A GENETIC AND IMMUNOLOGICAL STUDY OF MARSUPIALS, USING MARSUPIAL X EUTHERIAN SOMATIC CELL HYBRIDS

by

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SUMMARY

The two major extant groups of mammals, marsupials and eutherians, have evolved separately since they last shared a common ancestor some 130 million years ago. Detailed genetic maps already exist for several eutherian mammals, especially man and mouse. However, little gene mapping data are yet available for marsupials. The existence of such maps would be of special significance as it would enable comparisons to be made between the genetic maps of, for example, man and kangaroo, comparisons which would be of considerable evolutionary interest. Questions concerning the extent to which groups of genes have remained syntenic over very long evolutionary time spans could be answered. Gene mapping data to date suggest that the genetic content of the mammalian X chromosome has been conserved in a large number of mammals including marsupials. However, this chromosome probably represents a "special case" as the genes that reside on it are subject to a dosage compensation mechanism which, it has been argued, tends to conserve their location on this chromosome. With respect to the autosomes, there is already evidence that certain groups of genes which are syntenic in man are also syntenic in mouse and it would be of interest to see if the homologous counterparts to these genes are also syntenic in a marsupial.

Marsupial x mouse somatic cell hybrids containing subsets of marsupial chromosomes enable genes to be mapped to marsupial chromosomes. A cytogenetic, biochemical and immunological study has been carried out on *Macropus rufogriseus* (red necked wallaby) x mouse somatic cell hybrids with the aim of mapping enzymic and cell surface

antigenic markers to *M. rufogriseus* chromosomes.

Marsupial chromosomes were identified in marsupial x mouse somatic cell hybrids using various chromosome banding techniques. An interesting C-band variant was observed for a G-band identical *M. rufogriseus* translocation chromosome which was present in two subclones of an *M. rufogriseus* x mouse cell hybrid. Chromosome studies on these two clones using sequential C- and G-banding, fluorescent banding techniques, effect of culture in the presence of Hoechst 33258 and hybridisation of *M. rufogriseus* satellite DNA, indicated that the C-band variation was not due to an extensive loss of DNA but rather the non-C-band expression of DNA still present in the chromosome.

A number of enzymes were studied in the marsupial x mouse cell hybrids using electrophoretic techniques. The genes for the enzymes hypoxanthine phosphoribosyltransferase (HPRT) and phosphoglycerate kinase A (PGK-A) were shown to be syntenic in *M. rufogriseus* x mouse somatic cell hybrids. Family studies have shown PGK-A to be X-linked in a number of marsupial species closely related to *M. rufogriseus*, suggesting that PGK-A is also X-linked in *M. rufogriseus*. If so, then HPRT would be, by association, X-linked in *M. rufogriseus*. In the present study *M. rufogriseus Hpt* and *Pgk-A* were *provisionally* assigned to Xp. Some difficulty, however, was encountered in cytologically distinguishing, with absolute certainty, some of the smaller mouse acrocentric chromosomes from *M. rufogriseus* Xp. Glucose-6-phosphate dehydrogenase (G6PD), another enzyme universally X-linked in eutherians, and known from family studies to be X-linked in *M. rufogriseus*, was not found in any of the hybrids, including one

which appeared to have an intact marsupial X chromosome.

Attempts were made to immunologically identify cell surface antigens present on the marsupial x mouse cell hybrids. Classical methods involving the use of mouse antisera, as well as more recently developed procedures involving the production of monoclonal antibodies were used in these studies. A monoclonal antibody (GA-1), of Ig G_{2b} subclass was made by fusing mouse myeloma cells with spleen cells from a mouse immunized with marsupial x mouse hybrid cells. GA-1 bound to an *M. rufogriseus* cell surface antigen present on particular *M. rufogriseus* x mouse cell hybrids, *M. rufogriseus* fibroblasts and approximately 30% of *M. rufogriseus* lymphocytes. A gene coding for the GA-1 cell surface antigen or at least controlling its expression, was provisionally assigned to the long arm of the *M. rufogriseus* chromosome number 3 using *M. rufogriseus* x mouse somatic cell hybrids. This is the first gene determining a cell surface antigen to be assigned to a marsupial chromosome and is the second autosomal assignment to be made in a marsupial. GA-1 reacted with a cell surface antigen present on a number of *M. rufus* (red kangaroo) x mouse cell hybrids and was mapped to the *M. rufus* chromosome 5, the G-band identical chromosome to the 3q arm in *M. rufogriseus*.

Fibroblasts from a number of marsupial and eutherian species were tested for reaction with GA-1. Only marsupial species of the family Macropodidae and the sole member of the family Tarsipedidae reacted, indicating evolutionary homology between these two marsupial families.

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.

P.J. SYKES

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CHAPTER 1

INTRODUCTION

The number of genes that have been assigned to specific mammalian chromosomes has grown dramatically over the last decade, due largely to the application of somatic cell genetic techniques. In fact, the total number of confirmed gene assignments to human autosomes almost doubled in the period between the 1975 and 1981 Human Gene Mapping Conferences (HGM-3, 1975 and HGM-6, 1981) and about 2/3 of these assignments used methods involving somatic cell hybridisation. More recently, interest has spread to the mapping of chromosomes of mammals other than man, in particular mouse (550 genes assigned as of HGM-6, 1981), rat (44), rabbit (40) and the primates (e.g. chimpanzee (37) and gorilla (38)). Smaller numbers of genes have been mapped to chromosomes in a large variety of other eutherian mammals and for the first time chromosomes have been mapped in a marsupial using somatic cell genetics (HGM-6, 1981).

Genes which are located on the same chromosome are said to be syntenic (Renwick, 1971). It is becoming increasingly apparent that groups of genes that are syntenic in one species may also be syntenic in other species. The rapid growth of information on gene location has facilitated a "new" way of comparing the phylogenetic relationship between species. The closer the similarity between the distribution of genes amongst the chromosomes of two species, presumably the closer they are related phylogenetically. Considerable conservation of syntenic groups involving homologous loci has been found between man and the

primates (Garver *et al.*, 1980; Orkwiszewski *et al.*, 1976) and there is increasing information suggesting such conservation between man and more divergent species such as mouse, dog and cattle (HGM-6, 1981). An example of such conservation of synteny is provided by six enzymic loci on human chromosome 1p which are also syntenic on mouse chromosome 4. (HGM-6, 1981).

Man and the primates also show considerable homology in their G-banded chromosome patterns (Finaz *et al.*, 1977) suggesting that conservation of G-band pattern is accompanied by conservation of gene distribution amongst the chromosomes. Conservation of G-banding pattern has also been demonstrated for a number of different species in the marsupial family, Macropodidae (Rofe, 1978). However, to date, there is insufficient chromosome mapping information in marsupials to enable comparison of conservation of G-band pattern with conservation of gene content.

Why some groups of genes have remained syntenic over millions of years of separate evolution, while others have become separated onto different chromosomes has yet to be fully understood. The conservation of synteny of two or more loci over a long evolutionary time span may be the result of natural selection. Alternatively, such genes may have remained together on a chromosome because the opportunity for the repositioning of these genes on different chromosomes via chromosomal rearrangements has not occurred, or if it did occur, by chance the rearrangement did not become established. The earlier the divergence of two species the less likely is this second possibility.

Most chromosome mapping studies, to date, have been concentrated on eutherian mammals and particularly on man and mouse.

Another mammalian infraclass, the marsupials, diverged from eutherian mammals over 100 million years ago. (Air *et al.*, 1971) There has been a much greater period of time for chromosomal rearrangement to break up syntenic groups that occurred in a common ancestor prior to marsupial-eutherian divergence, than has passed between eutherian species. It is therefore of interest to compare syntenic groups of genes in eutherian and marsupial mammals. Any conservation of gene distribution over such an enormous period of time would indicate that natural selection favoured such gene arrangements. Of particular interest is the mammalian X chromosome, the gene content of which has been conserved in all mammals in which the X chromosome has been mapped. So far, the fairly sparse data on marsupials supports Ohnos' hypothesis of conservation of the gene content of the X chromosome throughout mammalian evolution (Ohno, 1967). As yet, there are no published linkage groups for any marsupial species. Family studies are hindered by problems associated with breeding marsupials in captivity and few useful genetic markers have been found. Apart from the assignment of some marsupial X-linked genes using family studies only a handful of genes (only one of which is autosomal) has been assigned to marsupial chromosomes using somatic cell genetic techniques. (Donald and Hope, 1981; Donald and Adams, 1981). There is, therefore, a need to map marsupial chromosomes in more detail to enable the study of homology of both X-linked and autosomal syntenys with other mammalian species.

Most genes that have been assigned to mammalian chromosomes code for enzymic markers. More recently, somatic cell hybrids have been used in the assignment to specific chromosomes, of genes coding

for cell surface antigenic markers (Kao *et al.*, 1976; Buck *et al.*, 1976; 1976a). The antigenic markers have been detected using both conventional polyclonal, and monoclonal antibodies (Milstein and Lennox, 1980). Cell surface antigens are particularly valuable genetic markers. They can be detected at the single cell level using fluoresceinated antibodies whereas this is not possible for most enzymes. Selection for or against somatic cell hybrids expressing cell surface antigens (and hence for or against the gene coding for the cell surface antigen or controlling its expression) can be achieved using cytotoxic antibodies (Puck *et al.*, 1971) or a fluorescence activated cell sorter (Parks *et al.*, 1979). Apart from their value as genetic markers, cell surface antigens are of interest in their own right as they appear to be involved in a number of important cell functions. They may act as receptors in cell-mediated immunity determined by histocompatability genes and in the determination of tissue and organ species specificity (Bodmer 1975; Klein 1975). They also play an important role in cell adhesion and recognition (Merrell *et al.*, 1975; Roth and White, 1972), differentiation (Bennett, 1975; Williams, 1977), tumour morphology (Andrews and Goodfellow, 1980) and as cell surface carrier proteins (Ho and Guidotti, 1975; Cabantchik and Rothstein, 1974). Cross-reaction of antibodies with a variety of species indicates antigenic homology between those species. There is considerable amino acid homology between the histocompatability antigens in man and mouse (Götze, 1977). In view of the early evolutionary divergence of eutherian and marsupial mammals it would be of interest to see if such homology exists for histocompatibility antigens in marsupials.

Genes determining the expression of X-linked cell surface antigens have been found in man, for example *Yg* (Mann *et al.*, 1962) and *SAX* (Buck and Bodmer, 1976 ; Dorman *et al.*, 1978; Schwab and Siniscalco, 1978). On the basis of Ohno's theory of conservation of the mammalian X chromosome, homologous loci coding for cell surface antigens would exist in other mammals. Apart from a tentative assignment of an X-linked gene determining a cell surface antigen in *Macropus rufus* (Sykes and Hope, 1978), no genes determining cell surface antigens have been assigned to any chromosome in any marsupial species.

The principle objective of this thesis was to use marsupial x eutherian somatic cell hybrids to:

(a) detect marsupial cell surface antigens, in particular X-linked antigens;

(b) map the genes controlling the expression of such antigens to specific chromosomal regions;

(c) investigate the possible homology of these genes, with genes determining cell surface antigens in eutherian mammals. It was also proposed to map enzymic genes (known to be determined by X-linked genes in other species) to the marsupial X chromosome, to see if the marsupial X chromosome has remained conserved over 100 million years of evolution, i.e. since the divergence from eutherian mammals.

Marsupials are well suited to somatic cell hybridisation studies, since they possess low numbers of easily distinguishable and well characterised chromosomes (Rofe, 1979; Hayman and Martin, 1974). The species, *Macropus rufogriseus*, was chosen for the present study for fusion with mouse cells because of the clearly distinguishable C-

banding patterns expressed by its chromosomes, enabling ready differentiation of these chromosomes from the mouse chromosomes. The *M. rufogriseus* X chromosome is of particular value for gene mapping studies because of its three interstitial C-bands on the long arm. Chromosome breaks between these three bands could be identified and employed in regional mapping studies.

In this project, three main approaches were taken in the analysis of the marsupial x mouse hybrids. First, the cell hybrids were analysed cytogenetically using various chromosome banding techniques to identify marsupial chromosomes present in the hybrids. Secondly, cell hybrid extracts were screened for enzymic markers using electrophoretic techniques. Thirdly, both conventional and monoclonal antibodies were induced against cell surface antigens present on marsupial x mouse cell hybrids and the hybrids were screened for the presence of antigen using an indirect immunofluorescence assay. The data derived from these three approaches were analysed to facilitate the mapping of genes, determining enzymic and antigenic markers, to marsupial chromosomes.

CHAPTER 2

LITERATURE REVIEW

2.1 GENE MAPPING

Genetic mapping involves determining, for a given genome, the positions of genes, either relative to one another, or relative to the positions of cytologically identifiable features of the chromosomes such as centromeres or G-bands. There are basically two types of genetic maps which differ fundamentally in the unit of measurement used in their construction. In *linkage* maps, which show the linear order and position of genes belonging to linkage groups, measurements are based on recombination frequencies. Data for the construction of linkage maps must be obtained from family studies. In what may be referred to as *chromosome maps*, the linear order and positions of genes are measured in terms of their physical positions on a chromosome. Obviously there is a relationship between these two types of genetic map. Such a relationship may be illustrated by referring to *Drosophila* where sufficient detail is available on both linkage and chromosome maps to enable meaningful comparisons to be made. While the order of a given set of genes on both types of map is of course the same, there is no direct or predictable proportionality between the distances measured on the two types of scale. This proportionality will be influenced by the distribution of chiasmata along the lengths of chromosomes. Renwick (1971) has coined the term 'syntenic' to refer to genes known to be located on the same chromosome. Genes that exhibit genetic linkage will be syntenic

but syntenic genes may not show genetic linkage.

In the classical genetic organisms in which family data pertaining to linkage may be readily obtained, including *Drosophila*, and *Mus musculus*, detailed genetic maps (linkage maps) have existed for many years. Up until recently, little detail existed on genetic maps in other species, including *Homo sapiens*. This lack of information is due largely to problems encountered in the collection of linkage data, e.g. long generation time, the need for large family size, three generation data and matings showing detectable variation at the loci concerned. These problems are overcome when collecting data for chromosome maps by using DNA transfer methods such as somatic cell hybridisation, chromosome mediated gene transfer, DNA transfer and molecular hybridisation to metaphase chromosomes (reviewed in Goss, 1978; Ruddle and Creagan, 1975; Siniscalco, 1979; Shows and Sakaguchi, 1980). Using these methods generation time is reduced to cell doubling time of cultured cells, cells of the desired mating can be fused without ethical problems and greater detectable variation exists for genes of different species. Hence, most chromosomal gene assignments have been made using these methods.

There is a large body of evidence demonstrating that rearrangement of the genetic material, without mutation or loss of genetic material can result in a change in the phenotype of an individual. Such effects are called "position effects" and include effects due to duplication of genetic material (Muller *et al*, 1936; Bridges *et al*, 1936), variegated position effects associated with interchange of genetic material (Catcheside, 1939, 1947; reviewed in Baker, 1968) and inactivation of genetic material due to the

artificial relocation of euchromatic segments of DNA near heterochromatic DNA segments (Cattanach and Isaacson, 1969). It is evident from these kinds of studies that neighbouring genes can influence one another and/or that their gene products can interact. Hence an increased knowledge of the arrangement of genes is crucial to the understanding of gene regulation and differentiation.

A knowledge of gene arrangement should also provide information on the evolutionary processes involved in conservation of syntenic groups of genes. In bacterial systems genes coding for enzymes involved in the same biochemical pathway are often under the control of an operon system. The main finding in eucaryotic genomes, so far, is that functionally related classes of genes need not be syntenic. Synteny of genes with related functions indicates that gene duplication has played an important role in evolution. This can be observed from amino acid data as has been shown for the globin genes (Ingram 1961; Dayhoff, 1972) and the immunoglobulin genes (Gally and Edelman, 1972) which evolved from a common ancestor by a process of gene duplication and subsequent evolutionary divergence. The major histocompatability systems (MHS) in a number of species have also been well studied. The MHS have been shown to consist of a bipartite structure in man, mouse, Rhesus monkey and guinea pig as a result of gene duplication (reviewed in Götze, 1977). The arrangement of genes can also give information on why some groups of genes have been conserved and others have not. For example, the group of genes controlling the same pathway for tryptophan synthesis has a different arrangement of these genes in nearly every group of organism (Crawford, 1975). The corresponding enzymes

show considerable sequence homology and so, probably have the same evolutionary origin but the gene cluster has been split up during evolution. This is not the situation for the MHS genes which are always observed in clusters even though there has been ample time for these clusters to have been broken up by evolutionary forces (Bodmer, 1972). There is some preliminary evidence that re-arrangements within the rat MHS are possible (Gill and Kung, 1976). Klein (1977) suggests that the MHS may need the same regulatory loci whereas genes such as those involved in the tryptophan pathway may not. It is evidence such as the above which has sparked interest in the compilation of chromosome maps in many different species in attempts to correlate gene arrangement with function, regulation and evolution.

The method most commonly used in the construction of chromosome maps is interspecies somatic cell hybridisation, first demonstrated by Barski *et al*, (1960). Interspecies cell hybrids can occur spontaneously in mixed cell culture but with very low frequency. They can also be made to occur with much higher frequency using cell fusagens such as inactivated Sendai virus (Harris and Watkins, 1965) or polyethylene glycol (Pontecorvo, 1975). Selection systems may be used to isolate the hybrid cells from the parent cells, the most commonly used of these being the HAT selection system (Szybalski *et al*, 1961; Littlefield, 1964). The most important aspect of interspecies somatic cell hybrids for the purpose of gene mapping is the preferential elimination of chromosomes of one of the parent species. This preferential chromosome loss enables a series of independently derived somatic cell hybrids to be examined for

correlation between the human chromosome retained and the human gene products expressed. The direction of chromosome loss can be controlled to some extent by the use of different parental cell types. In human x mouse somatic cell hybrids, the human chromosomes are usually preferentially eliminated (Weiss and Green, 1967). There are exceptions to this rule, where depending on cell type mouse chromosomes are eliminated, and hence the direction of chromosome loss is not solely determined by the parental species origin. (Croce and Koprowski, 1974; Minna and Coon, 1974). The reason for such chromosomal loss is not well understood. Ephrussi and Weiss (1967) have suggested that chromosome loss may arise through failure of one set of chromosomes to condense synchronously at mitosis. Hybrid cells resulting from the fusions contain all or most of the chromosomes of one species and only a few, one or fragments of chromosomes of the other species. The chromosomal constitution of the hybrids can be predetermined to an extent using a variety of selection systems. The gene for HPRT has been shown to be X-linked in those mammals where the gene has been assigned. Selection for the *HPRT* gene in human ($HPRT^+$) x mouse ($HPRT^-$) hybrids using the HAT selection system, therefore, effectively results in simultaneous selection for the human X chromosome. Different selective systems can be used to select for hybrids containing other specific human chromosomes e.g.; adenosine kinase and human chromosome 10 (Chan *et al*, 1978), serine hydroxymethyltransferase and chromosome 12 (Jones *et al*, 1972), adenosine phosphoribosyltransferase and chromosome 16 (Tischfield and Ruddle, 1974); and thymidine kinase and chromosome 17 (Weiss and Green, 1967; Miller *et al*, 1971). More recently, antibodies to cell surface antigens on somatic cell hybrids have been used to

select for or against cell hybrids containing genes coding for these antigens. (discussed in detail in Section 2.2).

Chromosome mapping is carried out by comparing the presence of specific chromosomes with the presence of a genetic marker in hybrid cells, for example, the presence of an enzyme or antigen, drug resistance or temperature sensitivity. The improved resolution obtained using new chromosome banding techniques (for example, Yunis, 1981 and Bobrow and Cross, 1974) alone, or in combination, has made possible easier identification of chromosomes and chromosomal fragments in interspecies hybrids. Cell repositories have now been established in which cell lines are stored and revived on request. Many of these cell lines contain specific translocations, deletions, etc., useful for gene mapping studies. Enzyme markers can be detected using a variety of biochemical and electrophoretic methods and antigenic gene markers can be detected using immunological techniques.

To assign a gene to a chromosome or chromosomal region, independent cell hybrids with known unique chromosome constitutions are screened for the presence of gene markers. Such a collection of independent hybrid cell lines constitutes what is termed a "clone panel" (Ruddle and Creagan, 1975). Different genes which segregate together in a hybrid clone panel are said to be syntenic.

Although somatic cell hybridisation provides a unique and convincing method of gene mapping, considerable caution must be exercised when interpreting the mapping data from somatic cell hybrids. For example, chromosomal fragments cannot always be confidently identified, with chromosome breakage resulting in both false positive and false negative results. An undetected chromosome translocation will result in non-random segregation of the translocated

genes, and hence those genes will appear, misleadingly, to be syntenic. Likewise an undetected chromosomal fragment or deletion will show lack of synteny of genes normally on the same chromosome. Also, without appropriate selection systems for each chromosome in a somatic cell hybrid, the chromosome constitution may often be determined by selection pressures beyond the control of the experimenter (Jonasson *et al*, 1977). Hybrids, with the required chromosome constitution for a particular study are, therefore, not always readily attainable or stable. Also, a structural locus may be present in a cell hybrid but may require the presence of a regulatory gene for its expression. Hence, before assignment can be confirmed independent studies are needed to verify assignment and synteny of genes to chromosomes.

2.2 CELL SURFACE ANTIGEN EXPRESSION IN SOMATIC CELL HYBRIDS

The eucaryotic cell surface membrane is composed of a complex mixture of lipids, carbohydrates and proteins which constitute macromolecules with antigenic properties (Bretscher, 1973). Cell surface antigens are known to play an important role in certain cellular functions, some of which were mentioned in Chapter 1. Little is known about the genetic control of structural and regulatory genes determining the expression of cell surface antigens.

Some antigens are ubiquitous in their cellular distribution, whereas others are tissue or organ specific, or are expressed only at certain stages of the cell cycle or at certain stages during differentiation. Some cell surface antigens appear only in a single species and are called species antigens, whereas others are found in many species, for example, heterophil antigens. A knowledge

of the chromosomal location and organisation of genes determining the molecular composition and expression of cell surface antigens may provide insight into their intrinsic biological functions.

Somatic cell genetic techniques have provided a valuable tool for studying the genetics of cell surface antigens. Surface antigens can be detected on somatic cell hybrids by a wide variety of methods including red-cell rosetting (Weiss and Green, 1967; Harris *et al*, 1969); radio-immuno assay (Smith *et al*, 1976); enzymic-linked immuno assay (Gasser *et al*, 1979); direct and indirect immuno-fluorescence (Shimizu *et al*, 1978) and cytotoxicity assays (Puck *et al*, 1971). The latter two methods have the advantage of revealing antigen expression at the single cell level. As an aid to gene assignment, it is possible to use these methods to compare the frequency of reacting cells with the frequency of identified retained chromosomes in somatic cell hybrids. It is also possible to detect simultaneously the presence of more than one antigen on the surface of single hybrid cells using antibodies conjugated to chromophores which fluoresce with different colours (Shimizu *et al*, 1978).

When cells from different species are fused the resultant heterokaryous usually exhibit codominant expression of the species cell surface antigens. (Watkins and Grace, 1967; Frye and Edidin, 1970). Codominant expression of cell surface antigens has also been demonstrated in interspecific (e.g. human x mouse) somatic cell hybrids (Kano *et al*, 1969; Fenyö *et al*, 1971) and in intraspecific hybrid cells (Barski, 1960; Spencer *et al*, 1964; Knowles and Swift, 1975), provided that chromosomes carrying the relevant genes are present. Weiss and Green (1967) found that the number of human species-specific cell surface antigens present was correlated with

the number of human chromosomes present in human x mouse somatic cell hybrids. They concluded that the genes coding for surface antigens are probably widely distributed amongst the human chromosomes providing valuable genetic markers for chromosome mapping. As chromosomes are lost from the hybrids so also are the antigens encoded by genes on those chromosomes. (Kano *et al*, 1969; Kano *et al*, 1972). If the chromosomal locations of genes determining cell surface antigens are known, then the loss of antigens from the hybrid cell surface can be used to indicate the loss of whole or parts of the relevant chromosomes. In this way, it is possible to study the pattern of chromosome loss in hybrid cells (Kennett *et al*, 1975).

The first use of interspecific somatic cell hybrids in the chromosomal assignment of a gene determining a human cell surface antigen was reported by Puck *et al* (1971). They made "antihuman" antibodies by injecting rabbits with different human cell types. Certain antisera were cytotoxic to human x hamster hybrids containing human chromosome 11 only, indicating the presence of a gene(s) on this chromosome determining a cell surface antigen(s). They called this antigen A_L, although they presented no evidence that the antigen was determined by a *single* structural gene.

The anti-A_L antiserum was used as a selective system to select against hybrid cells containing the A_L antigen and hence chromosome 11. (Puck *et al*, 1971). Puck *et al*, (1973) adsorbed the A_L antiserum with various human cell lines to determine the tissue distribution of the A_L antigen. The resulting adsorbed serum still reacted with some human tissues, hence the anti-A_L antiserum was detecting more than one antigen. Assuming that only one other antigen was being detected by the adsorbed serum, they called this antigen B_L. B_L did not segregate concordantly with A_L in the hybrids and was therefore presumably determined by a gene not present on human chromosome 11.

The A_L and B_L cytotoxic antibodies were used, either alone or in combination, as selective agents against hybrids expressing the A_L and/or B_L antigens, resulting in three cell types; $A_L^+ B_L^-$, $A_L^- B_L^+$ and $A_L^- B_L^-$.

The A_L system was further dissected into three antigens, $A_L a_1$ (= S_1), $A_L a_2$ (= S_2) (Jones *et al*, 1975) and $A_L a_3$ (= S_3) (Jones and Puck, 1977) using adsorbed sera. Kao *et al* (1977) regionally assigned the structural genes for $A_L a_1$, $A_L a_3$ and LDH-A to the short arm of chromosome 11 (11 p 13 → 11 p ter) and $A_L a_2$ to the long arm (11 q 13 → 11 q ter). These assignments were subsequently confirmed by Jones and Kao (1978). Using anti-a_1, anti-a_2 and anti-a_3 sera, six of the possible hybrid cell types containing various combinations of these genes and of LDH-A were selected. Killing by anti-a_1 serum was completely inhibited by adsorption with the major glycoprotein component of the human erythrocyte membrane, glycophorin. Hence the a_1 antigen had at least one cell surface antigen component in common with glycophorin (Moore *et al*, 1976). Further genetic and biochemical analysis of the a_1 cell surface antigen has been carried out by Jones *et al* (1979). Like a_1, but unlike a_2, a_3 is present on human erythrocytes. Kao *et al* (1977) suggested that the contiguity of the red blood cell antigenic loci (a_1 and a_3) may reflect participation in common regulatory processes.

Waldren *et al* (1979) utilized a cytotoxic selective system on these three cell surface antigens to measure mutagenesis in mammalian cells. Hybrid cells containing only human chromosome 11 were treated with a series of mutagenic agents. Only cells having lost the expression of one or more of the antigens, survived in the selective medium used, and these cells were assumed to be mutants at the

relevant genetic loci. Single cell survival curves were extrapolated from the results enabling estimates to be made of the forward mutation rate to loss of antigen expression caused by suspected mutagens. Because the presence of human chromosome 11 in the hybrids was not necessary for cell survival, large deletions could be detected that may not have been detected in other test systems. However, loss of antigen expression may possibly have been caused by changes at regulatory rather than structural loci. Regulatory genes could, for example, code for molecules necessary for transport to, and insertion of, the antigen into the cell surface membrane. The mutation estimates derived from the method of Waldren *et al* (1979) may, therefore, be suspect. If cytotoxic antibodies could be made against cell surface antigens coded by genes on each human chromosome, then the above approach could be extended to the comparative study of mutagenic effects on all the human chromosomes.

By using human x rodent somatic cell hybrids as immunogens in the syngeneic rodent host, the number of different antibodies produced is reduced to those directed against antigens coded for by chromosomes of the non-syngeneic species in the original target cells. Buck and Bodmer (1975) developed such an approach for mapping human cell surface antigens. They injected human x mouse hybrids into the syngeneic murine host. The resulting antiserum was directed against cell surface antigens coded for only by the few human chromosomes in the hybrid cells. The antiserum was adsorbed with mouse parental cells and human x mouse hybrid subclones containing selected human chromosomes to give a more specific antiserum which detected antigens determined by a gene(s) on human chromosome number 11. Although assuming that the antigen was the same as A_L (see above), they gave

it the more systematic nomenclature of SA-1 (species antigen 1, since renamed S4; Shows, 1979). SA-1 was found to be present on a wide variety of tissues including sperm and red blood cells (Buck and Bodmer, 1975). Using a balanced reciprical translocation a gene for expression of SA-1 was subsequently localised to the short arm of human chromosome 11 (Buck *et al*, 1976). *SA-1* was the first gene(s) coding for a cell surface antigen(s) to be assigned to a chromosome using interspecific hybrid cells.

Klinger and Ruoslahti (1980) have presented evidence suggesting that a structural locus for human fibronectin cell surface antigen (FN) is also on chromosome 11. It will be of interest to see if genes homologous to A_L and *FN* are found to be syntenic in other species, as chromosome 11 seems to have remained relatively conserved with respect to its gene map in other species (HGM-6, 1981), and, in the primates, conservation of the gene content of chromosome 11 has also been accompanied by conservation of G-band pattern (Finaz *et al*, 1977).

Using somatic cell hybrids and conventional antisera a number of genes coding for cell surface antigens have now been assigned to different human chromosomes including chromosome 7 (Aden and Knowles, 1976); chromosome 6 (Van Somaren *et al*, 1974); chromosome 12 (Seravalli *et al*, 1978); chromosome 21 (Slate and Ruddle, 1978) and chromosomes 10 and 14 (Owerback *et al*, 1979). If each human chromosome codes for at least one antigen and a cytotoxic assay is feasible, selection against any one or combination of chromosomes will be possible resulting in a method for the manipulation of the chromosomal content of somatic cell hybrids. Such a selective system could also be used to indirectly select against genes syntenic to particular antigenic

loci. For example, there is at present no known method to select directly against the LDH-A gene on chromosome 11. A_L is syntenic with *LDH-A* (Puck *et al*, 1971) and therefore by selecting against A_L using a cytotoxic anti-A_L antibody there would also be indirect selection against *LDH-A*.

Buck *et al* (1976a) assigned a cell surface antigen, SAX-1 (since renamed S10 (Shows, 1979)), to the human X chromosome by injecting human x mouse hybrid cells (1W1-5) into mice and testing the resulting sera for reaction with the hybrids. Subsequently, using radiation induced segregation, they regionally assigned the *SA-X* gene (*SAX-1*) to a region between *Hpt* and *Gpd* on the long arm of the X (Buck *et al*, 1976). Although these authors imply that SA-X is a single antigen coded for by a single gene, it is possible that more than one antigen is being detected by the SAX-1 antisera and that these antigens are determined by multiple loci. Further adsorptions of anti-SAX with human tissues may result in the detection of a series of antigens, as was found for the A_L system. Dorman *et al* (1978a) also described a cell surface antigen (SAX-2 = S11) determined by a gene on the human X chromosome. Unlike SAX-1, SAX-2 was found on human diploid fibroblasts (Dorman *et al*, 1978b). Neither SAX-1 nor SAX-2 appear to be associated with Xg, another X-linked human cell surface antigen present mainly on red blood cells (Mann *et al*, 1962). Schwab and Siniscalco (1978) also detected an antigen determined by a locus on the human X chromosome, SAX-3, and confirmed the regional mapping of the SAX-1 antigen(s) made by Buck *et al* (1976). Other human X-linked antigens, 12E7 and R1 have been defined by monoclonal antibodies and are discussed in Section 2.3.

It would be of interest to see if X-linked genes determining cell surface antigens have been conserved to the X chromosome to the same extent as X-linked genes determining enzymic markers. Additional evidence in favour of Ohno's hypothesis of conservation of the genetic content of the mammalian X chromosome would come from the observation that mammalian species other than man possess an antigen which shows cross-reaction with the human X-linked species antigen SA-X.

So far, only human X-linked antigens have been mapped using the above methods. There have been no comparative mapping studies in other mammals in search of homology for X-linked antigens apart from that of Sykes and Hope (1978) who showed that this method could be extended to a study of *M. rufus* (red kangaroo) x mouse somatic cell hybrids.

There have, however, been a number of reports illustrating antigenic conservation of the major histocompatability complexes (MHC) and H-Y in different species. Vitetta *et al* (1977) demonstrated that the homologous MHC genes of chicken, mouse, guinea pig and man share several amino acid residues in common.

Cross reaction of anti-mouse H-Y antisera with rats, guinea pigs, rabbits, humans, chickens, leopard frog and African clawed frog indicates antigenic similarity of H-Y in these different species. (Wachtel *et al*, 1974; Wachtel *et al*, 1975). The reason for conservation of the H-Y antigen is not obvious but presumably it has a sex-related function.

Lopo and Vacquier (1980) made a sperm specific antisera (SSA) against the plasma membrane of sperm of the sea-urchin, *Strongylocentrotus purpuratus*. SSA cross reacted with the surfaces of spermatozoa of

28 species representing 7 phyla of the animal kingdom. Reaction with SSA was not affected by adsorption with H-Y and hence SSA was not detecting H-Y.

2.3 MONOCLONAL ANTIBODIES

In the above experiments with polyclonal antisera, adsorption of sera with mouse cells and other cell types was required to restrict antibody reaction to only one human antigen. One of the problems with using conventional antisera is that regardless of the number of adsorptions carried out one can never be absolutely certain that the antisera contain only a single antigenic specificity. Extensive adsorption has been shown to be necessary to detect antibodies to polymorphic determinants, reducing considerably the titre of limited amounts of serum. (Staines *et al*, 1973; Greaves and Brown, 1973). Even if only one antigen is being detected many different antibodies can be made against a single antigen, each antibody reacting with an antigenic determinant or overlapping determinants of that antigen. Even though most antigenic determinants consist of no more than five or six amino acids (Van Vunakis *et al*, 1966), and can involve as small a difference as one amino acid substitution (Appella and Ein, 1967), the same antigenic determinant can be recognised by several different antibodies.

These problems were alleviated when Köhler and Milstein (1976) revolutionised classical immunology by employing hybridomas for the production of monoclonal antibodies. As their name suggests, monoclonal antibodies are antibodies secreted from a colony of cells derived from a single antibody secreting cell. Instead of isolating antibody from the blood serum of immunized mice, the spleen is

removed from an immunized mouse and the spleenic lymphocytes fused with a mouse myeloma cell line. Myeloma cells are tumours originating from a white blood cell of myeloid origin e.g. a plasma cell, which makes and secretes specific immunoglobulin against unknown antigens in an uncontrolled way. Myeloma cells continue to carry out their differentiated phenotype *in vitro*. Most myelomas produce immunoglobulin chains of their own but non-secreting myelomas have been isolated (Köhler and Milstein, 1976; Schulman *et al*, 1978). Non-secreting myelomas are more useful than secreting myelomas in fusions because they do not contaminate the monoclonal antibody with myeloma immunoglobulin species. According to Burnet's (1959) clonal selection theory any one spleenic lymphocyte secretes antibody of only one antigenic specificity, and a proportion of the lymphocytes in the spleen will be making antibody against the immunogen. If a lymphocyte secreting an antibody to the target cells fuses with a myeloma cell then the resulting hybridoma will produce and secrete that one antibody, thus, immortalising the antibody-secreting properties of the spleenic lymphocyte which would otherwise have undergone extinction if fused with other non-specialised cell types. Colonies of hybrid cells can be seen 7-14 days after the fusion. The antibody is secreted directly into the culture medium. The supernatants are then screened for antibody production against target cells. Although intraspecies hybrids are relatively stable with respect to chromosome retention, some chromosome elimination takes place in the hybridomas soon after fusion and can result in failure to secrete the required antibody due to overgrowth by non-secreting hybrids. For this reason, hybridomas are cloned at an early stage and positively secreting subclones selected for further use. Hence, the result is an unlimited supply

of monoclonal antibody which can be used as a pure and reliable diagnostic tool with the virtual elimination of non-specific binding. Of particular interest are human-human hybridomas such as that made by Olsson and Kaplan (1980). Human monoclonal antibodies could possibly be used as highly specific immunotherapeutic agents in the treatment of disease and cancer.

Monoclonal antibodies do, however, have some disadvantages. Often monoclonal antibodies are not cytotoxic because Ig G monoclonal antibodies react with only one site on an antigen and being typically bivalent can only form pairs of antibody/antigen complexes. Complement binding and activation necessary for a cytotoxic reaction usually depend on the availability of closely spaced clusters of four to five antigen/antibody complexes (Hyslop *et al*, 1970) and is hence dependent on the density and mobility of antigen on the cell surface (Howard *et al*, 1979). This problem can sometimes be overcome by using two or more monoclonal antibodies which bind non-competitively to different determinants on the same molecule resulting in a synergistic effect rendering the cell easier for complement binding (Howard and Coralen, 1978; Howard *et al*, 1979).

Monoclonal antibodies have been made against a vast variety of antigens over the last few years. Some of the types of monoclonal antibodies made and their applications are reviewed in Yelton and Scharff (1981); Milstein *et al*, (1979). The use of monoclonal antibodies for analysis of the HLA-system is reviewed in Brodskey *et al* (1979) and Charron and McDevitt (1979).

Goodfellow *et al* (1980), using human thymocyte x mouse thymoma somatic cell hybrids showed that the reaction of a monoclonal antibody, 12E7, raised against T-ALL cells from a human leukaemic patient was controlled by a human X-linked gene. The authors suggest that the

12E7 antigenic determinant is probably distinct from the previously described Xga, S10 and S12 X-linked determinants on the basis of different tissue and cell distribution. Although 12E7 monoclonal antibody reacted with red blood cells from both Xga-positive and Xga-negative cells, Goodfellow and Tippett (1981) were able to demonstrate a quantitative polymorphism which showed association with the *Xg* locus. 12E7 did not bind to 1W1-5 (the hybrid cells used as immunogen in the production of S10 serum - see Section 2.2) suggesting that 12E7 and S10 are not detecting the same antigenic determinant. Hope *et al* (1982) made a monoclonal antibody (R1) using 1W1-5 as immunogen in the search for a monoclonal antibody to the S10 antigen. R1 identified yet another antigenic determinant specified by an X-linked gene (*MIC-5*). Hope *et al* (1982) were unable to compare critically the specificities of the monoclonal antibody R1 and the antiserum S10 although they showed that R1 and 12E7 recognise different antigenic specificities. The question still remains as to whether R1, S10, S11 and S12 are detecting the same antigen. If each antibody is detecting a different antigen, this would suggest that the X chromosome may contain a large number of antigen determining genes. Andrews *et al* (1981) assigned to chromosome 12, a gene controlling a human cell surface antigen defined by a monoclonal antibody. Again, comparison between monoclonal and polyclonal antisera has proved difficult and it is not known whether the antibody is a component of the xenogeneic antiserum identifying the chromosome 12 - coded human cell surface antigen reported by Seravalli *et al* (1978).

Monoclonal antibodies enable the evolutionary study of single antigenic determinants by their cross-reaction with different species. The advantage of using monoclonal antibodies in evolutionary studies

is in their inherent extremely defined specificity. Any cross-reaction between different species should indicate conservation of the antigenic determinant detected. However, if the particular antigenic determinant detected by a monoclonal antibody has not been conserved in another species on an otherwise conserved antigen, the presence of the homologous antigen will go undetected. With a polyclonal antisera the non-conserved antigenic determinant would not be recognised but the conserved antigenic determinants would still be detected. Nevertheless monoclonal antibodies have been successfully used in comparison of the histocompatibility antigens in man and primates (reviewed in Brodsky *et al*, 1979) and in the demonstration of cross-reaction of a monoclonal antibody in rats and mice and humans (Gasser *et al*, 1979). In this latter study the antigen detected was shown to be determined by a gene syntenic to the major histocompatability complex in mice and rats, and the gene determining the antigen maps on human chromosome 6, the same chromosome that contains HLA. It seems unlikely that the same antigenic specificity would occur in the above species by chance alone and hence it is possible that a genetic segment has been conserved among these species. The antigen is not present in all rats and mice and hence is not necessary for survival. The reason for such conservation is unknown.

2.4 COMPARATIVE GENE MAPPING

Ohno (1967) postulated that the mammalian X chromosome has remained conserved throughout mammalian evolution. His theory was based on a number of observations. Genes which had been shown to be X-linked in one species were also found to be X-linked in all other

species where the gene mapping information was available. Although the size of the X chromosome varies greatly in different mammalian species, Ohno *et al* (1964) have suggested that the functional X chromosome of all mammals is similar in size relative to the entire complement (5-6% of the haploid female genome). It is postulated that the difference in overall size of the X chromosome in different mammalian species is probably due to the addition of genetically inert constitutive heterochromatin or a less common mechanism of X-autosome translocation. Pathak and Stock (1974) studied the G-banding pattern of the basic X chromosome in a large number of species and found two major bands persistently present in all X's irrespective of the gross morphology of the X chromosomes. Until the DNA composition of each band is identified it cannot be assumed that the genetic content of "homologous G-bands" in different species is the same. However, when such banding homology is shown in a large number of species the value of the data is increased. In female mammals, one X chromosome is randomly inactivated in each somatic cell. Once inactivated, it remains inactivated in all descendents of that cell. Ohno (1973) suggested that conservation of the mammalian X chromosome is due to the dosage compensation requirement of X-linked genes. It is possible that some X-linked genes have regulatory functions and hence an excess of X-linked products could result in a metabolic malfunction in the organism.

So far no exception has been found to Ohno's theory of conservation of the genetic content of the mammalian X chromosome. The four most widely studied X-linked genes are those coding for the enzymes, hypoxanthine phosphoribosyltransferase (HPRT), phyophoglycerate kinase-A (PGK-A), glucose-6-phosphate dehydrogenase (G6PD) and α-galactosidase (GLA). These genes have been mapped to

the X chromosome in a large number of mammalian species (6th Gene Mapping Conference, Norway, 1981).

As more gene mapping information has become available, it is apparent that there has also been some conservation of autosomal syntenic groups of genes during mammalian evolution. So far, however, there are also many examples of autosomal groups of genes which have not remained syntenic in different species. It is not yet apparent why some, but not other, syntenic groups of genes have been conserved in different species. Information from future comparative gene mapping studies should help to answer this question.

Most gene mapping work using somatic cell hybrids has been carried out in man and mouse, and hence conservation of syntenic groups of genes is more obvious in these species to date. For example, a large region of mouse chromosome 4 and the short arm of the human chromosome 1 has remained conserved throughout evolution (Lalley *et al*, 1978a). These chromosomes show homology for *ENO-1*, *PGD*, *PGM-2*, and *AK-2*. *DIP-1* is on the mouse chromosome 1 and the homologous *PEP-C* is on 1q in humans. Perhaps the human 1q is homologous to chromosome 1 in mouse. This homology of synteny is evidence for conservation of syntenic groups of genes over a period of 80 million years, the estimated time of divergence of man and mouse.

Lalley *et al* (1978b) list nine cases where two or more autosomal loci are known to be either linked or syntenic in man and *Mus*. There is evidence that genes which are tightly linked in man seem to have remained syntenic throughout mammalian evolution (Minna *et al*, 1976). Thymidine kinase and galactokinase have been shown to be syntenic in mouse (Kozak and Ruddle, 1977), man (Elsevier *et al*, 1974),

chimpanzee and African green monkey (Orkwiszewski *et al*, 1976; Chen *et al*, 1976). Evidence for other conserved syntenies in divergent species have been reported. (See Gene Mapping Conference, Norway, 1981).

The syntenic relationships of the major histocompatibility systems (MHS) have been studied closely in man, mouse and chimpanzee. *HL-A,B,C,Ir, MLR* and *C'* genes are syntenic in man and their homologous equivalents are also syntenic in mouse. (Bodmer, 1972; Klein, 1979). These two systems also exhibit some functional homology as well as genetic and structural homology. MHC systems have been found in many other species (Götze, 1977) and clusters of génes controlling graft rejection, mixed lymphocyte reaction, immune responsiveness and complement activity exist in species as different from mammals as chicken (Hála, 1977) and *Xenopus* (Du Pasquier *et al*, 1975).

Homologous genes in different primate species have been shown to reside on chromosomes also known to be homologous with respect to chromosome banding pattern (Warburton and Pearson, 1976; Finaz *et al*, 1977). The HLA genes are located on human chromosome 6. The equivalent MHC in the chimpanzee is found on the G-band equivalent chimpanzee chromosome number 5. The MHC systems in these two species show synteny with *SOD-2, ME-1, PGM-3* and *GLO-1*. The MHC of the gorilla, orangutan, and rhesus monkey are also located on the chromosomes homologous to the human 6, and, in the gorilla and orangutan syntenic with *PGM-3, SOD-2* and *ME-1,* and in the rhesus monkey with *PGM-3* and *SOD-2*. (Garver *et al*, 1980). This is another example of conservation of linkage groups during mammalian evolution.

Information on the conservation of syntenic groups of genes, both X-linked and autosomal, in a wide variety of different species

reveals constraints on karyotypic evolution and hence organisation of the genome. Therefore, comparative mapping is of importance in the study of the evolution of the mammalian genome and gene regulation.

Syntenic groups of genes in one species can act as a starting point when looking for syntenic groups in other species. However, difficulties can arise in the recognition of homologous gene products in different species without comparing their molecular structure.

Most gene mapping studies using somatic cell hybrids have been aimed at assigning genes to specific chromosomes ráther than determining the arrangement of genes on those chromosomes. If, along with gene synteny, gene arrangement has also been conserved for groups of genes in different species, this should give some insight into the importance of the arrangement of genes in evolution and differentiation.

It seems possible that genes that are closely linked in one species should remain so in other species if selection favours that gene combination (Fisher, 1958). Bodmer and Bodmer (1978) have suggested that the main selective force involved in the conservation of the major histocompatibility syntenic relationships is that of retention of genes involved in disease control. On the other hand, Ohno (1970) suggests that with the exception of the special case of the X chromosome, conservation of gene arrangement throughout evolution is due to chance. He argues that the most common mechanisms of chromosom al rearrangement in mammalian evolution are Robertsonian fusions, Robertsonian fissions and inversions. These changes do not tend to break up syntenic groups to a large extent and hence the conservation of autosomal regions might be expected due to chance. Any reciprocal translocations that might occur would, of course,

cause the breakage of linkage groups. The earlier the evolutionary divergence of species exhibiting conservation of gene synteny the less likely is the conservation due to chance as suggested by Ohno (1970).

2.5 GENETIC STUDIES IN MARSUPIALS

Marsupials are an extant branch of mammals estimated to have diverged from the eutherian mammals approximately 130×10^6 years ago (Air *et al*, 1971; Romero-Herrera *et al*, 1973). Because of this large evolutionary separation, it is of interest to see if groups of genes which are syntenic in eutherian mammals are also syntenic in marsupials; any such conservation of gene synteny presumably having existed over an enormous period of time.

The structural genes for the enzymes G6PD and PGK-A have been shown to be X-linked in kangaroos using family studies (Cooper, 1975), as is the case for all other mammals in which these genes have been mapped. Apart from these enzymes, there have been few gene mapping studies on marsupials, compared to the vast gene mapping literature on eutherian mammals especially on man and mouse (HGM-6). Satellite DNA's have been studied in the macropod marsupials *Macropus rufogriseus* (Dunsmuir, 1976) and *Macropus robustus robustus* (Venolia and Peacock, 1981). The *M. robustus robustus* satellite cross hybridised to the long arm of the *M. rufogriseus* X chromosome, indicating that this sequence has been conserved in the two species. Some aspects of the genetics of Australian marsupials are outlined in Cooper (1974).

Marsupials lend themselves well to gene mapping studies because of their low numbers of large easily distinguished chromosomes. Marsupial chromosomes have been well characterised cytogenetically

(Hayman and Martin, 1974; Hayman, 1977; Sharman, 1973 and Sharman, 1974). Rofe (1979) examined G-banded chromosome preparations from a large number of macropod marsupials and was able to show gross homology of G-banding pattern amongst the different species. She postulated a series of simple inversions and Robertsonian translocations to explain the evolutionary changes or variation and she was able to construct a phylogenetic pathway of the Macropodidae based on the smallest possible number of rearrangements required to transform the karyotype of one member of the Macropodidae to that of another. Studies on the genetic content of these G-band homologous marsupial chromosomes would help to determine if the genetic content of them has been conserved along with their gross G-banding pattern.

By studying the comparative serology of the Marsupialia, Kirsch (1968; 1977) constructed similar phylogenetic pathways to that of Rofe (1979). Serological data has also been used in classification and has provided evidence of speciation in the grey kangaroo (Kirsch and Poole, 1967; 1972).

Apart from their interest from an evolutionary viewpoint, another important reason for studying the genetic map of marsupials follows from their unique mechanism of X inactivation. X-inactivation occurs in each somatic cell of the mammalian female at an early stage of embryogenesis. In eutherian mammals the choice as to which chromosome is inactivated appears to be random, but once made is maintained in those cells and progeny cells (Lyon, 1961). In marsupial mammals, the paternal X chromosome seems to be preferentially inactivated (Sharman, 1971). Cooper (1975) suggests that marsupial paternal X-inactivation may be the ancestral mechanism of the random X inactivation found in eutherians. It is of interest to

see if the theory of conservation of the mammalian X holds as strongly for marsupial mammals as it does for eutherian mammals. It will be of particular interest to find homologous genes for the human *Xg* and microsomal steroid sulphatase genes in marsupials to see if they escape X-inactivation as is thought to occur in eutherian mammals (Race, 1971; Fialkow, 1970; Shapiro *et al*, 1979). So far, no gene linkage groups have been published for marsupials due largely to the problems associated with breeding marsupials in captivity for family studies. An alternative approach for mapping marsupial genes is to use somatic cell hybridisation techniques to assign marsupial genes to chromosomes and to investigate their syntenic relationships. Due to the evolutionary distance between marsupial and eutherian mammals the existence of a large number of potential isozyme differences between these two classes of mammals should be very useful for gene mapping using marsupial x eutherian somatic cell hybrids (Cooper, 1974; Graves and Hope, 1977a; Hope and Graves, 1977b).

Many difficulties were encountered in attempts to hybridise marsupial and eutherian cells, but after a series of detailed studies on the co-cultivation of marsupial and mouse cells (Graves and Hope, 1977a), fusion (Graves and Hope, 1977b), heterokaryon formation and activity (Graves *et al*, 1977; Graves and Hope, 1978) and the development of appropriate selective systems (Hope and Graves, 1978a), successful hybridisation of marsupial and mouse cells was achieved (Hope and Graves, 1978b). Marsupial chromosomes were preferentially eliminated in the marsupial x mouse cell hybrids and hence these hybrids have the potential to facilitate the mapping of marsupial genes. However, in order to map genes to marsupial chromosomes it is necessary to produce series of somatic cell hybrids containing a variety of different marsupial chromosomes. Problems have been

encountered in obtaining marsupial x rodent cell hybrids containing normal marsupial chromosomes (Wainwright p.c., Hope, p.c. and Graves, p.c.). Hope and Graves (1978b) observed extensive fragmentation of the marsupial genome soon after fusion with mouse cells. It is possibly as a result of such fragmentation that difficulties have been encountered in obtaining normal marsupial chromosomes in the hybrids.

Only two autosomal genes have been assigned to marsupial chromosomes. The first autosomal gene to be assigned in a marsupial was the gene for LDH-A to the *M. rufus* 5 in *M. rufus* x mouse cell hybrids (Donald and Adams, 1981). The second autosomal assignment, which is also the first gene coding for an antigen assigned in a marsupial, has been provisionally assigned to the long arm of the *M. rufogriseus* 3, as detected by a monoclonal antibody (Sykes and Hope, in preparation).

CHAPTER 3

MATERIALS AND METHODS

3.1 CELLS

3.1.1 Fusion Partners

Two mouse tumour cell lines, PG19 and 1R, were used as fusion partners in the marsupial x mouse hybridisation experiments. PG19 is an hypoxanthine phosphoribosyl-transferase deficient ($HPRT^-$), 6-thioguanine resistant ($6TG^r$) cell line derived from a C57BL/6J mouse melanoma (Jonasson *et al*, 1977). 1R is an $HPRT^-$, 8-azaguanine resistant ($8AG^r$) L cell derivative of C3H origin. (Nabholz *et al*, 1969).

Both marsupial lymphocytes and fibroblasts were employed in fusions. A primary fibroblast line (henceforth referred to as RNW ♀) was set up from ear tissue of a *Macropus rufogriseus* female. *M. rufogriseus*, *Macropus parryi* and *Macropus eugenii* lymphocytes were separated from whole blood using Ficoll/Hypaque density gradients.

P3/NS1/1Ag4/1 (referred to as NS1), an $HPRT^-$ ($8AG^r$) non-secreting myeloma cell line derived from a BALB/c mouse (Köhler *et al*, 1976) was used as the myeloma parent in the formation of hybridoma cell lines. NS1 synthesises its own κ light chains but is a non-secreting plasmacytoma.

Myeloma cells were fused with spleenic lymphocytes isolated from mice which had been pre-immunised with target cells.

3.1.2 Nomenclature of Marsupial x Mouse Somatic Cell Hybrids

The nomenclature and parental cell types of the marsupial x mouse primary hybrids are summarised in Table 3.1.

The nomenclature of the REP hybrids requires further explanation. *M. rufogriseus* (red-necked wallaby) lymphocytes x PG19 hybrid cells have been designated REP hybrids. The first two letters in REP, are derived from "red" in red-necked wallaby, and, P, from PG19. All the REP fusion culture plates contained a number of colonies. One independently isolated primary REP hybrid was obtained from each fusion culture plate with a cloning ring. These primary hybrids were distinguished by a number e.g. REP1, REP2 etc. The remaining cells from each fusion plate, consisting of a mixture of cells from other independent colonies and possibly also cells from the primary colony independently isolated from the plate, were harvested together and referred to as bulk (B) hybrids, e.g. REPB1, REPB2, etc. Hence these hybrid cells were not derived from single clones. REPB1 consists of all the remaining cells in the fusion plate from which the primary hybrid REP1 was originally isolated.

HAT selected subclones of REP hybrids are designated as subclasses of the primary or bulk hybrids, e.g. REP3-1, REP3-2, REPB3-1, REPB3-2 etc. REP3-7-1 is a subclone of REP3-7 which is itself a subclone of REP3.

6TG selected subclones of REP hybrids were referred to as revertant cell lines, designated by an R and distinguished by a number, e.g. REP3R1, REP3R2 etc. REP3R1 is a 6TG selected

TABLE 3.1 Summary of nomenclature of marsupial x mouse primary hybrids

HYBRID PARENTAL CELLS	PRIMARY HYBRID NOMENCLATURE
M. rufogriseus x PG19 (lymphocytes)	REP1
	REP2
	REP3
	REP4
M. rufogriseus x PG19 (fibroblasts)	PGRN-1
	PGRN-2
	PGRN-3
M. rufogriseus x 1R (fibroblasts)	1RRN-1
	1RRN-2
M. parryi x 1R (lymphocytes)	WT1R-1

subclone of the primary hybrid REP3.

All the REP hybrids used in the present study are listed in Table 3.2.

3.2 CELL CULTURE

3.2.1 Culture Medium

3.2.1.1 Standard medium

PG19 and 1R were grown in HAMS F10 (GIBCO) medium supplemented with 10% foetal calf serum (FCS) (Commonwealth Serum Laboratories or FLOW), 1% w/v glutamine, 50 µg/ml streptomycin sulphate and 60 µg/ml penicillin (Appendix 1). The medium was supplemented with additional glutamine every 7 days after storage at $4^{o}C$. 6TG (5 µg/ml) was periodically added to the medium of 1R and PG19 to select against the possible occurrence and growth of $HPRT^{r}$ cells in these cell cultures. Cells were incubated in a 5% CO_2 humid atmosphere at 36-$37^{o}C$.

Marsupial primary fibroblast cultures were grown in standard medium with 15% FCS.

3.2.1.2 Marsupial x mouse cell hybrids

Standard medium was supplemented with HAT (10^{-4} M hypoxanthine, 10^{-6} M aminopterin, 1.6×10^{-5} M thymidine) (Littlefield, 1964). When marsupial fibroblasts were used as parent cells, 1×10^{-6} M ouabain was also added to the medium for selection of hybrid cells, in order to slow down the growth of the more ouabain sensitive marsupial parental cells (Hope and Graves, 1978a).

TABLE 3.2 REP hybrids

PRIMARY HYBRIDS	BULK HYBRIDS	SUBCLONES		REVERTANTS
REP1				
REP2				
REP3		REP3-1		REP3R1
		REP3-2	REP3-2-1	REP3R2
			REP3-2-2	REP3R3
		REP3-3		REP3R4
		REP3-4		REP3R5
		REP3-5		REP3R6
		REP3-6		REP3R7
		REP3-7	REP3-7-1	REP3R8
			REP3-7-2	
			REP3-7-3	
			REP3-7-4	
			REP3-7-5	
REP4				
	REPB1			
	REPB2			
	REPB3	REPB3-1		
		REPB3-2		
		REPB3-3		
		REPB3-4		
		REPB3-5		
	REPB4			
	REPB5			

3.2.1.3 Myeloma cells

NS1 cells were grown in standard medium with 15-20% FCS. Periodically 5 µg/ml 6TG was added to prevent the possible occurrence and growth of $HPRT^r$ cells in the culture.

3.2.1.4 Hybridoma cells

After fusion of myeloma and spleenic lymphocytes, the cells were placed in standard medium supplemented with 15-20% FCS and BALB/c spleen feeder cells (2×10^6 viable cells/ml). Spleen cells were also used when subcloning hybridoma cells by limiting dilution. (1 or 2 hybrid cells/ml with 2×10^6 viable spleen cells/ml). About two weeks after fusion, cells were grown in standard medium with 15-20% FCS, 10^{-4} M hypoxanthine and 1.6×10^{-5} M thymidine.

3.2.2 Cold Storage and Revival of Cell Cultures

3.2.2.1 Monolayer cultures

Cells were harvested with trypsin-versene (Appendix 1), washed once in PBS (Appendix 1), resuspended in 5% DMSO in FCS and aliquoted into polypropylene freezing ampoules. Ampoules were either (1) placed in a liquid nitrogen programmed cooler to -100°C (0 to -25°C at 1°C/min, -25 to 100°C at 5°C /min) and then placed directly into liquid nitrogen or (2) placed directly into a -80°C freezer overnight in a polystyrene container and then placed in liquid nitrogen.

To thaw an ampoule, cells were removed from liquid nitrogen thawed quickly in a water bath at 37°C and placed directly into the appropriate culture medium.

3.2.2.2 Myeloma cells

NS1 cells were washed once in PBS and adjusted to 6×10^6/ml in standard medium with 50% FCS. One millilitre aliquots were placed in freezing ampoules and 1 ml of standard medium with 30% DMSO was slowly added to each ampoule. These were then frozen in the manner used for monolayer cultures.

Frozen ampoules were thawed rapidly in a 37°C water bath. The contents of each ampoule was diluted slowly with an equal volume of F10 medium over 5 mins, left to equilibrate for 15 mins at R.T. and the procedure repeated. The cells were then spun at 110g for 10 mins and resuspended in standard medium with 20% FCS.

3.2.2.3 Hybridomas

Considerable difficulty was encountered in attempting to revive frozen GA-1 stocks.† A number of different combinations of freezing/thawing procedures were tried, including various DMSO concentrations, the use of conditional medium, and the above method for NS1, all without success.

Finally, GA-1 was successfully thawed using the above freezing method for NS1 and thawing into standard medium, supplemented with 20% FCS, BALB/c feeder layer and FM stock (Appendix 1). Cells were distributed into 24 and 96 well plates directly after thawing. Cell growth occurred in most wells using this method.

3.2.3 Selection of Subclones

3.2.3.1 Marsupial x mouse cell hybrids

HAT selected hybrid cells were harvested with trypsin-versene, counted, and plated out at a density of 2×10^2 cells/

†This hybridoma secretes the GA-1 antibody described in Section 4.4.2.1 (page 112).

60×15mm culture plate in HAT medium. Approximately 2-3 weeks later colonies were picked with cloning rings. Subclones were maintained in HAT medium.

3.2.3.2 Hybridomas

GA-1 was subcloned by limiting dilution, with or without a spleen feeder layer, and the subclone supernatants screened for binding of antibody to target cells using an indirect immunofluorescence assay.

3.2.4 Selection of Revertants

HAT selected hybrid cells were harvested with trypsin-versene, counted and plated out at 2×10^5 cells/60×15mm culture plate in standard medium with hypoxanthine, thymidine and 5 µg/ml 6TG. Hypoxanthine and thymidine were added at this stage to dilute out, by cell growth, any residual aminopterin. After one week cells were cultured in standard medium with only 5 µg/ml 6TG. Colonies appeared 7-10 days later and were picked using cloning rings. Revertants were maintained in medium with 6TG.

3.2.5 *M. rufogriseus* and *M. rufus* Primary Fibroblast Cell Lines

A small sterile piece of shaved ear was removed using a punch and placed into a few millilitres of F10 medium without serum. The tissue was then cut into small pieces and placed in a culture plate containing just enough standard medium with 20% FCS to wet the bottom of the plate. When the pieces of tissue had attached, additional medium was added to the plate. In setting up the *M. rufus* (red kangaroo) primary, tissue attachment was aided by placing a sterile

coverslip over the pieces of ear to prevent them from floating in the medium. After about 5 days cells with an appearance resembling epithelial cells followed by fibroblast-like cells were observed.

3.3 MARSUPIAL x MOUSE CELL FUSIONS

3.3.1 Suspension Fusions

The marsupial was bled from the caudal-vein and the whole blood diluted to twice its volume with citrated saline. The lymphocytes were separated by layering the diluted blood onto a 4 Ficoll/Hypaque stock:1 H_2O (Appendix 1) density gradient and spun for 20 mins at 400g. The lymphocyte layer was removed from the gradient and washed 3× in PBS. Lymphocytes were incubated with FDA (fluorescein diacetate) and viable cells counted on a haemocytometer viewed under UV light (Miggiano *et al*, 1970).

The mouse cells, 1R or PG19, were harvested with trypsin-versene and washed three times in PBS. Parental cells were mixed in the ratio 3 marsupial :1 mouse and spun at 130g for 10 mins. The cell mixture was resuspended slowly in 1 ml 50% polyethylene glycol 1500 (PEG 1500, BDH) in F10 without serum (F10-S), over a period of 1 minute and then diluted slowly with 20 mls F10-S over 7 mins with gentle mixing. The cells were spun at 130g for 10 mins and then aliquoted into 50 ml 'Falcon' flasks in standard medium with 20% FCS. The next day, HAT was added to the medium.

3.3.2 Monolayer Fusions

M. rufogriseus fibroblasts and PG19 or 1R cells were harvested with trypsin-versene, mixed in a 1:1 ratio and

allowed to attach overnight in standard medium with 20% FCS in 'Falcon' flasks. The monolayers were washed twice with PBS and then 1 ml 50% PEG 1500 (BDH) in F10-S was placed over the monolayer for 55 secs. The PEG 1500 was removed by washing the monolayer three times with PBS. Standard medium with 20% FCS was then added to the culture vessels. Next day the medium was supplemented with HAT.

3.4 MYELOMA x SPLEEN CELL FUSIONS

A number of fusion methods were used throughout the project. The most successful procedure (described below) is an adaptation of Oi and Herzenbergs (1979) method and the method employed by workers at the Imperial Cancer Research Fund, London (Personal communication, R.M. Hope).

The mouse spleen was removed aseptically and teased into F10-S medium using 2 × 25 gauge needles. Large clumps of tissue were removed and the cells spun at 280g for 10 mins. The cells were washed 2 × in F10-S. In some fusions the red blood cells were lysed using Geys medium (Appendix 1) or with 1 part cell suspension : 9 parts 0.4% acetic acid. Viable cells were stained with FDA or dead cells with trypan blue and counted with a haemocytometer.

NS1 cells were harvested, and washed 2 × in F10-S. Cells were treated with trypan blue and viable cells counted on a haemocytometer. NS1 and spleen cells were mixed in the ratio of approximately 10 spleen : 1 NS1 and centrifuged at 280g for 10 mins. The pellet was resuspended in 0.8 ml 50% w/v PEG 1500 (BDH) in F10-S (prewarmed to 37°C) with gentle shaking over 1 min, and then held at 37°C for 1 min. The fusion mixture was diluted by slow addition of F10-S; 1 ml over 1 min

and then 20 ml over 5 min. Cells were centrifuged for 20 mins at 1800 rpm and resuspended *very* gently in standard medium with 20% FCS. One drop/well of the fusion suspension was plated into wells of Linbro 96 well (0.35 ml) flat bottomed plates. (Cat. No. 76-003-05).

Next day 1 drop of 2 × concentrated HAT medium was added to each well. For some of the fusions FM stock was also added to the medium (Appendix 1). Five to ten days later colonies appeared in a proportion of wells. The supernatants in these wells were screened for production of antibody to target cells using an indirect immunofluorescence assay.

3.5 CHROMOSOME PREPARATIONS

3.5.1 Standard Preparations

3.5.1.1 Monolayer cultures

Rapidly dividing cell cultures were incubated in the presence of 0.5 µg/ml colcemid for times ranging from 30-60 mins. Cells were washed once with PBS and harvested with trypsin-versene. After one wash in PBS the cells were resuspended in a solution of 1½ H_2O : 1 PBS for a few seconds and then centrifuged at 110g for 10 mins. Originally 0.075 M KCl for 20 mins was used as hypotonic treatment but the former method was faster and gave similar results. Preparations were fixed 3 × in 3:1 methanol:acetic acid, spotted onto slides and air-dried.

3.5.1.2 Lymphocyte cultures

The animal was bled with a syringe containing 0.02 ml of preservative free heparin (1000 units/ml), and the blood diluted

to twice its volume with citrated saline or PBS. Sometimes glass beads were used to defibrinate the blood. The diluted blood was layered onto a 4 Ficoll/Hypaque : 1 H_2O density gradient and centrifuged at 270g for 20 mins. The lymphocyte layer was isolated, washed 3 × with PBS and placed into 3 × 5 ml cultures of standard medium containing 0.05 ml PHA-M (difco-Phytohaemagglutinin form M) for two days. Colcemid was added to the cultures 1 hr prior to harvesting and chromosome preparations were made as described in Section 3.6.1.1.

3.5.2 Thymidine Pre-treatment

A final concentration of 5 mM thymidine was added to rapidly dividing cultures for 16 hours to synchronise cells in the S phase of the cell cycle. The thymidine was then removed by washing the monolayer once with PBS and the cells allowed to divide. Twelve hours later a final concentration of 0.05 µg/ml colcemid was added to the culture for 12 hours. Cells were harvested and the chromosome preparation made using the procedure already described.

3.5.3 Hoechst 33258 Pre-treatment

0.04 mg/ml Hoechst 33258 was added to cultures for from 5-12 hours prior to harvesting. During this period the culture vessel was wrapped in foil to prevent light degradation of Hoechst 33258.

3.5.4 Synchronisation of Cultures with Amethopterin

Synchronisation of *M. rufogriseus* lymphocytes in late prophase and prometaphase was achieved using the amethopterin

block method of Yunis (1978). Best results were obtained by adding 0.05 µg/ml colcemid for 4 hours 45 mins after release from the amethopterin block.

3.6 CHROMOSOME STAINING METHODS

3.6.1 General Stain

Slides were stained for 2 mins in 10% Giemsa v/v (Gurrs R66) in Sorensons phosphate buffer pH6.8, air-dried and mounted in Depex.

3.6.2 C-Banding

Chromosomes were C-banded using a modification of the method of Sumner (1972). Slides were treated with:

1. 0.02 M HCl for 30 mins at RT.
2. Rinsed 2 × in distilled H_2O.
3. Saturated $Ba(OH)_2$ at 56°C for ½ - 2 mins.
4. Rinsed 2 × in distilled H_2O.
5. 2 × SSC (Appendix 1) at 65°C for 30 mins.
6. Rinsed 2 × in distilled H_2O.
7. Stained with 10% v/v Gurrs R66 giemsa in Sorensons phosphate buffer, pH6.8 for 12 mins, air-dried and mounted with Depex.

3.6.3 G-Banding

Chromosomes were G-banded according to a modification of the method of Seabright (1971). Slides were immersed in a 0.01 - 0.05% w/v solution of trypsin for 20 - 200 secs, washed 3 × in PBS at 4°C, stained for 6 - 8 mins in 2% v/v Giemsa (Gurrs R66) in Sorensons phosphate buffer pH6.8, air-dried and mounted in Depex.

3.6.4 Sequential G and C-Banding

Kozak *et al* (1977) carried out sequential G and Hoechst 33258 banding in the analysis of interspecific hybrids. This method was modified for sequential G and C banding as follows.

Slides were G-banded as above, mounted in Depex and G-banded metaphase spreads photographed. Coverslips were removed using xylene and slides destained in 3:1 methanol: acetic acid. These slides were then C-banded as described above and the same metaphase spreads rephotographed.

3.6.5 N-Banding

Slides were incubated in distilled H_2O at 37°C for 1 hour and then allowed to dry. Cells were stained with filtered 50% silver nitrate and ½ coverslips placed sequentially along the slide to allow contact with air. Slides were incubated in a humid chamber for 24 hours at 37°C. Coverslips were washed off with distilled H_2O and slides counter-stained with 5% v/v Giemsa (Gurrs R66) in Sorensons phosphate buffer pH6.8 for 30 secs, dried and mounted (Bloom and Goodpasture, 1976).

3.6.6 In Situ Hybridisation

The *in situ* hybridisations of the *M. rufogriseus* major satellite (density 1.708g/cc) to REP 3-2 and REP 3-2-1 were kindly carried out by Ms. Abigail Elizir (Division of Plant Industry, C.S.I.R.O. Canberra, Australia) using the method outlined in Dunsmuir (1976).

3.6.7 R and Distamycin A(DA)-DAPI Banding

Simultaneous production of R and DA-DAPI bands was obtained by sequentially staining slides with Chromomycin A3,

DA and DAPI according to the method of Schweizer (1980). The DA-DAPI bands were viewed using a Leitz Ploem Opak Filter block A and the R bands using a Leitz Ploem Opak filter block E3.

3.6.8 Counterstaining of DAPI with Actinomycin D

Greatly enhanced DAPI bands were obtained by staining slides with DAPI followed by staining with actinomycin D according to a modification of the method of Schweizer (1976). The DAPI bands were viewed using a Leitz Ploem Opak filter block A.

3.7 PHOTOGRAPHY

Chromosomes were photographed with Copex Pan 35mm high contrast film (Agfa-Gaevert) using a Zeiss 60X Planapo, 1.4 numerical aperture oil immersion objective or a Zeiss 100X NPL Fluotar oil immersion objective. Films were developed in D19 or DEKTOL fine grain developer and printed onto Ilfospeed photographic paper - grades 1, 2 and 3.

3.8 ELECTROPHORESIS

3.8.1 Preparation of Cell Extracts

Cells were harvested with trypsin-versene, washed 3 × in PBS, counted and aliquoted into 300 µl Beckman tubes at 2×10^6 cells/tube. These were stored as cell pellets at -80°C. Immediately prior to loading the samples onto the gels, cell pellets were rapidly thawed and one drop of lysis solution (Appendix 2) added to the pellet. The cells were mascerated using a blunt dissecting probe and rapidly frozen and rethawed

twice. Lysed cells were pelleted using a Beckman 152 microfuge and the supernatant loaded onto the gels. Starch gels were loaded using a pasteur pipette and cellagels loaded with a fine draughtsmans pen.

3.8.2 HPRT (E.C.2.4.2.8)

Hypoxanthine phosphoribosytransferase

The procedure used to type hybrids for HPRT was basically that of Watson *et al* (1972) using starch gel electrophoresis followed by autoradiography, with the exception that the citric acid concentration for the gel buffer should read 0.0044 M rather than 4.4 M as given in their paper.

3.8.3 PGK-A (E.C.2.7.2.3)

Phosphoglycerate kinase-A

PGK-A isozymes were separated using a 0.1 M Tris-citrate buffer pH8.6 (Appendix 2) on cellulose acetate ("Cellogel-250"). After pre-running the gel for 10 mins at 200 V, samples were loaded onto the gel and run for three hours at 200 V at R.T. (Meera Khan, 1971).

The gel was stained and subsequently counterstained according to an adaptation of Meera Khan (1971). (Appendix 2).

3.8.4 G6PD (E.C.1.1.1.49)

Glucose-6-phosphate dehydrogenase

Mouse and *M. rufogriseus* G6PD have very similar electrophoretic mobilities and considerable difficulty was encountered in separating the two isozymes. A number of running buffers, times and voltages were tried including the Adams and Donald (Donald, 1980) discontinuous buffer system for separation of the mouse and *M. rufus* forms of G6PD. These attempts were

unsuccessful.

The best separation was obtained on "Cellogel-250" using a Tris-glycine buffer pH9.1 at 340 V for 35 mins at 4°C (Migeon *et al*, 1979). (Appendix 2).

The staining mixture was adapted from the method of Johnston *et al* (1975) (Appendix 2).

3.8.5 LDH (E.C.1.1.1.27)

Lactate dehydrogenase

LDH isozymes were separated on "Cellogel-250" using either a 0.02 M phosphate buffer pH7.0 or a 0.04 M sodium barbitone buffer pH9.9 (Meera Khan, 1971). The phosphate buffer gave slightly greater separation but sharper bands were obtained with the barbitone buffer. Both buffers gave the same overall results.

Both systems were run at 200 V for two hours at R.T. The staining mixture used was from Meera Khan (1971).

3.8.6 NP (E.C.2.4.2.1)

Purine nucleoside phosphorylase

NP was run on "Cellogel-250" in a 0.02 M phosphate buffer pH7.0 (Meera Khan, 1971) for 1½ hours at 200 V at 4°C. The reaction stain was a modification of Spencer *et al* (1968). (Appendix 2).

3.8.7 EsA_4 (E.C.3.1.1.1)

Esterase A_4

This enzyme has previously been run on starch gel electrophoretic systems. The method for starch gel electrophoresis given by Komma (1963), was adapted to cellulose acetate ("Cellogel-250") by running in either a sodium veranol buffer

pH8.0 (Shows, 1972) or Tris-maleate buffer pH6.5 for 1½ hours at 200 V at 4°C. The gel was stained using a modification of Markert and Hunter (1959). (Appendix 2).

3.8.8 PGM-3 (E.C.2.7.5.1)

Phosphglucomutase-3

PGM was run on "Cellogel-250" in a Tris-glycine buffer pH9.1 at 300 V for 1½ hours at 4°C. The reaction stain was modified from Meera Khan (1971) and Harris and Hopkinson (1976). (Appendix 2).

3.9 CONVENTIONAL ANTISERA

3.9.1 Production of Antisera

REP3 cells were harvested with trypsin-versene and washed 3 × in PBS. C57BL/6J mice were injected intraperitoneally with 5-10 × 10^6 freshly harvested cells in 0.2 ml PBS at an interval of 10 days for 30 - 40 days. Mice were bled from the retro-orbital venous plexus 4 - 5 days after the last injection and the blood left to clot at 37°C for 1 hour. The clot was rimmed and the blood centrifuged at 4,500g for 20 mins. The serum was collected and the clot allowed to contract over-night at 4°C. This was then centrifuged again and the serum collected and pooled with the initial lot of serum. Serum from C57BL/6J mice which had not been immunized was used as a negative control for the immunofluorescence assays.

3.9.2 Adsorptions

Antisera directed against marsupial x mouse hybrids were adsorbed with the mouse parent cell line from which the hybrids were originally made. Sera were adsorbed by incubating one

volume of undiluted antiserum with an equal volume of washed packed cells at 37°C for 1 hour and then at 4°C for approximately 18 hours with continuous shaking. This procedure was then repeated and the twice adsorbed sera checked for complete removal of unwanted antibodies.

3.10 MONOCLONAL ANTIBODIES

3.10.1 Injection Schedule for Hybridoma Production

C57BL/6J mice were injected intraperitoneally with approximately 1×10^7 REP3 cells every 10 days for a period of 30 days. Blood was collected from the retro-orbital venous plexus four days after the last injection and serum tested for reaction with REP3 cells, to check if an antibody response had been elicited. The spleen was removed for fusion of spleenic lymphocytes with NS1 cells.

3.10.2 *In Vivo* Culture of GA-1

Antibody producing hybridomas can be maintained in ascitic form in mice. C57BL/6J × BALB/c F1 hybrid mice were injected with 0.4 ml pristane (2,6,10,14 - tetramethyl-pentadecane) to destroy their immune response. After two weeks the mice were injected intraperitoneally with $0.8 - 1 \times 10^7$ GA-1 cells. These cells grew as solid tumours in the mice. Four to five weeks later when the mice were obviously large due to tumour growth, they were bled from the retro-orbital venous plexus and the peritoneal fluid collected. Both the peritoneal fluid and blood were centrifuged at 430g for 20 min and the resulting ascites fluid and blood serum collected as

above (Section 3.9.1) and stored in small aliquots at -20°C.

3.10.3 Concentration of GA-1 Supernatant

GA-1 culture supernatant was added to an equal volume of 3.69 M ammonium sulphate to give a 45% saturated solution. This concentration precipitates all γ globulins and some of the α and β globulins (Fruton and Simmonds, 1958). The mixture was allowed to precipitate for 1 hr. at 4°C and then spun at 280g for 10 mins. The pellet was redissolved in a small volume of PBS and the solution dialysed overnight against PBS at 4°C.

3.10.4 Purification of GA-1 Ascites

The monoclonal antibody, GA-1, was shown to be an IgG_{2b} subclass of immunoglobulin by Dr. P. Ey (Department of Microbiology and Immunology, University of Adelaide) using a radioimmunoassay with antibodies to known immunoglobulin subclasses. IgG_{2b} is an immunoglobulin subclass which binds to *Staphylococcus aureas* protein A. (Sandrin *et al*, 1978). The monoclonal antibody in ascites fluid obtained from *in vivo* growth of GA-1 cells was purified on a Protein A column according to the method of Ey *et al* (1978).

The GA-1 ascites was thawed quickly, diluted with 0.1 M tris buffer pH8.4 and the pH of the solution adjusted to pH8.4. The protein A column was equilibrated to pH8.4 and the ascites solution loaded onto the column. IgG_{2b} was eluted off the protein A column at pH3.5. The pH of the eluate was adjusted to 7.2 with 0.1 M Tris and then dialysed overnight in a large volume of PBS. The resulting antisera had a titre of ½ on

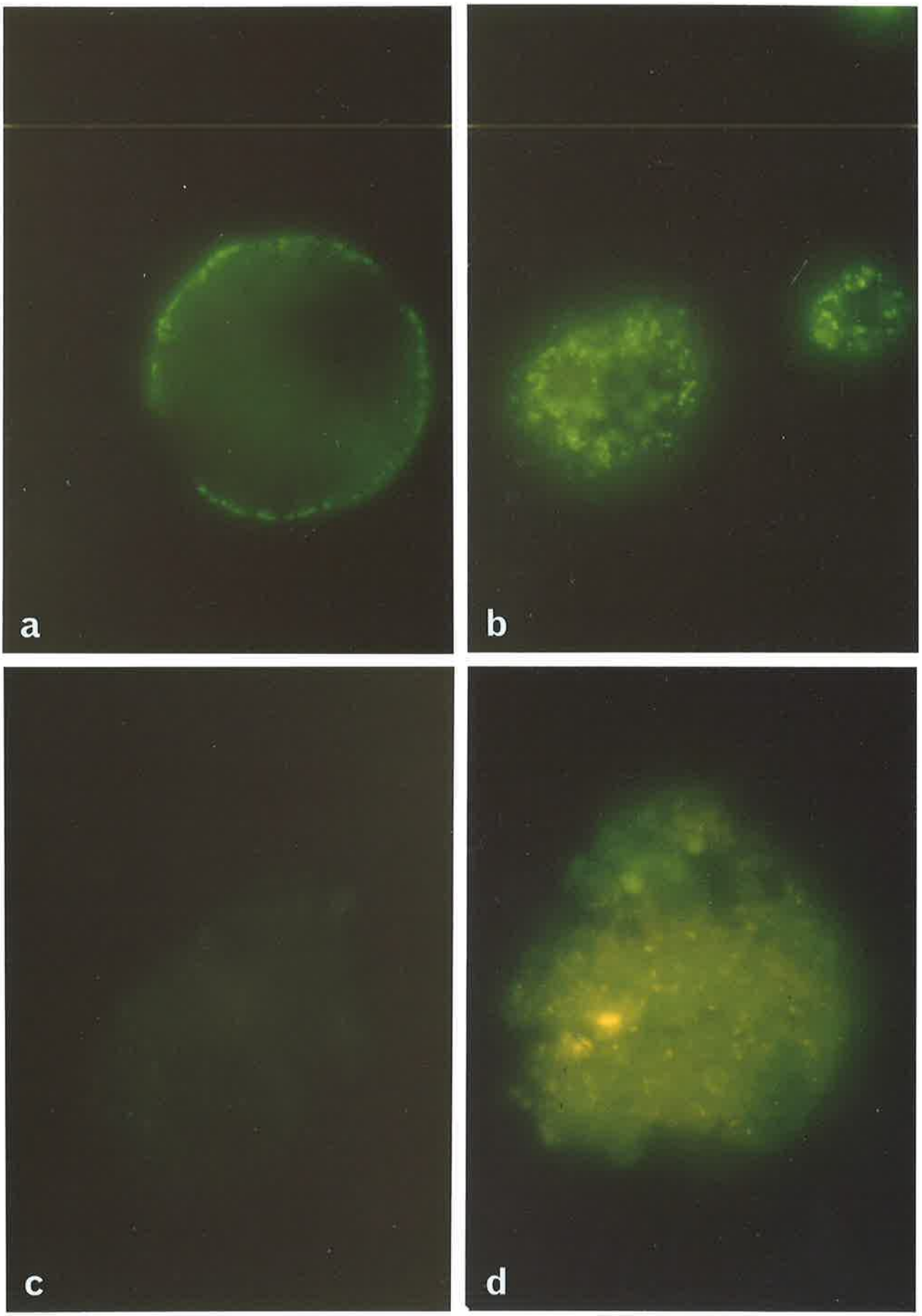

a
b
c
d

Fig. 3.1 - (a) A +++ cell

(b) A +++ cell

(c) A +/- cell (background autofluorescence)

(d) A dead cell.

REP3 cells using an indirect immunofluorescence assay.

3.11 INDIRECT IMMUNOFLUORESCENCE

5×10^5 PBS-washed cells were incubated with 50 µl of the appropriate dilution of serum, ascites fluid or hybridoma supernatant in 2 ml P8 precipitin tubes (Surgical and Medical) for 30 mins at 4°C. The cell suspension was centrifuged at 300g for 7 mins and the supernatant aspirated. The cells were then washed 3 × with PBS (containing 0.02 M sodium azide) to remove unbound antibody. Washing was carried out by resuspending the cells in two drops of PBS and then shaking on a 'Sybron Thermolyne Maxi-Mix'. Two mls of PBS were then added to the resuspended cells, the tubes were centrifuged at 300g for 7 mins and the supernatant aspirated. Cells were then incubated with 50 µl of a standard dilution of commercial fluorescein conjugated rabbit or goat anti-mouse immunoglobulin (Wellcome or Difco) for 30 mins at 4°C. A half dilution of commercial fluorescein conjugated sheep anti-mouse IgG $(Fab')_2$ fragment (Capell) was used when the target cells were lymphocytes. The dilution chosen for each batch of antisera and commercial conjugate was the highest dilution still giving bright fluorescence on the positive control cells. (False positive reactions could occur if either antisera was too concentrated). The cells were then washed 3 × as described above to remove unbound conjugate and resuspended in one drop of 50% glycerol in PBS pH7.4. Coverslips were placed on the slides and sealed with rubber gum to prevent evaporation. Cells were viewed under a Leitz Orthoplan Universal large field microscope fitted with a 200 watt high pressure

mercury lamp, a ploempak fluorescent vertical illuminator, a Leitz H exciter and barrier filter system and a 100X NPL Fluotar oil immersion objective lens.

Cells were scored as +/- (background autofluorescence), + (weak fluorescence), ++ (bright fluorescence) and +++ (very bright reaction, halo of fluorescence). Dead cells exhibited a bright homogeneous fluorescence (Fig. 3.1). Not all cells in all samples bound antibody and hence not all cells in a sample had the same fluorescence score. The percentage of total cells giving a fluorescence reaction was estimated. All other cells in the samples had +/- scores.

The inclusion of appropriate controls is of extreme importance for correct interpretation of results. The following controls were routinely used for each assay: (a) a negative control for antibody binding to cells (NS1 culture supernatant for first antisera incubation); (b) a positive control for antibody binding to cells (GA-1 on *M. rufogriseus* fibroblasts); (c) a binding specificity control for the fluorescence conjugate (PBS for first antisera incubation).

CHAPTER 4

RESULTS

4.1 CHROMOSOMES

4.1.1 Parental Cell Lines

Chromosomes of the mouse cell line, PG19, exhibited C-banded centromeric regions. Some cells also contained a marker chromosome characterised by an interstitial C-band. (Fig. 4.1). PG19 had a chromosome number ranging from 35 to 40 with a modal number of 38. Most of the chromosomes were acrocentric and the number of metacentrics ranged from 3 to 5 with a modal number of 4. A PG19 G-banded karyotype is presented in Figure 4.2.

The mouse cell line, 1R, had a modal number of 58 chromosomes with a range of 45 to 67 including 10 metacentrics (Fig. 4.3). C-banded centromeric regions were present on all chromosomes. The 1R isochromosome marker also expressed three interstitial C-banded regions.

Lymphocyte and fibroblast chromosome preparations from *M. rufogriseus* showed all 16 chromosomes to contain very large centromeric C-banded regions compared with PG19 or 1R chromosomes. Approximately $\frac{2}{3}$ of the short arm of the *M. rufogriseus* X chromosome was heterochromatic, the distal $\frac{1}{3}$ being euchromatic. The X chromosome also exhibited three interstitial C-bands on the long arm (Fig. 4.4b). These three bands were more clearly seen in *M. rufogriseus* x mouse somatic cell hybrids where the *M. rufogriseus* chromosomes appeared more extended (Fig. 4.5) than in C-banded fibroblast or lymphocyte preparations.

FIG. 4.1 - C-banded karyotype of PG19.

FIG. 4.2 - G-banded karyotype of PG19.

PG19

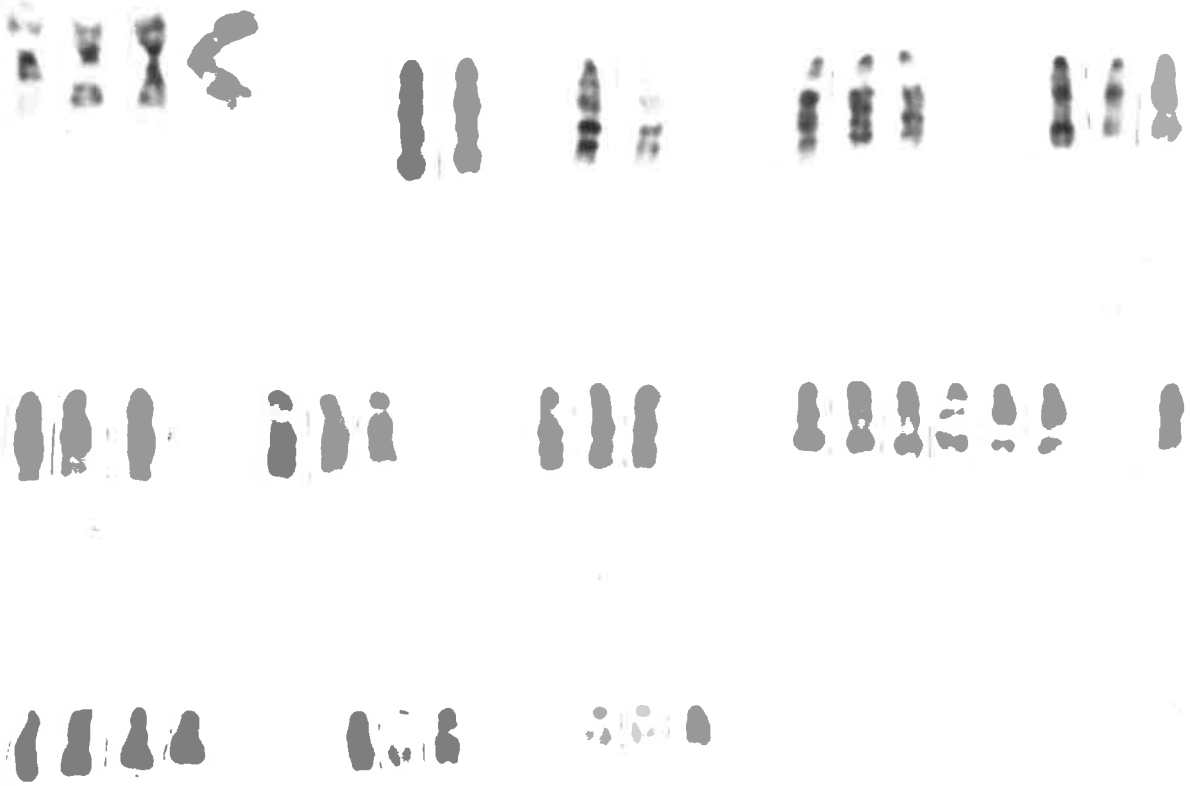
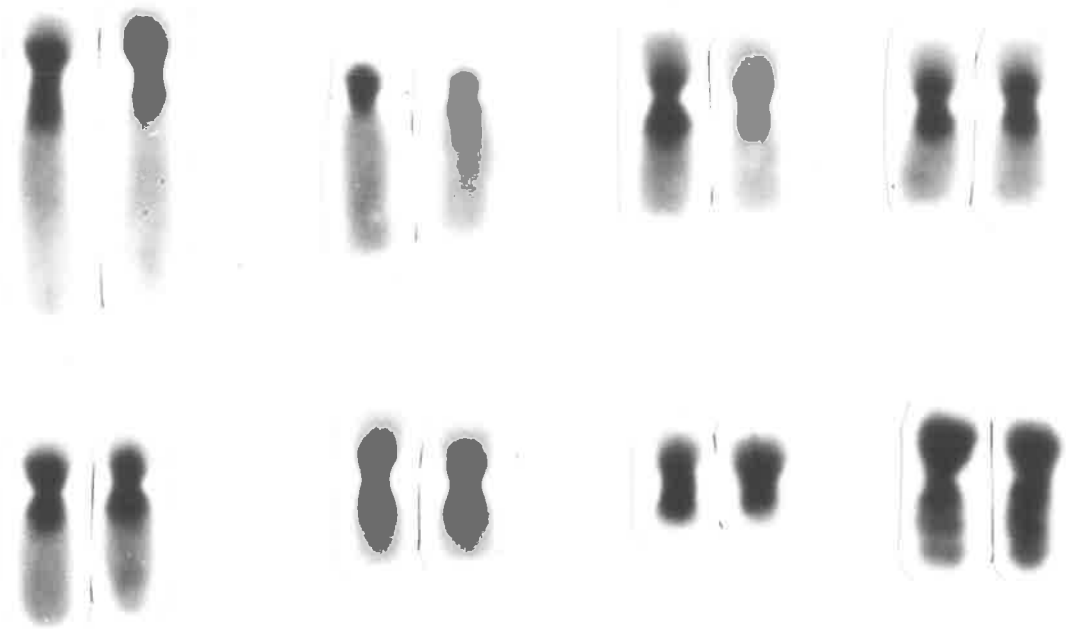


FIG. 4.3 - C-banded karyotype of 1R.

FIG. 4.4(a) - G-banded karyotype prepared from a diploid fibroblast culture of an *M. rufogriseus* ♀.

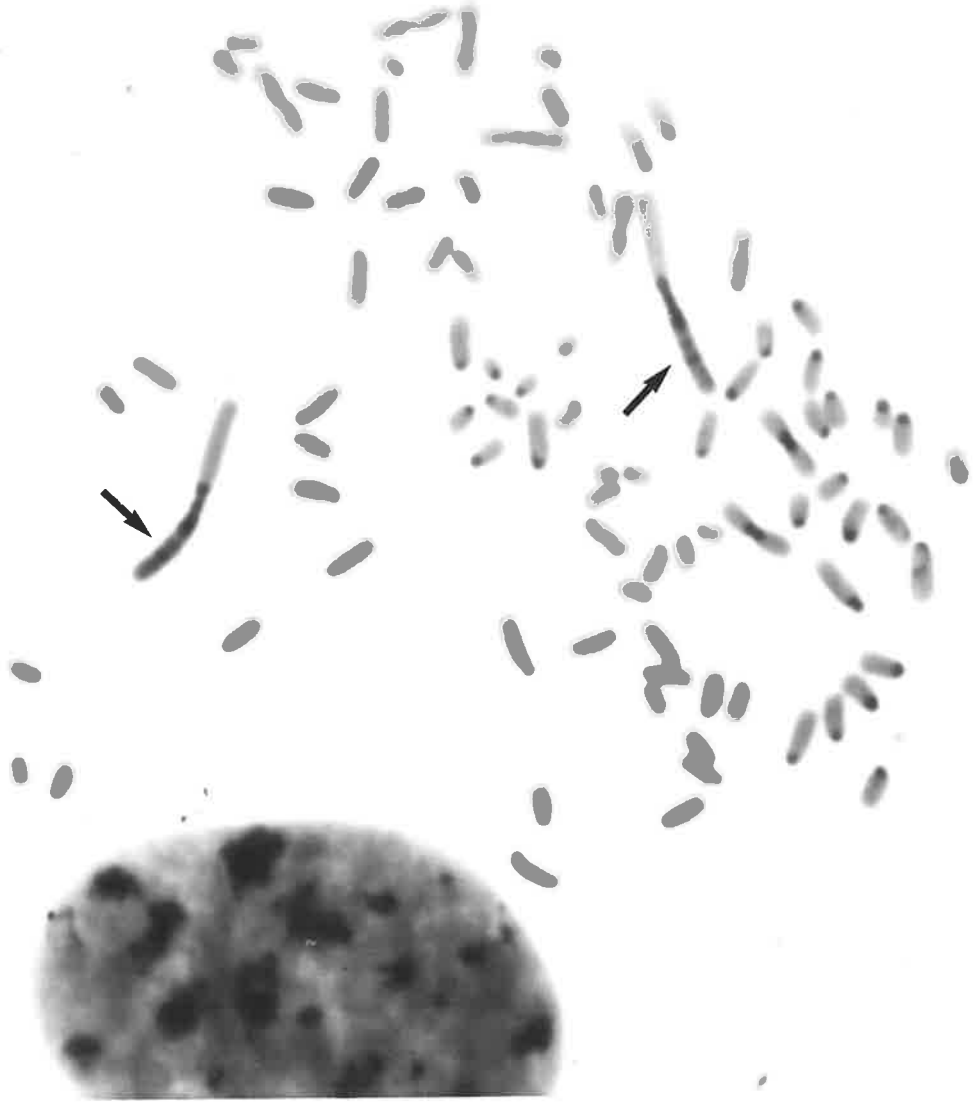
(b) - C-banded karyotype of an *M. rufogriseus* ♀.

a



b

FIG. 4.5 - C-banded *M. rufogriseus* x mouse somatic cell hybrid. The three interstitial C-bands of the long arm of the *M. rufogriseus* X chromosome can be seen clearly (arrowed).



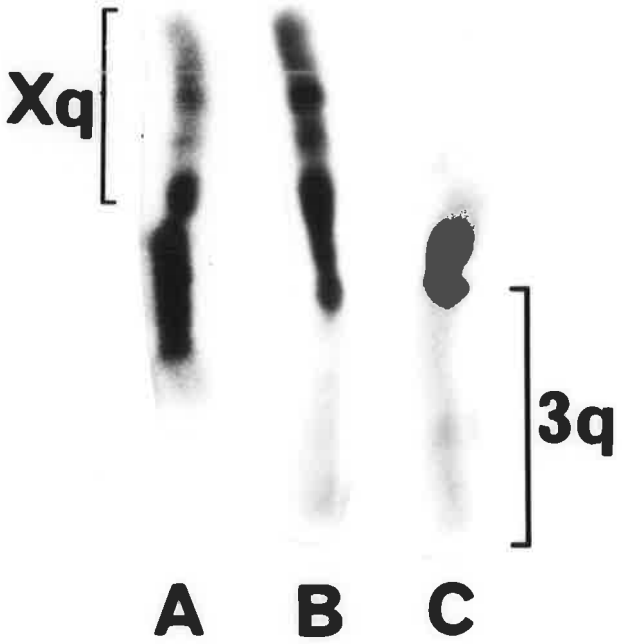
G-banding of fibroblast and lymphocyte *M. rufogriseus* chromosomes proved very difficult due to their large areas of centromeric C-banding material which caused compaction of the G-bands on either side of the centromere. Less contracted chromosomes, more suitable for G-banding were obtained using the method of Yunis (1978), but the G-banding still lacked the clarity observed in somatic cell hybrids, especially around the centromeric regions. The *M. rufogriseus* autosomes had large pale G-bands at the centromeric region whereas the X chromosomes had dark centromeric G-bands. Each pair of *M. rufogriseus* chromosomes can be identified by their G-banding pattern (Fig. 4.4a).

4.1.2 REP Cell Hybrids and Revertants

C-banding was carried out on all primary and bulk REP hybrids. *M. rufogriseus* chromosomes could be distinguished from PG19 chromosomes on the basis of their comparatively larger C-banded regions. The three interstitial C-bands on the long arm of the *M. rufogriseus* X chromosome could be used to see if the whole or only part of this chromosome arm was present in the hybrids.

All the primary hybrids, REP1, REP2, REP3 and REP4 contained chromosomes with very large blocks of C-banded material characteristic of *M. rufogriseus* chromosomes. A proportion of REP3 cells contained a chromosome similar to the *M. rufogriseus* X chromosome but with a longer than normal euchromatic short arm (Fig. 4.5 and Fig. 4.6). This chromosome appeared less condensed in REP3 than in *M. rufogriseus* fibroblasts and lymphocytes and gave clearer G-bands. The large chromosome with a pale G-banded centromeric region in Figure 4.7 was identified as a Robertsonian translocation chromosome

Fig. 4.6 - Comparison of the C- and G-banding patterns of *M. rufogriseus* t(3q,Xq) in REP3-2 with the *M. rufogriseus* X chromosome and the *M. rufogriseus* chromosome 3.
(A) *M. rufogriseus* X chromosome
(B) *M. rufogriseus* t(3q,Xq)
(C) *M. rufogriseus* chromosome 3.


Xq
3q
A
B
C

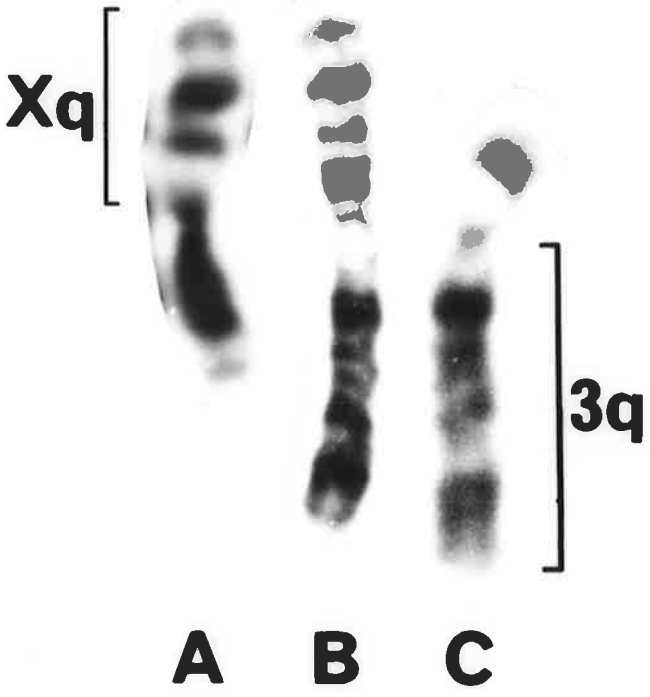
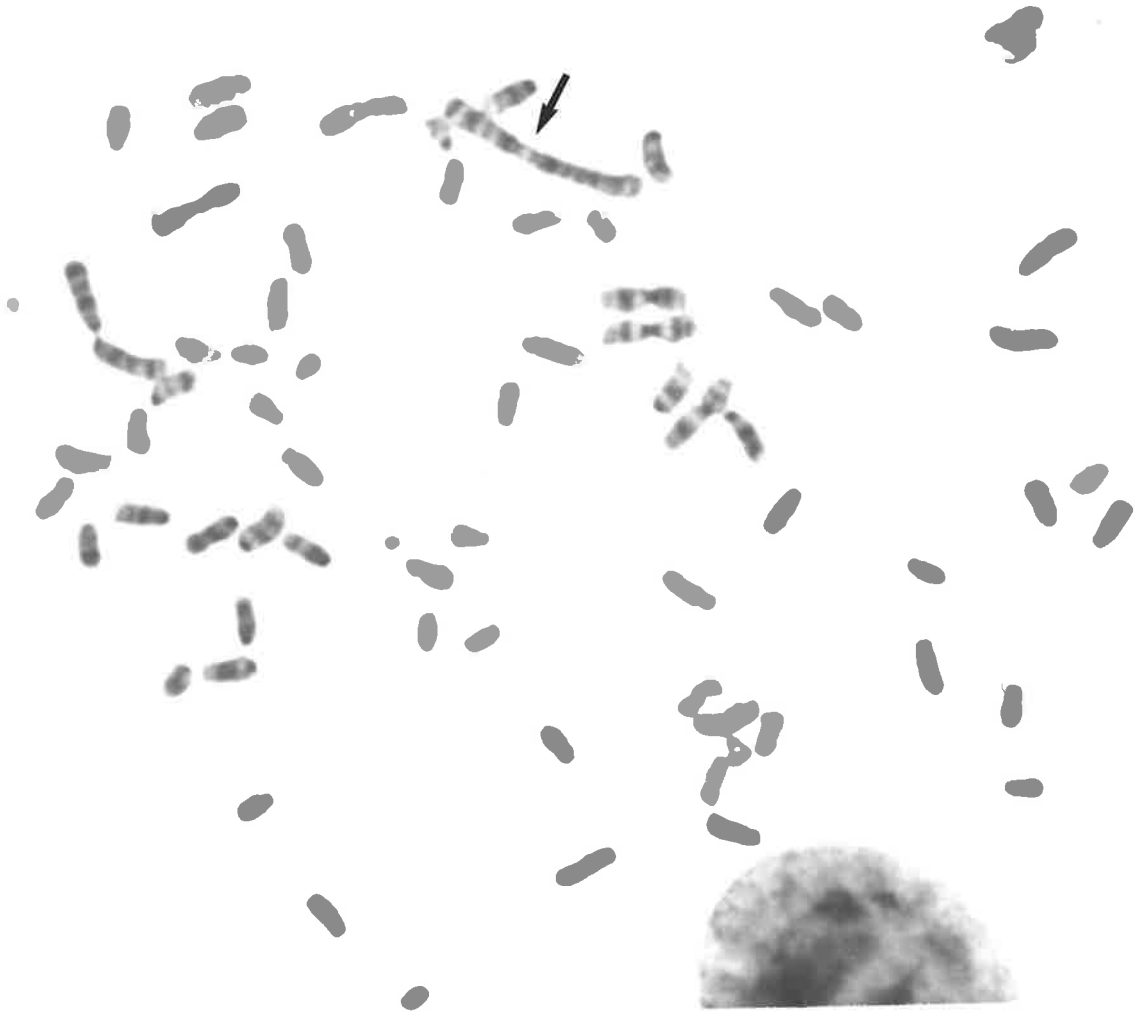

Xq
3q
A
B
C

FIG. 4.7 - A G-banded cell of REP3-2.
t(3q,Xq) is arrowed.



consisting of the long arm of the *M. rufogriseus* X chromosome and the long arm of the *M. rufogriseus* number three autosome. This chromosome is henceforth referred to as t(3q,Xq) (Fig. 4.6). The possible reciprocal translocation product of t(3q,Xq) i.e. t(3p,Xp) was not identified in any of the hybrids. Some REP3 cells contained only chromosomes which were indistinguishable from PG19 chromosomes, while others contained the *M. rufogriseus* Xq, or iXq identified by C-banding and *M. rufogriseus* chromosomes 3, 6 and 7 identified by G-banding. This chromosomal variation presumably resulted from ongoing chromosome loss from the hybrid cells in the early stages of REP3 chromosome evolution. This chromosomal heterogeneity prompted selection of REP3 subclones to provide a more homogeneous set of cell lines.

No marsupial chromosomes except for Xp were identified in REP3-3 or REP3-5 cells using C- or G-banding. All the chromosomes in these hybrids were indistinguishable from the PG19 parent (Fig. 4.8). Both cell lines did, however, have much larger chromosome numbers than PG19; REP3-3 (73(58-74)) and REP3-5 (71(65-72)).

REP3-1 contained a submetacentric chromosome resembling the *M. rufogriseus* chromosome 3 in size and arm ratio. This chromosome did not, however, have as large a block of centromeric heterochromatin as would have been expected for a chromosome of marsupial origin. G-banding studies confirmed the identity of this chromosome as the *M. rufogriseus* number 3 (Fig. 4.9b). Approximately 60% of REP3-1 cells contained a small acrocentric chromosome with a relatively large centromeric segment of heterochromatin. This chromosome which is presumed to be of marsupial origin (Fig. 4.9a), resembles the short arm of the

M. rufogriseus X chromosome. Such an identification is supported by G-banding studies but with some reservations (see end of this section).

Approximately 75% of REP3-2 cells contained a chromosome easily distinguishable from PG19 chromosomes due to its large size, large centromeric block of heterochromatin and three interstitial heterochromatic bands on its short arm. The three interstitial bands identify this arm as being the long arm of the *M. rufogriseus* X chromosome (Fig. 4.10a and see Fig. 4.6a for comparison). The identity of the other longer euchromatic arm could not be established using C-banding but was identified by G-banding to be the long arm of chromosome 3 (Fig. 4.10b). This is the same chromosome, therefore that was found in REP3 cells, i.e. t(3q,Xq). A few REP3-2 cells also contained a small submetacentric chromosome consisting almost entirely of C-banded material (Fig. 4.10a). This chromosome was identified by G-banding to be the *M. rufogriseus* 7 (Fig. 4.10b).

REP3-2-1 contained a G-band identical chromosome to the chromosome found in REP3-2, i.e. t(3q,Xq). (Fig. 4.11b). However, this chromosome lacked the three interstitial C-bands and nearly all the centromeric C-banded material found in t(3q,Xq) in REP3-2 cells (Fig. 4.11a). No marsupial chromosomes could be identified in REP3-2-2 by either C- or G-banding.

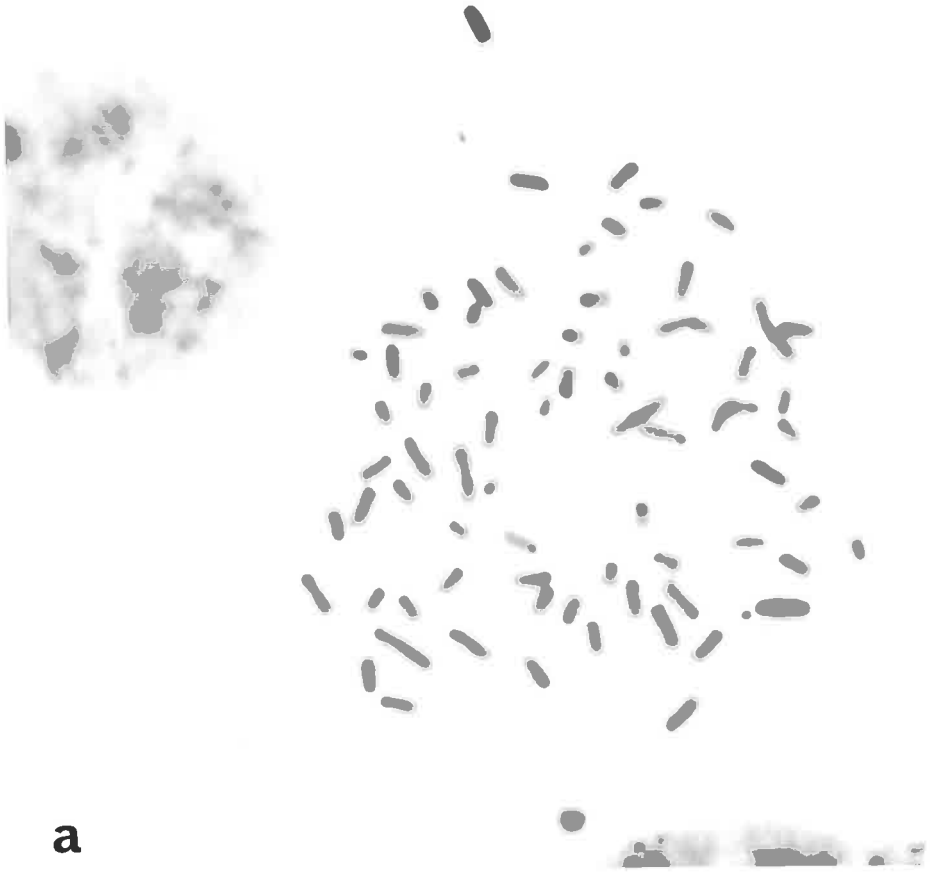
REP3-4 contained a chromosome with a large centromeric block of heterochromatin, shown to be the *M. rufogriseus* chromosome 6 by G-banding. REP3-4 also contained a translocation chromosome involving the *M. rufogriseus* 3q (Fig. 4.12). The origin of the short arm of this translocation chromosome was not

FIG. 4.8 - C-banded cell of

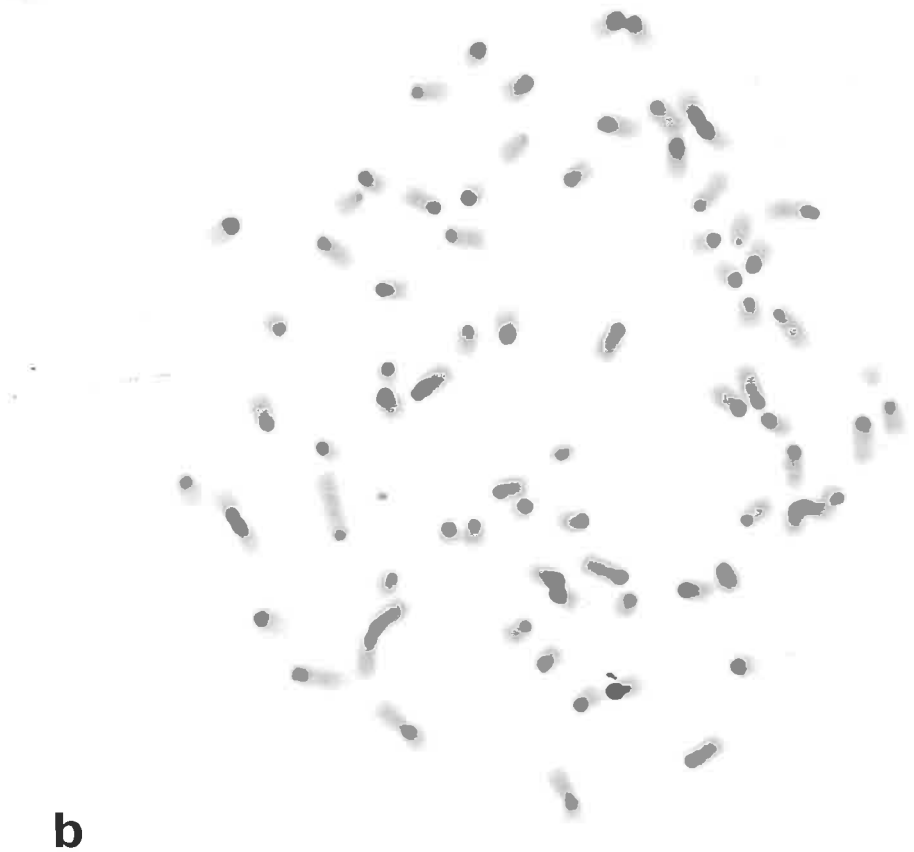
a) REP3-3

b) REP3-5

No marsupial chromosomes can be identified.



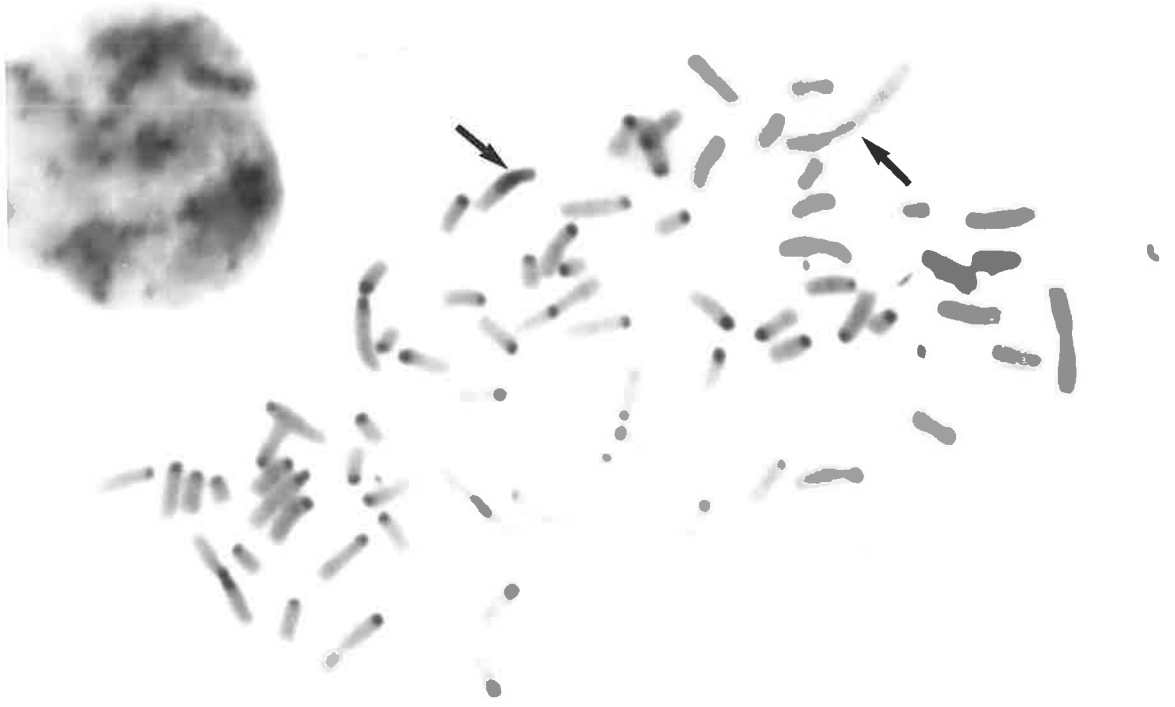
a



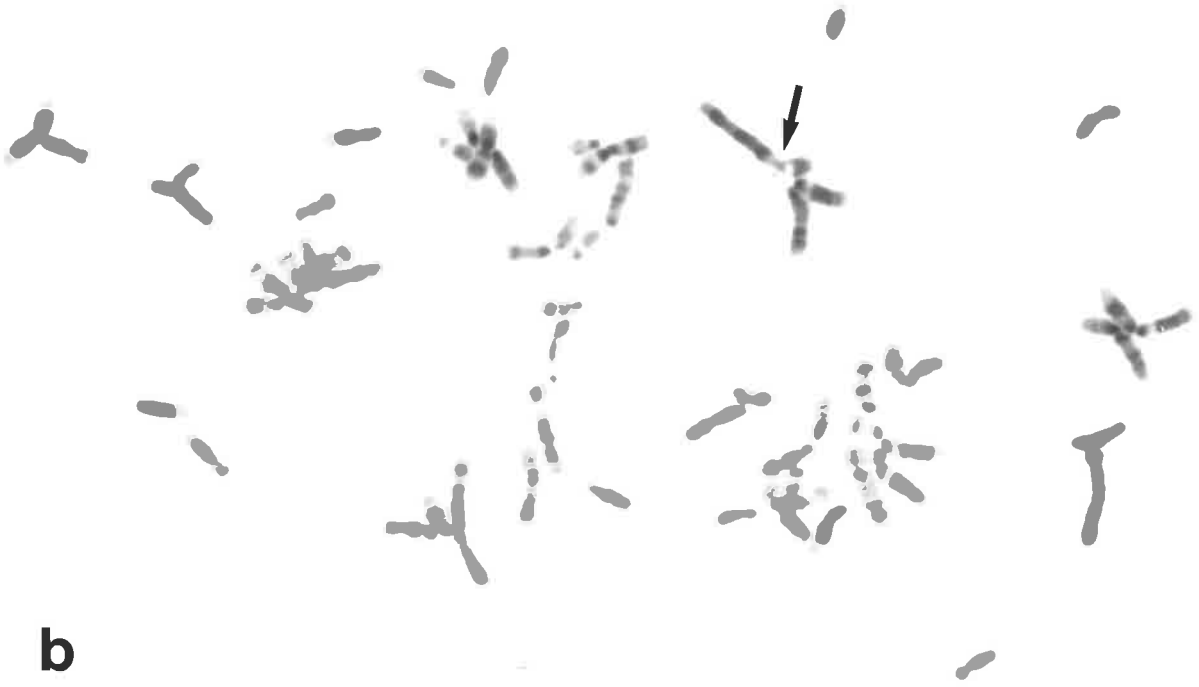
b

FIG. 4.9 - (a) A C-banded cell of REP3-1.
Marsupial chromosomes are arrowed.

(b) A G-banded cell of REP3-1.
The *M. rufogriseus* chromosome 3
is arrowed.



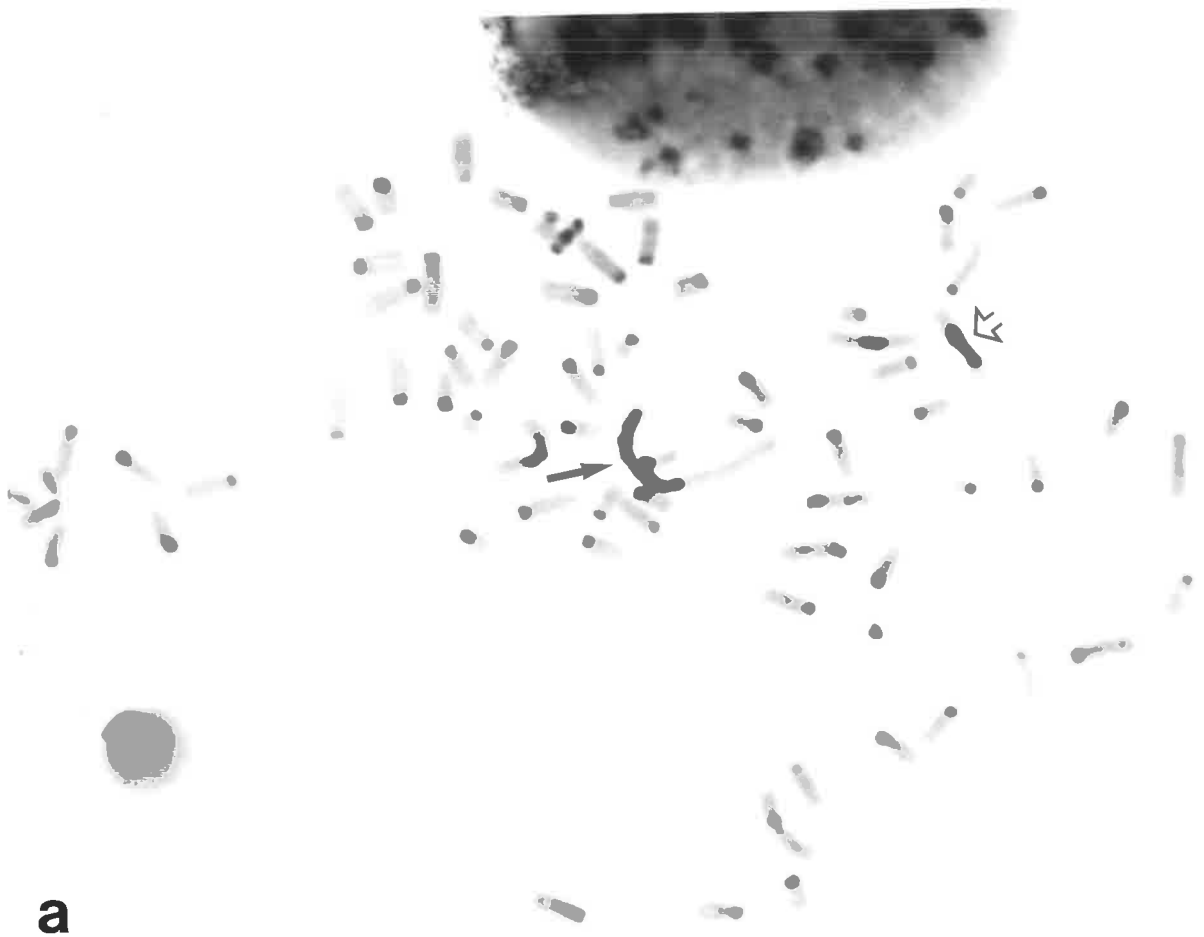
a



b

FIG. 4.10 - (a) A C-banded cell of REP3-2.
Marsupial chromosomes are arrowed.
Closed arrow - long arm of
M. rufogriseus X chromosome.
Open arrow - *M. rufogriseus*
chromosome 6 or 7.

(b) G-banded REP3-2 cell.
Closed arrow - *M. rufogriseus*
t(3q,Xq)
Open arrow - *M. rufogriseus*
chromosome 7.



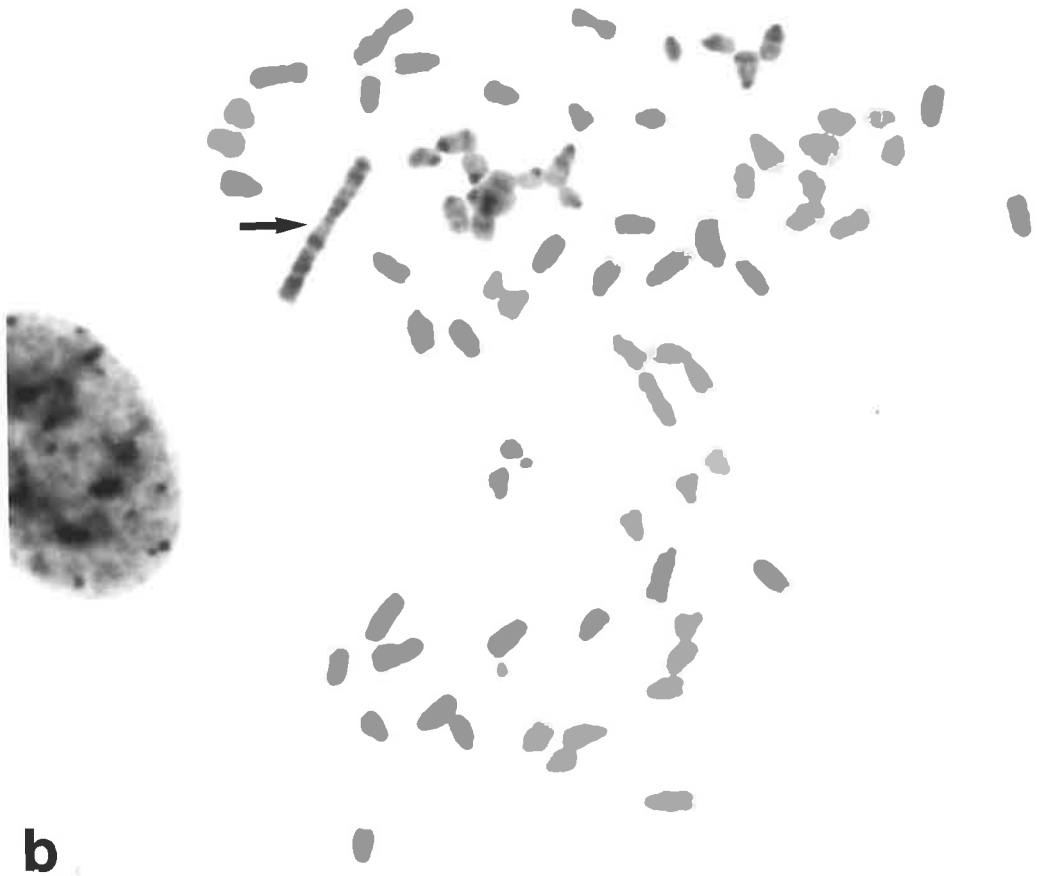
a

b

FIG. 4.11 - (a) A C-banded cell of REP3-2-1.
The marsupial chromosome is arrowed.

(b) A G-banded cell of REP3-2-1.
The *M. rufogriseus* t(3q,Xq) is
arrowed.

a



b

FIG. 4.12 - G-banded cell of REP3-4. A translocation chromosome involving the long arm of the *M. rufogriseus* chromosome 3 is arrowed.

established. It was possibly of marsupial origin because of its large pale G-banded region at the centromere.

Most REP3-6 cells contained a small acrocentric chromosome, almost half of which consisted of C-band material (Fig. 4.13). This chromosome was similar to the previously described small acrocentric seen in REP3-1 and was possibly the short arm of the *M. rufogriseus* X chromosome or an *M. rufogriseus* autosome arm. G-banded preparations supported the identity of this chromosome as being the *M. rufogriseus* Xp.

REP3-7 cells contained an isochromosome of 3q, and a number of cells contained the *M. rufogriseus* chromosome 6 as identified by C- and G-banding (Fig. 4.14). Of the five REP3-7 subclones, only REP3-7-1 and REP3-7-2 contained a C-banded chromosome resembling those of marsupial origin. Again, although lacking C-banded regions characteristic of *M. rufogriseus* chromosomes, the chromosome appeared to be the *M. rufogriseus* 3 in size and arm ratio as in REP3-1. Only REP3-7-1 and REP3-7-4 of the REP3-7 subclones were G-banded. A normal *M. rufogriseus* 3 could be identified in REP3-7-1 (Fig. 4.15a) and the long arm of the *M. rufogriseus* 3 in REP3-7-4 cells (Fig. 4.15b).

The *M. rufogriseus* chromosomes identified by G-banding as being present in the REP3 subclones are summarised in Table 4.1 and illustrated in Fig. 4.16. The only *M. rufogriseus* chromosome arm found in common in the REP3-1, REP3-2, REP3-4 and REP3-7 cell lines was the long arm of the *M. rufogriseus* chromosome 3 (Fig. 4.16). The *M. rufogriseus* 3q was also the only chromosome arm found in common in the REP3-2 and REP3-7 subclones.

FIG. 4.13 - A C-banded cell of REP3-6.
The *M. rufogriseus* chromosome, presumed to be Xp, is arrowed.

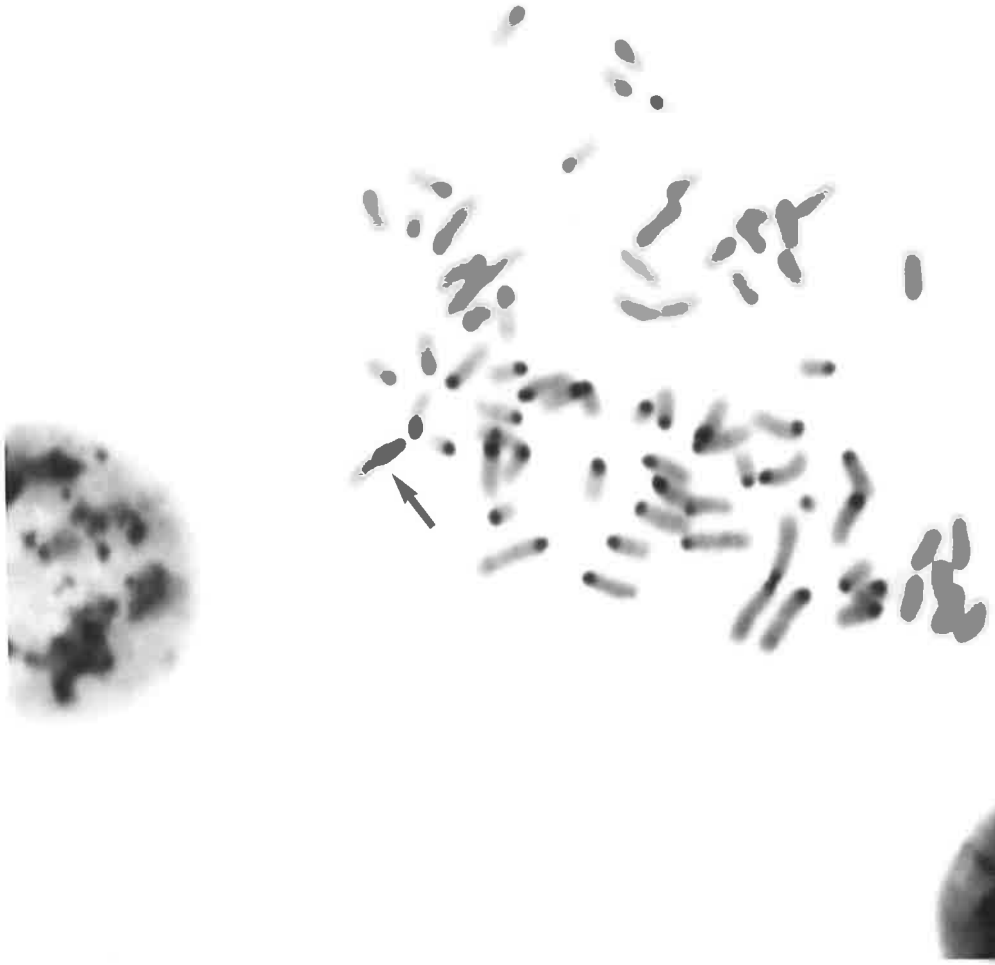
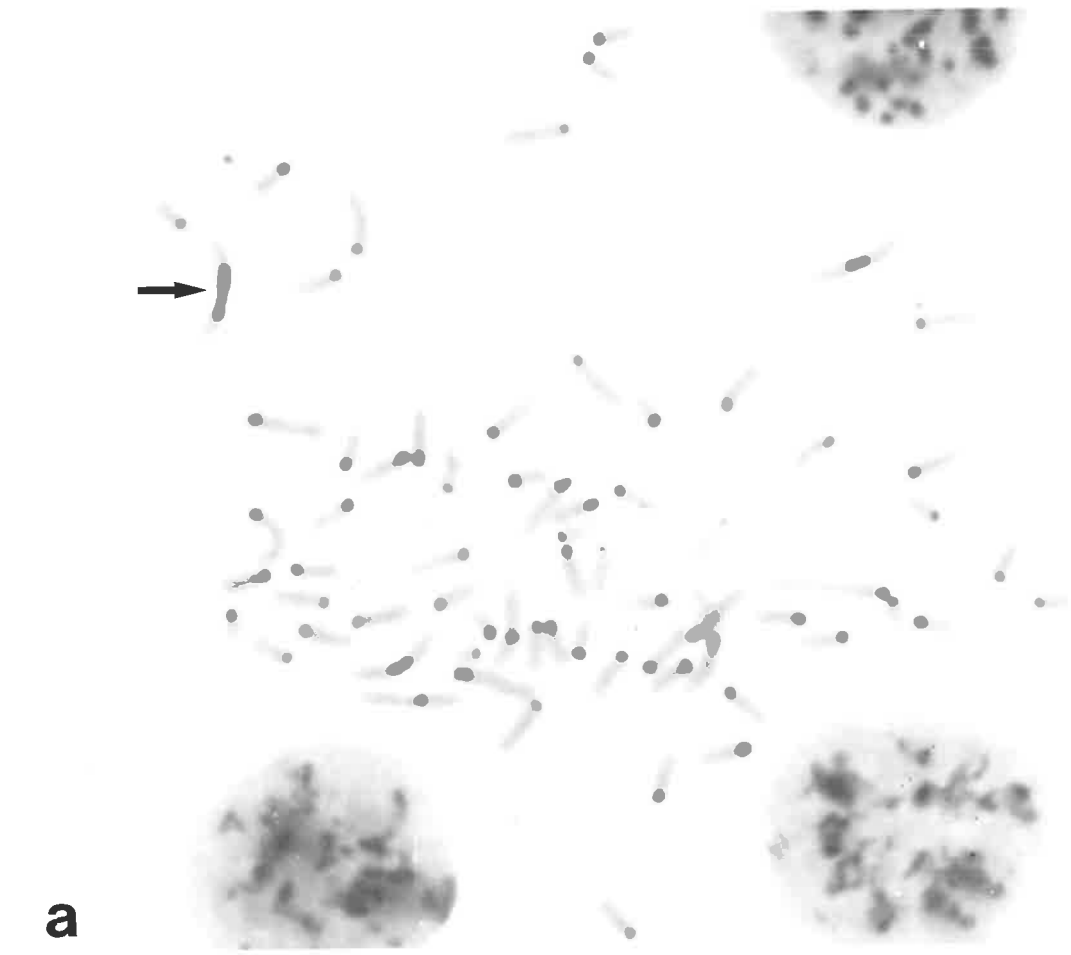
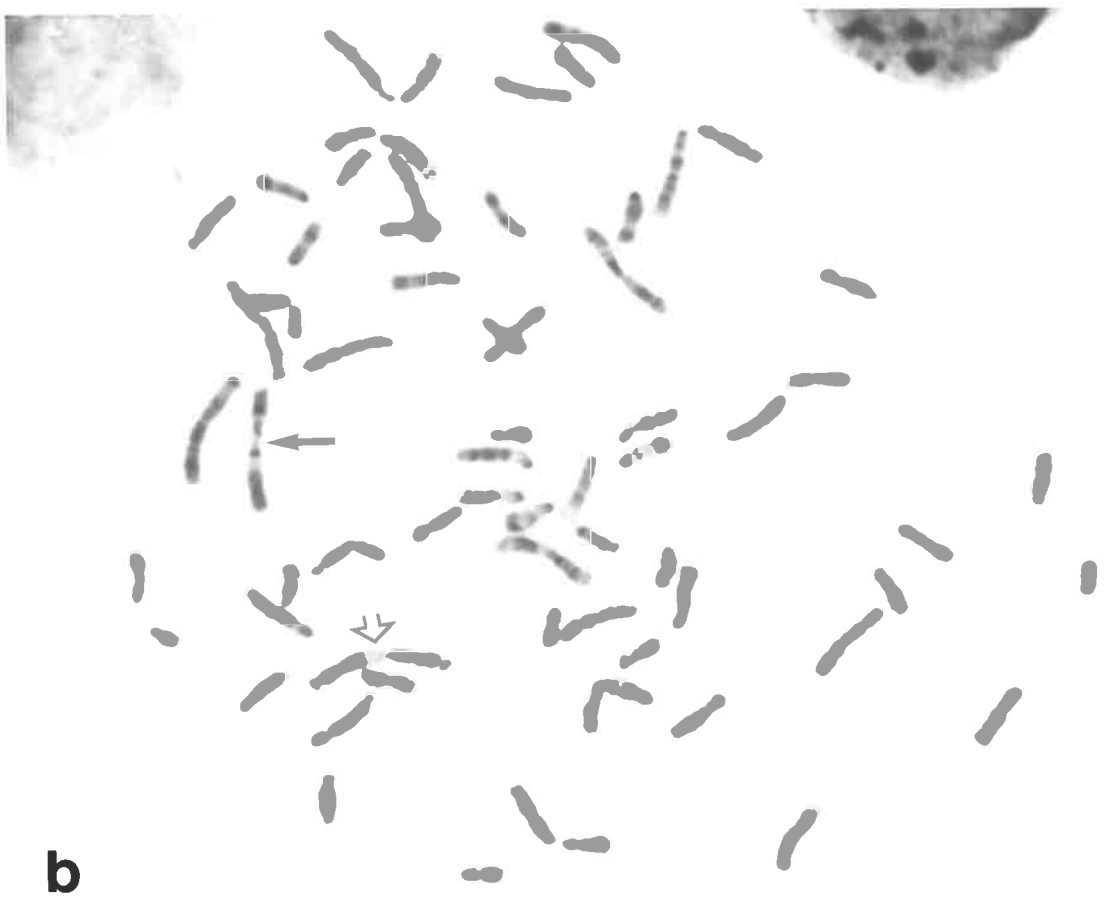


FIG. 4.14 - (a) A C-banded cell of REP3-7. The *M. rufogriseus* chromosome 6 is arrowed.

(b) A G-banded cell of REP3-7. The *M. rufogriseus* chromosomes 6 (closed arrow) and 3q (open arrow) are indicated.



a



b

FIG. 4.15 - (a) A G-banded cell of REP3-7-1.
The *M. rufogriseus* chromosome 3
is arrowed.

(b) A G-banded cell of REP3-7-4.
The long arm of the *M. rufogriseus*
chromosome 3 is arrowed.

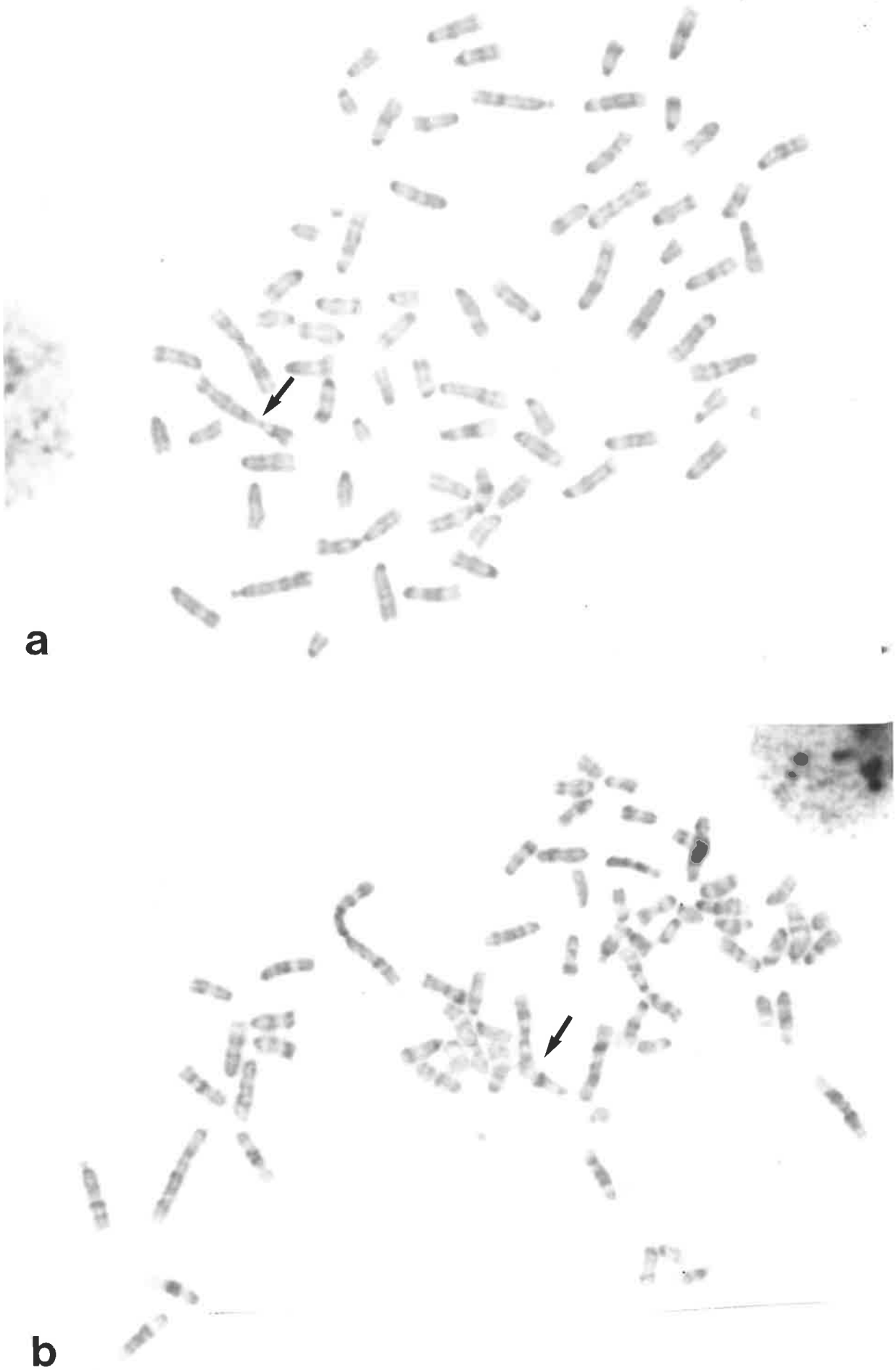
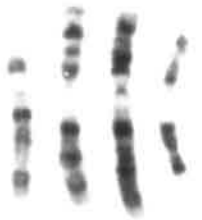

a
b

FIG. 4.16 - *M. rufogriseus* chromosomes present in REP3-1, REP3-2, REP3-4 and REP3-7 as identified by G-banding. The only common chromosome arm present is 3q.

① REP3-1

② REP3-2



① ② ③ ④

③ REP3-4

④ REP3-7

The five bulk hybrids REPB1 → REPB5 were chromosomally heterogeneous with a variety of chromosomes containing large heterochromatic segments characteristic of an *M. rufogriseus* origin. Five subclones were derived from REPB3 and their chromosomes analysed using C- and G-banding.

REPB3-1 and REPB3-2 have a large submetacentric chromosome resembling the *M. rufogriseus* 3 in size and arm ratio but lack the large centromeric heterochromatic region typical of *M. rufogriseus* chromosomes (Fig. 4.17a). G-banding of REPB3-1 confirmed the identity of this chromosome as the *M. rufogriseus* 3 (Fig. 4.17b). G-banding of REPB3-2 was not done. Some cells of REPB3-3 had a large chromosome with one arm almost entirely heterochromatic and the other with three interstitial C-bands characteristic of the *M. rufogriseus* X chromosome (Fig. 4.18a). G-banding confirmed the identity of this chromosome as a normal X, and the *M. rufogriseus* 3 was also present in some cells (Fig. 4.18b).

REPB3-4 contained the same C-banded chromosome found in REPB3-1 and REPB3-2 which was again shown to be the *M. rufogriseus* 3 by G-banding. Both G- and C-banding identified the *M. rufogriseus* Xq in most REPB3-4 cells (Fig. 4.19). The hybrid REPB3-5 contained a chromosome which on the basis of its C- and G-banding pattern proved to be the *M. rufogriseus* 6 (Fig. 4.20). The *M. rufogriseus* 3q was also identified in some cells by G-banding.

Table 4.1 summarises the chromosomes identified by C- and G-banding present in the REPB3 subclones. Again, the only *M. rufogriseus* chromosome arm in common in all the bulk hybrids was 3q.

The HAT selected REP hybrids analysed in Table 4.1 contained a small acrocentric chromosome with a G-banding pattern

TABLE 4.1

	APPROXIMATE PERCENTAGE OF CELLS CONTAINING *M. RUFOGRISEUS* CHROMOSOMES								TOTAL NO. OF CHROMOSOMES IN THE HYBRIDS		
	t(3q;Xq)	Xq	X	3	6	7	C	Xp G	MODE	RANGE	MEAN
REP3	27%	-	-	18%	18%	9%	-	43%	68	47-81	68.13
REP3-1	-	-	-	80%	-	-	60%	87%	78	49-78	70
REP3-2	75%	-	-	-	-	12%	-	65%	69	56-87	69.9
REP3-2-1	100%	-	-	-	-	-	-	66%	68	63-69	67.2
REP3-2-2	-	-	-	-	-	-	-	43%	75	60-76	70.83
REP3-3	-	-	-	-	-	-	-	25%	73	58-74	69
REP3-4	t(3q,?)55%	-	-	-	45%	-	-	83%	72	66-73	70.6
REP3-5	-	-	-	-	-	-	-	71%	71	65-72	69.1
REP3-6	-	-	-	-	-	-	85%	88%	62	62-69	65
REP3-7	-	-	-i3q	50%	36%	-	-	60%	73	62-77	70.11
REP3-7-1	-	-	-	89%	-	-	-	75%	69	67-70	68.43
REP3-7-2*	-	-	-	75%	-	-	-	ND	70	59-75	66.38
REP3-7-3*	-	-	-	-	-	-	-	ND	68	64-73	67.14
REP3-7-4	-	-	-	3q66%	-	-	-	80%	64	62-66	63.8
REP3-7-5*	-	-	-	-	-	-	-	ND	68	61-68	66.38
REP3R1	-	-	-	-	-	-	-	27%	37	33-74	52.88
REP3R2	-	-	-	-	-	-	-	25%	38	35-79	48
REP3R3	-	-	-	-	-	-	-	18%	38	35-74	41.91
REP3R4→REP3R8	-	-	-	-	-	-	-	ND	Similar to REP3R1, R2 & R3		
REPB3†	-	-	-	-	-	-	-	-	-	-	-
REPB3-1	-	-	-	90%	-	-	-	54%	73	69-74	71.74
REPB3-2*	-	-	-	66%	-	-	-	ND	74	71-77	73.71
REPB3-3	-	-	X	50%	-	-	-	43%	68	52-71	66.38
REPB3-4	-	Xq91%	-	70%	-	-	-	64%	64	61-70	65.2
REPB3-5	-	-	-3q	50%	95%	-	-	63%	75	61-75	70.25
REPB4	-	-	-	-	67%	-	-	33%	67	37-146	80.54
REPB5	-	-	-	-	36%	-	-	29%	73	62-73	66.86
REP1*	-	-	-	-	-	-	-	ND	63	60-70	64
REP2*	-	-	-	-	-	-	-	ND	74	63-78	71.27
REP4*	-	-	-	-	-	-	-	ND	ND	ND	ND
REPB1	-	-	-	-	-	-	-	43%	73	71-75	73.14
REPB2	-	-	-	-	13%	-	-	38%	70	36-77	36.38
1RRN-2*	-	-	-	-	-	-100%		ND	-	-	-

*These cell lines were only C-banded.

†REPB3 was neither C- nor G-banded.

FIG. 4.17 - (a) A C-banded cell of REP B3-1. The marsupial chromosome is arrowed.

(b) A G-banded cell of REP B3-1. The *M. rufogriseus* chromosome 3 is arrowed.

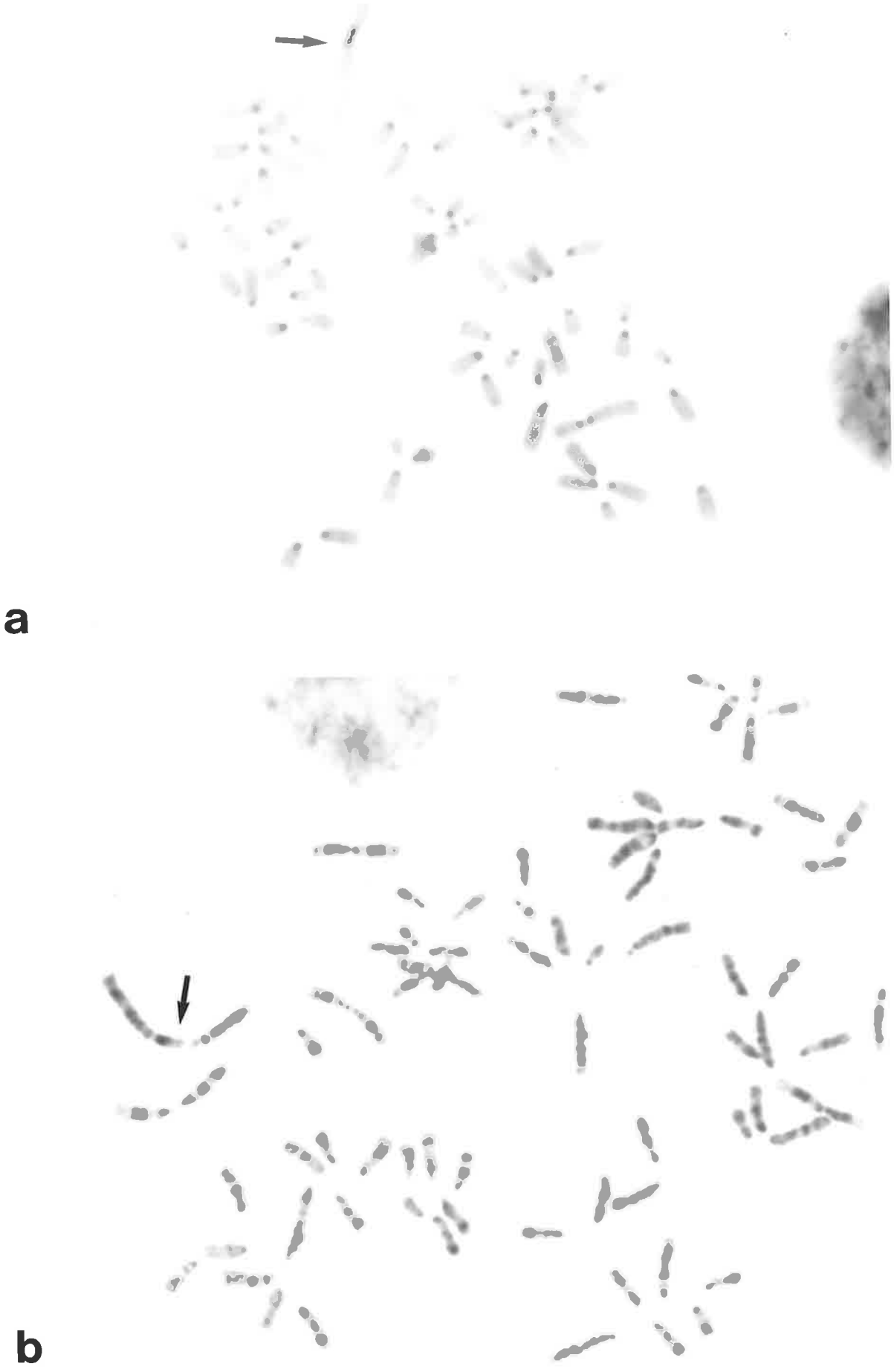
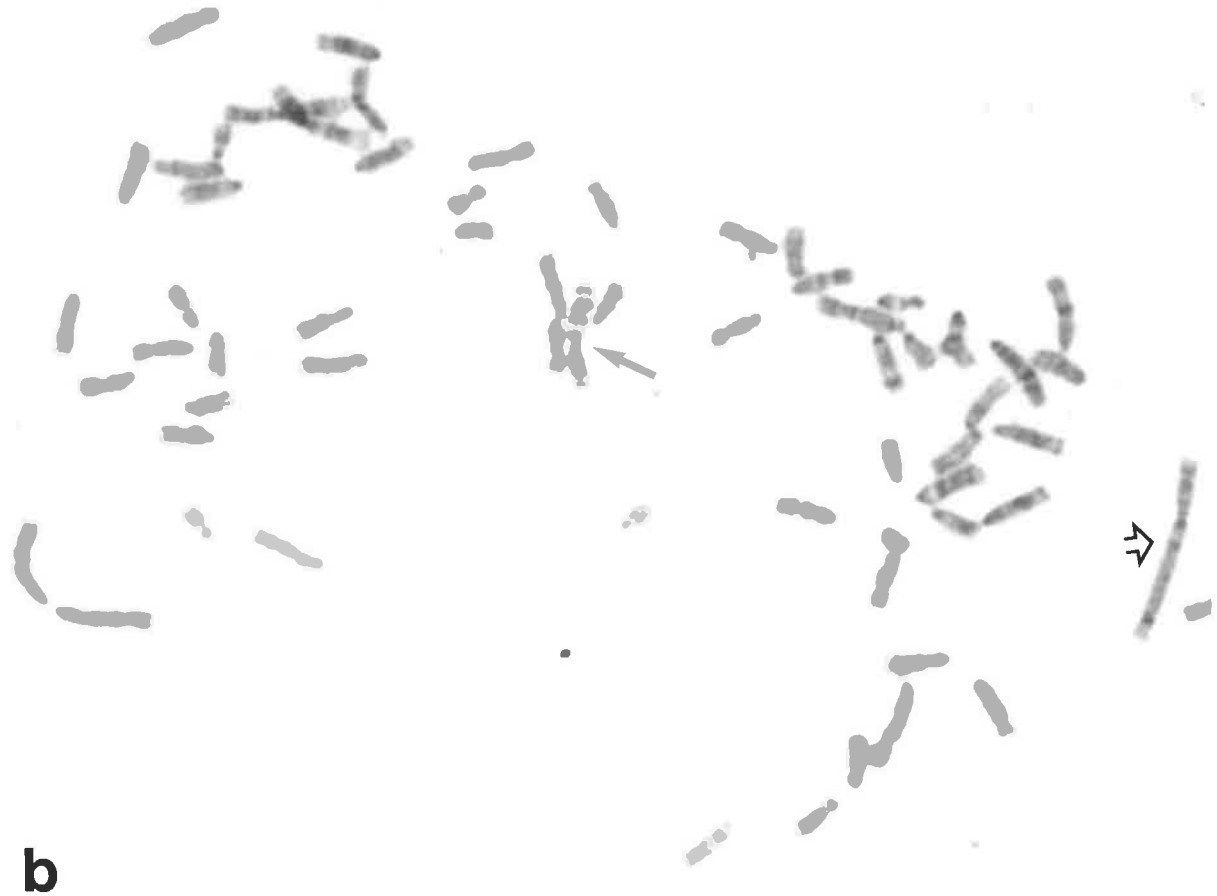

a
b

FIG. 4.18 - (a) A C-banded cell of REP B3-3 containing the *M. rufogriseus* X chromosome (arrowed).

(b) A G-banded cell of REP B3-3. The *M. rufogriseus* chromosome 3 is indicated by an open arrow and the *M. rufogriseus* X chromosome by a closed arrow.

a



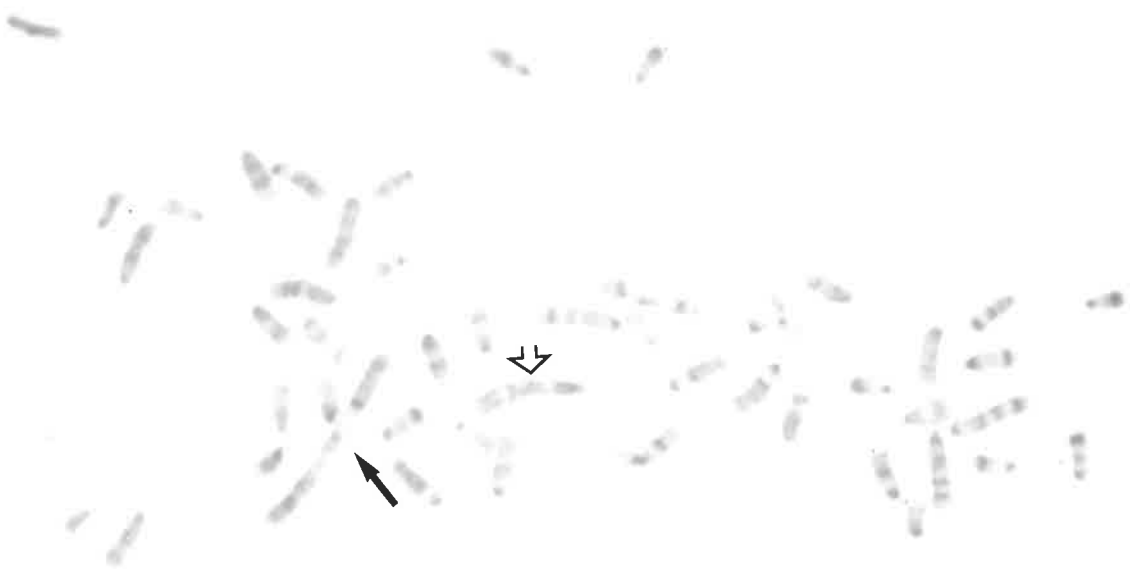
b

FIG. 4.19 - (a) A C-banded cell of REP B3-4.

(b) A G-banded cell of REP B3-4.

The *M. rufogriseus* chromosome 3 is indicated by a closed arrow and the *M. rufogriseus* Xq by an open arrow in both (a) and (b).

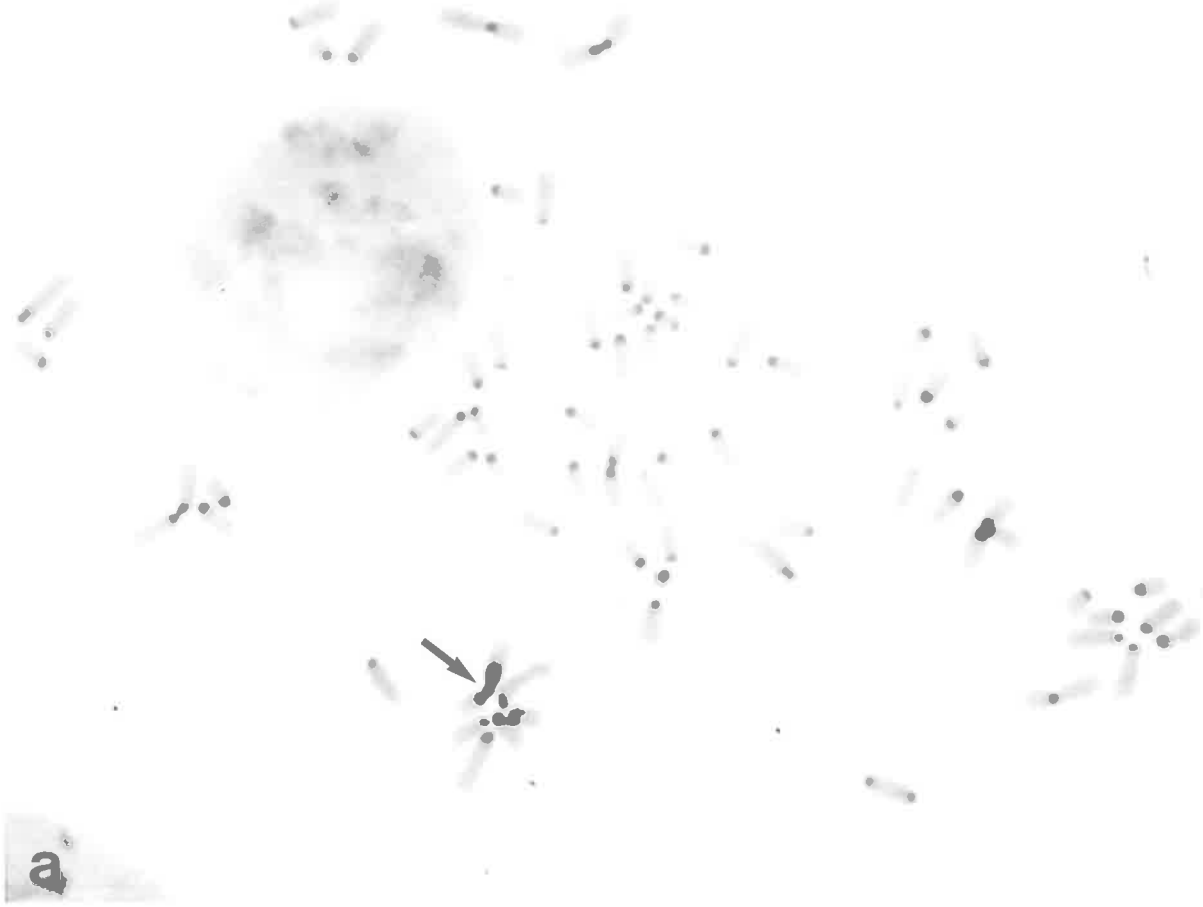
a

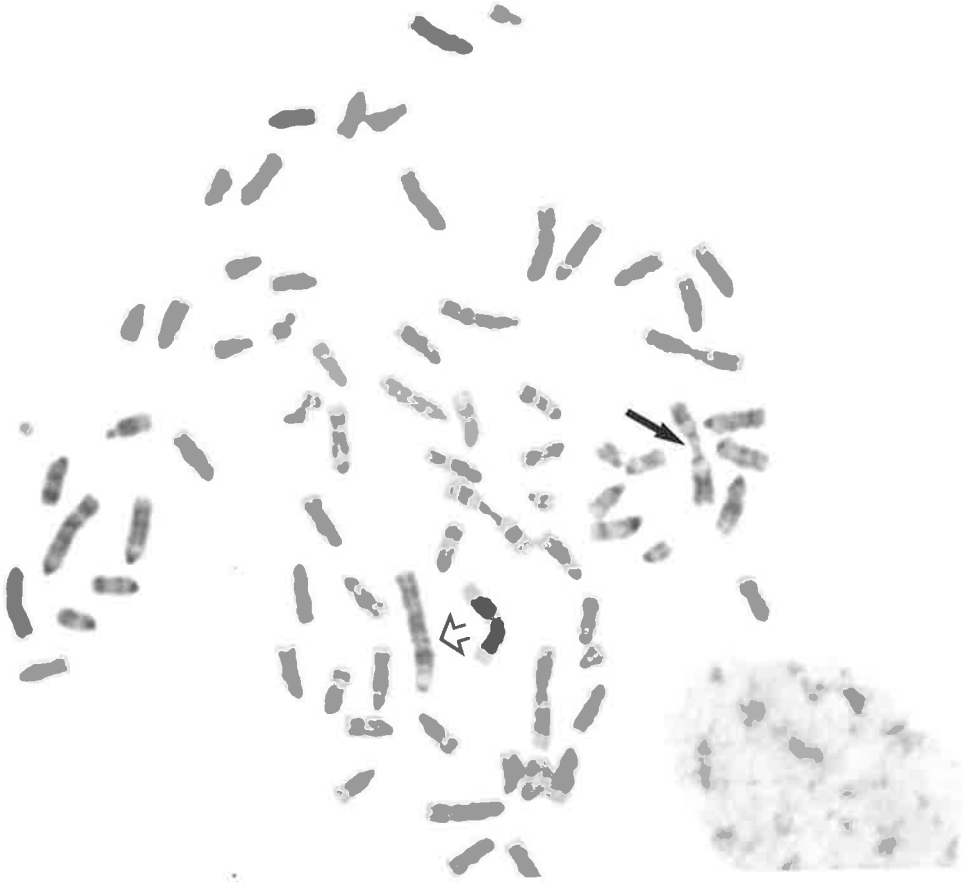


b

FIG. 4.20 - (a) A C-banded cell of REP B3-5. The *M. rufogriseus* chromosome 6 is arrowed.

(b) A G-banded cell of REP B3-5. Open arrow - *M. rufogriseus* 3q. Closed arrow - *M. rufogriseus* chromosome 6.


a


b

distinct from any of the PG19 or normal *M. rufogriseus* chromosomes (Fig. 4.21). The large dark G-band region near the centromere indicated that this chromosome was the short arm of the *M. rufogriseus* X as all *M. rufogriseus* autosomes expressed large light G-bands at the centromere and the mouse chromosomes expressed smaller light or dark G-bands at the centromere than Xp.

All the REP3 revertant cell lines had C- and G-banded karyotypes similar to PG19 cells. No normal *M. rufogriseus* chromosomes could be identified (Fig. 4.22).

4.1.3 Other Somatic Cell Hybrids

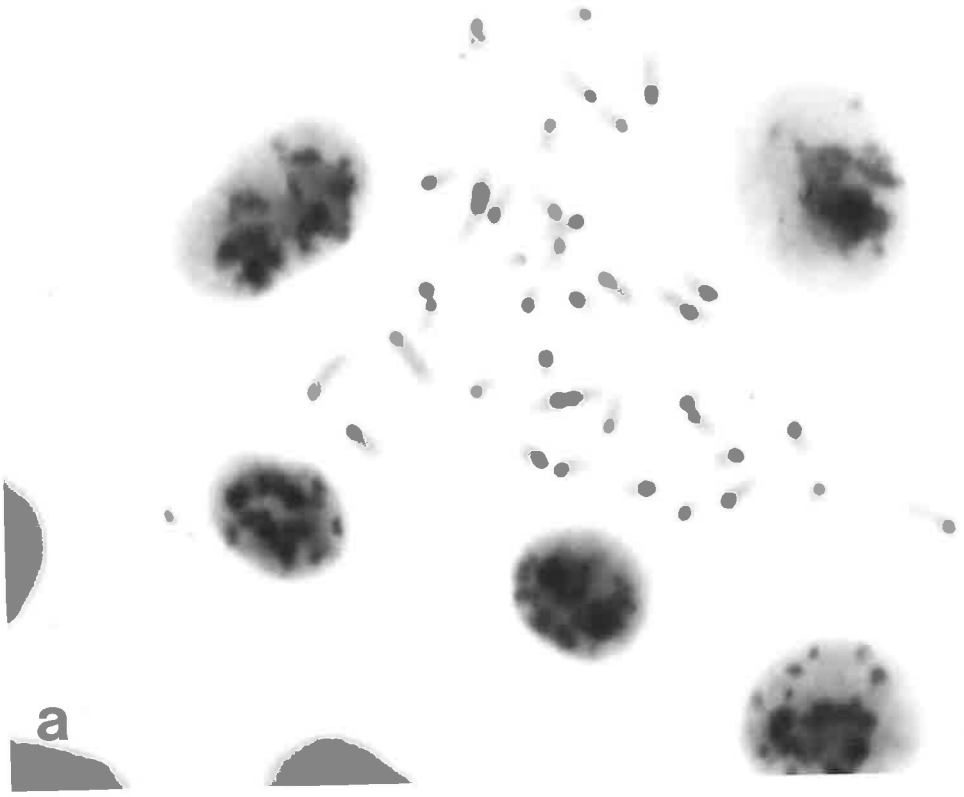
HYP-1 is an (*M. rufogriseus* x *Wallabia bicolor* interspecific hybrid) x PG19 HAT selected somatic cell hybrid (Hope, unpublished). Each cell contains a small submetacentric chromosome consisting almost entirely of C-banding material resembling the short arm of the *M. rufogriseus* X chromosome (Fig. 4.23a). The identity of the long arm of this chromosome was confirmed as the *M. rufogriseus* Xp by its G-banding pattern (Fig. 4.23b). The short arm of this chromosome could not be identified by C- or G-banding.

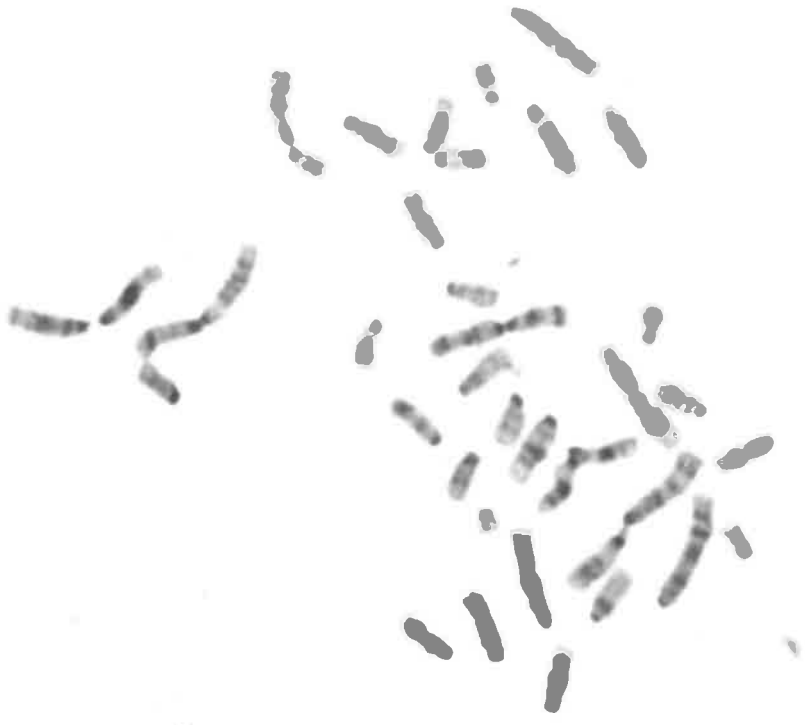
1RRN-1 cells only have C-banded chromosomes indistinguishable from those of 1R (Fig. 4.24a). 1RRN-2 contains an acrocentric chromosome consisting almost entirely of C-banded material and resembling the short arm of the *M. rufogriseus* X chromosome (Fig. 4.24b).

FIG. 4.21 - G-banded cell of REP3-1.
The putative *M. rufogriseus* Xp is arrowed.

FIG. 4.22 - (a) C-banded cell of REP3 R2.

(b) G-banded cell of REP3 R2.


a


b

The PGRN hybrid cells have very heterogeneous karyotypes. The C-banded chromosomes of PGRN-1 and PGRN-2 exhibit chromosomes with varying amounts of interstitial C-bands characteristic of neither parental cell type (Fig. 4.25). Only PGRN-1 was G-banded and although abnormal marsupial chromosomes were present, none could be positively identified.

PGMR2-4 is an *M. rufus* (red kangaroo) x PG19 somatic cell hybrid. (Sykes and Hope, 1978). The *M. rufus* X chromosome has been identified in this cell line by both C- and G-banding (Fig. 4.26).

No marsupial chromosomes were identified in the WT1R hybrid using C- or G-banding.

4.2 COMPARISON OF CHROMOSOME PROPERTIES OF REP3-2 AND REP 3-2-1

4.2.1 General Stain

Both REP3-2 and REP3-2-1 had an easily identifiable, large, submetacentric chromosome present amongst a chromosome background that was mainly of mouse origin.

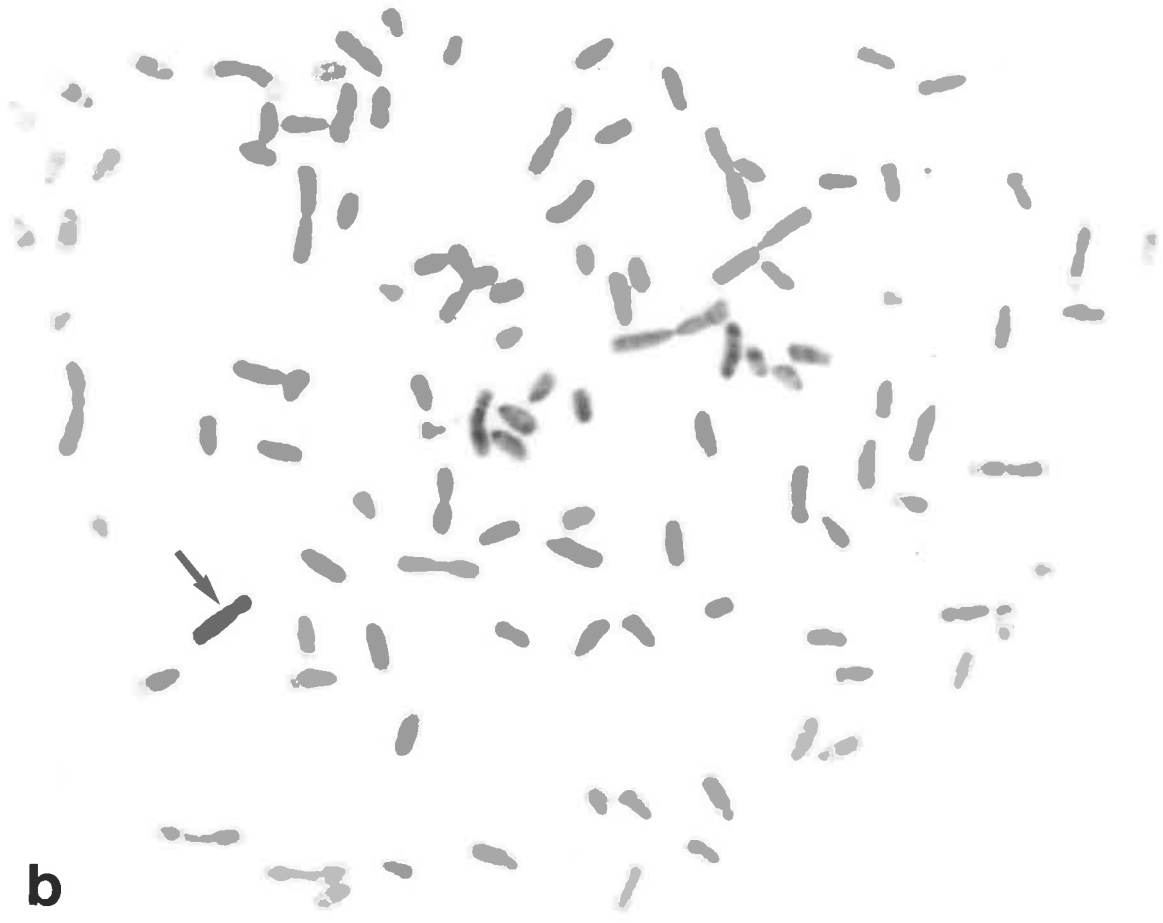
4.2.2 Sequential C- and G-Banding

The C- and G-banding patterns of REP3-2 and REP3-2-1 are described in Section 4.1.2. Figures 4.27(a) and (b) show a REP3-2

FIG. 4.23 - (a) C-banded cell of HYP-1.
The marsupial chromosome is arrowed.

(b) G-banded cell of HYP-1.
The short arm of the *M. rufogriseus* X chromosome is arrowed.

a



b

FIG. 4.24 - (a) A C-banded cell of 1RRN-1.
No marsupial chromosomes could be identified.

(b) A C-banded cell of 1RRN-2.
Marsupial chromosomes are arrowed.

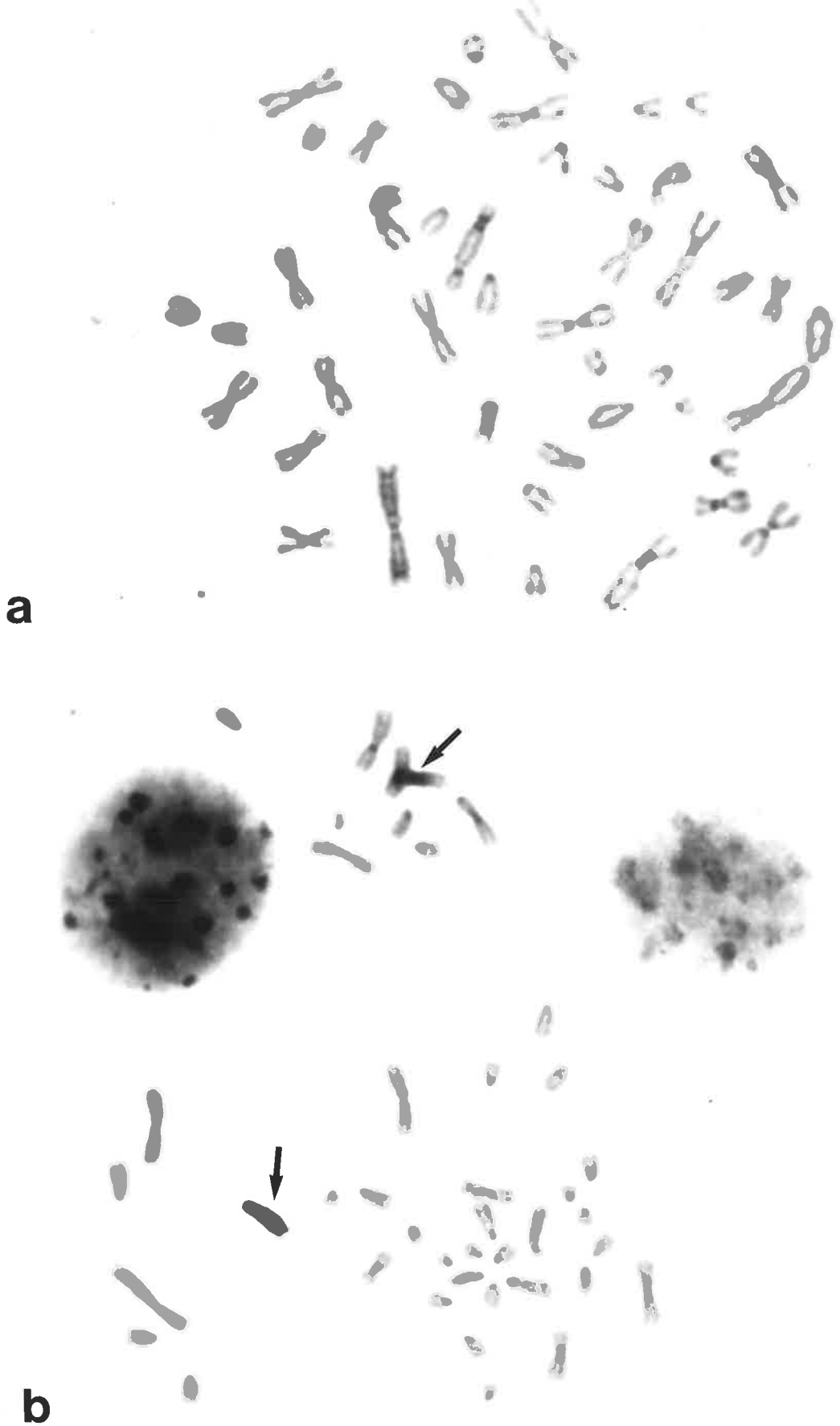
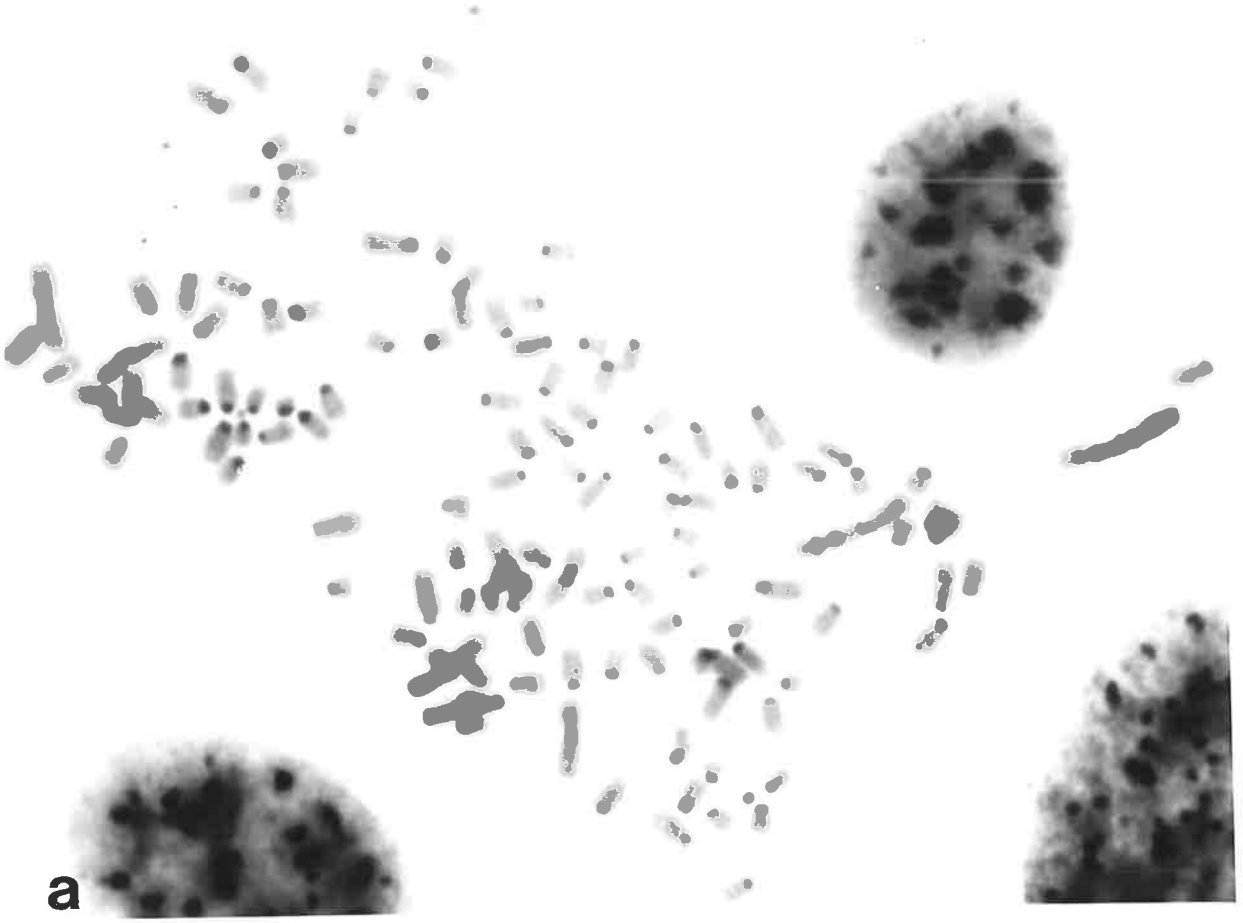

a
b

FIG. 4.25 - (a) A C-banded cell of PGRN-1.

(b) A C-banded cell of PGRN-2.


a

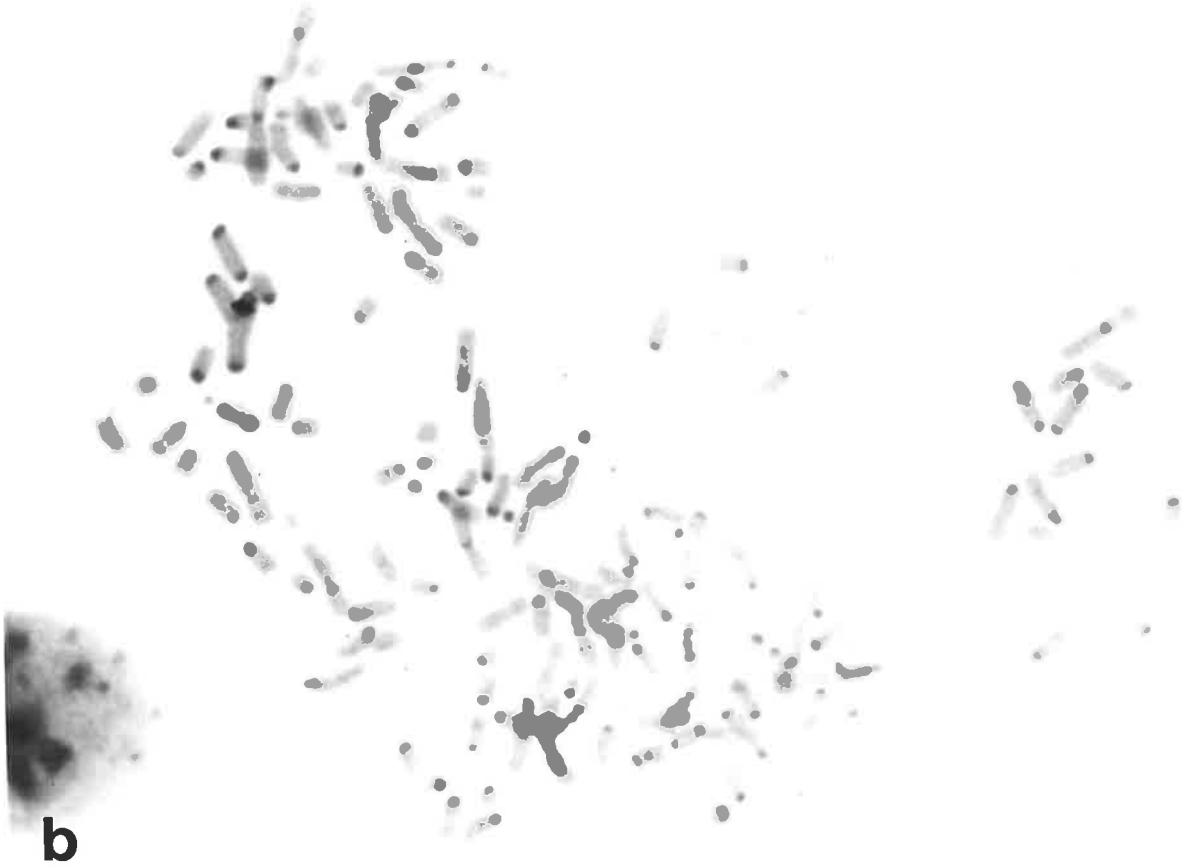
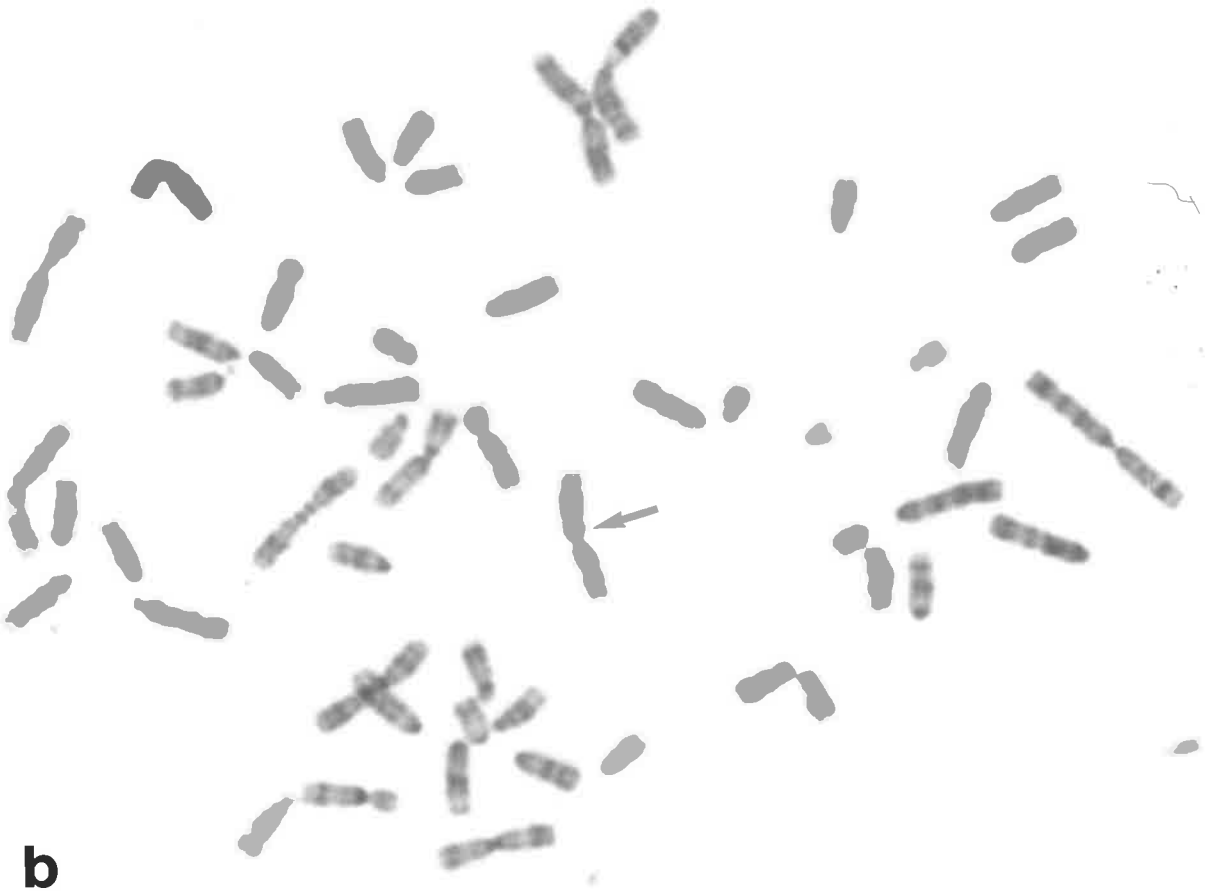

b

FIG. 4.26 - (a) A C-banded cell of PGMR2-4. The *M. rufus* X chromosome is arrowed.

(b) A G-banded cell of PGMR2-4. The *M. rufus* X chromosome is arrowed.

a



b

cell G-banded and the same cell subsequently C-banded. The same is shown for a REP3-2-1 cell (Figs. 4.27(c) and (d)). The *M. rufogriseus* translocation chromosome t(3q,Xq) exhibited identical G-banding in the two cell lines but the C-banding patterns differed. In REP3-2, t(3q,Xq) had a large centromeric C-band and three interstitial C-bands on the short arm. There was very little C-banding apparent on the t(3q,Xq) chromosome in REP3-2-1 and the interstitial C-bands were not cytologically visible.

The difference in C-banding patterns of t(3q,Xq) in REP3-2 and REP3-2-1 prompted further investigation of the chromosomes in these cell lines (Sections 2.4.3 - 2.4.7).

4.2.3 N.-Banding

Centromeric N-bands are present on four pairs of chromosomes in the C57BL/6J mouse strain (Dev *et al*, 1977). These mouse chromosomes exhibited N-bands in the REP3-2 and REP3-2-1 hybrid cell lines. No N-bands were found in t(3q,Xq) in either cell line (Fig. 4.28).

4.2.4 *In Situ* Hybridisation with the *M. rufogriseus* Major Satellite

These studies were carried out in collaboration with W. Peacock and A. Elizur (Division of Plant Industry, C.S.I.R.O., Canberra).

The major satellite (density 1.708g/cc) did not bind to any PG19 chromosomes. Any reaction represented background hybridisation (Fig. 4.29a). The satellite did, however, bind to the centromeric regions of all the *M. rufogriseus* autosomes but not the *M. rufogriseus* X chromosome (Fig. 4.29b). The satellite hybridised only to the 3q

FIG. 4.27 - Sequential C- and G-banding of REP3-2 and REP3-2-1.

(a) REP3-2 - G-banded.

(b) REP3-2 - C-banded.

(c) REP3-2-1 - G-banded.

(D) REP3-2-1 - C-banded.

t(3q,Xq) is arrowed in each case.

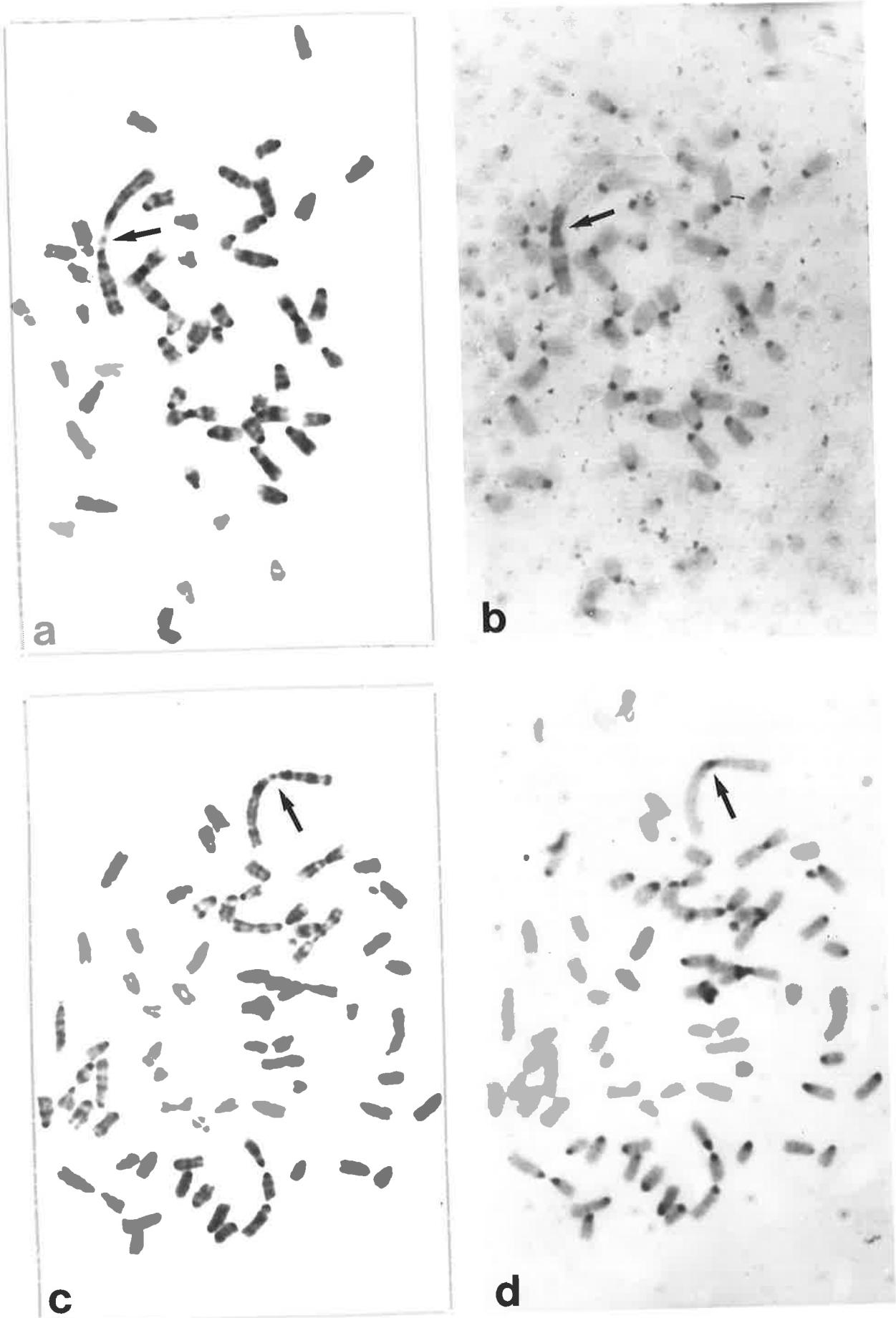
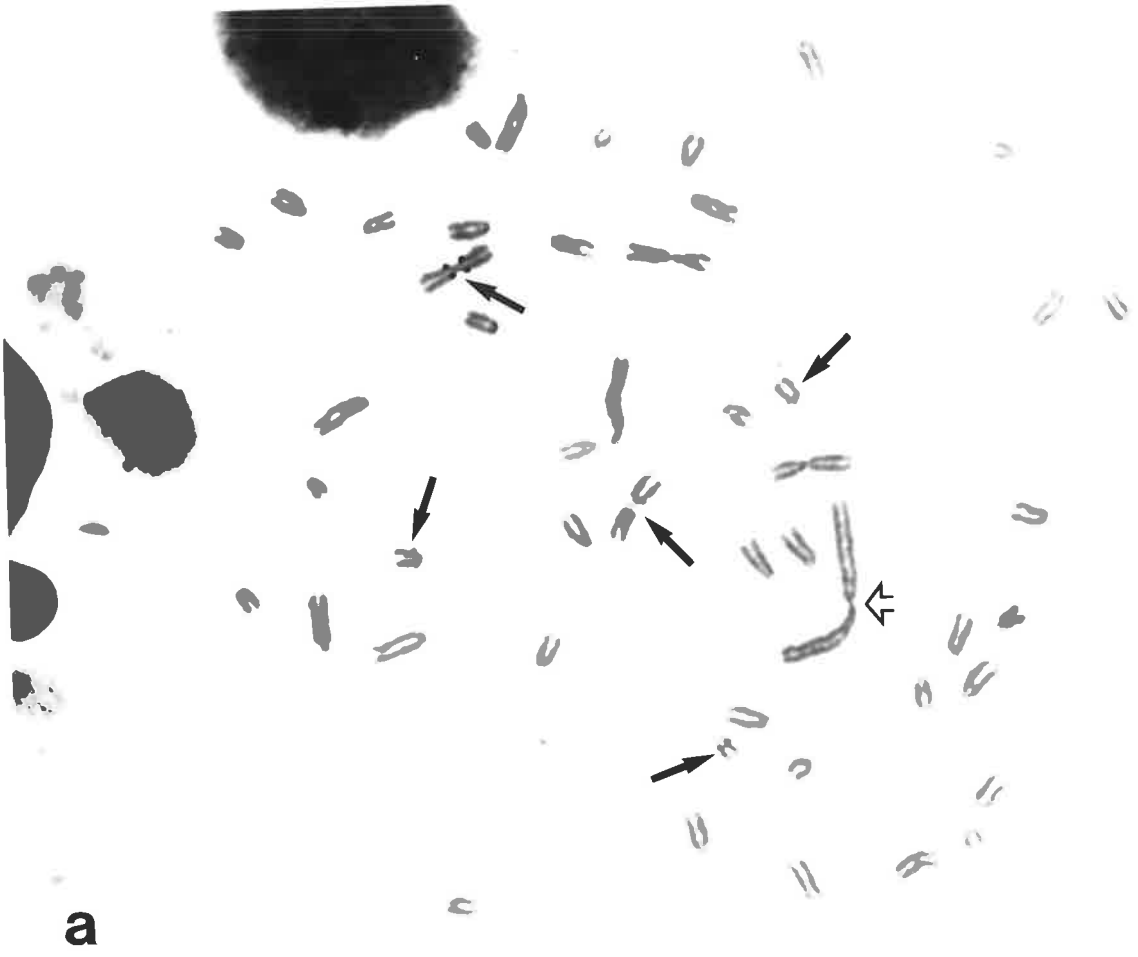

a
b
c
d

FIG. 4.28 - N-banded cells of

(a) REP3-2

(b) REP3-2-1

Silver stained mouse nucleolar organizer regions are arrowed (closed). t(3q,Xq) is also arrowed (open).



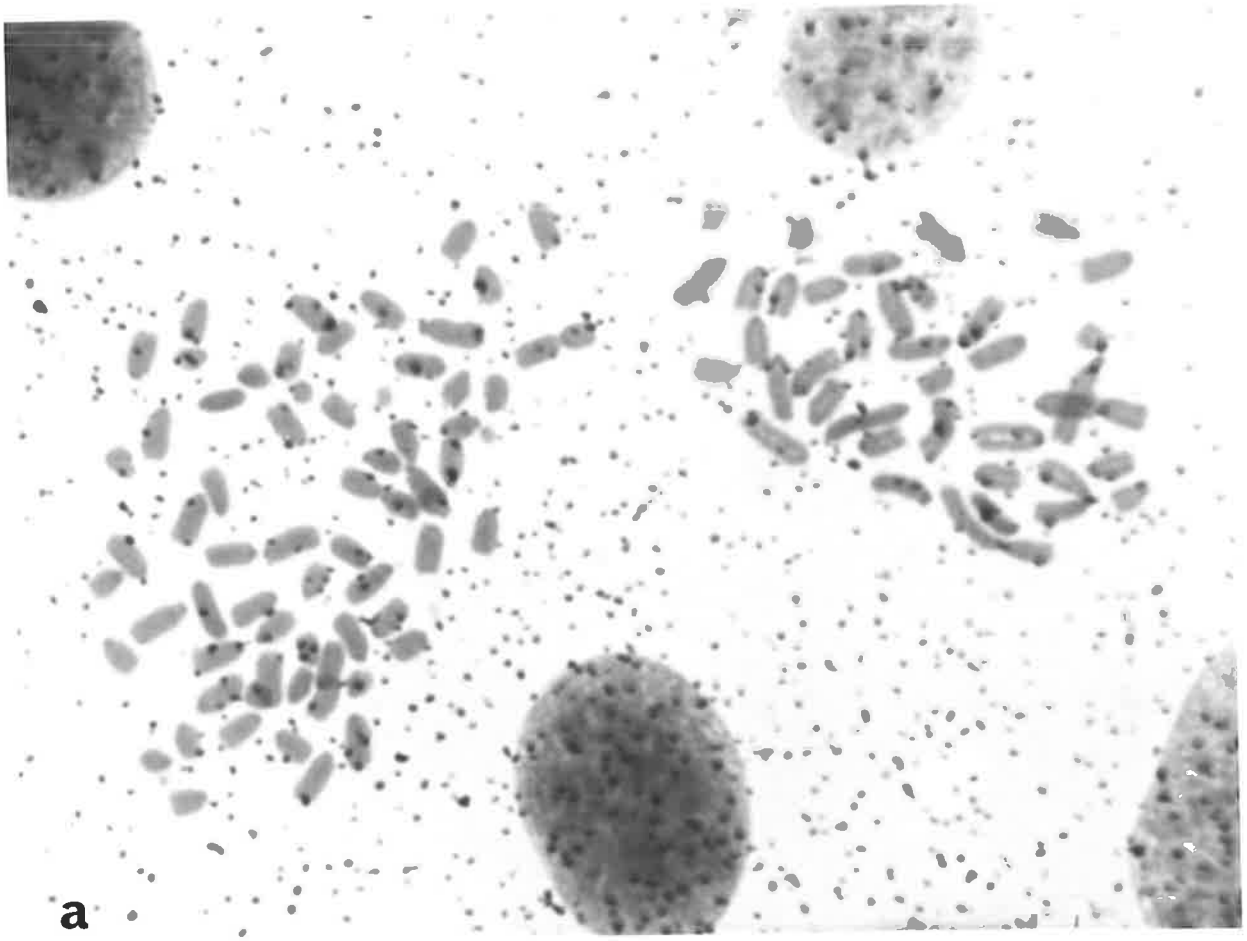
a

b

FIG. 4.29 - *In situ* hybridisation of the major *M. rufogriseus* satellite to:

(a) PG19

(b) *M. rufogriseus*. The X chromosomes are arrowed.


a

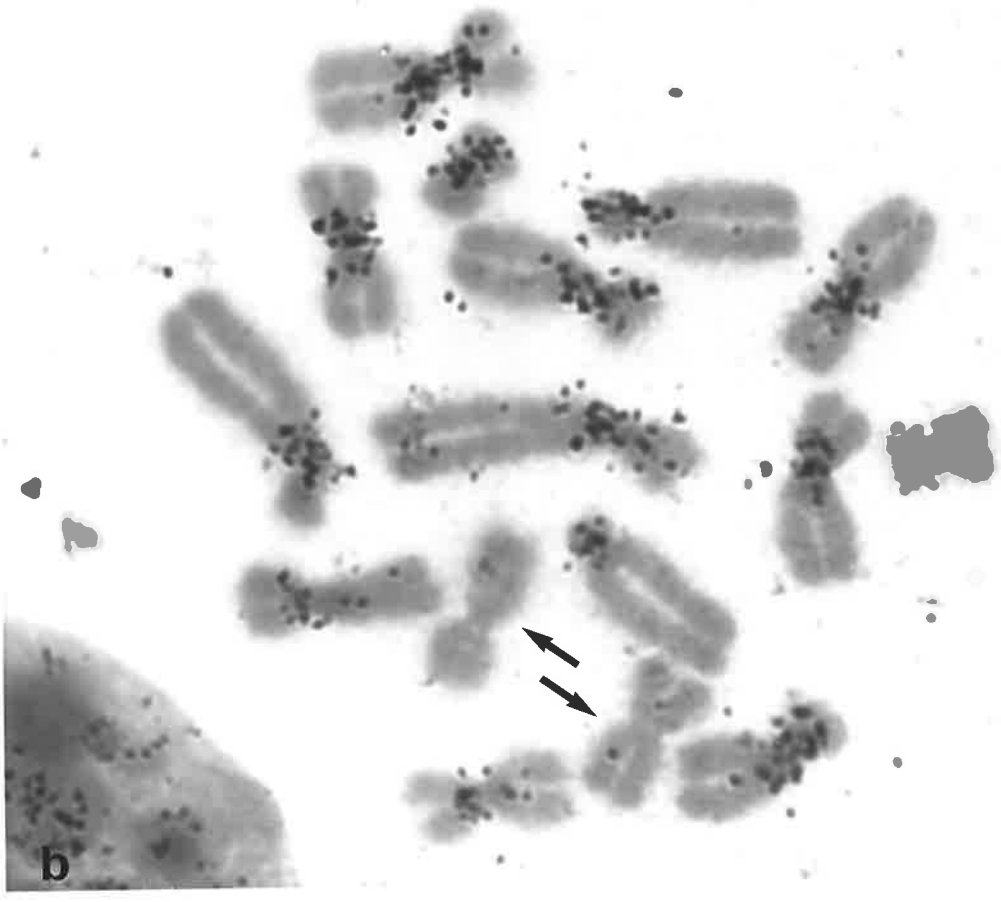

b

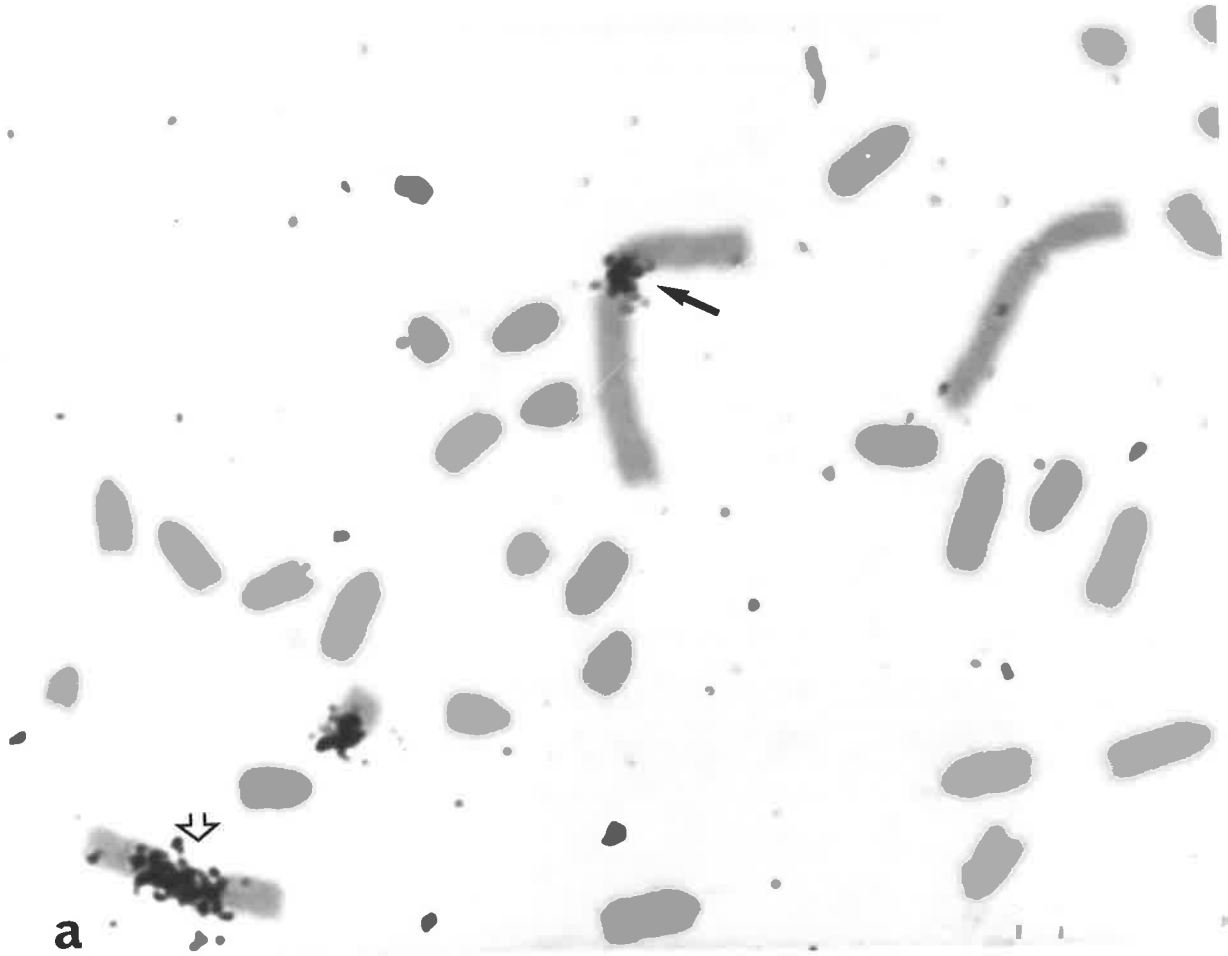
Fig. 4.30 - *In situ* hybridisation of the major *M. rufogriseus* satellite to:

(a) REP3-2

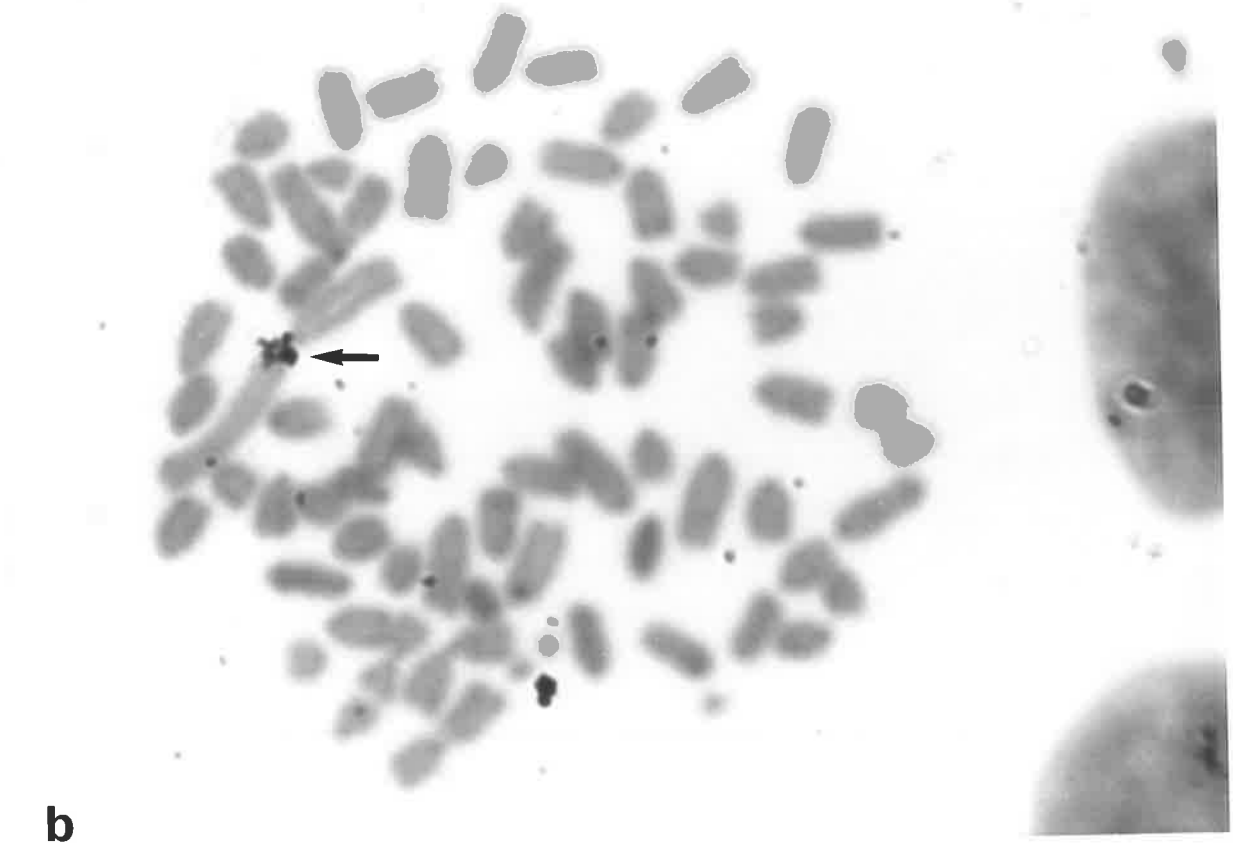
(b) REP 3-2-1

Closed arrow - *M. rufogriseus* t(3q,Xq)

Open arrow - *M. rufogriseus* 7.



a



b

arm of t(3q,Xq) in both REP3-2 and REP3-2-1 (Fig. 4.30).

4.2.5 Hoechst 33258

Culture of REP3-2 cells in the presence of Hoechst 33258 resulted in lack of condensation of the DNA in the region of the mouse centromeres. There was a particularly marked lack of condensation at the centromere of t(3q,Xq) compared with the mouse centromeres (Fig.4.31a).

The t(3q,Xq) chromosome in REP3-2-1 was not affected by culture in the presence of Hoechst 33258 even though the mouse chromosomes were affected as in REP3-2 (Fig. 4.31b).

The results for C, G, Hoechst 33258 and the *M. rufogriseus* major satellite for REP3-2 and REP3-2-1 are summarised in Fig. 4.32.

4.2.6 Counterstaining of DAPI with Actinomycin-D

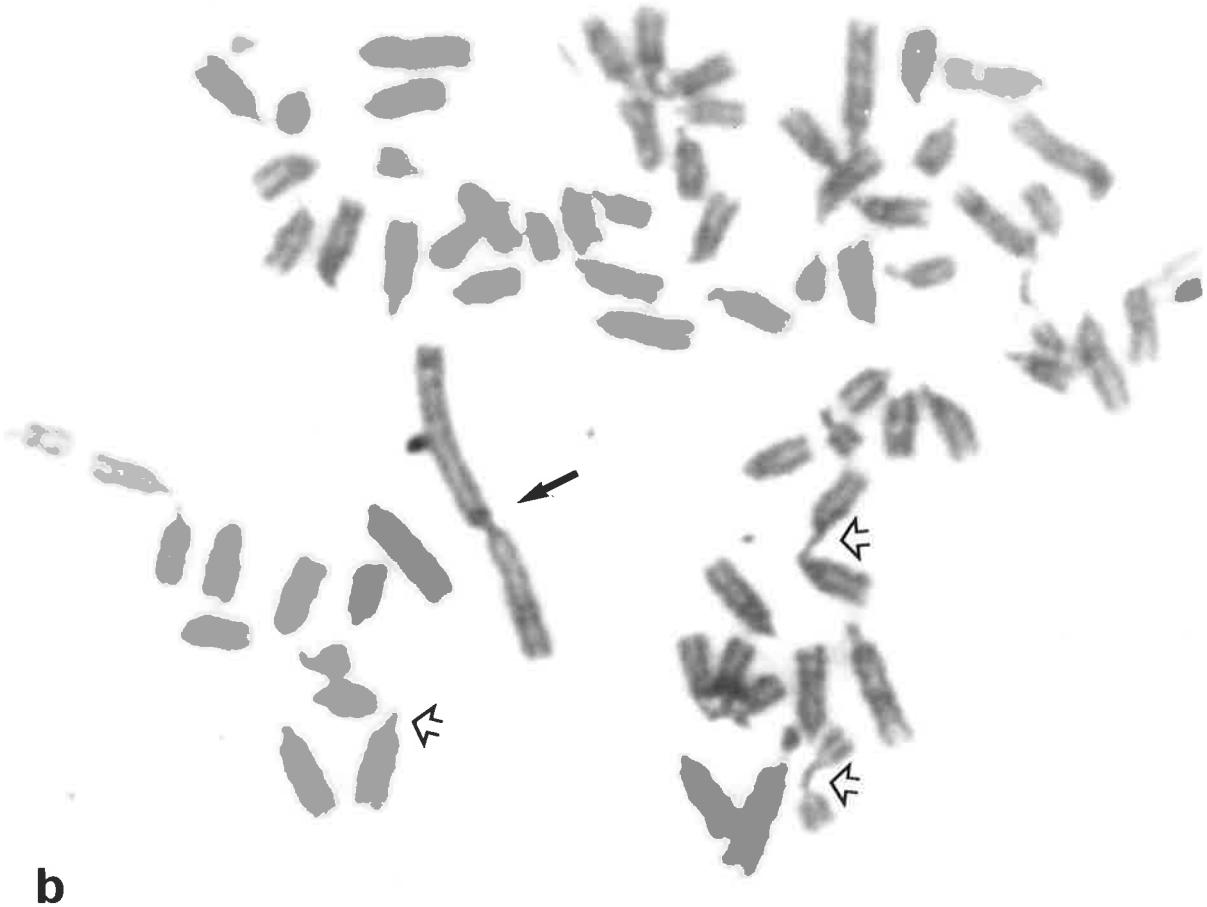
The *M. rufogriseus* t(3q,Xq) chromosome in REP3-2 showed bright DAPI-banding on the proximal half of the Xq arm and all but the centromeric region of the 3q arm (Fig. 4.33a). The *M. rufogriseus* autosomes had dull DAPI-banded centromeres and the centromeric region of the *M. rufogriseus* X expressed DAPI fluorescence of intermediate intensity compared with the very bright fluorescence of mouse centromeres (P.J. Sharp, personal communication). The dull centromere of the *M. rufogriseus* chromosome 7 could be distinguished on this basis from the bright DAPI centromeric bands of the mouse centromeres (Fig. 4.33a).

In REP3-2-1, t(3q,Xq) had a very dull centromere with faint fluorescence on the rest of the chromosome (Fig. 4.33b). The brightly stained proximal DAPI-band on the Xq arm in REP3-2 was not present in REP3-2-1. The mouse chromosomes in both REP3-2 and

FIG. 4.31 - (a) A REP3-2 metaphase spread after culture in the presence of Hoechst 33258. t(3q,Xq) shows a marked lack of condensation (closed arrow) compared with all the PG19 chromosomes (e.g. open arrows).

(b) A REP3-2-1 metaphase spread after culture in the presence of Hoechst 33258. All PG19 centromeres show lack of condensation (e.g. open arrows) whereas the *M. rufogriseus* t(3q,Xq) does not (closed arrow).

a



b

FIG. 4.32 - Comparison of C- and G-banding pattern, the affect of culture in Hoechst 33258 and *in situ* hybridisation with the *M. rufogriseus* major satellite of t(3q,Xq) in REP3-2 and REP3-2-1.

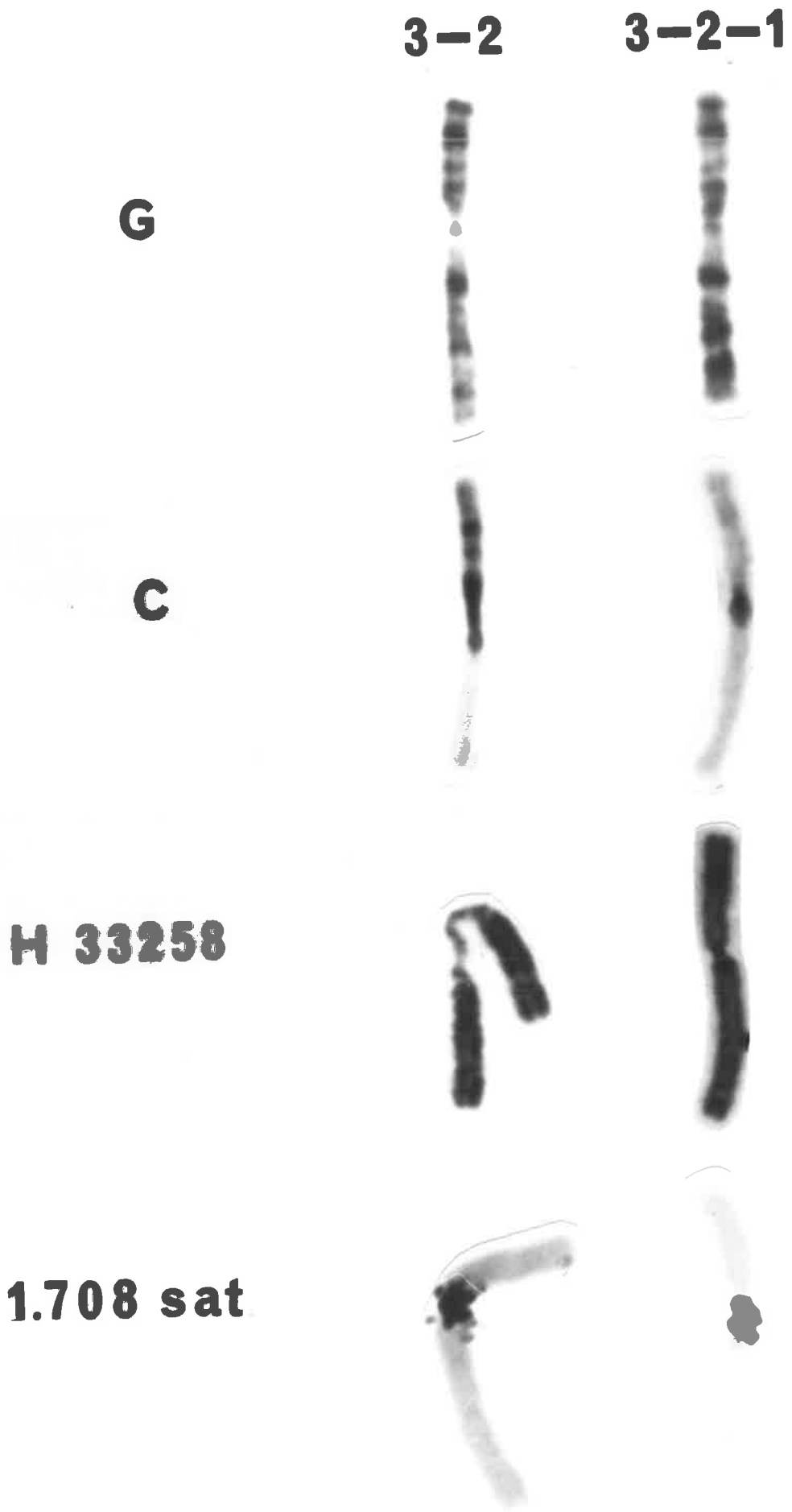
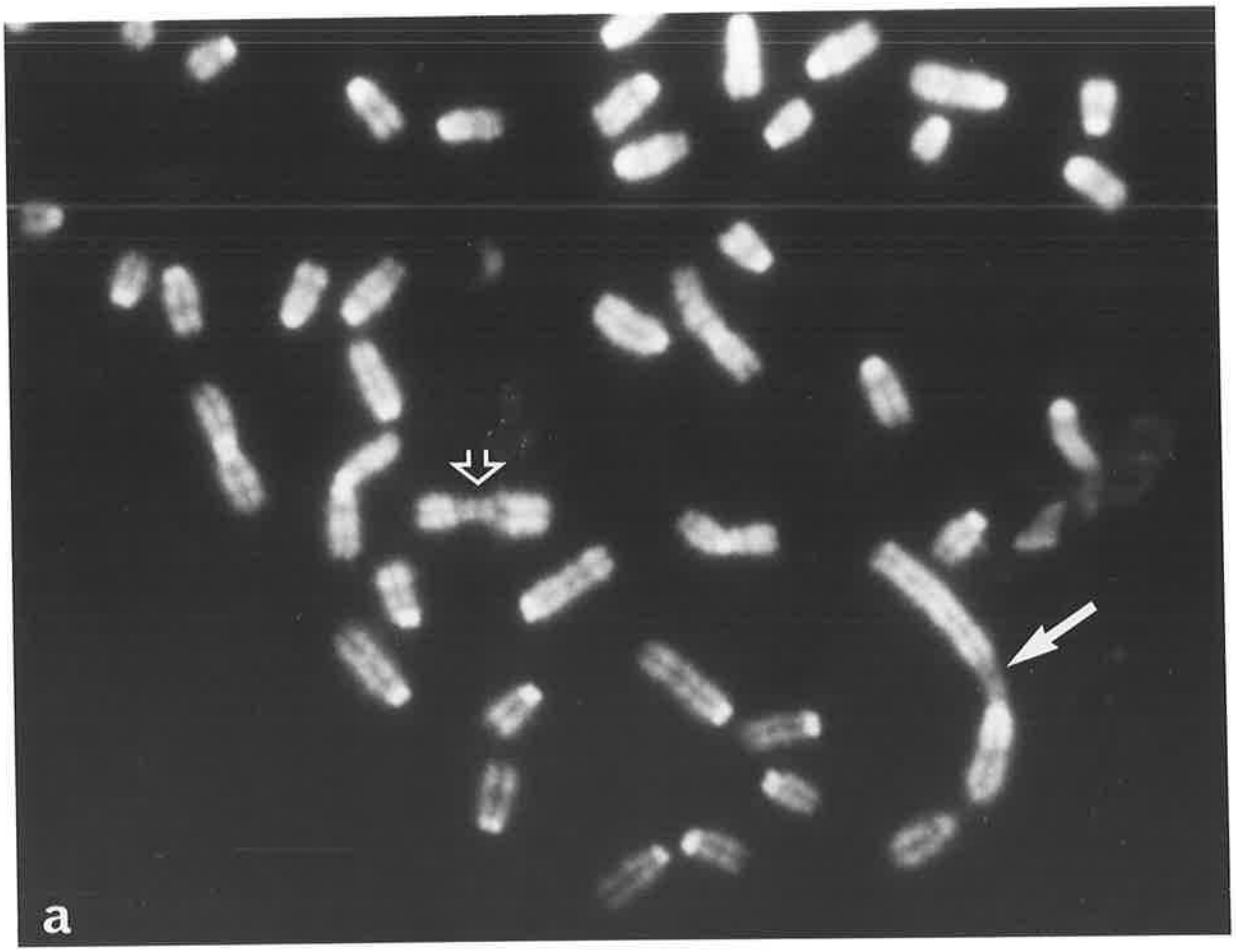

3–2
3–2–1
G
C
H 33258
1.708 sat

FIG. 4.33 - (a) REP3-2 stained with DAPI and Actinomycin D. t(3q,Xq) is arrowed (closed) as is the *M. rufogriseus* chromosome 7 (open).

(b) REP3-2-1 stained with DAPI and Actinomycin D. t(3q,Xq) is arrowed.


a

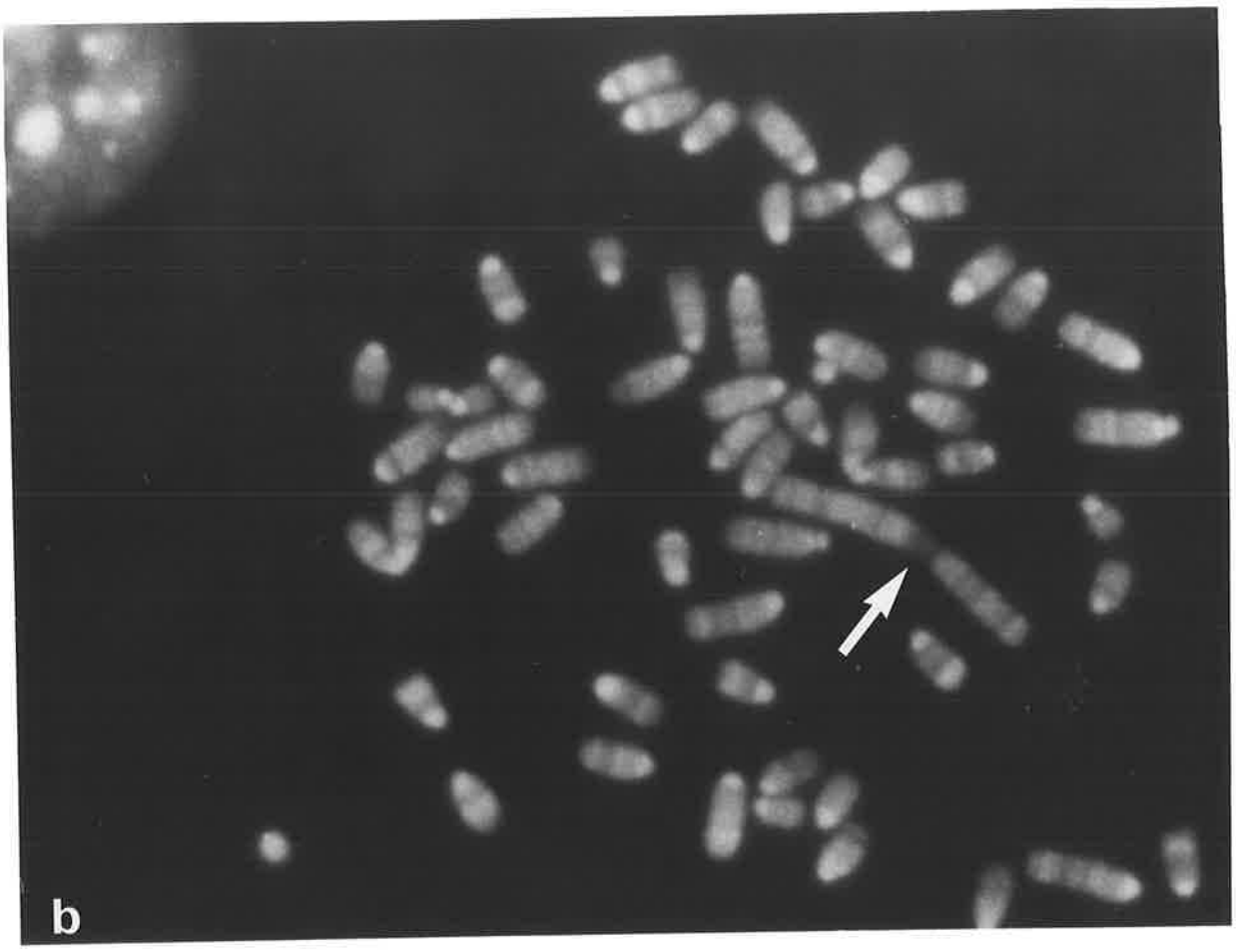

b

FIG. 4.34 - R and Distamycin A (DA) - DAPI banding.

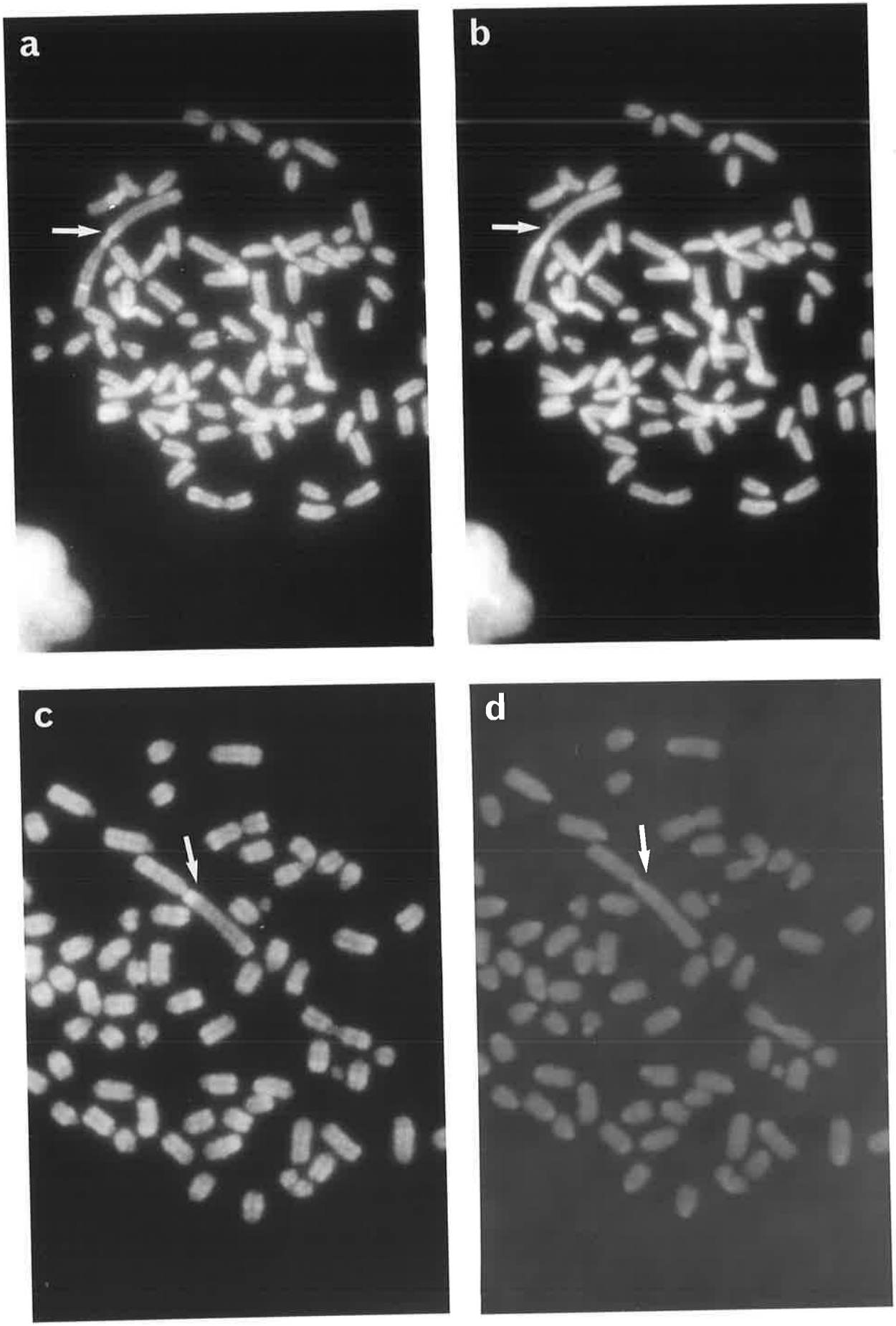
(a) REP3-2 - R-banded

(b) REP3-2 - DA-DAPI banded

(c) REP3-2-1 - R-banded

(d) REP3-2-1 - DA-DAPI banded

t(3q,Xq) is arrowed in each case.


a
b
c
d

REP3-2-1 expressed bright centromeric DAPI staining.

4.2.7 R and Distamycin-A (DA)-DAPI Banding

When chromomycin A3/DA/DAPI treated slides were viewed under a Leitz Ploemopak II Filter block E_3 (wavelength range 430-480 nm), the fluorescence R-banding patterns for REP3-2 and REP3-2-1 differed. REP3-2 had a bright fluorescent region on either side of the t(3q,Xq) centromere and a very bright interstitial band and 2 weaker bands on the Xq arm. REP3-2-1 had only one bright centromeric region on the 3q arm of t(3q,Xq) (Figs. 4.34(a) and (c)).

When these same metaphase spreads were then viewed with a Leitz Ploemopak II Filter block A (wavelength range 360-390 nm) the DA/DAPI-banding patterns of t(3q,Xq) in REP3-2 and REP3-2-1 were indistinguishable. (Figs. 4.34(b) and (d)).

4.3 ENZYMES

4.3.1 Hypoxanthine phosphoribosyltransferase (HPRT)

M. rufogriseus and mouse HPRT isozymes were easily separated by gel electrophoresis using the method of Watson *et al* (1972). The mouse cell lines PG19 and 1R did not express HPRT. The TK^-, $BrdU^r$ Swiss mouse embryo cell line, 3T3, was used as an $HPRT^+$ mouse control (Matsuya and Green, 1969). Hybrids were selected and maintained in HAT medium and hence selected for the presence of the marsupial HPRT gene. As expected all the REP and PGRN hybrid cell lines expressed marsupial HPRT. Revertants were selected and maintained in the presence of 6TG. Neither *M. rufogriseus* nor mouse HPRT was expressed in revertant cell lines. An autoradiograph of an HPRT gel is shown in Fig. 4.35.

The 1RRN-1 and WT1R "hybrids" lacked the *M. rufogriseus* HPRT isozyme but expressed mouse HPRT. (Discussed in Section 5.1).

FIG. 4.35 - An autoradiograph of a starch gel stained for HPRT. Channel.

1 - 3T3

2 - REP3-7

3 - REP3 R3

4 - REP3 R2

5 - REP3-4

6 - REP3 R8

7 - REP3 R4

8 - REP3-1

9 - REP3 R1

10 - REP3 R5

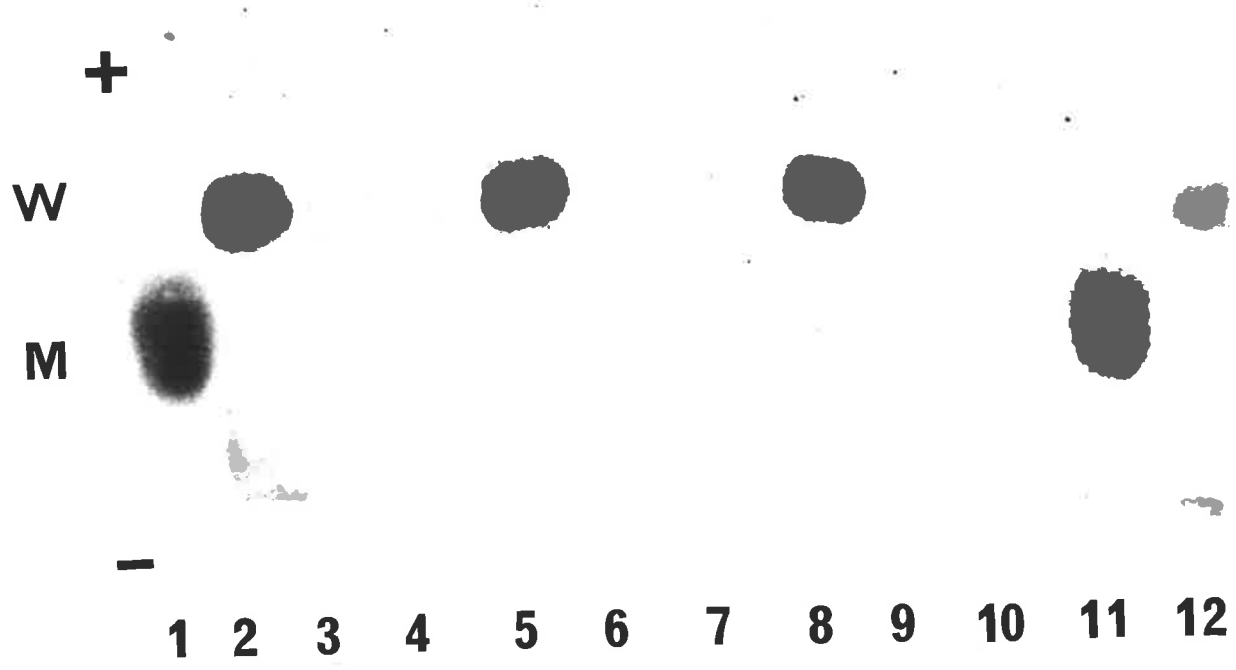
11 - 3T3

12 - REP3-2

3T3 expresses the slow mouse form of HPRT (Channels 1 and 11). All the hybrids (Channels 2,5,8 and 12) express the faster marsupial form of HPRT whereas the revertants (Channels 3,4,6,7,9 and 10) do not express either the mouse or marsupial forms of HPRT.

M = mouse mobility

W = red necked wallaby mobility.


+
W
M
−
1 2 3 4 5 6 7 8 9 10 11 12

The HPRT results for the REP, PGRN, 1RRN and WT1R cell lines are given in Table 4.2.

4.3.2 Phosphoglycerate kinase A. (PGK-A)

M. rufogriseus and PG19 PGK-A are easily separated using the electrophoretic method set out in Section 3.8.3. (See Fig. 4.36). All the HAT selected REP hybrids and their subclones expressed both *M. rufogriseus* and PG19 PGK-A. The band staining of *M. rufogriseus* PGK-A was less intense than that of PG19. *M. rufogriseus* fibroblasts, lymphocytes and red blood cells gave the same PGK-A mobility which was slightly faster than the *M. rufogriseus* PGK-A found in the hybrids. The reason for this mobility difference is not clear. It is possibly due to interaction with other molecules in the hybrid extracts not present in the *M. rufogriseus* control extracts. The 6TG selected REP3 revertants expressed only the mouse form of PGK-A. The PGK-A results for the REP hybrids are given in Table 4.2.

None of the PGRN hybrids nor the WT1R hybrid expressed marsupial PGK-A. Only one of the two 1RRN hybrids, 1RRN-2, expressed marsupial PGK-A (Table 4.2).

Two PGK bands were often found in the *M. rufogriseus* controls, with the less intensely staining slower band having a mobility very similar to the mouse band. Only one PGK-A allele is expected to be expressed in each cell due to paternal X-inactivation at this locus in female kangaroos (Cooper *et al*, 1971; Vandeberg *et al*, 1973). The slower band was *M. rufogriseus* PGK-B. PGK-B is controlled by an autosomal locus in kangaroos and is expressed in a wide range of *M. rufogriseus* somatic cells. (Vandeberg *et al*, 1978).

TABLE 4.2

	APPROXIMATE PERCENTAGE OF CELLS CONTAINING *M. RUFOGRISEUS* CHROMOSOMES								*M. RUFOGRISEUS* ENZYMES			
	t(3q,Xq)	Xq	X	3	6	7	Xp C	Xp G	HPRT	PGK-A	G6PD	LDH-A
REP1*	-	-	-	-	-	-	-	ND	+	+	-	-
REP2*	-	-	-	-	-	-	-	ND	+	+	-	-
REP3	27	-	-	18	18	9	-	43	+	+	-	-
REP4*	-	-	-	-	-	-	-	ND	+	+	-	-
REP3-1	-	-	-	80	-	-	60	87	+	+	-	-
REP3-2	75	-	-	-	-	12	-	65	+	+	-	-
REP3-2-1	100	-	-	-	-	-	-	66	+	+	-	-
REP3-2-2	-	-	-	-	-	-	-	43	+	+	-	-
REP3-3	-	-	-	-	-	-	-	25	+	+	-	-
REP3-4	t(3q,?)55	-	-	-	45	-	-	83	+	+	-	-
REP3-5	-	-	-	-	-	-	-	71	+	+	-	-
REP3-6	-	-	-	-	-	-	85	88	+	+	-	-
REP3-7	-	-	-	i3q50	36	-	-	60	+	+	-	-
REP3-7-1	-	-	-	89	-	-	-	75	+	+	-	-
REP3-7-2*	-	-	-	75	-	-	-	ND	+	+	-	-
REP3-7-3*	-	-	-	-	-	-	-	ND	+	+	-	-
REP3-7-4	-	-	-	3q66	-	-	-	80	+	+	-	-
REP3-7-5*	-	-	-	-	-	-	-	ND	+	+	-	-
REP3R1	-	-	-	-	-	-	-	27	-	-	-	-
REP3R2	-	-	-	-	-	-	-	25	-	-	-	-
REP3R3	-	-	-	-	-	-	-	18	-	-	-	-
REP3R4→REP3R8	-	-	-	-	-	-	-	ND	-	-	-	-
REPB1	-	-	-	-	-	-	-	43	+	+	-	-
REPB2	-	-	-	-	13	-	-	38	+	+	-	-
REPB3†	-	-	-	-	-	-	-	-	+	+	-	-
REPB3-1	-	-	-	90	-	-	-	54	+	+	-	-
REPB3-2*	-	-	-	66	-	-	-	ND	+	+	-	-
REPB3-3	-	-	X	50	-	-	-	43	+	+	-	-
REPB3-4	-	Xq91	-	70	-	-	-	64	+	+	-	-
REPB3-5	-	-	-	3q50	95	-	-	63	+	+	-	-
REPB4	-	-	-	-	67	-	-	33	+	+	-	-
REPB5	-	-	-	-	36	-	-	29	+	+	-	-
PGRN-1*	-	-	-	-	-	-	-	ND	+	-	-	-
PGRN-2*	-	-	-	-	-	-	-	ND	+	-	-	-
PGRN-3*	-	-	-	-	-	-	-	ND	+	-	-	-
1RRN-1*	-	-	-	-	-	-	-	ND	-	-	-	-
1RRN-2*	-	-	-	-	-	-	100	ND	+	+	-	-
WT1R*	-	-	-	-	-	-	-	ND	-	-	-	-

*These cell lines were only C-banded.

†REPB3 was neither C- nor G-banded.

4.3.3 Glucose-6-phosphate dehydrogenase (G6PD)

The *M. rufogriseus* form of G6PD was not found in any of the cell lines tested. Although there was little electrophoretic separation of the mouse and *M. rufogriseus* G6PD enzymes, with the *M. rufogriseus* G6PD having slightly faster electrophoretic mobility than the PG19 G6PD, the results of the electrophoretic gels were read with confidence as lacking a band in the region of the *M. rufogriseus* control band on the gel. (Fig. 4.37).

4.3.4 Other Enzymes

Four other enzymes apart from the X-linked enzymes HPRT, PGK-A and G6PD were studied in the *M. rufogriseus* x mouse cell hybrids. Lactate dehydrogenase - A, esterase - A_4 and phosphoglucomutase -3 were chosen because they are encoded by genes known to be syntenic with genes controlling antigenic systems in other species (see Discussion). Marsupial nucleoside phosphorylase has successfully been demonstrated in wallaroo x mouse hybrids containing a chromosome of similar G-band pattern to the *M. rufogriseus* 3q (G. Dawson, p.c) and this appeared a likely gene mapping candidate for the present study.

Lactate dehydrogenase (LDH)

PG19 LDH-A_4 has a much faster electrophoretic mobility than *M. rufogriseus* LDH-A_4 using the method set out in Section 3.8.5. LDH-A is the prevalent LDH subunit in *M. rufogriseus* fibroblasts and PG19. There are small amounts of LDH-B represented in the fainter, faster LDH-A/LDH-B hybrid bands in both *M. rufogriseus* and PG19 (Fig. 4.38). The mixture of *M. rufogriseus* and PG19 cell extracts demonstrates all the bands present in these two controls. If REP3-2 and REP3-7 expressed the *M. rufogriseus* LDH-A subunit as well as the PG19 LDH-A subunit,

FIG. 4.36 - Electrophoresis of PGK on cellogel.

Channel

1 - REP3-2-1

2 - PG19

3 - *M. rufogriseus* (fibroblasts)

4 - REP3-2-2

5 - REPB3-1

6 - REPB3-2

Channel 2: PG19 mouse PGK-A

Channel 3: *M. rufogriseus* PGK-A with much weaker PGK-B in mouse PGK-A region. The REP hybrid cell lines express both the mouse and marsupial forms of PGK-A (Channels 1,4,5 and 6).

M = mouse mobility

W = red necked wallaby mobility.

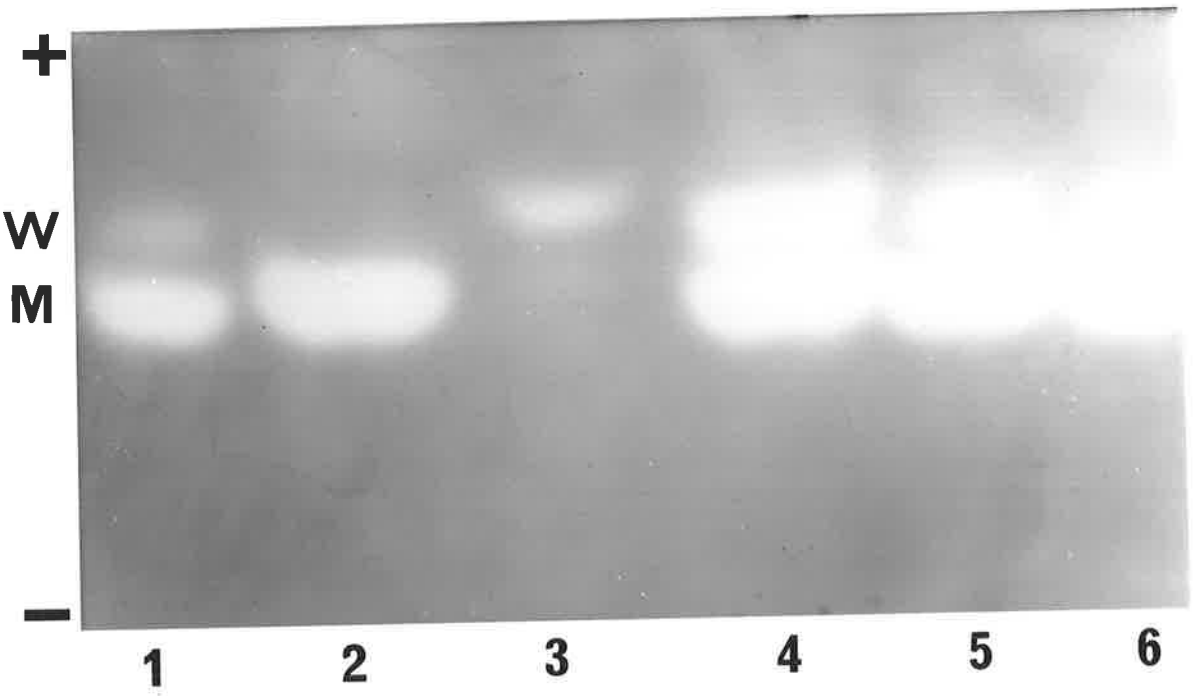

+
WM
−
1
2
3
4
5
6

FIG. 4.37 - A photograph of a gel stained for G6PD.

Channel

1 - *M. rufogriseus* (red blood cells)

2 - REP3-4

3 - REP3-4

4 - REP3-3

5 - REP3-3

6 - *M. rufogriseus* (red blood cells)

7 - PG19

8 - REP3-1

9 - REP3-1

PG19 G6PD is slower than *M. rufogriseus* G6PD (Channel 7). Only the mouse form of G6PD was found in the REP hybrids, REP3-4 (Channels 2 and 3), REP3-3 (Channels 4 and 5) and REP3-1 (Channels 8 and 9). The cathodal band in the *M. rufogriseus* red blood cell extract is haemoglobin.

M = mouse mobility

W = red necked wallaby mobility.

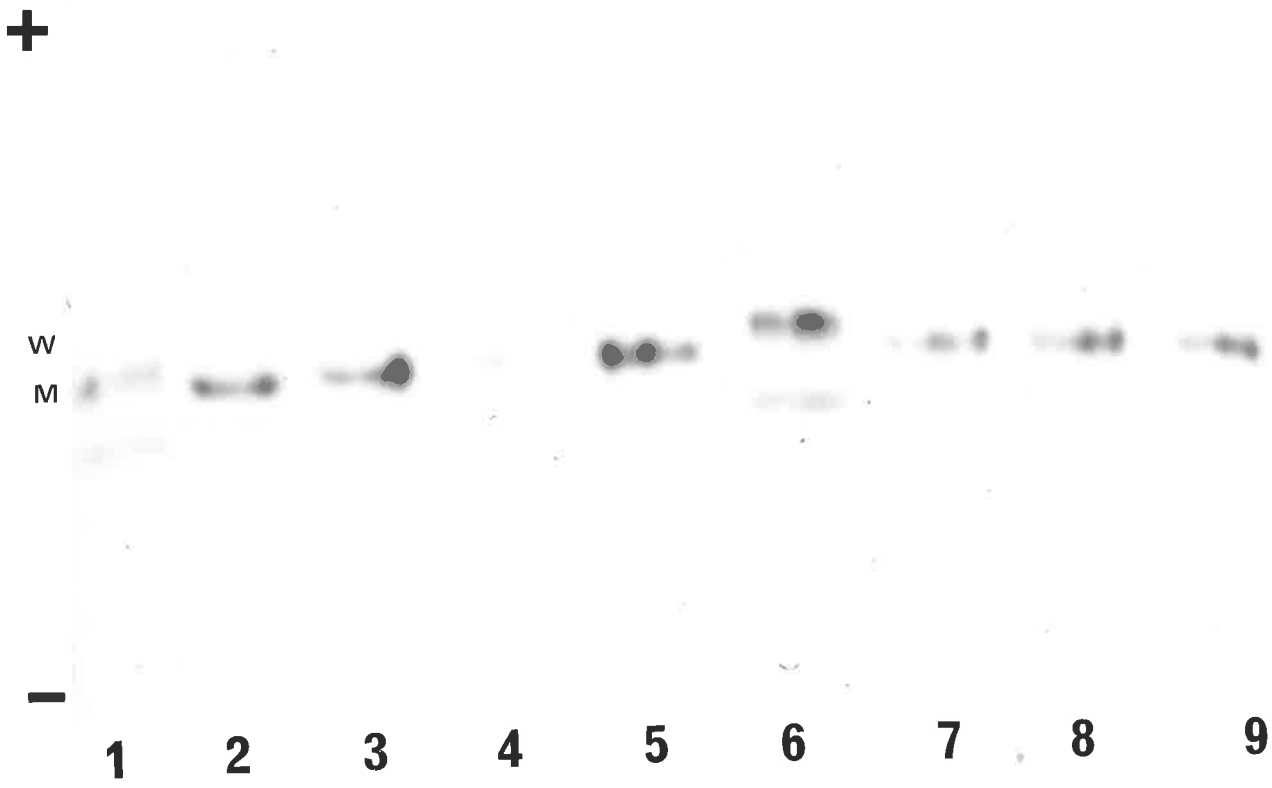
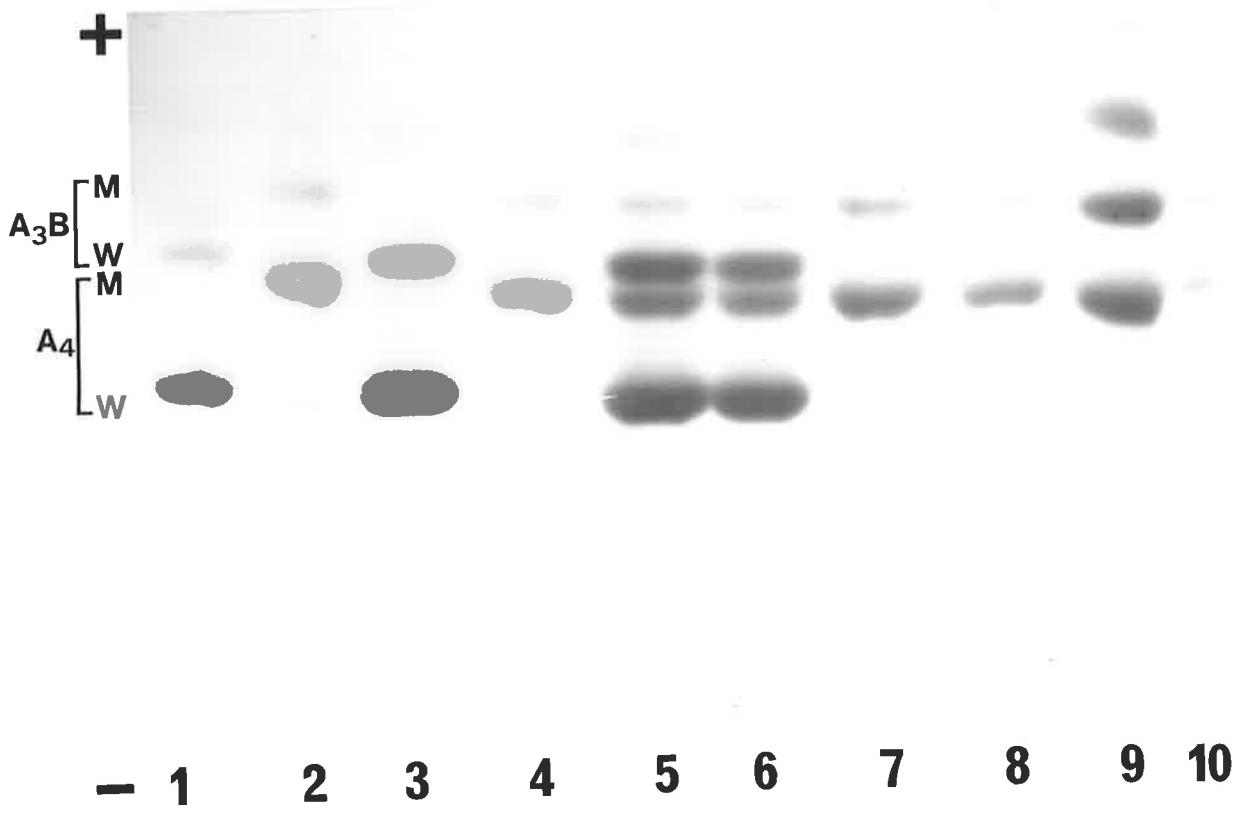

+
W
M
−
1
2
3
4
5
6
7
8
9

FIG. 4.38 - A photograph of a gel stained for LDH. PG19 LDH isozymes are shown in Channels 2 and 4. The slow *M. rufogriseus* LDH isozymes are shown in Channels 1 and 3. Channels 5 and 6 represent a 1:1 mixture of PG19 and *M. rufogriseus* LDH isozymes. The two hybrids REP3-2 (Channels 7 and 8) and REP3-7 (Channel 9) gave the same LDH pattern as PG19.

M = mouse mobility

W = red necked wallaby mobility.


+
M
A3B
W
M
A4
W
–
1
2
3
4
5
6
7
8
9
10

bands of intermediate mobility consisting of hybrid molecules between *M. rufogriseus* LDH-A_4 and PG19 LDH-A_4 would have been expected. These were not observed. (Fig. 4.38).

None of the hybrid clones, subclones or their revertants expressed the *M. rufogriseus* LDH-A gene. (Table 4.2).

Esterase A_4 (EsA_4)

The esterase isozymes consist of a complex series of bands when electrophoresed and stained on 'Cellogel' as described in Section 3.8.7 . It was not possible to identify which esterase band in the *M. rufogriseus* or PG19 samples constituted the EsA_4 isozymes. In human systems the EsA_4 isozymes move cathodally at the pH's used in Section 3.8.7. No *M. rufogriseus* or PG19 esterase isozyme moved cathodally at this pH. Also, there were no esterase bands in the somatic cell hybrids corresponding to any of the esterase bands in the *M. rufogriseus* control liver extract.

Phosphoglucomutase-3

The PGM-3 and PGM-2 isozymes could only be detected as very weakly staining bands using the cellogel method set out in Section 3.8.8 and could not be reliably identified in the REP hybrids tested. PGM-1 was identified in the REP hybrids on the basis of its intensely stained band compared with other PGM bands in the extracts. The *M. rufogriseus* and PG19 PGM-1 isozymes had very similar electrophoretic mobilities and would not be distinguishable in the hybrid cells.

Purine nucleoside phosphorylase (NP)

As mouse and *M. rufogriseus* NP have very similar electrophoretic mobilities it was not possible to separate the two forms sufficiently to enable detection of marsupial NP should it exist, in the REP hybrids.

4.4 ANTIGENS

4.4.1 Antigens Detected Using Conventional Techniques

SREP3 is blood serum derived from C57BL/6J mice immunised with REP3 hybrid cells. Using an indirect immunofluorescence assay, SREP3 bound to the surface of REP3 cells (+++ reaction) and also to PG19 and *M. rufogriseus* (fibroblasts). The non-immune control serum from C57BL/6J mice, NIS-C57, did not react with any of these cell types. These results are summarised in Table 4.3.

TABLE 4.3: Reaction of SREP3 with REP3 and REP3 Parental Species

	CELLS		
	PG19	*M. RUFOGRISEUS* ♀ Fibroblasts	REP3
ANTISERA			
SREP3	+++	+++	+++
NIS-C57	+/-	+/-	+/-

SREP3 was adsorbed twice with PG19 cells to remove the reaction of SREP3 against tumour antigens present on these cells. The resulting antiserum was assigned the nomenclature SREP3 AD PG19. The adsorbed serum no longer reacted with PG19 but still reacted with both *M. rufogriseus* (♀ fibroblasts) and approximately 20% of REP3 cells. The reaction with REP3 cells (++ → +++) was weaker than reaction with *M. rufogriseus* cells (Table 4.4).

TABLE 4.4: Reaction of SREP3 AD PG19 with REP3 and REP3 Parental Species

	CELLS		
	PG19	*M. RUFOGRISEUS* ♀ Fibroblasts	REP3
SREP3 AD PG19	+/-	+++	++ → +++
SERA			
NIS-C57	+/-	+/-	+/-

The fluorescence scores for all the REP hybrid cell lines which were tested with SREP3 AD PG19, are set out in Table 4.5.

Attempts to induce an antiserum in C57BL/6J mice against PG19 cells were unsuccessful.

4.4.2 Antigens Detected Using Monoclonal Antibodies

4.4.2.1 GA-1

The results of the five NS1 x C57BL/6J mouse spleen fusions in which the mouse had been immunised with REP3 cells are shown in Table 4.6. All 70 hybridoma supernatants were screened using an indirect immunofluorescence assay. The one positive hybridoma colony secreting antibody to a cell surface antigen on 10-20% of REP3 cells was called GA-1. (Genetics, Adelaide-1).

GA-1 was subcloned by limiting dilution approximately two weeks after it was first identified. This subcloning was carried out to select a cloned line of secreting cells from the possible mixture of secreting and non-secreting cells that may have existed in the

TABLE 4.5: REACTION OF SREP3 ADPG19 WITH REP CELL LINES

CELL LINE	REACTION WITH SREP3 AD PG19	CELL LINE	REACTION WITH SREP3 AD PG19†
PRIMARY REP HYBRIDS		BULK HYBRIDS	
REP1	+/- → +	REP B1	+/-
REP2	+/-	REP B2	+ → +/-
REP3	++ → +++	REP B3	+++
REP4	+ → ++	REP B4	+/-
REP3 SUBCLONES		REP B3 SUBCLONES	
REP3-1	++	REPB3-1	++ → +++
REP3-2	+ → ++	REPB3-2	+++
REP3-2-1	++	REPB3-3	+++
REP3-2-2	++ → +++	REPB3-4	+++
REP3-3	+/-	REPB3-5	++ → +++
REP3-4	+ → ++		
REP3-5	+/-	REP3 REVERTANTS	
REP3-6	+/-	REP3R1	+/-
REP3-7	+++	REP3R2	+/-
REP3-7-1	++	REP3R3	+/-
REP3-7-2	+++	REP3R4	+/-
REP3-7-3	++	REP3R5	+/-
REP3-7-4	+++	REP3R6	+/-
REP3-7-5	+ → ++	REP3R7	+/-
		REP3R8	+/-

†Where GA-1 bound to the hybrids, at least 75% of the cells fluoresced.

TABLE 4.6: Myeloma x Spleen (REP3 Primed) Fusions

FUSION	HYBRIDOMAS SCREENED	HYBRIDOMAS SECRETING ANTIBODY TO REP3 CELLS	
1	5	0	
2	0	0	
3	34	0	
4	12	0	
5	19	1	
5	70	1	TOTAL

original colony. Twenty-nine subclones were isolated and their supernatants screened for reaction with REP3 cells using an indirect immunofluorescence assay (Table 4.7).

TABLE 4.7: Immunofluorescence Scores of GA-1 Subclones

	NUMBER OF SUBCLONE SUPERNATANTS SCREENED	REACTION WITH REP3 CELLS
	14	++ → +++
	10	+ → ++
	4	+/- → +
	1	+/-
TOTAL	29	

All but one of the GA-1 subclone supernatants gave a brighter fluorescence score with REP3 cells than did the NS1 culture supernatant control (+/-). Therefore 28/29 of the culture supernatants secreted antibody which bound to REP3 cells. The extent

of reaction at such an early stage of screening is also dependent on the size of the colony when screened. Obviously colonies were screened when they had reached a standard size but this was difficult to control in a 96 well plate with rapidly growing cell lines.

4.4.2.2 HYP1 and PGMR2-4

None of the REP, PGRN or 1RRN cell hybrids contained a normal *M. rufogriseus* X chromosome in the majority of cells. For this reason, two other somatic cell hybrids, HYP1 and PGMR2-4 were used as immunogens in myeloma x spleen cell fusions in the search for a monoclonal antibody to a marsupial X-linked antigen. HYP1 is a somatic cell hybrid between lymphocytes from an *M. rufogriseus* x *Wallabia bicolor* hybrid animal, and PG19 (R.M. Hope, unpublished). All HYP1 cells contained at least the short arm of the *M. rufogriseus* X chromosome. PGMR2-4 is an *M. rufus* x PG19 somatic cell hybrid with the majority of cells containing a normal *M. rufus* X chromosome.

Three myeloma x spleen cell fusions were carried out where HYP1 cells were used as immunogen. All 36 hybridoma supernatants resulting from these fusions proved to be negative when tested against HYP1 cells using an indirect immunofluorescence assay. Likewise supernatants from 34 resulting hybrid colonies from two myeloma x spleen cell fusions with PGMR2-4 cells as immunogens, proved to be negative on PGMR2-4 cells.

4.5 SPECIFICITY OF THE MONOCLONAL ANTIBODY, GA-1

4.5.1 Reactions of Immunizing Hybrid Cells and Parental Cells with GA-1 .

GA-1 bound (+++) to 100% of *M. rufogriseus* (♀) fibroblasts, approximately 30% of *M. rufogriseus* (♂) lymphocytes and approximately

20% of REP3 cells. It did not show any reaction with PG19 (Table 4.8).. None of these cell types reacted with NS1 supernatant which was used as a control in all immunofluorescent assays.

TABLE 4.8: REACTION OF GA-1 WITH REP3, PG19 AND *M. RUFOGRISEUS*

CELL LINE	REACTION WITH GA-1	% CELLS REACTING
REP3	++ → +++	20
PG19	+/-	0
M. rufogriseus (♀ Fibroblasts)	+++	100
M. rufogriseus (♂ Lymphocytes)	++ → +++	30

Some lymphocytes and other peripheral blood cells have Fc receptors on their cell surface which may bind to the Fc region of immunoglobulin molecules and therefore interfere with the interpretation of indirect immunofluorescence assays (reviewed in Dickler, 1976). This was revealed by a +++ reaction of the 'negative' control i.e. when *M. rufogriseus* lymphocytes were tested with NS1 supernatant followed by fluorescein conjugate (whole molecule) (Table 4.9). When *M. rufogriseus* fibroblasts were used in the negative control a +/- fluorescence score was observed. Hence, when testing lymphocytes for reaction with GA-1 it was necessary to use a fluorescein conjugated $(Fab')_2$ fragment of sheep anti-mouse IgG, as second antibody. The fluorescein conjugate (whole molecule) showed no non-specific binding with any cell types tested other than lymphocytes.

TABLE 4.9 : NON-SPECIFIC BINDING OF FLUORESCEIN CONJUGATE (WHOLE MOLECULE) WITH LYMPHOCYTES

CELLS	ANTIBODY	FLUORESCEIN CONJUGATE	
		WHOLE MOLECULE	$(Fab')_2$ FRAGMENT
M. rufogriseus Fibroblasts	GA-1	+++	+++
M. rufogriseus Fibroblasts	NS1 Supernatant	+/-	+/-
M. rufogriseus Lymphocytes	GA-1	++ → +++	++ → +++
M. rufogriseus Lymphocytes	NS1 Supernatant	++ → +++	+/-

4.5.2 Reactions of REP Cell Hybrids and Revertants with GA-1

REP3 was the only primary cell hybrid in which a percentage of cells reacted with GA-1. There was a weak reaction with REP4 (+→+++). REP1 and REP2 did not react at all (Table 4.10).

TABLE 4.10: REACTION OF GA-1 WITH THE PRIMARY REP HYBRIDS

CELL LINE	REACTION WITH GA-1	% CELLS REACTING
REP1	+/-	0
REP2	+/-	0
REP3	++ → +++	20%
REP4	+ → ++	30%

The results of reaction of GA-1 with the REP3 subclones are given in Table 4.11. A large percentage of cells in REP3-1, REP3-2,

TABLE 4.11: REACTION OF GA-1 WITH REP3 SUBCLONES AND REVERTANTS

CELL LINE	REACTION WITH GA-1	% CELLS REACTING
REP3-1	+++	100
REP3-2	++ → +++	90
REP3-2-1	+++	95
REP3-2-2	+++	100
REP3-3	+/-	0
REP3-4	++ → +++	90
REP3-5	+/-	0
REP3-6	+++	25
REP3-7	+++	100
REP3-7-1	+	90
REP3-7-2	+++	100
REP3-7-3	+/-	0
REP3-7-4	+++	100
REP3-7-5	+++	100
REP3 R1	+/-	0
REP3 R2	+/-	0
REP3 R3	+/-	0
REP3 R4	+/-	0
REP3 R5	+/-	0
REP3 R6	+/-	0
REP3 R7	+/-	0

REP3-4 and REP3-7 reacted with GA-1. Only 25% of REP3-6 cells reacted with GA-1 but the reaction on those 25% of cells was strong (i.e. +++). REP3-3 and REP3-5 gave only background autofluorescence. REP3-2 and REP3-7 were subcloned and the reactions of these subclones with GA-1 are also listed in Table 4.13. The REP3-2 subclones reacted with GA-1 as did the REP3-7 subclones with the one exception of REP3-7-3 which did not react at all.

No reaction with GA-1 was observed for the REP3 revertants (Table 4.11).

Of the bulk REP hybrids only REPB3 reacted with GA-1. REP B3 was subsequently subcloned and the reactions of the REPB3 subclones are given in Table 4.12. All REPB3 subclones reacted (+++) with nearly 100% of cells reacting in each case.

TABLE 4.12: REACTION OF GA-1 WITH BULK REP CELL HYBRIDS

CELL LINE	REACTION WITH GA-1	% CELLS REACTING
REPB1	+/-	0
REPB2	+/-	0
REPB3	++ → +++	60
REPB3-1	+++	100
REPB3-2	+++	90
REPB3-3	+++	100
REPB3-4	+++	95
REPB3-5	+++	95
REPB4	+/-	0
REPB5	+/-	0

Table 4.13 summarises the cytological and immunological data for the REP hybrids.

4.5.3 Reactions of GA-1 with Other Cell Types

Marsupial Fibroblasts

Table 4.14 lists the reaction of GA-1 with fibroblasts of a variety of marsupial species ranging across the marsupial families. Only species in the Macropodidae and Tarsipedidae groups reacted. All other species listed gave only background autofluorescence.

M. rufus x Mouse Hybrids

Donald and Hope (1981) have made and analysed a series of *M. rufus* (red kangaroo) x 1R hybrids, called the 1RMR hybrids. A number of these were tested for reaction with GA-1. An *M. rufus* x PG19 hybrid PGMR2-4 (Sykes and Hope, 1978) was included in the analysis.

Table 4.15 summarises the chromosomal content of these hybrids along with their reaction with GA-1. GA-1 reacted with 100% of *M. rufus* fibroblasts but not with the mouse cell line 1R. It did react with two *M. rufus* x 1R Somatic cell hybrids, 1RMR-1 and 1RMR1-R5-A5 but not with 1RMR2-1 or the *M. rufus* x PG19 hybrid, PGMR2-4.

The data in Table 4.15 has been condensed into a 2×2 table (Table 4.16). From Table 4.16 it can be seen that reaction with GA-1 is dependent on the presence in the *M. rufus* x mouse hybrids of the *M. rufus* chromosome 5.

TABLE 4.13

	APPROXIMATE PERCENTAGE OF CELLS CONTAINING *M. RUFOGRISEUS* CHROMOSOMES								BINDING OF GA-1	
	t(3q,Xq)	Xq	X	3	6	7	Xp C	Xp G		
REP3	27%	-	-	18%	18%	9%	-	43%	++→+++	30%
REP3-1	-	-	-	80%	-	-	60%	87%	+++	90%
REP3-2	75%	-	-	-	-	12%	-	65%	++→+++	90%
REP3-2-1	100%	-	-	-	-	-	-	66%	+++	95%
REP3-2-2	-	-	-	-	-	-	-	43%	+++	100%
REP3-3	-	-	-	-	-	-	-	25%	+/-	-
REP3-4	t(3q,?)55%	-	-	-	45%	-	-	83%	++→+++	90%
REP3-5	-	-	-	-	-	-	-	71%	+/-	-
REP3-6	-	-	-	-	-	-	85%	88%	+++	25%
REP3-7	-	-	-	i3q50%	36%	-	-	60%	+++	100%
REP3-7-1	-	-	-	89%	-	-	-	75%	+	90%
REP3-7-2*	-	-	-	75%	-	-	-	ND	+++	100%
REP3-7-3*	-	-	-	-	-	-	-	ND	+/-	-
REP3-7-4	-	-	-	3q66%	-	-	-	80%	+++	100%
REP3-7-5*	-	-	-	-	-	-	-	ND	+++	100%
REP3R1	-	-	-	-	-	-	-	27%	+/-	-
REP3R2	-	-	-	-	-	-	-	25%	+/-	-
REP3R3	-	-	-	-	-	-	-	18%	+/-	-
REP3R4-REP3R8	-	-	-	-	-	-	-	ND	+/-	-
REPB3†	-	-	-	-	-	-	-	-	++→+++	60%
REPB3-1	-	-	-	90%	-	-	-	54%	+++	100%
REPB3-2*	-	-	-	66%	-	-	-	ND	+++	90%
REPB3-3	-	-	X	50%	-	-	-	43%	+++	100%
REPB3-4	-	Xq91%	-	70%	-	-	-	64%	+++	95%
REPB3-5	-	-	-	3q50%	95%	-	-	63%	+++	95%
REPB4	-	-	-	-	67%	-	-	33%	+/-	-
REPB5	-	-	-	-	36%	-	-	29%	+/-	-
REP1*	-	-	-	-	-	-	-	ND	+/-	-
REP2*	-	-	-	-	-	-	-	ND	+/-	-
REP4*	-	-	-	-	-	-	-	ND	+/-	-
REPB1	-	-	-	-	-	-	-	43%	+/-	-
REPB2	-	-	-	-	13%	-	-	38%	+/-	-
1RRN-2*	-	-	-	-	-	-	100%	ND	+/-	-

*These cell lines were only C-banded.

†REPB3 was neither C- nor G-banded.

TABLE 4.14 : REACTION OF GA-1 WITH MARSUPIAL FIBROBLASTS

MARSUPIAL DIPLOID FIBROBLASTS	COMMON NAME AND SEX	REACTION WITH GA-1
ORDER DIPROTODONTA		
Macropodidae		
Aepyprymnus rufescens	Rat Kangaroo (♂)	+++
Macropus rufogriseus	Red Necked or Bennetts Wallaby (♀)	+++
Macropus rufus	Red Kangaroo (♂)	+++
Macropus irma	Western Bush Wallaby (♂)	+++
Macropus parryi	Whip-Tail or Pretty-Face Wallaby (♀)	+++
Macropus eugenii	Tammar Wallaby (♀)	+++
Dendrolagus matschiei	Tree Kangaroo (♀)	+++
Macropus agilis x *M. rufogriseus*	Agile Wallaby x Red-Necked Wallaby (♀)	+++
Macropus dorsalis x *Wallabia bicolor*	Black Striped Wallaby x Swamp Wallaby (♂)	+++
Tarsipedidae		
Tarsipes spencerae	Honey Possum (♀)	+++
Vombatidae		
Vombatus ursinus	Common Wombat (♂)	+/-
Phascolarctidae		
Phascolarctos cinereus	Koala (♂)	+/-
ORDER POLYPROTODONTA		
Didelphidae		
Caluromys lanatus	Wooly Opossum (♀)	+/-
Dasyuridae		
Antechinus rosamonde	Little Red Antechinus (♀)	+/-
Sminthopsis crassicaudata	Fat-Tailed Dunnart (♀)	+/-
Peramelidae		
Parameles gunnii	Bandicoot (♀)	+/-

TABLE 4.15: REACTION OF GA-1 WITH *M. RUFUS* x MOUSE SOMATIC CELL HYBRIDS

CELLS	*M. RUFUS* CHROMOSOMES PRESENT IN HYBRID CELLS X	5	REACTION WITH GA-1	% CELLS REACTING
M. rufus Fibroblasts	+	+	+++	100
1R	-	-	+/-	0
1RMR1	+	+	++ → +++	100
1RMR1R5A5	-	+	++ → +++	100
1RMR2-1	-	-	+/-	0
PGMR2-4	+	-	+/-	0

TABLE 4.16: REACTION OF GA-1 WITH 1RMR & PGMR HYBRIDS ON BASIS OF CHROMOSOME CONTENT

		M. RUFUS X CHROMOSOME	
		+	-
M. RUFUS CHROMOSOME 5	+	++ → +++	++ → +++
	-	+/-	+/-

PGRN and 1RRN Hybrids

No reaction with GA-1 was observed for any of the PGRN, 1RRN, or WT1R hybrids (Table 4.17).

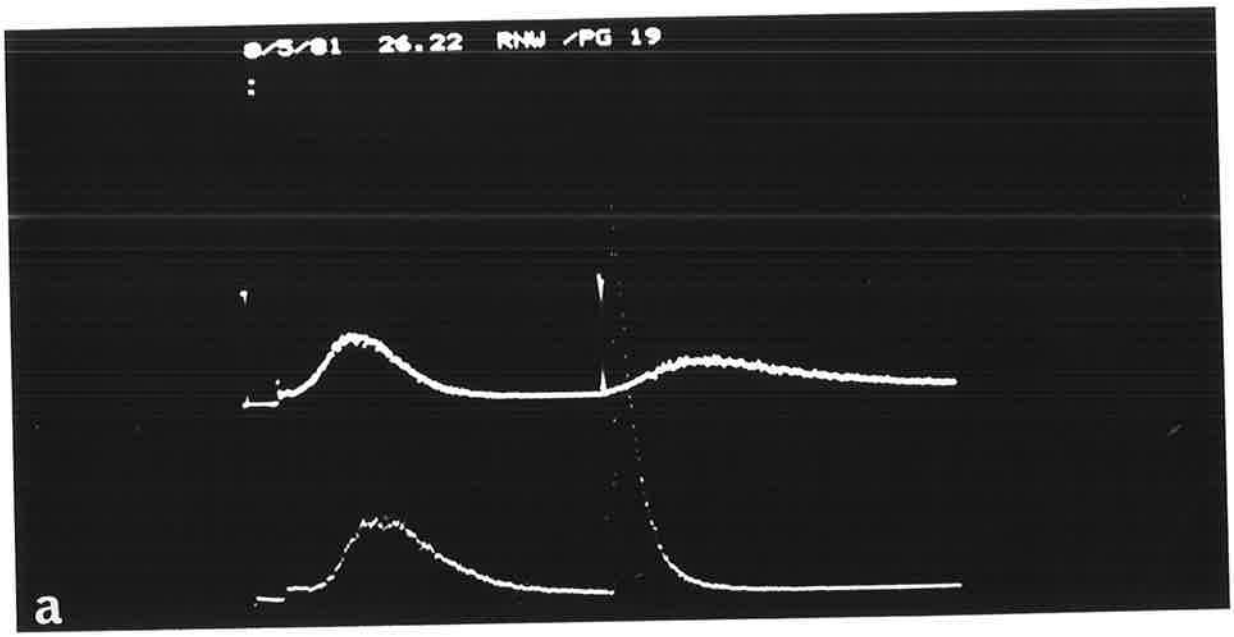
Fig. 4.39 - Forward light scatter (left) and individual fluorescence intensity distributions (right) of

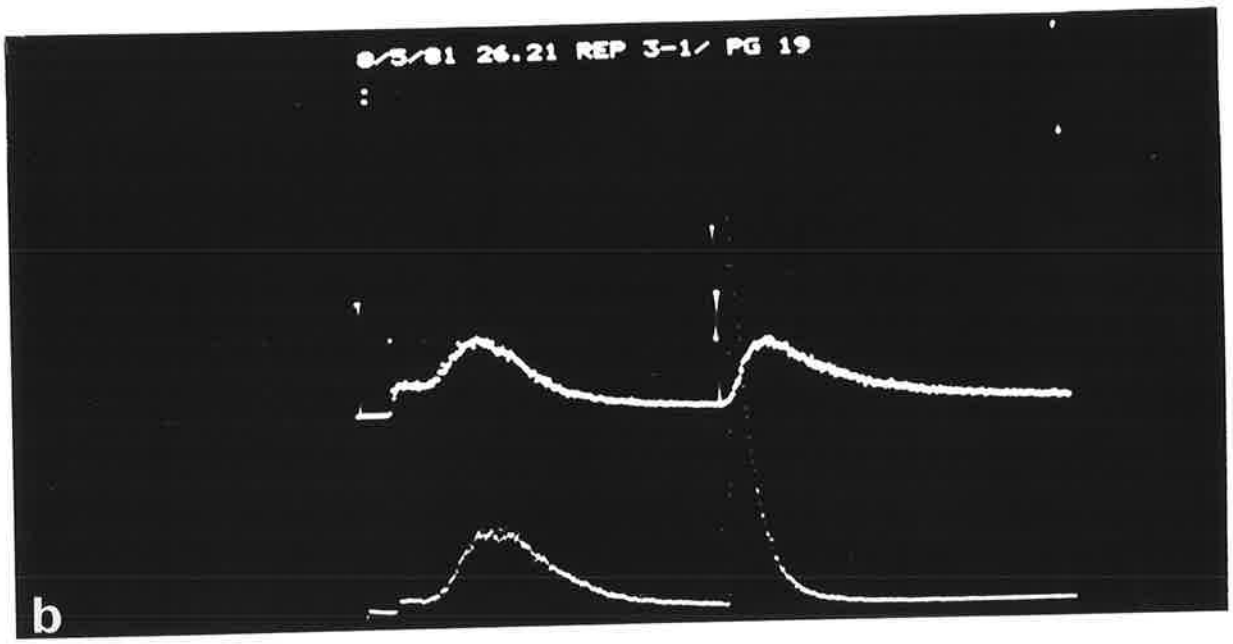
(a) *M. rufogriseus* fibroblasts

(b) REP3-1 cells

(c) REP3-2 cells

as measured by a fluorescence activated cell sorter. Each sample is compared with the size and fluorescence distribution of the PG19 negative control sample which is the lower set of distributions.


8/3/81 26.22 RNW /PG 19
a


8/3/81 26.21 REP 3-1/ PG 19
b

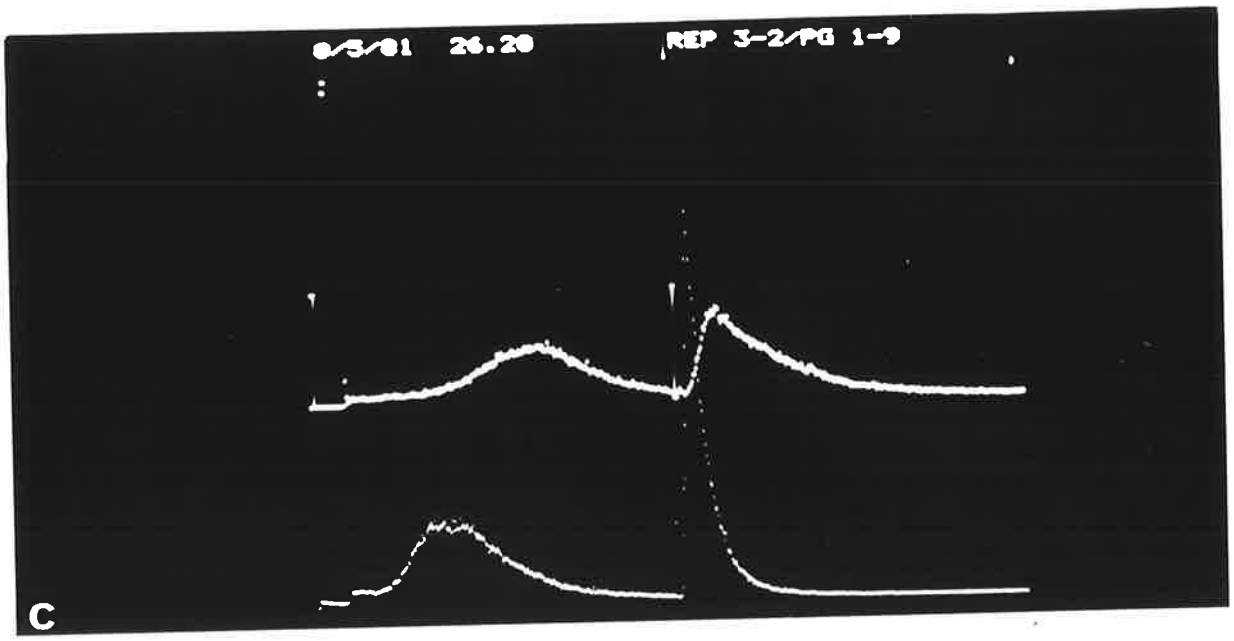

8/3/81 26.20 REP 3-2/PG 1-9
c

TABLE 4.17: REACTION OF GA-1 WITH PGRN, 1RRN AND WT1R CELL HYBRIDS

CELL LINE	REACTION WITH GA-1
PGRN-1	+/-
PGRN-2	+/-
PGRN-3	+/-
1RRN-1	+/-
1RRN-2	+/-
WT1R	+/-

Table 4.18 lists a collection of marsupial and eutherian cell lines tested for reaction with GA-1. Only the *Potorous tridactylus* cell lines reacted. *Potorous tridactylus* is a marsupial, a member of the Macropodidae.

4.5.4 Fluorescence Activated Cell Sorter

Following indirect immunofluorescence labelling with GA-1 and fluorescein conjugate, PG19 cells, *M. rufogriseus* fibroblasts and cells of two REP3 subclones, REP3-1 and REP3-2 were analysed using a fluorescence activated cell sorter. A Becton Dickenson FACS IV was used in this study to measure (1) forward light scatter and (2) the individual fluorescence intensity of each cell. The results of the FACS IV analysis are given in Fig. 4.39 as frequency histograms.

The frequency histogram on the left in each figure is the size distribution of the cells determined by forward light scatter. The amount of forward light scatter is directly proportional to cell size (Horan and Wheeless, 1977). The frequency histogram on the

TABLE 4.18 : REACTION OF VARIOUS MARSUPIAL AND EUTHERIAN CELL LINES WITH GA-1

CELL LINE	DESCRIPTION	REACTION WITH GA-1
LNSV	Human SV40 transformed fibroblasts ($HPRT^-$)[#]	+/-
MAR	Human (♂) fibroblasts*	+/-
PtI2	*Potorous tridactylus* fibroblasts	+++
PtK1	*Potorous tridactylus*	+++
PtK2	*Potorous tridactylus* kidney (aneuploid)	+++
Sc9/01	*Sminthopsis crassicaudata*[×] (♀) fibroblasts, pseudo-diploid	+/-
Chicken	Chicken fibroblasts	+/-
Sheep	Sheep fibroblasts	+/-
PGH/1	Human × PG19 cell hybrid[†]	+/-
R1P-6 & R1P-4	*Pseudocheirus peregrinus*[†] × PG19 cell hybrids	+/-
HYP1	(*M. rufogriseus* x *Wallabia bicolor*) x PG19 cell hybrid	+/-

[#]Croce *et al* (1973)

*Miggiano *et al* (1969)

[×]Ford *et al* (1978)

[†]Hope, unpublished.

right in each figure is the fluorescence intensity distribution of the same sample of cells. Each sample is compared with the size and fluorescence distributions of the PG19 negative control sample. An arbitrary background fluorescence cut off point, as determined by the fluorescence intensity of PG19 cells, is shown as a vertical line in each figure.

As can be seen in Fig. 4.39(a) nearly all the PG19 cells exhibited low levels of fluorescence relative to the fluorescence distribution of *M. rufogriseus* fibroblasts. That is, virtually all *M. rufogriseus* fibroblasts were reacting with GA-1.

Overall, neither REP3-1 nor REP3-2 cells fluoresced as intensely as *M. rufogriseus* fibroblasts but they had a much greater intensity of fluorescence than PG19 cells. Although covering a similar range of fluorescence intensities to *M. rufogriseus* fibroblasts, REP3-1 and REP3-2 had a higher proportion of cells expressing a less intense fluorescence.

CHAPTER 5

DISCUSSION

5.1 THE HYBRIDS AND THEIR CHROMOSOMES

The putative marsupial x mouse hybrids, which were selected using the HAT selection system (Littlefield, 1964) would be expected to express the marsupial HPRT isozyme. Preliminary screening for the presence of this enzyme was carried out to confirm the hybrid status of cells. 1RRN-2 and all the REP and PGRN cell lines, expressed *M. rufogriseus* HPRT. As these lines clearly contained mouse chromosomes and expressed a number of mouse enzymes, they were therefore "true" interspecies hybrids. The 6TG-resistant REP revertants were all $HPRT^-$ as expected.

The 1RRN-1 and WT1R HAT selected lines did not express marsupial HPRT but did express HPRT of mouse electrophoretic mobility. These two cell lines did not contain any identifiable marsupial chromosomes or express marsupial PGK-A, G6PD, LDH-A or GA-1 (Section 4.1 and Table 4.2). It appears, therefore, that these cell lines were not hybrids but were derivatives of 1R mouse cells which were expressing HPRT. Such re-expression of HPRT has been reported previously in a number of studies for $8AG^r$ mouse (Parsons *et al.*, 1976; Van Diggelen *et al*, 1979) and $8AG^r$ hamster (Gillen *et al.*, 1972) cell lines. A number of possible mechanisms have been proposed to account for this phenomenon. These include changes in the HPRT structural gene (Fenwick, 1977) and changes in cell membrane permeability and transport (Morrow *et al.*, 1973). Other possible mechanisms involving mutations at regulatory loci and re-expression of a repressed HPRT gene in the hybrid state are

reviewed in Caskey and Kruh (1979). The extent of reversion of 1R to express HPRT from any one series of interspecies fusions has been observed to vary greatly. (Bakay *et al.*, 1973; Donald, 1980). Without further biochemical investigation it is not possible to determine which mechanism caused "reversion" of 1R in the 1RRN1 and WT1R cell lines.

In order to analyse the chromosomal constitution of the hybrid cells it was necessary to obtain clear C- and G-banded karyotypes of both parental cell types to use as references. Considerable problems were encountered in attempts to obtain a sharply G-banded chromosome preparation of *M. rufogriseus* fibroblasts or lymphocytes due partly to compaction of the G-bands on either side of the large areas of centromeric heterochromatin. This particular problem was also encountered by Rofe (1979) in her attempts to G-band chromosomes from this species. In an attempt to overcome these problems, chromosome preparations were made using the method described by Yunis (1978) which is reported to yield pro-metaphase chromosome preparations of high quality. Although prometaphase chromosomes were obtained using this method, little improvement was observed in the definition and overall quality of G-bands. A similar problem was experienced in clearly differentiating the three interstitial C-bands on the long arm of the *M. rufogriseus* X chromosome in fibroblast and lymphocyte chromosome preparations. (Fig. 4.4a).

The C- and G-banding patterns were much clearer on the *M. rufogriseus* chromosomes in the hybrid cells compared with chromosomes from fibroblast and lymphocyte preparations. For example, in C-banded preparations of hybrid cells, the three

interstitial C-bands on the long arm of the X chromosome were clearly visible in t(3q,Xq) in REP3-2 (Fig. 4.10a) and on the normal X chromosome in REPB3-3 (Fig. 4.18a). The reason for the superior quality of the C and G-bands on marsupial chromosomes in hybrid cells is not known. It may be due, in part, to the fact that in hybrids, the *M. rufogriseus* chromosomes condense at a later stage than the PG19 chromosomes, and hence are less condensed in metaphase spreads. However, this cannot be the complete explanation. Even when the method of Yunis (1978) was employed to obtain prometaphase chromosome preparations of *M. rufogriseus* fibroblasts and lymphocytes, the chromosomes, although longer, appeared thicker and the G-banding blurred compared with the sharply defined *M. rufogriseus* chromosomes and G-bands in the hybrids. Possibly there was some interaction between the mouse and *M. rufogriseus* chromosomes which had enabled this improved G-banding of *M. rufogriseus* chromosomes in the hybrids. There was little, if any difference between the quality of the G-banded PG19 chromosomes in the PG19 cell line with those in the hybrids.

These difficulties in comparing the *M. rufogriseus* chromosomes in the hybrids with their counterparts in diploid fibroblasts and lymphocytes were partly overcome in the following manner. Any one chromosome preparation of the hybrid cell lines exhibited a range of chromosome condensation between metaphase spreads, enabling a progressive comparison of the same G-banded chromosome in different degrees of condensation. As a result of such comparison, confident identification of normal *M. rufogriseus* chromosomes in the hybrids was achieved.

The chromosomal constitutions of all the hybrid and revertant cell lines are described in detail in Section 4.1.2. As has been previously reported for mouse x marsupial cell hybrids, preferential elimination of marsupial chromosomes was observed (Hope and Donald, 1981; Hope and Graves, 1978b). Preliminary C- and G-banding studies on the primary and bulk REP hybrids indicated that these hybrids were chromosomally heterogeneous for both normal and abnormal mouse and marsupial chromosomes. Many cells contained translocated chromosomes (some of which appeared to be marsupial/mouse translocations), dicentrics, isochromosomes and acentric fragments. These cell lines, unless subcloned were too heterogeneous to be of much use for gene mapping studies. Of particular value to the present study would have been hybrids containing single *M. rufogriseus* chromosomes (especially the *M. rufogriseus* X or part thereof) to facilitate the mapping of marsupial genes determining antigenic and enzymic markers. Although a normal X chromosome was not observed in any of the primary or bulk hybrids, REP3 was chosen for more detailed study because it was the only primary hybrid containing a number of *M. rufogriseus* autosomes as well as at least the long arm of the *M. rufogriseus* X chromosome. The REP3 hybrid was subcloned with the aim of deriving a series of cell lines with different karyotypes (including subclones containing the long arm of the X chromosome), each being chromosomally homogeneous. The resulting REP subclones, REP3-1 → REP3-7 were chromosomally more homogeneous than REP3 but still exhibited some variation amongst cells in each subclone (Table 4.1). Two of the more heterogeneous subclones REP3-2 and REP3-7 were further subcloned in order to further partition this chromosome variation (Table 4.1).

REPB3 was the only hybrid other than REP3 to strongly bind the monoclonal antibody, GA-1 (see Section 5.4). This hybrid was subcloned to obtain a series of cell lines containing subsets of the *M. rufogriseus* chromosomes present in REPB3 (Table 4.1). Although a normal *M. rufogriseus* X chromosome was not observed in the REPB3 cells analysed, one subclone, REPB3-3, contained a complete *M. rufogriseus* X chromosome in a proportion of cells. With the exception of REPB3-3, none of the HAT selected hybrid clones or subclones used in the present study contained a normal *M. rufogriseus* X chromosome. However, all the hybrids and subclones analysed did contain a small acrocentric chromosome which on the basis of G-banding was identified as an abnormal *M. rufogriseus* chromosome. This chromosome had a large dark G-band in the centromeric region characteristic of *M. rufogriseus* Xp . (All *M. rufogriseus* autosomes have light G-bands at the centromeric region). *M. rufogriseus* Xp is somewhat similar in size and has a somewhat similar G-banding pattern to some of the smaller PG19 acrocentric chromosomes. Xp could, however, be distinguished in good quality, well differentiated G-banded chromosome preparations. It was not always possible to confirm the identity of the *M. rufogriseus* Xp based on its large centromeric heterochromatic segment due to the inconsistent C-band expression of this region in the hybrid cells (see later this section). *M. rufogriseus* Xp was identified by C-banding in REP3-1 and REP3-6 (Figs. 4.9a and 4.13) and was identified in a similar percentage of cells by G-banding (Table 4.1). *M. rufogriseus* Xp was certainly present in a proportion of all HAT selected hybrid cell lines that were G-banded. Nevertheless, the presence or absence of *M. rufogriseus*

Xp could *not* be scored with absolute certainty in 100% of G-banded metaphase spreads of all cell hybrid lines. About 10% of G-banded metaphase spreads from mouse PG19 cells contained a chromosome which could be misclassified as *M. rufogriseus* Xp. Given that the REP hybrids appeared to contain a double component of mouse PG19 chromosomes (Table 4.1) it would seem that the *maximum* level of misclassification of *M. rufogriseus* Xp in the hybrids would have been about 20%.

From Table 4.1 it can be seen that *M. rufogriseus* Xp was, overall, scored in a much higher percentage of HAT selected hybrid cells than in the 6TG resistant revertant cell lines. It seems highly unlikely that PG19 acrocentric chromosomes were misclassified as *M. rufogriseus* Xp in hybrids where from as high as 43% to 88% of cells were scored as possessing Xp. REP3-3 and the revertant cell lines contained *M. rufogriseus* Xp in percentages of cells similar to the misclassification percentage, and may not, therefore, contain any 'true' *M. rufogriseus* Xp chromosome arms. Although the revertant cell lines had modal chromosome numbers similar to PG19, the range varied in the revertants from numbers near the mode to numbers approximately double the mode. This variation in chromosome number of the revertants may have resulted in a percentage of misclassification intermediate between PG19 (10%) and the REP hybrids (20%).

The G11 technique has been used to identify human chromosomal fragments in human x rodent cell lines (Friend *et al.*, 1976; 1976a). This method would have been of value in identifying the Xp chromosome arm from the smaller mouse autosomes as well as other

M. rufogriseus chromosome fragments that may have been present in the hybrids. G11 staining was carried out at the ICRF, (London) by R. Hope (p.c.) on a number of REP hybrids known to contain *M. rufogriseus* chromosomes. Unfortunately, no differential colour staining between the mouse and marsupial chromosomes was observed. The lack of colour differentiation was not due to unsuccessful utilization of the G11 technique as human x mouse cell hybrids were G11 stained at the same time as the marsupial x mouse hybrids and clear colour differentiation between mouse and human chromosomes was achieved. As human chromosomes G11 stain differently to mouse, it will be of interest to see if they also stain differently to marsupial chromosomes in human x marsupial cell hybrids enabling identification of marsupial chromosome fragments. No human x marsupial cell hybrids have yet been isolated, despite numerous attempts to make them (Hope, p.c.)

On the basis of Ohno's theory of conservation of the gene content of the mammalian X chromosome, the HPRT gene would be expected to be X-linked in *M. rufogriseus* (discussed in Section 5.3) and therefore, selection of the hybrid clones and subclones in HAT medium would effectively result in simultaneous selection for the marsupial X chromosome, or at least that part of it containing the *Hpt* locus. Using similar methods for the selection of hybrids to that used in the present study, the presence of a normal *M. rufus* X chromosome *has* been observed in a number of *M. rufus* x 1R cell hybrids (Donald and Hope, 1980) and in an *M. rufus* x PG19 cell hybrid (Sykes and Hope, 1978). However, attempts to make wallaroo x eutherian cell hybrids containing the normal wallaroo X chromosome

(Graves *et al.*, 1979), and attempts to obtain normal marsupial autosomes in somatic cell hybrids (B. Wainwright, p.c.) have been unsuccessful. Hope and Graves (1978b) observed extensive chromosome fragmentation during the first week after marsupial x mouse cell fusions. This may be the reason for the difficulty encountered in obtaining a normal *M. rufogriseus* X chromosome in the *M. rufogriseus* x mouse hybrids. Perhaps the X chromosome was more susceptible to fragmentation than, for example, the normal *M. rufogriseus* chromosome 6 which was found in a number of the cell hybrids. The extent of fragmentation of the marsupial chromosomes in marsupial x mouse hybrids may also depend on the origin of the mouse parent. Human x mouse hybrids made with PG19 are known to contain human chromosomes exhibiting extensive re-arrangement (Hope, p.c.). However, PG19 yields higher numbers of hybrids with marsupials compared with other rodent cell lines. Hope and Graves (1978b) obtained *Pseudocheirus peregrinus* (ring-tail possum) x PG19 cell hybrids containing as many as ten *P. peregrinus* chromosomes in contrast to studies where only a few or no marsupial chromosomes have been found in hybrids with Macropodid species. Like *M. rufogriseus* chromosomes *P. peregrinus* chromosomes possess extensive regions of centromeric C-band material. Hope and Graves (1978b)suggested that there may be a tendency for the hybrids to retain marsupial heterochromatin. If this is the case then the retention of heterochromatic DNA may have been limited to the *M. rufogriseus* autosomes in the *M. rufogriseus* x mouse hybrids in the present study. The choice of *M. rufogriseus* as the marsupial parent of the hybrids was based on the belief that the large regions of conspicuous C-banding material in the chromosomes of this species would considerably aid in their identification. However, the C-banding

regions of G-band identified *M. rufogriseus* chromosomes were sometimes abnormal or even absent. This was particularly the case for *M. rufogriseus* chromosomes 3 (Figs. 4.9 and 4.17) Xq (Fig. 4.11) and Xp (see earlier this section). On the basis of chromosome size and G-banding, it appears that the C-band material of these chromosomes was, in fact, physically present but remained un-expressed cytologically. On the other hand, the C-banding patterns of the *M. rufogriseus* chromosomes 6 and 7 remained unaltered in all cell lines in which they were present. Therefore, this phenomenon of variable C-band expression in the hybrids was not common to all *M. rufogriseus* chromosomes. No change in C-band pattern was observed for any of the PG19 chromosomes.

The reason for the C-band variation of some *M. rufogriseus* chromosomes is not clear. It is possible that C-band expression on rearranged marsupial chromosomes in the hybrids was influenced by position affects associated with translocation in the case of t(3q,Xq) and chromosome fission for Xp and Xq. C-band variation of t(3q,Xq) in REP3-2 and REP3-2-1 is discussed in detail in the next section (Section 5.2). There is already some indication from the present study that placing marsupial chromosomes in the abnormal environment of a marsupial x mouse hybrid cell influences marsupial chromosomes (see earlier about improvement in banding patterns).

The *M. rufogriseus* 3q and Xp were the only chromosome arms found in common in most of the REP hybrid cells. The only other *M. rufogriseus* chromosomes identified in the hybrids were the numbers 6 and 7.

From Table 4.1 it can be seen that *M. rufogriseus* 3q was observed in a larger number of hybrids than would be expected on

the basis of random chromosome loss. The phenomenon of non-random chromosome loss in interspecies somatic cell hybrids has previously been observed in a number of studies. Both non-random retention of individual chromosomes (Norum and Migeon, 1974; Croce *et al.*, 1973) and combinations of chromosomes (Rushton, 1976) have been found. Of particular interest to the present study is the non-random retention of the *M. rufus* chromosome 5 in HAT selected *M. rufus* x mouse cell hybrids observed by Donald (1980). The G-band equivalent chromosome to the *M. rufus* 5 is the 3q arm in *M. rufogriseus* (Rofe, 1978). It would appear that these homologous G-banded chromosomes confer some selective advantage on the growth *in vitro* of the marsupial x mouse hybrids in HAT medium. Perhaps the HAT selection system, in addition to selecting for HPRT expression, indirectly selects for some gene or genes on these chromosomes. The fact that no marsupial chromosomes were observed in the REP revertants, supports this idea as selection against the marsupial HPRT gene was accompanied by the loss of *all M. rufogriseus* chromosomes including 3q.

5.2 COMPARISON OF CHROMOSOME PROPERTIES IN HYBRIDS REP3-2 AND REP3-2-1

A very interesting observation was made when the hybrid cell lines, REP3-2 and REP3-2-1, were examined cytologically. REP3-2 is a subclone of the primary *M. rufogriseus* x mouse somatic cell hybrid REP3, and REP3-2-1 is, in turn, a subclone of REP3-2. Both of these cell lines express the marsupial genes for HPRT, PGK-A and GA-1.

Using a general chromosome staining procedure, a submetacentric chromosome, easily distinguishable from the mouse chromosomes by its large size, could be observed in both REP3-2 and REP3-2-1. This chromosome was identified by G-banding in both cell lines as a Robertsonian translocation of the long arm of the *M. rufogriseus* X chromosome and the long arm of the *M. rufogriseus* chromosome three, i.e. t(3q,Xq) (Fig. 4.6). The C-banding pattern, however, of t(3q,Xq) differed markedly in the two cell lines. In REP3-2, this chromosome possessed a large centromeric C-banded region (similar to the C-banding regions seen on normal *M. rufogriseus* chromosomes) and the Xq arm of this chromosome showed three interstitial C-bands (Fig. 4.10a) (similar to the C-banding regions seen on Xq of a normal *M. rufogriseus* X chromosome). In REP3-2-1, however, this chromosome contained only a small amount of centromeric C-banding, comparable to the C-bands in the mouse chromosomes (Fig. 4.11a) and the interstitial C-bands were not observed cytologically. Sequential G- and C-banding verified the proposition that these two chromosomes which G-banded identically in the two clones, and hence would normally be considered as the "same" chromosome, gave

very different C-band patterns (Fig. 4.27).

The difference in magnitude of observed C-banding on t(3q,Xq) in the two cell lines predicted a detectable corresponding difference in G-band pattern. The fact that this difference was not observed raised the question of whether C-band material present in REP3-2 had been lost from REP3-2-1, or whether such material was present in both cell lines but not expressed in REP3-2-1.

As REP3-2-1 is a subclone of REP3-2, it is possible that t(3q,Xq) in a proportion of cells in REP3-2 did not possess the large centromeric C-banded pattern, but instead expressed reduced C-banding pattern characteristic of this chromosome in REP3-2-1. However, fifty metaphase spreads of REP3-2 were carefully examined for C-banding pattern of t(3q,Xq) and all possessed the same characteristic large C-band pattern (Fig. 4.10). Hence if REP3-2 did exhibit C-band variation for t(3q,Xq), then the reduced C-banding pattern probably occurred in only a small proportion of cells.

A number of additional cytological techniques (see Section 4.2) were carried out to further investigate the differences between the chromosome in question in REP3-2 and REP3-2-1. The results are discussed below.

Hoechst 33258 causes an unexplained failure of condensation around the centromeric regions of certain mouse (Hilwig and Gropp, 1973), Drosophila (Gatti *et al.*, 1976), Chinese hamster (Rocchi *et al.*, 1976) and human (Marcus *et al.*, 1979) chromosomes. More recently, Hayman and Sharp (1981) studied the effect of culturing a number of marsupial species in the family Macropodidae (including

M. rufogriseus) in the presence of Hoechst 33258. They found that the centromeric region of the *M. rufogriseus* X chromosome expressed a marked lack of condensation compared with all autosomal centromeric regions which were unaffected. A smaller effect was observed at the telomere of one of the *M. rufogriseus* autosomes.

In this study, a marked lack of condensation of the centromeric region of the t(3q,Xq) chromosome in REP3-2 was observed (Fig. 4.31(a)), indicating that one of the chromosome arms, or at least the centromeric heterochromatin, had originated from the *M. rufogriseus* X chromosome. No such lack of condensation was observed for t(3q,Xq) in REP3-2-1 (Fig. 4.31(b)). Hayman and Sharp (1981) were unable to determine which arm of the *M. rufogriseus* X chromosome was affected by culture in Hoechst 33258. The results from the present study suggest that at least the long arm of the *M. rufogriseus* X chromosome was affected. It would be of interest to see if Xq in REPB3-3 and REPB3-4 (Table 4.1) and Xp in the REP hybrids referred to in Table 4.1 are affected by culture in the presence of Hoechst 33258. If Xp was found to be affected to a much greater extent than the PG19 chromosomes, then this method could be used to differentiate the *M. rufogriseus* Xp from the smaller PG19 acrocentric chromosomes (see Section 5.1). Hoechst 33258 binds specifically to AT rich chromosome regions (Weisblum and Haenssler, 1974; Muller and Gautier, 1975; Comings, 1975). Perhaps t(3q,Xq) in REP3-2-1 has lost an AT-rich centromeric region present in REP3-2 or there has been a change in the proteins associated with the AT-rich DNA preventing Hoechst 33258 from binding. There is evidence that the H1 histone of one species is able to interact with the chromatin

of another species in human x mouse cell hybrids and still be biologically functional (Hsiung and Kucherlapati, 1980). Perhaps an interaction of *M. rufogriseus* and mouse histone proteins has occurred in REP3-2-1 but not in REP3-2, preventing the C-banding and binding of Hoechst 33258 to regions of t(3q,Xq) in REP3-2-1.

As was also observed by Hayman and Sharp (1981), the three interstitial C-bands on *M. rufogriseus* Xq were not affected by culture in Hoechst 33258. The change in C-band expression of t(3q,Xq) in REP3-2-1 involved both centromeric and interstitial C-bands and, therefore, this change appears to be more general than just an effect on A=T rich DNA. Whatever the change in REP3-2-1, the mouse chromosomes behaved the same way as in REP3-2 when cultured in the presence of Hoechst 33258 and hence if there has been a modification of chromosomal proteins it appears not to have affected the centromeric regions of the mouse chromosomes.

N-banding detects chromosomal nucleolar organiser (NO) regions. *Mus* has a number of centromeric NO regions detected by N-banding. The distribution is different in various mouse strains. In the C57BL/6J strain, pairs 12, 15, 18 and 19 are stained (Dev *et al.*, 1977). Dev *et al* (1977) found no correlation between the amount of silver stain and the presence or absence of C-band material. As the NO on the *M. rufogriseus* Xq is situated between the distal two interstitial C-bands (Hayman and Rofe, 1977), it was of interest to see if the changed expression of C-bands in REP3-2-1 had been accompanied by a change in the expression of the NO regions in these hybrid cells. Only the mouse NO's were expressed in REP3-2 and REP3-2-1 (Fig. 4.28). This is in accordance with the evidence of Miller *et al* (1976a) that the species which preferentially loses chromosomes in interspecies cell hybrids

shows suppression of rRNA synthesis. During the first few days after fusion, both sets of rRNA are expressed in human x mouse hybrids. As human chromosome loss progresses, so does the loss of human rRNA expression (Dev *et al.*, 1979). Perhaps a similar situation has taken place in the *M. rufogriseus* x PG19 hybrids. If so, then these hybrids would need to have been tested at a very early stage of chromosome segregation to compare rRNA activity and C-band variation.

C-banding is often, but not always entirely, associated with satellite DNA. For this reason, *in situ* hybridisation experiments with *M. rufogriseus* satellite DNA probes were carried out[†]. Dunsmuir (1976) isolated the major and minor satellites of *M. rufogriseus*. The major satellite hybridises to the autosomal centromeric heterochromatic regions but not to the X chromosome (Fig. 4.29(b)). The minor satellite binds to the centromeric regions of all the *M. rufogriseus* chromosomes. There was no hybridisation of the major satellite to PG19 cells (Fig. 4.29a) but the satellite hybridised to the centromeric region of t(3q,Xq) in both REP3-2 and REP3-2-1 (Fig. 4.30). Thus, the 3q centromeric satellite DNA must be present in both these hybrids regardless of the amount of C-band material observed cytologically.

The *M. rufogriseus* chromosome 3 does not express its characteristic large centromeric C-banded region in REP3-1 (Fig. 4.9a), REP3-7-1,

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[†]The *in situ* hybridisations were kindly carried out by Peacock and Elizur, C.S.I.R.O., Canberra.

REP3-7-2, REPB3-1 (Fig. 4.17a), REPB3-2 and REPB3-4 (Fig. 4.19a). Hybridisation of the major satellite to the *M. rufogriseus* 3 in these cell lines would provide further evidence for modification of the DNA rather than the loss of C-band material in REP3-2-1.

Hybridisation of the minor satellite to t(3q,Xq) in REP3-2 and REP3-2-1 may show if the centromeric C-banded DNA in REP3-2 is present in t(3q,Xq) in REP3-2-1. A problem here is that the minor satellite also binds to the autosomal C-banded regions and it may be difficult to determine if any resulting hybridisation was on the 3q or Xq arm of t(3q,Xq). A possible way to overcome this problem would be to culture REP3-2 and REP3-2-1 in the presence of Hoechst 33258 before hybridising with the minor satellite, but as t(3q,Xq) in REP3-2-1 was not affected by culture in Hoechst 33258, such differentiation would not be achieved for this cell line. It may, therefore, only be possible to demonstrate if the minor satellite is present on t(3q,Xq) in REP3-2.

Venolia and Peacock (1981) have isolated the major satellite of *Macropus robustus robustus* (wallaroo). This satellite hybridises to the interstitial heterochromatic regions of the long arm of the *M. rufogriseus* X chromosome. It would be of interest, therefore, to see if this satellite hybridises to the region of the three interstial C-bands of t(3q,Xq) in REP3-2 and REP3-2-1. Hybridisation of either the *M. rufogriseus* minor or the *M. robustus robustus* satellite to t(3q,Xq) in both REP3-2 and REP3-2-1 would support the evidence from studies using the *M. rufogriseus* major satellite, that the C-banded material of t(3q,Xq) is not lost in REP3-2-1 but rather modified in some way.

There are a number of different chromosome counter-staining methods which can be used to characterise, cytologically different

types of heterochromatin by modifying or enhancing chromosome banding (reviewed in Schweizer, 1981). In REP3-2 and REP3-2-1, t(3q,Xq) responded differently to treatment with DAPI/actinomycin D and chromomycin A_3/DA/DAPI staining (Figs. 4.33 and 4.34) DAPI is a fluorescent dye specific for A=T rich regions and actinomycin-D is a non fluorescent dye specific for G=C regions. The mouse chromosomes exhibited brightly fluorescent A=T rich centromeres and the centromere of t(3q,Xq) appeared dull in both REP3-2 and REP3-2-1 and therefore was G=C rich (Fig. 4.33). The substantial difference in DA/actinomycin-D banding pattern of t(3q,Xq) in the two cell lines indicated that a modification of the chromosome had occurred in REP3-2-1 rather than a loss of extensive A=T rich regions in t(3q,Xq).

Chromomycin A_3 is a fluorescent dye with G=C specificity. Both distomycin-A and DAPI show binding specificity for A=T rich regions, but only distomycin A fluoresces. Although DA and DAPI have similar base pair binding specificity their binding affinities are different. DA/DAPI staining has revealed a set of specific C-bands in the human karyotype (Schweizer, 1978). No such bands were found for the mouse or marsupial chromosomes in REP3-2 and REP3-2-1. Chromomycin A_3 staining resulted in a banding pattern similar to R-banding. t(3q,Xq) expressed a particularly brightly fluorescing centromeric region and a single sharp band and two much fainter bands on the Xq arm (presumably G=C rich). Only the centromeric region of t(3q,Xq) expressed brightly fluorescing G=C rich banding. The results from both DAPI/actinomycin-D and chromomycin/A_3/DA/DAPI banding techniques support a G=C rich centromeric region on t(3q,Xq). The differences in fluorescent banding pattern of this G-band equivalent chromosome in REP3-2 and

REP3-2-1 support a model of DNA modification rather than DNA loss to explain the difference in C-band content.

The functional aspects of C-band material are reviewed in John and Miklos (1979). The amount and distribution of C-banding material varies greatly with the organism concerned, and C-band polymorphisms are common within species in many natural populations. Position affects associated with translocation of heterochromatin are discussed in Section 2.1. Examples of loss of C-band material can be found in the literature but are usually accompanied by loss of DNA. For example, *in vitro* studies using *Microtis agrestus* cells by Cooper (1977a) showed that a loss of C-bands was accompanied by simultaneous loss of DNA and G-bands. The loss did not affect cell propagation (Cooper, 1977b). The presence of large areas of C-banding material is usually associated with the presence of either pale or dark G-bands in the same chromosome regions. It may be expected that a physical loss of C-banding material would be accompanied by a corresponding loss in G-banding material in that region. This was not found to be the case for t(3q,Xq) in REP3-2 and REP3-2-1.

Beermann (1977) described chromosome diminution in three species of *Cyclops*: *C. divulsus, C. furcifer* and *C. strenuus*. Telomeric and centromeric heterochromatic chromosomal segments were eliminated from the soma in all three species and so also were interstitial segments in *C. strenuus*. After chromosome diminution the DNA content of the soma in *C. strenuus* was 2/3 that in the germ-line. Unfortunately no G-banding studies of the germline or soma were carried out. In the present study, the non-expression of C-banded material in REP3-2-1 also involved not only

centromeric but also three interstitial C-bands. The loss of interstitial heterochromatic segments from *C. strenuus* did not affect the structural integrity of the chromosome, which implies that there must be a mechanism whereby the remaining fragments of the DNA were correctly joined together again. There is no known parallel to this unique type of interstitial DNA elimination in other organisms. Perhaps such an elimination mechanism has been involved in the loss from t(3q,Xq) in REP3-2-1 of the three interstitial C-bands present on t(3q,Xq) in REP3-2.

Motara and Rai (1977) found that the expression of a particular C-band in hybrid mosquitoes depended on the genetic background in which the C-band was placed. It seems that the C-band region was always present on the chromosome as it could be re-expressed in the progeny of matings where neither parent expressed this C-band. It is very difficult to compare the genetic background for REP3-2 and REP3-2-1 because of the heterogeneous nature of these cell lines. However, as REP3-2-1 was derived from REP3-2, the genetic background in which t(3q,Xq) existed in REP3-2-1 would be unlikely to contain components *not* present in REP3-2 i.e. the effect is likely to be due to loss rather than gain. REP3-2 had a modal chromosome number of 69 and REP3-2-1 had a modal number of 68. REP3-2 (chromosome number ranged from 56-87) was more heterogeneous than REP3-2-1 (63-69). t(3q,Xq) and Xp were the only marsupial chromosomes found in REP3-2-1 whereas REP3-2 also contained the occasional chromosome 7. It is possible that these differences may have affected C-band expression in the two cell lines.

Heterochromatic DNA is often late replicating. Late replication of the C-band regions of t(3q,Xq) in REP3-2 and not in REP3-2-1 would

indicate either the loss of late replicating DNA from these regions or some change affecting the time of replication of these regions in REP3-2-1. Preliminary BUdR and ^{3}H-thymidine late replication studies were inconclusive and hence further investigations in this area would be desirable.

One decisive experiment to determine whether the C-banded material was "lost" or just not expressed in REP3-2-1 would be to determine the DNA content of t(3q,Xq) in REP3-2 and REP3-2-1. If C-band material has been lost from t(3q,Xq) in REP3-2-1, this may be confirmed by a difference in DNA content.

5.3 X-LINKED ENZYMES

Of the 39 hybrid cell lines screened for the expression of *M. rufogriseus* HPRT and PGK-A, 36 showed concordance of expression of these two enzymes (Table 4.2). The three exceptions were the PGRN hybrids which only expressed *M. rufogriseus* HPRT. Although complete concordance for HPRT and PGK-A expression was not observed, the above results suggest synteny of the *Hpt* and *Pgk-A* loci. Some caution must be exercised when making such a deduction because of (1) the small number of primary hybrids studies (4 primary REP hybrids, 3 PGRN hybrids and the 1RRN-2 hybrid), (2) the fact that many of the hybrids were subclones of REP3, and (3) the fact that the 'bulk' hybrids were not isolated independently of the primary hybrid colonies. It is possible, for example, that REPB3 was composed of cells which had sloughed off from the original REP3 colony, in addition to cells derived from other primary hybrids. This lack of independence of origin of REP3 and REPB3 may have resulted in co-segregation of *Hpt* and *Pgk-A* in the REP3 and REPB3 subclones and revertants, even though these loci may be asyntenic. It is possible that *M. rufogriseus Hpt* and *Pgk-A* are on different chromosomes which are selected together in HAT medium and lost together in 6TG medium. If so, then this selection pressure may not have existed for the PGRN hybrids where only *Hpt* was retained in the hybrids. It will be necessary to use additional *M. rufogriseus* x eutherian cell hybrids from further fusions to confirm the synteny of these two genes. Nevertheless, the concordant expression of *M. rufogriseus Hpt* and *Pgk-A* in 36 hybrid cell lines does imply synteny of these two genes.

Expression of *M. rufogriseus* HPRT and PGK-A in the hybrids was not concordant with the presence of any *normal* identifiable *M. rufogriseus* chromosomes. However, for *M. rufogriseus* HPRT and PGK-A enzymes to be expressed, the hybrid cells must contain at least a fragment of the *M. rufogriseus* chromosome containing the relevant structural loci. A consideration of the cytological data presented in Section 4.1.2 and discussed in Section 5.1, suggests that Xp was the most likely location of these genes. *M. rufogriseus* Xp, as identified by G-banding, appeared to have been retained in a significant percentage of cells in the REP hybrids but not in the revertants (see Section 5.1 and Table 4.1). 1RRN-2 hybrid cells were not G-banded, but based on C-banding they contained a chromosome arm resembling the *M. rufogriseus* Xp (Fig. 4.24). Preliminary C-banding analyses failed to detect normal *M. rufogriseus* chromosomes in the PGRN hybrids. If HPRT and PGK-A are on *M. rufogriseus* Xp it appears that a smaller fragment of Xp containing only HPRT may be present in the PGRN hybrids. G-banding needs to be carried out on these hybrids to see if the *M. rufogriseus* Xp is present.

As the gene for HPRT has been found to be X-linked in all eutherian and marsupial species in which it has been mapped, presumably HPRT will also be found to be X-linked in *M. rufogriseus*. So far, marsupial family studies have been unable to demonstrate the X-linkage of HPRT in any species (including *M. rufogriseus*) due to a paucity of HPRT isozyme variants but it is known from family studies that PGK-A is X-linked in a number of kangaroo species, closely related to *M. rufogriseus* (Vandeberg *et al.*, 1973; 1977; Cooper *et al.*, 1971) PGK-A has been found to be monomorphic in all *M. rufogriseus* family studies carried out so far (P. Johnston, p.c.) and hence it has not

been possible to demonstrate possible X-linkage of PGK-A in this species. The gene for HPRT has been assigned to the *M. rufus* (red kangaroo) X chromosome and shown to be syntenic with the genes for PGK-A and G6PD using *M. rufus* x mouse somatic cell hybrids (Donald and Hope, 1981). Graves *et al.*, (1979) demonstrated co-transfer of these genes in *M. robustus* x mouse cell hybrids. They could not, however, identify cytogenetically any *M. robustus* chromosomes in their hybrids. The fact that *M. rufogriseus* is closely related phylogenetically to *M. rufus* and *M. robustus* (Rofe, 1978) suggests that the genes for HPRT, PGK-A and G6PD are also X-linked in *M. rufogriseus*. Although X-linkage of *M. rufogriseus Hpt* and *Pgk-A* could not be confirmed in the present study, these genes are at least syntenic, indicating conservation of gene synteny for *Hpt* and *Pgk-A* in *M. rufogriseus*, *M. rufus* and *M. robustus*.

The gene for G6PD is known to be X-linked in *M. rufogriseus* from population and family studies (Johnston *et al.*, 1975a). It was perhaps surprising, therefore, that *M. rufogriseus* G6PD was not found in any of the hybrid cell lines tested in this study (Table 4.2). G6PD is a dimer in eutherian species (Gartler *et al.*, 1973) and Johnston *et al.*, (1978) have shown G6PD to be a dimer in marsupials by *in vitro* hybridisation studies. If *M. rufogriseus* G6PD was present in the cell hybrids it would have been detected on the G6PD gels by the presence of a band representing the *M. rufogriseus* G6PD/PG19 G6PD heteropolymer and/or the less intensely staining *M. rufogriseus* G6PD homodimer. Neither of these two bands was detected in the hybrid lines. If the genes for HPRT and PGK-A are on the short art of the *M. rufogriseus* X chromosome as has been

suggested earlier, this would suggest that *Gpd* may be on the long arm of the X chromosome or that *Pgk-A* may be closer to *Hpt* than *Gpd*. Donald and Hope (1981) mapped all three genes, *Hpt*, *Pgk-A* and *Gpd*, to the euchromatic short arm of the *M. rufus* X chromosome but were unable to determine the order of these loci. Graves *et al.*, (1979) concluded that these X-linked markers lay in the order centromere - *Hpt* - *Pgk-A* - *Gpd* in the wallaroo. Assuming conservation of gene content is often accompanied by conservation of gene order for closely related species, the results from the present study support the hypothesis that the *Gpd* locus is on a chromosome region outside the *Hpt* - *Pgk-A* chromosome segment.

It is possible that the *M. rufogriseus Gpd* gene is present in the hybrids but is either (a) not expressed or (b) expressed at levels too low to be detected by the method used in Section 3.8.4. All extracts from REP and 1RRN-2 hybrids gave less intensely staining *M. rufogriseus* PGK-A bands than PG19 bands when electrophoresed and stained on cellogel (Fig. 4.36), possibly due to gene dosage differences for the PG19 and *M. rufogriseus Pgk-A* genes. The gels had to be loaded heavily with cell extract to enable *M. rufogriseus* PGK-A to be scored.

On this basis, the *M. rufogriseus* G6PD dimer would also give less intensely stained bands than PG19 G6PD. The hybrid band, however, may still be expected to be visualised but the gels had to be loaded extremely lightly with cell extract to allow separation of the mouse and marsupial control G6PD isozymes. Due to the small mobility difference between PG19 and *M. rufogriseus* isozymes, an increase in the amount of extract loaded onto the gels would result in encroachment of the mouse isozyme upon the region of the *M. rufogriseus*/PG19 heteropolymer and hence may not have been identified. Gene dosage

differences could result from chromosomal heterogeneity within the hybrid cell lines. For example, if the gene for G6PD is on Xp (with approximately 20% misclassification, see Section 5.1) then REP3-6 with the highest percentage (68% (88%-20%)) of cells containing the putative Xp would have about half the *M. rufogriseus* G6PD isozyme staining intensity compared with PG19 G6PD. If *Gpd* is on the long arm of the X-chromosome, then REP3-2-1 with 100% of cells containing Xq may be expected to express *M. rufogriseus* G6PD (Table 4.1). However, in this case, Xq was in a translocated state and lacked its characteristic C-bands, which could possibly affect the expression of G6PD. REPB3-3 was the only cell line to possess an intact X chromosome in some cells but again G6PD was not expressed. It is possible that a regulatory locus may be required for G6PD expression in REPB3-3 or that the X chromosome may contain a small deletion including the *Gpd* gene. More sensitive detection of *M. rufogriseus* G6PD may be possible using a temperature sensitive assay or an immunochemical approach similar to that used by Van Heyningen *et al.*, (1973).

Two *M. rufogriseus* G6PD phenotypes, $G6PD^F$ and $G6PD^S$, have been found in *M. rufogriseus* (Cooper , 1975). The *M. rufogriseus* parental cells of the hybrids used in the present study had the $G6PD^F$ phenotype. Greater separation of mouse and *M. rufogriseus* G6PD would be obtained if *M. rufogriseus* cells were of $G6PD^S$ phenotype. On the basis of Donald's (1980) result with *M. rufus*, hybrids from the fusion of *M. rufogriseus* x eutherian species other than mouse (e.g. Chinese hamster cells) would also give greater separation of the hybrid parental cell G6PD isozymes. This would allow heavier

application of extract for electrophoresis without loss of enzyme resolution on the stained gels and hence, facilitate the mapping of *M. rufogriseus Gpd.*

The absence of G6PD in the hybrids is indeed puzzling. As stated previously *M. rufogriseus* G6PD expression like G6PD in all other marsupial and eutherian species where this enzyme has been studied is known to be inherited as an X-linked trait, subject to random X-inactivation in eutherians (Lyon, 1961; 1974) and paternal X-inactive in marsupials (Sharman, 1971).

5.4 ANTIGENS

5.4.1 Comparison of Binding of Conventional and Monoclonal Antibodies

SREP3, an antiserum derived from C57BL/6J mice which had been immunised with REP3 cells, is expected to bind only to antigens on the REP3 hybrid cell surface which are foreign to C57BL/6J mice. As PG19 was derived from a melanoma originating in a C57BL/6J mouse, it was expected that antigens present on PG19 cells would not induce an immune response in their syngeneic strain of mouse. However, this was found not to be so. (Table 4.3). SREP3 not only bound to REP3 cells and *M. rufogriseus* fibroblasts as might be expected, but it also bound to PG19 cells implying the presence on these cells of tumour antigens recognised as foreign by C57BL/6J mice. Attempts were made to induce an antiserum to PG19 tumour antigens by immunizing C57BL/6J mice with PG19 cells. The resulting antiserum was to be used to mask the PG19 tumour antigens on the REP hybrid immunizing cells in order to restrict the immune response of the mouse to the marsupial antigens. These attempts were unsuccessful. Presumably C57BL/6J mice cannot recognise PG19 tumour antigens on PG19 cells but *can* recognise them as foreign when they are presented to the syngeneic host on the cell surface membrane of a somatic cell hybrid. A number of published studies indicate that adsorptions were required to remove antibodies to tumour antigens on hybrid cells, but no explanation has been offered to account for the induction of such antibodies (Buck and Bodmer, 1976). Perhaps the tumour antigens, when presented to the mouse immune system in the form of PG19/*M. rufogriseus* hybrid antigens, stimulate the mouse immune system to recognise and respond to the

mouse tumour antigens. Another possibility is that the presence of the "foreign" *M. rufogriseus* antigens on REP3 induce the initial immune response in the mouse and this is the cue for the production of antibodies in the form of a less specific secondary response to PG19 tumour antigens on the hybrid cells.

SREP3 was adsorbed with PG19 to determine if it contained antibodies to *M. rufogriseus* cell surface antigens, in addition to the mouse tumour antibodies. The resulting serum, SREP3 AD PG19, still reacted with REP3 cells and *M. rufogriseus* fibroblasts but had lost its reaction with PG19 cells, and hence the adsorbed antiserum was detecting an *M. rufogriseus* antigen(s) on REP3 cells (Table 4.4). The reactions of SREP3 AD PG19 with the REP hybrids are set out in Table 4.5. A number of the hybrids reacted with SREP3 AD PG19. The revertant cell lines only exhibited autofluorescence, and hence they lacked the *M. rufogriseus* antigen(s). Concordance of antibody binding with a single chromosome in the hybrids would indicate that there was at least one gene present on that chromosome determining a cell surface antigen. If the serum was detecting more than one antigen, determined either by different genes on the same chromosome, or, on different chromosomes, concordant relationships found in the hybrids may be difficult to interpret. In order to determine if SREP3 was reacting with only one antigen on the REP hybrids, extensive adsorption of SREP3 with different cell types (e.g. pairwise adsorptions between hybrids binding SREP3 AD PG19) would have been required. Large quantities of cells would have been needed for such adsorptions and a reduced antibody titre in the adsorbed serum would have been expected. Even after extensive

adsorption, it would have been difficult to determine if the resulting serum was actually monospecific. This problem was overcome by using Köhler and Milsteins (1976) method of myeloma x spleen fusions to produce a monoclonal antibody, GA-1 (described in Section 2.3).

GA-1 reacted with *M. rufogriseus* fibroblasts and lymphocytes as well as REP3 cells but not with PG19 (Table 4.8). This suggests that the reaction of GA-1 with REP3 cells is due to the presence of an *M. rufogriseus* cell surface antigen on these cells. Being a monoclonal antibody, GA-1 did not require adsorption to remove anti-PG19 antibodies as was the case for the polyclonal SREP3 sera. One hundred percent of *M. rufogriseus* fibroblast cells bound GA-1 whereas only about 20% of REP3 cells did (Table 4.8). The variation amongst REP3 cells was probably due to chromosomal heterogeneity rather than variation of antigen expression during the cell cycle. One assumes that only approximately 20% of REP3 cells contained the gene determining GA-1 antigen expression (Table 4.8).

The reactions of GA-1 with the REP hybrids and revertants are listed in Tables 4.10, 4.11 and 4.12. These reactions correlate almost exactly with the results obtained with SREP3 AD PG19 (Table 4.5). The degree of reaction (for any one cell line) was often one immunofluorescence score higher with GA-1 than with SREP3 AD PG19. For example, REP3-2 gave a + → ++ reaction with SREP3 AD PG19 and a ++ → +++ reaction with GA-1. This indicated that the neat GA-1 supernatant had a higher antibody titre against an *M. rufogriseus* antigen on the hybrid cells than neat SREP3 AD PG19. There are only two distinct discrepancies when comparing the reactions of GA-1 and SREP3 AD PG19 on the REP hybrids (see Tables 4.5, 4.10, 4.11 and 4.12): (1) REP3-6 did not react with SREP3 AD PG19 and yet GA-1

reacted strongly with approximately 25% of REP3-6 cells: (2) REP3-7-3 did not react with GA-1 but did react with SREP3 AD PG19. These discrepancies can be explained in several ways.

First, SREP3 AD PG19 and GA-1 may have been detecting the same *M. rufogriseus* antigen, that is, SREP3 AD PG19 was effectively monospecific. As the *M. rufogriseus* chromosomal contribution to the REP3 hybrid was restricted to only a few *M. rufogriseus* chromosomes it may be possible that only one *M. rufogriseus* antigen was being expressed on these hybrid cells. The small proportion of secreting hybridomas ($\frac{1}{70}$) when REP3 cells were used as immunogen, compared with results from some other studies using the same protocol for constructing hybridomas (but using different immunogens) supports this contention. Only 25% of REP3-6 cells reacted with GA-1. Absence of reaction of REP3-6 with SREP3 AD PG19 could be explained by lack of detection of a small percentage of cells using the lower titre antisera, SREP3 AD PG19. If SREP3 AD PG19 is detecting only the antigen detected by GA-1, then absence of reaction of GA-1 with REP3-7-3 could have resulted from chromosome loss in the period between screening of the hybrids with SREP3 AD PG19 and subsequently screening them with GA-1. Although the cells were stored in liquid nitrogen during this period, the thawing and growing procedures prior to the immunofluorescence assay with GA-1 could have resulted in further chromosome loss.

A second explanation for the discrepancies is as follows. GA-1, being a monoclonal antibody, reacted with one antigenic determinant whereas SREP3 AD PG19 may have detected other antigens in addition to GA-1. Possibly REP3-7-3 was the only cell line tested which did not express GA-1 but did express one or more of these "other antigens", whereas the positive reactions of the other hybrids with SREP3 AD PG19

represented the binding of antibody to both GA-1 and the other antigens on the cell surface. Again, the absence of reaction of REP3-6 could be explained as a lack of detection of a small percentage of cells using the lower titre antisera, SREP3 AD PG19.

Indirect immunofluorescence was chosen as the method of detection for antibody binding in this study because of the heterogeneous nature of the marsupial x mouse cell hybrids. Using this method the distribution of antibody binding amongst individual cells could be observed. Methods such as radio-immuno assays and enzyme-linked assays, although probably more sensitive and quantitative, give only an indication of the overall binding of antibody to cells in any one cell population. For example, using these methods it is not possible to distinguish whether all cells in a sample bind small amounts of antibody, or, only a subset of cells binds large amounts of antibody. A cytotoxic assay can be used to estimate the percentage of cells binding sufficient antibody to be lysed in the presence of complement. However, this method would require the use of a cytotoxic monoclonal antibody, and the assay would not necessarily indicate the amount of antibody bound to each cell in a sample. The GA-1 antibody is of immunoglobulin subclass IgG_{2b}, members of which are usually cytotoxic and hence a cytotoxic assay may have been applicable to the present study (Sandrin *et al.*, 1978) (see Section 5.5). When the positive (+++) and negative (+/-) controls can be clearly distinguished, the scoring of brightly fluorescing cells using a microscope can be an objective method of detecting cells binding antibody but scoring can be more subjective when measuring the *degree* of antibody binding measured by varying fluorescence intensity. Also, the estimation of percentages of fluorescing cells is both tedious and approximate. A more objective method of scoring the

intensity of fluorescence of cells and measuring the distribution of reacting cells is to make use of a fluorescence activated cell sorter (FACS). The FACS was used in the present study to obtain fluorescence intensity profiles of the reaction of GA-1 with PG19, *M. rufogriseus* fibroblasts, REP3-1 and REP3-2 (Fig. 4.39). These profiles were in agreement with the reactions of GA-1 scored with a fluorescence microscope (Table 4.13 and 4.8).

Information on the relative cell size distribution of different cell lines obtained from the FACS was useful (Fig. 4.39) for comparing antigen density on different cells. When viewed under a microscope, a large cell, with a low antigen density is likely to appear to fluoresce less brightly than a smaller cell with a higher antigen density, even though both cells contain the same total number of antigenic sites. The FACS measures the total fluorescence of each cell, and hence measures the fluorescence on the cells in a quantitative rather than a qualitative fashion. Therefore, the amount of antigen present on cells of two different cell lines can be compared, in relation to their size. PG19 cells were larger than *M. rufogriseus* fibroblasts (Fig. 4.39a) and therefore have a higher background fluorescence than marsupial fibroblasts. Theoretically, this moves the negative control cut-off point into an area of lower fluorescence intensity, thereby increasing the proportion of cells in *M. rufogriseus* analysed as reacting with GA-1. REP3-1 cells were approximately the same size as PG19 cells, and REP3-2 cells were larger than any of the other cell lines scanned (Fig. 4.39). On the basis of this size difference REP3-2 cells have a higher background fluorescence than PG19 and hence a more accurate PG19 negative control cut off point would be toward a region of more intense fluorescence, thereby decreasing the proportion of REP3-2 cells analysed as reacting with

GA-1. There was a higher proportion of REP3-1 cells with greater fluorescence intensities than in the REP3-2 sample (Fig. 4.39c), indicating a higher antigenic density of GA-1 on REP3-1 than on REP3-2 cells.

A most useful application of the FACS to the present study would have been to sterilely sort the hybrid cells according to their fluorescence intensity after reaction with GA-1. Chromosome preparations of each sorted population of cells could then be carried out and G-banded metaphase spreads analysed. In this way, it would be possible to analyse the chromosomal constitution of cells either binding or not binding GA-1 and hence verify the assignment of the GA-1 gene to *M. rufogriseus* 3q.

5.4.2 Chromosomal assignment of the gene for the antigen, GA-1

The PGRN and 1RRN-2 cell hybrids were not G-banded and preliminary C-banding studies did not allow identification of any normal *M. rufogriseus* chromosomes. These cell lines were therefore not included in an attempt to assign the gene determining GA-1 to an *M. rufogriseus* chromosome.

The chromosomal constitutions and reactions of the REP cell lines with GA-1 are set out in Table 4.13.

The possible concordance of GA-1 expression with any one *M. rufogriseus* chromosome in the hybrid and revertant cell lines was considered. None of the *M. rufogriseus* chromosomes showed absolute concordance with GA-1 expression. Non-concordance with GA-1 expression was particularly marked for the *M. rufogriseus* 6, 7 and X e.g. 14 hybrids expressed GA-1 but did not contain the *M. rufogriseus* chromosome 7. As discussed earlier in Section 5.3,

caution must be exercised when interpreting concordant relationships in the present study because of the lack of independent origin of many of the cell lines in Table 4.13. However, *M. rufogriseus* Xp and 3q showed strong concordance with GA-1 expression, and these two chromosome arms were therefore considered to be the two most likely locations of the gene determining GA-1 expression.

Of the 24 REP hybrids analysed 22 were concordant for *M. rufogriseus* 3q (identified by G-banding) and binding of GA-1 (Table 5.1).

TABLE 5.1

		PRESENCE OF *M. RUFOGRISEUS* 3q IDENTIFIED BY G-BANDING†		
		+	-	TOTAL
BINDING OF GA-1	+	13	2	15
	-	0	9	9
		13	11	24

†As identification of 3q alone required G-banded preparations, only hybrids which had been G-banded were included.

This result suggests that the gene coding for GA-1 (or a gene determining the expression of this antigen) is present on *M. rufogriseus* 3q. It is possible that the two discordant hybrids which bound GA-1 but did not contain *M. rufogriseus* 3q may have contained unidentified fragments of the *M. rufogriseus* 3q containing the GA-1 gene. One of these hybrids, REP3-6, possessed the lowest percentage of cells binding GA-1 and hence 3q may have remained unidentified in the sample of cells from this cell line. This is unlikely, however, to be the

explanation for REP3-2-2 where 100% of cells bound GA-1. It is possible that a fragment of 3q (containing the GA-1 gene) had been translocated onto a mouse chromosome in this cell line. *M. rufogriseus* 3q was similar to some of the larger mouse acrocentric chromosomes but was differentiated from these on the basis of its large pale centromeric region when G-banded. This region was easily identified in some metaphase spreads (Fig. 4.20) but not so clearly recognised in others (Fig. 4.19). There were no hybrids containing a clearly identifiable 3q arm which did not react with GA-1. The presence of a C-banded chromosome resembling the *M. rufogriseus* chromosome 3 in two GA-1$^+$ hybrids which were not G-banded, REP3-7-2 and REPB3-2, (Table 4.13) supports the assignment of GA-1 to 3q. REP3-7-5, another GA-1$^+$ hybrid which was C- but not G-banded did not contain the chromosome present in REP3-7-2 and REPB3-2. If only the *M. rufogriseus* 3q arm was present in REP3-7-5 it may have remained unidentified by C-banding. REP3-7-3, the one GA-1$^-$ hybrid which was not G-banded (Table 4.13) did not contain the normal *M. rufogriseus* chromosome 3 identified by C-banding. This observation is also in accordance with the assignment of the gene for GA-1 expression to 3q. However, G-banding needs to be carried out on this cell line to determine if any of the cells therein contain the *M. rufogriseus* 3q.

In all hybrids except REP3-2-1, the percentage of cells that reacted with GA-1 was higher than the percentage of cells which contained the *M. rufogriseus* 3q (see Table 5.2). Hybrid cell lines which showed the largest differences were those containing 3q alone, rather than the normal *M. rufogriseus* chromosome 3 or t(3q,Xq). For example, 80% of REP3-1 cells contained the *M. rufogriseus* chromosome 3 and 90% of cells bound GA-1 (a difference of 10%, see Table 5.2).

TABLE 5.2: Comparison of the percentage of cells binding GA-1 and the percentage of cells containing the *M. rufogriseus* 3q.

CELL LINE	PERCENTAGE OF HYBRIDS CONTAINING *M. RUFOGRISEUS*			% CELLS BINDING GA-1	% OF CELLS BINDING GA-1 -% CELLS WITH 3q
	3q	3	t(3q,Xq)		
REP3	-	18	27	20	
REP3-1	-	80	-	90	+10
REP3-2	-	-	75	90	+15
REP3-2-1	-	-	100	95	-5
REP3-4	55	-	-	90	+35
REP3-7	50	-	-	100	+50
REP3-7-1	-	89	-	90	+1
REP3-7-4	66	-	-	100	+44
REPB3-1	-	91	-	100	+9
REPB3-3	-	75	-	100	+25
REPB3-4	-	78	-	95	+17
REPB3-5	56	-	-	95	+39

Here the two percentages correspond closely. On the other hand only 66% of REP3-7-4 cells contained an identifiable *M. rufogriseus* 3q and yet 100% of these cells bound GA-1, a considerably larger difference (44%) than observed for REP3-1. It was harder to identify the 3q arm by itself in the hybrids than the larger *M. rufogriseus* chromosome 3 or t(3q,Xq). It is possible, therefore, that some of the hybrid cells e.g. in REP3-7-4, contained the unidentified 3q arm, and this may account for some of the differences observed in Table 5.2. As both figures (the percentage of cells possessing 3q and the percentage of cells binding GA-1) were subject to considerable sampling and other errors, little weight should be attached to the differences referred to in Table 5.2.

It appears that the *M. rufogriseus* 3q may confer a selective advantage of proliferation of the hybrid cells in culture as this chromosome arm was retained preferentially in the REP hybrids (Section 5.1). The hybrid cells had been expanded in numbers for extracts for electrophoresis at the time of chromosome preparation. They were then frozen and rethawed before they were screened for GA-1 binding (approximately 10 cell doublings). It is possible that there had been selection for cells containing 3q during this time interval, resulting in an increased proportion of 3q containing cells in any one cell line at the time of screening for GA-1 binding. Perhaps there was some interaction between the selected *Hpt* gene on the X chromosome and a gene or genes on 3q; perhaps the GA-1 gene itself confers a selective advantage on the growth of the hybrid cells. Another possibility is that there may be a regulatory locus on Xp controlling a structural locus on 3q which confers a selective advantage on the hybrid cells. As 3q was always absent in the absence

of Xp it is less likely that 3q contained a regulatory locus controlling a gene on Xp conferring a selective advantage on the hybrid cells than *vice versa*.

TABLE 5.3

		Xp	
		+ (> 30%)	- (< 30%)†
3q	+	12	0
	-	6	5

†(Xp was scored as absent if less than 30% of cells contained Xp, to account for misclassification).

The other likely location for the gene determining GA-1 was on *M. rufogriseus* Xp. The problems experienced in the identification of Xp in the hybrids (see Section 5.1) present difficulties in the interpretation of any concordant relationship with Xp. The most confident identification of *M. rufogriseus* Xp was made in REP3-1 and REP3-6 where both C- and G-banding confirmed the presence of Xp in over 3/4 of the cells (Fig. 4.21). Most REP3-1 cells and 25% of REP3-6 cells bound GA-1 (REP3-6 was the one exception which bound GA-1 but not SREP3 AD PG19, see earlier this section). REP3-5 did not bind GA-1 even though approximately 71% of REP3-5 cells contained Xp. The revertants contained 18-27% of cells with the putative Xp but like REP3-3 (25%) did not express GA-1. If the misclassification of the identity of *M. rufogriseus* Xp is of the order of 25% then the data may be showing concordance of Xp and GA-1 expression. However, if Xp did contain the GA-1 gene then REP3-5 with 71% of cells possessing Xp may have been expected to express GA-1, especially as a number of hybrids e.g. REP3-2 and REP3-7 expressed GA-1 with

less than 1% of cells containing Xp.

In section 5.3 it was suggested that the genes determining HPRT and PGK-A may be present on Xp. If the GA-1 gene is also on Xp' then concordance of GA-1 with HPRT and PGK-A would be expected. Concordance for these loci was not found (Table 5.4) suggesting that the genes for HPRT and PGK-A are on a different chromosome to the gene for GA-1.

TABLE 5.4.

		PRESENCE OF HPRT AND PGK-A IN THE REP HYBRIDS†		
		+	-	
BINDING OF				
GA-1	+	17	0	17
TO REP	-	11	8	19
HYBRIDS				
		28	8	36

†All the hybrids in Table 4.15 including 1RRN-2 are included in this table.

Amongst the REP hybrids, there were exceptions to the concordance of GA-1 expression with both *M. rufogriseus* 3q and Xp and therefore a firm assignment could not be based on these hybrids alone. However, further evidence for the assignment of GA-1 to *M. rufogriseus* 3q is presented below.

5.4.3 Cross-reaction of GA-1 with *M. rufus* x mouse hybrids

Donald (1980) and Donald and Hope (1980) have cytogenetically and enzymatically characterised a number of *M. rufus* (lymphocyte)

x 1R cell hybrids, referred to as the 1RMR hybrids. As GA-1 was shown to bind to *M. rufus* fibroblasts (Table 4.8), assays for the presence of GA-1 were carried out on the 1RMR cell hybrids. For the following reason it is of special interest that GA-1 reacted with only those 1RMR hybrids which contained the *M. rufus* chromosome number 5 (Tables 4.15 and 4.16).

Rofe (1978) has constructed a phylogeny of the Macropodidae inferred from the minimal number of chromosome changes possibly occurring in a basic karyotype, that of *Thylogale billardieri*. By comparing G-banding patterns, Rofe demonstrated that the karyotype of the autosomes of one species can be transposed into the karyotype of another species by Robertsonian translocations and/or inversions. Rofe clearly demonstrated that the G-banding pattern of the *M. rufus* number 5 is identical to that of the long arm of the *M. rufogriseus* number 3 chromosome and that the *M. rufogriseus* chromosome three is a Robertsonian translocation product of the *M. rufus* chromosomes 5 and 8.

This implies that GA-1 was reacting with a cell surface antigen present on 1RMR hybrids which is determined by a gene on *M. rufus* chromosome 5, the G-band identical chromosome to the *M. rufogriseus* 3q. These results provide strong support in favour of the assignment of the GA-1 gene to the *M. rufogriseus* 3q.

The G-band patterns of closely related species provide information on the homology of chromosomal segments. Until full DNA sequencing is accomplished for each chromosome, or at least until detailed genetic maps are available it is not possible to confirm if a particular chromosome G-band in one species is the "same" band as that occurring in another species, but close banding homology suggests

homology of genetic content. The genes determining the antigens in *M. rufogriseus* and *M. rufus* recognised by GA-1, seem to have been conserved on the same G-banded chromosomal segment.

Rofe (1978) also demonstrated that *M. rufogriseus* expresses the same G-banded karyotype as *M. eugenii* and *M. parryi*. Fibroblasts from both these species bind GA-1 (Table 4.14). An attempt was therefore made to construct *M. eugenii* x mouse and *M. parryi* x mouse cell hybrids containing the G-band equivalent chromosome arm of the *M. rufogriseus* 3q. If such hybrids bound GA-1 it would indicate that the homologous GA-1 gene in these species had remained conserved on the G-band equivalent arms to *M. rufogriseus* 3q. A number of marsupial x mouse fusions using the above species as the marsupial parent were carried out. Unfortunately, no *M. parryi* x mouse cell hybrids and only one putative *M. eugenii* x 1R cell hybrid was isolated (WT1R-1). This HAT selected cell line proved not to be a 'true' hybrid as it expressed HPRT of 1R origin (Section 5.1).

5.4.4 A eutherian counterpart of GA-1?

The fact that GA-1 did not bind to any of the eutherian species tested (Table 4.18) does not necessarily indicate the absence of an 'homologous' antigenic molecule in eutherian species. Presumably GA-1 only detects a single antigenic determinant on the marsupial GA-1 antigen. The GA-1 antigenic determinant may not be present in eutherian species, but the homologous eutherian molecule may be present. The best way to compare the homology of two proteins is to compare their amino-acid sequences, but the molecular techniques and time required for such comparisons were not available for the present study. Another possible approach for finding a eutherian

counterpart to GA-1 is to look for genes syntenic to the gene determining GA-1 expression. If the resulting syntenic group of genes shows homology with syntenic groups including genes determining antigens in eutherian species, this would imply conservation of these syntenic groups and hence indirect evidence for a eutherian counterpart of GA-1. The two most obvious eutherian candidates for comparison with GA-1 are the histocompatibility antigens in a number of eutherian species and the SA-1 locus in man. SA-1 is syntenic with lactate dehydrogenase-A (*Ldh-A*), esterase A_4 (*Es*A_4) and acid phosphatase-2 (*Acp-2*). The human major histocompatibility complex and phosphoglucomutase-3 (*Pgm-3*), together, or in combination with malic enzyme-1 (*Me-1*), glyoxylase (*Glo*) or superoxide dismutase-2 (*Sod-2*) have been shown to be syntenic in a number of primate species as well as in cat, dog, sheep and cattle (Human Gene Mapping - 5, 1979). Indirect evidence for the existence of a eutherian counterpart to GA-1 would result from synteny of the gene determining GA-1 expression and any of the above enzymic genes. As attempt was therefore made to screen the marsupial x mouse cell hybrids for some of these enzymes.

Donald and Adams (1981) assigned the gene for LDH-A to the *M. rufus* chromosome 5 using *M. rufus* x mouse cell hybrids. Using these same hybrids in this study, cross-reaction of GA-1 was found to be syntenic with LDH-A expression. The *M. rufogriseus* x mouse hybrids were therefore screened for the presence of *M. rufogriseus* LDH-A, to see if this syntenic group (i.e. LDH-A and GA-1) had been conserved in these two marsupial species.

All the hybrid and revertant cell lines shown in Table 4.2 were screened using electrophoresis for the *M. rufogriseus* LDH-A subunit.

M. rufogriseus LDH-A was not found in any of these cell lines. The absence of the gene for LDH-A in the *M. rufogriseus* x mouse hybrids implies that the syntenic relationship of LDH-A and cross-reaction with GA-1 of the *M. rufus* chromosome 5 has not been conserved in the *M. rufogriseus* G-band equivalent chromosome arm, 3q. Such a result was surprising as *M. rufus* and *M. rufogriseus* are closely related phylogenetically (Rofe, 1978). There may not have been a strong selective advantage favouring the retention during the separate evolution of these species of the genes for LDH-A and GA-1 as a syntenic pair.

Ldh-A has been regionally assigned to the *M. rufus* chromosome 5. It is possible that one or more of the REP hybrids had an unidentified deletion on the section of *M. rufogriseus* 3q containing the *Ldh-A* gene. This, however, seems unlikely, as it would need to apply to *all* the *M. rufogriseus* x mouse cell hybrids screened in this study to account for their LDH-A negativity. Also, whenever somatic cell hybrids are employed in a gene mapping study, it is possible that a gene is present but not expressed, or that genes are expressed but their products not detected in the hybrid cells.

Attempts were made to screen the *M. rufogriseus* x mouse hybrids with and without the *M. rufogriseus* 3q for marsupial EsA_4 activity. There was some difficulty encountered in identifying the *M. rufogriseus* EsA_4 band, but no esterase bands were identified in hybrid cell extracts which were not also present in the mouse control extracts. Further studies are obviously required before it can be concluded with certainty that EsA_4 of marsupial origin is absent from the hybrids. Acid phosphatase-2 (ACP-2) is also syntenic with LDH-A and SA-1 in man (Jones and Kao, 1978). It would be interesting to

screen the *M. rufogriseus* x mouse hybrids for ACP-2 and the LDH-A^+ *M. rufus* x mouse hybrids of Donald and Hope (1981) for both EsA_4 and ACP-2.

Preliminary studies for the detection of the marsupial PGM-3 and SOD-2 isozymes were carried out on cellogel. The PGM-1, PGM-2 and SOD-2 isozymes were identified on the gels for both the mouse and marsupial parental cell lines but it appears that starch gel electrophoresis may be required to detect the less intensely staining PGM-3 and SOD-1 isozymes. It was not possible to distinguish an electrophoretic mobility difference for the parental PGM-1, PGM-2 and SOD-1 isozymes and hence it was not possible to show if the *M. rufogriseus* genes coding for these enzymes were present in the hybrid cells.

In mouse, *Pgk-2* is closely linked to the *H-2* loci. The homologous *M. rufogriseus* gene, *Pgk-B*, (based on its tissue distribution) is controlled by an autosomal locus (Vandeberg *et al*, 1978). In the present study, however, the *M. rufogriseus* PGK-B isozyme band was completely overlapped by the PGK-A mouse isozyme band and hence it was not possible to detect *M. rufogriseus* PGK-B using the method in Section 3.8.3.

Temperature sensitive or immunochemical assays may be more successful in detecting marsupial PGK-B in the hybrids.

The REP hybrids were also screened for purine nucleoside phosphorylase (NP) because this enzyme has been identified in wallaroo x mouse cell hybrids containing a chromosome of similar G-band pattern to the *M. rufogriseus* 3q. (G. Dawson, p.c.). The mouse and *M. rufogriseus* NP isozymes had very similar mobilities and could not be separated sufficiently to confidently score the hybrids for

M. rufogriseus NP.

As stated earlier, it was thought that one of the advantages of working with marsupial x eutherian cell hybrids was the expected availability of isozyme differences between marsupial and eutherian mammals as a consequence of their early evolutionary divergence. However, in the present study detectable isozyme electrophoretic mobility differences between mouse and *M. rufogriseus* were not found for NP, PGM-1, PGM-2 and SOD-1 and the mobility difference for G6PD was small. Detectable mobility differences have been observed for these isozymes between different eutherian species e.g. human and mouse and human and hamster (Meera Khan, 1971; Harris and Hopkinson, 1976). It is somewhat surprising, therefore, that *M. rufogriseus* and mouse did not exhibit such mobility differences. It is possible for many mutations to have occurred in a gene resulting in changes in the amino acid sequence of an enzyme, without actually affecting the charge state of the enzyme to any great extent. Considering the large evolutionary distance between marsupial and eutherian mammals, this seems a more likely explanation than complete conservation of amino acid sequences in SOD-1, PGM-1, PGM-2 and NP in these two mammalian infraclasses.

5.4.5 Cross reaction of GA-1 with marsupial fibroblasts

Fibroblasts from representative species of a large range of marsupial families were assayed for binding of GA-1 (Table 4.14). GA-1 reacted with all species of the family Macropodidae that were tested and the only non-Macropodidae marsupial binding GA-1 was *Tarsipes spencerae* (honey possum), the sole member of the family Tarispedidae. The binding of GA-1 to these species demonstrates

a shared antigenic determinant and hence structural similarity of the relevant cell surface molecule. Although morphologically very different from the macropods, *Tarsipes spencerae* is unusual in that it resembles the Macropodidae species in a number of ways. First, the centromeric heterochromatin of all the *T. spencerae* chromosomes exhibits uncondensed regions in response to culture in the presence of Hoechst 33258 (Hayman and Sharp, 1982). The only other marsupial species showing such uncondensed regions belong to the family Macropodidae. Secondly, *T. spencerae* expresses nucleolar organiser regions on both the X chromosome and some autosomes, a feature previously found only in the super family Phalangeroidea (Hayman and Sharp, 1982). Hayman and Rofe (1977) presented evidence based on the number of nucleoli present in cells of some of the phalangeroid species, which suggested that the inactivation on the X chromosomes of these species does not involve the nucleolar organiser region. Thirdly, delayed implantation in marsupials has only been found in some macropods and *T. spencerae* (Renfree, 1980). There has been some controversy about the taxonomic affinities of *T. spencerae*. Both taxanomic and serological data indicate that *T. spencerae* is highly divergent from the rest of the marsupials (Kirsch, 1977). The binding of GA-1 to *T. spencerae* fibroblasts is another piece of supportive evidence for a close relationship of this species to those of the Macropodidae.

The only other cell types to react with GA-1 (Tables 4.17 and 4.18) were three *Potorous tridactylus* cell lines. *Potorous tridactylus* is also a member of the Macropodidae.

Cross reaction of a monoclonal antibody with antigens in different species demonstrates homology between those antigens and

hence a degree of similarity between species. The phylogenetic data, to date, indicate that *Tarsipes spencerae* diverged from the Macropodidae before Phoscolarctidae and Vombatidae diverged from the Macropodidae (Kirsch, 1977). The data for GA-1 in the present study support a much more recent divergence of the Macropodidae and Tarsipedidae (Table 4.14). It is unlikely that the GA-1 antigenic determinant evolved separately in the Macropodidae and Tarsipedidae because of the exact specificity required for binding of a monoclonal antibody. Assays for binding of GA-1 to cells of the Phalangeridae (which are known to be more closely related to the Macropodidae than the marsupials listed in Table 4.14), would be of interest as they may indicate if *Tarsipes spencerae* shows antigenic homology with this family as well as the Macropodidae.

The polymorphic region of most antigens probably constitutes only a small proportion of the entire antigenic molecule. It is probable therefore that most of the antibodies produced in response to xenogeneic immunization (which was used in this study) would be directed against strong species specific antigenic determinants, rather than polymorphic determinants. For example, less than 20% of monoclonals to HLA antigens so far characterised, recognise polymorphic determinants (Brodsky *et al.*, 1979; Lampson *et al.*, 1978). It is unlikely that GA-1 is polymorphic in *M. rufogriseus* on the basis of its specificity over the whole of the Macropodidae. As differentiated functions are generally not expressed in cell hybrids, perhaps the GA-1 antigen may be involved in a more fundamental or structural feature of the cells. A study of the tissue distribution of GA-1 may give further information about the function of the GA-1 antigen.

5.4.6 Myeloma x spleen fusions with HYP-1 and PGMR2-4 as immunogen

The difficulty encountered in isolating *M. rufogriseus* x mouse hybrids containing an identifiable *normal M. rufogriseus* X chromosome, prompted myeloma x spleen cell fusion experiments with different marsupial x mouse hybrids.

HYP1 is a somatic cell hybrid resulting from the fusion of PG19 with lymphocytes from an *M. rufogriseus* (♀) x *Wallabia bicolor* (♂) hybrid animal (♂) (Hope, unpublished). The female parent of this hybrid animal was *M. rufogriseus* and the hybrid animal therefore contained an *M. rufogriseus* X chromosome and a *W. bicolor* Y chromosome. This hybrid animal is particularly amenable to cytological study because of the difference in C-band content of the chromosomes of the two parental species. As previously described, all *M. rufogriseus* chromosomes possess large areas of centromeric C-banding as well as interstitial bands on the long arm of the X chromosome. The *W. bicolor* chromosomes are almost devoid of C-band material except for a small amount on the X chromosome (Hayman, 1980). PG19 chromosomes contain an intermediate amount of C-banding compared with the *M. rufogriseus* and *W. bicolor* chromosomes and can be distinguished from these two species using this criterion. In the HYP hybrids, therefore, chromosomes which expressed large areas of C-banded material were automatically identified as being of *M. rufogriseus* origin and those containing almost no C-banded material were identified as *W. bicolor* chromosomes. However, in view of the variable C-band expression observed for *M. rufogriseus* chromosomes (discussed in Sections 5.1 and 5.2), this approach for the identification of C-band material in the HYP hybrids may not have been entirely applicable. Nevertheless, the hybrid, HYP-1 was chosen as the immunising cell type on the basis

of preliminary cytogenetic C-banding of a series of HYP hybrids. Although HYP1 did not contain a normal *M. rufogriseus* X chromosome, the short arm of the *M. rufogriseus* X was identified by both C- and G-banding (Fig. 4.23). HYP1 also expressed *M. rufogriseus* PGK-A and HPRT (X-linked, see Section 5.1). Unfortunately none of the 36 hybrids generated from myeloma x spleen fusions with HYP1 as immunizing cells, secreted an antibody to HYP1 cells.

Another approach taken in the search for a marsupial X-linked antigen was an attempt to make monoclonal antibodies using an *M. rufus* x PG19 cell hybrid, PGMR2-4, as immunogen. C- and G-banding identified the presence of a normal *M. rufus* X chromosome in the majority of cells (Fig. 4.26). A conventional antiserum, SPGMR2-4, has already been made which binds to PGMR hybrids containing the *M. rufus* X chromosome and it has been proposed that SPGMR2-4 is detecting an *M. rufus* X-linked antigen (Sykes and Hope, 1978). Attempts to make monoclonal antibodies to *M. rufus* antigens on PGMR2-4 cells were unsuccessful. All 34 hybridoma supernatants screened from myeloma x spleen cell fusions with PGMR2-4 cells as immunogen were negative on PGMR2-4 cells. There was insufficient time available during this project to carry out further fusions with HYP1 and PGMR2-4 cells as immunogens. On the basis of the successful isolation of the GA-1 monoclonal antibody in the present study, it seems probable that monoclonal antibodies to antigens coded for by marsupial genes in HYP1 and PGMR2-4 cells could be made using the same methods.

5.5 GENERAL DISCUSSION AND FURTHER STUDIES

The gene determining the *M. rufogriseus* GA-1 antigen has been provisionally assigned to chromosome 3q in *M. rufogriseus*. The GA-1 antibody cross reacts with an antigen determined by a gene which has been provisionally assigned to *M. rufus* chromosome 5. Rofe (1978) has demonstrated the G-band homology of *M. rufogriseus* 3q and *M. rufus* 5. This chromosome homology suggests that the *M. rufogriseus* and *M. rufus* antigens detected by GA-1 are determined by homologous genes and that GA-1 is binding to a conserved antigenic determinant in these species. *Ldh-A* and the gene for GA-1 are syntenic in *M. rufus*, however LDH-A was not present in hybrids containing *M. rufogriseus* 3q. This was surprising in view of the close phylogenetic relationship of *M. rufogriseus* and *M. rufus*, and the apparent G-band identity of the two relevant chromosomes (discussed in section 5.4.4). It will be necessary to map many more gene loci to marsupial chromosomes in order to investigate in detail possible conservation of gene synteny both within the marsupials and with eutherian mammals. A first step may be to map GA-1 to chromosomes in other macropod species and *Tarsipes spencerae* to see if *Ldh-A* is syntenic with GA-1 expression in any of these species.

The non-random retention of *M. rufogriseus* 3q in this study, of *M. rufus* 5 in the study by Donald and Hope and of human chromosome 11 in human x rodent cell hybrids (Norum and Migeon, 1974) may be situations of some parity. *M. rufus Ldh-A* is on chromosome 5 and is syntenic with the gene for GA-1. The expression of human LDH-A is present in more human x mouse cell hybrids than expected on the

basis of random chromosome loss. *Ldh-A* is on human chromosome 11p and is syntenic with the *SA-1* locus (Kao *et al*, 1977). These results indirectly suggest that GA-1 may be the marsupial counterpart to the human SA-1 antigenic system. Evidence for or against such a speculation could be obtained in a number of ways. Cross reaction of SA-1 and GA-1 would be firm evidence for such a contention. The monoclonal antibody GA-1 did not bind to the human cell lines tested, although it is possible that GA-1 may bind to normal human fibroblasts or lymphocytes, as is the case for SA-1 antisera. Presumably, GA-1 binds to a single antigenic determinant which may not have been conserved since the divergence of marsupial and eutherian mammals even though GA-1 and SA-1 are homologous antigenic molecules. Since GA-1 only bound to cells from members of the Macropodidae and *Tarsipes spencerae* it is not surprising that GA-1 did not react with human cells. However, the polyclonal anti-SA-1 serum will probably contain antibodies to a number of antigenic determinants on the SA-1 antigen. If GA-1 is a counterpart to SA-1, reaction of SA-1 with marsupial cells and GA-1^{+} marsupial x mouse hybrids would indicate that at least one of the antigenic determinants on SA-1 may still be present on the GA-1 antigen. Similarly it may be more useful to use the polyclonal SREP3 AD PG19 serum rather than GA-1 to investigate cross-reaction with SA-1.

Other ways to compare the GA-1 and SA-1 antigens would be to evaluate their molecular weight, subunit structure, carbohydrate content etc. and investigate their tissue distribution. One preliminary study of the tissue distribution of GA-1 was carried out on kidney, heart and liver. The results suggested that GA-1 was present on kidney but the heart and liver preparations were not

scorable and need to be repeated. GA-1 was not present on red blood cells but was present on 20% of lymphocytes. SA-1 is present on red blood cells, fibroblasts and lymphocytes as is the case for the $A_L a1$ and $A_L a3$ antigens but the $A_L a2$ antigen is not present on red blood cells. (Buck and Bodmer, 1975; Jones *et al*, 1979). As was suggested in section 2.2, it is possible that the SA-1 antiserum was detecting a number of cell surface antigens determined by genes on human chromosome 11 as was found for the A_L series of antigens (Jones *et al*, 1979). On the basis of the known tissue distribution data for GA-1, *$A_L a2$* may be the most likely counterpart to *GA-1.*

Separation of the T and B lymphocyte subsets may identify if expression of GA-1 was confined to a particular subset. It would also be interesting to screen cell types during differentiation to determine the ontogeny of the expression of GA-1 on various cell types.

The SA-1 antibodies are cytotoxic.. Preliminary attempts to set up a reliable cytotoxic assay for GA-1 were plagued by technical problems. Further work along this line may enable the GA-1 antibody to be used as a selective system against cell hybrids containing the *M. rufogriseus* 3q. GA-1 is known to be of IgG_{2b} class, members of which are usually cytotoxic and so this idea seems feasible.

The fact that *M. rufogriseus* G6PD was not present in any of the marsupial x mouse cell hybrids was surprising (discussed in Section 5.3). It would be useful to screen the marsupial x mouse hybrids used in the present study for other enzymes, whose genes are known to be X-linked in other species, for example, α-galactosidase-A, 5-phosphoribosyl-1-pyrophosphate synthetase and steroid sulphatase. Such an approach would show if these genes have been conserved on the mammalian X-chromosome since marsupial-eutherian divergence. The

hybrids were not screened for α-galactosidase-A in the present study because the mouse isozyme is known to stain as a long anodal smear which completely envelops the area where the marsupial α-galactosidase-A stains on the gel. *M. rufogriseus* x hamster cell hybrids may be more useful here. Steroid sulphatase is known to be X-linked in man (Shapiro *et al*, 1979) and is of special interest because, like *Xg*, the steroid sulphatase locus is thought to escape X-inactivation. Investigation of the expression of these genes in marsupials should show if conservation of the gene content of the X chromosome in marsupials extends to genes which are not subject to X-inactivation. Hayman and Rofe (1974) found that the X chromosome of some marsupial species is smaller than the 'basic' X in humans (i.e. the original X chromosome of a common ancestor; 5-6% of the haploid gamete) postulated by Ohno (1967). This difference in size between the 'basic' X of marsupials and eutherians does not necessarily disagree with Ohno's hypothesis of conservation of the genetic content of the mammalian X chromosome. The size difference may, for example, be due to an increase of non-coding DNA in the human X rather than an increase in coding DNA. It could be postulated that the basic marsupial X does not contain genes homologous to those which escape inactivation on the short arm of the human X.

Screening of the hybrids used in the present study, for enzymes known to be encoded by autosomal genes in other species, may result in further gene assignments enabling comparison with syntenic groups of genes in eutherian mammals. Suggestions along these lines have been made in Section 5.4.4 but other possibilities for screening could include, for example, thymidine kinase and galactokinase, two enzymes known to be closely syntenic in a number of eutherian species (Section 2.4).

APPENDIX 1

FM Stock (P.N. Goodfellow, p.c.)

100 x concentration in 100 mls.

Bovine insulin	0.10g
Oxaloacetic acid	1.32g
Sodium pyruvate	0.45g

Add 1 ml FM stock/100 mls cell culture medium.

2 x SSC

Trisodium citrate	8.824g
Na Cl	17.532g

Made up to 1 litre with distilled water.

PBS (Phosphate-buffered saline)

NaCl	16.0g
K Cl	0.4g
Na_2HPO_4	2.3g
KH_2PO_4	0.4g

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

PENICILLIN-STREPTOMYCIN

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

FICOLL-HYPAQUE Stock

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

Warm Hypaque gently to dissolve crystals. Add Ficoll to Hypaque solution and stir. Add 34 mls distilled water

APPENDIX 1 (Cont.)

and stir. Sterilize by filtration. Store at room temperature in dark.

GEYS MEDIUM

Solution A: RPMI/Glutamine/P+S + 20% FCS

Solution B: RPMI/Glutamine/P+S/HAT + 20% FCS

Make up less than 30 mins. before use.

14.5 ml water

4 ml solution A

1 ml solution B

0.1 ml Biocarbonate (5.6% w/v)

Use 5 ml/spleen.

APPENDIX 2

PGK ELECTROPHORESIS BUFFER

0.1 M TRIS-CITRATE pH 8.6

Saturated citric acid 60g/100 ml

0.1 M Tris 12.1g/litre

Add saturated citric acid to 0.1 M Tris until pH 8.6.

Store at room temperature.

PGK STAIN (adapted from Meera Khan (1971))

1.2 ml 0.1 M Tris-HCl-EDTA pH 8.0

0.1 ml $MgCl_2$ (40 mg/ml)

0.1 ml NADH (9.9 mg/ml)

0.3 ml ATP (24.2 mg/ml)

0.3 ml PGA (7.4 mg/ml)

30µl G3PD (800 I.U./ml)

Counterstain with 0.2 ml PMS (2 mg/ml)

0.2 ml MTT (2 mg/ml)

G6PD ELECTROPHORESIS BUFFER

TRIS-GLYCINE pH 9.1 (Migeon *et al*, 1979)

Add 14.1g Tris to 22.6g glycine and make up to 1 litre with distilled water.

G6PD STAIN (Adapted from Johnston *et al*, (1975))

1 ml Tris-maleate buffer pH 8.0

0.2 ml NADP (5mg/ml)

0.2 ml G6P (25mg/ml)

0.2 ml MTT (2mg/ml)

0.2 ml PMS (2mg/ml)

APPENDIX 2 (Cont.)

NP STAIN (modification of Spencer *et al*, (1968))

2.0 ml 0.1 M Tris-HCl pH8.0

0.2 ml Inosine (25µg/ml)

0.2 ml 0.05 M sodium arsenate

0.2 ml MTT (2mg/ml)

0.2 ml PMS (2mg/ml)

5µl Xanthine oxidase 0.2 I.U. (Boehringer)

EsA_4 STAIN (modification of Markert and Hunter (1958))

2 ml 0.05 M phosphate buffer pH 6.5

0.8mg Fast blue RR

0.1ml 1% stock solution of α-naphthal acetate in 50% acetone.

PGM-3 STAIN (modification of Meera Khan (1971) and Harris and Hopkinson (1976))

0.8 ml 0.05 M Tris/HCl pH 8.0

0.2 ml 0.1 M Mg Cl

0.2 ml NADP (4mg/ml)

0.2 ml G-1-6 di P (1mg/6ml)

0.4 ml G-1-P (16mg/ml)

5µl G6PD (1mg/ml)

0.2 ml MTT (2mg/ml)

0.2 ml PMS (2mg/ml)

LYSIS SOLUTION

0.1% β mercaptoethanol

0.1% Triton X-100

1.19mM NADP.

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