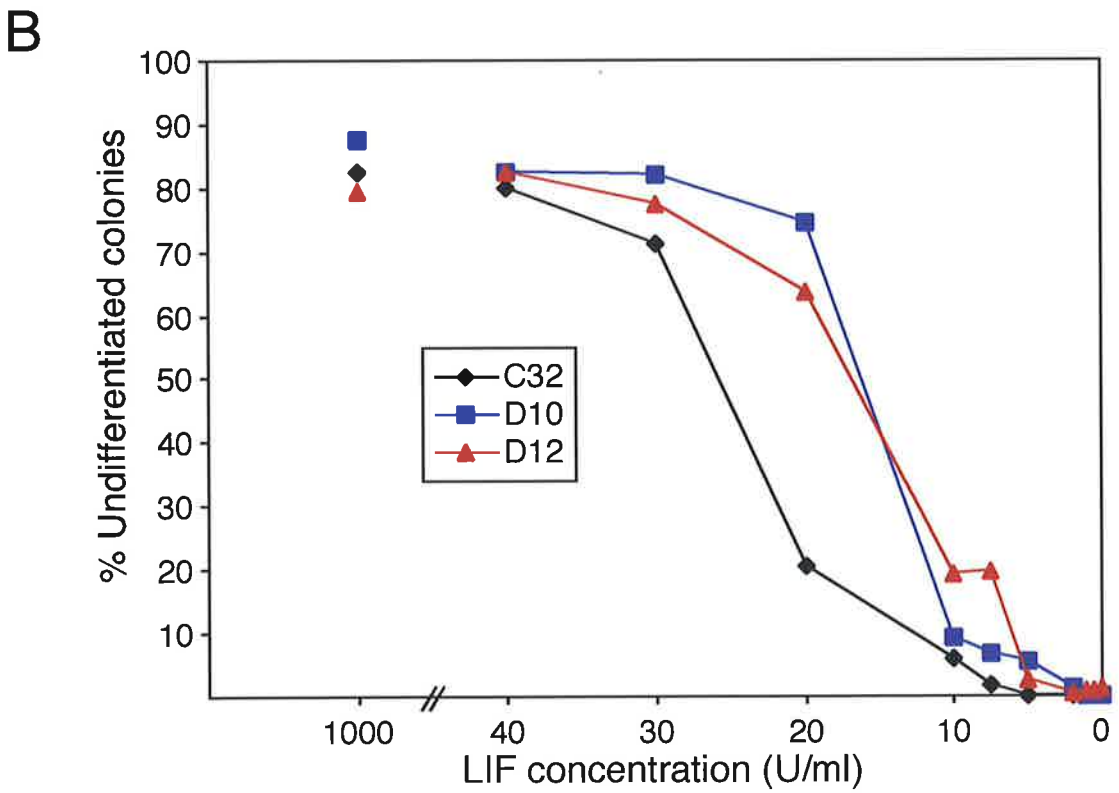
Figure 5.10

LIF dependence of cell lines D10 and D12.

A. LIF titration analysis of cell lines D10 and D12 (see table 5.1) and the parental line ES cell line C32. C32, D10 and D12 cells were seeded at 500 cells per well in ES cell medium containing LIF (1000 U/ml LIF) or 0, 0.5, 1, 2, 5, 7.5, 10, 20, 30 or 40U/ml of recombinant LIF and allowed to differentiate for 6 days prior to detection of AP activity. Plates were scanned using a Umax Powerlook II scanner.

B. LIF requirements of D10, D12 and C32 cells. Undifferentiated colonies, defined as those that lacked differentiated cells and retained AP activity, were scored following LIF titration assay (A) and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, C32 cells; purple squares, D10 cells; red triangles, D12 cells.


A
No LIF
LIF
40U/ml
30
20
10
C32
7.5
5
2
1
0.5
0
No LIF
LIF
40U/ml
30
20
10
D10
7.5
5
2
1
0.5
0
No LIF
LIF
40U/ml
30
20
10
D12
7.5
5
2
1
0.5
0


B
% Undifferentiated colonies
100
90
80
70
60
50
40
30
20
10
C32
D10
D12
1000
40
30
20
10
0
LIF concentration (U/ml)

Colonies were inspected by microscopy and scored as undifferentiated or differentiated. D10 and D12 cultures contained more undifferentiated colonies than C32 cells at LIF concentrations below 40 U/ml (Figure 5.10b). The distinction between D10 and D12 and C32 cells was particularly evident at 20 U/ml LIF where 74.4% of D10 and 63.5% of D12 colonies remained undifferentiated compared to 20.3% of C32 colonies. The LIF concentration at which 50% of the colonies were differentiated was 1.65-fold and 1.55-lower for D10 and D12 cells respectively, than C32 cells (C32, 25.5 U/ml; D10, 15.5 U/ml; D12, 16.5 U/ml) (Figure 5.10b). D10 and D12 therefore retain a capacity for differentiation but exhibit a reduced LIF requirement compared to the C32 parental cell line.

C25.1:

C25.1 (Figure 5.11a) also differed in the response to LIF compared with the parental C32 cell line. C32 colonies between 10 U/ml and 5 U/ml of LIF were spread and stained moderately for AP activity. At LIF concentrations lower than 5 U/ml C32 colonies stained lightly for AP activity (Figure 5.11b). C25.1 cultures grown in LIF concentrations between 0 U/ml and 10 U/ml LIF contained tight colonies, suggestive of undifferentiated morphology. These colonies were also more intensely stained for AP activity than C32 cultures grown in equivalent LIF concentrations (Figure 5.11b). Thus, the proportion of intense AP^+ colonies present in low LIF concentrations was higher in C25.1 cultures than C32 parental cells.

Scoring undifferentiated colonies (colonies made up entirely of undifferentiated cells) by microscopic inspection indicated that C25.1 cultures retained more undifferentiated colonies in the presence of 1000 U/ml LIF than C32 ES cells. In 30 U/ml and 40 U/ml LIF, a slightly higher percentage of undifferentiated colonies was observed in C25.1 cultures compared to equivalent C32 cultures (Figure 5.12a). Quantitation of the proportion of undifferentiated colonies in C25.1 LIF titration experiment did not however reflect the retention of AP activity in LIF concentrations lower than 10 U/ml that was observed macroscopically.

C25.1 LIF titrations were re-analysed by scoring colonies as undifferentiated if they contained greater than 90% undifferentiated cells judged by morphology and retention of AP activity. 100% of C25.1 colonies contained greater than 90% undifferentiated cells in the presence of 1000 U/ml LIF. In LIF concentrations between 2 U/ml and 10 U/ml C25.1 cultures

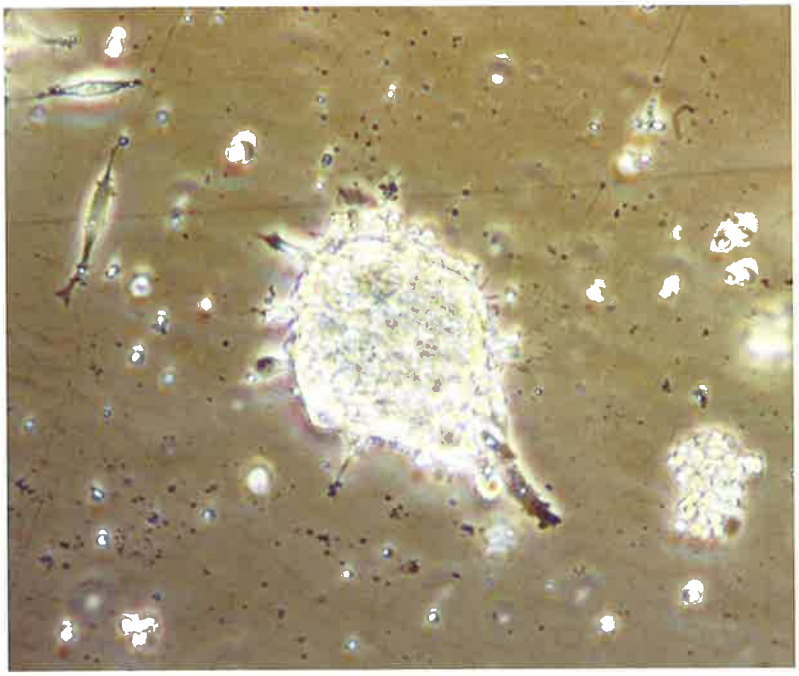
Figure 5.11

LIF dependence of cell line C25.1.

A. Morphology of selected cell line C25.1 following function based screening. C32 ES cells were supertransfected with library episome and subjected to function based screening (5.4.1 and Table 5.1). C25.1 maintained the undifferentiated state for 10 days in the absence of LIF and the presence of G418. The image was taken at 200 x magnification.

B. LIF requirements of C25.1 compared to the parental line ES cell line C32. C32 and C25.1 cells were seeded at 500 cells per well in ES cell medium containing LIF (1000 U/ml LIF) or 0, 0.5, 1, 1.5, 2, 5, 7.5, 10, 20, 30 or 40 U/ml of recombinant LIF and allowed to differentiate for 6 days prior to staining to detect AP activity. Plates were scanned using a Umax Powerlook II scanner.

A



B

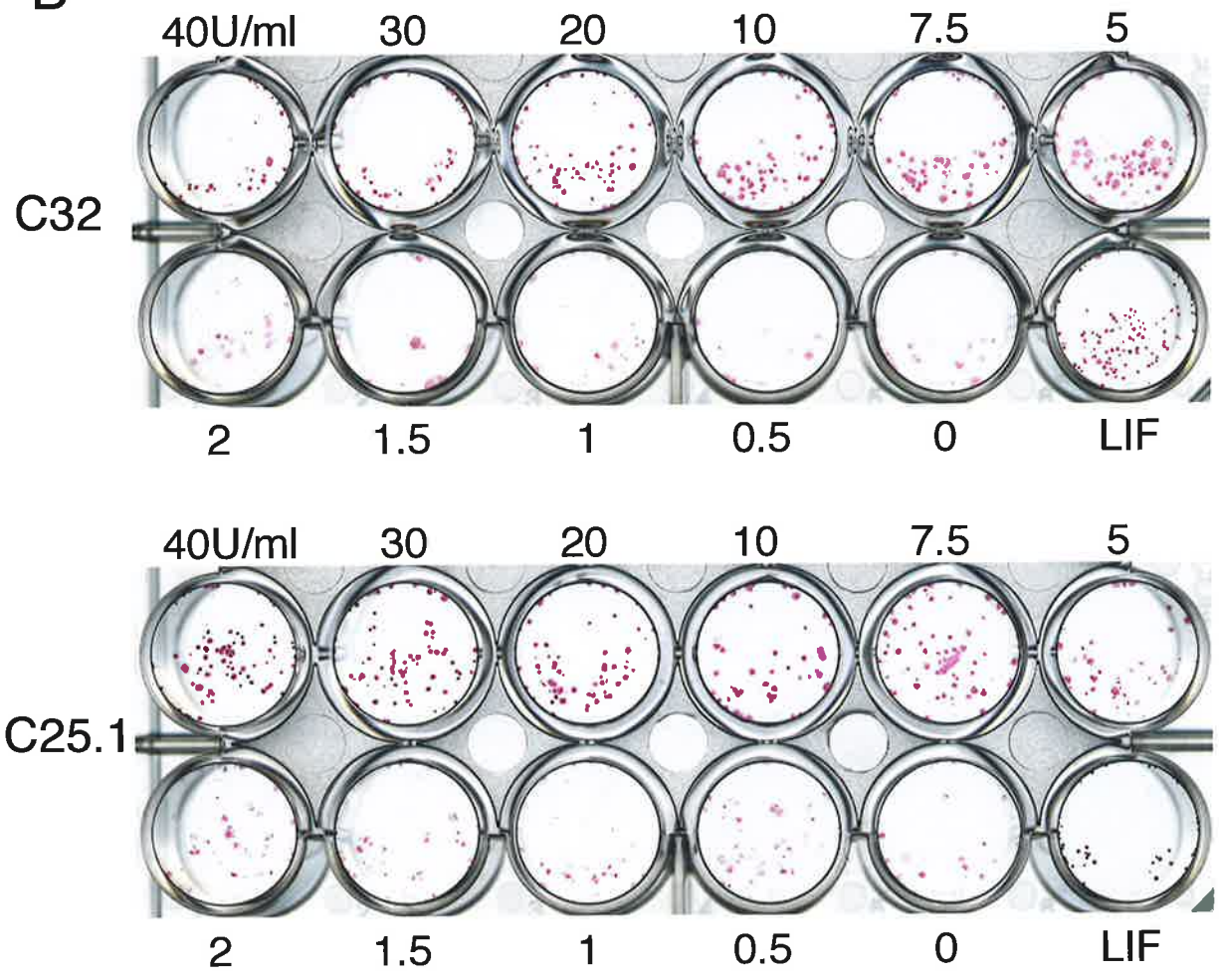

40U/ml
30
20
10
7.5
5
C32
2
1.5
1
0.5
0
LIF
40U/ml
30
20
10
7.5
5
C25.1
2
1.5
1
0.5
0
LIF

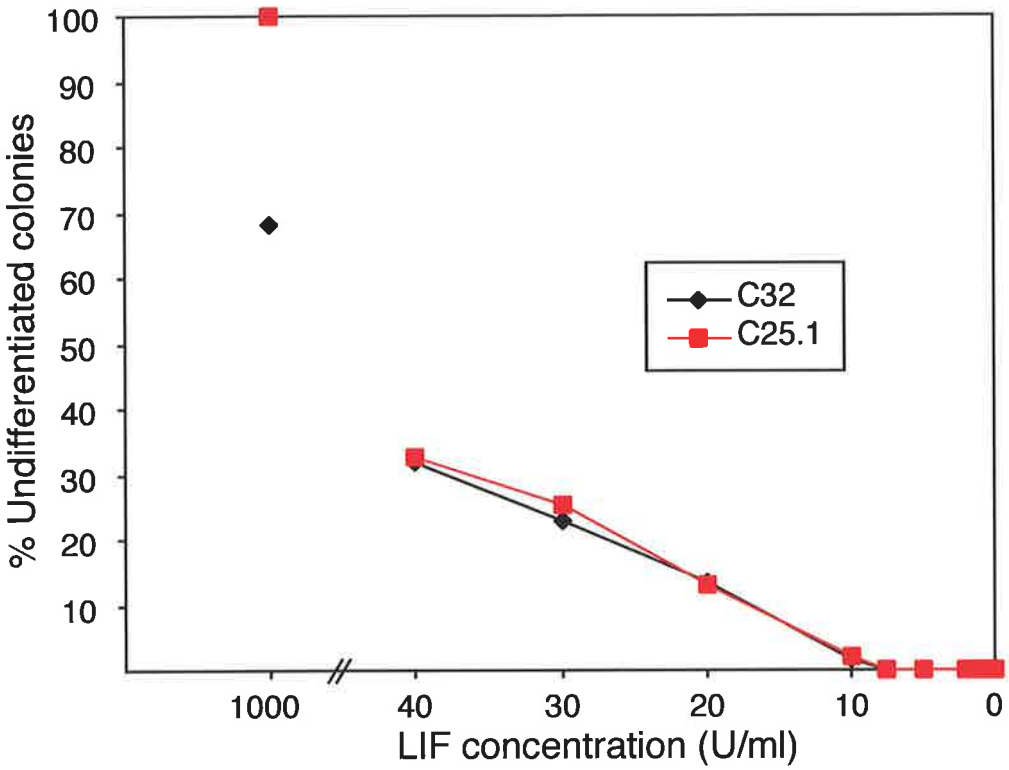
Figure 5.12

LIF requirements of C25.1 and C32 cells.

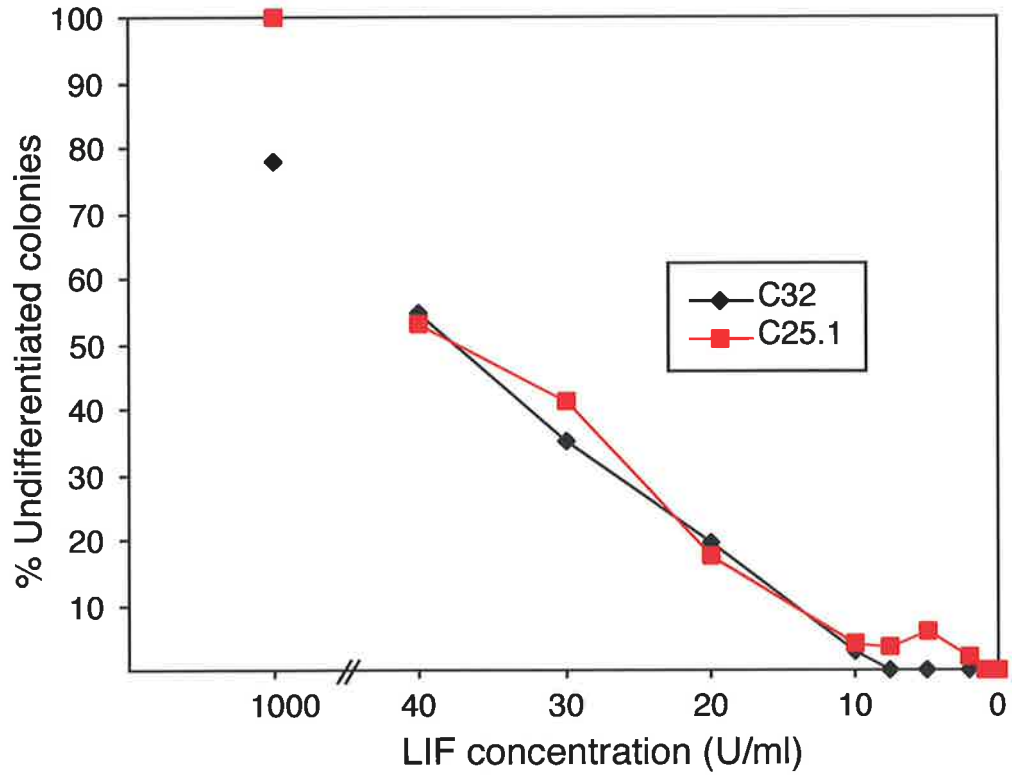
A. Undifferentiated colonies, defined as those that lacked differentiated cells and retained AP activity, were scored following LIF titration assay (figure 5.11b) and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, C32 cells; red squares, C25.1 cells.

B. Undifferentiated colonies, defined as those that retained AP activity and comprised more than 90% undifferentiated cells, were scored following LIF titration assay (figure 5.11b) and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, C32 cells; red squares, C25.1 cells.

A


100
90
80
70
60
50
40
30
20
10
% Undifferentiated colonies
C32
C25.1
1000
40
30
20
10
0
LIF concentration (U/ml)

B


100
90
80
70
60
50
40
30
20
10
% Undifferentiated colonies
C32
C25.1
1000
40
30
20
10
0
LIF concentration (U/ml)

contained more undifferentiated colonies (Figure 5.12b). Thus, the C25.1 cell line retained the capacity to differentiate and exhibited reduced pluripotent cell differentiation in low LIF concentrations.

C84 and C14.5:

A distinct phenotype was exhibited by C84 and C14.5 cell lines upon LIF titration analysis. As in previous LIF titration experiments, C32 cultures lost intense AP staining at approximately 10 U/ml of LIF (Figure 5.13a). C84 colonies were spread in LIF concentrations lower than 7.5 U/ml and, unlike C32 cultures, retained AP activity. Microscopic inspection of C84 colonies revealed that AP^+ cells were morphologically undifferentiated and were surrounded by AP^- differentiated cells (not shown). In the absence of LIF and in low LIF concentrations, significantly more AP staining was observed in C84 cultures than C32 cells (Figure 5.13a).

Quantitation of morphologically undifferentiated colonies (>90% undifferentiated) revealed that at LIF concentrations between 1.5 U/ml and 20 U/ml C84 cultures retained a greater percentage of undifferentiated AP^+ colonies than equivalent C32 cultures (Figure 5.13b). C84 cells therefore retain a capacity for differentiation, exhibit a reduced LIF requirement compared to C32 parental cell line and an altered AP^+ colony morphology in low LIF concentrations.

C14.5 cells were spread in LIF concentrations lower than 20 U/ml but retained AP activity, unlike C32 cell cultures. Microscopic inspection of these colonies indicated that AP^+ cells were morphologically undifferentiated and AP^- differentiated cells surrounded AP^+ regions of the colony. In the absence of LIF and in low LIF concentrations, more AP staining was observed in C14.5 cultures than in C32 cultures (Figure 5.14). Like C84 cells, C14.5 cells were still capable of differentiation but exhibited an altered AP^+ colony morphology in low LIF concentrations suggestive of pluripotence .

The function-based screen therefore identified a number of cell lines that exhibited a reduced LIF requirement associated with one of two distinct morphologies; those that expressed AP activity and resembled wild-type ES cells in low LIF concentrations and those

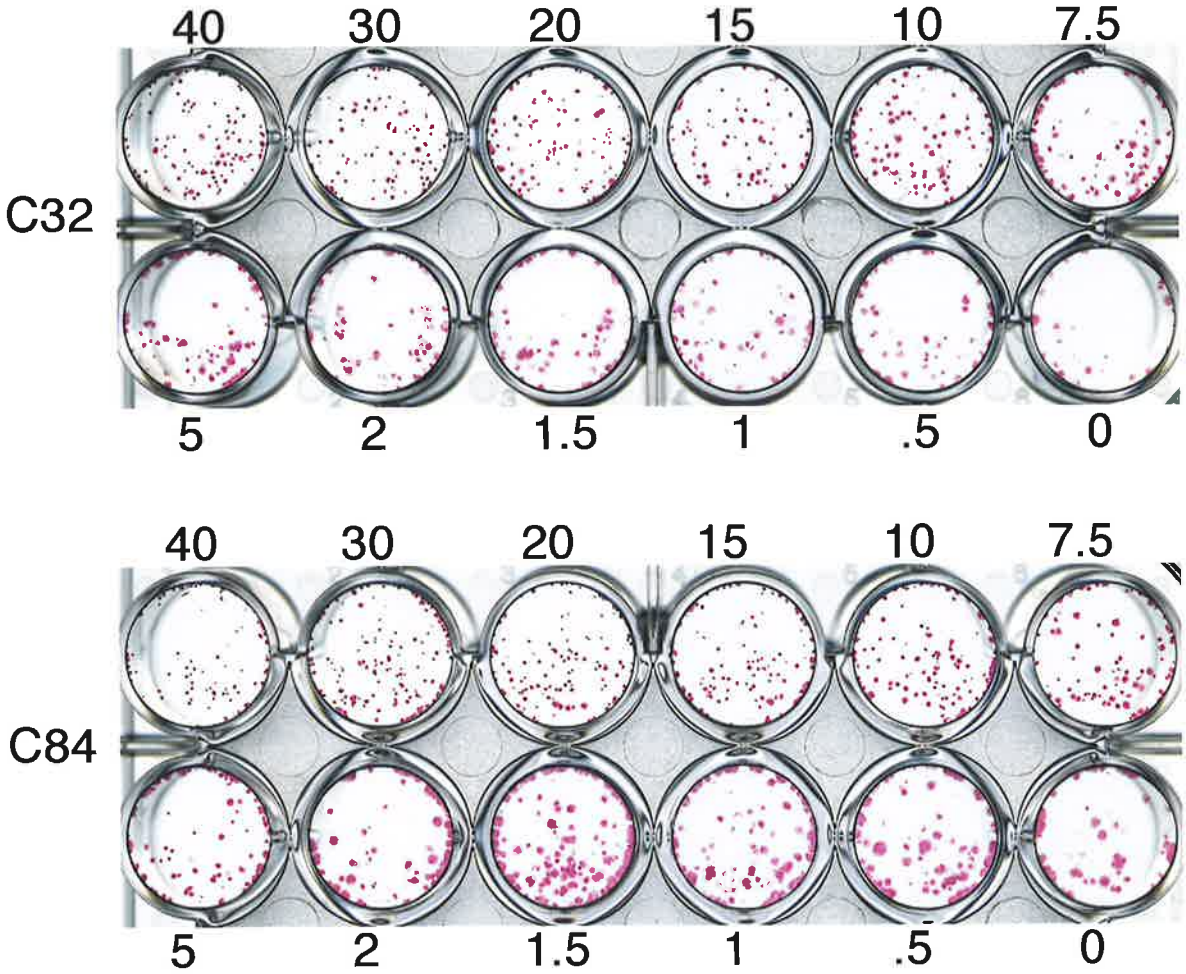
Figure 5.13

LIF dependence of cell line C84.

A. LIF titration analysis of C84 compared to the parental line ES cell line C32. C32 and C84 cells were seeded at 500 cells per well in ES cell medium containing 0, 0.5, 1, 1.5, 2, 5, 7.5, 10, 15, 20, 30, or 40U/ml of recombinant LIF and allowed to differentiate for 6 days prior to detection of AP activity. Plates were scanned using a Umax Powerlook II scanner.

B. LIF requirements of C84 in comparison to the parental line ES cell line C32. Undifferentiated colonies, defined as those that retained AP activity and comprised more than 90% undifferentiated cells, were scored following LIF titration assay (A) and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, C32 cells; red squares, C84 cells.

A


40
30
20
15
10
7.5
C32
5
2
1.5
1
.5
0
40
30
20
15
10
7.5
C84
5
2
1.5
1
.5
0

B

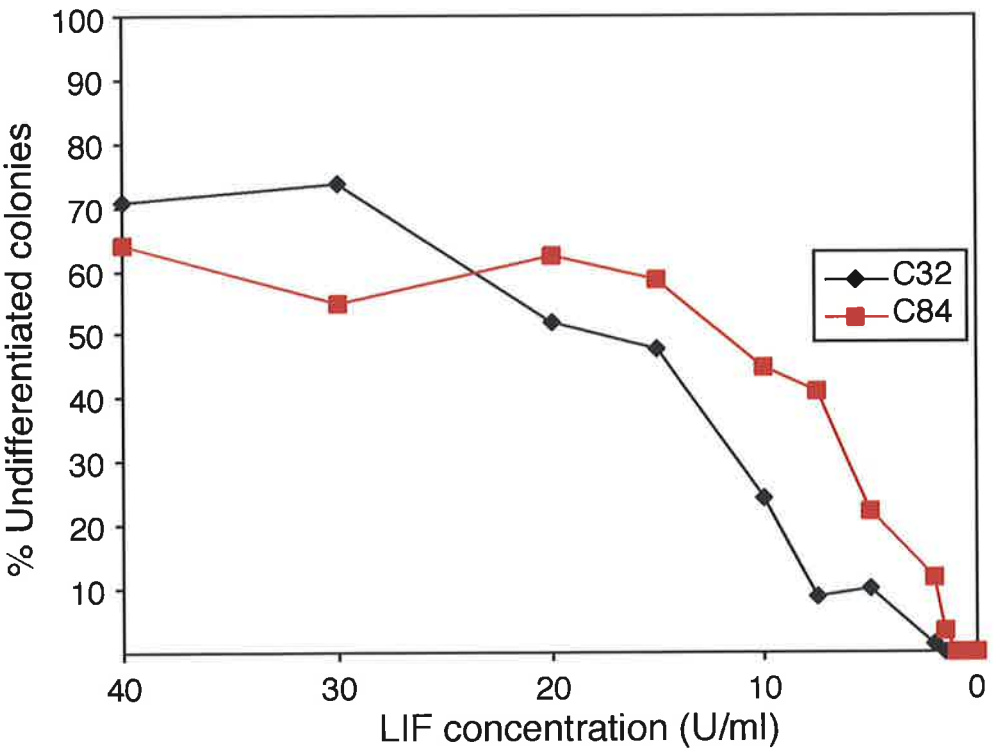
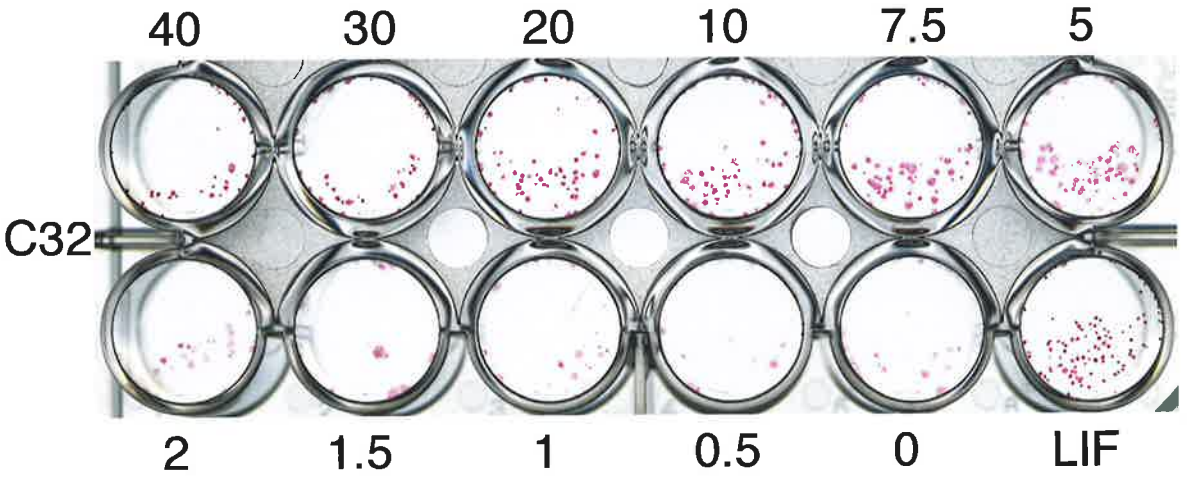
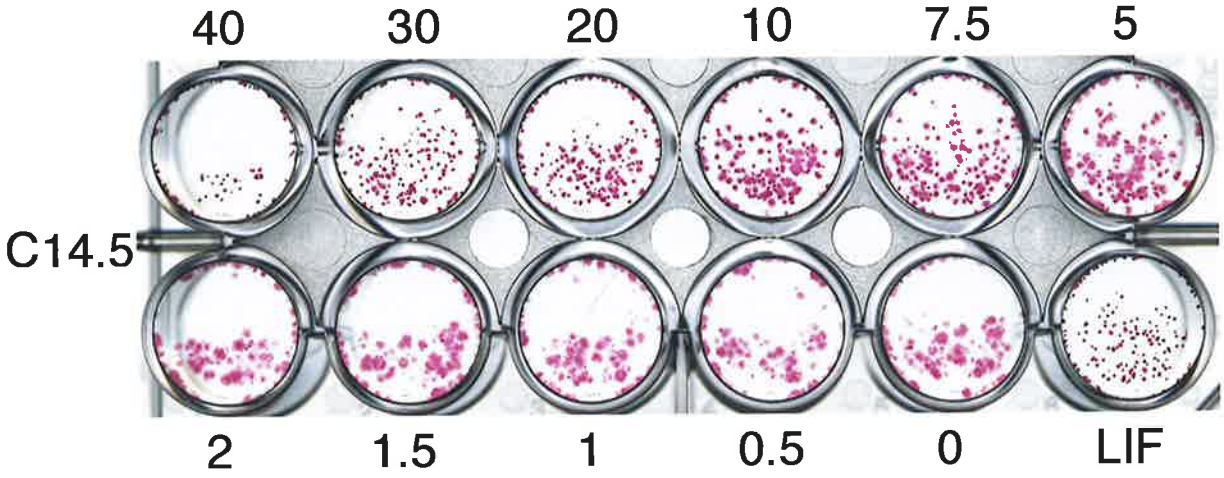

100
90
80
70
60
50
40
30
20
10
% Undifferentiated colonies
C32
C84
40
30
20
10
0
LIF concentration (U/ml)

Figure 5.14

LIF dependence of C14.5.

A. LIF titration analysis of C14.5 compared to the parental line ES cell line C32. C14.5 and C32 cells were seeded at 500 cells per well in ES cell medium containing LIF (1000 U/ml LIF) or 0, 0.5, 1, 1.5, 2, 5, 7.5, 10, 20, 30, or 40U/ml of recombinant LIF. Cells were allowed to differentiate for 6 days prior to AP staining. Plates were scanned using a Umax Powerlook II scanner.


40
30
20
10
7.5
5
C32
2
1.5
1
0.5
0
LIF


40
30
20
10
7.5
5
C14.5
2
1.5
1
0.5
0
LIF

that retained AP activity but were morphologically spread even in the absence of LIF. These cell lines were analysed further to identify the introduced cDNAs.

5.6 ISOLATION AND CHARACTERISATION OF cDNAS FROM PLURIPOTENT CELL LINES SELECTED DURING FUNCTION-BASED SCREENING

5.6.1 Detection of library episome in selected cell lines

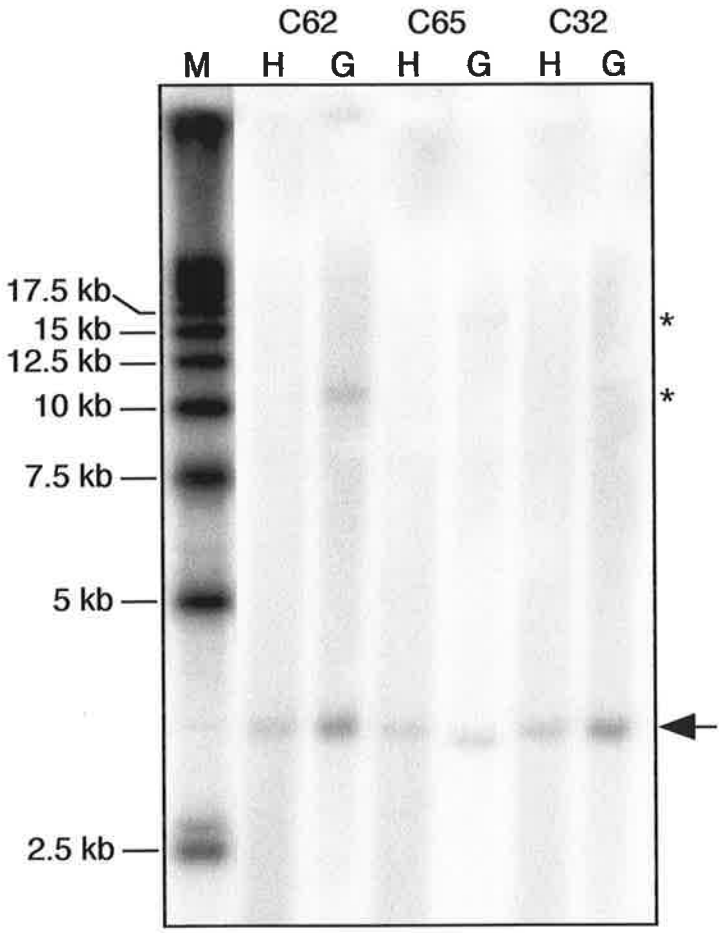
Grunstein analysis (2.3.11), shown previously to be the most sensitive method for detection of pPSDEneoΔLT20-based plasmids (4.3), was carried out using Hirt DNA from cell lines subjected to LIF titration analysis. Hirt DNA preparations were electroporated into electro-competent *E. coli*. Duplicate lifts were taken and probed with a 1 kb *Bgl*II *Oct4* promoter specific probe (see figure 3.1), washed to a stringency of 0.2 x SSC/0.1% SDS at 65°C and exposed to X-ray film. Of 36 cell lines screened by Grunstein analysis, only C2.5 and C18.6 had detectable library episome in Hirt DNA extracts (Table 5.1). Based on investigations described in Gassmann *et al,* (1995), G418-resistant lines without episome were predicted to have integrated at least the neomycin selection cassette of the library episome into the genome. It is also possible that plasmid rearrangements resulted in loss of bacterial replication sequences from the library episome.

To determine if chromosomal integration had occurred, genomic and Hirt DNA (2.3.25) from representative cell lines C62, C65 and the parental line C32 was subjected to Southern analysis. DNA was digested with *Bam*HI, electrophoresed on a 0.8% TAE agarose gel, Southern blotted onto a nylon membrane and probed with the 1 kb *Bgl*II *Oct4* promoter specific probe (see figure 3.1). The endogenous *Oct4* promoter was detected as a 3.3 kb band in genomic DNA as well as Hirt DNA (Figure 5.15). No other bands were observed in C32 ES cell genomic or Hirt DNA. Hybridisation was observed to bands of approximately 11 kb and 16 kb in genomic DNA of C62 and C65, respectively (Figure 5.15, asterix). These bands represent chromosomal integrants because they were not observed in Hirt DNA and were larger than pPSDEneoΔLT20 which is not cut by *Bam*HI. Therefore, integrated cDNA inserts are potentially responsible for the maintenance of pluripotence observed in some selected lines.

Figure 5.15

Southern analysis of ES cell lines C62 and C65.

*Bam*HI digested Hirt preparation DNA (H) and genomic DNA (G) from C62, C65 and the parental line C32 was electrophoresed on a 1% TAE agarose gel and Southern blotted (2.3.15). The filter was probed with a 1 kb *Bgl*II fragment derived from pF2KS specific for the *Oct4* promoter region present in pPSDEneoΔLT20-derived plasmids (see figure 3.1). The blot was washed to a stringency of 0.2xSSC/0.1% SDS at 65°C. The endogenous *Oct4* promoter was detected in genomic and Hirt DNA as a 3.3 kb band (arrow). Asterix indicate integrated pPSDEneoΔLT20-derived DNA bands present in C62 and C65 genomic DNA. M, 2.5 kb ladder molecular weight markers. Marker sizes are indicated.


C62
C65
C32
M
H
G
H
G
H
G
17.5 kb
15 kb
12.5 kb
10 kb
7.5 kb
5 kb
2.5 kb
*
*

5.6.2 Isolation of cDNA inserts from selected cell lines

Genomic and Hirt DNA purified from C62, C65, D10, D12, C25.1, C14.5 and C84 cell lines was subjected to PCR amplification (2.3.32) using primers designed against the sequences in pPSDEneoΔLT20 which flank the cDNA expression site. The primers UPGK2 and LOCT42 (2.2.7) which hybridise to the 3′ end of the *PGK-1* promoter and to the 5′ end of the *Oct4* promoter DE region respectively, were designed to amplify cDNA inserts cloned into pPSDEneoΔLT20 (Figure 5.16a). The amplified region encompassed the cDNA insert and an additional 460 bp containing the 3′ end of the *PGK-1* promoter, the SV40 polyadenylation region, and the 5′ end of the *Oct4* promoter. pβgalDEneoΔLT20 supertransfected lines 6 and 7 contain extrachromosomal pβgalDEneoΔLT20 plasmid (4.3) and were used as positive controls for amplification.

An expected product of approximately 4.3 kb was amplified from Hirt and genomic DNA from pβgalDEneoΔLT20 supertransfectants 6 and 7 (Figure 5.16b). Products of 0.95 kb and 2 kb were amplified from C65 and C84 Hirt DNA and genomic DNA, respectively (Figure 5.16b). C65 and C84 therefore contain cDNAs of 490 bp and 1.5 kb, respectively. A product of 1.5 kb was amplified from C25.1-derived Hirt DNA (Figure 5.20a), implying that C25.1 harboured a cDNA of 1.1 kb. PCR of Hirt and genomic DNA from other selected lines failed to amplify cDNA inserts and these cell lines were not analysed further.

5.6.3 Identification of the cDNAs present in cell lines C84 and C25.1

Cell line C84

The PCR amplified product from C84 Hirt DNA (2.3.32) was digested with *Eco*RI endonuclease, blunted and cloned into the *Eco*RV restriction site of pBluescript II KS to form p84KS (Figure 5.17a). The C84 cDNA sequence (Figure 5.18) was completed in both directions by sequencing reactions using primers 84U564, L84610 and L84940 (2.2.7) designed against sequence obtained from initial T3 and T7 sequencing reactions. The C84 cDNA was 1,471 bp long and contained a poly (A)-tail 17 bp downstream of a polyadenylation signal (AATAAA), within the limits of the typical positioning of the polyadenylation signal (Goulding *et al.*, 1991). The first ATG codon at position 62 partially matched the consensus sequence for translation initiation (Kozak, 1991) (Figure 5.18). The longest open reading frame

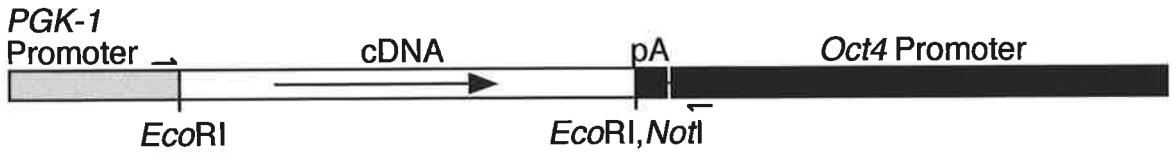
Figure 5.16

PCR amplification of cDNA sequences from Hirt DNA.

A. Schematic representation of the amplified region in the library episome. Primers UPGK2 and LOCT42 were designed against the 3′ end of the *PGK-1* promoter and the 5′ end of the *Oct4* promoter, respectively (2.2.7). Use of templates that contain pPSDEneoΔLT20 will result in the amplification of a 1,200 bp fragment that includes the 750 bp stuffer region, the SV40 poly adenylation sequences and regions of the *PGK-1* and *Oct4* promoters. Amplified library episome will result in a fragment that is 460 bp larger than any cDNA insert present.

B. Amplification of cDNA sequences from C62, C65 and C84 ES cell lines. Hirt and genomic DNA derived from selected lines C62, C65 and C84 were PCR amplified using primers UPGK2 and LOCT42. Hirt DNA and genomic DNA from pβ-galDEneoΔLT20 supertransfected lines 6 and 7, respectively, were also PCR amplified as positive controls (3.4.4). 1/20th of a Hirt DNA preparation or 100 ng of genomic DNA were amplified in a volume of 50 μl using a touch down protocol (2.3.32) through 35 cycles. 10 μl of each PCR was electrophoresed on a 1% TBE agarose gel. -, no DNA template; H, Hirt DNA PCR; G, genomic DNA PCR; M, *Eco*RI digested SPP-1 phage DNA markers. 0.99, 1.95, 3.6 and 4.9 kb marker bands are indicated.

A


PGK-1
Promoter
cDNA
pA
Oct4 Promoter
EcoRI
EcoRI,NotI

B

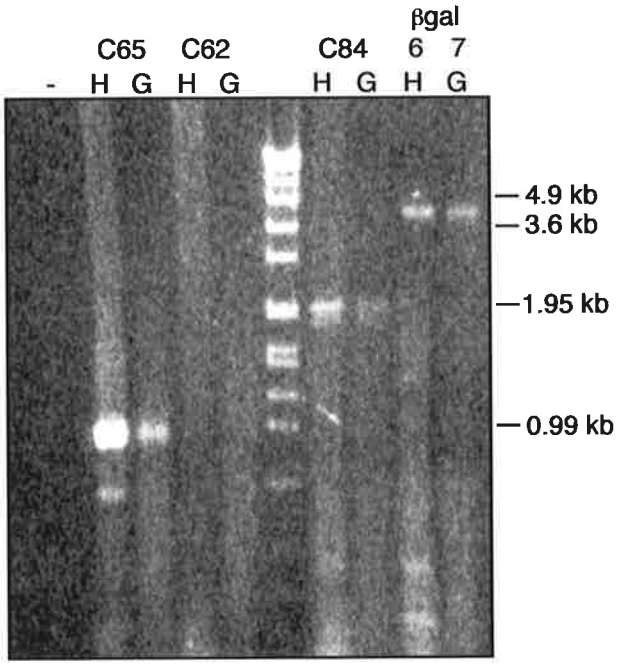

βgal
C65
C62
C84
6
7
-
H
G
H
G
H
G
H
G
4.9 kb
3.6 kb
1.95 kb
0.99 kb

Figure 5.17

Cloning of the PCR product derived from the C84 cell line.

A. Cloning of the C84 cDNA into pBluescript II KS. PCR carried out on Hirt DNA extracts from C84 ES cells produced a major species of 2 kb. The reaction was digested with *Eco*RI and blunted with the klenow fragment of DNA polymerase I. A fragment of 1.5 kb corresponding to the cDNA insert was blunt cloned into the *Eco*RV restriction site of pBluescript II KS forming p84KS.

B. Construction of p84DEneoΔLT20. p84DEneoΔLT20 plasmid can be supertransfected into episome harbouring ES cell lines to express truncated IQGAP3 from the constitutive *PGK-1* promoter. Shading is as described for figure 4.2.

C. Cloning of C84 cDNA into the mammalian expression vector pEFIRESneo. The resulting plasmid, pEF84IRESneo, expresses a truncated *IQGAP3* message corresponding to the C84 cDNA and neomycin resistance as a single dicistronic message from the constitutive *hEF1-a* promoter.

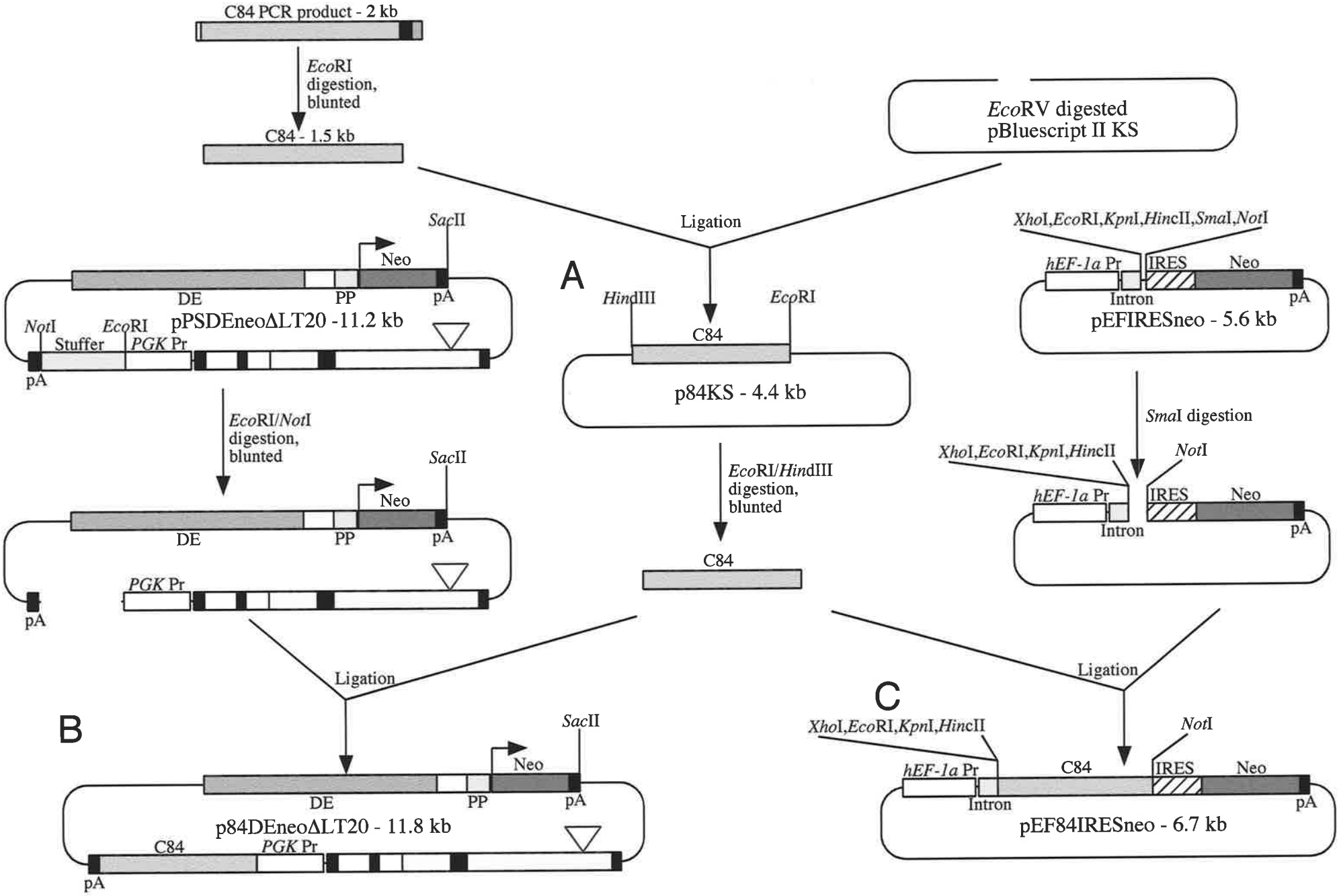

C84 PCR product - 2 kb
EcoRI digestion, blunted
C84 - 1.5 kb
EcoRV digested pBluescript II KS
Ligation
A
HindIII
EcoRI
C84
p84KS - 4.4 kb
SacII
Neo
DE
PP
pA
NotI
EcoRI
Stuffer
PGK Pr
pPSDEneoΔLT20 -11.2 kb
EcoRI/NotI digestion, blunted
XhoI,EcoRI,KpnI,HincII,SmaI,NotI
hEF-1a Pr
IRES
Intron
pEFIRESneo - 5.6 kb
SmaI digestion
XhoI,EcoRI,KpnI,HincII
NotI
EcoRI/HindIII digestion, blunted
B
C
pEF84IRESneo - 6.7 kb
p84DEneoΔLT20 - 11.8 kb

Figure 5.18

cDNA and putative amino acid sequence of C84.

The 3´ untranslated region is shown in lower case. The first ATG codon is underlined and translation initiation site is in an open box. Translation initiation from this site would result in the formation of a 206 amino acid polypeptide. The termination codon is indicated as an asterix and the polyadenylation signal is shaded in grey.

```
   1 ATAATTCCGTTGCTGTCGGACACCCAGAAACCAGAGCCACTTCGACGACACCACTCACTA    60

   1  M  A  H  S  L  L  S  L  S  E  K  Q  Q  R  V  L  R  N  L  R    20
  61 ATGGCACACAGTCTCCTGTCACTGTCGGAGAAGCAGCAACGCGTTCTGCGGAACCTGCGT   120

  21  R  L  Q  G  L  G  L  V  R  A  S  D  C  Y  Q  G  L  V  D  E    40
 121 CGGCTACAGGGCCTGGGGCTGGTCAGGGCCAGTGACTGCTACCAGGGACTTGTAGACGAG   180

  41  L  A  K  D  I  C  N  Q  R  R  H  R  Q  R  R  K  A  E  M  L    60
 181 CTGGCCAAGGACATCTGCAACCAGCGTAGGCACCGGCAACGGCGGAAGGCAGAGATGCTG   240

  61  R  L  R  T  T  L  Q  G  L  D  A  K  T  I  F  Y  E  E  Q  G    80
 241 AGGCTCCGGACCACACTGCAGGGCCTAGATGCAAAAACCATCTTCTATGAGGAACAGGGT   300

  81  D  Y  Y  N  Q  Y  I  Q  A  C  L  D  H  L  A  P  K  P  K  S   100
 301 GACTACTACAACCAATACATCCAGGCCTGCCTTGACCACCTGGCCCCCAAACCCAAGAGT   360

 101  S  G  K  G  K  K  Q  P  S  L  H  Y  T  A  A  Q  L  L  E  K   120
 361 TCTGGGAAGGGGAAGAAGCAGCCGTCTCTTCACTACACAGCCGCCCAGCTCCTAGAAAAG   420

 121  G  V  L  V  E  I  E  D  L  P  V  S  H  F  R  N  V  I  F  D   140
 421 GGCGTCTTGGTGGAAATTGAAGATCTCCCTGTTTCTCACTTCAGAAACGTCATCTTTGAC   480

 141  I  T  P  G  D  E  A  G  R  F  F  V  N  A  K  F  L  G  V  D   160
 481 ATCACTCCTGGAGACGAGGCAGGAAGGTTTTTCGTCAATGCCAAGTTCCTGGGTGTGGAC   540

 161  M  E  K  F  Q  L  H  Y  Q  D  L  L  Q  L  Q  Y  E  G  V  A   180
 541 ATGGAGAAGTTTCAGCTTCACTACCAGGACCTCTTGCAGCTGCAGTATGAGGGTGTGGCT   600

 181  V  M  K  L  F  N  K  A  K  V  N  V  N  L  L  I  F  L  L  N   200
 601 GTCATGAAACTCTTTAACAAAGCCAAAGTCAACGTCAATCTCCTCATCTTCCTGCTCAAC   660

 201  K  K  F  L  R  K  *                                         206
 661 AAGAAGTTCCTTCGGAAGtgacggatgcaggcggaactgaggcctctcagctgactggct   720
 721 gctgcgcttctccattacccagctcttttctccgaagaccagagctcgggccacaatgct   780
 781 tgaccctcccgcccaagaggaattgtcctacttggctgaagtgggccatgctccaggtcc   840
 841 ggccgggtggcttttaccacatccccgggaaccaggagcctgcactcagtcctgcaaaga   900
 901 accatggttttgtgtcgctactgctctcgtgtggtcaccacggtgtccgccagtcgtcct   960
 961 agcactgcccagcagccttatctcccttccttccacgggcttccaaagcccgggccagg  1020
1021 ctgtctgcccaactccgcttcataagccatgggtgtcgctgtcccctacagcccaggtcc  1080
1081 ctctgtgctgtgcccctcagccacctctatcatcctgtcctagccttgacttggtgcaag  1140
1141 cacttgctgatgtccatgacagctgagtgcacacctcaaatctcctttccccacttgtgg  1200
1201 tggttcttatgtgggtcactggggtatttattctaacctgattgttgtgtggccttaatc  1260
1261 atggttttaattctcactttctgaactcagagttcttgtcttgccatcttctcgtcacct  1320
1321 cagagctgacttccaggtggttctcatagacctgtaggccagccttggccatctctgtcc  1380
1381 tccccacatctcccatcttcctgcctcactgccctctagatttcctcttaataaaatgtt  1440
1441 atttcctatgttaaaaaaaaaaaaaaaaa                                 1470
```

in the sequence began at this position and would result in production of a polypeptide of 206 amino acids (Figure 5.18) terminating at position 679. The C84 cDNA therefore contained a 3´ UTR of 791 bp. A BLASTN (Altschul *et al.*, 1997) search of the Genbank Mouse EST database revealed that the C84 cDNA had 99% (473/475) identity to an EST sequence derived from mouse myotubes related to IQ-motif containing GTPase-activating protein 1 (IQGAP1).

IQGAP1 was cloned by RT-PCR from human osteosarcoma tissue and was the founding member of the IQGAP family of RasGAP proteins (Weissbach *et al.*, 1994). RasGAP proteins accelerate the weak intrinsic GTP-hydrolysing activity of GTPases and are thought to down-regulate GTPase signalling (Zhang *et al.*, 1990), although other data suggest that RasGAPs act downstream of activated Ras (Adari *et al.*, 1988; Cales *et al.*, 1988; Yatani *et al.*, 1990). The precise role of RasGap proteins remains to be elucidated.

IQGAP1, and the closely related protein IQGAP2 (Brill *et al.*, 1996) contain four complete IQ motifs (IQXXXRGXXXR) responsible for binding to Calmodulin in the absence of Ca^{2+} (Houdusse and Cohen, 1995), and a region with significant sequence identity to the catalytic domain of Ras-GAP (GRD) (Figure 5.19a) (Weissbach *et al.*, 1994). IQGAP1 and IQGAP2 also contain copies of a unique 50-55 amino acid repeat and a N-terminal Calponin Homology (CH) domain that in Spectrin family proteins, binds F-actin (Carugo *et al.*, 1997). IQGAP1 has also been shown to bind F-actin filaments (Bashour *et al.*, 1997). IQGAP1 appears to regulate cadherin-mediated cell-cell adhesion (Kuroda *et al.*, 1998) by interacting with β-Catenin and E-Cadherin. Overexpression of IQGAP1 dissociates α-Catenin from the cadherin-catenin complex reducing cadherin-mediated cell adhesion (Fukata *et al.*, 1999).

The putative amino acid sequence of C84 was compared to other mammalian IQGAP proteins using the CLUSTAL W alignment program (Thompson *et al.*, 1994). C84 exhibited highest amino acid identity to hIQGAP1 at 57.5% then mIQGAP1 at 56.6% followed by hIQGAP2 at 50.5 % (Figure 5.19b). Given that C84 exhibited similar identity to IQGAP1 and IQGAP2, and that human and mouse IQGAP1 are highly conserved (Figure 5.19b), the cDNA clone isolated from the C84 cell line represents a new member of the IQGAP family and will be referred to as mIQGAP3. The C-terminal 41 amino acids were highly conserved among all three proteins. Over this region C84 had four differences to human and mouse IQGAP1 (90% identity) and nine differences to human IQGAP2 (78% identity).

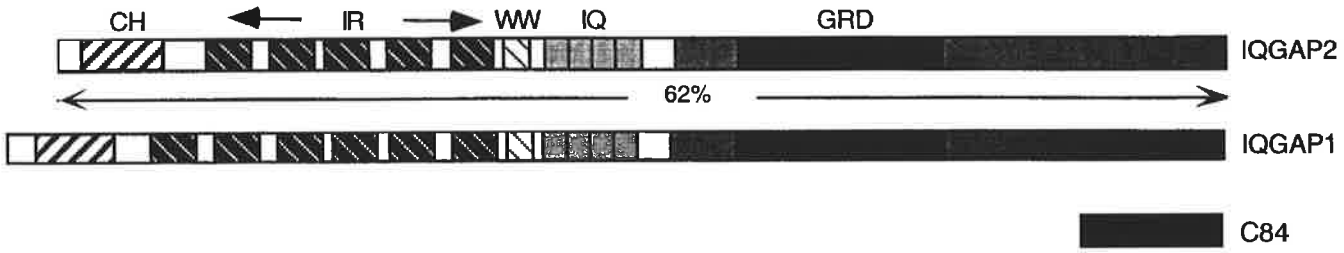
Figure 5.19

Comparison of C84 deduced protein sequence with mammalian IQGAP family members.

A. The schematic structure of IQGAP proteins. The domain structures of IQGAP1 and IQGAP2 are compared with the sequence of C84. IQGAP1 and IQGAP2 proteins exhibit 62% identity over their length. CH, Calponin Homology domain; IR, IQGAP repeats; WW, WW domain; IQ, IQ motifs; GRD, GTPase Related Domain. Adapted from Brill *et al*, (1996).

B. The putative amino acid sequence of C84 aligned with the C-terminal regions of mouse IQGAP1, hIQGAP1 and hIQGAP2. Amino acid sequence of IQGAP1 (mIQGAP1; accession No. NM_016721), human IQGAP1 (hIQGAP1; accession No. L33075; Weissbach *et al.*, 1994) and human IQGAP2 (hIQGAP2; accession No. U51903; Brill *et al.*, 1996) were aligned with the putative amino acid sequence of C84 using the CLUSTAL W alignment program. Amino acid identity is indicated as black boxes. Positions in respective protein sequences are shown and the first methionine residue is indicated with an arrow.

A



B

```
                    .    10    .    20    .    30    .    40    .    50
mIQGAP1   1421 QRAMQRRAIRDAKTPDKMKKSKPMKEDNNLSLQEKKEKIQTGLKKLTELG 1470
hIQGAP1   1421 QRAMQRRAIRDAKTPDKMKKSKSVKEDSNLTLQEKKEKIQTGLKKLTELG 1470
hIQGAP2   1336 ATDMVSRAMIDSRTPEEMKHSQSMIEDAQLPLEQKKRKIQRNLRTLEQTG 1385
C84          1 ----IIPLLSDTQKPEPLRRHHSLMAHSLLSLSEKQQRVLRNLRRLQGLG   46

                    .    60    .    70    .    80    .    90    .   100
mIQGAP1   1471 TVDPKNRYQELINDIAKDIRNQRRYRQRRKAELVKLQQTYSALNSKATFY 1520
hIQGAP1   1471 TVDPKNKYQELINDIARDIRNQRRYRQRRKAELVKLQQTYAALNSKATFY 1520
hIQGAP2   1386 HVSSENKYQDILNEIAKDIRNQRIYRKLRKAELAKLQQTLNALNKKAAFY 1435
C84         47 LVRASDCYQGLVDELAKDICNQRRHRQRRKAEMLRLRTTLQGLDAKTIFY   96

                    .   110    .   120    .   130    .   140    .   150
mIQGAP1   1521 GEQVDYYKSYIKTCLDNLASKGKVSKKPREMKGKKS---KKISLKYTAAR 1567
hIQGAP1   1521 GEQVDYYKSYIKTCLDNLASKGKVSKKPREMKGKKS---KKISLKYTAAR 1567
hIQGAP2   1436 EEQINYYDTYIKTCLDNLKRKNTRRSIKLDGKGEPKGAKRAKPVKYTAAK 1485
C84         97 EEQGDYYNQYIQACLDHLAPKPKSS-----GKGKKQ-----PSLHYTAAQ  136

                    .   160    .   170    .   180    .   190    .   200
mIQGAP1   1568 LHEKGVLLEIEDLQANQFKNVIFEIGPTEEVGDFEVKAKFMGVQMETFML 1617
hIQGAP1   1568 LHEKGVLLEIEDLQVNQFKNVIFEISPTEEVGDFEVKAKFMGVQMETFML 1617
hIQGAP2   1486 LHEKGVLLDIDDLQTNQFKNVTFDIIATEDVGIFDVRSKFLGVEMEKVQL 1535
C84        137 LLEKGVLVEIEDLPVSHFRNVIFDITPGDEAGRFFVNAKFLGVDMEKFQL  186

                    .   210    .   220    .   230    .   240
mIQGAP1   1618 HYQDLLQLQYEGVAVMKLFDRAKVNVNLLIFLLNKKFYGK 1657
hIQGAP1   1618 HYQDLLQLQYEGVAVMKLFDRAKVNVNLLIFLLNKKFYGK 1657
hIQGAP2   1536 NIQDLLQMQYEGVAVMKMFDKVKVNVNLLIYLLNKKFYGK 1575
C84        187 HYQDLLQLQYEGVAVMKLFNKAKVNVNLLIFLLNKKFLRK  226
```

The deduced protein did not contain any of the characterised domains present in IQGAP1 and IQGAP2. Given that IQGAP1 and IQGAP2 are 1,657 and 1,575 amino acids long, respectively, the C84 cDNA was likely to be a truncated cDNA clone (Figure 5.19b) which might potentially act as a constitutively active or dominant negative form of IQGAP3.

Cell line C25.1

The PCR product amplified from C25.1-derived Hirt DNA (2.3.32) was digested with *Eco*RI endonuclease and the resulting 1.1 kb fragment was endfilled with DNA polymerase I Klenow fragment. The fragment was gel purified and blunt end cloned into the *Eco*RV restriction site of pBluescript II KS forming pC25.1KS (Figure 5.20b).

Sequencing reactions with RSP and Ltbp1536 primers (2.2.7) were carried out to complete the sequence of the cDNA fragment in C25.1. The C25.1 cDNA was 1,074 bp long and contained a poly (A) tail. A BLASTN (Altschul *et al.*, 1997) search of the Genbank database revealed that the C25.1 cDNA shared 99.6% identity with mouse Tat Binding Protein-1 (*mTbp-1*), having four differences to the published *mTbp-1* sequence (Nakamura *et al.*, 1998)(accession No. D49686) (Figure 5.23). The C25.1 cDNA was 495 bp shorter at the 5′ end than the reported *mTbp-1* cDNA of 1,569 bp suggesting that it represented a truncated *mTbp-1* cDNA. The first in frame ATG codon at position 532 contained a consensus sequence for translation initiation (Kozak, 1991) (Figure 5.23 open box). Translation from this position would result in an N-terminally truncated form of mTbp-1 of 326 amino acids.

The yeast Tbp-1 homologue, YTA1A and tomato homologue, LeMa-1, have greater than 70% and 77% identity respectively, to TBP-1 and both contain a conserved ATPase domain of approximately 180 amino acids near the C-terminus that contains an ATP binding site and a helicase motif (Figure 5.23 shaded boxes) (Nakamura *et al.*, 1998). TBP-7, a related human protein, shares 57% identity to human TBP-1 over a 210 amino acid region that includes the ATPase domain (Ohana *et al.*, 1993). The entire ATPase domain was present in the deduced protein encoded by the C25.1 cDNA (Figure 5.23). Given that the N-terminal region of the protein was not encoded by the C25.1 cDNA, it was possible that the resulting truncated protein influenced ES cell differentiation because it was a functionally active, constitutively active or dominant negative form of mTbp-1.

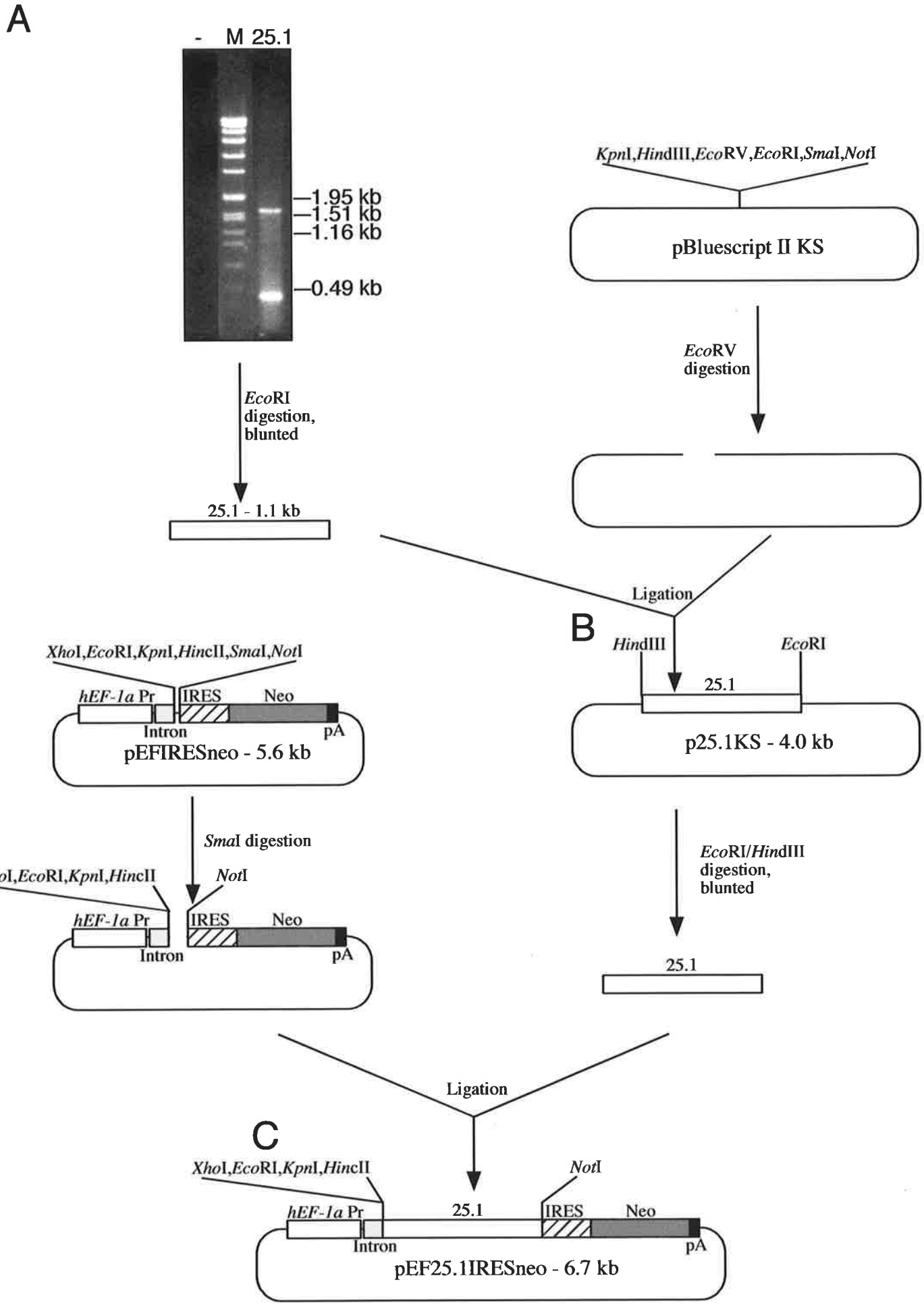
Figure 5.20

PCR amplification and cloning of the C25.1 cDNA.

A. Agarose gel electrophoresis of PCR performed on Hirt DNA extracted from C25.1 cells. 50 μl PCR reactions with primers, UPGK2 and LOCT42 (2.2.7) were performed with Pfu turbo polymerase for a total of 30 cycles. -, no DNA template PCR; 25.1, PCR on 1/20th of a Hirt DNA preparation purified from C25.1 cells; M, *Eco*RI digested SPP-1 phage DNA markers. The position of a 1.4 kb marker band is indicated.

B. Cloning of the C25.1 cDNA insert. The 25.1 PCR product (A) was digested with *Eco*RI, blunted and cloned into the *Eco*RV restriction site of pBluescript II KS to form p25.1KS.

C. Cloning of the C25.1 cDNA into the mammalian expression vector pEFIRESneo. The resulting plasmid, pEF25.1IRESneo, expresses a truncated *Tbp-1* message corresponding to the C25.1 cDNA and neomycin resistance as a single dicistronic message from the constitutive *hEF1-a* promoter.


A
- M 25.1
1.95 kb
1.51 kb
1.16 kb
0.49 kb
EcoRI digestion, blunted
25.1 - 1.1 kb
KpnI,HindIII,EcoRV,EcoRI,SmaI,NotI
pBluescript II KS
EcoRV digestion
Ligation
B
HindIII
EcoRI
25.1
p25.1KS - 4.0 kb
XhoI,EcoRI,KpnI,HincII,SmaI,NotI
hEF-1a Pr
IRES
Neo
Intron
pA
pEFIRESneo - 5.6 kb
SmaI digestion
XhoI,EcoRI,KpnI,HincII
NotI
EcoRI/HindIII digestion, blunted
25.1
Ligation
C
XhoI,EcoRI,KpnI,HincII
NotI
hEF-1a Pr
25.1
IRES
Neo
Intron
pA
pEF25.1IRESneo - 6.7 kb

HIV Tat binding protein-1 (TBP-1) was identified by screening a λgt11 expression library with biotinylated Tat protein (Nelbock *et al.*, 1990). The mouse *Tbp-1* cDNA was isolated from a newborn mouse brain cDNA library and it is expressed in the testis and to a lesser extent in all other organs (Nakamura *et al.*, 1998). Tbp-1 is a nuclear protein that is present in spermatocytes of the testis. Human TBP-1 binds to HIV Tat protein, and mouse and human Tbp-1 have been reported to inhibit Tat-mediated transactivation of the HIV-1 LTR (Nakamura *et al.*, 1998; Nelbock *et al.*, 1990) although TBP-1 has also been reported as a transcriptional activator (Ohana *et al.*, 1993). TBP-1 is also a component of the PA700 complex, that together with the 20S proteasome complex, reconstitutes the catalytic properties of purified 26S proteasome (DeMartino *et al.*, 1996). Recent work has implicated TBP-1 in tumour suppression as the product of the tumour suppressor gene VHL binds to TBP-1 (Tsuchiya *et al.*, 1996). Overexpression of TBP-1 caused reversion of $p185^{neu}$ transformed fibroblasts by an unknown mechanism (Park *et al.*, 1999).

5.6.4 Expression of the C84 cDNA in ES cells

In LIF titration experiments C84 cells were observed to retain AP activity even in the absence of LIF (5.5), although colonies were also observed to spread, suggestive of differentiation. To determine whether the truncated IQGAP3 cDNA was responsible for this phenotype, it was cloned into pPSDEneoΔLT20. A 1.5 kb *Eco*RI/*Hin*dIII fragment corresponding to the C84 cDNA was excised from p84KS, blunted (2.3.4) and cloned into *Eco*RI/*Not*I digested and blunted pPSDEneoΔLT20 to form p84DEneoΔLT20 (Figure 5.17b).

To establish if episomal IQGAP3 expression altered the efficiency of pluripotent colony establishment in the absence of LIF, p84DEneoΔLT20 or pPSDEneoΔLT20 supertransfected cells were selected in 200 μg/ml G418 in the presence or absence of LIF for 15 days (4.6.1). p84DEneoΔLT20 supertransfected C32 ES cells formed fewer AP^+ colonies in the absence of LIF (28%) than pPSDEneoΔLT20 supertransfected cells (35%) (Figure 5.21a). Therefore, supertransfection of an episome carrying the C84 cDNA did not appear sufficient for establishment of pluripotence in the absence of LIF. In experiments using ES cells transfected with pEF84IRESneo directing IRES-driven expression (2.2.6) of truncated IQGAP3, similar results were obtained (not shown).

Figure 5.21

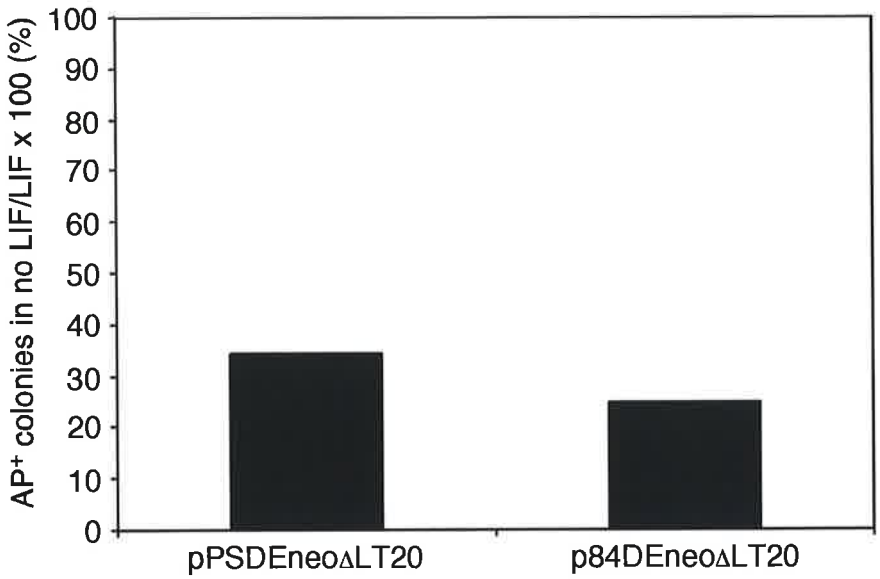
LIF dependence of ES cells expressing the truncated IQGAP3 cDNA.

A. Differentiation of p84DEneoΔLT20 supertransfected cells. 3 x 10^7 C32 ES cells were electroporated with 20 μg of CsCl purified p84DEneoΔLT20 or pPSDEneoΔLT20 plasmid. Half of the supertransfected cells were seeded onto a 10 cm plate and selected for 15 days in 200 μg/ml G418 in the presence or absence of LIF. Surviving colonies were stained to detect AP activity and colonies cultured with or without LIF were scored. Data are represented as the percentage of AP^+ colonies that survive in the absence of LIF over those selected in LIF.

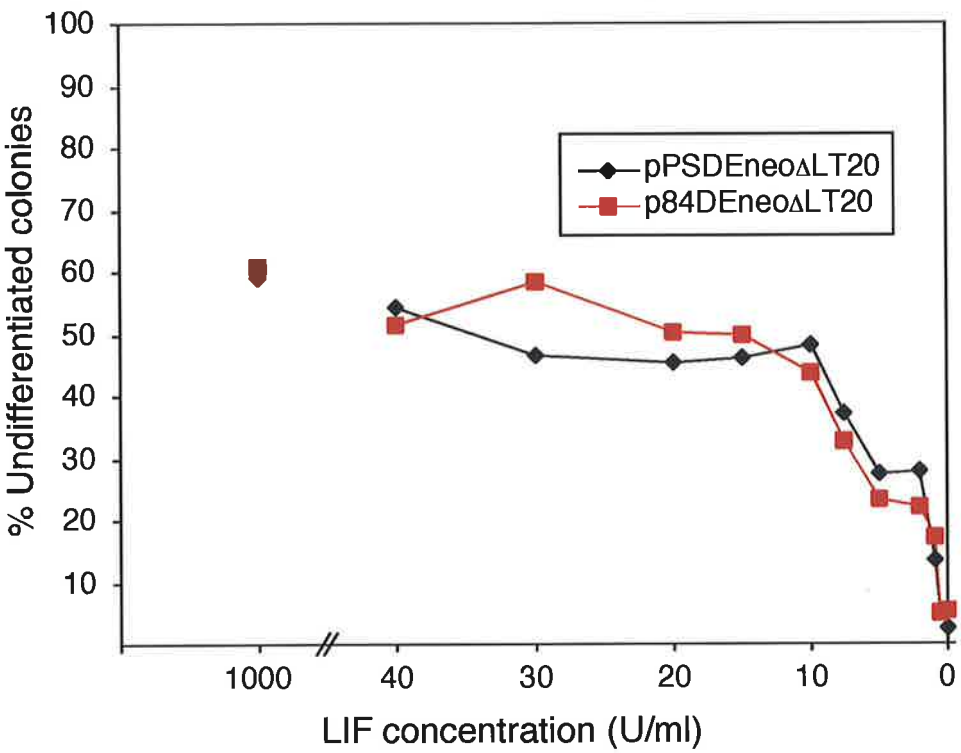
B. LIF titration analysis of p84DEneoΔLT20 supertransfected ES cells. ES cells stably supertransfected with p84DEneoΔLT20 or pPSDEneoΔLT20 were seeded at 500 cells per well in ES cell medium containing LIF or 0, 0.5, 1, 2, 5, 7.5, 10, 15, 20, 30 or 40U/ml of recombinant LIF and allowed to differentiate for 6 days prior to detection of AP activity. Undifferentiated colonies, defined as those more 90% undifferentiated and retained AP activity, were scored and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, pPSDEneoΔLT20 supertransfected ES cells; red squares, p84DEneoΔLT20 supertransfected ES cells.

C. LIF titration of pEF84IRESneo transfected ES cells. ES cells stably transfected with pEF84IRESneo or pEFIRESneo were seeded at 500 cells per well in ES cell medium containing LIF or 0, 0.5, 1, 2, 5, 7.5, 10, 15, 20, 30 or 40U/ml of recombinant LIF and allowed to differentiate for 6 days prior to detection of AP activity. Colonies containing more than 90% undifferentiated cells and retained AP activity were scored following LIF titration assay and graphed as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, pEFIRESneo transfected ES cells; red squares, pEF84IRESneo transfected ES cells.

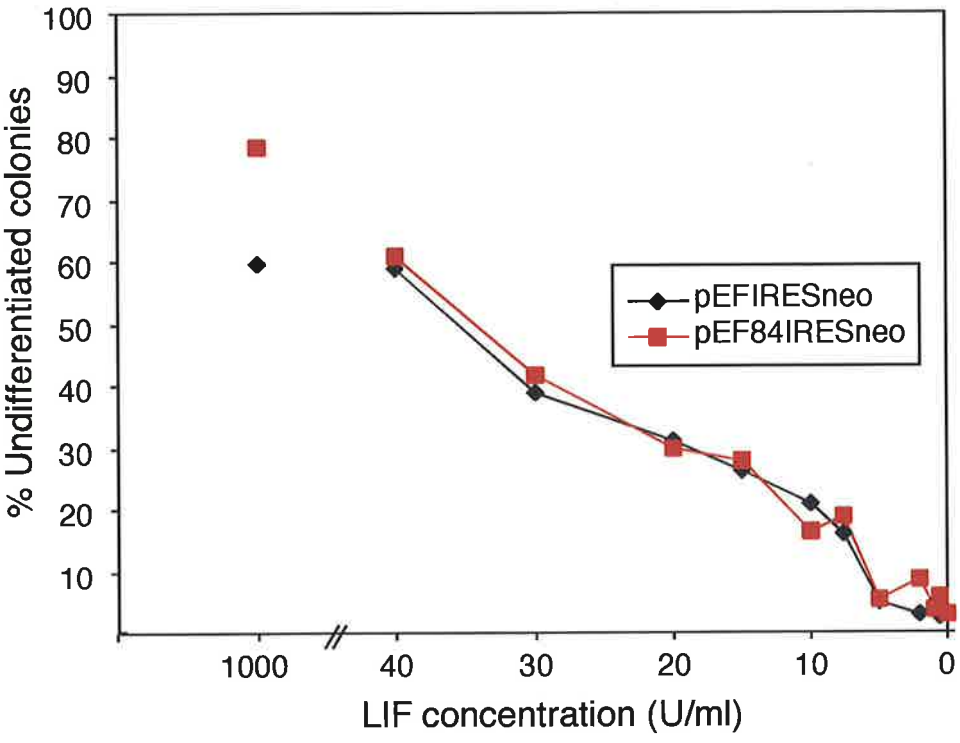
A


AP+ colonies in no LIF/LIF x 100 (%)
100
90
80
70
60
50
40
30
20
10
0
pPSDEneoΔLT20
p84DEneoΔLT20

B


% Undifferentiated colonies
100
90
80
70
60
50
40
30
20
10
pPSDEneoΔLT20
p84DEneoΔLT20
1000
40
30
20
10
0
LIF concentration (U/ml)

C


% Undifferentiated colonies
100
90
80
70
60
50
40
30
20
10
pEFIRESneo
pEF84IRESneo
1000
40
30
20
10
0
LIF concentration (U/ml)

To determine if the truncated IQGAP3 cDNA altered the LIF requirement of stably expressing supertransfected ES cells, p84DEneoΔLT20 was supertransfected into C32 ES cells. Cells were selected in 200 μg/ml G418 in the presence of LIF for 15 days, and stably supertransfected colonies were pooled and passaged in the presence of LIF and 200 μg/ml G418 to ensure episome maintenance. Approximately 70% of the G418-resistant clones in each pool were expected to express the truncated IQGAP3 cDNA (4.3). p84DEneoΔLT20 and pPSDEneoΔLT20 supertransfected pools were compared by LIF titration analysis (2.4.13). 500 cells were seeded in 24-well trays, cultured for 6 days in various concentrations of recombinant LIF and stained to detect AP activity.

pPSDEneoΔLT20 and p84DEneoΔLT20 supertransfectants progressively lost AP staining in lower LIF concentrations. Therefore, p84DEneoΔLT20 supertransfected cultures, like pEF84IRESneo transfected ES cells and C84 cells, remained responsive to LIF and retained differentiation capability. pPSDEneoΔLT20 and p84DEneoΔLT20 supertransfectants exhibited similar AP staining at all LIF concentrations tested (not shown), indicating that expression of truncated IQGAP3 was not responsible for retention of AP^+ cells in low LIF concentrations observed for C84 cells (see figure 5.13a). LIF titration cultures were scored under phase-contrast microscopy and expressed as a percentage of undifferentiated colonies (comprised of more than 90% undifferentiated cells) present in the culture. p84DEneoΔLT20 supertransfected ES cells retained a similar percentage of undifferentiated colonies to ES cells supertransfected with pPSDEneoΔLT20 in 0 U/ml to 10 U/ml LIF, and this proportion was only marginally higher than the control in LIF concentrations between 15 U/ml and 30 U/ml (Figure 5.21b). These data are not consistent with retention of pluripotent colonies in low LIF concentrations observed in C84 cultures and argue against a functional role for the truncated IQGAP3 cDNA in maintenance of the undifferentiated phenotype. It is possible that episome-driven expression of the truncated IQGAP3 cDNA is lower than in the C84 cell line (5.5) accounting for a lack of ES cell maintenance in this experiment.

The truncated IQGAP3 cDNA was cloned into pEFIRESneo (2.2.6) to examine if expression using a different expression vector could recapitulate the phenotype observed for the C84 cell line. The presence of an internal ribosome entry site (IRES) in pEFIRESneo means that greater than 90% of resistant cells should express the cDNA because neomycin

resistance and the cDNA will be expressed as a dicistronic message (reviewed in Mountford *et al.*, 1994). A 1.5 kb *Eco*RI/*Hin*dIII fragment that corresponds to the truncated IQGAP3 cDNA was excised from p84KS, blunted and cloned into *Sma*I restriction site of pEFIRESneo forming pEF84IRESneo (Figure 5.17c).

Wild-type ES cells were electroporated (2.4.10) with pEFIRESneo or pEF84IRESneo and selected in 250 μg/ml G418 for 10 days. Resistant colonies were pooled and passaged in the presence of LIF and G418 to reduce cell line specific variation. pEFIRESneo and pEF84IRESneo transfected ES cells were subjected to LIF titration analysis (2.4.13). 500 cells were seeded in 24-well trays, cultured for 6 days in various concentrations of recombinant LIF (ESGRO; Chemicon) and stained to detect AP activity.

AP staining of pEFIRESneo and pEF84IRESneo transfected ES cells was indistinguishable macroscopically over the LIF concentrations tested (data not shown), confirming that expression of truncated IQGAP3 did not recapitulate retention of AP^+ cells in low LIF concentrations observed for C84 cells (see figure 5.13a). Retention of undifferentiated colonies in the cultures was quantitated and expressed as a percentage of the total number of colonies. In the LIF concentrations tested, pEF84IRESneo transfected ES cells retained a similar percentage of undifferentiated colonies to ES cells transfected with pEFIRESneo (Figure 5.21c). These data support the finding that the truncated IQGAP3 protein encoded by the C84 cDNA is unlikely to be responsible for retention of pluripotent colonies in C84 cultures.

5.6.5 Expression of C25.1 and *Tbp-1* cDNAs in ES cells

Given that C25.1 potentially could have distinct activities from Tbp-1 (5.6.3) the effect of expressing both full-length *Tbp-1* and the C25.1 truncated cDNA in ES cells was investigated. Primers Utbp96 and Ltbp1536 (2.2.7) were designed against the published *mTbp-1* cDNA sequence (Nakamura *et al.*, 1998) in order to amplify the complete coding region of *mTbp-1*. A 1.4 kb product encompassing the *mTbp-1* coding region was PCR amplified (2.3.33) from reverse transcribed ES cell RNA (Figure 5.22a). The PCR product was endfilled with the Klenow fragment of DNA polymerase I, gel purified and blunt end ligated

Figure 5.22

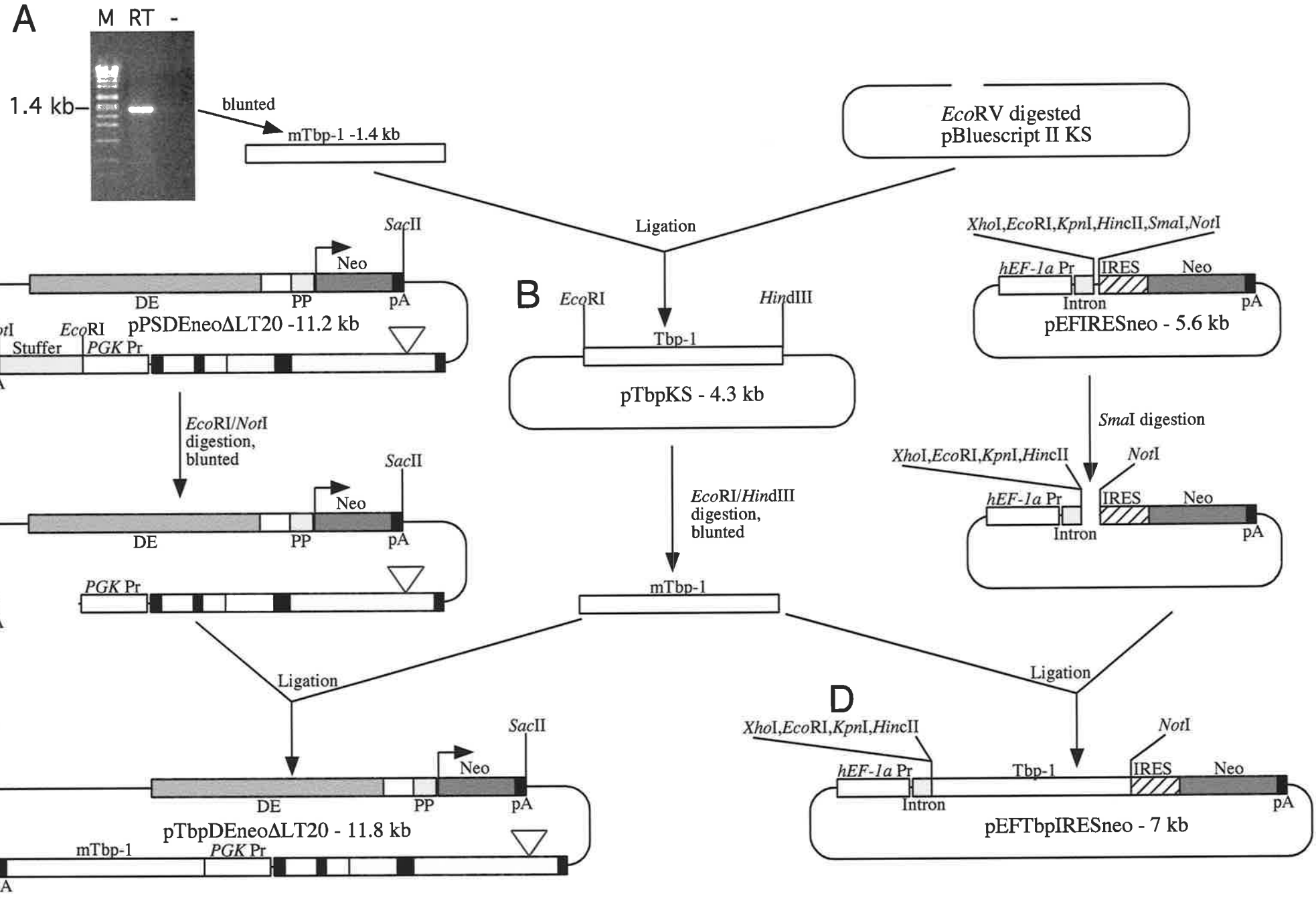
RT-PCR and cloning of the *mTbp-1* cDNA.

A. Agarose gel electrophoresis of mTbp-1 specific PCR. 50 μl PCR reactions with *Tbp-1* specific primers, Utbp96 and Ltbp1536 (2.2.7), on reverse transcribed ES cell RNA (RT) or no DNA template (-) were performed with Pfu turbo polymerase in a PTC-100 thermal cycler for a total of 30 cycles. M, *Eco*RI digested SPP-1 phage DNA markers. The position of a 1.4 kb marker band is indicated.

B. Cloning of the *mTbp-1* cDNA. *mTbp-1* PCR product was blunted and cloned into the *Eco*RV restriction site of pBluescript II KS forming pTbpKS.

C. Construction of pTbpDEneoΔLT20. The pTbpDEneoΔLT20 plasmid can be supertransfected into episome harbouring ES cell lines to express full-length mouse *Tbp-1* from the constitutive *PGK-1* promoter. Shading is as described for figure 4.2.

D. Cloning of *Tbp-1* cDNA into the mammalian expression vector pEFIRESneo. The resulting plasmid, pEFTbpIRESneo, expresses full-length *Tbp-1* and neomycin resistance as a single dicistronic message from the constitutive *hEF1-a* promoter.


A
M RT -
1.4 kb
blunted
mTbp-1 -1.4 kb
EcoRV digested
pBluescript II KS
Ligation
SacII
Neo
DE
PP
pA
NotI
EcoRI
Stuffer
PGK Pr
pPSDEneoΔLT20 -11.2 kb
pA
B
EcoRI
HindIII
Tbp-1
pTbpKS - 4.3 kb
XhoI,EcoRI,KpnI,HincII,SmaI,NotI
hEF-1a Pr
IRES
Neo
Intron
pA
pEFIRESneo - 5.6 kb
EcoRI/NotI
digestion,
blunted
SmaI digestion
XhoI,EcoRI,KpnI,HincII
NotI
EcoRI/HindIII
digestion,
blunted
mTbp-1
Ligation
C
pTbpDEneoΔLT20 - 11.8 kb
D
XhoI,EcoRI,KpnI,HincII
NotI
pEFTbpIRESneo - 7 kb

into the *Eco*RV restriction site of pBluescript II KS (Figure 5.22b). The identity of the resulting plasmid, pTbpKS, was confirmed by sequencing in both orientations.

The full sequence of the *Tbp-1* PCR product contained five nucleotide differences compared to the reported *mTbp-1* cDNA sequence (Figure 5.23). Of these, three substitutions at positions 510, 1196 and 1267 were also present in the C25.1 cDNA sequence and encoded histidine to tyrosine, serine to cysteine, and aspartic acid to glutamate amino acid changes respectively, compared with the reported *mTbp*-1 sequence. Given that C25.1 and the *Tbp-1* PCR product were isolated independently, the observed changes were not likely to represent errors due to sequencing or PCR amplification. Moreover, the altered amino acids were present in the human *TBP-1* cDNA sequence (Nelbock *et al.*, 1990) indicating that they may be polymorphisms or errors in the reported mouse *Tbp-1* sequence (Figure 5.24). The remaining change in the amplified *Tbp-1* product at position 346 (C to A, leucine to methionine) was not present in C25.1 or the reported *mTbp-1* cDNA (Figures 5.23 and 5.24). While this may represent an error introduced during PCR amplification, the change lies outside the ATPase domain and retains the hydrophobicity of the side chain making it unlikely to alter the activity of the encoded protein. An A to G nucleotide change (isoleucine to valine) present in C25.1 within the ATPase domain at position 853 (amino acid 285) (Figure 5.23) was not observed in the amplified *Tbp-1* or the reported *mTbp-1* sequence (Figure 5.24) and is likely to represent a PCR error specific to C25.1. This substitution is a conservative change from isoleucine to valine four amino acids N-terminal to the helicase motif.

The C25.1 and Tbp-1 cDNAs were cloned into pEFIRESneo (2.2.6) to generate a population of stably transfected ES cells that express neomycin resistance and the *Tbp-1* cDNAs. A 1.1 kb *Eco*RI/*Hin*dIII fragment derived from pC25.1KS corresponding to the C25.1 cDNA was blunted and cloned into *Sma*I restriction site of pEFIRESneo forming pEF25.1IRESneo (Figure 5.20c). The *Tbp-1* PCR product was excised from pTbpKS as a 1.4 kb *Eco*RI/*Hin*dIII fragment, blunted and cloned into *Sma*I digested pEFIRESneo to form pEFTbpIRESneo (Figure 5.22d).

Wild-type ES cells were electroporated (2.4.10) with pEFIRESneo, pEF25.1IRESneo or pEFTbpIRESneo and selected in 250 μg/ml G418 for 10 days. Resistant colonies were

Figure 5.23

cDNA and deduced protein sequence of the mouse Tat Binding protein–1 (*mTbp-1*) PCR product.

The coding region is represented in capital letters while the 3´ and 5´ untranslated regions are in lower case. The extent of clone 25.1 is indicated as a line at position 320 in the open reading frame. Possible initiation codons 3´ of this position are underlined, the Kozak sequence of the first in frame ATG is in an open box and the putative initiating methionine is in a black box. Translation initiation from this site would result in the formation of a 264 amino acid truncated form of mTbp-1. The base difference and amino acid change in the C25.1 sequence compared to the mTbp-1 PCR product are shown at position 853 bp and 285 amino acids in the coding region, respectively. Nucleotide and resulting amino acid substitutions in the mTbp-1 PCR product compared with the published mTbp-1 sequence (Nakamura *et al.*, 1998, accession No. D49686) are shown in bold. The conserved ATPase domain is boxed and contains an ATP binding motif (light grey box) and a DNA helicase motif (dark grey box). The positions of primers Utbp96 and Ltbp1536 (2.2.7) used to PCR the mTbp-1 cDNA are shown as arrowed lines.

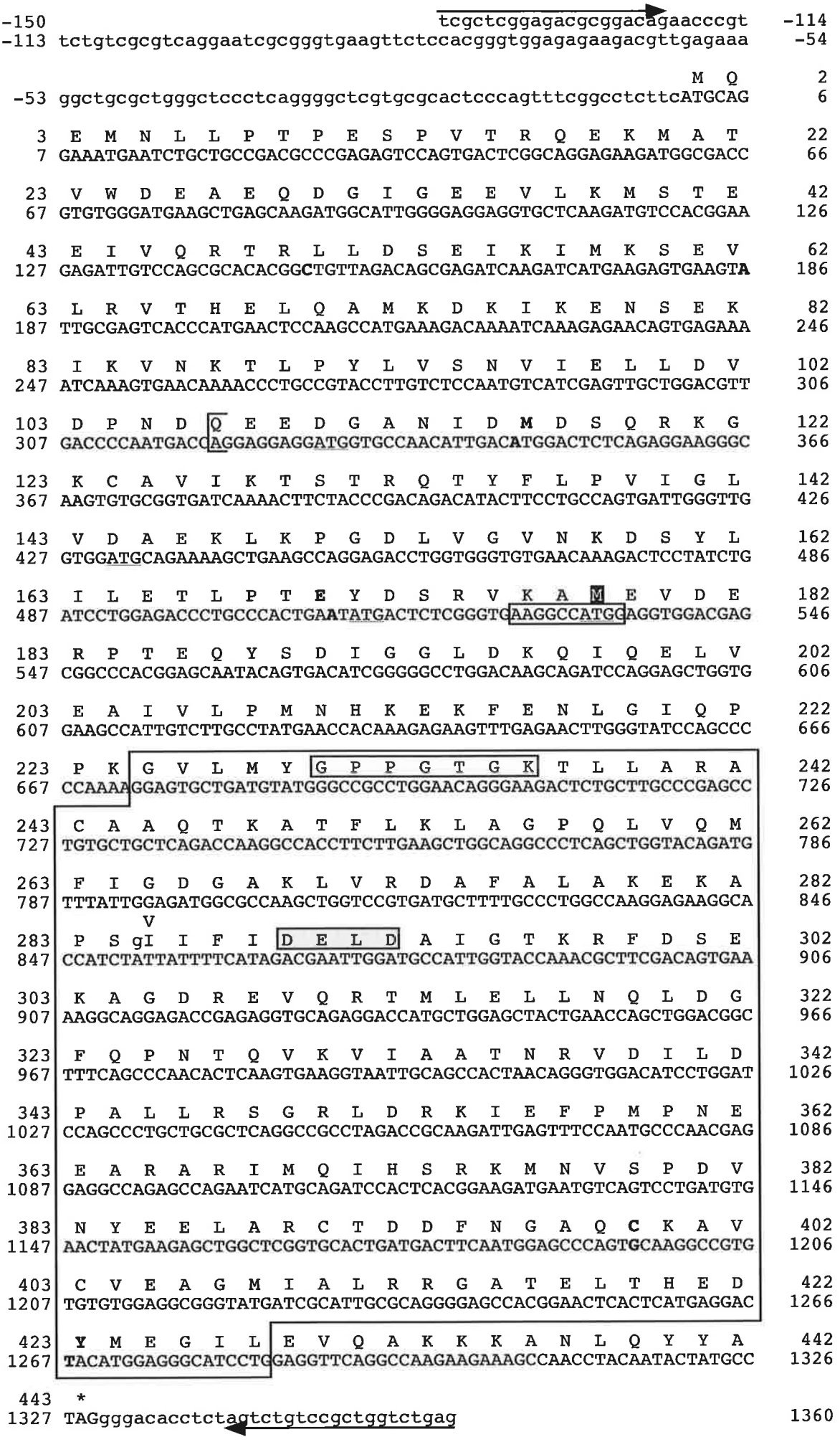

-150 tcgctcggagacgcggacagaacccgt -114
-113 tctgtcgcgtcaggaatcgcgggtgaagttctccacgggtggagagaagacgttgagaaa -54
M Q 2
-53 ggctgcgctgggctccctcaggggctcgtgcgcactcccagtttcggcctcttcATGCAG 6
3 E M N L L P T P E S P V T R Q E K M A T 22
7 GAAATGAATCTGCTGCCGACGCCCGAGAGTCCAGTGACTCGGCAGGAGAAGATGGCGACC 66
23 V W D E A E Q D G I G E E V L K M S T E 42
67 GTGTGGGATGAAGCTGAGCAAGATGGCATTGGGGAGGAGGTGCTCAAGATGTCCACGGAA 126
43 E I V Q R T R L L D S E I K I M K S E V 62
127 GAGATTGTCCAGCGCACACGGCTGTTAGACAGCGAGATCAAGATCATGAAGAGTGAAGTA 186
63 L R V T H E L Q A M K D K I K E N S E K 82
187 TTGCGAGTCACCCATGAACTCCAAGCCATGAAAGACAAAATCAAAGAGAACAGTGAGAAA 246
83 I K V N K T L P Y L V S N V I E L L D V 102
247 ATCAAAGTGAACAAAACCCTGCCGTACCTTGTCTCCAATGTCATCGAGTTGCTGGACGTT 306
103 D P N D Q E E D G A N I D M D S Q R K G 122
307 GACCCCAATGACCAGGAGGAGGATGGTGCCAACATTGACATGGACTCTCAGAGGAAGGGC 366
123 K C A V I K T S T R Q T Y F L P V I G L 142
367 AAGTGTGCGGTGATCAAAACTTCTACCCGACAGACATACTTCCTGCCAGTGATTGGGTTG 426
143 V D A E K L K P G D L V G V N K D S Y L 162
427 GTGGATGCAGAAAAGCTGAAGCCAGGAGACCTGGTGGGTGTGAACAAAGACTCCTATCTG 486
163 I L E T L P T E Y D S R V K A M E V D E 182
487 ATCCTGGAGACCCTGCCCACTGAATATGACTCTCGGGTGAAGGCCATGGAGGTGGACGAG 546
183 R P T E Q Y S D I G G L D K Q I Q E L V 202
547 CGGCCCACGGAGCAATACAGTGACATCGGGGGCCTGGACAAGCAGATCCAGGAGCTGGTG 606
203 E A I V L P M N H K E K F E N L G I Q P 222
607 GAAGCCATTGTCTTGCCTATGAACCACAAAGAGAAGTTTGAGAACTTGGGTATCCAGCCC 666
223 P K G V L M Y G P P G T G K T L L A R A 242
667 CCAAAAGGAGTGCTGATGTATGGGCCGCCTGGAACAGGGAAGACTCTGCTTGCCCGAGCC 726
243 C A A Q T K A T F L K L A G P Q L V Q M 262
727 TGTGCTGCTCAGACCAAGGCCACCTTCTTGAAGCTGGCAGGCCCTCAGCTGGTACAGATG 786
263 F I G D G A K L V R D A F A L A K E K A 282
787 TTTATTGGAGATGGCGCCAAGCTGGTCCGTGATGCTTTTGCCCTGGCCAAGGAGAAGGCA 846
V
283 P S gI I F I D E L D A I G T K R F D S E 302
847 CCATCTATTATTTTCATAGACGAATTGGATGCCATTGGTACCAAACGCTTCGACAGTGAA 906
303 K A G D R E V Q R T M L E L L N Q L D G 322
907 AAGGCAGGAGACCGAGAGGTGCAGAGGACCATGCTGGAGCTACTGAACCAGCTGGACGGC 966
323 F Q P N T Q V K V I A A T N R V D I L D 342
967 TTTCAGCCCAACACTCAAGTGAAGGTAATTGCAGCCACTAACAGGGTGGACATCCTGGAT 1026
343 P A L L R S G R L D R K I E F P M P N E 362
1027 CCAGCCCTGCTGCGCTCAGGCCGCCTAGACCGCAAGATTGAGTTTCCAATGCCCAACGAG 1086
363 E A R A R I M Q I H S R K M N V S P D V 382
1087 GAGGCCAGAGCCAGAATCATGCAGATCCACTCACGGAAGATGAATGTCAGTCCTGATGTG 1146
383 N Y E E L A R C T D D F N G A Q C K A V 402
1147 AACTATGAAGAGCTGGCTCGGTGCACTGATGACTTCAATGGAGCCCAGTGCAAGGCCGTG 1206
403 C V E A G M I A L R R G A T E L T H E D 422
1207 TGTGTGGAGGCGGGTATGATCGCATTGCGCAGGGGAGCCACGGAACTCACTCATGAGGAC 1266
423 Y M E G I L E V Q A K K K A N L Q Y Y A 442
1267 TACATGGAGGGCATCCTGGAGGTTCAGGCCAAGAAGAAAGCCAACCTACAATACTATGCC 1326
443 *
1327 TAGgggacacctctagtctgtccgctggtctgag 1360

Figure 5.24

Comparison of deduced protein sequence from the mouse Tat Binding protein–1 (*mTbp-1*) PCR product with mouse and human mTbp-1 amino acid sequences.

Differences between the deduced amino acid sequence of the amplified mouse Tbp-1 and the published mouse (Nakamura *et al.*, 1998, accession No. D49686) and human TBP-1 protein sequence (Nelbock *et al.*, 1990, accession No. M34079) are shown in black boxes.

```
                          10         20         30         40         50
RT-PCR (mTbp-1)     1 MQEMNLLPTP ESPVTRQEKM ATVWDEAEQD GIGEEVLKMS TEEIVQRTRL   50
mTbp-1              1 MQEMNLLPTP ESPVTRQEKM ATVWDEAEQD GIGEEVLKMS TEEIVQRTRL   50
hTBP-1                                                 MS TEEITQRTRL   12

                          60         70         80         90        100
RT-PCR (mTbp-1)    51 LDSEIKIMKS EVLRVTHELQ AMKDKIKENS EKIKVNKTLP YLVSNVIELL  100
mTbp-1             51 LDSEIKIMKS EVLRVTHELQ AMKDKIKENS EKIKVNKTLP YLVSNVIELL  100
hTBP-1             13 LDSEIKIMKS EVLRVTHELQ AMKDKIKENS EKIKVNKTLP YLVSNVIELL   62

                         110        120        130        140        150
RT-PCR (mTbp-1)   101 DVDPNDQEED GANIDMDSQR KGKCAVIKTS TRQTYFLPVI GLVDAEKLKP  150
mTbp-1            101 DVDPNDQEED GANIDLDSQR KGKCAVIKTS TRQTYFLPVI GLVDAEKLKP  150
hTBP-1             63 DVDPNDQEED GANIDLDSQR KGKCAVIKTS TRQTYFLPVI GLVDAEKLKP  112

                         160        170        180        190        200
RT-PCR (mTbp-1)   151 GDLVGVNKDS YLILETLPTE YDSRVKAMEV DERPTEQYSD IGGLDKQIQE  200
mTbp-1            151 GDLVGVNKDS YLILETLPTD YDSRVKAMEV DERPTEQYSD IGGLDKQIQE  200
hTBP-1            113 GDLVGVNKDS YLILETLPTE YDSRVKAMEV DERPTEQYSD IGGLDKQIQE  162

                         210        220        230        240        250
RT-PCR (mTbp-1)   201 LVEAIVLPMN HKEKFENLGI QPPKGVLMYG PPGTGKTLLA RACAAQTKAT  250
mTbp-1            201 LVEAIVLPMN HKEKFENLGI QPPKGVLMYG PPGTGKTLLA RACAAQTKAT  250
hTBP-1            163 LVEAIVLPMN HKEKFENLGI QPPKGVLMYG PPGTGKTLLA RACAAQTKAT  212

                         260        270        280        290        300
RT-PCR (mTbp-1)   251 FLKLAGPQLV QMFIGDGAKL VRDAFALAKE KAPSIIFIDE LDAIGTKRFD  300
mTbp-1            251 FLKLAGPQLV QMFIGDGAKL VRDAFALAKE KAPSIIFIDE LDAIGTKRFD  300
hTBP-1            213 FLKLAGPQLV QMFIGDGAKL VRDAFALAKE KAPSIIFIDE LDAIGTKRFD  262

                         310        320        330        340        350
RT-PCR (mTbp-1)   301 SEKAGDREVQ RTMLELLNQL DGFQPNTQVK VIAATNRVDI LDPALLRSGR  350
mTbp-1            301 SEKAGDREVQ RTMLELLNQL DGFQPNTQVK VIAATNRVDI LDPALLRSGR  350
hTBP-1            263 SEKAGDREVQ RTMLELLNQL DGFQPNTQVK VIAATNRVDI LDPALLRSGR  312

                         360        370        380        390        400
RT-PCR (mTbp-1)   351 LDRKIEFPMP NEEARARIMQ IHSRKMNVSP DVNYEELARC TDDFNGAQCK  400
mTbp-1            351 LDRKIEFPMP NEEARARIMQ IHSRKMNVSP DVNYEELARC TDDFNGAQSK  400
hTBP-1            313 LDRKIEFPMP NEEARARIMQ IHSRKMNVSP DVNYEELARC TDDFNGAQCK  362

                         410        420        430        440
RT-PCR (mTbp-1)   401 AVCVEAGMIA LRRGATELTH EDYMEGILEV QAKKKANLQY YA          442
mTbp-1            401 AVCVEAGMIA LRRGATELTH EDHMEGILEV QAKKKANLQY YA          442
hTBP-1            363 AVCVEAGMIA LARGATELTH EDYMEGILEV QAKKKANLQY YA          402
```

pooled to reduce cell line specific variation. Each pool was passaged in the presence of LIF and G418 and subjected to LIF titration analysis (2.4.13).

All three cultures progressively lost AP staining in lower LIF concentrations. Therefore, pEF25.1IRESneo and pEFTbpIRESneo transfected cultures, like the C25.1 cell line, remained responsive to LIF and retained the ability to differentiate. pEFIRESneo, pEF25.1IRESneo and pEFTbpIRESneo transfectants exhibited similar AP staining at all LIF concentrations tested (not shown). Undifferentiated colonies retained in LIF titration cultures were scored by phase-contrast microscopy and expressed as a percentage of the total number of colonies. pEF25.1IRESneo transfectants retained a greater proportion of undifferentiated colonies in LIF concentrations between 2 U/ml and 30 U/ml LIF as well as in the presence of 1000 U/ml LIF compared to pEFIRESneo and pEFTbpIRESneo transfectants (Figure 5.25a). By contrast, pEFTbpIRESneo transfectants contained similar proportions of undifferentiated colonies compared to pEFIRESneo transfectant cultures (Figure 5.25a). Between 7.5 U/ml and 20 U/ml LIF, pEFTbpIRESneo transfectants contained fewer undifferentiated colonies than control pEFTbpIRESneo transfectants. These data indicate that the truncated *Tbp-1* cDNA (c25.1 cDNA) may be responsible for reduced pluripotent cell differentiation observed for the C25.1 cell line. This property does not appear to be recapitulated by the full-length *Tbp-1* cDNA. The truncated *Tbp-1* cDNA may therefore be responsible for the selection of the C25.1 cell line during function-based screening.

To examine if episome-driven expression of *Tbp-1* influenced the establishment and maintenance of the pluripotent state, a 1.4 kb *Eco*RI/*Hin*dIII fragment corresponding to the *mTbp-1* PCR product was blunted and cloned into *Eco*RI/*Not*I digested and blunted pPSDEneoΔLT20 to form pTbpDEneoΔLT20 (Figure 5.22c). C32 ES cells were supertransfected with pPSDEneoΔLT20 or pTbpDEneoΔLT20 and selected in 200 μg/ml G418 for 15 days in the presence or absence of LIF. The cultures were stained to detect AP activity and scored colonies were expressed as the percentage reduction in AP^+ colonies due to culture in the absence of LIF. Pluripotent colony maintenance in the absence of LIF as assessed by AP expression was not affected by introduction of the pTbpDEneoΔLT20, indicating that expression of *Tbp-1* does not alter establishment of pluripotent colonies in the absence of LIF (Figure 5.25b).

Figure 5.25

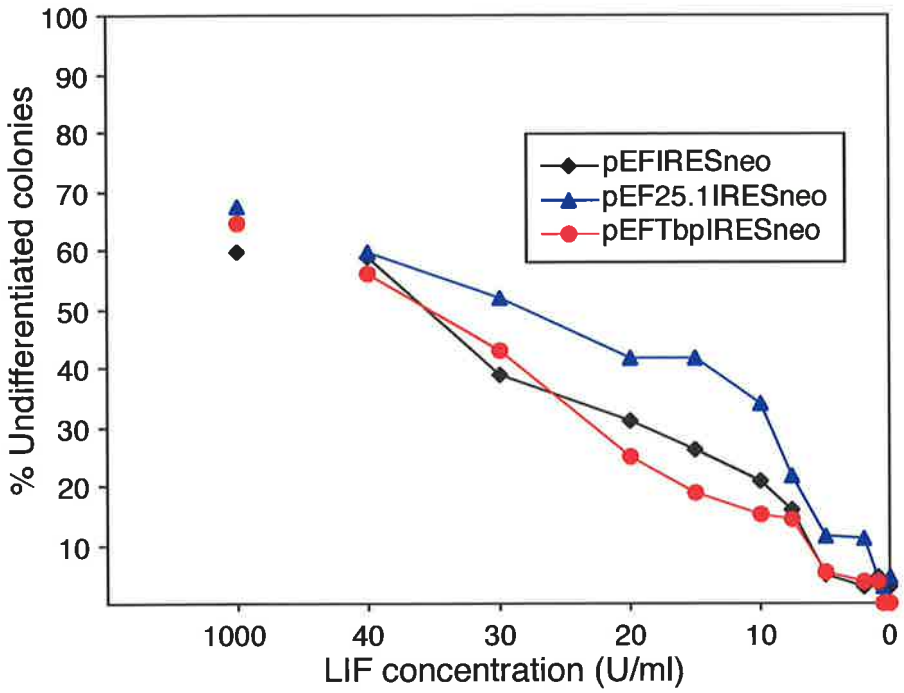
LIF dependence of ES cells expressing *Tbp-1* or C25.1 cDNAs.

A. LIF titration of p25.1IRESneo and pTbpIRESneo transfected ES cells compared to pEFIRESneo transfected ES cells. ES cells stably transfected with pEF25.1IRESneo, pEFTbpIRESneo or pEFIRESneo were seeded at 500 cells per well in ES cell medium containing LIF or 0, 0.5, 1, 2, 5, 7.5, 10, 15, 20, 30 or 40 U/ml of recombinant LIF and allowed to differentiate for 6 days prior to detection of AP activity. Undifferentiated colonies, defined as those that retained AP activity and comprised more than 90% undifferentiated cells, were scored and represented as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, pEFIRESneo-transfected ES cells; purple triangles, pEF25.1IRESneo-transfected ES cells; red circles, pEFTbpIRESneo-transfected ES cells.

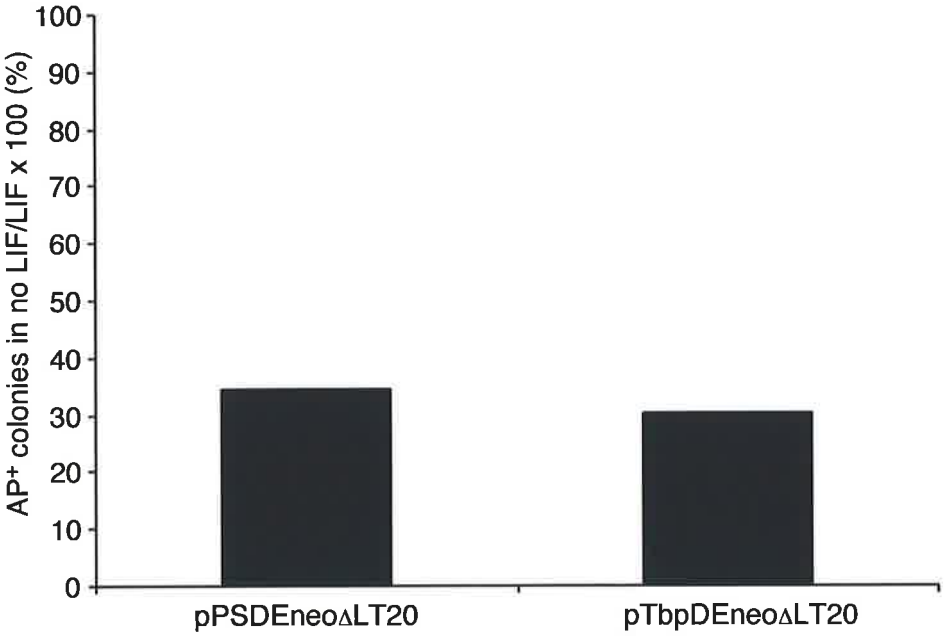
B. Differentiation of pTbpDEneoΔLT20 supertransfected cells. 3 x 10^7 C32 ES cells were electroporated with 20 μg of CsCl purified pTbpDEneoΔLT20 or pPSDEneoΔLT20 plasmid. Half of the supertransfected cells were seeded onto a 10 cm plate and selected for 15 days in 200 μg/ml G418 in the presence or absence of LIF. Surviving colonies were stained to detect AP activity and AP^+ colonies cultured with or without LIF were scored. Data are represented as the percentage of AP^+ colonies that survive in the absence of LIF over those selected in LIF.

C. LIF titration analysis of pTbpDEneoΔLT20 supertransfected ES cells. ES cells stably supertransfected with pTbpDEneoΔLT20 and pPSDEneoΔLT20 were subjected to LIF titration analysis (A). Undifferentiated colonies, defined as those that retained AP activity and comprised more than 90% undifferentiated cells were scored following LIF titration assay and graphed as the percentage of undifferentiated colonies for each LIF concentration. Black diamonds, pPSDEneoΔLT20 supertransfected ES cells; red squares, pTbpDEneoΔLT200 supertransfected ES cells.

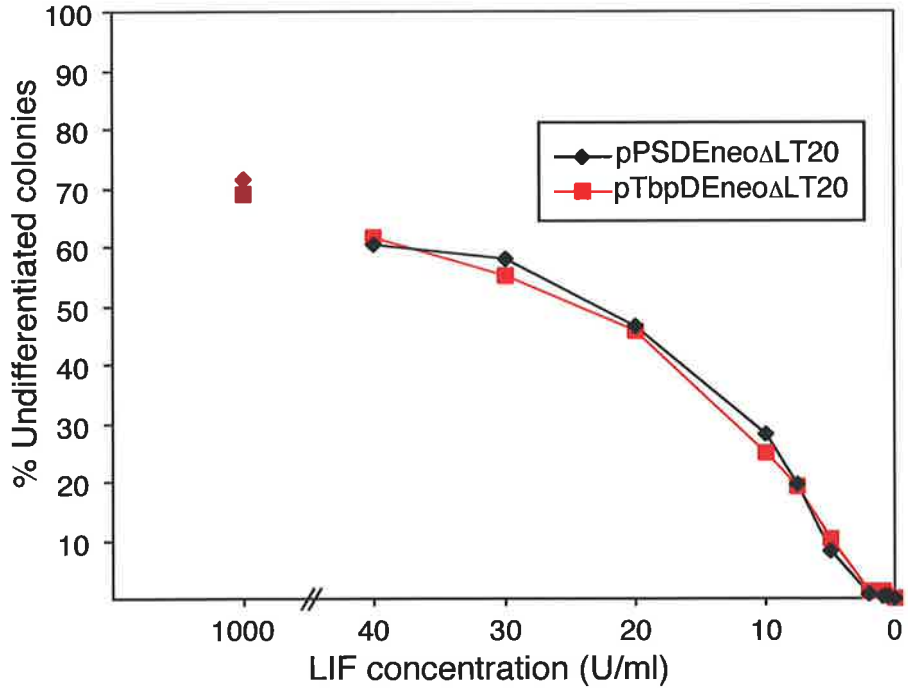
A


% Undifferentiated colonies
100
90
80
70
60
50
40
30
20
10
pEFIRESneo
pEF25.1IRESneo
pEFTbpIRESneo
1000
40
30
20
10
0
LIF concentration (U/ml)

B


AP+ colonies in no LIF/LIF x 100 (%)
100
90
80
70
60
50
40
30
20
10
0
pPSDEneoΔLT20
pTbpDEneoΔLT20

C


% Undifferentiated colonies
100
90
80
70
60
50
40
30
20
10
pPSDEneoΔLT20
pTbpDEneoΔLT20
1000
40
30
20
10
0
LIF concentration (U/ml)

Stably supertransfected pTbpDEneoΔLT20 pools were analysed to determine if they exhibited a reduced requirement for LIF. pPSDEneoΔLT20 and pTbpDEneoΔLT20 plasmids were supertransfected into C32 ES cells and selected in 200 μg/ml G418 and LIF for 15 days. Supertransfected C32 ES cells were passaged as pools in the presence of LIF and subjected to LIF titration analysis (2.4.13). Approximately 70% of colonies are expected to express Tbp-1 (4.3).

In the presence of LIF equivalent numbers of morphologically similar ES cell colonies formed from pTbpDEneoΔLT20 and pPSDEneoΔLT20 supertransfected cultures. In the absence of LIF, there was little difference between pTbpDEneoΔLT20 supertransfectants and cells supertransfected with pPSDEneoΔLT20, confirming that cells that express *Tbp-1* are still capable of differentiation and that *Tbp-1* expression is not sufficient for maintenance of the pluripotent state.

Undifferentiated colonies retained in LIF titration cultures were scored under phase-contrast microscopy. pTbpDEneoΔLT20 supertransfectant cultures retained a similar percentage of undifferentiated colonies in LIF concentrations tested compared to pPSDEneoΔLT20 supertransfectants (Figure 5.25c). Episomal expression of full-length *Tbp-1* therefore does not appear to inhibit differentiation of pluripotent cells, confirming findings of LIF titrations performed on ES cells with IRESneo-driven *Tbp-1* expression (Figure 5.25a).

5.7 DISCUSSION

Work presented in this chapter describes the production of a cDNA expression library, function-based screening, isolation and characterisation of cDNAs that affect maintenance of pluripotence.

5.7.1 Function-based screening

Numerous pluripotent colonies were isolated from the function-based screen. These lines were screened further by scoring pluripotent colony survival in the absence of LIF and by LIF titration analysis. Some clones that survived the screen showed few signs of morphological differentiation (Figure 5.7). Expanded clones were cultured in the absence of LIF to assess their differentiation potential and although differentiated cells were present in these cultures, some cell lines were observed to retain stem cell nests. Of these, seven had a reduced requirement for LIF or alternatively formed spread out colonies that retained AP activity in low LIF concentrations or in the absence of LIF. This demonstrates the ability of the function-based screen to select for cells that require lower concentrations of LIF.

A total of 7 x 10^5 clones were screened during function-based screening (5.4.1). 36 cell lines were identified based on retention of undifferentiated stem cell nests in the absence of LIF. Only 2 of 17 (12%) colonies analysed by Grunstein analysis were found to contain library episome (Table 5.1) compared with 69.5% of pβgalDEneoΔLT20 (4.3) and 88.8% of library episome (5.3.1) supertransfected colonies selected in the presence of LIF. Hirt DNA extracts from most selected colonies also lacked amplifiable cDNA sequences. G418 selection in the absence of LIF may therefore select for colonies that contain integrated copies of the library episome. Colonies containing integrated copies of the library plasmid may be selected because integration could lead to neomycin resistance gene expression in differentiated derivatives via positional effects. Constitutive neomycin resistance gene expression would mean that such cells do not require a functional cDNA to survive the selection protocol. Undifferentiated ES cells could be maintained due to paracrine LIF supply from G418-resistant differentiated derivatives as in stem cell nest formation (Mountford *et al.*, 1994; Rathjen *et al.*, 1990a).

Unrestricted neomycin resistance gene expression does not however account for the observed survival of pluripotent colonies not surrounded by differentiated cells. The majority of such colonies did not contain detectable extrachromosomal library DNA and did not exhibit a reduced requirement for LIF by LIF titration analysis. McWhir *et al,* (1996) found that embryoid body formation from ES cells transfected with an *Oct4*-neo construct in the presence of G418 resulted in retention of morphologically undifferentiated cells (McWhir *et al.*, 1996). When *Oct4*-neo ES cells were differentiated in suspension to form embryoid bodies, undifferentiated G418 resistant cells did not differentiate and the stem cell population expanded over time (Mountford *et al.*, 1998). The presence of differentiated progeny could induce elimination of remaining stem cells suggesting an autocrine mechanism by which ES cells can be maintained in the absence of exogenous LIF (Mountford *et al.*, 1998). Undifferentiated ES cells express the matrix-associated form of LIF, LIF-M (Mountford *et al.*, 1994; Rathjen *et al.*, 1990a), raising the possibility that LIF-M acts in an autocrine fashion to maintain ES cells.

Cell lines that are unable to differentiate upon LIF withdrawal were not isolated in the function-based screen. All isolated cell lines differentiated in the absence of LIF, but either exhibited a reduced LIF requirement or retained AP activity in low LIF concentrations. ES cells may not express proteins capable of inhibiting differentiation in isolation without activation by an external signal such as the LIF signalling pathway. This is consistent with the observation that, in general, constitutively active gene products (v-Ha-Ras and activated Hck) or gene deficiencies (*Shp-2*$^{-/-}$) impair ES cell differentiation but do not render ES cells LIF independent (Ernst *et al.*, 1994; Ernst *et al.*, 1996; Qu and Feng, 1998) (see table 1.1). Thus, master regulatory genes analogous to MyoD in muscle development may not act in ES cell maintenance.

It could be expected that LIF and other IL-6-type cytokines should be isolated using this screening approach given that LIF overexpressing ES cells have been identified in an insertional mutagenesis based screen (Gendall *et al.*, 1997) and that LIF acts in a relatively cell-autonomous manner to inhibit ES cell differentiation (4.4). *LIF* is expressed at low levels in undifferentiated ES cells and the *LIF* transcripts are 4.5 kb making it possible that a full-length *LIF* cDNA was not present in the library. However near full-length *mOSM* cDNAs were amplified from library DNA (5.2.2). The 7 x 10^5 clones screened may not be enough to subject

rare transcripts to the screening procedure. Alternatively, the *OSM* cDNA may have been expressed at insufficient levels in the context of the library plasmid to maintain ES cells. Finally, *OSM* may not act in a cell-autonomous manner to inhibit ES cell differentiation.

cDNAs were also isolated from Hirt DNA from a number of lines by PCR but not by colony screening (5.6) suggesting that recombination and/or loss of sequences essential for bacterial replication may have occurred. Thus it is possible that function-based screening introduced a selective pressure for smaller episomes.

Four cell lines isolated showed a quantitative or qualitative difference in the requirement for LIF (C62, C25.1, D10 and D12). Other cell lines cultured in low LIF concentrations (C84 and C14.5) formed flatter, spread colonies suggestive of differentiation yet retained AP^+ undifferentiated cells. Reduced LIF requirements may reflect alterations to the LIF signalling pathway or activation of pathways regulating pluripotence. Spread colonies which retained a large centre of AP^+ cells surrounded by AP^- differentiated cells may result from alteration of cytoskeletal functions such as cell adhesion, which may in turn be coupled to pathways regulating pluripotence.

5.7.2 Characterisation of cDNA inserts

LIF titration analysis revealed that six cell lines exhibited a reduced requirement for LIF. Two cDNAs were isolated from these. C84 contained a truncated cDNA encoding a protein related to IQGAP1 and IQGAP2 that are themselves related to RasGAP proteins. The observation that C84 shows equivalent identity to hIQGAP1 and hIQGAP2 indicates that C84 is not the mouse orthologue of hIQGAP2 but rather is a third member of the IQGAP family. This is supported by the fact that C84 also exhibits 79 % DNA identity and 71 % identity to the putative amino acid sequence of a 496 bp human EST sequence (accession No. AA252756) distinct from human IQGAP1 and IQGAP2.

Re-introduction of the C84 cDNA into ES cells using pEFIRESneo or episomal expression systems did not recapitulate the retention of AP activity observed for C84 cells upon withdrawal of LIF. These data imply that the truncated IQGAP3 cDNA encoded by C84 does not inhibit ES differentiation. The phenotype of C84 cells is likely to be due to cell line specific variation or an unknown genetic modification. Random genetic modifications to ES cells in

culture can result in altered LIF requirements as a LIF-independent ES cell line has been isolated that overexpresses LIF due to an unknown genetic modification (Gendall *et al.*, 1997). Such genetic modifications may also explain selection and reduced LIF requirement of C62, D10, D12 and C14.5 cell lines isolated during function-based screening that did not contain detectable cDNAs.

C25.1 was found to contain a cDNA corresponding to the 3´ two thirds of the mouse Tbp-1 transcript (5.6.3). Tbp-1 binds to HIV Tat protein and inhibits Tat-mediated transactivation (Nelbock *et al.*, 1990) and is also a component of the PA700 complex of the 26S proteasome (Nakamura *et al.*, 1998). ES cells stably transfected with pEF25.1IRESneo exhibited increased retention of undifferentiated cells in low LIF concentrations compared with pEFIRESneo transfected cells. This experiment was performed using pools of stable transfectants, eliminating clone-specific effects. This implies that selection of the C25.1 cell line and retention of AP activity in low LIF concentrations was not due to clonal variation but rather was due to expression of the truncated *Tbp-1* cDNA harboured by this cell line. Truncated *Tbp-1* may therefore function to inhibit ES cell differentiation. N-terminally truncated TBP-1 can still bind HIV Tat (Nelbock *et al.*, 1990) making it possible that the putative truncated protein encoded by the C25.1 cDNA retains some or all of the biological activities of the full-length protein.

The full-length cDNA of Tbp-1 was PCR amplified and expressed in ES cells. Supertransfection of an episome containing the full-length *Tbp-1* cDNA into C32 ES cells did not inhibit ES cell differentiation in the LIF concentrations tested. Further, expression of *Tbp-1* from the pEFIRESneo vector also had no effect on ES cell differentiation. Therefore, overexpression of full-length *Tbp-1* did not alter ES cell differentiation. Given that the C25.1 cDNA appears to inhibit ES cell differentiation, the truncated Tbp-1 protein it encodes may represent a dominant negative or constitutively active form of Tbp-1. This activity, not shared with the full-length Tbp-1, may be responsible for retention of AP activity by C25.1 expressing ES cells in low LIF concentrations. It is therefore possible that full-length Tbp-1 has a role in ES cell maintenance that requires prior stimulation/activation by other cellular factors. It remains a possibility that the single conservative amino acid substitution introduced during

PCR amplification of the full-length *Tbp-1* cDNA could be important for proper function of Tbp-1.

Ubiquitin-proteasome-mediated proteolysis is essential for cell growth, differentiation and survival (reviewed in Hochstrasser, 1995). A role for Tbp-1 in maintenance of ES cell differentiation is not surprising given the known functional links between the operation of the proteasome and Stat-dependent signalling. Stat1 is degraded by the proteasome (Kim and Maniatis, 1996). c-Jun is upregulated in ES cells exposed to LIF (Duval *et al.*, 2000) and has been shown to cooperate with Stat3β to activate transcription. c-Jun is also degraded by an ubiquitin-dependent mechanism (Musti *et al.*, 1997) which is inhibited upon MAP kinase activation (Musti *et al.*, 1997). In the human IL-6-dependent T cell line KT-3, exogenously added ubiquitin causes apoptosis and conjugates with Stat3 leading to proteasome dependent degradation of Stat3 (Daino *et al.*, 2000). The cyclin dependent kinase inhibitors $p21^{WAF1}$ and $p27^{Kip1}$ are degraded by ubiquitin-proteasome-mediated proteolysis (Pagano *et al.*, 1995; Rousseau *et al.*, 1999). $p27^{Kip1}$ is a downstream target of Stat3 (Kortylewski *et al.*, 1999) and $p21^{WAF1}$ interacts with Stat3 reducing Stat3-dependent transcriptional activation (Coqueret and Gascan, 2000). It is possible that degradation of Stat3 or its targets could alter the LIF response of ES cells leading to maintenance of the undifferentiated state. Protein degradation by the proteasome could be disrupted by expression of the truncated *Tbp-1* cDNA (C25.1), allowing build up of activated Stat3 proteins in the nucleus. Activated Stat3 dimers would continue to signal in the absence of LIF, leading to a reduction in ES cell differentiation. Proteins such as $p21^{WAF1}$ that negatively regulate Stat3 activity could be targeted for degradation in the presence of the C25.1 cDNA, again leading to increased Stat3 activity.

Transfection of oncogenes into differentiated immortal cells leads to uncontrolled cell growth, loss of contact inhibition, and growth in low serum concentrations. Introduction of oncogenes such as v-Src, L-myc, AML1-d and v-Ha-Ras inhibit ES cell differentiation (1.6.7). ES cells represent the only immortal cell type isolated without the use of immortalisation protocols. It is possible that uncontrolled growth conferred by oncogenes would be manifest in ES cells as continued proliferation and an inability to differentiate in the presence of differentiation inducing signals. In this regard it is worth noting that Tbp-1 has been associated with tumour suppressor functions (5.6.3). The truncated protein encoded by C25.1 could

antagonise putative tumour suppressor functions of Tbp-1 bringing about uncontrolled cell proliferation and/or loss of differentiation capability. Tbp-1 may also function to transactivate gene expression analogous to its function with HIV Tat. It is possible that the truncated Tbp-1 protein could inhibit transactivation of cellular genes that could lead to inhibition of pluripotent cell differentiation.

cDNA clones isolated from cell lines that survived the function-based screen represent genetic candidates for the observed cellular phenotypes. Moreover, expression of cDNAs in ES cells has implied a role for Tbp-1 in ES cell maintenance in low LIF concentrations. Further characterisation should elucidate the function of Tbp-1 in ES cell self-renewal and how it relates to components of the LIF signalling pathway.

CHAPTER 6:

FINAL DISCUSSION

6.1 A SYSTEM FOR IDENTIFYING GENES THAT INHIBIT ES CELL DIFFERENTIATION

This thesis describes the development of a function-based screening system for genes that maintain the pluripotent state. The function-based screen was designed to isolate genes that inhibit ES cell differentiation upon LIF withdrawal. An episome-based cDNA expression library containing an *Oct4*-neo selection cassette was supertransfected into episome harbouring ES cells and the cells were allowed to differentiate. Differentiated cells present in the population were removed by the addition of G418 and only undifferentiated ES cells that have lost the capacity to differentiate should have survived the screening procedure. Thus surviving ES cell colonies should have retained the undifferentiated state because the introduced cDNA encodes a protein that inhibits ES cell differentiation.

6.1.1 Episome replication and gene expression

The function-based screening strategy was complicated at the outset by several features including an inability to stably transfect large numbers of ES cells, loss of cDNA expression due to chromosomal integration of transfected plasmids and feedback maintenance of pluripotent stem cell nests by expression of LIF from differentiated cells. The work presented here has elucidated the dynamics of PyV-based episome replication and gene expression, and demonstrated that it can be combined with a selection cassette capable of specifically removing differentiated ES cell derivatives. These findings made it possible to use this system to screen random cDNAs for a role in ES cell maintenance.

Supertransfection of a second PyV-based episome into ES cells that already harbour an episome has been reported to be 100-fold more efficient than standard stable transfection (Gassmann *et al.*, 1995; Niwa *et al.*, 1998). Episome-harbouring lines were identified following transfection of the pMGD20hph episome. Supertransfection of a second plasmid containing the PyF101 ori into this episome harbouring ES cell line was found to be 10-fold more efficient than standard stable transfection. The difference reflects selection for 8 days used previously (Niwa *et al.*, 1998) versus 15 days in the present study (3.3.3) and therefore more accurately represents the true increase in stable transfection efficiency due to the episomal vectors.

Oct4 promoter-luciferase reporter constructs containing PE or DE regions were confirmed to drive expression in ES cells. In terminally differentiated NIH3T3 fibroblasts, DE and PE driven reporter expression was equivalent to basal levels driven by the PP region of the *Oct4* promoter in ES cells. The PP region was found to drive high level unregulated transcription in differentiated cells. These results suggest that the PE and DE upstream *Oct4* enhancers elicit positive and negative effects on PP-mediated expression, dependent on the cell type. Cell specific gene expression driven by the *Oct4* promoter appears to be due to both induction of gene expression in pluripotent cells and inhibition of expression in differentiated cell types controlled by the DE and PE regions. Therefore, the DE and PE regions may also contain repressor elements that downregulate transcription from the PP in differentiated cells. Downregulation of PP-dependent transcription in differentiated cells by the DE and PE regions has not been reported previously.

Consistent with results of luciferase reporter assays, *Oct4* promoter-neomycin gene constructs containing the DE region could be used to efficiently select against differentiated cells in the presence of G418. Specific ablation of differentiated cells by DEPPneo constructs occurred in the context of the episome system allowing selection of supertransfected ES cells that remain in a pluripotent state. This is the first demonstration that cell type specific gene expression can be recapitulated by episome-based vectors in ES cells.

6.1.2 Validation of the function-based screening system

The episome-based library vector contains an *Oct4*-neo selection cassette that drives neomycin expression specifically in undifferentiated ES cells and selects effectively against differentiated cells in the presence of G418 (3.4.2). This episome exhibits high efficiency supertransfection like the base episome pPGKhphΔLT20 from which it derives (Gassmann *et al.*, 1995). Constitutive β-galactosidase reporter expression was observed in 71% of ES cells supertransfected with episome-based vector containing the *β-gal* cDNA, and extrachromosomal episome was retained in these cells. Moreover, those lines that did not contain episome lacked reporter gene expression but harboured incomplete integrated copies of episome-based vector in the genome, confirming that cell lines that replicate episome also expressed the reporter gene. Reporter gene expression was constitutive and reliable from a supertransfected episome (4.3)

demonstrating that this system can be used to drive heterologous gene expression in supertransfected cells.

An episome-based plasmid containing the LIF coding region abrogated ES cell differentiation under selection in the absence of exogenous LIF. This demonstrates that the function-based screen was capable of selecting for ES cells containing functional cDNAs, validating the screening procedure as an effective system for isolating genes that inhibit ES cell differentiation. Moreover Stat3β, implicated as a constitutively active isoform of Stat3, also increased pluripotent colony survival upon LIF withdrawal when it was introduced into C32 ES cells on a episome-based plasmid implying that both intracellular and extracellular factors could be isolated by the screening approach.

6.2 SURVIVAL OF BACKGROUND PLURIPOTENT COLONIES DURING SCREENING

A background of pluripotent colonies was observed to survive function-based screening in the absence of LIF. Pluripotent cell survival and proliferation upon removal of differentiated cells from embryoid bodies using *Oct4*-neo selection cassettes (McWhir *et al.*, 1996; Mountford *et al.*, 1998) suggests that pluripotent cell differentiation requires the presence of differentiated cells and that ES cells have an autonomous ability to self-renew. These observations are in apparent conflict with other evidence indicating that stem cell nests form in differentiated ES cell cultures due, in part, to paracrine supply of LIF from differentiated progeny (Rathjen *et al.*, 1990a; Mountford *et al.*, 1994; Dani *et al.*, 1998). The observation that pluripotent cells remain when differentiated cells are removed from seeded embryoid bodies (McWhir *et al.*, 1996) indicates that the two mechanisms may not be mutually exclusive. Initial retention of stem cell nests is likely to be due to upregulation of LIF production following differentiation (Rathjen *et al.*, 1990a), however following a period of proliferation pluripotent colonies may reach a size where autocrine LIF supply is sufficient to maintain the undifferentiated state. This is supported by the observation that LIF or LIFR deficient ES cells cease to proliferate following 3-4 days culture in ESRF (Dani *et al.*, 1998) implying that large colonies that continue to proliferate in the absence of differentiated cells and exogenous LIF must do so due to autocrine LIF supply.

It is unknown why Mountford *et al*, (1998) observed stem cell maintenance in suspension culture but not when ES cells were differentiated attached to a substratum. One possibility is that attachment *per se*, or extracellular matrix signalling can disrupt pluripotence. Alternatively, this could be due to the differentiation in suspension of ES cell aggregates rather than individual seeded cells whereby differentiation of some ES cells within an aggregate supports survival of the remaining cells through paracrine LIF supply (Rathjen *et al.*, 1990a). G418 selection removes differentiated cells but not before remaining undifferentiated cells have divided sufficiently to support their own maintenance through production of LIF-M. This model could also explain pluripotent colony survival during function-based screening (5.3.2). Upon seeding ES cells in medium lacking LIF most cells differentiate immediately while some retain the undifferentiated state for some generations. This decision could be affected by the retention of some differentiated cells when cell aggregates are seeded and subjected to differentiation. G418 selection acts to remove differentiated cells but not before remaining ES cells establish autocrine self-renewal mechanisms. Thus microenvironmental factors present in the vicinity of seeded ES cells may impact on the propensity of individual ES cell colonies to differentiate.

The situation during functional selection in the absence of LIF appears more complicated given that formation and proliferation of stem cell colonies is often accompanied by episome loss (5.6.1 and Table 5.1). This implies that the screening procedure selects for stem cells that integrate the library episome into the genome. Genomic integration in or near actively transcribed loci is likely to alter neomycin gene expression rendering differentiated derivatives resistant to G418 selection. Stem cell colonies surrounded by resistant differentiated progeny may therefore survive due to LIF supplied by G418-resistant differentiated cells.

Although ES cell differentiation is a stochastic process at high density (Dani *et al.*, 1998) retention of stem cell nests is conceivably affected by microenvironmental factors such as seeding of ES cell aggregates and presence and proximity of differentiated cells, the concentration of LIF and serum components. Trypsinisation and re-seeding during G418 selection was found to significantly reduce survival of pluripotent colonies (5.3.3), presumably by destroying microenvironments and affording stem cells a second opportunity to differentiate. Given that most pluripotent colonies that survived the screen did not contain

extrachromosomal library DNA, episomes were harvested from surviving colonies and subjected to a second round of screening to allow amplification of episomes carrying functional cDNA clones (5.3.3).

6.3 ISOLATION OF cDNA CLONES BY FUNCTION-BASED SCREENING

A cDNA expression library was constructed using the episome-based vector and cDNA derived from undifferentiated ES cell RNA. A total of 7 x 10^5 colonies were subjected to function-based screening and numerous undifferentiated cell lines were isolated. Most isolated cell lines retained an unaltered capacity to differentiate in response to LIF withdrawal and did not harbour extrachromosomal library plasmids (5.6.1 and table 5.1). In contrast, supertransfected C32 ES cells selected in the presence of LIF retained extrachromosomal library plasmid (5.3.1), suggesting a correlation between background colony survival in the absence of LIF and a lack of library episome replication.

Even with strategies in place to minimise background pluripotent colony survival, some colonies that survived the screen differentiated upon further analysis in a manner indistinguishable from parental C32 ES cells. However, several ES cell lines exhibited distinct quantitative and qualitative responses to limiting LIF concentrations. In the absence of LIF C62, C25.1, D10, D12, C84, and C14.5 lines retained more AP^+ colonies indicating that they exhibited a reduced tendency to differentiate. C84 and C14.5 were observed to flatten and spread out in low concentrations of LIF but retain AP activity. C25.1 retained more intense AP activity than the parental line that was most obvious in low LIF concentrations and in the absence of LIF. D10 and D12 cells formed more AP^+ colonies in low LIF concentrations and in the absence of LIF compared to C32 cells. None of these ES cell lines contained detectable extrachromosomal library plasmid by Grunstein analysis, but episome specific PCR products were detected in Hirt DNA extracted from C25.1 and C84. C25.1 was found to contain a truncated *Tbp-1* cDNA while C84 harboured a truncated cDNA related to IQGAP1 and IQGAP2 that has been termed IQGAP3. Gain-of-function analysis by overexpression of these cDNAs in ES cells indicated that truncated IQGAP3 had no effect on ES cell differentiation (5.6.4) and suggested that C84 may carry unknown genetic modifications responsible for its phenotype. Overexpression of truncated *Tbp-1* but not the full-length cDNA resulted in

retention of AP^+ pluripotent colonies in low LIF concentrations (5.6.5) suggesting that the truncated *Tbp-1* cDNA was responsible for the reduced LIF requirement exhibited by C25.1.

6.4 COMPLEX CONTROL OF ES CELL DIFFERENTIATION

The complex process of pluripotent cell differentiation and loss of potency may not be controlled by single genes given that expression of v-Ha-Ras and activated Hck or gene deficiencies (*Shp-2*$^{-/-}$) partially relieve the requirement for LIF but do not render ES cells LIF independent (Ernst *et al.*, 1994; Ernst *et al.*, 1996; Qu and Feng, 1998) (see table 1.1). Supporting this view is the fact that this function-based screen did not isolate ES cell lines that were incapable of differentiating in response to LIF withdrawal, and instead lines with minor alterations in the requirement for LIF were isolated. Moreover, an alternative screening approach designed with the same aim succeeded in isolating an ES cell line that overexpressed LIF but no other factors were identified (Gendall *et al.*, 1997). A technical limitation of function-based screens is that it is only possible to screen for single gene effects. Function-based screens are perhaps more suited to specific phenotypic criteria that are likely to be encoded by a single gene such as screens for cell surface receptor genes (Rayner and Gonda, 1994).

Activation of Stat3α is necessary and sufficient for ES cell maintenance (Boeuf *et al.*, 1997; Matsuda *et al.*, 1999; Niwa *et al.*, 1998). ES cells supertransfected with a *Stat3β* containing episome retained pluripotency 3.5-fold better than controls in the absence of LIF (4.6.1) implying that Stat3β promotes ES cell pluripotency in the absence of LIF signalling. Stat3β is constitutively phosphorylated and therefore active in the absence of signalling but upon cytokine stimulation, relative to DNA binding capacity, activated Stat3α has a greater capacity to transactivate transcription than activated Stat3β (Schaefer *et al.*, 1995; Schaefer *et al.*, 1997). Stat3β overexpression may therefore act to inhibit ES cell differentiation by directly activating transcription of Stat3α target genes, circumventing LIF signal transduction. Although the effect of Stat3β expression appears to be minor, Stat3β may be functionally relevant in promoting pluripotent cell maintenance and self-renewal in the absence of LIF signalling. Given that the Stat3β message is expressed in undifferentiated ES cells (data not shown) it is

perhaps important for stem cell renewal in the absence of LIF such as in the phenomenon of stem cell nest formation.

It is worth noting that only constitutively active Stat3 (Matsuda *et al.*, 1999) and c-Src (v-Src) (Boulter *et al.*, 1991) and LIF overexpression (Shen and Leder, 1992) have been reported to render undifferentiated ES cells LIF independent. In general, addition or removal of components of the LIF signalling pathway impairs ES cell differentiation but cells do not exhibit LIF independence (see table 1.1). Expression of v-Ha-Ras and activated Hck have been reported to reduce the LIF requirement of ES cells by 8- and 15-fold, respectively (Ernst *et al.*, 1994; Ernst *et al.*, 1996), presumably by recapitulating some of the signal transduction events that follow LIF addition. The fact that activation of these signalling components does not render ES cells LIF independent implies the existence of multiple pathways, that in concert, result in maintenance of the pluripotent state. This view is also supported by the observation that ESRF can inhibit ES cell differentiation but not maintain ES cells without LIF (Dani *et al.*, 1998).

Cell lines selected during function-based screening differentiated in the absence of LIF but required less LIF to maintain the undifferentiated state (5.5). The C25.1 cell line contained a truncated cDNA of *Tbp-1* that when re-introduced into wild-type ES cells inhibited differentiation in low LIF concentrations but did not prevent differentiation in the absence of LIF (5.6.5). This is contrast to overexpression of Stat3β which inhibited pluripotent cell survival in the absence of LIF (4.6.1). This implies that truncated Tbp-1 promotes pluripotent colony survival only in limiting LIF concentrations and suggests that distinct responses to various LIF concentrations are observed upon expression of different genes. This work has therefore implicated a novel factor in maintenance of pluripotence.

6.5 FUTURE WORK

6.5.1 Roles for Stat3β and Tbp-1 in ES cell differentiation

To characterise further the action of Tbp-1 and Stat3β on ES cell maintenance, cell numeration or tritiated thymidine incorporation of undifferentiated and differentiated ES cells expressing Stat3β or Tbp-1 would determine the growth rate of these cells relative to control ES cells. In this manner it should be possible to determine if either factor has a mitogenic action on

ES cells. Increased rates of proliferation may result in retention of pluripotent colonies given the role that stem cell self-renewal has in the formation of stem cell nests (5.3.3).

To determine if Stat3β is expressed in the ICM and primitive ectoderm immunohistochemistry could be performed on early mouse embryos using a Stat3β specific antibody. ES cells capable of expressing Stat3α but not Stat3β (*Stat3β*$^{-/-}$ ES cells) could be generated using a 'knock in' gene targetting approach to fuse the exon or exons missing in *Stat3β* to an adjacent exon found in both transcripts. The ability of *Stat3β*$^{-/-}$ ES cells to differentiate in the absence of LIF could then be tested. ES cells capable of expressing Stat3β but not Stat3α (*Stat3α*$^{-/-}$ ES cells) could also be generated but may not be viable, necessitating the use of a system to remove Stat3α protein following gene targetting (see below). It should be possible to establish the ability of Stat3β to maintain ES cells in the presence or absence of LIF using such a cell line following selective removal of Stat3α expression.

To confirm a role for the truncated Tbp-1 protein in ES cell maintenance, FLAG tagged truncated Tbp-1 could be expressed from either episomal or IRES-based vectors under the control of the cytomegalovirus/chicken β-actin (CAG) promoter that drives strong expression in ES cells (Araki *et al.*, 1997; Niwa *et al.*, 1998). Western detection of FLAG tagged Tbp-1 and C25.1 would identify overexpressing lines and confirm expression in pools of transfectants. LIF titration analysis could then indicate an effect on LIF dependency of such ES cells and clearly determine the degree to which forced C25.1 protein expression inhibits pluripotent cell differentiation.

To determine the expression of Tbp-1 in pluripotent cells during development *in situ* hybridisation and immunostaining with anti-Tbp-1 antibodies could be performed on dissected morulae, blastocysts, and egg cylinder stage mouse embryos. Homozygous deletion of *Tbp-1* in mice using gene targetting techniques may reveal defects in the establishment or proliferation of the ICM and thus could support a role for Tbp-1 in pluripotent cell maintenance *in vivo*. Blastocyst outgrowth experiments could also determine the ability of *Tbp*$^{-/-}$ blastocysts to form ES cells in culture. Given that Tbp-1 is a component of the proteasome (DeMartino *et al.*, 1996), a complex critical for degradation of many diverse cellular proteins, an early embryonic lethal phenotype may not be unexpected. ES cells could be produced in which both *Tbp-1* alleles are conditionally functional using gene targetting and the Cre-loxP system (reviewed in

Ray *et al.*, 2000). Functional Tbp-1 could be removed from these ES cells by infection with an adenovirus capable of expressing Cre recombinase. In this manner, it should be possible to establish if *Tbp-1* is essential for ES cell survival or maintenance of the undifferentiated state.

It would be interesting to investigate the effect of expressing the truncated Tbp-1 (C25.1) protein on proteasome-dependent proteolysis. ES cells expressing truncated Tbp-1 could be analysed for accumulation of specific proteins involved in LIF signalling such as Jak and Src kinases and phosphorylated Stat3 as well as general protein degradation by quantitating levels of unrelated proteins whose degradation is proteasome dependent. It should also be possible to examine the effect of inhibiting ubiquitin-proteasome-dependent proteolysis on Stat3 activity and ES cell maintenance in ES cells no longer stimulated by LIF using chemicals such as PSI or MG132 that inhibit proteasome function. In this way it may be possible to determine if the function of Tbp-1 in the proteasome is important for inhibition of ES cell differentiation and if this is coupled to components of the LIF signalling pathway.

6.5.2 Improvements to the functional screening strategy

Even with the use of strategies to remove stem cell maintenance a background of pluripotent colonies formed during screening that did not harbour functional cDNAs. Stem cell nest formation is reduced upon differentiation of LIF deficient ES cells (Dani *et al.*, 1998). False positive pluripotent colonies that survive selection could be reduced by taking advantage of this observation. This would require introduction of the pMGD20hph plasmid into LIF deficient ES cells, testing for the presence of extrachromosomal plasmid and confirming that high efficiency supertransfection could be supported by such a cell line. This ES cell line could then be supertransfected with the cDNA expression library and subjected to the functional screen. This may significantly reduce background pluripotent colony formation and simplify isolation of genuine pluripotent cells with reduced LIF requirements.

The function-based screen could also be re-designed so that the *Oct4*-neo selection cassette is introduced into wild-type ES cells on an episome. By introducing an episome that includes the *Oct4*-neo selection cassette and also expresses PyV LT into ES cells a base ES cell line could be produced that lacks integrated neomycin cassette copies and specifically expresses neomycin resistance in undifferentiated cells. This would allow establishment of

conditions that eliminate differentiated and false positive undifferentiated cells prior to screening. Moreover, formation of G418-resistant differentiated cells would be minimised in such an approach because the *Oct4*-neo selection cassette is not introduced with the cDNA expression cassette on the supertransfected library episome. A cDNA expression library could be constructed by cloning double stranded cDNA into an IRESpuro constitutive expression cassette present on a second episome lacking PyV LT expression. Supertransfection of the second library episome followed by LIF withdrawal would differentiate ES cells and dual G418/puromycin selection would select for cells that maintain both plasmids, express the cDNA and retain the undifferentiated state (Figure 6.1).

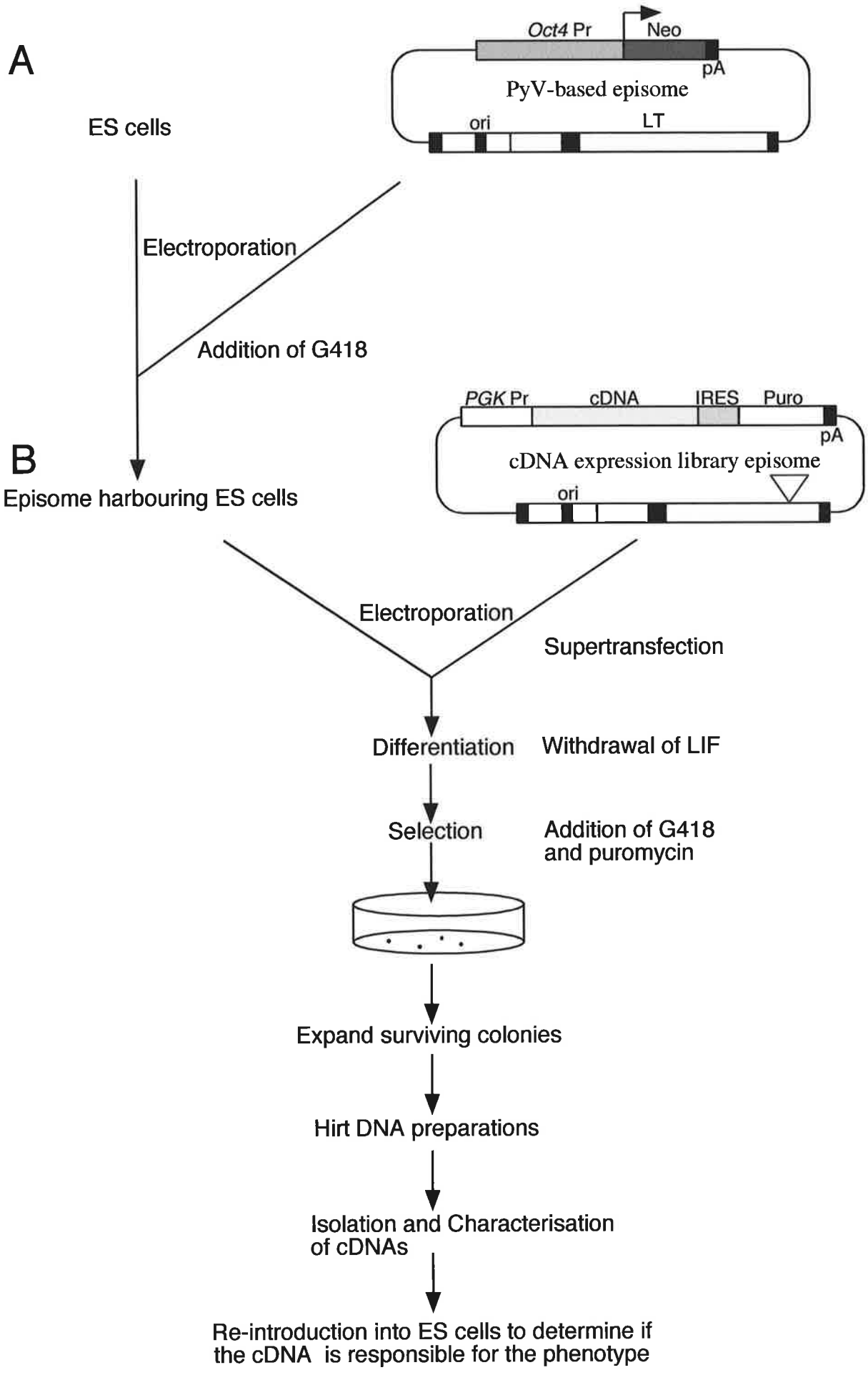
cDNAs that inhibit ES cell differentiation were not isolated from this screen. This is possibly because the target cDNAs may not be abundant and therefore to identify them, more clones must be screened. The average length of cDNA inserts within the cDNA expression library means that many would be truncated. Repeating the screen with a cDNA library comprising longer cDNAs may yield further functional cDNAs. Given that functionally important loss of protein expression by antisense expression has been identified in other function-based screens (Li and Cohen, 1996; Vito *et al.*, 1996) cDNAs could be cloned in both sense and antisense directions into the library vector. Intracellular factors and cell-autonomous extracellular ligands that comprise novel signalling pathways could be expected to be isolated from such a screen. Alternatively, Stat3 target genes responsible for maintenance of the undifferentiated state could also be isolated. Once such factors are identified and confirmed to have a role in ES cell maintenance they could be analysed to determine if they act in conjunction with Stat3 or in a Stat3-independent manner. The phosphorylation status of Stat3 could be ascertained in ES cells overexpressing isolated factors in the absence of LIF. It would also be possible to determine if Stat3 activity, by using constitutively active and dominant negative forms of Stat3, alters expression or activity of factors isolated during function-based screening. In this way it could be possible to determine if Stat3-independent signalling pathways exist that influence pluripotent cell maintenance.

Figure 6.1

Schematic representation of an improved function-based screening strategy.

A. Creation of an episome harbouring ES cell line. ES cells are electroporated with a PyV-based plasmid that also contains the neomycin resistance gene under the control of the DE and PP regions of the *Oct4* promoter. This neomycin selection cassette is capable of specifically expressing the neomycin resistance gene in undifferentiated ES cells but not differentiated derivatives. A line that harbours this episome but not integrated copies of the plasmid can be identified by Southern analysis. Differentiation and G418 selection conditions can be optimised for this line prior to function-based screening in order to minimise background survival of pluripotent colonies .

B. Function-based screening. Episome harbouring ES cells (A) can be electroporated with a second episome that constitutes a cDNA expression library. The episomal library vector constitutively expresses cDNA clones as a discitronic message with the puromycin resistance gene because it contains an IRES sequence. Supertransfected cells are differentiated by withdrawal of LIF and selected in G418 and puromycin. Differentiated cells are selected against as they lose expression of *Oct4* and therefore neomycin phosphotransferase. Undifferentiated ES cells should remain undifferentiated in the absence of LIF because they harbour a cDNA, the product of which is capable of inhibiting the differentiation of ES cells. Surviving colonies are picked, expanded and episomes are isolated from Hirt low molecular weight DNA preparations. cDNAs carried by episomes can be identified by colony screening, or PCR and sequencing. To confirm that isolated clones have a role in ES cell maintenance, they can be re-introduced into ES cells and the cells assayed for LIF dependency.


A
Oct4 Pr
Neo
pA
PyV-based episome
ori
LT
ES cells
Electroporation
Addition of G418
B
Episome harbouring ES cells
PGK Pr
cDNA
IRES
Puro
pA
cDNA expression library episome
ori
Electroporation
Supertransfection
Differentiation
Withdrawal of LIF
Selection
Addition of G418
and puromycin
Expand surviving colonies
Hirt DNA preparations
Isolation and Characterisation
of cDNAs
Re-introduction into ES cells to determine if
the cDNA is responsible for the phenotype

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