CHARACTERIZATION AND CLONING OF MHC CLASS I SEQUENCES FROM CHORIOCARCINOMA JEG-3 CELLS.

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This thesis is dedicated to my
mother and father
for all their love, support and encouragement.

DECLARATION.

The research reported in this thesis is my own and original work except where otherwise stated and has not been submitted for any other degree.

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SUMMARY.

1. The use of sera and monoclonal antibodies has permitted the serological definition of three polymorphic genetic loci HLA-A, HLA-B, and HLA-C, which encode the 45kDa heavy chains of the classical class I transplantation antigens. These molecules were identified because they elicited strong allograft rejection. Their true function is to serve as restriction elements by presenting peptidic antigens for recognition by cytotoxic T lymphocytes in MHC restricted responses. Molecular genetic analyses revealed that the vertebrate class I gene family was larger than expected, and there is evidence that at least some of these genes encode polypeptides whose structure and perhaps function is different from those of the classical class I loci.

2. Some forms of trophoblast express class I molecule(s) which raises the question of how this semi-allogeneic tissue is able to escape maternal immune recognition. The work described in this thesis is a study of class I molecules expressed by a malignant form of cytotrophoblast: the choriocarcinoma cell line JEG-3. Immunoprecipitation analysis of JEG-3 cells using two anti-class I monoclonal antibodies (W6/32 and 61D2) revealed the expression of two different molecular weight class I species (45kDa and 41kDa) which differed in their ability to bind 61D2. The smaller component was expressed to higher levels than the 45kDa polypeptide and did not cross react with 61D2.

3. Initially, it was thought necessary to induce JEG-3 cells with gamma-interferon in order to increase the level of class I transcripts and thereby facilitate their analysis and cloning. Total RNA was isolated from JEG-3 cells which had been treated with varying amounts of gamma-interferon (upto 0-10,000U/ml for 30hrs.). No obvious gamma-interferon induction of class I transcripts was apparent after hybridization of a class I HLA-B7 cDNA probe to quantitated RNA immobilized on dot-blot and Northern filters. The size of class I mRNA was shown to be approximately 1.6Kb.

4. In order to investigate these molecules further, a lambda gt10 cDNA library was constructed using mRNA isolated from JEG-3 cells treated with gamma-interferon (500U/ml for 30hrs.). The percentage of recombinants containing inserts was primarily calculated by a comparison of data with control experiments and subsequently determined empirically by analysis of random picked individual plaques. The insert frequency of the library was approximately 70% and thus from $1.8x10^5$ plaques, $1x10^5$ were true recombinants. The cDNA library was screened with the HLA-B7 cDNA probe under reduced stringency conditions and six independent class I positive cDNA clones (JW1-6) were recovered. After restriction endonuclease mapping, each was subcloned into M13mp18 for sequence determination. JW1 (1.4Kb) was identical to JW5 in both length and sequence, encompassing 15 bases of poly A tail and extending forward in the coding region as far as and including the alpha-2 domain. JW4 was shorter (1.2Kb) in the 5' end and differed further by a 7bp (AATCCGC) deletion 5' to the polyadenylation site.

5. Nucleotide and amino acid sequence comparisons of JW1, JW4 and JW5 with HLA-A, HLA-B and HLA-C locus sequences clearly indicated that these clones were derived from the HLA-C locus (Ward et al. submitted for publication). From the Northern data it was inferred that the largest clone was incomplete and a strategy was devised to clone the remaining 5' portion of the gene. Subsequent sequence analysis showed that the first 221bp of the 5' most region of JW1 was extremely homologous (>97%) to the intron 2 sequences of HLA-C locus genes. Thus JW1 was an incompletely spliced pre-mRNA. Although the cloning strategy was frustrated by the presence of intron-2 in JW1 it did generate a subclone that was necessary in order to complete sequencing.

JW1 as far as it extended was found to be identical to the cDNA BeWo C.1 cloned from the BeWo choriocarcinoma published this summer (Ellis et al. 1989). The latter is predicted to code for a 45kDa polypeptide.

6. Using an HLA-6.0 specific probe, work in collaboration with T.Rinke de Wit (Leiden) and Dr. P.L.Stern (Manchester)

showed that HLA-6.0 homologous transcripts were expressed in JEG-3 cells. It is possible that these HLA-6.0 homologous transcripts encode a 41kDa polypeptide corresponding to that immunoprecipitated from JEG-3 cells.

7. HLA-C antigens are polymorphic, and at least one allele (Cw3) has been shown to be capable of presenting antigens in MHC restricted responses. Those molecules derived from the maternal haplotype might present antigens to the maternal immune system as a means of protecting the foetus from infection whilst those derived from the paternal haplotype might act as allogeneic stimuli (if these differ from the maternal haplotype. The question which still remains to be answered is "What is the origin and nature of the 41kDa molecule?" The full length clones of both molecules are necessary in order to perform functional assays in an amenable system and to test whether they can present antigens to T lymphocytes.

Further cloning of a number of class I cDNA's ought to give an indication of the level of polymorphism at the loci considered. It may be possible to construct specific DNA probes to investigate the extent of expression of these transcripts in other tissues. This data may indicate whether the molecules have specialized functions (they might only be expressed on trophoblast) or whether they have more generalized roles in immunity.

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ABBREVIATIONS.

General

A	-	Angstroms
$A_{260/280}$	-	Optical Density, Absorbance 260nm/280nm
Ab	-	Antibody
ARS	-	Antigen Recognition Site
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytidine triphosphate
dd	-	dideoxy
dGTP	-	deoxyguanosine triphosphate
dTTP	-	deoxythymidine triphosphte
DNA	-	deoxyribonucleic acid
cDNA	-	complementary DNA
dsDNA	-	double stranded DNA
ssDNA	-	single stranded DNA
ER	-	endoplasmic reticulum
FCS	-	foetal calf serum
HLA	-	human leucocyte antigen
IFN	-	interferon
MHC	-	Major histocompatibility complex
MoAb	-	Monoclonal Antibody
NRS	-	normal rabbit serum
PAGE	-	polyacrylamide gel electrophoresis
PBS	-	phosphate buffered saline
PDB	-	phage dilution buffer
RNA	-	ribonucleic acid
SOS	-	save our souls
mRNA	-	messenger RNA
TCR	-	T cell receptor

Chemicals

ATP	-	adenosine triphosphate
BSA	-	bovine serum albumin
DEPC	-	diethyl pyrocarbonate
DTT	-	Dithiothreitol
EDTA	-	Ethylene diamine tetra-acetic acid (disodium salt)
FSB	-	final sample buffer
IPTG	-	isopropyl B-D thiogalactopyranoside
RNA	-	ribonucleic acid

RNase	-	Ribonuclease A
SDS	-	sodium dodecyl sulphate
TEMED	-	NNN' N'tetramethyl ethylenediamine
Tris	-	tris (hydroxylnethyl) amino ethane
X-gal	-	5-bromo, 4-chloro, 3-indolyl, B-D galactoside

Antibiotics

Ap	-	ampicillin
Tet	-	tetracycline

Measurements

mA	-	milliamps (10^{-3}A)
bp	-	base pair
Kb	-	Kilobase pair (10^3 bp)
kDa	-	kilodalton (10^3 dalton)
^oC	-	degrees centigrade
Ci	-	Curie
uCi	-	microcurie (10^{-6} curie)
g	-	centrifugal force = gravitational accelaration
g	-	gramme
mg	-	milligramme (10^{-3} g)
ug	-	microgramme (10^{-6} g)
l	-	litre
ml	-	millilitre (10^{-3} l)
ul	-	microlitre (10^{-6} l)
M	-	Molar
mM	-	millimolar
uM	-	micromolar
m	-	metre
cm	-	centimetre (10^{-2} m)
mm	-	millimetre (10^{-3} m)
mw	-	molecular weight
mins	-	minutes
pH	-	acidity ($-\log_{10}$ [molar concentration H^+ ions])
V	-	volts
W	-	watts

Miscellaneous

aa	-	amino acids
LMP	-	low melting point
moi	-	multiplicity of infection
%	-	percentage
pfu	-	plaque forming units
RSDF	-	restriction site directed fragment

rt	-	room temperature
SDR	-	site directed fragment
UV	-	ultra violet

GENERAL INTRODUCTION.

1.1.1.

The widespread application of the techniques of molecular cloning has complemented earlier classical immunogenetic studies and has enabled remarkable progress in understanding the molecular basis of immune recognition. The elucidation of the structure of the genes and cDNA's for antibody, T cell receptor and the Major Histocompatibility Complex (MHC) has enabled immunologists to gain understanding of some of the main ways in which the specificity of the immune system functions.

The immune system protects self by the recognition and elimination of non/altered-self. This task of discrimination relies upon antibodies that are produced by B lymphocytes and T lymphocyte receptors. Self or non-self antigens bind to antibodies, or are presented in association with cell surface MHC class I and class II molecules for recognition by T cells. The MHC is known as such for historical reasons since class I molecules were first discovered because they elicited strong allograft rejection. The class I molecules (which are the subject of this thesis) are known as the major classical transplantation antigens and are used in surgery as the major determinants in directing choice of tissue grafts. There are also a large number of minor histocompatibility loci scattered throughout the genome including H-X and H-Y, on the sex chromosomes which individually evoke a relatively weak rejection response.

Specific alloantisera and monoclonal antibodies prepared by the appropriate cross-immunizations of inbred and recombinant strains have provided much information about the mouse MHC complex (H-2). MHC studies in man (HLA - human leucocyte antigen) originated with the discovery of antibodies in pregnant women and polytransfused individuals. Serological MHC analysis of inbred strains is performed differently from outbred species because the latter necessitates the use of a number of antisera which is much more complicated, and thus serological analyses of inbred mouse strains has enabled the definition of some class I loci in advance of their human equivalents. These analyses demonstrated that the classical class I transplantation antigens were extremely polymorphic.

Protein (Orr et al. 1979; Parham et al. 1988), DNA

sequence analyses (Strachan et al. 1984; N'Guyen et al. 1985; Sodoyer et al. 1985) and X-ray crystallographic studies (Bjorkman et al. 1987a;b) are uncovering the overall molecular organization of the class I antigens, and the nature and location of their polymorphic regions. Sequence comparisons between strongly serologically cross-reactive specificities have provided a basis for evaluating the contributions of these polymorphic regions to the domains recognized by specific antibodies and T-cell receptors (Cowan et al. 1987; Lopez de Castro et al. 1982; 1985).

Two particularly active areas of research on MHC are the characterization of class I molecules, and those that lie outside of the classical loci (Koller et al. 1987, 1988, 1989; Orr et al. 1983; Srivastava et al. 1987) and investigation into the mechanism by which foreign antigens are presented to T cells (Bjorkman et al. 1987a;b; Long and Jacobson, 1989; Townsend and Bodmer, 1989; Townsend et al. 1989).

MAJOR HISTOCOMPATIBILITY COMPLEX (MHC).

1.1.2. Classes of Genes in the MHC.

The genes in this complex fall into three classes (I, II and III) which all play central roles in the functioning of the immune system. The class I genes encode the polymorphic alpha chain of a heterodimer, some of which are involved in the presentation of endogenously synthesized foreign (viral) antigens to cytotoxic T lymphocytes in both MHC-restricted and allogeneic responses. They are expressed on many but not all cell types (Daar et al. 1983). The class Ib molecules are largely similar to class I in organization but their precise functions are as yet unknown and their expression seems to be limited to very few cell types (reviewed Strominger, 1989). Class II genes code for alpha/beta heterodimers which are involved in the presentation of exogenously acquired (endocytosed) foreign antigens. They are expressed mainly on B cells. The class III genes code for some of the plasma complement components.

In addition to these three classes of molecules there are other genes located in this area of the human (Carroll et al. 1987) and mouse chromosome (Abe et al. 1988).

Some of the molecules encoded within the MHC show homology with the immunoglobulins (Ig) at the nucleotide (exon), amino acid and structural levels. It has been suggested that molecules which exhibit this degree of homology (MHC class I and class II, B_2-m, Thy-1, T-cell receptor, CD2, 4 and 8, and others) are members of an "Ig-superfamily" (Williams et al. 1984; 1985), and it is likely that the gene fragments encoding these homology regions arose from the duplication of a common ancestral gene segment. Some members of the group interact with each other at the cell surface suggesting that a possible primordial domain diverged into a sort of ligand and receptor recognition system. Lutz and Cresswell (1987) showed by Western blot analysis that a monoclonal antibody PAC.M1 reacted with a determinant shared by some members of the Ig superfamily although its precise location was not known.

1.1.3. Genomic Organization of the MHC.

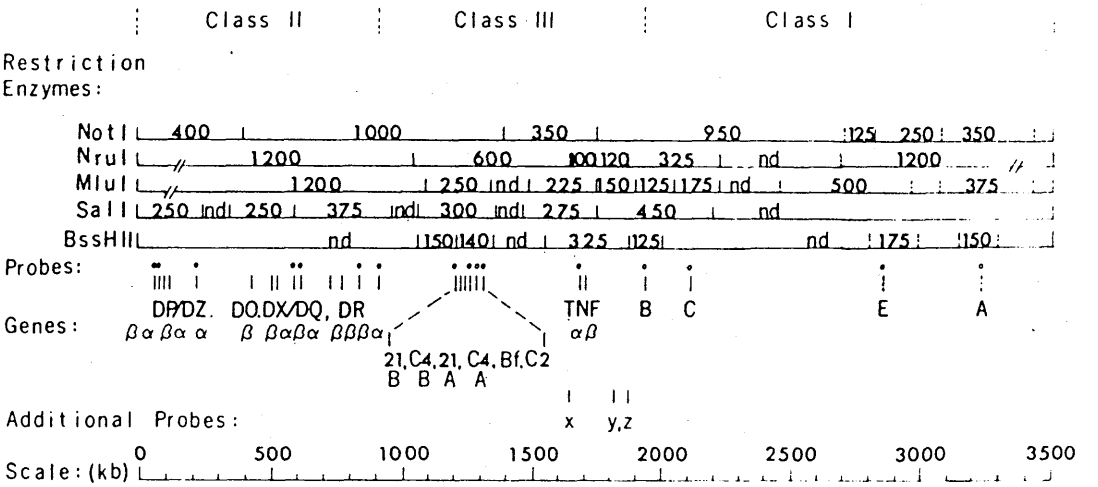
The molecules of interest in this study are the Class I antigens which fall within the MHC. The classical class I genes map to three loci in man (HLA-A, HLA-B and HLA-C) and in mouse (H-2K, H-2D and H-2L). The genes carried on an individual chromosome form a haplotype. Each individual inherits one paternal and one maternal haplotype which are designated by a superscript after H-2 in the mouse, and by numbers after the appropriate locus (in letters) in humans.

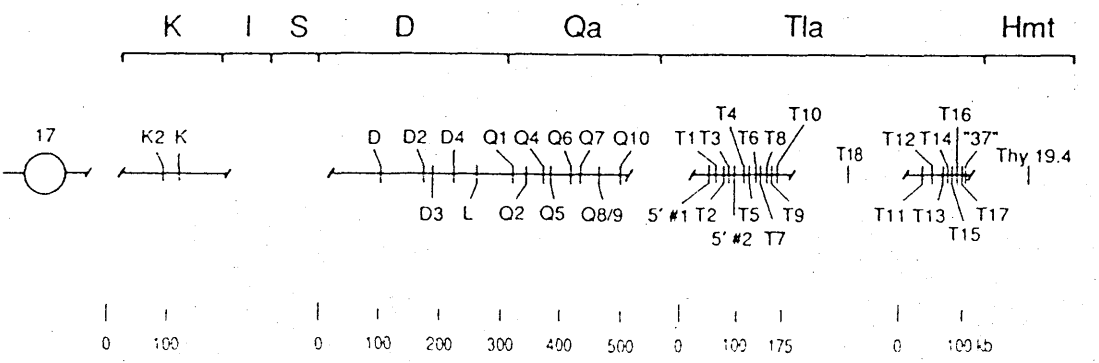
Pulse field gel electrophoresis and molecular hybridization techniques using various DNA probes have been used to construct a linkage map (see figure 1.1.3a) of the human MHC (Carroll et al. 1987; Lawrance et al. 1987). The complex is located on the short arm of chromosome 6 at 6p21.1 to 6p21.3 (Morton et al. 1984) and occupies approximately 3500Kb (approx. 1/3000th human genome). The analogous region in mouse (H-2) is located on chromosome 17.

Subsequent to the purification of HLA polypeptides, sequencing of the N-terminus (Orr et al. 1979) allowed the preparation of specific oligonucleotides. The oligonucleotides were used to isolate various genomic and cDNA clones by homologous hybridization (Ploegh et al. 1980; Sood et al. 1981). Southern blotting of genomic DNA and the

Figure 1.1.3a. Organization of genes within the MHC on chromosome 6. nd - regions not completely identified by overlapping and contiguous fragments. This region also contains pairs of genes for Tumour Necrosis Factor (TNF alpha and beta) and 21-hydroxylase (21A and 21B). From Carroll et al. (1987(. HLA-F (5.4) and HLA-G (6.0) map more telomeric to HLA-A (see Koller et al. 1989).

Figure 1.1.3b. Genetic Map of the Murine H-2 MHC (Balb/c mouse). The class I genes map to the K, D, Q (Qa) and TL (Tla) regions. The I and S regions correspond to class II and class III (complement) genes respectively. The distance between the K, D and T regions is unknown (Brorson et al. 1989).


Class II
Class III
Class I
Restriction Enzymes:
NotI
NruI
MluI
SalI
BssHII
Probes:
Genes:
DP/DZ.
DO.DX/DQ, DR
TNF
B
C
E
A
21,C4,21, C4,Bf,C2
B B A A
Additional Probes:
x
y,z
Scale: (kb)
0
500
1000
1500
2000
2500
3000
3500


K
I
S
D
Qa
Tla
Hmt
17
K2 K
D D2 D4 Q1 Q4 Q6 Q7 Q10
D3 L Q2 Q5 Q8/9
T1 T3 T4 T6 T8 T10
5' #1 T2 T5 T9
5' #2 T7
T18
T12 T14 T16 "37" Thy 19.4
T11 T13 T15 T17
0 100
0 100 200 300 400 500
0 100 175
0 100 kb

use of various interspecies cross-hybridizing class I probes has revealed that the class I genes constitute a larger than expected gene family than that indicated from allo-serological analyses. There are in the order of 30 class I related sequences in mouse (Cami et al. 1981, Goodenow et al. 1982), although fewer so far in man (Malissen et al. 1982), horse (Alexander et al. 1987) and chicken, (Guillemot et al. 1989). Koller et al. (1987) mapped seventeen distinct class I sequences in the human lymphoblastoid cell line LCL. The number of class I genes in the BALB/c H-2^d haplotype is 33 (Steinmetz et al. 1982) and 26 in C57BL/10 (B10) H-2^b haplotype mouse (Weiss et al. 1984). In actual fact, most class I genes are located outside the classical transplantation loci of mouse (H-2K, D and L) and man (HLA-A, -B and -C). In the mouse these "non-classical" loci map to the Q and TL regions (Winoto et al. 1983) while the majority of the other human class I genes are situated more towards the telomere from HLA-A (Orr and DeMars, 1983). The discrepancy between the number of phenotypically characterized class I molecules and gene sequences is under investigation.

The availability of inbred strains of mice and the linking up of cosmid clones (Weiss et al. 1984) has enabled the study of the overall gene organization of the mouse H-2 in advance of the human MHC. The general arrangement of genes differs between mouse and human. In the HLA complex the class II loci are positioned between the centromere and class I loci, whereas the H-2 class II genes are between the H-2K locus and class III region (see figure 1.1.3b).

The genes are widely spaced, especially so in the human (Malissen et al. 1982a) and a comparison of their surrounding sequences shows both conserved and polymorphic regions which have probably resulted from deletions, insertions and translocations. However, Chimini et al. (1988) found very little size polymorphism in humans implying that expansion/contraction events due to unequal crossing over (as in the mouse) were comparatively infrequent. A difference in the number of class I genes seems to be tolerated between different strains of mice and across species (Weiss et al. 1984; Hammerling et al. 1985). If any of the genes have important and/or separate functions, then it might be expected that their sequences be conserved during

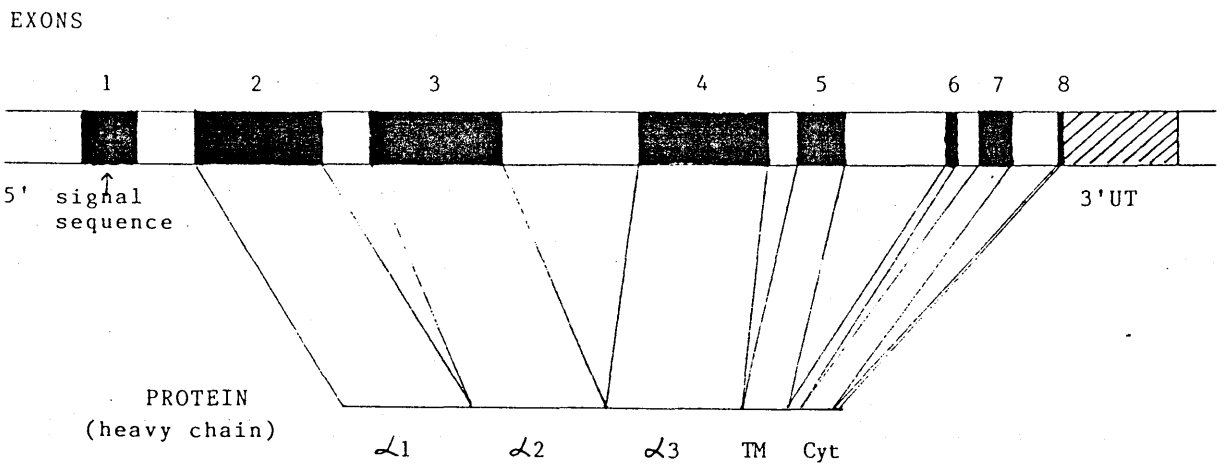


Figure 1.1.3c. Exon/intron Organization of a Class I Gene. Exon 1 encodes the signal peptide, exons 2-4 specify the alpha-1, alpha-2 and alpha-3 extracellular domains. Exon 5 codes for the transmembrane (Tm) region and exons 6-8 code for the cytoplasmic region (C). The hatched box represents the 3'UT region (see also Malissen et al. 1982; Sodoyer et al. 1984 and Strachan et al. 1984).

evolution.

Exon-intron Organization of a Class I Gene.

Figure 1.1.3c shows the exon-intron organization of a typical classical class I gene (see also Malissen et al. 1981; Sodoyer et al. 1984; Strachan et al. 1984). The class I genes encode alpha heavy chains which vary in size (approximately 40-45kDa) depending upon the locus from which they are derived. The signal/leader peptide (of approximately 21-24 amino acids) is removed co-translationally in the ER and is coded for by the first 5' most exon. The three extracellular domains (alpha-1,- 2 and -3) are approximately 90 amino acids and are encoded by exons 2-4. Exon 5 corresponds to the transmembrane region which is approximately 40 amino acids. Depending upon the class I gene, the cytoplasmic region can be made up from between one to three exons (6, 7 and 8). In general the total number of exons in a class I gene varies between six and eight although novel alternative splicing combinations do occur (see section 1.1.7)

1.1.4. Structure of a Classical Class I Molecule in relation to Function.

Class I transplantation molecules are highly polymorphic integral membrane glycoproteins formed by the non-covalent interaction between a heavy chain and B_2-microglobulin (B_2-m) (Silver and Hood, 1974). Structurally, the heterodimer is characterized by the presence of four extracellular regions; three alpha chain domains and B_2-m. B_2-m is an invariant non-glycosylated 12kDa protein encoded on chromosome 15 in man (Goodfellow et al. 1975). Murine and human cDNA's for B_2-m have been obtained (Parnes et al. 1981; Suggs et al. 1981). Figure 1.1.4a depicts the basic conformation of a class I molecule. The structure of a class I molecule (HLA-A2) has now been elucidated using X-ray crystallography (see below). The hydrophobic transmembrane region of the alpha chain is responsible for anchoring the whole complex into the plasma membrane. This region extends intracellularly to form the cytoplasmic region. Studies on H-2 (Steinmetz et al. 1981) and HLA (Malissen et al. 1982)

Antigen Recognition Site (Groove)

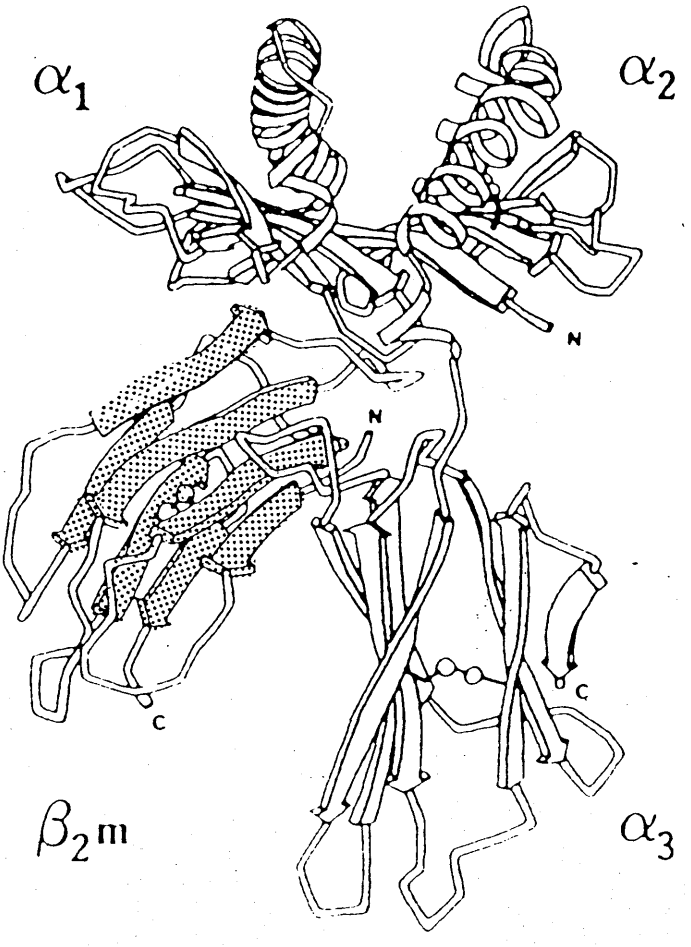


Figure 1.1.4a. Schematic Representation of the four domains of **HLA-A2.** The alpha-1 and alpha-2 domains can be seen to form a platform and groove structure (Bjorkman et al. 1987a).

have shown that each domain of the class I protein is largely encoded by a separate exon. Sequence comparisons have indicated that the alpha-1 and alpha-2 domains are highly polymorphic in defined regions within a common framework structure, whilst the proximal alpha-3 domain is less variable and Ig-like in structure (Steinmetz and Hood, 1983). The degree and nature of this variation is thought to reflect the function of the molecule, indeed they are now known to interact with a very large number of antigens (see section 1.1.10). The high level of conservation in the alpha-3 domain is thought to reflect its importance in the association with the B_2-m subunit.

Alpha-1 and Alpha-2 Domains of Class I Molecules Interact to form Unique epitopes.

Exon shuffling of class I alpha-1 and alpha-2 domains and subsequent transfection into L cells by DNA-mediated gene transfer have enabled the identification of possible sites on different exons which are involved in the formation of class I epitopes. Ajitkumar et al. (1988) presented data which illustrated that multiple residues on both the alpha helices of the class I MHC molecule were simultaneously recognized by the T-cell receptor. Much evidence has accumulated recently demonstrating that class I molecules whose alpha-1 and alpha-2 coding exons have been interchanged are no longer recognised by T-lymphocytes specific for the parental molecules. This loss of recognition and reactivity by monoclonal antibodies specific for the parental products is accompanied by the gain of immunodominant epitopes (Kanda et al. 1987).

X-Ray Crystallographic Studies.

The above functional assays were informative but the three-dimensional structure of the histocompatibility antigen was required in order to locate the polymorphic residues on the structure and to gain understanding of how and where antibodies and T cell receptors recognize the molecules and the foreign antigens that are presented.

The three-dimensional structure of a human class I antigen (HLA-A2) has been elucidated using X-ray crystallography at 3.5A resolution (Bjorkman et al. 1987a;b). This work represents a valuable contribution in furthering

our understanding of how these molecules perform their immune function. After papain cleavage of HLA-A2 (encompassing alpha-1, -2, -3 and B_2-m), the polypeptides were purified from 200 litres JY culture (homozygous at the HLA-A locus) and subsequently crystallized. The overall structure (figure 1.1.4a) can be described as follows: alpha-3 and B_2-m domains (which are closest to the cell membrane) support the alpha-1 and alpha-2 domains, with B_2-m interacting with all three alpha domains.

Nature of the Antigen Recognition Site (ARS) and T cell recognition regions.

The alpha-1 and alpha-2 domains both have similar secondary amino acid structure. The first 50 residues encompass four regions of beta-strands, followed by an alpha-helix in the remaining 40 residues. X-ray crystallography studies have illustrated the nature of the interaction between the alpha-1 and alpha-2 domains. This close association results in the formation of a platform/floor of an antiparallel single beta-pleated sheet which is topped by two long alpha helices (figure 1.1.4b). The resulting long pocket or groove is secured by disulphide links in the alpha and beta strands. This shallow area is aproximately 25A long, 10A wide and 11A deep and faces away from the cell surface.

The structural information together with the knowledge of the polymorphic residues within the alpha-1 and alpha-2 sequences have indicated the regions which might be involved in presenting the peptidic antigens. It is likely that the prominent groove on the surface of class I molecules is the foreign antigen recognition site since its polymorphic nature, location, size and shape are consistent with the binding of a processed peptide. A continuous region of electron density within this groove seemed likely to be the image of a peptide or mixture of peptides that had co-purified and co-crystallized with HLA-A2.

The known positions of the polymorphic residues within the alpha-1 and alpha-2 sequences can largely be divided into three groups. One group of variable residues is located on the inner sides of both domain alpha helices and point into the groove. Another group lies within the beta-strands and the residues point upwards into the pocket. These two groups

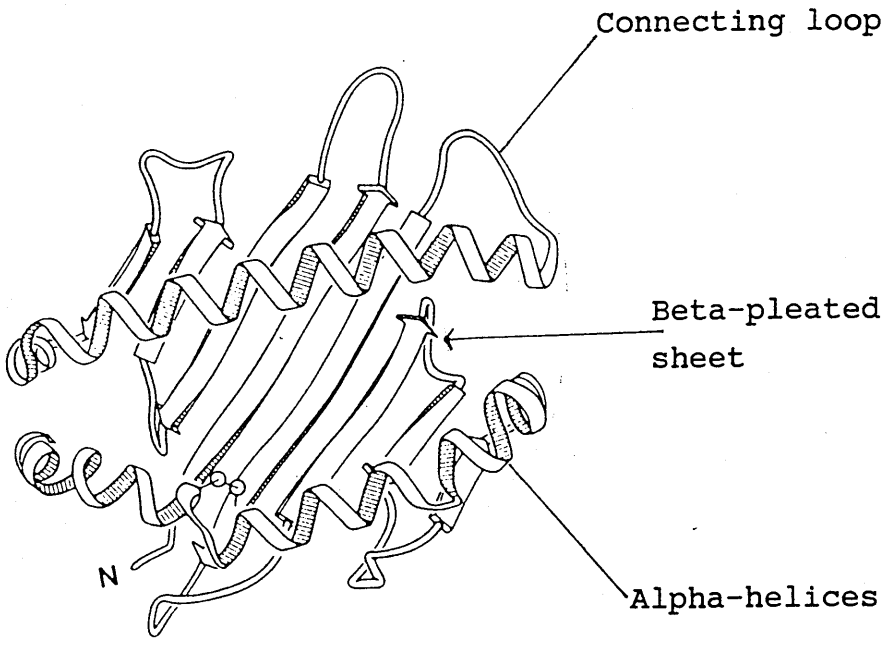


Figure 1.1.4b. Representation of the upper surface (looking down into the groove) of the alpha-1 and alpha-2 domains. The platform is made up from four alpha-1 and four alpha-2 strands of beta-pleated sheet which are topped by alpha-1 and alpha-2 alpha helices (Bjorkman et al. 1987a).

are involved in peptide binding. A third group on the top face of both alpha helices pointing away from the cell are thought to be the ligands involved in specific recognition by the T cell receptor. Polymorphism at some amino acid residues is thought to direct the specificity of recognition by T-cells and the degree of responsiveness to various foreign antigens.

A number of residues pointing into the ARS are absolutely conserved in at least 22 human sequences and are nearly all conserved in at least 13 mouse sequences. These residues may recognize a common feature of the short peptide. Other conserved residues which face away from the ARS may form the structural framework of the class I molecule or may be candidates for interaction with T cells.

Other functional residues are located on the site edges where they may be recognized by a T-cell receptor. Specific residues involved in the recognition by CTL's have been identified by characterization of H-2K^b class I spontaneous mutants and occur both within and outside of the ARS (reviewed Bjorkman et al. 1987b).

Interaction with B_2-microglobulin.

Various studies using somatic hybrids and cell lines (human Daudi and mouse RI) with defective B_2-m genes indicate that B_2-m is required for the cell surface expression of class I heavy chains (Arce-Gomez, 1978; Parnes et al. 1982; de Preval et al. 1983). The latter accumulate in the ER in a glycosylated form and can not be processed any further. B_2-m is thought to influence the correct conformation of the class I heavy chain such that it can be translocated from the ER to the trans-Golgi compartment where it can be terminally glycosylated (reviewed Maloy and Coligan, 1985; Lancet et al. 1979). Section 1.1.11 describes ways in which B_2-m might affect the regulation of class I molecule expression. X-ray crystallography has shown that B_2-m interacts with the central beta-strands and loops of the alpha-1-alpha-2 beta-pleated-sheet. Ferrier et al. (1985) had previously suggested that critical interactions between B_2-m and both the alpha-1 and alpha-2 domains were required since some mouse B_2-m/human A3, B7 and Cw3 hybrids could not be serologically detected (Ab recognition) and certain human alloimmune CTL's failed to recognize such hybrid molecules

(Bernabeu et al. 1983).

Glycosylation of Class I molecules.

Human class I molecules are glycosylated at position Asn_{86} which is found in the connecting loop between the alpha-1 and alpha-2 domains and extends away from the molecule. Both amino-terminal domains are glycosylated in mouse class I antigens but some, such as H-$2L^d$ are also glycosylated in the alpha-3 domain at position 256_{Asn} (Kimball and Coligan, 1983). Miyazaki et al. (1986) showed that non-glycosylated H-$2L^d$ was unchanged in its overall serological specificities and was recognized by alloreactive T cells, suggesting that carbohydrates were not essential for the immunological function of class I antigens. Since the cell surface expression of nonglycosylated antigen was much reduced compared to the fully processed H-$2L^d$ polypeptide (non attributable to degradation or rapid shedding) they concluded that carbohydrate facilitated intracellular transport of the nascent protein to the plasma membrane. In addition to this, it is possible that carbohydrate is involved in molecular recognition processes in ways that are not dependent on antigen specificity.

The characteristics of the class I genes and their products from individual loci have been described below - some of which will be referred to again in later sections.

1.1.5. Characteristics of individual Class I Loci of Mouse H-2.

The class I genes of the mouse H-2 complex have been mapped to four genetic loci/regions; H-2K, H-2D/H-2L, Q and TL.

The "classical" H-2K, D and L molecules are highly polymorphic, expressed on many nucleated somatic cells and are considered to be the 45kDa major classical class I transplantation antigens. The mouse Qa and Tla products of the Q and TL genes respectively are considerably less polymorphic (Mellor et al. 1984; Steinmetz et al. 1981), have a lower molecular weight (approximately 40kDa) (Michaelson et al. 1981) and their expression is limited to certain

subpopulations of haemopoetic-derived cells (reviewed by Lew et al. 1986). Q gene products (Qa) have been shown to act as very weak transplantation antigens since they can stimulate graft rejection in vivo (Flaherty, 1976), although the function of Q and TL genes is largely unknown.

The H-2K Region.

Overlapping cosmid clones of the C57BL/10 mouse have shown that two H-2K region genes (H-2K and H-2K1) are separated by approximtely 15Kb and are arranged in a head to tail configuration (Weiss et al. 1984).

The H-2D/H-2L Region.

In most laboratory strains of mice two classical transplantation antigens are usually co-expressed and these are coded for by the H-2K and H-2D loci. Transformation of L cells with cosmid clones and analysis with specific antibodies has allowed the identification of the H-2L products on $H\text{-}2^d$ and $H\text{-}2^q$ haplotypes. The number of class I genes located in the D/L region of the BALB/c ($H\text{-}2^d$) and B10 ($H\text{-}2^b$) mouse differs (Weiss et al. 1984; Flaherty et al. 1981). Five genes are present in the $H\text{-}2^d$ mouse and two of these encode serologically detectable $H\text{-}2D^d$ and $H\text{-}2L^d$ antigens. Apparently there is no equivalent B10 "$H\text{-}2D^b$" molecule in this mouse. Whether the other three BALB/c $H\text{-}2^d$ genes ($D2^d$, $D3^d$ and $D4^d$) are functional remains to be seen, although Mann and Forman (1988) have a cytotoxic T lymphocyte clone which recognizes one of these molecules.

The Q Region.

This region spans approximately 200Kb and is distal to the D/L region. Molecular genetic analyses have shown that the number of genes within the Q region differs between mouse strains. There are eight BALB/c and ten B10 class I Q genes (Q1-Q10) all arranged in a head to tail, 5' to 3' orientation. The B10 Q3 gene is absent in BALB/c mice (Goodenow et al. 1982). Analysis of surrounding sequences suggests that the BALB/c Q8/9 gene has resulted from a non-reciprocal recombination event of the individual Q8 and Q9 genes (Mellor, 1985). Strong homology between the Q6-Q7 and Q8-Q9 class I gene pairs in the C57Bl/10 Q region suggests that this region has evolved by the duplication of the gene

pairs.

Some distinct class I gene products have been identified by serological and biochemical studies; Qa-1 (Stanton and Boyse, 1976), Qa-2,3 (Flaherty, 1976), Qb-1 (Robinson, 1985) and Q10 (Devlin et al. 1985a). The heavy chains of some Q gene products are approximately 40kDa (Michaelson et al. 1981). The Qa-2,3 serological determinant was originally thought to be a single species until variations in molecular weight and isoelectric point revealed that there was more than one gene involved, i.e. the Q6, Q7, Q8 and Q9 genes in the B10 mouse (Sherman et al. 1984). The non-polymorphic Q10 protein lacks a complete transmembrane region and is secreted specifically in mouse liver (Devlin et al. 1985a; Maloy et al. 1984). The Qa-1 antigens are an allelic series of molecules (Stanton et al. 1981), and seem to be expressed on most tissues (Aldrich et al. 1988). At least three products from this region are ultimately secreted from the cell and these are discussed in section 1.1.7.

The TL Region.

The TL (Tla-thymus leukaemia antigen) region is located most distal in the MHC and contains the largest number of class I genes in mice; thirteen in B10 and eighteen (T1 - T18) in BALB/c (Hammerling et al. 1985; Fisher et al. 1985), although T18 is an incomplete class I gene.

The TL antigens are expressed on thymocytes, activated T lymphocytes and on some thymic leukaemias which suggests a different/modified role in immunity although T18 has been found to be expressed to 10-25% of the levels of the classical loci (reviewed by Strominger, 1989). Six antigenic specificities have been observed on thymocytes and T cell leukaemias. Amongst the Tla haplotypes (a-f), the levels of expression of TL antigens varies both qualitatively and quantitatively (see Pampeno and Meruelo, 1988).

The integration of TLev1 (a retroviral element) into the Tla locus of a common ancestor of the species Mus musculus, with its subsequent loss from certain mouse genomes e.g. C57Bl/10, reflects evolutionary rearrangements or polymorphisms (Pampeno and Meruelo, 1988). Pontarotti et al. (1986) have suggested that the $T3^c$ and $T13^c$ genes of the Balb/c mouse (Tla^c haplotype) may have arisen by duplication since they show extraordinary high sequence conservation.

The $T3^b$ gene contains only six exons, the first coding for a signal peptide that is five amino acids longer than all other class I molecules whilst the last exon encodes a complete cytoplasmic tail. A unique feature of the $T3^b$ and $T13^c$ third intron is a 1.1Kb insertion flanked by a 10bp inverted inexact repeat which suggests that these may be homologues or alleles. Generally there are high levels of homology between Tla^a, Tla^b and Tla^c haplotypes (Fisher et al. 1985; Obata et al. 1985; Pontarotti et al. 1986).

Further non-classical class I genes have recently been identified. Singer et al. (1988) have reported a subfamily of mouse class I genes mapping telomeric to Q which so far consists of two to three members. One of these, Mb-1, is predicted to contain five exons and its expression seems to be highly regulated. The overall nucleotide homology with other class I sequences is very low (60%) and it is no more related to class I genes from other species suggesting that it represents a primordial class I sequence. Fischer Lindahl et al. (1983) cloned the Hmt gene which encodes a non-classical class I molecule that associates with a mitochondrial peptide to form the maternally transmitted antigen (Mta). The Hmt gene lies within the Hmt region which is telomeric to TL. A further non-classical class I gene (Thy19.4) has very recently been cloned and also maps to the Hmt region (Brorson et al. 1989). The overall homology of Thy19.4 with other class I loci sequences ranges from 51-70%, and reaches 86-88% in the conserved alpha-3 domain. The overall homology of class I sequences in exons 1-5 is between 82-93%. Thus, Mb-1 seems to be the most divergent member of the class I gene family so far (Singer et al. 1988).

1.1.6. Characteristics of Individual HLA Class I Loci.

Classical Class I Loci.

The human equivalents of the genes encoding the classical class I molecules map to the HLA-A, -B and -C loci and they are arranged as in figure 1.1.3a. Some of the expressed human class I genes identified are:

HLA-A2 Koller et al. (1985).
HLA-Aw24 N'Guyen et al. (1985).
HLA-A3 Strachan et al. (1984).
HLA-A32 Wan et al. (1986).
HLA-A11 Cowan et al. (1987).

HLA-B44 Kottman et al. (1986).
HLA-B7 Sood et al. (1985).
HLA-Bw58 Ways et al. (1985).
HLA-B27 Weiss et al. (1985).

HLA-Cw1 Gussow et al. (1987).
HLA-Cw2 Gussow et al. (1987).
HLA-Cw3 Sodoyer et al. (1984).
HLA-Cw6 Mizuno et al. (1989).
HLA-Cw7 Pohla et al. (1989).
HLA-Cx52 Takata et al. (1988).

Although the class I sequences show an overall high degree of sequence homology and are highly polymorphic, there are regions within the gene which show locus specificity, that is, those regions which are highly conserved between alleles, but which differ significantly from other loci in these corresponding regions. Locus specificity can be defined as the percentage divergence between non-allelic genes minus that which occurs between allelic genes.

Koller et al. (1984) described probes isolated from the 3'UT region of the HLA-A an -B genes that hybridized only to one locus under high stringency conditions (15mM NaCl/1.5mM NaCitrate, pH7, 65^{o}C). Similar studies were also completed for HLA-B (Coppin et al. 1985). Strachan et al. (1986) analysed nucleotide sequence data of class I HLA genes and identified the seventh intron of HLA-C as being one of the regions which exhibited the highest degree of locus

specificity. Indeed, Ronne et al. (1985) used differences in the sequences of introns in order to establish evolutionary relationships between various class I sequences. The HLA-C locus specific probe (pC250 - encompassing exon and intron 7) is largely lacking exon sequences and so specific oligonucleotide probes directed towards the transmembrane region are expected to be useful in mRNA/cDNA work (Strachan et al. 1986). At the amino acid level, locus specific regions are obvious in the transmembrane regions between HLA-A, -B and -C products (see figure 6.9.1 in chapter 6).

The preparation of locus specific probes and their use on genomic Southern blots to investigate restriction fragment length polymorphisms has indicated that the regions surrounding the loci are quite conserved (Coppin et al. 1985b; Koller et al. 1984; Strachan et al. 1986).

Non-Classical Class I Loci.

Extensive efforts are underway to discover the human equivalents of the mouse non-classical loci. Class I genes that do not map to the classical loci have been broadly termed "New" (Holmes, 1989), or "Class Ib" (Strominger, 1989), and their function(s) is unknown. Much less is known about the precise location of the various HLA class I genes since complete cosmid linking of human MHC chromosome remains to be done. However, many class I genes do map to a region telomeric to the HLA-A locus (Orr and DeMars, 1983). Chimini et al. (1988), discovered that most class I sequences were contained in a 340Kb MluI fragment which carries the HLA-A gene. Srivastava et al. (1987) also suggested that this region could harbor the human equivalents of the mouse Q and TL regions.

Both genomic and cDNA clones of some genes have been obtained. Firstly, two class I pseudogenes, HLA12.4 (Malissen et al. 1982) and LN11 (Biro et al. 1983) were cloned and found to be more than 80% homologous to the HLA-A, -B and -C genes. Srivastava et al. (1987) cloned a genomic class I gene, RS5, which was shown to have a number of distinct features which included promoter structure, a different translation initiation site, a carboxyl terminus frameshift mutation and Alu repeat elements. The derived amino acid sequence of the three external domains of RS5 (Srivastava et al. 1987) revealed a level of divergence from

HLA-A, -B and -C locus proteins comparable to that noted between classical H-2 and some of the Q and TL region genes (Fisher et al. 1985).

Harry Orr's laboratory and colleagues have recently cloned and sequenced three genomic class I genes from a T-lymphoblastoid cell line LCL-721, HLA-6.0 (Geraghty et al. 1987), HLA-5.4 (Shimizu et al. 1988) and HLA-E (Koller et al. 1988). All associate with B_2-m and upon transfection, translate into 38.5, 40 and 41kDa proteins respectively. The degree of polymorphism is unknown at these loci. HLA-E maps between HLA-C and HLA-A and is known to produce two classes of mRNA which are predicted to code for an identical protein product. The HLA-E gene is quite related to RS5 since it shares an aberrant translation start site and an Alu insertion in the 3'-UT region. However, RS5 lacks a further Alu element found in HLA-E intron 5 and a direct repeat in exon 8, and its overall amino acid sequence homology with HLA-E is 90%.

Although HLA-6.0 is similar in organization to classical class I genes, it may be a structural homologue of a murine Q class I gene since the cytoplasmic region is predicted to be considerably shorter than usual and its COOH terminus is most similar to that of the Q7 gene. In addition to this, the HLA-6.0 promoter region very much resembles those of the Qa genes (Geraghty et al. 1987).

Paul et al. (1987) developed an 800 base pair probe (JF11), flanking the 5' region of HLA-A in order to identify non-classical HLA genes. This probe detected a limited number of bands other than HLA-A, -B or -C associated fragments on Southern blots of genomic DNA. Cosmid transfectants of a mouse fibroblast cell line already expressing human B_2-m were subsequently tested with the alloantiserum HA2 (human activated). HA2 is an antiserum (see Fauchet et al. 1986) that reacts with a component on HLA-A2 PHA-activated T-cells and Epstein-Barr virus (EBV)-transformed B cells but does not react with resting T or B cells. A cosmid clone P2B2 was isolated and subsequently mapped outside of the HLA-A locus. It was found not to react with W6/32. Paul proposed that this gene, specifically expressed on activated lymphocytes, could be related to HLA-A locus genes and be a possible analogue of a mouse Qa region gene. This gene seems to provide a link between the newly

cloned genes and previous reports of alloantigens resembling class I molecules human T or HT (Gazit et al. 1980a;b) HA (Fauchet et al. 1986) and TCA (van Leeuwen et al. 1985).

According to a brief review by Holmes, (1989), Orr's group have isolated 14 other class I genes and by sequence analyses, 11 of these are pseudogenes and the other three remain to be analyzed. The function of these expressible "non-classical" genes remains to be determined, as does the extent of the class I gene family. Strominger, (1989) described the CD1 molecules (Calabi et al. 1989) as being members of the class Ib set of loci (see final discussion for details).

1.1.7. Soluble and Alternatively Spliced Forms of Class I Molecules.

Class I antigens are normally anchored into the membrane via a sequence of hydrophobic amino acids. Shedding (Emerson et al. 1981), and proteolytic removal of antigens from the membrane have been thought of as mechanisms producing soluble class I molecules. To date, soluble class I molecules are known to be generated by three mechanisms. Products of the Q and TL region may be secreted because they have shortened transmembrane segments (due to a STOP codon). The Q10 product is interesting because it is secreted specifically in the liver at a high rate (Devlin et al. 1985a). Some mouse genes actually encode anchorless forms (Kress et al. 1983a,b). Soloski et al. (1986) studied the expression of the Qa-2 molecule in both resting and activated T-cell populations and noted upto a four-fold increase in biosynthesis after activation, but no concomitant increase in cell-surface levels. After activation, approximately 70% of newly synthesized Qa-2 were secreted as soluble molecules. This process is so far unique to Qa-2 and suggests a different functional role for this antigen. Further studies of resting and conA activated T and B spleen cells and their supernatants by surface and metabolic labelling has established that the Qa-2 molecule is inserted into the cell membrane and processed to a soluble form whereas the Qb-1 antigen is secreted directly into the supernatant (Robinson, 1987). Stroynowski et al. (1987) have found that the Q7

product (Qa-2 antigen) is anchored into liver cell membranes by a phosphatidyl inositol tail (Low et al. 1986). This feature is thought to be responsible for the expression of soluble/secreted and membrane forms of the Qa-2 antigen. Two alternatively spliced genes (excluded transmembrane region) are also secreted (Krangel et al. 1986; Cianneti et al. 1989).

The function of soluble class I molecules is unknown to date, and whether the transmembraneless gene of Krangel has a different function from its membrane counterpart remains to be seen. In general, it is not known whether alternative spliced class I molecules will have specific or different functions. The functional importance of Q10 must be viewed with caution since not all H-2 haplotypes express such a gene (Lew et al. 1986). Alternative splicing is known to direct the synthesis of membrane bound versus sereted IgM (Early et al. 1980), fibronectin (Kornblihtt et al. 1985) and the products of viral genomes. A series of alternatively spliced class I molecules have been discovered and are listed below.

Kress et al. (1983) discovered the use of an alternative splice acceptor site in intron 7 of the $H\text{-}2K^q$ gene leading to an addition of nine amino acids to the cytoplasmic tail. Similar results were obtained for the $H\text{-}2K^b$ and $H\text{-}2K^k$ molecules by Archibald et al. (1986). McCluskey et al. (1986) reported a deletion of exon 7 of the $H\text{-}2D^d$ gene which contains the important serine residue which is specifically phosphorylated <u>in vivo.</u> Variation in the first amino terminal antigen binding domain has been achieved by alternative splicing within the second exon causing the formation of a hybrid alpha-1 domain (Transy et al. 1984). Cianneti et al. (1989) observed mature transcripts where alternative splicing had caused the absence of exon 5 corresponding to the transmembrane region. Vogel et al. (1989) reported that 15 nucleotides from the 3' end of intron 5 were inserted between the complete exons of the transmembrane and first cytoplasmic domain.

It is possible that some of the splicing events represent the error in pre-mRNA processing, since alternative forms often occur at low frequency (Kress et al. 1983b; Transy et al. 1984).

The normal function of class I molecules is to present

antigen, and their polymorphism ensures interaction with a large number of different antigens. The lack of polymorphism for Q region products, whether they are membrane bound or soluble, certainly indicates that they may function in a different manner, but whether they do or not remains to be seen. Kress et al. (1983a) thought that soluble molecules might play a role in inducing self tolerance.

1.1.8 Evolution And Polymorphism.

The necessity for the immune system to detect different viruses in association with different MHC antigens could constitute the evolutionary pressure which has maintained not only the polymorphism but also a multiplicity of class I genes. Initially it was suggested that all class I genes were represented by a set of polymorphic variants present in the genomes of individuals (Bodmer, 1973), and that the diversity among cell surface antigens was achieved by a form of regulation but this is now known not to be the case. The occurrence of somatic gene rearrangements in the generation of diversity (c.f. immunoglobulin) have also been excluded (Tonegawa, 1983).

Several conclusions can be drawn concerning the evolution/origin of class I genes even though the sequence data is somewhat limited and unevenly distributed amongst alleles and species. Of the vertebrates studied, (man, mouse, chickens, dogs, pigs, hamsters, horses, monkeys, sheep, rabbits, goats and cows), class I antigens are highly related in sequence homology at both the nucleic acid and protein level although this is more obvious in the former. Extensive sharing of amino acids between species indicates that the structure of the molecule has been conserved and that only a small degree of remodelling has occurred since the evolution of placental mammals.

There are many different class I loci and the genes expressed are the most polymorphic known in vertebrates. The gene sequences have three features; complexity (many class I loci), diversity (since these molecules differ between individuals of the same species, that is, the H-2K,-D and -L do not have the same sequences as those of HLA-A, -B and -C) and polymorphism (a large number of alleles at a given locus).

Klein and Figueroa (1986), proposed the existence of an original single ancestral molecule based on the presence of invariant amino acids throughout the sequences of current class I vertebrate genes. The presence of "two ancestral" residues at a given position indicates the early duplication of an ancestral molecule followed by tolerated mutations subsequent to the duplication. In such instances, the codons of the two amino acids differed by a single nucleotide and therefore supported this interpretation. They also proposed that the ancestral gene duplicated (probably more than once) before the separation of the evolutionary branches of the contemporary primitive lagomorphs and rodents. Subsequent to this, the duplicated genes began to diversify to form two gene clusters (the proto-Q and proto-T genes). A number of these sequences were selected to become the functional molecules which have been identified (H-2K, D and L, HLA-A, B and C and possibly others). These sequences came under selective pressure and became much more polymorphic. Mouse/rat-specific and human/rabbit-specific sequences are found to be distributed throughout the heavy chain and this led Klein and Figueroa (1986) to suggest that one ancestral gene gave rise to the mouse and rat sequences and another gave rise to the human and rabbit sequences, although this is a minimal hypothesis. They further proposed that the H-2K allele would be no more related to HLA-A than -B or -C.

The TL sequences are in fact the most divergent members of the class I gene family (Fisher et al. 1985; Hammerling et al. 1985; Obata et al. 1985; Pontarotti et al. 1985; Rogers, 1985; Weiss et al. 1984;) since they contain some unique amino acid residues throughout the molecule (with the exception of the alpha-3 domain). However the TL sequences still share residues at normally invariant positions which clearly identify them as class I genes. This means either that the evolutionary branch leading to the TL genes separated early on or that they were able to accumulate more mutations than other class I genes. Rogers, (1985) reported that non-classical class I genes seemed to be poorly conserved in mammalian species since cross-hybridization studies showed much weaker homology suggesting that the Q and TL genes were not conserved even in rodents. This discovery led Rogers to propose that these genes were by-products of evolution and he expected some to have relatively unimportant

but not worthless functions.

A comparison of class I sequences between humans and chimpanzees has revealed that polymorphism at the classical loci predates the divergence of these two species (Lawlor et al. 1988). Single HLA-A or -B alleles have been found to be more closely related to certain ChLA alleles than other human sequences and generally there are no special features that differentiate the two. This indicates that they existed as an ancestral sequence before diversification 3.7-7.7 million years ago into two separate species (trans-species evolution). One chimpanzee (CHla) sequence Ch28 seems to be the homologue of the non-classical human class I HLA 5.4 gene; no function has yet been assigned to either of these two genes.

Mechanisms Known to Generate Polymorphism.

Three mechanisms are known to contribute to the diversity of class I molecules; single nucleotide substitutions, reciprocal recombination and gene conversion (non-reciprocal recombination). Gene conversion is a mechanism for rapid rearrangement and relies initially on the duplication and divergence as a source of variation. The size of the MHC class I gene family (upto 35 genes) would be able to provide a repertoire of sequences for these events. Here are examples of the above mechanisms generating diversity:-

(1)-The nucleotide substitutions between HLA-A2.1 and HLA-A3 alleles are evenly distributed among the three extracellular exons (Koller et al. 1985).

(2)-Reciprocal recombination was shown to be the basis for the derivation of the Aw69 allele from Aw68.1 and Aw.1 (Holmes and Parham, 1985).

(3)-Substitutions seem to occur in clusters and this is much more apparent at the nucleotide level. Sodoyer et al. (1985), illustrated this when comparing the allele sequences A2, A3, Aw24, A28 and pHLA12.4 (pseudogene) and proposed that an event analagous to gene conversion had occured. On this occasion over a span of eight amino acid residues (positions 77-84) in Aw24 was found to be completely different from other classical HLA-A allele sequences but identical to pHLA12.4 in this region.

The discovery of spontaneous <u>in vivo</u> H-2K^{b} mutants

(bm1, bm3 and bm8) also led Pease et al. (1983) to suggest that a copy mechanism analagous to gene conversion contributed to the polymorphism of class I molecules. Synthetic oligonucleotides with sequences to the clustered mutations were able to detect a class I gene on Southern blots of genomic DNA (presumably a donor gene). Four characteristics were consistent with the gene conversion hypothesis: firstly, the variant amino acids were present in other class I molecues in the same positions, secondly, the mutations were clustered which would otherwise require multiple base substitutions (very unlikely). Thirdly, the nucleotide changes are identical to other sequences and lastly, identical and complex changes have occurred repeatedly. Thus it appeared that some sequences could behave as donors and others as recipients. Bregegere, (1983), used a computer model to simulate the effect of gene conversion in a population of limited size and discussed gene conversion promoting allelic polymorphism whilst homogenizing the gene sequences. This mechanism would only generate polymorphism if it acted unidirectionally. The effect of gene conversion would be to generate diversity whilst maintaining a reasonable overall identity.

1.1.9 Nature and Extent of Class I Sequence Polymorphism.

Polymorphism Detected at the Amino Acid Level.

At the amino acid level, a consensus sequence of class I molecules is made possible since single residues predominate at most positions. The most up to date comparison of thirty-nine class I amino acid sequences (Parham et al. 1988) has shown that the polymorphism is derived from 20 high variability and 71 low variability residues. The majority of substitutions are located in clusters in the alpha-1 (positions 62-83) and alpha-2 (105-116) domains and across the residues 177-194 which span the alpha-2-alpha-3 boundary causing the homology to drop to as little as 30%, 50% and 50% respectively (Lopez de Castro, 1983). Monoclonal antibodies reacting with one allele at a locus have been isolated, and also with more than one allele (from more than one locus), but none have been generated which defines an allele according to its locus. A comparison

of the three external domains between alleles at a single locus shows that they differ considerably from the consensus by 12-30/270 amino acids, whereas in other systems, alleles usually differ by only one or two residues.

The first amino acid and nucleotide sequence comparisons indicated that the HLA-A locus alleles were more closely related to each other than to alleles from other loci (N'Guyen et al. 1985). As more sequences became available the A-ness and B-ness at the amino acid level became apparent. There may be species specific residues although this is uncertain at the moment because of the limited sample sizes. To date, there seem to be locus specific residues located mostly in the transmembrane regions (see section 6.9).

Nucleotide Sequence Polymorphism.

Nucleotide sequence comparisons show that high conservation extends throughout both the introns, exons and untranslated regions within loci (Jordan et al. 1985; Sodoyer et al. 1985; Srivastava et al. 1985). The polymorpism in the alpha-1 and alpha-2 domains is qualitatively different from that in the alpha-3 domain (N'Guyen et al. 1985). The nucleotide substitutions in the alpha-3 domain largely appear in the third position of the codon, which means that the substitution is conserved, silent or synonymous i.e. same amino acid. Replacement substitutions are abnormally frequent in the alpha-1 and alpha-2 domains and thus it appears that variation is selected for in the first two domains and against in the third. A comparison of the substitutions in HLA-A3 and HLA-Aw24 (N'Guyen et al. 1985) gives an idea of the magnitude of the polymorphism. There are fourteen relacement and one silent substitution in the alpha-1 domain, and fourteen and none respectively in the alpha-2 domain. In contrast, the silent substitutions in the alpha-3 domain outnumber the replacement substitutions by four to one.

Pairwise comparisons between sequences of six species (Lawlor et al. 1988) human, mouse, Syrian hamster, miniature swine, rabbit and cow exhibit a bimodal distribution in the number of nucleotide differences and give an indication of the levels of polymorphism. There is a range of 1-95 nucleotide substitutions within a species (1-50 for allelic

sequences and 45-100 for intra-locus comparisons) and a range of 100-200 substitutions when inter-species comparisons are made.

Molecules of the K and D loci are highly polymorphic whereas both clusters of Q and T loci display only limited polymorphism. Loci in the Q cluster are more homologous to each other than to the loci in the T cluster and vice versa.

The class I heavy chains vary in length according to the locus from which they were derived which is often due to the variation in the length of the cytoplasmic tail. Locus specificity can be observed also in the nucleotide sequence of the 3' UT region (see section 1.1.6) The environment surrounding a particular locus is recognizably similar between alleles and in this respect the genes at these loci can be considered to be classical alleles, meaning that they have not evolved from a single original sequence without its prior duplication.

1.1.10. Aspects of Antigen Presentation.

General Considerations.

Zinkernagel and Doherty, (1979) proposed that when a virus infected a cell, viral glycoprotein antigens which were inserted into the plasma membrane as a normal part of the viral life cycle subsequently associated with MHC molecules that were already resident. The work of Townsend et al. (1984) demonstrated that the majority of mouse anti-influenza virus CTL's had specificity for self H-2 molecules in association with viral nucleocapsid rather than haemmaglutinin or neuraminidase (which are coat proteins) which is in contrast with the earlier proposal of Zinkernagel and Doherty, (1979). Further work (Townsend et al. 1985; 1986a) suggested that proteins were digested intracellularly and presented as peptides in association with MHC antigens.

It is becoming clear that the natural function of class I and II molecules is to bind endogenously synthesized and exogenously acquired (endocytosed) antigens (peptides) respectively and present them to T lymphocyte receptors. Receptors on T-lymphocytes (TCR's) recognise antigens in the context of an MHC molecule (MHC restriction/recognition) CD3 and various other cell surface accessory molecules (reviewed

Allison and Lanier, 1987). Inducer T lymphocytes (T_h) recognise antigen in association with a class II molecule and CD4, whilst cytotoxic T lymphocytes (T_c) recognise antigen in the context of a class I molecule and CD8. The glycoprotein accessory molecules are thought to establish cell adhesion, play a role in T-cell recognition and possibly contribute either positive or negative signals which assist in T-cell activation. Immunochemistry with anti-CD8 MoAbs and W6/32 showed that the CD8 (38kDa) accessory molecule was non-covalently associated with the HLA class I heavy chain on the surface of Con A activated human T-cells (Bushkin et al. 1988). The accessory molecules do not reveal any significant polymorphism and it is assumed that they associate with the monomorphic regions of MHC molecules. The experiments of Connolly et al. (1988) have suggested that CD8 binds to the alpha-3 domain of class I molecules, and this binding can be affected by a small amount of polymorphism in the alpha-3 domain (Salter et al. 1989).

The vast diversity of the antibody repertoire derives largely from the fact that the heavy and light chain variable regions are encoded by a part of the chain that are diversified somatically from component germline segments (Tonegawa, 1983). The heterodimeric T cell antigen receptor (TCR) is also encoded by genes that are assembled somatically. In contrast with antibodies that are found to bind to free soluble antigens or virus, T-cell receptors recognize foreign antigens associated with a particular HLA molecule (Zinkernagel and Doherty, 1979). The nature of the association remains an uncertainty because it appears unlikely that the limited haplotype of an individual could complex with a vast number of foreign antigens. Nagy et al. (1989) proposed that it would appear reasonable to expect that the antigen binding site should not exhibit a high degree of affinity/specificity (as do Ab's) [see below]. The variation of the immune response to particular antigens and the association of histocompatibility antigens with susceptibility to diseases (Zinkernagel, 1979) may be a direct result of the ability of particular HLA products to effectively bind foreign antigens. Some antigens/peptides are indeed presented better than others and it is not known whether they share any physical or structural properties or whether the class I molecule imposes a common conformation on

the peptide in order to facilitate complex formation (reviewed Bjorkman et al. 1987b).

Alloreactivity.

A high percentage of CTL are capable of lysing uninfected cells bearing a foreign or allo class I molecule, a phenomenon known as alloreactivity, but it is still unclear as to whether this type of recognition involves peptide binding. There is the possibility that this peptide could be derived from the HLA molecule itself but this may not be so likely since at least six classical class I molecules are being expressed at the one time in an individual. The recognition of foreign HLA by alloreactive T cells is thought to be a cross reaction occurring because the structure of a self MHC molecule plus antigen resembles non-self or (allo) MHC molecule (Bevan, 1977). About $1:10^{-3}$ $H\text{-}2^{b}$ lymphocytes respond to variant class I molecules that differ in only three or less amino acids from the wild type, whereas $1:10^{-5}$ T cells in unimmunized mice recognize influenza virus antigens, implying that a very small difference in the MHC protein makes it about 100 times more immunogenic than a viral antigen (reviewed by Townsend and McMichael, 1987). A T cell receptor could directly contact the polymorphic residue on an allo HLA molecule to distinguish it from self, or the receptor could recognize the molecule complexed with a peptide. Although either is possible, the observation that the majority of alloreactive CTL variant residues are in the peptide binding site rather than on surfaces facing towards outside, suggests that alloreactivity may involve the recognition of a complex between allo HLA and some peptide (Bjorkman et al. 1987a;b). Lysis of a virus-infected cell, involves TCR recognition of viral antigen and class I molecule and it is not yet clear how exactly the T-cell receptor recognizes both together. It may be necessary to experimentally determine the three-dimensional structure of ternary TCR-MHC-peptide complexes.

Direct Binding between MHC Class I Molecules and Immunogenic Peptides.

Peptide binding to class II MHC proteins and T cell recognition of these complexes at the functional level has been demonstrated (see Bouillot et al. 1989 for references).

Direct binding of class I molecules to peptides has been difficult to demonstrate, although the crystallographic structure of HLA-A2 (Bjorkman et al. 1987a;b) and peptide sensitization experiments (Gotch et al. 1987; Townsend et al. 1986b) provide evidence for this. Chen and Parham, (1989), have described selective binding of an influenza matrix peptide to HLA-A2 and that of influenza nucleoprotein peptides to HLA-B37 using a gel filtration assay. Both peptides were previously shown to act as targets for HLA-A2 and HLA-B37 restricted influenza-specific CTL's (Townsend et al. 1986; Gotch et al. 1987). Not all allospecificities can present these peptides to CTL's. Only 0.3% of the HLA molecules bound the peptides and this was thought to be limited by previously bound peptides which have also been found by Bjorkman et al. (1987 a;b). Collectively, this data indicates that the ARS has quite a high level of affinity for the peptides with which it interacts since they seem to have persisted throughout both isolation procedures. Subsequently, Bouillot (1989) described a simple quantitative and competition assay to measure the binding of radiolabelled MHC class I molecules to influenza specific peptides (known to be immunogenic) and some of their derivatives fixed on a solid phase support. Peptides bound preferentially to the restricting MHC class molecule as well as to others, whereas the control peptides (not known to be CTL epitopes) gave negative results. When the same test peptides were used in a cytolytic assay there was a correlation between binding of the peptides to (e.g. HLA-A2) class I and its capacity to sensitize HLA-A2+ cells for lysis by anti-influenza A virus CTL. The differences of binding are thought to reflect the patterns of MHC restriction <u>in vivo</u>. At a more functional level, by measuring the release of serine esterase, Kane et al. (1989) reported that purified class I proteins pulsed with influenza peptides triggered MHC restricted antigen-specific, TCR-mediated degranulation of CTL.

Townsend et al. (1989), have studied a mouse lymphoma, RLB-5 (RMA) and its EMS-induced mutant, RMA-S. The latter only expresses 1/20th of the amount of H-2D^b, H-2K^b and B_2-m at the cell surface when compared with its parent. RMA-S synthesizes both heavy and the light chains, although the heavy chains do not bear high mannose oligosaccharides, do not associate with B_2-m and thus are not expressed at the

cell surface. Exposure of RMA-S to certain influenza nucleocapsid peptides restored the association and cell surface expression of the class I complex suggesting that the association with the binding site of class I molecules may be required for correct folding of the heavy chain and its association with B_2-m. The peptides may become enveloped within the class I binding site and indeed by instrumental in determining the final conformation. This may explain why various workers (Bouillot et al. 1989; Chen and Parham, 1989) have found it difficult to demonstrate peptides binding in vitro (Parham, 1989).

Pathways of Antigen Processing and Presentation.

Exogenous antigens are endocytosed and taken into the cell as endosomes and are supposedly unlikely to be presented by class I molecules, but do interact with class II molecules (see Long and Jacobson, 1989). These results suggested distinct requirements for class I and class II restricted presentation, and it was suggested that there were two separate pathways involved in presenting exogenously acquired and endogenously synthesized antigens. The association of the invariant chain (Sekaly et al. 1988) with MHC class II molecules has indicated its involvement in processing (Long and Jacobson, 1989). Townsend et al. (1986) showed that viral antigens synthesized in the cytoplasm could be processed and presented by class I molecules. How the processed antigens are generated, reach their site of association and associate with class I or class II and how the whole complex is translocated to the relevant site(s) is unknown.

Role of Class I in the Elimination of Somatic Mutants?

The results of Townsend et al. (1984) led Rajan (1987) to raise a fundamental evolutionary question which was "Why would the CTL system have evolved to deal with viral intracellular proteins rather than viral glycoprotein antigens that are inserted in the cell membrane?" Nucleocapsid proteins are synthesized early in infection whereas neuraminidase and hemagglutinin are synthesized late and so it makes sense to eliminate the infected cell before it truly begins to support the production of virus. Such studies indicated the importance of dealing with

novel/foreign intracellular proteins. This led Rajan to suggest that the presentation of viral antigen to T lymphocytes was only one aspect of a more general function of class I antigens. He proposed that class I molecules may also have a role in elimination of somatic mutants. The evidence which most supports this proposal comes from the tum- mutants (reviewed Boon and van Pel, 1989; Rajan, 1987). Subcutaneous injection of the DBA/2 mastocytoma P815 (diploid cells) into syngeneic mice normally results in local tumours. However, 50% mutagenized P815 cells failed to grow, and although the mice did not produce antibodies they generated CTL's to the tumour variants which did not cross-react with any other tumour isolate. Rajan proposed that this could be explained if one assumed that random mutations in the genome generate at least a heterozygote mutation. This novel peptide from the mutated genome would be presented to T cells in the context of the cell's MHC molecules and the cell would be eliminated. The mutated form of the protein would not be present in intact form on the membrane (especially if not a membrane protein anyway) and so antibodies would not be made. However, most somatic mutants would be heterozygous and those few which would be homozygous may simply die. In addition to this, not all cells have been found to express class I antigens (Daar et al. 1984) although this could be due to very low levels of expression?

Boon and van Pel, (1989) have hypothesized that antigenic peptides are generated by autonomous transcription and translation of short subgenic regions (peptons) and not by degradation of intracellular proteins.

1.1.11. Regulation of Class I Antigen Expression.

The levels of class I antigens vary substantially between different cell types (Cosman et al. 1982; Kress et al. 1983b). Some cells do not constitutively express class I, namely certain exocrine cells of some glands, smooth or striated muscle cells, corneal endothelium and neurons of the central nervous system (Daar et al. 1984). Fibroblasts, muscle and nerve cells express very low levels while cells of the immune system have relatively high cell surface levels. During differentiation of embryonic cells and tumorigenesis

the expresson of class I changes.

The modulation of HLA expression could potentially have dramatic biologic effects e.g. oncogenicity of certain tumours is closely associated with the degree of class I gene expression (Tanaka et al. 1988). The levels are thought to affect the ability of a cell to activate or be lysed by cytotoxic T cells, for example, transplanted AKR leukaemia cells are rejected after MHC DNA-mediated transfer (Hui et al. 1984). Expression of class I genes can be modulated by exogenous factors like mitogens, immunomodulators and infectious agents, and it would be quite interesting to define the regulatory regions involved.

Cis and Trans Regulation of Class I Gene Transcription.

Studies on the regulation of eukaryotic gene expression have focused on both the sequences (cis-regulation) required for expression and the cellular factors (trans-regulation) that specifically interact with these cis-elements. The expression of a gene is influenced by a wide variety of mechanisms acting at many control points.

Both positive and negative regulatory cis elements characteristic of eukaryotic promoter regions have been identified 5' of class I genes (Shirayoshi et al. 1987). Mouse genes have perfect CAAT and TATAAA promoter elements 5' of the cap site whilst some promoters of human class I genes have a variant TATAAA sequence; TCTAAA. The lower levels of HLA-C expression compared to HLA-A and -B may be explained by the variant CGGT and TCTGAA sequences (Sodoyer et al. 1985). Kimura et al. (1986) described the detailed analysis of the H-2K^b promoter in an attempt to decipher the way in which this gene was regulated. At least some of the important sequences were confirmed by a comparison with other H-2 class I genes and also B_2-m since their expression seems to be co-regulated (Croce et al. 1981; Morello et al. 1982). The class I enhancer-like sequences function poorly in undifferentiated embryonal carcinoma cells and are not the target of H-2 inhibition which was observed in Adenovirus 12 transformed primary cells (Schrier et al. 1983). No sequence homology to these enhancer regions was found in the 5'-flanking region of the B_2-m gene although enhancer activity was detected. The conclusion from these studies was that the lack of homology between sequences may reflect differentially

regulated programs of expression during embryonic development. These regions were not found to be conserved in the Qa region class I promoters. These sequences however are not "true" enhancers since a reversal in orientation or positioning further upstream decreased their ability to facilitate transcription. Shirayoshi et al. (1987), discovered a conserved class I regulatory element (CRE) in the 5' region of the H-2L^d gene which was approximately 40 base pairs long. The CRE acted as a negative regulatory element in undifferentiated F9 embryonal carcinoma cells which were HLA-ve. The same element acted as a positive regulator in cells expressing class I genes at high levels. The CRE bound three factors on three distinct sequences which corresponded to inverted and direct repeats. F9 cells seem to lack one of these factors and this may account for the above observations.

Fusion of two different somatic cell types which express different amounts of class I, T-LCL (low levels) and B-LCL (higher levels) results in increased expression of T-LCL molecules. This suggested that the T-LCL lacked an important factor that was able to diffuse between the two. Using gel retardation assays and <u>in vitro</u> "footprinting" Israel et al. (1987) analyzed the relationship between nuclear proteins, the 5' enhancer and interferon response sequences of the H-2K^b gene. The binding of a number of proteins to the class I enhancer and to a similar sequence (in opposite orientation) in the B_2-microglobulin gene suggested a common regulatory mechanism.

Regulation of Class I Gene Expression by Immune Modulators.

Certain immune modulators have been shown to increase the level of class I mRNA. Collins et al. (1986). have shown that TNF (tumour necrosis factor) elevates both mRNA and cell surface levels of HLA-A, -B and -C antigens in human endothelial cells and dermal fibroblasts. This effect is thought to be mediated via the expression of a newly synthesized intermediate.

Interferons (also immune modulators) are a highly species specific family of secreted protein factors which are involved in the defence against viral infections and in the regulation of cell growth and differentiation. The interferons induce a common set of proteins as well as others

which are only induced by the particular type and subtype of interferon.

Gamma-interferon has been used in this study (chapter 4). Recombinant DNA technology has enabled the confirmation of the individual effects of type I (leucocyte IFN-alpha and fibroblast IFN-beta) and type II (immune IFN-gamma) interferons, i.e. specific gene activation mediated by a distinct set of receptors. Interferons alpha and beta increase class I mRNA levels after one hour (Fellous et al. 1982).

Gamma-interferon is produced by both T-helper (T_h) and T-cytotoxic (T_c) cells under appropriate stimulation. It appears to be both a modulator as seen by its ability to enhance the level of class I and an inducer, able to activate the expression of MHC genes. Baldwin and Sharp, (1987) have found a cis-acting regulatory sequence that contributes to basal levels of gene expression and also to stimulation by gamma-interferon. Momburg et al. (1986) were able to show that <u>in vivo</u> application of IFN-gamma in B10.BR mice led to both induction and enhancement of MHC class I antigens in a variety of tissues although some were refractive to this stimulation. Beta-interferon is known to be one of the developmental factors switching on class I expression during differentiation (Yarden et al. 1984).

Though interferon is species specific, stably transfected human class I genes in mouse cells can be induced by treatment with mouse interferon (Rosa et al. 1983). This suggests that the effects of interferon in this instance are modified by a trans-acting factor which does not discriminate between mouse and human class I genes.

Chen et al. (1986) studied the effects of IFN-gamma on HLA class I gene expression, differentiation and proliferative capacity of K562 human leukaemia cells. They found that IFN-gamma increased class I transcripts selectively in a dose dependent manner after 48 hours in the absence of differentiation and no change in proliferative capacity. Their kinetic analyses suggested that the mechanism by which IFN-gamma induced transcription might be different from that for IFN-alpha or IFN-beta. There was a longer delay before transcription activation after IFN-gamma induction than with type I IFN's although IFN-gamma can induce rapid transcriptional activation of an early response

gene which may be involved in this process. In addition there is the possibility that the down regulation of a labile repressor may be necessary before the activation of class I transcription takes place.

Vogel et al. (1986) dissected the H-2L^d gene in order to identify the potential control regions and located the presence of a transcriptional enhancer mapped within 350 base pairs of the transcriptional start site. Although the accumulation of class I transcripts increases after interferon treatment it is not yet clear from Vogel's studies whether the enhancement is entirely the result of an increased rate of transcription or of a combination of increased transcription and stabilization of mRNA. Israel et al. (1986) showed in a transient assay that the H-2K^b promoter could be induced by both types I and II interferons and that the interferon responsive sequence (IRS) was necessary for this induction. This IRS was active only when associated with a functional enhancer in the H-2K^b promoter and that of other class I genes.

Post-transcriptional Regulation of Class I Expression.

The formation of a cap at the 5' end of the mRNA and addition of poly(A) residues at the 3' end of the mRNA, intron excision and exon splicing form the basis of post-transcriptional control mechanisms in eukaryotes.

Shimizu et al. (1986) and references within, transferred and cloned class I genes into gamma-ray induced HLA-mutant lymphoblastoid cells. Although the steady state levels of class I mRNA were not reduced, cell surface expression was deficient. The conclusion drawn from these experiments was that expression of class I required a post-transcriptional function.

The independent expression of B_2-m may be a potential step in the regulation by which the cell surface expression of class I molecules can be controlled although no cellular system has been identified in which this situation occurs (Morello et al. 1985). A more indirect control mechanism has been suggested by Klar and Hammerling (1989), who have shown that the association of the class I heavy chain with B_2-m requires an active cellular mechanism. They demonstrated that mouse class I and B_2-m chains could co-exist in the cell without assembling spontaneously and that assembly could be

stimulated by gamma-interferon. The mechanisms required for this interaction probably involve either the induction of a positive factor or the down-regulation or neutralization of blocking/competitive factors.

Viral Regulation of Class I Expression.

Studies of cell lines transformed by mutant E1 region viruses showed that inactivation of class I gene expression is a function of the product of the Ad12 13S E1a mRNA. Bernards et al. (1983) and accompanying work of Schrier et al. (1983) showed that rejection of transformed cells correlated with the expression of adenovirus type 12 E1a sequences. A reduction of class I cell surface expression is often accompanied by a reduction in steady state mRNA levels. Vaessen et al. (1987), showed that Ad12 interfered with processing of nuclear precursors causing much reduced cell surface levels. The Rous sarcoma virus also reduces levels of class I mRNA (Gogusev et al. 1988).

Oncogene Influence on Class I Expression.

Both N-myc and c-myc oncogenes reduce the expression of MHC class I in neuroblastomas and melanomas, respectively (Versteeg et al. 1988; Lenardo et al. 1989). Versteeg et al. (1988), showed an inverse correlation between c-myc and class I mRNA's. In addition to this, B_2-m mRNA levels were reduced concomitantly with class I mRNA and treatment with gamma-interferon restored both levels. Lenardo et al. (1989), identified two distinct class I promoter elements which were susceptible to N-myc-mediated suppression and one of these was an enhancer. Elevated expression of N-myc oncogene correlated with a reduction in binding of a transcription factor to the enhancer.

Other

Lambert et al. (1989) demonstrated that human fibroblast and keratinocytes were induced to express class I by several classes of DNA damaging agents. Cycloheximide, a protein synthesis inhibitor also induced class I suggesting that this may be a component of a derepressible cellular SOS pathway.

1.2. CLASS I EXPRESSION ON PLACENTAL TROPHOBLASTS.

1.2.1 Immunological Paradox of Pregnancy.

A prerequisite of pregnancy is that no homozygous lethal genes be present in the conceptus and having satisfied this, the question of an immunological relationship with the mother may become critical. Mammals exhibit a high degree of tissue antigen polymorphism and as a consequence of outbred matings produce semi-allogeneic foeto-placental units. The fate of tissue grafts surgically transplanted across genetically dissimilar individuals has established the universality of allograft rejection. The ability of the maternal immune system to recognize and destroy non-self does not seem to be drastically impaired;indeed it is implied in the literature that maternal recognition of the embryo is essential for the maintenance of pregnancy (reviewed Billington, 1988). This coexistence highlights the paradoxical success of the foetus and has stimulated studies of placental trophoblast antigen expression and local uterine responses (Bulmer et al. 1988; Bulmer and Sunderland, 1984).

Transplantation immunology of pregnancy was initiated by Peter Medawar in 1953. He asked how a pregnant mother could nourish a foetus that was antigenically foreign and how might the foetus avoid presenting itself as such to the mother? There are many factors which are thought to enhance the success of a pregnancy and these have been reviewed (Billington, 1988; Hill and Anderson, 1988; Bulmer, 1988). One important suggestion made by Medawar to explain this mystery was the anatomical separation of the foetus from the mother by the placenta. Visualization of the placental interface under the light microscope shows that it separates foetal and maternal blood circulations. The absence of a combined circulatory system and the existence of the trophoblastic barrier effectively prevents the passage of any significant numbers of maternal immunocompetent cells into the foetus. Just as important is the traffic of foetal lymphocytes in the opposite direction which again has been shown to occur only to a limited degree (Hunziker et al. 1984).Understandably, the placental trophoblast cells in their key position at the maternal-foetal interface have been the major focus of attention. Some placental trophoblasts are in direct contact with maternal cells and blood. It

would therefore seem necessary to arrest the expression of trophoblast antigens capable of eliciting a harmful immune response which would be ultimately aimed at the foetus. Obvious candidates for such antigens are the classical transplantation molecules that have been discussed earlier. The modification or loss of function, or total lack of class I antigen expression on the placenta might be the key answer to this "immunological paradox". Immunostaining experiments on placental sections using monoclonal antibodies have revealed that the simple lack of class I expression is not the case and thus it seems that they are likely to play an essential role during pregnancy. It is now clear that only some trophoblast subpopulations express class I transplantation antigens and there are reasons to believe that these may not always be of the classical type. Obviously the presence of MHC antigens on some populations of trophoblast raises questions concerning the susceptibility of these cells to maternal effectors such as cytotoxic T lymphocytes and alloantibodies.

The following material discusses the structure of the placenta, and the nature and expression pattern of class I molecules.

1.2.2. Placental Development.

The human haemochorial placenta is an extremely complex organ of extra-embryonic origin and is comprised of different trophoblast populations and other foetal tissues such as endothelium, stroma and blood cells (Boyd and Hamilton, 1979). The junctional area is an admixture of foetally derived and maternal cells.

After fertilization and development as far as the morula (32-cell stage) there is further differentiation into the unilaminar blastocyst by a process of cavitation (see figure 1.2.2). The blastocyst can be divided into two parts, the inner cell mass which becomes the embryo proper and the trophoblast which differentiates into the placenta and other extra-embryonic membranes.

After implantation of the blastocyst the trophoblast differentiates into a peripheral layer of primitive syncytiotrophoblast (which eventually lines the intervillous blood space) and an inner layer of primitive

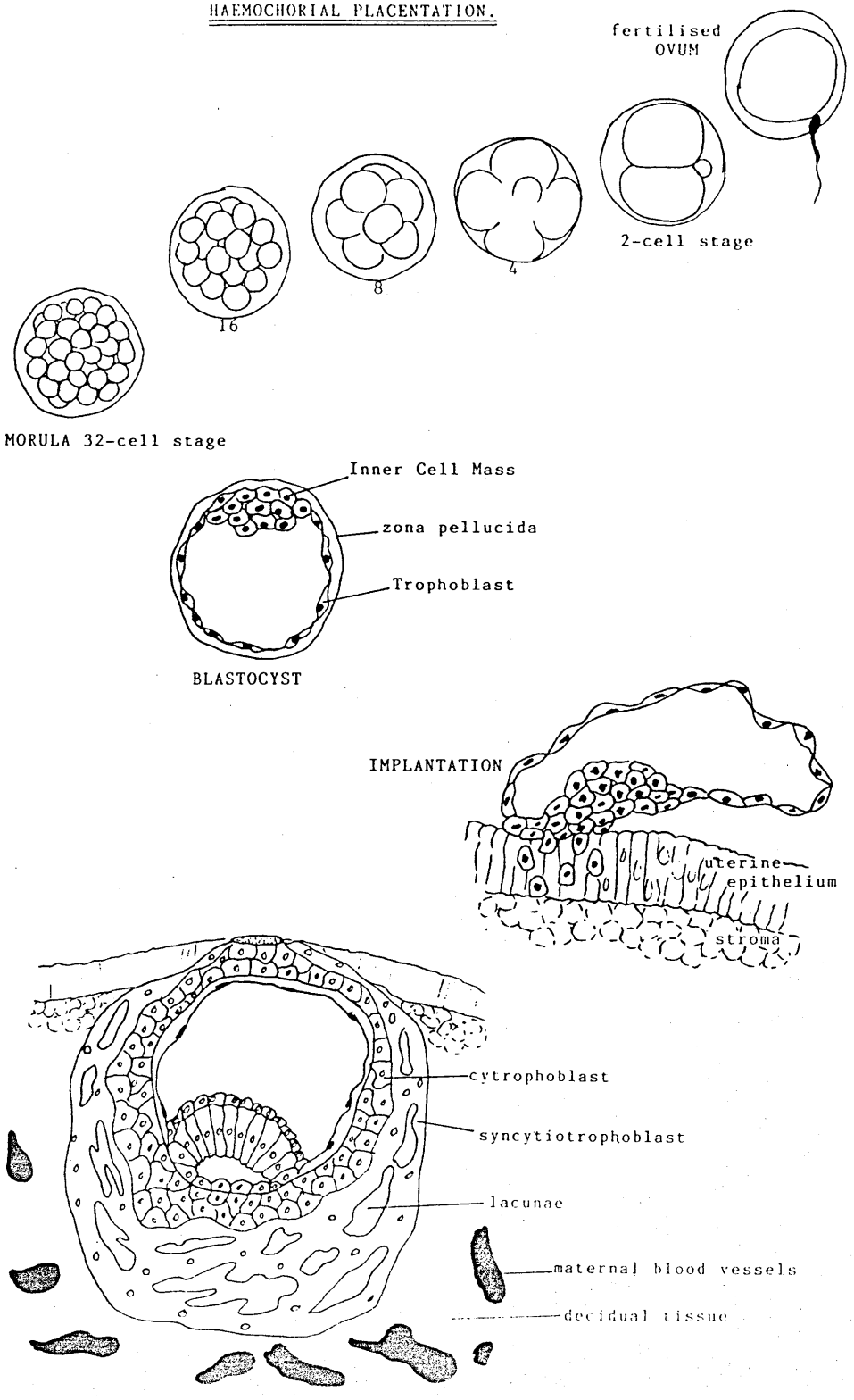


Figure 1.2.2. Development of a fertilized ovum into an implantation blastocyst. Differentiation of trophoblast to form the syncytiotrophoblast and cytotrophoblast after implantation.

cytotrophoblast which starts proliferating to form the precursors of the primary villi at approximately 11-12 days post-conception (reviewed by Pijnenborg et al. 1981a;b). Syncytiotrophoblast cells (syncytium) are recognized to be the differentiated derivative of cytotrophoblasts. Mesoderm growth leads to the formation of the secondary and tertiary villi, and cytotrophoblastic cell columns extend through the peripheral syncytium, fanning out to form the cytotrophoblastic shell.

After eight weeks, the continuity of the cytotrophoblast shell is broken down and trophoblast proliferation is mainly confined to the tips of the anchoring villi which make contact with the basal decidua. Intravascular retrograde migration of trophoblast into decidual spiral arteries begins with the cytotrophoblasts plugging into their distal tips. The walls of these arteries are converted into tubes of fibrinoid material embedded with trophoblast cells.

So in the human, the cytotrophoblastic elements are dispersed in the uterine basal plate, decidua and the subjacent myometrium in what is now termed the placental bed. After approximately four months the non-placental materno-foetal interface is composed of the amniochorion (amnion epithelium and chorionic cytotrophoblast) which now fills the uterine cavity and is in contact with maternal decidua. Amniotic epithelial cells arise from the inner cell mass.

1.2.3. Nature, Extent and Properties of the Trophoblast.

The villous syncytium (syncytiotrophoblast) is in direct contact with maternal blood. Not restricted to a villous form, trophoblast is found at several extra-villous locations (Earl et al. 1985). These include the **cytotrophoblast cell islands** of the chorionic plate at the foetal side of the placenta, **interstitial trophoblast** in the placental bed, **decidual trophoblast** in the placental bed nearest the uterine decidua and the **membranous chorion (chorionic cytotrophoblasts)** cells of variable size between the decidua parietalis (uterine wall) and connective tissue underlying the amniotic epithelium (figure 1.2.3).

When placental tissue (but not trophoblast alone) is moved to an extra-uterine site it is rejected in the

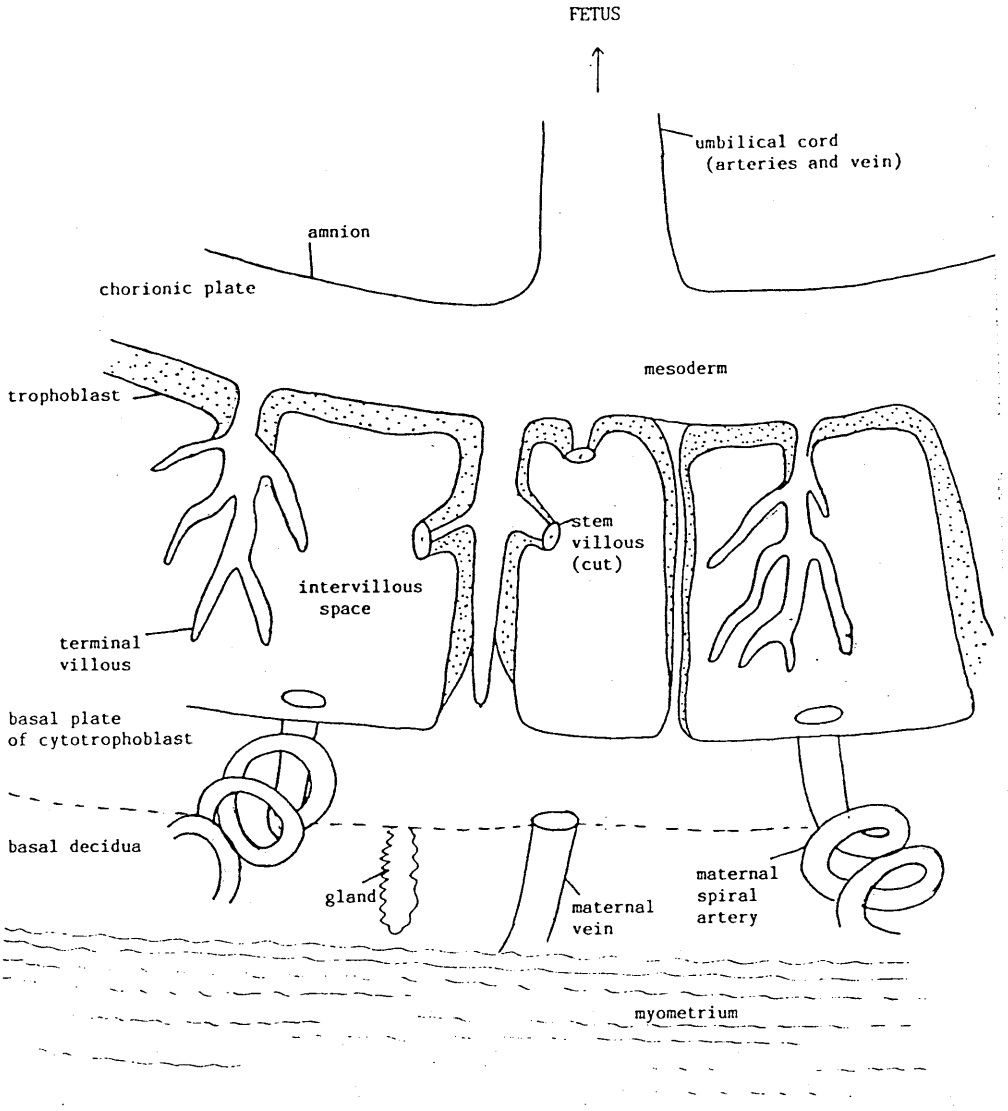


Figure 1.2.3. Cross-Section of the Human Maternal-Placental Interface, illustrating the finger-like villi projecting into the intervillous blood space. See text for details.

normal immunological fashion (in Gill, 1984). Thus, the trophoblast seems able to survive by virtue of its own properties. This tissue is normally resistant to immune damage by lymphocytes and cytotoxic antibodies and is thought to form a physical barrier preventing immune effectors (except some antibody) from reaching the foetus (reviewed Billington, 1988). Trophoblasts are intrinsically resistant to lysis possibly due to low alloantigen density or efficient membrane repair mechanisms. They are thought to recruit or signal suppressor lymphocyte populations into the uterine decidua and produce progesterone (an immunosuppressive agent) and other hormones in high local concentrations. Progesterone is important since it has an anti-inflammatory role and allows the trophoblast to penetrate and anchor to the uterine decidua.

1.2.4. Immunostaining Analysis revealing the Pattern and Nature of HLA Class I Expression in the Human Extra-Embryonic Membranes.

Immunohistological staining of placental sections using monoclonal antibodies has illustrated the absence of Class II antigens throughout pregnancy both in mouse (Chatterjee-Hasrouni et al. 1981) and man (Goodfellow et al. 1976; reviewed by Redman, 1983) and these will not be mentioned further. Syncytiotrophoblast (major area of exchange) has been shown to be HLA-ve by immunohistology (Bulmer and Johnson, 1985; Faulk et al. 1977; Sunderland et al. 1981b) and by recent *in situ* hybridization studies (Hunt et al. 1988b). They are also resistant to gamma-interferon-mediated induction of class I (Hunt et al. 1987). Villous cytotrophoblasts are also negative for class I (Sunderland et al. 1981b) and contain very low levels of class I HLA mRNA (Kawata et al. 1984b).

It is now very clear that class I antigens are expressed on certain trophoblast subpopulations which are in intimate contact with both maternal decidua and blood. Their low levels of expression have brought slight confusion to the literature, but such situations have now been clarified. Early work reported the lack of detectable class I antigens in human chorionic villous trophoblast (Faulk et al. 1977) which unfortunately led to extrapolation of this

data to other trophoblast populations. However, Goodfellow et al. (1976) had already found very low levels of class I antigens on whole placental cell membranes.

The amnion epithelial cells and chorionic cytotrophoblasts compose two distinct layers of the extra-placental amniochorion membrane. Initial work of Hunt and Wood (1986) reported lack of class I surface expression on amnion epithelial cells at least in a form or density that could be detected immunohistologically. The cell surface expression of these antigens could be induced with gamma-interferon. Hunt et al. (1988b) have now confirmed that some amnion epithelial cells do express class I antigens.

Hsi et al. (1982) reported that the chorionic cytotrophoblasts of the amniochorion were negative for class I whereas Hunt et al. (1988a) have often found that, like nearby amnion epithelial cells, chorion membrane cytotrophoblasts were class I HLA positive. However, although the amnion cells seem very responsive to gamma-interferon, chorionic cytotrophoblasts do not seem to be (Hunt et al. 1987).

HLA antigens have been found in the villous stroma (Redman et al. 1984; Sunderland et al. 1981 a;b). The antibodies used in this study were W6/32 (directed against a common class I determinant (Barnstable et al. 1978) and NDOG, specific for a villous syncytiotrophoblast antigen which distinguishes maternal and foetal tissues. Using an indirect immunoperoxidase staining technique these studies showed that extravillous trophoblast in the cytotrophoblastic shell of an 8 week placenta stained positive. Redman et al. (1984) further investigated the expression of class I antigens in placental tissues taken from first, second and third trimester pregnancies. A battery of monoclonal antibodies was used in order to thoroughly distinguish maternal and foetal cell types (see Redman et al. (1984) for these antibodies). Monoclonal antibodies directed against monomorphic (W6/32) and polymorphic determinants of HLA-A and HLA-B antigens revealed that the class I antigens failed to bind HLA-A and HLA-B antibodies specific for the foetal phenotype (paternal antigens). No antibodies for HLA-C loci antigens were available and so they concluded that the antigen expressed may be restricted to HLA-C or some other serologically undefined class I antigen.

Hsi et al. (1984) studied class I expression on normal human term amniochorions and found that the extravillous cytotrophoblasts (called metatrophoblasts) could be stained with anti-B_2-m and W6/32, but not with 61D2 (Ugolini et al. 1980). 61D2 is a monoclonal antibody which recognizes a different class I determinant to W6/32. They suggested that class I antigen expression may be incomplete (probably meaning that the molecule was incomplete), or that there may be a contribution of an antigen from another region of the MHC - possibly extra-embryonic. Wells et al. (1984) used both W6/32 and 61D2 to stain the cytotrophobast shell of the placental basal plate. Although class I antigens were detected here with W6/32, no staining with 61D2 was observed.

Using trophoblast-reactive monoclonal antibodies and anti-class I antibodies Earl et al. (1985) compared the antigen expression of villous and extra-villous trophoblasts of ectopic (extra-uterine) implantations with that of normal pregnancy. Identical staining patterns were observed between both, and these studies also confirmed the lack of reactivity when using 61D2. Hsi et al. (1984) suggested that the determinant recognized by 61D2 may be situated nearer to the distal end of the class I heavy chain than the epitope recognized by W6/32. The absence of 61D2 reactivity with these trophoblast antigens may indicate a modified structure. This is an interesting proposal since an incomplete or modified molecule may be only weakly immunogenic. Earl et al. (1985) suggested that such a molecule might stimulate a local "protective immune response". Similar patterns of expression were identified on chorionic villi of complete or partial molar pregnancies; the staining being confined to the villous mesoderm and absent from the syncytiotrophoblast layers (Fisher and Lawler, 1984; Sunderland et al. 1985).

1.2.5. Pattern of HLA Expression on Mouse, Rat and Baboon Placentae Investigated by Immunostaining.

There are two instances in which the pattern of staining of class I antigens differs from that of human placenta. Firstly, *in vivo* studies by Chatterjee-Hasrouni and Lala (1982) using radiolabelled anti-class I antibodies showed positive signals in the labyrinthine trophoblast (exchange area) rather than in the spongiotrophoblast but

these studies have not been confirmed. Secondly, Stern et al. (1987) found class I molecules on baboon villous syncytiotrophoblast using monoclonal antibodies raised to human MHC determinants.

Jenkinson and Owen (1980) detected very low levels of H-2 antigens on 13-14 day labyrinth (counterpart of human villous trophoblast) and clear expression on the spongiotrophoblast (major placental cytotrophoblast region) which is accessible to the maternal circulation. Rat placenta expresses an apparently non-classical class I antigen (Pa) which is found predominantly in the spongiotrophoblast region (Ho et al. 1987; MacPherson et al. 1986) and seems to have the classical molecular weight (Ghani et al. 1984). The use of allospecific mouse anti-rat monoclonals has led to the detection of paternally-inherited class I antigens on spongy-zone trophoblast. No staining of rat labyrinthine trophoblast was observed even with a monoclonal recognizing a monomorphic determinant of $RT1^a$ (Billington and Burrows, 1989).

1.2.6. Immunoprecipitation and Western Blot Studies of Class I Expression on Placentae and Choriocarcinomas.

It is now apparent that the antigens expressed on certain trophoblast subpopulations in some species are not always classical MHC structures. The first indication of this was when 61D2 did not register any reactivity with class I positive cells as described above. Immunoprecipitation (using W6/32 and 61D2) and electrophoretic studies have added weight to this observation since some of the class I molecules have a lower molecular weight (40-41kDa) than do the classical class I antigens. Immunoprecipitation studies have shown that the class I heavy chains on both human chorionic cytotrophoblasts, a human choriocarcinoma cell line BeWo (Ellis et al. 1986) and on baboon syncytiotrophoblast (Stern et al. 1987) are approximately 40kDa and 41kDa respectively. Stern et al. (1988), immunoprecipitated 45kDa and 41kDa components from the human choriocarcinoma cell line, BeWo which were both associated with B_2-m. Billington and Burrows, (1989) also have evidence for the existence of a 40kDa class I molecule as well as a 45kDa component detected by Western blotting on trophoblast enriched rat placental

cells.

Together these data suggest that trophoblast expresses a novel class I antigen in addition to an apparently classical antigen. There is the possibility that the smaller molecule may be the product of a common but alternatively spliced classical mRNA or that post-transcriptional modifications such as differential glycosylation have occurred. Stroynowski et al. (1987), have reported that the Q7 gene product is anchored into liver cell membrane by a phosphatidyl inositol tail rather than the more conserved transmembrane region (section 1.1.5 and 1.1.7). The Q7 gene product has a molecular weight of 40kDa and so it may also be possible that the smaller 40kDa component has been inserted into the trophoblast membrane by a similar mechanism. It is notable that the products of the mouse H-2 Q region genes are of a lower and similar molecular weight (reviewed Lew et al. 1986a). This work will be referred to in the final discussion.

1.2.7 Regulation of Class I Expression on Trophoblast.

Anderson and Berkowitz (1985) found that the weakly HLA positive human choriocarcinoma cell line, BeWo, showed a marked increase in class I expression after incubation with the supernatants of PHA activated lymphocytes. Neither PHA alone or non-activated lymphocyte supernatant reproduced this effect. Subsequent to this they illustrated that IFN-gamma enhanced the expression of class I MHC antigen in BeWo after long term exposure (upto 30 days with 1,000 or 5,000U/ml). The HLA negative choriocarcinoma cell line, Jar, was not responsive to this lymphokine, although it is not known whether this cell line has gamma-interferon receptors. This implied that some degree of constitutive MHC expression may be a pre-requisite for IFN mediated antigen modulation in some trophoblast cell lines. These observations suggest that some trophoblast subpopulations regulate the expression of class I molecules by different mechanisms. Although this area of research is still not fully understood it is likely to involve complex regulatory mechanisms which vary between cell types and location.

Hunt et al. (1987) showed that syncytiotrophoblast

explants of normal placental villi could not be induced to express class I antigens with IFN-gamma although enhancement of mRNA synthesis occurred in foetal stromal cells and JEG-3 choriocarcinoma. Hunt et al. (1987) suggested that at least in one respect a parallel could be drawn since some malignant (BeWo and JEG-3) and normal (primarily extravillous) trophoblasts expressed class I antigens, whereas other malignant (Jar) and normal (villous) trophoblasts did not. Zuckermann and Head, (1986) was able to show that gamma-interferon enhanced class I expression on short term cultures of mouse spongiotrophoblast from mid-gestation.

Methylation of cytosine residues of DNA has been proposed as a mechanism for permanent inactivation of eukaryotic genes (Razin and Riggs, 1980). Genomic DNA purified from the normally MHC-negative Jar cell line would not support the expression of class I MHC genes after transfection into recipient L cells as would other genomic DNA. Further work in this area using 5-azacytidine inhibition of DNA methylation in Jar cells restored the ability of the DNA to render L cells positive for class I MHC antigens (reviewed in Head et al. 1987). If the class I molecules that are expressed are capable of eliciting a destructive immune response, then their permanent switching off on certain spatially vulnerable trophoblasts would certainly be beneficial to the foetus, since it is known that high levels of type I interferons are now known to exist in human placentae (Chard et al. 1986; Duc-Goiran et al. 1986).

Thus it appears that class I antigens are expressed on trophoblasts of several species and they appear to be regulated at least in some cases to trophoblast outside the major materno-foetal exchange area. The functional implications of restricted expression are unknown. However, it is important to remember that the lack of MHC antigens on some trophoblast cell populations may not be so exceptional since other cell types show regulation to varying degrees (Daar et al. 1984) and so it becomes clearly important that the exact function of class I molecules on trophoblast needs to be determined.

1.2.8 Aims of the Present Work.

Since class I antigens are known to evoke allograft rejection, their expression on placenta has raised many interesting questions concerning the immunological relationship between the mother and semi-allogeneic foetus. At the beginning of this study, our knowledge extended to the fact that the class I antigen expressed on extravillous trophoblast did not cross-react with 61D2 or antibodies specifically directed towards the foetal HLA phenotype. The work described in this thesis was concerned with the characterization and molecular cloning of human class I cDNA's expressed on a malignant form of cytotrophoblast; the choriocarcinoma JEG-3. It was hoped that the sequence of the class I cDNA(s) obtained would shed light on the nature of the molecule(s) expressed and ultimately afford clues as to the function.

2.1 STERILIZATION

Growth media were sterilized by autoclaving at 120°C for 15 minutes, supplements and buffer solutions at 108°C for 10 minutes and $CaCl_2$ at 114°C for 10 minutes. Heat sensitive reagents were sterilized by filtration using disposable 0.45um membrane filters. Plastic-ware was sterilized by autoclaving at 120°C for 90 minutes.

2.2 CHEMICALS

Chemicals	Source
General Chemicals and solvents	B.D.H., Hopkins and Williams, Kochlight Laboratories, May and Baker.
Media	Difco, Oxoid
Biochemicals	Sigma, Pharmacia, BRL.
Agarose(s)	BRL.
Radiochemicals	NEN
Antibiotics	Sigma
Restriction Enzymes	BRL, Boehringer mannheim Pharmacia.
DNA-modifying Enzymes	BRL, Boehringer mannheim
En^3hance	Dupont
Tissue Culture Media	Gibco, Northumbria Biologicals.

2.3 BUFFER SOLUTIONS

TE: 10mM Tris-Cl, 1mM EDTA pH8

20xSSC: 3M NaCl, 300mM Sodium Citrate, pH7.

20xSSPE: 3.6M NaCl, 200mM NaH_2PO_4, 20mM EDTA (pH 7.4).

10xTAE: 0.04M Tris-acetate, 0.001M EDTA

Protein Loading Gel Buffer: 5% SDS, 50% Glycerol, 0.01% Bromophenol Blue, 50mM Tris (pH6.8), made upto 5% beta-mercaptoethanol immediately prior to use.

2.4 BACTERIAL STRAINS

All are derivatives of Escherichia coli K-12 and are listed below.

HB101: F^-, hsdS20 (r_B, m_B) recA13, ara-14, proA2, lacY1, galK2, rpsL20 (Sm^r), xyl-5, mtl-1, supE44, $lambda^-$. Boyer, H

W and Roulland-Doussoix, D. (1969).

JM101: (supE, thi (lac proA,B)/F' TraD36 proA,B $lacI^q$ Z _M15) (Yannish-Perron et al. 1985) was used for experiments with M13mp-derived bacteriophage vectors.

L87: in Huynh et al. (1985); Scherer et al. (1981).

NM514: _Hfl Murray et al. (1977).

2.5 ANTIBIOTICS

The concentrations of antibiotics used for both liquid and plate selections were as follows:-

Antibiotics	Selective $conc^n$	Stock $conc^n$	Storage temp.(^oC)
Ampicillin	50ug/ml	5mg/ml (water)	-20
Tetracycline	10ug/ml	1mg/ml (50% EthOH)	-20

2.6 BACTERIOPHAGES

Lambda gt10: genotype lambda srIlambda 1^o b527 srI lambda 3^o imm^{434} ($srI434^+$) srI lambda 4^o srI lambda 5^o. Lambda gt10 was constructed by *in vitro* techniques using the left arm of lambda 518 and the right arm of lambda 607. (Hyunh et al. 1985). Lambda gt10 (imm^{434} b 527) contains a single EcoRI site within the phage repressor gene, cI.

M13mp18: Cloning vector derived from M13 and used for sequencing. (Norrander et al. 1983; Yannish-Perron et al. 1985). The vector contained the alpha-fragment of the lac Z gene that was able to complement the lac Z, _M15 deletion in bacterial host strains JM101. The loss of lac Z complementation (beta-galactosidase activity) by insertion mutagenesis allowed the selection of recombinant (white) plaques and colonies according to the blue/white colour test depending on the action of beta-galactosidase on the chromogenic substrate, X-gal.

2.7 INDICATORS

X-gal (5-Bromo-4-chloro-3-indolyl-B-galactoside) was used with the inducer IPTG and the host strain JM101 with M13 vectors. This provides a screen for insert containing vectors in the polylinker region. Recombinants are white against blue coloured parental vector. X-gal was stored in dimethylformamide (DMF) at -20^oC and added to top agar at a concentration of 300ug/ml. IPTG was added to a top agar to a final concentration of 150ug/ml.

2.8 MAMMALIAN CELL CULTURE

Cell Lines: JEG-3 Choriocarcinoma (Kohler and Bridson, 1971).
KN_2 Namalva Lymphoblastoid Cell Line.

Culture Media: Both cell lines were maintained in RPMI Medium supplemented with 10% foetal calf serum and made upto 5 litres as follows -

3850ml	H_2O
450ml	Foetal calf serum (Gibco)
450ml	10 x RPMI
75ml	1M NaOH
112.5ml	Hepes (1M pH7.5)
50ml	Glutamine (200mM)
50ml	$NaHCO_3$ (7.5%)
90ml	Penicillin/Streptomycin (10^3U:10^3ug/ml)
9ml	Gentomycin Sulphate (50mg/ml)
2.25ml	B_2-mercaptoethanol (50mM)

Culture conditions: Cells in routine culture were maintained in 25cm^3 plastic culture flasks (Falcon) under 10ml of medium at 37^{o}C in an atmosphere containing 7%CO_2. The substrate cells were passaged (x1/4) when approaching confluency approximately every three to four days.

In order to pass cells the medium was apirated away. After washing with 10ml PBSA, the cells were subsequently detached by treatment with 1ml trypsin-versein for one minute. The cells were dislogded by gently tapping and were subsequently divided appropriately into new flasks with 10ml fresh medium. Fresh cultures were initiated with 1/4 dilutions.

Large 75cm^3 culture flasks were used for quickly growing stocks - all solutions used were scaled up accordingly.

PBSA	5mM $NaHPO_4$
	7.5mM KH_2PO_4
	2.75mM KCl
	170mM NaCl
Trypsin-Versein	0.025% Trypsin
	500um EDTA
	0.0015% Phenol Red in PBSA

2.9 MONOCLONAL ANTIBODIES.

W6/32 - Mouse anti-human leucocyte antigen (HLA) directed against a HLA-A, -B and -C shared determinant (Sera-lab).

61D2 - Mouse anti-human leucocyte antigen (HLA) obtained from BRL, Inc. Ugolini et al. (1981).

BM-63 - Mouse anti-human $Beta_2$-microglobulin (Bio Yeda).

2.10 IMMUNOPRECIPITATION

This method followed the procedure outlined in Thompson et al. (1984) and is approximated for immunoprecipitation for ten samples.

Near confluent cells were removed from the tissue culture flask by trypsinization as described in section 2.8, and washed three times in a generous volume of PBS by repeated low speed centrifugation and resuspended to $2x10^7$ cells/ml PBS. Surface labelling was done collectively. 5ul 100uM KI, 20ul 9% glucose (fresh) and 40ul glucose oxidase (G6500 Asp Nigeria type V from Sigma) were added to the cells followed quickly by 20ul 1mg/ml lactoperoxidase in PBS (Sigma). 5ul (500uCi) ^{125}I-NaI (IM530-Amersham) was added and the contents mixed well before incubating at room temperature for 45 minutes. The labelled cells were washed four times with PBS and transferred to a fresh tube. The supernatant was poured off and the cells finally resuspended in 1ml 0.5% NP40 in TBS (pH8.1) and 1ul PMSF. After incubation on ice for ten minutes, and centrifugation, the supernatant (approx. 1ml) was divided equally amongst the ten tubes (for ten antibodies). To 100ul supernatant, 10ul of the appropriate antibody was added and this was left to incubate at room temperature for 30 minutes. 50ul of a 50% (w/v) suspension of protein A-Sepharose (Pharmacia) in 1% (v/v) NP40 in PBS and the samples were incubated again at room temperature for 30 minutes. Unbound material was gently centrifuged away from the Sepharose beads by five washes at 4^oC in 2.5ml 1% (v/v) NP40 in 50mM Tris.HCl (pH7.4), 0.15M NaCl, 5mM EDTA and 0.02% (w/v) NaN_3. The antigen-antibody complexes were released from the beads by 50ul 2x SDS/PAGE sample buffer (no 2-mercaptoethanol), and the supernatants were boiled after the addition of 5% (v/v) 2-mercaptoethanol before loading for electrophoresis.

2.11 PROTEIN GEL ELECTROPHORESIS

Protein electrophoresis followed the procedure of Laemmli (1970). The samples were prepared by adding half the volume of protein gel loading buffer. Gel plates were separated with 0.8mm spacers. The running gel was poured leaving a 2cm gap between the top of the running gel and the top of the rabbit-eared smaller glass plate. 2% SDS was gently layered on top of the running gel to create a sharp interface. When the running gel had set, the stacking gel was poured and the comb put in position. After removing the comb the running buffer was poured into the gel tank to cover the wells. Residual acrylamide was flushed gently out of the wells. The gel was run until the blue dye had reached the bottom of the gel (approximately 40mA for 4 hours). The gel was then fixed in 35% methanol/10% glacial acetic acid for 1 hour before being soaked in EN^{3}HANCE for a further hour. The EN^{3}HANCE was removed and the gel soaked in water for 30 minutes before being vacuum dried onto Whatman 3MM paper and autoradiographed at room temperature against Kodak X-Omat S film.

Resolving Gel Buffer (RGB): 3M Tris-HCl pH8.8

Stacking Gel Buffer (SGB): 0.5M Tris-HCl pH6.8

10% Acrylamide Gel (30ml):

15.75ml water
225ul 10% fresh ammonium persulphate
0.3ml 10% SDS
3.75ml RGB
10ml 30% Acrylamide:Bisacrylamide (30:0.8)
+
15ul TEMED

Mix well.

4% Stacking Gel (20ml):

12.15ml Water
150ul 10% fresh ammonium persulphate
0.2ml 10% SDS
5ml SGB
2.5ml 30% Acrylamide:Bisacrylamide (30:0.8)
+
15ul TEMED

Running Buffer: 14.4g glycine, 1g SDS, 3.03g Tris-base in 1 litre of distilled water.

2.12 PREPARATION OF RNase-FREE SOLUTIONS AND APPARATUS

Ribonuclease activity was minimised during the preparation of RNA by treatment of solutions with 0.1% diethylpyrocarbonate (DEPC - Sigma) overnight and autoclaving twice. DEPC is unstable in the presence of tris buffers and so these solutions were made up with chemicals which were kept out of general use and so less likely to be contaminated with ribonucleases.

Glassware was baked overnight at 200^{o}C.

Where possible sterile disposable plastic-ware was used (free from RNase).

2.13 PREPARATION OF RNA

This procedure is outlined in Maniatis et al. (1983) pp196 and takes advantage of the powerful denaturing properties of guanidinium isothiocyanate. This is a very dangerous substance - wear gloves to cover the wrists and a full face mask!

4ml ice cold guanidinium isothiocyanate solution were added to a small cell pellet and vortexed until the pellet had completely dissolved. Leave on ice for 15 minutes. Meanwhile prepare 1.2ml CsCl cushion in a 6ml SW50 tube carefully so that none of this solution splashes up the sides of the tube.

1g CsCl per 2.5ml cell homogenate was added and this was layered very slowly/carefully onto the cushion. Keep tubes upright and steady. Centrifuge at 35,000 rpm for 12 hours at 20^{o}C using the SW50.1 swing out rotor.

After centrifugation keep tubes upright and steady (use a rack and a piece of plasticeine) and remove the lipid "plug" which invariably forms (best done with a small rolled up piece of tissue) and carefully suck off the rest of the homogenate (in one attempt) using a wide pipette. Chromosomal DNA can be seen at this stage. With a fresh pipette remove the cushion very carefully and then tip the tube upside down on tissue paper to drain.

Use a new scalpel to cut off the bottom of the tube to form a small cup. The RNA is usually visible as a glassy pellet. Carefully flush the pellet with 80% ice cold ethanol (to flush away some of the CsCl). Usually, the pellet becomes white around the edges at this stage (due to contaminating proteins etc.) and is easily removed into a

large 1.5ml eppendorf. Dry very briefly under vacuum. Resuspend the pellet in 0.6ml TE at 55°C and extract and re-extract with an equal volume of phenol:chloroform:iso-amyl alcohol, (v/v) 25:24:1. The remaining aqueous phase (0.4ml) is precipitated with 0.1 volumes Na-Acetate pH5.2 and 2.5 volumes ethanol at -20°C for 2 hours. Microcentrifuge for 30 minutes and wash twice with 70% ethanol. Re-centrifuge for 5 minutes and vacuum dry (do not overdo this!). Resuspend in either water or 0.1xTE and store at -70°C. Determine the amount of RNA by spectrophotometry; $O.D._{260}$ =1 is equivalent to 40ug/ml. Pure preparations of RNA have an $O.D._{260/280}$ =2; contaminating proteins reduce this value. Eukaryotic RNA of high quality shows three distinct bands corresponding to 28s, 18s ribosomal and tRNA. Messenger RNA (mRNA) comprising 5% of total RNA can be seen as a background glow around the ribosomal RNA bands.

Guanidinium isothiocyanate solution:

4M Guanidinium isothiocyanate
5mM Na-Citrate pH7
10mM EDTA
0.1M beta-mercaptoethanol
0.5% sarkosyl (Na-N-laurylsarcosine)

Caesium Chloride cushion:

5.7M CsCl
10mM EDTA

2.14 AGAROSE GEL ELECTROPHORESIS

Various sizes of agarose gel were used in this study and agarose concentrations varied from 0.5% to 1.5% (w/v) depending on the size of the fragments to be resolved (see below).

Amount of agarose in gel (%)	Efficient range separation of linear DNA molecules (Kb).
0.3	60 - 5
0.6	20 - 1
0.7	10 - 0.8
0.9	7 - 0.5
1.2	6 - 0.4
1.5	4 - 0.2
2.0	3 - 0.1

The rate of linear duplex migration is inversely proportional to the $\log_{10}$ molecular weight. The extent of

superhelical twist in covalent closed circular DNA, and whether the DNA is in the single stranded form affects its electrophoretic mobility. The concentration of ethidium bromide (used for visualization with ultra-violet light) in the gel was 0.5ug/ml.

Applied voltages varied between 2-10 Vcm^{-1} depending on the time and resolution required.

Agarose was dissolved by boiling in electrophoresis buffer (TBE) replacing lost volume due to evaporation with distilled H_2O. DNA samples were mixed with 1/5th volume of 5xFSB.

10 x TBE pH8.3 89mM Tris-base
89mM boric acid
2mM EDTA

5 x FSB 0.25% bromophenol blue
0.25% xylene cyanol
30% glycerol in water

2.15 NORTHERN FORMALDEHYDE DENATURING GEL ELECTROPHORESIS FOR RNA

Agarose (1-1.5%) was boiled in water and cooled to 60°C. The appropriate volumes of 10xMOPS and 37% formaldehyde (deionized) were prewarmed in a 60°C water bath before pouring into the agarose. The final concentration of these components in the gel was 1xMOPS and 6% formaldehyde. Electrophoresis running buffer was 1xMOPS.

10xMOPS 0.2M Morpholinopropanesulphonic Acid (41.8g/l)
0.05M NaAcetate (4.1g/l)
0.01M Na_2EDTA, pH7 (1.86g/l)
Store in the dark - do not autoclave!

FGLB 50% Glycerol
1mM EDTA
0.4% Bromophenol Blue
0.4% Xylene Cyanol

MMF

Formamide	500ul
37% Formaldehyde	162ul
10x MOPS	100ul
H_2O	238ul

RNA samples (usually between 5-20ug) in small volumes (not greater than 5ul) were heated for 15mins at 60°C after the addition of 30ul MMF. 10ul formaldehyde gel loading buffer (FGLB) was added to the samples on ice. The

samples were electrophoresed at 100-150volts for approximately 4hrs making sure that the buffer was recirculated. RNA samples and linear DNA markers (not absolutely accurate) were visualized by staining additional sample tracks with ethidium bromide after flushing formaldehyde out of the gel with five changes of water over a one hour period.

2.16 NORTHERN TRANSFER AND DOT BLOTTING.

After electrophoresis, the formaldehyde gel was laid upside down (samples have less distance to travel during blotting) on a support fed with transfer buffer (20xSSC). Filter paper or nappies were used to absorb the transfer buffer through the gel. Pal Biodyne^{TM} membrane (nylon) was used to collect samples. The membrane was baked for one hour at 80^{o}C.

20xSSC ph7 3M Nacl
0.3M Sodium citrate

For dot blotting, RNA samples were resuspended in 200ul 50% Formamide, 5xSSPE and denatured by heating (65^{o}C for ten minutes) before applying to the membrane. The membrane was baked for one hour at 80^{o}C.

2.17 NORTHERN FORMALDEHYDE PREHYBRIDIZATION AND HYBRIDIZATION

See Chapter 4 for optimization of this protocol.

The following prehybridization/hybridization solution was empirically derived from existing protocols by varying the concentration of some of the components. It was hoped that the background level of hybridization could be reduced to a minimun as this was a serious problem when detecting very low levels of mRNA with probes of high specific activity. Prehybridization of filters was at 42^{o}C in a plastic bag placed in a gently shaking water bath for at least 4 hours. Fresh hybridization solution was prepared and the denatured probe (by boiling for 5 minutes) added to it before pouring over the filters. Do not pour undiluted probe onto filters because this often results in splodgy background after autoradiography.

Pre/hybridization solution: 5x SSPE
50% Formamide
10x Denhardts

200ug/ml denatured salmon sperm

100x Denhardts: 2% (w/v) BSA (bovine serum albumin)
2% (w/v) Ficoll
2% (w/v) Polyvinyl pyrollidone
Dissolve the BSA first!

It is very necessary to recirculate the gel tank buffer during formaldehyde gel electrophoresis since MOPS is known to be a weak buffering agent. Although fairly tight signals were obtained upon hybridization, these can be apparently improved by recirculating the buffer much more quickly.

Since mRNA constitutes only 5% (approximately) of total RNA, large amounts (upto 20ug) are required for electrophoresis especially if the mRNA of interest is expressed at relatively low levels. 20ug total RNA are generally loaded onto the gel in volumes of approximately 40ul and consequently the gel wells become quite full. Unfortunately, formaldehyde leaches out of the gel during the running period and results in a concentration gradient. Consequently, part of the sample at the top of the well/gel (less formaldehyde due to leaching) migrates slightly quicker than the part of the sample which has sunk to the bottom of the well/gel (higher concentrations of formaldehyde). Visualization of DNA markers on formaldehyde gels using ethidium bromide revealed that they sloped in the direction of travel. This effect was not due to sloping gel wells and can be seen in figure 4.2.4. Formaldehyde was included in the running buffer and was found to alleviate the problem.

2.18 CULTURE MEDIA AND MICROBIOLOGICAL GROWTH CONDITIONS

Culture Conditions: All liquid cultures were grown at 37^{o}C with vigorous shaking. Plates were incubated overnight at 37^{o}C upside down.

Storing Bacterial Cultures: Bacterial strains were stored in 85% L-broth, 15% glycerol (v/v) at -20^{o}C in a tightly sealed bijoux.

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g Glucose, 20mg thymine, made upto 1 litre in distilled water and adjusted to pH7 with NaOH.

L-Agar: As L-Broth without glucose and the addition of 15g/l agar.

Reagents used with Lambda phage

L-Broth: 10g bactotryptone, 5g bacto-yeast extract, 10g NaCl, dissolve in 800ml, pH to 7 and make up to 1 litre.

L-agar plates: As L-Broth with the addition of 15g bacto-agar.

Top agar: 1g bactotryptone, 0.5g bacto-yeast extract, 0.5g NaCl, 0.25g $MgSO_4$, 1g bacto-agar - adjust to pH7 and make upto 100ml.

SM Buffer: 5.8g NaCl, 2g $MgSO_4.7H_2O$, 5ml 1M Tris HCl pH7.5, 5ml 2% gelatin - dissolve in 800ml water, adjust volume to 1 litre and sterilize.

Reagents used with M13 phage

Minimal Agar: 7g K_2HPO_4, 2g KH_2PO_4, 4g NH_4SO_4, 0.25g trisodium citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made upto 1 litre with distilled water.

M9 Salts (x10): 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl in 1 litre of distilled water.

M9 Minimal Medium: 10ml M9 salts, 2ml glucose (20% w/v), 0.1ml 1M $MgCl_2$, 0.1ml 100mM $CaCl_2$, 0.1ml (1mg/ml) vitamin B1, 2.5ml 20% casamino acids, made upto 100ml with distilled water.

When required, supplements were added to minimal media to the following concentrations:- glucose 2mg/ml, thiamine vitamin B1 20ug/ml.

YT Broth (x2): 10g tryptone, 10g yeast extract, 5g NaCl made upto 1 litre with distilled water.

Phage Buffer: 7g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4$, 15mg $CaCl_2.2H_2O$, 1ml 1% gelatin made up to 1 litre in distilled water.

2.19 PREPARATION OF COMPETENT CELLS FOR TRANSFORMATION

Plasmids were introduced into strains by genetic transformation. A fresh culture of the recipient strain was grown from an overnight in 50ml L-Broth to an O.D. of $2.5x10^8$ cells/ml. The cells were harvested (12000g, 10 minutes, 4^oC) and resuspended in 10mL 50mM $CaCl_2$ and kept on ice for at least for 15 minutes. After recentrifugation, the cells were resuspended in 2.5ml 50mM $CaCl_2$ and kept on ice for 15 minutes before use. 200ul of this cell suspension was added to plasmid DNA to be transformed and left on ice for one hour. After heat shocking (2 minutes at 42^oC) the cells were returned to ice for a further 15 minutes. An equal volume of

L-broth was added and the cells were incubated for 1 hour at 37°C to allow for expression of the plasmid resistance genes. Recovery for ampicillin selection is not necessary.

2.20 DNA PROBES

pDP001: Class I HLA (Sood et al. 1981) partial (1400bp) cDNA clone HLA-B7 allele in the Pst1 site of pBR322.

pmS3: Actin incomplete (900bp) cDNA clone for mouse skeletal muscle actin (Leader et al. 1986) in the Pst1 site of pBR322.

2.21 LARGE SCALE PREPARATION OF PLASMID

The following protocol is a modification of the method developed by Birnboim and Doly, 1979.

This particular method is based on the differences between the large linear chromosome and the closed circular covalent plasmid DNA, and uses the differential precipitation and selective alkaline denaturation of each.

500ml cultures of stationary phase cells were harvested by centrifugation (12000g, 5 minutes at 4°C). The pellet was resuspended in 8ml Quick Mix (GTE) containing 10mg/ml lysozyme and mixed very gently on ice for 5 minutes. 16ml (freshly made) NaOH/SDS were added, gently mixed and left on ice for a further 5 minutes. 12ml cold 5M Na Acetate pH4.8 were added gently and the mixture was left on ice for 5 minutes. The mixture was transferred to 50ml plastic Oak Ridge centrifuge tubes and centrifuged twice to precipitate a white floccular pellet (cell debris and most of the chromosomal DNA) at 32000g, for 15 minutes at 4°C. The supernatant was equilibrated to room temperature and 0.6 volumes isopropanol was added. This was left to stand for 15 minutes. A JA-20 rotor was prewarmed (so no salts were precipitated which contaminate the pellet) and centrifuged at 39200g, for 30 minutes. The supernatant was poured off and the pellet flushed with 5ml 70% ethanol before vacuum drying. The pellet was resuspended in 8ml TE pH8 at 37°C if necessary and 1g CsCl per 1ml of solution was added. For every 10ml of this salt/DNA solution, 0.8ml 10mg/ml ethidium bromide was added. The final density was checked and adjusted to 1.56-1.60g/ml. The gradients were centrifuged in a Beckman Ti70 fixed angle rotor at 200,000g for 16 hours at 20°C. Two bands were visible, a lower supercoiled plasmid band and an upper chromosomal band and relaxed plasmid DNA band. The

lower band was obtained using a 5ml syringe and the ethidium bromide was removed by repeated extractions with water saturated iso-amyl alcohol. The salts were dialysed with repeated 500ml TE pH8 changes at 4°C.

Quick Mix (GTE):
50mM Glucose
25mM Tris.Cl (pH8)
10mM EDTA
add lysozyme fresh (10mg/ml)

NaOH:
0.2M NaOH
1% SDS
make up fresh

5M K-Acetate pH4.8:
60ml 5M K-Acetate
11.5ml glacial acetic acid
28.5ml H_2O

2.22 PHENOL/CHLOROFORM EXTRACTION

Proteins and lipids were removed from nucleic acid samples by extraction with buffer-saturated phenol, phenol:chloroform (1:1, v/v) and chloroform. Each extraction was centrifuged for 5 minutes to partition the organic and aqueous phase. Residual chloroform was removed by ether extraction, and in turn, this waw removed by either incubating for a brief period at 55°C or ethanol precipitation.

2.23 ETHANOL PRECIPITATION

One tenth volume of 3M Na-Acetate pH5.2 and 2.5 volumes of -20°C ethanol were added to the nucleic acid solution and stored at -20°C for at least 1 hour. Nucleic acids were pelleted by micro-centrifugation for 15-30 minutes depending upon the concentration of the sample and are washed with cold 70% ethanol. After re-centrifugation, the pellet was vacuum dried briefly and resuspended in the appropriate buffer or water.

2.24 RESTRICTION OF DNA

Restriction endonuclease digests of DNA were routinely carried out in the appropriate buffers specified below.

10x Low Salt Buffer:
100mM Tris-Cl pH7.5
100mM $MgCl_2$
10mM DTT

10x Medium Salt Buffer: 100mM Tris-Cl pH7.5
50mM NaCl
100mM $MgCl_2$
10mM DTT

10x High Salt Buffer: 500mM Tris-Cl pH7.5
1M NaCl
100mM $MgCl_2$
10mM DTT

10x Sma I Buffer: 100mM Tris-Cl pH8
200mM KCl
100mM $MgCl_2$
10mM DTT

The following KGB buffer can be used sequentially for different enzymes and was extremely useful when ligating fragments after their restriction. Both potassium and glutamate are effective buffering ions and most restriction endonucleases, polymerases and ligases show broad K-Gluc concentration optima and all enzymes function in 1xKGB (McClelland et al. 1988).

10x KGB: 1M K-Glutamate
250mM Tris-acetate pH7.6,
10mM Mg-Acetate
500ug/ml BSA
5mM 2-mercaptoethanol.

2.25 ENZYMES AND STANDARD BUFFERS USED IN THIS STUDY TO SYNTHESIZE, MODIFY AND RADIOACTIVELY LABEL NUCLEIC ACIDS

<u>DNA Dependent DNA Polymerases</u>

E. coli DNA Polymerase I - all DNA polymerases add deoxyribonucleotides to the 3'-hydroxyl terminus of a primed double-stranded DNA molecule - synthesis is exclusively in a 5' to 3' direction with respect to the synthesized strand. Many DNA polymerases have 3' to 5' exonuclease activity inherently associated with polymerase activity, though this is influenced by the concentration of free dNTP's. Some DNA polymerases e.g. E. coli DNA Polymerase I, also have an associated 5' to 3' exonuclease activity which degrades double-stranded DNA from a free 5' hydroxyl end. The large fragment of E. coli DNA Polymerase I which has been modified so that it does not retain the 5' to 3' exonuclease activity is known as the Klenow fragment. The Klenow fragment has been used to label 3'ends of DNA molecules and in random

oligonucleotide primed synthesis. E. coli DNA Polymerase I has been used in the synthesis of second strand of cDNA.

10x E. coli DNA Polymerase I or klenow fragment buffer:

0.5M Tris.Cl, pH 7.5
0.1M $MgCl_2$
10mM DTT
0.5mg/ml BSA

T4 DNA Polymerase has a very active single- and double-stranded 3' to 5' exonuclease activity (though lacks 5' to 3' exonuclease activity). This enzyme has been used in this study to convert the ends of cDNA to blunt ended structures.

10x T4 DNA Polymerase buffer

0.5M Tris.Cl, pH8
50mM $MgCl_2$
50mM DTT
0.5mg/ml BSA

<u>Template-Independent DNA Polymerases</u>

Terminal Deoxynucleotidyltransferase (terminal transferase) This enzyme catalyzes the incorporation of deoxynucleotides to the 3'-hydroxyl termini of DNA (releasing inorganic phosphate). A template is not required.

This enzyme would have been used in the strategy which has been described in Chapter 7.

10x Terminal Transferase buffer

1M sodium cacodylate, pH7
10mM $CoCl_2$
1mM DTT
0.5mg/ml BSA

<u>RNA-Dependent DNA Polymerases</u>

Avian myeloblastosis virus (AMV) reverse transcriptase was used in this work to reverse transcribe mRNA into first strand cDNA. Typically derived from retroviruses the reverse transcriptases use a primer on RNA to extend the complementary DNA strand.

10x Reverse Transcriptase buffer

0.5M Tris.Cl pH8
50mM $MgCl_2$
50mM DTT
0.5M KCl
0.5mg/ml BSA

<u>Phosphatases and Kinases</u>

Calf Intestinal Phosphatase (CIP) catalyzes the hydrolysis of

5'-phosphate residues of DNA, RNA and ribo and deoxyribonucleoside triphosphates, leaving 5'-hydroxyl termini. CIP was used in this study for the dephosphorylation of M13 linear DNA to prevent self-ligation during subcloning.

10x CIP buffer

0.2M Tris.Cl, pH8
10mM $MgCl_2$
10mM $ZnCl_2$
0.5mg/ml BSA

T4 Polynucleotide Kinase catalyzes the transfer of the terminal (gamma) phosphate of ATP to the 5'-hydroxyl termini of DNA and RNA. This reaction is very efficient and was used to label 5' ends of the dephosphorylated oligonucleotide used for primer extension experiments.

10x Kinase buffer

0.5M Tris.Cl, pH7.5
0.1M $MgCl_2$
50mM DTT
0.5mg/ml BSA

DNA Ligases

T4 DNA Ligase catalyzes the repair of single strand nicks in duplex DNA and joins duplex DNA restriction fragments having either blunt or cohesive ends. It uses ATP as a cofactor. This enzyme was used for cloning cDNA into lambda gt10 and for subcloning the cDNA clones into M13mp18.

10x T4 DNA Ligase buffer

0.5M Tris.Cl, pH7.5
0.1M $MgCl_2$
0.1M DTT
0.5mg/ml BSA

2.26 RANDOM PRIME LABELLING

This method follows the procedure outlined in Feinberg and Vogelstein, 1983. The restricted DNA fragment was cut from a low melting point gel after sufficient separation so as to reduce contamination from other bands. Excess gel was removed and the fragment weighed (1ug of gel slice has an equivalent volume of 1ul) before heating the sample to 65°C. At least five equivalent volumes of either dH_2O or TE were added. The concentration of the final solution was adjusted to 1ng/ul for convenience. It was

important to know the amount of DNA in the band isolated. The random prime labelling was set up as follows -

Aul dH_2O
Bul DNA solution (10ng)
Cul [alpha-^{32}P] dCTP (usually 40uCi)
10ul "reaction mix" excluding dCTP's
2ul BSA
1ul Klenow fragment of DNA polymerase I (5 units).

50ul total

A+B+C = 37ul.

"reaction mix" 250mM Tris-HCl
25mM $MgCl_2$
50mM 2 mercaptoethanol
50uM dATP, dGTP and dTTP
1M Hepes pH6.6
1.5mg/ml hexadeoxyribonucleotides

The labelling mixture was incubated O/N or for 5 hours at room temperature. Unincorporated nucleotides were removed using a sephadex G-50-150 column and Amersham column buffer. The probe was denatured by boiling for ten minutes.

2.27 SEPARATION OF mRNA FROM TOTAL RNA USING OLIGO-dT CELLULOSE AFFINITY MATRIX

Polyadenylated mRNA can be separated from other RNA species using oligo-thymidylic (dT) cellulose (BRL) which is a stable affinity matrix specifically designed for this purpose (Aviv and Leder, 1972). Oligo (dT) cellulose typically has a poly rA binding capacity of 50 O.D. units/g under appropriate high concentration ionic conditions at room temperature. Low ionic buffers used at an elevated temperature (37^{o}C) are sufficient to effect elution of mRNA by disrupting hydrogen bonds formed between oligo (dT) and the poly A tail of mRNA.

A 5ml plastic syringe, 1 inch soft tubing, 0.5cm^3 syringe volume of glass wool and 0.5cm^3 sephadex G-50-150 beads were treated with dimethyl-dichloro silane solution (repelcote) and baked overnight.

An appropriate but generous (0.4g) amount of dried oligo (dT) cellulose powder was suspended in Binding Buffer (BB) over a period of 1 hour and poured into the syringe

(fixed upside down by clamping). A clip was used to regulate the flow by attaching it to the soft tubing which was in turn attached to the end of the syringe.

Wash the column with 30 volumes Elution Buffer (EB) and equilibrate with 30 volumes BB.

An equal volume of 2xBB was added to the RNA sample (typically 0.5-1mg) which was heat denatured at 70°C for 1 minute and rapidly chilled on iced water. After equilibration to room temperature the sample was applied to the column at a rate of 1ml/minute. The column was washed with BB until no U.V. absorbing material eluted from the column. BB was used for the blank standard. The mRNA was eluted with EB at 37°C using spectrophotometry to locate the mRNA-containing fractions. EB was used as the blank standard. The fractions were pooled and precipitated overnight with 0.1 volumes 4M NaCl and 2 volumes ethanol at -20°C.

After microcentrifugation for 30 minutes, the pellet was washed with 80% ethanol, vacuum dried briefly and resuspended the pellet in sterile RNase free water and frozen immediately at -70°C. It was advisable to precipitate using a potassium salt if the mRNA was required for cell-free translation.

Binding Buffer (BB):	0.5M NaCl
(make up 2x)	10mM Hepes pH7.5
	1mM EDTA
	0.1% Sarcosine
Elution Buffer (EB):	10mM Hepes pH7.5
	1mM EDTA

2.28 IN VITRO TRANSLATION

mRNA can be translated in a cell-free system Pelham and Jackson (1976). Translation of mRNA is carried out in the presence of 1uCi/ul L-[^{35}S]-methionine (NEN) using a rabbit reticulocyte lysate obtained from Amersham International plc. mRNA is added to the lysate (the lysate should comprise 80% of the total volume) with label and incubated at 30°C for 90 minutes. Half the volume of protein gel electrophoresis buffer (30% glycerol, 6% SDS, 15% 2 mercaptoethanol, 150mM Tris HCl pH6.7, 0.05% bromophenol blue) was added and the samples were boiled for 2 minutes before loading on a 10% SDS-PAGE gel.

2.29 PREPARATION OF cDNA

The preparation of double stranded cDNA from mRNA is based on the method outlined in Gubler and Hoffman, 1983. Biological materials, chemicals and various buffers were obtained from Amersham International plc. The composition of some buffers and solutions are undisclosed. It is important to add the components in the order specified. The synthesis procedure is divided into two parts.

The synthesis of the first strand cDNA was executed in a small 20ul volume whilst the second strand synthesis was performed in 100ul (necessary to adjust the buffer concentrations). The incorporation of [alpha-^{32}P]dCTP during the synthesis of first and second strand cDNA enabled the estimation of the amount of cDNA made and its visualization using denaturing gel electrophoresis and subsequent autoradiography. Although the labelling is optional it is very advisable to monitor the reaction during the synthesis of both strands and therefore two separate reactions are usually set up. The cDNA from each can be pooled after analysis and treated singularly in the following steps.

First Strand Synthesis - First strand synthesis buffer (typically, 100mM Tris-HCl pH8.3, 140mM KCl, 6mM $MgCl_2$, 10mM DTT) with sodium pyrophosphate, 20units human placental ribonuclease inhibitor, 1mM dATP, 1mM dGTP, 1mM dTTP and 0.5mM dCTP, 0.1ug/ul oligo-$dT_{(12-18)}$ primer, 20uCi [^{32}P]dCTP (optional), 1ug mRNA, 20units (avian) reverse transcriptase and water upto a total volume 20ul. Incubate at 42^{o}C for 40 mins. 1ul (1/20th total volume) sample was removed for analysis and placed on ice.

Second Strand Synthesis - The following materials were added to the first strand synthesis mix on ice - second strand synthesis buffer (typically 50mM Tris.Cl pH8, 10mM $MgCl_2$, 10mM DTT, 50ug/ml BSA), 20uCi [^{32}P]dCTP (optional), 0.8units E. coli Ribonuclease H and 23units E. coli DNA Polymerase I with water upto 100ul. This was mixed gently and incubated sequentially at 12^{o}C for 1 hour, 22^{o}C for 1 hour and 70^{o}C for ten minutes. 2units of T4 DNA Polymerase (per ug of mRNA used) were added and the tube incubated at 37^{o}C for exactly ten minutes. The reaction was stopped by adding 10ul 0.25M EDTA pH8 and 10ul 10% SDS. 6ul (1/20th total volume) sample was removed for analysis.

Purification of cDNA - Double stranded cDNA was extracted twice with an equal volume of phenol:chloroform (1:1;v/v) saturated with TE buffer. Two volumes of diethyl ether were added to the aqueous phase in order to remove traces of phenol:chloroform. An equal volume of 4M ammonium acetate was added to the remaining aqueous phase followed by twice the combined volume of ethanol. This was chilled on dry ice for 15 minutes and microcentrifuged for at least 30 minutes. The supernatant was removed and resuspended in TE. After reprecipitation with ammonium acetate, the pellet was washed twice with -20^{o}C ethanol. The pellet was dried, resuspended in TE buffer and stored frozen. frozen.

Analysis of cDNA synthesis products - After removing 1ul first strand synthesis (1ss) reaction and 6ul second strand synthesis (2ds) reaction, the volumes were made upto 20ul with TE. 10ul of each was used for analysis on alkaline agarose gels (see section 2.29). End-labelled DNA markers were used for sizing (e.g. lambda Pst1 and phi X 174 HaeIII).

Calculation of cDNA reaction yields - The remaining 10ul of the samples removed for analysis were used for the calculation of cDNA synthesis yields. For each sample, 2ul was spotted onto the centre of each of five 2.4cm discs Whatman DE81 and left for 15 minutes to dry. Three of these were washed six times in 0.5M $NaHPo_4$, (5 minutes per wash), twice in water (1 minute per wash) and twice in 95% ethanol (1 minute per wash). The filters were dried thoroughly. The two unwashed filters measured the total radioactivity in the sample, the three washed filters measured only the radioactivity incorporated into nucleic acid. The filter discs were counted by liquid scintillation, averages and % incorporation of radioactivity (precipitable counts/total counts x 100%) for first and second strand synthesis. The second strand synthesis % incorporation value (**X**) was used for the calculations outlined below. This value enabled the calculation of the amount of cDNA synthesized and hence the efficiency of the process.

Percentage of labelled [alpha-^{32}P]dNTP incorporated = **X%**

Amount of unlabelled dCTP in standard (20ul) first strand and (100ul) second strand mix* = **10nmoles**

Therefore assume amount of unlabelled dCTP incorporated	= **X% of 10nmoles**
	= **Ynmoles**
Total amount of dNTP's incorporated	= **4Ynmoles**
Assume residue molecular weight of dNTP's (1mole)	= **350g**
Therefore weight of cDNA synthesized **4Ynmoles**	= **350ng x**
	= **Ang**
Weight of input mRNA	= **Zng**
Therefore % yield of cDNA	= **A/Z x 100%**

* The amount of unlabelled dNTP's does not change significantly in the second strand mix.

For good preparations, 15-30% mRNA is transcribed into first strand cDNA and this is usually efficiently (90%) converted into double stranded cDNA.

2.30 ALKALINE AGAROSE GEL ELECTROPHORESIS

Agarose gel solution 1.2%	50mM NaCl
	1mM EDTA
Alkaline tank electrophoresis buffer	30mM NaOH
	1mM EDTA
Alkaline loading buffer	50mM NaOH
	1mM EDTA
	2.5% Ficoll
	0.025% bromocresol

This denaturing gel was used in order to electrophorese radioactive single stranded DNA. Electrophoresis was carried out at voltages upto 7.5v/cm until the dye had migrated one third of the length of the gel. At the end of the run the gel was soaked in 10% (tri-chloro acetic acid) TCA for 2x30 minutes at room temperature. The gel was dried down onto Whatman 3MM paper and the position of the radioactive samples visualized by autoradiography using X-Omat S film.

2.31 CLONING cDNA INTO LAMBDA gt10 - cDNA LIBRARY

Lambda gt10 is an ideal vector for cloning and retrieving cDNA's (see Chapter 5). cDNA is normally cloned into the EcoRI site of lambda gt10 and so the following protocols describe the necessary preparation of the cDNA before cloning. Basically, any EcoRI sites that occur within

the cDNA need to be methylated before the addition of blunt-ended EcoRI linkers.

The materials used were purchased from Amersham International plc - the composition of some of the buffers are undisclosed.

Methylation: 1ug cDNA (or control blunt ended DNA) was methylated in a final volume of 20ul containing s-adenosyl-methionine, 8ug BSA in typically (50mM Tris HCl pH7.5, 1mM EDTA, 5mM DTT) and 20units EcoRI methylase, incubated at 37^{o}C for 1 hour and inactivated at 70^{o}C for ten minutes.

Ligation of Linkers: 1ug methylated cDNA was ligated to 1ug EcoRI linkers and 5 units T4 DNA ligase in a total volume of 30ul. This was incubated at 15^{o}C overnight (20 hours). The ligase was heat inactivated at 70^{o}C for ten minutes. For ligation buffer, see section 2.24.

EcoRI Restriction: The buffer was adjusted in order to restrict DNA with EcoRI and made up to a final volume of 100ul (including 100units EcoRI). This was incubate at 37^{o}C for 5 hours. EcoRI was heat denatured by incubating to 70^{o}C for 10 minutes. After EcoRI digestion (to remove multiple ligated linkers) the excess linkers were removed using a sephacryl column. The linkered cDNA was be ligated into the arms of lambda gt10.

Separation of EcoRI linkers: A sephacryl column was equilibrated with STE buffer (100mM NaCl, 10mM Tris, 1mM EDTA, pH7.5) and allowed to empty until the meniscus arrived at the top of the column bed. All of the 100ul sample was loaded and allowed to enter the column bed. The 100ul eluate was collected in tube 1 (of 10 1.5ul microcentrifuge eppendorf tubes). 200ul STE was added and eluate collected in tube 2. These latter steps were repeated until 10 eluates had been collected. The radioactive cDNA was located by Cerenkov counting of total ^{32}P dpm. The major two fractions which contributed to the cDNA peak were pooled. If samples were not radioactive they were run on an agarose gel. The cDNA elutes first from the column so it was important not to include fractions of the trailing edge since these contained progressively more linkers.

Ligation into lambda gt10: The linkered and methylated cDNA was ethanol precipitated by adding 1/10th volume 3M NaAcetate pH5.2 and 2.5 volumes of cold ethanol,

Left at -20°C for at least 2 hours and microcentrifuged for at least 30 minutes. The pellet was washed with 80% ethanol, vacuum dried and resuspended in 6ul dH_2O. The cDNA sample was divided into tubes with a volume ratio of 1:2:3 (e.g. 50ng:100ng:150ng) and each was ligated into 1ug lambda gt10 arms using 2.5 units T4 DNA Ligase in appropriate buffer (typically 50mM Tris-HCl ph7.6, 10mM $MgCl_2$, 5% (w/v) polyethylene glycol 8000, 1mM ATP, 1mM DTT) in a volume of 10ul for 15 hours at 15°C. After ethanol precipitation each was carefully resuspended in 2.5ul TE buffer.

Packaging of lambda gt10: The ligation reaction was packaged using packaging reactions obtained from Amersham International plc following the protocol provided. 0.5ml SM buffer (see section 2.18) was added to each followed by 10ul chloroform - mix very gently and store at 4°C.

2.32 PLATING OF PACKAGED PHAGE

Preparation of phage plating cells: In this case the appropriate cells (see Chapter 5) were E. coli L87 and NM514 and were grown fresh from an overnight culture in L broth supplemented with 0.4% maltose until the $O.D._{600}$ reached 0.5 ($2.5x10^8$cells/ml). The culture was cooled on ice and centrifuged at 3000 rpm for 10 minutes at 4°C. The cells were resuspended in ice cold 10mM $MgSO_4$ to a concentration of $8x10^7$cells/ml and stored at 4°C.

Titration of recombinants: In order to assess the number of successfully packaged lambda bacteriophage a proportion of the "cDNA library" was plated out. 30 ul (out of a total 500ul) was serially diluted on ice in 270ul SM buffer (10^2) until the 10^7th dilution. 100ul of each type of plating cells were aliquoted out for 100ul of each of the dilution series above in 5ml glass tubes (100ul of each remained from the dilution series), incubated at 37°C for 15 minutes and 4ml of liquid top agar was added at 45°C. The contents were poured onto prewarmed L-agar plates and after setting, the plates were incubated upside down at 37°C overnight. The number of plaques on those plates with upto 1000 plaques was counted The phage titre per ml was calculated by multiplying the numbers of plaques by the dilution number (see Chapter 5.4 for calculations and explanations).

2.33 PLAQUE LIFTS

After the appearance of plaques, the plates were removedfrom the incubator and chilled for 1 hour at 4°C. The plates were returned to room temperature and appropriately numbered and position marked filters were placed onto the plate surface. After 30 seconds, the filter was peeled off with blunt forceps in a controlled manner. Replica filters were taken and left on the plates longer by 30 seconds each time. The filters were laid plaque side up for 5 minutes on Whatman 3MM soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 5 minutes and then transferred to neutralizing solution (3M Na-Acetate ph7, 1.5M NaCl) for a further 5 minutes. The filters were blotted dry on Whatman 3MM for 30 minutes and baked at 80°C for one hour.

2.34 SOUTHERN BLOTTING

After electrophoresis and photography, agarose gels were soaked in denaturing solution and subsequently in neutralizing solution each for 30 minutes. DNA was transferred to nylon membranes by capillary action due to migrating with 20xSSC from a reservoir through the gel and filter into layers of absorbent material (modified from Southern, 1975). After transfer overnight, the filter was air dried and baked for 1 hour at 80°C.

2.35 AQUEOUS PREHYBRIDIZATION AND HYBRIDIZATION CONDITIONS

The procedures which follow are adapted from Southern, 1975 and apply to DNA immobilized on filters either after plaque lifting or Southern blotting. The temperature at which hybridization occurs (usually 65°C) may have to be estimated empirically if the sequences to be detected are expected to have relatively low levels of homology to the probe being used.

Aqueous pre/hybridization solution

5x Denhardts
5x SSPE
0.2% SDS
100ug/ml salmon sperm DNA (non-homologous).

The Tm of hybridization can be estimated using the following formula.

$Tm = 81.5 + 0.5(\%G+C) + 16.6 \log[Na^+] - (600/L) - (0.6(\%formamide)) - (\%mismatch)$

Hybridization was performed at Tm-25°C (often 55-65°C) depending on the probes used.

2.36 WASHING OF FILTERS AFTER HYBRIDIZATION

Firstly, filters (either Northern, plaque or Southern) were washed 3x for 2 minutes at room temperature with a small volume of 1xSSPE, 0.1%SDS to remove free probe.

The stringency of washes was increased by reducing the ionic concentration to 0.1xSSPE, 0.1%SDS and by increasing the temperatures from 42°C, 55°C to 65°C. After washing filters, they were sealed damp in a plastic bag and autoradiographed using Kodak X-Omat S film for a variable length of time depending on the counts. 10 counts per second was usually adequate for an overnight exposure. The stringency of washes was increased according to the number of counts retained on the filter. Depending on the nature of the experiment and the estimated/expected degree of sequence homology, it was often necessary to autoradiograph the filters overnight and wash at a higher stringency later. If the degree of sequence homology was expected to be low and if convenient, it was time-saving to do the experiment in triplicate and wash at different stringencies.

2.37 PREPARATION OF LAMBDA DNA

Phage can be obtained from a plate lysate or a liquid culture lysate - the latter is preferable. The procedure can be scaled up for large preparations of this material.

A single plaque usually contains 10^6 phage. To 20ml exponentially growing culture ($O.D._{600}$ = $8x10^8$cells/ml) (a multiplicity of infection (m.o.i.) of 1/1000) single plaque was addeed and incubated overnight or until lysis occurred. The culture was transferred to a 20ml Oak Ridge centrifuge tube and the cell debris spun down at 10,000rpm for 10 minutes. The supernatant was decanted into a new tube, DNase I and RNAase I were added to final concentrations of 1ug/ml and 2ug/ml respectively and incubates at room temperature for 1 hour.

The concentration of the supernatant was adjusted to 1M NaCl and centrifuged at 20,000 rpm for 2.5 hours. The phage pellet (glassy in appearance) as resuspended in 2ml phage buffer, transferred to a Sarstedt tube and SDS added to

a final concentration of 0.1%, with protease K to 100ug/ml. These were incubates at 65°C for 1 hour. The sample was extracted twice with phenol/chloroform as usual by slowly roll-mixing for twenty minutes and extracted with chloroform/iso-amyl alcohol (24:1,v/v). 0.4 volumes 5M Ammonium acetate and 1 volume isopropanol was added and the solution was left for 1 hour at 4°C before centrifuging in the Centra 4X at maximum speed, 20 minutes. The supernatant was removed and the DNA pellet washed with 70% ethanol before very briefly vacuum drying before resuspending in TE at 37°C.

2.38 PREPARATION OF SINGLE STRANDED AND REPLICATIVE FORM (RF) M13 DNA

Single strand and replicative forms of M13 DNA can be prepared at the same time.

20ml of an overnight culture of JM101 diluted 100-fold in 2xYT broth was infected with a single M13 plaque (stored in 0.5ml phage buffer, 4°C section 2.18) and incubated at 37°C with vigorous shaking for 5 hours. After centrifugation of JM101 cells in 50ml plastic Oak Ridge tubes at 10,000 rpm for 5 minutes at room temperature, the supernatant was transferred (containing the virus particles) to a fresh tube and centrifuged at 14,000 to remove any remaining cells and debris. The large cell pellet was used to prepare ds RF DNA by the boiling method described in 2.39 This allowed the identification of recombinant clones by restriction enzyme analysis.

The viral supernatant was adjusted to 5% polyethylene glycol (PEG 6000) and 0.5M NaCl and after no more than ten minutes (since longer incubation periods precipitate free cellular chromosomal DNA) the pellet was centrifuged for 30 minutes at 14,000 at room temperature. All traces of supernatant were removed and the viral pellet resuspended in 700ul 0.1xTE, transferred to a 1.5ml eppendorf tube and extracted twice with phenol:chloroform and once with chloroform. The interface was avoided by 100ul each time and the remaining 400ul aqueous phase was ethanol precipitated. The pellet was resuspended in 30ul water and frozen immediately. Aliquots were removed beforehand for observation on agarose gels.

2.39 TRANSFECTION OF JM101 WITH M13

This followed the procedure described in section 2.19 except that after heat-shock, the cells were incubated on ice for 15 minutes after which 200ul of fresh log phase JM101 culture was added. 5ml of molten soft-agar (pre-cooled to 45^{o}C) which contained IPTG (150ug/ml) and X-Gal (300ug/ml) was mixed with the cells and plated out onto dried (2 hours) plates containing D&M minimal agar, vitamin B1 and glucose.

2.40 MINIPREPS OF PLASMID DNA

Plasmid mini-preps were performed according to the method of Holmes and Quigley (1981) which is outlined in Maniatis et al. 1983 pp336.

1.5ml of a stationary phase culture containing the plasmid of interest was harvested by centrifugation for 1 minute in an eppendorf. The cells were quickly resuspended in 350ul STET buffer (8% sucrose, 0.5% Triton X-100, 50mM EDTA, 10mM Tris-Cl, pH8). 25ul freshly prepared lysozyme solution (10mg/ml in 10mM Tris-Cl, pH8) was added and the mixture vortexed briefly. After boiling for 40 seconds the contents were centrifuged for 15 minutes at 4^{o}C. The pellet was discarded using a toothpick and 40ul 2.5M Na-Acetate pH5.2 and 420ul isopropanol was added to the supernatant. After equilibration to room temperature the supernatant was centrifuged and the resulting pellet washed with 70% ethanol, re-centrifuged, and vacuum dried briefly. After resuspending in 20-50ul TE the DNA was suitable for various <u>in- vitro</u> manipulations. If necessary this preparation was phenol extracted and treated with 50ug/ml DNase-free RNase.

2.41 DNA SEQUENCING

Sequencing was performed using reagents supplied in the BRL M13 Sequencing System and according to the instructions provided in the "M13 Cloning/dideoxy sequencing Instruction Manual" with one exception. The working dideoxynucleotide concentrations were altered to:

0.1mM ddATP
0.05mM ddCTP
0.5mM ddGTP
1mM ddTTP

The routine concentrations of ddNTP's were doubled to ameliorate reading of the first few bases and diluted to

ten-fold for reading upto 350 bases.

Also the Sequenase System Version 2.0 (United States Biochemical Corporation (USB)) was used according to the instructions supplied. Both systems were adapted for the incorporation of deoxyadenosine 5'-[alpha-THIO] triphosphate, [^{35}S] (Dupont).

Samples were electrophoresed on 0.4mm, 5-8% polyacrylamide/urea gels as described in the BRL manual. Instagel mix was deionised using analytical grade mixed bed resin beads (Bio Rad). Sequencing gels were run on a home-made apparatus and the IBI Model STS 45 gel apparatus using a sharks tooth comb. After overnight polymerization, the gels were pre-run at a constant 110 W for 1 hour. After loading (multiple loadings) the samples were electrophoresed until the bromophenol blue dye-front reached the base of the gel (approximately 2 hours) or for upto 6 hours to extend the reaadable sequence. Wedge gels were also used to increase the number of readable bases for a single 2 hour run. It is important to realise that the concentration of polyacrylamide in the sequencing gel alters the rate of co-migration of both dyes with polynucleotides. For 8% gels, the rate of migration of bromophenol blue and xylene cyanol corresponds to 20 and 73 bases whereas for 5% gels, these values are 31 and 130-140 bases respectively.

After electrophoresis, gels were fixed by soaking in 5% methanol/5% acetic acid for 15 minutes and transferred onto damp Whatman 3MM backing paper. The gel was dried under vacuum at 80^{o}C for 1 hour. The dried gel was autoradiographed using Kodak X-omat S X-ray film.

2.42 SYNTHETIC OLIGONUCLEOTIDES

Oligo-359: 5'-GGTGGATCTCAGACGG-3' (16mer)

Complementary (antisense) to the intron of cDNA JW1 and JW5. Used initially for primer extension

Oligo-386: 5'-GGCTGTCTCTCCACCTCC-3' (18mer)

Complementary (antisense) to the 3'UT region of mRNA and cDNA JW1, JW4 and JW5.

Oligo-387: 5'-AGTGGGCTGGATGTCTCC-3' (18mer)

Complementary (sense) to the 3'UT region of cDNA JW1, JW4 and JW5.

Oligo-400: 5'-GGTCGCAGCCAAACATCCTCTGG-3' (23mer)

Complementary (antisense) to alpha-2 domain (exon

3), and used for primer extension.

Oligo-407: 5'-GAGGTGCTGGGCCCTGG-3' (17mer)

Complementary (sense) to the alpha-3 domain (exon 4) and used in sequencing.

Oligo-SCREENING: 5'-GGTCACGACCCCTCCCCATCCCCCA-3' (25mer)

Complementary (sense strand) to intron 2 of unspliced mRNA and used for screening extended recombinants (see chapter 6.6 for explanation of this).

Oligo-RSDF: 5'-TAGAGGATCCCCGGGTCGCCCCGA-3' (24mer)

Complementary (sense) to intron 2 encompassing the Sma I site of the cDNA clones JW1 and JW5. Used to restrict single stranded DNA (see chapter 6.6 for explanation of this).

Oligo-SDF: 5'-GACTCTAGAGGATCCCCGGGG-3' (21mer)

"Bridgeing oligo". Complementary (sense) to M13 polylinker (see chapter 6.6 for explanation of this).

2.43 ^{32}P-LABELLING OF OLIGONUCLEOTIDE PRIMERS

5'-dephosphorylated synthetic oligonucleotides (to be used as primers) were gamma^{32}P-phosphate labelled using T4 polynucleotide kinase at 37^{o}C for 30 minutes. Oligonucleotides stocks were adjusted to 50ng/ul in distilled water (and stored frozen).

Labelling reaction:

100uCi gamma^{32}P-ATP	10ul
50ng oligonucleotide	1ul
T4 polynucleotide kinase (10 units)	1ul
10x oligo labelling buffer	1.3ul
	13.3ul

10x oligo labeling buffer: RNase free!

50mM Tris pH7.4

10mM $MgCl_2$

5mM DTT

0.1mM spermidine

Afterwards, the kinase was heat inactivated at 65^{o}C for 5 minutes. It was not absolutely necessary to remove free label although this could be done using ammonium acetate/ethanol precipitation.

2.44 PRIMER EXTENSION

10ng (2ul) of the specific ^{32}P-gamma labelled oligonucleotide primer was mixed with 5ug mRNA as outlined in section 2.28 (first strand cDNA synthesis). Incubate 40uCi ^{32}P-dCTP if internal labeling of the synthesized strand wasrequired. The addition of 0.8ug/ul actinomycin D (Sigma) to mRNA before annealing this to the primer may prevent self hydrogen bonding. This creates looping out of mRNA and hinders primed extension.

The sample was incubated at 42^{o}C for 90 minutes, ethanol precipitated as usual, and washed with 80% ethanol. After vacuum drying briefly the sample was resuspended in 2ul TE and 3ul formamide sequencing gel loading buffer (BRL manual). A sequencing reaction (to size the synthesized strands) and free oligonucleotide (to check labeling) was run alongside the sample on an 8% polyacrylamide/urea sequencing gel. Autoradiograph at -70^{o}C overnight using Kodak X-Omat S film.

2.45 GENE CLEANING PROCEDURE.

After visualization of the DNA within the type I agarose gel on a long wave transilluminator (300-360nm), the band was excised. This was trimmed and added to 2-3 volumes of "NaI" solution (approximately 500ul) and heated to 55^{o}C until the agarose had completely dissolved. 5ul of "Glassmilk" suspension was added quickly and the contents were placed on ice for 5 mins. After a 5 second microcentrifugation, the supernatant was discarded and the pellet washed in ice-cold "New" solution by resuspending, re-spinning and discarding the supernatant. Any traces of "New" were removed using a micropipettor. The DNA was eluted from the glass beads by adding a volume of TE (pH8.0) and heating to 50^{o}C. Spin for 10 second to pellet the glass beads and remove the supernatant. Repeat. Pool the supernatants and finally centrifuge to remove all traces of glass. The DNA can be ethanol precipitated but this is not necessary. The compositions of "NaI", "Glassmilk" and "New" were undisclosed.

CHAPTER THREE.

CLASS I IMMUNOPRECIPITATION ANALYSIS OF THE CHORIOCARCINOMA CELL LINE JEG-3.

INTRODUCTION

3.1.1.

The introduction to this chapter gives a brief review of the literature landmarks at the start of the research period.

Sunderland et al. (1981a;b) illustrated that class I molecules were not expressed by villous syncytiotrophoblast but that extra-villous trophoblast forming the cytotrophoblast cell columns clearly exhibited class I molecules. Redman et al. (1984) subsequently discovered that these molecules consistently failed to bind antibodies specific for the foetal HLA-A and -B phenotype and suggested that MHC antigen expression may be restricted to HLA-C or some other unidentified class I antigen. Wells et al. (1984) noted differences in reactivity with W6/32 and 61D2 and it was suggested that class I molecules may have undergone alterations in immunogenicity.

On the basis of these results, the initial aim of the research set out was to isolate HLA class I homologous cDNA clones from a cytotrophobolast cDNA library in order to investigate the nature of the molecule(s) expressed. However, attempts to detect class I homologous sequences were unsuccessful. Subsequently, immunoprecipitation studies showed that the class I heavy chains of human chorionic cytotrophoblasts, a human choriocarcinoma cell line BeWo (Ellis et al. 1986) and also baboon syncytiotrophoblast (Stern et al. 1987), had lower molecular weights, 40kDa and 41kDa respectively, than did the classical 45kDa molecules. The aim of the research focused on isolating such class I cDNA's from a choriocarcinoma library.

3.1.2 JEG-3 Choriocarcinoma Cell Line.

Current work in this area focuses on the precise nature of the stimulus and this necessitates good preparations of the cell type in question, namely the extra-villous cytotrophoblast. Since the latter are difficult to

separate from uterine decidua and sustain in culture, a model choriocarcinoma (JEG-3) known to be derived from extra-villous cytotrophoblast was used in this study.

Hertz, (1959), developed an in vivo model of human choriocarcinoma by its hetero-transplantation into cortisone-conditioned hamster cheek pouches (WO strain). From this original material, Patillo and Gey, (1968), Kohler and Bridson, (1971) and Patillo et al. (1971), succeeded in establishing in vitro growth of three separate clones, BeWo, JEG and Jar respectively. From eight subclones of JEG cultured on fibroblast feeder layers, JEG-3 was found to produce the highest levels of the placental hormone human chorionic gonadotrophin (Kohler and Bridson, 1971). Hamilton et al. (1979) have shown that JEG-3 cells can be induced to produce placental alkaline phosphatase by various biological chemicals including some corticosteroid hormones and sodium butyrate. Progesterone (also a placental hormone), is the major steroid synthesized by JEG-3 but there is no 17 beta-hydroxylase or 17-20 desmolase activity which is typical also of placental cells (Bahn et al. 1981). As well as this, the cells display a large number of high affinity EGF receptors. Takamizawa and Sekiya, (1984), reviewed the cell biology of choriocarcinoma. In culture, mononuclear cells (cytotrophoblast like cells) with a prominent nucleus seem to be in excess over a relatively small number of giant multinuclear ones which are syncytiotrophoblast-like. Thus, the choriocarcinoma (JEG-3) is a highly malignant tumour and exhibits many of the steroidogenic capabilities and enzyme blocks of the normal placenta.

Even though class I expression is evident on some choriocarcinomas, both BeWo (Ellis et al. 1986) and JEG-3 (P.L.Stern pers. comm.) cells have proven difficult to tissue-type.

3.1.3. Aims

The aim of the immunoprecipitation experiment carried out in section 3.2 was to investigate the nature of the class I molecules expressed on the JEG-3 choriocarcinoma cell line which is known to be weakly positive for HLA (Hunt et al. 1987). Section 1.2.6 of the introduction outlines similar work carried out previously in other laboratories in

detail and will be referred to in the discussion of this chapter.

RESULTS

3.2. Immunoprecipitation Analysis of Surface Labelled JEG-3 Cells.

Choriocarcinoma JEG-3 cells were grown in RPMI medium in large 75cm^3 tissue culture flasks. (For very nearly confluent growth, there are approximately 4x10^4 cells/cm^2). The expression of class I or class I-like molecules was investigated by immunoprecipitation with two anti-class I monoclonal antibodies (W6/32 and 61D2) and anti-B_2-microglobulin (BM.63)(Figure 3.2.1). JEG-3 cells were surface labelled by the 125-I-lactoperoxidase-glucose-oxidase technique and the immunoprecipitates were electrophoresed on a 10% SDS polyacrylamide gel (see sections 2.10 and 2.11).

W6/32, a monoclonal antibody directed towards a common framework determinant on class I heavy chains was shown to immunoprecipitate two class I heavy chains of 45kDa and 41kDa; the latter molecule being the major component. The surface immunoprecipitation of these heavy chains with BM.63 demonstrated clearly that both molecules were associated with B_2-microglobulin. The monoclonal antibody 61D2 (directed against one other class I heavy chain determinant) precipitated the 45kDa molecule but did not react with the major 41kDa component.

DISCUSSION

3.3.

The immunoprecipitation results show that there are at least two class I molecules expressed on JEG-3 cells which differ in size. The pattern of the immunoprecipitation suggests that they differ in their ability to bind W6/32 and 61D2. Similar results to the above have been obtained with BeWo cells (Stern et al. 1988), as might have been expected since both cell lines were derived from the same choriocarcinoma material. Although both JEG-3 and BeWo cell lines have previously been shown to be weakly positive for HLA a tissue type has been difficult to obtain. It might be expected that at least one of the molecules may be serologically recognized but the sparsity of sera and low levels of surface expression has probably prevented their

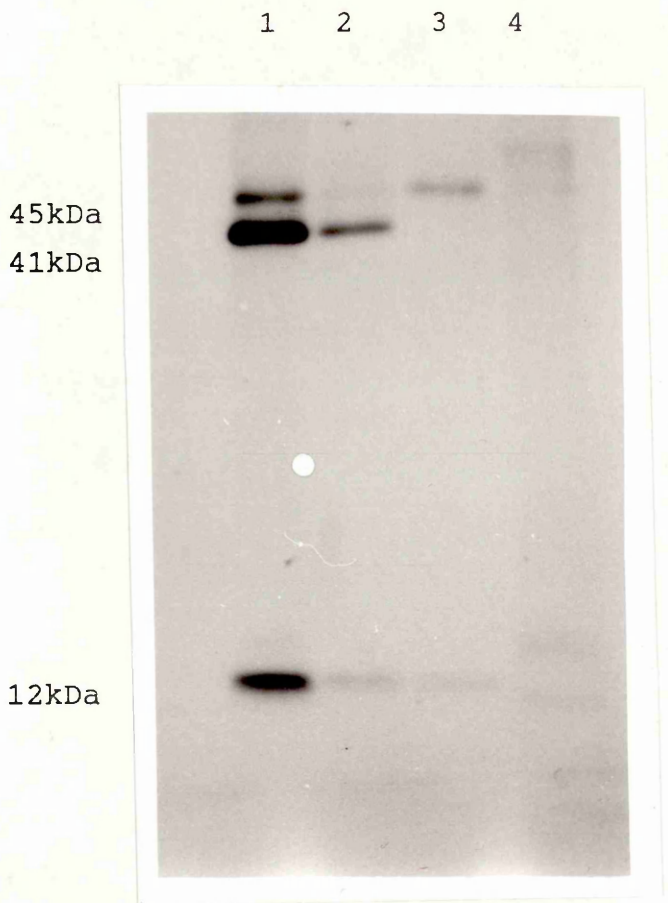


Figure 3.2.1 Class I Immunoprecipitation Analysis of JEG-3 Cells.

(1)-W6/32 anti-class I

(2)-BM.63 anti-B_2-microglobulin

(3)-61D2 anti-class I

(4)-Normal Rabbit Serum

specific typing.

Cloning and sequencing a number of class I cDNA clones would distinguish whether the class I proteins were derived from a common but alternatively spliced transcript (see section 1.1.7), or whether they originated from two separate loci. It is possible that the 41kDa molecule is the result of alternative splicing of the mRNA coding for the 45kDa protein. However, to date, no one has shown that alternatively spliced transcripts code for MHC proteins which exhibit modified/different or loss of function. It was thought that the difference in size of the two immunoprecipitated molecules may have been due to differing degrees of glycosylation. Although both heavy chains in BeWo were found to be glycosylated, treatment with N-glycanase did not convert these molecules into a single smaller component (P.L.Stern, pers. comm.).

Interestingly, Ellis et al. (1986) did not immunoprecipitate a classical 45kDa heavy chain from either the BeWo choriocarcinoma or chorionic cytotrophoblast cell membranes and had therefore concluded that classical class I molecules (usually of 45kDa), were not expressed by these cell types. Thus, the results of the two experiments are apparently qualitatively different. There is the possibility however, that the concentration of polyacrylamide (12%) used in the SDS gel (Ellis et al. 1986), may have been slightly too high and prevented the resolution of the two components. Subsequent studies by Stern et al. (1988) have confirmed the presence of the 45kDa molecule in BeWo cells. However, where the expression of the 45kDa molecule is concerned, one should not extrapolate the class I immunoprecipitation results for BeWo and JEG-3 cells to those of chorionic cytotrophoblasts. Further studies are necessary to confirm whether or not the 45kDa heavy chain is present on chorionic cytotrophoblast membranes. The lack of 61D2 immunostaining on various trophoblast populations (Hsi et al. 1984; Wells et al. 1984; Earl et al. 1985) supports the immunoprecipitation data of Ellis et al. (1986) if one accepts that 61D2 normally reacts with classical MHC structures. However, the immunoprecipitation data show at least a 5-fold (as assessed by eye) predominance of the 41kDa component and it could well be that the immunostaining studies using 61D2 were not sensitive enough to detect the low levels of the 45kDa cell

surface protein. Indeed, the experiments of Hunt et al. (1987) agree with this suggestion, since only after gamma-interferon induction of JEG-3 cells could class I cell surface expression be detected clearly using 61D2, whereas W6/32 and BB7.5 detection of class I did not require further induction with gamma-interferon. These results will be referred to again with reference to additional collaborative data in the final discussion.

At this stage, it could be argued that only the choriocarcinoma cells express 45kDa molecules though clearly the situation needs clarifying. It is probably worth being reminded at this point that the class I molecules found on baboon syncytiotrophoblast are 41kDa (Stern et al. 1988), and those from mixed rat placental cells are found to be 41 and 45kDa (Billington and Burrows, 1989).

If the two molecules indeed are different, then it would be reasonable to predict that they also have different functions but what these are and whether they are both absolutely required remains to be determined. Another interesting point is that the molecules are clearly expressed to very different levels and this may also be a requirement and be related to their individual functions. This latter point will be taken further in the final discussion.

CHAPTER FOUR

PREPARATION AND ANALYSIS OF RNA FROM UNINDUCED AND GAMMA-INTERFERON TREATED CHORIOCARCINOMA CELLS.

INTRODUCTION

4.1.1

Preparations of JEG-3 RNA were analyzed for the presence of intact HLA mRNA since these samples were to be used in cDNA synthesis. The gamma-interferon induction profile of HLA mRNA was investigated since this might lead to an increased representation of the number of class I cDNA clones, and so increase the chance of obtaining such a clone. Work published recently also shows JEG-3 gamma-interferon induction profiles (Hunt et al. 1987).

RESULTS

4.2. Preparation of RNA from uninduced and Gamma-Interferon Treated JEG-3 cells and also from KN_2 cells.

Maniatis et al. (1982) estimates that the yield of total RNA from a single cell is 10^{-5}ug. Depending upon the experiment, appropriate numbers of cells were grown (for very near confluency they are $40x10^3$cells/cm^2). RNA was prepared from both uninduced and gamma-interferon (varying concentrations) treated JEG-3 cells, and also from a lymphoblastoid cell line KN_2 (strongly HLA^+).

The quantity of RNA was estimated by $A_{260/280}$ and checked on agarose gels as outlined below. Different amounts (1-10ug) of total RNA prepared from untreated JEG-3 cells were electrophoresed on a 1% non-denaturing agarose gel alongside BglII/HincII digested pBR322 DNA markers (figure 4.2.1). All samples showed intact RNA (not degraded) as illustrated by the presence of three sharp ribosomal RNA bands (28S, 18S rRNA and tRNA), and a background smear of mRNA. The actual size of eukaryotic 28S and 18S rRNA is 3614 bases and 2244 bases respectively, tRNA is 70 bases. The rRNA bands were visible indicating that the RNA was intact.

Unpublished data of Joan Hunt indicated that induction of class I with gamma-interferon was optimal at 500-700U/ml and so these levels were covered in preliminary induction experiments. Initially, (first preparation) the concentration of gamma-interferon varied in culture from 0-700U/ml and in the second preparation ranged from 0-

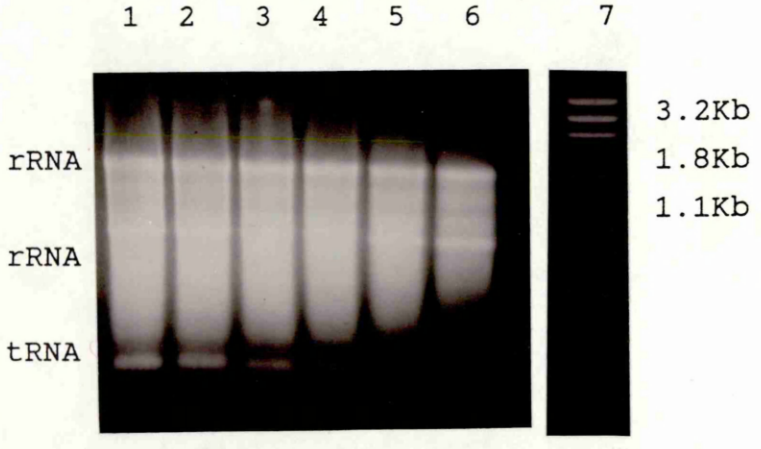


Figure 4.2.1 1% Agarose gel showing increasing amounts of JEG-3 total RNA.
Tracks (1-6)-loaded respectively with 10,5,4,3,2 and 1ug total RNA, (7)-HindIII/BglII pBR322 DNA markers.

10,000U/ml. These preparations used a different batch of gamma-IFN which had been obtained on separate occasions (gift from Dr. Page, Wellcome Biotech). 5ug samples from each preparation were electrophoresed on a 1% agarose gel (see figures 4.2.2 and 4.2.3 respectively). All samples showed very nearly equal loadings of intact RNA, although slight variation can just be discerned.

10ug JEG-3 RNA was electrophoresed on a formaldeyde northern gel and visualized by ethidium bromide staining in order to check their condition (figure 4.2.4). EcoRI/HindIII Lambda DNA markers (denatured to single strands) were used although these are not strictly accurate for RNA. Both rRNA bands and a background smear of mRNA are visible.

4.3. Optimization of background levels of hybridization.

At first the background level of hybridization on Northern blots was too high and it became necessary to empirically optimize pre-/hybridization conditions. The level of HLA mRNA is known to be typically very low (less than 0.1% from my own studies presented in chapter 5 and other workers) and as such, did not help this situation. Non-specific background hybridization levels were reduced to a minimum by varying the concentration of some of the components of the solution in existing protocols. A formaldehyde gel (no RNA samples) was blotted with PAL Biodyne^{Tm} nylon membrane as usual, cut into quarters and probed using the conditions outlined below (figure 4.3.1). The salt concentration (5xSSPE) and that of formamide (50% v/v) were unaltered during the analysis whist other parameters were adjusted as follows:

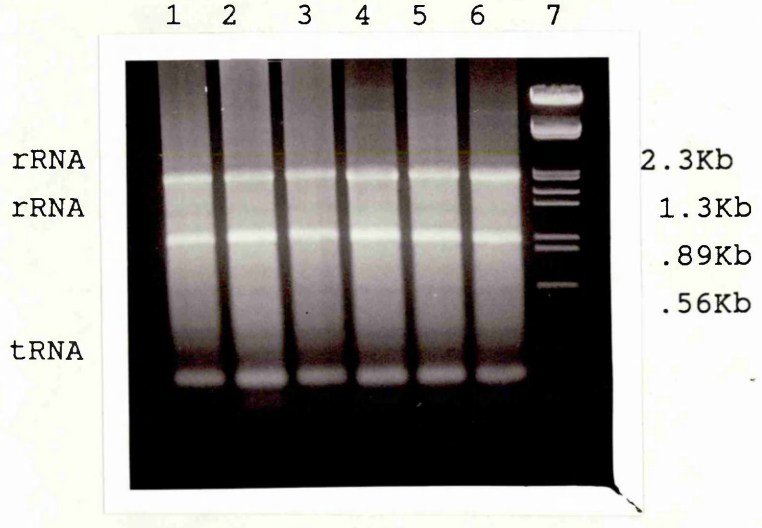


Figure 4.2.2 1% Agarose gel illustrating 5ug samples of total RNA from the first preparation of uninduced and upto 700U/ml Gamma-IFN treated JEG-3 cells.
Track (1-5)-700,500,350,200,100U/ml Gamma-IFN induced RNA respectively (6)-uninduced JEG-3 (7)-lambda HindIII/EcoRI DNA markers.

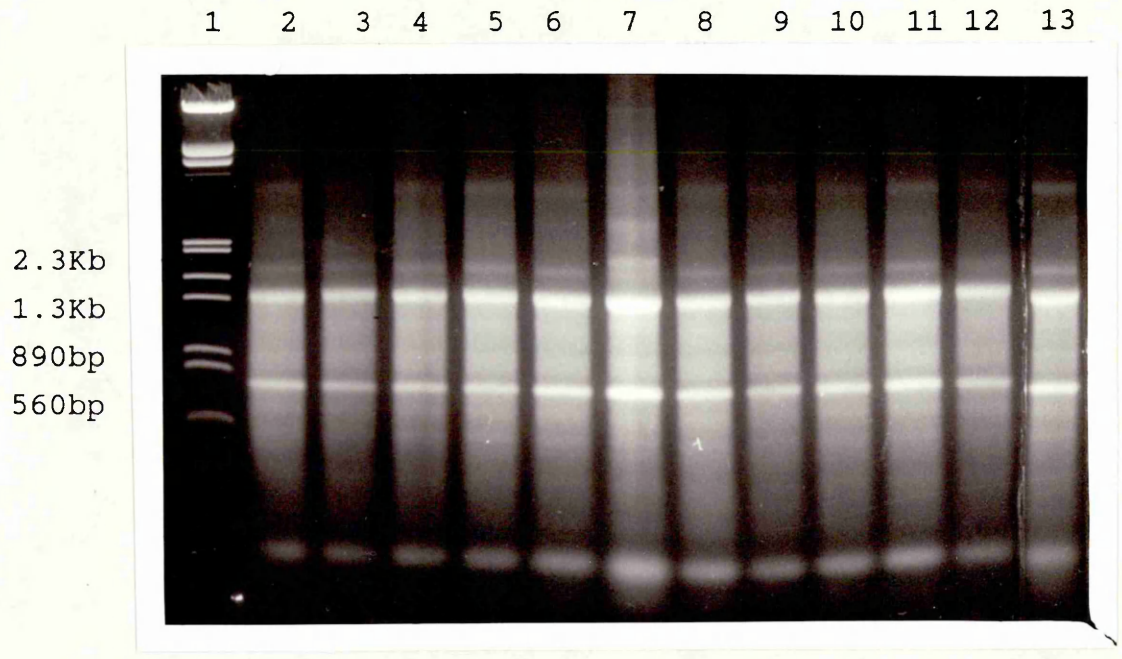


Figure 4.2.3 1% Agarose gel illustrating 5ug samples of total RNA from the second preparation of upto 10,000U/ml Gamma-IFN treated JEG-3 cells.
Tracks (1)-lambda HindIII/EcoRI markers (2-12)-10,000, 5000, 2000, 1000, 700, 500, 300, 150, 100, 50 and 25U/ml Gamma-IFN induced samples (13)-uninduced JEG-3.

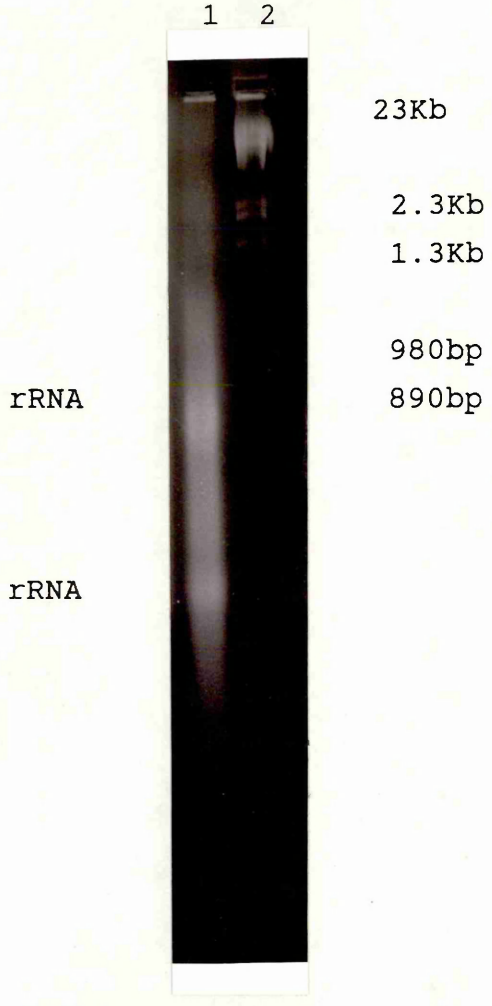


Figure 4.2.4 Ethidium bromide stained JEG-3 total RNA on a Northern Formaldehyde gel.
Track (1)-10ug total JEG-3 RNA, (2)-lambda HindIII/EcoRI DNA markers.

Hybridization solution	Comments
1 - 50% Formamide	Conventional solution
5x SSPE	
5x Denhardts	
10% Dextran Sulphate	
100ug/ml salmon sperm DNA	
2 - 50% Formamide	
5x SSPE	
5x Denhardts	No Dextran Sulphate
100ug/ml salmon sperm DNA	
3 - 50% Formamide	
5x SSPE	
5x Denhardts	No Dextran SO_4 or s/sperm.
4 - 50% Formamide	
5x SSPE	
10x Denhardts	2x Denhardts and s/sperm.
200ug/ml salmon sperm	No Dextran SO_4

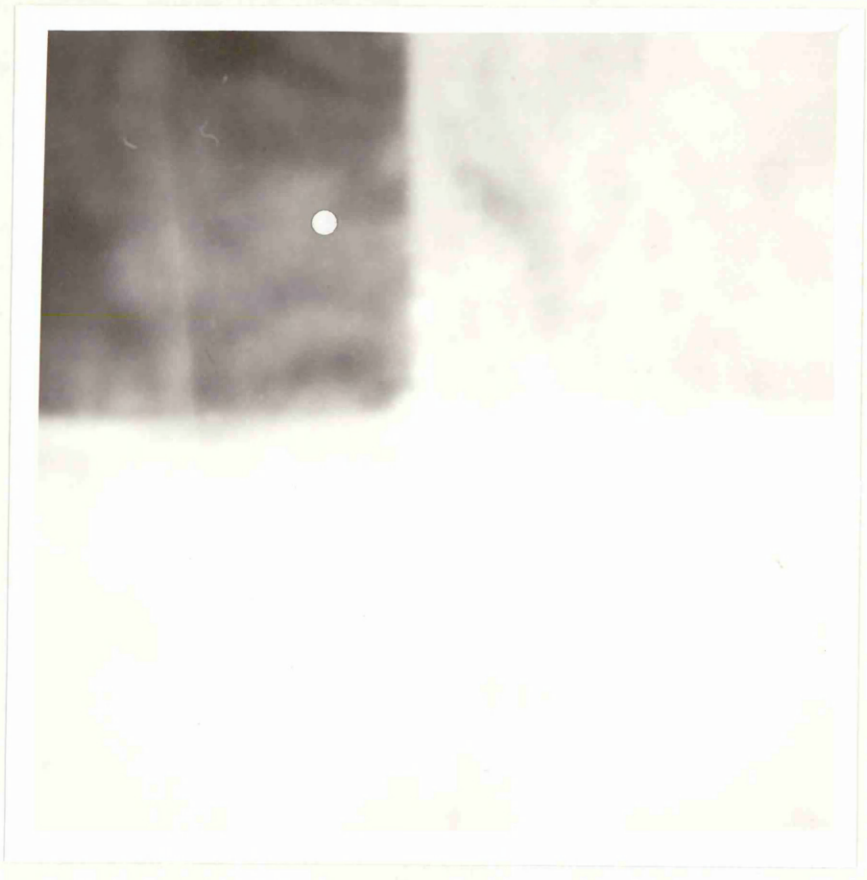
As can be seen from figure 4.3.1 the conventional pre-/hybridization solution gave rise to the highest levels of background hybridization. The conditions used with filters 2 and 4 proved to be the most effective in reducing background levels of hybridization and are fully described in section 2.17. Indeed, the signal to noise ratio was much improved in subsequent Northern experiments.

4.4. Hybridization Analysis using HLA Class I DNA Probes.

4.4.1. Dot Blot Hybridization.

Gamma-interferon induced JEG-3 (0-700U ml shown in figure 4.2.2) and KN_2 total RNA (0.05ug-20ug) and mRNA samples (1-200ng) were dot blotted onto PAL Biodyne^{Tm} nylon membrane and hybridized to the 1.4Kb HLA-B7 cDNA probe (figure 4.4.1). The membrane included both positive * **(pDP 1.4Kb 0.5ng-100ng** with pBR322 4.3Kb 1.5ng-300ng) and negative **(pBR322 1.5ng-300ng)** control samples. The amounts of RNA loaded onto the dot blot was controlled for by a comparison with the ethidium fluorescence of electrophoresed samples shown in figure 4.2.2 (see below and discussion). A

1 3



2 4

Figure 4.3.1 Northern background levels of radioactivity after using various hybridization conditions.
Blocks 1-4 show the hybridization background levels when using the solutions described in section 4.3.

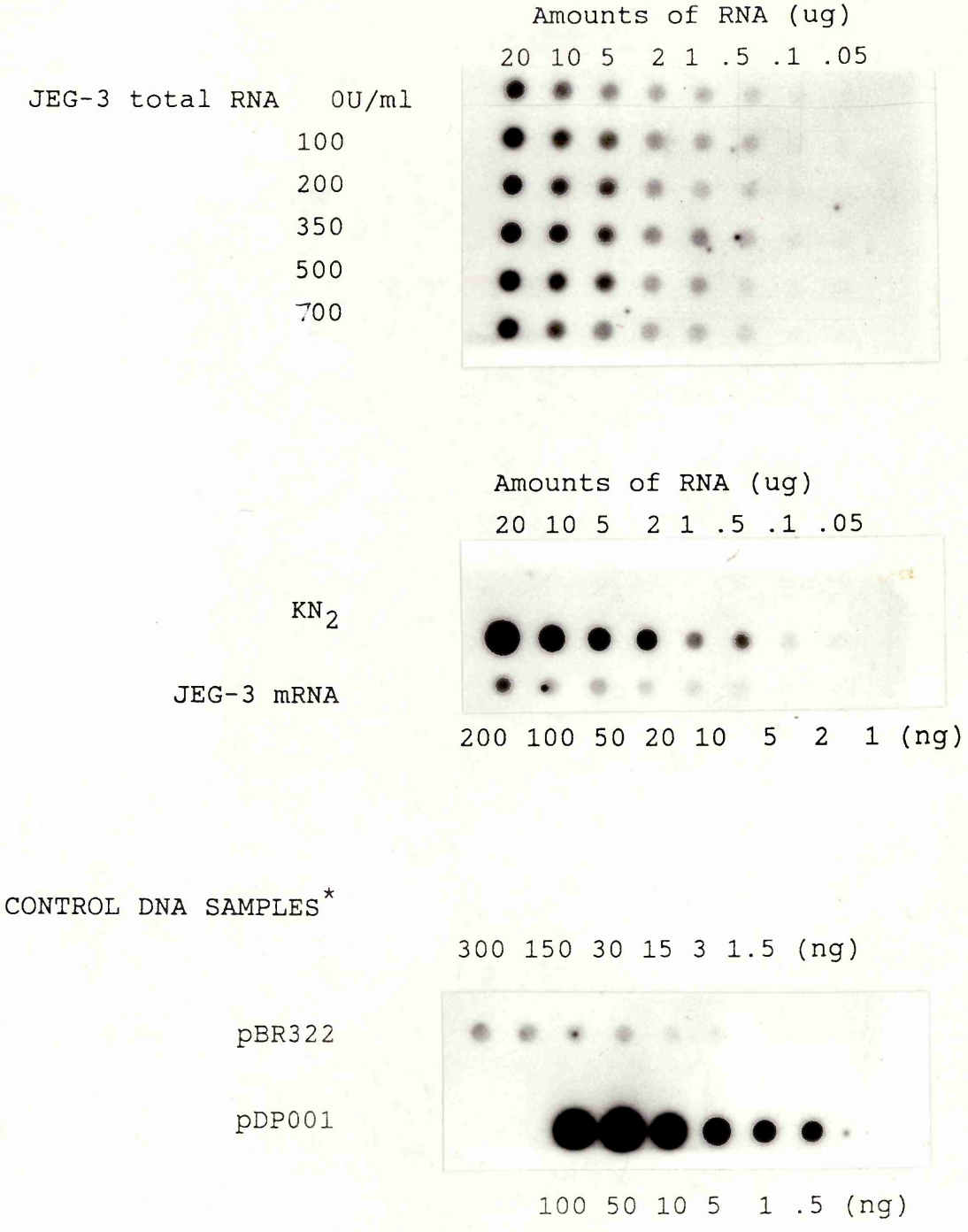


Figure 4.4.1 Dot Blot Arrangement of KN_2 and Gamma-IFN induced JEG-3 RNA samples hybridized with a 1.4Kb class I HLA-B7 cDNA probe. * - see text.

comparison of equal amounts of each RNA from both uninduced and gamma-IFN treated JEG-3 samples indicates no obvious induction levels of HLA mRNA resulted from when any concentration of gamma-IFN is used for 30 hours.

4.4.2. Northern Hybridization.

10ug total RNA samples from KN_2 and gamma-interferon induced (0-10,000U ml shown in figure 4.2.3) JEG-3 cells were electrophoresed on a Northern formaldehyde gel (figure 4.4.2) and after transfer, were hybridized to the 1.4Kb HLA-B7 cDNA probe. The amounts of RNA loaded onto the dot blot was controlled for by a comparison with the ethidium fluorescence of electrophoresed samples shown in figure 4.2.3 (see below and discussion).A duplicate filter was probed with pmS3, a 900bp incomplete actin cDNA probe (section 2.20) in order to normalize levels of RNA, since this is not induced by gamma-interferon. Unfortunately, the actin probe did not hybridize as clearly as expected and the Northern autoradiograph could not be photographed. This latter control was repeated and filters washed at reduced stringency but with the same results.

For definitive quantitation of class I mRNA levels, such controls are most important. The level of fluorescence of ethidium bromide staining of rRNA bands as an indication of the amounts of RNA loaded is a poor way of controlling the latter, especially since rRNA is not as readily degraded as mRNA.

Both the dot-blotting and Northern transfer experiments showed the presence of class I mRNA but that gamma-interferon did not appreciably increase these levels as assessed by hybridization with a class I HLA-B7 cDNA probe. There does appear to be some induction of transcripts when the concentration of gamma-interferon is increased to 50U/ml (track 5). This induction is variable upto 1,000U/ml and is much reduced when the concentration of interferon is increased further. It is unwise to estimate the degree to which induction occurs since it is low. The amount of RNA in track 7 of figure 4.2.3 is slightly higher than the other samples and could account for the increased hybridization attained in track 8 (500U/ml) of figure 4.4.2. These experiments were only preliminary and the collective data suggested no dramatic increase of class I mRNA levels after

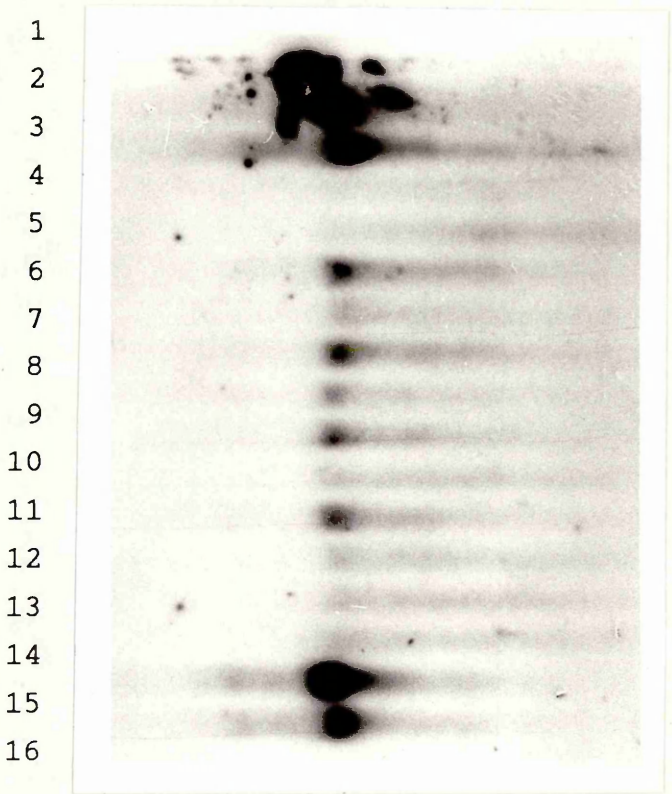


Figure 4.4.2 Northern Formaldehyde Gel of KN_2 and Gamma-IFN induced JEG-3 RNA samples (shown in 4.2.3) probed with a 1.4Kb class I HLA-B7 cDNA probe.

20ug total RNA samples. Tracks (1)-KN_2, (2)-500U/ml Gamma-IFN induced JEG-3 (large preparation), 5μg (3)-uninduced JEG-3, (4-14)-25, 50, 100 150, 300, 500, 700, 1000, 2000, 5000, and 10,000U/ml Gamma-IFN induced JEG-3, 20μg (15)-KN_2, (16)-500U/ml Gamma-IFN induced JEG-3 (large preparation).

gamma-interferon induction. Since class I mRNA was detected, attempts to clone the corresponding cDNA's were made immediately. Possible reasons for not obtaining induction are given in the final discussion. The positive control, KN_2-LCL gave stronger signals.

Northern gels (figure 4.4.3) using BglII/HincII restricted pBR322 end labelled radioactive markers showed the size of the mRNA to be approximately 1.6Kb. Figure 4.4.2, shows a slight difference in the mobility of the mRNA, with the JEG-3 band being slightly smaller than the KN_2 band. The resolution generally attained on Northern gels is known to be relatively poor. To see whether the class I mRNA was the same or different size in KN_2 and JEG-3 cells, RNA from each was electrophoresed as single and mixed samples and subsequently hybridized to the HLA-B7 probe (figure 4.4.4). Unfortunately the signal from the KN_2 sample was relatively too strong and masked that of the JEG-3 sample. This Northern however, does show that single KN_2 and JEG-3 total RNA samples migrate similarly. The reason for the difference in mobility of the two mRNA's in figure 4.4.2 is not clear but it could have been due to differing salt concentrations in the samples.

4.5. Preparation of mRNA using Oligo-dT Cellulose Affinity Matrix.

Gamma-interferon induced (500U/ml) total JEG-3 RNA was used to prepare mRNA (section 2.27). Initially, this separation was attempted using Amersham's messenger affinity paper (mAP), but this was relatively unsuccessful since the mRNA was not completely separated from the rRNA. In addition to this, the ethanol precipitated mRNA pellet was an orange colour (same as the paper) and this did not instill any confidence in this method! Oligo-dT cellulose affinity matrix gave much better results. 1ug JEG-3 mRNA (prepared using oligo-dT cellulose) was electrophoresed on a 1% agarose gel with 5ug total JEG-3 RNA from the original sample, and HindIII/EcoRI digested lambda markers (figure 4.5.1). The mRNA appears as a background glow as expected and does not appear to be degraded or contaminated with rRNA bands. On a Northern gel (figure 4.5.2) this mRNA can be hybridized to the HLA-B7 cDNA probe. The mRNA was used for in vitro

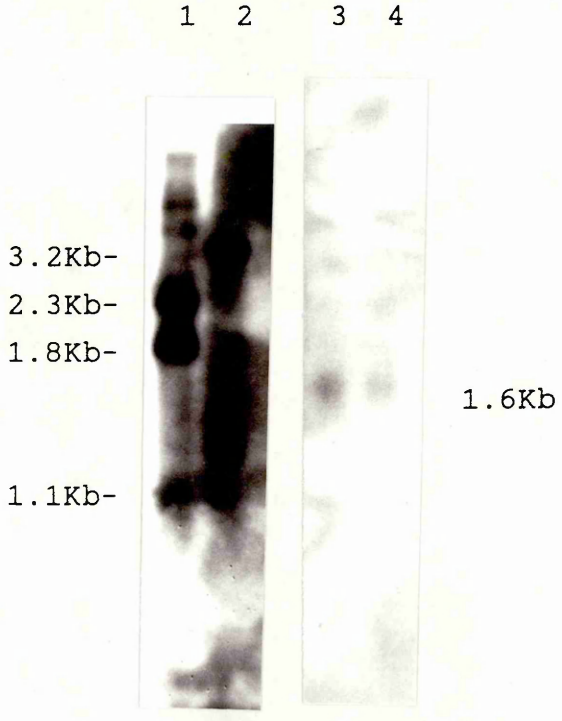


Figure 4.4.3 Northern Formaldehyde Gel of KN_2 and Gamma-IFN induced JEG-3 RNA illustrating the size of the class I HLA mRNA expressed in both.
Tracks (1)-pBR322 BglII markers, (2)-pBR322 HincII markers (3)-KN_2 RNA, (4)-JEG-3 RNA.

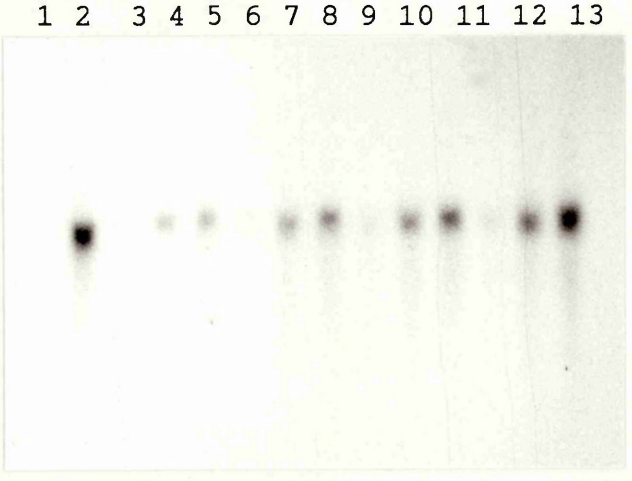


Figure 4.4.4 Northern Formaldehyde Gel of mixed RNA samples probed with a class I HLA-B7 cDNA probe. K=KN_2, J=JEG-3

Tracks (1)	-	20ug K.	
(2,3,4)-	5ug J,	2.5ugK/2.5ugJ,	5ug K
(5,6,7)-	10ug J,	5ugK/5ugJ	10ug K
(8,9,10)-	15ug J,	7.5ugK/7.5ugJ	15ug K
(11,12,13)-	20ug J,	10ugK/10ugJ	20ug K

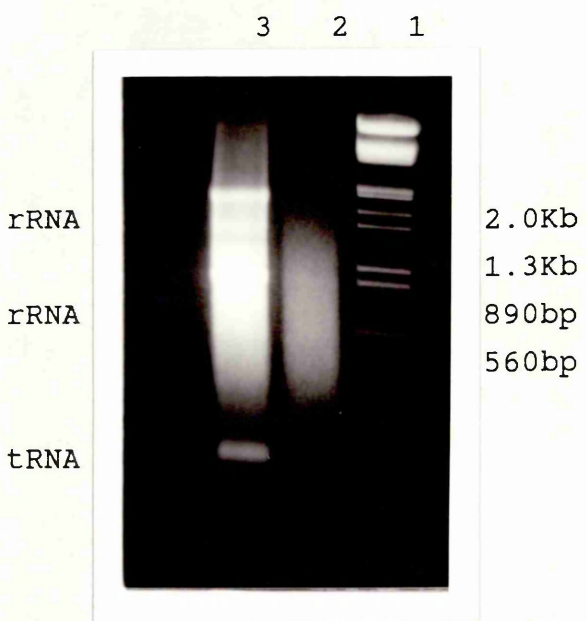


Figure 4.5.1 Agarose gel electrophoresis of Gamma-IFN induced JEG-3 mRNA isolated by oligo-dT cellulose affinity matrix.
Track (1)-lambda HindIII/EcoRI DNA markers, (2)-1ug JEG-3 mRNA, (3)-5ug JEG-3 total RNA.

Figure 4.5.2 Northern Formaldehyde Gel of mRNA from Gamma-IFN induced JEG-3 cells probed with a class I HLA-B7 cDNA probe.
Tracks (1-6)- 0.5, 0.25, 0.1, 0.065, 0.05, and 0.025ug mRNA.

translation analysis (see below) and for cDNA synthesis (chapter 5).

4.6. In vitro Translation of messenger and total RNA from JEG-3 cells.

Total RNA from untreated and gamma-interferon induced (500U/ml for 30 hours) JEG-3 cells and mRNA from induced cells was in vitro translated (figure 4.6.1) as described in section 2.28. The samples were exposed to X-ray film for only three days and consequently the signals are quite weak. During vacuum drying under heat, the gel cracked but it is clear that all samples have been translated since protein bands are visible upto the 92kDa protein marker. Discrete bands indicated that the mRNA samples were intact and had not been degraded. The H_2O blank gave the expected results.

DISCUSSION

4.7.

Gamma-interferon is known to increase the levels of class I mRNA (reviewed by Rosa and Fellous, (1984) see also section 1.1.11. - Regulation of Class I Gene Expression by Immune Modulators). It was used in this study to increase the levels of class I mRNA species among the total mRNA population in order to elevate the chance of detecting the corresponding cDNA clone (to be described in chapter 5). Whether or not this lymphokine preferentially up or down regulated the expression of existing molecules on JEG-3 cells was not known at the time of the experiment and this point will be referred to in the final discussion, since collaborative data completed later sheds light on the results obtained here.

Figure 4.2.1. illustrates very simply the appearance of ethidium bromide stained RNA on an agarose gel and indicates that over a range of 1ug to 10ug (previously assessed by spectrophotometry) one can assess the variability in the loading between samples and that the most visible range of variability is between 2-5ug. Figures 4.2.2 and 4.2.3 showed that the 5ug amounts of total RNA loaded were comparable. The Dot Blot and Northern data (figures 4.4.1 and 4.4.2 respectively) suggested that gamma-interferon did not appreciably increase the levels of class I mRNA and that

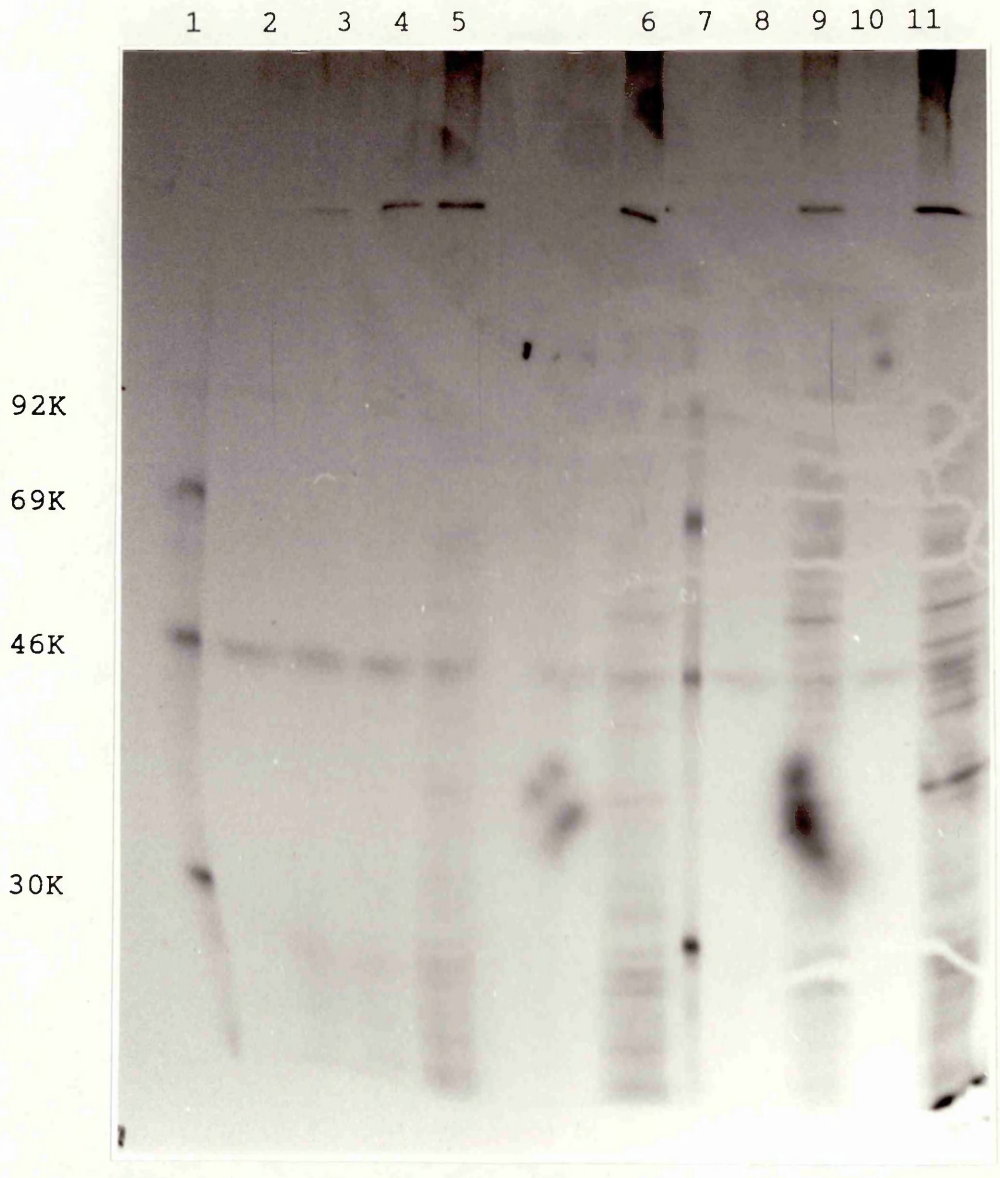


Figure 4.6.1 Autoradiograph of [^{35}S]-methionine labelled <u>in vitro</u> translated protein products of untreated and Gamma-IFN induced JEG-3 messenger and total RNA samples.
Track (1)-10n/Ci mw markers, (2)-water blank, (3,4,5,6)-0.005, 0.01, 0.05, 0.2ug mRNA from induced JEG-3 cells (7)-10nCi mw markers, (8,9)-0.5ug, 1ug 500U/ml Gamma-IFN total JEG-3 RNA, (10,11)-0.5ug, 1ug uninduced total JEG-3. The unlabeled middle two tracks were not used for technical reasons.

there was no further induction in samples treated with more than 300U/ml. The hybridization signals were relatively weak and did not "blacken" the autoradiograph to saturation. The radioactivity of individual dots was not counted since there was no obvious induction and these results were therefore assessed by eye. Subsequent to this, an actin probe was acquired in order to properly control for the relative amounts of RNA, but unfortunately the levels of hybridization attained were extremely low and the signals were diffuse and a decent photograph could not be obtained. The signal was quite even across the samples. These quantitative data should be properly controlled for as explained earlier. Since the amounts of total RNA appear to be reasonably comparable on agarose gels, the levels of class I induction by gamma-interferon were assessed. Induction was not particularly apparent although it was quite clear that class I mRNA was present and these experiments were taken over by attempts to clone the corresponding cDNA.

Hunt et al. (1987) subsequently published very clear Northern data of a time course and induction profile of class I JEG-3 gamma-interferon induction. First of all, it is true to say of Hunts results that there is definite induction of transcripts upto 1000U/ml and that over a period of 24hrs when using 1000U/ml the transcripts do accumulate overall. However, the two sets of data are inconsistent in two samples from each. The two control samples show differing amounts of class I transcripts, as do the samples treated with 1000U/ml for 24hrs. This could be due to a number of reasons (including different exposure times) which may or may not be a result of the following. Previous literature (Takamizawa and Sekiya, 1984) had reported syncytial elements present in the JEG-3 choriocarcinoma (see section 3.1.2), and it could be that the cells differentiate in <u>in vitro</u> culture (if they reach high density) as do normal cytotrophoblasts differentiate into syncytiotrophoblast. The latter are known not to express class I and so the effect of differentiation may effectively reduce the amounts of class I mRNA in a preparation.

Recently, a comparison of class I immunoprecipitation studies on BeWo cells before and after treatment with 2000U/ml gamma-interferon for 24 hours showed at most a three fold increase, and this was accompanied by

the appearance of a 51kDa immunoprecipitable component, the nature of which was unknown (Stern et al. 1988). Hunt et al. (1988) used the elaboration of renin to evaluate the viability of chorionic cytotrophoblasts in culture. Cultures exposed to interferon for 48hours resulted in a reduction in the amount of renin produced (reduced viability) especially under the influence of both 100 and 1000U/ml interferon.

The conditions that were finally chosen to induce class I mRNA for the cDNA library were considered to be moderate: 500U/ml gamma-interferon for 30 hours.

CHAPTER FIVE

SYNTHESIS, CLONING AND ISOLATION OF CLASS I cDNA CLONE DERIVED FROM GAMMA-INTERFERON INDUCED JEG-3 CHORIOCARCINOMA CELLS.

INTRODUCTION

5.1.

To isolate HLA class I cDNA clones from JEG-3 a cDNA library representing the mRNA population of these cells was constructed in lambda gt10. The frequency at which the cDNA clones of a particular mRNA occur should be proportional to the abundance of that species in the population. Levels of class I mRNA are known to be very low (rare) and in order to isolate such a cDNA it was necessary to choose a vector which would allow efficient cloning and selection of a large number of recombinants (at least 10^5). The use of a lambda vector in preference to a plasmid for the construction of a cDNA library takes advantage of the high efficiency and reproducibility of *in vitro* packaging of lambda DNA and the increased efficiency of nucleic acid screening of plaques over colonies.

Lambda gt10 (see section 2.6) is a good cloning vector for this purpose since it can accept DNA inserts up to 7.6Kb and its propagation on two different strains of *E. coli* also allows for an efficient biological selection system for recombinant phage.

Lambda gt10 contains a unique EcoRI restriction cloning site within the phage repressor gene (cI) into which the cDNA is inserted (see figure 5.1.1). The disruption of cI forces the bacteriophage to follow the lytic pathway (leads to cell lysis) and gives the effect of a clear plaque in the areas of infection. Lysogenic integration of lambda into the *E. coli.* chromosome results in the formation of a turbid plaque. The bacterial cell survives the lysogenic bacteriophage and does not produce phage particles. Both bacteriophage and host factors are involved in this process. Bacteriophage cII and cIII protein products negatively regulate the lytic process by positively regulating cI early transcription and hence any bacteriophage mutant in either the cI, cII or cIII genes follows the lytic pathway.

Parental lambda gt10 are cI^+ and produce turbid plaques on wild type *E. coli.* and on the L87 strain used in

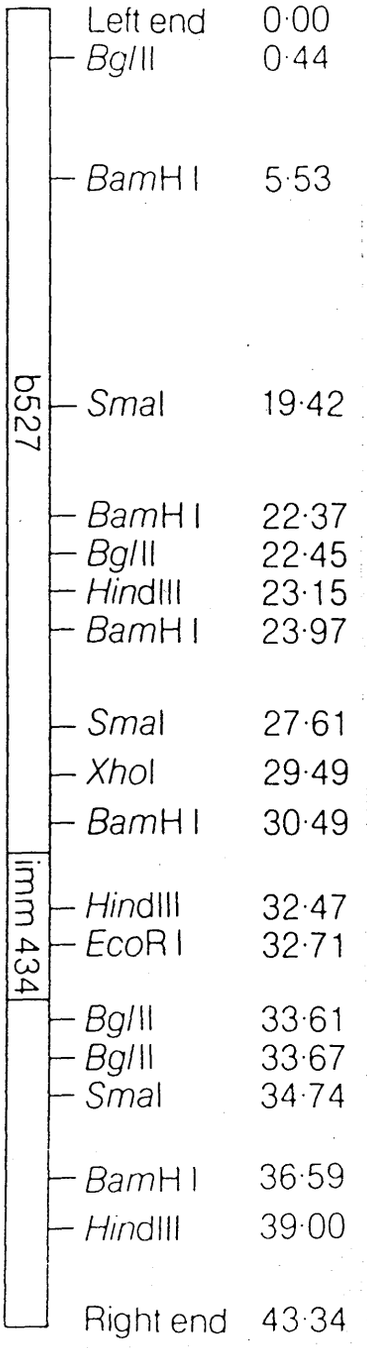


Figure 5.1.1 Restriction endonuclease cleavage map of Lambda gt10. Hyunh et al. (1985).

this study. Parental phage are also forced into the lysogenic pathway on high frequency lysogeny (hfl^+) strains (NM514 used in this study). However, when cI^- bacteriophage are grown with wild type and L87 strains, lysogeny cannot occur and clear plaques result. Growing cI^- bacteriophage on NM514 (Murray et al. 1977), adds a stringent biological selection since these phage replicate and lyse the cells efficiently.

RESULTS

5.2. Synthesis of cDNA.

Messenger RNA obtained from (500U/ml 30 hours) gamma-interferon induced JEG-3 cells was reverse transcribed into first strand cDNA as described in section 2.29. Double stranded cDNA was prepared using the RNase H method of Gubler and Hoffmann, (1983) which involves the nicking of mRNA strands in mRNA.cDNA hybrids followed by the synthesis of second strand cDNA using the mRNA fragments as primers for DNA polymerase I. Globin mRNA (600bp) was used as a control.

The radioactive first and second strand cDNA products of cDNA synthesis were visualized by autoradiography after alkaline gel electrophoresis (see 2.30). cDNA samples were run alongside end-labelled DNA markers (lambda Pst1 and HaeIII phiX174) and globin cDNA on an alkaline agarose gel (see figure 5.2.1). The alkaline agarose gel illustrated that radioactivity was being incorporated into both first and second strand cDNA and that the products ranged in size from below 300bp to 5Kb. Globin cDNA (600bp) was seen with two unexpected lower molecular weight fragments whose presence cannot be explained with very much certainty, but one possible explanation could be that reverse transcription was arrested at sites of secondary structure yielding shorter products. The molecular weight species (above 600bp) can only be explained by suggesting that globin mRNA (commercially acquired) was synthesized from a vector and that the synthesis carried on past the DNA clone or that *in vivo* splicing of pre-mRNA was incomplete.

The presence of a strong band of approximately 1.8Kb in JEG-3 cDNA may represent abundant mRNA species of this particular size - although this does not seem to be mirrored during the second strand synthesis. The latter statement cannot be fully supported since overexposure of this track

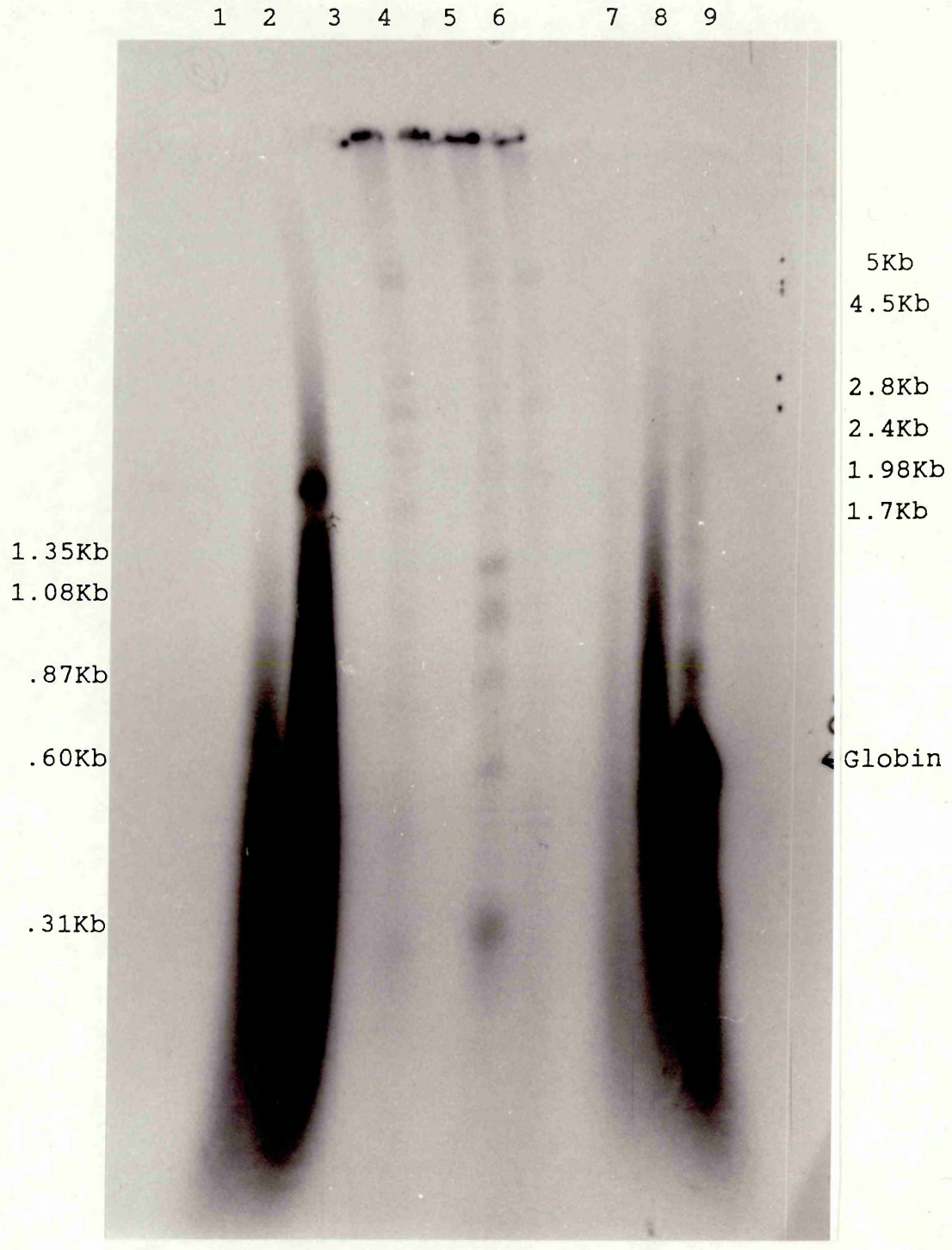


Figure 5.2.1 Alkaline agarose gel electrophoresis of radioactive first and second strand labelled JEG-3 cDNA products.

Track (1)-second strand labelled JEG-3 cDNA, (2)-first strand labelled JEG-3 cDNA, (3,4)-lambda Pst1 markers, (5,6)-HaeIII Phi X 174 markers, (7)-second strand labelled globin cDNA (8)-first strand labelled globin cDNA, (9)-double stranded (labelled first and second strand) globin cDNA after ethanol precipitation.

may reveal such a band.

The amount of cDNA synthesized (see section 2.29 for calculations) varied a great deal between the different mRNA preparations used throughout the study. This is probably due to the purity and quality of the mRNA if all other parameters are considered to be constant. The efficiency of reverse transcription varied between 10-20% whilst that of second strand synthesis varied between 60-75%. On average the amount of cDNA synthesized from 1ug mRNA was 200ng. This was stored frozen in water or as an ethanol precipitate at -70^{o}C.

5.3. Cloning of JEG-3 cDNA into Lambda gt10.

1ug cDNA was used as starting material and *in vitro* manipulated for cloning as described in detail in section 2.31. After methylation and separation of EcoRI linkers, 0.5ug cDNA was removed after ethanol precipitation resuspended in 6ul water and divided into 1ul, 2ul and 3ul volumes. These three samples were individually ligated into 1ug EcoRI cut arms of lambda gt10 (see section 2.31). Two other ligations were prepared: 50ng EcoRI cut control DNA ligated into 1ug lambda gt10 arms and 1ug lambda gt10 arms self ligated with no other DNA present (see section 5.4 for explanation of these controls).

The ligation reactions were packaged *in vitro* (as was 0.5ug uncut parental lambda gt10) and subsequently plated out as described in section 2.32 and 5.4.

5.4. Titration of Lambda gt10 recombinants.

In effect, 2/50th of each preparation of the library has been used up in titrating recombinants and 1/50th has been used in the dilution series (see section 2.32). 30ul phage solution was removed from a total of 500ul and serially diluted 10-fold into 270ul phage buffer. 100ul of each dilution was plated out onto both types of plating cells. Thus, 47/50th's of the library remained to be plated and screened.

After overnight incubation, the number of plaques was counted on both sets of plates whose phage were nearly confluent. If the phage are virtually confluent then these plates must be ignored since errors arise too easily when counting merged plaque forming units (pfu's). Likewise, ignore plates with too few plaques because the experimental

error increases with the progression of the dilution series for a number of reasons.

The numbers of plaques obtained on each E. coli host were normalized for the 10^2 dilution series for subsequent calculations. The % of clear plaques in each in vitro packaged reaction was calculated by using the following;

% of clear^c plaques = no. of clear^c plaques appearing on NM514 / (no. of clear^c plaques + no. of turbid^t plaques appearing on L87) x 100%.

This has been done for the five ligations (tubes 2-6) that were set up in section 5.3 and also for the packaged parental lambda gt10 (tube 1). The raw data is presented below with an accompanying explanation of the controls.

Tube	**% clear^c plaques**
Controls	
1 - 0.5ug lambda gt10 (parental)	0.38
2 - 1ug lambda gt10 arms (self-ligated)	0.8
3 - 1ug lambda gt10 arms + 50ng control DNA	12
Experimental	
4 - 1ug lambda gt10 arms + 50ng (1ul) JEG-3 cDNA	1.4
5 - 1ug lambda gt10 arms + 100ng (2ul) JEG-3 cDNA	2.0
6 - 1ug lambda gt10 arms + 150ng (3ul) JEG-3 cDNA	2.0

6ul total volume	

Calculate the total number of plaques (clear^c and turbid^t) in tube 1. This figure reflects the efficiency of the in vitro packageing reaction and should be greater than $2x10^8$pfu/ug. The % of clears in this tube gives an indication of the degree of biological selection and mutated forms of lambda, and should be between 0.1-1%.

The number of plaques counted on (or normalized) to the 10^2 dilution (remember to add the figures obtained from both sets of plates) plate represents 1/50th total number (strictly this is 0.9ul / 50 on 10^2 dilution plate but for our purposes such accuracy is not necessary). For tube 1, only 0.5ug parental lambda gt10 was packaged and so therefore the figure must be doubled.

Tube 1 - Total no. plaques = (4410^t + 17^c) x 50ul x 2

= 442700

= 4.4 x 10^5pfu/ug.

This figure is exceptionally low and may be because of problems in the particular packaging mix.

Repeat the calculation for tube 2 as for tube 1. This is an important control, since the titre gives a measure of the efficiency of ligation and should be approximately 10^7pfu/ug.

Tube 2 - Total no. plaques = (1140000^t + 9200^c) x 50ul

= 5746000

= 5.7 x 10^7pfu/ug.

Two other numbers can be obtained by comparing the ratio of the titre on L87 and NM514. The arms selective ratio should always be >100 and is almost always lower than the parental DNA ratio (in tube 1).

In this case, the value for the arms selective ratio was 124 (or 0.8%, percentage of clears). This is an invariant property of the specific batch of lambda gt10 arms and the host cells taken together. It is directly applicable to tubes 3-6 since the same batch of lambda gt10 and the host cells were used. The L87 titre represents the total products of the ligation/packageing reaction which are mainly recreated parental lambda gt10 (in this experiment 99.2%). If this titre is divided by the arms selective ratio, a figure is obtained for the true background on NM514 if no recombinants have been formed (no insert DNA added to the ligation reaction). Subtracting the background figure from the actual titre obtained on NM514 cells in the cDNA reaction should give a reasonable count of the number of recombinants obtained and also the % of true recombinants out of the clear plaques. Obviously, if the same amount of lambda gt10 arms has been used in each cDNA ligation then values can be worked out more easily as percentages. The figures obtained here for the % of true recombinants are in agreement with empirical data described in section 5.6.

Tube 3 - Total no. of plaques = (369000^t + 50900^c) x 50ul
50ng control insert = 20995000

= 2.1 x 10^7pfu/ug.

The percentage of clears is 12%, and the background (always

taken from tube 2) is 0.8%. The percentage of true insert containing recombinants out of the total number of clears is:

(12 - 0.8 / 12) x 100% = 93%

So the expected number of

true recombinants = 0.93 x 50900 x 50ul

= 2.4 x 10^6pfu/ug.

Tube 3 calculations should give an idea of the performance of the cloning procedure and should show definitive cloning of inserts. Success in cloning of this DNA (which can either be EcoRI linkered or EcoRI restricted DNA fragments) alongside failure of cloning experimental cDNA indicates that the latter could be the problem.

The same calculations have been completed on data from tubes 4, 5 and 6 where increasing amounts of JEG-3 cDNA (1ul, 2ul and 3ul = 500ng) were ligated into 1ug lambda gt10 arms. Figures have been adjusted for the 10^2 dilution series on both E. coli. hosts.

Tube 4 - Total no. plaques = (37300^t + 560^c) x 50ul

1ul cDNA = 1893000

= 1.9 x 10^6pfu/ug.

The percentage of clears is 1.4% (background = 0.8%).

The percentage of true insert containing recombinants out of a total number of clears is:

(1.4 - 0.8 / 1.4) x 100% = 43%

So the expected number

of recombinants is = 0.43 x 560 x 50ul

= 1.2 x 10^4 true recombinants.

Tube 5 - Total no. of plaques = (79200^t + 1670^c) x 50ul

2ul cDNA = 404350

= 4 x 10^5pfu/ug.

The percentage of clears is 2.0% (background = 0.8%).

The percentage of true insert containing recombinants out of a total number of clears is:

(2 - 0.8 / 2) x 100% = 60%

So the expected number

of recombinants is = 0.60 x 1670 x 50ul

= 5 x 10^4 true recombinants.

Tube 6 - Total no. of plaques = (63300^t + 1320^c) x 50ul

3ul cDNA = 3231000

$= 3.2 \times 10^6$pfu/ug.

The percentage of true insert containing recombinants out of a total number of clears is:

(2 - 0.8 / 2) x 100% = 60%

So the expected number

of recombinants is $= 0.66 \times 1320 \times 50$ul

$= 4 \times 10^4$ true recombinants.

Tubes 4, 5 and 6 were pooled together and so:

the total number of clears would be (560 + 1670 + 1320) x 50ul

$= 177500$

$= 1.8 \times 10^5$

The total number of true recombinants of the pooled library should be approximately $(1.2 \times 10^4 + 5 \times 10^4 + 4 \times 10^4)$

$= \mathbf{1 \times 10^5}$

Thus, the percentage of true recombinants in the pooled library (tubes 4 + 5 + 6) will be approximately 57%.

5.5 Probing JEG-3 cDNA Library with cDNA to check for inserts.

In order to see if the cDNA library actually contained inserts, it was probed with radioactive labelled cDNA. The levels of hybridization were compared to the background hybridization levels on parental lambda gt10.

A small proportion of the library was plated out at low density such that individual, medium sized plaques were well spaced out (less than 500 plaques per $100cm^2$ square plate). Parental lambda gt10 plaques were plated out to confluency. Duplicate plaque lifts were taken and probed with radioactively labelled reverse transcribed first strand cDNA using 1ug mRNA starting material as described in section 2.28 but with two exceptions. Firstly, 40uCi [alpha-^{32}P]-dCTP were used and secondly, no cold dCTP's were included in the synthesis. Unfortunately the probe made in this manner had too few counts to give a reasonable signal after autoradiography (data not shown). Subsequently, a preparation of cDNA was labelled using random hexadeoxynucleotide primers and synthesis with Klenow as described in section 2.26.

The primary plaque lifts were washed at 65°C with 1xSSPE, 0.1%SDS while the secondary lifts were washed at 75°C. Figure 5.5.1 shows strong hybridization of the cDNA probe to some of the plaques on the experimental filter, with medium and weak hybridization to others. The levels of hybridization of the cDNA probe to parental lambda gt10 control filter lifts was very much lower (could not be photographed using the same exposure times) and could be compared to some of the plaques on the experimental filter. The exact frequency of insert containing plaques could not be estimated because of the differing levels of hybridization (as expected). This is a direct consequence of the nature of the mRNA population and hopefully its true representation as cDNA (whether as probe or that ligated into lambda gt10). mRNA's that are quite rare in the population will not contribute greatly to the total signal of the probe and in addition to this, the probability of such a labelled species hybridizing to a plaque represented at low frequency will be much reduced. The relative hybridization signal achieved from a plaque on inspection might be an indication of how common this mRNA species is - indeed this could be a method of isolating more abundant mRNA's (using low levels of probe and limiting the time for hybridization). Filters washed at 75°C had relatively low levels of hybridization and were not used in or necessary for the analysis (data not included).

The results appeared to be encouraging and consequently lambda DNA was prepared from six plaques which exhibited strong hybridization levels ($lambda_{i-vi}$) and from two plaques with the lowest levels of hybridization levels ($lambda_{vii \text{ and } viii}$) - see figure 5.5.1 for examples of such plaques.

5ug $lambda_{i-viii}$ DNA was EcoRI digested to see if an insert was present (figure 5.5.2a). An EcoRI digest should remove the insert from within the original EcoRI cloning site in the cI gene. Figure 5.5.2 shows that $lambda_{vi}$ contains an insert of approximately 800bp.

Inserts will not be seen on agarose gels if they are too small even if large amounts of lambda DNA is restricted. A direct consequence of their relative small size will be a small amount of each (since they are restricted from lambda gt10 which is 43Kb). There is also the possibility that inserts may contain an internal restricion site and this

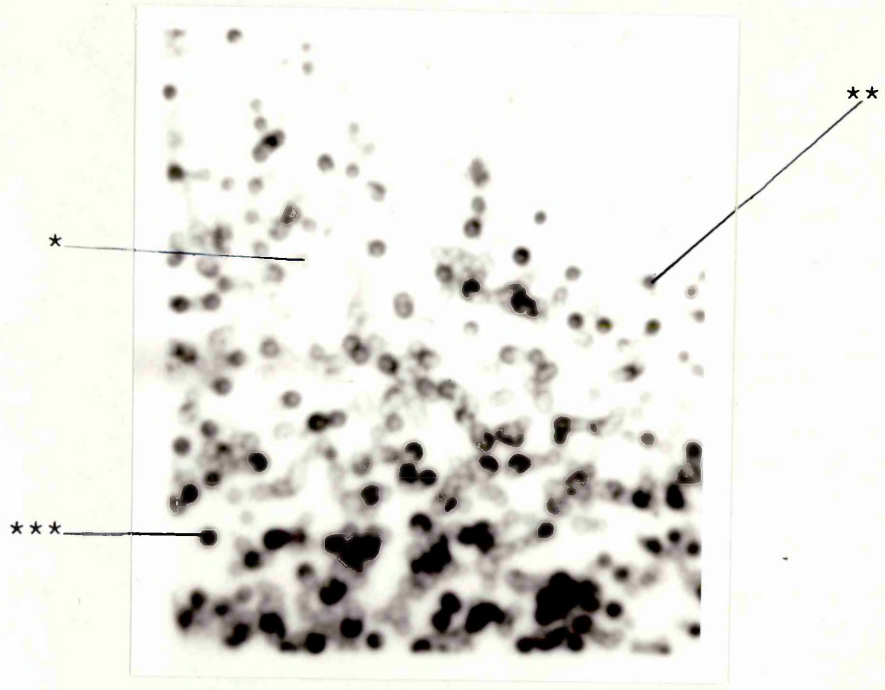


Figure 5.5.1 Hybridization of random primed cDNA to lambda gt10 JEG-3 cDNA library and a comparison with background hybridization to purely parental lambda gt10 plaques.

***- Strong plaque hybridization to cDNA

**- Medium " " "

*- Weak " " "

- Background levels of hybridization to parental lambda gt10 (not visible using the exposure times for the above).

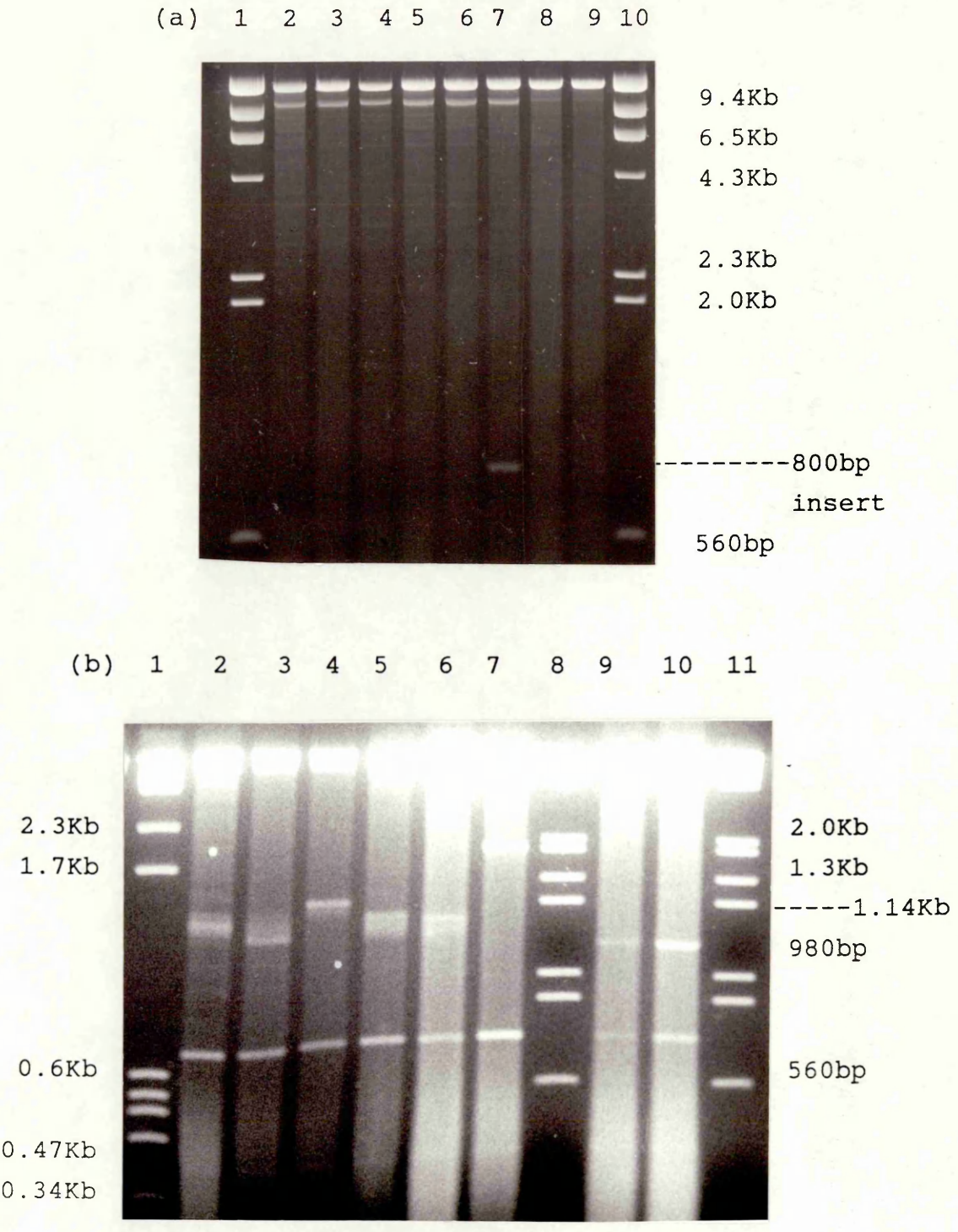


Figure 5.5.2 (a and b) Agarose gel electrophoresis of (a)-EcoRI and (b)-HindIII/BglII restricted $Lambda_{i-viii}$ DNA samples.

a-Tracks (1,10) lambda HindIII markers; (2-9)-$lambda_{i-viii}$EcoRI.

b-Tracks (1) lambda PvuII markers, (8,11) lambda HindIII/EcoRI markers; (2-7)-$lambda_{i-vi}$HindIII/BglII; (9,10)-$lambda_{vii}$ and viii.

would hinder their visualization on gels for the reasons outlined above. In order to detect any of the possible remaining inserts, the samples could have either been Southern blotted and probed with cDNA or end-labelled after EcoRI restriction. Instead, the samples were restricted with HindIII/BglII which generates a 1.14Kb band encompassing the EcoRI cloning site (Figure 5.5.2b and 5.5.1). Providing that the inserts themselves do not contain one or both of these sites then the 1.14Kb band should show a reduced mobility on agarose gels.

The plaques (lambda$_{vii \text{ and } viii}$) which showed only very weak / background levels of hybridization (figure 5.5.1) appeared to have no inserts. Indeed, the lambda arms themselves do not appear to have been restricted and possible explanations for this might be the disruption of the EcoRI site during <u>in vitro</u> manipulation or a deletion of a small fragment encompassing the EcoRI site. All of the plaques (lambda$_{i-vi}$) showed the presence of small inserts (approximately 150bp) within the 1.14Kb HindIII/BglII fragment. One puzzling aspect of these fragments is that they appear to be diffuse when compared to lambda DNA fragments of sizes greater and smaller than 1.14Kb.

5.6. Analysis of Random Picked cDNA Clones for the presence and size of inserts.

Lambda gt10 DNA was prepared from twenty-four random picked and well isolated plaques (lambda$_{A-X}$). 1ug of each (and also 1ug of lambda gt10 - containing no insert) was EcoRI digested in KGB buffer and subsequently heated to 70^{o}C for 10 minutes. The EcoRI cohesive ends generated were radioactively labelled by filling in with Klenow, [alpha-^{32}P]-dATP, cold dCTP, dGTP and dTTP. The radioactive samples were electrophoresed on an agarose gel along with HindIII/EcoRI digested lambda DNA markers and photographed. The gel was TCA precipitated (section 2.30) and vacuum dried before autoradiography. Although each lambda preparation had been successfully restricted (presence of two lambda gt10 arms), the labelling reaction repeatedly did not work and so the samples were treated as follows. 5ug of each lambda$_{A-X}$ were subsequently digested with HindIII/BglII as outlined in section 5.5, and electrophoresed on a 2% agarose gel (figure 5.6.1).

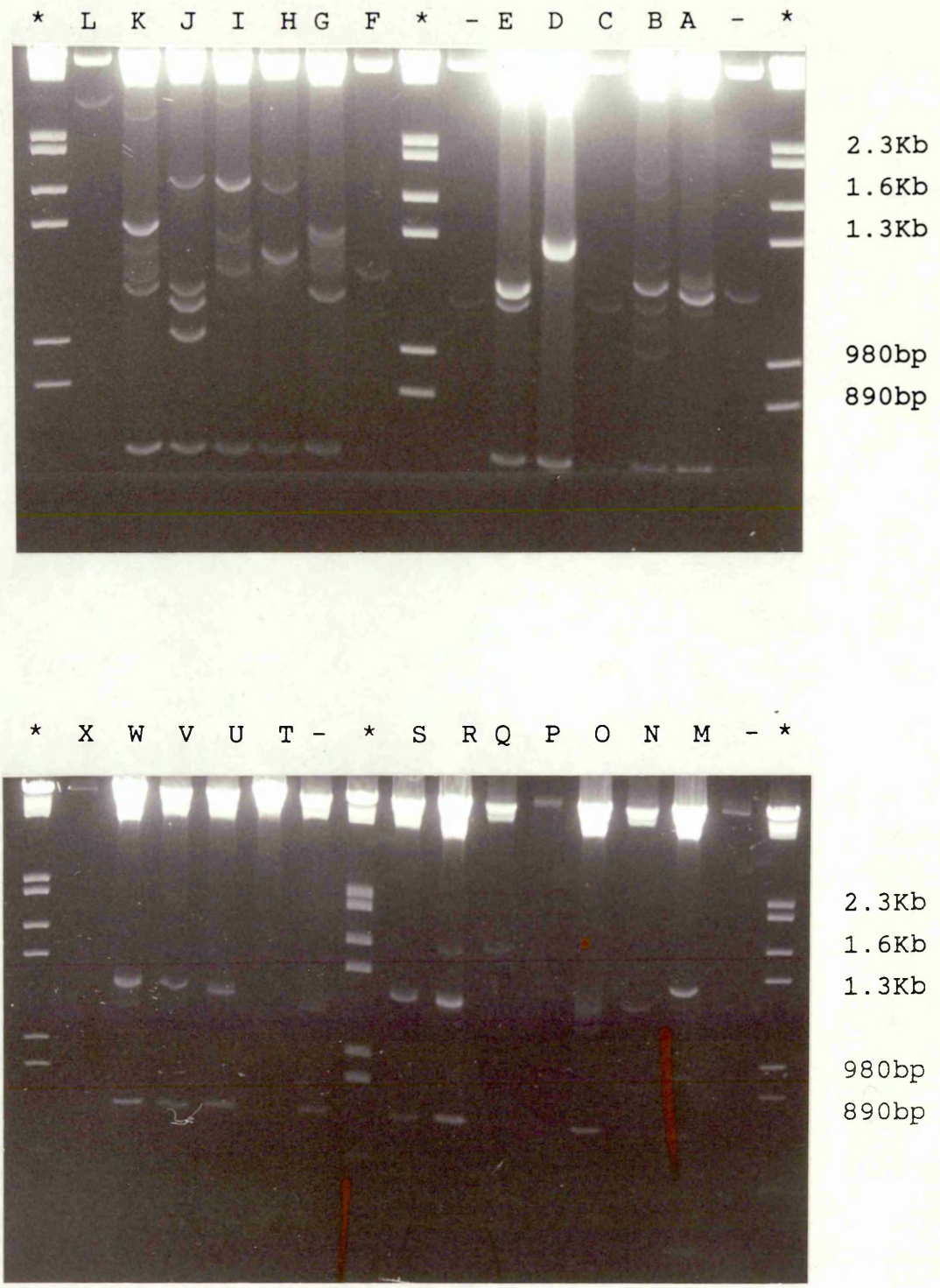


Figure 5.6.1 Agarose gel electrophoresis of HindIII/BglII restriction digests of $Lambda_{A-X}$ DNA samples. Tracks (*) are Lambda HindIII/EcoRI markers, tracks (-) are parental lambda gt10 DNA samples.

Not all of the samples could be used in the analysis since some preparations were poor (samples P, X) and T did not restrict at all. Just to remove any confusion, the low levels of fluorescence in the control sample next to sample M is because of leakage from a hole in the gel well.

As can be seen from figure 5.6.1, the samples that appear to have inserts are B,D,E,F,G,H,I,J,K,L,M,Q,U,V and W. The percentage of insert containing recombinants is probably 15/21 x 100% = 71%. Before the frequency of insert containing recombinants is completed, certain points must be discussed. Firstly, it appears that in some samples (A and J are good examples) there is more than one 1.14Kb band and one explanation for this is contamination with other lambda phages. Although these plaques were not left to diffuse for any length of time and they were plated out at low density, it seems that the requirements to keep a plaque well isolated were underestimated. However, the relative intensity of these bands (within a certain limit) should largely discriminate between (1) more than one lambda phage in the preparation and (2) a large insert (multiple inserts) which contains either HindIII or BglII or both restriction sites. Thus, in sample A the band which shows greater fluorescence is likely to represent the actual sample, whereas the two other bands (one smaller, one larger) are more likely to be contaminating phage (situation 1). Sample J is more representative of situation 2 and shows four bands of equal intensity. The size of the insert here would be (1.7Kb + 1.1Kb + 1.3Kb + .98Kb) - [1.14Kb]) = approximately 4Kb. It would be unwise to comment on the average size of the library inserts for the reasons above, but the fact that some samples do display inserts, even though there is variation, indicates that the cDNA library was worth screening. The average size of cDNA's obtained from commercial preparations is usually 1Kb.

5.7. Screening the cDNA Library using a Class I HLA-B7 cDNA Probe.

The remaining proportion of the library was plated out at a density of approximately 10-11,000 clear pfu's per plate in two stages (plates labelled A-J and K-P) and plaque lifted as described in 2.33. The filter lifts were hybridized with a classical class I HLA-B7 cDNA probe as described in section

2.34 at 55°C.

Six positive plaques lambda$_{JW1-6}$ remained after primary, secondary and tertiary screening (see figure 5.7.1). The filters were autoradiographed between washing progressively with increasing stringencies. This aspect of the work will be discussed fully in the discussion in section 5.8. Lambda DNA was prepared from each.

EcoRI digests of each positive lambda clone showed the size of the inserts to be either approximately 1.2 or 1.4Kb (figure 5.7.2). This gel was Southern blotted and probed with HLA-B7 (figure 5.7.3). All inserts hybridized clearly with the class I probe (upto 0.1xSSPE 0.1%SDS at 70°C). No hybridization was observed with a non-specific (negative control) pBR322 probe.

Lambda$_{JW}$ Clone	Size (Kb)	1° screen plate of origin	2° screen
1	1.4	A	A,3
2	1.2	G	G,1
3	1.2	N	N,1
4	1.2	N	N,2
5	1.4	N	N,3
6	1.2	3	3,1

The clones lambda$_{JW1}$ and $_{JW2}$ were analyzed primarily since these were obtained from the first round of screening (plates A-J). Since the clones were to be sequenced in M13 (polylinker mp18 and mp19), polylinker compatible restriction enzyme were used in their restriction analysis as well as others.

Many restriction enzymes were used (single and double digests) to map lambda$_{JW1}$ (1.4Kb) and after agarose electrophoresis followed by Southern transfer, the membrane was probed with the 1.4Kb HLA-B7 cDNA probe. The results of the mapping were confusing since the molecular weight of the fragments in some of the samples did not always sum up to 1.4Kb. This data has not been included and an explanation of this will be given in section 6.8.

The single SstI (see figure 5.7.4) and PstI sites in all clones were useful in subcloning into M13 (see chapter 6). The two longer clones lambda$_{JW1}$ and $_{JW5}$ (1.4Kb) seemed to generate similar patterns of restriction as each other as

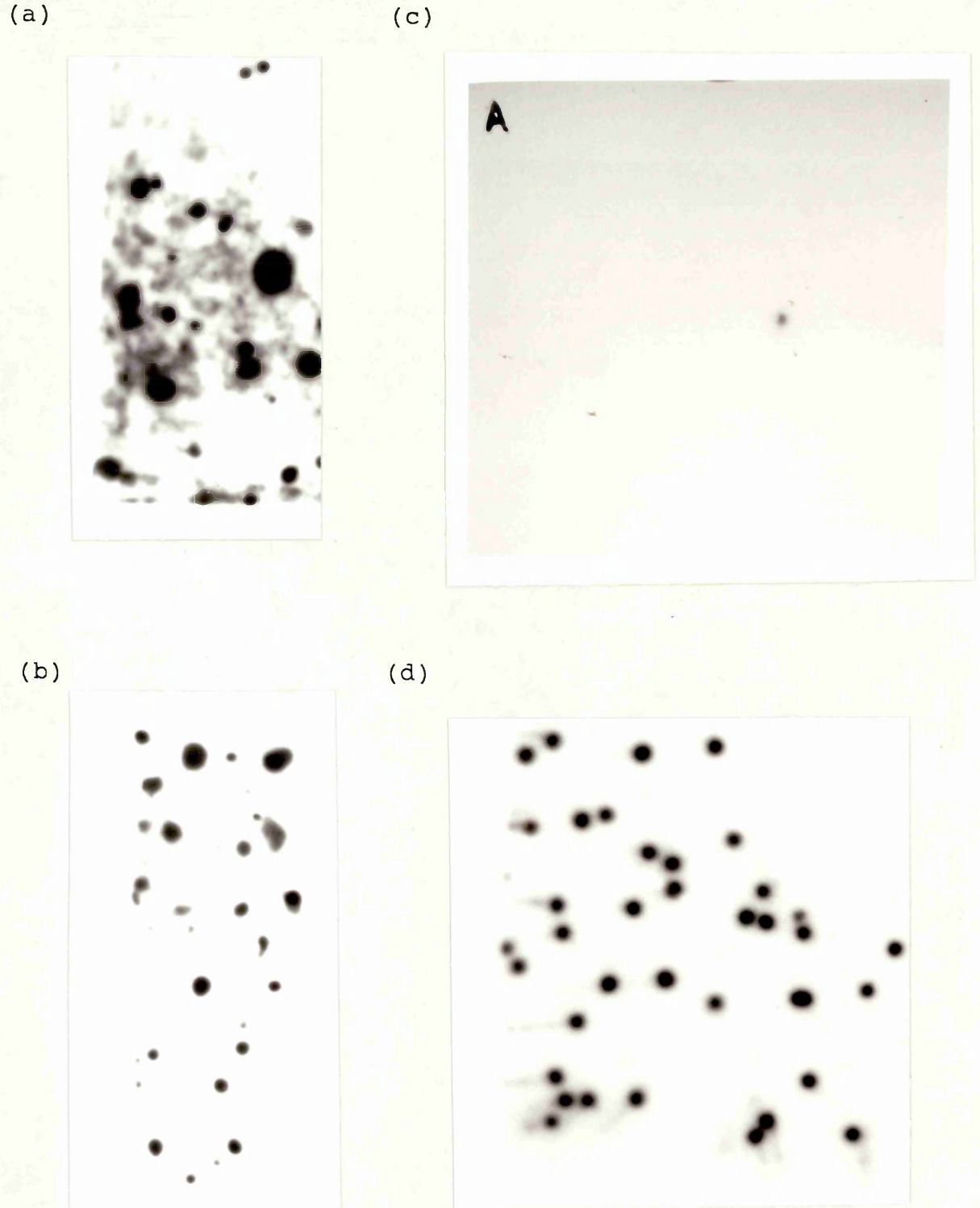


Figure 5.7.1 Primary and secondary screening of Gamma-IFN induced JEG-3 lambda gt10 cDNA library, with a class I HLA-B7 cDNA probe.

Primary Screen

(a)-washed with 1xSSPE, 0.1%SDS 55^{o}C e.g. plate A

(b)- " " " " e.g. background parental lambda

(c)- " " " 65^{o}C

Secondary Screen

(d)-washed with 0.1xSSPE, 0.1%SDS at 65^{o}C.

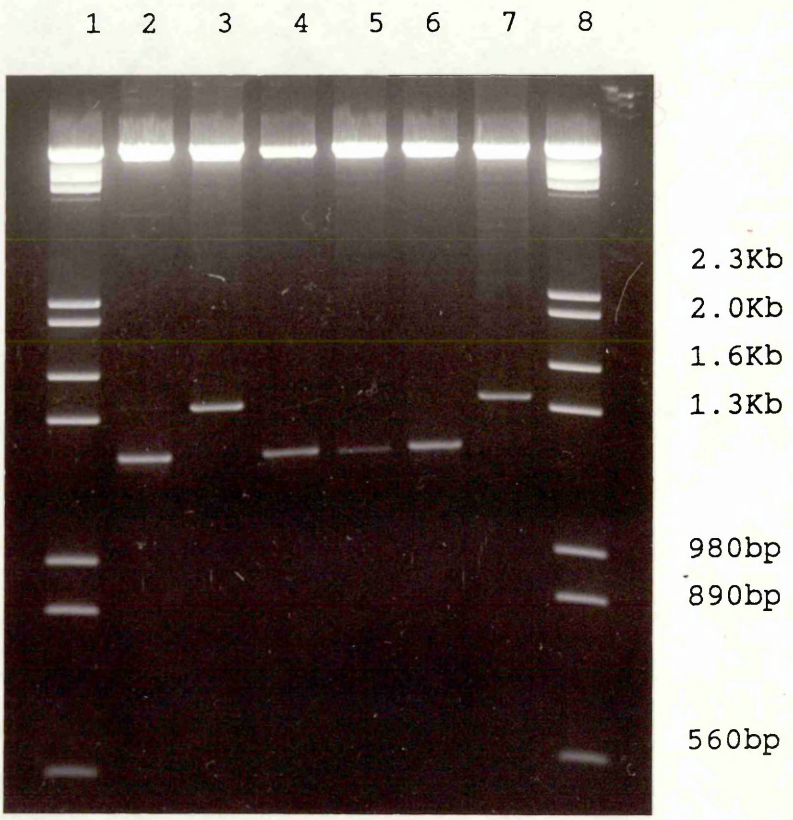


Figure 5.7.2 Agarose gel electrophoresis of six positive cDNA lambda gt10 clones showing a size range 1.2 - 1.4Kb. Tracks (1,8)-lambda HindIII/EcoRI markers, (2-7)-EcoRI $lambda_{JW6-1}$ cDNA

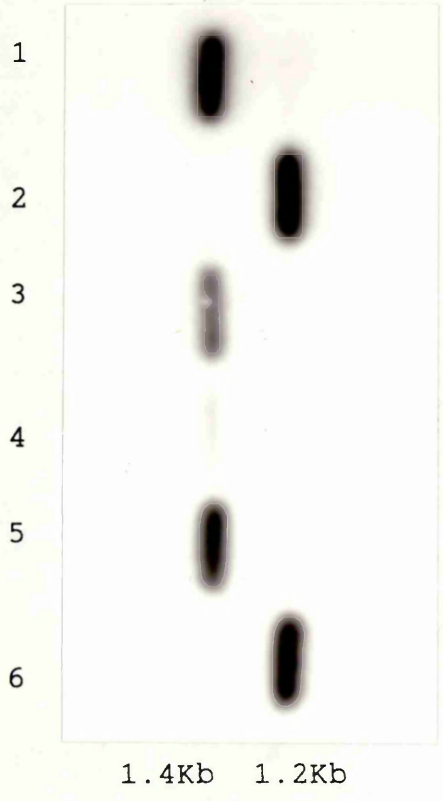


Figure 5.7.3 Southern hybridization of six positive lambda cDNA clones (JW1-6) hybridized with HLA-B7 cDNA probe. Tracks (1-6)- EcoRI lambda$_{JW1-6}$ cDNA clones. N.B. Track 3 is a partial digest.

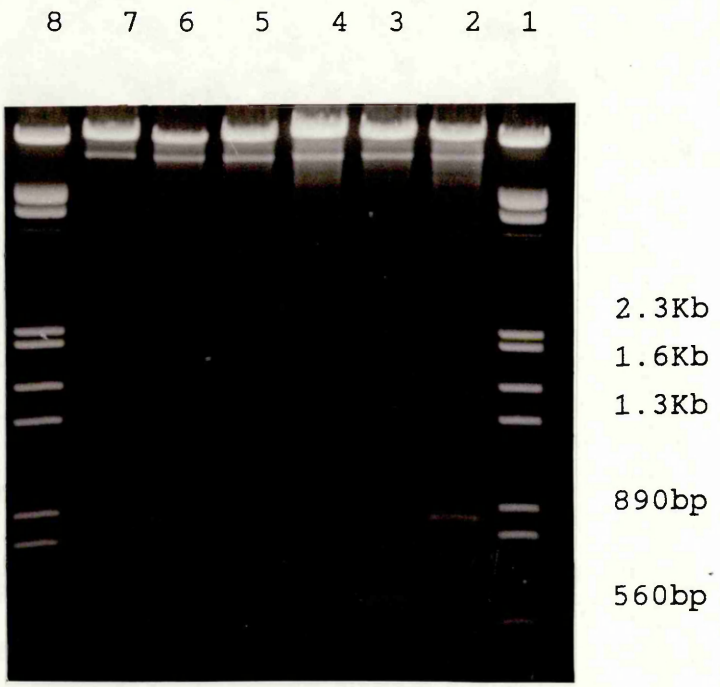


Figure 5.7.4 Sst1 digest of lambda clones JW1-6 illustrating similar restriction patterns.
Tracks (1,8)-lambda HindIII/EcoRI markers (2-7) Sst1 $lambda_{JW1-6}$ cDNA clones.

did the four shorter clones lambda$_{JW2,3,4 \text{ and } 6}$ (1.2Kb).

DISCUSSION.

5.8.

The total number of clear pfu's plated out was $1.8x10^5$ of which $1x10^5$ were by comparison with control experiments calculated to be true recombinants (section 5.4). Six positive cDNA clones were obtained which indicates that the frequency of class I inserts is 1:16,666 or 0.006%. If one assumes the library has an insert frequency of 71% (determined empirically) as assessed in section 5.6 then the frequency of class I inserts is 1:21,300 or 0.005%.

The longest (1.4Kb) cDNA clone is obviously shorter than the size of the mRNA detected on northern formaldehyde gels (section 4.4.2.). Obviously, full length mRNA is required at the beginning of the cloning procedure. Using total RNA for cDNA cloning would eliminate the mRNA isolation step and hence reduce the chance of degradation. Ribosomal RNA could be removed after the synthesis of the second strand by treatment with DNAse free RNAse and preparations of cDNA could be pooled before ligating into lambda gt10. The attempted cloning of the remainder of the class I cDNA molecule is described in section 6.6 although a different procedure from the above was employed.

After autoradiography of the library filters that were washed at 55^oC with 1xSSPE, 0.1%SDS, a high level of background was observed, and also on the primary plaque lifts of parental lambda gt10 (figure 5.7.1a;b). The filters had to be washed with increasing stringencies in order to remove the background levels of hybridization. The unfortunate consequences of this will be discussed in the concluding discussion. Screening secondary plaque lifts of the second part of the cDNA library was found to give much lower background hybridization levels and "cleaner" results, and it is advisable to screen both sets of plaque lifts. The high background levels on primary lifts are almost certainly due to an excess of E.coli debris, DNA and possibly agarose. Such high backgrounds were not observed on filters that were probed with random primed cDNA buth this is probably because the filters were washed at 65^oC first (the hybridization conditions were the same in both experiments).

CHAPTER SIX

SEQUENCING OF CLASS I HLA cDNA CLONES OBTAINED FROM A cDNA LIBRARY MADE FROM GAMMA-INTERFERON INDUCED JEG-3 CELLS.

INTRODUCTION

6.1.

Sequencing of DNA has undergone rapid improvement since introduction of the chain terminating DNA sequencing method (Sanger et al. 1977) and the construction of convenient single-stranded DNA cloning vectors (Messing, 1983).

Initially, the M13 BRL sequencing system was used until the components were finished and subsequent to this the USB Sequenase^{TM} Version 2.0 sequencing system was purchased since it claimed to have improved qualities. In actual fact, there was no difference in the quality of the sequence produced, but the latter system was easier to use and required much less [alpha-^{35}S]thio-dATP.

There are two main differences between both systems; the enzyme used and the timing of sequence termination. BRL uses the Klenow fragment of E. coli. DNA Polymerase I whilst USB uses Sequenase^{TM} (Tabor and Richardson, 1987) which is a modification of bacteriophage T7 DNA Polymerase. The synthesized strand is continuously labelled and terminated throughout the sequencing procedure in the BRL system, whereas in the USB protocol, the dideoxynucleotides are added further on in the reaction. A consequence of the latter is that the concentration of dideoxynucleotides need not to be primarily optimized in order to influence the average length of the synthesized strand (refer to section 2.41).

Properties of T7 DNA Polymerase include high processivity (ability of the polymerase to incorporate nucleotides continuously without dissociating from the template), low 3' to 5' exonuclease activity, high speed and the efficient use of nucleotide analogues important for DNA sequencing (ddNTP's, alph-thio dNTP's, dITP, 7-deaza dGTP and others).

6.2. Preparation of Double-Stranded Replicative Form (RF) M13 DNA.

The vector used for sequencing was M13. Single plaques of both M13mp18 and M13mp19 were obtained and grown in 250ml cultures. Replicative form (RF) double stranded DNA (to be used for sub-cloning) was prepared as described in section 2.21. These preparations were tested for their efficiency in transfecting JM101/JM109 as described in 2.19 and 2.39. The efficiency of transfection of M13 DNA is typically lower than that described for plasmids ($1x10^5$/ug as compared to $1x10^8$/ug respectively) and also varies between JM strains of E. coli. as outlined in the BRL manual.

Stocks M13mp18 300ug/ml

M13mp19 160ug/ml

Initially, the transformation efficiency of each preparation was extremely low on JM101: $1x10^3$pfu/ug. After phenol extraction and ethanol precipitation this was increased to $7.5x10^4$ pfu/ug. It is worthy to note here that if too much M13 is used to transfect JM101 no blue plaques appear on the plate. Instead, a green tinge can just be discerned. This can easily be mistaken for no transfectants at all. Reducing the amount of transfecting DNA sees the individual blue plaques re-appearing.

6.3. Subcloning of JW1-6 into M13mp18 and M13mp19.

All six class I cDNA clones were EcoRI excised from 10ug lambda gt10 and electrophoresed on a tris-acetate-EDTA (TAE) agarose (type I) gel. The inserts (approx. 300ng) were cut out of the gel and purified using the gene-clean procedure described in section 2.45. After ethanol precipitation they were resuspended in ligation buffer and ligated into 500ng EcoRI digested M13 (in duplicate ligations the vector was treated with calf-intestinal phosphatase - see section 2.25). After transfection, clear plaques were picked and minipreps of M13 RF DNA were made (STET prep, section 2.40) and tested for the presence of inserts on an agarose gel. Since there is an Sst1 restriction site within each clone and also in the M13 polylinker, an Sst1 digest reveals the orientation of the clone (in JW1 and JW5 but not the others). A large proportion of the clear plaques did not

contain inserts, and it became much less time consuming and more efficient to filter lift the plaques and hybridize the cDNA clone to detect the true recombinants. Most of the recombinant clones obtained were in M13mp18 and some were in both orientations. Because of this, M13mp19 was not used any further.

6.4. Sequence Strategy.

JW1 was subcloned into the EcoRI site of M13mp18 in both orientations, JW1a and JW1b (see below and figure 6.4.1).

Orientation (a) - antisense strand is located on the single +ve strand of M13 and the 5' end of the gene is nearest the sequencing primer. Consequently, the sense strand is synthesized during sequencing.

Orientation (b) - sense strand is located on the single +ve strand of M13 and the 3' end of the gene is nearest the sequencing primer. Consequently, the antisense strand is synthesized during sequencing.

JW1EcoRI,a/b (JW1 EcoRI fragment in orientation a or b.)

Replicative forms of these recombinants were Sst1 and Pst1 digested in 2xKGB buffer and heated to 70^{o}C for 10 minutes. After cooling on ice, the buffer was adjusted to include 1mM ATP and 5units of T4 DNA ligase in 0.5x KGB buffer. The ligations were incubated overnight and were used to transfect JM101 as usual. The reason for doing this was to delete out the region between the sites (the 5' or 3' end of the gene depending on its orientation) since both sites were present once in the polylinker and once in the cDNA.

JW1SstI,a/b (JW1 EcoRI/SstI fragment, these are formed from the SstI deletion digest of the JW1EcoRI a/b clone).

JW1PstI,a/b (JW1 EcoRI/PstI fragment, these are formed from the Pst1 deletion digest of the JW1EcoRI a/b clone.

JW1PvuII,a/b 300bp PvuII fragment from JW1.

An EcoRI/PvuII digest generated three restriction fragments of 600, 300 and 400 base pairs, whilst a PvuII digest generated only the 300 base pair fragment. This

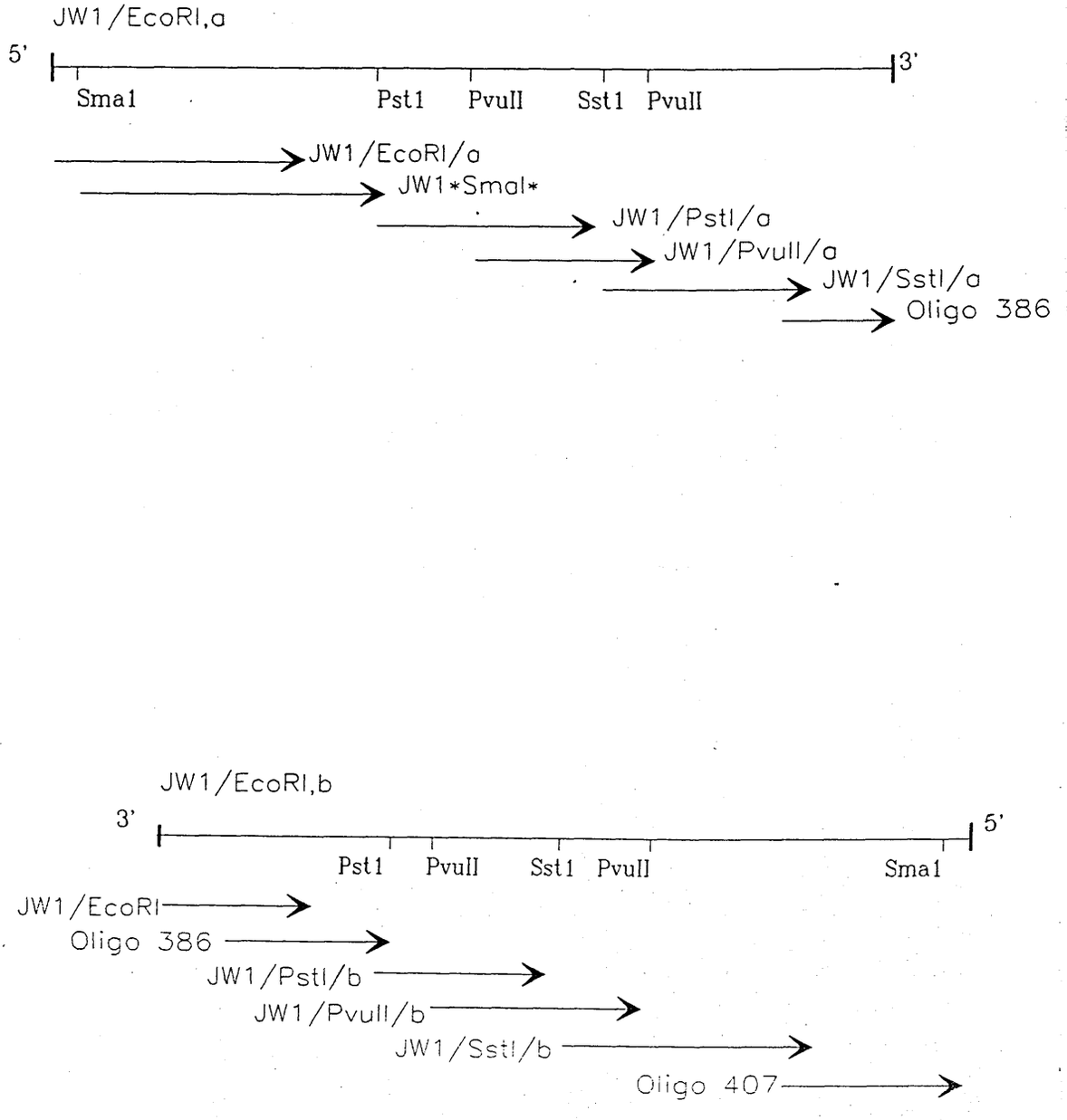


Figure 6.4.1 Sequence strategy used in sequencing JW1,4 and 5. See section 6.4 foR details.

central 300bp PvuII fragment was cloned into the SmaI (blunt) site of the M13 polylinker (M13 contains 3 PvuII sites).

Other subclones were designated **JW1SmaI,a** and **JW1*Sma*,a** and will be described in section 6.6 in detail.

Unfortunately, no other useful restriction sites were in any way compatible (nor any isoschizomers) with the M13mp18 polylinker restriction sites and it was necessary to use synthetic oligonucleotides (386, 387 and 407, see section 2.42) to sequence the remaining part of the clone (see figure 6.4.1).

6.5. Preparation and Sequencing of Single Stranded Recombinant M13.

Single stranded M13 DNA containing inserts were prepared from subclones obtained at that stage and a sample of each (JW1EcoRI,a/c and JW1Sst1,a/c) electrophoresed on an agarose gel in order to estimate the amount of DNA prepared since this varies between preparations (see figure 6.5.1.). Sequencing reactions were electrophoresed on 5-8% wedge or uniform sequencing gels as described in the BRL Manual and in section 2.41.

The JW1EcoRI,a/c subclones were sequenced at the same time as preparing the other subclones, and this initial information was analyzed. The first 250bp of sequence from the 5' and 3' ends of cDNA clone JW1 were compared by eye to the published HLA-B7 cDNA sequence (Sood et al. 1985). The 3' region (extending from the poly A tail) could be position matched (lined up) quite easily but that of the 5' region could not be. Being aware that the cDNA clone (1.4Kb) was incomplete (Northern data indicated a 1.6Kb mRNA) and now obviously lacking the 5' end, a strategy based around the 5' sequence information was devised to obtain the remaining section of this cDNA (see section below).

6.6. Cloning Strategy to Obtain the Remaining 5' Part of the Class I cDNA.

This cloning strategy was based upon the method of Dale et al. (1985), and required knowledge of the orientation of the clone and preliminary 5' sequence. The method and strategy are represented in figure 6.6.1. The idea behind

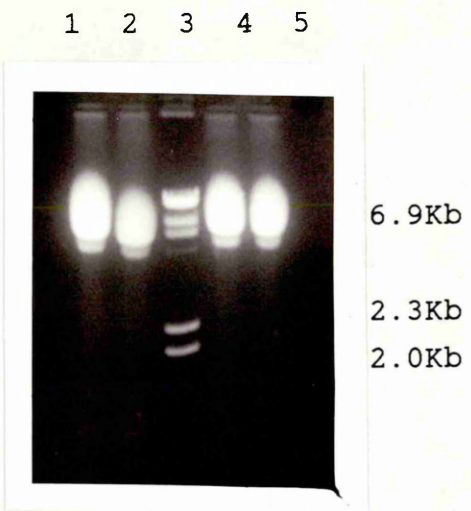


Figure 6.5.1 Agarose gel electrophoresis of ssM13 DNA samples containing various subclones of JW1.
Tracks (1,2)-lambdaJW$_{EcoRIa/c}$ (3)-lambda HindIII markers, (4,5)-lambdaJW$_{SstIa/c}$.

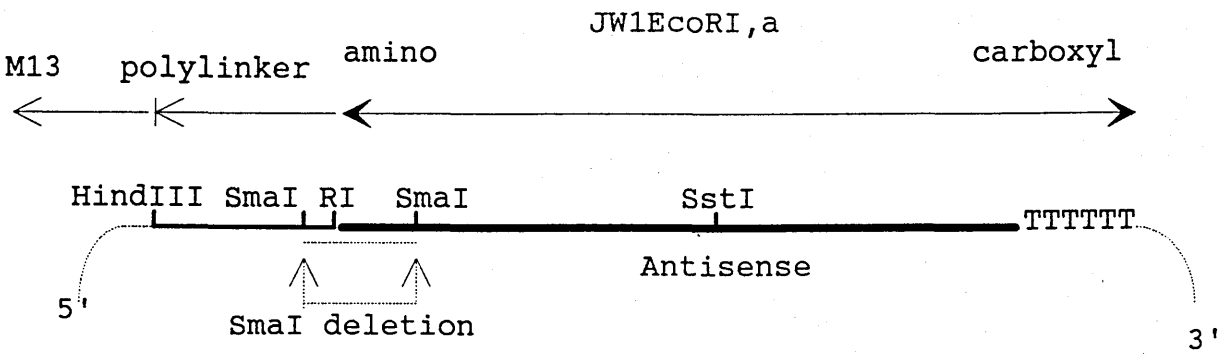
this was to hybridize the antisense single strand of the partial cDNA (cloned into M13mp18 +ve strand) to its complementary mRNA and use this as a primer/template complex in order to extend the 5' end. The antisense strand of the cDNA must be cloned within the +ve phage single strand in order that it should hybridize to the homologous mRNA (sense). Single strand M13 phage DNA is circular and so this must be linearised at a point within the clone and preferably as near to the 5' end as possible. Linearization of the strand can be accomplished by the appropriate combination of a restriction site directed synthetic oligonucleotide (RSDF see below) and its compatible restriction endonuclease. Indeed, some restriction endonucleases are able to cleave single strand sequences (Bischofberger et al. 1987). If no convenient restriction sites are present, the endonuclease (FokI) can be used with a specific oligonucleotide to restrict a non-specific dsDNA sequence (Kim et al. 1984; Podhajsska and Sybalski, 1985). The use of FokI represents a more universal approach.

One practical consideration, is the removal of the EcoRI site from the 5' end of the cDNA clone (which has arisen from the addition of EcoRI linkers to the cDNA before cloning). It is most unlikely that this site will be present here (unless methylation during cDNA preparation was unsuccessful). Fortunately, a SmaI site within the polylinker and a single SmaI site 40bp into the 5' end of the cDNA enabled the deletion of the EcoRI site and the formation of the SmaI modified clone, JW1SmaI,a from JW1EcoRI,a (see figure 6.6.1). The deletion of the SmaI fragment obviously retained a 5' most SmaI site to which a restriction site directed fragment (RSDF) or oligonucleotide could be made. This was used as a means of linearizing the modified, single stranded cloned. The deletion of the SmaI fragment to form the modified clone and the knowledge of the cDNA sequence within it enabled the design of the screening oligo (see section 2.42). This was used to detect extended recombinants, since the blue/white selection is no longer useful.

Obviously there are problems associated with extending sequences which belong to a polymorphic multigene family, since hybrid alleles could be generated. A number of clones would be required to confirm results.

JW1,EcoRI,a.

Deletion of the SmaI fragment from RF JW1,EcoRI,a (to remove the EcoRI site, see figure 6.6.3). Religate and transfect. Select for the subclone by T-tracking and sequencing (see figure 6.6.2).



JW1,SmaI(Modified Clone). Prepare ssM13 DNA and anneal SmaI oligo. Linearize by restriction with SmaI.

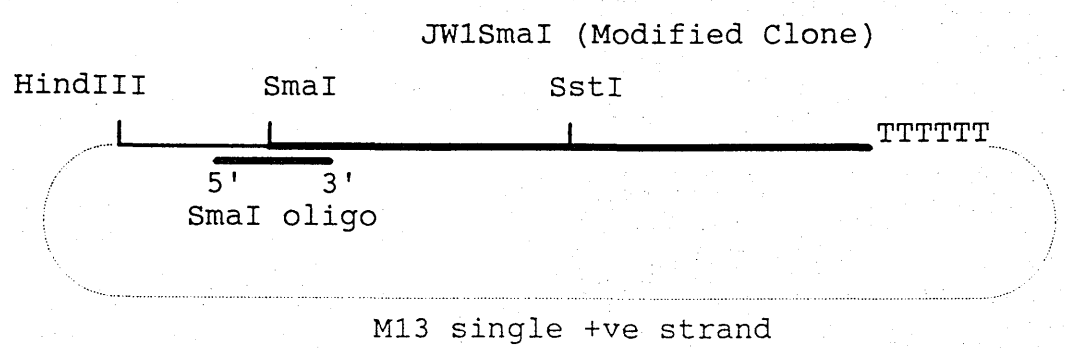
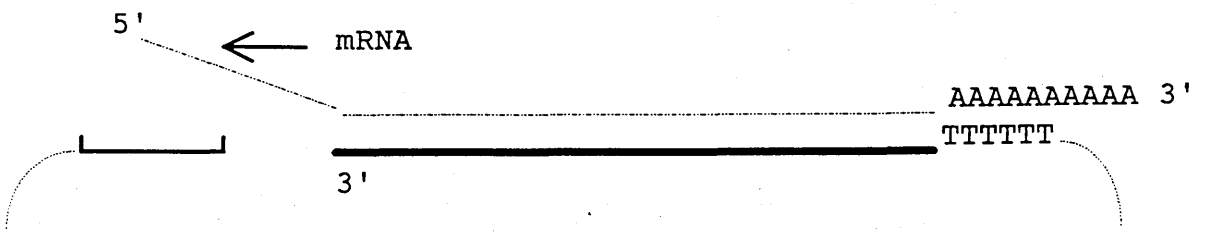


Figure 6.6.1 Outline of the cloning strategy to obtain the remaining 5' part of the JW1 Class I cDNA. This was based around the method of Dale et al. (1985).

JW1,SmaI (Modified Clone).

Hybridize the linearized single stranded form or JW1,SmaI to JEG-3 mRNA.



Synthesize cDNA using reverse transcriptase (XXX). Terminal transferase using deoxycytidine(C). Hybridize the annealing (bridgeing) oligo-RSDF.

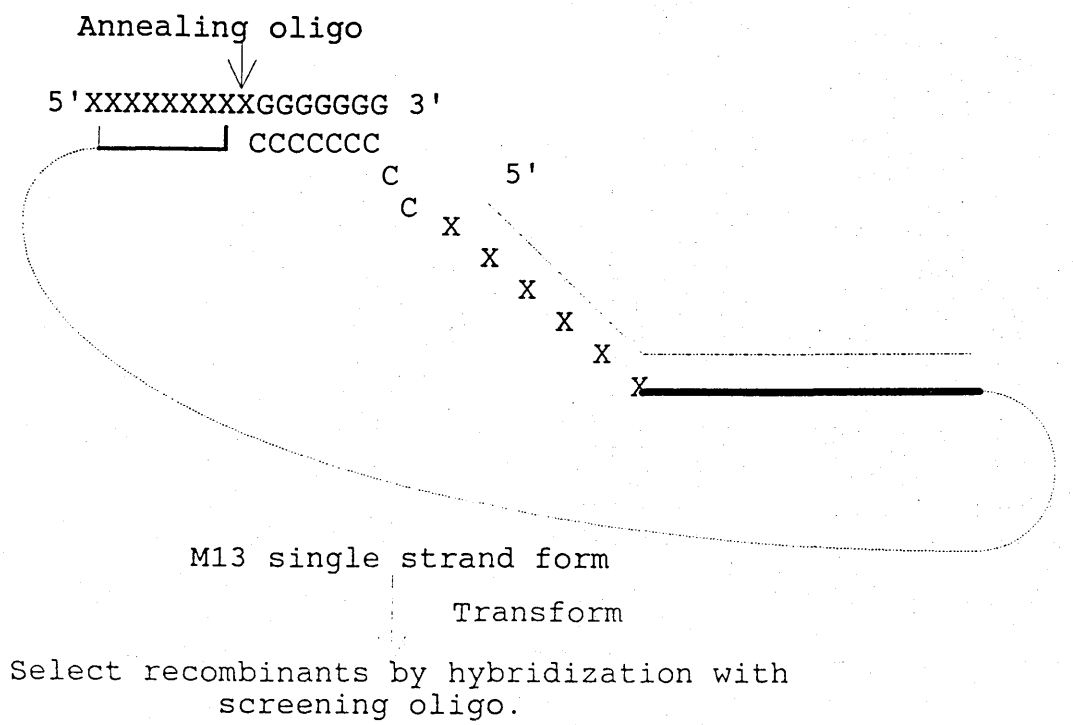
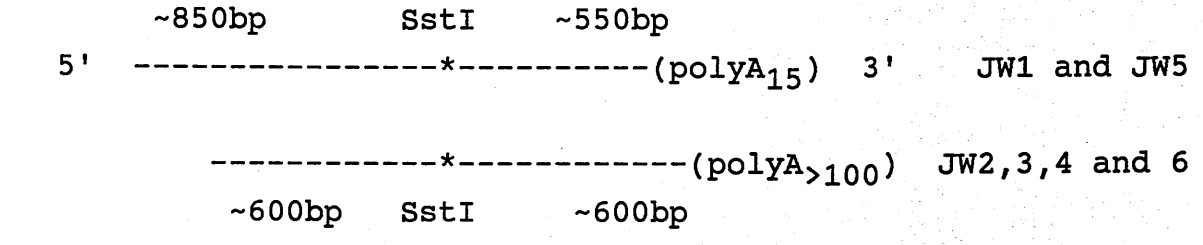


Figure 6.6.1 contd.

During the preparation of the modified clone, two other different "spurious" deletion clones were obtained, and the sequences are shown in figure 6.6.2. The possible sites for the spurious SmaI restriction seem to be different and are represented in figure 6.6.3. The first of these clones (JW1*SmaI*) was useful in completing the sequence of JW1, since it allowed progress through to the PstI site.

6.7. Nucleotide Sequence Comparison of JW1, JW4 and JW5.

The complete sequence of both strands of JW1 was obtained and that for JW4 and JW5 was completed by overlapping information obtained from alternative strands. Overlapping sequence data within the coding region (as far as they extend) of clones JW1, 4 and 5 suggest that all are derived from the same allele (see figure 6.7.1.). Primarily, before any sequence data had been obtained a simple diagram was drawn (shown below) from the Sst1 restriction pattern (figure 5.7.4) with reference to this site (although there was no evidence to suggest that this site was in the same position in all of the clones because of the possibility of there being two or more genes/alleles expressed. The size (bp) was estimated from mobility on agarose gels.



Sequence alignments have confirmed that this is how the clones JW1, JW4 and JW5 overlap.

Clones JW1 and JW5 were found to be identical in both sequence and length (1418bp) and extended forward from the polyadenylation tail (15bp). This was very unusual since these clones were independently picked and they originated from different plates (refer to end of section 5.7).

JW4 was shorter than JW1, beginning at nucleotide 268 (see figure 6.7.1) and having a much longer polyadenylation tail (>100bp assumed partly from the Sst1 digest). JW6 which gives the same SstI restriction pattern as JW4 was found to have a >100bp polyadenylation tail on sequencing gels. JW1

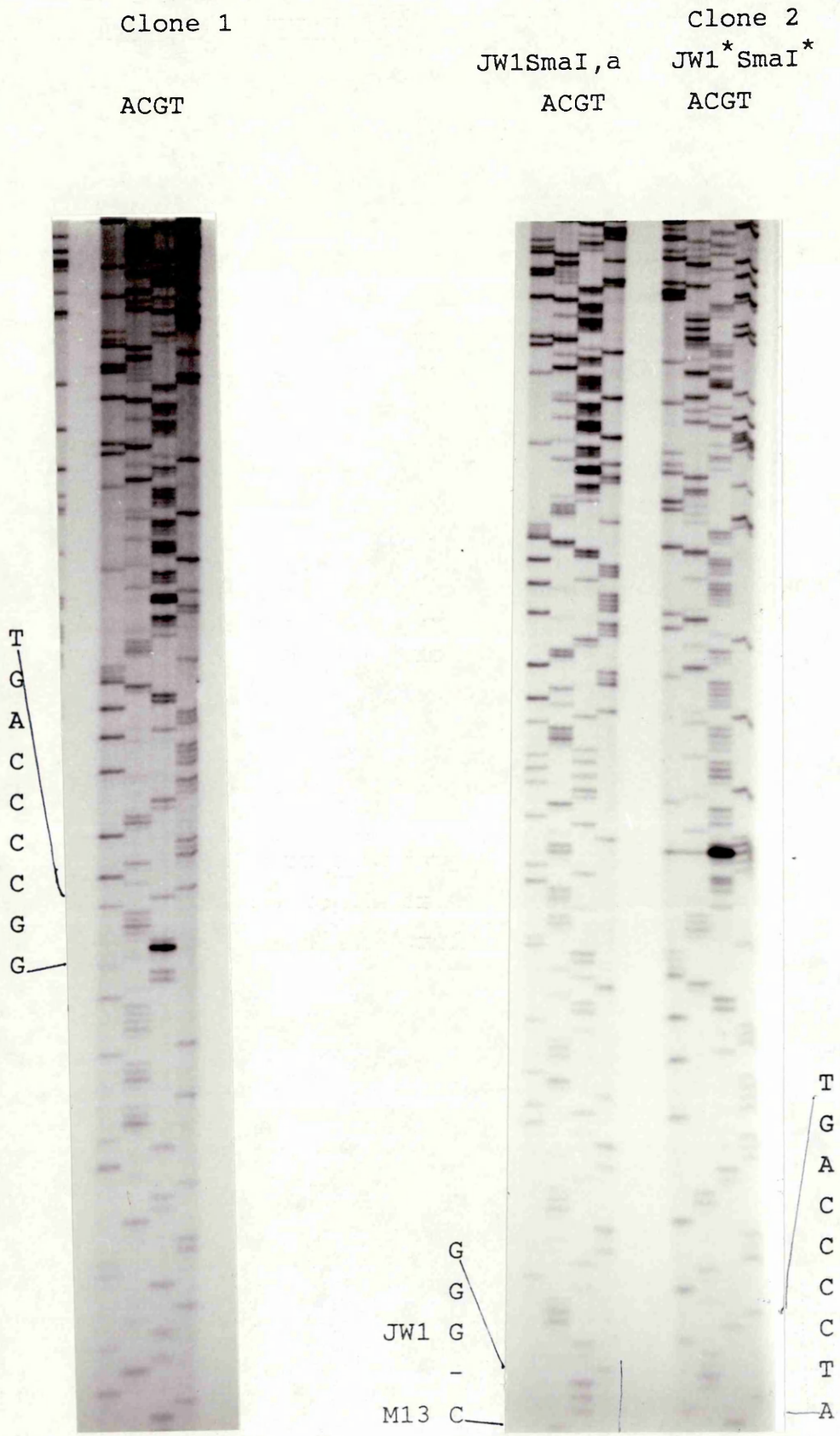


Figure 6.6.2 Sequence Autoradograph of SmaI deletion subclones.

The strand represented below is the sense strand which is synthesized during sequencing of $JW1_{EcoRI,a}$ and so corresponds to the 5'most part of the cDNA.

```
      M13 polylinker---- ----------HLA JW1 cDNA clone
   SmaI            EcoRI            SmaI            *SmaI?*

atcCCCGGGtaccgagctcGAATCCcgg~~~cggCCCGGGtcg~~~~~~gagccCCAGT
     --------------------------------

atcCCCGGGtcg~~~~~~gagccCCAGT     SmaI deletion clone!
```

This SmaI deletion clone (see figure 6.6.1) was created solely for the purpose of obtaining the remaining 5' end of the existing cDNA clone. It represents the starting material for the cloning strategy outlined in section 6.6.2.

However, two different spurious SmaI deletions were obtained unexpectedly, the first of which enabled sequencing of the 5' region of the clone.

```
1:
atcCCCGGGtaccgagctcGAATCCcgg~~~cggCCCGGGtcg~~~~~~gagccCCAGT
     ------------------------------------------------

atcCCCAGT   JW1*SmaI* useful for completing the sequence.


2:
atcCCCGGGtaccgagctcGAATCCcgg~~~cggCCCGGGtcg~~~~~~gagccCAGT
                                          ----------------?

atcCCCGGGtaccgagctcGAATTCcgg~~~~cggCCC^CAGTCAC
                                      ?
```

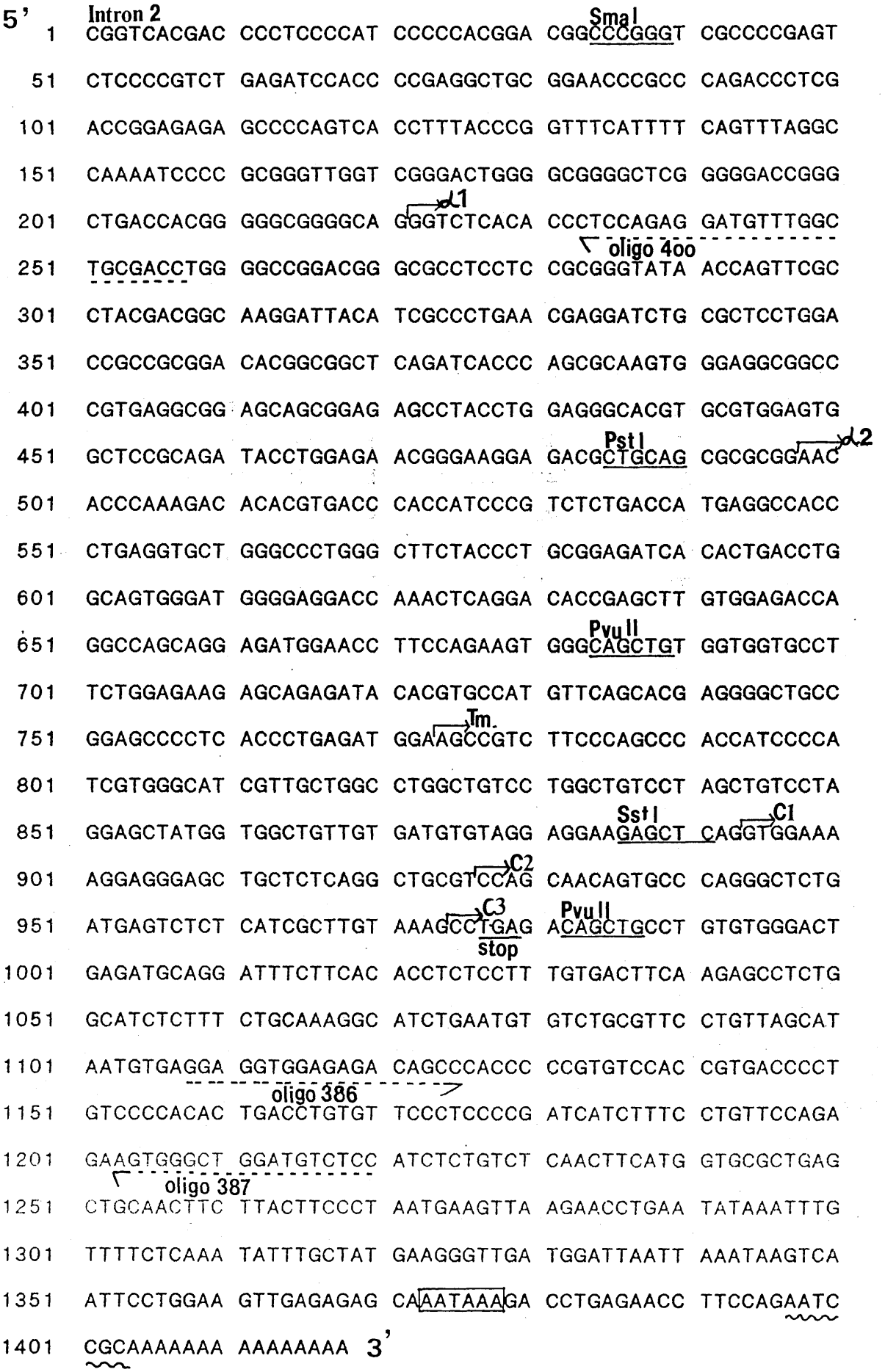
Figure 6.6.3. SmaI deletion subclone of JW1 - JW1*SmaI* used in the cloning strategy described in section 6.6. Two further "spurious" subclones are outlined.

and JW4 differed in the 3' untranslated region by a 7 base pair deletion (AATCCGC) 5' to the polyadenylation site in JW4 (see figure 6.7.1. and 6.7.2). Only one common polyadenylation signal could be identified. The significance of a different polyadenylation site in clones JW1 and JW4 is not known. Cianneti et al. (1989) reported an alternative polyadenylation site in the HLA-A2 gene which probably resulted from the use of a less efficient polyadenylation signal located 100 base pairs 5' to the canonical one. Multiple polyadenylation sites and signals have been reported for several other genes and are known to contribute to the overall regulation of gene expression (reviewed by Birnstiel et al. 1985).

6.8. Comparison of JW1 and HLA-A, -B and -C locus nucleotide sequences.

Computer alignments of the sequences of JW1 compared to HLA-A, -B and -C genomic and cDNA revealed that the first 221 bp of the 5' region were in fact part of intron 2 (figure 6.8.1). Thus JW4 began 47 base pairs into the alpha-2 domain. JW1 and JW5 were thought to be cDNA products of polyadenylated nuclear precursor mRNA. The frequency of incompletely spliced mRNA's (two out of six clones) is unusually high. The presence of intron 2 sequence explains the confusion in restriction mapping outlined in section 5.7. Primarily, restriction digests of the HLA-B7 cDNA were Southern blotted and hybridized to JW1 insert probe since the restriction map of the former was known. Some of these results were confusing. Obviously, no sequences hybridized to intron 2 and the restriction digests that separated the latter from the alpha-2 domain sequence could not be understood fully. Similar concurrent work (where HLA-B7 cDNA was radiolabelled and hybridized to a series of restriction digests of JW1) was equally confusing and for this reason none of the data was included in section 5.7. This work was to be repeated using self (JW1) hybridization of the lambda clones, however, initial sequence information indicating the presence of intron 2 excluded the need for this data. As a consequence of this, the strategy developed to clone the remaining 5' of the gene (outlined in section 6.6) was abandoned although it has been represented diagramatically

Figure 6.7.1 Complete Sequence of JW1, 4 and 5. The first 221bp of JW1 are intron 2 followed thereafter by coding sequence. Note that Jw4 begins at position 268 and differs from JW1 and JW5 by a 7bp deletion 5' to the polyadenylation site. Restriction sites used in subcloning are underlined and the oligonucleotides used in completing the sequence are marked in . The exon boundaries are marked with a note of the protein domain that follows (refer to figure 1.1.4c).


5'
Intron 2
SmaI
1 CGGTCACGAC CCCTCCCCAT CCCCCACGGA CGGCCCGGGT CGCCCCGAGT
51 CTCCCCGTCT GAGATCCACC CCGAGGCTGC GGAACCCGCC CAGACCCTCG
101 ACCGGAGAGA GCCCCAGTCA CCTTTACCCG GTTTCATTTT CAGTTTAGGC
151 CAAAATCCCC GCGGGTTGGT CGGGACTGGG GCGGGGCTCG GGGGACCGGG
λ1
201 CTGACCACGG GGGCGGGGCA GGGTCTCACA CCCTCCAGAG GATGTTTGGC
oligo 400
251 TGCGACCTGG GGCCGGACGG GCGCCTCCTC CGCGGGTATA ACCAGTTCGC
301 CTACGACGGC AAGGATTACA TCGCCCTGAA CGAGGATCTG CGCTCCTGGA
351 CCGCCGCGGA CACGGCGGCT CAGATCACCC AGCGCAAGTG GGAGGCGGCC
401 CGTGAGGCGG AGCAGCGGAG AGCCTACCTG GAGGGCACGT GCGTGGAGTG
PstI
λ2
451 GCTCCGCAGA TACCTGGAGA ACGGGAAGGA GACGCTGCAG CGCGCGGAAC
501 ACCCAAAGAC ACACGTGACC CACCATCCCG TCTCTGACCA TGAGGCCACC
551 CTGAGGTGCT GGGCCCTGGG CTTCTACCCT GCGGAGATCA CACTGACCTG
601 GCAGTGGGAT GGGGAGGACC AAACTCAGGA CACCGAGCTT GTGGAGACCA
PvuII
651 GGCCAGCAGG AGATGGAACC TTCCAGAAGT GGGCAGCTGT GGTGGTGCCT
701 TCTGGAGAAG AGCAGAGATA CACGTGCCAT GTTCAGCACG AGGGGCTGCC
Tm.
751 GGAGCCCCTC ACCCTGAGAT GGAAGCCGTC TTCCCAGCCC ACCATCCCCA
801 TCGTGGGCAT CGTTGCTGGC CTGGCTGTCC TGGCTGTCCT AGCTGTCCTA
SstI
C1
851 GGAGCTATGG TGGCTGTTGT GATGTGTAGG AGGAAGAGCT CAGGTGGAAA
C2
901 AGGAGGGAGC TGCTCTCAGG CTGCGTCCAG CAACAGTGCC CAGGGCTCTG
C3
PvuII
951 ATGAGTCTCT CATCGCTTGT AAAGCCTGAG ACAGCTGCCT GTGTGGGACT
stop
1001 GAGATGCAGG ATTTCTTCAC ACCTCTCCTT TGTGACTTCA AGAGCCTCTG
1051 GCATCTCTTT CTGCAAAGGC ATCTGAATGT GTCTGCGTTC CTGTTAGCAT
1101 AATGTGAGGA GGTGGAGAGA CAGCCCACCC CCGTGTCCAC CGTGACCCCT
oligo 386
1151 GTCCCCACAC TGACCTGTGT TCCCTCCCCG ATCATCTTTC CTGTTCCAGA
1201 GAAGTGGGCT GGATGTCTCC ATCTCTGTCT CAACTTCATG GTGCGCTGAG
oligo 387
1251 CTGCAACTTC TTACTTCCCT AATGAAGTTA AGAACCTGAA TATAAATTTG
1301 TTTTCTCAAA TATTTGCTAT GAAGGGTTGA TGGATTAATT AAATAAGTCA
1351 ATTCCTGGAA GTTGAGAGAG CAAATAAAGA CCTGAGAACC TTCCAGAATC
1401 CGCAAAAAAA AAAAAAAA 3'

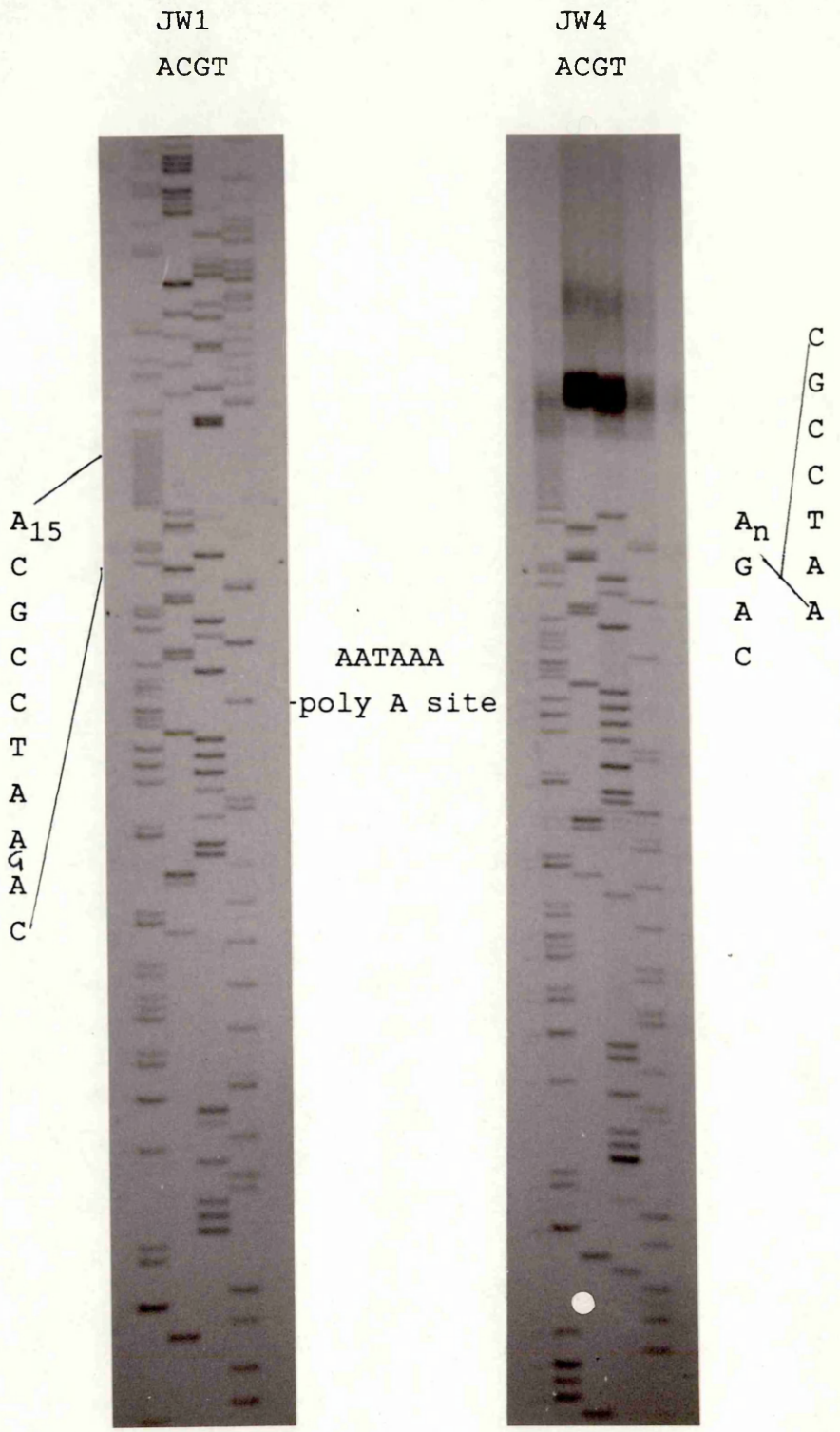


Figure 6.7.2 Sequence autoradiograph illustrating the presence and absence of 7 base pairs (AATCCGC) in the 3' UT region of clones JW1 and JW4 respectively.

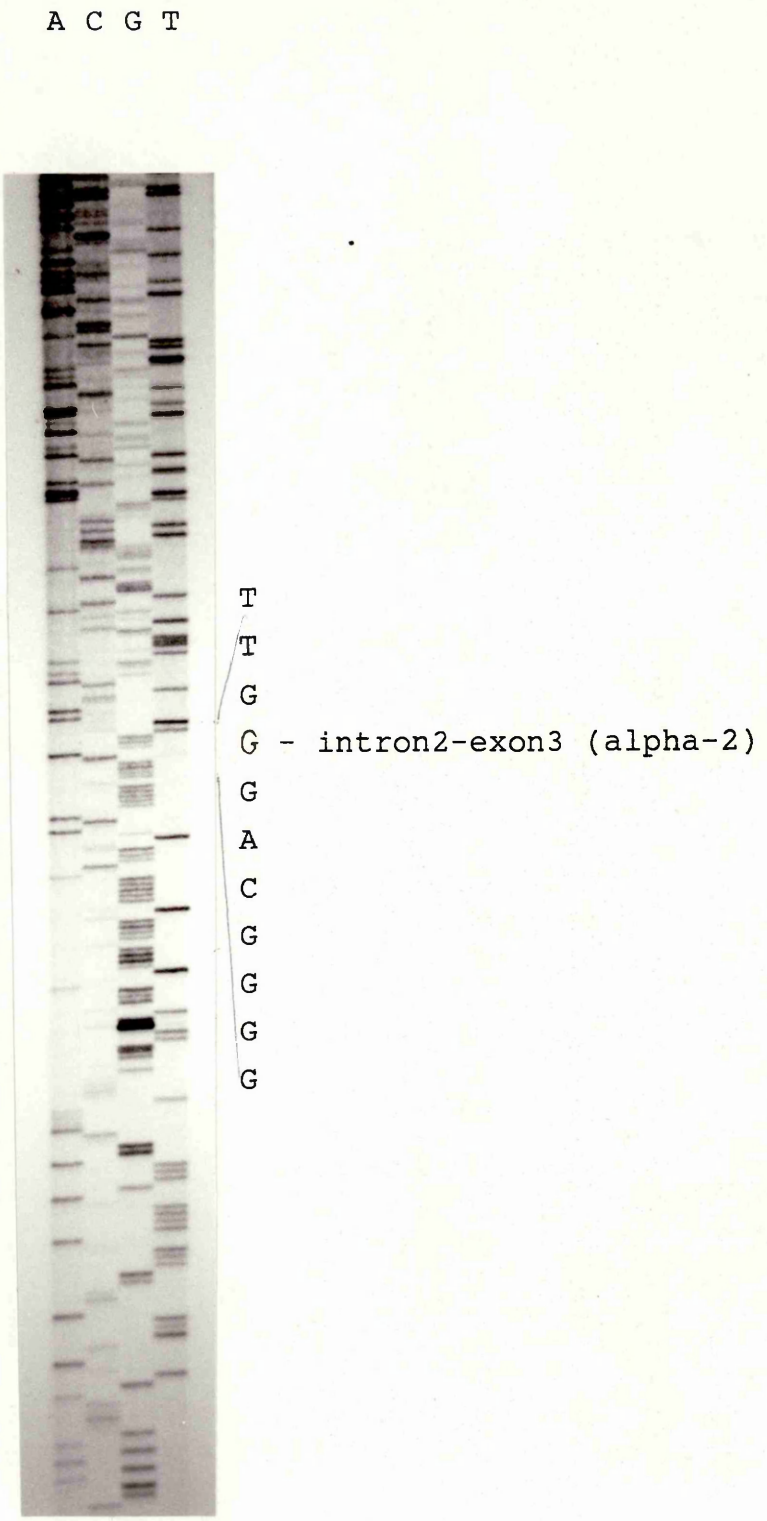


Figure 6.8.1 Sequence autoradiograph of the intron2/exon3 boundary of clones JW1 and JW5.

because both the Sma subclone, JW1SmaI,a and a spurious Sma subclone, JW1*SmaI* from this strategy were very useful in completing the sequence of JW1.

It is important to mention at this point that further attempts were being made to clone the remaining 5' end of the cDNA since all the sequence data was complete. However, Ellis and co-workers in Oxford had submitted a paper which described the full length sequence of a class I cDNA clone obtained from a BeWo cDNA library. The sequence of JW1 as far as it extended seemed to be (see discussion) identical to that of BeWo C.1 described in this recent publication (Ellis et al. 1989). This is indeed not surprising since BeWo and JEG-3 seem to be derived from the same choriocarcinoma, see section 3.1.1.

The information below has been derived from the sequence comparison (using the GCG Sequence Analysis Software Package) of the coding and 3'UT regions of JW1 and HLA-A3 (Strachan et al. 1984), HLA-B7 (Sood et al. 1985), HLA-Cw1; HLA-Cw2 (Gussow et al. 1987), HLA-Cw3 (Sodoyer et al. 1984) and HLA-Cw4 mRNA (Davidson et al. 1985) which were obtained from the GenBank and EMBL databases. A comparison of JW1 or BeWo C.1 with HLA-A, -B and -C locus sequences clearly shows that they are both more homologous with sequences derived from the HLA-C locus (see figure 6.8.2 and Ellis et al. (1989), respectively).

A detailed comparison of nucleotide substitutions in the coding region, shows that JW1 differs from Cw1-4 in five positions (393, 418, 844, 904, 991, these numbers do not correspond to the positions in the actual genomic sequence) where the latter are in full agreement, and in two positions (374, 425) where a Cw1-4 consensus cannot be derived. At eight positions (465, 492, 545, 565, 566, 611, 736, 912) when a consensus cannot be derived, the corresponding nucleotide of JW1 is always the same as one other sequence. The % homology between the exonic coding sequences of JW1 with HLA-A3, HLA-B7 and four C-locus (Cw1-4) is illustrated in figures 6.8.3 and 6.8.4. These figures give a detailed summary of where these differences lie with an exact account of the number of nucleotides used in the calculations.

Overall, the four alleles Cw1-4 are 98.5% homologous to each other, while JW1 is 99.6% homologous to a C-locus consensus! This figure however is obviously biased because

```
          1 signal peptide                                                  50
 Cw3ex    ATGCGGGTCA TGGCGCCCCG GACCCTCATC CTGCTGCTCT CGGGAGCCCT
 Cw2ex    ATGCGGGTCA TGGCGCCCCG AACCCTCATC CTGCTGCTCT CGGGAGCCCT
 Cw1ex    ATGCGGGTCA TGGCGCCCCG AACCCTCATC CTGCTGCTCT CGGGAGCCCT
Hla-B7          A TGGCGCCCCG AACCGTCCTC CTGCTGCTCT CGGGG.CCCT
Hla-A3          A TGGCGCCCCG AACCCTCCTC CTGCTACTCT CGGGGGCCCT

          51                          α-1                                  100
Cw4rna                               T.●.GCTC CCACTCCATG AGGTATTTCG
 Cw3ex    GGCCCTGACC GAGACCTGGG CCG.●.GCTC CCACTCCATG AGGTATTTCT
 Cw2ex    GGCCCTGACC GAGACCTGGG CCT.●.GCTC CCACTCCATG AGGTATTTCT
 Cw1ex    GGCCCTGACC GAGACCTGGG CCT.●.GCTC CCACTCCATG AAGTATTTCT
Hla-B7    GGC..TGACC GAGACCTGGG CGG.●.GCTC CCACTCCATG AGGTATTTCT
Hla-A3    GGCCCTGACC CAGACCTGGG CGG.●.GCTC CCACTCCATG AGGTATTTCT

         101                                                              150
Cw4rna   ACACCGCCGT GTCCCGGCCC GGCGCCGGAG AGCCCCGCTT CATCTCAGTG
 Cw3ex   GCACCGCTGT GTCCCGGCCC GGACGCGGGG AGCCCCACTT CATCGCCGTG
 Cw2ex   ACACCGCTGT GTCCCGGCCC AGCCGCGGAG AGCCCCACTT CATCGCAGTG
 Cw1ex   TCACATCCGT GTCCCGGCCT GGCCGCGGAG AGCCCCGCTT CATCTCAGTG
Hla-B7   ACACATCCGT GTCCCGGCCC NNNNNNGGGG AGCCCCG.CT CATCTCAGTG
Hla-A3   TCACATCCGT GTCCCGGCCC GGCCGCGGGG AGCCCCGCTT CATCGCCGTG

         151                                                              200
Cw4rna   GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGCCGCGAG
 Cw3ex   GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGACGAGAG
 Cw2ex   GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGCCGCGAG
 Cw1ex   GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGCCGCGAG
Hla-B7   GGCTACGTGG ACGACACCCA GTTCGTGAGG TTCGACAGCG ACGCCGCGAG
Hla-A3   GGCTACGTGG ACGACACGCA GTTCGTGCGG TTCGACAGCG ACGCCGCGAG

         201                                                              250
Cw4rna   TCCGAGAGGG GAGCCGCGGG CGCCGTGGGT GGAGCAGGAG GGGCCGGAGT
 Cw3ex   TCCGAGAGGG GAGCCGAGGG CGCCGTGGGT GGAGCGGAAG GGGCCGGAGT
 Cw2ex   TCCAAGAGGG GAGCCGCGGG GCCGCTGGGT GGAGCAGGAG GGGCCGGAGT
 Cw1ex   TCCGAGAGGG GAGCCGCGGG CGCCGTGGGT GGAGCAGGAG GGGCCGGAGT
Hla-B7   TCCGAGAGAG GAGCCGCGGG CGCCGTGGAT AGAGCAGGAG GGGCCGGAGT
Hla-A3   CCAGAGGATG GAGCCGCGGG CGCCGTGGAT AGAGCAGGAG GGGCCGGAGT

         251                                                              300
Cw4rna   ATTGGGACCG GGAGACACAG AAGTACAAGC GCCAGGCACA GGCTGACCGA
 Cw3ex   ATTGGGACCG GGAGACACAG AAGTACAAGC CCCAGGCACA GACTGACCGA
 Cw2ex   ATTGGGACCG GGAGACACAG AAGTACAAGC GCCAGGCACA GACTGACCGA
 Cw1ex   ATTGGGACCG GGAGACACAG AAGTACAAGC GCCAGGCACA GACTGACCGA
Hla-B7   ATTGGGACCG GAACACACAG ATCTACAAGG CCCAAGCACA GACTGACCGA
Hla-A3   ATTGGGACCA GGAGACACGG AATGTGAAGG CCCAGTCACA GACTGACCGA
```

Figure 6.8.2. Exon and 3'UT Sequence Comparison of JW1 with HLA-A3 (Strachan et al. 1984); HLA-B7 (Sood et al. 1985); HLA-Cw1 and HLA-Cw2 (Gussow et al. 1987); HLA-Cw3 (Sodoyer et al. 1984) and HLA-Cw4mRNA (Davidson et al. 1985).
The exons boundaries are marked by (.●.) with a note of the protein domain that follows (refer to figure 1.1.4c).

```
          301                                                       350
Cw4rna    GTGAGCCTGC GGAACCTGCG CGGCTACTAC AACCAGAGCG AGGACG.●.G
 Cw3ex    GTGAGCCTGC GGAACCTGCG CGGCTACTAC AACCAGAGCG AGGCCG.●.G
 Cw2ex    GTGAACCTGC GGAAACTGCG CGGCTACTAC AACCAGAGCG AGGCCG.●.G
 Cw1ex    GTGAGCCTGC GGAACCTGCG CGGCTACTAC AACCAGAGCG AGGCCG.●.G
 Jw1ex                                                     .●.G
Hla-B7    GAGAGCCTGC GGAACCTGCG CGGCTACTAC AACCAGAGCG AGGCTG.●.G
Hla-A3    GTGGACCTGG GGACCCTGCG CGGCTACTAC AACCAGAGCG AGGCCG.●.G

          351 α-2                                                   400
Cw4rna    GTCTCACACC CTCCAGAGGA TGTCTGGCTG CGACCTGGGG CCCGACGGGC
 Cw3ex    GTCTCACATC ATCCAGAGGA TGTATGGCTG CGACGTGGGG CCCGACGGGC
 Cw2ex    GTCTCACACC CTCCAGAGGA TGTACGGCTG CGACCTGGGG CCCGACGGGC
 Cw1ex    GTCTCACACC CTCCAGTGGA TGTGTGGCTG CGACCTGGGG CCCGACGGGC
 Jw1ex    GTCTCACACC CTCCAGAGGA TGTTTGGCTG CGACCTGGGG CCGGACGGGC
Hla-B7    GTCTCACACC CTTCAGAGCA TGTACGGTTG CGACGTGGGG CCGGACGGGC
Hla-A3    TTCTCACACC ATCCAGATAA TGTATGGCTG CGACGTGGGG TCGGACGGGC

          401                                                       450
Cw4rna    GCCTCCTCCG CGGGTATGAC CAGTCCGCCT ACGACGGCAA GGATTACATC
 Cw3ex    GCCTCCTCCG CGGGTATGAC CAGCACGCCT ACGACGGCAA GGATTACATC
 Cw2ex    GCCTCCTCCG CGGGTATGAC CAGTCCGCCT ACGACGGCAA GGATTACATC
 Cw1ex    GCCTCCTCCG CGGGTATGAC CAGTACGCCT ACGACGGCAA GGATTACATC
 Jw1ex    GCCTCCTCCG CGGGTATAAC CAGTTCGCCT ACGACGGCAA GGATTACATC
Hla-B7    GCCTCCTCCG CGGGCATGAC CAGTACGCCT ACGACGGCAA GGATTACATC
Hla-A3    GCTTCCTCCG CGGGTACCGG CAGGACGCCT ACGACGGCAA GGATTACATC

          451                                                       500
Cw4rna    GCCCTGAACG AGGACCTGCG CTCCTGGACC GCGGCGGACA CCGCGGCTCA
 Cw3ex    GCCCTGAACG AGGATCTGCG CTCCTGGACC GCCGCGAACA CGGCGGCTCA
 Cw2ex    GCCCTGAACG AGGACCTGCG CTCCTGGACC GCCGCGGACA CAGCGGCTCA
 Cw1ex    GCCCTGAACG AGGACCTGCG CTCCTGGACC GCCGCGGACA CCGCGGCTCA
 Jw1ex    GCCCTGAACG AGGATCTGCG CTCCTGGACC GCCGCGGACA CGGCGGCTCA
Hla-B7    GCCCTGAACG AGGACCTGCG CTCCTGGACC GCCGCGGACA CGGCTGCGCA
Hla-A3    GCCCTGAACG AGGACCTGCG CTCTTGGACC GCGGCGGACA TGGCGGCTCA

          501                                                       550
Cw4rna    GATCACCCAG CGCAAGTGGG AGGCGGCCCG TGCGGCGGAG CAGCTGAGAG
 Cw3ex    GATCACCCAG CGCAAGTGGG AGGCGGCCCG TGAGGCGGAG CAGCTGAGAG
 Cw2ex    GATCACCCAG CGCAAGTGGG AGGCGGCCCG TGAGGCGGAG CAGTGGAGAG
 Cw1ex    GATCACCCAG CGCAAGTGGG AGGCGGCCCG TGAGGCGGAG CAGCGGAGAG
 Jw1ex    GATCACCCAG CGCAAGTGGG AGGCGGCCCG TGAGGCGGAG CAGCGGAGAG
Hla-B7    GATCACCCAG CGCAAGTGGG AGGCGGCCCG TGAGGCGGAG CAGCGGAGAG
Hla-A3    GATCACCAAG CGCAAGTGGG AGGCGGCCCA TGAGGCGGAG CAGTTGAGAG

          551                                                       600
Cw4rna    CCTACCTGGA GGGACTGTGC GTGGAGTGGC TCCGCAGATA CCTGGAGAAC
 Cw3ex    CCTACTTGGA GGGCCTGTGC GTGGAGTGGC TCCGCAGATA CCTGAAGAAT
 Cw2ex    CCTACCTGGA GGGCGAGTGC GTGGAGTGGC TCCGCAGATA CCTGGAGAAC
 Cw1ex    CCTACCTGGA GGGCACGTGC GTGGAGTGGC TCCGCAGATA CCTGGAGAAC
 Jw1ex    CCTACCTGGA GGGCACGTGC GTGGAGTGGC TCCGCAGATA CCTGGAGAAC
Hla-B7    CCTACCUGGA GGGCGAGTGC GTGGAGTGGC TCCGAAGATA CCTGGAGAAC
Hla-A3    CCTACCTGGA TGGCACGTGC GTGGAGTGGC TCCGCAGATA CCTGGAGAAC

          601                                α-3                    650
Cw4rna    GGGAAGGAGA CGCTGCAGCG CGCA..●.AA CCCCCAAAGA CACACGTGAC
 Cw3ex    GGGAAGGAGA CGCTGCAGGG CGCGG.●.AA CACCCAAAGA CACACGTGAC
 Cw2ex    GGGAAGGAGA AGCTGCAGCG CGCGG.●.AA CACCCAAAGA CACACGTGAC
 Cw1ex    GGGAAGGAGA GCCTGCAGCG CGCGG.●.AA CACCCAAAGA CACACGTGAC
 Jw1ex    GGGAAGGAGA CGCTGCAGCG CGCGG.●.AA CACCCAAAGA CACACGTGAC
Hla-B7    GGGAAGGACA AGCTGGAGCG CGCTG.●.AC CCCCCTAAGA CACACGTGAC
Hla-A3    GGGAAGGAGA CGCTGC.... ......●.AC CCCCCCAAGA CACATATGAC
```

```
          651                                                       700
Cw4rna    CCACCACCCC CTCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTGG
 Cw3ex    CCACCATCCC GTCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTGG
 Cw2ex    CCACCATCCC GTCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTGG
 Cw1ex    CCACCATCCC GTCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTGG
 Jw1ex    CCACCATCCC GTCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTGG
Hla-B7    CCACCACCCC ATCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTTG
Hla-A3    CCACCACCCC ATCTCTGACC ATGAGGCCAC CCTGAGGTGC TGGGCCCTGG

          701                                                       750
Cw4rna    GCTTCTACCC TGCGGAGATC ACACTGACCT GGCAGCGGGA TGGGGAGGAC
 Cw3ex    GCTTCTACCC TGCGGAGATC ACACTGACCT GGCAGTGGGA TGGGGAGGAC
 Cw2ex    GCTTCTACCC TACGGAGATC ACACTGACCT GGCAGCGGGA TGGCGAGGAC
 Cw1ex    GCTTCTACCC TGCGGAGATC ACACTGACCT GGCAGTGGGA TGGGGAGGAC
 Jw1ex    GCTTCTACCC TGCGGAGATC ACACTGACCT GGCAGTGGGA TGGGGAGGAC
Hla-B7    GTTTCTATCC TGCGGAGATC ACACTGACCT GGCAGCGGGA TGGCGAGGAC
Hla-A3    GCTTCTACCC TGCGGAGATC ACACTGACCT GGCAGCGGGA TGGGGAGGAC

          751                                                       800
Cw4rna    CAGACCCAGG ACACCGAGCT TGTGGAGACC AGGCCAGCAG GAGATGGAAC
 Cw3ex    CAAACTCAGG ACACTGAGCT TGTGGAGACC AGGCCAGCAG GAGATGGAAC
 Cw2ex    CAAACTCAGG ACACCGAGCT TGTGGAGACC AGGCCAGCAG GAGATGGAAC
 Cw1ex    CAAACTCAGG ACACCGAGCT TGTGGAGACC AGGCCAGCAG GAGATGGAAC
 Jw1ex    CAAACTCAGG ACACCGAGCT TGTGGAGACC AGGCCAGCAG GAGATGGAAC
Hla-B7    CAAACTCAGG ACACTGAGCT TGTGGAGACC AGACCAGCAG GAGATAGAAC
Hla-A3    CAGACCCAGG ACACGGAGCT CGTGGAGACC AGGCCTGCAG GGGATGGAAC

          801                                                       850
Cw4rna    CTTCCAGAAG TGGGCAGCTG TGGTGGTGCC TTCTGGACAA GAGCAGAGAT
 Cw3ex    CTTCCAGAAG TGGGCAGCTG TGGTGGTGCC TTCTGGAGAA GAGCAGAGAT
 Cw2ex    CTTCCAGAAG TGGGCAGCTG TGGTGGTGCC TTCTGGAGAA GAGCAGAGAT
 Cw1ex    CTTCCAGAAG TGGGCAGCTG TGATGGTGCC TTCTGGAGAA GAGCAGAGAT
 Jw1ex    CTTCCAGAAG TGGGCAGCTG TGGTGGTGCC TTCTGGAGAA GAGCAGAGAT
Hla-B7    CTTCCAGAAT TCGGCAGCTG TGGTGGTGCC ATCTGGAGAA GAGCAGAGAT
Hla-A3    CTTCCAGAAG TGGGCGGCTG TGGTGGTGCC TTCTGGAGAG GAGCAGAGAT

          851                                                       900
Cw4rna    ACACGTGCCA TATGCAGCAC GAGGGGCTGC AAGAGCCCCT CACCCTGAGC
 Cw3ex    ACACGTGCCA TGTGCAGCAC GAGGGGCTGC CGGAGCCCCT CACCCTGAGA
 Cw2ex    ACACGTGCCA TGTGCAGCAC GAGGGGCTGC CGGAGCCCCT CACCCTGAGA
 Cw1ex    ACACGTGCCA TGTGCAGCAC GAGGGGCTGC CGGAGCCCCT CACCCTGAGA
 Jw1ex    ACACGTGCCA TGTTCAGCAC GAGGGGCTGC CGGAGCCCCT CACCCTGAGA
Hla-B7    ACACATGCCA TGTACAGCAT GAGGGGCTGC CGAAGCCACT CACCCTGGGA
Hla-A3    ACACCTGCCA TGTGCAGCAT GAGGGTCTGC CCAAGCCCCT CACCCTGAGA

          901  Tm                                                   950
Cw4rna    TGGG.●.AGC CATCTTCCCA GCCCACCATC CCCATCATGG GCATCGTTGC
 Cw3ex    TGGG.●.AGC CGTCTTCCCA GCCCACCATC CCCATCGTGG GCATCGTTGC
 Cw2ex    TGGG.●.AGC CATCTTCCCA GCCCACCATC CCCATCGTGG GCATCGTTGC
 Cw1ex    TGGG.●.AGC CGTCTTCCCA GCCCACCATC CCCATCGTGG GCATCGTTGC
 Jw1ex    TGGA.●.AGC CGTCTTCCCA GCCCACCATC CCCATCGTGG GCATCGTTGC
Hla-B7    TGGG.●.AGC CGTCTTCCCA GTCCACCGTC CCCATCGTTG GCATTGTTGC
Hla-A3    TGGG.●.AGC TGTCTTCCCA GCCCACCATC CCCATCGTGG GCATCATTGC

          951                                                      1000
Cw4rna    TGGCCTGGCT GTCCTGGTTG TCCTAGCTGT CCTTGGAGCT GTGGTCACCG
 Cw3ex    TGGCCTGGCT GTCCTGGCTG TCCTAGCTGT CCTAGGAGCT GTGGTGGCTG
 Cw2ex    TGGCCTGGCT GTCCTGGCTG TCCTAGCTGT CCTAGGAGCT GTGGTGGCTG
 Cw1ex    TGGCCTGGCT GTCCTGGCTG TCCTAGCTGT CCTAGGAGCT GTGGTGGCTG
 Jw1ex    TGGCCTGGCT GTCCTGGCTG TCCTAGCTGT CCTAGGAGCT ATGGTGGCTG
Hla-B7    TCGCCCGGCT GTCCTAGCAG TTGTGGTCAT CGGAGCTGTG GTCGCTGCT.
Hla-A3    TGGCCTGGTT CTCCTTGGAG CTGTGATCAC TGGAGCTGTG GTCGCTGCC.
```

```
                1001                                    C1                  1050
Cw4rna  CTATGATGTG TAGGAGGAAG AGCTCAG.●. GTGGAAAAGG AGGGAGCTGC
 Cw3ex  TTGTGATGTG TAGGAGGAAG AGCTCAG.●. GTGGAAAAGG AGGGAGCTGC
 Cw2ex  TTGTGATGTG TAGGAGGAAG AGCTCAG.●. GTGGAAAAGG AGGGAGCTGC
 Cw1ex  TTGTGATGTG TAGGAGGAAG AGCTCAG.●. GTGGAAAAGG AGGGAGCTGC
 Jw1ex  TTGTGATGTG TAGGAGGAAG AGCTCAG.●. GTGGAAAAGG AGGGAGCTGC
Hla-B7  ..GTGATGTG TAGGAGGAAG AGTTCAG.●. GTGGAAAAGG AGGGAGTTAC
Hla-A3  ..GTGATGTG GAGGAGGAAG AGCTCAG.●. gtAGAAAAGG AGGGAGTTAC

                1051                C2                                  1100
Cw4rna  TCTCAGGCTG CGT.●.GCAG CAACAGTGCC CAGGGCTCTG ATGAGTCTCT
 Cw3ex  TCTCAGGCTG CGT.●.CCAG CAACAGTGCC CAGGGCTCTG ATGAGTCTCT
 Cw2ex  TCTCAGGCTG CGT.●.CCAG CAACAGTGCC CAGGGCTCTG ATGAGTCTCT
 Cw1ex  TCTCAGGCTG CGT.●.CCAG CAACAGTGCC CAGGGCTCTG ATGAGTCTCT
 Jw1ex  TCTCAGGCTG CGT.●.CCAG CAACAGTGCC CAGGGCTCTG ATGAGTCTCT
Hla-B7  TCTCAAGCTG CGT.●.GCAG CGACAGTGCC CAGGGCTCTG ATGTGTCTCT
Hla-A3  ACTCAGGCTG CAA.●.GCAG TGACAGTGCC CAGGGCTCTG ATGTGTCCCT

                1101    ●           C3      stop    3'UT                    1150
Cw4rna  CATCACTTGT AAAG.●.CC. ●.TGA.●.GA CAGCTGCCT. GTGTGGGACT
 Cw3ex  CATCGCTTGT AAAG.●.CC. ●.TGA.●.GA CAGCTGCCT. GTGTGGGACT
 Cw2ex  CATCGCTTGT AAAG.●.CC. ●.TGA.●.GA CAGCTGCCT. GTGTGGGACT
 Cw1ex  CATCGCTTGT AAAG.●.CC. ●.TGA.●.GA CAGCTGCCT. GTGTGGGACT
 Jw1ex  CATCGCTTGT AAAG.●.CC. ●.TGA.●.GA CAGCTGCCT. GTGTGGGACT
Hla-B7  CACAGCTTGA AAAG.●.CC. ●.TGA.●.GA CAGCTGTCTT GTGAGGGACT
Hla-A3  CACAGCTTGT AAAG.●.TG. ●.TGA.●.GA CAGCTGCCTT GTGTGGGACT

                1151                ◆                                   1200
Cw4rna  GAGATGCAGG A.TTTCTTCA CACCTCTCC. TTTGTGACTT CAAGAGCCTC
 Cw3ex  GAGATGCAGG A.TTTCTTCA CACCTCTCC. TTTGTGACTT CAAGAGCCTC
 Cw2ex  GAGATGCAGG A.TTTCCTCA CACCTCTCC. TTTGTGACTT CAAGAGCCTC
 Cw1ex  GAGATGCAGG A.TTTCCTCA CACCTCTCC. TTTGTGACTT CAAGAGCCTC
 Jw1ex  GAGATGCAGG A.TTTCTTCA CACCTCTCC. TTTGTGACTT CAAGAGCCTC
Hla-B7  GAGATGCAGG A.TTTCTTCA CGCCTCCCC. TTTGTGACTT CAAGAGCCTC
Hla-A3  GAGAGGCAAG AGTTTGTTCC TGCCCTTCCC TTTGTGACTT GAAGAACC.C

                1201                                                        1250
Cw4rna  TGGCATCTCT TTCTGCAAAG GCACCTGAAT GTGTCTGCGT TCCTGTTAGC
 Cw3ex  TGGCATCTCT TTCTGCAAAG GCATCTGAAT GTGTCTGCGT TCCTGTTAGC
 Cw2ex  TGGCATCTCT TTCTACAAAG GCATCTGAAT GTGTCTGCGT CCCTGTTAGC
 Cw1ex  TGGCATCTCT TTCTGCAAAG GCATCTGAAT GTGTCTGCGT TCCTGTTAGC
 Jw1ex  TGGCATCTCT TTCTGCAAAG GCATCTGAAT GTGTCTGCGT TCCTGTTAGC
Hla-B7  TGGACTCT.T TTCTGCAAAG GCACCTGAAT GTGTCTGCGT CCCTGTTAGC
Hla-A3  T.GACTTTGT TTCTGCAAAG GCACCTGCAT GTGTCTGTGT TCGTGTAGGC

                1251                                                        1300
Cw4rna  ATAATGTGAG GAGGTGGAGA GAC.AGCCCA CCCCCGTGTC CACCGTGACC
 Cw3ex  ATAATGTGAG GAGGTGGAGA GAC.AGCCCA CCCCC.TGTC CACCGTGACC
 Cw2ex  ATAATGTGAG GAGGTGGAGA GACCAGCCCA CCCCCGTGTC CACCGTGACC
 Cw1ex  ATAATGTGAG GAGGTGGAGA GAC.AGCCCA CCCCCGTGTC CAC.GTGACC
 Jw1ex  ATAATGTGAG GAGGTGGAGA GAC.AGCCCA CCCCCGTGTC CACCGTGACC
Hla-B7  ATAATGTGAG GAGGTGGAGA GAC.AGCCCA CCCTTGTGTC CACTGTGACC
Hla-A3  ATAATGTGAG GAGGTGGGGA GACCACCCCA CCCCCATGTC CACCATGACC

                1301                                                        1350
Cw4rna  CCTGTCCCCA CACTGACCTG TGTTCCCTCC CCGATCATCT TTCCTGTTCC
 Cw3ex  CCTGTCCCCA CACTGACCTG TGTTCCCTCC CCGATCATCT TTCCTGTTCC
 Cw2ex  CCTGTCCCCA CACTGACCTG TGTTCCCTCC CCGATCATCT TTCCTGTTCC
 Cw1ex  CCTGTCCCCA CACTGACCTG TGTTCCCTCC CCGATCATCT TTCCTGTTCC
 Jw1ex  CCTGTCCCCA CACTGACCTG TGTTCCCTCC CCGATCATCT TTCCTGTTCC
Hla-B7  CCTGTTCCCA TGCTGACCTG TGTTTCCTCC CCAGTCATCT TTCTTGTTCC
Hla-A3  C.TCTTCCCA CGCTGACCTG TGCTCCCTCC CCAATCATCT TTCCTGTTCC
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          1351                                                      1400
Cw4rna    AGAGAGGTGG GGCTGGA.TG TCTCCATCTC TGTCTCAAAT TCATGGTGCA
 Cw3ex    AGAGAAGTGG G.CTGGA.TG TCTCCATCTC TGTCTCAACT TCATGGTGCG
 Cw2ex    AGAGAAGTGG G.CTGGA.TG TCTCCATCTC TGTCTCAACT TTA.CGTGTA
 Cw1ex    AGAGAAGTGG G.CTGGA.TG TCTCCATCTC TGTCTCAACT TCATGGTGCG
 Jw1ex    AGAGAAGTGG G.CTGGA.TG TCTCCATCTC TGTCTCAACT TCATGGTGCG
Hla-B7    AGAGAGGTGG GGCTGGA.TG TCTCCATCTC TGTCTCAACT TTA.CGTGCA
Hla-A3    AGAGAGGTGG GGCTGAGGTG TCTCCATCTC TGTCTCAACT TCATGGTGCA

          1401                                                      1450
Cw4rna    CTGAGCTGCA ACTTCTTACT TCCCTAATGA AGTTAAGAAC CTGAATATAA
 Cw3ex    CTGAGCTGCA ACTTCTTACT TCCCTAATGA AGTTAGGAAC CTGAATATAA
 Cw2ex    CTGAGCTGCA ACTTCTT... .CCCTACTGA AAATAGGAAT CTGAATATAA
 Cw1ex    CTGACGTGCA ACTTCTTACT TCCCTAATGA AGTTAAGAAC CTGAATATAA
 Jw1ex    CTGAGCTGCA ACTTCTTACT TCCCTAATGA AGTTAAGAAC CTGAATATAA
Hla-B7    C.GAGCTGCA ACTTCTTACT TCCCTACTGA AAATAAGAAT C.GAATATAA
Hla-A3    CTGAGCTGTA ACTTCTTCCT TCCCTATTAA AATTAGAACC TT.AGTATAA

          1451                                                      1500
Cw4rna    ATTTGTGTTC TCAAATATTT GCTATGAAGC GTTGATGGAT TAATTAAATA
 Cw3ex    ATTTGTTTTC TCAAATATTT GCTATGAAGG GTTGATGGAT TAATTAAATA
 Cw2ex    ATTTGTTTTC TCAAATATTT GCTATGAGAG GTTGATGGAT TAATTAAATA
 Cw1ex    ATTTGTTTTC TCAAATATTT GCTATGAAGG GTTGATGGAT TAATTAAATA
 Jw1ex    ATTTGTTTTC TCAAATATTT GCTATGAAGG GTTGATGGAT TAATTAAATA
Hla-B7    ATTTGTTTTC TCAAATATTT GCTATGAGAG GTTGATGGAT TAATTAAATA
Hla-A3    ATTTACTTTC TCAAATTCTT GCCATGAGAG GTTGATGAGT TAATTAAAGG

          1501                                                      1550
Cw4rna    AGTCAATTCC TAAAAAC
 Cw3ex    AGTCAATTCC TGGAAGTTGA GAGAGCAAAT AAAGACC... ..TGAGAAGC
 Cw2ex    AGTCAATTCC TGGAAGTTGA GAGAGCAAAT AAAGACC... ...GAGAACC
 Cw1ex    AGTCAATTCC TGGAAGTTGA GAGAGCAAAT AAAGACC... ..TGAGAACC
 Jw1ex    AGTCAATTCC TGGAAGTTGA GAGAGCAAAT AAAGACC... ..TGAGAACC
Hla-B7    AGTCAATTCC TGGAATTTGA GAGAGCAAAT AAAGACC... ..TGAGAACC
Hla-A3    AGAAGATTCC TAAAATTTGA GAGACAAAAT AAATGGAAGA CATGAGAACC

          1551                                                      1600
 Cw3ex    TTTCCAGAAT CCGCATGTTC TCTGTGGCTG AGTCTGTTGC AGGTGGGGGT
 Cw2ex    TT.CCAGAAT CCGC
 Cw1ex    TT.CCAGAAT CCGC
 Jw1ex    TT.CCAGAAT CCGCAAAAAA AAAAAAAAA
Hla-B7    ...CCAG
Hla-A3    TT.CCAGAGT CCACGTGTTT CTTGTGCTGA TTTGTTGCAG GGGAGGAGAG
```

Allele	Sig	α-1	α-2	α-3	Tm	C1	C2	C3	3'UT
Cw4	-	4	3	8	6	0	2	0	9*
Cw3	2	8	8	1	0	0	0	0	2
Cw2	0	8	2	2	0	0	0	0	18
Cw1	0	4	2	1	0	0	0	0	3
JW1	-	-	2	2	1	0	0	0	0
No. positions where there is no consensus	0	5	8	1	1	0	0	0	3
No. of nucleotides	73	270	276	276	120	33	48	2	428
Total no. of variable pos^{ns}.	2	29	25	15	8	0	2	0	35
Overall Cw1 - 4 % Homology	99.5	98.0	98.2	99.0	98.7	100	99.2	100	98.4

SUMMARY

Allele	Tot. No. Nuc' Diffs.	Tot. No. Nucleotides.	% Homology with the consensus.
Cw4	31	1412	97.8%
Cw3	21	1526	98.7%
Cw2	30	1520	98.0%
Cw1	10	1524	99.3%
JW1	5	1182	99.6%

Figure 6.8.3. Comparison of HLA-C-locus Nucleotide

JW1/HLA-A3

	Sig	α-1	α-2	α-3	Tm	C1	C2	C3	3'UT
Diff. Nucleotides	-	-	28	22	30	3	2	2	80
No. Nucleotides	-	-	276	278	121	30	48	2	435
% Homology	-	-	89.9%	92.1%	75.3%	90%	95.9%	0%	82.7%

JW1/HLA-B7

	Sig	α-1	α-2	α-3	Tm	C1	C2	C3	3'UT
Diff. Nucleotides	-	-	17	17	9	3	6	2	37
No. Nucleotides	-	-	276	278	121	30	48	2	419
% Homology	-	-	94%	93.9%	92.6%	90.0%	87.5%	0%	91.2%

HLA-A3/HLA-B7

	Sig	α-1	α-2	α-3	Tm	C1	C2	C3	3'UT
Diff. Nucleotides	6	36	32	26	19	4	3	2	83
No. Nucleotides	64	270	276	276	118	34	48	2	428
% Homology	90.7%	86.7%	88.4%	90.6%	83.9%	88.2%	93.7%	0%	80.6%

Figure 6.8.4 Comparison of Inter-Locus Nucleotide Sequence Homologies.
JW1 represents a HLA-C locus derived sequence.
These figures are similar to those of Sodoyer et al. 1984.

the likely variable alpha-1 domain can not be included in the analysis. The data has been presented per exon in a tabulated form for this reason. All C-locus sequences are extremely homologous over the transmembrane and cytoplasmic domains, indeed there is complete identity with Cw1, Cw2 and Cw3 in exons 6-8 which includes the HLA-C locus specific region (Strachan et al. 1986 and Smeaton et al. 1987).

In the non-coding region, there are three positions (1167, 1400, 1436) where a consensus cannot be derived, but the nucleotides of JW1 agree with one or more nucleotides of Cw1-4.

Comparisons of JW1 (C-locus) with HLA-A3 and HLA-B7 and of HLA-A3/HLA-B7 are shown in figure 6.8.4. The percentage homologies are typical of interlocus comparisons made elsewhere (Sodoyer et al. 1985; Strachan et al. 1984).

Intron 2 of JW1 was nearly full length and over 221bp differed at six positions (>97% homology) when compared to other HLA-C-locus intron 2 sequences (see figure 6.8.5). Where Cw1-3 are in full agreement, JW1 differs at positions 26, 83 and 250. In two of these positions (74, 205) the nucleotide of JW1 was the same as another sequence which also differed from the consensus. The nucleotide of JW1 at position 228 is different from the other sequences which are not in agreement. The % homology of intron 2 when compared to intron 2 of HLA-Aw24, HLA-B27, HLA-6.0 and HLA-E is 76.2%, 82.4%, 73% and 84% respectively.

6.9. Comparison of JW1 and HLA-C amino acid sequences.

A comparison of the amino acid sequences from the alpha-1, -2 and -3 domains of class I HLA-A, -B and -C molecules shows an overall conservation of 183/273 residues. There are only six locus-specific residues (HLA-A 138_{Met} and 189_{Met}, HLA-B 239_{Arg}, HLA-C 52_{Val}, 183_{Glu} and 268_{Glu}), though at no one position are all three loci different (reviewed by Parham et al. 1988). Initially, the comparison of few sequences enabled the identification of locus specific sequences however, this is becoming increasingly difficult since the publication of new alleles often abolishes this specificity. As single residues predominate at most positions, a consensus sequence can be deduced. Individual molecules from the three loci are seen to differ from the

Figure 6.8.5. Nucleotide sequence comparison of JW1 Intron 2 with those from HLA-Aw24 (N'Guyen et al. 1984), **HLA-B27** (Weiss et al. 1985), **HLA-Cw1 and HLA-Cw2** (Gussow et al. 1987) and **HLA-Cw3** (Sodoyer et al. 1984). JW1 intron 2 is 221bp and begins at position 27 in this comparison. The positions where JW1 differs from those sequences of HLA-Cw1-3 are marked in blue. See section 6.8 for a description of these differences.

```
            1                                                      50
 Hla-Aw24   GTGAGTG.AC CCCGGCCCGG GGCGCAGGTC ACGACCC.T. ....CATCCC
  Hla-B27   GTGAGTG.AC CCCGGCCCGG G.CGCAGGTC ACGAC...TC CC..CATCCC
  Hla-Cw3   GTGAGTGGAC CCCGGCCCGG GGCGCAGGTC ACGACCCCTC CT..CATCCC
  Hla-Cw2   GTGAGTG.AC CCCGGCCCGG GC.GCAGGTC ACGACCCCTC CC..CATCCC
  Hla-Cw1   GTGAGTG.AC CCCGGCCCGG GGCGCAGGTC ACGACCCCTC CT..CATCCC
Intronjw1                                  CGGTC ACGACCCCTC CC..CATCCC
    Hla-6   GTGAGTA.AC TCCGGCCCAG GGAGCAGATC ACGACCCCCA CCTCCATGCC
    Hla-E   GTGAGTG.AC CCCGGCC.AG G.AGCAGGTC ACGACCCCTC CC..CATCCC

            51                                                    100
 Hla-Aw24   CCACGGAGGG CCGGGGCGCC CACAGGCTCC GGGGCCT.AG ATCCACCCC.
  Hla-B27   CCACGTACGG CCCGGGTCGC CCCGAGTCTC CGGGTCCGAG ATCCGCCCCC
  Hla-Cw3   CCACGGACGG CCCGGGTCGC CCCAAGTCTC CCGGTCTGAG ATCCACCCCG
  Hla-Cw2   CCACGGACGG CCCGGGTCGC CCCGAGTCTC CGGGTCTGAG ATCCACCCCG
  Hla-Cw1   CCACGGACGG CCCGGGTCGC CCCAAGTCTC CCGGTCTGAG ATCCACCCCG
Intronjw1   CCACGGACGG CCCGGGTCGC CCCGAGTCTC CCCGTCTGAG ATCCACCCCG
    Hla-6   CCACGGACGG CCCGGGTACT CCCGAGTCTC CGGGTCTGGG ATCCACCCCG
    Hla-E   CCACGGACGG CGCGGGTCCC CTCGAATCTT CGGGTCCCAG ATTCACCCCA

            101                                                   150
 Hla-Aw24   GAAGCCGCGG GACCCCG..A GACCCTTGCC CTGGGAGAGG CCCAGGCGCC
  Hla-B27   GAGGCCGCGG GACCCGCCCA GACCCTCGAC CGGCGAGAG. CCCAGGCGCG
  Hla-Cw3   AGGCTGCGGA ACCCG....A GACCCTCGAC CGGAGAGAGC CCCAGTCACC
  Hla-Cw2   AGGCTGCGGA ACCCGCCC.A GACCCTCGAC CGGAGAGAGC CCCAGTCACC
  Hla-Cw1   AGGCTGCGGA ACCCGCCC.A GACCCTCGAC CGGAGAGAGC CCCAGTCACC
Intronjw1   AGGCTGCGGA ACCCGCCC.A GACCCTCGAC CGGAGAGAGC CCCAGTCACC
    Hla-6   AGGCCGCGGG ACCCGCCC.A GACCCTCTAC CTGGGAGAAC CCCAAGGCGC
    Hla-E   AGGCTGCGGA ACCCGCCC.A GACCCTAGAC CGGGGAGAGT CTCAGGC.GC

            151                                                   200
 Hla-Aw24   .TTAACCCGG TTTCATTTTC AGTTTAGGCC AAAAATCCCC CCGGGTTGGT
  Hla-B27   .TTTACCCGG TTTCATTTTC AGTTGAGGCC AAAA.TCCCC GCGG.TTGGT
  Hla-Cw3   .TTTACCCGG TTTCATTTTC AGTTTAGGCC AAAA.TCCCC GCGGGTTGGT
  Hla-Cw2   .TTTACCCGG TTTCATTTTC AGTTTAGGCC AAAA.TCCCC GCGGGTTGGT
  Hla-Cw1   .TTTACCCGG TTTCATTTTC AGTTTAGGCC AAAA.TCCCC GCCGGTTGGT
Intronjw1   .TTTACCCGG TTTCATTTTC AGTTTAGGCC AAAA.TCCCC GCGGGTTGGT
    Hla-6   CTTTA..... .......... ........CC AAAA.TCCCC GCGGGTGGGT
    Hla-E   CTTTACCCGG TT.CTTTTTC AGTTTAGGCC AAAA.TGCCC ACAGGGTGGT

            201                                                   250
 Hla-Aw24   CGGGGC.CGG GCGGGGCTCG GGGGACT.GG GCTGACCGCG GGGTCGGGGC
  Hla-B27   CGGGGC.GGG GCGGGGCTCG GGGGGACGGG GCTGACCGCG GGGGCGGGTC
  Hla-Cw3   CGGGGC.GGG GCGGGGCTCG GGGGAC.GGG GCTGACCGCG GGGGCGGG.C
  Hla-Cw2   CGGGGCTGGG GCGGGGCTCG GGGGAC..GG GCTGACCACG GGGGCGGGGC
  Hla-Cw1   CGGGACTGGG GCGGGGCTCG GGGGAC.GGG GCTGACCACG GGGGCGGGGC
Intronjw1   CGGGACTGGG GCGGGGCTCG GGGGAC.CGG GCTGACCACG GGGGCGGGG.
    Hla-6   CCGGGCGAGG GCGAGGCTCG GTGGGC.GGG GCTGACCGAG GGGGTGGGGC
    Hla-E   GGCGACGGGG GCGGGGCTTG GTGGGC.GGG ACTGACTAA. GGGGCGGGGC

            251
 Hla-Aw24   CAG
  Hla-B27   CAG
  Hla-Cw3   CAG
  Hla-Cw2   CAG
  Hla-Cw1   CAG
Intronjw1   CAG
    Hla-6   CAG
    Hla-E   CAG
```

Figure 6.9.1 Amino acid sequence comparison of JW1 (from the beginning of the alpha-2 domain) with ten class I sequences from different loci. HLA-A3 (Strachan et al. 1984), HLA-A2ex28 (Koller et al. 1985), HLA-B7 (Sood et al. 1985), HLA-B27 (Weiss et al. 1985), HLA-Cw1 and HLA-Cw2 (Gussow et al. 1987), HLA-Cw3 (Sodoyer et al. 1984), HLA-Cw4mRNA (Davidson et al. 1985), HLA-6.0 (Geraghty et al. 1987) and HLA-E (Shimizu et al. 1988). The "Parhcons" represents the consensus sequence derived from a comparison of 39 class I sequences (Parham et al. 1988). The hypervariable positions are marked X. The row "Structure" represents the conformation of the corresponding polypeptide regions, "A" is alpha helix, "B" is beta-pleated sheet and "T" represents a turn. The row "Pclocspec" shows the positions of the three HLA-C locus specific residues (see section 6.9 for details). The residues where JW1 differs from C-locus sequences have been marked .

```
                -23                                  α1                  26
   HLA-A3       MAPRTLL LLLSGALALT QTWAGSHSMR YFFTSVSRPG RGEPRFIAVG
   HLA-A2                                    MR YFFTSVSRPG RGEPRFIAVG
   HLA-B7    MLVMAPRTVL LLLSGPW.LT ETWAGSHSMR YFYTSVSRPX XGEPRFISVG
  HLA-B27    MRVTAPRTLL LLLWGAVALT ETWAGSHSMR YFHTSVSRPG RGEPRFITVG
HLA-Cw4mR                                 CSHSMR YFDTAVSRPG AGEPRFISVG
  HLA-Cw3    MRVMAPRTLI LLLSGALALT ETWAGSHSMR YFCTAVSRPG RGEPHFIAVG
  HLA-Cw2    MRVMAPRTLI LLLSGALALT ETWACSHSMR YFYTAVSRPS RGEPHFIAVG
  HLA-Cw1    MRVMAPRTLI LLLSGALALT ETWACSHSMK YFFTSVSRPG RGEPRFISVG
    HLA-6    MVVMAPRTLF LLLSGALTLT ETWAGSHSMR YFSAAVSRPG RGEPAFIAMG
    HLA-E    MVDGTLLLLL SEALALTQTW AGSHSLKYFH TSVSRPGRGE PRFISVGYVD
Parhmcons                                 GSHSMR YFYTSVSRPG RGEPRFIAVG
   Phyvar                                       X.......  .......X..
Structure                                 TBBBBB BBBBBBBBTT TTTBBBBBBB

               27                                                    76
   HLA-A3    YVDDTQFVRF DSDAASQRME PRAPWIEQEG PEYWDQETRN VKAQSQTDRV
   HLA-A2    YVDDTQFVRF DSDAASQRME PRAPWIEQEG PEYWDGETRK VKAHSQTHRV
   HLA-B7    YVDDTQFVRF DSDAASPREE PRAPWIEQEG PEYWDRNTQI YKAQAQTDRE
  HLA-B27    YVDDTLFVRF DSDAASPREE PRAPWIEQEG PEYWDRETQI CKAKAQTDRE
HLA-Cw4mR    YVDDTQFVRF DSDAASPRGE PRAPWVEQEG PEYWDRETQK YKRQAQADRV
  HLA-Cw3    YVDDTQFVRF DSDDESPRGE PRAPWVERKG PEYWDRETQK YKPQAQTDRV
  HLA-Cw2    YVDDTQFVRF DSDAASPRGE PRGRWVEQEG PEYWDRETQK YKRQAQTDRV
  HLA-Cw1    YVDDTQFVRF DSDAASPRGE PRAPWVEQEG PEYWDRETQK YKRQAQTDRV
    HLA-6    YVDDTQFVRF DSDSACPRME PRAPWVEEQG PEYWEEETRN TKAHAQTDRM
    HLA-E    DTQFVRFDND AASPRMVPRA PWMEQEGSEY WDRETRSARD TAQIFRVNLR
Parhmcons    YVDDTQFVRF DSDAASPRME PRAPWIEQEG PEYWDRETQI VKAQSQTDRE
   Phyvar    .......... .........X. .......... .....X..XX X.XXX..X.X
Structure    BTTTTBBBBB BTTTTTTBBB BBTAAAAAAT TAAAAAAAAA AAAAAAAAAA
Pclocspec                                 V.... .......... ..........

               77                  α2                                126
   HLA-A3    DLGTLRGYYN QSEAGSHTIQ IMYGCDVGSD GRFLRGYRQD AYDGKDYIAL
   HLA-A2    DLGTLRGYYN QSEAGSHTVQ RMYGCDVGSD WRFLRGYHQY AYDGKDYIAL
   HLA-B7    SLRNLRGYYN QSEAGSHTLQ SMYGCDVGPD GRLLRGHDQY AYDGKDYIAL
  HLA-B27    DLRTLLRYYN QSEAGSHTLQ NMYGCDVGPD GRLLRGYHQD AYDGKDYIAL
HLA-Cw4mR    SLRNLRGYYN QSEDGSHTLQ RMSGCDLGPD GRLLRGYDQS AYDGKDYIAL
  HLA-Cw3    SLRNLRGYYN QSEAGSHIIQ RMYGCDVGPD GRLLRGYDQH AYDGKDYIAL
  HLA-Cw2    NLRKLRGYYN QSEAGSHTLQ RMYGCDLGPD GRLLRGYDQS AYDGKDYIAL
  HLA-Cw1    SLRNLRGYYN QSEAGSHTLQ WMCGCDLGPD GRLLRGYDQY AYDGKDYIAL
       JW1                 SHTLQ RMFGCDLGPD GRLLRGYNQF AYDGKDYIAL
    HLA-6    NLQTLRGYYN QSEASSHTLQ WMIGCDLGSD GRLLRGYEQY AYDGKDYLAL
    HLA-E    TLRRYYNQSE AGSHTLQWMH GCELGPDRRF LRGYEQFAYD GKDYLTLNED
Parhmcons    SLRTLRGYYN QSEAGSHTLQ RMYGCDVGPD GRLLRGYHQY AYDGKDYIAL
   Phyvar    X..X...... .........X. X.......... .......X.X ..........
Structure    AAAAAAAATT TTTTTBBBBB BBBBBBBBTT TTTTTBBBBB TTTTTTTBBB
Pclocspec    .......... .......... .......... .......... ..........

               127                                                   176
   HLA-A3    NEDLRSWTAA DMAAQITKRK WEAAHEAEQL RAYLDGTCVE WLRRYLENGK
   HLA-A2    KEDLRSWTAA DMAAQTTKHK WEAAHVAEQL RAYLEGTCVE WLRRYLENGK
   HLA-B7    NEDLRSWTAA DTAAQITQRK WEAAREAEQR RAYLEGECVE WLRRYLENGK
  HLA-B27    NEDLSSWTAA DTAAQITQRK WEAARVAEQL RAYLEGECVE WLRRYLENGK
HLA-Cw4mR    NEDLRSWTAA DTAAQITQRK WEAARAAEQL RAYLEGLCVE WLRRYLENGK
  HLA-Cw3    NEDLRSWTAA NTAAQITQRK WEAAREAEQL RAYLEGLCVE WLRRYLKNGK
  HLA-Cw2    NEDLRSWTAA DTAAQITQRK WEAAREAEQW RAYLEGECVE WLRRYLENGK
  HLA-Cw1    NEDLRSWTAA DTAAQITQRK WEAAREAEQR RAYLEGTCVE WLRRYLENGK
       JW1    NEDLRSWTAA DTAAQITQRK WEAAREAEQR RAYLEGTCVE WLRRYLENGK
    HLA-6    NEDLRSWTAA DTAAQISKRK CEAANVAEQR RAYLEGTCVE WLHRYLKNGK
    HLA-E    LRSWTAVDTA AQISEQKSND ASEAEHQRAY LEDTCVEWLH KYLEKGKETL
Parhmcons    NEDLRSWTAA DTAAQITQRK WEAARVAEQL RAYLEGTCVE WLRRYLENGK
   Phyvar    .......... .......... .........X ......X
Structure    BTTTTBBBBB TAAAAAAAAA AATTTAAAAA AAAAATAAAA AAAAAAATAA
Pclocspec    .......... .......... .......... .......... ..........
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                177                                                    226
   HLA-A3   ETL...HPPK THMTHHPISD HEATLRCWAL GFYPAEITLT WQRDGEDQTQ
   HLA-A2   ETLQRTDAPK THMTHHAVSD HEATLRCWAL SFYPAEITLT WQRDGEDQTQ
   HLA-B7   DKLERADPPK THVTHHPISD HEATLRCWAL GFYPAEITLT WQRDGEDQTQ
  HLA-B27   ETLQRADPPK THVTHHPISD HEATLRCWAL GFYPAEITLT WQRDGEDQTQ
HLA-Cw4mR   ETLQRAEPPK THVTHHPLSD HEATLRCWAL GFYPAEITLT WQRDGEDQTQ
  HLA-Cw3   ETLQGAEHPK THVTHHPVSD HEATLRCWAL GFYPAEITLT WQWDGEDQTQ
  HLA-Cw2   EKLQRAEHPK THVTHHPVSD HEATLRCWAL GFYPTEITLT WQRDGEDQTQ
  HLA-Cw1   ESLQRAEHPK THVTHHPVSD HEATLRCWAL GFYPAEITLT WQWDGEDQTQ
       JW1  ETLQRAEHPK THVTHHPVSD HEATLRCWAL GFYPAEITLT WQWDGEDQTQ
     HLA-6  EMLQRADPPK THVTHHPVFD YEATLRCWAL GFYPAEIILT WQWDGEDQTQ
     HLA-E  LHLEPPKTHV TH...HPISD HEATLRCWAL GFYPAEITLT WQQDGEGHTQ
Parhmcons   ETLQRADPPK THVTHHPISD HEATLRCWAL GFYPAEITLT WQRDGEDQTQ
Structure   AAAATTTTTB BBBBBTTTTT TTBBBBBBBB TTTTTTTBBB BTTTTTTTTT
Pclocspec   ......E... .......... .......... .......... ..........

                227                                          276  Tm
   HLA-A3   DTELVETRPA GDGTFQKWAA VVVPSGEEQR YTCHVQHEGL PKPLTLRWEL
   HLA-A2   DTELVETRPA GDGTFQKWAA VVVPSGQEQR YTCHVQHEGL PKPLTLRWEP
   HLA-B7   DTELVETRPA GDRTFQKSAA VVVPSGEEQR YTCHVQHEGL PKPLTLGWEP
  HLA-B27   DTELVETRPA GDRTFQKWAA VVVPSGEEQR YTCHVQHEGL PKPLTLRWEP
HLA-Cw4mR   DTELVETRPA GDGTFQKWAA VVVPSGQEQR YTCHMQHEGL QEPLTLSWEP
  HLA-Cw3   DTELVETRPA GDGTFQKWAA VVVPSGEEQR YTCHVQHEGL PEPLTLRWEP
  HLA-Cw2   DTELVETRPA GDGTFQKWAA VVVPSGEEQR YTCHVQHEGL PEPLTLRWEP
  HLA-Cw1   DTELVETRPA GDGTFQKWAA VMVPSGEEQR YTCHVQHEGL PEPLTLRWEP
       JW1  DTELVETRPA GDGTFQKWAA VVVPSGEEQR YTCHVQHEGL PEPLTLRWKP
     HLA-6  DVELVETRPA GDGTFQKWAA VVVPSGEEQR YTCHVQHEGL PEPLMLRWKQ
     HLA-E  DTELVETRPA GDGTFQKWAA VVVPSGEEQR YTCHVQHEGL PEPVTLRWKP
Parhmcons   DTELVETRPA GDRTFQKWAA VVVPSGEEQR YTCHVQHEGL PKPLTLRWEP
Structure   TTBBBBBBBB TTTTBBBBBB BBBTTTTTTT BBBBBBTTTT TBBBBB
Pclocspec   .......... .......... .......... .......... .E

                277                                          C1  326
   HLA-A3   SSQPTIPIVG IIA.GLVLLG A..VITGAVV AAVMWRRKSS EKRRELHSGC
   HLA-A2   SSQPTIPIVG IIA.GLVLFG A..VITGAVV AAVMWRRKSS DRKGGSYSQA
   HLA-B7   SSQSTVPIVG IVARPAVLAV V..VI.GAVV AAVMCRRKSS GGKGGSYSQA
  HLA-B27   SSQSTVPIVG IVA.GLAVLA VV.VI.GAVV AAVMCRRKSS GGKGGSYSQA
HLA-Cw4mR   SSQPTIPIMG IVA.GLAVLV VLAVL.GAVV TAMMCRRKSS GGKGGSCSQA
  HLA-Cw3   SSQPTIPIVG IVA.GLAVLA VLAVL.GAVV AVVMCRRKSS GGKGGSCSQA
  HLA-Cw2   SSQPTIPIVG IVA.GLAVLA VLAVL.GAVV AVVMCRRKSS GGKGGSCSQA
  HLA-Cw1   SSQPTIPIVG IVA.GLAVLA VLAVL.GAVV AVVMCRRKSS GGKGGSCSQA
       JW1  SSQPTIPIVG IVA.GLAVLA VLAVL.GAMV AVVMCRRKSS GGKGGSCSQA
     HLA-6  SSLPTIPIMG IVAAGLVVLA VV...TGAAV AAVLWRKKSS D*
     HLA-E  ASQPTIPIVG IIA.GLVLLG SV.VS.GAVV AAVIWRKKSS GGKGGSYSKA
Parhmcons   SSQPTIPIVG IVA.GLAVLA VLAVL.GAVV AVVMCRRKSS GGKGGSCSQA

              327  C2
   HLA-A3   KQ*..... .....  C3
   HLA-A2   ASSDSAQGSD VSLTACKV*.
   HLA-B7   ACSDSAQGSD VSLTA*....
  HLA-B27   ACSDSAQGSD VSLTA*....
HLA-Cw4mR   ACSNSAQGSD ESLITCKA*
  HLA-Cw3   ASSNSAQGSD ESLIACKA*
  HLA-Cw2   ASSNSAQGSD ESLIACKA*
  HLA-Cw1   ASSNSAQGSD ESLIACKA*
       JW1  ASSNSAQGSD ESLIACKA*.
     HLA-E  EWSDSAQGS. ESHSL*
Parhmcons   ASSNSAQGSD ESLIACKA*
```

consensus by 7 - 26 residues.

Since the nucleotide sequence of JW1 (limited as far as the alpha-2 domain) more closely resembled sequences of members of the HLA-C locus, a more detailed comparison of the amino acid alignment was made (see figure 6.9.1.). JW1 does not specify any A- or B-locus specific residues but codes for glutamic acid residues at positions 183 and 268. JW1 does not extend as far as residue 52, but BeWo C.1 has a valine locus specific residue at this position. In exon 5, which codes for the transmembrane region, there is a degree of HLA-A, -B and -C interlocus amino acid divergence over positions 292-300. In this region, JW1 codes for amino acids which are specific only for HLA-C derived molecules. When the amino acid sequences of Cw1-4 are in full agreement, JW1 differs from the consensus at only 3/242 positions. At position 114 a change from aspartic acid to asparagine occurs in the beta-pleated sheet portion of the alpha-2 domain. This has previously been designated a hypervariable residue. The other substitutions normally common to A, B and C locus products, (Glu to Lys-275, Val to Met-303) both occur within the transmembrane region. At positions 99 and 116, JW1 codes for an amino acid that is different from all other C-locus amino acids although there is no consensus at these positions. BeWo C.1 is predicted to code for a 45kDa protein. From the complete amino acid sequence of BeWo C.1, Ellis et al.(1989), suggested that a substitution for tryptophan at position 14 (in the alpha-1 domain) from the highly conserved arginine may bring about a conformational change, or alter a recognition site.

The extent to which the amino acid sequence of JW1 (not including the signal peptide or alpha-1 domain) differs from other class I polypeptides is shown in figure 6.9.2.

Allele	No. of different amino acids	No. of residues considered	% Homology
HLA-A2	44	250	82.4
HLA-A3	48	231	79.3
HLA-B7	34	247	87.2
HLA-B27	27	247	89.1
HLA-Cw1	7	251	97.2
HLA-Cw2	10	251	96.1
HLA-Cw3	12	251	95.3
HLA-Cw4mRNA	21	251	91.7
HLA-Cw6	10	251	96.1
HLA-Cw7	16	251	93.4
HLA-Cw11	9	251	96.4
HLA-Cx52	9	251	96.4

Figure 6.9.2. Table of Amino Acid Homologies between JW1 (not including the signal peptide or the alpha-1 domain) and various class I polypeptides. The homology between JW1 and HLA-C derived molecules is higher than that when compared to HLA-A and HLA-B. HLA-A2 (Koller et al. 1985); HLA-A3 (Strachan et al. 1984); HLA-B7 (Sood et al. 1987); HLA-B27 (Weiss et al. 1985); HLA-Cw1 and HLA-Cw2 (Gussow et al. 1987); HLA-Cw3 (Sodoyer et al. 1984); HLA-Cw4mRNA (Davidson et al. 1985); HLA-Cw6 (Mizuno et al. 1989); HLA-Cw7 (Pohla et al. 1989); HLA-Cx52 (Takata et al. 1988).

6.10 Discussion.

The initial aim of the research was to clone and characterize a class I molecule that was most certainly present on some subpopulations of extravillous trophoblast. At this time, our knowledge extended only to the fact that the molecule did not cross react with antibodies which were specific for the foetal HLA-A or HLA-B phenotype. Since anti-HLA-C antibodies were not available, the assumptions made were simply that the molecule expressed was either derived from the HLA-C locus or from some other locus.

Subsequent work illustrating the lack of reactivity of 61D2 with the trophoblast class I molecule suggested tentatively that it may be a "different" or slightly modified form of class I. Class I immunoprecipitation studies of chorionic cytotrophoblasts and BeWo choriocarcinoma revealed for the first time that the molecule expressed was a 40kDa heavy chain associated with B_2-M (Ellis et al. 1986).

cDNA libraries of JEG-3 (this work) and amplified cDNA libraries of BeWo (Ellis, Oxford) were prepared solely for the purpose of obtaining class I cDNA clones corresponding to the 40kDa molecule (although data presented in chapter 3 shows the expression of 45kDa and 41kDa molecules). A number of class I clones have been obtained from JEG-3 (JW1-6) and also from BeWo.

Comparisons between the exonic nucleotide and amino acid sequence of JW1 and other HLA sequences undoubtedly placed this cDNA amongst the HLA-C locus alleles. High homology (97.7%) of intron 2 when compared to a consensus C-locus intron 2 added strength to this observation. A formal designation would however require the full length cDNA clone. The coding sequence of JW1 (although incomplete) seems to be identical to that of BeWo C.1 and since JEG-3 and BeWo cell lines are derived from the same choriocarcinoma material, these cDNA's might be expected to be derived from the same gene. The sequence of JW1 has been assumed to be identical to that of the published BeWo C.1 sequence. The sequence of BeWo C.1 has not been written out in the publication but has been represented by dashes (-) indicating its identity to HLA-Cw1. A comparison of the HLA-Cw1 sequence in the Ellis et al. (1989) publication with that obtained from the data base for this analysis (figure 6.8.1) indicates that this

sequence has been differently represented in the coding region both at the nucleotide (see positions 541 and 1109 in figure 6.8.1) and amino acid (see position 167 in figure 6.9.1) level. The translation of BeWo C.1 is identical to that of JW1 which supports possible typing errors in the nucleotide sequence.

Ellis et al. (1989), predicted that BeWo C.1 encoded a 45kDa heavy chain and this was surprising at the time, since previous immunoprecipitation and IEF analyses indicated only the presence of a 40kDa molecule (Ellis et al, 1986). No transcriptional start signals were found in JW1 intron 2 which could have ultimately accounted for the translation of the smaller 40kDa protein product. The translational stop codon (TGA) if in frame would prevent the synthesis of a class I polypeptide. However, concurrent to the sequencing of JW1 and BeWo C.1, anti-class I immunoprecipitation studies on BeWo cells showed without a doubt that two class I molecules of 45kDa and 41kDa were expressed and that the latter was more abundant (Stern et al. 1988). Similar results (chapter 3) have been seen with JEG-3 cells. The presence of the larger 45kDa component could account for the predicted amino acid sequence of BeWo C.1.

As mentioned in section 6.6, there may be a source of confusion when extending sequences which belong to a polymorphic multigene family, since hybrid alleles could be generated. The strategy shown in section 6.6 was adopted because IEF data indicated the presence of a single 40kDa component. This points out the difficulties encountered when researching a multigene family. Progress can be hampered by the limited resolution of the technology employed. A number of clones would be required to assess the situation.

At this point speculations of the origin of the two molecules included 1: that two separate genes were being expressed, 2: or that a common pre-mRNA was alternatively spliced leading to the translation of two differently sized molecules. The molecules are not glycosylation variants (P.L.Stern pers. comm.). Since the smaller component was 41kDa, it was thought that it might be a homologue of a Q region gene in the mouse and that it could be inserted into the membrane via a phosphatidyl inositol anchor (Low et al. 1986) as is the Qa-2 product of the Q-7 gene (Stroynowski et

al. 1987).

It might have been expected that the size of their respective mRNA's be different. The Northern hybridization results in chapter 4 show a single molecular weight class I mRNA (species) although it must be said that the resolving power of formaldehyde gel electrophoresis is not as high as that for non-denaturing gels. Class I mRNA's generally have 3'UT regions that are approximately 450 bases long which means that a reduction (if this occurs) within the 1Kb coding region of approximately 150 bases mRNA (equivalent to 4kDa) would be difficult to resolve. Although the difference between approximately 1Kb (coding region) and 850 bases (reduced coding region) may be electrophoretically resolved, the presence of the 3'UT region and polyadenylation tail (totalling approximately 1.6Kb) prevents such resolution. In addition to this there would be a degree of mRNA degradation during purification. As was also pointed out in section 2.15 the inclusion of formaldehyde in the gel and its diffusion out during the running period, might affect electrophoretic resolving power under these denaturing conditions.

Since BeWo C.1 (and possibly JW1) is predicted to code for a 45kDa molecule, then it seems very likely that it corresponds to the minor 45kDa component immunoprecipitated from BeWo cells. Eight cDNA clones obtained from the amplified BeWo cDNA library (Ellis et al. 1989) and at least three from that of JEG-3 are identical in sequence which suggests that only one allele at the C-locus is being transcribed unless the cell line is homozygous at this locus. One other explanation for this might be that choriocarcinomas are usually derived from hydatidiform moles (diploid) which are often of a homozygous androgenetic origin (reviewed Bagshawe and Lawler, 1982; Lawler and Fisher, 1987a,b). Thus the possibility remains that both HLA-C alleles may be expressed in normal trophoblast.

The 41kDa molecule appears to be approximately in a five fold excess after immunoprecipitation. This can not (or would be unlikely) be due to the relative affinities of W6/32 for either, since the same results were obtained when using BM.63 (anti-B_2-M). One might be tempted to suggest that the levels of mRNA representing each would be in these relative proportions whether alternative splicing had occurred or not - however, nothing is known about the

absolute or relative turnover rates of either mRNA's or their heavy chains. Pulse chase experiments would resolve such a situation at the protein level. Measurements on mRNA stability would be much more complicated and would require specific probes (which obviously are not available yet!).

The inconsistency between obtaining only C-like sequences which may code for 45kDa molecules and a predominance of the immunoprecipitable 41kDa component is surprising. One would have expected the cDNA clones corresponding to the 41kDa molecule to have been represented in both the JEG-3 cDNA library (this work) and the BeWo cDNA library (Ellis et al. 1989). Classical class I probes are normally expected to hybridize to most class I sequences under relaxed stringency conditions, since the alpha-3 domains are highly conserved within the class I gene family, indeed, the C-locus cDNA clones were isolated using an HLA-B7 cDNA probe. One explanation might be that these transcripts have a short polyadenylated tail and would therefore not be favourably selected during oligo-dT chromatography and oligo-dT primed cDNA synthesis.

A further and more likely explanation for not obtaining a sequence corresponding to the 41kDa molecule is that the stringency of washing <u>after</u> hybridization was too high. Preliminary filter washes were carried out at 55^{o}C with 1xSSPE, 0.1%SDS, but after autoradiography, high background levels forced the temperature of subsequent washes to be raised (see figure 5.7.1a;b). If the sequences coding for the 41kDa molecule have reduced homologies, then hybridization of HLA-B7 would be much reduced on elevating the washing temperatures. Hybridization of the HLA-B7 probe to the HLA-C-like clones that were obtained persisted to 65^{o}C with 0.1xSSPE, 0.1%SDS. Further screening of the second half of the library using secondary filter lifts did actually result in a much lower background and filters were washed at a lower stringency. The clones obtained from this screen (JW4 and JW5) were HLA-C derived. The higher levels of background hybridization on primary filter lifts was probably due to an excess of <u>E. coli.</u> chromosome, protein and agarose fragments which would not be in such great quantities on secondary filter lifts

Neverless, the HLA-C-like clones that have been obtained deserve careful consideration.

In general, C-locus products are expressed at much lower levels than HLA-A or HLA-B locus antigens which is probably why there is a lack of specific typing sera (Gussow et al. 1987; Mizuno et al. 1989). However, if the total number of C-locus alleles is much reduced when compared to HLA-A and HLA-B then it will be relatively more difficult to accrue specific sera between individuals for use in typing. Indeed, there are only eleven serologically defined alleles (in Ellis et al. 1989). The promoter sequences found upstream of the HLA-Cw3 gene are also different to those found at HLA-A and HLA-B loci (Sodoyer et al. 1985). If the efficiencies of these respective promoters are different then this may explain the lower levels of HLA-C products at least at the transcriptional level. Thus, if the above proposal is correct then it is very surprising that the clones obtained are all from the HLA-C locus. This implies that their expression may be governed by different regulatory factors and that they may have an altered and important role in immunity when compared to HLA-A and HLA-B locus products. The absence of clones derived from the HLA-A and HLA-B loci is consistent with earlier serological studies (Redman et al. 1984). Both BeWo and JEG-3 cells could not be tissue typed (Ellis et al. 1986 and P.L.Stern pers. comm.).

Several recent reports have illustrated some interesting features associated with C-locus antigens. Guild et al. (1983) reported the conserved Tyr^{321} (substituted for Cys in C-locus products) to be the site of phosphorylation by tyrosine kinases, although the significance of phosphorylation is as yet unknown. IEF gels have shown that multiple sized products (upto five) are produced upon transfection of Cw1, Cw2 genomic and Cw6 cDNA clones (Gussow et al. 1987; Mizuno et al. 1989) which is in contrast to HLA-A and HLA-B gene single sized products. The differential focusing is probably due to multiple post-translational modifications (possibly tissue specific) in the extracellular domains and Mizuno et al. (1989) have suggested that it is possible that these may confer an altered function on the molecule. Ellis et al. (1989) had previously completed IEF studies of BeWo class I products and although the 40kDa component was electrophoretically non-polymorphic the 45kDa which probably corresponds to BeWo C.1 was not detected. Ellis is now undertaking similar studies to those of Gussow

and Mizuno with the BeWo C.1 cDNA clone. The overall data suggests the possibility of a distinct functional role for HLA-C antigens. Dill et al. (1988) demonstrated that the transgenic HLA-Cw3 allele functioned as an immunologically competent MHC restriction element.

There is no evidence to date that HLA-C antigens are present on placenta (unless one extrapolates the choriocarcinoma immunoprecipitation experiments). However, the inability to type for HLA-A and HLA-B (and also HLA-C but with only few sera) suggests that the 45kDa molecule expressed at the surface is more likely to be HLA-C. It must also be remembered that the cloning experiments have been carried out on choriocarcinomas and <u>not</u> on normal trophoblasts expressing class I. If the HLA-C antigens are expressed as classical class I molecules, then they may be capable of presenting antigens to the maternal immune system and those derived from the paternal haplotype might act as allogeneic stimuli (if these differ from the maternal haplotype). Ellis et al. (1989) suggested that one or more amino acid substitutions in BeWo C.1 may bring about a conformational change or alter a recognition site and that this may account for the antibody binding pattern between W6/32 and 61D2. Even though this may be true, the immunoprecipitation studies in chapter 3 and those of Stern et al. (1988), largely disprove the latter possibility concerning the patterns of antibody binding. A change in the conformation of the recognition site may impair antigen presentation or recognition by T cells. Furthermore, Ellis et al. (1989) propose that if HLA-C can be shown to be expressed at higher levels than HLA-A or HLA-B, or in their absence, this might point towards a specialized role for HLA-C antigens - which may or may not be involved in the maintenance of pregnancy.

Finally, the question which still remains to be answered is "What is the origin, nature and function of the 41kDa molecule?" This question will be addressed in the final discussion.

FINAL DISCUSSION

Since class I antigens are known to evoke allograft rejection, their expression on some extra-villous trophoblasts (which are genetically semi-allogeneic) has raised the question of the nature of the antigen(s) and the immunological relationship between the mother and foetus. The existence of the trophoblast barrier and hence lack of a vascular connection limits the transfer of maternal cells into the foetus (and vice versa) to a very large extent (Hunziker et al. 1984). There are cases where the traffic of foetal cells (e.g. blood) provokes the maternal immune system to produce antibodies (reviewed Jones and Need, 1988). These antibodies pass through the placenta via Fc-gamma receptors and cause deleterious effects on target cells; such an example of this is the anti-Rh(D) response. The syncytiotrophoblast constitutes most of this barrier and its lack of class I expression (or any other antigens that may provoke a response) is important in avoiding sensitization of the mother to foetal/placental antigens (Sunderland et al. 1981a) and thus it is thought to contribute to the success of pregnancy.

Maternal alloantibodies against foetal class I and II MHC antigens have been found frequently during normal human pregnancy demonstrating that these antigens are not completely hidden from the competent maternal immune system. These alloantibodies (perhaps blocking antibodies) have been found in first pregnancies and can be eluted from placentas. Therefore it appears that their formation is not dependent on antigen exposure during delivery and in this respect differs from rhesus isoimmunization. Billington and Burrows (1989) also detected alloantibodies in pregnant sera directed against rat classical class I MHC structures but were unable to observe a concomitant cell-mediated immune response to these antigens. Thus, the molecules (possible alloantigens) expressed at the interface have been proposed to function as "absorbing material" for any antibodies that are accidentally generated. The production of antibodies might arise by leakage between the interface, and their binding activity may be directed towards both classical and modified structures. Depletion of these antibodies by trophoblast antigens would reduce the severity of any adverse response made towards

foetal antigens. Any cell-mediated response may be overcome by the intrinsic properties of the trophoblast cells. The immunogenicity of any antigens will obviously influence the maternal response and thus it is of prime importance to establish the nature of the molecule(s) involved.

Pregnancy is not normally disrupted by pre-immunization against paternal or third party antigens (Chaouat et al. 1984b), indeed some types of recurrent spontaneous abortion have been treated successfully in this way - presumably from the development of a "protective" immune response (Mowbray, 1985). However, abortion can be induced in mice by maternal immunization with tumour cells and it is thought that the response elicited is directed towards cross-reacting foetal (onco-foetal) antigens (Tartakovsky, 1987). The mechanism whereby immunization confers a beneficial effect is not very well understood, but it is thought to require appropriate recognition of some antigens. One candidate though to be involved in this are the trophoblast-leucocyte common (TLX) antigens which are expressed on all populations of peripheral blood lymphocytes and foetal trophoblasts (Bulmer and Johnson, 1985) and may be encoded on chromosome 1 (Stern et al. 1986). There is the possibility that trophoblast lymphocyte cross-reactive antigens (Faulk et al. 1978; McIntyre et al. 1983; Bulmer and Johnson, 1985) stimulate the production of maternal antibody in normal pregnancy, but there is conflicting data on the nature of the responses to these antigens (reviewed Billington, 1989).

It has been difficult to ascertain whether trophoblasts are susceptible to cellular and humoral immune effectors. Recent work has shown that class I positive mouse trophoblasts were lysed by specific alloantibody but not by allospecific cytotoxic lymphocytes (Drake and Head, 1989; Zuckerman and Head, 1987). The basis of this insusceptibility is unknown but it will obviously be beneficial if trophoblasts are implicitly resistant to a maternal immune attack.

Certain studies have shown that maternal lymphocyte populations arrive in the decidua and suppress the induction of a response towards foetal antigens (Bulmer and Sunderland, 1984; Kabawat et al. 1985). Immunosuppressive activity has been detected in extracts of decidua and trophoblast

(Golander et al. 1981), in the supernatants of some human pre-implantation embryos (Daya and Clark, 1986), in mouse mid-term placental supernatants (Chaouat et al. 1986) and in human syncytiotrophoblast plasma membranes (Degenne et al. 1986). If immunosuppression is important in the maintenance of pregnancy, then it must act locally since retroplacental blood inhibits lymphocyte activation whereas peripheral blood does not (Redman et al. 1986).

Sunderland et al. (1981b;c) showed that class I molecules were not expressed by villous syncytiotrophoblast but that extra-villous trophoblast forming the cytotrophoblast cell columns clearly stained positive. Generally, class I antigens are restricted to those forms of trophoblast outside the major maternal-foetal exchange area in mature placentae. Obviously, the easiest solution to escaping immunological recognition would be the complete absence of class I antigens from all trophoblasts (as is the case for class II molecules, reviewed Redman, 1983). Thus, their low level of expression on some forms of extra-villous trophoblasts implies that they may have an important function. This restricted expression may have implications in the immune interactions during pregnancy and may influence the physiological function of this organ in growth and differentiation.

Some earlier studies have documented parental and thus foetal sharing of classical HLA alleles and have attempted to describe the significance of this in terms of pregnancy failure (Bolis et al. 1984; Komlos et al. 1977; Ober et al. 1983;1987; Unander and Olding, 1983). Given that recent studies have now described the expression of apparently non-classical class I molecules, the significance of such results should be reassessed.

The class I molecules expressed on extra-villous trophoblast consistently failed to bind antibodies specific for the foetal HLA-A and -B phenotype and 61D2 (Redman et al. 1984, Wells et al. 1984) suggesting that MHC antigen expression may be restricted to HLA-C or some other unidentified class I antigen. Similar results were observed in both early, mature and molar pregnancies (Fisher and Lawler, 1984; Sunderland et al. 1985).

The nature of the molecules expressed on extravillous trophoblast will hopefully be resolved to a

certain extent after their corresponding cDNA's are obtained. By comparing their amino acid sequences to other known classical and non-classical molecules, it is hoped that any real differences may be discerned. These may indicate a structural change which may in turn modify or completely change their function. Such comparisons may be informative in indicating whether these molecules are able to present antigen (do they have a discernible antigen binding site?) although this would have to be formally proven using in vitro assays. As has been suggested, they both may have a role in re-directing a potentially harmful immune response directed towards the foetus by acting as blocking molecules in a sort of "buffering zone" and so preventing any immune response from reaching the foetus.

The work presented in this thesis describes the characterization of class I molecules expressed on a form of extra-villous trophoblast. Pure preparations of class I positive trophoblasts are required to study such molecules, but since they are difficult to separate from uterine decidua and sustain in culture, a choriocarcinoma cell line (JEG-3) derived from extra-villous cytotrophoblast was used (Kohler and Bridson, 1971). Class I expression was initially investigated by immunoprecipitation followed by the use of recombinant DNA techniques in order to clone the genes expressed. The high level of sequence conservation within the MHC class I gene family has made it difficult to identify products of a given class I locus independent of other sequences. Thus a cDNA library representing only the genes specifically expressed by trophoblasts was constructed.

Class I immunoprecipitation data had already proven that the BeWo choriocarcinoma and chorionic cytotrophoblast cell membranes expressed a 40kDa molecule and Ellis et al. (1986) had therefore concluded that classical class I molecules were not expressed by these cells. Immunoprecipitation studies on JEG-3 cells (chapter 3) using W6/32 showed that two class I heavy chains of molecular weight 45kDa and 41kDa were expressed. Both were associated with B_2-microglobulin 12kDa, and the 41kDa polypeptide was the major component, being in a five fold excess. Notably, 61D2 did not cross react with, and therefore did not immunoprecipitate the 41kDa component. Similar results were obtained with BeWo cells (Stern et al. 1988) as expected

since both cell lines were probably derived from the same choriocarcinoma material (Hertz et al. 1959).

So in addition to the 40-41kDa (hereafter referred to as 41kDa) component, the more recent data suggests that a classical 45kDa molecule can be detected by 61D2. If the immunoprecipitation results of the JEG-3 choriocarcinoma are extrapolated to normal class I positive extravillous trophoblasts then it is very likely that the 61D2 immunostaining experiments of Redman et al. (1984) and Wells et al. (1984) and the IEF experiments of Ellis et al. (1986) were limiting in their sensitivity and consequently did not detect the low levels of the 45kDa component. It is also likely that the high percentage (12%) polyacrylamide SDS gel prevented Ellis et al. (1986) from resolving these two different molecular weight species, or that exposure to autoradiographic film was too short to detect the minor 45kDa polypeptide. Indeed, the subsequent cloning of a complete HLA-C class I molecule (BeWo C.1 from BeWo choriocarcinoma predicted to encode a 45kDa polypeptide), also lends support to this interpretation (Ellis et al. 1989). Further confirmatory studies are necessary to determine whether or not the larger polypeptide is indeed present on human chorionic cytotrophoblast membranes. This seems likely since Billington and Burrows, (1989) have recently immunoprecipitated both 41kDa and 45kDa polypeptides from rat placenta and have evidence for the presence of paternally encoded class I antigens on rat spongiotrophoblast (counterpart of human cytotrophoblast) and endovascular trophoblast.

The collective data suggests that there are two class I products expressed on trophoblast. These polypeptides could be derived from two different genes. However, if only one gene is transcribed then there is the possibility that the two different molecular weight heavy chains could represent the products of a common but alternatively spliced mRNA or they could be the result of differential glycosylation. Since one of the components is 40kDa, it may represent a human analogue of a Q region gene product. Furthermore, the Q7 gene product (Qa-2) is known to be inserted into the cell membrane via a phosphatidyl inositol anchor (Stroynowski et al. 1987; Low et al. 1986; see also section 1.1.7). N-glycanase treatment of BeWo class

I heavy chains has excluded the possibility of these two molecules being glycosylation variants (P.L.Stern unpublished results), and phospholipase C treatment of JEG-3 class I molecules shows that they are not inserted into the membrane via a phosphatidyl inositol anchor (Rinke de Wit pers. comm.).

Screening the JEG-3 gamma-interferon cDNA library yielded six independent class I positive clones, JW1-6. Sequence data of three of the cDNA clones JW1, 4 and 5 suggest that they are all derived from the same allele at the HLA-C locus. JW1 intron-2 allowed the assignment of the sequence to the HLA-C locus. The JW1 exonic sequence as far as it extends seems to be (see chapter 6) identical to that of the complete BeWo C.1 cDNA (Ellis et al. 1989). JW1 intron 2 does not contain a translational start codon which could re-direct the synthesis to a 41kDa heavy chain. Two of the six clones contained intron 2 which was quite unexpected. Interestingly, studies by Matsuura et al. (1987) show that incompletely spliced (containing intron 1 and 2) Tla transcripts are present at higher levels in leukaemia cells than in thymocytes.

The immunoprecipitation data taken together with the nucleotide sequence indicate that the 45kDa component may be coded for by BeWo C.1 and possibly by JW1. Eight cDNA clones obtained from BeWo (Ellis et al. 1989) and at least three (of six) from JEG-3 are identical in sequence which implies that only one allele at the C-locus is being transcribed. Unless the cell line is homozygous at this locus, one other explanation for this might be that choriocarcinomas are usually derived from hydatidiform moles (diploid) which are often androgenetic in origin (Bagshawe and Lawler, 1982; Lawler et al. 1987a,b). Thus the possibility remains that both HLA-C alleles may be expressed in normal trophoblast. The data of Ellis et al. (1989) and this work has largely ruled out alternative splicing as the mechanism generating two molecules of different molecular weight. Thus three of the four proposals of the origin of both components have largely been ruled out. The remaining possiblity that two separate genes code for these two molecules becomes much more likely and evidence for this is given below.

The absence of clones derived from the HLA-A and

HLA-B loci is consistent with earlier serological studies (Redman et al. 1984), indeed, both BeWo and JEG-3 cells could not be tissue typed (Ellis et al. 1986 and P.L.Stern pers. comm.). In general, C-locus products are thought to be expressed at much lower levels than A- or B-locus antigens which is probably why there is a lack of specific typing sera (Gussow et al. 1987; Sodoyer et al. 1984). The lack of typing sera may not be necessarily due to their low levels of expression, but may be a result of their relatively low polymorphism, since the chance of generating a serum to a molecule that is less polymorphic is relatively much lower. Thus it was quite unexpected that only HLA-C clones were obtained although this had always been a possibility. Why there should be a reversal in the expression of HLA-A, HLA-B with HLA-C is itself intriguing and may imply that HLA-C has a different function. However, Dill et al. (1988) have shown that the one allele tested (Cw3) was able to act as a restriction element. The function of the class I antigen(s) expressed by extravillous trophoblast remains obscure to date. There is no firm evidence to date that HLA-C antigens are present on placenta and obviously further studies need to be done. If the HLA-C locus derived molecules function as classical class I antigens then the paternal haplotype might act as an allogeneic stimulus (if this differs from the maternal haplotype).

Some of the characteristics of HLA-C locus genes and products have been described in chapter 6. One further point about HLA-C locus derived genes has been made by Ronne et al. (1985). The intron sequences of eleven class I genes (human, mouse and rabbit) were used to ascertain the evolutionary relationships between each although only one allele (HLA-Cw3) at the C-locus was used. Ronne used a dendrogram to show that the HLA-C genes were as divergent from other class I genes as H-2 was from Qa-2,3. As mentioned before, the polymorphism at the HLA-C locus seems to be relatively lower than that at HLA-A and HLA-B loci. This is consistent with the proposal of Pohla et al. (1989) who suggest that the HLA-C locus evolved much more recently.

Having obtained cDNA's that seem to encode a 45kDa molecule, the origin and nature of the 41kDa component still remains obscure.

Much more progress has been made in cloning and

sequencing the mouse MHC and various "non-classical" Q and TL genes (see section 1.1.5) have been found to be considerably less polymorphic than H-2K, D and L class I classical sequences. However, the function of these non-classical antigens is largely unknown but their limited expression in some somatic cells (Q10 in the liver, Tla in thymocytes and T-cell leukaemias) suggests that they may have specialized roles in immunity e.g. in lymphoid differentiation (Mellor et al. 1986). Some relatively invariant human class I-like molecules HT (Gazit et al. 1980a;b), HA (Fauchet et al. 1986) and TCA (van Leeuwen et al. 1986) have been detected serologically (see section 1.1.6) and the genes corresponding to these polypeptides are currently being cloned. TCA molecules are not expressed on JEG-3 cells (Rinke de Wit. pers. comm.) or on BeWo cells (Stern et al. 1988).

One particularly active area of MHC research involves cloning all members of the human class I gene family. Shimizu et al. (1988), cloned three genomic class I genes from a T lymphoblastoid cell line; HLA-5.4 (Shimizu et al 1988), HLA-6.0 (Geraghty et al. 1987) and HLA-E (Koller et al. 1988). All three associate with B_2-microglobulin and code for 40, 38.5 and 41kDa molecules respectively. The function of these products and the level of polymorphism at these loci is unknown but the latter is thought to be low since no sera are known to have been generated. HLA-E and HLA-6.0 genes show some unusual features in their nucleotide sequence and at the amino acid sequence level are clearly different from classical HLA-A, -B and -C antigens (see figure 6.9.1). Geraghty et al. (1987) suggested that HLA-6.0 may be a structural homologue of a murine class I Q gene since its cytoplasmic region is predicted to be considerably shorter than expected and its COOH terminus is most similar to that of the Q7 gene. In addition to this, the HLA-6.0 promoter region very much resembles those of the mouse Q genes. The lower molecular weight (41kDa) of one of the trophoblast class I molecules may signify that it is encoded by a human analogue of the murine Q genes, which also yield similar sized class I molecules. (Lew et al. 1986).

Hybridization with an HLA-E specific probe showed that trancripts homologous to HLA-E were not expressed by JEG-3 cells (Rinke de Wit pers. comm.). Using an HLA-6.0 specific probe, work in collaboration with with T. Rinke de

Wit (Leiden) and P.L.Stern (Manchester) showed that homologous transcripts were expressed in JEG-3 cells (Ward et al. 1990). The HLA-6.0 specific probe hybridized to a 1.6Kb transcript which was present in both JEG-3 and EBV transformed lymphoblastoid cells. Subsequent work showed that the level of the HLA-6.0 homologous transcripts in JEG-3 cells was not induced by gamma-interferon (Rinke de Wit pers. comm.), whereas several reports have documented the induction of class I transcripts by this lymphokine (reviewed Rosa and Fellous, 1984).

That HLA-6.0 homologous transcripts have been detected in JEG-3 cells is interesting because HLA-6.0 itself has a much shortened cytoplasmic region. Studies of Murre et al. (1984) showed that CTL lysis of vesicular stomatitis virus-infected cells was significantly reduced when the cytoplasmic domain of $H\text{-}2L^{d}$ was removed. However, the removal of this domain was previously found not to effect the surface expression levels of the this gene nor effect its interaction with CTL's, (Zuniga et al. 1983).

The lack of sera to the HLA-6.0 molecule is intriguing and suggests that the level of polymorphism at this particular locus is relatively low. One might suggest that the lack of sera could be due to the HLA-6.0-like product being expressed on only certain tissues at a particular stage of development; trophoblast is a good example of this. HLA-6.0 homologous transcripts are also present on EBV transformed lymphocytes (see Ward et al. 1990).

One would have expected HLA-6.0 homologous transcripts to have been represented in both the JEG-3 cDNA library and the BeWo cDNA library (Ellis et al. 1989). Classical class I probes are normally expected to hybridize to most class I sequences under relaxed stringency since the alpha-3 domains are highly conserved within the class I gene family, indeed, the HLA-C locus cDNA clones were isolated in this way. The possible reasons for HLA-6.0 homologous transcripts not being isolated here or by Ellis (Oxford) have been discussed in chapter 6. The JEG-3 cDNA library filters are currently being screened with the HLA-6.0 specific probe.

The expression of class I on some populations of trophoblast and not others has raised questions of their regulation, for example, whether MHC expression is

irreversibly down regulated on normally negative trophoblasts.

The molecular mechanisms responsible for the regulation of MHC gene expression are beginning to be understood (see section 1.1.13). The effect of interferons on trophoblast cell lines has been described in section 1.2.7 and chapter 4. Class I antigens can be induced by exposure to interferons on some populations of trophoblasts and not at all on others suggesting that regulation of these genes differs between trophoblast populations (Hunt et al. 1987). Interferons regulate class I expression in vitro as well as in vivo (Halloran et al. 1986) and may therefore influence class I gene expression at the placental interface (Chard et al. 1986; Duc-Goiran et al. 1986). Zuckerman and Head, (1986) have shown that class I expression on non-malignant mouse trophoblasts can be induced with interferon.

The quantitative studies outlined in chapter 4 did not detect a significant increase in class I induction after gamma-interferon treatment in contrast to those studies of Hunt et al. (1987). The collaborative work described above indicates the presence of an HLA-6.0 homologous transcript which was subsequently found not to be induced by gamma-interferon. The immunoprecipitation data show that the 41kDa component is approximately in a 4-5 fold excess over the 45kDa polypeptide. If one assumes that the relative levels of the corresponding mRNA's for these polypeptides are proportional to surface expression levels and that the 41kDa polypeptide is coded for by the HLA-6.0 homologous transcript, then the low levels of class I mRNA induction described in chapter 4 can possibly be explained. Any induction of classical class I mRNA would be masked by the more abundant HLA-6.0 homologous transcripts (under the assumption placed above). This would be particularly accentuated if the hybridization and washing procedures of Northern filters were carried out at relaxed stringency with the HLA-B7 cDNA probe. However, as mentioned previously, nothing is known of the actual or relative rates of turnover of either mRNA or protein of the two components.

If classical class I molecules are expressed on some trophoblasts (from extrapolation of choriocarcinoma data in chapter 3 and Stern et al. 1988), then this might render them susceptible to immune effectors such as cytotoxic

lymphocytes and antibodies. Power et al. (1988) have shown that the antibodies present in rat pregnancy sera are directed against multiple epitopes on conventional/classical RT1A molecules rather than to the Pa antigenic determinant. Billington and Burrows (1989) suggested that foetal survival was ensured because no cell mediated immune reactions were generated against the paternal class I antigens.

In general, classical class I molecules provide a context for the recognition of foreign antigens by CD8+ T cells. Studies on T-lymphocyte subsets and their receptors (TCR's) have shown that this recognition is largely dependent on the alpha/beta T-cell receptor heterodimer which is associated with the CD3 complex on the cell surface (reviewed Allison et al. 1987). However, the exact function of a second gamma/delta TCR present on 1-10% of peripheral T cells is still unknown (reviewed, Strominger, 1989). Janeway et al. (1988) proposed that relatively non-polymorphic MHC Class I-like molecules might serve as ligands for gamma/delta TCR's and there are examples of genes derived from both the TL (Bluestone et al. 1988) and Q region (Vidovic et al. 1989) which have been shown to act as restriction elements for this receptor.

One other example of molecules interacting with gamma/delta TCR's are the surface glycoprotein cluster of differentiation 1 (CD1) molecules which have a low but significant level of homology to MHC class I molecules (Bradbury et al. 1988; Calabi and Milstein, 1986;Calabi et al. 1989a,b). They are also associated with B_2-m. Three homologous human proteins CD1a,b and c have been detected serologically, but five potential CD1 genes (CD1a,b,c,R2 and R3 falling into two classes) reside on chromosome 1 (Calabi et al. 1989b). Porcelli et al. (1989) suggested that CD1 molecules may have a similar function to MHC class I molecules, since CD8 +ve alpha/beta and gamma/delta TCR cells recognize CD1 molecules (although antigen presentation has not yet been demonstrated). As yet, the function of the relatively non-polymorphic class I gene products (which might include the 41kDa class I molecule on trophoblast) is largely unknown. As has been proposed above, non-polymorphic MHC class I-like moleules (Janeway et al. 1988) and CD1 products (Porcelli et al. 1989) may serve as ligands for gamma/delta TCR's. Strominger, (1989) suggested two reasons why class

"Ib" polypeptides could be functionally linked to TCR gamma/delta cells. The first concerned the low numbers of receptor variants expressed, indicating that only a few are required to interact with the relatively small number of class Ib molecules. Secondly, the majority of gamma/delta TCR positive cells lack CD8 and CD4 which suggests that classical class I and class II antigens may not be their appropriate ligands. However, CD1 molecules are not expressed on JEG-3 cells (Rinke de Wit per. comm.).

The consequences of restricted expression of MHC class I to certain trophoblast populations are largely unknown. It seems that class I antigens may have other physiological functions as well as presenting antigens to T cells, such as binding peptide hormones and epidermal growth factor (reviewed, Head et al. 1987). The implication here is that binding of such molecules may influence the growth and differentiation of the placenta. Head postulated that an aberrant class I molecule might continue to have such a function without eliciting an allogeneic immune response. This idea is supported by the fact that mainly proliferating trophoblasts express class I antigens. One further example of a different function for class I is the cloning of two Fc receptor subunits which are used in rats to remove immunoglobulin G from mother's milk (Simister and Mostov, 1989). Sequence analysis indicates that this molecule, (FcRn) belongs to the class I gene family.

Hopefully, the localization of expression of individual class I genes will afford clues as to whether their products have specific or more generalized functions. First of all it is necessary to obtain a number of cDNA clones from the choriocarcinoma cell line which correspond to the gene that codes for the 41kDa molecule. After sequencing has been completed, and a comparison made to other class I sequences, specific probes may be designed for screening cDNA libraries of class I positive extravillous trophoblasts. The use of these probes for <u>in situ</u> hybridization experiments on placental sections would enable the tissue localization of the expressed genes and would circumvent the problems of lymphocyte and maternal cell contamination. Indeed, the fact that HLA-6.0 homologous transcripts are also detected in EBV transformed lymphocytes reinforces the point of this problem (see Ward et al. 1990).

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