3 Analysis of the Human Cell Surface by Somatic Cell Genetics

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EDITOR'S INTRODUCTION

Genetic analysis using classical Mendelian techniques suffers from several drawbacks when applied to *Homo sapiens*. First, humans have a long generation period. Second, because of personal choice in mating, laudable ethical constraints on experimenters and small family sizes, matings are frequently less informative than would be the case for experimental animals. Third, the paucity of genetic markers present at polymorphic levels (i.e. greater than 1%) in human populations also limits Mendelian analysis (however, the recent introduction of restriction fragment polymorphisms is beginning to obviate this last limitation). The solution to these problems was the introduction of somatic cell genetic analysis using human–rodent hybrids.

Hybrid cells produced by artificially fusing rodent and human cells with sendai virus or polyethylene glycol retain the rodent chromosomes but spontaneously segregate human chromosomes. Correlation of the presence of human markers with the presence of human chromosomes or chromosomal fragments forms the basis of rapid gene mapping by somatic cell genetics. The technique has the added advantage that many more phenotypic differences can be detected between species (i.e. between humans and rodents) than within species, thereby, greatly expanding the number of markers which can be studied.

Surprisingly, analysis of cell surface molecules by somatic cell genetics has lagged behind that of other markers. This is particularly strange because traditionally human genetics has employed cell surface markers (see Chapter 2), and cell surface molecules defined by antibodies have several advantages. In Chapter 3, Alan Tunnacliffe and I describe the methods and technology of somatic cell genetics.

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3.1 INTRODUCTION

Genetic analysis can be used to define the components and functions of complex systems. A preliminary to this analysis is the identification of the genes responsible and their linkage relationship to each other. This process is known as gene mapping and results in the assignment of a gene to an individual chromosome and the ordering of genes on each chromosome. The cell surface is a complex organelle, responsible for communication between the cell and its environment, which is amenable to genetic analysis. Many components of the human cell surface have been identified immunologically: coupling the immunological approach with standard somatic cell genetic techniques can lead to the chromosomal assignment of genes which govern cell surface biochemistry. In a complementary manner, genetically characterized cell surface components can be used to manipulate the genome of somatic cell hybrids.

The past decade has resulted in an explosion in the size of the human gene map: by 1981 over 300 genes had been mapped to the autosomes (Sixth International Workshop on Human Gene Mapping, Oslo 1981) and journals continue to publish further additions at an increasing rate. The reason for this burgeoning is the development of somatic cell genetics. Cells when fused together form, at low frequency, hybrid cells, which combine the chromosomes from both parental cells in one nucleus. Hybrid cells produced between human and rodent cells randomly lose human chromosomes. The presence of a human gene product can be correlated with the presence of a specific human chromosome thereby assigning the gene to that chromosome (see Fig. 3.1). Gene assignment requires a gene assay, or an assay for a product of that gene: it is also necessary that the human gene or gene product be distinguishable from the corresponding rodent gene in hybrids. The specificity of an enzyme for its substrate, or a related compound, coupled with the ability to separate many human and rodent isoenzymes by gel electrophoresis (Harris and Hopkinson, 1976) have allowed the chromosomal mapping of numerous enzyme-encoding loci.

Assays for cell surface receptors have traditionally involved the binding of a (labelled) ligand to the receptor, and although this satisfies the first requirement for a gene assay (i.e. specificity), it often fails to satisfy the second requirement (i.e. *species*-specificity). Thus, human transferrin will bind to both human and mouse transferrin receptors (Goodfellow *et al.*, 1982a). It is understandable, therefore, why the mapping of receptor-encoding loci has lagged behind the mapping of genes for enzymes. A possible solution to this problem involves the use of suitable mutants as rodent parent of hybrids and this is how the gene coding for the epidermal growth factor receptor (*EGFR*) was first mapped (Shimizu *et al.*, 1980; Davies *et al.*, 1980). Hybrids were made between various mouse lines, negative for EGF-binding activity, and human cells. Then, binding of labelled EGF to hybrids was correlated with the presence of

human chromosome 7. However, the limited availability of mutants, the problem of a possible re-expression of the rodent gene and the need to determine the nature of the genetic lesion restrict the value of this approach.

The treatment of the cell surface component as an antigen has proved to be a valuable approach to the problem. Originally with conventional antisera and more recently with monoclonal antibodies, it has been shown that species-specific recognition of surface determinants by antibodies satisfies the criteria for gene mapping. In this review, we shall describe this approach and outline the techniques involved and show how they are applicable to systems familiar to the cell biologist and also how new systems may be defined.

3.2 HYBRID PRODUCTION AND CHARACTERIZATION

3.2.1 Choice of hybrid parents

The production of hybrids intended for use in gene mapping requires a careful choice of parent cells and there are two particularly important aspects. First, unless a DNA probe is available, there is the problem of tissue-restricted expression. For example, if the molecule of interest is only found on, say, the surface of fibroblast cells, then it is advisable to choose those cells as human parent. Equally important is the choice of rodent parent, since the rodent cell must allow expression of the human gene. Extinction of human gene expression can result if human and rodent parents are incompatible and, in the above example, it would be wise to choose, say, an L cell or 3T3 line as a mouse parent. Having said this, expression of a human gene, silent in the human parent cell, can sometimes be seen in hybrids if the rodent cell can reprogram that gene. For example, several human liver-specific enzyme functions were exhibited in hybrids between human skin fibroblasts or neuroblastoma cells and a rat hepatoma line which allowed mapping of two enzyme loci (Kielty *et al.*, 1982a,b).

Secondly, since characterizing hybrids will involve chromosome analysis, it is preferable that a human cell with a normal karyotype is used as hybrid parent since the presence of markedly rearranged chromosomes can make a definitive gene assignment difficult, although well-defined chromosome translocations or deletions are used extensively for regional assignments. It should be noted that most immortal human cell lines have abnormal karyotypes and, where possible, fresh human tissue or primary cultures should be used in fusions.

3.2.2 Cell fusion and hybrid selection

Having made the choice of parent cells, hybrids are made by fusion with polyethylene glycol (PEG) (Pontecorvo, 1975) or, less frequently nowadays,

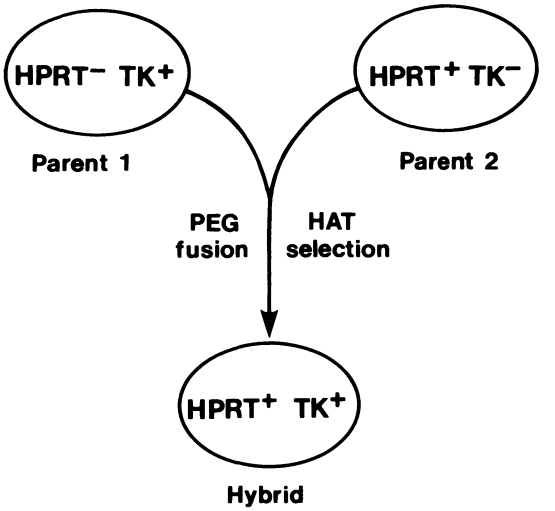

Fig. 3.1 Schematic production and selection of hybrids from $HPRT^-$ and TK^- parent cells by fusion with polyethylene glycol (PEG) and selection in HAT medium.

inactivated sendai virus (Okada, 1958, 1962; Harris and Watkins, 1965; Neff and Enders, 1968). Fusion conditions with PEG have been optimized since the technique was first introduced and have been reviewed extensively (Kennett *et al.*, 1981; Mercer and Baserga, 1982; Norwood and Zeigler, 1982). Hybrids are isolated by selection conditions which allow preferential growth of fusion products. The HAT selection system (Szybalski *et al.*, 1962) is most frequently employed for hybrid selection (Littlefield, 1964) and involves the use of mutant parents (Fig. 3.1): Parent 1 is $HPRT^-$ (hypoxanthine phosphoribosyltransferase; EC 2.4.2.8) and Parent 2 is TK^- (thymidine kinase, EC 2.7.1.21). Hybrids complement each others' defects and are $HPRT^+$ TK^+ allowing growth in HAT medium (hypoxanthine, aminopterin or methotrexate and thymidine), whereas parents are killed, since the activity of both enzymes is required: aminopterin inhibits dihydrofolate reductase and thus blocks *de novo* synthesis of purines and one-carbon-transfer reactions, including the conversion of deoxyuridylic acid to thymidylic acid. Cells growing in the presence of aminopterin cannot therefore survive, unless precursors for DNA synthesis are provided in the growth medium. In practice, when making hybrids for gene mapping, full selection is not always employed since 'normal' cells are often used as the human parent. (An exception to this is the use of fibroblasts from a patient with Lesch-Nyan syndrome, which are $HPRT^-$.) Thus, whilst mouse parents are either $HPRT^-$ or TK^- and are killed in HAT, selection against human parents is often due to growth properties: thymocytes and unstimulated lymphocytes will not grow in culture, whilst fibroblasts are usually outgrown by

hybrids (Nabholz *et al.*, 1969). The increased resistance of rodent and hybrid cells to ouabain allows its use in selection medium to kill off human parent cells if desired (Kucherlapati *et al.*, 1975).

3.2.3 Determination of human chromosomes in hybrids

When continuous cultures of human–rodent somatic cell hybrids were first made, it was noted that human chromosomes were preferentially lost from hybrids, while the full rodent genome was retained (Weiss and Green, 1967). It is on this observation that the ability to map human genes in hybrids is based. Retention of human genetic material usually relies on selection for a single gene (*HPRT* or *TK*), and whilst this can ensure that either the human chromosomes carrying these genes (X or 17, respectively) are present in hybrids, other chromosomes are lost approximately randomly. Thus, different hybrids will have different numbers and distributions of human chromosomes and by performing gene assays on many hybrid clones, gene assignment is possible. This is illustrated in Section 3.4.2.

The initial instability of the karyotype of heterokaryons is followed by a period of relative stability and the human genetic complement of a hybrid can be constant for many cell generations, although ultimately we would expect that hybrids would lose all but the selected human chromosome or gene. Once a hybrid is established, we need to know which human chromosomes are present. The most reliable method involves karyotype analysis where spreads of mitotic hybrid cells are examined for human chromosomes. One standard procedure involves G11 staining (Bobrow and Cross, 1974) followed by quinacrine staining (Caspersson *et al.*, 1971) of the same spread. The G11 technique differentially stains human and rodent chromosomes, whilst quinacrine gives banding patterns characteristic of each human chromosome. Karyotype techniques are reviewed in Dev and Tantravahi (1982). These methods ensure that normal human chromosomes are present in hybrids and allow confidence in hybrids used for gene assignments. Hoever, karyotyping is labour- and time-intensive and requires highly skilled personnel. Other methods of determining the human contribution to hybrids involve the detection of markers specific for each of the human chromosomes. Since at least one enzyme has been mapped to each of the human chromosomes except Y, marker isozyme analysis is routinely used to detect human chromosomes in hybrids (reviewed in O'Brien *et al.*, 1982). This review highlights the use of antibodies in somatic cell genetics: one use is of a panel of monoclonal antibodies recognizing marker antigens for each of the human chromosomes. We are attempting to assemble such a panel (Section 3.4.2; Table 3.2) and so far have antibodies covering almost half of the human chromosomes. In the near future, it should be possible also to use cloned DNA fragments from each chromosome which hybridize to restriction fragments distinguishable from

rodent counterparts. The drawback with the use of chromosome markers for hybrid analysis is that only one or a few genes (or restriction fragments) are assayed and if chromosome translocations or rearrangements have occurred in hybrids, these might not be detected. For this reason, karyotypic analysis is presently indispensable.

Having determined which human chromosomes are present in hybrids, gene assays can be carried out, but there are several caveats. (a) It is, of course, essential that hybrids are clones from separate culture vessels, although the appearance of subpopulations from a clonal isolate is almost inevitable. For this reason (b) periodic checks of the human chromosome content of hybrids should be carried out to detect any segregation which is occurring. This can conveniently be done by marker analysis. (c) Frozen stocks of hybrids should be made at regular intervals after characterization, or a large pool of cells grown up and characterized and then frozen in small aliquots. (d) Several independent sets of hybrids should be used for gene assignments to protect against possible genetic eccentricities of the human parent cell or undetected chromosomal rearrangement in hybrids.

3.3 ANTIBODY PRODUCTION AND CHARACTERIZATION

Antibodies as reagents for gene mapping need to be (a) specific for a particular gene product, and (b) species-specific or species-restricted, unless suitable rodent mutants are available. These criteria are achieved differently for conventional polyclonal antisera and monoclonal antibodies.

3.3.1 Polyclonal antisera

The production of useful polyclonal antibodies requires a substantially purified or genetically isolated immunogen. Animals are immunized according to simple protocols and subsequently bled. Antisera must then be adsorbed against rodent cells to remove any cross-reacting antibodies. This produces an antiserum which is species-specific for the gene product of interest. The technique of genetic isolation for the production of antisera against human cell surface determinants was developed by Buck and Bodmer (1974). A human–mouse hybrid with only a few human chromosomes is used to immunize a strain of mouse isogenic with that from which the mouse parent of the hybrid was derived. Thus, antibodies are raised against the human cell surface material only, unless the hybrid also expresses C-type virus tumour antigens. In this case, adsorption with hybrid parent mouse cells should remove contaminating antibodies. The value of the technique lies in the ability to produce antibodies against human material encoded by single chromosomes or chromosomal regions isolated from the remainder of the human genome. This is described in Section 3.4.1.

3.3.2 Monoclonal antibodies

Monoclonal antibody production (Kohler and Milstein, 1975; reviewed in Kennett *et al.*, 1981; Bastin *et al.*, 1982) in theory requires a less stringent purity of immunogen, since appropriate screening can isolate the desired antibody. Thus, whole human cells can be used to immunize mice and individual hybridomas screened. In practice, certain antibodies will predominate and it can be difficult to find a particular antibody since the antigen of interest may be present in only small amounts or lost in the immunological pecking order of a complex immunogen. Again, purification or genetic isolation of the antigen are helpful. We have used the latter method to produce monoclonal antibodies recognizing antigens encoded by the X (Hope *et al.*, 1982) and 11 (Tunnacliffe *et al.*, 1983; see Table 3.2) chromosomes. Abnormal expression or presentation of an antigen on the surface of a particular cell type may enhance the immunogenicity of the antigen. This phenomenon has been used to produce monoclonal antibodies against the EGF receptor (Waterfield *et al.*, 1982) and the insulin receptor (Roth *et al.*, 1982; Kull *et al.*, 1982) for example. Binding of monoclonal antibodies to human and rodent cells measured by radioimmunoassay or ELISA will determine whether the cognate antigenic determinant is species-specific. Strictly, this does not demonstrate true species-specificity, but merely a polymorphism between comparable molecules on human and rodent cell surfaces. It is often found that monoclonal antibodies directed against human determinants which do not react with mouse counterparts (assuming they exist) will also bind to some primate cells. An example is the antigen recognized by the monoclonal antibody 12E7 (Levy *et al.*, 1979), which is present on human, chimpanzee and gorilla fibroblasts, but not on orang-utang, muntjac, rabbit or rodent cells (Goodfellow, 1983; and unpublished results). Thus, the 12E7 antigenic determinant is species-restricted rather than truly species-specific. However, its absence from mouse cells allowed assignment of the controlling locus initially to human Xp (Goodfellow *et al.*, 1980), and subsequently to Xp22.3 → Xpter (Goodfellow *et al.*, 1983). An analogous gene was also found on the human Y chromosome (Goodfellow *et al.*, 1983 and see Section 3.5.2). Further investigation of the genetics of these loci in primates and other mammals might prove useful in studies of sex chromosome evolution.

Polyclonal, heterospecific antisera have proved of value in human gene mapping by somatic cell genetics (Section 3.4.1; Table 3.1) and also in other genetic systems: e.g. the use of alloantisera against HLA region (see Chapter 4 and Parham and Strominger, 1982) and red blood cell (Chapter 2) surface determinants in man, and against lymphocyte determinants in mouse (see Chapter 1 and McKenzie and Potter, 1979). However, there can be numerous problems with such antisera, some of which are: the presence of contaminating antibodies, low titre, paucity of material and non-reproducibility. In this

Table 3.1 Genes for cell surface antigens characterized by polyclonal antisera

Chromosomal location	Antigen	Reference
6	HLA (S5)	a
7	EGFR (S6)	b
11	S1, S2 S3, S4	c,d,e,f
12	S8	g
15	β_2m	h
17	S9	i
21	Interferon receptor (S14)	j
X	S10 (SAX)	k

* References: (a) van Someren *et al.* (1974); (b) Aden and Knowles (1976); Carlin and Knowles (1982); (c,d) Jones *et al.* (1975); (e) Jones and Puck (1977); (f) Buck and Bodmer (1974); (g) Seravalli *et al.* (1978); (h) Goodfellow *et al.* (1975); (i) Cicurel and Croce (1977); (j) Chan *et al.* (1979); (k) Buck and Bodmer (1976).

respect, monoclonal antibodies are superior reagents in that they are monospecific, can be obtained with high titres and are available in theoretically unlimited quantities. Monoclonal antibodies are therefore the reagents of choice.

3.4 THE USE OF ANTIBODIES IN SOMATIC CELL GENETICS

3.4.1 Initial mapping studies using polyclonal antibodies

When the first human–mouse somatic cell hybrid cell lines were made in 1967 by Weiss and Green, it was noted that antisera made in rabbits against human fibroblasts, after adsorption with mouse cells, would promote agglutination of hybrid cells and human erythrocytes. The extent of this activity was dependent upon the number of metacentric chromosomes, most of which would be human, in different hybrids. Thus, human antigens are present on the hybrid cell surface. This was confirmed later by others (Nabholz *et al.*, 1969; Kano *et al.*, 1969), and the Stanford group suggested that genes for human antigens might be segregating in hybrids together with one of the lactate dehydrogenase

(LDH) loci. Indeed, this was shown to be the case by Puck and colleagues (Puck *et al.*, 1971) who demonstrated that genes for human antigens and LDH_A were on the same human chromosome (i.e. showed synteny). These antigens were called A_L, for 'lethal' antigens, since the method of assay was cytotoxicity (Oda and Puck, 1961). In this assay, cells are plated at low dilutions in the presence of antiserum and complement. Those cells with A_L (in this case) are lysed by the antibody–complement system, whereas those without A_L survive to form colonies. In the early seventies, methods for identifying each of the human chromosomes became available (Caspersson *et al.*, 1971; Seabright, 1972; Bobrow and Cross, 1974). This allowed identification of human chromosomes in hybrids, and subsequently syntenic loci (Santachiara *et al.*, 1970; Puck *et al.*, 1971) could be assigned to particular chromosomes. Thus, when *LDHA* was assigned to chromosome 11 (Boone *et al.*, 1972), the A_L loci were simultaneously placed on that chromosome. By the use of selected mutants and antisera from a range of animals immunized with several human or hybrid cell types, it was possible to split the A_L system into at least three distinct antigenic identities (Jones *et al.*, 1975; Jones and Puck, 1977). These were named a_1, a_2 and a_3, and by using hybrids with deletions in chromosome 11, it was possible to assign a_1 and a_3 to 11p13→11pter and a_2 to 11q13→11qter (Kao *et al.*, 1977). The A_L antigens were later renamed S1, S2 and S3.

Using the immunization protocol outlined in the previous section, Buck and Bodmer (1974) were also able to produce conventional antisera to antigens coded by chromosome 11. They used the genetic isolation procedure outlined in Section 3.1 and immunized mice with a hybrid containing only human chromosomes 11, 13 and X, and after adsorption with mouse cells, produced an antiserum whose cytotoxic activity against hybrids segregated with chromosome 11. It was later shown (Buck *et al.*, 1976) that only the short arm of chromosome 11 was necessary for antigen expression. This antigen (or antigens) was called SA-1 (species antigen 1) and subsequently renamed S4, and may be identical, or related to S1 of the A_L system, since both of these antigens have been shown to be on glycolipids (Jones *et al.*, 1979; D. Marcus, unpublished results). It is interesting that when whole human cells, or hybrids with more than one chromosome, are used as immunogens, the dominant immune stimulus is provided by antigens encoded by chromosome 11. Evidently, this is not simply due to all or most cell surface determinants being coded on this chromosome as Table 3.1 demonstrates. This gives a list of surface determinants mapped by techniques involving polyclonal antibodies: at least a third of the human chromosomes harbour genes controlling surface antigens mapped by these methods. Four of these antigens have partially characterized functions: HLA, β_2-microglobulin (β_2m), EGF receptor (EGFR) and interferon receptor. Possible biological functions of the other surface molecules identified genetically remain to be determined and it is possible that monoclonal antibodies to these determinants will be valuable in this respect.

3.4.2 Mapping with monoclonal antibodies

The genetic use of monoclonal antibodies against cell surface molecules was first demonstrated by Barnstable *et al.* (1978), who immunized mice with human tonsil leucocytes and isolated several useful antibodies: W6/1, specific for blood group A erythrocytes; W6/32, anti-HLA-A, B, C heavy chain; three monoclonal antibodies whose binding correlates with the presence of human chromosome 11 in hybrids, including one W6/34, which was cloned and shown to recognize an antigen on a glycolipid. The gene controlling W6/34 antigen expression was further mapped to the short arm of chromosome 11.

The use of monoclonal antibodies has been exploited further such that now antigenic markers for approximately half the human chromosomes are available (Table 3.2), although not all of these are expressed on all human cell types. An example of the mapping techniques is shown for the transferrin

Table 3.2 Genes for cell surface antigens identified by monoclonal antibodies

Chromosomal Location	Gene	Antibody	Antigen	Reference†
3	*TFRC*	OKT9	Transferrin receptor	a
4	—	OKT10	45 K Protein	b
6	*HLA-A, B, C*	W6/32	HLA-A, B, C	c
7	*EGFR*	EGFR1	Epidermal growth factor receptor	d
11	*MIC1**	W6/34	Glycolipid	c
11	*MIC4*	F10.44.2	105 K Glycoprotein	e
11	*MIC9*	4D12	100 K Protein	f
11	*MIC8*	TRA1.10	80 K, 40 K Protein	f
11	*MIC11*	163A5	200 K, 150 K, Protein	g
12	*MIC3*	602	21 K Protein	h
15	*B2M*	BBM1	β_2-Microglobulin	i
15	*MIC7*	28.3.7	95 K Protein	j
15	*MIC12*	302	—	k
17	*MIC6*	H207	125 K Protein	l
X	*MIC2X*	12E7	34 K Protein	m
X	*MIC5*	RI	—	n
Y	*MIC2Y*	12E7	34 K Protein	o

*The designation *MIC* is a provisional local name for genes mapped by monoclonal antibodies at ICRF, a nomenclature agreed upon at the 6th International Human Gene Mapping Workshop, Oslo, 1981.

†References: a Goodfellow *et al.* (1982a); b Katz *et al.* (1983); c Barnstable *et al.* (1978); d Goodfellow *et al.* (1981); Waterfield *et al.* (1982); e Goodfellow *et al.* (1982b); f Tunnacliffe *et al.* (1983); g Woodruffe *et al.* unpublished; h Andrews *et al.* (1981); i Brodsky *et al.* (1979); j Blaineau *et al.* submitted; k Walsh *et al.* unpublished; l Bai *et al.* (1982); m Goodfellow *et al.* (1980); n Hope *et al.* (1982); o Goodfellow *et al.* (1983).

receptor (TFR) in Tables 3.3 and 3.4 (Goodfellow *et al.*, 1982a). The antibody used against TFR was OKT9 (Reinherz *et al.*, 1980), which was proved to recognize the receptor after co-precipitation by the antibody of labelled transferrin with TFR (Sutherland *et al.*, 1981). For mapping the *TFR* gene, initially a panel of independent hybrids was tested (Table 3.3) by indirect radioimmunoassay (IRIA; Williams, 1977; Footnote to Table 3.3), followed by confirmation with a set of secondary subclones of a primary hybrid which segregates the

Table 3.3 Testing a panel of independent hybrids for OKT9 antigen

Cell*	Human chromosomes present	$10^{-3}\times$ Radioactivity bound (cpm)	
		OKT9†	P3.X63.Ag8 (negative control)
MOG7	1,3,4,5,7,8,10,11,12,13,15,16,18,21,X	13.8	1.8
SIR2	1,2,3,4,5,6,7,8,10,11,12,13,14,15,16,17, 18,19,20,21,22,X	11.2	0.7
HORL4.1.1.B6	1,3,10,11,13,15,18,X	8.9	0.5
MOG13/17	3,21,22,X	7.3	0.4
DUR4.3	3,5,10,11,12,13,14,15,(17),18,20,21,22,X	3.9	0.5
DUR5	3,5,10,11,12,13,14,15,17,18,20,21,22,X	5.1	1.5
3W4C1.5	7,10,11,12,14,15,21,X	2.3	1.3
HORL9D2	11,15,X	1.8	1.5
HORP9.5	10,11,12,14,21,X	1.3	0.6
THYB.133	21,X	0.7	0.4
F4SC13.CL12	1,9,14,X	2.0	1.0
4W10.R3	8,20,21	2.2	1.1
CL21	7	2.7	1.9
FIR5R3	14,18	1.1	0.7
MOLT-4	Human T cell line	12.1	0.5
G3.32.2	Burkitt's lymphoma line	22.2	0.6
HFL121	Human fibroblast	6.4	0.4
1R	Mouse L cell	2.1	1.5
3T3	Mouse cell (fibroblast-like)	1.6	1.5
RAG	Mouse adenocarcinoma	1.8	1.6
BW5147	Mouse thymoma	1.6	1.0

*References to hybrids and cell lines are given in Goodfellow *et al.* (1982a).

†2×10^5 attached or 5×10^5 suspension cells are incubated for 1 h at room temperature with saturating titres of first antibody, washed three times and then incubated with 2×10^5 cpm of iodinated rabbit anti-(mouse IgG for a further hour at 4°C. Cells are washed four times and counted. Values greater than three times background (with P3.X63.Ag8) are taken as positive and are underlined.

Table 3.4 Testing subclones of DUR4 and MOG13 for OKT9 antigen

Hybrid	Human chromosomes	10^{-3} × Radioactivity bound (cpm)	
		OKT9	P3.X63.Ag8
DUR4.3*	*3*,5,10,11,12,13,14,15,17,18,20,21,22,X	3.9	0.5
DUR4.4*	*3*,11,12,13,14,15,17,18,21,22,X	5.1	0.4
DUR4.5*	*3*,5,10,11,12,13,14,15,17,18,20,21,22,X	4.4	0.7
DUR4R.1	5,11,12,13,14,17,18,20,21,22	1.2	0.8
DUR4R.3	*3*,5,11,12,13,14,17,18,20,21,22	7.5	1.2
DUR4R.4	*3*,5,11,12,13,14,17,21,22	10.3	1.0
MOG13.9	X	0.9	0.7
MOG13.10	1,*3*,21,22,X,Y	7.3	0.4
MOG13.17	*3*,21,22,X	8.4	0.6
MOG13.22	1,21,22,X	0.9	0.5

*Contains an active X→15 translocation.

chromosome in question (Table 3.4). It can be seen by inspection that only human chromosome 3 segregates with OKT9-binding activity in both the hybrid panel and the two sets of hybrid subclones. This assignment has been confirmed independently by Enns *et al.* (1982) using different techniques.

The assignment of *EGFR* to chromosome 7 using a monoclonal antibody (Table 3.2) confirms a previous result (Shimizu *et al.*, 1980; Davies *et al.*, 1980), where EGF binding to hybrids was measured, the rodent parent of which lacked receptor. It was also shown recently (Carlin and Knowles, 1982) that the 165 K marker for chromosome 7 identified with polyclonal antisera (Aden and Knowles, 1976) is also EGFR. Thus, the experiments with the monoclonal antibody have added weight to the assignments made using polyclonal antibodies and labelled ligand binding.

Table 3.2 shows that at least five different genes coding surface antigens are present on chromosome 11. Their possible relationship to genes identified using polyclonal antisera has been discussed previously (Tunnacliffe *et al.*, 1983b): it seems likely that $A_L a_1$ is identical, or closely related to W6/34 antigen. There may also be a relationship between $A_L a_3$ and F10.44.2 and 163A5 antigens, and between TRA1.10 and 4D12 antigens and $A_L a_2$. It has also been shown recently that the monoclonal antibody 4F2 recognizes an antigen mapping to 11 (Peters *et al.*, 1982). This may be binding the same molecule(s) as TRA1.10, since both antibodies precipitate bands at 80 K and 40 K on SDS/PAGE.

The question arises as to why so many genes controlling cell surface antigens exist on chromosome 11. It is possible that there are only one or a few genes

responsible which code for glycosyltransferases which create novel (to the mouse) antigenic determinants on mouse surface molecules. However, the five antibody-binding activities are genetically separable using hybrids with differing fragments of chromosome 11 present and also show differing distributions on cell lines and tissues (Tunnacliffe *et al.*, 1983a). In the case of W6/34, the antigenic determinant definitely resides on a glycolipid which suggests a human glycosyltransferase as the active agent in creating the epitope. Indeed, if W6/34 antigen is identical to S1 (A_La_1) as it seems to be, then mouse genes in at least three complementation groups are necessary for its expression (Jones *et al.*, 1979). This molecule is thus probably found on hybrid cell surfaces as a result of the action of mouse and human genes in concert. It would be of interest to compare in detail, the human and hybrid antigen-carrying molecules responsible for W6/34 and anti-S1 binding. The other four chromosome 11-specific monoclonal antibodies precipitate proteins, however, and it is likely that at least a part of the antigenic determinants they recognize are due to proteins whose coding sequences are on human chromosome 11.

It is probable that a monoclonal antibody recognizing a marker antigen for each of the human chromosomes will soon be available, in which case such a panel of antibodies will be useful for the rapid characterization of human chromosomes present in hybrids. It is evident that antigenic analysis of hybrids using monoclonal antibodies and IRIA or ELISA can produce a result in a few hours, whereas karyotype or isozyme analysis (where 23 gels need to be run per hybrid) take considerably more time and effort.

A panel of monoclonal antibodies, in conjunction with the fluorescence-activated cell sorter (FACS), can also be used for hybrid manipulation and studies of antigen expression, as described in the next section.

3.5 THE USE OF THE FACS

3.5.1 Quantitative and qualitative analysis of cell surface antigens in hybrid cell populations

The FACS (Loken and Herzenberg, 1975) allows the objective determination of the percentage of a cell population expressing an antibody-defined cell surface determinant, and in the case of a hybrid population, immediately provides data on the percentage of cells containing the marker-defined chromosome. An example is shown in Fig. 3.2. Scatter plots are shown of FACS analysis of a hybrid HORL9D2RM (Goodfellow *et al.*, 1982b). This mass culture was derived from a hybrid HORL9D2 (Goodfellow, 1975) which contains human chromosomes X,11 and 15 against a mouse $HPRT^-$ L-cell background. The X chromosome has been removed by selection in 6-thioguanine (6TG) to give HORL9D2RM. Panel (a) shows the negative control

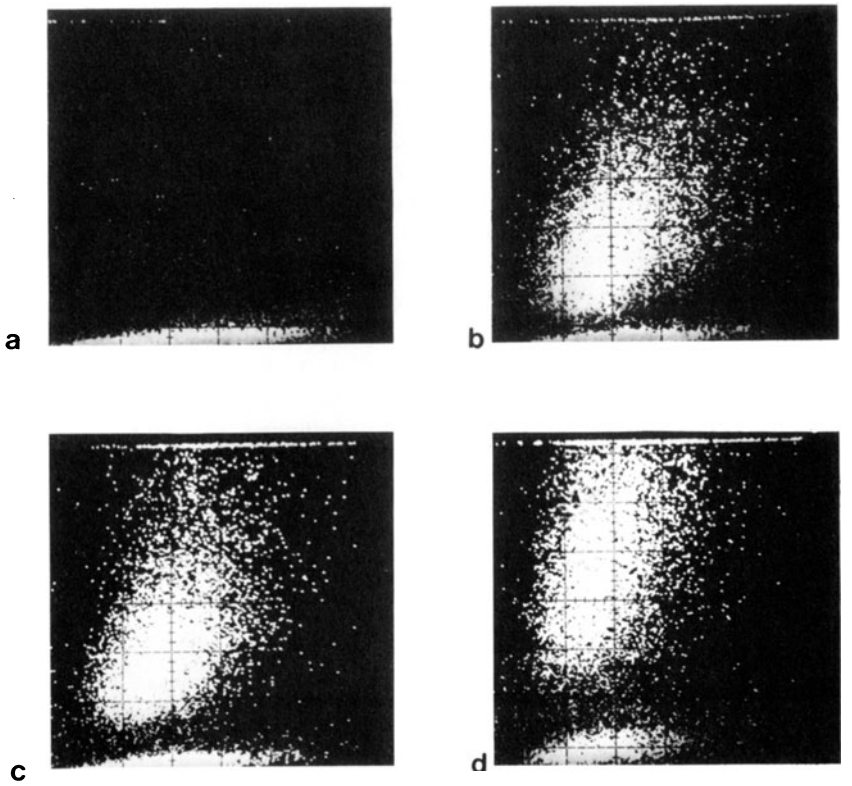

Fig. 3.2 FACS analysis of hybrid HORL9D2RM with monoclonal antibodies (a) P3.X63.Ag8; (b) F10.44.2; (c) W6/34; (d) BBM1.

reaction with mouse myeloma P3.X63.Ag8 (Kohler *et al.*, 1976). Panels (b) and (c) show the scatter plots after indirect immunofluorescence (IIF) with monoclonal antibodies F10.44.2 (Dalchau *et al.*, 1980) and W6/34 (Barnstable *et al.*, 1978) respectively. Incubation of hybrid cells with these antibodies was followed, after washing, by reaction with fluorescein-labelled rabbit anti-mouse IgG to label positive cells. Both F10.44.2 (Goodfellow *et al.*, 1982b) and W6/34 (Barnstable *et al.*, 1978; Goodfellow *et al.*, 1982b) recognize antigens whose expression is controlled by chromosome 11 (Table 3.2). Panel (d) shows staining with BBM1, a monoclonal anti-β_2m (Brodsky *et al.*, 1979), a marker for human chromosome 15 (Goodfellow *et al.*, 1975). It can be seen by the presence of negative populations that not all cells of HORL9D2RM have chromosomes 11 and/or 15. When the hybrid is stained with combinations of two antibodies it is found that both 11-markers together give the same size positive populations as an 11-marker alone, but that staining with an 11- and

15-marker together increases the size of the positive population. This means that some hybrids have only chromosome 11 or 15 and also suggests synteny of the genes encoding W6/34 and F10.44.2 antigens.

An extension of this technique could be used to demonstrate synteny between two antigen-controlling loci. The first antibody might be used to sort a mass culture into two populations, and then each of these populations restained with the second antibody and a different fluorochrome. If the two loci are indeed syntenic, the positive and negative populations from the first sort should be approximately 100% and 0% positive after the second staining (assuming the second fluorescent stain is distinguishable from the first).

It is possible to demonstrate the co-existence of two antigens in a population by the use of two-colour fluorescence (Loken *et al.*, 1977). Here, stepwise incubations and stainings are carried out: antibody 1 followed by a fluorescein-conjugated second antibody, then antibody 2 followed by a rhodamine-linked

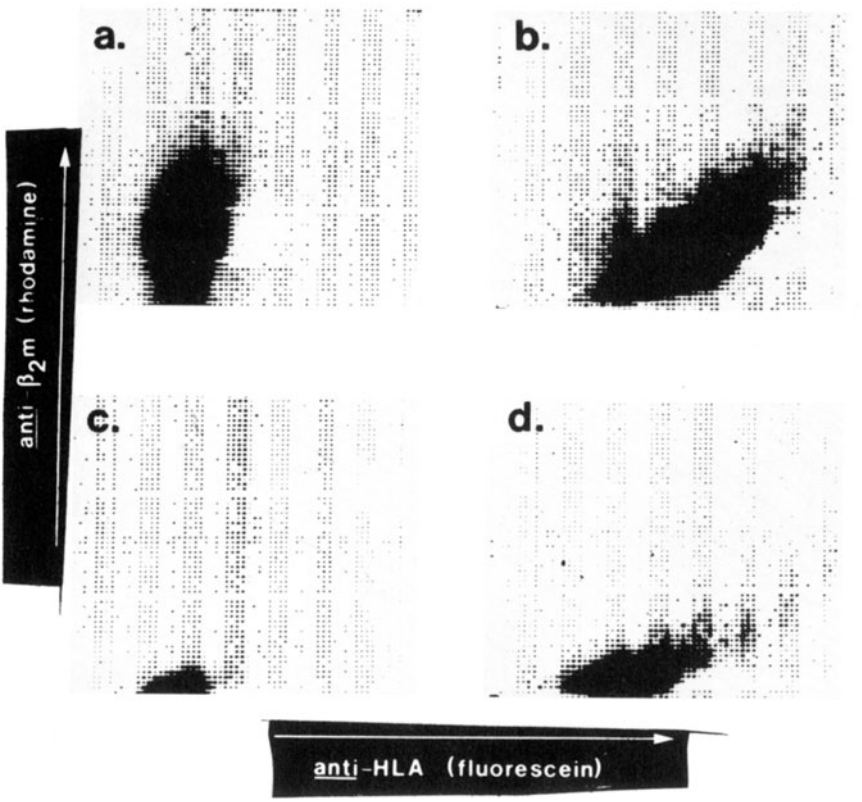

Fig. 3.3 Two-colour FACS analysis of β_2-microglobulin and HLA on subpopulations of hybrid FRY4 which are (a) HLA$^-$ β_2m$^+$; (b) HLA$^+$ β_2m$^+$; (c) HLA$^-$ β_2m$^-$ and (d) HLA$^+$ β_2m$^-$. Taken from Kamarck *et al.* (1982) with permission.

second reagent. This technique has been used by Kamarck *et al.* (1982) to examine the co-expression of HLA and human β_2m on a hybrid cell surface. HLA was indirectly stained with fluorescein and β_2m with rhodamine and then both fluorochromes were simultaneously excited. After filtering out common fluorescent wavelengths, it is possible to identify separate signals from each fluorescent stain and hence from HLA and β_2m. The authors examined four populations which had previously been sorted from a hybrid FRY4 by one-colour fluorescence, for the various combinations of HLA and human β_2m, namely (a) $HLA^- \beta_2m^+$, (b) $HLA^+ \beta_2m^+$, (c) $HLA^- \beta_2m^-$ and (d) $HLA^+ \beta_2m^-$. Two-colour staining confirmed the one-colour results, as shown in Fig. 3.3, where the panels correspond to the phenotypes given above. It is also possible to two-colour sort for a subpopulation with any of the four combinations. Kamarck *et al.*, using FACS technology, were also able to show that the presence of human β_2m increased the level of expression of HLA and mouse H-2 antigens on the hybrid cell surface. Two-colour analysis could also be used to show synteny of genes controlling cell-surface determinants: syntenic genes should give only ++ and −− populations.

Synteny in a single population between the loci for a surface antigen and an enzyme can be shown by sorting on the FACS: positive and negative populations are obtained from a hybrid segregating an antigenic marker, cell extracts made and enzyme assays carried out. This has been used to show synteny between F10.44.2 and W6/34 antigens, and LDHA (EC1.1.1.27), and between β_2m and pyruvate kinase (PK; EC2.7.1.40) (Table 3.5; Goodfellow *et al.*, 1982b). Sorting for a particular antigen, followed by DNA extraction from subsequent populations should also enable synteny tests for a cloned DNA fragment to be performed. These techniques are considerably more rapid than

Table 3.5 FACS sorting followed by isozyme analysis of HORL9D2RM

		Isozyme testing*	
Selecting antibody	Antigen population	LDHA	PK
F10.44.2	Positive	++	++
	Negative	−	+
W6/34	Positive	++	++
	Negative	−	++
BBM1	Positive	++	++
	Negative	++	−

*++, Strong expression of human isozyme; +, moderate expression of human isozyme; −, no expression of human isozyme.

conventional subcloning techniques and individual analysis of resultant clones, which were mandatory before FACS technology was available.

3.5.2 Hybrid manipulation

Antibodies to chromosome-specific cell surface antigens are of use not only for quantifying the human chromosome contribution to a somatic cell hybrid population and for synteny tests, but also for altering the human contribution. Polyclonal antibodies to HLA, β_2m and SA-1 have previously been used to select against hybrids containing chromosome 6, 15 and 11 by cytotoxicity in the presence of complement (Jones *et al.*, 1976; Goodfellow, 1975). Thus, in a hybrid population which is segregating a particular non-selected human chromosome, only those cells lacking the chromosome, and consequently the respective antigen, will survive. Hybrid manipulation in this way is essentially a negative selection system and has largely been superseded by the use of the FACS. The FACS allows selection of populations which are either positive or negative for a surface antigen when used in conjunction with the appropriate antibody. Dorman *et al.* (1978) used a conventional antiserum against the X-linked antigen SAX (Buck and Bodmer, 1976) to successfully sort a hybrid mass culture into two viable populations, positive and negative for the X chromosome. These initial experiments showed the potential of the approach: current work has employed monoclonal antibodies in place of heterospecific antisera.

Cell sorting followed by cloning of the resultant populations can lead to directed and stable hybrid manipulation: subclones with or without a particular chromosome can be isolated. Following the example of HORL9D2RM again, it was possible to isolate '15-only' hybrids by selecting a population negative for W6/34 from which clones were derived. As assayed by IRIA and isozyme testing, these had lost chromosome 11 markers, but retained markers for chromosome 15 (Goodfellow *et al.*, 1982b; and unpublished results). We have used these techniques to demonstrate the Y-linkage of a gene controlling expression of the 12E7 antigen (Levy *et al.*, 1979). A hybrid, AMIR2, made between fibroblasts from a male with X-linked icthyosis (46; t(X:Y;Xqter→Xp22.3:Yqter→Yp1.1);Y) and mouse HPRT$^-$ L cells, was shown to be 12E7-positive by IRIA. When examined on the FACS, however, only 24% of cells were positive (Goodfellow *et al.*, 1983). Sorting for a 12E7-negative population, followed by karyotype analysis, showed that these cells had lost the normal Y chromosome, present in the original hybrid, although still retaining the X→Y translocation chromosome in 94% of cells. This simple experiment provides several pieces of information. (a) 12E7 expression is coincident with the presence of the normal Y chromosome in AMIR2; (b) although a 12E7-controlling gene had previously been assigned to Xp (Goodfellow *et al.*, 1980), the presence of the region Xqter→Xp22.3 in hybrids was not sufficient for 12E7

expression. This suggests that the X-linked locus, *MIC2*, controlling antigen expression, is in the region Xp22.3→Xpter. (c) By the same logic, the Y locus must be in the region Yq1.1→Ypter, which is not included in the translocation chromosome.

Other data from different hybrids are concordant with the results of this experiment (Goodfellow *et al.*, 1983) and present an intriguing picture of two similar, if not identical, structural genes on the X and Y chromosomes. Furthermore, the regional mapping places these genes in the regions which pair with each other during meiosis (Pearson and Bobrow, 1970), and *MIC2X* is in the region of the X chromosome thought not to be inactivated in females (Race and Sanger, 1975; Shapiro *et al.*, 1979; Muller *et al.*, 1980). The identification of potentially homologous functional genes on the X and Y chromosomes may thus have important implications for sex chromosome evolution, X-inactivation and sex determination.

3.5.3 Other uses of the FACS

The FACS is also proving useful in other areas, for example, for identifying the products of cloned genes: Barbosa *et al.* (1982) identified cosmid clones for HLA-A2 and HLA-B7 after transfecting those clones from a genomic library which hybridized to an HLA cDNA probe into L cells, and monitoring HLA expression with allospecific monoclonal antibodies. There is also a report (Stanners *et al.*, 1981) of the use of the FACS to isolate by sorting of a population of rodent cells transfected with a hybrid genomic library, a clone responsible for the expression of a cell surface antigen associated with human chronic lymphocytic leukaemia, and inducing a more malignant phenotype in hybrids and transfectants. The authors judge the gene to be present in 100–1000 copies in the hybrid genome, and this technology may prove more difficult for the cloning of low copy number genes. Recently, however, Kavathas and Herzenberg (1983) transfected mouse L cells with human DNA and isolated transfectants positive for HLA, β_2-microglobulin, Leu-1 (OKT1) and Leu-2 (OKT5/8) by FACS sorting. This should allow identification of the specific human sequences responsible for the Leu-1 and Leu-2 antigens after further rounds of transfection and sorting.

The use of the FACS is not limited to the analysis of cell surface determinants only: we have used a monoclonal antibody to a cytokeratin to examine, in fixed, permeablized cells, the differential expression of this protein in differentiating human–mouse teratocarcinoma hybrids (Benham *et al.*, 1983). Expression of SV40 T-antigen in SV40-transformed human cells and somatic cell hybrids has also been investigated with these techniques. It has also been possible to demonstrate SV40 large T in an SSEA-1-positive population of embryonal carcinoma hybrids after assaying a FACS-sorted cell extract (unpublished results).

3.6 CONCLUSIONS AND PROSPECTS

We have reviewed briefly the contribution of the use of antibodies to somatic cell genetic analysis, and in particular noted the progression from the use of polyclonal to monoclonal antibodies, which parallels a general trend in the use of antibodies as analytical tools in cellular and molecular biology. In the near-future, there is a high probability that monoclonal antibodies will be available which recognize at least one marker antigen for each human chromosome. The current picture, summarized in Table 3.2, indicates that, to date, almost half of the human chromosomes are covered. A complete panel will allow rapid analysis of the human chromosome content of hybrids and will further potentiate the use of the FACS in mapping and expression studies using hybrids. The use of antibodies for gene mapping has meant that species-restricted functional gene assays are not required. Instead, structural and immunological properties of the gene product are exploited; further use of this approach should lead to the identification of new genetic systems. An example is provided by the 12E7 antigen-controlling locus, *MIC2*: gene mapping has shown the existence of two related forms of *MIC2*, one on the tip of the short arm of the X chromosome, probably not X-inactivated and having some relationship with the *Xg* locus (Goodfellow and Tippett, 1981), and the other in the euchromatic region of the Y chromosome (Goodfellow *et al.*, 1983). This is the first demonstration of a structural gene on the human Y chromosome. Many other gene products identified by monoclonal antibodies and listed in Table 3.2 have no known function as yet. Possibly, some of these molecules have already been identified by other methods, but others may represent novel identities which can be explored further for functions.

Most of the monoclonal antibodies in Table 3.2 have a general tissue distribution, but it is also possible to investigate the genetics of specialized functions by use of the appropriate hybrid parents. Also, many monoclonal antibodies against haematopoietic cells have been produced (see Foon *et al.*, 1982; Reinherz and Schlossman, 1980 for reviews) and some of the genes controlling cognate antigen expression are now being mapped, for example, OKT10 antigen is encoded by human chromosome 4 (Katz *et al.*, 1983).

The antibody/hybrid approach is not, of course, restricted to the analysis of surface components. For example, complement component C3, secreted by fibroblast hybrids, has been mapped to human chromosome 19 using a monoclonal antibody (Whitehead *et al.*, 1982). It is also possible to map enzyme loci using antibodies: Vora *et al.* (1982) used a monoclonal antibody to human muscle-type phosphofructokinase (PFKM) to map the *PFKM* gene to chromosome 1. The conventional approach of isozyme separation by gel electrophoresis was not possible in this case due to at least three structural loci, in both humans and rodents, expressing different forms of PFK activity. This would

give an extremely complex gel pattern, whereas the monoclonal antibody could be used to precipitate enzyme from hybrid cell extracts.

As the number of gene assignments increases, it is possible to ask questions about the overall organization of the human genome. One question pertinent to the work described here is: are genes for cell surface determinants clustered? This question should be refined slightly to emphasize the structural genes of surface proteins, since one possible complication of the antigenic recognition of cell surface molecules is that the determinant may be produced by the addition of carbohydrate to a nascent protein chain. In this case, if the addition is species-specific, or, in combination with a protein structure, produces a species-specific determinant, it may be the gene for a glycosyltransferase which is mapped. In the case of a surface glycolipid (such as W6/34 antigen), this is almost certainly what is happening. A corollary of this is that one glycosyltransferase may produce several different epitopes on different recipient molecules. To guard against this, it may be necessary to take the approach of Whitehead *et al.* (1982) where it was demonstrated that the monoclonal antibody recognizing human C3 also precipitated a non-glycosylated form of the molecule from poly-A^+ RNA extracts translated *in vitro*. Having refined the question in this way, we can immediately answer that no one chromosome carries all the surface protein genes, but that these appear to be spread over a number of chromosomes. This is expected from analysis of other known gene assignments. For example, whereas in *E. coli,* genes of a particular biochemical pathway are often contiguous and expressed in the order dictated by the biochemistry, no such organization is found in the human genome. Indeed, functionally related genes are scattered over several or many chromosomes, although four enzymes of the glycolytic pathway map to chromosome 1.

Neither are evolutionarily related genes necessarily linked: genes for the subunits of the same enzyme can be found on different chromosomes (e.g. *LDHA* on 11, *LDHB* on 12), as can other related structural loci (e.g. β-globin cluster on 11, α-globin cluster on 16). A similar pattern is seen for surface protein genes: thus, while sequence-related HLA and DR genes are linked on chromosome 6, β_2-microglobulin (15) and immunoglobulin (2, 14, 22) genes, whilst having some sequence relationships, are clearly not linked. However, both polyclonal and monoclonal antibodies have identified a number of antigens encoded by chromosome 11 which appear to be on distinct molecules and which are genetically separable. It is known that one of these antigens is present on a glycolipid, although the others are defined by antibodies which precipitate proteins. However, the possibility remains that genes for a small number of glycosyltransferases are being mapped and further experiments will be necessary to clarify this point. In the mouse, it is now emerging that a large cluster of surface antigen genes is grouped around the β_2m locus on chromosome 2 (Meruelo *et al.*, 1982; see also Chapter 1). In humans, β_2m is on chromosome 15 (Goodfellow *et al.*, 1975) and recently we have used

monoclonal antibodies to map two further genes for surface determinants to this chromosome (Table 3.2; Blaineau *et al.*, in press; unpublished work). In addition, Sakaguchi and Shaws (1982) have mapped the gene conferring coronavirus 229E susceptibility, probably surface-mediated, to chromosome 15. This makes at least four determinants encoded by this chromosome and it may be that a comparable picture to that of the mouse is forming for this locus. The next few years should answer the question of clustering, and possible relationships of members of clusters to each other in terms of evolution, structure and function.

A far-reaching consequence of gene mapping in general is the possible application to medical genetics. The most dramatic example is that of the oncogenes, genes implicated in cell transformation, approximately fifteen of which have been mapped in the last two years to particular chromosomes and chromosomal regions. It has been found in most cases that these regions are involved in rearrangements or deletions in specific cancers, which may reflect a role of oncogenes in the aetiology of these diseases (reviewed in Rowley, 1983). If it is not possible to determine the presence of a genetic defect directly or when the nature of the defect is not known (e.g. muscular dystrophy), it may be possible to identify a genetic polymorphism or trait which is linked to the disease locus. A classical example is that of haemophilia and its linkage to *G6PD* on the X chromosome: if a mother carrying the haemophilia defect is heterozygous for *G6PD*, family studies will show which allele is associated with the disease locus, and by determining the allelic form of G6PD (glucose 6-phosphate dehydrogenase) in the mother's male offspring *in utero*, it can be determined whether the child might be affected. Genetic counselling along these lines could help to eliminate rapidly unwanted genes from the population if such pregnancies were terminated. An important consideration in such cases is the genetic distance between loci: the larger this distance, the greater the likelihood of cross-over between loci, which would give an incorrect evaluation of whether a child will carry a genetic defect when the above methods were followed. To date, the best-characterized heritable condition involving a defect in a cell surface molecule is familial hypercholesterolaemia. This is a condition associated with defective low-density lipoprotein receptors (LDLR). There is reported to be weak linkage between *C3* and *LDLR* (Ott *et al.*, 1974; Berg and Heiberg, 1976; Elston *et al.*, 1976), which would place *LDLR* on chromosome 19. However, more recently, using hybrids between human fibroblasts and hamster cells lacking functional LDLRs, one of these groups has suggested chromosomes 5 or 21 or both as being involved in LDLR activity in hybrids (Maartmann-Moe *et al.*, 1982). The situation is, therefore, not clear at present and the use of available monoclonal antibodies (Beisiegel *et al.*, 1981) may solve the problem. Once a chromosomal assignment is made, it might be possible to show linkage in family studies with a polymorphic locus which would be useful for genetic counselling.

Conversely, it might be possible with genetic diseases of unknown cause, but where the disease locus has been mapped, to inspect the human gene map for linked or syntenic genes, and then to assay these functions in affected individuals for a relationship. Negative mapping data may also be useful, at least in eliminating certain candidates for the gene defect. For example, haemochromatosis, an iron-storage deficiency, is associated with a defect on chromosome 6. This suggests that the condition is not due to a defect of the transferrin receptor, whose gene maps to chromosome 3. A defect in the ferritin structural gene is also ruled out, since this gene maps to chromosome 19 (Caskey *et al.*, 1983). However, linkage of the defect to *HLA-A* has been shown (Simon *et al.*, 1980; Edwards *et al.*, 1980), and this allows pre-morbid identification of patients, who should then be protected from iron overloading. An expansion of the human gene map by methods including those reviewed here should allow some of these suggestions to be realized and further possibilities explored.

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