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GENE ASSIGNMENT:

INTERSPECIFIC SOMATIC CELL HYBRIDIZATION

Maria do Rosário N. dos Santos

Submitted in fulfilment of the requirements
for the Degree of Master of Science, in the
Faculty of Science, University of the Witwatersrand,
JOHANNESBURG.

1986

DECLARATION

I hereby declare that this is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

.....

9th day of October 1986

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LIST OF ABBREVIATIONS

ACD	Citric acid and dextrose (anti-coagulant)
ACP 1	Red cell acid phosphatase
8-AG	8-azaguanine
APRT	Adenine phosphoribosyl transferase
APRT[-]	APRT-deficient
ATP	Adenosine triphosphate
BR2	A mouse TK[-] cell line
B95-8	An EBV-secreting Marmoset cell line
BrdU	Bromodeoxyuridine
°C	Degrees Centigrade
$CaCl_2$	Calcium chloride
C-bands	Centromeric banding pattern
Cl.1D	A mouse TK[-] cell line
cm(s)	Centimetre(s)
CMST	Chromosome-mediated gene transfer
dCD	Deoxycytidine deaminase
dCK	Deoxycytidine kinase
del	Deletion of chromosomal material
der	Derivative chromosome
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dup	Duplication of chromosomal material
EBV	Epstein-Barr Virus
E- group	Human chromosome numbers 16, 17 and 18.
FBS	Foetal bovine serum
G-11	Giemsa-11 (staining technique)

G-bands	Giemsa banding pattern
G-group	Human chromosome numbers 21, 22 and Y
HAT	Hypoxanthine/aminopterin/thymidine
HATO	Hypoxanthine/aminopterin/thymidine/ouabain
HCℓ	Hydrochloric acid
H-F	Hypaque-ficoll
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
HGPRT[-]	HGPRT-deficient
hr(s)	Hour(s)
HT	Hypoxanthine/thymidine
i.u.	International units
kb	Kilobase(s)
KCℓ	Potassium chloride
KOH	Potassium hydroxide
µg	Microgram(s)
µℓ	Microlitre(s)
µm	Micrometre(s)
M	Molar
mat	Maternal chromosome
mg	Milligram(s)
min(s)	minute(s)
mℓ(s)	Millilitre(s)
mm	Millimetre(s)
Mol.wt.	Molecular weight
N	Normal
NaCℓ	Sodium chloride
NaOH	Sodium hydroxide
Na_2HPO_4	*di*-Sodium hydrogen phosphate

NH_4Cl	Ammonium chloride
NOR	Nucleolar organizing region
NP	Nucleoside phosphorylase
p	Short arm of chromosome
pat	Paternal chromosome
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
PRPP	Phosphoribosyl pyrophosphate
q	Long arm of chromosome
Q-bands	Quinacrine banding pattern
RAG	A mouse HGPRT[-] cell line
R-bands	Reverse banding pattern
rec	Recombinant chromosome
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
R.T.	Room Temperature
sec	Second(s)
SRO	Smallest region of overlap
SSC	Sodium chloride + *tri*-sodium citrate
SV40	Simian Virus 40
t	Translocation
ter	Terminal end of chromosome
TG	Thioguanine
TK	Thymidine kinase
TK[-]	TK-deficient
UMPK	Uridine monophosphate kinase
UV	Ultraviolet light
wg3-h	A Chinese hamster HGPRT[-] cell line.

ABSTRACT

A multiple chromosome mapping panel was constructed with hybrids isolated from various human-rodent somatic cell hybridization experiments. The rodent parental cells used were from the mouse permanent lines RAG (HGPRT[-]), C1.1D (TK[-]) and B82 (TK[-]) and the Chinese hamster permanent line wg3-h (HGPRT[-]). The human parental cells were obtained from amniotic fluid samples, skin biopsies or peripheral blood specimens, presenting normal or abnormal karyotypes.

Reliable fusion protocols were established for fusions involving the different combinations of parental cell types. Preliminary cytogenetic characterization of the resulting hybrid cell lines was carried out by means of sequential G- and Hoechst 33258-banding. A reliable modification for the G-11 differential staining technique was then established, and the lines were subsequently analysed with the aid of the sequential G-banding → G-11 banding techniques.

Of the twenty-nine hybrid cell lines isolated and analysed, a few combinations could be used to assemble multiple chromosome mapping panels. The best combination consisted of nine cell lines which provided unique bimodal signatures for all human chromosomes, with the exception of number 11 and X which shared the same pattern of presence and absence. This hybrid panel will be used in the preliminary assignments of random, polymorphic, single-copy DNA sequences to specific human chromosomes.

1. INTRODUCTION

1.1 Gene Mapping

1.1.1 Definition

Mapping the human genome entails not only the assignment of genes to one of the twenty-four nuclear chromosomes, but also the linear ordering of genes in relation to one another and concomitantly their localization to a specific region on a particular chromosome.

1.1.2 General Principles of Gene Mapping

The main strategies employed in the assignment and regional localization of genes are (i) family pedigree analysis and/or linkage studies, based on classical Mendelian genetics, and (ii) parasexual genetics in the form of (a) gene dosage studies, (b) somatic cell hybridization, (c) fluorescence-activated chromosome sorting, and (d) *in situ* hybridization.

1.1.2.1 Family Linkage Studies

Family studies have led to the assignment of a number of genes to specific chromosomes. X-linked genes are assigned after studying the characteristic mother to son (and possibly father to daughter) pattern of inheritance in a pedigree, while autosomal gene assignments rely on the segregation of a particular trait with a serological, biochemical or chromosomal variant over several

generations. In this manner, the first human gene to be mapped was the colour blindness trait, assigned to the X-chromosome by Wilson, in 1911, and thereafter the demonstration of linkage with haemophilia (see McKusick and Ruddle, 1977). Examples of autosomal gene assignments using family studies, are the Duffy blood group, which was found to be linked to a heterochromatic size variation on chromosome 1q designated 1qh, commonly referred to as the "uncoiler" locus (Donahue *et al.*, 1968), and the linkage of the nail-patella syndrome with the ABO blood group system (see McKusick and Ruddle, 1977). Between 1911 and 1967, this approach was responsible for the assignment of approximately one hundred genes to the X-chromosome and a few to specific autosomes.

Family linkage studies have obvious drawbacks. Firstly, there is a need for dominant or co-dominant inheritance, and allelic variation of the trait as well as the linked marker is necessary. Furthermore, this strategy is limited by the relatively small number of siblings in human pedigrees and the long generation time involved.

1.1.2.2 Parasexual Genetic Methodologies

(a) Gene Dosage Mapping:

Chromosomal deletions and duplications may be used in gene assignments and regional mapping, by quantitatively assaying enzyme activity levels. The principle of this gene dosage mapping method is the following: Assuming that the expression of two normal alleles gives rise to a theoretical enzyme activity of 100%,

the monosomic state (deleted chromosome) would then provide only 50% enzyme activity, while the trisomic state (duplicated chromosome) would result in 150% enzyme activity. This approach has led to the assignment of several genes, including red cell acid phosphatase to 2p23→2pter (Ferguson-Smith *et al.*, 1973), LDH B to 12p (Mayeda *et al.*, 1974) and the Rhesus locus to 1p (Marsh *et al.*, 1974).

A significant drawback to this approach is that enzyme activity does not always reflect gene dosage. This may be due to regulatory mechanisms, such as feedback inhibition, controlling the expression of certain enzymes. An example of an incorrect assignment using this method, is that of galactose-1-phosphate-uridyltransferase to chromosome 3 (Allderdice and Tedesco, 1975), which was later correctly assigned to chromosome 9, using interspecific somatic cell hybrids (Mohandas *et al.*, 1979).

(b) Somatic Cell Hybridization:

The number of gene assignments increased exponentially with the advent of interspecific somatic cell hybridization. The first assignment made using this approach, was that of thymidine kinase to human chromosome 17 (Migeon and Miller, 1968; Miller *et al.*, 1971). To date, at least 85 percent of all mapped human genes have been assigned with the aid of this technique.

As a result of the more than eighty million years of evolutionary divergence between man and rodent, virtually every gene or gene product identified in human-rodent somatic cell hybrids can be

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As a result of the more than eighty million years of evolutionary divergence between man and rodent, virtually every gene or gene product identified in human-rodent somatic cell hybrids can be

distinguished as being either human- or rodent-derived (Shows *et al.*, 1982). Since these hybrids retain only a few human chromosomes in random combinations, assignments can be made by correlating the presence or absence of a particular human genomic sequence, or gene product, with the presence or absence of a specific human chromosome. Once an assignment has been made, the gene may be localized to a particular region of that chromosome by means of a subchromosomal mapping panel. This consists of a collection of hybrids, each containing different segments of the chromosome to which the gene has been assigned. Following the same principle as in chromosomal assignments, only one region of the relevant chromosome would unambiguously correlate with the distribution of the gene in question.

(c) Fluorescence-activated Chromosome sorting:

Human mitotic chromosomes have been successfully resolved and sorted into discrete size groups by means of the fluorescence-activated cell sorting technique, which is in fact the "mechanical" counterpart of somatic cell hybridization. The procedure is particularly useful for regional mapping; translocations alter the size of the chromosomes in a predictable way, and their respective size classes are readily distinguished from the normal sorting pattern. This approach has enabled the regional localization of human β-, γ- and δ-globin genes to the distal portion of 11p (Lebo *et al.*, 1979). Although it promises to add another dimension to gene mapping methodologies, the full potential of this approach is yet to be realized. Current drawbacks include

the long sorting time for the recovery of sufficient material, the high cost of the sorting equipment, and the inability to clearly resolve and separate chromosomes of the same size (especially the D-group chromosomes).

(d) *In situ* Hybridization:

In situ hybridization, initially used to locate amplified single-copy genes in *Drosophila* polytene chromosomes, has recently been refined to detect unamplified single-copy genes. (Malcolm *et al.*, 1981; Harper *et al.*, 1981; Harper and Saunders, 1981; Harper *et al.*, 1982). This technique, which involves the hybridization of ^{3}H-labelled DNA probes to mitotic chromosome preparations, has made a substantial contribution to the mapping of human genes and single-copy DNA fragments. Statistical analysis of the results not only provides a chromosomal assignment, but also reveals the precise region of the sequence in question.

1.1.3 The Use of Human DNA Polymorphic Markers

Gene mapping should be particularly accelerated through the mapping of restriction fragments involving single-copy DNA sequences that are highly polymorphic within the human species. Botstein and co-workers first described the basis of a genetic linkage map using polymorphic loci detected by random single-copy DNA probes. The establishment of linkage relationships among restriction fragment length polymorphisms (RFLPs) should eventually lead to a set of well-spaced polymorphic markers covering the entire human genome (Botstein *et al.*, 1980). Numerous polymorphic

loci have already been detected by such single-copy DNA probes (Wyman and White, 1980; Shows *et al.*, 1982; Naylor *et al.*, 1984).

As these random markers are isolated, they are being tested for linkage with disease conditions segregating in members of families. This immediate application is of great importance; the RFLP need not encompass the disease locus in order to be useful, and the gene itself does not have to be isolated in order to be mapped. The clinical application of such linkage relationships has been well illustrated in reports of prenatal diagnosis in families at risk for sickle cell anaemia (Kan and Dozy, 1978a,b; Phillips *et al.*, 1980) and certain types of thalassemia (Kazazian *et al.*, 1983). Other polymorphic markers which have already been linked with disease loci include those associated with the X-linked Duchenne muscular dystrophy (Murray *et al.*, 1982), Becker muscular dystrophy (Kinston *et al.*, 1983), X-linked retinitis pigmentosa (Bhattacharya *et al.*, 1984), Huntington's disease (Gusella *et al.*, 1983), fragile X-linked mental retardation (Camerino *et al.*, 1983) and cystic fibrosis (Knowlton *et al.*, 1985; White *et al.*, 1985; Wainwright *et al.*, 1985).

However, single polymorphic sites are not always informative in families at risk for a given disease; the availability of various closely linked polymorphic markers increases the probability of detecting the marker combinations within a family. The search for multiple RFLPs in the vicinity of a given locus is exemplified by Gusella's group, who have isolated and mapped numerous random, polymorphic single-copy sequences on human chromo-

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some 11, from cloned gene libraries of a man-mouse hybrid line carrying only that human chromosome (Gusella *et al.*, 1980).

To date, over 500 polymorphic DNA markers have been reported (de la Chapelle, 1985). In addition to predicting the transmission of a defective gene to offspring, these may enable the eventual isolation of the gene and subsequent identification of the molecular defect.

1.2 Somatic Cell Hybridization

1.2.1 Historical Review

As early as 1960, Barski, Sorieul and Cornefert had reported that occasionally cell fusion occurred, when two different mouse cell lines were mixed in culture. They were able to show that the fusion products contained a summation of the two parental karyotypes. Similar observations by other groups of workers confirmed the occurrence of fusion and provided further evidence for the hybrid nature of these cells (Gershon and Sachs, 1963; Defendi *et al.*, 1964). It was soon realized that this phenomenon could be exploited to provide a valuable tool for the genetic analysis of mammalian cells; and so dawned the era of important technical developments in the field of somatic cell hybridization.

The first major contribution was made by John Littlefield, who established a selective system for the isolation of somatic cell hybrids (Littlefield, 1964, 1966). After the demonstration that

mutant cells defective in specific enzymes could be obtained by subjecting a normal cell population to selection with drugs (Szybalski *et al.*, 1962), Littlefield obtained two sublines of the permanent mouse L-line, carrying different drug-resistance markers. Hybrids of these two sublines were isolated following the hypoxanthine/aminopterin/thymidine (HAT) selection system devised by Szybalski's group. These selective conditions would allow proliferation of hybrids but not parental cells (see Section 1.2.2.1).

Littlefield's system was soon used as a model in several other laboratories, with similar drug-resistance markers being introduced into different permanent mouse cell lines.

The next significant technical development came from Davidson and Ephrussi in 1965, with the establishment of the "half-selective" system. This modification of Littlefield's system permitted the isolation of hybrids between drug-resistant permanent cell lines and normal diploid cells. The parental mutant cells could be eliminated using HAT medium, while the parental diploid cells, which were relatively slow-growing, could be distinguished as thin colonies compared to the thicker colonies characteristically formed by hybrid cells (Davidson and Ephrussi, 1965).

In the same year, three independent groups demonstrated that cells from different animal species could be fused to produce hybrids (Harris and Watkins, 1965; Okada and Murayama, 1965; Ephrussi and Weiss, 1965). In their experiments, Harris and

Watkins, and Okada and Murayama introduced the usage of UV-inactivated Sendai virus as a fusogen. The use of Sendai virus became the standard method of fusion when, a year later, it was shown that the resulting heterokaryons could generate long-lived hybrid cell lines (Yerganian and Nal, 1966). Another important discovery made at this stage, was that both parental genomes were expressed in the hybrid cells (Weiss and Ephrussi, 1966a,b).

Up to this point then, it had been shown that fusion could take place between different permanent aneuploid mouse cell lines as well as between these and diploid lines, and that the resulting hybrids were capable of indefinite proliferation. The persistence of two functionally active genomes in these hybrids made it possible to establish allelism, dominance, co-dominance, recessiveness and complementation of various genetic traits.

The stage was set for what could be considered the most exciting finding in the history of somatic cell hybridization. After effecting fusion between a TK[-] mouse line known as Cl.1D and a human diploid cell line denoted WI-38, Weiss and Green found that the resulting interspecific hybrids rapidly and preferentially lost most of the human chromosomes (Weiss and Green, 1967). This segregation phenomenon, not observed in intraspecific hybrids, now enabled the mapping of human genes in human-mouse hybrids by correlating the expression of a particular trait with the retention of a specific human chromosome.

Indeed, one of the clones isolated from this experiment led to

the assignment of the thymidine kinase (TK) gene. This particular clone had lost all but three human chromosomes during continuous culture in HAT medium. Since the mouse parental cell line was deficient for thymidine kinase, it could be assumed that the gene was carried on one of the three human chromosomes retained in these hybrid cells. Counterselection was performed by growing the cells in medium supplemented with BrdU. Under these conditions, only those cells which had lost the numan TK-encoding chromosome, and were subsequently BrdU-resistant, could survive. The chromosome identified in this manner was first thought to be a member of group C (Weiss and Green, 1967), but was then shown to be an E-group chromosome (Migeon and Miller, 1968). With the aid of the quinacrine fluorescence banding technique for the identification of human chromosomes (Caspersson *et al.*, 1971a,b; Paris Conference, 1971), this E-group chromosome was later identified as number 17 (Miller *et al.*, 1971).

1970 saw the beginning of several attempts to discover a suitable fusing agent which could replace Senda[illegible]rus, thereby eliminating the risk of infection from incompletely inactivated virus. After being introduced as a regular agglutinating and fusing agent for plant protoplast formation (Bonnett and Eriksson, 1974; Constabel and Kao, 1974; Kao and Michayluk, 1974), polyethylene glycol was tested as an animal cell fusogen (Pontecorvo, 1975). The results were very encouraging; the efficiency of fusion with polyethylene glycol proved to be many times higher than that obtained with inactivated Sendai virus (Pontecorvo, 1975; Davidson and Gerald, 1975; Davidson *et al.*, 1976).

Before polyethylene glycol was introduced as a regular fusogen in somatic cell hybridization, several independent groups had already begun to use human-rodent hybrids routinely for gene mapping studies. The difference in electrophoretic mobility between many homologous enzymes of man and rodent was exploited, providing a means of correlating defined human gene products with the presence of specific human chromosomes. This led to tremendous progress in gene mapping, with the result that by 1972 some fifty human genes had been reliably assigned to specific whole chromosomes (Ruddle 1972).

This, however, remained a rather crude form of mapping, and more specific localization was required. Human parental cells containing deleted or translocated chromosomes proved to be an important source of material for such regional mapping (Grzeschik *et al.*, 1972; Ricciuti and Ruddle, 1973; Gerald and Brown, 1974; Pearson *et al.*, 1974; Mohandas *et al.*, 1979). A second source was in the form of spontaneous *in vitro* chromosomal rearrangements such as intra- and interspecific translocations, that often occur during hybrid stabilization (Boone *et al.*, 1972; Elsevier *et al.*, 1974; Hamerton *et al.*, 1975). The "disruptive strategy", first employed by Goss and Harris, where massive chromosome fragmentation and rearrangement is induced with chemical or physical mutagens, has also contributed to high resolution mapping. The treatment may be applied either to one of the parental cell lines (Goss and Harris, 1975, 1977a,b; Law and Kao, 1978), or to the hybrids themselves (Burgerhout *et al.*, 1977; Burgerhout, 1978).

Somatic cell hybrids have been advantageously used in combination with recent advances in the field of recombinant DNA technology. In particular, the cloning of genes, their insertion into vectors and amplification in suitable hosts, gave rise to specific DNA probes which could be hybridized to chromosomal DNA by restriction endonuclease digestion and Southern blot techniques. This permitted rapid detection of the presence of specific gene sequences without the need for their expression in hybrid cells, whereas previously only biochemically defined gene products could be studied. The human α- and β-globin structural gene sequences were the first to be mapped using a combination of somatic cell hybridization and molecular genetic techniques (Deisseroth *et al.*, 1977, 1978). Other gene assignments made with the aid of these techniques include those of the insulin, prolactin and proopiocortin genes, and the growth hormone and interferon gene complexes (Owerbach *et al.*, 1980a,b, 1981a,b,c). More recent assignments include that of the interleukin-1 gene and a cystic fibrosis antigen gene (see Human Gene Mapping 8, 1985). The establishment of hybrid panels also enabled the mapping of numerous random single-copy DNA fragments that reveal polymorphic sites (Naylor *et al.*, 1984).

All these chromosomal assignments of cloned DNA sequences may be confirmed by *in situ* hybridization, which also provides data on their regional localization. For regional localization alone, the use of an appropriate hybrid line will facilitate the *in situ* analysis, since relatively few human chromosomes will then require scrutinizing (Ruddle, 1981; Kamarck *et al.*, 1984). However, grain scatter (or non-specific hybridization of the probe)

limits *in situ* hybridization resolution to approximately 10 000 kb (10 centiMorgans). It is believed that chromosome deletions and translocations have a potential resolution of between 1-2 orders of magnitude less (Ruddle, 1981), making subchromosomal mapping panels more definitive and accurate as regional mapping tools.

1.2.2 Selection Systems and Isolation of Hybrid Cells

The phenomenon known as "hybrid vigour", where hybrid cells proliferate more rapidly than either of the parental cell types, was responsible for the initial discovery and isolation of hybrid cells (Barski *et al.*, 1961). However, this occurs very rarely, and usually it is the unfused parental cells which outgrow the hybrid cells. Nonetheless, it is possible to isolate hybrid cells from a cell mixture by nonselective means. This entails the expansion of an array of randomly isolated single cells, from which clones with a hybrid karyotype are then selected (Scaletta and Ephrussi, 1965). Although hybrid cells can sometimes be distinguished from the parental cells on the basis of morphology, this procedure remains laborious, and the use of selective systems developed over the years, is a far easier way to isolate hybrids.

Non-specific selection, based on differences in the inherent properties of animal cells derived from different sources, may be used in the isolation of hybrid cells. These differences are found in the form of temperature optima for growth, nutri-

tional requirements, ability to grow in suspension, adhesiveness or "anchorage dependence", sensitivity to contact inhibition, and senescence phenomena. These, however, are properties for which the molecular background remains undefined to a large extent, hence the advantage of using more specific selection mechanisms.

1.2.2.1 Selection of Hybrids Using Drug Resistant Markers

Drug resistant cells arise spontaneously in cell cultures, but the mutation rate may be increased by applying X-irradiation or chemical mutagens (Szybalski *et al.*, 1962).

On the basis of these observations, Littlefield (1964, 1966) developed the first selective system for the isolation of hybrid cells, using parental cells which were resistant to azaguanine (AG^R) or thioguanine (TG^R), and parental cells resistant to bromodeoxyuridine ($BrdU^R$). These drugs will kill normal cells, because they are metabolized and converted to "abnormal" nucleotides. In the case of AG and TG, this effect is mediated by the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), while the enzyme thymidine kinase (TK) is responsible for the phosphorylation and incorporation of BrdU. Mutant cells which lack the enzyme HGPRT are therefore resistant to AG and TG, and those lacking the enzyme TK are resistant to BrdU.

In Littlefield's system, two sublines of the mouse L-line were used; one resistant to 8-azaguanine (i.e. HGPRT[-]) and the other resistant to 5'-bromodeoxyuridine (i.e. TK[-]). Hybrids between

these sublines were isolated using the hypoxanthine/aminopterin/thymidine (HAT) medium devised by Szybalski *et al.* (1962).

The basis of the HAT selection system is the following: Mammalian cells have the choice of two pathways for purine synthesis; the *de novo* pathway whereby purines are synthesized from amino acids and sugars, and the salvage or "scavenger" pathway where these nucleotides are synthesized from the preformed nucleosides hypoxanthine and thymidine. The *de novo* pathway is blocked by the addition of aminopterin, which prevents the conversion of dihydrofolate to tetrahydrofolate by inhibiting dihydrofolate reductase activity. The cofactor tetrahydrofolate is essential for purine synthesis, methylation of dUMP to dTMP and conversion of serine to glycine (Hitchings and Burchall, 1965; Kiebe *et al.*, 1970). In the presence of aminopterin, and provided that exogenous hypoxanthine, thymidine and glycine are available, the cells resort to the salvage pathway for purine synthesis. This, however, requires the simultaneous presence of HGPRT and TK for phosphorylation and subsequent incorporation of the base analogues (Figure 1.1). Thus, in Littlefield's experiments, the enzyme deficient parental cells would die in HAT medium, while hybrids between these two would grow unhampered due to intergenic complementation for HGPRT and TK.

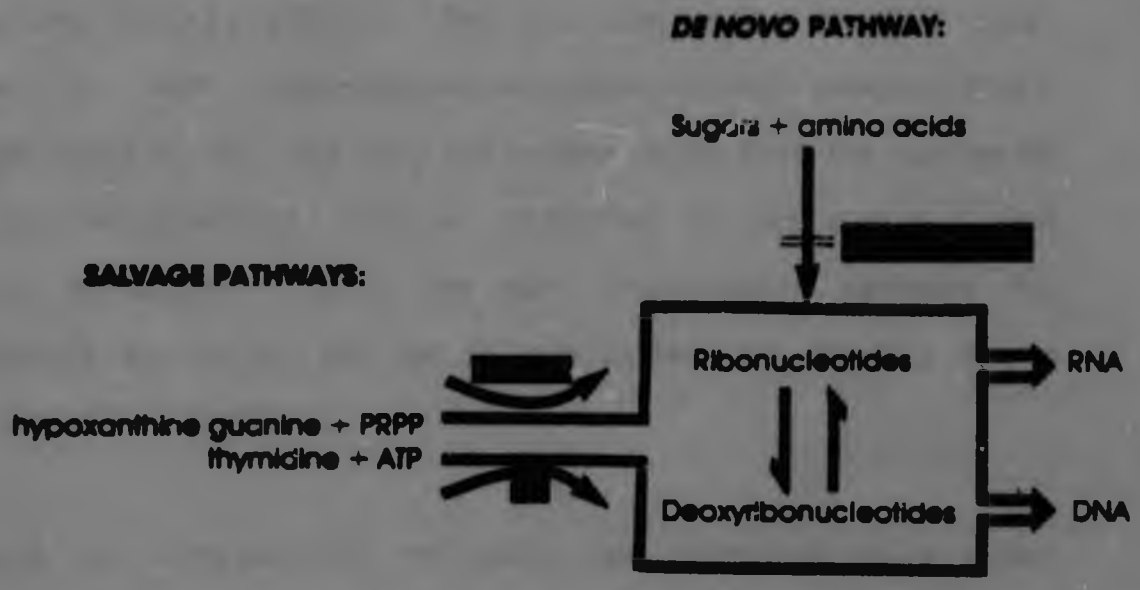


FIGURE 1.1 Diagrammatic representation of the de novo and salvage pathways for nucleotide synthesis in mammalian cells (Modified from Ringertz and Savage, 1976).

Other selection systems, based on the fusion of drug resistant cells and selection in special media, emerged later. One such system, which operates in much the same way as does the HAT system, involves the use of cells deficient in adenine phosphoribosyl transferase (APRT[-]), obtained after selection with fluoroadenine, instead of HGPRT[-] cells (Kusano *et al.*, 1971). Hybrid cells have also been obtained from mixtures of two diploid Chinese hamster lines, one highly resistant to amethopterin and the other to actinomycin D (Sobel *et al.*, 1971). These hybrids were selected in medium containing elevated concentrations of both drugs.

The system devised by Chan *et al.* (1975), makes use of an HGPRT[-] parental line and a double-mutant parental cell line which is both deoxycytidine kinase deficient and deoxycytidine deaminase deficient (dCK[-], dCD[-]). The selective medium in this case, known as HAM (hypoxanthine/aminopterin/methyl-deoxycytidine), is analogous to HAT, the only difference being that the nucleoside 5-methyl-deoxycytidine replaces thymidine in the medium. Here again, aminopterin blocks the main biosynthetic pathways for nucleotide synthesis, and the salvage pathway is the only option for pyrimidine synthesis.

Ouabain (or strophanthin) resistance was introduced as a selective marker for the first time in 1974. This drug, a cardiac glycoside, inhibits the membrane-mediated ATPase-dependent potassium intake and sodium output (Kucherlapati *et al.*, 1975a). In the system suggested by Jha and Ozer (1976), only one of the parental lines is "marked", carrying both azaguanine and ouabain resistance. The great advantage of this method lies in the fact that the other parental cell line need not be a mutant, since animal cells are normally ouabain-sensitive. Hybrids arising from such crosses will therefore be able to grow on HAT medium supplemented with ouabain, whereas cells from both parental lines will die.

A most valuable finding which emerged from the above studies was the differential sensitivity of human and rodent cells to ouabain. Both mouse and Chinese hamster permanent cell lines show sensitivity only at concentrations of at least 1×10^{-3} M ouabain, whereas early-passage human fibroblast lines are sen-

sitive at concentrations as low as 3-10 x 10^{-8} M (Baker *et al.*, 1974; Kucherlapati *et al.*, 1975a).

Prior to this finding, "half-selective" systems had been used in the isolation of hybrids between heteroploid permanent rodent cell lines and normal diploid human cells (Davidson and Ephrussi, 1965). Human late-passage fibroblasts could be used in such crosses, since their nuclei, if unfused, would remain in the G1 stage of the cell cycle (Norwood *et al.*, 1975). Alternatively, lymphocytes could be used as the human parental cell type, since these are non-dividing, non-adherent cells which would eventually be lost from the culture. With ouabain selection it now became possible to eliminate unfused human fibroblasts, regardless of age, by simply including the drug in the selective medium at a concentration which would kill the human but not the rodent cells.

1.2.2.2 Selection of Hybrids Using Auxotrophic Mutants

Several methods are available for the isolation of auxotrophic mutants. These methods, first developed for bacteria, have been adapted for eukaryotic cells and have led to the isolation of mutants that require either a specific amino acid, carbohydrate, purine or pyrimidine (Kao and Puck, 1967, 1968, 1970, 1972a, b; Kao *et al.*, 1969a, b; Chu *et al.*, 1969, 1972).

The most commonly used method of auxotroph isolation is that described by Kao's group (Kao and Puck, 1967). Here, the cells

are allowed to grow rapidly on minimal medium which lacks an amino acid. Under these conditions, normal cells will proliferate, whereas any spontaneously-arising mutant cells, which cannot synthesize the missing [illegible] acid, will be unable to grow. When BrdU is subsequently added to the culture, it is only incorporated into the DNA of the actively-growing non-mutant cells. Since BrdU renders the DNA highly sensitive to light-induced chromosome breakage, these normal cells can be selectively killed by irradiating the culture with UV light. Alternatively the normal cells can be eliminated by the addition of ^{3}H-thymidine (Siminovitch, 1974). The surviving auxotrophic mutants are then rescued and shifted to an enriched medium.

Auxotrophic markers not only provide a mechanism for the isolation of hybrids, (illustrated in Figure 1.2), but also facilitate many kinds of experimental approaches to the study of linkage. One such approach is well demonstrated in the experiment conducted by Kao and Puck (1972b). Mutants isolated from a subline of the Chinese hamster line CHO, designated CHO-K1, which were doubly deficient either for inositol and proline (*ino*$^{-}$, *pro*$^{-}$) or for glycine and proline (*gly*$^{-}$, *pro*$^{-}$), were fused with normal human cells. The mixed cultures were placed in medium containing dialysed foetal calf serum (to inhibit the growth of human parental cells), and supplemented with proline but no inositol in the first case, and with proline but no glycine in the second. Hybrid clones, which were *ino*$^{+}$ and *gly*$^{+}$ as a result of the selective system, were expanded and tested for their proline requirement. Results showed that these hybrids could be either *pro*$^{+}$ or *pro*$^{-}$,

indicating that there was no close linkage between proline and glycine or between proline and inositol.

A similar approach is to isolate the hybrids in medium containing either one or the other metabolite required to compensate for the nutritional deficiencies of the doubly mutant Chinese hamster parental cells. If clones grown in the two different media have only one chromosome in common, it can be said that the genes causing the double auxotrophy are syntenic (i.e. on the same chromosome).

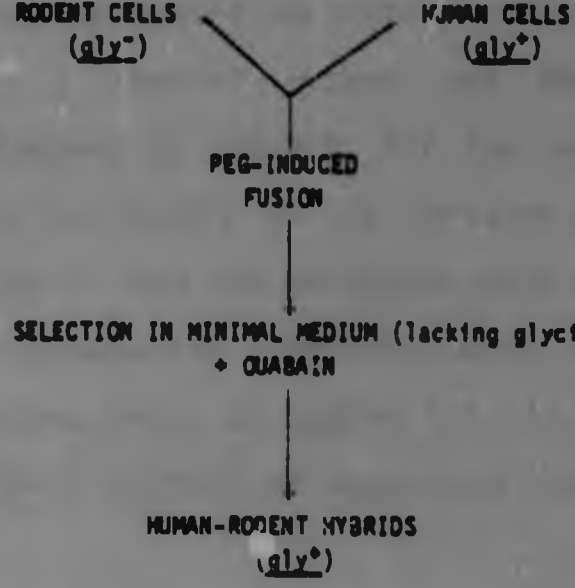


FIGURE 1.2: Scheme for isolation of human-rodent cell hybrids using auxotrophic mutants. In this example, a gly⁻ rodent cell line is used, which cannot grow on minimal medium. Ouabain is added to eliminate human parental cells. Those hybrids which retain the human glycine-encoding chromosome will survive.

1.2.2.3 Selection of Hybrids Using Temperature Sensitive Mutants

The optimal temperature for mammalian cell growth in culture is 37°C, the permissive temperature ranging from 32°C to about 40°C. Temperature sensitive (ts) mutants have been isolated, which are unable to grow at either the higher or the lower permissive temperatures ("hot-sensitive" and "cold-sensitive" mutants respectively) (Naha, 1969; Thompson *et al.*, 1970, 1971, 1975; Siminovitch, 1974, 1976).

Temperature sensitive rodent mutants are isolated by methods analogous to those employed in the selection of auxotrophs. Cells are exposed to a chemical mutagen and then grown at a non-permissive temperature (38-39°C for the isolation of hot-sensitive mutants and 33-34°C for the isolation of cold-sensitive mutants). Here again, only the non-mutant cells will proliferate, and these are subsequently eliminated after incorporation of BrdU or ^{3}H-thymidine (refer to Section 1.2.2.2). The ts mutants are then recovered by shifting the temperature back to a permissive level.

As well as providing yet another mechanism for hybrid isolation (as shown in Figure 1.3), these mutants may be used for the selective retention of specific whole human chromosomes and their translocation derivatives.

When propagated at the non-permissive temperature, hybrids between

ts rodent cells and normal human cells should retain the chromosome (or chromosomes) carrying the genes necessary to correct the lesion. In view of the wide variety of biochemical functions which may be affected when isolating ts mutants, it should be possible to map many human gene loci by studying the patterns of human chromosome segregation in such hybrids, even when the biochemical nature of the mutation is not known. In this way several traits have been assigned, including a cell division block to chromosome 3 (Ming *et al.*, 1976), a leucyl-tRNA synthetase deficiency to chromosome 5 (Giles *et al.*, 1977) and a DNA synthesis lesion to the X-chromosome (Giles and Ruddle, 1979).

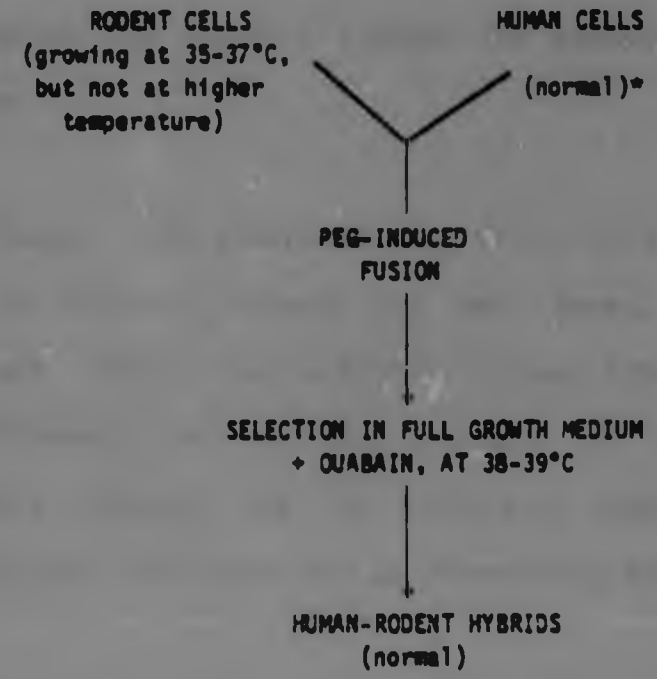


FIGURE 1.3: Scheme for isolation of human-rodent cell hybrids using temperature sensitive mutants. In this example, a "hot"-sensitive rodent cell line is used, which cannot grow at the non-permissive temperature of 38-39°C. Ouabain is added to eliminate human parental cells.

*able to proliferate at a wider temperature range (‡ 35-39°C).

1.2.3 Agents for Cell Fusion

Initially, the isolation of somatic cell hybrids was totally dependent on infrequent spontaneous fusion events. Okada's group demonstrated that Sendai virus — known also as parainfluenza-I or the haemagglutinating virus of Japan (HVJ) — could be used in the fusion of suspended mononucleated cells to yield non-proliferating multinucleated cells (Okada, 1962a, b; Okada and Murayama, 1965). However, it was only in 1966 that the feasibility of using Sendai virus in generating long-lived somatic cell hybrids, was described (Yerganian and Nel, 1966). This virus, either UV-inactivated (Harris *et al.*, 1967), or β-propiolactone-inactivated (Neff and Enders, 1968; Klebe *et al.*, 1970), subsequently became the standard fusogen for promoting somatic cell hybridization.

The use of Sendai virus presented significant difficulties: fusion could not be effected between all cell types, preparation of the virus was tedious and activity varied from one batch to another. Moreover, the possible inclusion of viral components in the fusion products was, in principle, undesirable. These problems motivated the search for an alternative fusing agent.

One of the first chemical compounds investigated for fusogenic properties was lysolecithin (Poole *et al.*, 1970). This lipoidal substance was found to induce hybrid formation about 13 to 30 times more frequently than that occurring spontaneously. However, as it was extremely harmful to cell membranes, lysolecithin had

a toxic effect which drastically reduced the yield of viable hybrid cells.

Other lipoidal substances which were tested included fatty acids, fatty acid alcohols (Ahkong *et al.*, 1972; Ahkong *et al.*, 1975), fatty acid amines (Bruckdorfer *et al.*, 1974) and glycerol derivatives (Cramp and Lucy, 1974). Among the most promising were oleylamine and glycerol monooleate. Nevertheless, the results obtained with these compounds did not warrant dispensing with Sendai virus.

In 1974, polyethylene glycol (PEG) was introduced as an agglutinating agent leading to extensive fusion of higher plant protoplasts (Bonnett and Eriksson, 1974; Constabel and Kao, 1974; Kao and Michayluk, 1974). In the following year, Pontecorvo demonstrated that this polyol could also induce fusion between mammalian cells (Pontecorvo, 1975). Not only were "effective" fusion rates (i.e. rates of viable hybrid formation) found to be higher than those obtained with inactivated Sendai virus, but it was also shown that fusion could be effected between most mammalian cell types (Pontecorvo, 1975; Davidson and Gerald, 1975; Wang *et al.*, 1979).

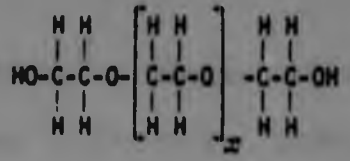


Figure 1.4: Structural formula of polyethylene glycol. When the molecular weight of the polymer is about 7 500 daltons, x = 170. (From Ringertz and Savage, 1976).

Although PEG has since been widely used in diverse studies involving the technique of cell fusion, the mechanism by which it mediates fusion remains unclear. Generally, the necessary conditions for membrane fusion are membrane aggregation, close apposition and destabilization. Studies carried out by Wojcieszyn *et al.* (1983), strongly suggest that PEG brings the adjacent plasma membranes into very close apposition (by virtue of its dehydrating ability) and then destabilizes these "close contact" areas. The destabilization process, which leads directly to fusion, is thought to be provoked not by the polymer itself, but by the additives found in commercial PEG, such as antioxidants and polymerization agents.

Several factors, other than duration of treatment, determine the efficiency of PEG-mediated cell fusion; these include pH, molecular weight and concentration of PEG in the fusion mixture, as well as the level of cytotoxicity which varies between different batches of the polymer. Optimal conditions are reported to be a 50% concentration of high molecular weight PEG (6 000 and 4 000 daltons) which has been adjusted to pH 7,8 - 8,2 (Davidson and Gerald, 1975; Davidson *et al.*, 1976; Brahe and Serra, 1981).

As the optimal effective concentration of PEG is approached, cytotoxicity becomes increasingly pronounced. Various compounds have therefore been used in conjunction with PEG, in order to reduce the level of toxicity. In this way dimethyl sulphoxide (DMSO) has been used to protect the cells from the harmful effects

of PEG (Norwood *et al.*, 1976). The plant lectin phytohaemagglutinin has also been used successfully, not as a means of protection against highly toxic levels, but rather as an enhancer of fusion efficiency using PEG concentrations which are well below the optimum (Mercer and Schlegel, 1979).

1.2.4 The Clone Panel

1.2.4.1 Single Chromosome Mapping Panels

A mapping panel of hybrids may be defined as a collection of hybrids that subdivide the donor chromosomes (i.e. chromosomes from the parental complement which undergoes segregation) in such a way that allows unambiguous mapping assignments to be made. It follows that the ideal system for assigning genes to chromosomes would be to construct a set of twenty-four hybrid lines, each containing a single and different human chromosome. At present, few mechanisms exist whereby a particular human chromosome can be selectively retained in a hybrid line. In terms of drug resistance selection, only a limited number of enzymes, such as HGPRT, TK and APRT can be used as selectable markers for the specific retention of chromosomes X, 17 and 16, respectively. In addition, genetic complementation for auxotrophic and temperature sensitivity markers may lead to the isolation of hybrids with single human chromosomes.

Different approaches have been taken in efforts to sidestep this shortage of selectable markers. For example, when very few human chromosomes are retained, the hybrid line may be

subcloned in an attempt to isolate any cells which carry only one human chromosome. This is particularly effective if a selectable marker is involved, since the selectively retained human chromosome can also be eliminated by appropriate counter-selection, prior to subcloning.

Another approach is to generate human microcells, containing small numbers of chromosomes, and to fuse these with whole rodent cells. Following cryogenic treatment, mitotic cells sometime undergo aberrant cleavage, producing clusters of protruberances resembling a bunch of grapes (referred to as "BOGs"). The mini-segregants, or microcells, remaining after the separation of these buds, often contain reduced amounts of DNA, and may be resolved into different size fractions following separation through a density gradient column (Schor *et al.*, 1975). In this way, a human microcell-mouse hybrid line, containing only human chromosome 17, has been used to confirm the assignment of type I procollagen to this chromosome (Raj *et al.*, 1977).

Two other alternatives to biochemical selection have been suggested (Kamarck *et al.*, 1984). Firstly, by means of DNA-mediated gene transfer (see Section 1.2.4.3), single, dominant selectable genes, such as the *E.coli* gene which codes for xanthine-guanine phosphoribosyltransferase (*Eoogpt*) and the neomycin resistance gene, could be introduced into the human parental cell line (Mulligan and Berg, 1980). On the assumption that random, stable integration into any of the human chromosomes would occur, a uniform selection system could result in the isolation

of hybrids containing each individual human chromosome. For example, after the transfer of the gene conferring neomycin resistance, human-rodent cell fusion products would be subjected to elevated concentrations of neomycin, and the surviving clones would presumably differ with respect to the human chromosome which was selectively retained. The second possibility involves the use of immunological procedures such as complement-mediated cytotoxicity and antibody binding to cell surface antigens in hybrid cells (Puck *et al.*, 1971). Chromosomes 2, 3, 5, 6, 7, 10, 11, 12, 14, 15, 17, 19, 21 and X are known to encode surface antigens (Creagan *et al.*, 1975; Kao *et al.*, 1977; Kamarck *et al.*, 1983; Rettig *et al.*, 1984), and selection and counter-selection can therefore be performed with the aid of specific antisera.

In practice, every approach other than biochemical selection still presents great difficulties and is time consuming, and most of the stable single chromosome hybrids isolated without the use of such markers have simply been chance events.

1.2.4.2 Multiple Chromosome Mapping Panels

The single chromosome mapping panel, although discussed for many years, has not been fully realized. To overcome this problem, Creagan and Ruddle (1975) suggested a system which has proved to be extremely useful. This approach, which may be designated the multiple chromosome mapping system, entails the assembly of a set of hybrids, each of which contains a unique combination of human chromosomes.

Such a multiple chromosome mapping panel was used for the first time in the assignment of uridine monophosphate kinase (UMPK) (Satlin *et al.*, 1975). This required the development of an electrophoretic technique for separation of the rodent and human enzyme. Concordant segregation subsequently led to the assignment of UMPK to human chromosome 1. DNA fragment-chromosome linkage has also been established following the same principle of concordant segregation in hybrids, using Southern blot analysis (Kao *et al.*, 1982).

To illustrate the principle of such a panel, the minimum number of hybrid lines required to give a unique pattern of presence or absence (i.e. the bimodal signature) must be considered for a given number of chromosomes. Table 1.1 illustrates this when two, four and eight chromosomes are involved. It can be seen that the number of hybrid members sufficient for mapping purposes may be stated as a geometric progression: $C = 2^m$, where C is the number of donor chromosomes involved and m is the minimum number of panel members required (i.e. $2 = 2^1$; $4 = 2^2$; $8 = 2^3$). It follows that at least five hybrid lines are necessary to accommodate the unique bimodal signatures required for all twenty-four human chromosomes.

In practice, it is unlikely that anyone would be able to isolate a set of five clones retaining the perfect distribution so as to give unique bimodal signatures to each particular chromosome, and a somewhat larger number of clones is usually required. The redundancy built into the panel through the use of a larger

number of hybrid clones actually increases the accuracy of the mapping panel.

HYBRID LINES	CHROMOSOMES							
	1	2	3	4	5	6	7	8
A	+	-						
A	+	+	-	-				
B	+	-	+	-				
A	+	+	+	+	-	-	-	-
B	+	+	-	-	+	+	-	-
C	+	-	+	-	+	-	+	-

Table 1.1: Bimodal signatures in panels of 1, 2 and 3 hybrid lines.
+ indicates presence; - indicates absence.
(Modified from Creagan and Ruddle, 1975).

Some interacting variables should be considered in the design of a reliable and efficient multiple chromosome mapping panel, namely the number of donor chromosomes in each hybrid clone, the number of hybrid clones and the combination of chromosomes within each panel member. With the aid of computer simulation, Ruddle's group has worked out that, for human mapping studies, approximately ten hybrid clones should be used, each containing around twelve randomly permuted human chromosomes (Kamarck *et al.*, 1984). If these conditions are met, then incorrect assignments can be made only if at least three or four false-negative results are generated.

Two significant drawbacks are encountered in the assembly of reliable hybrid mapping panels in general. The first is the

heterogeneity, with respect to human chromosome content, often found within a hybrid line. In view of this, it is necessary to establish the minimal frequency of a particular human chromosome in a hybrid population, required to yield a detectable expression of a specific gene (Burgerhout, 1978). The same applies to Southern blot analyses, where the detection of hybridization to a given probe may require a minimal frequency of the human complementary sequences. In order to ensure greater homogeneity and higher percentages of each human chromosome, secondary rather than primary clones should be used (Creagan and Ruddle, 1975). Ultimately, percentages greater than 90 percent are desirable, but much lower percentages have been accepted (Satlin *et al.*, 1975; Fox, 1983, Knowlton *et al.*, 1985).

The second drawback is that, althougn intact human chromosomes are easily identified in hybrid cells, it is difficult to prove that no material of another human chromosome is present. For this reason, Burgerhout (1978) has suggested that an assignment to a particular chromosome should be followed by the exclusion of all other chromosomes. This requires that all other chromosomes be present in at least two clones where the gene or gene product has not been detected.

1.2.4.3 Subchromosomal Mapping Panels

Once a gene has been assigned to a particular chromosome, it then has to be mapped to a specific region within that chromo-

some. This can be accomplished with the aid of subchromosomal mapping panels, where each hybrid member contains a different segment of the human chromosome of interest. The position of the gene can then be delimited by the resulting pattern of concordance and discordance observed using the panel. The "smallest region of overlap" (SRO) refers to the region common to all segments with which the gene has been associated.

Four strategies are commonly employed in the assembly of subchromosomal mapping panels. The first makes use of translocations and deletions detected in the human population. Hybrids constructed using these abnormal parental cells are screened for the presence of the derivative chromosomes. Particularly useful translocations are those involving chromosomes which carry selectable markers (for example, chromosomes 17 and X), since these can be isolated with specific selective media (Grzeschik *et al.*, 1972; Picciuti and Ruddle, 1973; Scott *et al.*, 1979; Fox, 1983).

A second strategy takes advantage of interspecific translocations that arise spontaneously in human-rodent cell hybrids (Boone *et al.*, 1972; Hamerton *et al.*, 1975; Kamarck *et al.*, 1983). It has been shown that donor chromosomes, under selection in foreign cells, often undergo translocation to recipient cell chromosomes during the hybrid stabilization phase (Kamarck *et al.*, 1983; Friend *et al.*, 1976b). The stabilizing factor is believed to be the acquisition of a centromere by the donor chromosome fragment.

Both of these approaches, however, are constrained by the availability and identification of translocations involving the chromosome being studied. Since a large variety of widely-distributed chromosomal breaks and deletions is required for extensive regional mapping, both chemical and physical chromosome-breaking agents have been used to induce such alterations (Goss and Harris, 1975, 1977a,b; Burgerhout *et al.*, 1977; Law and Kao, 1978, 1979). Statistical mapping from data obtained using this "disruptive strategy", was first described by Goss and Harris (1975). By measuring the co-transfer frequency between pairs of genes, and assuming that the distance between two loci is directly proportional to their segregation frequency, the linear order and distances between the genes may be determined.

Hybrids of this sort may be generated either by treating the human parental cell line prior to fusion, or by treating the fusion products themselves. The first alternative rests heavily on the presence of a selective marker on the chromosome of interest, which may then be used to isolate hybrids containing the relevant chromosome segments. This method also lends itself to counterselection, upon which "marker-deletion" hybrids are generated and subsequently used for regional mapping by exclusion, or for confirmation of mapping results (Becker *et al.*, 1979; Law and Kao, 1978, 1979). In the second method, where previously isolated hybrid lines are subjected to treatment, the presence of selectable markers is not essential. Here, treatment is applied to a hybrid line which contains

the required human chromosome. Hybrids carrying the various chromosome segments are then isolated by sequential subcloning. As well as eliminating the need for detailed cytogenetic analysis, induced chromosome breakage and co-transference analysis can extend the resolving power beyond that currently possible by cytogenetic techniques.

Finally, chromosome-mediated gene transfer (CMGT) has been used to generate regional mapping panels. In this process, the transfer of genetic material between cultured mammalian cells is accomplished using purified metaphase chromosomes as vectors (McBride and Ozer, 1973; Miller and Ruddle, 1978). Following metaphase arrest, the donor cell membranes are disrupted in order to release the chromosomes. The chromosomes are subsequently separated from most of the debris and any remaining intact cells by ultracentrifugation, and immediately incubated with recipient (rodent) cells, in the presence of $CaCl_2$. Usually only subchromosomal amounts of material are retained in the transformed cells, the size of these fragments ranging from under 10 kb to greater than 1 000 kb (Miller and Ruddle, 1978), with stabilization coinciding mostly with a decreased fragment size (Klobutcher and Ruddle, 1979). Regional localization of several genes has been accomplished with the aid of CMGT (Francke and Pellegrino, 1977; Klobutcher and Ruddle, 1979; Kamarck *et al.*, 1983).

1.2.5 Hybrid Properties

1.2.5.1 Direction and Timing of Chromosome Loss

Extensive chromosome loss in hybrid cell lines has been observed since the earliest studies on interspecific hybrids (Weiss and Green, 1967; Migeon and Miller, 1968; Nabholz *et al.*, 1969). From these and subsequent observations, it was established that the pattern of chromosome elimination seems to follow some general empirical rules: (i) the chromosomes of one parental species are preferentially eliminated while those of the other species are selectively retained, (ii) the direction of chromosome loss depends on the species of origin of the parental cells, and (iii) the extent of chromosome loss is also a function of the parental species combination — for example, very few chromosomes are lost from rodent-rodent hybrids (Littlefield, 1966; Graves and Koschel, 1980), rodent-human hybrids lose the majority of the human chromosomes (Weiss and Green, 1967; Kao and Puck, 1970; Westerveld *et al.*, 1971), and rodent-marsupial hybrids usually lose all identifiable marsupial chromosomes (Graves *et al.*, 1979).

Although human chromosomes are almost invariably lost from rodent-human hybrids, reversal of this pattern of loss has been observed in a number of crosses. An unusual line from a human VA2-mouse C1.1D hybrid was found to have retained most of the human chromosomes, whereas the mouse component was drastically reduced (Jami and Grandchamp, 1971). Similar "reverse chromosome segregation" phenomena were observed in hybrids between VA2 and freshly isolated

mouse embryonic tissue (Minna and Coon, 1974), and between HT-1080 human fibrosarcoma cells and mouse peritoneal macrophages (Croce, 1976). The reversal in these last two crosses has been attributed to dominance of the transformed over the normal parental contribution. More recently, it has been suggested that, rather than the states of transformation, it is the chromosome input ratio which determines the direction of chromosome loss, since transformed lines are usually hypotetraploid. Experiments designed to study the effect of parental cell ploidy on chromosome segregation (Graves, 1984), seem to indicate that the direction and extent of chromosome loss is correlated with either the chromosome input ratio or the cell volume ratio (a possible cytoplasmic determinant being the dosage of parental mitochondria, as is discussed in Section 1.2.5.2).

Attempts have been made to influence the direction of chromosome elimination by damaging the chromosomes of one of the parental cell lines before fusion (Pontecorvo, 1971). The idea stemmed from work carried out in the early 40's on *Drosophila* hybrids, where sperm of one species was X- or γ-irradiated to invoke extensive chromosome breakage and subsequent elimination after fusion with eggs from triploid females of another species. As an alternative to irradiation, Pontecorvo also used BrdU exposure followed by visible light treatment. Although this strategy enables one to direct chromosome loss in hybrid cells, the chromosomes tend to undergo extensive rearrangements and are therefore difficult to identify.

The rate at which chromosomes are eliminated from hybrid cell lines may, to some extent, reflect the phylogenetic distance between the parental cell lines.

In mouse-human cell hybrids, rapid elimination often occurs very early in the evolution of the hybrid lines, and this phase may then be followed by a stabilization period during which chromosome elimination is much slower (Weiss and Green, 1967; Nabholz *et al.*, 1969; Allderdice *et al.*, 1973). There may, however, be great variations between clones derived from the same hybrid line; for instance, one clone may retain a full human chromosome complement while another may exhibit loss of some or most of its human chromosomes, during the same period of time (Nabholz *et al.*, 1969).

In hybrids between Chinese hamster and human cells, two general patterns have been observed: rapid and often complete loss in all or a proportion of the cells, and a more gradual loss resulting in stable hybrid lines (Wang *et al.*, 1979). Rapid chromosome loss is the more common of the two, with hybrids often losing most of the human chromosome complement within the first two weeks of fusion (Kao and Puck, 1970; Puck, 1974).

1.2.5.2 Mechanism of Chromosome Elimination

The mechanism of chromosome elimination in cell hybrids remains quite obscure, although several hypotheses have been presented.

The first is asynchronous DNA replication, where the parental cells represent different stages of the cell cycle at the time of fusion. This will result in premature chromosome condensation of the one complement, as well as in other mitotic abnormalities. Moreover, the different generation times of the parental cells may lead to nuclear asynchrony. Here, the chromosomes of the relatively slow-growing species are invariably lost, and there appears to be a correlation between increased hybrid growth rate and increased elimination of chromosomes from the slower parent (Matsuya *et al.*, 1968; Kao and Puck, 1970; Marin and Pugliatti-Crippa, 1972).

Mitotic abnormalities could be due to species incompatibility between chromosomes of the one species and components of the mitotic apparatus of the other. Although there is no evidence for this in interspecific hybrids, faulty interactions between centrioles, spindle threads and kinetochores have been observed in other multinucleate cells (Oftebro and Wolf, 1967; Heneen *et al.*, 1970).

Another attractive hypothesis is that chromosome loss is influenced by, and is a consequence of, incompatible interactions between chromosomal and mitochondrial gene products of the different species. Croce's group has reported the presence of a single species of mitochondrial DNA belonging to the parent whose nuclear chromosomes were retained, in a series of man-mouse cell hybrids (de Francesco *et al.*, 1980). This pattern was consistent regardless of whether the human or the mouse chromosome sets

were retained. Further support for this hypothesis has been presented by Weide and co-workers. This group constructed nuclear hybrids, or "karyobrids", which are the products of viable nuclei and whole cells, in order to study the effect of mitochondria on hybrid cell survival (Weide *et al.*, 1992). Not only was the long-term survival rate of these karyobrids increased in relation to that of whole cell hybrids, but chromosome loss was more rapid. The interpretation of their results was that cellular components encoded by mitochondrial genes may be responsible for the incompatibility, whereas those encoded by nuclear genes do not interfere with hybrid cell survival.

Enzymes involved in chromosome replication and repair, and chromosomal proteins, may also be incompatible between species. Chromosome damage, in the form of fragmentation or pulverization, is yet another possible factor leading to chromosome loss (Graves, 1984).

1.2.5.3 Randomness of Chromosome Segregation

Studies on the selective elimination or retention of specific human chromosomes have yielded contradictory results in both human-mouse and human-Chinese hamster hybrids, so that no predictions can be made as to the segregation pattern which will be found in a given fusion.

Preliminary cytogenetic analyses, carried out prior to the establishment of banding techniques, yielded data suggestive of

both random chromosome segregation (Weiss and Green, 1967; Kao and Puck, 1970; Fox and Retief, 1986) and non-random chromosome segregation (Santachiara *et al.*, 1970; Ruddle *et al.*, 1970; Marin and Pugliatti-Crippa, 1972). Since the new banding techniques came into general use, more evidence has accumulated supporting both schools of thought.

Selection for chromosomes X and 17 has been seen to result in the frequent retention of certain autosomes (Croce *et al.*, 1973; Norum and Migeon, 1974). In these studies the retention of chromosomes 7, 11 and 12 was almost as frequent as that of the selected marker chromosomes. Contrary to the earlier suggestion that the larger human chromosomes are preferentially eliminated (Allderdice *et al.*, 1973), it has since been reported that chromosome 3 is usually present in stable hybrid lines (Donald *et al.*, 1982).

There has been no agreement as to which chromosomes are involved in non-random segregation patterns. The basis for the reported directed loss may be related to the nature of the gene products specified by the chromosomes involved, so that the patterns of elimination could vary with the parental cells and culture conditions employed. There is also the possibility that some rodent chromosomes are lost, and specific human chromosomes would then be retained to compensate for the deficiencies of the rodent gene products. Nonetheless, there is evidence that at least in the early stages of hybrid cell growth, there is no consistent retention pattern of specific human chromosomes (Chen, 1979; Wang *et al.*, 1979).

1.2.5.4 Hybrid Stability

Stability of a hybrid line is believed to depend on the integration of the two parental genomes, to produce a balanced hybrid genome which is capable of survival in culture. Until this balance is reached, hybrids will show continued chromosome loss and rearrangement, with multinucleate cells, micronuclei, and fragmented human and rodent chromosomes being an indication of instability (Wang *et al.*, 1979).

After approximately 15 cell generations, by which time cytogenetic analysis can be performed, the hybrid lines usually begin to stabilize. This has been shown by cytogenetic analyses performed on hybrids at this stage of development and then again after several passages (Creagan and Ruddle, 1975; Francke *et al.*, 1976; Wang *et al.*, 1979). Hybrid lines are therefore considered to be stable if, after 30 to 40 cell generations, no further chromosome loss has occurred and the percentages of the human chromosomes present remain the same or are only slightly altered.

The ploidy level of hybrids may also be implicated in hybrid stability, as suggested by different investigations (Chen, 1979; Klebe *et al.*, 1970). The most frequent stable hybrid cell lines reported in these studies are of the type derived from fusions involving one human and two mouse chromosome complements.

Regardless of stability, hybrid lines almost invariably lose all human chromosomes if maintained in culture for several months

(Nabholz *et al.*, 1969; Ruddle, 1972). This is particularly so when no selective pressure is being exerted on the culture. When grown under selective conditions, gene transfer may take place between the human and rodent chromosomes. This phenomenon was first observed by Migeon and co-workers (see Ephrussi, 1972) when, after four months in continuous culture, human chromosome 17 disappeared in some of the hybrid clones which required this TK-encoding chromosome for survival in HAT medium. Electrophoretic mobility and heat sensitivity tests showed that the human enzyme was still being synthesized in these clones. The only plausible explanation for this was that a cytogenetically undetectable fragment of human chromosome 17, carrying the TK gene, had been translocated to one of the mouse chromosomes.

1.3 Chromosome Banding Techniques

1.3.1 General

In the "pre-banding" era, the analysis of metaphase chromosomes was based on criteria such as chromosome length, arm ratio, presence of secondary constrictions and labelling pattern in autoradiography. These parameters, however, did not allow accurate identification of morphologically similar chromosomes, neither did they allow the recognition of specific chromosome segments involved in rearrangements, such as reciprocal translocations.

Longitudinal differentiation of chromosomes was first observed using quinacrine mustard, which produced clear banding patterns

in both plant and mammalian chromosome preparations (Caspersson *et al.*, 1967, 1970, 1971a, b). On the basis of their characteristic Q-bands, each individual chromosome of the human genome could be identified. Soon afterwards, several groups of workers demonstrated that banding patterns could also be obtained by staining with Giemsa, after the preparations had been subjected to various types of treatment (Sumner *et al.*, 1971; Drets and Shaw, 1971; Seabright, 1971). The banding patterns observed using these methods became known as G-bands, and were very similar to Q-banding patterns.

Other banding techniques which have been developed since 1971 include (i) A-banding, for regions near the centromeres and telomeres of several chromosomes, (ii) C-banding, for constitutive heterochromatin, (iii) D-banding, whereby daunomycin or adriamycin produces bands similar to those obtained by the quinacrine method, (iv) N-banding, which stains the nucleolar organizing regions, (v) R-banding, where the pattern is the reverse of G-bands, and (vi) T-banding, for the telomeric regions of certain chromosomes (for review, see Pathak, 1976).

1.3.2 Differential Stains for Hybrids

Although the above-mentioned techniques are suitable for routine cytogenetic analyses, they are often inadequate for the analysis of interspecific hybrid cells. Difficulties lie in the distinction between morphologically similar chromosomes from the two parental components, and in the identification of inter-

species rearrangements. In an effort to increase the accuracy of hybrid karyological characterization, special staining techniques have been developed, which may be combined with one or more of the above cytogenetic procedures, to allow distinction between, and identification of, human and rodent chromosomes.

1.3.2.1 Differential Staining with Hoechst 33258

The fluorochrome Hoechst 33258, a benzimidazol compound, has been used to distinguish between mouse and human chromosomes in hybrids. This fluorochrome preferentially stains mouse constitutive heterochromatin, such that the mouse chromocentres fluoresce brightly, whereas the human chromosomes show a dull, uniform fluorescence. Although some human centromeric regions may also be positively stained (particularly those of chromosomes 1, 9 and 16), they still fluoresce less intensely than those of the mouse (Hilwig and Gropp, 1972).

Centromeric staining with Hoechst 33258 facilitates the identification of a few structural rearrangements, particularly of the chromosomal breakage and Robertsonian translocation types (Kozak *et al.*, 1977). However, most intra- and interspecific rearrangements, especially those which do not involve the centromeric regions, cannot be detected.

Although the stain often reveals faint Q-banding patterns, this method is routinely preceded or followed by a standard banding method, such as G- or Q-banding, to allow positive identification

of the human chromosomes (Kozak *et al.*, 1977; Mohandas *et al.*, 1979; Fox, 1983; Moser *et al.*, 1975).

1.3.2.2 Differential Staining with Alkaline Giemsa

The alkaline Giemsa technique -- also known as the Giemsa-11 or G-11 technique — where the stain is adjusted to pH 11,0-11,5, differentially stains specific regions of paracentric constitutive heterochromatin (Bobrow *et al.*, 1972). The human heterochromatic regions stain magenta against the blue euchromatic chromosome arms, while the rodent chromosomes show a reciprocal pattern.

Because these staining properties are retained in hybrid cells, the G-11 method is also useful in the analysis of human-rodent hybrid cell lines (Bobrow and Cross, 1974a, b). Not only does this staining specificity enable the species origin of individual chromosomes to be determined, but it also provides a reliable means of identifying interspecies translocations (Friend *et al.*, 1976b).

This staining process is regularly combined with other methods, such as G-, Q- and/or Hoechst-banding (Bobrow and Cross, 1974a,b; Friend *et al.*, 1976a,b), providing a firm characterization of the hybrid karyotypes. The technique is somewhat erratic: differential staining almost always requires adjustments in staining time, pH and/or dye concentration.

1.3.2.3 Biotinylated DNA Probes

The synthesis of biotinylated polynucleotides and their use as affinity probes was first reported by Ward's group (Langer *et al.*, 1981). Recently, a method has been described in which biotin-labelled DNA probes are used to detect human chromosomes and assess interspecific rearrangements occurring in human-rodent hybrids (Durnam *et al.*, 1985; Pinkel *et al.*, 1985).

The procedure involves nick-translation of total human DNA in the presence of biotinylated dUTP, which is then hybridized *in situ* to the chromosome preparations. The probe is detected using fluoresceinated avidin (i.e. avidin molecules with the yellowish-green fluorescing tags). To enhance the signal, alternating layers of fluoresceinated avidin and biotinylated anti-avidin antibody may be applied. The chromosomes are then counterstained with a red dye, either propidium iodide (Pinkel *et al.*, 1985) or Giemsa (Durnam *et al.*, 1985). In this way the rodent DNA stains red, whereas the hybridized human DNA stains yellow or green depending on the relative amounts of dye applied. Inter-species translocations are readily detected as bi-coloured chromosomes.

It is believed that this technique can detect as little as $1\text{-}5\times10^{6}$ base pairs of translocated human DNA. The intensity and specificity of this procedure make it significantly more powerful than the G-11 technique. Possibly the only drawback in using this method is the time involved in the preparation of probes and antibodies.

1.4 Biochemical Techniques

Different molecular forms of certain enzymes exist in man and rodent, due to evolutionary divergence. The different forms, or isozymes, often exhibit different net surface ionic charge, and can therefore be detected by electrophoretic techniques. Upon migration to their specific positions along the carrier substrate, the isozymes are visualized by specific histochemical staining.

Several laboratories have found it convenient to use isozyme markers on each of the chromosomes, to test the human chromosome content in human-rodent cell hybrids. Specific isozyme markers, which apply to both man-mouse and man-Chinese hamster hybrids, have been chosen for each chromosome and in some cases for each chromosome arm. Table 1.2 is a list of such markers, which can all be identified by starch-gel electrophoresis (Shows *et al.*, 1982). Some of the isozymes listed can also be identified on cellulose acetate gels.

Biochemical analysis is accomplished fairly rapidly. However, there are a couple of significant limitations in using isozyme markers for the analysis of interspecific hybrids. The first is the detection level of the isozyme test: as mentioned previously, some human chromosomes may be present at a low frequency in a particular hybrid line; if the isozyme markers used to detect these chromosomes have a relatively low enzymatic activity, these chromosomes may be scored as 'absent'. The second limitation

is that chromosomal aberrations such as deletions, duplications and translocations cannot always be detected. For these reasons, isozyme analysis should not be used as an alternative to cytogenetic analysis, but rather as a supporting technique to confirm hybrid karyological characterization.

1.5 Aim of Study

The recent isolation, in our laboratory, of numerous random single-copy DNA sequences revealing polymorphic sites, prompted the establishment of a rapid and reliable mapping system.

The aim of the present study was to construct a panel of human-rodent somatic cell hybrids which could be used to assign these and other sequences to specific human chromosomes. Interspecific somatic cell hybridization was chosen in view of the fact that it is the single methodology responsible for the majority of human gene assignments made to date. This required acquaintance with various aspects of tissue culture and routine cytogenetic techniques, as well as the establishment of reliable protocols for the more specialized procedures pertaining to somatic cell hybridization, including cell fusion, hybrid selection and isolation, and differential chromosome staining.

Once established, these techniques could also be used to isolate "translocation" hybrids towards the assembly of subchromosomal mapping panels, for the regional localization of the sequences.

TABLE 1.2: Isozyme markers for biochemical analysis of human-rodent cell hybrids (Shows *et al.*, 1982)

CHROMOSOME/ CHROMOSOME ARM		ENZYME MARKER*
1	p	Enolase-1 (ENO1)[a]
	p	Phosphoglucomutase-1 (PGM1)[b]
	q	Peptidase-C (PEPC)[c,d]
	q	Fumarate hydratase (FH)[e]
2	p	Malate dehydrogenase-1 (MDH1)[b]
	q	Isocitrate dehydrogenase-1 (IDH1)[b]
3	p	Aminoacyclase-1 (ACY1)[f]
4	?	Peptidase-S (PEPS)[c]
	p?	Phosphoglucomutase-2 (PGM2)[g]
5	q	β-Hexosaminidase-B (HEXB)[h,i]
6	p	Glyoxalase-1 (GLO)[j]
	q	Superoxide dismutase-2 (SOD2)[j]
	q	Malic enzyme-1 (ME1)[k]
7	q	β-Glucuronidase (GUSB)[m]
8	p	Glutathione reductase (GSR)[n]
9	p	Aconitase-1 (ACO1)[o]
	q	Adenylate kinase-1 (AK1)[p]
10	q	Glutamic-oxaloacetic transaminase-1 (GOT1)[r]
11	p	Lactate dehydrogenase-A (LDHA)[b]
12	p	Triosephosphate isomerase-1 (TPI1)[s]
	q	Pepidase-B (PEPB)[d]
13	q	Esterase-D (ESD)[t]
14	q	Nucleoside phosphorylase (NP)[u]
15	q	Mannose phosphate isomerase (MPI)[h,i]
	q	Pyruvate kinase-M2 (PKM2)[q]
16	q	Adenosine phosphoribosyl transferase (APRT)[v]
17	q	Galactokinase (GALK)[f]
18	q	Peptidase-A (PEPA)[c,d]
19	?	Glucose phosphate isomerase (GPI)[w]
	?	Peptidase-D (PEPD)[v]
20	q	Adenosine deaminase (ADA)[w]
21	q	Superoxide dismutase-1 (SOD1)[j]
22	q	Aconitase-2 (ACO2)[o]
X	q	Glucose-6-phosphate dehydrogenase (G6PD)[b]
	q	Phosphoglycerate kinase (PGK)[b]

* References are to methodology used in detecting the isozyme markers:

(a) Meera Khan *et al.*, 1973; (b) Meera Khan, 1971a,b; (c) Shows *et al.*, 1978;
(d) Lewis and Harris, 1967; (e) van Someren *et al.*, 1974; (f) Naylor *et al.*, 1982;
(g) Wijnen *et al.*, 1977; (h) Kucherlapati *et al.*, 1975b; (i) Bender and Grzeschik, 1976;
(j) Brewer, 196 (k) Shows *et al.*, 1970; (l) Lalley *et al.*, 1977;
(m) Lalley *et al.*, 1977; (n) Overbach *et al.*, 1978; (o) Shows *et al.*, 1979;
(p) Fildes and Harris, 1966; (q) Shows, 1974a; (r) Shows, 1974b;
(s) Ricciuti and Ruddle, 1973; (t) Mowbray *et al.*, 1972; (u) Detter *et al.*, 1968;
(v) McAlpine *et al.*, 1976; (w) Koch and Shows, 1978.

2. MATERIALS AND METHODS

2.1 Cell Lines

2.1.1 Human Cell Lines

The human cell lines were of both normal and abnormal karyotypes, from cases which had been referred for cytogenetic investigations. They were established either from ammiotic fluid samples, skin biopsies, or peripheral blood specimens (separation and transformation of white blood cells is described in Section 2.4). All cultures used in fusion experiments are listed in Table 2.1. Some of these cases were particularly interesting, and warrant a brief description.

Patient FB: The father of FB was the carrier of a balanced deletion-insertion involving chromosome 5, where the interstitially deleted portion of the short arm was inserted into the long arm of the same chromosome 5 homologue (Figure 2.1). Analysis of FB amniotic cells revealed a recombinant chromosome 5, with an interstitial deletion of the short arm and an intact long arm (Figure 2.2). This pregnancy was terminated on the assumption that the recombinant chromosome 5 [del(5)(p14:p15)] would result in physical and mental abnormalities such as those associated with cri-du-chat syndrome.

Patients CR and DR: These siblings both carried an abnormal recombinant X-chromosome, with a duplication of the region q26.3→qter and deletion of p22.3→pter (Figures 2.3 and 2.4).

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Patients CR and DR: These siblings both carried an abnormal recombinant X-chromosome, with a duplication of the region q26.3→qter and deletion of p22.3→pter (Figures 2.3 and 2.4).

The mother of CR and DR carries an inversion in one of her X-chromosomes, [inv(X)(p22.3 q26.3)], and Figure 2.5 illustrates how this inversion presumably gave rise to the rearrangement found in the offspring. The male infant, DR, who suffered from multiple congenital abnormalities, died at the age of 2 years 3 months. His younger sister, CR, appeared to be developing normally up to the age of 2 years, although she showed a few physical features similar to those of DR and was of short stature.

Patient BL: The features of this patient, who was of female-gender identity, included ambiguous genitalia, with no uterus or cervix, and an enlarged phallus. A micromarker was found in a large proportion of the dividing blood cells. Techniques such as Q-banding, C-banding and NOR staining failed to reveal the origin of this marker. However, the clinical manifestations suggested that the marker consisted of Y-chromosome material (in particular, Yp material, since testicular differentiating genes have been localized to this region). G-banded and Q-banded karyotypes are shown in Figures 2.6 and 2.7.

Patient JN: JN presented yet another unusual karyotype, with a normal X-chromosome and a dicentric chromosome composed partly of X and partly of Y material (Figures 2.8 and 2.9). This patient was investigated because of infertility, and was found to have secondary amenorrhea, streak gonads and slight hirsutism, but was otherwise a healthy phenotypic female of normal intelligence. Although X;Y translocations are relatively common, most cases documented to date describe the Y material joined to the short arm of the X-chromosome, whereas in this case the Y was

joined to the long arm of the X-chromosome. For a detailed description, the reader is referred to the case report "A unique dicentric X;Y translocation involving the X long arm and the Y short arm: Cytogenetic and molecular studies" (Bernstein *et al.*, submitted 1986).

TABLE 2.1: Human cells used in fusion experiments

PATIENT CODE	TYPE OF SPECIMEN	KARYOTYPE
FB	Amniotic fluid	46,XX,rec(5).del(5)(p14;p15)pat
DR	Skin biopsy	46,rec(X)(qter→q26.3::p22.3→qter)mat,Y
CR	Skin biopsy	46,X,rec(X)(qter→q26.3::p22.3→qter)mat
BL	Peripheral blood	45,X(30 cells)/46,X,+ micromarker G(19 cells)/47,X,+ 2 micromarker G(1 cell)
JN	Skin biopsy	46,X,t(X;Y)(q22.1;p11.3)
FK	Skin biopsy	47,XY,+ 18
FK	Skin biopsy	46,XX
DK	Peripheral blood	46,XX,t(16;18)(q24;q21)
MP	Peripheral blood	46,XX
TL	Peripheral blood	46,XY

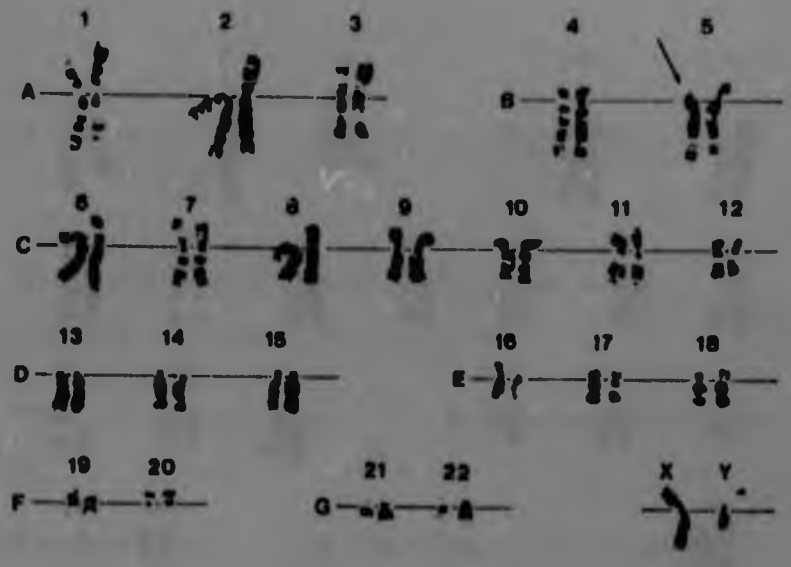


FIGURE 2.1: G-banded karyotype of father of patient FB. Arrow points to rearranged chromosome 5.

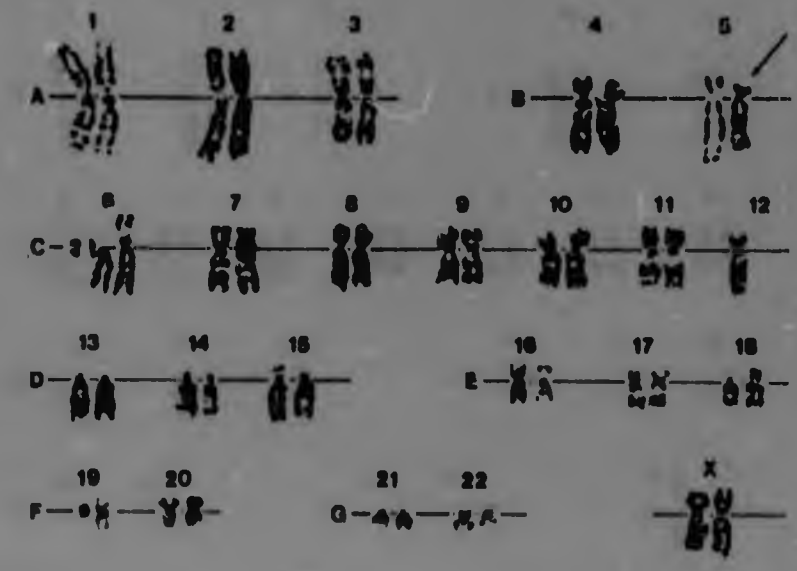


FIGURE 2.2: G-banded karyotype of patient FB. Arrow points to recombinant chromosome 5. (Chromosome 12 is randomly lost).

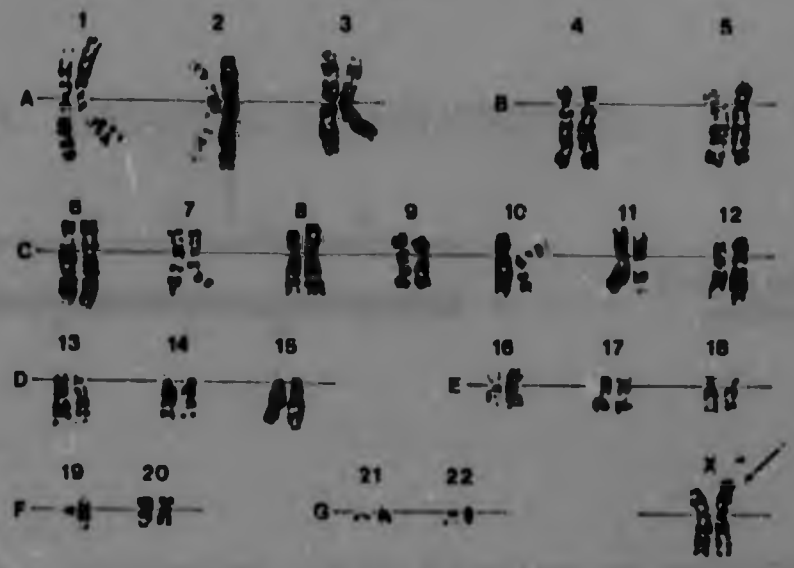


FIGURE 2.3: G-banded karyotype of patient CR.
Arrow points to the recombinant X-chromosome.

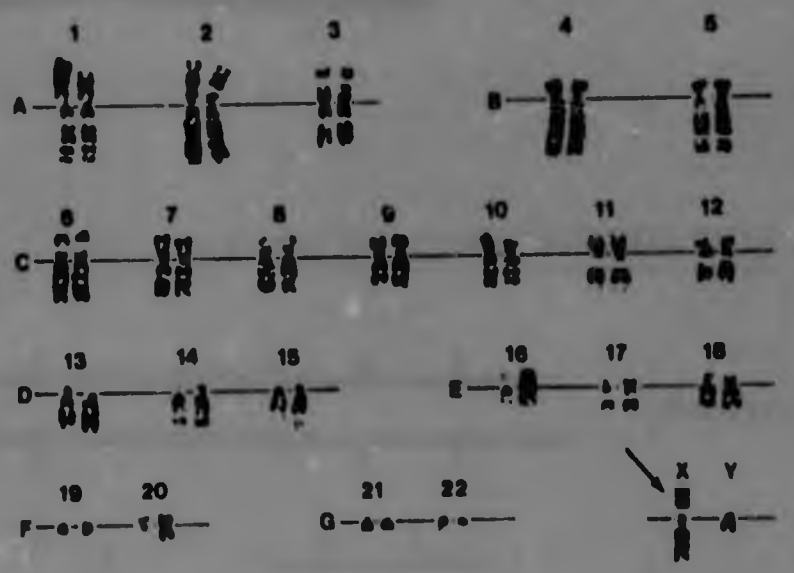
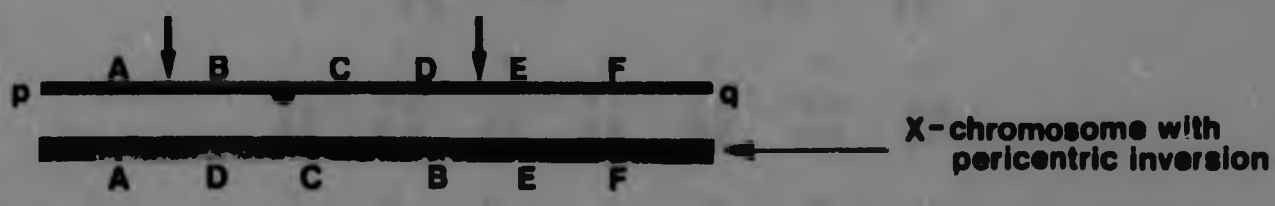
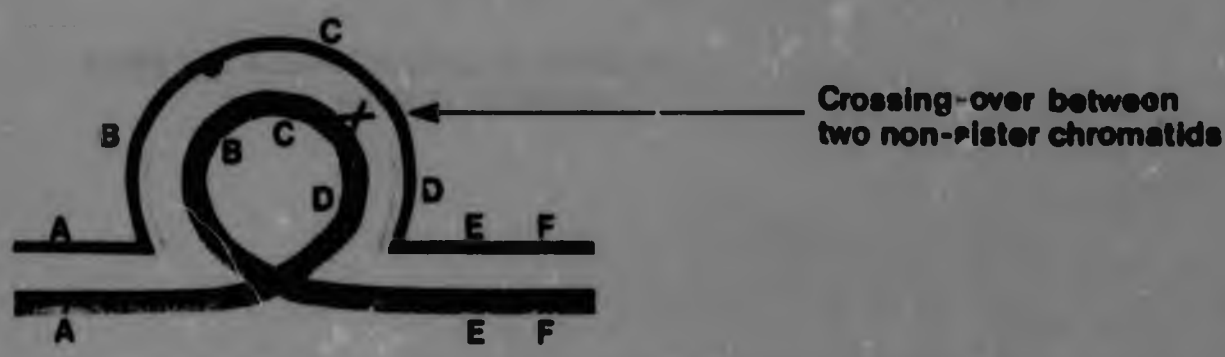


FIGURE 2.4: G-banded karyotype of patient DR.
Arrow points to the recombinant X-chromosome.

A. MATERNAL X-CHROMOSOME



B. INVERSION LOOP FORMED AT MEIOSIS



C. THE FOUR RESULTING CHROMATIDS

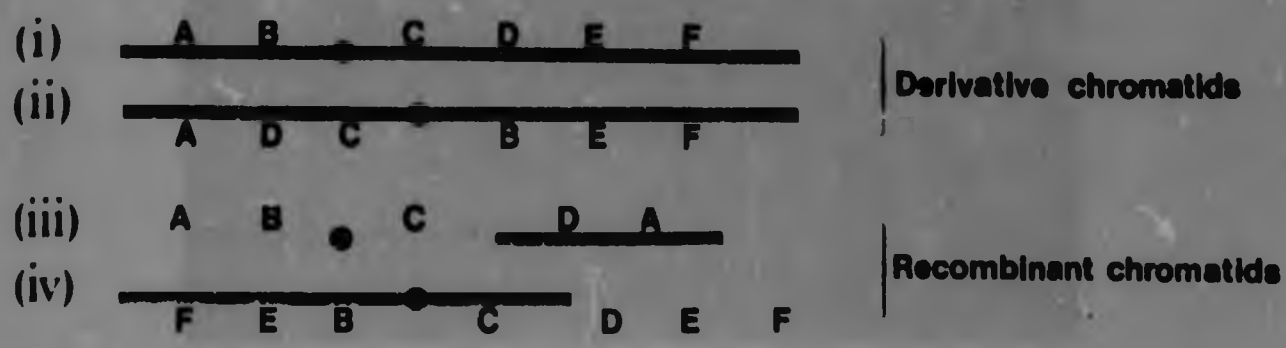


FIGURE 2.5: Origin of abnormal X-chromosome in patients CR and DR. Recombinant chromatid number (iv) represents the re-arrangement transmitted to these individuals.

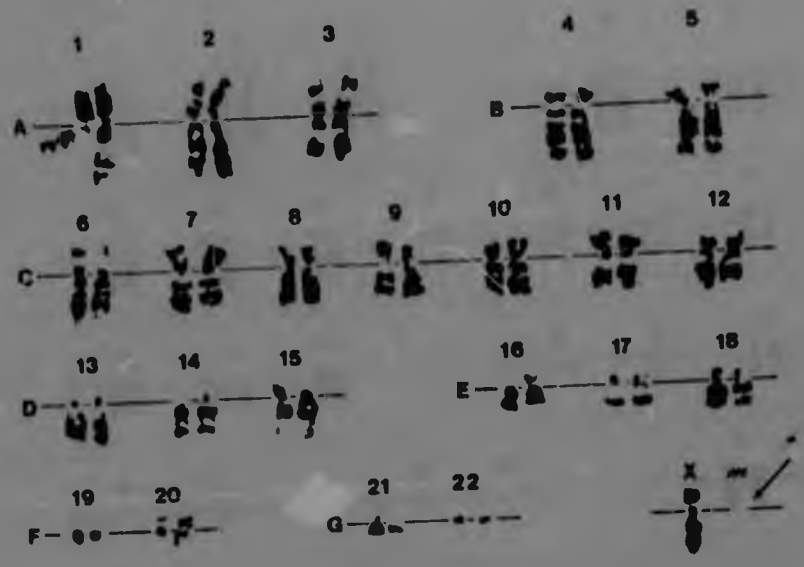


FIGURE 2.6: G-banded karyotype of patient BL.
Arrow points to micromarker.



FIGURE 2.7: Q-banded karyotype of patient BL.
Arrow points to micromarker.

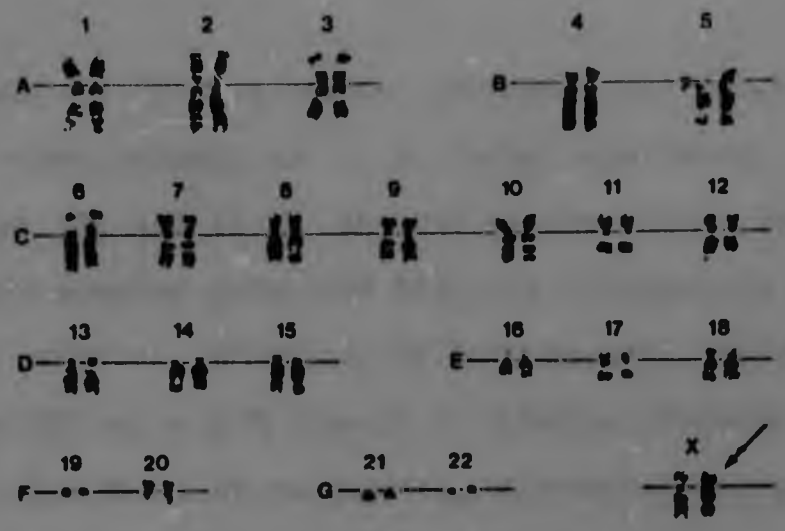


FIGURE 2.8: G-banded karyotype of patient JN.
Arrow points to t(X;Y) chromosome.

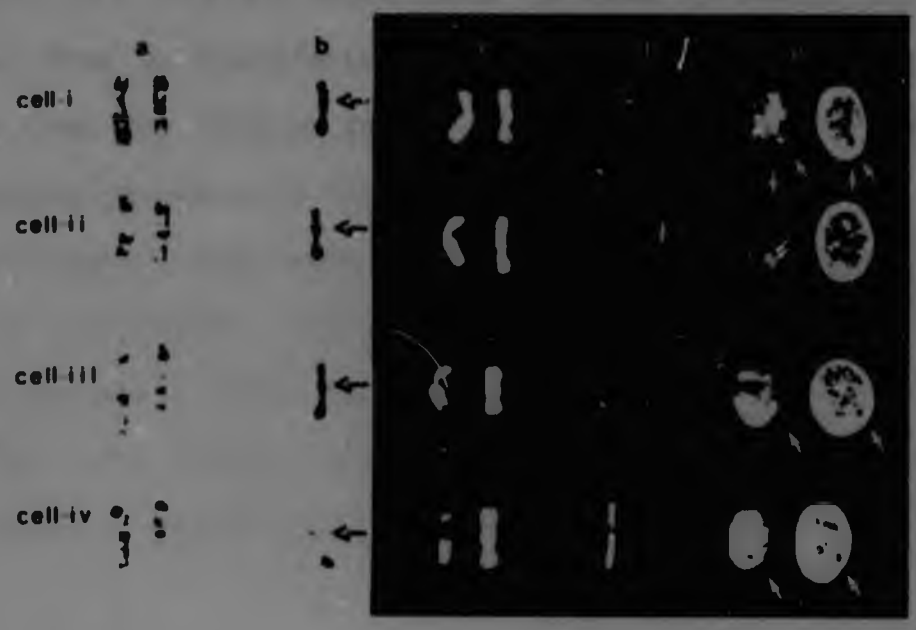


FIGURE 2.9: Cytogenetic studies on patient JN

(a) G-banding of the normal X (left) and the t(X;Y) (right)
(b) C-banding of the t(X;Y), showing the suppressed X centromere and the Yq12→qter
(c) Q-banding, showing the brightly-fluorescing Yq12 on the t(X;Y) chromosome (right)
(d) R-banding, showing inactivation of the whole t(X;Y) except the two X pericentric bands (arrowed)
(e) Sequential quinacrine and cresyl violet staining of buccal epithelial nuclei, showing associated X and Y chromatin bodies.

2.1.2 Rodent Cell Lines

Both mouse and Chinese hamster permanent cell lines were used as the rodent parental cells in fusion experiments. The mouse cell lines RAG and Cl.1D, as well as the Chinese hamster line wg3-h, were generous gifts from Dr M. Fox (Cytogenetics Department, Tygerberg Hospital, University of Stellenbosch), while the mouse cell line B82 was a gift from Dr T. Mohandas (Division of Medical Genetics, Department of Paediatrics, UCLA-Harbor General Hospital, Torrance, California). Descriptions of these lines appear either in the American Type Culture Collection catalogues or in the Camden Human Genetic Mutant Cell Repository catalogues.

RAG: This cell line was established in 1968, by Ruddle and colleagues, from a transplantable renal adenocarcinoma originally derived from the BALB/cd strain of mice. It was isolated as an 8-azaguanine-resistant clone (i.e. an HGPRT[-] mutant), which remained free of revertants despite prolonged growth under non-selective conditions. RAG was submitted to the American Type Culture Collection (ATCC) where it was registered as the certified cell line (CCL) number 142. It was characterized as having a modal number of 68 chromosomes per cell.

Cl.1D: The mouse cell line Cl.1D ("clone 1D") was one of many sublines indirectly derived from Strain L (one of the first cell strains to be established in continuous culture). Earle's group, who were responsible for the establishment of Strain L, in 1940, later developed the first clone strain from this line - clone

929 – which was registered with the ATCC under the number CCL 1. Clone 929 gave rise to the line L-M, which in turn gave rise to the BrdU-resistant line known as L-M(TK[-]) (Kit *et al.*, 1963). Cl.1D was a subclone of this line, and was also deficient for thymidine kinase activity. It was found to have a modal number of 50 chromosomes per cell.

B82: This heteroploid line, exhibiting a modal chromosome number of 50, was one of two clonal sublines isolated directly from strain L, by John Littlefield (1966). It was also a TK[-] mutant, obtained after subjecting L-cells to BrdU. B82 was submitted to the Camden Human Genetic Mutant Cell Repository, where it was registered under the code GM0347.

wg3-h: The HGPRT[-] Chinese hamster line denoted wg3-h, was one of three primary clones derived from the DON line (Westerveld *et al.*, 1971). DON, a non-mutant fibroblast line, was originally established from the lung of a normal male adult Chinese hamster. The diploid and modal chromosome number of both DON and its subline wg3-h was found to be 22.

2.1.3 Marmoset Cell Line B95-8

This cell line was derived by Miller and Lipman (1973), while studying replication and latency of Epstein-Barr Virus (EBV). B95-8 was a transformed line of Marmoset peripheral blood B-lymphocytes, obtained by exposure to an extract of yet another transformed line known as 593L – a B-lymphocyte line established from

a human patient with infectious mononucleosis. This Marmoset cell line was found to release extracellular infectious EBV with particularly high titres of transforming activity. It became most useful as a source of EBV not only for extensive studies on the virus itself, but also for further transformation of B-lymphocytes.

2.2 Media and Culture Conditions

Two types of synthetic liquid media were used throughout this study: DMEM (Dulbecco's Modified Eagle's Medium) and RPMI-1640 (Roswell Park Memorial Institute series). DMEM was used for culturing human fibroblasts and the rodent permanent cell lines, while RPMI-1640 was used to support the growth of transformed human lymphocytes and the EBV-secreting lymphocyte line B95-8.

Penicillin and streptomycin were added to the media at concentrations of 100 i.u./mℓ and 100 µg/mℓ, respectively. This combination of antibiotics was chosen in order to inhibit the growth of both Gram-negative and Gram-positive organisms.

RPMI-1640 was routinely supplemented with 10% foetal bovine serum and DMEM with 15% foetal bovine serum. When cell growth appeared to be very poor, this serum was substituted by high grade foetal bovine serum used for hybridomas. Medium to which serum had been added shall be referred to as full growth medium.

All cultures were grown at 37°C. Closed cultures were gassed with 5% CO_2: 95% air, while open cultures were kept in a humidified incubator with a 5% CO_2 atmosphere.

2.3 Cell Storage and Reconstitution

Subcultures of the different lines were frozen and stored in liquid nitrogen. The process involved pelleting and resuspending the cells in ice-cold freezing mixture. This consisted of full growth medium (either RPMI-1640 or DMEM) supplemented with 10% dimethyl sulphoxide (to prevent crystal formation in the cells). The cells were resuspended to a concentration of 10^6-10^7 cells/mℓ freezing mixture. 1 mℓ aliquots were dispensed into appropriate freezing ampoules. These could be sequentially placed at -20°C for 4 hours, -65°C for approximately 16 hours and finally in the liquid nitrogen freezer (± -170°C). Alternatively, the ampoules could be kept on dry ice for about 1 hour and then stored in liquid nitrogen.

When these cultures were again required, the respective ampoules were removed from the liquid nitrogen and immediately warmed in a 37°C waterbath. Once all but a small portion of the sample had thawed, the suspension was quickly but carefully diluted, drop by drop, in 10 mℓs prewarmed Hanks' balanced salts solution (37°C). This was immediately spun down at about 1 000 rpm, and the supernatant fluid removed. The cell pellet was then resuspended in appropriate full growth medium, gassed and transferred to a 50 mℓ culture flask.

2.4. Cell Immortalization

The B-lymphocytes of leukaemic patients and of individuals both with constitutionally normal and abnormal karyotypes were transformed with Epstein-Barr virus (EBV) in order to establish immortal cell lines.

2.4.1 Harvesting of Epstein-Barr Virus

The virus was collected in the medium which supported the growth of B95-8, an EBV-secreting marmoset cell line (see Section 2.1.3). This line was generously donated by Dr M. Joffe (Department of Immunology, SAIMR, Johannesburg). Cells were placed in full growth medium (RPMI-1640) to a final concentration of approximately $1x10^6$ cells/mℓ, and incubated for 7-10 days. The cells were then pelleted and used for further subcultures. The EBV-containing supernatant fluid was filtered through a 0,22 um millipore filter to ensure that the medium was free of cells, and divided into 1 mℓ aliquots which were then stored at -20°C until required.

2.4.2 Lymphocyte Isolation

To obtain pure samples of lymphocytes, blood specimens were separated by the hypaque-ficoll density sedimentation method of Boyum, 1968 (see Gahrton *et al.*, 1980). The hypaque-ficoll (H-F) solution was prepared to a density of 1,077 as follows:
Using distilled water, ficoll 400 was made up to a 9% solution, and hypaque 65% was made up to a 32% solution. The two solutions

were mixed in a 24:10 proportion of ficoll to hypaque. Density was then tested with a densitometer; mixtures that were too dense or too light could be adjusted by adding distilled water or hypaque, respectively. The mixture was autoclaved and stored at -4°C.

Separation was carried out in sterile 15 or 50 mℓ round-bottomed centrifuge tubes, using an H-F to heparinized (or ACD) whole blood ratio of 2:3. The blood sample was carefully layered on the column and spun at 3900-4000 rpm for 40-50 minutes. The band containing the mononucleate cells (lymphocytes and monocytes) was removed using a pasteur pipette, and diluted out in an equal volume of either RPMI-1640 or Hanks' Balanced Salts Solution. A portion of the surrounding plasma and H-F layers was collected together with the mononucleate band, to ensure maximal lymphocyte yield.

The cells were pelleted at 2 000 rpm for 5 minutes, washed again in medium or balanced salts solution, and pelleted at 1 000 rpm for 5 minutes. After the last wash, they were resuspended in full growth medium (RPMI-1640) and transferred to a large culture flask. They were left in the incubator overnight so that the monocytes would adhere to the bottom of the flask.

Those cell suspensions which contained an undesirably large proportion of red blood cells were treated with NH_4Cl. The cells were pelleted, resuspended in 5 mℓs NH_4Cl and left on ice for 5 min. to allow red blood cell lysis. They were then again pelleted and returned to full growth medium.

The following variables were assessed during the procedures of lymphocyte isolation:

- the type of anti-coagulant (ACD or heparin) in which blood specimens were collected;
- the quality of the specimens (eg. clotted; haemolysed);
- the type of H-F used (the non-commercial H-F as opposed to the commercial product Histopaque-1077);
- the temperature of the H-F solution;
- the centrifugation speed and time;
- the incubation period required for mononucleate cell separation.

2.4.3 Transformation

The lymphocytes left in suspension were transferred to a clean flask and a viability test and cell count was carried out. A 1:10 dilution of the sample in trypan blue was used, so that only viable cells were counted. About $2x10^6$ viable cells were pelleted in a small round-bottomed centrifuge tube, and the supernatant fluid discarded. Either of two methods could be used to infect the cells:

(i) The cell pellet was resuspended in 1 mℓ of the EBV-containing medium and incubated at 37°C for approximately 1 hour, with frequent shaking. The cells were then pelleted gently and resuspended in 1 mℓ frush full growth medium. 0,1 mℓ aliquots (i.e. $2x10^5$ cells) were dispensed into flat-bottomed wells of microtitre plates. A further 0,1 mℓ medium was added to each well. The cells were placed in a humidified incubator with a 5% CO_2:95% air mixture. Every week 0,1 mℓ

medium from each well was replaced with 0,1 mℓ fresh medium.

(ii) The cell pellet was resuspended in 1 mℓ full growth medium and 0,1 mℓ aliquots dispensed into microtitre wells. 0,1 mℓ EBV-containing medium was added to each of the wells. The cells were incubated for 24 hours in a humidified incubator with a 5% CO_2 atmosphere. 0,1 mℓ of the well contents was then discarded and replaced with 0,1 mℓ fresh medium. The medium was replaced on a weekly basis, as in the previous method.

The cells were examined under an inverted microscope where, after 3-4 weeks, successfully transformed cells appeared as clumps. When large clumps were visible, the contents of the wells were pooled and transferred to a 50 mℓ culture flask containing 5 mℓ full growth medium. During the next 4-6 weeks, the medium was supplemented with 10% crude B-cell growth factor, on every alternative feeding. The cultures were split according to the extent of growth.

An experiment was carried out to determine how long blood specimens could be kept after collection, without the lymphocytes losing their ability to transform. Approximately 400 mls of whole blood was collected in ACD tubes and kept at -4°C. 30 mℓ samples were then used for lymphocyte isolation and transformation, on 12 consecutive days, with "day 0" being the day of collection. EBV from two batches was used throughout, and the infection period was 2-2¼ hrs.

2.4.4 B-cell Growth Factor

A crude preparation of B-cell growth factor was obtained as follows:

Preservative-free, heparinized whole blood was separated on a hypaque-ficoll column as previously described. The mononucleate cells were collected, washed twice in Hanks' Balanced Salts Solution, and subsequently placed in full growth medium at a concentration of approximately $2x10^6$ cells/mℓ. Pokeweed mitogen was added, to a final concentration of 10 µg/mℓ. After incubation for either 24 or 48 hours, the cells were removed by high-speed centrifugation. The supernatant fluid, which contained B-cell growth factor, was collected and filtered through a 0,22 µm millipore filter, and divided into 1 mℓ aliquots which were then stored at -20°C until required.

2.5 Cell Fusion

2.5.1 Fusogen

Polyethylene glycol (PEG) of molecular weights 4 000, 1 500 and 1 000 was used as the fusogen. It was prepared at a 30%, 40% and 50% concentration (weight:volume) in the fusion mixture. The PEG was autoclaved two or three times and then allowed to cool at room temperature. Before it solidified, a premixed sterile solution of DMEM with 10% DMSO was added. (The first fusion mixtures were prepared using DMEM alone, but these did not lead to viable hybrid formation - see Section 1.2.3). About half

a drop of 1N NaOH was added to the mixture in order to increase its alkalinity. The pH was not measured; instead, a pink colour produced by the phenol red in DMEM was accepted as an indication of slight alkalinity.

Each batch of PEG prepared was tested for toxicity before being used in fusion experiments. The toxicity tests were carried out on both rodent and human cell cultures, as follows:
Sub-confluent cultures were washed three times in serum-free medium. PEG was added to completely cover the monolayers, and left for 60 or 90 seconds. Serum-free medium was then added to dilute and wash out the PEG. After washing the monolayers two more times, full growth medium was added. The cultures were gassed with 5% CO_2 and incubated. Toxicity would be evident within a few hours, in the form of morphologically distorted cells detaching from the surface of the flask.

2.5.2 Cell Fusion Using Human Fibroblasts

Sub-confluent cultures were given fresh medium the day before setting up hybridization experiments, so that the cells would be actively dividing at the time of fusion. Single-cell suspensions of the parental lines had to be obtained. To this end, the monolayers were first washed three times in serum-free DMEM and then treated with 1-2 mls 0,05% trypsin (made up in either serum-free DMEM or Hanks' Balanced Salts Solution) for 3-10 minutes - treatment time varied according to the cell lines. Once the cells were in suspension, the trypsin reaction was stopped by adding 5-10 mls full growth medium. Cell counts were then carried

out, so that the recommended number of cells could be collected for the fusion experiments.

Several fusion methods were attempted. The first was a method devised by Galfré *et al.* (1977) and modified by Sheer (personal communication, 1983). This could be divided into the following steps:

1. Equal numbers of parental cells — approximately 10^7 each — were washed and pelleted three times in RPMI-1640 to remove the serum (DMEM was also used instead of RPMI-1640, throughout this procedure). The cells were combined in a conical tube before the last spin.
2. The supernatant fluid was removed and the pellet suspended by tapping the tube lightly.
3. 0,8 mℓ PEG (prewarmed to 37°C) was added over a period of 1 minute, and the tube was then placed in a 37°C waterbath for 45 seconds.
4. Serum-free medium (also at 37°C) was added immediately; 1 mℓ added over 1 minute, and then another 20 mℓs added over 5 minutes.
5. The cells were pelleted at 2 000 rpm for 7 minutes.
6. The supernatant fluid was removed and 1 mℓ full growth medium was added very gently. The volume was then made up to 20 mℓs, and this was divided between 2-4 x 50 mℓ culture flasks, such that the cells were not too confluent when attached.
7. Selection began the following day, with HAT medium being changed approximately every 2-3 days until hybrid clones were "picked".

A second method for cell fusion using human fibroblasts was that

devised by Duckels (personal communication, 1984), and was based on methods employed in hybridoma formation:

1. As in the foregoing method, parental cells were washed in serum-free medium, and the two cell suspensions combined before the last spin.
2. All the medium was aspirated off, leaving the pellet as dry as possible.
3. 0,1-0,2 mℓ PEG solution was added and timing of treatment began immediately. The cells were suspended in the PEG by gently flicking the tube, and then centrifuged at very low speed (about 100 rpm) for approximately 5 minutes. (Total time of exposure to PEG should not exceed 7-8 minutes).
4. 5 mℓ serum-free medium was added drop by drop in order to disperse the cells.
5. 5 mℓ full growth medium was then added. The suspension was left to stand at room temperature for 10 minutes, after which time the cells were pelleted at 500 rpm.
6. The cells were then resuspended in 40 mℓ full growth medium and plated out over 5-6 x 24-well tissue culture trays. These were then placed in a humidified incubator with a 5% CO_2 atmosphere.
7. After 24 hours, HATO selection commenced.

The following fusion protocol was described by Fox (1983) and is a modification of the method devised by Davidson *et al.* (1976).

1. The parental cells were seeded into 35 mm petri dishes in various proportions (1:1, 3:1 or 4:1, totalling $5{,}0 \times 10^5$ cells). The mixed cultures were incubated in full growth medium for 24 hours.

2. Prior to PEG treatment, the cell monolayers were washed three times in serum-free medium.
3. 0,5-1,0 mℓ 50% PEG was added. The cells were exposed to the PEG for variable lengths of time, ranging from 30 seconds to 2 minutes.
4. The PEG was removed by quickly rinsing the monolayers three times in serum-free medium.
5. 1-2 mℓs full growth medium was then added. The cells were incubated for a further 24 hours to allow completion of fusion.
6. The monolayers were washed in serum-free medium, trypsinized and transferred to a series of 50 mℓ tissue culture flasks containing 5 mℓs full growth medium. The cells from each petri dish were divided among 2-5 such flasks, depending on fusion efficiency and cell recovery rate after PEG treatment.
7. HATO selection began the following day. Occasionally ouabain selection began only at the next medium change.

A fourth method tested for fibroblast fusions was that developed and presently used by Mohandas (personal communication, 1984). It is similar to the foregoing method and also involves fusion in a cell monolayer system.

1. $4x10^6$ cells of each parental line were seeded into a 100 mm petri dish and incubated overnight in 10 mℓs full growth medium.
2. The medium was removed and the cell monolayer washed once with approximately 4 mℓs Hanks' Balanced Salts Solution.
3. The cells were treated with 4 mℓs 50% PEG 1500 for 90 seconds, and then washed three times with Hanks' Balanced Salts Solution.

4. 10 mℓs full growth medium was added to the cells, which were then incubated overnight.
5. The cells were trypsinized as usual and resuspended in 20 mℓs selective medium (HATO). To each of twenty 60 mm petridishes, 1 mℓ of this cell suspension and a further 2,5 mℓs selective medium was added.
6. The medium was changed on a weekly basis until hybrid colonies could be distinguished, and then twice weekly until the colonies were "picked".

2.5.3 Cell Fusion Using Human Lymphocytes

Human lymphocytes were collected as described previously, and used immediately in fusion experiments. Cell counts were carried out after washing three times in serum-free RPMI-1640.

As in the case of fusions with human fibroblasts, both suspension and monolayer fusion protocols for human lymphocytes were tested. The suspension fusion procedures followed were the two already described in the preceding section (viz. the modified method of Sheer and Duckels' method), as well as that developed by Klein (personal communication, 1983). RPMI-1640 was the basic medium used throughout the fusion procedures, and fusion products were then selected and grown in DMEM.

Klein's suspension fusion method was as follows:

1. $2x10^6$ human lymphocytes and $2x10^5$ rodent cells were mixed, centrifuged, and the supernatant fluid removed to leave the pellet as dry as possible.

2. 0,5 mℓ 50% PEG solution was carefully added to the pellet. PEG treatment proceeded at room temperature for 5 minutes.
3. 0,5 mℓ serum-free medium wa. added slowly, and then a further 10 mℓs was added.
4. The cells were pelleted and washed three times in serum-free medium.
5. After the last spin, the cells were resuspended in 10 mℓs full growth medium and divided between two x 50 mℓ culture flasks.
6. Selective medium was added after 24 hours.

Brahe and Serra (1981) described a method for lymphocyte fusion which resembles the monolayer fusion methods used for fibroblasts:

1. 1-2x10^5 rodent cells were seeded into each 60 mm petri dish.
2. About 24 hours after innoculation, 0,5-1,0x10^6 lymphocytes were added to each dish and the mixed cell cultures were incubated for a further 3-4 hours.
3. The medium was then carefully aspirated, and 1 mℓ 50% PEG solution was added.
4. After 1 or 2 minutes, the PEG solution was removed.
5. The cells were washed three times by carefully adding Hanks' Balanced Salts Solution by running it down the wall of the dishes, and they were then incubated with full growth medium.
6. After 24 hours the cells were again washed and HAT medium was added. The medium was changed every 2-3 days.

2.6. Hybrid Selection, Cloning and Subcloning

2.6.1 Subdivision Ratios

After fusion, the cells were left to recover in full growth medium and incubated for approximately 24 hrs before being subdivided among several flasks. The subdivision ratios were determined according to the number of parental cells used in each experiment, fusion efficiency and cell survival rates after exposure to PEG.

At this stage, fusion efficiency could be evaluated by observing the extent of polykaryon formation, under the inverted microscope. It was not always possible to distinguish between homokaryons and heterokaryons (i.e. intraspecies as opposed to interspecies fusion products). However, in order to facilitate hybrid isolation, it was preferable to assume that all polykaryons represented an interspecies fusion event, so that more flasks were used than were perhaps necessary.

Cell survival rates had to be taken into consideration, regardless of the efficiency of fusion, because cell confluency would seriously hamper the process of selection. This meant that, even when fusion rates were low, the cells had to be distributed so as not to form a confluent layer once they had adhered to the flask.

2.6.2 Selection

In order to eliminate unfused rodent parental cells as well as

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2.6.2 Selection

In order to eliminate unfused rodent parental cells as well as

intraspecific hybrids of these, the cells were cultured in HAT medium. This consisted of whole medium to which hypoxanthine, aminopterin and thymidine (HAT) were added to final concentrations of 5,0 mM. 20 µM and 0,8 mM, respectively. HAT could be purchased as a 50x concentrated solution, which was appropriately diluted in whole medium prior to use. The principle of HAT selection has been described previously (see Section 1.2.2.1).

In addition to rodent-human cell hybrids, human cells, whether fused or unfused, would proliferate under HAT selection alone. These were eliminated by adding ouabain to the culture medium to a final concentration of $1\text{-}2\times10^{-6}$ M. When human lymphocytes were used as parental cells, ouabain selection was unnecessary, since these non-adherent cells would be lost from culture after the first few medium changes.

Ouabain selection was a relatively rapid process, and was discontinued once the human fibroblasts had been destroyed. HAT selection, on the other hand, was continued at least until the hybrid colonies had been "picked", and in a few cases continued indefinitely (i.e. when chromosomes X and 17 were being selected for). Discontinuation of HAT selection was a gradual process, in order to avoid chromosomal instability and rearrangements in the hybrids. HAT medium was therefore initially replaced by HT medium (i.e. full growth medium, hypoxanthine and thymidine). After about two weeks, HT could be omitted, and the cultures returned to full growth medium only.

2.6.3 Cloning and Subcloning

Each distinct colony presumably originated from a single interspecific fusion event. A "colony-picking" method was used to isolate these colonies, with the aid of an inverted microscope, which was placed in the laminar flow cabinet and used to locate colonies and monitor the dislodging of cells. The method entailed firstly removing the culture medium, rinsing the surface of the flask with serum-free medium and aspirating it, leaving the surface as dry as possible. Pasteur pipettes were bent and rounded off at the tip, over the flame of a burner. A small amount of full growth medium was drawn into a prepared pipette and then carefully dropped onto the colony which was to be cloned. The cells were gently lifted from the surface with the aid of the rounded pipette tip, and then drawn up together with the medium. They were distributed among a series of microtitre wells, each containing 2-3 drops of full growth medium. Basically, a half-dilution series was set up along each row of wells. The trays were incubated in a 5% CO_2 atmosphere. The wells containing single cells were marked and chosen for cell expansion.

When sufficient cells were present in the marked wells, they were trypsinized and transferred to 50 mℓ flasks. After approximately one week, isolated colonies again became visible. These were subcloned by returning individual colonies to microtitre trays and diluting them in the same manner as for the primary clones. Subclones, or secondary clones, were grown as before, and then further expanded in 250 mℓ flasks.

Cytogenetic analysis of each hybrid line was carried out when sufficient cells were available for harvesting, biochemical studies and storage. A sub-confluent culture was chosen for metaphase arrest, while approximately $5x10^7$ cells were collected for isozyme analyses and for freezing in liquid nitrogen.

2.6.4 Nomenclature of Hybrid Lines

There is no standard system for hybrid nomenclature, and a method similar to that employed by Fox (1983) was chosen for the coding of hybrids in this study.

First the parental cell types were indicated; the first two letters denoted the human cell line (i.e. the patient code), while the third was the first letter of the rodent cell line. For example, JNC indicates that the hybrid originated from a cross between human cells from patient JN and mouse cells from the line Cl.1D. This triplet applied to fusions involving human fibroblasts. When human lymphocytes were used, a fourth letter was added to the code, namely "L" (for "lymphocytes"). An example is BLRL, which indicates that BL lymphocytes were fused with RAG cells.

The letter code was followed by a number which represented the number of the hybridization experiment carried out between that particular combination of parental cells. This was then followed by the clone and subclone code, which was a letter and a number, respectively. CRR 4E2 would therefore be the second subclone of the fifth clone from the fourth cross between the human fibroblast line CR and the mouse line RAG.

When further subcloning was carried out at a later stage (after hybrid analysis), the original code was given, followed by an individual subline number; the first subline of CRR 4E2 would therefore be denoted CRR 4E2.1.

Those lines which were maintained on HAT medium, for the specific retention of human chromosomes 17 or X, were given the symbol "H" at the end of their code; for example, JNB 1D1-H.

2.7. Cytogenetic Techniques

2.7.1. Cell Harvesting and Slide Preparation

Sub-confluent cultures were given fresh medium approximately 24 hours before metaphase arrest. The cells were exposed to colchicine at a concentration of 0,5 ug per 1 mℓ medium. After 3-4 hours the medium was aspirated, and hypotonic treatment commenced. 5 mℓs prewarmed 0,075 M KCℓ were added, and the cultures were returned to the incubator for 20-30 minutes. The cells were then gently suspended by shaking the flask – when firmly adherent cells were being harvested, a silicon scraper was used to loosen the cells. About 7 drops of ice-cold 3:1 fixative (3 parts methanol to 1 part glacial acetic acid) were added to the cell suspension which was then kept on ice for 30 minutes. The cells were transferred to a centrifuge tube and pelleted at 1600 rpm for 10 minutes. The supernatant fluid was drawn off and the cells were slowly resuspended in fresh 3:1 fixative. Two further fixative changes were carried out, and the fixed cell suspension was left at -4°C overnight.

The fixative was changed once more, prior to slide preparation. Pre-cleaned slides, which had been soaking in absolute alcohol for at least 24 hours, were flooded with fresh ice-cold fixative. The excess fixative was flicked off, and a few drops of the cell suspension were dropped onto the horizontal slides from a variable height. As the suspension began to evaporate, the slides were again flooded with fixative. After about 1 minute, the excess fixative was tipped off and the slides were dried in front of a hot fan.

For each harvest, one slide was first prepared so that the quantity and quality of the metaphase spreads could be evaluated, before preparing the rest of the slides. The test slides were screened after staining with either 10% Giemsa for 5 minutes or quinacrine mustard for 20 minutes. If chromosome spreading was poor, this could sometimes be improved by doing one or a combination of the following:

(i) standing the clean slides in the freezer for at least 30 minutes prior to slide preparation;

(ii) holding the slides at an angle when dropping the cell suspension;

(iii) increasing the height from which the cell suspension was dropped

(iv) gently flicking the slides immediately after dropping the cell suspension;

(v) flame-drying the slides instead of drying them in front of a hot fan.

2.7.2 Staining Techniques

All parental cell lines were karyotyped after trypsin-Giemsa banding.

Cytogenetic analysis of the hybrid lines required a combination of staining procedures. Chromosome preparations were either fluoresced with quinacrine mustard (Q-banding) or trypsin-Giemsa banded (G-banding) to enable positive chromosome identification. About 25-30 banded metaphases of each hybrid line were photographed. Since only the human chromosome content of the lines required detailed characterization, chromosome banding was followed by differential chromosome staining in the form of Hoechst fluorescence or G-11 staining. After relocating the photographed metaphases, the human chromosomes could be distinguished and subsequently identified on the corresponding photographs. Differential staining was usually carried out within two weeks of G- or Q-banding.

2.7.2.1 Trypsin-Giemsa Banding

Trypsin-Giemsa banding was carried out on slides which had been aged in one of the following ways; (i) 4 hours at 60°C, (ii) overnight by incubation at 37°C, or (iii) 48 hours at room temperature. The method used routinely was a modification of that documented by Seabright (1971):

1. The slides were dipped into a 0,05% trypsin solution in phosphate buffered saline (PBS), which had been prewarmed to 37°C.

Trypsin treatment time varied between 18 and 60 seconds, depending on the quality of the chromosome preparations.

2. To stop the trypsin reaction, the slides were immediately rinsed in 5% foetal calf serum (in PBS) and then in PBS alone.
3. The preparations were stained in a solution of 4% Giemsa in phosphate buffer, pH 6,8, for 4-5 minutes.
4. They were rinsed briefly in distilled water and blotted dry.

Before differential staining could be carried out, G-banded preparations had to be destained by rinsing in two changes of 3:1 fixative for 5 minutes, followed by 4-5 changes of deionized water over a period of two hours. Spare slides (of the same harvest) were also destained and used as test slides to establish the optimal conditions for differential staining.

2.7.2.2 Quinacrine Fluorescence Banding

This was done according to a modification of the method devised by Caspersson *et al.* (1970):

1. MacIlvaine's buffer, pH 5,5, was prepared by mixing approximately 4 parts 0,1 M citric acid and 5 parts 0,2 M *di*-sodium phosphate.
2. The slides were stained for at least 20 minutes in a 0,05% solution of quinacrine mustard dissolved in this buffer.
3. Once the slides had taken up the dye, they were rinsed twice and mounted, using fresh MacIlvaine's buffer in each case.

Those slides which were to be kept for subsequent Hoechst or G-11 staining were stored at room temperature after sealing the

coverslips with translucent nail varnish.

2.7.2.3 G-11 Staining

This technique was first described, and adapted for differential staining in interspecific cell hybrids, by Bobrow and Cross (1972, 1974a,b). Since then, numerous modifications of the original technique have appeared in the literature. The original method, as well as several modifications thereof, were applied in the present study.

According to the original method, slides were aged for about one week at room temperature, or in a dry oven at 60°C for two days, before staining.

1. The staining solution consisted of a 2% aqueous dilution of Giemsa, adjusted to pH 11,0 with concentrated NaOH.
2. Staining time varied between 10 and 20 minutes.
3. Slides were then rinsed in phosphate buffer, pH 6,8, and allowed to dry.

The same authors also proposed staining for 12-15 minutes in a 2% dilution of Giemsa in 0,007 N NaOH. Alternatively the slides could be stained for 15-20 minutes in a 2% solution of Giemsa in 1 mM NaOH before being rinsed in tap water.

Friend *et al.* (1976a,b) made the following modifications to the original procedures of Bobrow and Cross:

1. Freshly prepared slides were aged by soaking in 5-6 changes of distilled water for 1-2 hours.

2. 0,05 M sodium phosphate buffer, adjusted to pH 11,3 by the addition of 1 N NaOH, was prewarmed to 37°C.
3. Giemsa stain was prepared immediately prior to use, and added to the buffer to a final concentration of 1,6-2,0%. This staining solution was mixed gently.
4. Staining of the slides was monitored by removing them at various time intervals, and in most instances 3-5 minutes was adequate.
5. Once stained, the slides were rinsed in distilled water at 37°C for 30 seconds, air-dried and mounted.

Another modification, described by Fox (1983), was the following:

1. A solution of 0,05 M Na_2HPO_4 was brought to pH 11,3 with concentrated KOH.
2. 50 mℓs of this buffer was heated to 37°C; 2 mℓs of Giemsa stain was added and the solution stirred gently.
3. 8-14 day old slides were immediately stained for 7 minutes, rinsed briefly (2-3 seconds only) in distilled water prewarmed to 37°C, and air-dried in front of a hot fan.

Yet another procedure for alkaline Giemsa staining employed in this study was that communicated by Stanley (as carried out at the Human Cytogenetics and Somatic Cell Genetics Laboratory, Imperial Cancer Research Fund, London):

1. The slides were aged for one day at 60°C and washed in 2xSSC at 57°C for about 5 minutes.
2. They were immediately stained for 3½ minutes in a solution of 1 mℓ Giemsa + 50 mℓs 0,14 N NaOH, kept at room temperature.

2. 0,05 M sodium phosphate buffer, adjusted to pH 11,3 by the addition of 1 N NaOH, was prewarmed to 37°C.
3. Giemsa stain was prepared immediately prior to use, and added to the buffer to a final concentration of 1,6-2,0%. This staining solution was mixed gently.
4. Staining of the slides was monitored by removing them at various time intervals, and in most instances 3-5 minutes was adequate.
5. Once stained, the slides were rinsed in distilled water at 37°C for 30 seconds, air-dried and mounted.

Another modification, described by Fox (1983), was the following:

1. A solution of 0,05 M Na_2HPO_4 was brought to pH 11,3 with concentrated KOH.
2. 50 mℓs of this buffer was heated to 37°C; 2 mℓs of Giemsa stain was added and the solution stirred gently.
3. 8-14 day old slides were immediately stained for 7 minutes, rinsed briefly (2-3 seconds only) in distilled water prewarmed to 37°C, and air-dried in front of a hot fan.

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1. The slides were aged for one day at 60°C and washed in 2xSSC at 57°C for about 5 minutes.
2. They were immediately stained for 3½ minutes in a solution of 1 mℓ Giemsa + 50 mℓs 0,14 N NaOH, kept at room temperature.

3. The slides were then rinsed under the tap and blotted dry.

The protocol devised by Smith (personal communication, 1984) was as follows:

1. The buffer was prepared by dissolving 3,52 g Na_2HPO_4 in 500 mls distilled water (i.e. 0.05 M Na_2HPO_4), and the pH adjusted to 11,3-11,5 with 1 N NaOH. (This was prepared immediately prior to use).
2. In two separate coplin jars, 50 mls buffer and 50 mls distilled water were heated to 37°C.
3. As soon as the buffer reached 37°C, 4,0 mls freshly prepared Giemsa stain was added and mixed very well, very quickly.
4. Slides were immediately placed in the staining solution, and the coplin jar covered with parafilm to avoid oxidation. Optimal staining time varied from 1-10 minutes.
5. Following staining, the slides were quickly rinsed in the prewarmed distilled water, and then for 1-2 minutes in distilled water at room temperature.

Several brands of Giemsa, both liquid and powder forms, were assessed in the above protocols; these included Gurr, Merck, BDH Chemicals, Fisher Scientific, Clinical Sciences Diagnostics and Harleco.

2.7.2.4 Giemsa Stain Preparation

The Giemsa stain used in most of the above-mentioned G-11 protocols was made up from the powdered form, as follows:

1 g of Giemsa powder was d'ssolved in 66,0 mℓs (83,16 g) of glycerol, in a foil-covered container. This was stirred for 4-5 hours at approximately 60°C. It was then allowed to cool to room temperature, and 66,0 mℓs of 100% methonol was added. The mixture was stirred at room temperature overnight in a light-tight stoppered jar.

With minimal exposure to light, the stain was usually good for 2-3 months.

2.7.2.5 Hoechst 33258 Staining

Hoechst 33258, a benzimidazol derivative, was also used to distinguish between mouse and human chromosomes in hybrid metaphase preparations. Staining was carried out according to the method described by Kozak *et al.* (1977):

1. Destained, dry slides were placed in a coplin jar containing Hoechst 33258 at a concentration of 0,05 µg/mℓ in 0,09% NaCℓ.
2. After 15 minutes, the slides were rinsed twice in distilled water, air-dried, and wet-mounted in 0,08 M sodium phosphate, 0,12 M sodium citrate (pH 4.1).

After examination and photography of the metaphase spreads, the coverslips were removed and the slides rinsed in distilled water, air-dried, and stored in a covered light-tight box. Under these conditions, the fluorescence would not fade appreciably for several weeks.

These authors suggested that the slides be G-banded using a Viokase-Giemsa protocol devised by Friend. In the present study however, the Hoechst stain was applied to chromosome preparations which had been G-banded using the trypsin pretreatment method (as described in Section 2.7.2.1). Hoechst staining could also be followed by G-11 staining, after destaining the slides.

2.7.3 Microscopy and Photography

Metaphase spreads were examined and photographed using a Leitz Wetzlar microscope, with the WILD Photoautomat MPS 55 electronic control unit for automatic exposure.

G-banded and G-11-banded preparations were photographed with a 1,0x camera objective and an NPL FLUOTAR 100x oil-immersion objective, on Agfapan 25 film. Fluorescence microscopy was done with the aid of a 200 W mercury lamp and a Leitz Wetzlar excitor filter. Photography in this case was with a 0,8x camera objective, and on Kodak Technical Pan film 2415 (ESTAR-AH Base).

Promicrol ultra-fine grain developer was used for both types of film, and printing was done on Ilford or Agfa paper, of grade 3 or 4 depending on the degree of contrast required.

3. RESULTS

3.1 Cell Immortalization

3.1.1 Isolation of Lymphocytes

The pattern obtained upon separation of whole blood through a hypaque-ficoll column is illustrated in Figure 3.1. A total of 236 blood specimens were separated in this manner, and Tables 3.1 to 3.3 provide the general assessment of the variables tested during these separations. Best results were obtained when samples were layered on prewarmed H-F, regardless of whether the commercial or non-commercial product was used. It was essential to have clot-free blood samples with minimal haemolysis, preferably collected in ACD. Centrifugation speed and time could be varied considerably, without affecting the quality of separation. It was also found that the majority of monocytes had settled out of suspension after an incubation period of only 20 minutes.

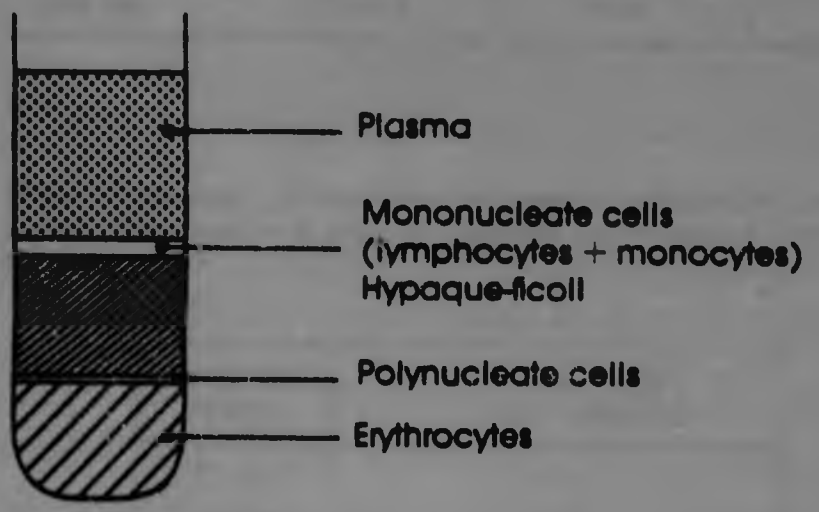


FIGURE 3.1: Diagrammatic representation of the pattern obtained upon separation of whole blood through a hypaque-ficoll column.

TABLE 3.1: General assessment of variables tested in separation of 236 blood specimens. Quality of separation is expressed on a scale of +(very poor) → +++++(very good)

BLOOD SPECIMEN		HYPAQUE-FICOLL NON-COMMERCIAL ±4°C	HYPAQUE-FICOLL NON-COMMERCIAL ±25°C	HYPAQUE-FICOLL COMMERCIAL ±4°C	HYPAQUE-FICOLL COMMERCIAL ±25°C
HEPARIN:	Partially clotted	+	+	+	+
	Partially haemolysed	+	+++	+	+++
	Clot-free and non-haemolysed	++	++++	++	++++
ACD:	Partially clotted	+	+	+	+
	Partially haemolysed	++	++++	++	++++
	Clot-free and non-haemolysed	+++	+++++	+++	+++++

TABLE 3.2: Effect of centrifugation speed and time on quality of blood separation. Expressed on a scale of + → +++++ (as above)

CENTRIFUGATION SPEED	CENTRIFUGATION TIME 30 min	45 min	50-60 min
2500 rpm	+++++	+++++	+++++
3500 rpm	+++++	+++++	+++++
3900 rpm	++++	+++++	+++++

TABLE 3.3: Extent of monocyte and macrophage adherence in relation to incubation period. Expressed on a scale of +(minimal separation) → +++++(maximal separation)

INCUBATION PERIOD	MONONUCLEATE CELL SEPARATION
± 17 hrs	+++++
3 hrs	+++
20-30 min	++++

3.1.2 Transformation

The two methods for B-lymphocyte transformation (Section 2.4.3) were tried on a total of 20 lymphocyte samples. The success rate in each case, with and without the addition of B-cell growth factor, is shown in Table 3.4. Although both methods could lead to lymphocyte immortalization, the second method, where the EBV infection period was much longer, proved to have a higher success rate. The addition of B-cell growth factor also appeared to have a positive influence on transformation.

TABLE 3.4.: Transformation rate in 20 lymphocyte samples

METHOD:	B-CELL GROWTH FACTOR	NUMBER OF SPECIMENS	SUCCESSFUL TRANSFORMATIONS
(I) (infection period:1hr)	+	4	2
	-	2	1
(II) (infection period:24hrs)	+	13	12
	-	1	0
	TOTAL:	20	15

The experiment depicted in Table 3.5 indicates that blood specimens may be stored for 5 days after collection, without the lymphocytes losing their ability to transform. (The effect of freezing H-F separated lymphocytes and subsequent transformation is currently being investigated).

TABLE 3.5: Transformation results on 11 consecutive days, with a blood sample which was being stored at -4°C. (Day "0" = day of collection of specimen - presumed to give positive result).

DAY	AMOUNT OF BLOOD SEPARATED	EBV CATCH	INFECTION PERIOD	TRANSFORMATION
1	30 mls	03/04/85	2 hrs	+
2	30 mls	03/04/85	2½ hrs	+
3	30 mls	03/04/85	2½ hrs	+
4	30 mls	23/04/85	2½ hrs	+
5	30 mls	23/04/85	2½ hrs	+
6	30 mls	23/04/85	2½ hrs	-
7	30 mls	23/04/85	2½ hrs	-
8	30 mls	23/04/85	2½ hrs	-
9	30 mls	23/04/85	2½ hrs	-
10	30 mls	23/04/85	2½ hrs	-
11	30 mls	23/04/85	2½ hrs	-

3.2 Rodent Cell Lines

3.2.1 Growth Characteristics

All the rodent cell lines were fast-growing compared to the human cell cultures. The Chinese hamster line wg3-h was particularly fast-growing. This line, as well as the mouse lines B82 and Cl.1D, were more adherent and exhibited less contact inhibition than the line RAG. The four lines were tested for revertants by growth in HAT medium for variable lengths of time. No back-mutation was observed; within two weeks most of the cells had died. Prolonged culture under these conditions resulted in eventual death of all cells.

3.2.2 Chromosomal Constitution

Cytogenetic analysis of the rodent cell lines was carried out, as described previously (Section 2.7.1 and 2.7.2). All the preparations were trypsin-Giemsa banded. Representative karyotypes of the four lines are shown in Figures 3.2 to 3.5, where the chromosomes are arranged according to the normal mouse and Chinese hamster karyotypes (Nurster, 1972; Nesbitt & Francke, 1973; Cowell, 1984). The normal mouse diploid number is 40, while that of the Chinese hamster is 22.

From 34 to 52 cells of each line were analysed, in order to determine their modal numbers. These results are depicted in Figures 3.6 to 3.9; the values include characteristic marker chromosomes and any *de novo* rearrangements which occurred in culture.

RAG was found to be hyperdiploid, with a distribution peaking at 66 chromosomes per cell. Lines C1.1D and B82 were also hyperdiploid, and showed distributions with distinct peaks at 47 and 53 chromosomes per cell, respectively. The Chinese hamster line wg3-h was the most homogeneous with respect to chromosome number, showing a spread of values from 19 to 22 chromosomes per cell. Approximately 60% of the cells were found to have 22 chromosomes. A second and smaller peak is also evident at 44 chromosomes per cell.



FIGURE 3.2: Representative karyotype of the mouse cell line RAG (64 chromosomes).

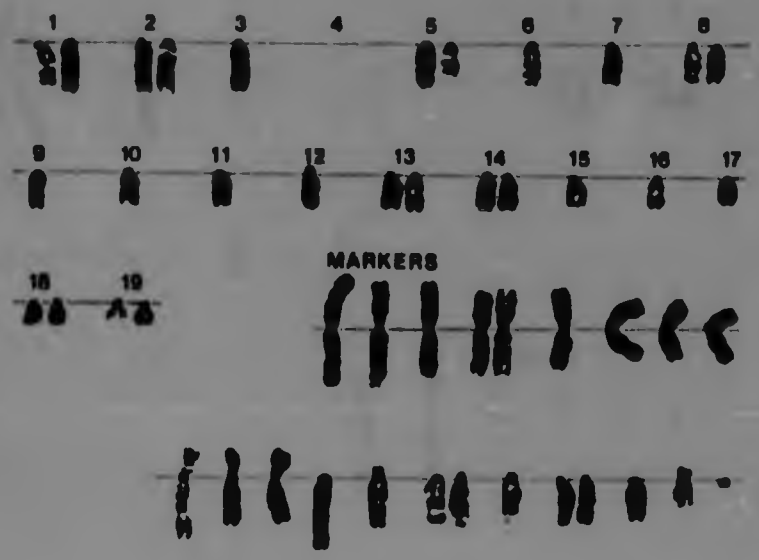


FIGURE 3.3: Representative karyotype of the mouse cell line Cl.1D (48 chromosomes).

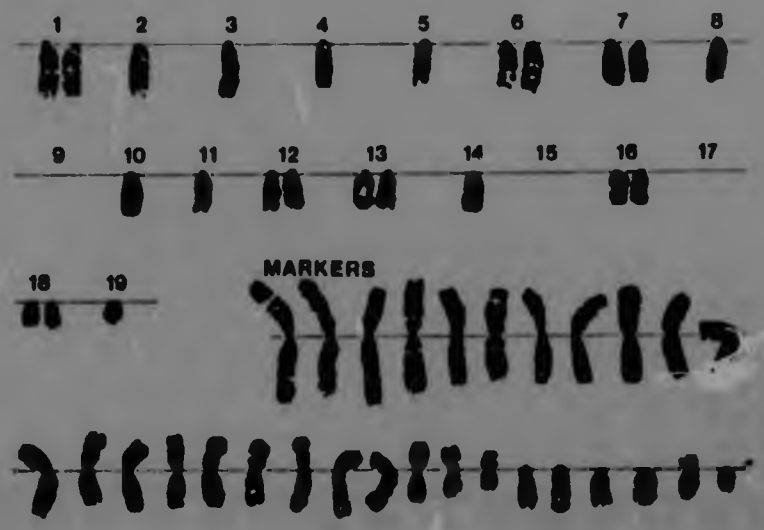


FIGURE 3.4: Representative karyotype of the mouse cell line B82 (52 chromosomes).

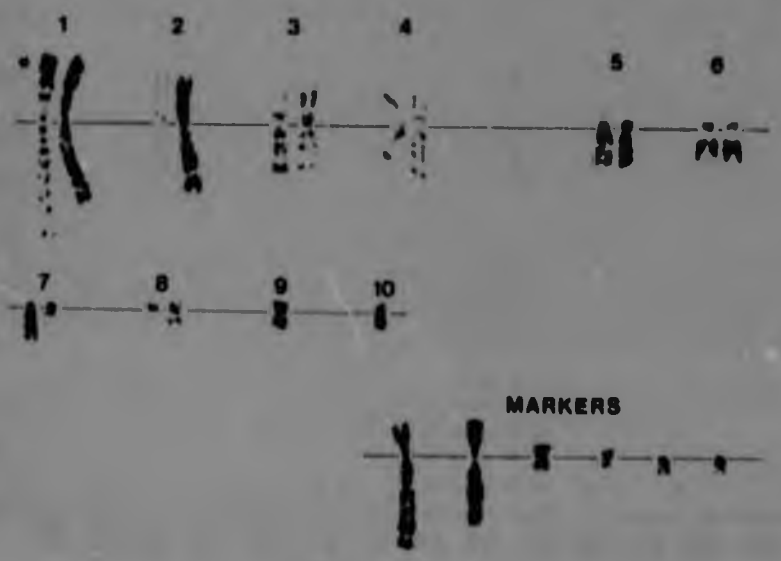


FIGURE 3.5: Representative karyotype of the Chinese hamster line wg3-h (24 chromosomes).
* Variant chromosome 1

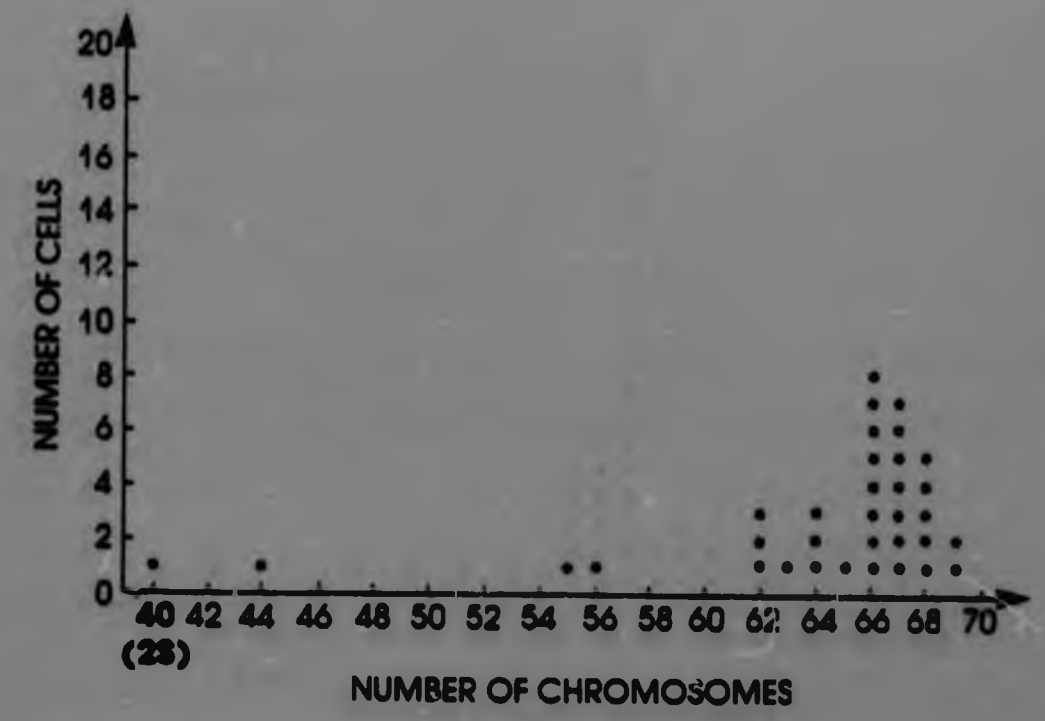


FIGURE 3.6: Chromosome distribution of line RAG (34 metaphases).

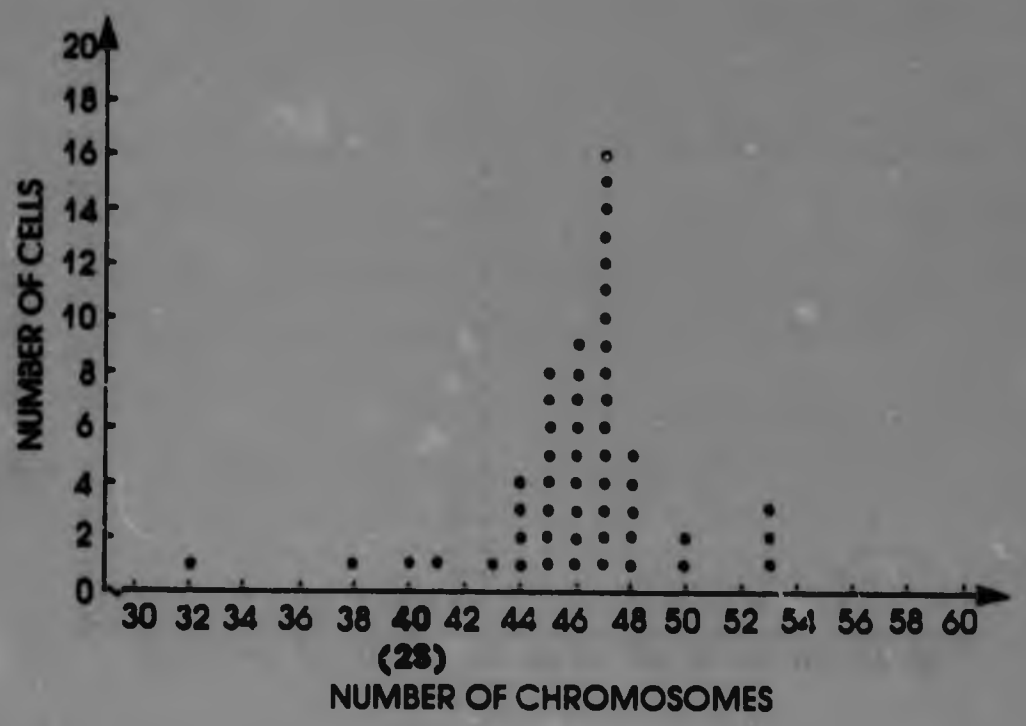


FIGURE 3.7: Chromosome distribution of line Cl.1D (52 metaphases).

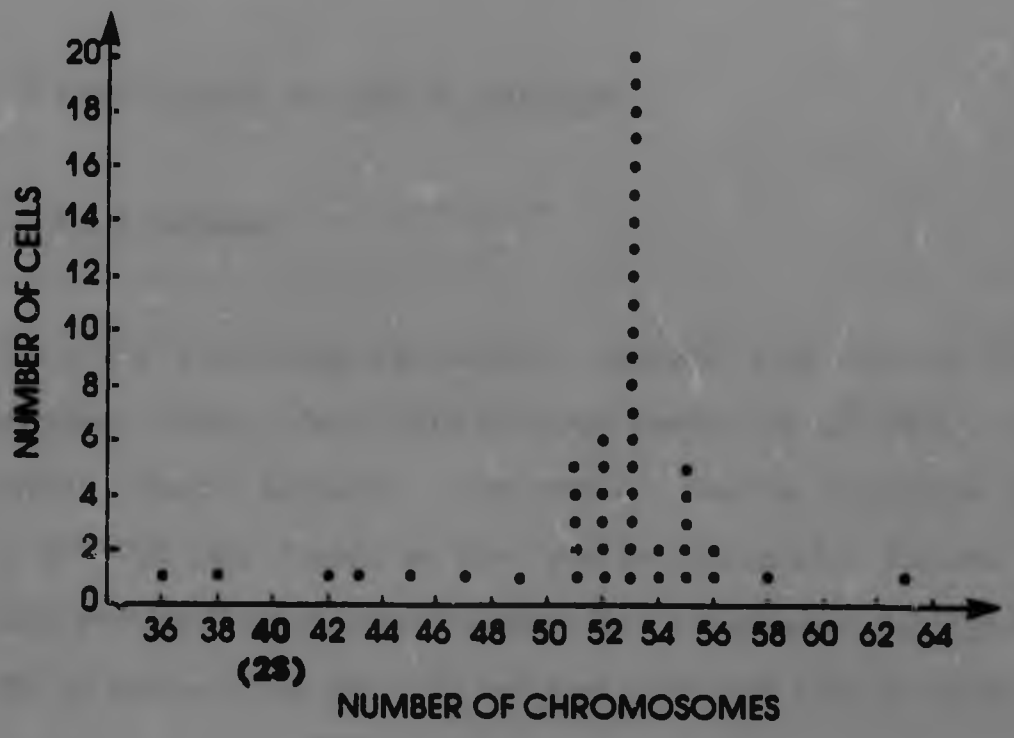


FIGURE 3.8: Chromosome distribution of line B82 (49 metaphases).

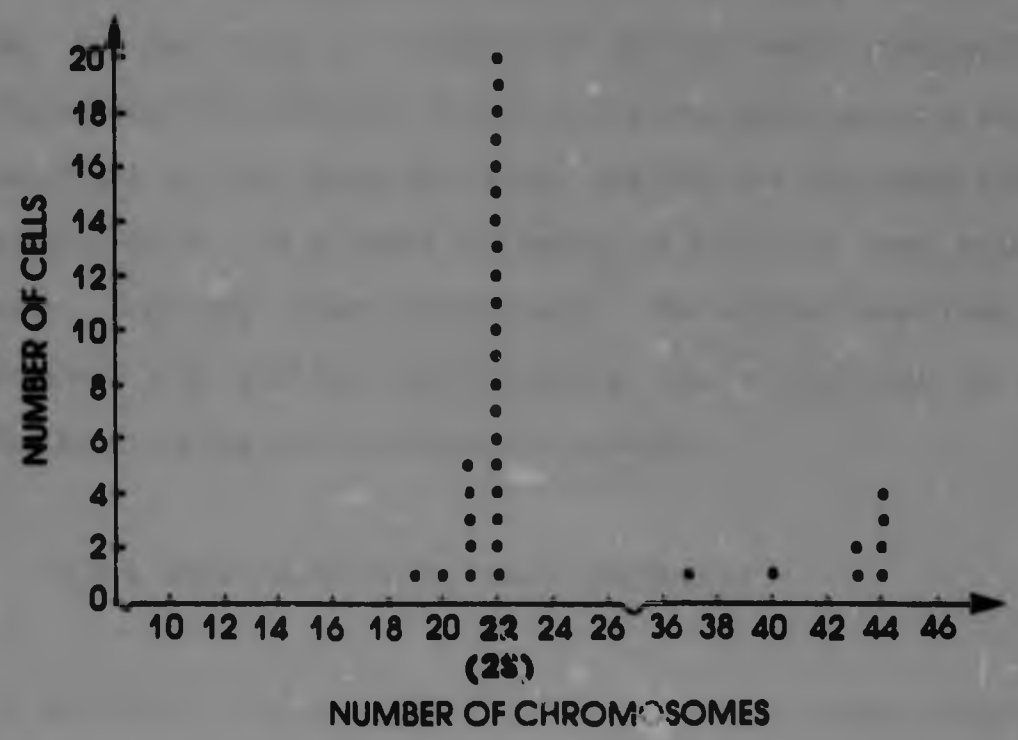


FIGURE 3.9: Chromosome distribution of line wg3-h (35 metaphases).

3.3 Cell Fusion and Hybrid Isolation

3.3.1 Fusogen

Table 3.6 summarizes the results obtained with various fusion mixtures of PEG. Only those mixtures containing 10% DMSO yielded viable fusion products. Preliminary results indicated that a 50% PEG 1500 fusion mixture yielded successful fusions and this protocol was therefore adhered to in subsequent experiments. PEG of molecular weight 1540 was also used with similar success.

3.3.2 Cell Fusion Using Human Fibroblasts

Table 3.7 is an assessment of the different fusion protocols involving human fibroblasts (described in Section 2.5.2). It may be seen that all suspension fusions were unsuccessful. Fusion was first achieved following the monolayer fusion protocol described by Fox, using the mouse line RAG and the human fibroblast line FK. This became the method of choice for most experiments involving human fibroblasts. The method described by Mohandas also yielded viable hybrids. Fusion could not be effected using the Chinese hamster line wg3-h.

3.3.3 Cell Fusion Using Human Lymphocytes

As described in Section 2.5.3, three suspension fusion procedures were evaluated in crosses involving human lymphocytes. Of

these, the modified method of Galfré *et al.* (i.e. the method described by Sheer) was the only one which led to the formation of fusion products (as indicated in Table 3.8). Upon cloning, however, these hybrids were no longer viable. Viable hybrids were obtained following the method described by Brahe and Serra, which resembles the monolayer fusion methods described for human fibroblasts.

TABLE 3.6: Success rate with various PEG fusion mixtures. (N.D. - not done).

MOLECULAR WEIGHT	CONCEN-TRATION	10% DMSO	TOTAL NO. OF EXPERIMENTS	SUCCESSFUL NO OF FUSIONS
4000	30%	-	1	0
		+	2	2
	40%	-	N.D.	
		+	N.D.	
	50%	-	1	0
		+	N.D.	
1500	30%	-	1	0
		+	1	1
	40%	-	6	0
		+	7	4
	50%	-	4	0
		+	14	12
1000	30%	-	3	0
		+	1	0
	40%	-	N.D.	
		+	1	0
	50%	-	6	0
		+	1	0
1540	30%	-	N.D.	
		+	N.D.	
	40%	-	N.D	
		+	N.D.	
	50%	-	N.D.	
		+	17	16

TABLE 3.7: Assessment of fusion protocols for human fibroblasts

FUSION PROTOCOL	PARENTAL CELL COMBINATIONS	PARENTAL CELL RATIOS (RODENT:HUMAN)	TOTAL NUMBER OF PARENTAL CELLS	PEG MIXTURE* AND TREATMENT TIME	RESULT**
SUSPENSION FUSIONS:					
(i) Method of Sheer (1983):	RAG x FN	1:1	$1,0 \times 10^7$	40% PEG 1500, 7 min	Unsuccessful
(ii) Method of Juckels (1984):	RAG x FK	1:1	$2,0 \times 10^6$	50% PEG 1500, 7 min	Unsuccessful
	ug3-h x FK	1:1	$2,0 \times 10^6$	50% PEG 1500, 5 min	Unsuccessful
	ug3-h x FK	1:1	$2,0 \times 10^6$	40% PEG 1500, 5 min	Unsuccessful
MONOLAYER FUSIONS:					
(i) Method of Fox (1983):	Cl.1D x FK	1:1	$5,0 \times 10^5$	80% PEG 1500, 65 sec	Unsuccessful
	Cl.1D x FK	1:1	$5,0 \times 10^5$	50% PEG 1500, 60 sec	Successful
	Cl.1D x JH	1:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	Cl.1D x JH	1:1	$5,0 \times 10^5$	50% PEG 1540, 90 sec	Successful
	Cl.1D x JH	3:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	RAG x FK	3:1	$5,0 \times 10^5$	30% PEG 1000, 60 sec	Unsuccessful
	RAG x FK	3:1	$5,0 \times 10^5$	80% PEG 1500, 60 sec	Unsuccessful
	RAG x FK	3:1	$5,0 \times 10^5$	50% PEG 1500, 45 sec	Successful
	RAG x FK	3:1	$5,0 \times 10^5$	50% PEG 1500, 60 sec	Successful
	RAG x FK	3:1	$5,0 \times 10^5$	50% PEG 1500, 90 sec	Successful
	RAG x FK	3:1	$5,0 \times 10^5$	30% PEG 4000, 60 sec	Successful
	RAG x FH	3:1	$5,0 \times 10^5$	50% PEG 1500, 90 sec	Successful
	RAG x FH	3:1	$5,0 \times 10^5$	80% PEG 1500, 60 sec	Successful
	RAG x FH	3:1	$5,0 \times 10^5$	50% PEG 1500, 90 sec	Successful
	RAG x FH	3:1	$5,0 \times 10^5$	50% PEG 1500,120 sec	Successful
	RAG x CR	3:1	$5,0 \times 10^5$	80% PEG 1500, 90 sec	Successful
	RAG x CR	3:1	$5,0 \times 10^5$	50% PEG 1500, 60 sec	Successful
	RAG x CR	3:1	$5,0 \times 10^5$	50% PEG 1500, 90 sec	Successful
	RAG x FB	3:1	$5,0 \times 10^5$	40% PEG 1500, 60 sec	Successful
	RAG x FB	3:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Successful

/Continued

TABLE 3.7: Continued

FUSION PROTOCOL	PARENTAL CELL COMBINATIONS	PARENTAL CELL RATIOS (RODENT:HUMAN)	TOTAL NUMBER OF PARENTAL CELLS	PEG MIXTURE* AND TREATMENT TIME	RESULT**
MONOLAYER FUSIONS:					
(i) Method of Fox (1983):	BAG x DR	3:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	BAG x JW	3:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	BB2 x JW	1:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	BB2 x JW	1:1	$5,0 \times 10^5$	50% PEG 1540, 90 sec	Successful
	BB2 x JW	1:1	$6,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	BB2 x JW	4:1	$6,0 \times 10^5$	50% PEG 1540, 60 sec	Successful
	BB2 x DR	3:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Unsuccessful
	BB2 x DR	3:1	$5,0 \times 10^5$	50% PEG 1540, 90 sec	Unsuccessful
	wg3-h x FK	1:1	$5,0 \times 10^5$	40% PEG 1500, 60 sec	Unsuccessful
	wg3-h x FK	1:1	$5,0 \times 10^5$	40% PEG 1500, 90 sec	Unsuccessful
	wg3-h x FK	1:1	$5,0 \times 10^5$	50% PEG 1500, 40 sec	Unsuccessful
	wg2-h x FK	1:1	$5,0 \times 10^5$	50% PEG 1500, 60 sec	Unsuccessful
	wg3-h x JW	3:1	$5,0 \times 10^5$	50% PEG 1540, 60 sec	Unsuccessful
(i) Method of Mohandas (1984):	BAG x CR	1:1	$8,0 \times 10^6$	50% PEG 1540, 90 sec	Successful

* Cases listed are only those in which the fusion mixture contained 10% DMSO
** Successful results reflect fusion, but not necessarily viable hybrid isolation.

TABLE 3.8: Assessment of fusion protocols for human lymphocytes

FUSION PROTOCOL	PARENTAL CELL COMBINATIONS	PARENTAL CELL RATIOS (RODENT:HUMAN)	TOTAL NUMBER OF PARENTAL CELLS	PEG MIXTURE* AND TREATMENT TIME	RESULT**
SUSPENSION FUSIONS					
(i) Method of Sheer (1983):	RAG x [illegible]	1:2	1.5×10^7	40% PEG 1500, 7 min	Unsuccessful
	RAG x BL	1:1	2.0×10^6	50% PEG 1500, 7 min	Unsuccessful
	RAG x DK	1:1	1.0×10^7	40% PEG 1600, 7 min	Successful
(ii) Method of Duckels (1984):	RAG x TL	1:1	2.0×10^6	50% PEG 1540, 7 min	Unsuccessful
	RAG x MP	1:1	2.0×10^6	50% PEG 1540, 7 min	Unsuccessful
(iii) Method of Klein (1983):	RAG x DK	1:10	2.2×10^6	40% PEG 1000, 5 min	Unsuccessful
	RAG x DK	1:10	2.2×10^6	50% PEG 1000, 5 min	Unsuccessful
MONOLAYER FUSIONS					
(i) Method of Brahe & Serra (1981):	RAG x BL	1:5	5.0×10^5	50% PEG 1540, 120 sec	Successful
	RAG x BL	1:5	6.0×10^5	50% PEG 1540, 60 sec	Successful

* Cases listed are only those in which the fusion mixture contained 10% DMSO

** Successful results reflect fusion, but not necessarily viable hybrid isolation.

3.3.4 Hybrid Selection

3.3.4.1 HAT Selection

The rodent cell lines used in all fusion experiments were either HGPRT[-] or TK[-], so that selection could be effected using HAT medium alone. As may be seen in Table 3.9, selective treatment for about two or three weeks was sufficient to eliminate most unfused rodent cells, as well as rodent-rodent fusion products. RAG cells sometimes required a much longer period of treatment for total elimination.

Termination of HAT selection required treatment with HT medium for one or two weeks, before the cultures could be returned to full growth medium. By this time, hybrid colonies were large and distinctly visible, so that usually the substitution of HAT by HT medium was done during or after hybrid cell cloning.

3.3.4.2 Ouabain Selection

Ouabain effectively eliminated the human parental cells. An average of ten days was sufficient for total elimination, as may be seen from the data in Table 3.9. Amniotic cells were found to be particularly sensitive to this drug, and were usually destroyed within one week.

TABLE 3.9: Duration of selective treatment for total elimination of unfused parental cells and intraspecific fusion products. Data excludes (i) lymphocyte fusions, (ii) unsuccessful fusions, and (iii) experiments where cultures were kept on HAT medium indefinitely.

PARENTAL CELL COMBINATION	HUMAN SPECIMEN TYPE	RODENT CELL PHENOTYPE	DURATION OF SELECTIVE TREATMENT (DAYS)		
			HAT	HT	OUABAIN
C1.1D x FK	Skin biopsy	TK⁻	16	6	8
C1.1D x JN	Skin biopsy	TK⁻	18	10	11
C1.1D x JN	Skin biopsy	TK⁻	18	15	15
RAG x FK	Skin biopsy	HGPRT⁻	21	7	12
RAG x FK	Skin biopsy	HGPRT⁻	36	10	10
RAG x FK	Skin biopsy	HGPRT⁻	31	7	12
RAG x FK	Skin biopsy	HGPRT⁻	21	15	9
RAG x FN	Amniotic fluid	HGPRT⁻	15	7	7
RAG x FN	Amniotic fluid	HGPRT⁻	16	8	6
RAG x FN	Amniotic fluid	HGPRT⁻	18	7	6
RAG x FN	Amniotic fluid	HGPRT⁻	18	10	6
RAG x FB	Amniotic fluid	HGPRT⁻	21	7	6
RAG x CR	Skin biopsy	HGPRT⁻	21	12	21
RAG x CR	Skin biopsy	HGPRT⁻	21	12	21
RAG x JN	Skin biopsy	HGPRT⁻	37	15	11
B82 x JN	Skin biopsy	TK⁻	2?	10	12
B82 x JN	Skin biopsy	TK⁻	22	10	12

3.4 Cytogenetic Analysis of Hybrid Lines

3.4.1 Harvesting and Slide Preparation

The procedures employed in cell harvesting and slide preparation applied to both rodent and hybrid cell lines (see Section 2.7.1). Optimal duration of colchicine treatment was found to be 2½-3 hrs, and that of hypotonic treatment 20-35 min. Sequential changes of 6:1, 3:1 and 1:1 fixative did not improve chromosome morphology or spreading; 3:1 fixative was therefore used throughout the fixation steps. Spreading was improved to a large extent by dropping the fixed cell suspension from a height of approximately 75-100 cms onto slides that were held at an angle. Flame-dried slides showed somewhat better chromosome spreading, but subsequent banding was of very poor quality.

3.4.2 Chromosome Banding Techniques

Different combinations of chromosome banding techniques were evaluated, as described in Section 2.7.2. Acceptable results were obtained with G-banding followed by Hoechst 33258 staining (Figure 3.10). The Q-banding → Hoechst staining sequence was also tried but was abandoned, since Q-banded metaphases tended to fade rather rapidly and proved relatively more difficult to analyse.

A

B

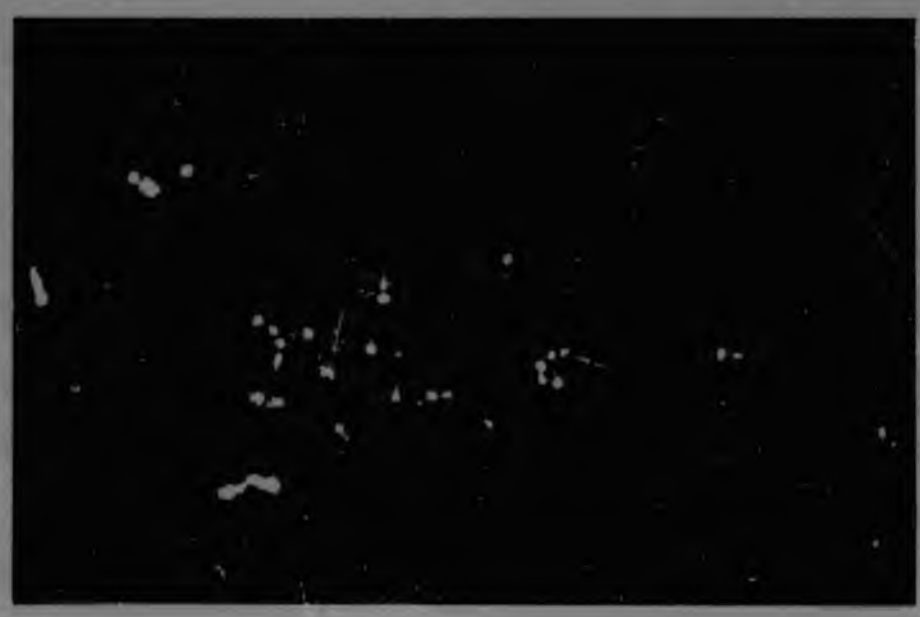


FIGURE 3.10: Partial metaphase with s al G-banding (A) → Hoechst 33258 staining (B). Arrow poin o human chromosome 18.

Numerous protocols for the G-11 stain were tested (Section 2.7.2.3), but most were found to be highly inconsistent. The assessment of the variables tested in each method is given in Table 3.10. The most consistent method was found to be that devised by Smith. Provided that Fisher Scientific (or BDH) Giemsa powder was used to make up the stain, all other variables could be adjusted to yield differential staining. However, different batches of the powder had to be tested with respect to staining efficiency. The G-banding → G-11 banding combination (Figure 3.11) was subsequently chosen for routine cytogenetic analysis of the hybrid cell lines.

For best results, only three slides were stained at a time. New staining solution had to be prepared for every three slides, since the stain was found to oxidize very rapidly once the lids of the coplin jars had been removed.

Once prepared, the Giemsa stain itself was only effective for about two months, but its age was otherwise not a significant variable. As for the age of the chromosome preparations, it was found that these could be differentially stained from 48 hrs (or 4 hrs at 60°C) to two months following trypsin-Giemsa treatment.

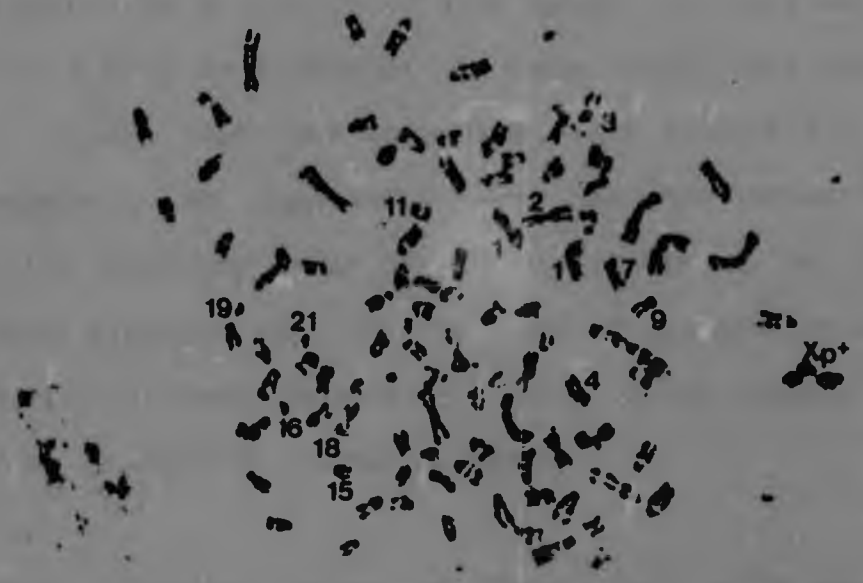
Overcrowding of the cells resulted in inconsistent staining across the slides. With well-spaced cells, on the other hand, a 90-100% differential staining efficiency could usually be achieved.

TABLE 3.10: Methods and variables tested for the G-11 staining technique

PROTOCOL	BUFFER	pH	GIEMSA CONCEN-TRATION	GIEMSA BRAND	AGE OF SLIDES	STAINING TIME	RESULT
BOBROW & CROSS (1974)	H_2O	11,0 with NaOH	2%	Gurr	1 week at R.T.	10-20 min	Unsuccessful
	0,007N NaOH	11,0 with NaOH	2%	Gurr	1 week at R.T.	12-15 min	Unsuccessful
	1 mM NaOH	11,0 with NaOH	2%	Harleco	1 week at R.T.	15-20 min	Unsuccessful
FRIEND et al. (1976)	0,05M Na_2HPO_4	11,3 with 1N NaOH	1,6-2,0%	Fisher	Fresh,1-2 hrs in distilled water	3-5 min	± 20% staining efficiency
	0,05M Na_2HPO_4	11,3 with 1N NaOH	1,6-2,0%	Clinical Sciences Diagnostics	Fresh,1-2 hrs in distilled water	3-5 min	Unsuccessful
FOX (1983)	0,05M Na_2HPO_4	11,3 with KOH	4%	BDH	8 days at R.T.	7 min	± 50% staining efficiency
	0,05M Na_2HPO_4	11,3 with KOH	4%	BDH	14 days at R.T.	7 min	± 50% staining efficiency
	0,05M Na_2HPO_4	11,3 with KOH	4%	Fisher	8 days at R.T.	7 min	± 50% staining efficiency
STANLEY (1984)	0,14N NaOH	11,0 with NaOH	2%	Merck	24 hrs at 60°C	3½ min	Unsuccessful
SMITH (1984)	0,05M Na_2HPO_4	11,3 - 11,5 with NaOH	8%	Fisher	2 days + 2 months at R.T.	3-7 min	± 90-100% staining efficiency
	0,05M Na_2HPO_4	11,3 - 11,5 with NaOH	8%	Merck	2 days + 2 months at R.T	3-7 min	Unsuccessful
	0,05M Na_2HPO_4	11,3 - 11,5 with NaOH	8%	BDH	2 days + 2 months at R.T	3-7 min	± 90-100% staining efficiency

R.T. - room temperature

A



B



FIGURE 3.11: Metaphase with sequential G-banding (A) → G-11 banding (B). The human chromosomes are labelled in (A).

3.4.3 Human Chromosome Constitution of Hybrids

An average of 25 metaphases of each hybrid line were analysed. and Table 3.11 is an example of how these results were recorded. It may be seen that some chromosomes were present at a very low frequency. As suggested by Fox (personal communication, 1985), the arbitrary value of 15% was chosen as the cut-off point when assessing the lines in terms of presence or absence of a particular human chromosome. Thus, in the example shown, chromosome 13 would be considered "absent".

The human chromosome content of those lines which were analysed, is given in Table 3.12. These results reflect the first cytogenetic analysis of each line. About 20% of the lines were found to have intra- or interspecific rearrangements, an example of which is shown in Figure 3.12.

Some hybrid lines were again analysed following freezing, reconstitution and expansion. On average, this represented a time interval of 6-8 weeks (excluding the storage time). Table 3.13 compares the human chromosome content of these lines on both occasions.

Hybrid line JNB 2D1-H was subcloned after the first cytogenetic analysis, in an attempt to obtain more homogeneous sub-populations. This proved to be effective as may be seen from the results depicted in Table 3.14.

TABLE 3.11: Human chromosome constitution of hybrid line CRR 266-H. Chromosomes were tabulated as "present" (+), regardless of whether one or two copies were retained in the cell.

METAPHASE NUMBER	HUMAN CHROMOSOMES																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Other	Total
1					+	+				+	+			+		+		+		+	+	+	+			11
2										+	+		+	+						+	+	+	+			8
3										+	+			+	+	+			+		+	+	+			9
4	+				+	+				+	+			+	+			+		+	+	+	+			12
5											+							+		+	+		+			5
6	+					+					+				+	+			+		+	+	+			9
7	+				+								+			+					+	+	+			7
8	+					+					+			+		+				+	+		+			8
9					+	+				+				+		+		+			+	+	+			9
10	+					+					+		+		+	+		+		+	+	+	+			11
11					+						+					+										3
12															+	+				+			+			4
13					+					+	+			+		+		+				+				7
14	+				+						+			+							+	+	+			7
15																		+		+	+	+	+			5
16	+				+						+					+			+	+	+	+	+			9
17	+									+	+							+	+		+		+			7
18	+					+				+	+					+			+		+	+				8
19											+								+			+				3
20					+					+	+					+			+				+			6
21					+						+										+	+	+			5
22	+										+									+	+	+	+			6
23											+					+		+	+	+	+		+			7
TOTAL	10				10	7				9	19		3	8	5	14		9	8	11	18	16	19			15
PERCENTAGE	44				44	30				39	83		13	35	22	61		39	35	48	78	70	83			

TABLE 3.12: Human chromosome constitution of somatic cell hybrids. Results are from the first cytogenetic analysis of the lines, and the values indicate percentage of cells containing the particular human chromosomes.

HYBRID CODE	INTACT HUMAN CHROMOSOMES																							OTHER		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	a	b	c
CRR 1C4	27	53			80	47	73	53		27	93	80	27	60	47			20	80	60	80	80	67				
CRR 1G4-H	64			18	27	55	82	55		64	73	82			18	46			73		64	27	82				
CRR 1G6					50					20	85	80			35	15		75	35	10	70	30	60				
CRR 1G9					45	70	10		5	50	75	60		60	45			45	50				80			85	
CRR 2F7	55	55	55		80	45	50				75	60	30	35	45	60		40	50	55	75	80	60				
CRR 2G6-H	44				44	30				39	83		13	35	22	61		39	35	48	78	70	83				
CRR 4B5					72	79	79	36	29	36	79		21	21	29	43				14	36		79				
CRR 4E2						22	67	22		11	61	83			19	29			33	83	55	44	72				
DRR 1D5-H	91	77	77	55	64	64	27	41	18	59	73	55	32	50	77	69	18	36	86	32	68	82		46	77*		
DRR 1E5-H	80	80	70	10	50	70	60	40	70	20	70	60	40	30	20	90	40	50	80	30	70	90			60*		
FKR 2C6												75		38							31	25					19
FKR 2C7					33					25		17		17					58		17						
FKR 3J1					10									55					10		70	30					
FKR 3J2***																					19					38	
FKR 3J3				33				22						33			78	67			56	39					
JNB 1C1-H																	17									60	
JNB 1D1																											
JNB 2A1																											
JNB 2B1-H			30				75			30		60	85	95	70	15	85	25	60	60	75	75					
JNB 2D1-H		70		52	13			87		61	22	91	70	74			87		9		74	65					
BLRL 1B1				50	25		10				45	20						55		15			10			70	
FBR 3K1-H			50				67			10		56				67					50	39	72				
JNR 2C1				7																						7	
JNC 1A1-*																	52		*							8	71
JNC 2B1			35													19	77		19		35	12					66**

a - Constitutional rearrangement in human cell lines.
* recombinant duplication-deleted X-chromosome (see Section 2.1.1).
b - Undefined de novo (in vitro) rearrangement of human chromosomes.
c - Interspecific rearrangement.
** interspecific translocation involving human chromosome 1p (1pter→1cen).

*** hybrid line with no cytogenetically detectable human chromosomal material.



FIGURE 3.12: G-11 banded metaphase, showing an interspecific translocation involving an unidentified segment of a human chromosome.

TABLE 3.13: Comparison of human chromosome content of hybrid lines analysed on two occasions. (i) results from first cytogenetic analysis; (ii) results from second cytogenetic analysis. Time interval = 6-8 weeks (excluding storage time.)

HYBRID CODE	PERCENTAGE HUMAN CHROMOSOMES*																								NUMBER OF METAPHASES ANALYSED
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	Other	
BLRL 1B1: (i)				50	25		10				45	20						55		15			10		70**	20
(ii)				50			5			15	65	30						55					5		90**	20
FKR 3J1: (i)					10									55					10		70	30				20
(ii)					14									54							68	32				28
JHB 281-H (i)			30				75			30		60	85	95	70	15	85	25	60	60	75	75				20
(ii)			20				46		26	20		46	86	86	73		86	33	46	60	73	60				30

* Where no values are given, this indicates that the chromosome was not detected in the cells which were analysed.

** undefined de novo (in vitro) rearrangement involving human chromosomes.

TABLE 3.14: Human chromosome constitution of hybrid line JNB 2D1-H* and its four sublines

HYBRID CODE	PERCENTAGE HUMAN CHROMOSOMES																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	TOTAL
JNB 2D1-H		70		52	13			87		61	22	91	70	74			87		9		74	65			13
JNB 2D1.1-H		86		86				91				91	82	86			91				77	82			9
JNB 2D1.2		40		100				60		40		100	60	80			80				80	80			10
JNB 2D1.3				92				77		69		85	54	38			62				69	69			9
JNB 2D1.5-H				77				88		77		88	59	65			94				82	71			9

*"H" indicates maintenance on HAT medium. The rodent cell line used in this cross (B82) is TK!-!, resulting in the retention of human chromosome 17 in hybrids maintained on HAT medium.

3.5. The Chromosome Mapping Panel

Of the available hybrids, a few combinations could be used to assemble multiple chromosome mapping panels. Possibly the best combination is shown in Table 3.15. Here the nine panel members provide unique bimodal signatures for all human chromosomes, with the exception of number 11 and X which have the same pattern of presence and absence.

The ninth member of the panel, DRR 1D5-H, contains at least one copy of all the human chromosomes; it was the only line which had retained the human Y-chromosome, and was therefore included in the panel. Apart from this line, which carries a recombinant X-chromosome, all hybrids in the panel are free of cytogenetically detectable rearrangements involving the human chromosomes.

A requirement which has not been met in this panel is the consistently high frequency of each human chromosome. In some instances, the frequency is only 15%.

Three hybrid cell lines were generously donated by Professor U. Francke (Department of Human Genetics, Yale University School of Medicine), to complement the hybrids obtained in this study. These hybrids were isolatec from crosses involving an HGPRT[-] Chinese hamster line denoted 380-6, which was a subline of V79. Although no specific values were provided, the human chromosomes were present in at least 80% of the cells. With

these three lines, a more efficient panel can be assembled, as shown in Table 3.16. Here, a total of eight panel members provide unique bimodal signatures for all human chromosomes. It may be seen that, overall, human chromosome frequencies are higher in this panel.

TABLE 3.15: Proposed multiple chromosome mapping panel with hybrids isolated in this study

HYBRID CODE	PERCENTAGE HUMAN CHROMOSOMES																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
CRR 4E2						22	67	22			61	83			19	28			33	83	55	44	72	
CRR 1C4	27	53			80	46	73	53		27	93	80	27	60	47			20	80	60	80	80	67	
CRR 2G6-H	44				44	30				39	83			35	22	61		39	35	48	78	70	83	
JNB 2B1-H			20				46		26	20		46	86	86	73		86	33	46	60	73	60		
FKR 2C7					33					25		17		17					58		17			
CRR 1G6					50					20	85	80			35	15		75	35		70	30	60	
JNB 2D1.1-H		86		86				91				91	82	86			91				77	82		
CRR 2F7	55	55	55		80	45	50				75	60	30	35	45	60		40	50	55	75	80	60	
DRR 1D5-H	91	77	77	55	64	64	29	41	18	59	73	55	32	50	77	68	18	36	86	32	68	82	77*	46

* constitutional recombinant duplication-deleted X-chromosome (See Section 2.1.1).

TABLE 3.16: Proposed multiple chromosome mapping panel with three hybrid lines donated by U. Francke.

HYBRID CODE	PERCENTAGE HUMAN CHROMOSOMES																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
XII-4A-1d-E			+								+			p^a		+		+					p^b	+
XII-2D-1d			+										+	+	+	p^c					+			
XII-2D-1f	+	+	+	+		+		+	+			+	+	+	+	+		+	+		+	+	p^b	
JNB 2D1.1-H		86		86				91				91	82	86			91				77	82		
JNB 2D1.5-H				77				88		77		88	59	65			94				82	71		
JNB 2B1-H			20				46		26	20		46	86	86	73		86	33	46	60	73	60		
CRR 4E2						22	67	22			61	83			19	28			33	83	55	44	72	
CRR 2G6-H	44				44	30				39	83			35	22	61		39	35	48	78	70	83	

\+ - >80
p - partial chromosome
a - 14qter → 14q21 | constitutional t(14;X)(14q21;Xp22)
b - Xp22 → Xqter |
c - 16pter → 16q23 (constitutional deletion)

4. DISCUSSION

4.1 Cell Immortalization

4.1.1 Epstein-Barr Virus

Epstein-Barr Virus (EBV) is responsible for polyconal-growth stimulation of B-lymphocytes, the phenomenon underlying the pathogenesis of infectious mononucleosis and the diffuse lymphomas that occur in immunodeficient patients. This is related to its ability to immortalize normal B-lymphocytes *in vitro*, where it remains latent (Miller, 1984). Latency in this case is believed to be imposed by the B-lymphocytes, which restrict the production of structural components of the virus such as the capsid and the envelope that are needed for infectivity. The virus may therefore replicate, but only rarely are the infectious viral particles produced. It appears that latency of the virus and growth stimulation of the host cell are linked. However, the regulation of the replicative and immortalizing modes of infection by EBV is poorly understood.

Earlier studies on the release of infectious EBV by transformed lymphocytes have provided evidence that production of extracellular EBV is due to factors inherent in the transformed cell type (Miller and Lipman, 1973). During these experiments it was found that transformed Marmoset lymphocytes yielded considerably larger quantities of infectious virus than did transformed human lymphocytes. In particular, the Marmoset

line denoted B95-8 was found to yield a maximum of about 0,3 infectious units per cell. This cell line has subsequently become an invaluable source of EBV.

In the present study, B95-8 cells were subcultured to a density of approximately 10^6 cells/mℓ, and the EBV was collected after 7 or 8 days. No infectivity titres were carried out; this subculture cell density and incubation period was chosen on the basis of observations by Miller and Lipman (1973) who found that maximal EBV yield from B95-8 cells occurred under these conditions.

4.1.2 Transformation

The number of cells used in each experiment was found to be a crucial factor determining the efficiency of transformation. Too few cells would decrease the probability of contact with, and subsequent infection by, EBV virions. On the other hand, overcrowding would sometimes hamper cell division upon successful transformation.

Cell density remained an influencing factor once lymphocyte proliferation was under way. It was found that a low cell density often resulted in extensive cell death and even loss of the transformed cell line. For this reason, the initial cell clumps had to be transferred from the microtitre wells (96-well trays) to slightly larger wells (24-well trays), and only later were they pooled and transferred to 50 mℓ culture

flasks. The addition of a crude preparation of B-cell growth factor also prevented cell death at this stage, and enhanced cell division.

Successful transformation was evident only 2 to 3 weeks after EBV infection, when the immortalized cells had undergone several divisions to form distinct, round clumps. Until then, immortalization could not be ascertained, since the single live cells often observed could represent non-transformed lymphocytes — the life span of these being about 10 to 20 days (de Gruchy, 1970).

Although most lymphocyte samples were immortalized, some transformation experiments were not successful until the second or third attempt. Lymphocytes from patient DK, who presented a 46,XX,t(16;18) karyotype, could not be immortalized. In this particular case variables such as method of transformation and EBV harvest batch were tested. Both methods of transformation proved unsuccessful. The possibility of loss of viral infectivity in the different EBV batches was also ruled out, since the same batches would transform other lymphocyte samples. It is unlikely that the karyotype influenced transformation ability, since the translocation in these cells was of the balanced type, where it is presumed very little, if any, relevant chromosomal material had been lost (the carrier of the translocation was phenotypically normal).

The immortalized cell lines were frozen and not used in any

fusion experiments during the course of this study. However, those presenting abnormal karyotypes are potentially useful for the construction of subchromosomal mapping panels. The other immortalized lines were source material for the San (formerly called "Bushmen"), who live in very inaccessible areas of the Kalahari Desert and whose numbers are dwindling.

4.2 Rodent Cell Lines

4.2.1 Growth Characteristics

The faster growth rate of rodent cells compared to human cells could be expected, since the former were permanent lines which had been derived from malignant cells. Moreover, rodent cells in general have a shorter cell cycle than do human cells — about 16-20 hrs as opposed to 22-24 hrs in human cells (Kao and Puck, 1970). Chinese hamster lines are reported to have a generation time of approximately 12 hrs. (Puck, 1974), which explains why the Chinese hamster line used in this study (wg3-h) was found to be particularly fast-growing.

None of the four mutant rodent cell lines showed reversion to wild-type. This was desirable, as the presence of revertants would interfere with the recovery of hybrids during the selection procedure. Because no back-mutation was observed, it became unnecessary to isolate drug-resistant sublines by selection in AG or BrdU, prior to fusion.

4.2.2 Chromosomal Constitution

The modal chromosome numbers were found to be in agreement with documented analyses of the lines (refer to Section 2.1.2). For example, the line RAG showed a chromosome distribution peaking at 66 chromosomes per cell (Figure 3.6), and modal chromosome numbers of 67 (Fox, 1983) and 68 (ATCC Catalogue of Strains II, 1983) have been observed in this line.

The majority of wg3-h cells were found to have 22 chromosomes, which is the reported diploid and modal chromosome number of wg3-h, as well as of wild-type Chinese hamster cells. It could be expected that the chromosomal constitution of wg3-h would resemble that of wild-type Chinese hamster cells, since this line was derived from a non-mutant fibroblast line (refer to Section 2.1.2). The second, smaller peak at 44 chromosomes per cell (Figure 3.9) possibly represents either endoreduplication or spontaneous intraspecific fusion.

4.3. Somatic Cell Hybridization

4.3.1 Cell Fusion

4.3.1.1 Polyethylene Glycol

Polyethylene glycol (PEG) of various molecular weights is known to be capable of inducing fusion between most mammalian cell types (Davidson *et al.*, 1976). In this study, fusion mixtures

with PEG of molecular weights 4 000, 1 500 and 1 000 were compared with respect to fusion efficiency. With each molecular weight, preparations consisting of 30%, 40% and 50% PEG were evaluated. Fusion could be effected with all combinations, except with those involving PEG of molecular weight 1000. Although most mixtures induced cell fusion, the most effective was found to be 50% PEG 1500 (or 1540). At this concentration the mixture became rather viscous, and thorough rinsing was essential to minimize cytotoxicity due to prolonged exposure to the fusogen. Thorough rinsing often meant that a large number of cells were dislodged and lost. However, the increased fusion efficiency would compensate for this loss.

Like other known chemical fusogens, PEG has a cytotoxic effect. Cytotoxicity becomes increasingly pronounced as the optimal concentration of 50-55% is approached. To moderate this toxic effect, DMSO or PHA may be included in the fusion mixture (Norwood *et al.*, 1976; Mercer and Schlegel, 1979). In this study, attempts to recover viable fusion products using PEG in DMEM alone met with little success. When DMSO was included in the fusion mixtures, the toxic effect was dramatically reduced (as evidenced by the reduction in amount of cell destruction), and high rates of fusion could be induced.

In addition to molecular weight and concentration of PEG, pH plays a role in fusion efficiency, with an environment of pH 7,8 to 8,2 being more conducive to fusion (Davidson and Gerald, 1975; Davidson *et al.*, 1976; Brahe and Serra, 1981; Fox, 1983).

The effect of pH was not tested in the present study — instead, the fusion mixtures were routinely made slightly alkaline by the addition of NaOH.

Once prepared, the fusion mixtures could be kept at -20°C for several months, without any noticeable loss of fusion efficiency, upon thawing.

4.3.1.2 Fusion Methods and Parental Cell Types Compared

A variety of procedures were tested for fusion involving both human fibroblasts and human lymphocytes. Suspension fusions with either types presented great difficulties, as has been found by a number of workers in the field of somatic cell hybridization (O'Malley and Davidson, 1977; Brahe and Serra, 1981; Fox, 1983). The only suspension fusion protocol with which some degree of success was achieved, was the modified method of Galfré *et al*. On this single occasion, peripheral blood lymphocytes from subject DK were successfully fused with RAG cells (this is the individual on whom the attempts at transforming lymphocytes with EBV were unsuccessful). However, upon cloning, the hybrid cells were no longer viable.

The monolayer fusion protocols, on the other hand, all led to viable hybrid formation, regardless of the human parental cell type used. Fusion between rodent cells and human fibroblasts could be induced in monolayers containing either 1:1, 3:1 or 4:1 ratios of rodent to human cells. PEG treatment

for 45 sec. was often sufficient, but this could be extended to 90 sec. and even 120 sec. without resulting in considerable cell destruction. The amount of PEG added to these monolayers had no influence on the efficiency of fusion, provided that sufficient was added to cover all the cells. With human lymphocytes, however, minimal amounts of PEG had to be added, as it was found that large amounts of PEG decreased the rate of fusion. This probably reflected an increasing disruption of the loose cell-cell contacts with an increasing volume of solution.

The majority of fusions were carried out using human fibroblasts, as these yielded larger numbers of hybrids than did fusions using lymphocytes. Ultimately, there is no difference between the hybrids obtained from either type of cross. However, it is much more convenient to establish an efficient fusion procedure for lymphocytes, because blood specimens are more readily obtained than skin biopsy specimens. Furthermore, leukaemic patients often present unusual chromosomal rearrangements in their leukaemic peripheral blood cells, which, if retained in cell hybrids, could be extremely useful for the regional localization of genes.

The rodent cell line with which fusion was first achieved was RAG. This line proved to be one of the easiest to fuse with both types of human cells. Line B82 was also readily fused with human fibroblasts. The Chinese hamster line wg3-h could not be fused under the various conditions which were tested

in this study (refer to Sections 2.5.2, 2.5.3 and Table 3.7). This was unfortunate, since it is well documented that hybrids obtained from fusions with Chinese hamster cells retain relatively few human chromosomes (Kao and Puck, 1970; Puck, 1974; Wang *et al.*, 1979; Fox and Retief, 1986). Furthermore, the resulting hybrids are known to be faster-growing than those obtained from mouse-human fusions. It is believed that wg3-h is best suited to suspension fusions with human lymphocytes (Mohandas, personal communication, 1984). This combination was not tested in the present study.

4.3.2. Hybrid Selection and Isolation

Hybrid selection with HAT medium could usually be terminated after two or three weeks. Where the retention of human chromosome X or 17 was desired, the cells were kept on HAT medium indefinitely. This sometimes led to extensive cell death (presumably as a result of spontaneous loss of the human chromosome being selected), and many of these lines exhibited continuous instability, manifested in the form of chromosomal rearrangements and pulverization (Figure 4.1).

Ouabain selection was discontinued as soon as human cells were no longer visible, since drug accumulation in the surviving hybrid cells could be toxic. Any remaining human cells would presumably be outgrown by the hybrid colonies. Furthermore, human fibroblasts could usually be distinguished from hybrid cells on the basis of morphology. On no occasion were human fibroblasts mistaken for hybrid cells during the process of hybrid cell cloning.

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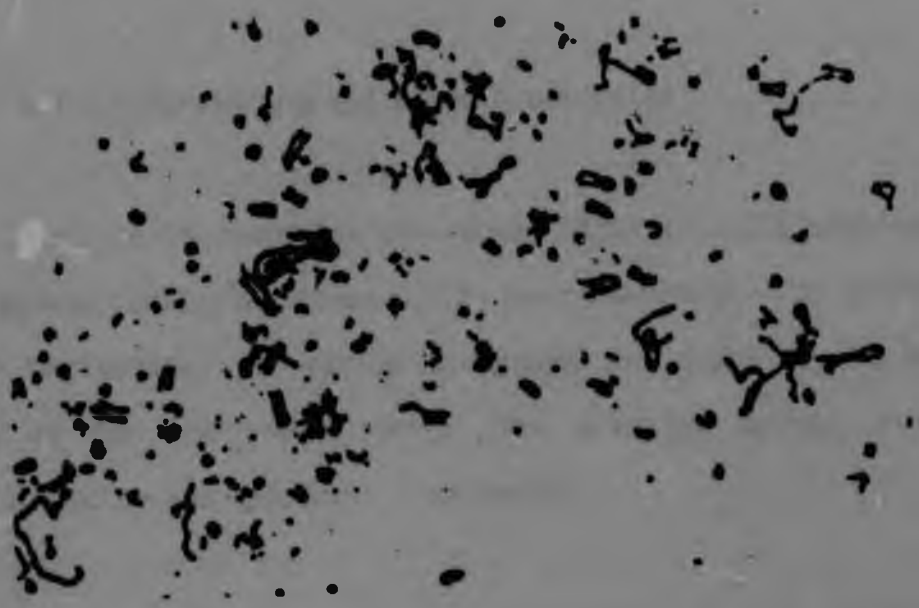


FIGURE 4.1 Chromosomal rearrangements and pulverization in an unstable hybrid line.

A problem initially encountered during the process of cloning was the frequent death of single cells deposited in the individual wells of the microtitre tray. This was eventually overcome by supplementing the medium with 10% "conditioned" medium. This was filter-sterilized medium which had supported the growth of the rodent parental cell line for at least three days, and presumably contained undefined growth factors which had been secreted by these cells. The mechanism by which such medium promoted cell growth is probably analogous to that of medium containing B-cell growth factor, which was added to transformants (see Section 2.4.4).

4.4 Analysis of Hybrid Lines

4.4.1 Cytogenetic Analysis of Hybrid Lines

4.4.1.1 Harvesting and Slide Preparation

The hybrid cells resembled the rodent parental cells with respect to growth characteristics. It was therefore not surprising that the optimal harvesting procedure for these should be the same as for the rodent cells (for example, optimal duration of colchicine and hypotonic treatments).

Initially, difficulties were encountered in the process of slide preparation, as the unusually large number of chromosomes would not spread well. Chromosome spreading was eventually improved using a combination of techniques, as described in Sections 2.7.1 and 3.4.1. In addition, the final fixed cell suspension had to be diluted out sufficiently, so as not to have overcrowding of cells on the slide; this was essential for optimal G-11 staining.

4.4.1.2 Staining Methods Compared

Trypsin-Giemsa banding is still the most commonly used chromosome banding technique; it offers a good permanent record of the banded structure of the chromosomes, and does not require expensive reagents. In this study, it was found that Q-banding did not provide the same resolution as G-banding, so the latter

was chosen for routine analysis of the cell lines.

Although high resolution banding was possible with trypsin-Giemsa treated preparations, this was often not adequate to allow distinction between human and rodent chromosomes in the hybrid nuclei; in particular, it was difficult to distinguish between the acrocentric chromosomes of the two complements. For this reason it became necessary to use a differential staining technique.

The fluorochrome Hoechst 33258 binds preferably to the constitutive heterochromatin of the mouse, resulting in brightly fluorescing centromeres, and thus provides a means of distinguishing the chromosomes in human-mouse hybrids. In the present study, karyological analysis of hybrids was facilitated through the sequential use of G-banding and Hoechst 33258 staining. The protocol was simple and highly reliable. However, some mouse heterochromatic regions did not bind the fluorochrome; the chromosomes involved could then be mistaken for rearranged human chromosomes. This was only a minor drawback, because it was found that, within a particular mouse cell line, these non-fluorescing chromosomes were usually constant.

Since the staining involved only the centromeric (and sometimes pericentromeric) regions, this technique had a significant flaw: the inability to reveal interspecific rearrangements. Nonetheless, it proved extremely valuable in the early inspection of fusion products, to determine whether these were indeed

interspecific hybrids.

Although not applicable in this study, it should be noted that Hoechst 33258 does not bind to Chinese hamster heterochromatin (Kozak *et al.*, 1977), and is therefore of no use in the analysis of human-Chinese hamster hybrids.

The G-11 staining technique was found to be a most effective means of differentiating between mouse and human chromosomal material. Although this technique was somewhat temperamental, the quality of information obtained compensated for the trouble in obtaining it. Numerous protocols were tested — the number of modifications described attest to the erratic nature of the technique!

As mentioned in Section 3.4.2, a relatively consistent protocol was eventually applied in the routine analysis of hybrid cell lines. Here the pH was routinely adjusted to 11.3, and the first test slide stained for 5 minutes. When all the chromosomes appeared blue, it was an indication that either the pH was too high or the staining time too short. If they were all magenta, the pH was too low or the staining time too long. Variables such as temperature and dye concentration were also important, but if these were kept constant (37°C and 8% Giemsa), differential staining could be obtained by adjusting only the pH of the buffer and/or staining time.

Whereas most G-11 staining methods are only effective on fresh

chromosome preparations or preparations which have been Q-banded (Bobrow and Cross 1974a; Friend *et al.*, 1976a), the method used in the present study was found to yield differential staining even after G-banding. The age of the preparations was also not a very limiting factor, as these could be stained from two days to about two months after G-banding.

The mechanism by which alkaline Giemsa produces a differential staining pattern remains obscure. Studies on human chromosomes have revealed that the formation of the azure-eosinate complexes (responsible for the magenta colouring), depends on (i) the absolute concentrations and ratio of azure (A or B) and eosin Y, (ii) the pH and, to a lesser extent (iii) the buffer composition of the staining solution (Wyandt *et al.*, 1976). The presence of both azure and eosin in precisely the right proportions was found to be especially critical for achieving G-11 banding. This may explain why differential staining is possible using only certain batches of specific brands of Giemsa, as was found in the present study. Alkalinity may play a dual role of (i) partially removing specific DNAs and/or proteins, and (ii) stabilizing the binding of the azure-eosinate complexes. Removal of structural proteins could disrupt the compact coiling of the chromatin fibrils in particular regions of the chromosomes, allowing accumulation of the red dye complexes (Bobrow and Cross 1974a).

4.4.2 Biochemical Analysis of Hybrid Lines

Isozyme tests were carried out on a number of hybrid lines,

to confirm their cytogenetic characterization or to assist in the identification of the D-group chromosomes. These assays were carried out by Mr S. Hart and Dr A. B. Lane (of the department of Human Genetics), who also set up the systems for each enzyme (according to Table 1.2). In most cases, the results confirmed those obtained by cytogenetic analysis. Where cytogenetic analysis had revealed very low frequencies of certain human chromosomes, the respective isozyme assays did not always detect the presence of these chromosomes. The detection level was found to vary between the different isozyme tests.

Figure 4.2 is an example of an isozyme assay; in this case nucleoside phosphorylase (NP), which is encoded by human chromosome 14, was tested. The electrophoretic mobility of NP on cellogel is shown, with lysates from mouse, human, *in vitro* mixed mouse + human, and seven human-rodent cell lines. It may be seen that the mouse isozyme travels faster than that of man. Hybrids which had retained the human gene for NP produced a four-band pattern consisting of the slow human, the fast mouse, and two intermediate bands corresponding to the two different heteropolymeric forms of this trimeric enzyme (i.e. H-H-M and H-M-M, where H is the human and M the mouse component). As expected, the *in vitro* mixture of human and mouse cell lysates failed to produce the two intermediate bands.

The band patterns observed in these samples confirm the results from cytogenetic analyses (Table 3.12). From the intensity of the different bands in lane C it is evident that hybrid

line CRR 2F7 has a low percentage of human enzyme compared to that of mouse. This too is in agreement with cytogenetic findings where chromosome 14 was seen to be present in only 35% of the analysed cells.



FIGURE 4.2: Zymogram of nucleoside phosphorylase, encoded by gene on human chromosome 14.

Description of Samples:

Lane A: Mouse cells (line RAG)
Lane B: Human-mouse hybrid line CRR 1C4
Lane C: Human-mouse hybrid line CRR 2F7
Lane D: Human-mouse hybrid line CRR 2G6-H
Lane E: Human-Chinese hamster hybrid line XII-4A-1d-E
Lane F: Mouse + human cells
Lane G: Human-mouse hybrid line CRR 4B5
Lane H: Human-mouse hybrid line CRR 4E2
Lane I: Human-mouse hybrid line DRR 1D5-H
Lane J: Human cells

The pattern produced by hybrid line XII-4A-1d-E (lane E) is particularly interesting. The single band in this case corresponds to that of the Chinese hamster enzyme. By cytogenetic methodologies, this line was characterized as having a partial human chromosome 14 (14q21→14qter) (Francke *et al.*, 1976). From the band pattern obtained, it may therefore be concluded that the human gene for NP maps to the region 1→pter→14q21. Indeed, this line was used in one of the first regional localizations of NP. Since then, NP has been localized more specifically to band q13.1 (as illustrated in Figure 4.3). This test clearly illustrates the drawback in isozyme analysis of hybrid cell lines; namely that human chromosomal rearrangements, deletions and duplications may be undetected.

Nevertheless, isozyme analyses provide a rapid means of confirmation of hybrid karyotypes. This should be particularly useful when hybrid lines need to be reconstituted and expanded for mapping purposes, to ascertain whether or not they have changed with respect to human chromosome content. In addition, isozyme tests may be carried out to assist in the identification of interspecific translocations. For example, cytogenetic analysis of hybrid line JNC 2B1 revealed an interspecific translocation in which the short arm of human chromosome 1 was joined to a mouse chromosome. This line was subsequently tested and found to be positive for PGM 1, which maps to 1p. Because no other human chromosome 1 material is present (see Table 3.12), this isozyme assay confirmed the identity of the translocated human chromosome segment.

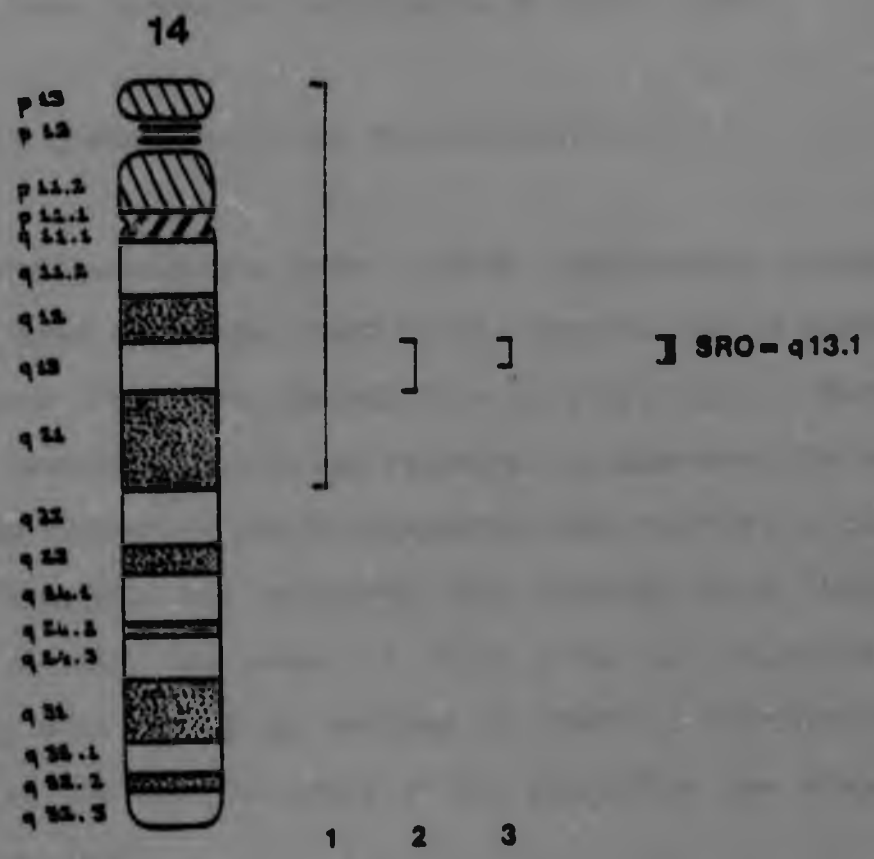


FIGURE 4.3: Regional localization of nucleoside phosphorylase on human chromosome 14.

1. (Francke *et al.*, 1976)
2. (Aitken and Ferguson-Smith, 1978) see Human Gene Mapping 8
3. (Ramos *et al.*, 1984)

SRO — Smallest region of overlap.

4.4.3 Human Chromosome Constitution of Hybrid Lines

4.4.3.1 Heterogeneity and Hybrid Stability

The hybrid populations often showed considerable variation in their human chromosome constitution, from one cell to another, even though they were derived from a single cell. Because of this heterogeneity, it was essential to determine the human chromosome content in 20-30 metaphases when analysing a hybrid line. Allderdice and colleagues have proposed using "heterogeneity curves", the shape of which gives an indication of how many cells should be analysed to obtain a representative picture of the mean karyotype of the population (see Ringertz and Savage, 1976).

More homogeneous populations were obtained by subcloning the lines, once these had stabilized. Not only did the sublines exhibit higher percentages of each human chromosome, but they had also lost more chromosomes (as may be seen in Table 3.14). The reduced number of human chromosomes could be expected, in view of the heterogeneity in the primary hybrid lines. Since these sublines were again derived from a single cell, the retained human chromosomes should be present in all the cells (i.e. a frequency of 100% for all human chromosomes retained). It is evident, however, that further chromosome loss occurred during expansion of these sublines. This may be considered as evidence that hybrid cell lines, regardless of "stability", invariably lose all the human chromosomes if maintained in culture continuously.

Although no serial analyses were carried out on the hybrid lines, some were analysed twice: the first analysis when sufficient cells were available for isozyme studies, cytogenetic studies and storage, and the second following cell reconstitution and further expansion. One such line analysed on two occasions was BLRL 1B1 (Table 3.13). At the second cytogenetic analysis, chromosomes 5 and 20 were no longer detected, whereas chromosome 10, previously undetected, was now found in 15% of the cells. The percentages of the remaining chromosomes were only slightly altered. These changes highlight the necessity of analysis of cells from the same passage as those which are to be used in mapping studies.

4.4.3.2 Intraspecific and Interspecific Translocations

Approximately 20% of the hybrid cell lines were found to have intraspecific and/or interspecific rearrangements. Although these were more frequent in some crosses than in others, there appeared to be no correlation between rodent or human parental cell line and frequency of chromosomal rearrangements. However, they were usually found in those lines which had retained relatively fewer human chromosomes.

The interspecific rearrangements were easily detected using the G-11 staining technique, but were difficult to identify, even on the G-banded preparations. In order to identify the chromosomal segments involved, isozyme analyses could perhaps be carried out, or DNA probes, that hybridize to known loci,

could be used. Until these segments are identified, the respective lines are of little use. Once they have been fully characterized, the lines may then be included in mapping panels. Moreover, the segments involved in the translocations may prove invaluable in the regional localization of genes.

4.4.3.3 Chromosome Segregation

The randomness of human chromosome segregation in somatic cell hybrids remains a controversial point. Among the results supporting non-random segregation, the least frequently retained human chromosomes are reported to be numbers 8, 9, 14 and 16, while the most frequently retained are numbers 3, 5, 7, 11, 12, 13, 19, 20, 21, 22 and Y (see Wang *et al.*, 1979). In the present study, the least frequently retained chromosomes were found to be numbers 1, 2, 3, 4, 9 and 17 (where the latter was not selected). The Y-chromosome was only present in one hybrid line; however, this does not necessarily indicate preferential elimination of the Y-chromosome, since only two of the human cell lines used in these experiments contained a copy of this chromosome. In general, the smaller human chromosomes were retained more often; in particular, chromosome 21 was present in about 85% of the lines. All of these results are in agreement with those obtained by Allderdice and co-workers (1973), where the smaller human chromosomes appeared to be retained more frequently than the larger chromosomes. Considering that any redundant chromosomal material may impose a burden on the cells, it should not be surprising that the larger human chromosomes are preferentially lost.

It has also been reported that selection for chromosome X or 17 is commonly accompanied by the retention of chromosomes 7, 11 and 12 (see Section 1.2.5.3). In the present study, all of these autosomes were found in 75% of the lines which were under selective pressure for the X-chromosome. However, there appeared to be no correlation between selection for chromosome 17 and the retention of these autosomes.

Line FKR 3J2 did not retain any human chromosomes. It could be argued that this was not a "hybrid" line as such, but rather a mouse revertant which had been able to grow in HAT medium. However, this is unlikely, since FKR 3J2 originated from a colony from which two other lines (FKR 3J1 and FKR 3J3) had been cloned, both of which contained human chromosomes (see Table 3.12).

Another interesting finding was that in crosses involving CR and JN human parental cells, selection for the X-chromosome always resulted in the retention of the normal, and not the abnormal, X-chromosome. In the case of CR, this may be attributed to non-random X-inactivation of the abnormal recombinant X, whereas the single, abnormal X-chromosome from her brother DR could be selectively retained because it was not inactivated. The X;Y translocation chromosome in JN, with an Xq22.3 breakpoint, did not contain the HGPRT locus, which maps to Xq26-Xq27 (see Figure 4.1). This means that even pretreatment of the cells with an agent which inhibits methylation, such as azacytidine, would not increase the probability of this chromosome being retained. Retention of this chromosome would thus remain a chance event.

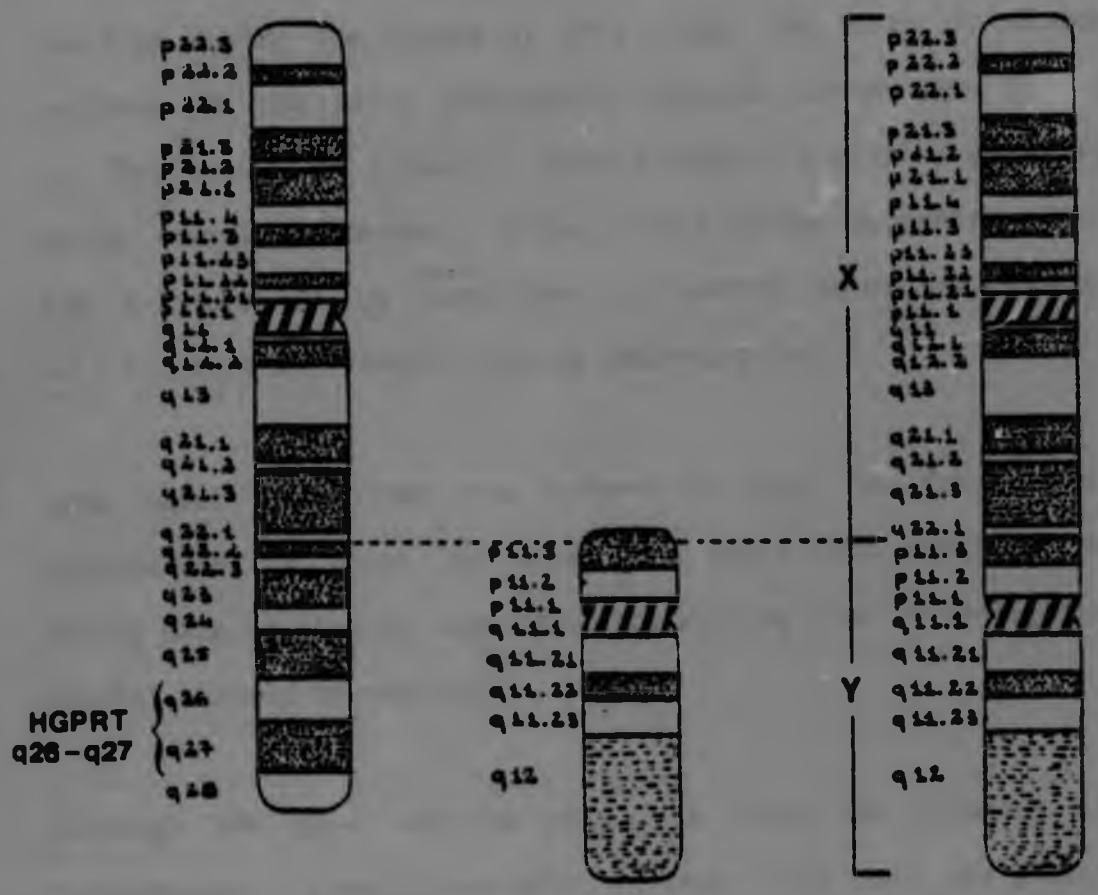


FIGURE 4.4: Diagram of the normal X- and Y-chromosomes, and the X;Y chromosome from patient JN, illustrating the breakpoints Xq22.1:Yp11.3.

4.5 The Chromosome Mapping Panel

In the proposed panel shown in Table 3.15, consisting of hybrids isolated during the course of this study, the human chromosome patterns do not allow distinction between chromosomes 11 and X. This should not present a major drawback in mapping sequences which reveal polymorphic sites, since those sequences which are X-linked can be identified by testing males and females — i.e. where only females can be heterozygotes.

Some human chromosomes are present in very low frequencies. Nonetheless, if these low values are taken into consideration during mapping studies, any ambiguity arising from false-negative results, should be resolved.

Although DRR 1D5-4 was the only line which had retained the Y-chromosome, it was dispensable. Without this line, assignments to the Y-chromosome could be made on the principle of exclusion (i.e. by excluding all other human chromosomes). Indeed, total absance of a particular human chromosome in a hybrid panel is accepted as that chromosome's bimodal signature (see Table 1.1). However, it may be desirable to always have some positive correlation of genes or gene products with specific chromosomes. As a member of the panel, this line could also serve as a positive control in assignments to any other human chromosome.

Lines carrying detectable intra- or interspecific translocations were not included in this panel. This is not an essential feature of multiple chromosome mapping panels; the interpretation of results should not be hampered by such rearrangements, provided that these are well defined. In the present study the majority of rearrangements could not be defined by cytogenetic methodologies.

Within each hybrid line, human chromosomes were considered "absent" when their frequency fell below the arbitrary value of 15%. The validity of this value as the cut-off point can only be established once the panel has been used in a few mapping tests. After the initial Southern blot analyses, it should be possible to ascertain whether or not, at this frequency, the human DNA becomes diluted to such an extent that it can no longer be detected. With the mapping of enzymes, the cut-off point would have to be determined each time on a trial-and-error basis, since the detection level of enzymes varies according to their activity and the amount produced by each cell.

Undetected human chromosomal material may also generate incorrect mapping results. It has therefore been suggested than an assignment by positive correlation should be accompanied by the exclusion of all other chromosomes, requiring that these be present in at least two hybrid lines where the gene or gene product has not been detected (Burgerhout, 1978). For example, when the presence of a gene correlates with the presence of

chromosome 1, then the gene should not be detected in at least two hybrid lines containing chromosome 2 (but not chromosome 1), at least two hybrid lines containing chromosome 3 (but not chromosome 1), and similarly for each of the other "excluded" chromosomes. The proposed panel does not contain appropriate chromosome combinations for such exclusion mapping. This, however, is by no means a prerequisite in the assembly of efficient mapping panels. Those chromosomes whose bimodal signatures in the panel do not accommodate exclusion mapping, may subsequently be excluded using other, appropriate hybrid lines.

A combination of the two proposed panels (Tables 3.15 and 3.16) is currently being used to assign random single-copy DNA sequences isolated in this Department. These sequences were isolated by screening a human λ Charon 4A DNA library for recombinant bacteriophage containing unique sequence human inserts, and were subsequently found to be highly polymorphic within the South African caucasian population. Thus far, one such sequence has been provisionally assigned to human chromosome 16. This particular sequence (DM8), when used as a radio-labelled probe, detects a two-allelic *SacI* polymorphism with a heterozygote frequency of 0,5.

Among the hybrids used to map DM8 was the line CRR 1C4, which contained human chromosome 16 in only 13% of the cells. The lane with digests of this cell line still showed faint hybridization of the probe, suggesting that a 15% cut-off point

for human chromosome frequency in hybrids may indeed be acceptable.

The panel member XII-2D-1d, which also hybridized to the probe, provided additional information with respect to the mapping of DM8. This line contained only a partial human chromosome 16 — 16pter-16q23. Although very little of the chromosome was deleted, it made some contribution towards the regional assignment of the sequence.

Regional assignment of DM8 may be facilitated by the fact that human chromosome 16 carries the adenine phosphoribosyl transferase (APRT) gene, which maps to 16q22.2-16q22.3. By subjecting rodent cells to fluoroadenine, APRT-deficient clones can be isolated which, when fused to human cells, allow for selective retention of human chromosome 16 (see Section 1.2.2.1). This selection mechanism would thus enable the retention of chromosome 16 derivatives, which could lead to further regional assignments of DM8. In addition, the resulting hybrids could be counterselected, by treating the cells with fluoroadenine. Confirmation of the assignments would be obtained if the APRT-deficient sublines then failed to hybridize to the probe.

5. CONCLUDING REMARKS

The preferential loss of human chromosomes from human-rodent somatic cell hybrids has provided a most powerful tool for both chromosomal and subchromosomal human gene assignments. While the ideal mapping system consisting of twenty-four single human chromosome hybrid lines has not yet been realized, the multiple human chromosome mapping system has, in the interim, been highly useful. It may be argued that this "clone panel" (as it was first described) is more reliable, in view of the fact that it provides internal controls in the form of human chromosome redundancy. With the single chromosome mapping system, on the other hand, controls cannot be set up unless another, independent group of twenty-four hybrid lines is generated.

It can be envisaged that, by making these panels of hybrids appropriately available, the definitive construction of the gene map would be accelerated.

The concerted application of Mendelian, somatic cell and molecular genetics can be expected to yield not only a detailed human genomic map. but also information which can be applied in genetic counselling, preclinical diagnosis and prenatal diagnosis of an increasing number of genetic disorders.

6. APPENDIX: SOURCES OF MEDIA AND CHEMICAL REAGENTS

Acetic acid (glacial)	Merck
Citric acid	Merck
Colchicine	Merck
Dimethyl sulphoxide	Merck
Dulbecco's Modified Eagle's Medium	GibCo
Ficoll 400	Pharmacia Fine Chemicals
Foetal Bovine Serum	M.A. Bioproducts
Foetal Bovine Serum (for hybridomas)	M.A. Bioproducts
Giemsa stain : liquid form:	Gurr Microscopy Materials/ Clinical Sciences Diagnostics/ Harleco
powder form:	BDH Laboratory Reagents/ Fisher Scientific/Merck
Glycerol	BRL Inc.
Hanks' Balanced Salts Solution	Flow Laboratories
HAT solution (50x)	Flow Laboratories
Histopaque-1077	Sigma
Hoechst 33258 fluorochrome	Hoechst Pharmaceuticals
HT solution (50x)	Flow Laboratories
Hydrochloric acid	Merck
Hypaque -65%	Winthrop Laboratories
Methanol	Merck
Ouabain	Merck
Penicillin (Crystapen)	Glaxo
Phosphate buffered saline	Oxoid

Pokeweed mitogen	GibCo
Polyethylene glycol	Koch Light
Potassium chloride	Koch Light
Potassium hydroxide	Merck
Quinacrine mustard	Sigma
RPMI-1640	GibCo
Scdium chloride	SMM Chemicals
Sodium citrate	Merck
Sodium hydroxide	Merck
di-Sodium orthophosphate (anhydrous)	BDH Laboratory Reagents
Streptomycin (Novc-Strep)	Novo Industries
Trypan blue	Sigma
Trypsin (1:250)	Flow Laboratories

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