CHROMOSOME MAPPING OF THE RED KANGAROO, *MACROPUS RUFUS*, USING MARSUPIAL x EUTHERIAN SOMATIC CELL HYBRIDS.

by

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TABLE OF CONTENTS

SUMMARY

Chromosome maps provide information on the arrangement and localization of genes on particular chromosomes. This study was concerned with chromosome mapping in a species of marsupial, the red kangaroo (*Macropus rufus*), using the techniques of somatic cell genetics.

A number of somatic cell hybrids were produced by fusion between red kangaroo and rodent cells. The hybrid cells retain rodent chromosomes and lose marsupial ones, a property which enables them to be used for mapping red kangaroo chromosomes. Most of these hybrid cells were selected to retain the kangaroo gene for hypoxanthine phosphoribosyl transferase (HPRT), which is known to be X-linked in eutherian mammals. Some retained the complete red kangaroo X chromosome, while others appeared to possess only a small fragment of that chromosome, bearing the gene for HPRT.

Revertant derivatives, selected for loss of expression of HPRT, were obtained from these hybrids. Some revertants were found to possess partially deleted kangaroo X chromosomes of a number of different types. Consequently, concordance between expression of certain genes and presence of a particular part of the kangaroo X chromosome in the hybrids and revertants could be used for the assignment and localization of marsupial X-linked genes.

In this manner, the genes for glucose-6-phosphate dehydrogenase (G6PD), phosphoglycerate kinase (PGK-A) and HPRT were localized to the terminal portion of the euchromatic arm of the red kangaroo X chromosome. The finding that these genes are all contained within a small region of the red

kangaroo X chromosome has possible implications for the evolution of marsupial X chromosomes and raises questions as to the nature and mode of regulation of genes located in the remainder of the X chromosome.

In addition to the X chromosome, some of the hybrids also contained one or two copies of a marsupial autosome, number five in the complement. There was a striking association between the presence of this particular autosome and the presence of the red kangaroo X chromosome in the hybrids, which was suggestive of interaction between genes on the X chromosome and autosomal genes. The retention of this autosome in revertants which lost the X chromosome and failed to express HPRT, G6PD or PGK-A enabled a distinction to be made between autosomal and X chromosomal gene assignments. Analysis of hybrids and revertants showed that the kangaroo gene for lactate dehydrogenase-A (LDH-A) was located on this autosome. This is the first autosomal gene assignment in a marsupial, and provides a starting point for the examination of autosomal linkage groups in this group of mammals.

One additional red kangaroo x rodent somatic cell hybrid was obtained that had been selected for the retention of the kangaroo gene for thymidine kinase, an autosomal gene in eutherian mammals. This putative hybrid clone, in which no red kangaroo chromosomes were detectable, did not possess the marsupial form of galactokinase, the gene for which is very closely linked to that for thymidine kinase in eutherians. However, it expressed the kangaroo gene for G6PD. This finding and the non-random retention of a kangaroo autosome,

mentioned above, can both be interpreted as evidence for interaction between X-linked and autosomal genes in somatic cell hybrids, possibly concerned with the regulation of gene expression.

DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and that to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due reference is given.

J.A. DONALD.

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ABBREVIATIONS

ATP	adenosine-5-triphosphate
HCl	hydrochloric acid
KCl	potassium chloride
KH_2PO_4	potassium dihydrogen phosphate
MTT	3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADP	β-nicotinamide adenine dinucleotide phosphate
NaCl	sodium chloride
Na_2EDTA	ethylene diamine tetraacetic acid, disodium salt
Na_2HPO_4	di-sodium hydrogen orthophosphate
PMS	phenazine methosulphate
Tris	tris(hydroxymethyl)aminomethane

CHAPTER 1

INTRODUCTION

The study of the linkage relationships between genes and the arrangement of such linkage groups on mammalian chromosomes is a rapidly expanding area of research. Extensive linkage maps, based on recombination between genes, are available for man and the mouse. In addition, chromosome maps, which show the physical location of genes on chromosomes, are now being constructed for man and a variety of other mammalian species, including many species for which a paucity of genetic information has prevented the production of conventional linkage maps. Progress in this field is exemplified by recent preliminary reports from the 5th International Human Gene Mapping Workshop, Edinburgh, 1979, in which data are presented on gene maps, encompassing information on both linkage and chromosomal location, for 21 mammalian species.

There are a number of reasons for the extension of interest in the arrangement of genes on chromosomes to species besides man. Information obtained from other species may be directly useful for investigations of medical and genetic problems in man, by providing animal models for human diseases and predicting human gene linkages that could be of use in genetic counselling. At a more fundamental level, knowledge of the organization of genes on chromosomes may be relevant to an understanding of the manner in which the genes of eukaryotes are regulated. It is now becoming clear that the mechanism of genetic

regulation in higher organisms is much more complex than that found in bacteria. Location of genes in close proximity appears not be necessary for their co-ordinate regulation (Hughes *et al.*, 1979), although instances have been found of very close linkage between genes involved in a common metabolic pathway (Spandidos and Siminovitch, 1977). This fragmentary knowledge needs to be considerably expanded before the relative contributions of different modes of regulation can be assessed.

Perhaps the major impetus behind comparative gene mapping to date has been an interest in the evolutionary conservation of linkage groups. Extensive homologies have been demonstrated between the chromosome maps of man and the mouse (Searle, 1976; Lalley *et al.*, 1978; Lundin, 1979) and numerous instances have also been found of genes that are linked in man being located together on the chromosomes of other species (preliminary report of the Committee on Comparative Gene Mapping, 5th International Human Gene Mapping Workshop, Edinburgh, 1979).

That quite striking conservation of linkage groups has occurred over many million years of evolution is now beyond doubt. There is considerable debate, however, over the significance of such conservation. Fisher (1930) was the first to propose that there may be a selective advantage to certain combinations of alleles in double heterozygotes, and hence that selection could act to reduce recombination and favour closer linkage between interacting genes. In support of this suggestion, Bodmer (1975) has argued that chromosomal rearrangements are fixed sufficiently often

during evolution for any extensive linkage homologies between widely divergent species to be disrupted unless they confer a selective advantage. On the other hand, Ohno (1970) maintained that the conservation of autosomal linkage groups occurs essentially by chance, as the most common chromosomal changes seen in vertebrate evolution, Robertsonian fissions and fusions, do not alter linkage relationships within a chromosome arm. For the merits of these two views to be assessed, a considerable body of data will have to be assembled on linkage homologies between different species. Only when it becomes clear whether linkage groups are rearranged at random or whether certain linkage groups remain inviolate will the possible selective advantages of certain combinations of genes be revealed.

A special case of conservation of a linkage group throughout mammalian evolution is provided by the X chromosome. Ohno first proposed in 1967 that genes that are located on the X chromosome in one mammal will be X-linked in all other mammalian species. His proposal was based on the similarity in size of most mammalian X chromosomes (5 - 6% of the haploid genome) and a limited amount of evidence about X-linked genes. The evidence in support of the hypothesis at the time at which it was first proposed was scanty. Since then, however, the list of genes that are X-linked in more than one mammalian species has grown considerably, and no exceptions to the hypothesis of conservation of X-linkage have yet been found (Ohno, 1973a; Pearson and Roderick,

1978). Pathak and Stock (1974) claimed that the similarities in G-banding patterns of the X chromosomes of over 60 species of mammals provide cytogenetic support for this hypothesis. However, comparisons of banded chromosomes between species in widely separated mammalian groups are probably not valid, as the limits of resolution of G-banding techniques are not sufficient to allow any adequate reflection of genetic content, and gross similarities in appearance could easily occur by chance. This same reservation applies to the suggestion made by Ohno (1973a), on the basis of such similarities in banding pattern, that not only the genetic content of the X chromosome, but also the linear order of genes, may have been conserved to a remarkable extent during mammalian evolution. In the absence of a theoretical basis for the proposal of conservation of gene order on the X chromosome, the hypothesis should be regarded with scepticism.

The apparent "evolutionary freeze" on the mammalian X chromosome has been explained by Ohno (1973a) as a consequence of the development of a unique dosage compensation mechanism for X-linked genes. The necessity for such a dosage compensation mechanism can be traced back to the probable origin of the XY sex chromosome pair in mammals. Ohno (1967) has proposed that this pair arose from an ordinary pair of chromosomes, in which assumption by the Y chromosome of the responsibility for heterogametic sex determination was accompanied by loss of genetic material homologous to the X. As a corollary of this hypothesis, Lyon (1974a) has proposed that the genetic material lost

from the Y chromosome was transferred to the X, giving rise to a duplication of most of the genes on the X chromosome. The resulting disparity between the two sexes for genes located on the X chromosome could be compensated for by the development of X chromosome inactivation. Regardless of the mode of origin of dosage compensation, once it was established any fragmentation of the X chromosome would disturb the dosage compensation mechanism, and hence be eliminated by natural selection.

The main features of this system of dosage compensation by X-inactivation were outlined by Lyon (1961), who proposed that early in embryonic development one of the two X chromosomes of female mammals is inactivated in somatic cells. The choice of which X chromosome is inactivated in any cell is random, and once differentiation of the X chromosome has occurred, the pattern is stably maintained in descendant cells. This model has subsequently been supported by further studies, in which the inactivity of one X chromosome has been detected by lack of genetic activity, chromosome condensation and late DNA replication. These properties are characteristic of facultative heterochromatin (Brown, 1966), for which inactivity is reversible, as opposed to constitutive heterochromatin, which appears to be completely genetically inert. The mechanism of X chromosome inactivation is not known, although many models have been proposed. Various models, and the evidence in support of X chromosome inactivation, have been discussed in detail in a number of reviews (Lyon, 1972, 1974b; Cattanach, 1975; Gartler and Andina, 1976).

These basic principles of the X chromosome inactivation system stated above apply to all eutherian mammals that have been examined. However, the process appears to differ in several important respects in the other major group of mammals, the marsupials. Biochemical evidence indicates that marsupial and eutherian mammals have probably evolved separately for at least 100 million years (Air *et al.*, 1971). Consequently, the mechanisms of gene organization and regulation in this group are of especial interest from an evolutionary point of view, although it may prove difficult to evaluate whether differences from eutherians in aspects of the biology and genetics of marsupials reflect primitive characters from which the eutherian systems were derived, or are the products of independent evolutionary paths.

Such a difficulty applies to interpretation of X chromosome inactivation in marsupials, which appears to differ more from the eutherian system than was at first apparent. The fundamental difference is that, unlike the random X-inactivation system in eutherians, the paternally-derived X is preferentially inactivated in female marsupials. This conclusion was based on studies of DNA replication patterns (Sharman, 1971) and of two enzymes coded for by X-linked genes (Richardson *et al.*, 1971; Cooper *et al.*, 1971). However, subsequent research has shown the situation to be more complex. In some tissues and in cultured fibroblasts, inactivation of paternal genes is either incomplete or absent and the degree to which this occurs differs for the two X-linked genes studied (Vandeberg *et al.*, 1977b;

Cooper *et al.*, 1977a; Johnston *et al.*, 1978). These findings have led Cooper *et al.* (1977b) to postulate that dosage compensation in marsupials does not operate at the level of the whole chromosome, but rather that different segments of the X chromsome may be under independent control.

In the light of such speculations about the organization, regulation and evolution of linkage groups, in particular that located on the X chromosome, it is clearly essential to obtain detailed chromosome maps for many mammalian species over as wide an evolutionary range as possible. As detailed pedigree and population data are not available for most of these species apart from man and the mouse, alternative methods must be used to investigate the linkage relationships between genes and the arrangement of these genes on chromosomes. It is in this capacity that the techniques of somatic cell genetics have played a very important role. Many comprehensive reviews of somatic cell hybrids and their use in gene mapping are now available (Ruddle, 1972; Davidson and de la Cruz, 1974; Ruddle and Creagan, 1975; Ringertz and Savage, 1976; Creagan and Ruddle, 1977); only a brief outline of the salient points will be given here.

Since the discovery by Barski *et al.* (1960) of spontaneous *in vitro* somatic cell hybridization between related mouse cell lines, several important developments have contributed to the rapid advance of this field. The first was the invention by Littlefield (1964) of a method by which hybrid cells could be preferentially

selected from a mixture of parental cells, the HAT selective system. Even with selective systems to improve the recovery of hybrids, the frequency of spontaneous hybrid formation remained very low. This problem was alleviated when Harris and Watkins (1965) showed that the use of Sendai virus could induce much greater fusion frequencies. It was at this time that the first proliferating interspecific (rat x mouse) hybrids were obtained by Ephrussi and Weiss (1965).

The next step, which was of major importance in revealing the potentialities of somatic cell hybrids for gene mapping, was the production by Weiss and Green (1967) of the first human x rodent somatic cell hybrids. These hybrids underwent rapid and preferential loss of human chromosomes, although genes on the remaining human chromosomes were still expressed. The phenomenon of preferential chromosome loss, referred to as chromosome segregation, has since been shown to be characteristic of interspecific somatic cell hybrids. It appears to occur in two phases: an initial rapid loss of the chromosomes of one species from a heterokaryon containing both parental genomes, and a later slow continual evolution of the hybrid karyotype (Creagan and Ruddle, 1977; Schall and Rechsteiner, 1978). While the latter process can probably be explained in terms of the known cellular mechanisms of nondisjunction or anaphase lag, the cause of the initial directional chromosome segregation is unknown. Various theories for the mechanism of chromosome loss are reviewed by Handmaker (1973).

The production of hybrid cells containing partial chromosome complements of one species provided the means for analysis of linkage groups. This can be done in two ways. The first relies on the concordance in presence or absence of two biochemical markers in a number of hybrid cell lines to deduce that the genes responsible are located on the same chromosome. This relationship between two genes, termed "synteny" by Renwick (1971), is not equivalent to genetic linkage, which is deduced from meiotic recombination frequencies. Two syntenic genes located far apart on the same chromosome may not be demonstrably linked, even through an intermediate.

The other method of gene assignment, "assignment by association" (Creagan and Ruddle, 1977), is based on concordance between the expression of a particular gene product and retention or loss of a particular chromosome. This obviously depends on the ability to identify individual chromosomes, which has been provided by chromosomal banding techniques, the first such being the Q-banding technique discovered by Caspersson *et al.* (1970). The two procedures for gene assignment can be combined, so that assignment of a gene to a particular chromosome allows all genes syntenic with that gene to be assigned to the same chromosome. Refinements of these techniques using parental cells containing chromosome rearrangements have enabled genes to be localized to particular regions of chromosomes.

Somatic cell genetic techniques, combined with family studies, have produced very rapid advances in the

field of gene mapping, for man in particular. The number of provisional and confirmed human gene assignments to autosomes rose from 59 at the first International Human Gene Mapping Workshop in 1973 to 165 at the fourth Workshop in 1977 (Donald and Hamerton, 1978), and detailed human chromosome maps are now available. However, two points about gene mapping using somatic cell hybrids need to be borne in mind. The first is that such chromosome maps are not equivalent to linkage maps produced by conventional genetic means. While linkage maps can be used to predict the recombination between two gene loci, chromosome maps only provide information on the physical location of genes. Both types may have advantages in specific situations, provided their limitations are respected.

The second point concerns the assumptions on which gene mapping using somatic cell hybrids is based. These assumptions for gene mapping in human x mouse hybrids, which apply to most gene mapping studies, have been expounded in detail by Ruddle (1970), and can be summarized as follows: human chromosomes segregate randomly and progressively, no appreciable human chromosome rearrangement occurs, hybrid clones are independently derived and homogeneous, human genes are constitutively expressed, human and mouse gene products can be distinguished, and human and mouse chromosomes can be cytologically identified. It is now clear that in many cases these assumptions do not hold and, consequently, results obtained from somatic cell hybrids must be subjected to careful scrutiny.

Although the main thrust of somatic cell genetic

studies has been towards mapping human chromosomes, information is now accumulating for many other species of eutherian mammals, as discussed earlier. The order Marsupialia, comprising approximately 250 species in 8 superfamilies (Kirsch and Calaby, 1977), is much less well characterized in somatic cell genetic terms. The chromosomes of a wide range of marsupials have been studied by conventional cytogenetic techniques and DNA measurements (Sharman, 1973, 1974; Hayman and Martin, 1974; Hayman, 1977) and hypotheses advanced concerning chromosomal evolution within this group. Recent studies of G-banded chromosomes of 26 species of Australian marsupials (Rofe, 1979) have clarified some of the pathways of chromosomal evolution within and between Australian superfamilies and indicated that chromosome arms have usually remained intact during the rearrangements separating different species. However, the arrangement of genes on chromosomes in marsupials, which would be of interest both for establishing a clearer picture of marsupial evolution, and for assessing the forces governing linkage relationships between genes over the greater evolutionary distance separating marsupial and eutherian mammals, is only beginning to be investigated.

Besides the evolutionary interest of marsupials, they have many advantages for genetic and somatic cell genetic analysis, which have been pointed out previously (Cooper, 1974; Graves and Hope, 1977a; Hope and Graves, 1978b). These advantages include low numbers of large, easily distinguished chromosomes and the potential availability of a large number of isozyme differences from eutherian

cells. Apart from an unconvincing report of a hamster x potoroo cell hybrid by Jakob and Ruiz (1970), the only somatic cell genetic studies of marsupials have been those of Hope and Graves. After a series of detailed studies of co-cultivation, fusion, heterokaryon formation and selective systems (Graves and Hope, 1977a, b; Graves *et al.*, 1977; Graves and Hope, 1978; Hope and Graves, 1978a), the production of a number of marsupial x eutherian cell hybrids was reported by Hope and Graves (1978b). These hybrids between cells of a variety of marsupial species and established rodent cell lines were shown to possess marsupial isozymes, and in some cases, marsupial chromosomes. In all these hybrids, marsupial chromosomes were rapidly and preferentially lost. Segregation of marsupial chromosomes enables these cell hybrids to be used for chromosome mapping studies. The first such study has been reported by Graves *et al.* (1979). Although these marsupial x mouse somatic cell hybrids retained no detectable marsupial chromosomes, a syntenic relationship could be established between three genes, one of which had previously been shown to be X-linked.

The aim of this project was to perform a detailed analysis of somatic cell hybrids between eutherian cells and cells of one species of marsupial, the red kangaroo, *Macropus rufus* (= *Megaleia rufa*, Desmarest). This species, which belongs to the family of kangaroos and wallabies, the Macropodidae, has a diploid number of twenty chromosomes and a clearly distinguishable X chromosome (Sharman, 1961). Particular attention was paid to mapping genes located on the X chromosome in the hope of providing

information that would be useful in consideration of the conservation and arrangement of genes on the X chromosome, and their regulation by X chromosome inactivation.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

Cell Lines

A number of established rodent cell lines were used as the eutherian parent cells in hybridizations. They are listed in Table 2.1, with a summary of their characteristics. Also included is a marsupial primary cell line which was used as a source of fibroblasts for some of the enzyme studies. For all the fusions, however, fresh marsupial lymphocytes were used.

Routine Culture Methods

Cells were grown as monolayers in plastic flasks (Falcon) or glass prescription bottles, maintained at 36°C in a humid incubator with a 5% - 10% CO_2 atmosphere. Cells were subcultured by washing with phosphate-buffered saline (PBS)*, dissociating with 0.1% trypsin-versene* and transferring an inoculum to a fresh vessel. Most of the cell lines studied grew rapidly, and were subcultured once or twice per week. The standard (non-selective) culture medium used was RPMI 1640 (Gibco), supplemented with 10% foetal calf serum, 50 µg/ml streptomycin, 60 µg/ml penicillin and 2×10^{-6} M glutamine. For the isolation and propagation of hybrid cells this was converted to HAT medium (Littlefield, 1964) by the addition of 10^{-4} M hypoxanthine, 10^{-6} M aminopterin and 1.6×10^{-5} M thymidine.

* See Appendix I

TABLE 2.1

CELL LINES USED IN THIS STUDY

Cell Line	Origin	Modal No. Chromosomes	Characteristics	Reference
1R	Mouse (C3H), L-cell derivative	58	$HPRT^-(8AG^r)$	Nabholz *et al.* (1969)
PG19	Mouse (C57BlJ) melanoma	41	$HPRT^-(6TG^r)$	Jonasson *et al.* (1977)
BIo	Chinese hamster derivative of $B_{14}I_{50}$	22 or 23	$TK^-(BrdU^r)$ ouabain resistant	Humphrey and Hsu, (1965)
3T3	Mouse embryo (Swiss)	68 or 69	$TK^-(BrdU^r)$	Matsuya and Green, (1969)
Cl 1D	Mouse (C3H) L-cell derivative	51	$TK^-(BrdU^r)$	Weiss and Green (1967)
K2	Red kangaroo ♀ ear fibroblasts	20	Primary diploid line	Graves and Hope, (1977b)

Cell lines were subcloned by plating 50 - 200 cells in 60 mm plastic tissue culture Petri dishes (Lux). When colonies had grown, they were isolated in metal cloning rings greased with paraffin and picked with a Pasteur pipette. The cells were then transferred to a 35 mm Petri dish until sufficient cells were obtained to be transferred to a routine culture flask.

Revertant derivatives were obtained from the hybrid cell lines by plating cells in medium containing 4 µg/ml 6-thioguanine (6TG) or 30 - 100 µg/ml 5-bromodeoxyuridine (BrdU) at a concentration of 10^5 cells per 60 mm Petri dish. Colonies were isolated as described above.

HAT sensitivity tests were carried out by plating cells at a concentration of 5×10^4 cells per 60 mm Petri dish in HAT medium. Duplicate plates were used for each cell line tested and were scored for growth after two weeks. To obtain a record of the number of colonies, plates were washed in PBS, fixed by successive washes in 70% and 100% ethanol and stained for 10 min with 10% Giemsa (Gurr's R66).

Cells in foetal calf serum containing 5% dimethyl-sulphoxide (DMSO) were stored in heat-sealed ampoules in liquid nitrogen. They were frozen slowly using a liquid nitrogen programmed cooler (Paton Industries).

Fusions

All the fusions were between cells from an established rodent cell line and marsupial lymphocytes. The lymphocytes were obtained from male and female red kangaroos (*Macropus rufus*). Approximately 10 ml of blood

was taken from a lateral tail vein and defibrinated by agitation on a magnetic stirrer for 20 - 30 minutes. The blood was then diluted 1:1 with PBS and lymphocytes were separated by density gradient centrifugation as follows: two volumes of diluted blood were gently layered onto one volume of Ficoll-Hypaque solution (4 parts Ficoll-Hypaque*:1 part water), and the tubes were centrifuged at 1400 r.p.m. (400 g) for 20 min to separate the lymphocyte layer. This layer was then removed and the lymphocytes were washed with PBS and counted.

The ratio of kangaroo lymphocytes to rodent cells in the fusions was approximately 3:1. The cell suspensions were mixed, washed twice in alkaline medium without serum, resuspended in 0.5 ml of medium and chilled before adding 1 ml of chilled β-propriolactone-inactivated Sendai virus (Graves and Hope, 1977b), at a concentration of 2,000 haemagglutination units per ml. The mixture was chilled for 20 min to allow agglutination to occur, then incubated at 37°C for 35 min, after which the cells were plated out in non-selective medium at a concentration of approximately 2.5×10^6 cells per 60 mm Petri dish or 25 cm^2 plastic flask. The medium was changed to HAT medium with 12% foetal calf serum after 24 to 48 hours.

2.2 Nomenclature

The nine hybrid clones which were the principal subject of this study fall into two groups: those derived from fusions between red kangaroo cells and 1R, and those

* See Appendix I

for which PG19 was the mouse parent cell line. The clones were named according to the fusion and colony number, as set out in Table 2.2.

The other cell lines referred to in detail are the 6-thioguanine resistant revertants of 1RMR-1. These were derived from mass revertant cultures, obtained by allowing the numerous 6TG-resistant colonies in several Petri plates to grow to confluence. Subclones were then isolated in medium with 6TG or non-selective medium from four mass cultures produced in this way. The nomenclature of these revertant subclones is illustrated in Table 2.3.

2.3 Chromosomes

Chromosome Preparations

Chromosome preparations were obtained from logarithmically growing cultures, which were usually sub-cultured 20 to 21 h before harvesting to achieve partial synchronization and increase the yield of mitoses. They were treated with 0.05 µg/ml colcemid (Gibco) for 15 to 20 min before harvest in the case of derivatives of 1R (including all 1RMR hybrids and revertants) and for 30 to 40 min for all other cell lines. The chromosomes of 1R and its derivative cell lines become very condensed with colcemid treatment, as was noted by Bobrow and Cross (1976), and this brief colcemid treatment was necessary to obtain chromosome preparations sufficiently elongated for banding studies. Cells were then harvested, washed once with PBS, and suspended in hypotonic KCl (0.075 M) at 37°C for 35 min. The cell suspensions were fixed in three changes of fixative (3 methanol:1 glacial acetic

TABLE 2.2

DERIVATION AND NOMENCLATURE OF HYBRID CELL LINES

Parental Cells	Fusion Number	Hybrid Clone
M. rufus x 1R	1	1RMR-1
	2	1RMR-2/1
		1RMR-2/2
		1RMR-2/3
	4	1RMR-4/1
		1RMR-4/2
		1RMR-4/4
M. rufus x PG19	1	PGMR-1/1
		PGMR-1/4

TABLE 2.3

DERIVATION AND NOMENCLATURE OF REVERTANT CELL LINES

Mass Revertant Culture	Subclones Isolated in:	
	Medium with 6TG	Non-selective Medium
1RMR-1-R1	A1 - A10	B1 - B10
1RMR-1-R2	A1 - A10	B1 - B10
1RMR-1-R3	A1 - A10	B1 - B10
1RMR-1-R5	A1 - A10	B1 - B10

acid), dropped onto clean microscope slides and air-dried.

C-Banding

Chromosome preparations were C-banded according to the method of Sumner (1972): slides were pretreated with 0.2 N HCl at room temperature for 30 min, followed by 1 - 5 min in 5% (w/v) $Ba(OH)_2$ at 50°C and 30 min in 2 x SSC* at 60°C. They were then stained for 30 min in a 10% solution of Giemsa (Gurr's R66) in Sorenson's phosphate buffer pH 6.8*. Good C-banding could be obtained on chromosome preparations aged from a few days to several years.

G-Banding

G-banding of chromosome preparations was performed by a modification of the trypsin technique of Seabright (1971). Slides were treated for 30 - 200 seconds with a 0.05% solution of trypsin (Difco) in Sorenson's phosphate buffer, pH 6.8, at room temperature. They were then rinsed in cold physiological saline and stained with 5% - 10% Giemsa for 15 - 20 min. Chromosome preparations generally needed to be aged for at least two months before they would G-band satisfactorily.

N-Banding

Chromosomes were silver N-banded using the "Ag-I" method of Bloom and Goodpasture (1976), which involves treating the slides with 50% w/v silver nitrate ($AgNO_3$). Slides covered with a thin film of silver nitrate under a coverslip were incubated at 37°C for from

* See Appendix I

one to four days in a humid box, rinsed and counter-stained with 5% Giemsa for 10 to 30 seconds.

Photography

Banded chromosome preparations were photographed with Copex Pan 35 mm high contrast film (Agfa-Gaevert), using a 63 x or 100 x oil immersion objective. The film was developed with a fine grain developer, Kodak D11, and printed at a standard magnification on Ilfospeed photographic paper.

2.4 Enzymes

Cell Extracts

Subconfluent cell cultures were harvested and washed twice in cold PBS. Cells were suspended in an equal volume of lysis buffer containing 10 mg NADP and 0.1 ml β-mercaptoethanol in 10 ml distilled water (modified from Johnston *et al.*, 1975), distributed as aliquots of 2 x 10^6 cells in 0.5 ml plastic tubes, and stored at -80°C. Samples were subsequently thawed, spun in a Beckman Microfuge and the supernatant used fresh for electrophoresis.

Enzyme Electrophoresis

Hypoxanthine phosphoribosyl transferase

(HPRT, E.C. 2.4.2.8.): Mouse and kangaroo HPRT were separated by starch gel electrophoresis, according to the method of Watson *et al.* (1972). Electrophoresis was performed for approximately 5 h at 200 - 280 volts, while the gel was maintained at 30° - 40°C. HPRT was detected by the autoradiographic technique described by Watson *et al.*, using an overlay of Whatman DE81 paper. Kodak RP/S

X-Omat X-ray film was exposed to the overlay for one week and developed with Kodak liquid X-ray developer, Type 2.

Glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49): Considerable difficulty was experienced in separating the mouse and kangaroo forms of this enzyme, which had very similar mobilities under a variety of conventional electrophoreticprocedures (see Section 7.1). They were eventually successfully distinguished by cellulose acetate electrophoresis (Cellogel, Chemetron, Milan) with a discontinuous buffer system described by Adams and Donald (1980) (Appendix II). Gels were run for 2 h at 300 volts, the loading and electrophoresis being performed at 4°C. They were stained with a reaction mixture adapted from Johnston *et al.* (1975)*.

Phosphoglycerate kinase-A (PGK-A, E.C. 2.7.2.3): Electrophoresis on Cellogel was used to distinguish mouse and marsupial forms of this enzyme. Electrophoresis in Tris-citrate buffer, pH 8.6*, was carried out for 3½ h at 200 volts. The stain mixture was adapted from Meera Khan (1971)*. Gels were observed under ultraviolet light (3660 Å) and when bands had appeared fully (10 - 20 min), were counterstained as described by Meera Khan (1971) and rinsed under hot running water. This hastened the reverse staining procedure and resulted in sharp bands.

Lactate dehydrogenase (LDH, E.C. 1.1.1.27): The mouse and kangaroo forms of LDH were separated by

* See Appendix I

Cellogel electrophoresis, using a 0.02 M phosphate buffer, pH 7.0 (Meera Khan, 1971). Gels were run at 4°C for 1½ - 2 h at 200 volts, and were stained with the reaction mixture given by Meera Khan.

Galactokinase (GALK, E.C. 2.7.1.6): Starch gel electrophoresis by the method of Nichols *et al.* (1974) was used to distinguish hamster and kangaroo GALK. Gels were electrophoresed at 150 volts for 5½ h at 4°C. They were subsequently incubated for 2 h at 37°C with the reaction mixture described by Nichols *et al.* However, rather than using lanthanum chloride precipitation to detect the product, as these authors did, the gels were incubated with an overlay of DE81 paper and treated subsequently according to the method for HPRT gels described by Watson *et al* (1972).

Autoradiography

In addition to enzyme studies using electrophoretic methods, the activity of one enzyme, thymidine kinase (TK, E.C. 2.7.1.75) was detected by *in situ* autoradiography of cells. The method was adapted from that of Migeon *et al.* (1968). A number of 60 mm Petri plates were seeded at a concentration of 5×10^4 cells per plate. Twenty-four hours later, when the cells had attached to the plates and resumed metabolism, 0.1 ml of ^{3}H-thymidine (200 µCi/ml) was added to the medium in each plate, giving a final concentration of 5 µCi/ml. After 6 h exposure to the radioisotope, the plates were washed twice with physiological saline, fixed by successive washes with 30%, 70% and 100% ethanol and dried. The

plates were then treated with ice-cold trichloroacetic acid (TCA) for 15 min, rinsed three times in distilled water and dried. They were subsequently dipped in Ilford Nuclear Emulsion (Type L4) and developed after 10 days with Kodak D11 developer.

CHAPTER 3

ISOLATION AND IDENTIFICATION OF HYBRIDS

3.1 Production And Selection Of Hybrids

The somatic cell hybrids with which this thesis is concerned were produced by fusions between red kangaroo lymphocytes and cells of a number of established rodent lines. Hybrid clones resulting from Sendai virus-induced fusion were isolated from the parental cells by the use of a "half-selective" system, so called because no selection is necessary to eliminate the marsupial lymphocytes, which do not attach to the flasks and are simply washed away. The rodent cells were killed by biochemical selection with the HAT selective system developed by Littlefield (1964). This is based on the use of aminopterin, a folic acid analogue which blocks *de novo* purine biosynthesis and pyrimidine interconversions. Cells can survive in the presence of aminopterin, if additional thymidine and hypoxanthine are present, by using the purine and pyrimidine salvage pathways. These require the activity of two enzymes: hypoxanthine phosphoribosyl transferase (HPRT) and thymidine kinase (TK). Consequently, if the rodent parent cell line lacks either HPRT or TK, the only cells which survive in HAT medium after hybridization should be those in which the deficiency is complemented by the presence of the marsupial gene for HPRT or TK.

Many HPRT-deficient or TK-deficient cell lines are now available for use in cell hybridization. They have been

produced by growing cells in the presence of a base analogue which will kill the cells if incorporated into the DNA. Thus only cells with particular enzyme deficiencies can survive. The base analogues 6-thioguanine (6TG) and 8-azaguanine (8AG) are used to select for HPRT deficiency and 5-bromodeoxyuridine (BrdU) to select for TK deficiency. As well as producing mutant cell lines suitable for use as parent cells in hybridizations, this approach can also be used to reverse the direction of the selection pressure applied to hybrid cells, and produce revertant derivatives lacking the previously selected enzymes.

Most of the research in this project dealt with hybrids resulting from fusions beween kangaroo lymphocytes and HPRT-deficient mouse cell lines. Hybrids selected for the marsupial gene for TK are discussed separately in Chapter 9. Initially, three hybrid clones were isolated by Dr. R.M. Hope; two from a fusion between red kangaroo and PG19 cells and one from a fusion with the 1R cell line. I subsequently performed three further fusions between kangaroo and 1R cells, the two that were successful each yielding three colonies. In both of these fusions, the three colonies were isolated from a total of four or five flasks, each containing 2.5×10^6 cells in a ratio of 3 kangaroo:1 mouse. From this, the frequency of hybrid formation can be calculated to be approximately 3.75×10^{-6}. Only one clone was picked from each flask to ensure that the hybrids were independently derived. All clones resembled the mouse parent in morphology. The nine hybrid clones isolated are listed in Table 3.1, together with their parental cell lines

TABLE 3.1

CHROMOSOME CONSTITUTION OF HYBRIDS AND PARENTAL CELLS; MODE AND RANGE OF CHROMOSOME COUNTS OF 10 CELLS

Cell Line	Total Chromosome Number	Number of Biarmed Chromosomes	Number of Acrocentric Chromosomes	Presence of Kangaroo X Chromosome
Marsupial Parent:				
M. rufus	20	10	10	+
Mouse Parent:				
1R	58 (54 - 67)	18 (15 - 22)	40 (38 - 46)	
Hybrids:				
1RMR-1	59 (55 - 61)	21 (18 - 21)	38 (33 - 41)	+
1RMR-2/1	61 (52 - 67)	18 (17 - 21)	43 (35 - 47)	-
1RMR-2/2	66 (62 - 67)	20 (19 - 21)	45 (42 - 46)	+
1RMR-2/3	64 (59 - 68)	21 (19 - 22)	43 (37 - 49)	-
1RMR-4/1	57 (51 - 58)	19 (17 - 20)	38 (34 - 38)	-
1RMR-4/2	63 (58 - 64)	20 (18 - 21)	43 (37 - 45)	+
1RMR-4/4	58 (54 - 59)	20 (16 - 20)	41 (35 - 41)	+
Mouse Parent:				
PG19	38 (37 - 40)	4 (3 - 4)	34 (33 - 36)	
Hybrids:				
PGMR-1/1	65 (54 - 71)	9 (6 - 12)	56 (45 - 59)	-
PGMR-1/4	71 (65 - 79)	5 (4 - 6)	66 (60 - 73)	Part*

* See Section 3.2

3.2 Preliminary Cytogenetic Identification Of Hybrids

Initial cytogenetic characterization of the hybrids was performed on C-banded chromosome preparations. One of the main advantages of the use of the red kangaroo as the marsupial parent in these hybrids was the distinctive C-banding pattern of the red kangaroo X chromosome, as shown in Figure 3.1a. The long arm of the X chromosome, which bears a secondary constriction and appears to be largely constitutive heterochromatin, stains darkly with this method and often shows characteristic sub-banding. This C-banding region extends a short distance past the centromere into the euchromatic arm. Neither 1R (Figure 3.1b) nor PG19 (Figure 3.4a) possess any chromosomes with a similar C-banding pattern. Consequently, the presence in the hybrids of the red kangaroo X chromosome, or at least the heterochromatic arm, could be easily determined.

C-banded cells of each of the hybrids are shown in Figures 3.1, 3.2, 3.3 and 3.4, and the presence or absence of the red kangaroo X is recorded in Table 3.1. Four of the hybrids possessed a complete kangaroo X chromosome, and in PGMR-1/4 an isolated heterochromatic arm was detected. The hybrid origin of these five clones was thus confirmed. C-banding analysis, however, gave no information on the nature of the other four colonies. Enzyme studies (Section 3.3) were necessary to establish whether these colonies were also hybrids.

Counts of the total chromosome number and of the number of biarmed and acrocentric chromosomes were made

FIGURE 3.1

C-banded cells of

a) the red kangaroo, *Macropus rufus*

b) a mouse parent line, 1R

c) a hybrid, 1RMR-1

The red kangaroo X chromosome is indicated by an arrow.

a

b

c

FIGURE 3.2

C-banded cells of

a) a hybrid, lRMR-2/1

b) a hybrid, lRMR-2/2

c) a hybrid, lRMR-2/3

The red kangaroo X chromosome is indicated by an arrow.

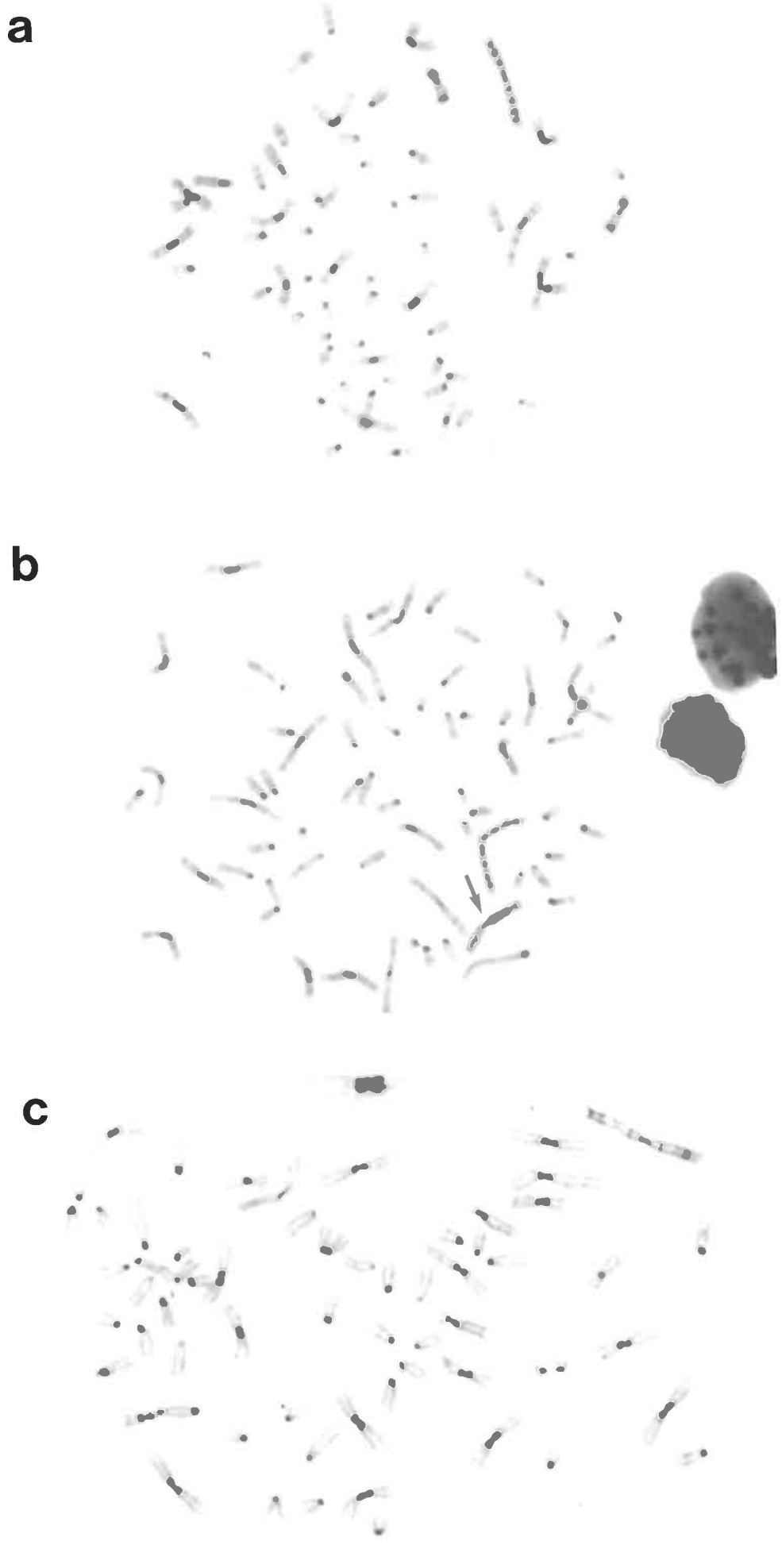

a
b
c

FIGURE 3.3

C-banded cells of

a) a hybrid, lRMR-4/1

b) a hybrid, lRMR-4/2

c) a hybrid, lRMR-4/4

The red kangaroo X chromosome is indicated by an arrow.

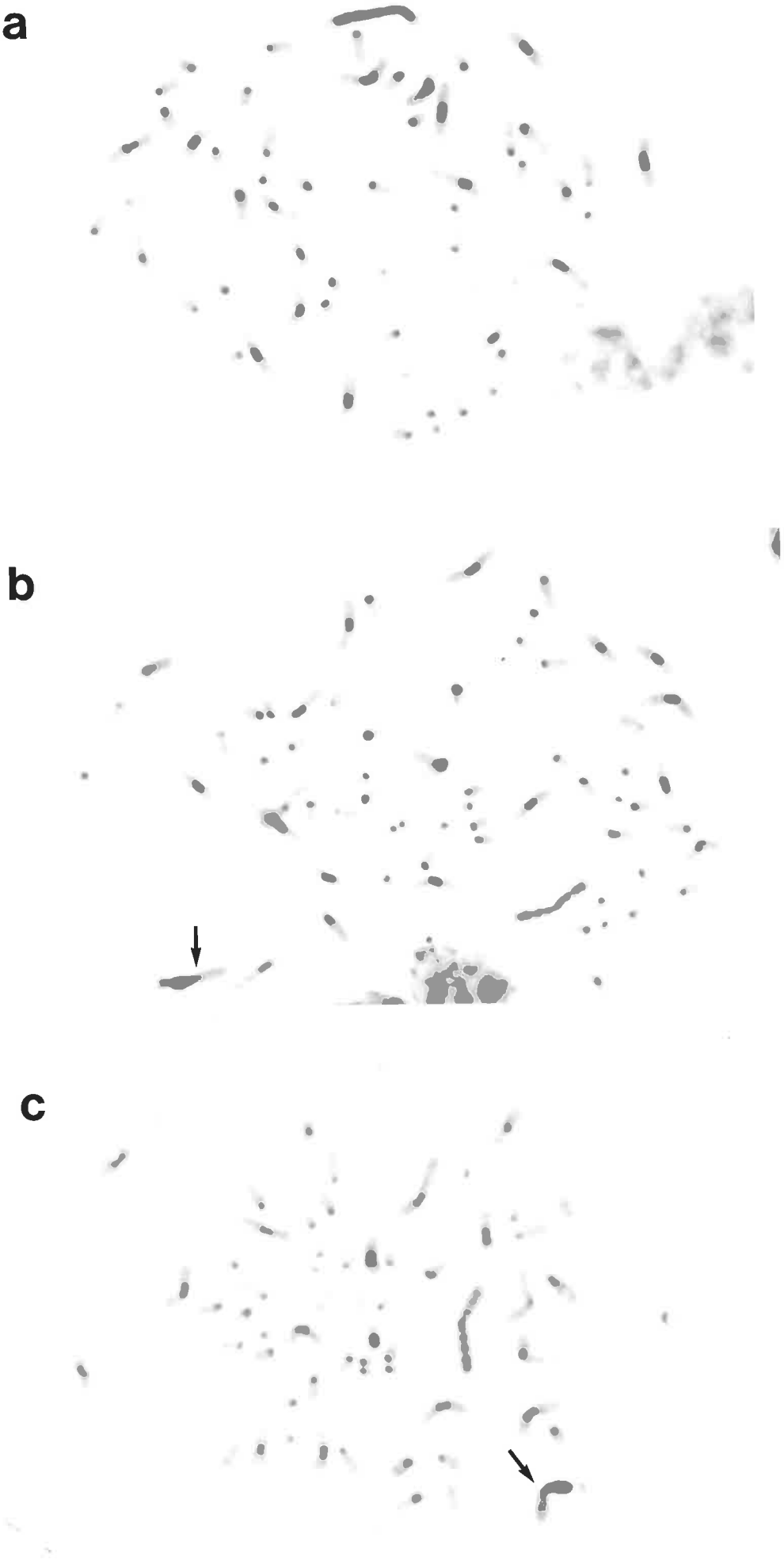

a
b
c

FIGURE 3.4.

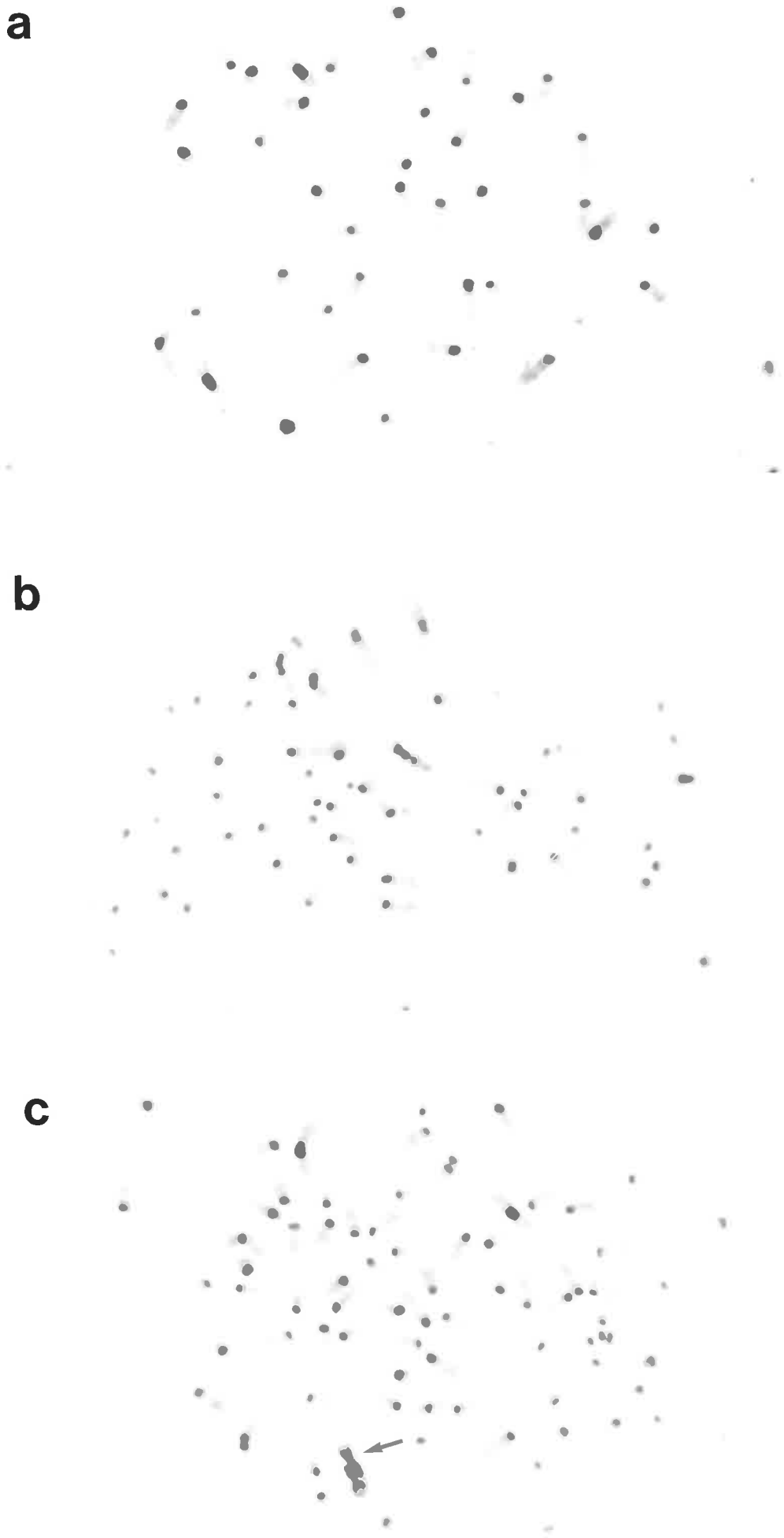
C-banded cells of

a) a mouse parent line, PG19

b) a hybrid, PGMR-1/1

c) a hybrid, PGMR-1/4

The partial red kangaroo X chromosome is indicated by an arrow.


a
b
c

on ten cells from each of the hybrids and the parental mouse cell lines. These are shown in Table 3.1 as the mode and range of counts in the ten cells. It is immediately apparent that there is a great deal of variability between individual cells of both the hybrids and their mouse parents. For this reason, the modal numbers of biarmed and acrocentric chromosomes do not always add up to the mode of the total chromosome numbers in the ten cells.

If each hybrid cell contained all the chromosomes of one mouse and one kangaroo cell, lRMR hybrids would be expected to have approximately 80 chromosomes and PGMR hybrids 60 chromosomes, an expectation which Table 3.1 shows is clearly not fulfilled. Although most mouse and kangaroo chromosomes, unlike the kangaroo X chromosome, do not have distinctive C-banding patterns, the larger kangaroo autosomes are distinguishable from mouse chromosomes on the basis of size and morphology. None of these chromosomes are present in the hybrids (Figures 3.1 to 3.4), which suggests that kangaroo chromosomes are lost in these kangaroo x mouse hybrids. Loss of marsupial chromosomes in marsupial x eutherian somatic cell hybrids has been previously reported by Hope and Graves (1978b). PGMR-1/1 and PGMR-1/4 have much higher chromosome numbers than their mouse parent, PG19, and probably have been formed by fusion between two mouse cells and one kangaroo cell, with subsequent chromosome loss. The lRMR hybrids, on the other hand, appear to result from fusion of one mouse with one kangaroo cell.

The variation in chromosome number between 1RMR clones and between cells in each clone is not unexpected in the light of the range of chromosome counts in 1R. However, the PGMR hybrids do not reflect the relative stability in chromosome number shown by PG19. This is probably a result of the presence of two copies of the mouse genome and the consequent tolerance of the cells to loss of mouse chromosomes.

The retention of a large part or all of the red kangaroo X chromosome in five of the nine hybrids discussed above is in marked contrast to the chromosome constitution of the series of 41 marsupial x eutherian hybrids characterized by Graves *et al.* (1979), none of which retained any cytologically detectable part of the X chromosome. As very similar hybridization techniques were used in obtaining each group of hybrids, the most probable cause of the difference between them is the difference in parental cell lines. Very little is known about the effects of different parental mouse cell lines on hybrids derived from them. It is perhaps significant that the four hybrids possessing a complete kangaroo X chromosome were all derived from 1R. However, the observation that one of the two PGMR hybrids contained a portion of the red kangaroo X chromosome, the heterochromatic arm, while no marsupial chromosomes were detected in the 19 hybrid clones derived from PG19 by Graves *et al.*, indicates that the source of marsupial parent cells may be important. The active X chromosome in the marsupial cells used by Graves *et al.* was the

wallaroo X chromosome, an acrocentric chromosome with little detectable constitutive heterochromatin, unlike the red kangaroo X chromosome. It has been suggested previously (Hope and Graves, 1978b) that the pattern of chromosome loss in marsupial x eutherian somatic cell hybrids may reflect a tendency of such hybrid cells to retain marsupial constitutive heterochromatin. This possibility is discussed further in Section 4.2, in a consideration of the chromosomal behaviour of revertants obtained from these hybrid cells.

3.3 HPRT In Hybrids

The growth of the nine clones in HAT medium suggests that they possess the kangaroo form of HPRT. Other means by which cells could become resistant to HAT medium include re-expression of mouse HPRT or development of insensitivity to aminopterin as a result of membrane changes. In order to distinguish these alternatives, the HPRT of all the putative hybrids was examined by electrophoresis.

HPRT is a trimer, and has a widespread tissue distribution which includes red blood cells and cells in tissue culture. The mouse and kangaroo forms of this enzyme can be readily distinguished by their different mobilities with starch gel electrophoresis, as can be seen in Figure 3.5. The HPRT of Cl 1D shows the triple-banded pattern characteristic of mouse L cells (Watson *et al.*, 1972). HPRT from red blood cells of the red kangaroo always migrated a little faster than the form

FIGURE 3.5

Starch gel electrophoresis of HPRT of mouse, kangaroo and hybrid cells.

Channel 1: lRMR-2/3, a hybrid lacking a detectable kangaroo X chromosome.

Channel 2: Cl 1D, a mouse L-cell derivative.

Channel 3: lRMR-1, a hybrid containing a kangaroo X chromosome.

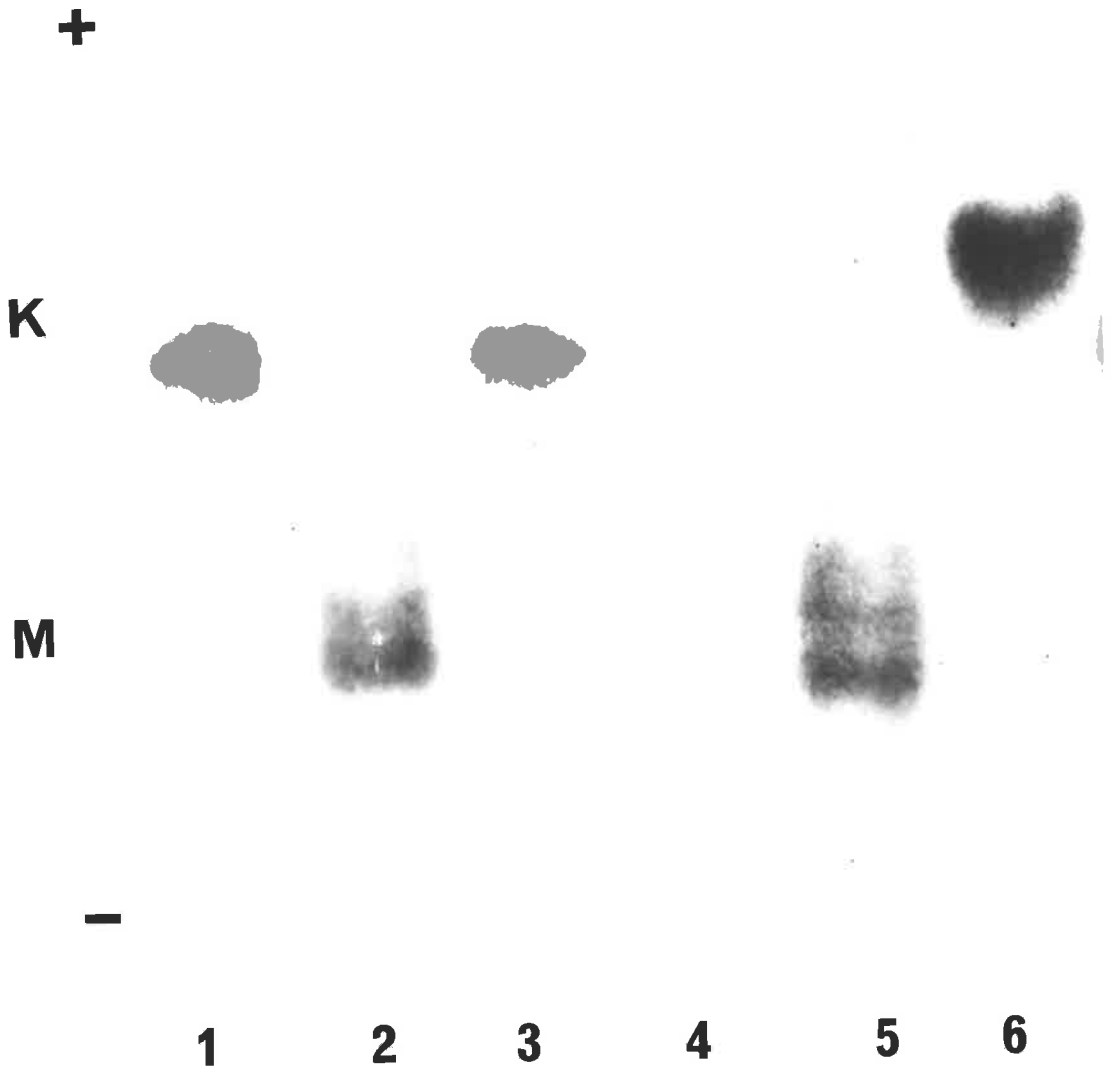
Channel 4: lRMR-1-R1 B5, a 6TG-resistant revertant, lacking detectable HPRT.

Channel 5: lRMR-4/1, a hybrid lacking a detectable kangaroo X chromosome, which possessed HPRT of mouse mobility.

Channel 6: Red kangaroo red blood cells.

M = mouse mobility

K = kangaroo mobility.


+
K
M
–
1
2
3
4
5
6

found in fibroblasts. As expected, all hybrids expressed HPRT, which was clearly of kangaroo mobility for all except one of the clones.

The exception, 1RMR-4/1, appeared to possess the mouse form of HPRT. There are a number of possible explanations for this observation. One is that the mobility of the kangaroo form of HPRT has been changed by mutation to resemble that of the mouse enzyme, although this is extremely unlikely. An alternative hypothesis is that 1RMR-4/1 could be a revertant of 1R, in which a back mutation in the defective gene for HPRT has restored the original enzyme activity. This assumes that the cause of the HPRT deficiency in 1R is a structural gene mutation, resulting in an inactive gene product. However, HPRT deficiency in cultured cells cannot be satisfactorily explained in such a simple fashion.

A large number of studies have been conducted which use 6-thioguanine or 8-azaguanine resistance as a means of obtaining cells lacking HPRT. It has become apparent that 8AG resistance, in particular, can result from changes unconnected with HPRT, such as alterations in cell permeability and transport mechanisms which prevent the uptake of azaguanine or mutations in some of the other enzymes involved in purine synthesis (Gillin *et al.*, 1972; Morrow *et al.*, 1973). Cells have been obtained which have near-normal HPRT activity and yet are resistant to 8AG, presumably owing to altered substrate affinity (Parsons *et al.*, 1976; Van Diggelen *et al.*, 1979). Nevertheless, there is evidence that a considerable proportion of 6TG-

and 8AG-resistant cell lines do result from mutations in the structural gene for HPRT, both missense (Fenwick *et al.*, 1977; Fox *et al.*, 1976) and nonsense (Wahl *et al.*, 1975; Capecchi *et al.*, 1977).

The remainder of the HPRT-deficient cell lines reported in the literature are more difficult to interpret. The production of $HPRT^+$ hybrid cells from the fusion of 8AG- and 6TG-resistant cells has led Sekiguchi *et al.* (1974, 1975) to propose inter-allelic complementation between multiple cistrons for HPRT. However, these results can be more plausibly explained in terms of the heterogeneity of 8AG- and 6TG-resistant mutants discussed above. Although an explanation in terms of multiple structural loci for HPRT is unlikely, the existence of additional regulatory loci for this enzyme cannot be dismissed. There is evidence from studies of cell lines lacking cross-reacting material (Shin, 1974) or with reduced amounts of apparently normal enzyme (Skaper *et al.*, 1977) that some HPRT-deficient cells may be regulatory mutants. These could arise either by mutation of a gene responsible for the control of HPRT activity or, as suggested by Morrow (1977), by repression or derepression of genes as a result of chromosomal rearrangements and position effects.

Therefore, a third hypothesis concerning the presence of mouse HPRT in the hybrid clone 1RMR-4/1 must be considered: that the HPRT deficiency in the 1R parent cells results from the inactivation or repression of the mouse structural gene, which is reversed when these cells

are fused with normal marsupial cells. Such an explanation has been suggested previously to account for similar findings of re-expression of rodent HPRT in a number of human x mouse (A9) hybrids (Watson *et al.*, 1972), chick x mouse (lR) hybrids (Bakay *et al.*, 1973), human x mouse (lR) hybrids (Bakay *et al.*, 1975) and human x Chinese hamster (Wg3-h) hybrids (Bakay *et al.*, 1978). The occurrence of this phenomenon several times with two closely related cell lines, lR and A9, supports the contention that HPRT deficiency in these lines is the result of a mutation in a regulatory gene, or repression by position effect. The restoration of rodent HPRT is independent of the fusing agent used (Bakay *et el.*, 1978). However, the frequency of re-expression does vary in the different hybridizations. Whereas all the hybrid clones produced by Bakay *et al.* (1973) and most of those produced by Watson *et al.* (1972) expressed mouse HPRT, lRMR-4/l represents the first instance of reactivation or reversion of the gene for HPRT in several years' work with lR in this laboratory.

The presence of the kangaroo form of HPRT in the remaining eight hybrids indicates that they possess at least part of the kangaroo chromosome bearing the structural gene for this enzyme. The gene for HPRT has been shown to be located on the X chromosome in man (summarized in Harris and Hopkinson, 1976) and a number of eutherian mammals (Pearson and Roderick, 1978). There are no population or pedigree studies on this enzyme in marsupials, owing to a lack of allozymic variants. However, an X-linked

mode of inheritance in some kangaroos can be inferred from the study of Graves *et al.* (1979), in which this enzyme was shown to be syntenic with a known X-linked gene, that for G6PD. The finding of a red kangaroo X chromosome in a number of these hybrids suggests that the gene for HPRT may also be X-linked in the red kangaroo. This hypothesis can only be evaluated, however, in the light of more thorough cytogenetic examination of the hybrids, as well as of revertants which were selected for loss of expression of this gene.

CHAPTER 4

REVERTANT DERIVATIVES OF HYBRIDS

4.1 Isolation And Identification Of Revertants

The chromosomal assignment of a gene by somatic cell genetic techniques is usually based on the correlation between the presence or absence of the gene product and the presence or absence of a particular chromosome. Consequently, it was necessary to determine not only which kangaroo chromosomes were present in the hybrids that expressed the kangaroo gene for HPRT, but also whether loss of a particular chromosome occurred when this gene was selected against. To obtain the appropriate cell lines lacking HPRT, hybrid cells were back-selected by growth in medium containing 6-thioguanine (6TG), in which only "revertant" cells that do not express the enzyme can survive.

For 1RMR-1, four mass revertant cultures were obtained by allowing cells in four Petri plates to grow to confluence in the presence of 6TG. When cells from these plates were examined by C-banding, each mass culture was found to consist of a mixture of cells with differing portions of the kangaroo X chromosome. The types of deleted red kangaroo X chromosomes found are shown in Figure 4.1, with their classification. Table 4.1 lists the proportion of cells in each of the mass cultures containing these various X chromosome types. In order to obtain cell lines homogeneous for each of these types, twenty subclones were

FIGURE 4.1

C-banded partial or complete red kangaroo X chromosomes of lRMR-l and its revertant derivatives. Xr: a complete kangaroo X chromosome found in lRMR-l. I, II, III, IV and V: types of partial kangaroo X chromosomes found in revertants.

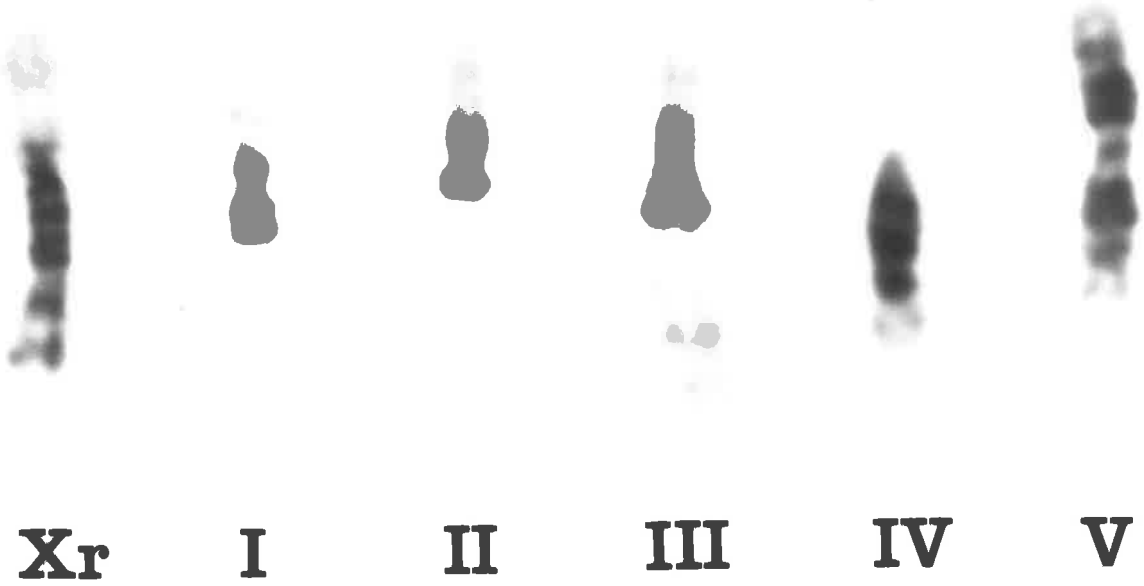

Xr
I
II
III
IV
V

TABLE 4.1

PROPORTION OF CELLS WITH DIFFERENT KANGAROO X CHROMOSOME CONSTITUTIONS IN MASS REVERTANT CULTURES OF 1RMR-1

Mass Revertant Culture	Percentage of Cells with:					
	No Xr	Xr I	Xr II	Xr III	Xr IV	Xr V*
1RMR-1-R1	7	87		6		
1RMR-1-R2	17	15	68			
1RMR-1-R3	29				56	5
1RMR-1-R5	84				6	10

* See Figure 4.1

isolated from each mass culture, ten in medium with 6TG(A) and ten in non-selective medium (B). Unfortunately, only seven of the subclones of 1RMR-1-R5 survived, as a result of problems experienced at that time with foetal calf serum and contamination. Each of the 67 subclones obtained was C-banded to determine which type of X chromosome it possessed. As can be seen in Table 4.2, recovery of the different X chromosome types in the subclones corresponded to the proportions found in the initial mass revertant cultures. Twenty-four subclones were chosen for further study as being representative of the variety of cell types. They are listed in Table 4.3 with their X chromosome constitution and counts of total chromosome number. On further examination, it became apparent that one of the subclones, 1RMR-1-R3 A4, was in fact a contaminant, as it had a higher chromosome number than the other subclones, lacked the 1R marker chromosome and possessed no metacentric chromosomes.

The occurrence of these partially deleted X chromosomes in the revertants derived from 1RMR-1 was unexpected. In the face of selection pressure exerted against a gene on the X chromosome, it would be expected that most hybrid cells would simply lose the chromosome. However, in four separate revertant cultures, a substantial proportion of cells retained part of the X chromosome, most notably the heterochromatic arm. Part or all of the euchromatic arm was deleted, and in some cells the remaining hetero-chromatic arm was found as an isochromosome. Although the initial event of X chromosome breakage may have been caused by the presence of 6TG in the medium, 6TG apparently

TABLE 4.2

KANGAROO X CHROMOSOME CONSTITUTIONS OF SUBCLONES ISOLATED FROM MASS REVERTANT CULTURES OF 1RMR-1

Mass Revertant Culture	Sub-Clone Series‡	Number of Subclones with: No Xr	Xr I	Xr II	Xr III	Xr IV	Xr V*
1RMR-1-R1	A	2	8				
	B	2	8				
1RMR-1-R2	A	1	2	6	1		
	B	2	4	3	1		
1RMR-1-R3	A	2				6	2
	B					10	
1RMR-1-R5	A	1					
	B	5					1

‡ See Table 2.3

* See Figure 4.1

TABLE 4.3

CHROMOSOME CONSTITUTION OF SOME REVERTANT SUBCLONES OF 1RMR-1

Revertant Subclone	Kangaroo X Chromosome Constitution*	Total Number of Chromosomes in Three Cells
1RMR-1-R1 A2	Xr I	56,60,60
R1 A3	Xr I	54,59,60
R1 A10	No Xr	55,55,56
R1 B4	No Xr	52,54,57
R1 B5	Xr I	56,57,58
R1 B6	No Xr	57,58,60
R2 A1	No Xr	55,57,57
R2 A3	Xr II	57,58,58
R2 A5	Xr III	58,59,59
R2 A6	Xr I	57,58,59
R2 B1	Xr III	51,59,61
R2 B2	Xr I	51,52,55
R2 B9	Xr II	58,59,60
R2 B10	No Xr	56,59,59
R3 A3	No Xr	56,56,58
R3 A4	No Xr	66,66,67
R3 A7	Xr V	56,57,58
R3 A8	Xr IV	57,58,59
R3 B6	Xr IV	55,59,60
R3 B10	Xr IV	57,58,60
R5 A5	No Xr	52,52,54
R5 B2	No Xr	59,59,60
R5 B4	Xr V	58,58,59
R5 B10	No Xr	58,59,59

* See Figure 4.1

did not affect subsequent retention of the deleted X chromosomes, as can be seen from the results of subcloning in both selective and non-selective medium (Table 4.2).

Six revertant clones were also selected from another hybrid bearing the complete red kangaroo X chromosome: 1RMR-2/2. However, none of these revertants possessed any detectable part of the kangaroo X chromosome. Whether the failure to observe a similar phenomenon in the 1RMR-2/2 revertants results from selecting a small number of clones rather than growing mass revertant cultures is unclear. Thus it is not known whether or not retention of the heterochromatic part of the red kangaroo X chromosome is a general tendency in hybrids between 1R and *M. rufus*. However, the occurrence of the phenomenon independently in four separate revertant cultures from 1RMR-1 suggests that it is unlikely to be simply due to chance. Some possible explanations are considered in Section 4.2.

No estimate of reversion rate was obtained for 1RMR-1, as colonies in the plates were allowed to become confluent. A record was kept, however, of the number of revertant colonies that appeared when the 1RMR-2 hybrids were back-selected. Seven colonies arose from 6×10^5 cells of 1RMR-2/2, a reversion rate of approximately 1×10^{-5}. The frequency of revertant colonies differed considerably between the two hybrids lacking a detectable X chromosome. For 1RMR-2/1, 80 colonies appeared from 4×10^5 cells plated in 6TG, which gives a reversion rate of approximately 2×10^{-4}. On the other hand, no 6TG-resistant colonies were obtained from 4×10^5 cells of

lRMR-2/3. The difference in frequency of revertant colonies is probably related to the stability with which the kangaroo HPRT gene is integrated into the hybrid genome, which is discussed further in Chapter 6.

As the revertants grew in the presence of 6TG, they would be expected to have lost the capacity to express HPRT. To verify this supposition, all revertant clones were examined for HPRT by starch gel electrophoresis. No HPRT activity was detected in any of the revertants with this method; the absence of HPRT in one of these cell lines is demonstrated in the HPRT autoradiograph shown in Figure 3.3. Low levels of HPRT activity might not be detected by electrophoresis, so all the revertants were tested in addition for HAT sensitivity. When 10^5 cells of each revertant were plated in HAT medium no HAT-resistant colonies appeared, indicating that loss of HPRT expression was complete and stable.

The behaviour of these 6TG-resistant revertants is in marked contrast to that of the 8AG-resistant revertants obtained by Graves *et al.* (1979), which re-express HPRT when grown in HAT medium. Whether the use of 6TG, which has been shown to be a much more stringent selective agent against HPRT than 8AG (Morrow, 1977), is responsible for the difference in behaviour of the revertants is not known.

4.2 Retention Of Part Of The X Chromosome In Revertants

The retention of the heterochromatic arm of the kangaroo X chromosome in the revertants in the face of

selection against a gene on that chromosome is puzzling. One possible explanation concerns the ribosomal RNA genes, which are located in the nucleolar organizer regions on this chromosome arm in the red kangaroo (Hayman and Rofe, 1977). It could be argued that this part of the X chromosome is kept as a means of increasing the number of copies of ribosomal RNA genes, in a manner analogous to that observed in a rat hepatoma cell line (Miller *et al.*, 1979). This hypothesis presupposes that the kangaroo ribosomal genes are active in the cell hybrids, an assumption which can be tested.

Nucleolus organizer regions (NORs) can be selectively stained using a number of techniques, collectively known as "N-banding". The sites stained by silver N-banding correspond to the sites of the ribosomal genes (rDNA), as revealed by *in situ* hybridization, in a number of species (Goodpasture and Bloom, 1975). The technique appears not to stain the DNA of NORs, but rather acidic proteins associated with the ribosomal RNA (rRNA) at these sites (Howell, 1977). A close correlation has been found between silver staining and ribosomal RNA synthesis in a variety of cell types (Schmiady *et al.*, 1979), and it appears that only NORs actively engaged in rRNA synthesis are stained.

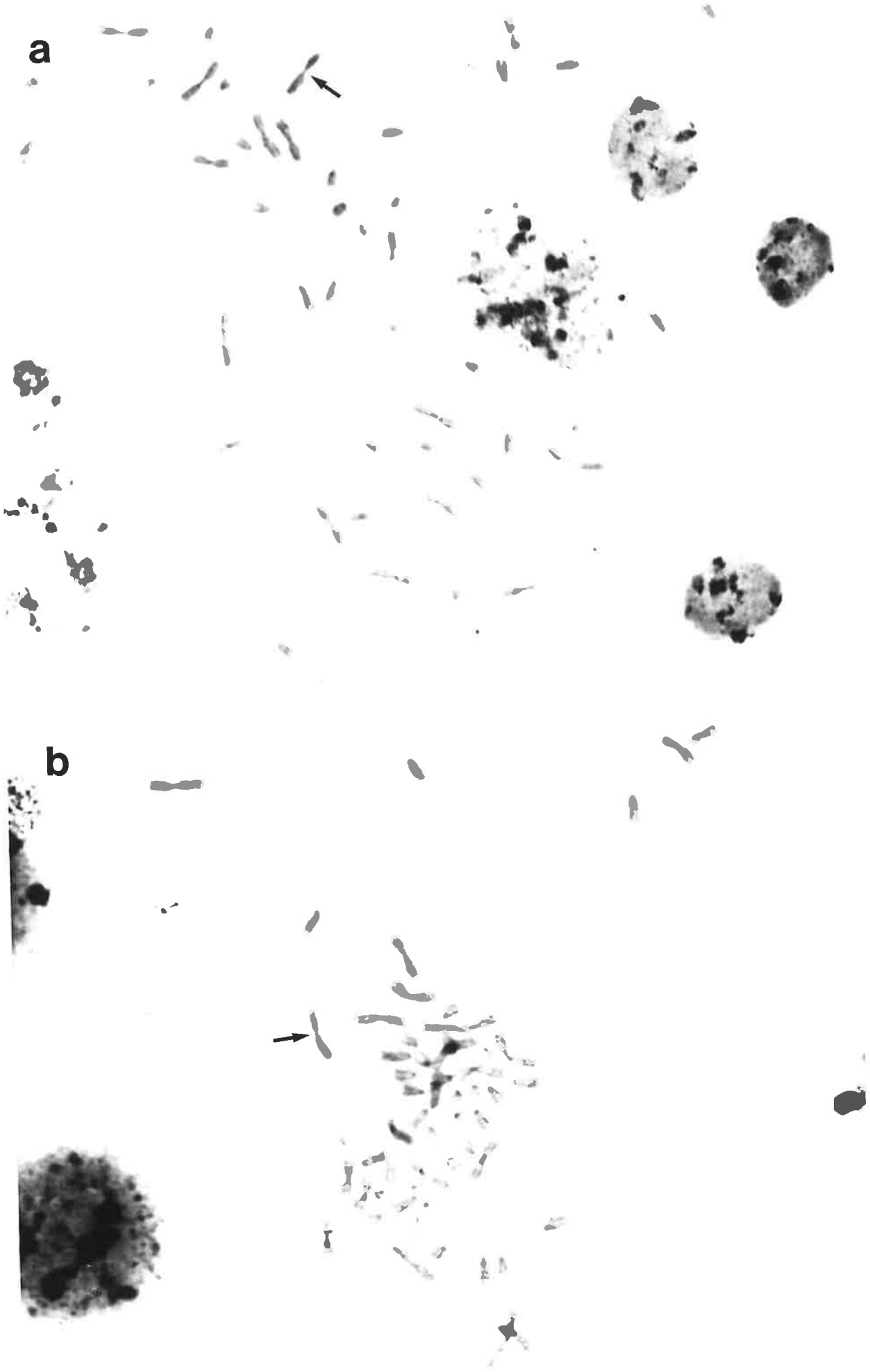
Consequently, silver N-banding by the "Ag-I" method of Bloom and Goodpasture (1976) was used to determine whether the NORs of the red kangaroo X chromosome were active in the hybrid cells. These sites have been previously shown to N-band in red kangaroo cells (Hayman

and Rofe, 1977). The cell hybrid studied was 1RMR-4/4, in chromosome preparations of which the kangaroo X chromosome possessed very prominent secondary constrictions, enabling it to be easily identified. N-banding is a technique of variable efficiency, and only a small proportion of cells on a slide responded to the treatment. Forty cells that possessed a distinguishable kangaroo X chromosome and at least one clearly N-banded chromosome were examined. The number of chromosomes per cell showing distinct N-bands ranged from one to six; Figure 4.2 shows some examples. In none of these cells did the kangaroo X chromosome possess a detectable N-band. Thus, it can be concluded that the NORs of the kangaroo X chromosome do not synthesize ribosomal RNA when they are in a hybrid cell environment.

This finding is consistent with results from a variety of other somatic cell hybrids. In human x mouse hybrids that segregate human chromosomes, no human NORs are silver stained (Miller *et al.*, 1976a); when the direction of chromosome loss in such hybrids is reversed, it is the mouse chromosomes which fail to silver N-band (Miller *et al.*, 1976b). Similar suppression of silver N-banding in the species whose chromosomes are lost is seen in rat x human hybrids (Tantravahi *et al.*, 1979). On the other hand, somatic cell hybrids between more closely related species, such as Syrian hamster and mouse, show silver N-bands on both sets of chromosomes (Miller *et al.*, 1978a). That this phenomenon is not simply an artefact of the cytological technique, but does in fact reflect real

FIGURE 4.2

N-banded cells of 1RMR-4/4. The red kangaroo X chromosome is indicated by an arrow.


a
b

differences in activity, has been established by parallel studies of the rRNA of such hybrid cells. Elicieri and Green (1970) first demonstrated that the 28S rRNA of a number of species could be distinguished by polyacrylamide gel electrophoresis. Subsequent studies on human x mouse hybrids using this method have detected 28S rRNA of only the species whose chromosomes are retained (Bramwell and Handmaker, 1971; Croce *et al.*, 1977). However, hamster x mouse hybrids possess both forms of 28S rRNA (Elicieri, 1972) and both species' types of ribosomes (Stanners *et al.*, 1971). There is evidence that suppression of the ribosomal genes in cell hybrids is mediated at the level of transcription (Perry *et al.*, 1979).

Suppression of ribosomal RNA genes appears to occur not only in somatic cell hybrids, but also in interspecific plant and animal hybrids. Inactivation of the NORs of one species, known as "nucleolar dominance", has long been recognised in a variety of interspecific plant hybrids (reviewed by Rieger *et al.*, 1979). Similar observations have been made in intrageneric *Xenopus* hybrids in which repression of nucleolus formation by the chromosomes of one species has been shown to occur through repression of transcription (Honjo and Reeder, 1973). It would seem probable that selective inactivation of ribosomal genes is effected by basically similar mechanisms in interspecific hybrids and in somatic cell hybrids, although this can not be confirmed until the molecular mechanisms are understood.

The significance of such species-specific suppression is not clear. There is some evidence that this phenomenon

may not be restricted to ribosomal RNA genes. Huebner *et al.* (1977) found that replication of DNA tumour viruses for which the "recessive" or segregating species is permissive was suppressed in human x mouse cell hybrids. In the same hybrids, the histone genes of the recessive parent are also suppressed (Ajiro *et al.*, 1978). Thus, it appears that while most loci coding for enzymes are constitutively expressed in interspecific somatic cell hybrids, there exist a number of functions which are sufficiently distinct in different species for selective inactivation of one set of genes to take place. This incompatibility does not occur in more closely related combinations such as rodent x rodent hybrids. Selective inactivation is not found in heterokaryons, in which both parental rRNA types are synthesized (Marshall *et al.*, 1975), but occurs once the chromosome sets are united in a single nucleus and chromosome segregation has begun (Dev *et al.*, 1979). Whether it is the selective inactivation of certain genes which determines the direction of chromosome loss, or the reverse, is not known.

The studies on somatic cell hybrids discussed above have provided compelling evidence that silver N-banding is an accurate indicator of the state of activity of ribosomal genes. Consequently, the failure of the red kangaroo X chromosome in a hybrid to show N-bands can be taken to reflect suppression of the ribosomal genes. This is the result which would be expected on the basis of other studies, because of the direction of chromosome loss in these hybrids. Although N-banding was not performed on any

of the revertants, it is reasonable to assume that suppression of the kangaroo NORs seen in a cell hybrid would also extend to the revertants. This could be confirmed by electrophoresis of ribosomal RNA from hybrid and revertant cells. Therefore, the conclusion from this investigation is that it is unlikely that the heterochromatic arm of the kangaroo X chromosome is retained in the revertants as a means of increasing ribosomal RNA synthesis.

Consequently, alternative explanations must be considered. It could be postulated that the cells are preferentially retaining heterochromatin, because the part of the kangaroo X chromosome that is kept is predominantly constitutive heterochromatin. There is evidence that such a phenomenon does occur in cultured cells. Heterochromatin increase *in vitro* by addition and saltatory replication has been reported for a *Drosophila* cell line by Halfer (1978). In a similar result to this study, Farrell and Worton (1977) found that, although most 6TG-resistant revertants of intraspecific Chinese hamster hybrids lost the entire X chromosome concomitant with loss of HPRT, some retained the heterochromatic long arm of the hamster X chromosome. That this is not a universal tendency for species possessing an X chromosome with a heterochromatic arm, however, has been demonstrated by Cook (1976). In his hybrids between field vole and mouse, the heterochromatic long arm of the vole X chromosome was often lost and replaced by an isochromosome of the short arm.

Some understanding of the role of heterochromatin and its effects on cells is clearly necessary before hypotheses of selective increase or decrease of heterochromatin can be evaluated. In a recent review, John and Miklos (1979) maintain that the only function of satellite DNA and heterochromatin that has been satisfactorily established is the regulation of recombination between homologous chromosomes at meiosis. Very little is known of the somatic effects of heterochromatin. Bennett (1971) has proposed that the quantity of DNA in a nucleus may influence cellular parameters such as cell size, cell cycle and generation time by physical mechanical effects of its mass, independent of its informational content. There is some evidence that heterochromatin may act in such a manner to increase cell cycle time in both plants and cultured cells (Barlow, 1973). However, the relevance of these observations to the preferential retention of heterochromatin in mammalian cells *in vitro* is uncertain.

If it is not the heterochromatin *per se* which is the cause of the retention of part of the kangaroo X chromosome, then the possibility remains that there exist genes of unknown function in this otherwise heterochromatic arm which can confer a selective advantage on certain cells. Until our understanding of the genetics and physiology of cultured cells is much more advanced, this question must remain open.

CHAPTER 5

G-BANDING STUDIES

The preliminary cytogenetic analysis of the hybrids was accomplished by the use of the C-banding technique, as discussed in Chapter 3. The kangaroo X chromosome was identified with this technique, but few other chromosomes, either mouse or marsupial, could be individually distinguished, and analysis was limited to chromosome number and gross morphology (Table 3.1). In order to obtain more detailed information about these cell lines, a number of hybrids and revertants were G-banded. Although G-banding allows chromosomes to be individually identified by their banding pattern, it is a far from reliable technique and additionally difficult to use on cells containing approximately 60 chromosomes. For this reason, G-banding was restricted to selected hybrids and revertants, those of the 1RMR series. Ten G-banded cells of each of these cell lines were photographed and used as the basis for analysis.

5.1 G-Banding of 1R

The first step in the G-banding analysis was to characterize the parental mouse cell line, 1R. It was already apparent from counts of chromosome number (Table 3.1) that this cell line was highly heterogeneous, with total chromosome numbers ranging from 54 to 67 in ten cells. This impression was confirmed when G-banded cells were examined. Figures 5.1a and 5.1b present the G-banded chromosomes of six 1R cells, with chromosomes which are

apparently homologous between cells arranged beneath each other, for comparison. The chromosomes of each cell are presented in two parts: the larger chromosomes in Figure 5.1a and the smaller in Figure 5.1b. For some of the smaller chromosomes, in particular, no attempt has been made to identify individual pairs, but chromosomes of similar size and appearance have been grouped together.

The variability shown in Figures 5.1a and b appears to be characteristic of mouse cell lines. Allerdice *et al.* (1973) made a detailed Q-banding analysis of two cell lines of A9, an L-cell derivative like 1R. Both were highly heterogeneous, with ranges of chromosome counts as broad as those found for 1R. Of eleven cells examined, no two had the same chromosome content, and only 40% of the chromosomes found in these cells could be identified as normal mouse chromosomes. Similar findings were made by Hashmi *et al.* (1974) when they examined two other unrelated mouse cell lines, RAG and MSWBS. Aside from the range in total chromosome number between cells, even cells with the same total number of chromosomes had very different karyotypes, and each of the twenty RAG cells studied was unique. The patterns of karyotype evolution appear to differ in the various cell lines. In the RAG and A9 cell lines, as can also be seen in the 1R cells presented here (Figure 5.1a), most of the biarmed chromosomes are obviously isochromosomes. On the other hand, no isochromosomes were found in MSWBS, in which biarmed chromosomes appeared to arise by translocation.

The conclusion which can be drawn from these invest-

FIGURE 5.1

G-banded chromosomes of six cells of 1R; the larger chromosomes are shown on the opposite page and the smaller chromosomes on the following page.

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

[illegible]

igations is that permanent mouse cell lines are not well suited to somatic cell genetic studies in which cytogenetic analysis is necessary. The high variability and apparent continual evolution of such lines makes the task of detecting rearrangements involving parts of foreign chromosomes virtually impossible. Superficially, Chinese hamster cell lines which retain a pseudodiploid karyotype (Worton *et al.*, 1977) would appear to be a better choice, from a cytogenetic point of view. Nonetheless, the apparent stability of such lines may be misleading, as a pseudodiploid chromosome constitution may conceal a considerable amount of internal rearrangement resulting from the continuing process of karyotype evolution seen in all permanent cell lines (Terzi, 1972; Worton, 1978). The reason why mouse permanent cell lines appear to be so unstable is not clear, although a contributing factor could be the considerable amount of chromosome damage induced in mouse cells by fluorescent light (Parshad *et al.*, 1978).

One result of the heterogeneity of 1R is that translocations of small fragments of kangaroo chromosomes onto mouse chromosomes are unlikely to be identifiable. However, entire or partially deleted kangaroo chromosomes should be recognizable in G-banded cells. For the purposes of comparison with karyotypes of the hybrid cells presented in Section 5.2, the karyotype of a "typical" 1R cell is shown in Figure 5.2.

FIGURE 5.2

Karyotype of a G-banded cell of 1R.

[illegible]

5.2 G-Banding of 1RMR Hybrids

In order to identify kangaroo chromosomes in the hybrid cells, the G-banding pattern of the red kangaroo chromosomes was first established. A G-banded karyotype of the red kangaroo was kindly provided by Dr. R. Rofe, and is shown in Figure 5.3.

The six 1RMR hybrids which had been shown to possess the kangaroo form of HPRT (Section 3.3) were G-banded, and karyotypes of a "typical" cell of each of these are presented in Figures 5.5 to 5.10. In addition, for one of these hybrids, 1RMR-1, a chart was made comparing the chromosome constitution of six cells (Figures 5.4a and 5.4b), as was done for 1R. The heterogeneity which the chart reveals is very similar to that found for 1R, despite the much more recent clonal origin of this cell line, a fact which suggests that the process of karyotype evolution in such cell lines is continual and comparatively rapid.

The karyotypes of the hybrids (Figures 5.5 to 5.10) are arranged in the same manner as that of 1R (Figure 5.2), with the identifiable kangaroo chromosomes placed in a separate row at the bottom. The differences in the mouse chromosome complements between the hybrid cell lines are no greater than would be expected of clonal derivatives of 1R. It can be seen immediately that the conclusion reached in Section 3.2 that kangaroo chromosomes are lost in these cell hybrids is confirmed by G-banding analysis. No cells were found to have more than three kangaroo chromosomes, including the X. Two of the hybrids, 1RMR-2/1 (Figure 5.6) and 1RMR-2/3 (Figure 5.8), do not contain any recognizable

FIGURE 5.3

Karyotype of a G-banded cell of the red kangaroo, *Macropus rufus* (courtesy of Dr. R. Rofe).

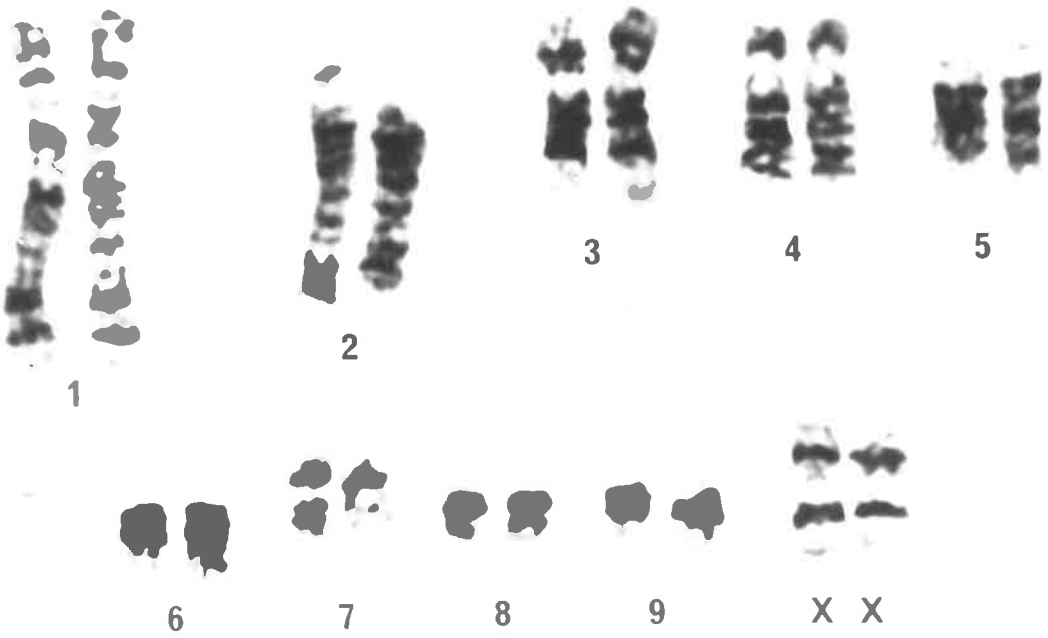

1
2
3
4
5
6
7
8
9
X X

FIGURE 5.4

G-banded chromosomes of six cells of lRMR-1; the larger chromosomes are shown on the opposite page and the smaller chromosomes on the following page, with the kangaroo X chromosome on the extreme right of the second page.

[illegible]

FIGURE 5.5

Karyotype of a G-banded cell of 1RMR-1. Kangaroo chromosomes are in the bottom row, with the X chromosome on the right.

[illegible]

FIGURE 5.6

Karyotype of a G-banded cell of lRMR-2/l.

FIGURE 5.7.

Karyotype of a G-banded cell of lRMR-2/2. Kangaroo chromosomes are in the bottom row, with the X chromosome on the right.

FIGURE 5.8

Karyotype of a G-banded cell of lRMR-2/3.

FIGURE 5.9

Karyotype of a G-banded cell of 1RMR-4/2. Kangaroo chromosomes are in the bottom row, with the X chromosome on the right.

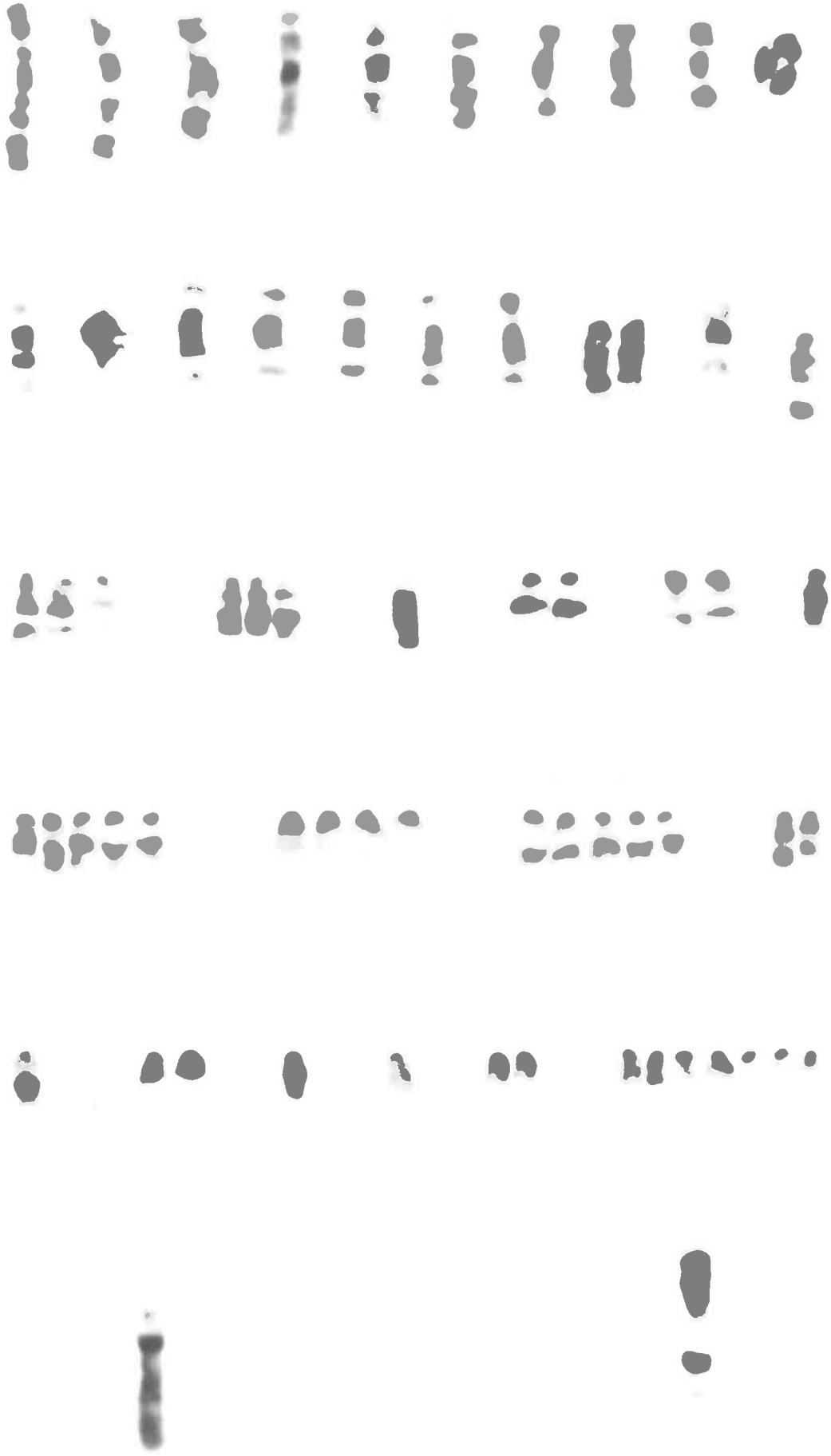


FIGURE 5.10

Karyotype of a G-banded cell of 1RMR-4/4. Kangaroo chromosomes are in the bottom row, with the X chromosome on the right.

kangaroo chromosomes. As these clones have been shown to express the marsupial form of HPRT, they must presumably contain a fragment of a kangaroo chromosome, possibly translocated onto a mouse chromosome, which is too small to be detected with this technique.

The remaining four hybrids, 1RMR-1 (Figure 5.5), 1RMR-2/2 (Figure 5.7), 1RMR-4/2 (Figure 5.9) and 1RMR-4/4 (Figure 5.10), contain, in addition to the X chromosome, one or two copies of a kangaroo autosome. An examination of the red kangaroo karyotype (Figure 5.3) showed that this autosome is the second largest acrocentric, number 5 in the karyotype. All four of the hybrids possess this same autosome and no other marsupial chromosomes can be detected, apart from the X chromosome. The presence of the same kangaroo autosome in four cell hybrids which possess the kangaroo X chromosome, and its absence in two other hybrids lacking the X chromosome, is clearly non-random. This observation will be discussed further in Section 5.4.

5.3 G-Banding of 1RMR Revertants

Ten of the twenty four 1RMR-1 revertants were G-banded, primarily to confirm their classification by C-banding and to provide more detailed information about the partially deleted X chromosomes. Three of these clones, 1RMR-1-R1 B6, 1RMR-1-R2 B10 and 1RMR-1-R5 A5 had been typed by C-banding as lacking an X chromosome. This was confirmed when G-banded cells were examined; a G-banded cell of 1RMR-1-R2 B10 is shown in Figure 5.11a. 1RMR-1-R2 A3 and 1RMR-1-R2 B9 possessed a chromosome classified as a

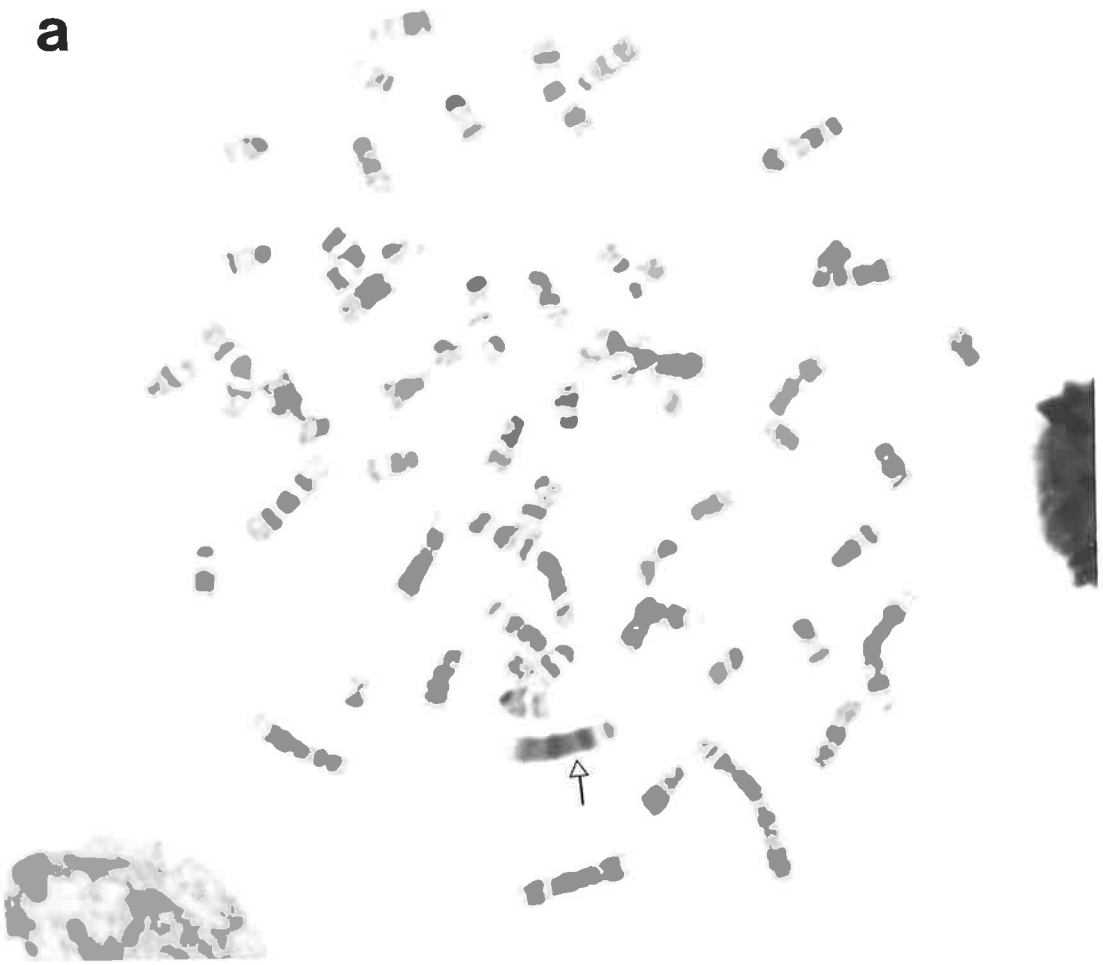
FIGURE 5.11

G-banded cells of

a) 1RMR-1-R2 B10

b) 1RMR-1-R2 B9

The partial kangaroo X chromosome is indicated by a closed arrow, the kangaroo autosome by an open arrow.


a

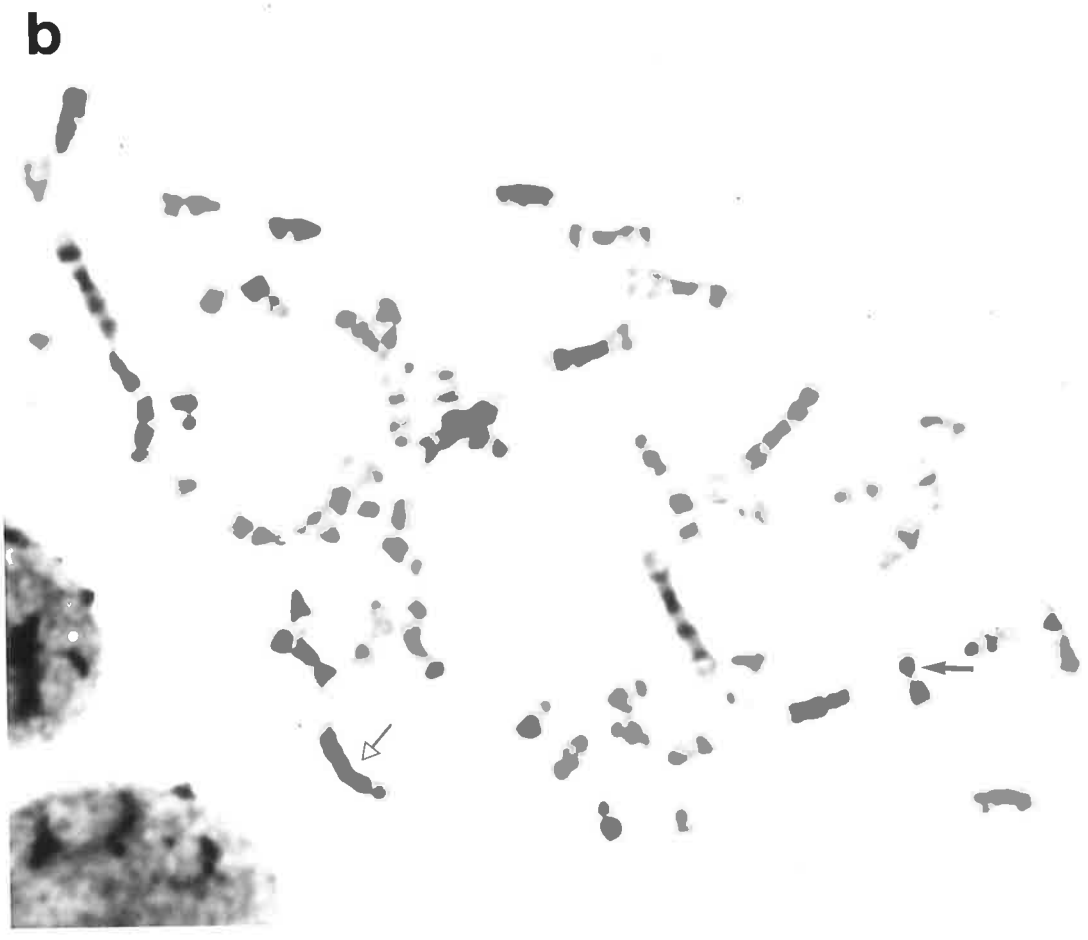

b

FIGURE 5.12

G-banded cells of

a) 1RMR-1-R3 B10

b) 1RMR-1-R5 B4

The partial kangaroo X chromosome is indicated by a closed arrow, the kangaroo autosome by an open arrow.

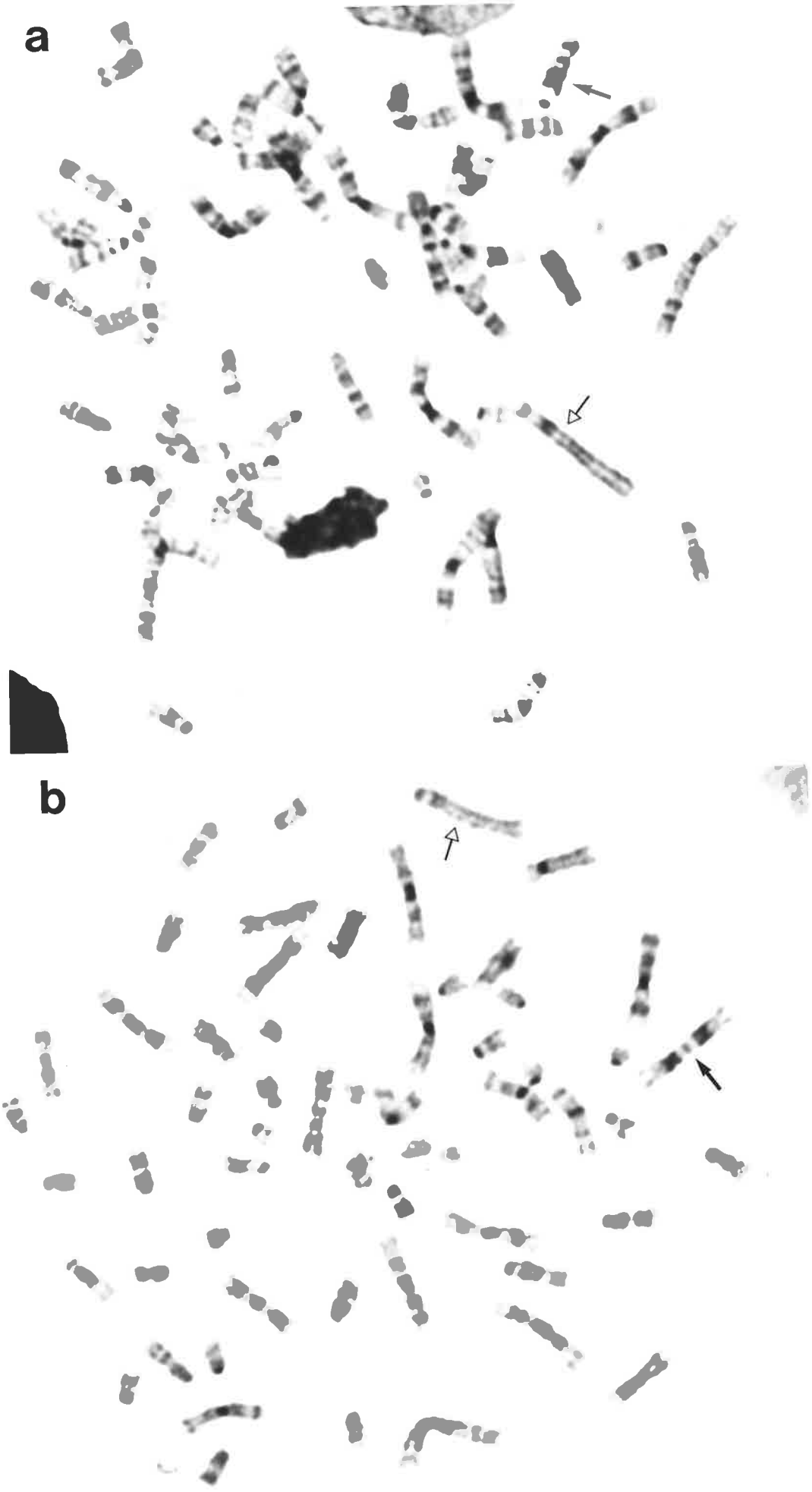

a
b

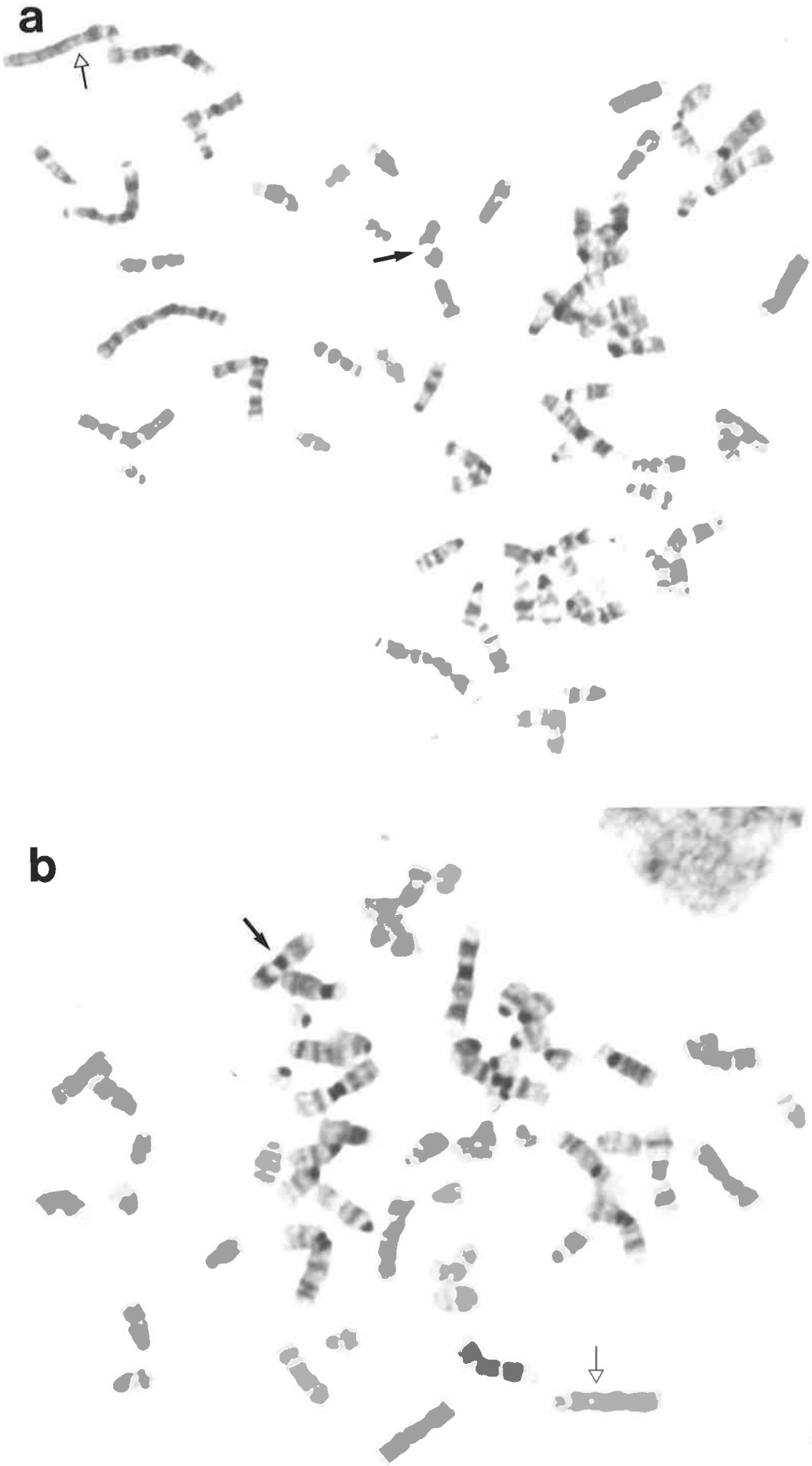
FIGURE 5.13

G-banded cells of

a) 1RMR-1-R1 B5

b) 1RMR-1-R2 A6

The partial kangaroo X chromosome is indicated by a closed arrow, the kangaroo autosome by an open arrow.


a
b

"Type II" partial X chromosome (see Figure 4.1). When cells of these revertants were G-banded, a chromosome with a banding pattern compatible with a deleted X chromosome was found. A cell of lRMR-1-R2 B9 showing this chromosome is presented in Figure 5.11b. However, the G-banding pattern of this small chromosome is not sufficiently distinctive to establish conclusively that it is indeed part of the kangaroo X chromosome. Consequently, data from revertants possessing this chromosome type were not used in constructing the red kangaroo X chromosome map (Chapter 8). For all the other partial X chromosome types, however, G-banding analysis confirmed the interpretation based on C-banding. Only the heterochromatic arm of the X chromosome was kept in lRMR-1-R3 B10 (Figure 5.12a), and this part of the X chromosome was found as an isochromosome in lRMR-1-R5 B4 (Figure 5.12b). A "Type I" partial X chromosome was demonstrated in lRMR-1-R1 A3, lRMR-1-R1 B5 and lRMR-1-R2 A6; cells of two of these revertants are shown in Figures 5.13a and b. In all of these cells, the red kangaroo X chromosome is indicated by a closed arrow. A more detailed analysis of these partial X chromosomes can be found in Chapter 8 and Figure 8.1.

The general chromosome constitution of the lRMR-1 revertants, as revealed by G-banding, is not unlike that of lRMR-1, from which they were derived. In addition to the mouse chromosomes, most of these revertants also possess the kangaroo autosome found in lRMR-1. The one or two copies of the autosome are indicated by open arrows in Figures 5.11, 5.12 and 5.13. Hence, although the presence

of this kangaroo autosome is closely correlated with that of the kangaroo X chromosome in the original hybrids, part or all of the X chromosome can be lost without affecting the subsequent retention of the autosome.

5.4 Non-random Chromosome Loss in Hybrids

One of the chief premises on which gene assignment using somatic cell hybrids has been based is that loss of chromosomes from a hybrid cell is random, and hence that segregation of one chromosome from a hybrid is independent of the segregation of any other chromosome. Thus, concordance between two biochemical markers is assumed to occur only when the genes responsible for both are located on the same chromosome.

However, it is now apparent that chromosome loss in hybrids may be far from random. Even before chromosome banding techniques were available, Nabholz *et al.* (1969), on the basis of gross chromosome morphology, and Santachiara *et al.* (1970), on the basis of enzyme markers, concluded that some chromosomes have a higher probability of retention than others. This has subsequently been confirmed in more detailed cytogenetic studies (Norum & Migeon, 1974; Rushton, 1976). As well as variation in the frequency of retention of individual chromosomes, there is also evidence for non-randomness in the occurrence of certain chromosome combinations in hybrids. Rushton (1976), in a statistical analysis of the chromosomes of fifteen different hybrid strains, found significant concordances between the presence of certain pairs of human chromosomes. The danger that

such associations between chromosomes may result in spurious genetic linkages has been pointed out by several of these authors (Nabholz *et al.*, 1969; Santachiara *et al.*, 1970; Rushton, 1976).

A case in point was the 89% concordance between two enzyme markers found by Shows and Brown (1975), which at first was used to assign the two genes to the same chromosome. It was later shown that the genes were in fact located on different chromosomes, which co-segregated in 90% of the cells. Another instance of specific association between two chromosomes has been described by Croce *et al.*, (1973) in a study of 96 human x mouse hybrid clones selected for human thymidine kinase. All clones retained human chromosome 17, on which the gene for TK is located; 82% also retained a number 7 chromosome, although this same chromosome was only kept in 7.5% of clones selected for HPRT. However, when the clones were back-selected in medium containing BrdU, chromosome 7 was not lost along with chromosome 17.

The retention of a red kangaroo autosome in these lRMR hybrids appears to be an extreme case of such specific chromosome association. Only those hybrids possessing the kangaroo X chromosome retain this autosome; the presence of the kangaroo gene for HPRT alone is not sufficient to ensure its retention. This finding, and those of the studies cited above, can only be explained in terms of selective interactions between genes located on the two chromosomes. Such interactions appear to be important mainly in the early stages of evolution of a hybrid karyotype, as loss of the

selected chromosome was not accompanied by loss of the associated chromosome, either in the 1RMR revertants, or in the back-selected clones of Croce *et al.* (1973). In the absence of a much more complete knowledge of the genetic content of certain chromosomes and the nature of gene interactions in cells in general and in somatic cell hybrids in particular, the nature of such selective interactions remains obscure. Some possible interpretations of these findings will be considered in Chapter 10.

CHAPTER 6

STABILITY OF HYBRIDS

Somatic cell hybrids between kangaroo cells and HPRT-deficient mouse cells will grow in HAT medium provided the marsupial gene for HPRT is expressed. The continual selection for the expression of this marsupial gene results in indirect selection for the presence of the kangaroo X chromosome, on which the gene for HPRT is located. In the absence of such selection pressure, this marsupial gene and the chromosome on which it is located may be lost if the hybrid genome is unstable. An investigation was made of the stability in non-selective medium of a number of the somatic cell hybrids, both those possessing and those lacking a red kangaroo X chromosome.

6.1 Stability of Hybrids With A Complete Kangaroo X Chromosome

The stability of hybrid cells containing a cytologically detectable red kangaroo X chromosome can be simply assessed by counting the number of cells containing the X chromosome, as revealed by C-banding, before and after growth in non-selective medium. The results of such an experiment on two of the hybrids are shown in Table 6.1. It can be seen that, while the frequency of cells containing the X chromosome did not change in lRMR-1, there was a significant decrease in lRMR-2/2. This was accompanied by the appearance of a few cells (4/100) containing only the heterochromatic arm of the kangaroo X chromosome.

TABLE 6.1

STABILITY IN NON-SELECTIVE MEDIUM OF TWO CELL HYBRIDS POSSESSING A COMPLETE KANGAROO X CHROMOSOME

Cell Line	No. days in non-selective medium	No. cells containing a kangaroo X chromosome	
		Before growth in non-selective medium	After growth in non-selective medium
1RMR-1	29 days	91/100	93/100
1RMR-2/2	43 days	89/100*	74/100*

* These values are significantly different,

$\chi^2_1 = 6.5$, $0.02 > P > 0.01$.

The reasons for this difference in behaviour are not clear. It is possible that the observed difference results from the greater period of time for which 1RMR-2/2 was grown in non-selective medium. An alternative explanation concerns the previous culture history of these two hybrid cell lines. 1RMR-1 was the first red kangaroo x mouse somatic cell hybrid obtained, approximately two years before 1RMR-2/2, and it has been grown in cell culture for extended periods. Consequently, 1RMR-1 would have had considerably greater opportunity than 1RMR-2/2 to evolve a stabilized genome. During this process, there may have arisen deficiencies in the mouse genome which have been complemented for by marsupial genes, thus establishing a requirement for certain marsupial genes or chromosomes to be retained.

This hypothesis could only be evaluated in the light of some understanding of the factors involved in stabilizing a hybrid genome. However, just as the mechanisms of chromosome loss in somatic cell hybrids are not understood, neither are the reasons for the eventual stability of the genome of a hybrid cell, nor the processes by which this stage is reached. It is likely that interactions are involved, both within each parental set of chromosomes and between parental sets, but the nature and dynamics of such processes are not known.

6.2 Stability of Hybrids Lacking A Complete Kangaroo X Chromosome

The stability of hybrids lacking a complete kangaroo X chromosome cannot be assessed cytologically,

and hence an alternative approach is required. The method used involved plating the hybrid cells at very low cell densities in non-selective medium and isolating subclones, which were subsequently grown in non-selective medium until sufficient cells were obtained for analysis of HPRT by electrophoresis. This process took approximately six weeks. Three somatic cell hybrid lines were analysed in this manner. Two of them, lRMR-2/1 and lRMR-2/3, possessed no cytologically detectable portion of the kangaroo X chromosome, while the third, PGMR-1/4, possessed an isolated heterochromatic arm of the X chromosome. All three hybrids expressed kangaroo HPRT, which, in lRMR-2/1 and lRMR-2/3 at least, is presumably carried on a small fragment of the X chromosome.

In the course of this subcloning experiment, it was discovered that cells of PGMR-1/4 failed to grow in standard non-selective medium. This problem was investigated by plating cells at a concentration of 10^5 cells per 60 mm Petri plate in "H", "A", and "T" media: non-selective medium supplemented respectively with hypoxanthine, aminopterin and thymidine at the concentrations used in HAT medium. The results are shown in Figure 6.1, from which it can be seen that growth is greatest in medium supplemented with thymidine and negligible in medium with aminopterin. Thus it appears that after a period of growth in HAT medium this hybrid cell line requires additional exogenous purine or pyrimidine bases. This may simply be an after-effect of an accumulation of aminopterin in these cells. Alternatively, a defect may

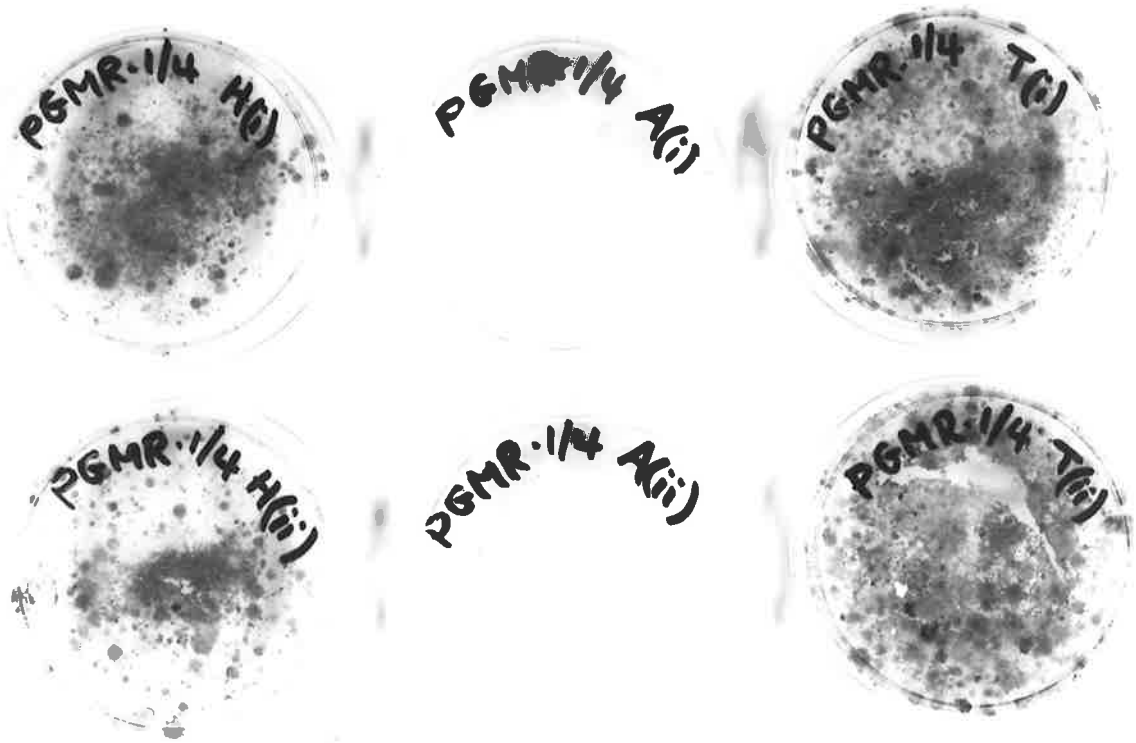
FIGURE 6.1

Petri plates containing cells of PGMR-1/4 grown in media supplemented with (from left to right)

"H" hypoxanthine

"A" aminopterin

"T" thymidine


PGMR·1/4 H(i)
PGMR·1/4 A(i)
PGMR·1/4 T(i)
PGMR·1/4 H(ii)
PGMR·1/4 A(ii)
PGMR·1/4 T(ii)

have arisen in the *de novo* purine or pyrimidine biosynthetic pathways while these pathways were inoperative in the presence of aminopterin. Consequently, future subcloning of this cell line was performed in "HT" medium, containing additional thymidine and hypoxanthine.

Cell extracts from subclones of these three hybrid lines were examined for HPRT by starch gel electrophoresis. The results, shown in Table 6.2, indicate a considerable difference in stability between the hybrids, ranging from PGMR-1/4, which appeared to be completely stable, to 1RMR-2/1, in which marsupial HPRT was not detectable in any of the subclones. However, as starch gel electrophoresis is not a very sensitive method for detecting low levels of enzyme activity, all subclones were additionally tested for HAT sensitivity, as described in Section 2.1. This involved plating cells in HAT medium at a concentration of 10^5 cells per plate and observing the subsequent growth. Results of the HAT sensitivity tests are shown in Table 6.2.

All subclones of PGMR-1/4 were HAT-resistant, as expected from their typings for HPRT. Therefore, this hybrid appears to be completely stable in the absence of selection. These subclones were C-banded and examined for the presence of the partial X chromosome, consisting of the heterochromatic arm alone, that had been found in the parent hybrid. The presence of part of the X chromosome in the hybrid had initially raised the possibility that the gene for HPRT was located in this portion. However, six of the ten subclones were found to have lost

TABLE 6.2

STABILITY IN NON-SELECTIVE MEDIUM OF THREE CELL HYBRIDS LACKING A COMPLETE KANGAROO X CHROMOSOME

Cell Line	Subclones isolated in non-selective medium	
	HPRT	Growth in HAT medium*
PGMR-1/4	10 clones $HPRT^+$	HAT resistant
1RMR-2/1	10 clones $HPRT^-$	6 clones HAT sensitive, 4 clones had a low frequency (1 in 10^3 - 10^4) of HAT resistant cells.
1RMR-2/3	5 clones $HPRT^-$	HAT sensitive
	4 clones $HPRT^+$	HAT resistant

* For details, see text.

the partial X chromosome, while continuing to express kangaroo HPRT. This suggests that the gene for HPRT is not in fact located on the heterochromatic arm of the red kangaroo X chromosome, but rather on a cytologically undetectable piece of the X chromosome which was retained independently in the original hybrid, presumably in a manner analogous to 1RMR-2/1 and 1RMR-2/3.

1RMR-2/1 showed the lowest stability of the three hybrids examined and all ten subclones appeared to lack HPRT. However, while six of these subclones were completely sensitive to HAT medium, the remainder possessed a very low frequency of HAT-resistant cells: 10 to 100 colonies out of 10^5 cells per plate, a frequency of 1 in 10^3 to 10^4. This result can be interpreted in terms of a gradual process of loss of the gene for HPRT, which has not reached completion in all subclones at the stage studied. On this basis, it would be expected that all HAT-resistant cells would disappear with further growth in non-selective medium, and hence that no stabilization of the gene for HPRT would have occurred in this hybrid. Alternatively, the few HAT-resistant cells could have resulted from stabilization occurring late during the period of growth in non-selective medium. These possibilities could be distinguished by continued growth of the subclones in non-selective medium followed by further HAT sensitivity tests, or isolation and growth in non-selective medium of some of the rare HAT-resistant colonies.

1RMR-2/3 produced two types of subclones upon growth

in non-selective medium: five clones lacking HPRT and uniformly HAT-sensitive, and four HAT-resistant clones expressing marsupial HPRT. It would appear that the gene for HPRT has become stably integrated into the hybrid genome in a proportion of the cells and is lost from the remainder when selective pressure is removed. Thus lRMR-2/3 can be seen as a mixture of cells in which the gene for HPRT is stably or unstably associated with the hybrid genome. Another possibility is that, rather than consisting of a mixture of stable and unstable cells, this hybrid is composed of a mixture of $HPRT^+$ and $HPRT^-$ cells, with the $HPRT^-$ cells being maintained in HAT medium by metabolic co-operation. This is the process, first described by Subak-Sharpe *et al.* (1966), by which molecules can be transferred between cells through intercellular contacts known as gap junctions. The transfer of the nucleotide products of the enzyme HPRT from $HPRT^+$ to $HPRT^-$ cells can result in the continued growth of $HPRT^-$ cells in HAT medium (Cox *et al.*, 1972). However, this is unlikely to affect any of the lRMR hybrids, as derivatives of mouse L cells, of which lR is one, lack gap junctions and consequently fail to engage in metabolic co-operation (Gilula *et al.*, 1972).

The mechanism by which a chromosomal fragment bearing a selectable marker is stabilized in such hybrid cells is not known. It appears likely, however, that the processes operating are similar to those involved in chromosome-mediated gene transfer. This technique uses purified metaphase chromosomes as vectors to transfer genetic

information into cultured recipient cells. Chromosome-mediated gene transfer was first convincingly demonstrated by McBride and Ozer (1973). The principal features of such experiments have been reviewed by Willecke (1978). Clones expressing transferred genes (transformants) appear to contain small fragments of the donor chromosomes, possibly in multiple copies. The donor chromosome fragments or transgenomes are usually sufficiently small to exclude other than very closely linked markers, and are not cytologically detectable. Hence these transformants appear similar in many respects to the somatic cell hybrids described here which lack an observable X chromosome.

The majority of transformants obtained in gene transfer experiments are unstable, but stabilization can occur with continued growth in selective medium. Loss of the transgenome in unstable transformants probably occurs by physical loss of the chromosomal fragment at mitosis, resulting from failure of the fragment to replicate or failure to attach effectively to the spindle (Degnen *et al.*, 1976). Klobutcher and Ruddle (1979) have examined the process of stabilization in transformants, using clones containing a fragment large enough to be cytologically detectable. They found that stabilization involves a physical association of the transgenome with a recipient chromosome by centric fusion or terminal addition. This would ensure the orderly distribution of transferred genes at mitosis. The fragment was associated with a different recipient chromosome in different stable

subclones.

When techniques are available for cytologically identifying fragments which are at present not detectable, it will be possible to determine whether similar mechanisms are involved in the stabilization of very small trans-genomes in transformants and in somatic cell hybrids, as seems probable. Although small quantities of foreign DNA can be detected in hybrid cells by molecular hybrid-ization (Rodgers, 1979), such studies do not reveal the nature of its association with the parental genome. A hypothesis of physical integration of a marsupial chromosomal fragment into a mouse chromosome in some subclones and its gradual loss at mitosis in other sub-clones would be consistent with the results obtained for the cell hybrids discussed above.

CHAPTER 7

ENZYME STUDIES

As discussed in Section 3.3, all hybrid cell lines were examined for the presence of the enzyme HPRT in order to confirm their hybrid status. In this chapter, results are presented for studies on a number of other enzymes, in particular those known to be coded for by X-linked genes in some mammals. Correlations between the presence of HPRT and the expression of other unselected markers provide evidence for syntenic relationships and can be used to map the genes coding for these enzymes. The typings of the hybrids and revertants for HPRT are presented again in Table 7.1, together with the results for some of the other enzymes, in order that comparisons may be made.

7.1 Glucose-6-Phosphate Dehydrogenase (G6PD, E.C. 1.1.1.49)

G6PD, an enzyme involved in the metabolism of pentose sugars, is found in all tissues. It is coded for by a gene on the X chromosome in man (summarized in Harris and Hopkinson, 1976) and a number of other eutherian mammals (Pearson and Roderick, 1978). The gene for G6PD has been shown to be X-linked in several species of kangaroos: in the red necked wallaby, *Macropus rufogriseus*, by population and family data (Johnston *et al.*, 1975), and in wallaroos, *Macropus robustus robustus*, euros, *Macropus robustus erubescens*, and red kangaroos by studies of interspecific hybrids (Richardson *et al.*, 1971; Johnston

TABLE 7.1

PRESENCE OF THE KANGAROO FORM OF THREE ENZYMES IN HYBRIDS AND REVERTANTS

Cell line	HPRT	G6PD	PGK-A
Hybrids:			
1RMR-1	+	+	+
1RMR-2/1	+	-	-
1RMR-2/2	+	+	+
1RMR-2/3	+	-	-
1RMR-4/1	*	-	-
1RMR-4/2	+	+	+
1RMR-4/4	+	+	+
PGMR-1/1	+	-	-
PGMR-1/4	+	-	-
Revertants:			
1RMR-1-R 24 clones	-	-	-
1RMR-2/2-R 6 clones	-	-	-

* This cell line possessed HPRT, but not of kangaroo mobility - see Section 3.3.

and Sharman, 1975). *In vitro* hybridization studies have demonstrated that G6PD is a dimer in marsupials (Johnston *et al.*, 1978), as it is in eutherians.

When the G6PD of kangaroo, mouse and hybrid cells was examined using a standard system for electrophoresis of G6PD on cellulose acetate gels (Johnston *et al.*, 1978), only a very slight difference in mobility was found between the kangaroo and mouse forms of the enzyme. As a result, it was extremely difficult to obtain reliable G6PD typings for the hybrids, some of which could be expected to show an intermediate phenotype. A similar problem was encountered by Graves *et al.* (1979) with another group of marsupial x mouse somatic cell hybrids, although there was a slightly greater separation in mobility between the mouse band and that of the G6PD allozyme in the kangaroo species that they used. In this study, a variety of electrophoretic buffers for cellulose acetate were tried, in addition to several other electrophoretic systems, including polyacrylamide electrophoresis and isoelectric focusing, but without success.

An alternative approach to distinguishing the two forms of the enzyme is to examine some of the quantitative biochemical properties of the enzyme in the two species. Preliminary experiments with heat inactivation indicated that the kangaroo form of G6PD is more stable at high temperatures than the mouse form. However, although kangaroo and mouse G6PD could be distinguished by their residual activity after 4 min exposure to a temperature of 47°C, typings of the hybrid cell lines were unreliable.

This is most probably a result of the preponderance of the mouse form of the enzyme in hybrid cells, as discussed later. Furthermore, the heat stability of the hetero-polymer in the hybrid cells cannot necessarily be assumed to be the mean of the parental types. These problems would be likely to affect any attempt to determine the G6PD phenotypes of the hybrids based on properties such as Km and substrate specificity. In addition, quantitative assays are not suitable for the routine typing of a considerable number of cell lines.

The problem was eventually solved with the development by Mr. M. Adams of a discontinuous buffer system for cellulose acetate electrophoresis (Adams and Donald, 1980). The use of discontinuous buffer systems is widespread in starch gel electrophoresis, but the principle has not been generally applied to cellulose acetate. By the simple expedient of soaking gels in a buffer that differs from the electrophoresis buffer, much greater separation was achieved between mouse and kangaroo forms of G6PD, without reducing the sharpness of the bands. The basis of this effect is not known, but the principle may have widespread applicability for revealing cryptic mobility differences between isozymes. With the discontinuous buffer system, a mouse-kangaroo heterodimer could be clearly distinguished in some hybrid cell lines (Figure 7.1). The kangaroo G6PD homodimer was much more weakly staining than the mouse form, and while it could be faintly seen in some hybrids, it was consistently undetectable in others. The results of G6PD typings for all the hybrid

FIGURE 7.1

Cellulose acetate electrophoresis of G6PD of mouse, kangaroo and hybrid cells.

Channnel 1: red kangaroo red blood cells

Channel 2: lRMR-1, a hybrid containing a complete kangaroo X chromosome.

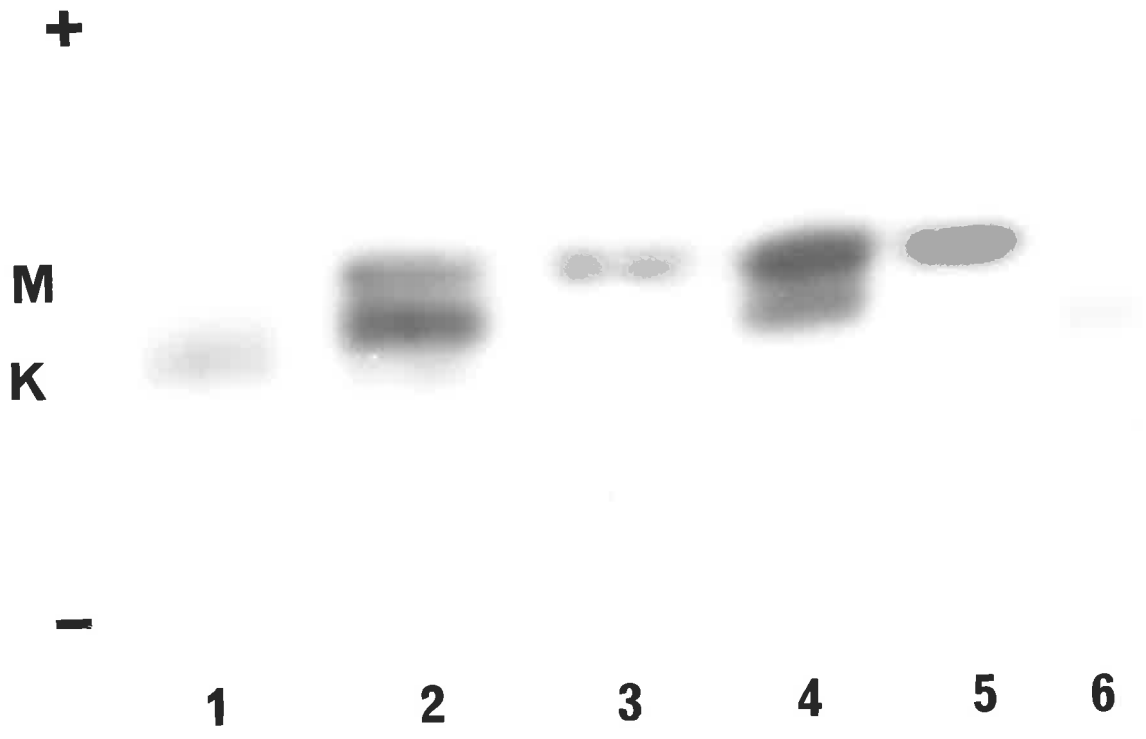
Channel 3: lR, the parental mouse cell line.

Channel 4: lRMR-2/2, a hybrid containing a complete kangaroo X chromosome.

Channel 5: lRMR-1-R2 B10, a 6TG-resistant revertant, lacking the kangaroo X chromosome.

M = mouse mobility

K = kangaroo mobility


+
M
K
−
1
2
3
4
5
6

and revertant cell lines are shown in Table 7.1. Four of the hybrids expressed the kangaroo gene for G6PD; the rest of the hybrids and all the revertants showed only the mouse form of the enzyme.

7.2 Phosphoglycerate Kinase-A (PGK-A, E.C. 2.7.2.3)

PGK-A is a glycolytic enzyme found in many tissues including red blood cells and cells in tissue culture. The enzyme is a monomer and has been shown to be determined by an X-linked gene in man (summarized in Harris and Hopkinson, 1976), and several other eutherian mammals (Warburton and Pearson, 1976). Population and pedigree studies have shown that the gene for PGK-A is also X-linked in a number of marsupial species: the eastern grey kangaroo, *Macropus giganteus*, the western grey kangaroo, *Macropus fuliginosus* (Cooper *et al.*, 1971; Vandeberg *et al.*, 1977a), the pretty-face or whiptail wallaby, *Macropus parryi* (Vandeberg *et al.*, 1973b) and the brush-tailed possum, *Trichosurus vulpecula* (Vandeberg *et al.*, 1979). There is an additional form of PGK in mammals, PGK-B, which is a minor component in all tissues except testis. PGK-B has been observed in red blood cells of a large number of marsupial species, and has been shown to be autosomally controlled (Vandeberg *et al.*, 1973a). Only PGK-A was observed in the red kangaroo red blood cells used as controls in this study.

Unlike the situation for G6PD, there was no difficulty in distinguishing between mouse and kangaroo forms of PGK-A, which possess different mobilities with conventional

FIGURE 7.2

Cellulose acetate electrophoresis of PGK-A of mouse, kangaroo and hybrid cells.

Channels 1 and 2: 1R, the parental mouse cell line.

Channels 3 and 4: 1RMR-2/2, a hybrid containing a complete kangaroo X chromosome.

Channel 5: red kangaroo red blood cells.

Channels 6 and 7: 1RMR-2/1, a hybrid lacking a detectable kangaroo X chromosome.

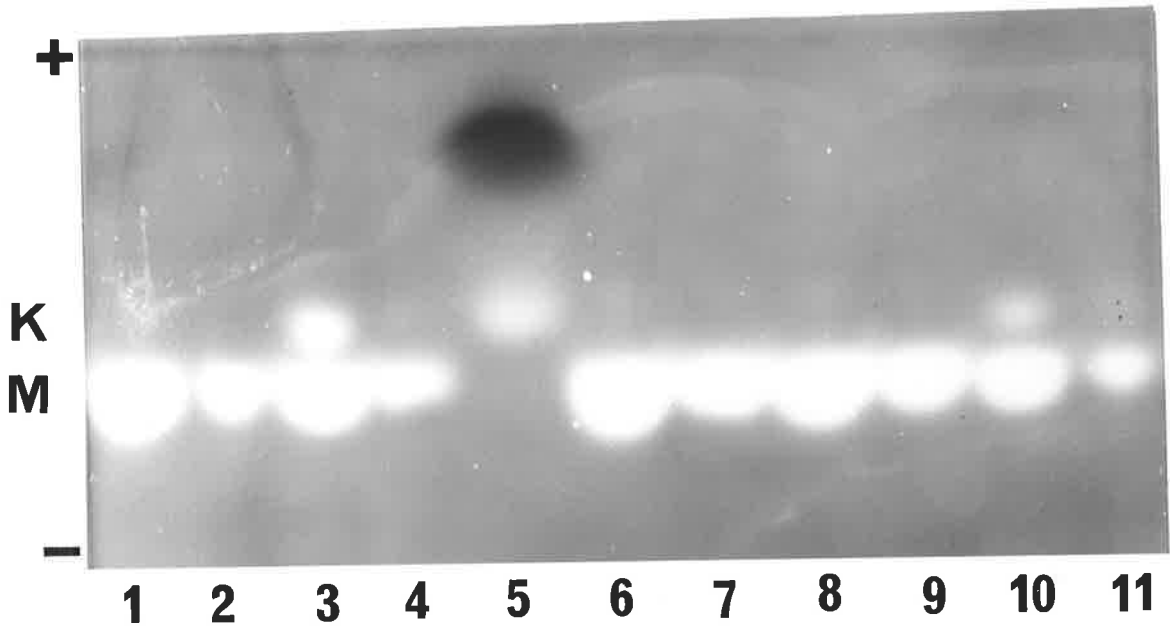
Channels 8 and 9: 1RMR-2/3, a hybrid lacking a detectable kangaroo X chromosome.

Channels 10 and 11: 1RMR-1, a hybrid containing a complete kangaroo X chromosome.

M = mouse mobility

K = kangaroo mobility

The mouse minor band with a similar mobility to kangaroo PGK-A (visible in Channels 1, 6 and 8) is sufficiently faint to avoid confusion with the kangaroo form.


+
K
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1
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10
11

cellulose acetate electrophoresis. Hastening the counter-staining procedure by rinsing the gel in hot water (D.A. Briscoe, pers. comm.) improved the resolution of the bands. As can be seen in Figure 7.2, the mouse band is much stronger than the kangaroo band in the hybrids. Hence, in order to detect the kangaroo PGK activity, samples were loaded heavily on the gel at different concentrations in adjoining channels. One potential source of difficulty was the presence of a faster migrating minor band of mouse PGK, presumably mouse PGK-B, which has very similar mobility to that of the kangaroo form of the enzyme. However, it was always considerably fainter than the kangaroo band (Figure 7.2), and did not confuse the typings. All hybrids and revertants were typed for PGK-A (Table 7.1). The typings paralleled those for G6PD in that only four of the hybrids expressed kangaroo PGK-A as well as the mouse form. The other hybrids and all the revertants tested showed only the mouse isozyme.

7.3 α-Galactosidase A (α-GAL A, E.C. 3.2.1.22)

α-Galactosidase is a lysosomal enzyme found in most tissues except red blood cells. There are two distinct forms, called α-galactosidase A and α-galactosidase B, which are determined by different gene loci. α-GAL A is a dimer and is governed by an X-linked gene in man (summarized in Harris and Hopkinson, 1976) and a number of eutherian mammals (Pearson and Roderick, 1978). There is unconfirmed evidence that this gene may also be X-linked in kangaroos (Cooper *et al.*, 1977b).

The method of Meera Khan *et al.* (1975) for cellulose acetate electrophoresis was used in an attempt to detect α-GAL A in the hybrids. Unfortunately, although a sharp band of α-GAL A was obtained from kangaroo cells, mouse α-GAL A activity was poorly resolved, and showed a long anodal smear. This is apparently caused by the presence of sialated derivatives of the enzyme, and has been observed in other species (Harris and Hopkinson, 1976). As mouse α-GAL A migrates faster than the kangaroo enzyme, the presence of this smear makes it impossible to detect a kangaroo band in the hybrids.

Two approaches could be used to overcome this problem and enable the gene for α-GAL A to be mapped in kangaroos. One is to construct somatic cell hybrids between red kangaroo cells and a eutherian cell line possessing a more clearly resolved form of α-GAL A. Chinese hamster cell lines have been used for studies of this enzyme (Meera Khan *et al.*, 1975), and one line in particular, E36, has been shown to possess a single sharp band of α-GAL A activity. Repeated attempts to obtain this cell line for hybridizations were, regrettably, unsuccessful. Alternatively, antibodies specific for kangaroo α-GAL A could be used to detect the presence of the kangaroo enzyme in kangaroo x mouse cell hybrids. Such a method was used by Hamers *et al.* (1977) to investigate the expression of human α-GAL A in cell hybrids. Preparation of a suitable antibody was not feasible, however, in the time available for this project.

7.4 5-Phosphoribosyl-1-pyrophosphate synthetase (PRPP synthetase, E.C. 2.7.6.1)

PRPP synthetase is an enzyme involved in purine metabolism. The gene for this enzyme has recently been assigned to the X chromosome in man by family data (Yen *et al.*, 1978) and somatic cell genetic studies (Becker *et al.*, 1979). No reports have yet been published on the chromosomal assignment of this gene in other mammals.

It would be of interest to determine whether the gene for PRPP synthetase is located on the X chromosome in marsupials. Consequently, an attempt was made to detect PRPP synthetase activity in these kangaroo x mouse hybrid cells, using the technique for cellulose acetate electrophoresis described by Yen *et al.* (1978). This technique relies on comparisons between strips stained with and without the substrate PRPP to distinguish PRPP synthetase activity from a considerable amount of non-specific staining. One gel showed a very faint band of possible PRPP synthetase activity, but the activity of this rather unstable enzyme (Johnson *et al.*, 1974) was too low, and the resolution insufficient, for possible differences between the mouse and kangaroo forms to be detected. Until a better electrophoretic system is developed it may be necessary to use an antibody directed against PRPP synthetase to type for this enzyme, as was done by Becker *et al.* (1979).

7.5 Other Enzymes

The presence of a marsupial autosome in some of the hybrid cell lines raised the possibility of assigning autosomal genes to a particular chromosome. To date there have been no such assignments in marsupials, and any information on the location of genes on specific chromosomes would be a very useful starting point for a chromosome map.

To this end, cellulose acetate electrophoresis was used to examine some of the somatic cell hybrids for the presence of 22 enzymes known to be coded for by autosomal genes in eutherian mammals. The preliminary screening was kindly performed by Mr M. Adams of the Institute of Medical and Veterinary Science. Sixteen of these enzymes were detectable in the hybrid cells, but with one exception, the hybrid cell lines did not show any bands not present in the mouse parent. This does not necessarily imply that the structural genes for these 15 enzymes cannot be located on this chromosome, for reasons discussed in Section 7.6. Rather, the only conclusion that can be drawn is that none of the genes responsible can be assigned to the marsupial autosome on the basis of these data.

The exceptional enzyme of those tested was lactate dehydrogenase (LDH, E.C. 1.1.1.27). LDH is a key enzyme in carbohydrate metabolism and is found in almost all tissues. The enzyme is a tetramer, and is made up of two different kinds of subunits, A and B. Various combinations of these subunits produce a five-banded

pattern upon electrophoresis, the relative amounts of the different isozymes varying markedly between tissues and during development (Markert, 1968). The two types of subunits are coded for by genes on different chromosomes in man: the gene for LDH-A is located on chromosome 11 (Boone *et al.*, 1972) and the gene for LDH-B on chromosome 12 (Chen *et al.*, 1973). These genes have also been located on autosomes in a number of other eutherian mammals (Pearson and Roderick, 1978). A third locus, that for LDH-C, is active only in spermatocytes. A comprehensive study of the LDH isozymes of species from five families of marsupials by Holmes *et al.* (1973) showed a high correlation between the LDH isozymes of marsupials and eutherians in biochemical and immunological properties, subunit structure and tissue distribution. Variations in LDH isozyme patterns in erythrocytes have been used as a basis for discussions of marsupial phylogeny (Clarke, 1972; Holmes *et al.*, 1973).

When LDH of the parental and hybrid cells was subjected to cellulose acetate electrophoresis, a clear difference between kangaroo and mouse was apparent in the mobility of the A_4 isozyme, which is the predominant form in cultured fibroblasts and the liver extract used as a control (Figure 7.3). Some of the hybrids showed a distinct multiple banded pattern for the A_4 isozyme, which resulted from the formation of hybrid molecules. The kangaroo form stained much less intensely than mouse, as was noted for G6PD and PGK-A. A faint sub-band could also be seen for the A_3B isozyme.

The results of typing all the 1RMR hybrids and

FIGURE 7.3

Cellulose acetate electrophoresis of LDH of mouse, kangaroo and hybrid cells.

Channel 1: 1RMR-2/1, a hybrid lacking a detectable kangaroo X chromosome or any kangaroo autosome.

Channel 2: 1RMR-2/2, a hybrid containing a complete kangaroo X chromosome and a kangaroo autosome.

Channel 3: red kangaroo liver

Channel 4: 1RMR-1-R2 B10, a 6TG-resistant revertant lacking a kangaroo X chromosome, but containing a kangaroo autosome.

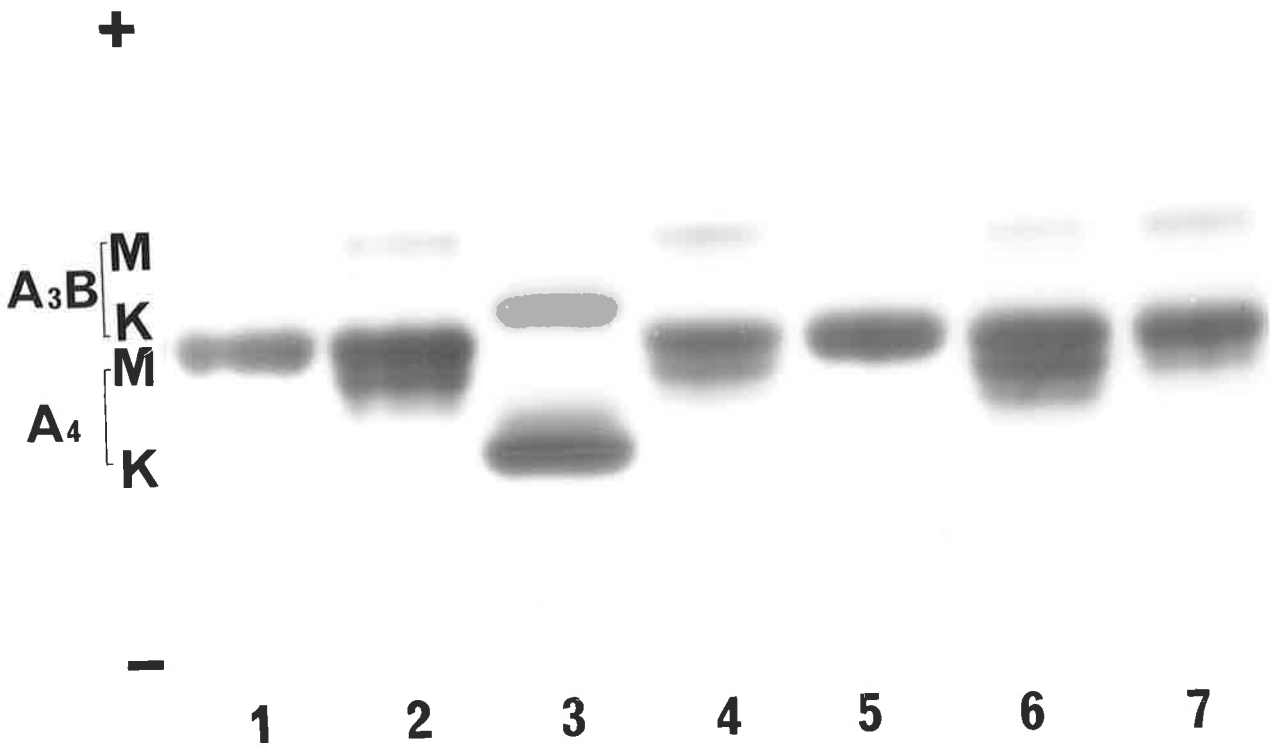
Channel 5: 1R, the mouse parental line.

Channel 6: 1RMR-14/4, a hybrid containing a complete kangaroo X chromosome and a kangaroo autosome.

Channel 7: 1RMR-1-R5 A5, a 6TG-resistant revertant lacking a kangaroo X chromosome, but containing a kangaroo autosome.

M = mouse mobility

K = kangaroo mobility


+
A3B
M
K
A4
M
K
−
1
2
3
4
5
6
7

selected 1RMR-1 revertants for this enzyme are shown in Table 7.2, in which the marsupial chromosome constitutions of the cell lines are also presented. All the cell lines tested express kangaroo LDH-A, except for two cell hybrids that lack both the kangaroo X chromosome and the kangaroo autosome. The expression of marsupial LDH-A in revertants of 1RMR-1 that entirely lack the kangaroo X chromosome, but possess the autosome, establishes that the gene for LDH-A must be located on this marsupial autosome.

The assignment of the gene for LDH-A to chromosome 5 of the red kangaroo complement is the first autosomal gene assignment in a marsupial. A number of possibilities for future work are immediately suggested. One is to examine these hybrid and revertant cells for the expression of genes known to be syntenic with LDH-A in man and other mammals, to determine whether such syntenies are found in the red kangaroo. This will provide an opportunity for examining the conservation of a linkage group over a greater evolutionary distance than has previously been possible. In addition, the location of a genetic marker on this chromosome, which is present as an entity recognizable by G-banding not only in kangaroos, but also in members of the three other families belonging to the superfamily Phalangeroidea (Rofe, 1979), should provide a starting point for autosomal chromosome maps in many other marsupial species. It may even be possible to use this genetic marker as a means of extending the mapping to other marsupial chromosomes, as this chromosome has been involved in centric fusions with three other acrocentric chromosomes

TABLE 7.2

PRESENCE OF KANGAROO CHROMOSOMES AND THE KANGAROO FORM OF LDH-A IN HYBRIDS AND REVERTANTS

Cell Line	Kangaroo chromosomes		Kangaroo Form of LDH-A
	X Chromosome	Autosome	
Hybrids:			
1RMR-1	+	+	+
1RMR-2/1	-	-	-
1RMR-2/2	+	+	+
1RMR-2/3	-	-	-
1RMR-4/2	+	+	+
1RMR-4/4	+	+	+
Revertants:			
1RMR-1-R2 B10	-	+	+
1RMR-1-R5 A5	-	+	+

in different species of kangaroos. Such data will also allow the conclusion, based on banding studies, that marsupial evolution has been characterized by the conservation of large blocks of genetic material, often whole chromosome arms (Rofe, 1979), to be assessed at a genetic level.

7.6 General Considerations.

Enzyme studies in somatic cell hybrids, such as those discussed in this chapter, pose a number of problems in interpretation. While the detection of an isozyme provides clear evidence that the gene responsible is present and functioning, failure to do so may have a number of different implications.

In a substantial proportion of cases, it may not be possible to distinguish the isozyme of one parental species from that of the other. Although such a problem could be expected to be reduced in cell hybrids between species widely separated on an evolutionary scale, the difficulties encountered with G6PD in this study demonstrate that it can still be significant. Even if electrophoretic mobility differences do exist, problems of resolution, such as those found for α-GAL A, may interfere with typings. Several of the autosomal enzymes examined by M. Adams fall into this class of enzymes for which no useful information can be obtained.

Leaving aside non-informative enzyme markers, there remain those for which the two parental forms can be clearly distinguished. Absence of an isozyme in this case may

signify absence of the structural gene. On the other hand, it could also result from complete or partial repression of the structural gene. The markedly lower activities of the kangaroo forms of G6PD (Figure 7.1), PGK-A (Figure 7.2) and LDH-A (Figure 7.3) when compared to the mouse isozyme support this concept. Some of this difference in activity may be attributed to a gene dosage effect, although the observed difference is considerably greater than the ratio of two mouse X chromosomes to one kangaroo X chromosome observed in these hybrid cells. Admittedly, in a highly variable cell line such as 1R it is not possible to be certain that rearrangements have not resulted in duplication or changes in regulation of mouse genes. Nonetheless, the consistent difference between 1RMR-1 and 1RMR-2/2 in the strength of the G6PD homodimer (Figure 7.1) suggests that modulation of expression of kangaroo genes occurs in a hybrid cell environment. Whether this difference between the hybrids reflects differing control over expression of the gene for G6PD or differing metabolic states of the cells at the time at which extracts were made is not clear.

The result of these considerations is that caution must be exercised in interpreting absence of a gene product as indicating absence of the corresponding gene. Because regulation of expression is likely to differ between different hybrid cell lines depending on their genomic balance, typing a large number of independent hybrid clones provides some safeguard against "false negatives". The consistency of the results obtained for the G6PD and PGK-A

typings both between gels and between similar but independent hybrids and revertants argues for their validity. Hence the absence of these two enzymes in a number of the cell lines can be reasonably assumed to result from loss of the corresponding structural genes. It is on this assumption that the map of the red kangaroo X chromosome is based.

CHAPTER 8

MAP OF THE RED KANGAROO X CHROMOSOME

8.1 Construction of Map

A syntenic relationship between the kangaroo genes for G6PD and PGK-A can be inferred from the correlation found between the presence of these two enzymes in the hybrids. Two approaches can be used to extend the data and determine a chromosomal assignment for these genes, and the gene for HPRT. One is to combine the chromosomal assignment of one of the markers, previously established by conventional genetic means, with the observed synteny to deduce that all genes syntenic with the marker must also be located on the same chromosome. This method was used by Graves *et al.* (1979) in their study of hybrids containing part of the wallaroo X chromosome. They concluded that the genes for HPRT and PGK-A were X-linked in the wallaroo because they were syntenic with a known X-linked gene in this species, that for G6PD.

A more direct approach employs correlations between the presence of a particular chromosome or part of a chromosome and the presence of specific gene products in cell hybrids and revertants. This is, of course, only possible when the chromosome in question can be cytologically identified, as was the case for these kangaroo x mouse hybrids. The cytological and biochemical data for the hybrids and revertants are summarized in Table 8.1. Only cell lines which had been G-banded are included in this analysis, to avoid any possible ambiguities in inter-

TABLE 8.1

PRESENCE OF KANGAROO CHROMOSOMES AND ENZYMES IN HYBRIDS AND REVERTANTS.

Cell Line	Kangaroo Chromosomes		Kangaroo Enzymes		
	X Chromosome	Autosome	HPRT	G6PD	PGK-A
Hybrids:					
lRMR-1	+	+	+	+	+
lRMR-2/1	-	-	+	-	-
lRMR-2/2	+	+	+	+	+
lRMR-2/3	-	-	+	-	-
lRMR-4/2	+	+	+	+	+
lRMR-4/4	+	+	+	+	+
Revertants:					
lRMR-1-R (24 clones)	Part*	+	-	-	-

* See Figure 8.1

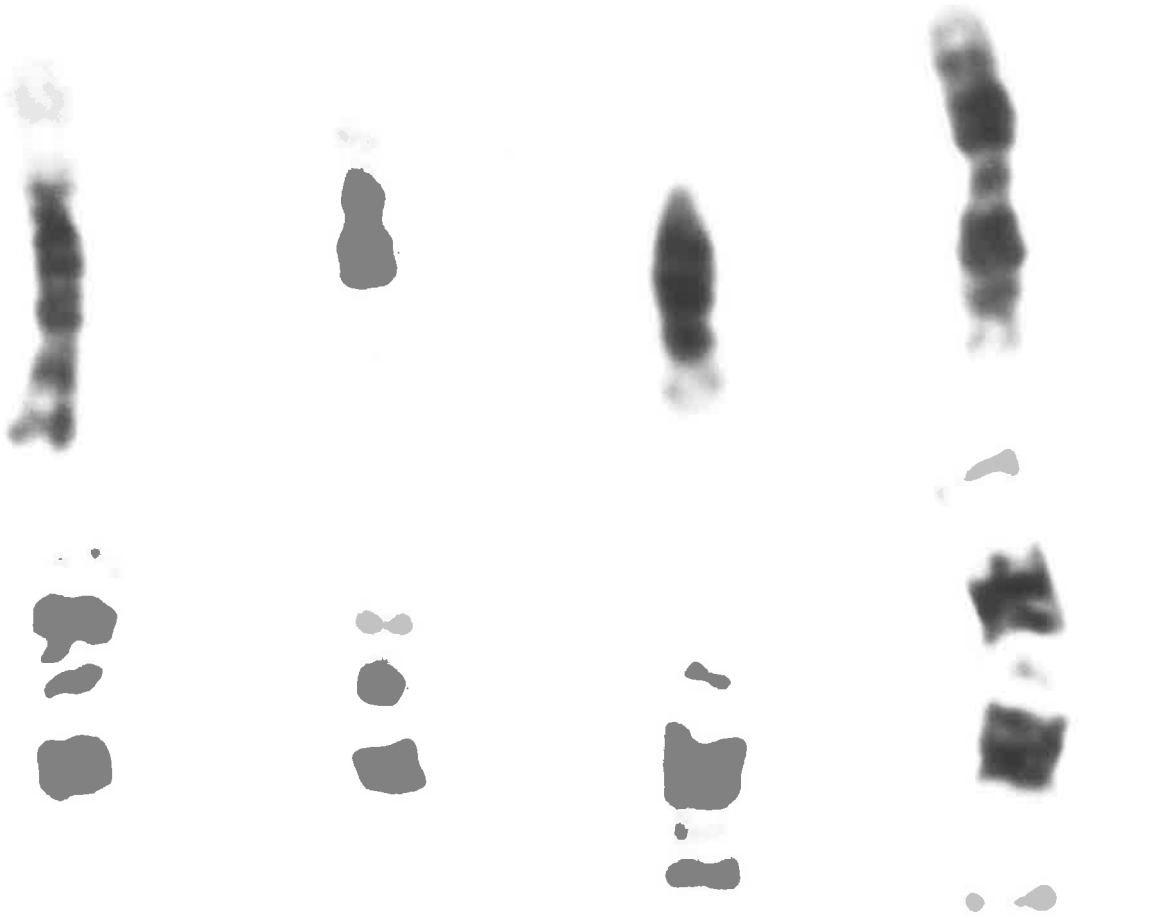
pretation of the cytogenetics.

The close correlation between the presence of the X chromosome and a kangaroo autosome in the hybrids could have caused problems in assigning these genes. However, the presence of the autosome in revertants which do not express the three kangaroo enzymes shows that the genes reponsible are not located on this chromosome. There is, on the other hand, perfect concordance between presence of the X chromosome and expression of G6PD and PGK-A in the hybrids. The situation is slightly more complicated for HPRT, as some of the hybrids appear to possess an undetectable chromosome fragment bearing this gene. However, the loss of HPRT expression in the revertants of lRMR-l together with the absence of G6PD and PGK-A and loss of part of the X chromosome confirms that the gene for this enzyme is also X-linked. Thus, the data in Table 8.1 establish that the genes for HPRT, G6PD and PGK-A are located on the X chromosome in the red kangaroo.

The position of these three genes on the red kangaroo X chromosome can be determined by a more detailed examination of the partial X chromosomes found in some of the revertant clones. Some of these X chromosome types, as visualized by both C-banding and G-banding techniques, are shown in Figure 8.1. A complete red kangaroo X chromosome is also included for comparison. Under each partial X chromosome are listed the cell lines containing this chromosome type, as confirmed by G-banding analysis. A number of independent revertant clones were G-banded in

FIGURE 8.1

C-banded (top row) and G-banded (bottom row) red kangaroo X chromosomes from 1RMR hybrids and revertants.



1RMR-1

1RMR-2/2

1RMR-4/2

1RMR-4/4

1RMR-1-R1 A3

1RMR-1-R1 B5

1RMR-1-R2 A6

1RMR-1-R3 B10

1RMR-1-R5 B4

case the break points in some of the deleted X chromosomes varied between clones; however, chromosomes classified as belonging to a particular type appeared identical within the limits of resolution of the G-banding technique. An examination of the partial X chromosomes shows immediately that the three genes in question cannot be located in the heterochromatic long arm of the X chromosome. Neither are they in the proximal two-thirds of the euchromatic arm. Consequently, the three genes can be localized to the terminal third of the euchromatic arm of the red kangaroo X chromosome, as illustrated in Figure 8.2.

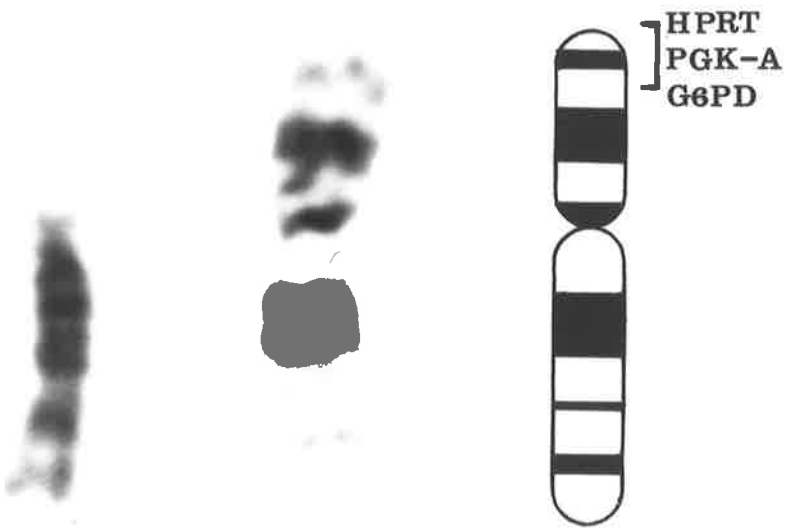
This study yields no information on the order of the genes within this segment of the X chromosome, owing to the lack of break points between genes. Hence, it is not possible to confirm the tentative gene order of HPRT - PGK-A - G6PD suggested by Graves *et al.* (1979). In contrast to their study, however, the chromosomal location of these three genes has been directly demonstrated in a marsupial for the first time.

8.2 Comparison With Other Species

It is of interest to compare this map of the red kangaroo X chromosome with those available for other species. The human X chromosome is undoubtedly the best characterized in terms of localization of genes, and serves as a point of reference for comparisons. At the date of the report by Miller *et al.* (1978b) the four genes for HPRT, G6PD, PGK-A and α-GAL A had been assigned to the long arm of the X chromosome and a provisional order established: centromere - PGK-A - α-GAL A - HPRT - G6PD. This order has

FIGURE 8.2

The red kangaroo X chromosome, showing (left to right) the C-banding pattern, G-banding pattern and a diagrammatic representation of the G-banding pattern, on which the location of the genes for HPRT, PGK-A and G6PD is indicated.


HPRT
PGK-A
G6PD

now been confirmed, and PRPP synthetase assigned to the region between α-GAL A and HPRT (preliminary report of the Committee on the Genetic Constitution of the X and Y Chromosomes, 5th International Human Gene Mapping Workshop, Edinburgh, 1979).

Among the other mammalian species, considerable attention has been paid to the group of non-human primates. Not unexpectedly, extensive homologies have been found between the chromosome maps of man and a number of these species, including the chimpanzee, gorilla, orangutan, rhesus monkey, baboon and African green monkey (Seuánez (1979). However, from an evolutionary point of view, it is of interest to examine conservation of linkage groups over the greater evolutionary distance separating man from non-primate mammals, and it is on other mammalian species that the following discussion concentrates.

Information is now accumulating rapidly for mammalian species other than primates. At the time of the 4th International Human Gene Mapping Workshop in 1977, the mouse was the only species in which the four genes for HPRT, G6PD, PGK-A and α-GAL A had been shown to be X-linked. X-linkage of three of these genes had been demonstrated in two species, and X-linkage of two genes in a further three species (Pearson and Roderick, 1978). Preliminary reports from the 5th International Human Gene Mapping Conference, Edinburgh, 1979, have considerably expanded this list (summarized by Pearson and Roderick in the report of the Committee on Comparative Mapping). HPRT, G6PD, PGK-A and α-GAL A have now been shown to be coded

for by X-linked genes in the mouse, rat, rabbit, cattle and sheep. X-linkage of the genes for HPRT, G6PD and PGK-A has been established in the Chinese hamster, horse and pig, and of the genes for HPRT and G6PD in the cat, dog and Indian muntjac. No exceptions have yet been found to the principle of conservation of X-linkage of certain genes (Ohno, 1967). With the exception of the mouse, in which assignment of genes to the X chromosome has been achieved by a variety of techniques, including somatic cell genetic, family and gene dosage studies, demonstration of X-linkage of these genes in other species has largely relied on the use of somatic cell hybrids. In some cases, only a syntenic relationship between the genes has been established, without direct proof of location of the genes on the X chromosome.

Although there are now many species in which genes have been assigned to the X chromosome, information on the localization of the genes within this chromosome is very limited. The gene for HPRT has been localized to the terminal part of the short arm of the Chinese hamster X chromosome, which possesses a heterochromatic long arm (Farrell and Worton, 1977). In the field vole, which also has an X chromosome with a heterochromatic long arm, the genes for HPRT and G6PD are on the euchromatic short arm (Cook, 1975). Although Shows *et al.* (1976) were not able to localize genes to a particular region of the X chromosome of the Indian muntjac, their finding that HPRT and G6PD segregated concordantly in hybrids that showed breakage of muntjac chromosomes, while PGK-A did not, suggests

that the genes for HPRT and G6PD are located closer to each other than either is to the gene for PGK-A in this species.

The mouse X chromosome is the only one for which any gene order can be deduced. Francke and Taggart (1979) have recently assigned the gene for HPRT to the proximal two-thirds of the mouse X chromosome, in the region designated Xcen - XD. The gene for α-GAL A had previously been localized to a distal part of the X chromosome, XE - XF (Francke *et al.*, 1977). Consequently, the order of genes must be centromere - HPRT - α-GAL A. This order is inverted compared to that on the human X chromosome, and provides the first evidence that the arrangement of genes on the X chromosome has not necessarily been conserved during mammalian evolution.

The map of the red kangaroo X chromosome does not furnish any further information on the question of conservation or rearrangement of the order of genes on the X chromosome. However, the red kangaroo is now one of the few mammalian species for which the position of genes on the X chromosome has been determined. Regional mapping of the X chromosomes of many other species will be necessary before useful comparisons can be made between the X chromosome maps of various mammals.

CHAPTER 9

HYBRIDS SELECTED FOR THYMIDINE KINASE

The major concern of this research project was with somatic cell hybrids selected for the expression of the kangaroo form of HPRT. However, a subsidiary investigation was also undertaken in which hybrids were selected for thymidine kinase. Thymidine kinase (TK; E.C.2.7.1.75) is an enzyme of nucleotide metabolism which can be selected for with the HAT selective system, as was discussed in Section 3.1. The gene coding for TK has been assigned to an autosome in man (Miller *et al.*, 1971), several other primates (Orkwiszewski *et al.*, 1976) and the mouse (Kozak and Ruddle, 1977; McBreen *et al.*, 1977). Its location in marsupials is not known. Consequently it was of interest to attempt to obtain hybrids which could be useful for assigning this gene to a chromosome in the red kangaroo and determining its syntenic relationships. The results of this investigation are only preliminary, as sufficient time was not available to pursue the topic in more detail. Reference will also be made in this chapter to the results of Mr. B. Wainwright (Honours thesis, University of Adelaide, 1979), who undertook a related study using the dasyurid marsupial, *Sminthopsis crassicaudata*.

9.1 Isolation and Behaviour of Hybrids and Revertants

Twelve fusions were performed between TK-deficient rodent cells and kangaroo lymphocytes. Three rodent cell lines were used: two mouse lines, 3T3 and Cl 1D, and one

Chinese hamster line, BIo. The results of these fusions are summarized in Table 9.1. Six of the twelve fusions were contaminated as a result of the difficulty of obtaining kangaroo blood in sterile conditions. Of the remainder, four yielded no hybrid colonies at all. In one fusion between kangaroo cells and Cl 1D, two colonies were found which were composed of large, flat cells, unlike either parent in morphology. These colonies grew well initially, but after they were isolated and transferred to new vessels, their growth rate diminished and they subsequently died. This behaviour appears to be similar to that of the "aberrant colonies" described by Hope and Graves (1978b) in their marsupial x eutherian fusions. Similar difficulties with fusions between marsupial cells and TK-deficient mouse cells had been experienced previously by Dr. R.M. Hope (pers. comm.). The reason why these fusions were so much less successful than those involving HPRT-deficient cell lines is not clear, although the results would suggest that marsupial cells are unable to complement adequately the defect found in some rodent cells.

Despite these problems, one colony was eventually obtained from a fusion between kangaroo cells and the Chinese hamster cell line, BIo. This clone, BMR-1, grew vigorously and was very similar in morphology to the hamster parent. BMR-1 was back-selected in medium containing BrdU in order to obtain revertants lacking expression of TK. Unexpectedly, most cells of BMR-1 grew in media containing BrdU at concentrations between 30 µg/ml and 200 µg/ml. Although no counts were made, it appeared that

TABLE 9.1

FUSIONS BETWEEN KANGAROO CELLS AND TK-DEFICIENT RODENT CELLS

Parental Cells	Fusion No.	No. Flasks	No. Cells per Flask Kangaroo	Rodent	Results
M. rufus					
x 3T3	TMR 1	10	2.5×10^6	0.5×10^6	Contaminated
	TMR 2	9	4.7×10^6	1.25×10^6	No colonies
M. rufus					
x Cl 1D	DMR 1	5	2.1×10^6	0.8×10^6	No colonies
	DMR 2	5	3.8×10^6	1.0×10^6	2 aberrant colonies*
	DMR 3	6	2.4×10^6	0.45×10^6	Contaminated
M. rufus					
x BIo	BMR 1	6	1.75×10^6	0.4×10^6	Contaminated
	BMR 2	6	1.9×10^6	0.6×10^6	Contaminated
	BMR 3	6	1.9×10^6	0.6×10^6	1 Colony
	BMR 4	5	2.0×10^6	0.7×10^6	No colonies
	BMR 5	5	1.5×10^6	0.6×10^6	Contaminated
	BMR 6	8	2.0×10^6	0.6×10^6	Contaminated
	BMR 7	7	1.9×10^6	0.9×10^6	No colonies

* see text

more than half of the cells were capable of growth in the presence of BrdU. This is in contrast to the 1RMR hybrids which had reversion rates of 1×10^{-4} to 1×10^{-5}. Reversion rates of a similar order of magnitude to that for BMR-1 were also found in four colonies, called BIS, obtained from fusions between *Sminthopsis crassicaudata* and BIo by B. Wainwright (Table 9.2).

The very high apparent reversion rate of these clones raised the possibility that the putative hybrids did not express TK, but had acquired resistance to HAT medium by other means, such as membrane changes affecting uptake and transport of aminopterin. This hypothesis was tested by treating BMR-1 cells, which had been growing in HAT medium, with ^{14}C-thymidine for six hours and making autoradiographs. When these plates were developed and examined, the nuclei of most of the cells (39/50) were heavily labelled, as illustrated in Figures 9.1a and b. This indicates that ^{14}C-thymidine is taken up by these cells, and hence that thymidine kinase is present. The few unlabelled or lightly labelled cells observed were presumably either dead or not in an appropriate stage of the cell cycle to take up ^{14}C-thymidine. Revertant cells (called BMR-1-R) obtained by growth of BMR-1 in BrdU, were unlabelled when grown in non-selective medium or medium with BrdU and treated similarly (Figures 9.1c and d), confirming that TK is not expressed in these cells.

Although autoradiography established that BMR-1 possessed TK activity, it did not exclude the possibility that this was the hamster form of the enzyme, which had

TABLE 9.2

BACK-SELECTION AND SUBSEQUENT HAT SENSITIVITY TESTS OF COLONIES FROM BIo X MARSUPIAL FUSIONS

HAT-resistant Colonies	Reversion Rate (Growth in BrdU)	Growth of Revertants in HAT
BMR-1	>50%	2.5×10^{-4}
BIS* 4 clones	6% - 60%	-

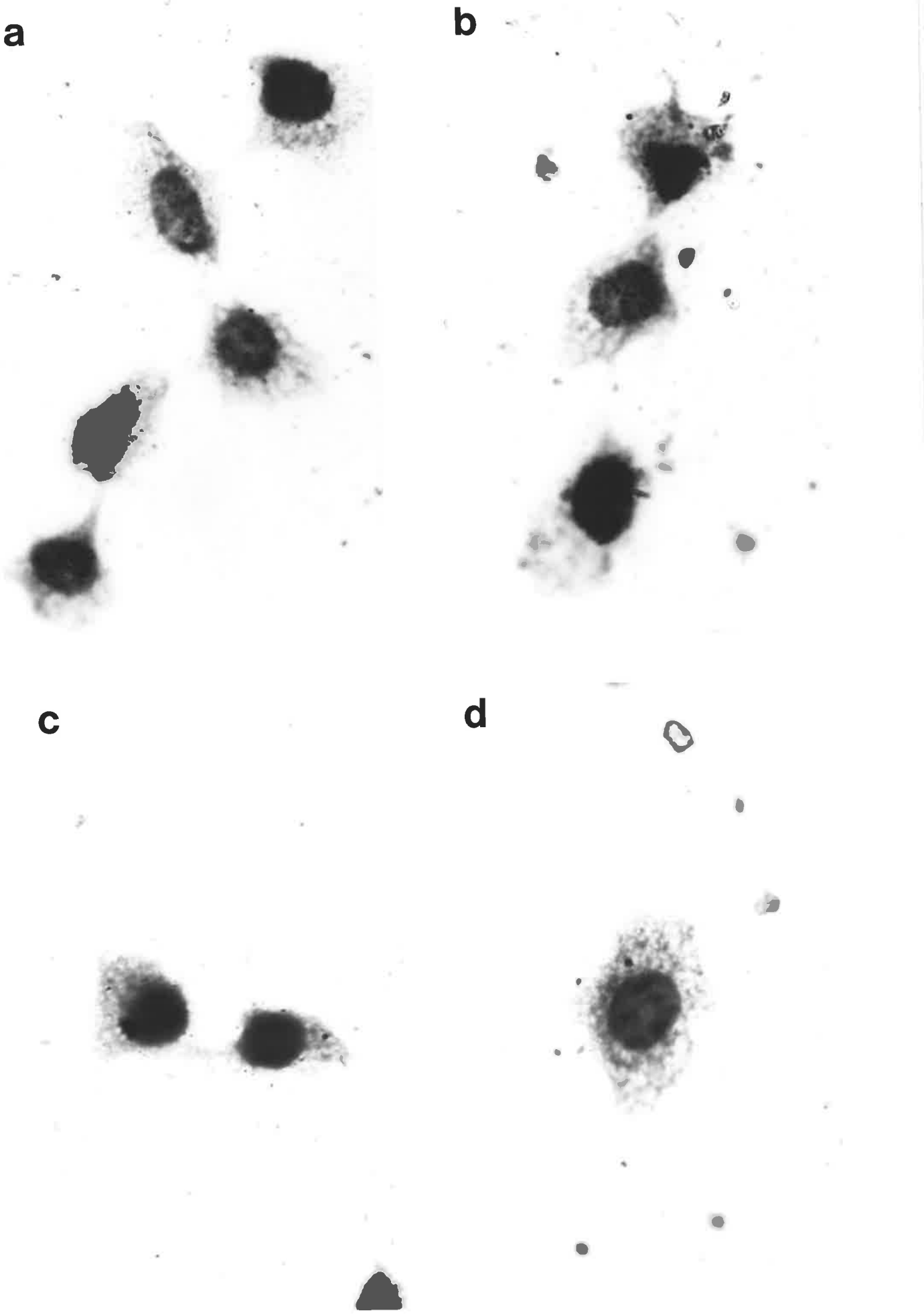
* Data of B. Wainwright; see text.

FIGURE 9.1

Autoradiography of cells grown in the presence of ^{14}C-thymidine.

a) and b): cells of BMR-1, showing heavily labelled nuclei.

c) and d): cells of BMR-1-R, showing unlabelled nuclei.


a
b
c
d

been re-expressed in an analogous manner to the re-expression of mouse HPRT in lRMR-4/1 (Section 3.3). In this case, the very high frequency of growth of BMR-1 cells in medium with BrdU could be seen as a result of the hamster gene simply being "switched off" again. Alternatively, the hybrid could possess a kangaroo gene for TK which is repressed in the hybrid cell in the presence of BrdU. Irrespective of its origin, if the gene for TK in BMR-1 is capable of being reversibly expressed, one would expect that revertant cells growing in BrdU should also grow in HAT medium. When BMR-1-R cells were plated in HAT medium, this was found not to be the case. Twenty-five HAT-resistant colonies (designated BMR-1-R-H) appeared from 10^5 cells plated (Table 9.2). No HAT-resistant colonies were found when the same experiment was performed by B. Wainwright.

These results suggest that it is loss of the gene for TK, rather than repression, which accounts for the growth of the putative hybrid clones in the presence of BrdU. Why the loss of TK expression should occur so much more rapidly and completely in these clones, as compared to reversion rates for the lRMR hybrids, is difficult to understand. The most immediate explanation is that the mode of integration of the gene for TK into the genome differs from, and is considerably less stable than, that of many other hybrids, including the lRMR hybrids. However, this explanation poses almost as many questions as it answers, and is not supported by preliminary investigations on the stability of BMR-1. There was no obvious decrease in the capability

of BMR-1 cells to grow in HAT medium after they had been grown in non-selective medium for a week. If the gene for TK is extremely unstable in BMR-1, a much more rapid loss of expression in non-selective medium might have been expected. The significance of the finding of a very low frequency of HAT-resistant cells in BMR-1-R is also unclear.

None of the investigations of the behaviour of BMR-1 discussed above demonstrated conclusively that this clone is a hybrid containing the kangaroo gene for TK. Cytogenetic and enzyme studies were therefore undertaken in an attempt to establish this point.

9.2 Cytogenetic Studies

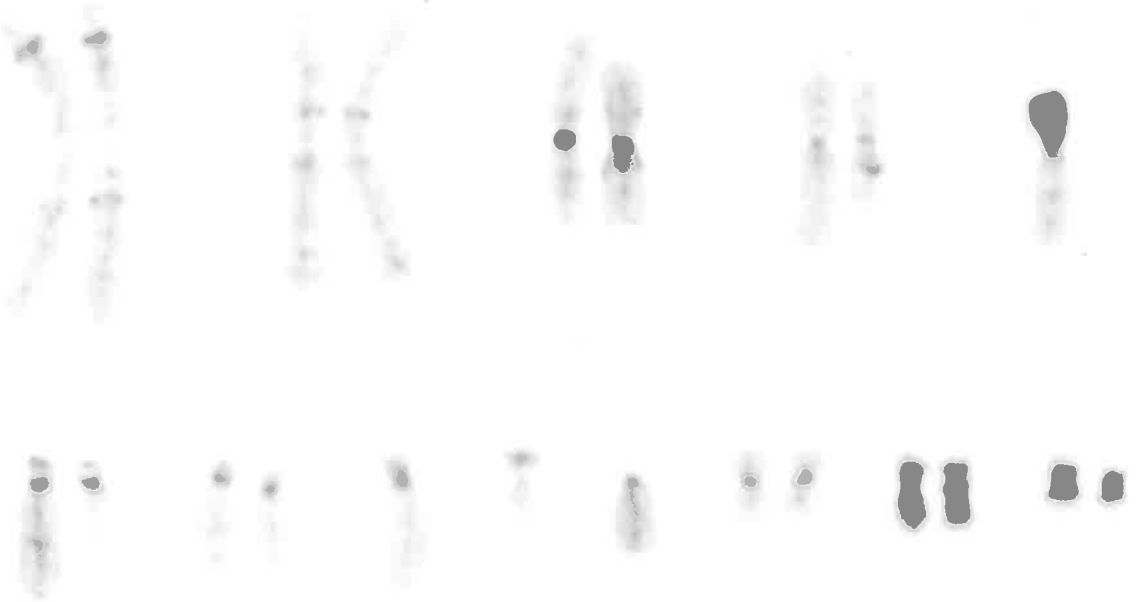
BIo is a pseudodiploid Chinese hamster cell line with a modal chromosome number of 22 or 23. Despite its apparent stability of karyotype in retaining close to the diploid number of chromosomes, there is evidence that considerable internal rearrangement and evolution of the karyotype may occur in Chinese hamster cell lines, resulting in the production of new pseudodiploid lines (Terzi, 1972; Worton, 1978). C-banded karyotypes of cells containing 22 and 23 chromosomes are shown in Figures 9.2a and 9.2b, respectively.

When C-banded cells of BMR-1 were examined, no recognizable kangaroo chromosomes could be detected. However, counts of total chromosome number in 50 cells indicated that the most common chromosome numbers had shifted to 23 and 24, as demonstrated in Figure 9.3b. Karyotypes of cells with these total chromosome numbers are

FIGURE 9.2

Karyotypes of C-banded cells of BIo containing (a) 22 chromosomes and (b) 23 chromosomes.

a



b

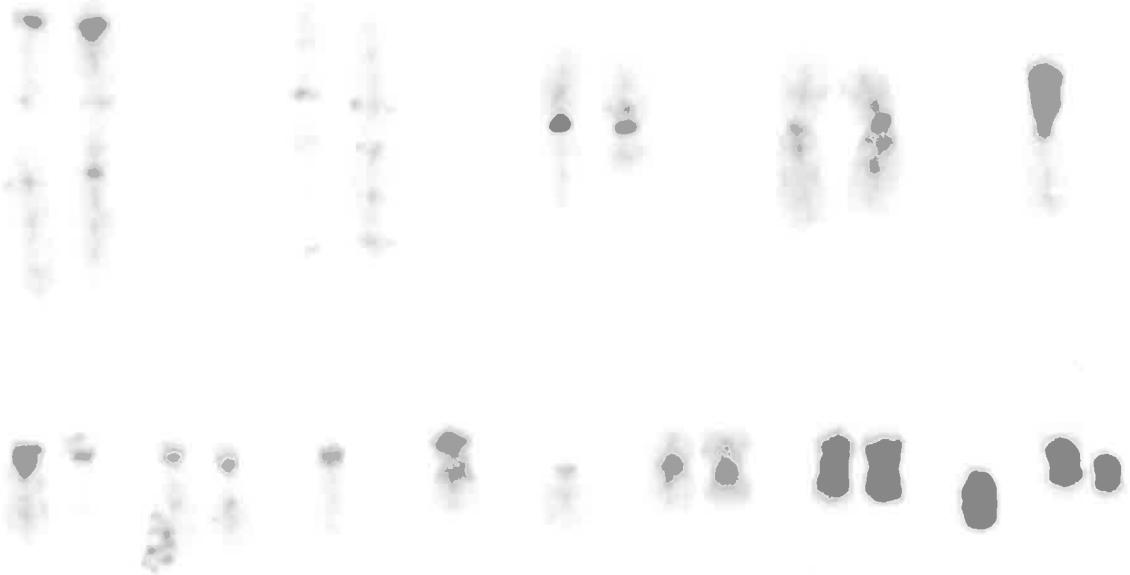


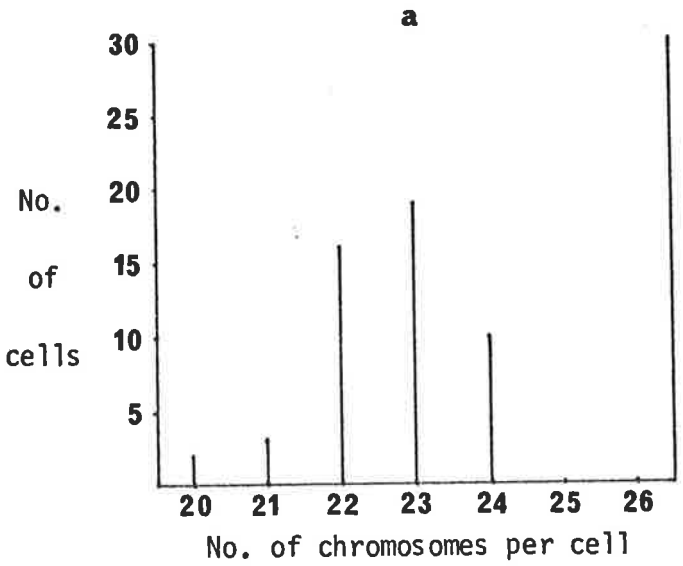
FIGURE 9.3

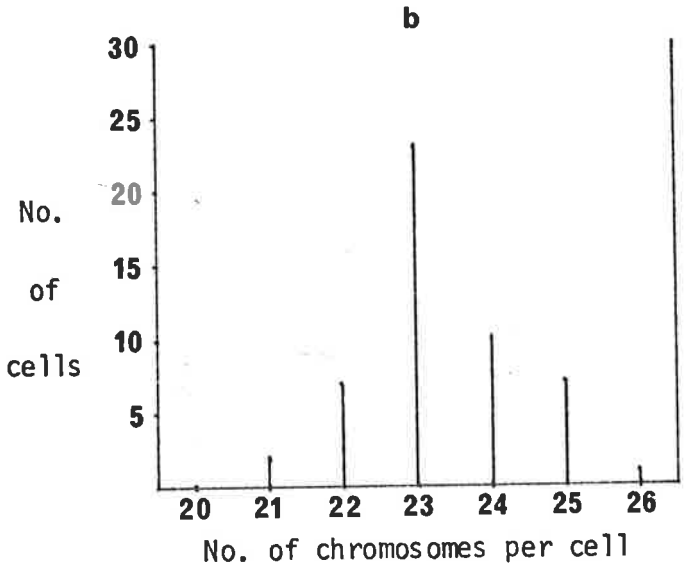
Frequency distribution of chromosome numbers in 50 cells of:

a) BIo, the parental hamster line.

b) BMR-1, a putative hybrid

c) BMR-1-R, a BrdU-resistant revertant derivative of BMR-1.


a
30
25
20
15
10
5
No.
of
cells
20 21 22 23 24 25 26
No. of chromosomes per cell


b
30
25
20
15
10
5
No.
of
cells
20 21 22 23 24 25 26
No. of chromosomes per cell

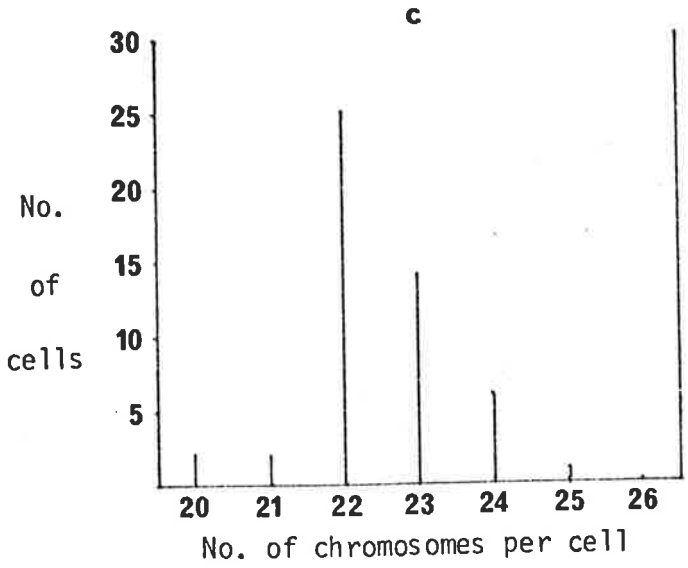
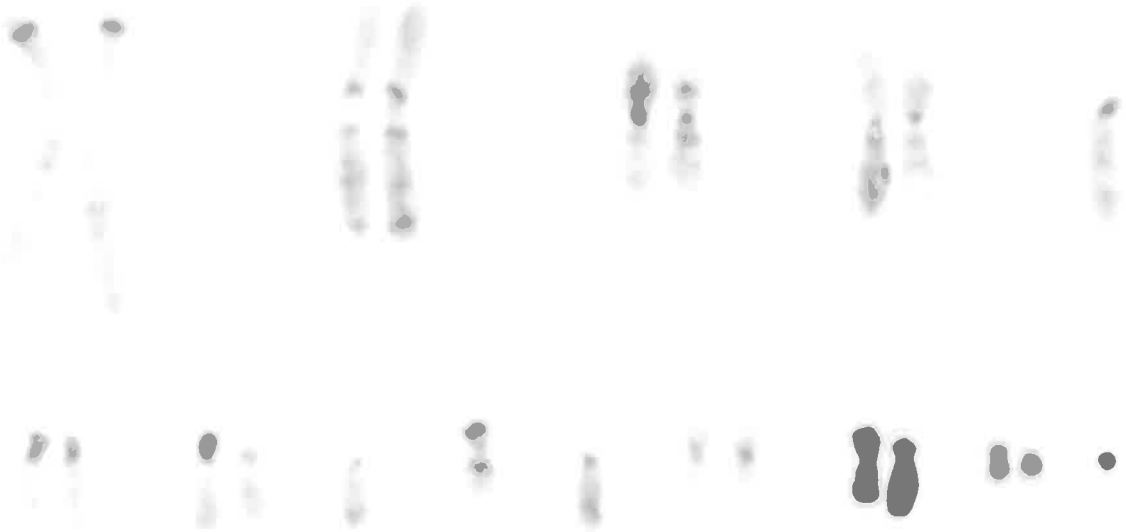

c
30
25
20
15
10
5
No.
of
cells
20 21 22 23 24 25 26
No. of chromosomes per cell

FIGURE 9.4

Karyotypes of C-banded cells of BMR-1, containing (a) 23 chromosomes and (b) 24 chromosomes.

a

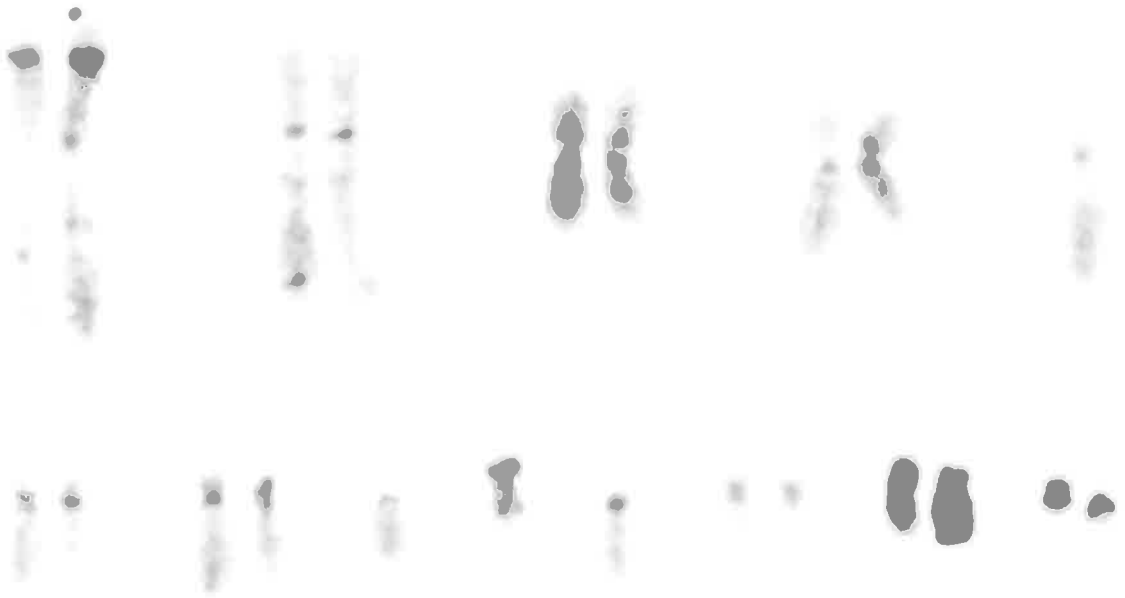


b

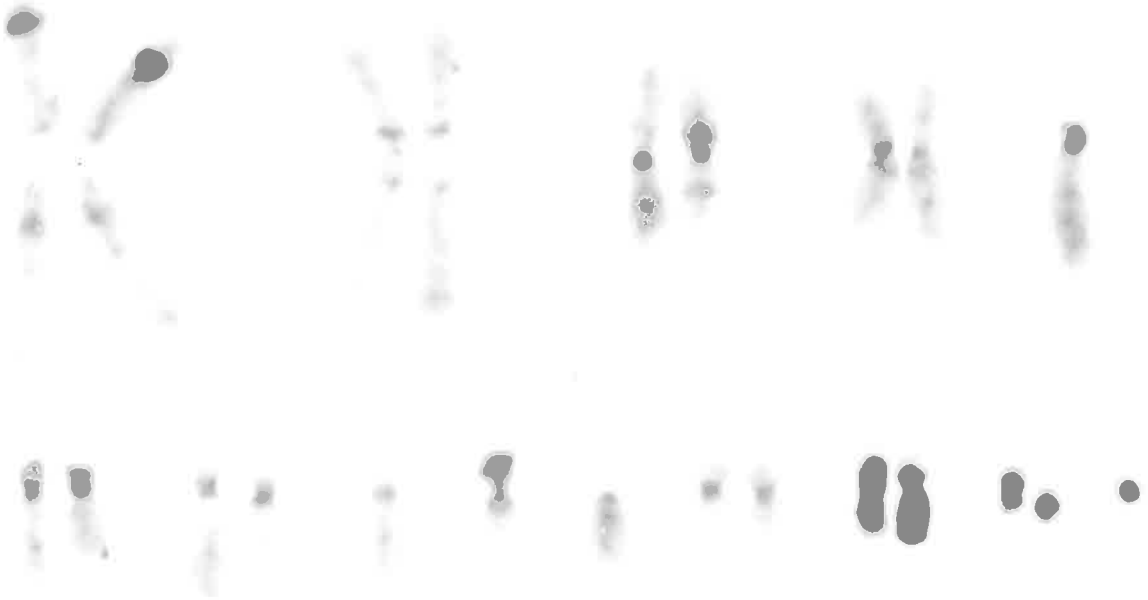
FIGURE 9.5

Karyotypes of C-banded cells of BMR-1-R, containing (a) 22 chromosomes and (b) 23 chromosomes.

a



b



presented in Figures 9.4a and b. Examination of individual cells revealed that this increase in number cannot be attributed to the consistent presence of one particular extra chromosome. The additional chromosomes in both BIo and BMR-1 are very similar, and appear to have been produced by breakdown of existing chromosomes. Thus, it appears likely that the higher chromosome number of BMR-1 results from rearrangement of hamster chromosomes, perhaps initiated by the presence of a foreign chromosome fragment.

The shift to higher chromosome numbers appears to be reversed in BMR-1-R cells, in which the most frequent total chromosome numbers were again 22 and 23 (Figure 9.3c). Individual cells of this line also showed no consistent karyotypic change that could be identified (Figures 9.5a, b).

These cytogenetic studies did not enable any kangaroo chromosome or part of a chromosome to be identified. G-banding was not performed on these cells, but even detailed G-banding is unlikely to reveal the presence of a small piece of a marsupial chromosome. Consequently, the hybrid nature of BMR-1 cannot be proved by cytogenetic means. However, the fact that the chromosome constitution of BMR-1 is altered relative to the hamster parent, and that this change may be reversed in the back-selected cells of BMR-1-R, supports the contention that this clone is distinct from BIo and, thus, possibly of hybrid origin.

9.3 Enzyme Studies

Direct proof that BMR-1 is a cell hybrid could be provided by demonstration of the kangaroo form of TK in these cells. Consequently, an attempt was made to distinguish

the hamster and kangaroo forms of TK by electrophoresis on cellulose acetate gels, using a technique adapted from that of Wullems *et al.* (1977) (B. Wainwright, pers. comm). Unfortunately, TK is very unstable when cells are stored as extracts, even with thymidine added to stabilize the enzyme (Littlefield, 1965), and no TK activity could be detected when extracts of BMR-1 were subjected to electrophoresis. However, B. Wainwright did find TK activity with a mobility clearly distinct from the hamster form in two of his four BIS hybrids. This is most probably the *Sminthopsis* form of TK. Although this form of TK has not been detected in BMR-1 or the two other BIS hybrids, the consistency in behaviour between the five cell lines (discussed in Section 9.1) strengthens the conclusion that they may all be hybrid clones expressing marsupial TK.

One of the main reasons for producing somatic cell hybrids containing a marsupial gene for TK was to examine the syntenic relationships of this gene. The gene for galactokinase (GALK; E.C. 2.7.1.6) has been shown to be located very close to the gene for TK in a small region on the long arm of chromosome 17 in man (Elsevier *et al.*, 1974). The linkage of these two genes in man is sufficiently close for them to be co-transferred in chromosome-mediated gene transfer experiments, in which only very small fragments of foreign chromosomes are incorporated (Willecke *et al.*, 1976; Wullems *et al.*, 1977; McBride *et al.*, 1978). The two genes are also syntenic in the chimpanzee and African green monkey (Orkwiszewski *et al.*, 1976) and in the mouse (Kozak and Ruddle, 1977; McBreen *et al.*, 1977). As this

very close linkage appears to have been conserved in groups as evolutionarily divergent as primates and rodents, it was of interest to determine whether it is also found in marsupials.

Consequently, galactokinase of the presumed hybrid clones was examined by starch gel electrophoresis, using the electrophoretic method of Nichols *et al.* (1974). The results are shown in the GALK autoradiograph presented in Figure 9.6. The mobility of GALK in the marsupial control, a *Sminthopsis* cell line, is considerably less than that of the BIo form, and it is plain that none of the hybrid clones expressed marsupial GALK. On the assumption that the hybrid clones do contain a marsupial gene for TK, this result implies that either the marsupial chromosome fragment bearing the gene for TK is extremely small in all these clones, or that the very close syntenic relationship between the genes for TK and GALK seen in eutherians does not hold for marsupials.

When the lRMR hybrids and revertants were being typed for G6PD, a sample of BMR-l was loaded on one of the gels. Unexpectedly, this clone showed a clear hybrid band of G6PD activity, indicating that it possessed the kangaroo form of this enzyme. The G6PD heteropolymer was also present in BMR-l-R (Figure 9.7). BMR-l did not express the kangaroo form of either of two other enzymes coded for by X-linked genes, HPRT and PGK-A. B. Wainwright subsequently examined his BIS hybrids for G6PD and two of the four were also shown to possess marsupial G6PD activity. However, revertants of these cell lines, in contrast to BMR-l-R, did

FIGURE 9.6

Starch gel electrophoresis of GALK of hamster, marsupial and hybrid cells.

Channel 1: BIo, the hamster parental line.

Channel 2: BIS-3, a putative *Sminthopsis* x hamster hybrid of B. Wainwright.

Channel 3: a putative *Sminthopsis* x mouse hybrid of B. Wainwright.

Channel 4: a *Sminthopsis crassicaudata* cell line.

Channel 5: BMR-1-R, a BrdU-resistant revertant of BMR-1.

Channel 6: BMR-1, a putative kangaroo x hamster hybrid.

H = hamster mobility

M = marsupial mobility

FIGURE 9.7

Cellulose acetate electrophoresis of G6PD of hamster and hybrid cells.

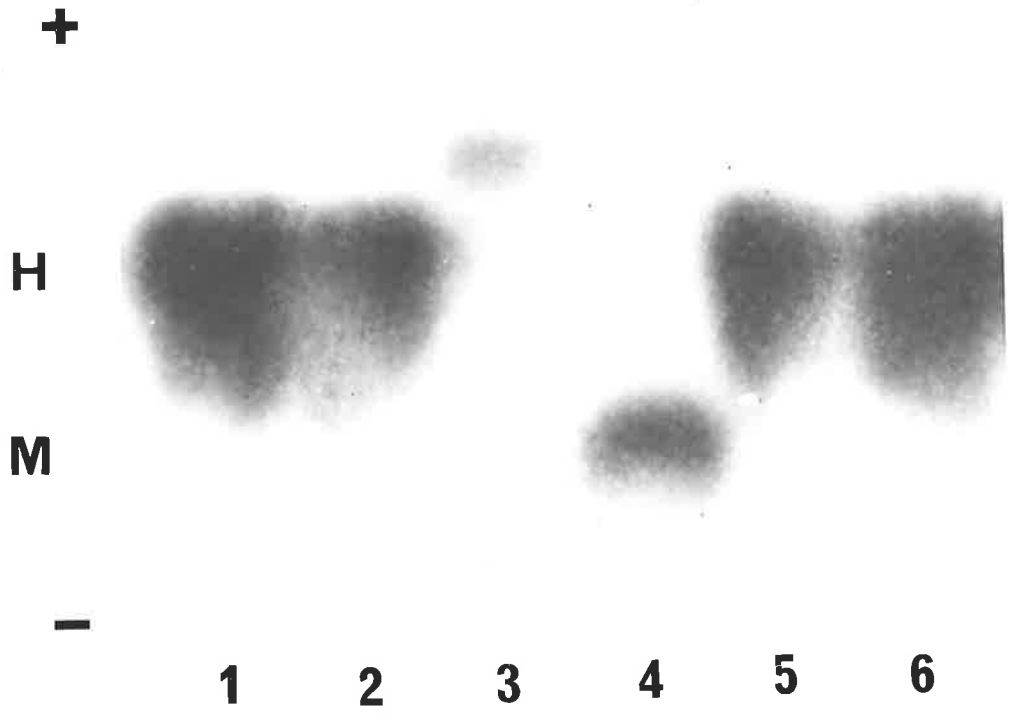
Channels 1 and 2: BIo, the hamster parental line.

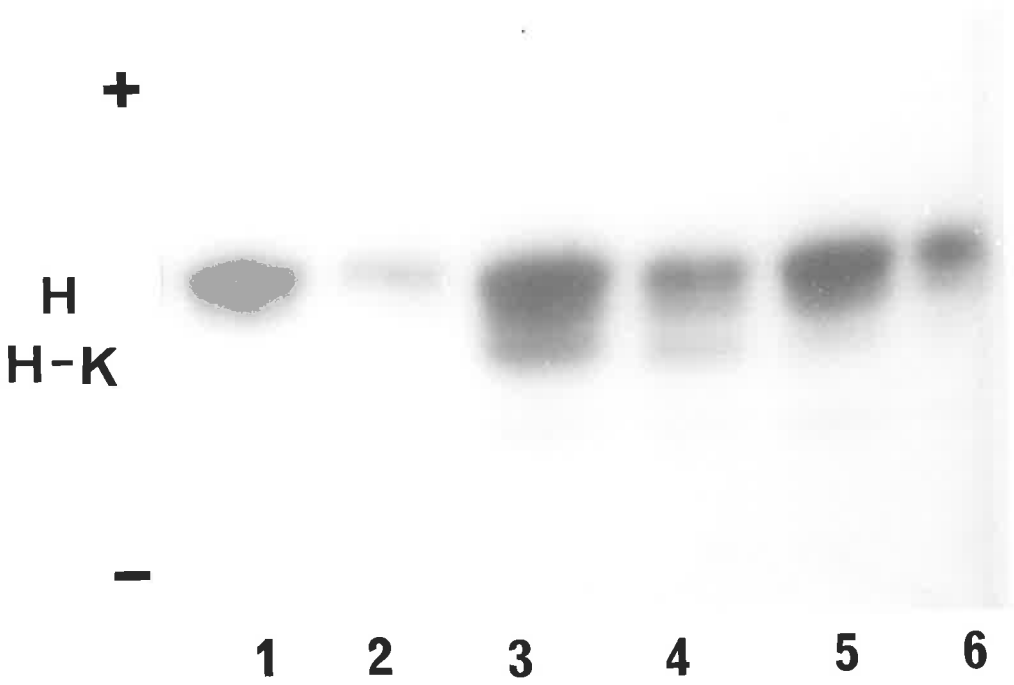
Channels 3 and 4: BMR-1, a putative kangaroo x hamster hybrid

Channels 5 and 6: BMR-1-R, a BrdU-resistant revertant of BMR-1.

H = hamster mobility

H-K = hamster-kangaroo heterodimer


+
H
M
−
1
2
3
4
5
6


+
H
H-K
−
1
2
3
4
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6

not express the *Sminthopsis* form of G6PD. Similar observations of expression of marsupial G6PD in hybrid cells selected for TK have been made previously by MacGregor (1978) in one of four clones derived from a fusion between *Sminthopsis crassicaudata* and BIo, and recently by A. Dobrovic (pers. comm.) in some *Sminthopsis crassicaudata* x BIo and *Phascogale maculata* x BIo cell hybrids.

The finding of the marsupial form of G6PD in a number of independent hybrids selected for the gene appears unlikely to be simply a chance phenomenon. Possible interpretations of this observation are considered further in Chapter 10. One immediate implication, however, is that the contention that BMR-1 and the other clones possess the marsupial gene for TK is strengthened. The evidence that these clones possess at least one marsupial gene proves that they are not merely revertants of BIo, but rather are hybrids between marsupial and hamster cells.

CHAPTER 10

GENERAL DISCUSSION

10.1 Implications of the X Chromosome Map

The map of the red kangaroo X chromosome presented in Chapter 8 of this thesis provides the first direct evidence for the chromosomal location of three genes known to be X-linked in eutherian mammals. Although the genetic content of this chromosome can be compared with that of the X chromosomes of other mammals, direct comparisons of gene arrangement are not possible, partly because the order of genes on the kangaroo X chromosome is not known, and also because of the scarcity of detailed chromosome maps for species other than man. However, the information that three X-linked genes are located within a small segment of the kangaroo X chromosome does have implications for the evolution of the X chromosomes of marsupials.

Ohno's thesis of the conservation of the mammalian X chromosome was based, in part, on observations that the X chromosomes of most mammals constitute 5 - 6% of the haploid genome (Ohno, 1967). He has postulated that this is the size of the "basic unit" of the X chromosome, and that larger X chromosomes are produced by the addition of genetically inert constitutive heterochromatin. There is evidence, however, that the "basic unit" of marsupial X chromosomes may be considerably smaller. Hayman and Martin (1974) found that sizes of the X chromosomes of various marsupial species varied over an approximately 10-fold

range, and the larger ones have been shown to result from the addition of constitutive heterochromatin, as Ohno had proposed. The smallest X chromosomes, on the other hand, constitute approximately 3% of the genome and appear to be half the size of the human X chromosome, which has been taken as representative of the basic eutherian X.

The red kangaroo X chromosome occupies a position in the upper half of this size distribution. The long arm, which stains heavily with C-banding and appears to consist largely of constitutive heterochromatin, clearly is not part of any basic marsupial X chromosome. The euchromatic arm is equivalent in size to the euchromatic portions of X chromosomes of other kangaroos in the genus *Macropus*, but is still very much larger than the X chromosomes of some other species of marsupials, including other members of the family Macropodidae. If a "basic unit" does exist in marsupial X chromosomes, it must comprise only a portion of the euchromatic arm of kangaroo X chromosomes.

The results reported in this thesis can be seen as supporting this contention. Three of the genes known to be X-linked in mammals, those coding for HPRT, G6PD, and PGK-A, have been shown to lie within the distal third of the euchromatic arm of the red kangaroo X chromosome. Although the cytological location of these genes on the X chromosome of the wallaroo was not determined in the study of Graves *et al.* (1979), the finding that they were co-transferred in hybrids in which no chromosomal fragment could be detected suggests that they may also be located in close proximity in a related species of kangaroo. These

three genes, along with two others, those for α-GAL A and PRPP synthetase, have all been localized to the long arm of the human X chromosome, where their locations extend from PGK-A near the centromere to G6PD near the tip of the long arm (Preliminary report from the 5th International Human Gene Mapping Workshop, Edinburgh, 1979). This sample of genes is clearly too small to provide adequate coverage of the genetic content of the X chromosome. Nonetheless, if their location is accepted as representative of mammalian genes subject to X-inactivation and conservation of X-linkage, for the sake of argument, then one could postulate that a "basic unit" of the marsupial X is located in the distal part of the euchromatic arm of the red kangaroo X chromosome.

If this should prove to be the case, questions are immediately raised concerning the nature of genes in the remainder of the X chromosome. It is conceivable that only genes that form part of the conserved "basic unit" of the X chromosome are regulated by X-inactivation. Support for this concept is provided by the results of Hayman and Rofe (1977), which suggest that the nucleolus organizer regions located in the heterochromatic long arm of the red kangaroo X chromosome may not be subject to X-inactivation. In this regard, the finding that none of the well-known X-inactivated genes are located on the short arm of the human X chromosome may be significant. The only genes that have been provisionally assigned to this arm of the human X are Xg and the locus for steroid sulphatase, both of which may escape X-inactivation (Fellous *et al.*,

1974, 1975; Shapiro *et al.*, 1979), although the evidence for both their location and lack of inactivation is conflicting (Campana *et al.*, 1978; Balazs *et al.*, Preliminary report, 5th International Human Gene Mapping Workshop, Edinburgh, 1979).

Whether the X-linkage of genes that may not be subject to dosage compensation is conserved as strictly as that of X-inactivated gene loci is not known. If the "freeze" on the genetic content of the mammalian X chromosome is a consequence of its unique method of regulation, as Ohno suggested, then non-inactivated genes need not be located on this chromosome in all species. Marsupials would provide useful material to test this hypothesis, as their considerable separation from man on an evolutionary timescale may have provided the opportunity for chromosomal rearrangement to have disrupted any non-essential linkages.

It is very difficult, however, to assess the strength of dosage compensation as a force in conserving a linkage group. The recent findings of partial paternal X-inactivation in marsupials cast doubt on the assumption that dosage compensation is necessary for all X-linked genes. Considerable differences have been demonstrated between the levels of G6PD activity in fibroblasts of male and female kangaroos (Raphael and Cooper, 1978). If such differences can be tolerated, then the requirement for dosage compensation for all genes located on the X chromosome must be questioned. Admittedly, partial paternal X-inactivation has only been demonstrated in certain cells and tissues of kangaroos, and there may be an absolute requirement for complete X-inactivation in

certain organs or at certain stages of development.

Arguments such as these highlight the inadequacy of our present knowledge of the mammalian X chromosome and its genetic content. Discussions of X-inactivation are based on the behaviour of only a handful of gene loci, which cannot safely be assumed to represent adequately all the genetic material of the X chromosome. This applies particularly to marsupials, in which conclusions regarding X-inactivation have been drawn from studies of only two loci, those for G6PD and PGK-A, in a very limited number of species. Those properties of inactive X chromosomes that appear to function on a larger scale, such as late DNA replication and chromosome condensation, are not reflections of primary genetic events, but rather correlations in gross chromosome behaviour which cannot always be relied upon as accurate indicators of genetic inactivity. The need for many more X-linked markers over a wider range of species is clearly pressing. Although the process of X-inactivation has not so far proved amenable to manipulation in somatic cell hybrids, somatic cell genetic techniques can contribute significantly by providing a means for assigning additional genes to the X chromosome.

10.2 Gene Interaction in Hybrids

Most of the somatic cell genetic studies discussed in this thesis have been concerned with the use of cell hybrids to assign structural gene loci to chromosomes. Somatic cell hybrids can also be used to investigate another aspect of the genetics of cultured mammalian cells: the regulation of gene expression. Regulatory interactions

between specific genes, which are obscured by the complexity of cellular mechanisms in normal cells, may be revealed when the genome can be dissected in a somatic cell hybrid.

Two of the findings reported in this thesis may have a bearing upon the question of genetic regulation in a cell hybrid. The first of these is the non-random retention of a specific kangaroo autosome in those hybrids that possess the kangaroo X chromosome (Section 5.4). This phenomenon cannot be explained simply as selection for a gene located on this autosome that confers an advantage upon the hybrid cell, because the autosome is not retained in cell hybrids possessing only a small fragment of the kangaroo X chromosome. It appears that the presence of a part of the kangaroo X chromosome, other than the locus for HPRT, is necessary for retention of the autosome to become advantageous to the cell.

This phenomenon could be explained in various ways. One hypothesis is that the autosome bears a regulatory locus for a gene on the X chromosome, the activity of which confers a selective advantage upon the hybrid cell. Alternatively, the situation may be reversed, and a regulatory locus on the X chromosome may control a structural gene on the autosome. It is not possible to decide between these alternative hypotheses on the basis of the data available. Under both of these hypotheses, if the requisite portion of the X chromosome is not present, the gene on the autosome will no longer be necessary, either because a structural locus will not function or because a regulatory gene will become redundant.

However, the autosome was not lost from most of the revertants concomitant with loss of part or all of the kangaroo X chromosome. This apparent difference in behaviour between the revertants and the primary hybrid clones most probably results from the different nature of the process of chromosome loss at the two stages. Initial chromosome loss is rapid, and strongly directed against the marsupial chromosomes, as is shown by the absence of any other marsupial chromosomes in the hybrids. Unless there is a definite selective advantage in retaining a certain gene or combination of genes, all kangaroo material is likely to be lost in this early phase. The revertants, however, were selected at a much later stage in the history of the hybrid cells, when initial chromosome loss has ceased, and a stable hybrid karyotype has evolved. Loss of portions of the X chromosome may remove the necessity of retaining the autosome, but in the absence of direct selection against this chromosome, it will only be lost slowly by random processes such as non-disjunction.

The other observation in this study which is suggestive of interaction between genes is the discovery that marsupial G6PD is expressed in a number of hybrids selected for the marsupial gene for TK (Section 9.3). The occurrence of the marsupial form of G6PD in three of five hybrids from this laboratory, and a number of hybrids reported elsewhere, all of which were independently derived from fusions involving several marsupial species, is clearly not a result of chance. It should be noted, however, that in all cases, the eutherian parent was the Chinese hamster cell line,

BIo. Whether such a phenomenon will also occur in hybrids with other rodent cell lines is presently unknown.

There are a number of possible explanations for the apparent preferential retention of the gene for G6PD in these hybrids. The first and most obvious explanation is that the structural genes for TK and G6PD are syntenic, and located on the X chromosome. If this were the case, it would confound Ohno's law of conservation of X-linkage, as the gene for TK has been shown to be located on an autosome in a number of eutherian mammals. The finding that the marsupial genes for HPRT and PGK-A, which are located in the same region of the red kangaroo X chromosome as that for G6PD, are not expressed in these hybrids renders this explanation less likely. This hypothesis could readily be proved or disproved by determining whether marsupial TK is expressed in 1RMR hybrids which contain the kangaroo X chromosome. As these cells will also possess the mouse gene for TK, determination of the type of TK present will require the successful electrophoretic separation of mouse and kangaroo forms of the enzyme. Alternatively, marsupials may possess a second autosomal locus for G6PD, located close to the gene for TK on an autosome. However, this hypothesis is not supported by evidence from population and pedigree studies of the inheritance of the gene for G6PD in kangaroos (Johnston and Sharman, 1975; Johnston *et al.*, 1975).

A more plausible explanation is that the hybrids contain a marsupial chromosome fragment bearing an X-linked controlling locus for TK that has activated a previously inactive hamster structural gene. This hypothesis could be

easily proved or disproved by determining with electrophoresis whether the hybrids possess the marsupial or hamster form of the enzyme. B. Wainwright's finding of a form of TK with a mobility clearly distinct from hamster in two of his four hybrids tends to discount this explanation.

The fourth and most likely explanation is that there is a regulatory locus for TK located in close proximity to the gene for G6PD on the X chromosome. In order for the marsupial TK gene to be expressed, this regulatory locus must also be present. Consequently, the hybrids would contain two marsupial chromosome fragments. Under this hypothesis, the absence of G6PD in some of the other hybrid clones would indicate that a break had occurred between the gene for G6PD and the regulatory locus when the marsupial chromosomes were fragmented and lost. The necessity for two independent marsupial chromosome fragments for expression of TK perhaps accounts for the low frequency of recovery of hybrids. The finding that the revertants obtained by B. Wainwright lost marsupial G6PD, while the revertant of BMR-1 still expressed the kangaroo form of the enzyme, can be explained in a similar manner to retention of the kangaroo autosome in lRMR-1 revertants. In the absence of the structural locus for TK, a regulatory gene would no longer be necessary and could be randomly lost.

This appears to be the most probable explanation for these observations. It is, however, very difficult to prove in the absence of any cytologically detectable chromosome fragments in the hybrid cells. The development of a DNA

probe which could be used to detect small pieces of marsupial DNA, perhaps on the basis of characteristic differences in repetitive DNA between eutherians and marsupials, would be extremely useful for investigating situations like this, and related topics such as the mode of integration of small chromosome fragments into the genome of a hybrid cell.

Both the association between the kangaroo X chromosome and an autosome and the association between the marsupial genes for TK and G6PD are suggestive, therefore, of specific gene interactions concerned with regulation of gene expression. Such an explanation would imply that at least some regulatory interactions in cellular metabolism involve species-specific signals. If marsupial regulatory loci need to be selectively retained in hybrid cells, then clearly rodent genes are not capable of fulfilling the same role. How many other functions in hybrid cells are not constitutively expressed but require the presence of specific regulatory genes is not known. The implications for gene mapping studies, however, are significant and highlight the need for caution in concluding that absence of a particular gene product results from loss of the corresponding structural gene.

It is of considerable interest that both of these suggested instances of genetic regulation involve genes located on the X chromosome. This may be relevant to Ohno's (1973b) postulate that one of the reasons for the development of dosage compensation could have been a concentration of regulatory gene loci on the mammalian X chromosome. In the case of retention of the autosome, it is not clear

whether the X chromosome is controlling an autosomal gene or vice versa. If an autosomal gene should prove to be regulating an X-linked locus, it is tempting to speculate that this autosomal gene might be one of those postulated to be involved in control of inactivation of the X chromosome.

10.3 Further Studies

The results obtained in this study with somatic cell hybrids containing red kangaroo chromosomes have raised questions concerning evolution and regulation of mammalian linkage groups which can only be answered after a considerable amount of detailed investigation in a number of species. Clearly, one of the main priorities should be extension of the red kangaroo X chromosome map by the assignment and localization of many more genetic markers, as they are discovered. With the accumulation of such data, the proposal of a basic unit of the marsupial X located in the terminal portion of one arm of the red kangaroo X chromosome could be evaluated.

If the existence of such a unit is verified, it would be of interest to determine whether the block of genes has been kept intact during the chromosomal rearrangements involved in marsupial evolution, and whether the location of this block within the X chromosome has changed relative to the position of the centromere. This would require the construction of detailed chromosome maps for a variety of marsupials, both closely related species such as other kangaroos, and members of more distant families and superfamilies. Comparison between species with large and small

X chromosomes may reveal the nature of the additional genetic material in the larger X chromosomes.

Knowledge of the order of genes on marsupial chromosomes is a crucial factor. Not only would this enable Ohno's suggestion of conservation of the order of genes on the X chromosome to be evaluated, but such information may also be relevant for understanding the mechanism of X chromosome inactivation. Most models of X chromosome inactivation postulate one or more centres from which inactivation spreads. Correlation of gene order with differences in dosage compensation for marsupial X-linked loci could shed light on this point.

No order for the genes on the X chromosome of the red kangaroo could be deduced in this study because no breaks occurred between genes. If detailed information on gene order and position is to be amassed, it is insufficient to rely on the haphazard occurrence of spontaneous breaks. Application of the technique developed by Goss and Harris (1975, 1977) could help in this respect. This method involves irradiation of the cells of one parent, which greatly increases chromosome breakage and allows the relative locations of genes to be statistically mapped.

Most of the research reported in this thesis has been concerned with X-linked genes. However, the opportunities for analysis of autosomal linkage groups in somatic cell hybrids are vast. This applies both to selectable genes such as that for TK and also to genes on other autosomes which may be retained in hybrid cells. With the assignment of the gene for LDH-A to chromosome 5 of the red kangaroo,

a starting point has been established for autosomal gene mapping in marsupials, as discussed in Section 7.5. During such studies, it may be possible to determine whether the observations pertaining to gene interaction are of general occurrence.

Before these sorts of somatic cell genetic studies are undertaken in future, careful consideration should be given to the choice of parental cells for the hybridizations. Mouse cell lines such as 1R clearly have drawbacks for research involving cytogenetic analysis, because of their chromosomal heterogeneity and instability. The use of parent cell lines containing chromosomes that are easily identifiable by a technique like C-banding can save a great deal of time in preliminary screening of hybrid clones. In this respect, the red kangaroo had considerable advantages as a marsupial parent, especially for the mapping of X-linked genes. Another factor influencing the choice of cells for hybridization should be the availability of the appropriate isozyme differences. The difficulties experienced in this project because of the very similar electrophoretic mobilities of the G6PD isozymes of the two species and the inability to resolve the two species' forms of α-GAL A show that it is unsafe to assume that enzymes of two widely divergent species will necessarily be distinguishable.

A final point should be made concerning the use of somatic cell hybrids for gene mapping studies. The assumptions on which such studies are based, detailed by Ruddle (1970) and listed in Chapter 1, must now be acknowledged to be rarely satisfied. In addition to the

technical difficulties discussed above, this investigation has provided evidence for non-random chromosome loss, possible interactions between structural and regulatory loci and re-expression of enzymes in presumably deficient cells. These complexities can confound an apparently straightforward gene mapping study. At the same time, they may provide unexpected opportunities for insight into mechanisms of gene regulation in somatic cell hybrids and in mammalian cells in general.

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APPENDIX I

RECIPES

Phosphate-buffered saline (PBS)

NaCl	16.0 g
KCl	0.4 g
Na_2HPO_4	2.3 g
KH_2PO_4	0.4 g

Made up to 2 litres with distilled water; pH 7.4.

Trypsin-versene solution.

10 x Versene stock solution	100 ml
12.5% Trypsin stock solution	8 ml
Penicillin-streptomycin solution	891 ml

pH 7.7

Trypsin stock solution

Trypsin	12.5 g
NaCl	0.8 g
KCl	0.04 g
Na_2HPO_4	0.006 g
KH_2PO_4	0.005 g
Glucose	0.5 g

Made up to 100 ml with distilled water; pH 7.7.

Versene Stock solution

NaCl	8.0 g
KCl	0.2 g
KH_2PO_4	0.2 g
Na_2HPO_4	1.15 g

Na_2 EDTA 0.2 g

Made up to 100 ml with distilled water; pH 7.2

Penicillin-streptomycin solution

Streptomycin sulphate	1 g
Penicillin	1,000,100 I.U.
Distilled water	10 ml.

Ficoll-Hypaque stock solution

Ficoll (Pharmacia)	9 g
85% Hypaque (Winthrop)	20 ml
Distilled water	50 ml

Warm and stir until dissolved. Add 34 ml distilled water and stir. Sterilize by filtration; store at room temperature in dark.

2 x SSC

Tri-sodium citrate	8.824 g
NaCl	17.532 g

Made up to 1 litre with distilled water.

Sorenson's phosphate buffer

Solution A: 9.08 g KH_2PO_4 per litre

Solution B: 9.464 g Na_2HPO_4 per litre

Equal volumes of A and B give pH 6.8.

G6PD stain

(adapted from Johnston *et al.*, 1975)

Tris-maleate buffer, pH 8.0		1 ml
NADP	(5 mg/ml)	0.2 ml
Glucose-6-phosphate	(25 mg/ml)	0.3 ml
MTT	(2 mg/ml)	0.3 ml
PMS	(2 mg/ml)	0.2 ml

PGK-A electrophoresis buffer

0.1 M Tris	12.1 g per litre
Saturated citric acid	60 g per 100 ml

Add citric acid to Tris solution until pH = 8.6.

PGK-A stain

(adapted from Meera Khan, 1971)

Tris-HCl-EDTA buffer, pH 8.0		1.2 ml
Magnesium chloride	(40 mg/ml)	0.1 ml
NADH	(9.9 mg/ml)	0.1 ml
ATP	(24.2 mg/ml)	0.3 ml
3-phosphoglyceric acid, sodium salt	(7.4 mg/ml)	0.3 ml
Glyceraldehyde-3-phosphate dehydrogenase (800 I.U./ml)		30 µl

APPENDIX II

THE USE OF DISCONTINUOUS BUFFERS IN CELLULOSE ACETATE ELECTROPHORESIS: ELECTROPHORETIC SEPARATION OF MOUSE AND KANGAROO GLUCOSE-6-PHOSPHATE DEHYDROGENASE.

by

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Cellulose acetate electrophoresis is frequently used to distinguish enzyme markers of different species in somatic cell hybrids (Meera Khan, 1971; Van Someren *et al.*, 1974). The standard procedure includes soaking strips of cellulose acetate in the electrolyte buffer before loading the samples and commencing electrophoresis. However, we have found that, in certain circumstances, electrophoretic resolution and separation are improved by presoaking the gel in a buffer different from that used for electrophoresis. We have used such a discontinuous buffer system to distinguish electrophoretically the mouse (*Mus musculus*) and red kangaroo (*Macropus rufus*) forms of glucose-6-phosphate dehydrogenase (G6PD, E.C. No. 1.1.1.49) in mouse x kangaroo somatic cell hybrids. Separation of these two forms has previously proved difficult, both on cellulose acetate (Graves *et al.*, 1979) and other electrophoretic media (Donald and Hope, unpublished), despite the estimated 100 million years of evolutionary divergence between the two species (Air *et al.*, 1971).

The hybrid cells used were obtained by fusion of *M. rufus* lymphocytes with cells of 1R, a mouse cell line deficient in hypoxanthine phosphoribosyltransferase (Hope and Graves, 1978b). Cell culture and harvest techniques were as described in Graves & Hope (1977) and Hope & Graves (1978a). Electrophoresis on cellulose acetate strips ("Cellogel", Chemetron, Milan) was performed by the method of Baverstock *et al.* (1977), except that gels were loaded and run at 4°C. The discontinuous buffer system used involved soaking gels in Tris-borate buffer (15 mM Tris,

5 mM disodium EDTA, 3.5 mM boric acid, 0.01 M $MgCl_2$, pH 7.8), and running gels in tanks containing Tris-maleate buffer (0.05 M Tris; pH adjusted to 7.8 with saturated maleic acid solution). Gels were run for 2 hours at 200 volts and then stained for G6PD by the method of Johnston *et al.*, (1978).

As mentioned above, it is not possible with conventional cellulose acetate electrophoresis to achieve the required combination of resolution and separation necessary to consistently distinguish between the mouse and kangaroo enzyme forms in somatic cell hybrids (Fig. 1). Hybrids expressing both loci tend merely to give a slightly broader band of G6PD activity, and are therefore difficult to distinguish from an overloaded sample of mouse G6PD (Graves *et al.*, 1979). However, by using both buffers in a discontinuous system, it is possible to obtain bands of mouse and kangaroo G6PD which are both sharp and well separated (Fig. 2). Somatic cell hybrids which retain the kangaroo X chromosome show three bands of G6PD activity, the most cathodal (kangaroo) being relatively weakly staining. Hybrids which lack the kangaroo X (Donald, unpublished) possess a single band corresponding to mouse G6PD (Fig. 2). Consequently, this system can be used to detect the presence or absence of kangaroo G6PD in these hybrids, and will enable the position of the kangaroo G6PD locus to be mapped.

Discontinuous buffers are commonly used in starch gel electrophoresis (Harris & Hopkinson, 1976). The results obtained here suggest that the use of discontinuous buffers has general applicability and may facilitate the separation of other isozymes on cellulose acetate, a support medium

especially suited for gene mapping in somatic cell hybrids. This approach may allow the differentiation of enzymes from a number of species combinations in which the forms are presently not separable by electrophoresis.

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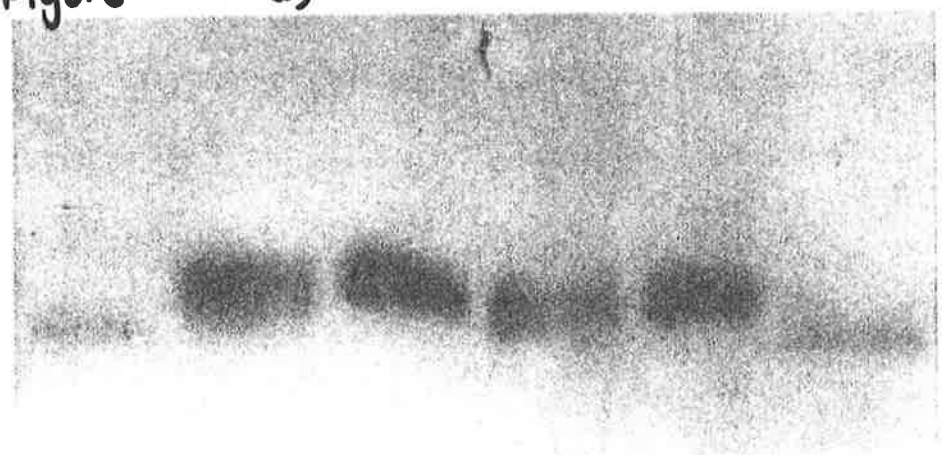
Fig. 1. G6PD phenotypes obtained by electrophoresis in a) Tris-borate buffer and b) Tris-maleate buffer. 1 and 6, *M. rufus*. 2 and 4, independent somatic cell hybrids possessing the kangaroo X chromosome. 3, mouse. 5, somatic cell hybrid lacking the kangaroo X chromosome.

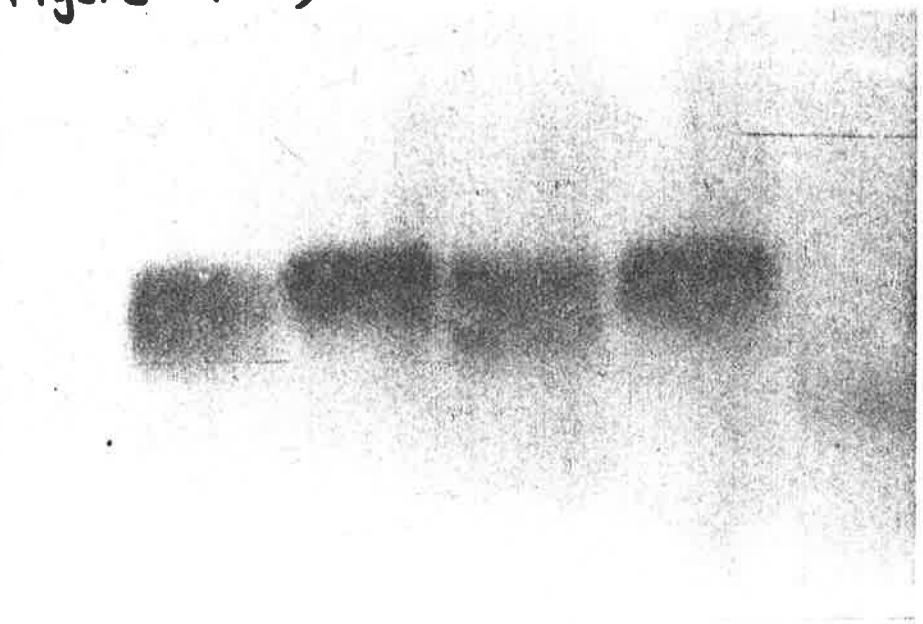
Fig. 2. G6PD phenotypes obtained using the discontinuous buffer system. Legend as in Figure 1.

Figure 1 a)



1. 2. 3. 4. 5. 6.

Figure 1 b)



1. 2. 3. 4. 5. 6.

FIGURE 2

See Figure 7.1 in main body of thesis.