

THE REGULATION OF MHC CLASS II GENE EXPRESSION
BY TUMOURS OF THE B LYMPHOCYTE LINEAGE

by

Ashok Ramakrishnan VENKITARAMAN

The Charing Cross Sunley Research Centre,
Lurgan Avenue,
London.

Thesis submitted to the University of London for the degree of
Doctor of Philosophy; August 1988.

कर्मण्येवाधिकारस्ते मा फलेषु कदाचन ।
मा कर्मफलहेतुर्भूर्मा ते सङ्गोऽस्वकर्मणि ॥

To Rajini, for her unfailing support; and my parents, for
giving me that all-important start

ABSTRACT

MHC Class II gene expression is related to the stage of differentiation in the B lymphocyte lineage. A number of murine and human tumour cell lines representing cells 'frozen' in various B lymphocytic differentiation stages were used as a model for the analysis of Class II gene regulation. The Class II antigen phenotypes of these lines were found to correspond to those of the normal cell types they represented. Further, the expression of Class II antigens correlated with the presence and amount of mRNA for the Class II genes. Messenger RNA was not found in Class II antigen non-expressing cell lines, while cell lines that expressed Class II antigens also expressed mRNA in amounts that corresponded to the level of antigen expression. Thus these tumour cell lines regulate Class II expression at the level of gene transcription.

The mechanism of suppression of Class II gene expression in a BALB/c strain derived plasmacytoma, NS1, was studied in further detail. NS1 cells did not express Class II antigens, or their constituent polypeptide chains, either at the cell surface or intracellularly. However the closely related Class I antigens were expressed. Twenty uncloned hybrid cell lines obtained by the fusion of NS1 with Class II expressing splenic B lymphocytes prepared from CBA, SJL and BALB/c strain mice were all found to express Class I antigens of either or both parental haplotypes; however none expressed Class II antigens of either haplotype. These results were further validated using one such (NS1 x splenic B lymphocyte) hybrid cell clone, K3. When K3 was fused to the Class II expressing B lymphoma A20, the seven resulting hybrid cell lines were again Class I positive but Class II negative. These results indicate that NS1 cells suppress Class II gene expression by an active, highly specific mechanism.

Evidence was also found that another murine plasmacytoma, X63, suppressed Class II gene expression by a similar mechanism. One X63 x splenic B lymphocyte hybrid clone, Sp2/0, and two Sp2/0 x splenic B lymphocyte hybrid clones, were found to express Class I but not Class II antigens. This demonstration was less rigorous than in the case of NS1 as only a small number

of cloned, rather than uncloned, hybrids were tested.

X63 cells, which expressed neither Class II antigens or mRNA, were similar to other B cell tumours in that they regulated Class II gene expression at the level of transcription. It was therefore surprising that NS1 cells expressed correctly-sized, poly-A tailed transcripts for the Class II genes A α , E α and the associated Ii chain gene in both the nucleus and cytoplasm. The amounts of RNA were equivalent to those in an unfractionated splenocyte population, and approximately one-fifth those in A20 cells. A known inducer of Class II gene transcription, IFN γ , enhanced mRNA levels but did not result in Class II antigen expression. These results imply that NS1 cells suppress Class II gene expression by a novel post-transcriptional regulatory mechanism operating subsequent to RNA processing and nuclear-cytoplasmic transport.

Further strong evidence for post-transcriptional Class II gene regulation was found. NS1 cells were transfected with an expression construct containing 8 copies each of the complete human DR α and DR β gene cDNA sequences under the control of the strong viral SV40 early promoter/enhancer sequences which are transcriptionally active in most eukaryotic cells. None of the seven NS1 transfectants which had integrated the construct expressed DR antigens intracellularly or at the cell surface, although preliminary evidence for DR α gene mRNA expression was found. In contrast, DR antigens were expressed by six of nine transfectants of the Class II expressing A20 cell line; as well as by transfectants of Class II non-expressing P815 cells.

Two important conclusions can be drawn from these results : first, that plasma cells suppress Class II expression by an active, specific mechanism; and second, that this suppression may operate either transcriptionally or by a novel post-transcriptional mechanism. These conclusions are discussed in the context of the mechanisms that regulate gene expression during B lymphocyte differentiation.

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ABBREVIATIONS

2D	-	2-dimensional
β -ME	-	β -mercaptoethanol
μ g	-	microgram
μ l	-	microlitre
μ m	-	micron
μ M	-	membrane immunoglobulin M heavy chain
μ S	-	secretory immunoglobulin M heavy chain
APC	-	antigen presenting cell(s)
bp	-	base pair(s)
BSA	-	bovine serum albumin
cDNA	-	complementary deoxyribonucleic acid
CHX	-	cycloheximide
CYT	-	cytoplasmic tail
(d)A(TP)	-	(deoxy)adenine (triphosphate)
(d)C(TP)	-	(deoxy)cytidine (triphosphate)
(d)G(TP)	-	(deoxy)guanine (triphosphate)
(d)T(TP)	-	(deoxy)thymidine (triphosphate)
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease I
EBV	-	Epstein Barr virus
EDTA	-	ethylene diamine tetra-acetic acid (sodium salt)
FACS	-	fluorescence-activated cell sorter
FCS	-	foetal calf serum
GAMIg	-	goat antibody to mouse immunoglobulins
HAT	-	hypoxanthine, aminopterin, thymidine
HBSS	-	Hanks' buffered salt solution
HEPES	-	2-4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
HGPRT	-	hypoxanthine guanosine phosphoribosyl transferase
IFN(γ)	-	interferon (γ)
Ig	-	immunoglobulin(s)
Ii	-	invariant chain of MHC Class II
IL-4	-	interleukin-4
Ir	-	immune response (gene or antigen)
kbp	-	kilobase pair(s)
kD	-	kilodalton(s)

LMP	-	low melting point (agarose)
LT	-	lymphotoxin (tumour necrosis factor β)
mAb	-	monoclonal antibody(ies)
MHC	-	Major Histocompatibility Complex
mg	-	milligram
ml	-	millilitre
mRNA	-	messenger ribonucleic acid(s)
NP-40	-	Nonidet P-40
PAGE	-	polyacrylamide gel electrophoresis
PEG	-	polyethylene glycol
PBS	-	phosphate buffered saline
PMSF	-	phenyl methyl sulphonyl fluoride
poly-A	-	polyadenylated
PVP	-	polyvinylpyrrolidone
RBCs	-	red blood corpuscles
RNA	-	ribonucleic acid(s)
RNase	-	ribonuclease
SD	-	standard deviation of the mean
SDS	-	sodium dodecyl sulphate
sIg	-	surface immunoglobulin
SSC	-	standard sodium citrate
TM	-	transmembrane
TNF	-	tumour necrosis factor α
Tris	-	tris(hydroxymethyl)aminomethane
u	-	unit(s)
v/v	-	volume/volume

ACKNOWLEDGEMENTS

I would like to thank Marc Feldmann for providing me with the facilities and encouragement I needed to perform this work. I am very grateful to Benny Chain and Phil Askenase : they spent a lot of time initiating me to the laboratory, teaching a clinician how to perform research. Maija Kissonerghis has been an invaluable fount of practical information, and I must thank her for putting up with my incessant queries. Mel Kahan helped with using, and getting to grips with, the FACStar. Past and present staff members at the ICRF Tumour Immunology Unit, UCL and the Charing Cross Sunley Research Centre have made the last three years both enjoyable and profitable : I hope that I will be forgiven for not naming them individually. In particular I thank Kathy Barrett, Glenn Buchan, Udo Hartwig and Colin Hewitt for their help. Thanks also to Kaisa Harylowicz for her comments on this thesis.

The Lady Tata Memorial Trust for Research on Leukaemia and the Cancer Research Campaign provided financial support for this work.

I gratefully acknowledge the following specific contributions to the work presented here :

- Dr Eric Culbert, for preparing four CBA splenocyte x NS1 hybrid cell lines and testing them for MHC antigen expression (section 3:3.3)
- Kathy Barrett, for purifying the 7B6 insert (section 3:4.4)
- Dr Hitoshi Ikeda, for preparing the cosmid construct pHD8R/RW and providing P815 cells transfected with R/RW (section 3:5.2)
- Udo Hartwig, for help with maintaining transfectant cell lines in tissue culture (section 3:5.2); and providing RNA from A20/DR5 (section 3:5.7)
- Maija Kissonerghis, for purifying the DR α chain insert (section 3:5.6); and providing RNA from JURKAT (section 2:9.3)

CHAPTER 1

REGULATION OF MHC CLASS II GENE EXPRESSION

1:1 INTRODUCTION

Two groups of integral cell membrane glycoproteins - the Class I and Class II antigens - that play a pivotal role in regulating interactions between cells of the immune system are among the molecules encoded by the genes of the major histocompatibility complex (MHC). One of the most striking differences between these closely related groups of molecules is their tissue distribution. The MHC Class I antigens are widely distributed, being expressed by most nucleated cell types in varying amounts. In contrast the Class II antigens have a highly restricted tissue distribution : they are found primarily on certain cells of the immune system, and can be induced on a few other cell types. The restricted tissue distribution of the Class II antigens is closely related to their function; indeed the maintenance of tissue specific expression appears to be critical for the normal function of the immune system.

The pivotal role of the MHC Class II antigens in the regulation of the immune response has directed much attention to understanding their structure and function. Studies over the past twenty years have contributed to their serological and biochemical characterization, the cloning of the Class II genes, elucidation of the tertiary structure of the Class II antigens and its relation to their function. The stage has thus been set for studies on the mechanisms that regulate MHC Class II gene expression in a tissue-specific manner, about which little is known. This is the focus of the research presented in this thesis.

In this introductory section, I will review current knowledge of the mechanisms that regulate MHC Class II gene expression. Because the body of literature on the structure and function of MHC Class II is so vast, these aspects will be dealt with only briefly.

1:2 THE MURINE MHC

1:2.1 Definition of the murine MHC. H-2

The murine MHC, H-2 (histocompatibility-2), was first described as an autosomally dominant locus controlling the

expression of a determinant expressed on the RBCs of some but not all mouse strains (Gorer, 1936 and 1937). Incompatibility at this locus was later shown to be a major factor controlling the rejection of transplanted tumours (Gorer, 1938; Gorer et al., 1948). The construction of congenic mouse strains (Snell, 1948) enabled the locus to be mapped to murine linkage group IX (chromosome 17) by Gorer and coworkers (1948).

It was soon apparent that the H-2 determinants controlling tumour rejection (the 'transplantation antigens') were extremely polymorphic, and encoded by at least two loci, K and D (Gorer and Mikulska, 1959; Stimpfling, 1971). Many other traits in congenic mouse strains were also mapped to H-2. Two traits, the quantitative variation in a serum protein Ss (Shreffler and Owen, 1963) and the ability to mount an immune response against foreign antigens (McDevitt et al., 1972), defined third and fourth H-2 loci clearly different from K and D. Klein (1981) first subdivided the H-2 loci into "Classes" on the basis of structural and functional homologies - Class I (K, D) encoding the classical 'transplantation antigens'; Class II (I), the immune response (Ir) genes; and Class III, serum complement proteins. The current genetic map of the murine MHC on the short arm of chromosome 17 (Figure 1.1) is based not only on information from the analysis of recombinant mouse strains (Shreffler and David, 1974; Murphy, 1981) but also on more recent molecular genetic analyses (Goodenow et al., 1982a; Steinmetz et al., 1982a and 1982b). It includes six regions - K, D, L (Class I), I (Class II, including the I-A and I-E subregions), S (Class III) and Qa/Tla (Class I-like) - besides a number of other genes not included within the MHC "Classes" : the genes for the lymphokines tumour necrosis factor (TNF) and lymphotoxin (LT), and the enzyme 21 hydroxylase which plays a role in steroid hormone biosynthesis.

The H-2 haplotypes of inbred mouse strains are designated by superscripted lower case letters, for example, H-2^d. The products of the genes within various regions are designated by a capital letter (region) with a superscripted lower case letter (haplotype), for example D^d or I-A^d.

The Class I and Class II genes and gene products are considered in more detail.

1:2.2 The Class I antigens

The Class I antigens are heterodimers of a polymorphic 44 - 45 kD integral membrane glycoprotein (the α chain) in association with a 12 kD nonpolymorphic polypeptide (β_2 microglobulin) (Dobberstein et al., 1979) that is not encoded by the MHC (Michaelson, 1983). The α chains consist of 5 protein domains - three external domains ($\alpha 1$, $\alpha 2$ and $\alpha 3$) of about 90 to 100 residues each, a 40 amino acid transmembrane (TM) portion and a short (50 amino acid) cytoplasmic tail - with two (sometimes three) glycosylation sites located in the first and second external domains (Lopez de Castro et al., 1979; Coligan et al., 1981; Nathenson et al., 1981). This domain structure is characteristic of molecules belonging to the Ig superfamily.

Recent X ray crystallographic studies on the human Class I antigen HLA-A2 (Bjorkmann et al., 1987) show that two apposing α helices form a ligand-binding groove on the membrane-distal end of the molecule; which is supported from below by β sheet structures formed by the membrane-proximal protein domains. The α helices contain those amino acid residues identified as the most polymorphic.

1:2.3 The Class I genes

The K and D loci, initially defined on the basis of the segregation of serological H-2 determinants in recombinant mice (Gorer and Mikulska, 1959), lie on either side of the Class II locus, I (Klein and Shreffler, 1971). More loci have been defined serologically, for example L (Demant et al., 1975) and R (Hansen et al., 1981) but the mapping of the genes that encode them is unclear : some of them may lie within the K/D loci (Klein and Shreffler, 1971; Hansen et al., 1981). Several Class I-like genes lie in the Qa/Tla cluster (Flaherty, 1980) which is telomeric to the D locus (Figure 1.1).

The best data on gene organization within the Class I loci has come from the isolation and sequencing of gene clusters in series of overlapping cosmid clones (Steinmetz et al., 1982a; Weiss et al., 1984). In the BALB/c mouse (H-2^d haplotype) 36 Class I genes are organized in 12 distinct clusters which range in size from 40 to 215 kbp, and contain from 1 to 8 genes each

(Steinmetz et al., 1982a). Thirty one of these map to the Qa/Tla region, and five map to the classical Class I loci (two each to K and L, and one to D). The same number of genes are found in the K and D loci of the C57Bl/6 mouse (H-2^b haplotype) (Weiss et al., 1984).

Sequence analysis of these genes shows the same basic structure (Figure 1.2) (Steinmetz et al., 1981; Goodenow et al., 1982b; Moore et al., 1982). They contain eight exons that correlate exactly with structural domains of the proteins they encode - one exon each for the leader peptide, the three extracellular domains, and the TM domain; and three exons encoding the short cytoplasmic tail. Additional sequences code for the short 5' and the long 3' untranslated regions of the mRNA.

1:2.4 The Class II antigens

The Class II antigens, I-A and I-E, are also heterodimers (Figure 1.4). Discontinuous SDS-PAGE of detergent solubilized Class II antigens shows that each of the two antigens consists of a relatively acidic α chain of apparent molecular weight 34 to 36 kD and a more basic β chain of apparent molecular weight 26 to 29 kD which vary slightly in size between different haplotypes (Cook et al., 1978). A third, non-polymorphic, Invariant (Ii) chain is found in association with α and β in the cytoplasm but not the cell membrane (Jones et al., 1978).

Alkylation experiments indicate that the α and β chains are associated non-covalently with each other (Cook et al., 1978; Dancey et al., 1979). Each chain has two external domains (Kaufman and Strominger, 1983) which are shaped into Ig-like folds by cysteine-cysteine disulphide bridges (apart from the $\alpha 1$ domain), a TM region of hydrophobic amino acids, and a short cytoplasmic tail (Mathis et al., 1983b; Larhammar et al., 1984; Saito et al., 1983; Choi et al., 1983). The external domains, excepting $\beta 1$, contain potential sites for glycosylation (Mengle-Gaw and McDevitt, 1985) (Figure 1.4). Recent studies on the tertiary structure of the Class II antigens reveal close similarity to Class I - a ligand-binding pocket is formed by apposing α helices contributed by each of the membrane-distal domains ($\alpha 1$, $\beta 1$), which is supported by β sheet structures

formed by the membrane-proximal domains (Brown et al., 1988).

Rates of turnover of the polypeptide chains vary. I-E β , I-A α and I-A β chains are the most rapidly synthesized, while I-E α synthesis is slower. Once synthesized and assembled, I-A α / β complexes are turned over more rapidly than I-E α / β (Emerson et al., 1980). Besides these quantitative differences, each of the four polypeptides also display differences in structure. Both β chains are highly polymorphic in N-terminal peptide sequence, while the α chains are less so (Freed, 1981; Klein and Figueroa, 1981). I-E α in particular is not very polymorphic (Klein and Figueroa, 1981). Intra-allelic heterogeneity also exists. Multiple spots for each chain are found on 2D gels, probably due to differences in glycosylation (Jones et al., 1978b). The significance of glycosylation is not clear although it may play a role in intracellular transport or function of these molecules. Indeed, glycosylation patterns differ between different cell types (Cullen et al., 1981).

1:2.5 The Class II genes

The Class II locus, I, which determines the ability to mount an immune response to foreign protein antigens maps between the K and S loci (Figure 1.3) (McDevitt et al., 1972). The locus comprises at least two subloci (David et al., 1973), designated I-A and I-E, which were initially postulated to contain the genes encoding the α and β chains of the I-A and I-E antigens respectively (Cook et al., 1979; Silver and Russell, 1979). However the finding that I-E β chain polymorphisms were controlled by a locus at the telomeric end of I-A (Jones et al., 1978b; Jones, 1980) was at odds with this hypothesis.

The organization of the I region (Figure 1.3) has been finally resolved by analysis of overlapping cosmid clones (Steinmetz et al., 1982b). In the BALB/c mouse, a single cluster of about 270 kbp of DNA contains the I-A and I-E subloci. The recombination separating the subloci occurs within the E β gene (Figure 1.3). In fact all intra-I region recombinations in 9 mouse strains occur within an 8 kbp 'recombinational hot-spot' overlapping the 3' end of the E β gene (Steinmetz et al., 1982b), which has been localized to the second intron (Kobori et al., 1984). An additional gene, A β 2, is found 5' to the A β gene

(Larhammar et al., 1984) - it consists of a single (second domain) exon and is probably a pseudogene. A similar pseudogene, E β 2, is found between E α and E β (Steinmetz et al., 1982b). Yet another pseudogene, A β 3, has been detected in certain haplotypes (H-2^{t1} and H-2^{y1}) (Widera and Flavell, 1985). It lies at least 75 kbp 3' of H-2K, and 160 kbp 5' of A β 2 (Steinmetz et al., 1986). There is also evidence that one more gene, E β 3, lies to the far 3' end of the I-E sublocus (Flavell et al., 1985).

The lack of polymorphism observed with E α chain polypeptides is confirmed at the gene level : there is almost no difference between three completely sequenced alleles (McNicholas et al., 1982; Mathis et al., 1983b; Mengle-Gaw et al., 1985). However A α gene alleles are, in contrast, quite polymorphic (Benoist et al., 1983a; Benoist et al., 1983b) with the variability concentrated in 3 hypervariable regions (amino acid residues 11-15, 53-59 and 69-77) in the membrane-distal domain. Similar regions of allelic hypervariability have been found in the sequences of the membrane-distal domains of the A β and E β chains (Choi et al., 1983; Larhammar et al., 1984 for A β ; Saito et al., 1983; Malissen et al., 1983; Mengle-Gaw and McDevitt, 1983 for E β). Sequence comparisons show that the I-E genes are most similar to the human DR genes (Mathis et al., 1983b); and I-A to DQ (Benoist et al., 1983a). E β 3 shows great homology to a DP β gene (Widera and Flavell, 1985).

The considerable sequence homologies between the corresponding α and β chain genes of I-A and I-E, as well as the fact that they are in opposite 5' to 3' orientation in the genome (Figure 1.3) suggests that an ancestral β - β - α group was duplicated to give rise to the I-A and I-E subloci. The presence of additional β chain pseudogenes may suggest that duplication occurred more than once, as in the case of the human Class II genes.

1:2.6 Structure of Class II genes

Analysis of the sequence of the Class II genes reveals a similar exon-intron organization (Figure 1.3). The α chain genes have a leader exon separated by a long intron (approximately 2.3 kbp) from the first and second protein domain exons (Mathis et al., 1983b), followed by a single exon encoding

both the TM region and cytoplasmic tail. A long sequence encoding the 3' UT region of the mRNA is split into two by another long intron. This structure is slightly different from that of the β chain genes (Malissen et al., 1983) where the TM and cytoplasmic tail regions are separated by an intron. The human DR α gene has the same intron-exon organization as EQ (Lee et al., 1982).

1:2.7 The Class II antigens are products of the Ir genes and serve as restriction elements for T cell activation

Three lines of evidence indicate that the Class II antigens are the products of the immune response (Ir) genes which were first identified by their function in regulating the ability to mount an immune response to foreign protein antigens (McDevitt et al., 1972; Bennaceraf and McDevitt, 1972; Bennaceraf, 1981). First, monoclonal antibodies (mAb) against the Class II antigens specifically block immune responses to antigens which are under Ir gene control (Lerner et al., 1980; Baxavenis et al., 1980). Second, mutant mouse strains with an altered Ir gene phenotype also show concomitant changes in Class II antigen phenotype (Lin et al., 1981; Huber et al., 1982; Matis et al., 1982). The most convincing data has come from the restoration of responsiveness to antigens under Ir gene control by re-expression of the EQ gene in transgenic mice produced from I-E antigen deficient strains (LeMeur et al., 1985).

The Class II antigens accomplish their immune regulatory function by serving as restriction elements for the activation of antigen-specific T lymphocytes which recognize antigen exclusively in the context of Class II upon the surface of "antigen presenting cells" (APC) (Katz et al., 1973; Rosenthal and Shevach, 1973; Sredni et al., 1981). Recent evidence indicates that Class II molecules directly bind peptide antigens (Babbitt et al., 1985) that have been partially proteolysed or "processed" (Unanue, 1984; Watts et al., 1984) and that this complex is recognized by the antigen-specific T cell receptor (Watts et al., 1984; Buus et al., 1986a; Siliciano et al., 1986). Thus the capacity to respond to a given antigen depends critically (but not exclusively) upon its ability to bind Class II molecules (Buus et al., 1986b). In addition, quantitative

variations in the level of Class II expression on APC may influence the type and outcome of the immune response elicited (Janeway et al., 1984).

There is a clear functional need for Class II expression to be tightly restricted to certain cell types. There is increasing evidence that aberrant Class II expression may be related to the induction of organ-specific autoimmunity (Bottazzo et al., 1986). Aberrant high-level Class II expression is a prominent histopathological feature of tissues from sites of autoimmunity (Bottazzo et al., 1986), and there is evidence - both in vitro (Londei et al., 1984) and more recently, in vivo in transgenic mice (Sarvetnick et al., 1988) - that this aberrant expression may activate autoantigen specific T lymphocytes causing tissue destruction.

1:3 REGULATION OF MHC CLASS II GENE EXPRESSION

1:3.1 I-A and I-E antigens are co-expressed in a cell type specific manner

Class II antigen expression is restricted primarily to cells of the immune system : macrophages, B lymphocytes, activated human T lymphocytes, dendritic cells and thymic epithelia (Frelinger, 1982). Constitutive expression is a prominent feature of mature B cells (Sachs and Cone, 1973), and many investigators have shown that both I-A and I-E antigens are expressed on Ig-bearing B lymphocytes from a variety of sources including central lymphoid tissues, lymph nodes and spleen (Sachs and Cone, 1973; Hammerling et al., 1974; Hammerling, 1976a). Terminally differentiated plasma cells are either negative or express Class II at low levels (Hammerling et al., 1976b; Warner et al., 1979; Lanier et al., 1981). This has also been shown to be true of murine pre-B lymphocytes (Hammerling et al., 1976; Lala et al., 1979; Dasch and Jones, 1986). Thus in the B lymphocyte lineage, Class II antigen expression is related to the stage of differentiation; with progression from a Class II negative pre-B cell phenotype through an expressing mature B lymphocyte phenotype, finally to a non-expressing terminally differentiated plasma cell phenotype. Ii chain polypeptide expression is related in a

similar manner to B cell differentiation (Koch and Harris, 1984).

Class II expression in cells of the B lymphocytic lineage is not only controlled in a differentiation linked manner. Mediators such as interleukin-4 (IL-4) can induce expression on murine pre-B cells (Polla et al., 1986) or enhance expression on mature B cells (Noelle et al., 1984).

Antigen presenting accessory cells such as macrophages (Schwartz et al., 1976), dendritic cells (Steinman and Nussenzweig, 1980), Langerhans cells (Tamaki et al., 1979), Kupffer cells (Richman et al., 1979) and thymic dendritic cells and epithelia (van Ewijk et al., 1980) express both I-A and I-E antigens, but exhibit different patterns of expression. Expression by dendritic cells is at a high level, and constitutive; whereas macrophage Class II expression is inducible with mediators such as interferon γ (IFN γ) (Steeg et al., 1982; King and Jones, 1983).

IFN γ has been shown to induce Class II expression on a wide variety of nonlymphoid tissues, including brain astrocytes (Wong et al., 1984a), a variety of epithelial and fibroblast cell lines (Wong et al., 1984b), and vascular endothelium (Pober et al., 1983) both in vitro and in vivo (Skoskiewicz et al., 1985). Ii chain expression is co-induced by IFN γ (Collins et al., 1984; Koch et al., 1984).

An additional conclusion is apparent from the studies summarized in the preceding paragraphs : cell types that express I-A antigens also express I-E; suggesting that these antigens are co-expressed as well as co-regulated by mediators like IFN γ . The major exception to this generalization occurs in certain strains of inbred mice which do not express I-E antigens or express them at low levels, because of a deficiency in the synthesis of E α or E β polypeptides, or both (Murphy et al., 1980; Jones et al., 1981). In these strains, the defect is at the gene level, and all Class II expressing cell types are of the same (I-E negative or low) phenotype.

1:3.2 Expression is subject to multifaceted control

It is clear from the above discussion that Class II gene expression is tightly controlled in a cell type-specific way.

Expression in a given cell type may be constitutive, or induced by lymphokine mediators like IFN γ . In some lineages, expression is linked to the stage of cell differentiation. These data persuasively argue that Class II gene expression must be regulated in a multifaceted manner.

Several recent observations further support this point. One facet of regulation appears to affect the Class II genes but not Ii : cells from patients with Class II negative severe combined immunodeficiency (SCID) express Ii but not Class II mRNA (de Preval et al., 1985). This is also true of Class II negative mutant B cell lines (Accolla, 1983; Long et al., 1984; Calman and Peterlin, 1987).

Another facet of regulation may distinguish Class II genes derived from different cell types (or species) : in murine macrophage x human B cell hybrids, the macrophage derived (but not B cell derived) genes are upregulated by murine (but not human) IFN γ (Maffei et al., 1987). However the fact that human Class II antigens are re-expressed in hybrids of Class II negative mutant human B cells with murine B cells (Accolla et al., 1985; Salter et al., 1985; Accolla et al., 1986) suggests that regulation is not strictly species specific.

The final facet of regulation may be gene specific : transcripts of the pseudogene A β 2 are not induced in macrophages treated with IFN γ , in contrast to the other Class II genes (Wake and Flavell, 1985). This form of differential regulation has not been demonstrated for any other murine Class II genes, suggesting that it is not a prominent feature of regulation.

1:3.3 Expression is regulated primarily at the level of gene transcription

Two lines of evidence indicate that Class II gene expression is regulated primarily at the level of gene transcription. First, cells that do not express Class II antigens also fail to express mRNA for the α and β chain genes (Mathis et al., 1983b; Davis et al., 1984; Flavell et al., 1985). This is further supported by studies on E α polypeptide deficient mice (Mathis et al., 1983a) : strains of the H-2^b and H-2^s haplotypes have a 650 bp deletion in the 5' flanking sequence of the E α gene, and fail to transcribe this gene. Second, stimuli which increase or

decrease the level of Class II antigen expression also correspondingly alter the amounts of mRNA expressed. For example, recombinant IFN γ causes a 10-fold upregulation in the level of Class II mRNA in macrophages (Nakamura et al., 1984) corresponding to the increased cell-surface expression of Class II antigens. Similar alterations in mRNA levels are produced by different mediators (IL-4, tumour necrosis factor α (TNF α)) that induce or enhance Class II antigen expression in other cell types - for example pre-B cells (Polla et al., 1986), resting B cells (Noelle et al., 1984), fibroblasts and endothelial cells (Collins et al., 1984) and pancreatic islet cells (Pujol-Borrell et al., 1987).

There are some exceptions to this precept. Mice of the I-E antigen deficient mouse haplotype H-2^f synthesize an aberrantly sized E α gene mRNA which does not appear to be translated into functional protein, while those of the H-2^d haplotype have a defect in mRNA processing or stability (Mathis et al., 1983a). Post-translational defects may also occur : a conserved structural mutation (Asn to Ser at position 83 in the first domain) in the β chains of I-A and I-E has recently been shown to inhibit intracellular transport and cell surface expression of these antigens (Griffith et al., 1988). These exceptions are however, not frequent - the first two are restricted to a particular mouse strain while the third is restricted to a certain cell line - and their physiological relevance is not clear.

Transcriptional regulation of Class II gene expression will therefore be discussed in detail. I will begin by considering the components of the eukaryotic gene promoter.

1:3.4 The eukaryotic gene promoter is a modular structure whose components interact with DNA binding proteins

The analysis of point mutations and deletions in the 5' flanking sequences of well characterized eukaryotic genes such as β -globin has led to the formulation of a generalized model for the eukaryotic gene promoter which contains three major functional elements (Dyran and Tijian, 1985) (see Figure 1.5 for a specific example). The TATA box, an element with the consensus sequence 'TATAAA', is located 25 to 40 bp upstream of the

transcription start site. It is a substrate for RNA polymerase II binding, and serves to fix the site of transcription initiation. Mutation in this region leads to the production of aberrant transcripts with incorrect start sites, but does not affect the overall level of transcription (Grosschedl and Birnstiel, 1980; Ghosh et al., 1981). One or more upstream elements lying upto 200 bp upstream function to increase the rate of transcription from the promoter, and in addition, may also help to fix the transcription start site. Several short sequence motifs are known to function as upstream elements, including the 'CCAAT' (CAT) box (McKnight and Tijian, 1986) and the 'GGGCGGG' (CG) box (Dyanan and Tijian, 1985). Enhancer elements may lie upstream or downstream of the transcription start site, very close to it, or many kilobases away, in the same or reverse orientation (Banerji et al., 1981; de Villiers and Schaffner, 1981; Moreau et al., 1981). They serve to upregulate the rate of transcription from the promoter. Some enhancers, for example the Ig enhancer, are tissue-specific (Banerji et al., 1983; Queen and Baltimore, 1983); while others, for example the SV40 enhancer, are active in a wide range of tissues (Banerji et al., 1981; Gruss et al., 1981).

All of these DNA sequence elements interact with sequence-specific DNA binding proteins (Ptashne, 1986; Sassone-Corsi and Borrelli, 1986). The transcriptional activity of the promoter depends upon these protein-DNA interactions, although a common fundamental mechanism is not yet apparent. Activity may involve the exclusion or displacement of other DNA binding proteins ('repressors') (Barberis et al., 1987); local changes in chromatin structure resulting in hypersensitivity to DNase I digestion (Bird, 1986); and/or looping or folding of the DNA molecule (Ptashne, 1986).

This model of the eukaryotic promoter has certain qualifications. First, not all eukaryotic genes have all three promoter elements. Second, many enhancers and upstream elements share the same sequences (since their function is so similar); and may be difficult if not impossible to distinguish. For example the Ig octamer motif - ATTTGCAT - occurs in the Ig heavy chain enhancer and V region upstream element (Falkner and Zachau, 1984; Parslow et al., 1984) as well as in the SV40 enhancer (Gruss et al., 1981). Both the model and the

qualifications do however fit into the same general picture : the eukaryotic promoter is a modular structure containing repetitive sequence motifs, with their number and arrangement determining promoter activity in various tissues. The individual activity of each component is modulated by the context (that is, tissue) in which the gene is expressed. Genes with similar expression patterns may not share greater promoter homologies than genes which have quite distinct expression patterns, because there are two roles to be considered - that of the DNA sequence elements of the promoter in each gene, and that of the specific binding proteins that interact with them in each cell type.

Three approaches have been taken to define the sequence elements and binding proteins that are important in Class II gene regulation : sequence analysis to identify putative regulatory sequences, assays of their function by expression in transfected cells and transgenic mice, and analysis of protein-DNA interactions by biochemical methods. These approaches will be considered in the next few sections.

1:3.5 Molecular mechanisms for Class II gene regulation : identification of cis-acting DNA sequence elements

Highly conserved DNA sequence motifs are found in the 5' flanking and intronic regions of all known human and murine Class II genes (Mathis et al., 1983b; Saito et al., 1983; Kelly and Trowsdale, 1985; Sullivan et al., 1987) as well as the Ii chain gene (O'Sullivan et al., 1986). These correspond to the components of the typical eukaryotic gene promoter. Most studies on Class II transcription in the murine system have focused on the E α gene; and hence I will use this as a representative example in the subsequent discussion.

1:3.5.1 The E α 5' flanking region contains the components of the eukaryotic gene promoter

Three highly conserved regulatory sequences have been defined in the 5' flanking sequence of the E α gene immediately upstream of the RNA cap site : the TATA consensus sequence

(GATTTTAAT) between positions -32 bp to -24 bp; and two highly conserved 14 bp motifs termed the X and Y boxes (Figure 1.5) (Mathis et al., 1983b; Dorn et al., 1987a; Thanos et al., 1988). The EQ TATA box does not match very well with the consensus sequence, which may explain the observation that there are multiple transcription initiation sites (Mathis et al., 1983b). The Y box sequence (CTGATTGGCCAAAG) which lies between positions -48 bp and -61 bp, contains an inversion (ATTGG) of the CAT box consensus sequence. The CAT box motif is found in the upstream elements of many eukaryotic genes (McKnight and Tijian, 1986), and is believed to bind an ubiquitous (or highly similar family of) transcription factor(s) (Dorn et al., 1987b; Raymondjean et al., 1988). The X box sequence (CCTAGCAACAGATG) is separated from the Y box by an invariant spacer element of 17 or 18 bp. In addition to the X and Y boxes, three repeats of the Ig octamer motif (ATTTGCAT) - with 1 or 2 base mismatches - are also found in the immediate 5' flanking region shown in Figure 1.5. This motif has been related to the B lymphocyte specific activity of the Ig gene enhancer (Mason et al., 1985), but is also found in other promoters. A heptamer sequence (GGACCTG) between positions -112 and -118, together with the X and Y boxes, may be involved in the transcriptional response to IFN γ (Thanos et al., 1988). This region is highly homologous to the IFN γ response element identified in the human DR α gene (Basta et al., 1987).

There is growing evidence that regulatory elements that lie as far as 2 kbp upstream of the transcription start site are involved in cell type-specific Class II gene expression in B cells (Widera et al., 1987). Sequence analysis shows that an enhancer core sequence (GTGGAAAG) (Sassone-Corsi and Borrelli, 1986) common to many viral and eukaryotic enhancers occurs at position -1965, and six similar repeats occur at -133, -178, -1310, -1480, -1680 and -1949 (Figure 1.5). These enhancer motifs are found in both orientations with respect to the transcription start site.

1:3.5.2 The 5' flanking region of other Class II genes : the significance of gene-specific sequence elements is not clear

Systematic comparison of the 5' flanking regions of the Class II genes of mouse and man reveals that E α /DR α and A β /DQ β are the most closely homologous pairs although similar sequence elements do occur in all of the genes. A 22 bp sequence, termed the W box, with consensus sequence TTTCAGAANNNNNCCTTCANA is found between positions -125 to -145 in HLA Class II β chain genes; in addition to the X and Y boxes (Miwa et al., 1987). It is part of a consensus IFN γ response element present in both human and murine β chain genes (Boss and Strominger, 1986; Miwa et al., 1987), and is similar although not identical to a motif found in the α chain genes. Sequences farther (1-2 kbp) 5' of the transcription start site are fairly divergent between the α and β chain genes (Widera et al., 1987). The significance of these differences is unclear; first, because the functionally active sequence elements are small (8 to 20 bp) in relation to the rest of the flanking regions, and second, because the context (that is, tissue) of gene expression also plays a determining role. Certainly the I-A and I-E antigens seem to be co-expressed and co-regulated (see section 1:3.1). Minor regulatory differences (for example, non-induction of A β 2 transcription by IFN γ (Wake and Flavell, 1985)) may be explained by the observed sequence differences.

1:3.5.3 Regulatory sequences identified by DNase I hypersensitivity analysis

Putative regulatory sequences have been identified in the first intron of the DR α gene by DNase I mapping (Peterlin et al., 1987). These include three 10 bp sequences homologous to those found in the SV40 and polyoma virus enhancers, other sequences homologous to the Ig heavy chain switch regions, and a consensus topo-isomerase II binding site (Sullivan et al., 1987). The function of these sequences is not known. Intronic regulatory sequences have not been found in the E α gene (D. Mathis, personal communication).

Apart from these results, DNase I mapping has tended to confirm the information obtained by sequence analysis - the DR α upstream elements are always DNase I hypersensitive in expressing cell types, but are not in non-expressing cells (Peterlin et al., 1987). The same is true of the A α gene (Liou

et al., 1988).

1:3.6 Molecular mechanisms for Class II gene regulation :
cis-acting sequence elements are responsible
for tissue-specific expression

The function of regulatory DNA sequence elements has been studied primarily by deletion analysis : analysis of the expression in transfected cells or transgenic mice of modified class II genes from which putative regulatory sequence elements have been removed.

For both the E α and DR α genes, those sequence elements that lie between positions -1 and -100 bp from the transcription start site are both necessary and sufficient for expression in transfected cells (Sherman et al., 1987; Thanos et al., 1988). Deletions encompassing this region (which includes the TATA, X and Y boxes) are transcriptionally inactive, and do not direct the synthesis of mRNA. Deletion of sequences 5' of the X box, upto about position -160 to -200, results in an enhancement of the basal level of transcription in the E α (Thanos et al., 1988), DR α (Basta et al., 1987) and DQ β (Boss and Strominger, 1986) genes; suggesting that negative regulatory element(s) are present in this region. It is noteworthy that this region includes the IFN γ response element. It is therefore possible that the transcriptional enhancement caused by this mediator is the result of displacement of a repressor protein bound to the IFN γ response element (Boss and Strominger, 1986). Sequence deletions further 5' reveal the presence of both negative and positive regulatory elements (Thanos et al., 1988) but without a remarkable pattern.

It is difficult to ascribe tissue-specific activity to regulatory elements from the results of cell transfection assays, since a limited number of cell types has been used (fibroblasts, HeLa cells, the WEHI-3 macrophage or K562 leukaemia cell lines). It is, however, noteworthy that the basal transcription level of identical E α gene 5' deletions (-837 bp to -353 bp) is different between fibroblasts and WEHI-3; indicating that at least some of the regulatory elements in this region are tissue specific (Thanos et al., 1988).

Data from transgenic mice provides much more information

regarding the sequence elements responsible for the tissue-specific transcription of the E α gene. Most of these studies have been performed with I-E deficient H-2^b or H-2^s haplotypes, enabling the expression of the transgene to be detected easily. Clean deletions of either the X or Y box result in superficially the same phenotype : tissue specific expression in B cells and macrophages, but with reduced levels of transcription and aberrant transcription initiation (Dorn et al., 1987a). Detailed examination shows compartmentalization of I-E expression : X box deletions result in the loss of expression in the thymic cortex but not the medulla, while Y box deletions have the converse effect (van Ewijk et al., 1988). B cell expression is heterogenous in both cases, with only 20-50% positivity with Y box deletions. Another difference is in expression by peritoneal exudate macrophages, which are negative with Y but not X box deletions. These striking results, obtained with clean deletions of small, highly conserved sequences that are very close together argue that the X and Y boxes act in concert rather than independently. It is tempting to speculate that this interdependency is due to the interaction of X and Y box binding factors that also bind each other (Takahashi et al., 1986; van Ewijk et al., 1988).

Data from transgenics also confirms that sequences in the -1 to -2 kbp region of the E α gene are important for B cell specific transcriptional activity (Widera et al., 1987; van Ewijk et al., 1988). It is interesting that when this region is deleted most, but not all, B cells do not express I-E : B cells in the germinal centre of the spleen and lymph nodes retain expression. Thus these cells, activated lymphoblasts for the most part, may have a different transcriptional control from other B lymphocytes. Differences in the germinal centre micro-environment, such as the presence of a cytokine mediator, may also be important.

1:3.7 Molecular mechanisms for Class II gene regulation : trans-acting proteins that interact with cis-acting DNA sequence elements

The fact that cis-acting elements are necessary for tissue specific Class II gene expression might be expected to predicate

the existence of tissue-specific nuclear proteins that interact with them. Two biochemical techniques have been used to identify such proteins. Gel retardation assays permit the detection of protein-DNA complexes that move more slowly than free DNA on nondenaturing polyacrylamide gel electrophoresis. DNase I footprinting assays reveal sites of DNA-protein interaction as bands protected from digestion with DNase I. The X and Y boxes have been shown by both these techniques to bind different proteins (Dorn et al., 1987a; Dorn et al., 1987b). NF-Y, a sequence specific DNA-binding protein which binds the Y box, is clearly different from any other known CAT box binding protein such as CBP or CTF/NF-1 (McKnight and Tijian, 1986; Dorn et al., 1987b). NF-Y binding activity is critically dependent on the central 5 nucleotides of the Y box sequence, which includes the inverted CAT box sequence. Preliminary biochemical analysis shows that it is a large (>250 kD), multimeric glycoprotein (D. Mathis, personal communication). NF-Y has also been demonstrated to bind to CAT boxes from other genes (Dorn et al., 1987b), but its affinity is less than for the Y box.

Are the Class II gene promoter-binding proteins found only in Class II expressing cell types? Unfortunately, this simple explanation for the tissue-specificity of gene expression cannot be invoked: proteins binding elements of the Class II gene promoter are present in nuclear extracts from several non-expressing cell types (Dorn et al., 1987a; Sullivan et al., 1987). In this regard the situation is similar to that for other tissue-specific genes like Ig (Sen and Baltimore, 1986a).

What is not yet clear, however, is whether the proteins that bind the Class II gene promoter in non-expressing cell types are the same as those that bind it in expressing cell types. There is evidence to suggest that this is not the case: the DNase I footprinting pattern of the DR α gene is different with nuclear extracts from fibroblasts in comparison to B cells (Sullivan et al., 1987). In addition, there are quantitative differences between fibroblast and B cell nuclear extracts in binding to each of the DR α promoter elements (Sullivan et al., 1987). It is tempting to speculate that these differences, quantitative and qualitative, account for the observed differences in gene expression. However further analysis is necessary to resolve this problem.

The actors - DNA sequence motifs and DNA-binding proteins - that play a role in MHC Class II gene regulation have been identified. However the manner in which they interact in physiological situations is still far from clear. The B lymphocyte lineage, in which Class II expression is related to the stage of differentiation, is a model system for analysis of this interaction.

1:3.8 Molecular mechanisms for Class II regulation : analysis of regulation in the B lymphocyte lineage

As discussed in detail in section 1:3.1, MHC Class II expression is related to the stage of differentiation in the B lymphocyte lineage. Murine pre-B cells are MHC Class II negative, mature B cells express at high levels, and plasma cells are either Class II negative or express at very low levels (Figure 1.6). This makes the B lymphocyte lineage a unique model for the study of Class II gene regulation : the genes are expressed and suppressed in a predictable manner during the life history of a single cell.

It is likely that differences in the expression of Class II genes during B cell differentiation are due to specific regulatory mechanisms, perhaps related to the presence or absence of trans-acting regulatory proteins that bind to specific sequence motifs. This does appear to be the case with Ig gene transcription in the B lymphocyte lineage, which is also related to the stage of cell differentiation (Sen and Baltimore, 1986b); suggesting that Class II gene regulation is but one facet of a complex developmental programme.

Most data on trans-acting regulatory proteins has come from work on a series of non-expressing mutants derived from Class II expressing B lymphocyte lines representing the mature B cell phenotype (Accolla, 1983; Accolla et al., 1985; Salter et al., 1985; Calman and Peterlin, 1987). The mutants in these series have distinct defects due to the lack of the activity of one or more regulatory proteins that permit the initiation of gene transcription. The defects can be corrected by fusion with normal human or murine cells, either non-expressing T lymphocytes or expressing B lymphocytes (Accolla et al., 1985;

Salter et al., 1985). Segregation analysis of a series of human-mouse fusions has assigned one such trans-acting element to murine chromosome 16 (Accolla et al., 1986).

Evidence from transfection and DNase I footprinting assays indicates that the mutants do not always lack the regulatory proteins per se; instead subtle mutations in the proteins may result in the loss of transcriptional regulatory activity although DNA-binding activity persists (Sullivan et al., 1987). Alternatively, the defect may be in unidentified 'cooperative' regulatory elements that do not themselves directly bind to DNA sequence motifs. For example, defective DR and DQ expression in one such mutant was partially complemented by transfection of the v-Ha-ras or n-ras oncogenes; although the original defect is not a ras mutation (Hume et al., 1987).

1:4 AIMS OF THE RESEARCH

The data discussed here indicate that Class II gene regulation in cells of the B lymphocytic lineage is a distinct facet of a complex developmental programme. It is not clear how this programme is implemented in the case of Class II genes in each of the different B lymphocytic differentiation stages. Understanding this is clearly of great importance in unravelling the complex interactions of the different types of Class II gene regulatory elements in a physiological situation.

The research presented in this thesis is an attempt to approach these questions : what are the mechanisms that are responsible for the expression and suppression of Class II genes during B lymphocyte differentiation ? Are they similar in cells at different developmental stages ? Are expression and suppression two sides of the same regulatory mechanism, or are they distinct ?

THE MURINE MHC

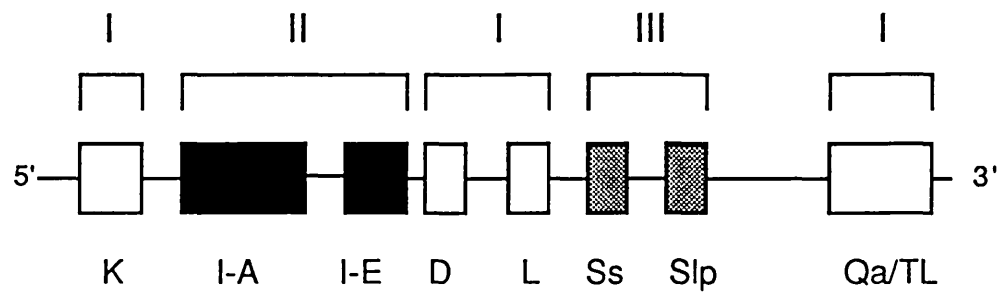


FIGURE 1.1

(Based on data from Steinmetz et al., 1982b and 1986; Flavell et al., 1985).

This figure is a schematic representation of the murine MHC locus on the short arm of chromosome 17. Roman numerals indicate Class I, II and III genes; while letters refer to the MHC regions and sub-regions. MHC associated passenger genes (TNF/LT, 21 hydroxylase) are not shown.

MURINE CLASS I GENES AND ANTIGENS

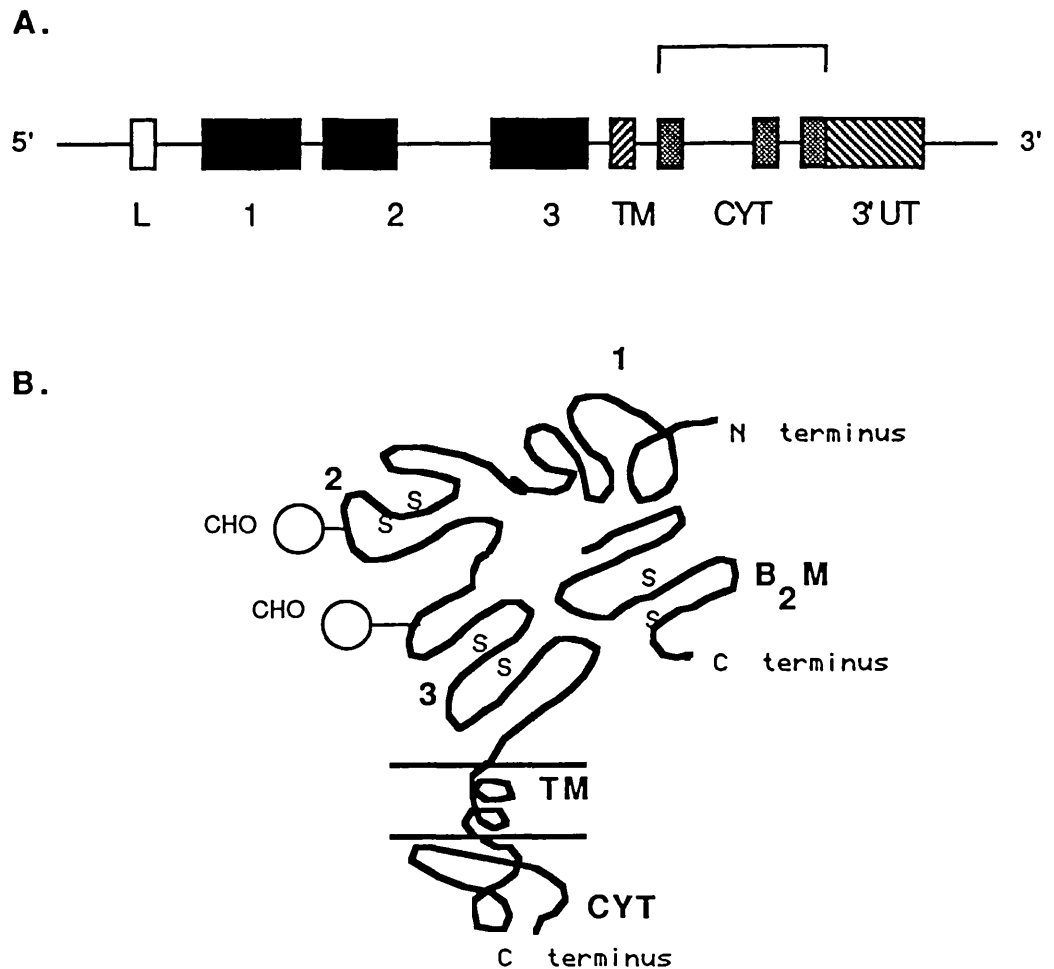


FIGURE 1.2

(Adapted from Flavell et al., 1985).

A. shows the organization of a typical Class I gene, while B. shows the structure of a Class I antigen in complex with β_2 -microglobulin (B_2M). Numerals 1 to 3 refer to the α chain extra-cytoplasmic domains, L to the leader peptide, TM to the transmembrane domain and CYT to the cytoplasmic tail region.

In B., the position of intra-chain disulphide bonds is shown by S S; and potential glycosylation sites by CHO.

MURINE CLASS II GENES

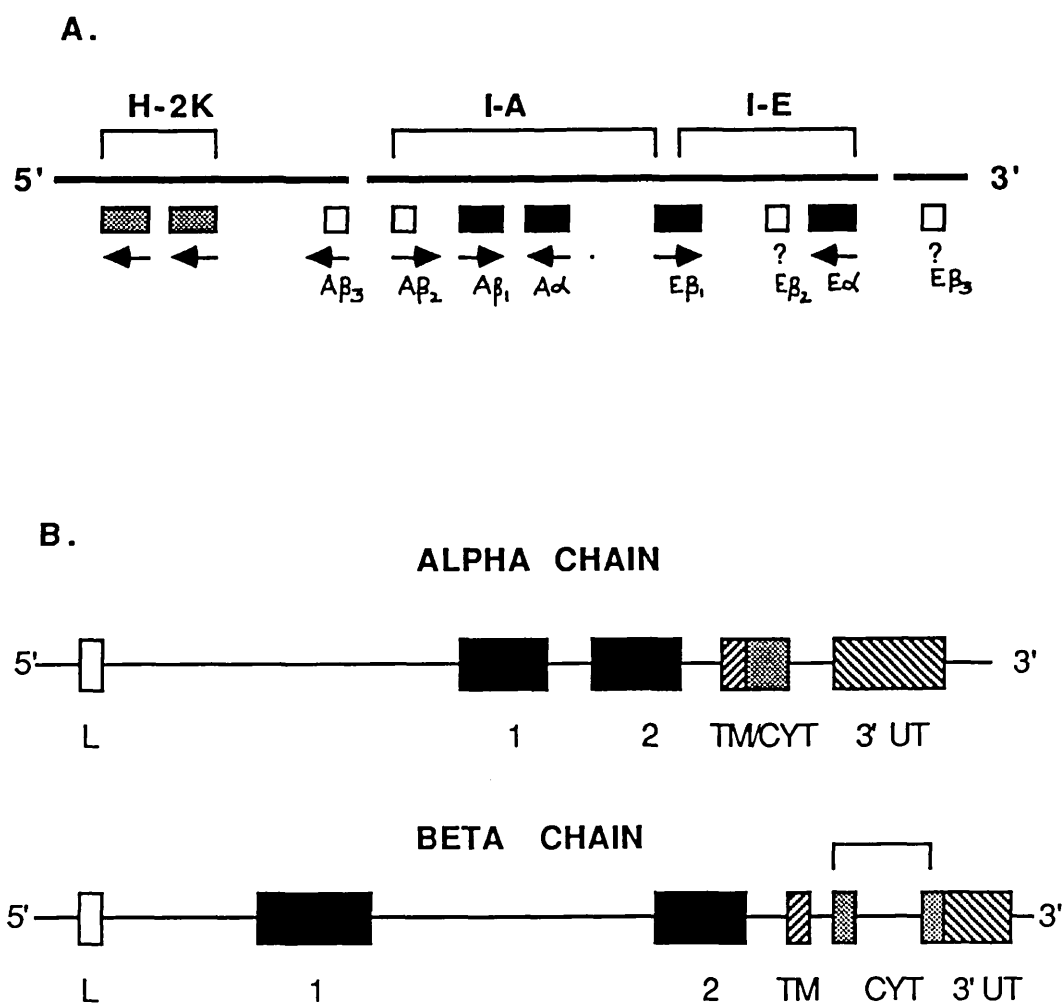


FIGURE 1.3

(Adapted from Flavell et al., 1985)

A. shows the detailed organization of the I region of the MHC. Boxes indicate known genes (open boxes are pseudogenes). The pseudogene $A\beta_3$ lies close to the H-2K region, which is also shown. Arrows show gene orientation, if ascertained.

B. shows the typical structure of α and β chain genes. Numerals refer to extra-cytoplasmic domain exons, L to the leader peptide, TM to the transmembrane region, CYT to the cytoplasmic tail and UT to untranslated regions of the mRNA.

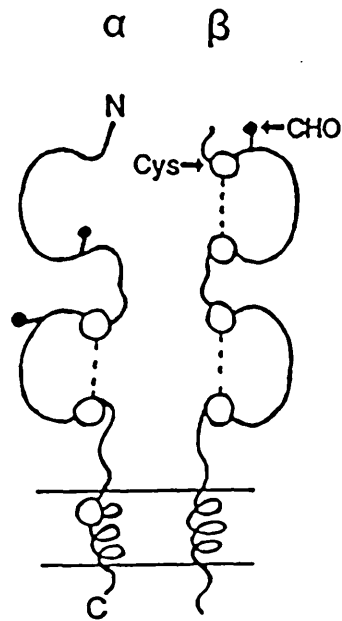


FIGURE 1.4

CLASS II ANTIGENS

(Based on information from Mengle-Gaw and McDevitt, 1985)

This figure is a schematic representation of the structure of Class II antigens. α and β chain polypeptides have intra-chain disulphide links (-S-S-) and potential glycosylation sites (CHO).

E alpha GENE REGULATORY SEQUENCES

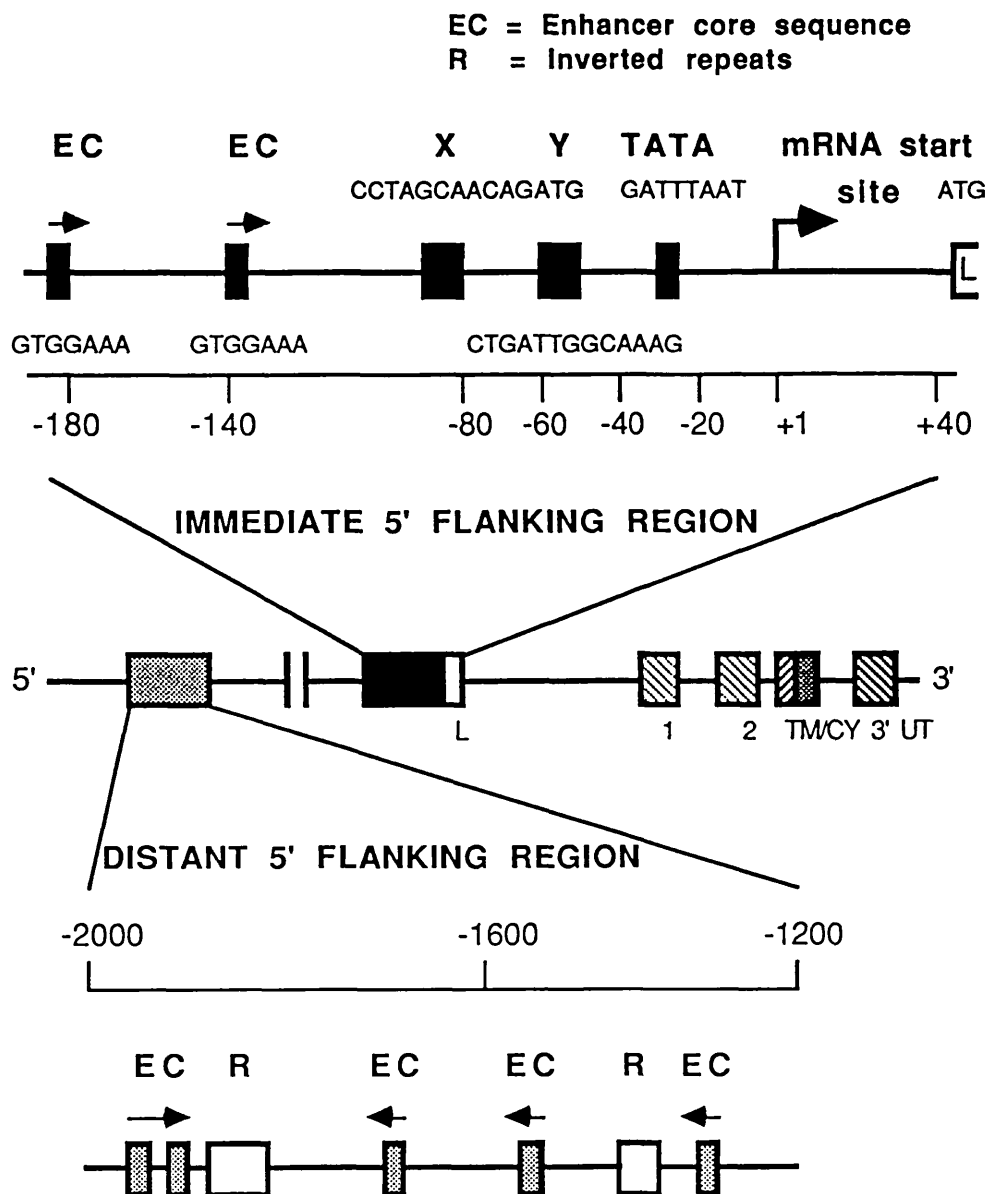


FIGURE 1.5

(Based on information from Dorn et al., 1987a; Widera et al., 1987)

The sequences that regulate E α gene transcription are shown. Arrows show the orientation of the enhancer core sequence GTGGAAAG (EC). Other regulatory sequences are indicated by boxes. The scales are in bp, counting from the main E α mRNA start site (+1).

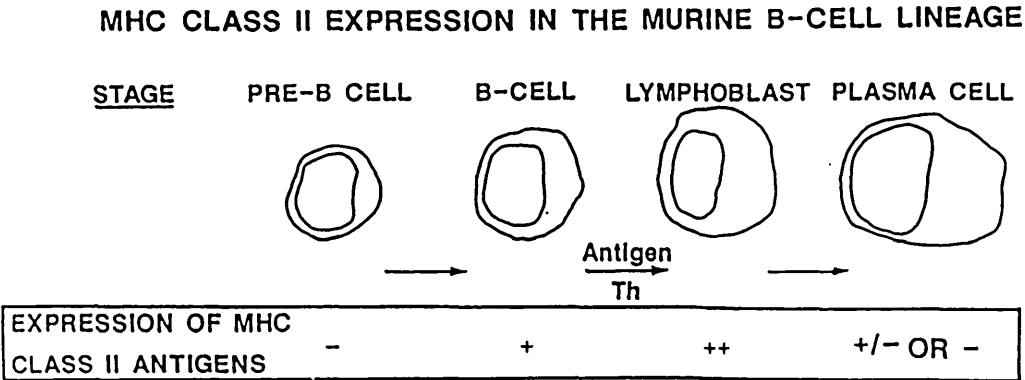


FIGURE 1.6
CLASS II EXPRESSION IN THE MURINE B LYMPHOCYTE LINEAGE

This figure is based on the data of many workers mentioned in section 1:3.1.

CHAPTER 2

MATERIALS AND METHODS

Except where otherwise specified, all chemicals used for cell culture and immunochemistry were AnalaR grade supplied by BDH Chemicals Ltd., Poole, Dorset. Materials used for nucleic acid molecular biology are specified separately.

2:1 CELLS AND CELL CULTURE

2:1.1 Cell lines

NFS 8.18 and NFS 1.3 are two of a series of B lymphoid tumours derived from NFS/N strain mice and propagated in cell culture by Davidson and co-workers (1984). These tumours were the kind gift of Dr. W.F. Davidson (Laboratory of Virology, NIAID, NIH, Bethesda, USA).

A20 and M12 are spontaneous B cell lymphoma cell lines derived from the BALB/c mouse strain (Kim et al., 1979). A HGPRT⁻, HAT medium sensitive variant of A20 (originally termed L10A2J; McKean et al., 1981) was selected for resistance to 10 mM ouabain by Dr B.M. Chain (Department of Biology (Medawar Building), University College London). This cell line (A20.01.5) was used in the experiments described here.

P3X63Ag8 (X63) is a HAT medium sensitive, IgG1 secreting plasmacytoma that was cloned from the mineral-oil induced MOPC-21 tumour of BALB/c mice (Kohler and Milstein, 1975). X63 cells were the kind gift of Dr D. Mathis (Centre de Genetique Moleculaire des Eukaryotes, Strasbourg, France). P3-NS1/1Ag4.1 (NS1) is a HAT medium sensitive, non-secreting plasmacytoma cell line cloned from X63 (Kohler et al., 1976). The NS1 cells used in these experiments were originally obtained from the American Type Culture Collection (ATCC, Bethesda, Maryland, USA) and had been maintained for several years in our laboratory prior to use.

K3, a cloned hybridoma cell line produced by the fusion of NS1 with CBA strain splenic cells, was provided by Dr Chain. These cells are resistant to HAT medium but are sensitive to 10 mM ouabain.

Sp2/0-Ag14 (Sp2/0) is a nonsecreting hybrid cell derived from the fusion of X63 with BALB/c strain splenic cells (Schulman et al., 1978). Sp2/0 is HAT sensitive and is used as a fusion partner for the production of murine hybridomas. Sp2/0 and two cloned hybridoma cell lines, HB124 and HB125, produced by the fusion of Sp2/0 with BALB/c strain splenic cells (Schroer et al., 1983), were obtained from the ATCC.

The human Burkitt's lymphoma cell lines RAJI and RAMOS, which are respectively EBV positive and negative (Pulvertaft et al., 1964; Giovanella et al., 1975), were obtained from the ATCC. Another EBV positive lymphoblastoid cell line RPMI 7666 (Moore et al., 1967), and the human plasmacytoma HS-Sultan (Harris et al., 1974), were obtained from the same source.

The murine macrophage-like tumour cell line, WEHI-3, and a variant of the mouse mastocytoma, P815, were the gifts of Dr P. Kaye (London School of Tropical Medicine and Hygiene) and Dr H. Ikeda (ICRF, London) respectively. These lines were originally obtained from the ATCC.

2:1.2 Cell culture

In general, the methods used were as described by Mishell and Shiigi (1980).

RPMI 1640 medium buffered with 25 mM 2-4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES) and containing 2 mM l-glutamine (RPMI 1640; Gibco Europe, Paisley, Scotland) was used for the culture of cell lines. Before use, β -mercaptoethanol (β -ME, Sigma Chemical Co., Poole, Dorset) was added to a final concentration of 5×10^{-5} M. Gentamycin sulphate (50 μ g/ml; Sigma) and Amphotericin B (25 μ g/ml; Fungizone, Gibco) were sometimes added.

Batches of foetal calf serum (FCS; Gibco) were tested prior to purchase to ensure that they supported the growth of cell lines. Serum was heat-inactivated at 56° C for 30 minutes before use.

Hank's buffered salt solution (HBSS, Gibco) was used for preparing and washing cell suspensions. Phosphate buffered saline (PBS, pH 7.2; Gibco) containing 0.14 M sodium chloride, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate and 8.1 mM disodium hydrogen phosphate was used for the same purposes.

Cells were grown in RPMI 1640 with 10% FCS (growth medium) at 37⁰ C in a humidified atmosphere of 5% carbon dioxide in air.

They were maintained at a density of 10⁴ to 10⁵ cells/ml in horizontal flasks (25 to 175 cm² surface area; Nunc A/S, Roskilde, Denmark) and subcultured every 2 to 5 days. Cell number and viability was routinely measured by trypan blue exclusion (Kruse and Patterson, 1973) before use in any experiment.

Cultures were periodically tested for the presence of Mycoplasma infection at the Department of Microbiology, Charing Cross Hospital.

2:1.3 Mice

Specific pathogen free BALB/c, CBA and SJL strain mice of either sex at 4-12 weeks of age were obtained from the Animal Unit, Charing Cross Hospital, London and used as a source of splenic lymphoid cells. The animals were maintained under conventional conditions with food and water provided ad libitum. Nu/nu mice of either sex at 4 weeks of age kept in specific pathogen free conditions were used for the preparation of mAb containing ascites.

2:1.4 Preparation of single cell suspensions of murine splenic cells

Spleens were aseptically removed from animals, pressed through sterile nylon mesh (125 mesh; Cadish Ltd., London) and washed twice in HBSS (Mishell and Shiigi, 1980). Red blood cells were removed by lysis in erythrocyte lysis buffer (17 mM

Tris-HCl, pH 7.2; 0.144 M NH_4Cl) for 2 minutes at room temperature followed by two washes in HBSS containing 10% FCS (Mishell and Shiigi, 1980). This unfractionated splenic cell population was used for somatic cell fusions within 1 hour, and for other experiments, within 8 hrs.

2:2 SOMATIC CELL HYBRIDIZATION

2:2.1 Preparation of cells

Cultures ($5-8 \times 10^4$ cells/ml) of NS1, A20 and K3 cell lines were subcultured 1 in 2 twenty-four hours before use to ensure that they were in the log phase of growth. The following day cells were harvested, washed twice in HBSS and viability determined by trypan blue exclusion. Cells were not used unless viability was > 95%.

2:2.2 NS1 x splenic cells

NS1 cells were fused with single cell suspensions of murine splenic cells in a ratio of 1:10 (typically, 10^7 NS1 cells to 10^8 splenic cells) by a modification of the method of Galfre and coworkers (1977) using PEG (Pontecorvo, 1976). The cells were washed twice in HBSS to remove serum and pelleted together at $500 \times g$. All supernatant fluid was carefully aspirated and the cells were gently but thoroughly resuspended in 1 ml of 50% PEG (w/v) ($M_r=1500$; tissue culture tested, Sigma) warmed to 37°C . The mixture was incubated at 37°C for 6 minutes and then 25 ml of RPMI 1640 was added drop by drop over 5 minutes with constant gentle mixing. This cell suspension was plated out at 0.5 to 1×10^6 cells/ml in 24 well tissue culture plates (Nunc), and RPMI 1640 containing 20% FCS added to a final volume of 1 ml/well. After 24 hours' culture at 37°C , 1 ml of 2x HAT selective medium (50x concentrate, Sigma, diluted into growth medium) was added to each well to give a final concentration of 1x HAT (100 μM hypoxanthine, 16 μM aminopterin, 100 nM thymidine). The

cells were maintained in HAT selective medium until growth of HAT resistant hybrids was apparent (usually 8-12 days after fusion). The hybrid cell lines were then expanded in HT selective medium (HAT medium without aminopterin) for 3-4 days and finally in growth medium. One ml aliquots of these uncloned hybrid cell lines were cryopreserved in liquid nitrogen 15-25 days after fusion.

In every experiment, mixtures of NS1 and splenic cells treated in an identical manner except for the addition of PEG served as controls. In no experiment were viable cells present in these controls 8-12 days after commencement of HAT selection.

2:2.3 A20 x K3

In a single experiment 10^7 A20 cells were fused with 10^7 K3 cells as described in the preceding paragraph. The selective medium used was HAT medium containing 10 mM ouabain (Sigma). The growth of 7 resistant hybrid cell lines was apparent 12 days after fusion. They were expanded for 4 days in HT medium without ouabain, and finally for 4 to 6 days in growth medium before cryopreservation in liquid nitrogen.

2:2.4 Ploidy determinations of parent and hybrid cells

Nuclear ploidy determinations were performed by standard methods (Taylor, 1980) to ensure that the cell lines obtained from fusion were in fact hybrids.

Between 1 and 5×10^5 cells were incubated for 5 minutes on ice in 50 µg/ml propidium iodide, 0.1% Triton X-100 (BDH) and 10 µg/ml Ribonuclease A (for molecular biology, Sigma) in PBS and the suspension filtered through nylon mesh (125 mesh; Cadish). Stained nuclei were analysed by flow cytometry as described in section 2:3.3.

2:3 IMMUNOCHEMISTRY

2:3.1 Monoclonal antibodies and antisera

A number of hybridomas secreting mAb against the murine MHC antigens were obtained from Prof. G.J. Hammerling (Deutschkrebsforschung, Heidelberg, FR Germany), and from the ATCC (HB and TIB series). Hybridomas secreting mAb against human HLA antigens (W6/32 and L243) were obtained from Dr. P. Beverley (University College Hospital, London) and the ATCC respectively; while mAb against HLA-DR 4 antigens were provided by Dr H. Ikeda (ICRF, London). The characteristics of these mAb are listed in Table 2.1.

Culture supernatants of these hybridomas were prepared by growing the cells to near extinction ($2-3 \times 10^6$ cells/ml) in RPMI 1640 with 5% FCS. Cells and debris were removed by centrifugation at 3000 rpm for 20 minutes followed by filtration through a 0.22 μ m Millipore filter. Supernatant was then concentrated 10-fold using an Amicon concentrator (Amicon Corp., Lexington, Mass., USA) and used without further dilution for immunofluorescence staining experiments. Stock supernatants were stored at -20° C and aliquots in use kept at 4° C.

The hybridomas used in these experiments were found to secrete between 5 and 20 μ g/ml of mAb into the culture supernatant as determined by an inhibition radio-immunoassay (personal communication; Dr P.M. Kaye, London School of Tropical Medicine and Hygiene).

Ascitic fluid containing mAb was prepared by injecting 10^6 hybridoma cells in 0.5 ml RPMI 1640 intraperitoneally into nu/nu mice 5 to 7 days earlier. Ascitic fluid was harvested aseptically 10 to 20 days later after sacrificing the mice, spun at 3000 rpm for 20 minutes to remove erythrocytes and debris, and stored at -20° C. Ascitic fluids were spun at 13000 rpm for 2 minutes in an Eppendorf Microfuge (Eppendorf GmbH, Hamburg, FR Germany) prior to use.

Rabbit antiserum to murine MHC Class II antigens, the kind gift of Dr B.M. Chain (University College London), was stored in aliquots at -20° C. The antiserum was centrifuged at 13000 rpm

for 2 minutes immediately prior to use.

2:3.2 Indirect immunofluorescence staining

Cells were analysed for the expression of MHC antigens at the cell surface by standard methods of indirect immunofluorescence (Parks et al., 1986) using the murine mAb listed in Table 2.1. In order to diminish non-specific staining due to binding of the second layer fluorochrome conjugate with F_c receptors, goat antibodies, which do not bind strongly with human and mouse F_c receptors (Alexander and Sanders, 1977) were used throughout. Although some of the cells used in these experiments do express sIg (for example, A20) high backgrounds were not found to be a problem in the interpretation of results. As shown in Table 2.2, the intensity of staining with the second layer conjugate alone, while positive with respect to unstained cells, was only 1/5 th to 1/10 th the intensity of staining with mAb to MHC antigens. This suggests that the major specificity of the second layer fluorochrome conjugate is for the F_c portion of mouse Ig.

Optimal dilutions of mAb ascites and second layer fluorochrome conjugates were determined by checkerboard titrations using splenic cell suspensions (data not shown).

Between 10^5 and 10^6 cells per sample were stained for analysis in sterile 4.5 ml round bottom tubes (Falcon). Washing buffer (WB) was ice-cold HBSS containing 1% FCS and 0.01% sodium azide. Cells were washed by resuspending in 2 ml WB followed by centrifugation at 1000 rpm for 3 minutes. All reagents (blocking sera, second layer antibodies, etc.) were diluted in WB.

Cells in culture were harvested by centrifugation at 1000 rpm for 3 minutes, resuspended in 100 μ l 10 % heat-inactivated normal goat serum (NGS; Sigma) and incubated at room temperature for 30 minutes. Cells were washed once and resuspended in 25 μ l of the appropriate or negative control mAb. After 30 minutes incubation at 4^0 C, the cells were washed

thrice.

The cell pellet was then resuspended in 25 μ l of second layer fluorochrome conjugate. In the experiments described here this was either FITC- or PE-conjugated goat anti-mouse immunoglobulin (GAMIg; Southern Biotechnology Associates, Birmingham, Alabama, USA) at 1:100 dilution. After 30 minutes incubation at 4⁰ C, the cells were washed thrice.

Stained cells were either resuspended in 200-500 μ l of WB and immediately analysed, or fixed for later analysis. Fixation was carried out by resuspending the cell pellet in 100 μ l of 2% paraformaldehyde (Sigma) in PBS, followed by overnight incubation at 4⁰ C (Lanier and Warner, 1981). The next morning cells were washed thrice and resuspended in WB. Fixed cells were stored in the dark at 4⁰ C and analysed within 7 days. It was found (as reported previously by Lanier and Warner, 1981) that this treatment had no effect on results obtained.

2:3.3 Fluorescence activated cell sorter (FACS) analysis and cell sorting

Analysis was carried out with the assistance of Dr M. Kahan on a FACStar cell sorter (Becton Dickinson, Mountain View, California, USA) with a single argon laser excitation system and four detectors (for forward and sideward light scatter (FSC, SSC), green and red fluorescence (FL1, FL2)).

2:3.3.1 Analysis of stained cells

Initial alignment of the flow nozzle and calibration of the detection system was performed using standard suspensions of glutaraldehyde-fixed chicken erythrocytes (Gibco), and the signals from these calibration particles adjusted to a standard output level. Fine tuning of the detection system for each type of cell being analysed was performed by adjusting amplifier gain settings for FSC and SSC measurements such that viable cells could be clearly distinguished from dead cells and debris. Gain

settings for FL1 and FL2 measurements were adjusted using cells stained with GAMIg alone (negative control) and a positive control antibody. If required a data acquisition gate was required to exclude dead cells from analysis - this was used only where > 50% of a sample consisted of dead cells.

Ten thousand events were measured for each sample, with FSC/SSC measurements on a linear scale and FL1/FL2 measurements on a logarithmic scale. Acquired data were analysed using the Consort 30 computer programme (Becton-Dickinson), with appropriate gates set to exclude dead cells and cell debris. These analyses were performed in accordance with the standard methods outlined by Parks and coworkers (1986).

Where the mean fluorescence intensities of different cell populations stained with the same mAb were compared, histograms of relative cell number to fluorescence intensity were plotted (Figure 2.2), and the mean fluorescence intensity per cell determined according to the formula :

$$\text{Mean fluorescence intensity} = \frac{\text{Total fluorescence (ie., area under histogram)}}{\text{Number of cells analysed}}$$

Standard deviations from the mean fluorescence were calculated from the frequency distributions shown by the fluorescence histograms (Sokal and Rohlf, 1981a).

In all the indirect immunofluorescence experiments performed, saturating concentrations of both first layer mAb and second layer conjugates were used. Under these conditions the mean fluorescence intensity of stained cells is directly proportional to the amount of mAb bound, which is in turn proportional to the number of antigen molecules expressed at the cell surface. Similar methods have been used with other immunoassays (for example Mason and Williams, 1980) in determining the number of antigenic sites expressed/cell.

Percentages of negative and positive cells were also determined (Figure 2.3). In most cases, the fluorescence histograms of cells stained with negative control mAb or GAMIg

alone did not intersect with the histograms of positively stained cells. The point of termination of the negative histogram was then used to mark the division between negative and positive staining. Where the histograms did intersect, the point of intersection was used as the dividing marker.

On occasion, the Kolmogorov-Smirnov statistical test was used to compare fluorescence histograms (Young, 1977). The method and its application are described where it has been used (for example, see Figure 3:2.3).

2:3.3.2 Cell sorting

Further calibration steps were performed before use of the FACS for cell sorting. The fluidics system was pre-sterilized by passing absolute ethanol, followed by washing with sterile PBS to remove traces of ethanol. The optimal sort delay (time between cell detection and drop charging) was set by trial-and-error to maximise cell recovery without losing separation efficiency.

After these machine settings were optimised, sort windows were set on cells stained for the surface antigen of interest, using cells stained with negative control mAb or GAMIg alone for comparison. Cell populations within the sort windows were then separated. Sorted cells were collected in sterile tubes containing a drop of 50% FCS, and were immediately re-analysed to validate separation.

2:3.4 Radioimmunoassay

Cells were also analysed for MHC antigen expression using a modification of a standard radioimmunoassay method (Tsu and Herzenberg, 1980). Assays were carried out in sterile 4.5 ml plastic tubes (Falcon). All washes were performed in ice-cold WB as described in section 2:2.2. MAb were used at the same concentrations as for indirect immunofluorescence. All reagents were diluted in WB.

One hundred thousand cells were used per sample. They were resuspended in 100 μ l of 10% heat-inactivated normal rabbit serum (Gibco) and incubated at room temperature for 30 minutes. Cells were washed once, and resuspended in 100 μ l of appropriate or negative control mAb. After an hours' incubation at 4⁰ C, the cells were washed thrice, resuspended in 100 μ l of WB containing 50000 cpm of ¹²⁵I-labelled Protein A (Amersham International Plc., Amersham, Buckinghamshire), and further incubated for 1 hour at 4⁰ C. The cell suspension was then washed thrice and finally resuspended in 500 μ l of WB. Bound radioactivity was quantitated using a Minigamma γ -counter (LKB Instruments, Croydon, Surrey).

Murine mAb to the β -chain of ricin (the kind gift of Dr E. J. Culbert, ICI Plc., Alderley Edge, Cheshire) was used as a negative control in these experiments.

2:3.5 Biosynthetic labelling and immunoprecipitation

MHC Class II antigens were biosynthetically radiolabelled with ³H amino acids prior to immunoprecipitation. This method was chosen in preference to ¹²⁵I surface labelling because it labels both intracellular and membrane forms of the antigens; and because some murine Class II antigens do not surface label (Jones, 1977).

RPMI 1640 medium deficient in l-lysine, l-leucine and l-serine (RPMI DEF) was prepared using the RPMI 1640 Select-amine kit (Gibco). This medium was identical in every other way with the medium routinely used for tissue culture.

Immunoprecipitation was carried out by a modification of the method described by Cullen and Schwartz (1976) for the purification of surface labelled H-2 glycoproteins. Lentil lectin affinity chromatography was not carried out, as this reduces recovery 3-fold but does not greatly improve the specificity of detection (Moosic et al., 1980).

Ten million cells were washed thrice in serum-free RPMI DEF and resuspended in 10 ml RPMI DEF containing 2.5% FCS (10⁶

cells/ml). L-[4,5-³H] lysine monohydrochloride, L-[4,5-³H] leucine monohydrochloride and L-[3-³H] serine (all at 1 mCi/ml; Amersham) were added to a final concentration of 20 µCi/ml each; and the cells were incubated at 37⁰ C for 8 hours. The cells were washed thrice in RPMI DEF with 5% FCS, and the pellet resuspended in 1 ml of lysis buffer (0.5% Nonidet P-40 (BDH), 10 mM Tris-Cl pH 7.4, 1 mM EDTA, 0.15 M sodium chloride) containing 1 mg/ml of bovine serum albumin (BSA; Fraction V, Sigma) and 1 mM PMSF (Sigma). The suspension was kept for 10 minutes on ice and the lysis monitored by periodic examination under the microscope (it was > 95% at the end of the incubation). Nuclei were removed by centrifugation for 60 seconds in an Eppendorf microfuge, and the supernatant removed. The supernatant was pre-cleared by centrifugation for 30 minutes at 13000 rpm in an Eppendorf microfuge at 4⁰ C.

Polyacrylamide beads coated with goat anti-mouse Ig (ImmunobeadsTM, 10 mg/ml in PBS; Bio-Rad Laboratories Ltd., Watford, Herts.) were used as the mAb cross-linking reagents. Fifty µl of bead suspension was added to the pre-cleared lysate, which was rotated end-on-end at 4⁰ C for 30 minutes. The beads were removed by a brief centrifugation in a microfuge.

The cleared lysate was divided into two 0.9 ml aliquots (corresponding to 5 x 10⁶ cells each), and supplemented to 0.5 M sodium chloride by adding 100 µl of a 5 M solution. Five µl of rabbit antiserum against murine Class II antigens was added to one aliquot, the other received an equal volume of normal rabbit serum (Gibco). These mixtures were incubated for 1 hour at 4⁰ C with constant mixing, 50 µl of Immunobeads suspension added, and then incubated for a further hour under similar conditions.

Immunobeads were separated from the mixture by a brief centrifugation, and then washed first in 1 ml lysis buffer with 0.5 M sodium chloride, next in 1 ml lysis buffer with 0.1% SDS and finally in 1 ml of 0.1% NP-40 in 10 mM Tris-HCl, pH 7.4.

Immunoprecipitated antigen was removed from the Immunobeads and reduced by boiling in sample buffer (0.02% bromphenol blue, 10% glycerol, 2% SDS, 80 mM Tris-Cl, pH 6.8 and 10 mM

dithiothreitol). Immunobeads were removed from the sample by centrifugation, and the immunoprecipitates subjected to electrophoresis on 10% SDS-polyacrylamide gels as described in the following section. One μ l of each sample was measured for ^3H decay in a β counter (Rack-Beta, LKB Instruments, Milton Keynes, Bucks.).

2:3.6 Discontinuous SDS-polyacrylamide gel electrophoresis

Immunoprecipitated proteins were analysed by SDS-PAGE using a discontinuous buffer system (Laemmli, 1970; Laemmli and Favre, 1973).

Glass plates (200 mm x 200 mm) were cleaned in detergent, rinsed in DDW and wiped with absolute ethanol to remove grease and dirt. The plates were assembled using a 0.3 mm plastic spacer, and the edges sealed.

A 30% stock solution of acrylamide (30 gms acrylamide, 0.8 gm NN'-methylene bis-acrylamide (both electrophoresis grade, BDH) per 100 ml DDW) was diluted to 10% in 30 ml of separating gel buffer (0.375 M Tris-HCl, pH 8.8; 0.1% SDS). The gel was degassed, and 100 μ l of freshly prepared 10% ammonium persulphate and 20 μ l of NNN'-N' tetramethylenediamine (TEMED) gently mixed in. The separating gel was carefully poured between the plates and allowed to set. A thin layer of water-saturated butanol was poured above the gel to ensure that its surface was level : this was removed once the gel had set. The stacking gel was made by diluting the stock acrylamide solution to 5% in stacking gel buffer (0.125 M Tris-HCl, pH 6.8; 0.1% SDS); polymerizing agents were added to the same final concentration and the gel was allowed to set. A sufficient volume of stacking gel was poured to allow a distance of at least 15 mm between the end of the sample slots and the beginning of the separating gel. Electrophoresis was performed in 0.05 M Tris-HCl, 0.384 M glycine, pH 8.3, 0.1% SDS in a Pharmacia vertical gel tank using a voltage gradient of 120 V.

2:3.7 Visualization of protein by staining and fluorography

The gel was fixed by immersion in 40% methanol, 7% acetic acid in DDW for 30 minutes, followed by staining in 0.0125% Coomassie Blue (BDH) in the same solution for 2 hours. Molecular weight markers (Phosphorylase b (94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD) and α lactalbumin (14.4 kD)) were visualized by destaining for 2-3 hours in 5 changes of 40% methanol, 7% acetic acid.

To enhance fluorographic detection of ^3H , the gel was washed thrice in 7% acetic acid to remove methanol, immersed for 2 hours in AMPLIFYTM (Amersham) and then washed thrice again in 7% acetic acid. This procedure has been shown to enhance detection of ^3H by about 1000-fold (Laskey and Mills, 1975). The gel was then dried under vacuum at 80⁰ C and exposed to X-ray film (Kodak X-AR 5, Kodak Ltd., Hemel Hempstead) at -70⁰ C. Development was as described in section 2:4.4.

2:4 MATERIALS FOR NUCLEIC ACID MOLECULAR BIOLOGY

2:4.1 Chemicals

Except for the following, all chemicals were AnalaR Grade supplied by BDH. Bromophenol blue, ethidium bromide, sodium chloride and Trizma base (Tris) were from Sigma. Phenol (glass-distilled, water saturated) was from Rathbone Chemicals, Walkerburn, Scotland. PEG 6000 was from Koch-Light, Slough.

2:4.2 Radioactive isotopes

α - ^{32}P dCTP and α - ^{32}P dGTP (both 3000 Ci/mmol) were purchased from Amersham.

2:4.3 Materials for chromatography, electrophoresis, Northern/Southern blotting and nucleic acid

hybridizations

Sephadex G50 was supplied by Pharmacia Fine Chemicals (Milton Keynes, Bucks). Agarose and LMP agarose were from Bethesda Research Laboratories (BRL, Cambridge). Nitrocellulose membrane filters were from Schleicher and Schuell, Dassel, FR Germany. Nylon membrane filters were Hybond-N from Amersham. PVP and BSA (Fraction V) were from Sigma, as was salmon sperm DNA. Amberlite monobed resin MB-1 was from BDH.

2:4.4 Photography and autoradiography

Polaroid type 52 Polapan film was from Polaroid (UK) Ltd., St Albans. X-ray films were either R_x from Fuji Photo Film Co., Tokyo, Japan) or X-AR 5 from Kodak. Calcium tungstate phosphor intensifying screens and light-tight film cassettes were from X-ograph Ltd., Malmesbury, Wilts. Autoradiographs were developed using a Durr Med Medicine 260 automatic developer. A short-wave UV transilluminator from UV Products Inc., San Gabriel, California, USA was used to illuminate ethidium bromide stained nucleic acid gels.

2:4.5 Commonly used buffers

TE buffer was 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. STE buffer contained, in addition, 100 mM sodium chloride. 1x SSC contained 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0. Buffers were prepared as described by Maniatis et al., 1982.

2:5 GENERAL METHODS IN NUCLEIC ACID MOLECULAR BIOLOGY

2:5.1 Restriction enzyme digests

All restriction enzymes were purchased from Bethesda Research Laboratories and used in conditons recommended by the

manufacturer. The 10x reaction buffer concentrates for each enzyme (React™) were also supplied by BRL.

Genomic or recombinant DNAs were digested in a total volume of 50-75 µl in 1x reaction buffer. Genomic DNA samples were digested with 20-50 u of restriction enzyme/µg DNA, while recombinant DNA samples were digested with 1-5 u/µg. The volume of enzyme added never exceeded 10% of the total reaction volume to avoid inhibition due to glycerol : where necessary highly concentrated enzyme preparations (50 u/µl) were used for genomic DNA digests.

Genomic DNAs were digested for 2 to 4 hours, and recombinant DNAs for 30 to 90 minutes. Digestion was analysed by agarose gel electrophoresis.

2:5.2 Agarose gel electrophoresis

Genomic and recombinant DNA digests were resolved by electrophoresis in horizontal slab gels in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA; made as 5x stock). 0.7 to 1.2% gels were prepared by boiling the appropriate amount of agarose in TBE, cooling to 55⁰ C and adding 0.5 µg/ml ethidium bromide. Samples were loaded in 5% sterile glycerol, 0.04% bromophenol blue and electrophoresed for a predetermined distance. Nucleic acids were visualized by UV transillumination, and photographed through a Wratten type 16 filter.

Fully digested genomic DNA samples for Southern blotting were electrophoresed in a similar manner on 1% agarose gels, except that electrophoresis was in TAE buffer (40 mM Tris-acetate, 1 mM EDTA; made as 50x stock).

Precautions were taken to remove RNases by washing all apparatus in a 1:3 dilution of concentrated hydrogen peroxide (30 volumes%, BDH) and rinsing in DDW before use for RNA electrophoresis. Denaturing gels were made by boiling agarose in NG buffer (50 mM morpholinopropanesulphonic acid, 10 mM sodium acetate, 1 mM EDTA; made as 10x stock and stored in the

dark), and adding formaldehyde (12.3 M stock solution) to 2.2 M after the gel had cooled to 60⁰ C (Lehrach et al., 1977). Gels were run in NG buffer.

In all cases, phage λ DNA completely digested with HindIII (BRL) was used as a molecular weight marker. Marker bands (23.1, 9.4, 6.7, 4.4, 2.3, 2.0, 0.56, 0.125 kbp) were visualized by photography under UV transillumination before blot transfer, or by staining the transferred marker DNA on nitrocellulose or nylon membrane filters after blot transfer. Filters were stained (Efstratiadis, unpubl., cited in Maniatis et al., 1982) by soaking in 5% acetic acid for 15 minutes, followed by 0.5 M sodium acetate, pH 5.2, 0.04% methylene blue for 30 minutes and destained in DDW until marker bands were seen.

2:5.3 Phenol extraction of DNA/RNA

Glass distilled, water saturated phenol (Rathbone Chemicals) equilibrated with 100 mM Tris-HCl, pH 8.0 was used to precipitate proteins contaminating samples of nucleic acids. An equal volume of phenol was added to the sample, mixed thoroughly, and centrifuged (for 2 minutes in a microfuge for small samples, or at 2500 rpm for 10 minutes for large ones). The upper aqueous phase containing nucleic acids was carefully aspirated and transferred to a fresh tube. Traces of phenol were removed by two similar extractions with an equal volume of chloroform : isoamyl alcohol (24:1).

2:5.4 Ethanol precipitation of DNA/RNA

Nucleic acids were precipitated from aqueous samples by adding 1/10 sample volume of 3 M sodium acetate, pH 5.5 and 2 sample volumes of absolute ethanol, followed by overnight incubation at -20⁰ C or a 30 minute incubation in a dry ice/methanol bath. Nucleic acids were recovered by centrifuging for 20 minutes in a microfuge at 4⁰ C, pellets washed in 70% ethanol kept at -20⁰ C, dried in a vacuum dessicator and

dissolved in an appropriate volume of sterile TE (DNA) or DDW (RNA).

2:5.5 Precautions for handling RNA

Plastic gloves were worn at all times when working with RNA samples to avoid contamination with ribonucleases. Glassware, plasticware (tips, etc.) and all solutions (except guanidinium isothiocyanate, SDS and caesium chloride density gradient buffer) were autoclaved before use.

2:6 EXTRACTION OF NUCLEIC ACIDS FROM EUKARYOTIC CELLS

2:6.1 Extraction of high molecular weight DNA

High molecular weight DNA was extracted from cells growing in tissue culture by the method of Blin and Stafford (1976). Fifty million cells were harvested by centrifugation, washed twice in PBS and once in STE. The cell pellet was resuspended to a concentration of 5×10^6 cells/ml in STE, and SDS was added to a final concentration of 2%. Two hundred μ g of Proteinase K (Sigma) was added per ml of this mixture, and it was incubated overnight at 37⁰ C with gentle shaking. The mixture was phenol extracted thrice, and finally with chloroform/isoamyl alcohol alone. The DNA was ethanol precipitated at room temperature, and recovered by gently rolling the thread-like precipitate around the bent tip of a sterile pasteur pipette. The DNA was dissolved to a final concentration of 0.5 to 1 mg/ml in TE buffer by gentle shaking at 4⁰ C overnight. Great care was taken at all times to avoid shearing the DNA molecules.

DNA preparations were quantitated and their purity assessed by measuring their optical densities at 1 nm intervals between 220 and 320 nm on a recording spectrophotometer (Uvikon 80, Kontron Analytical Instruments, St Albans, Herts.). The concentration was determined, assuming that an optical density of 20 units at 260 nm represented a DNA concentration of 20

mg/ml. In all cases the ratio of the optical density at 260 nm to that at 280 nm was > 1.8 , indicating negligible protein contamination.

2:6.2 Extraction of RNA

RNA fractions were prepared from cells growing in culture by a modification of the guanidinium isothiocyanate (GuSCN) lysis method (Glisin et al., 1974; Chirgwin et al., 1979). At least 5×10^7 cells were harvested, washed twice in ice-cold PBS and used for the preparation of various RNA fractions.

Total cellular RNA was prepared by lysing the cell pellet in 5 ml of GuSCN lysis buffer (4 M GuSCN (Fluorochem Ltd., Glossop, Derbyshire), 0.1 M β -ME, pH 5.5). The lysate was either processed immediately or stored at -20°C for not longer than 6 weeks. RNA was separated from other cellular macromolecules by density gradient ultracentrifugation over a gradient of 5.7 M caesium chloride in 0.1 M EDTA (CsCl buffer) at $100000 \times g$ for 24 hours. DNA and protein bands were carefully removed, and the last remaining 3 ml of CsCl buffer overlaid with DDW to remove traces of GuSCN. The supernatant was removed from the RNA pellet, which was dissolved in 400 μl DDW and ethanol precipitated overnight at -20°C .

Nuclear RNA was separated by gently resuspending the washed cell pellet in 0.5 ml of 0.5% NP-40, incubating on ice for 5 minutes and pelleting the nuclei by centrifugation for 2 minutes in a microfuge. The nuclear pellet was lysed in 5 ml of GuSCN lysis buffer, and nuclear RNA isolated by density gradient ultracentrifugation. The cytoplasmic fraction of the NP-40 lysate was mixed with 4.5 mls of lysis buffer and used to prepare cytoplasmic RNA.

RNA concentration and purity were determined by spectrophotometry as in the case of DNA, except that an optical density of 25 units at 260 nm was taken to represent an RNA concentration of 1 mg/ml.

2:6.3 Purification of poly-A⁺ RNA

Poly-A⁺ mRNA was purified from total cellular RNA by affinity chromatography on oligo-dT paper (Hybond MAP, Amersham). This is a modification of the original method of chromatography on oligo-dT cellulose columns (Edmonds et al., 1971; Aviv and Leder, 1972). The manufacturer's recommended protocol was used.

Half to one mg of total cellular RNA was resuspended in 0.36 ml of DDW, boiled for 5 minutes and chilled on ice to denature the RNA. Forty µl of 5 M sodium chloride was added, bringing the final concentration to 0.5 M. Three cm² of Hybond MAP paper cut into narrow strips was prewetted for 5 minutes in 0.5 M sodium chloride and added to the RNA solution in a 1.5 ml sterile Eppendorf tube which was then rotated end-on-end for 60 minutes at 4⁰ C. The paper (carrying bound poly-A⁺ mRNA) was removed from the solution of poly-A⁺ RNA, washed twice in 20 mls of 0.5 M sodium chloride for 5 minutes and once in 20 mls of 70% ethanol for 2 minutes at room temperature. The paper was air-dried at room temperature and tightly packed into the bottom of a 1.5 ml Eppendorf tube. The minimum amount of DDW required to completely immerse the paper was then added (usually 0.4 ml), the mixture was heated to 70⁰ C for 5 minutes and the tube vortexed briefly to release the poly-A⁺ mRNA into the solution. The paper strips were jammed into the cap of the tube which was centrifuged at 13000 rpm for 45 seconds in a microfuge to remove all traces of mRNA solution from the paper.

2:7 SOUTHERN, NORTHERN AND SLOT BLOTTING

2:7.1 Analysis of restriction fragments by Southern blotting

High molecular weight DNA digested to completion with restriction enzymes was analysed by electrophoresis on 1% agarose gels in TAE buffer as described earlier. Ten µg of DNA was loaded per track. Southern blotting was performed by a

modification of standard methods (Southern, 1975 and 1980). The gel was denatured by soaking in two changes of 1.5 M sodium chloride, 0.5 M sodium hydroxide for 1 hour with gentle shaking. and washed twice in 1 M Tris, pH 8.0.

A glass plate was laid across a large glass tray containing 20x SSC and a sheet of Whatman 3M filter paper folded over the plate with both ends in the solution to act as a wick. The gel was placed on the wick and all air bubbles gently smoothed out. A sheet of Hybond N nylon membrane cut to the exact size of the gel was carefully laid over it. This was covered by 16-20 sheets of Whatman 3M paper, a 12-15 cm thickness of folded paper towels and finally a glass plate carrying a 500 gm weight. Saran Wrap was carefully placed around the edges of the gel to prevent short circuiting of the fluid path through the gel. DNA transfer was allowed to proceed for 18-24 hours, before the nylon membrane was removed, washed once in 5 x SSC and baked at 80⁰ C for 3 hours. The positions of the tracks were marked on the membrane prior to removal from the gel.

2:7.2 Analysis of RNA by Northern blotting

Five to 30 µg of purified RNA (per track) was denatured by heating to 65⁰ C for 5 minutes with 50% formamide (BDH; deionised by reaction with Amberlite monobed resin), 6% formaldehyde in 1x NG buffer; and electrophoresed on 1% denaturing agarose gels as described previously. Size-separated RNA species were transferred to nitrocellulose or Hybond-N membrane filters by Northern blotting (Thomas, 1980) using 20x SSC as the eluent. The procedure used was identical to that described in the previous paragraph for Southern blots, except that the gel was not denatured prior to blotting. Transferred RNA was fixed onto the filters by baking at 80⁰ C for 2 hours.

2:7.3 Analysis of RNA by slot blotting

Ten µg of purified RNA per sample was denatured as described

in the preceding paragraph, and three serial dilutions containing 5, 1 and 0.2 µg of RNA respectively were prepared using ice-cold 15x SSC as the diluent. RNA was applied directly to nitrocellulose membrane filters using a Minifold slot blot apparatus (Schleicher and Schuell) connected to a vacuum pump, and fixed onto the filters by baking at 80⁰ C for 2 hours.

2:8 PREPARATION OF NUCLEIC ACID PROBES

2:8.1 Probes

Table 2.2 lists the characteristics of the nucleic acid probes used in these experiments. They were all obtained in the form of purified circular plasmid DNA in solution in TE buffer from various sources (Dr B. Malissen, CNRS Unite d'Immunologie, Marseilles, France (pCDN17, pCA39); Dr D. Mathis, Laboratoire de Genetique Moleculaire des Eukaryotes, Strasbourg, France (pSPEAC₁); Dr N. Koch, Deutschkrebsforschung, Heidelberg, FR Germany (pIi-1); Dr R Baserga, University of Rochester, NY, USA (p7B6) and Dr J. Trowsdale, ICRF, London (pDRH2)).

2:8.2 Transformation of bacteria by the calcium chloride method

The E. coli strains DH1 (F⁻, recA⁻, recA1, λ⁻; Low, 1968) and HB101 (F⁻, recA13, λ⁻; Boyer and Rouland-Dussoix, 1969) were used in transformations.

Stocks of competent E. coli were prepared by the calcium chloride procedure (Mandel and Higa, 1970). One hundred ml of Luria Bertani (LB) medium (10 gms tryptone, 5 gms yeast extract (both Difco, Detroit, Michigan, USA), 10 gms sodium chloride per litre) in a 500 ml conical flask was inoculated with 1 ml of an overnight culture of bacteria and kept at 37⁰ C with vigorous shaking for 3-4 hours. The culture was chilled on ice for 10 minutes, and the bacteria harvested by centrifugation. The cell pellet was resuspended in 50 ml of ice-cold 50 mM CaCl₂, 10 mM

Tris-HCl, pH 8.0 and incubated in an ice bath for 15 minutes before harvesting the bacteria by centrifugation. The pellet was then resuspended in 6.5 ml of ice-cold 50 mM CaCl_2 , 10 mM Tris-HCl, pH 8.0, dispensed into 200 μl aliquots in pre-chilled sterile vials and left at 4⁰ C overnight (14-18 hours). The competent bacteria were stored at -70⁰ C until use.

For each plasmid to be transformed, 2 vials of competent bacteria were thawed at room temperature until just melted, very carefully mixed and incubated on ice for 10 minutes. About 10-20 ng of circular plasmid DNA in not more than 20 μl of TE was added to one vial, the second vial serving as a control. The suspensions were mixed, incubated on ice for 30 minutes, and heat-shocked at 42⁰ C for 90 seconds followed by a further 2 minute incubation on ice. One ml of LB medium was added, and the vials incubated at 37⁰ C for 1 hour without shaking.

Bacteria from both vials were streaked out onto the surface of LB agar (LB medium with 1.5% Bacto-agar (Difco)) plates containing an appropriate concentration of antibiotic (50 $\mu\text{g/ml}$ Ampicillin or 12.5 $\mu\text{g/ml}$ tetracycline (both Sigma)) and incubated for 12-16 hours at 37⁰ C. Experiments in which the control plates had bacterial colonies were discarded, otherwise 2-4 single colonies were picked with a sterile loop and grown up separately in 10 ml of LB medium with antibiotic for further analysis.

2:8.3 Small and medium scale preparations of plasmid DNA

Small to medium quantities of plasmid DNA were prepared by an identical adaptation of the alkaline SDS method (Ish-Horowicz and Burke, 1981). Only the quantities of reagents used varied : those for the small-scale preparations are listed, with medium-scale quantities in brackets. An overnight culture of transformed bacteria in 10 ml of LB medium with the appropriate antibiotic was used as the starting material. Cells from 1 ml (10 ml) of this culture were harvested by centrifugation and thoroughly resuspended in 0.1 ml (0.2 ml) of 50 mM glucose, 25

mM Tris-HCl, pH 8.0, 10 mM EDTA containing 10 mg/ml lysozyme (Sigma). The cells were lysed by the addition of 0.2 ml (0.4 ml) of freshly prepared 0.2 M NaOH, 1% SDS, incubated on ice for 5 minutes, and 0.15 ml (0.3 ml) freshly prepared 5 M potassium acetate, pH 4.8 was added. After a further 5 minute incubation on ice, bacterial chromosomal DNA and cell debris were removed by centrifugation for 5 minutes in a microfuge. The supernatant was transferred to a fresh tube, and 0.8 ml of ethanol was added to precipitate the plasmid DNA. DNA was recovered by ethanol precipitation and redissolved in 50 μ l (200 μ l) of TE. RNA was removed by adding 1 μ g/ml RNase A (Sigma), incubating at 37⁰ C for 10 minutes, followed by phenol extraction and reprecipitation.

2:8.4 Validation and preservation of transformed bacteria

Small scale preparations of plasmid DNA from transformed bacteria were checked for the presence of the correct plasmid insert by digestion with the appropriate restriction enzyme(s), followed by agarose gel electrophoresis. Aliquots from a single culture of transformed bacteria containing the correct plasmid were cryopreserved at -70⁰ C in 15% sterile glycerol, and used for further experiments.

2:8.5 Large scale preparation of plasmid DNA

Large scale preparations were performed by a modification of the above procedure incorporating further purification by PEG precipitation. Two hundred and fifty to 500 ml of an overnight bacterial culture was used as the starting material. The cells were harvested by centrifugation at 5000 rpm for 15 minutes, and the pellet thoroughly resuspended in 6 ml of freshly prepared 25 mM Tris-HCl, pH 7.5, 10 mM EDTA, 15% sucrose (Ultra-pure, BRL) containing 2 mg/ml lysozyme. The suspension was incubated on ice for 20 minutes, and 12 ml of 0.2 M NaOH, 1% SDS was added.

The suspension was thoroughly mixed by inversion and incubated on ice for 10 minutes. Seven and a half ml of 3 M sodium acetate, pH 4.6 was added, the mixture incubated on ice for a further 20 minutes and centrifuged at 15000 rpm for 15 minutes to remove bacterial chromosomal DNA and debris. The supernatant was carefully aspirated, once again subjected to centrifugation at 15000 rpm for 15 minutes, and transferred to a fresh tube. RNase A was added to a final concentration of 2 µg/ml and the supernatant was incubated at 37⁰ C for 20 minutes. Proteins were removed by three rounds of phenol extraction, and the DNA recovered by ethanol precipitation. The pellet was redissolved in 1.6 ml DDW, and 0.4 ml 4 M NaCl, 2 ml 13% PEG 6000 were added with gentle mixing. After a 60 minute incubation on ice, the mixture was centrifuged at 12000 rpm for 10 minutes to recover the plasmid DNA. The pellet was washed in 70% ethanol, dried, and redissolved in TE. The purity and quantity of the preparation was determined by spectrophotometry.

2:8.6 Purification of cloned DNA inserted into plasmid vectors

Cloned genomic and cDNA probes used for radiolabelling by the random oligo-priming method were cut from purified plasmid DNA by complete digestion with the appropriate restriction enzyme(s). The probes were purified by electrophoresis; either in LMP agarose gels (in which case the appropriate DNA band was cut out of the gel, melted and aliquoted) or in standard agarose gels (in which case the appropriate DNA band was harvested by electrophoresis into a shallow trough filled with TE). Aliquots containing approximately 100 ng of purified insert DNA in 70 µl TE were stored at -20⁰ C until use.

Plasmids containing cloned DNA probes which were to be radiolabelled by the SP6 transcription method were linearized by digestion to completion with the appropriate restriction enzyme, protein removed by phenol extraction followed by ethanol precipitation, and redissolved in TE at a concentration of 0.3 to 1 µg/µl.

2:9 RADIOLABELLING OF NUCLEIC ACID PROBES AND FILTER HYBRIDIZATION

2:9.1 Radiolabelling by random oligo-priming

Double stranded DNA probes for the A α , E α (pCA39), Ii and DR α chains and for 7B6 were labelled with α -³²P dCTP (3000 mCi/mmol; Amersham) by the random oligopriming method (Feinberg and Vogelstein, 1984) using the Klenow fragment of *E. coli* DNA polymerase I.

Oligopriming reactions were performed in OP buffer. Stocks of OP buffer (5x) were prepared by mixing three solutions - solution A (1.25 M Tris-HCl, pH 8.0, 0.125 M MgCl₂, 0.25 M β -ME, and 0.5 mM each of dATP, dGTP, dTTP (stock at 0.1 M in 3 mM Tris-HCl, pH 7.0, 0.2 mM EDTA)), solution B (2 M HEPES, pH 6.6) and solution C (90 OD u/ml random hexadeoxynucleotide primers (Pharmacia) in TE) - in the ratio 100:250:150. Stocks were stored at -20⁰ C in 100 μ l aliquots.

One hundred ng of DNA in 70 μ l TE was denatured by boiling for 4 minutes, cooled (on ice for DNAs in TE, at 37⁰ C for those in LMP agarose), and the polymerase reaction allowed to proceed for 16-24 hours at room temperature in 1 x OP buffer containing 0.5 μ g/ μ l BSA (DNase/RNase free; Boehringer Mannheim), with 5 μ Ci ³²P dCTP and 1 u Klenow polymerase (Boehringer Mannheim). Radiolabelled DNA was separated from free dCTP by chromatography over a 1 ml Sephadex G50 column equilibrated with STE buffer. The specific activity of probes labelled in this way was 10⁸ to 10⁹ cpm/ μ g of DNA : this was determined for each experiment using a β -counter.

2:9.2 Radiolabelling by SP6 transcription

Single stranded, full length RNA probes for the E α gene (pSPEAC₁) were prepared by linearizing the plasmid with HindIII (BRL) and transcribing the insert using SP6 polymerase (Promega

Biotec, Madison, Wisconsin, USA) as previously described (Melton et al., 1984) in the presence of α - ^{32}P GTP (3000 mCi/mmol; Amersham).

300 to 1000 ng of linearized plasmid was transcribed in a volume of 20 μl in 1x transcription buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl_2 , 2 mM spermidine, 10 mM NaCl), 10 mM dithiothreitol, 1 u/ μl RNasin RNase inhibitor (Promega), 2.5 mM each of ATP, CTP, UTP, 12 μM GTP, 50 μCi ^{32}P GTP and 5 u Riboprobe SP6 polymerase. The reaction was allowed to proceed for 1 hour at 37 $^{\circ}$ C, and the plasmid template destroyed by digestion with 1 u RQ1 DNase (Promega) for 15 minutes at the same temperature. The mixture was phenol extracted, and the labelled RNA probe recovered by ethanol precipitation. The specific activities obtained were in the same range as the DNA probes. The size and purity of the RNA probes was confirmed by electrophoresis on 7% urea-polyacrylamide gels followed by autoradiography.

2:9.3 Validation of probe specificity on Northern blots

Each batch of probes was checked for specificity by hybridization with a Northern blot containing a positive and negative control RNA sample. Figure 2.3 shows that the probes used in these experiments were specific. The procedures used are described below.

2:9.4 Hybridization of Southern blots

Southern blots were pre-hybridized at 65 $^{\circ}$ C for 4-5 hours in 6x SSC, 0.5% SDS, 5x Denhardt's solution (1x = 0.02% each of BSA, Ficoll (Pharmacia) and polyvinylpyrrolidone) and 100 $\mu\text{g}/\text{ml}$ spermidine. Denatured radiolabelled probes were diluted to 1-5 $\times 10^7$ cpm/ml in fresh pre-hybridization solution containing 0.01 M EDTA. Blots were hybridized for 18 hours at 65 $^{\circ}$ C with gentle shaking. This is the method described by Maniatis et al., 1982.

Hybridized blots were washed for 15 minutes in each of 2

changes of 2x SSC, 0.1% SDS at room temperature; and for 30 to 45 minutes in each of two changes of 0.2x SSC, 0.1% SDS at 65⁰ C (total 60 to 90 minutes). Excess fluid was removed before the moist blots were sealed in Saran Wrap for autoradiography.

2:9.5 Hybridization of Northern and RNA slot blots

Northern and RNA slot blots were pre-hybridized at 43⁰ C for 4-6 hours in 50% deionized formamide, 50 mM sodium phosphate, pH 6.5, 2x Denhardt's solution, 0.5 mg/ml spermidine and 0.2% SDS. Denatured radiolabelled probes were added to a final concentration of $2-4 \times 10^6$ cpm/ml in fresh pre-hybridization solution, and the blots were hybridized for 18 hours at 43⁰ C with gentle shaking.

Hybridized blots were washed and autoradiographed in a similar manner to Southern blots except that the washes in 0.2x SSC, 0.1% SDS were carried out at 50⁰ C, and further washes in 0.1x SSC, 0.1% SDS (50⁰ C for 20-30 minutes) were performed if necessary.

2:9.6 Quantitation of filter hybridization

The level of RNA expression was quantitated on slot and Northern blots by densitometry. X-ray films were pre-flashed before exposure to reduce errors due to the non-linear response of film at -70⁰ C to the light emitted by intensifying screens (Laskey and Mills, 1977). Autoradiographs of blots hybridized with radiolabelled probes to the MHC Class II genes were densitometrically scanned on an automated Chromoscan III (Joyce-Loebl, Gateshead, Tyne-and-Wear) to quantitate the amount of radioactivity bound in each track or slot. Where such quantitations were performed, at least three RNA concentrations were used to ensure that densitometric measurements were made in the linear range of the X-ray film exposure curve (Laskey, 1984).

The blots were then stripped free of bound radioactivity by

washing for 2-3 hours in two changes of 5 mM Tris-HCl, pH 8.0, 0.05% sodium pyrophosphate, 0.1x Denhardt's solution (Thomas, 1980), re-exposed to X-ray film for 2 to 4 days to ensure that all bound radioactivity had been removed, and hybridized with radiolabelled 7B6 probe which detects an RNA species that is invariant in the cell cycle (Kaczmarek et al., 1985). Autoradiographs were scanned to quantitate the amount of 7B6 hybridization in each slot or track, which is a measure of the amount of RNA loaded.

Comparisons between the levels of RNA expressed were made by standardizing the MHC Class II-specific hybridization signal with respect to 7B6 in order to eliminate errors due to sample preparation, dilution or loading. One of the three RNA concentrations on the slot blot was chosen where the densitometric readings for hybridization with MHC and 7B6 probes were between 0.75 and 1.5 units to ensure that film exposure was on the linear part of the exposure curve. Due to experimental errors in RNA loading, the 7B6 reading for each of the slots containing this RNA concentration was slightly different. The least of these 7B6 readings (ie., the slot that had been loaded with the least amount of RNA), was taken to be 1 arbitrary unit (of RNA) and the readings for the other slots divided by the appropriate correction factor to standardise the amount of RNA loaded into each slot with respect to 7B6 hybridization. Densitometric readings for hybridization of these slots with MHC probes were then multiplied by the same correction factor : thus the final value represented intensity of the hybridization signal per unit amount of RNA loaded.

Comparisons were only made between different tracks or slots of the same autoradiograph showing hybridization with the same gene probe to eliminate errors due to differences in binding affinity, specific activity, etc. of the probe.

2:10 GENE TRANSFECTION

2:10.1 Cosmid expression construct

The cosmid expression construct pHD8R/RW (R/RW) was the kind gift of Dr H. Ikeda (Imperial Cancer Research Fund, London). Its construction has been described in detail (Ikeda et al., 1988). Briefly, it consists of 8 tandemly linked copies of an expression cassette (Figure 2.4) cloned into the SfiI site of a cosmid vector containing the Hygromycin B resistance gene (Blochlinger and Diggelman, 1984) as a dominant selection marker. Each copy of the expression cassette contains one copy each of the DR α and DR β gene cDNAs cloned from an EBV transformed human lymphoblastoid line derived from a HLA-DR4, Dw15 homozygous individual (Ikeda et al., 1988). The Hygromycin B resistance gene and each of the 8 tandem copies of the DR α and DR β cDNAs are under the control of individual SV40 early promoter fragments. Transcription from the early promoter fragments is enhanced by twin copies of the SV40 enhancer (PvuII-BglI fragment containing the 72 bp repeat sequences (Benoist and Chambon, 1981)) which are within the cosmid vector.

This expression construct has a number of advantages. It contains multiple copies of both the DR α and β chain genes, ensuring that they are simultaneously expressed. The SV40 promoter and enhancer elements are transcriptionally active in a wide range of eukaryotic cells. The dominant selection marker for Hygromycin B resistance enables selection of transfected cells. It has been shown (Ikeda et al., 1988) that the construct can be transfected both by protoplast fusion and electroporation. When transfected cells are maintained in Hygromycin B, DR expression is stable and at high levels.

2:10.2 Titration of Hygromycin B resistance

Before transfection, cell lines were tested for Hygromycin B resistance. Briefly, cells were cultured at 10^5 cells/ml in the wells of a 24-well culture plate in 2 ml of complete growth medium containing 0, 100, 200, 400, 600 and 1000 μ g/ml of Hygromycin B (Boehringer). Culture was continued for 4 days,

with samples taken each day for viability testing by trypan blue exclusion. In all cases, no viable cells were seen after 4 days in 400 µg/ml or higher concentrations of Hygromycin B.

2:10.3 Preparation of cosmid DNA for transfection

E. coli which had been transformed with the cosmid construct by in vitro packaging were inoculated into 250 ml of LB medium containing 50 µg/ml of Ampicillin and cultured overnight at 37⁰ C. Cosmid DNA was purified by an appropriately scaled-up variation of the alkaline lysis method described in section 2:8.3.

Cosmid DNA was then linearized by complete digestion with the enzyme KpnI. KpnI cuts the vector at a single unique site, and does not cut the inserted cDNA expression cassette (Figure 2.4). The completeness of digestion was checked by electrophoresis before the DNA was repurified by phenol:chloroform extraction and ethanol precipitation. The linear DNA was dissolved to a concentration of 5 µg/µl in autoclaved TE buffer which had been passed before use through a 0.22 µ Millipore filter.

2:10.4 Gene transfection by electroporation and selection of transfectants

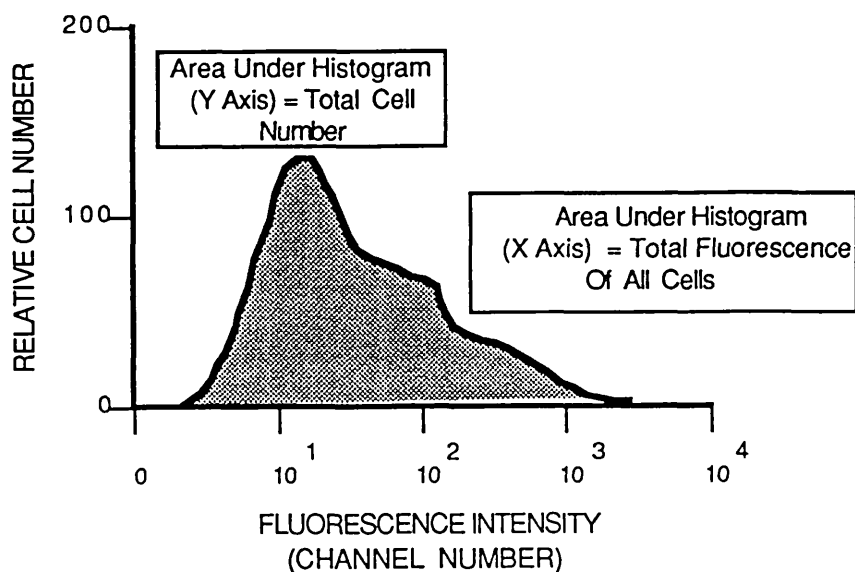
A modification of the procedure of Wong and Neumann (1982) was used. Briefly, 2×10^7 cells were washed once with cold PBS, resuspended in 200 µl, and incubated on ice for 15 minutes. Fifteen µl of the linearized cosmid DNA solution (=75 µg) was added, and the cells were subjected to 15 pulses of 2.0 kV each with one second intervals between pulses using an Apelex electroporator (EMBL, Heidelberg). The cells were chilled on ice for a further 20 minutes before dilution to 5×10^5 cells/ml in complete growth medium containing 50 µg/ml of gentamycin sulphate (Gibco) and 12.5 µg/ml of Amphotericin B (Gibco). Cells were plated out at 1 ml/well in a 24-well tissue culture plate

and incubated at 37⁰ C overnight.

After 24 hours culture, 100 µg/ml of Hygromycin B was added to the growth medium. This was increased to 200 µg/ml after 3 days' culture, 400 µg/ml after 6 days culture and finally to 600 µg/ml after 9 days culture. Medium was changed every third day. The growth of drug-resistant transfectant cell lines was apparent 12 to 15 days after transfection, when the Hygromycin B concentration was increased to 1000 µg/ml. Cell lines were expanded in complete growth medium containing 1000 µg/ml of Hygromycin B and cryopreserved in liquid nitrogen between 25 and 28 days after transfection.

The gradual increase in Hygromycin B concentration during the initial phase of selection was found to be necessary to maximise the yield of transfected cell lines. As a control to verify that this procedure did not result in the creation of drug-resistant, untransfected cells, at least four wells containing mock transfected cells (which were electroporated without cosmid DNA being added) were included in each tissue culture plate and subjected to the same treatment. In no experiment did mock transfected cells survive the first week of culture.

Calculation Of Mean Fluorescence Intensities



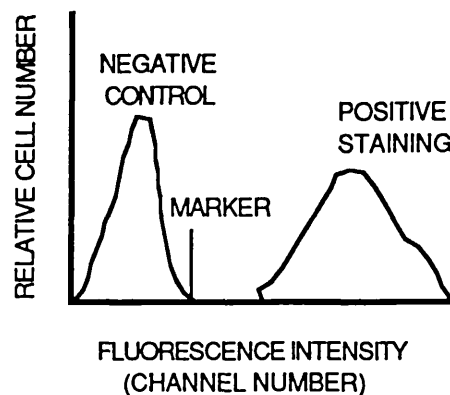
$$\text{MEAN FLUORESCENCE INTENSITY PER CELL} = \frac{\text{TOTAL FLUORESCENCE}}{\text{TOTAL CELL NUMBER}}$$

FIGURE 2.1

Where the mean fluorescence intensities of different cell populations stained with the same mAb were compared, histograms of relative cell number to fluorescence intensity were plotted, and the mean fluorescence intensity per cell determined according to the formula shown using the B/DIS Consort computer programme. Standard deviations from the mean fluorescence were calculated from the frequency distributions shown by the fluorescence histograms (Sokal and Rohlf, 1981a).

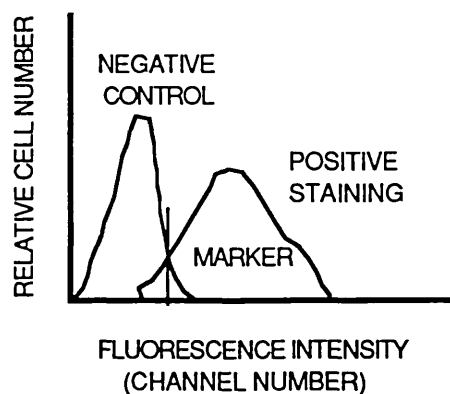
MARKING THE DIVISION BETWEEN NEGATIVE AND POSITIVE STAINING

A. NON-INTERSECTING HISTOGRAMS



MARKER = POINT OF TERMINATION OF
NEGATIVE CONTROL
HISTOGRAM

B. INTERSECTING HISTOGRAMS



MARKER = POINT OF INTERSECTION OF
NEGATIVE CONTROL AND
POSITIVE HISTOGRAMS

FIGURE 2.2

Markers were set as depicted when marking the division between negative and positive staining during analysis of FACS results. Negative control staining was with fluorochrome conjugated GAMIg alone.

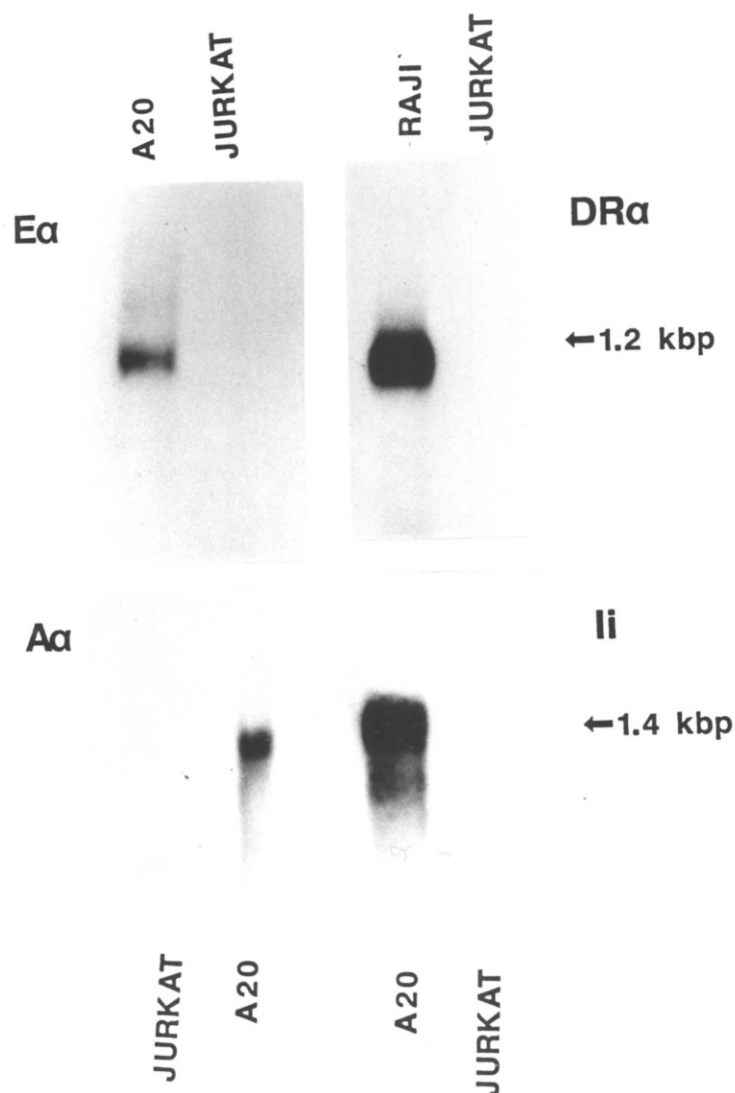


FIGURE 2.3

SPECIFICITY OF NUCLEIC ACID PROBES

The specificity of radio-labelled nucleic acid probes was tested by hybridization to a Northern blot containing 15 µg of total cellular RNA prepared from an appropriate negative and positive control cell line. For the murine MHC gene probes (Aα, Eα and Ii) the positive control was the B lymphoma A20, while for the DR probe it was the Burkitt lymphoma RAJI. RNA from the human T cell lymphoma JURKAT was used as the negative control (kind gift of Maija Kissonerghis, Charing Cross Sunley Research Centre). Both Eα probes (see Table 2.3) gave similar results, only pCA39 is shown.

COSMID EXPRESSION CONSTRUCT

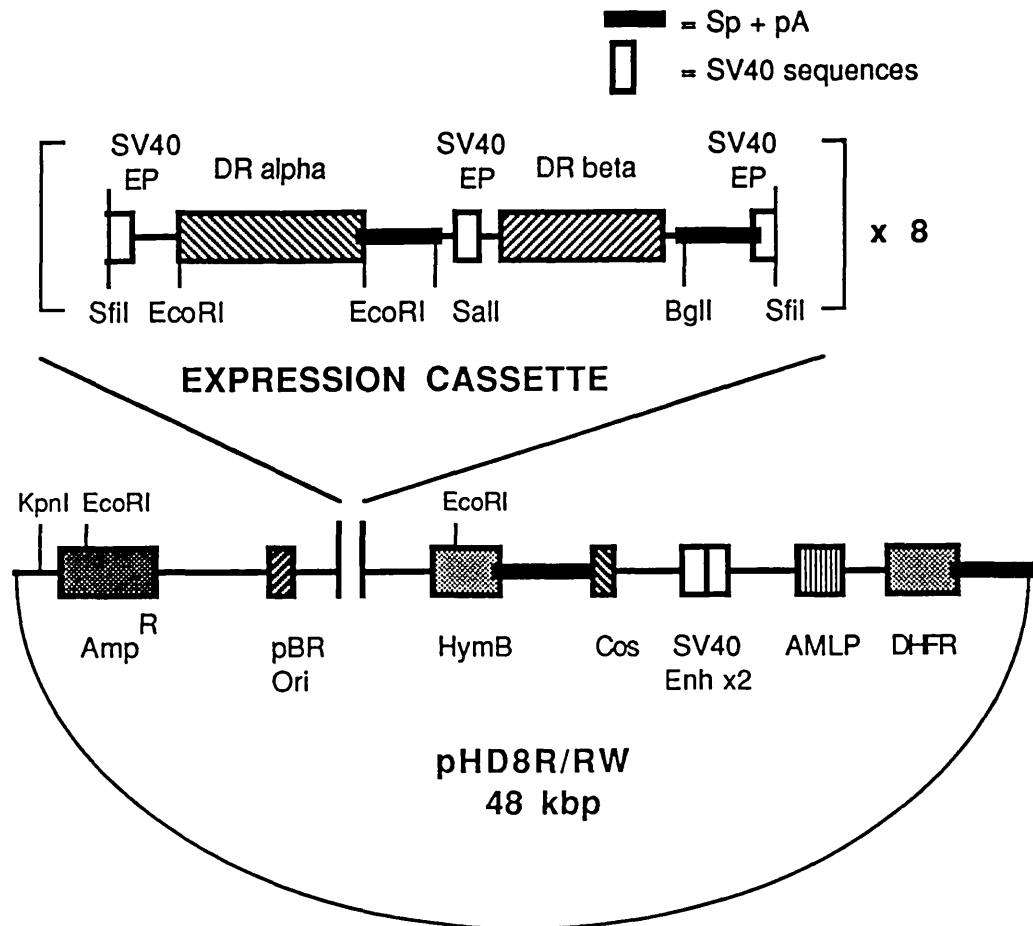


FIGURE 2.4

This construct was provided by Dr H. Ikeda (ICRF, London). Boxes indicate the major genes. Abbreviations used are : Sp+pA - SV40 splice site and poly-A signal; EP - early promoter; Amp^R - ampicillin resistance gene; Ori - pBR origin of replication; HymB - hygromycin B resistance gene; Cos - cos site; Enh - SV40 enhancer sequence (PvuII-BglI fragment); AMLp - adenovirus 2 major late promoter sequence and DHFR - dihydrofolate reductase gene.

TABLE 2.1

MONOCLONAL ANTIBODIES USED IN THESE STUDIES

Designation	Specificity	Ig Class	Reference
H100-5	K ^k , D ^k	IgG2a	Lemke et al., 1979
K9-18	K ^d , D ^s	"	"
T17.5	D ^{sb}	"	"
T19.191	D ^d , L ^d	"	"
17.227	I-A ^d , I-E ^d	"	"
13/4	I-E ^{dk}	"	"
K25-8.7	I-A ^{sq}	"	Koch et al., 1982c
HB75	D ^d	"	Ozato et al., 1980
HB3	I-A ^d	"	Kappler et al., 1981
TIB92	I-A ^k	"	Oi et al., 1978
TIB154	I-A ^{dk_{sqr}}	IgG3	Lemke et al., 1979
W6/32	pan-HLA A,B,C	IgG2a	Barnstable et al., 1978
L243	pan-HLA-DR	"	Lampson & Levy, 1980
HU.43	HLA DR 1,4,7,9	"	Bodmer et al., 1984
HU.48	HLA DR 4,7	"	"

TABLE 2.2BACKGROUND sIg STAINING OF A20 CELLS

CELLS STAINED WITH :	MEAN FLUORESCENCE INTENSITY ($\pm$ SD)
Unstained cells	14.0 $\pm$ 7.8
GAMIg*PE alone	60.5 $\pm$ 8.4
HB75 (anti-D ^d) plus GAMIg*PE	315.8 $\pm$ 9.7
HB3 (anti-I-A ^d) plus GAMIg*PE	615.9 $\pm$ 10.5

The Table shows the mean fluorescence intensities of staining of A20 cells with phycoerythrin (PE) conjugated GAMIg alone in comparison to staining with mAb against murine MHC antigens. Background sIg staining (with GAMIg*PE alone) is 1/5 to 1/10 that with the mAb.

TABLE 2.3

NUCLEIC ACID PROBES USED IN THESE STUDIES

Specificity	Designation	Type	Description
A α chain	pCDN17	cDNA	650/400 bp PstI fragments with complete mRNA sequence ¹
E α chain	pCA39	Genomic	3.4 kbp SalI fragment encompassing α 1, α 2 and TM/CY exons ²
E α chain	pSPEAC ₁	cDNA	1.2 kbp EcoRI fragment with complete mRNA sequence cloned into pSP65 ³
Ii chain	pIi-1	cDNA	920 bp PstI fragment containing part of mRNA sequence ⁴
???	7B6	cDNA	700 bp DraI/KpnI fragment hybridising to mRNA that does not vary during the cell cycle ⁵
DRO α chain	pDRH2	cDNA	700/600 bp PstI fragments with complete mRNA sequence ⁶

¹ Davis et al., 1984² McNicholas et al., 1982³ Mathis et al., 1983b⁴ Singer et al., 1984⁵ Kaczmarek et al., 1985⁶ Lee et al., 1982b

CHAPTER 3

ANALYSIS OF MHC CLASS II GENE REGULATION IN THE B LYMPHOCYTIC LINEAGE

3:1 MHC CLASS II GENE EXPRESSION IS CONTROLLED
AT THE LEVEL OF GENE TRANSCRIPTION IN SEVERAL
TUMOURS OF THE B LYMPHOCYTIC LINEAGE

3:1.1 Introduction

As discussed earlier, MHC Class II expression is related to the stage of differentiation in both the human and murine B lymphocytic lineages (section 1:3.1 and Figure 1.6). In this section I present the results of experiments which suggest that MHC Class II gene expression is controlled at the level of gene transcription in B lymphocytic tumour cell lines representing several stages of differentiation.

Tumour cell lines were studied because of the difficulty in isolating pure populations of subsets of B lymphocytes in sufficient numbers. This approach has been used successfully in studies of B lymphocyte differentiation (Warner et al., 1979; Morse, 1983). The underlying assumption is that each tumour cell line represents a homogenous, discrete subpopulation. The detailed analysis of surface antigen expression by a wide range of such tumours by several groups (Kim et al., 1979; Lanier et al., 1981; Davidson et al., 1984) has, in general, supported this assumption. Most pertinent to this discussion, these workers found that the cell type-specific expression of surface antigens in B cell tumours closely resembled that in their normal counterparts. In addition the tumours did not express genes encoding differentiation antigens corresponding to other (non-B cell) haemopoietic lineages. Although exceptions to these statements, both qualitative and quantitative, did occur (Warner et al., 1979; Lanier et al., 1981), these findings suggest that tumour cell lines are a valid model for the study of gene regulation during B lymphocyte differentiation.

3:1.2 Murine B lymphocytic tumour cell lines

Currently held concepts of the pathway of B cell differentiation recognize at least five stages (summarized in

Table 3:1.1) which are correlated primarily with Ig gene rearrangement and expression and with surface antigen phenotypes (Hammerling et al., 1976; Davidson et al., 1984). Tumours corresponding to several of these stages were chosen for analysis.

The tumours NFS 8.18 and NFS 1.3 were derived from the NFS/N strain of mice (Davidson et al., 1984). NFS 8.18 resembles an immature B lymphocyte in being surface Ig positive, but lacking complement receptor expression and Ig secretion. In contrast NFS 1.3 represents a B lymphoblast : it secretes Ig and is complement receptor positive.

A20 is a spontaneous B cell lymphoma derived from the BALB/c mouse strain (Kim et al., 1979; McKean et al., 1981). This cell line, and the similar M12 tumour (Kim et al., 1979; Hamano et al., 1982) represent mature, Ig secreting B lymphocytes.

P3X63Ag8 (X63) is an IgG1 secreting plasmacytoma that was derived from the mineral-oil induced MOPC-21 tumour of BALB/c mice (Kohler and Milstein, 1975).

All the above tumour cell lines have been demonstrated to be near-diploid, with a low frequency (< 10%) of aneuploidy.

3:1.3 MHC antigen expression at the cell surface

MHC antigen expression by these tumour cell lines was determined by indirect immunofluorescence and FACS analysis using the methods described earlier. The two NFS tumours are of the H-2^{s^q4} haplotype (Klein et al., 1983) in which the subloci K, I-A and I-E are H-2^s derived while the D sublocus is H-2^q derived. The E α gene in this haplotype is not expressed (Mathis et al., 1983a). The NFS tumours were therefore stained with the mAb K9-18 (anti-D^q) and K25-8.7 (anti-I-A^{s^q}). The remaining tumours are of the H-2^d haplotype, and were stained with the mAb HB75 (anti-D^d) and HB3 (anti-I-A^d). These mAb have been described in section 2:3.1.

The results obtained (in one of three similar experiments) are represented in Figure 3:1.1. All the tumours express MHC Class I antigens, albeit in varying amounts. The mean

fluorescence intensity of staining of the tumours with mAb to MHC Class I ranged from 297 to 487.8 in the case of K9-18, and 361.2 to 653.3 in the case of HB75. These ranges represent approximately 2-fold differences in fluorescence intensity.

Not all tumours were stained with mAb to MHC Class II antigens when compared to the negative control (NFS 8-18 and X63, Figure 3:1.1). In addition the mean fluorescence intensities of Class II expressing tumours (NFS 1-3, A20, M12) were approximately 10-fold higher than those of Class II negative cells stained with the same mAb (143 to 1379.4 in the case of K25-8.7, and 118.8 to 1480 in the case of HB3).

Bearing in mind the caveat that mean fluorescence intensities of staining with different mAb cannot be directly compared, it is nonetheless clear that there is little difference in MHC Class I expression between tumours representing various B lymphocyte differentiation stages. In contrast MHC Class II expression varied markedly. The tumours representing immature lymphocyte (NFS 8-18) and plasma cell (X63) stages did not express MHC Class II antigens, while mature B lymphocytic (A20, M12) and lymphoblastic (NFS1.3) tumours expressed at high levels. This data is in concordance with that of other workers (Hammerling et al., 1976; Kim et al., 1979; Davidson et al., 1984).

It has recently been shown (Erusalimsky et al., 1987) that MHC Class I antigen expression in human lymphoid cell lines is dependent on cell cycle stage; with G_1/S arrested cells displaying 2 to 3-fold higher levels of expression than cells in G_2/M . Other workers have observed that smaller (0.25-0.3-fold) increases in Class I expression occur in G_2 , as a result of increases in cell size (Harris and Sekaly, 1984; Muller et al., 1986). Such cell cycle related variations appear to be the most probable cause of the observed small differences in Class I expression between various tumour cell lines, as asynchronous cell cultures were used for the experiments reported here. It is also clear, however, that variations of this magnitude cannot account for the lack of MHC Class II expression in NFS 8-18 and X63.

3:1.4 Expression of mRNA for the MHC Class II genes

Fifteen µg of total cellular RNA prepared from cultures of the murine B lymphocytic tumour lines was denatured and size-separated on 1% agarose gels containing 16% (v/v) formaldehyde before Northern blotting as described in section 2:7.2. Northern blots were hybridized with ³²P-labelled nucleic acid probes for the murine MHC Class II gene A α and the Ii chain; and autoradiographed.

Figure 3:1.2 shows the results of these experiments. Both A α and Ii gene mRNAs were found in the Class II antigen expressing cell lines NFS 1.3, A20 and M12. No A α mRNA was detected in the cell lines (NFS 8.18 and X63) which did not express Class II antigens. While NFS 8.18 was also Ii chain mRNA negative, X63 expressed low levels of Ii chain mRNA. Similar results were obtained even when the autoradiographs were exposed for up to 14 days.

Thus the differences observed between cell types by serology are also found at the mRNA level. Similar results have been obtained in the case of A α and E α chain genes by other groups (Mathis et al., 1983b; Davis et al., 1984) who tested murine T cell lines, plasmacytomas and B cell lymphomas. The results presented here reinforce the conclusion that in several tumours of the murine B cell lineage as well as in certain other cell types, MHC Class II gene expression is regulated at the level of gene transcription.

It must be noted that Ii chain transcripts were found at low levels in the plasmacytoma X63, which does not express Class II antigens, although another Class II antigen negative cell line, NFS 8.18, did not express Ii chain mRNA. This accords with the results of Koch and Harris (1984) who detected very low levels of Ii chain polypeptide in plasmacytomas, but not other Class II antigen negative B cell tumours.

3:1.5 Human B lymphocytic tumours

Do these observations extend to human B lymphocytic tumours? Several human tumour cell lines were obtained in order to determine this.

RAJI and RAMOS are cell lines established from EBV positive and EBV negative Burkitt's lymphoma tumours respectively (Pulvertaft et al., 1964). The cells represent early, surface IgM positive lymphocytes.

RPMI 7666 is one of a large series of EBV positive lymphoblastic cell lines initiated from the blood of a normal human (Moore et al., 1967). These cells represent mature, Ig secreting lymphocytes.

HS-Sultan (Harris et al., 1974) is a plasmacytoma cell line established from the blood of a patient with IgG multiple myeloma. Cellular morphology is typical of a mature Ig secreting plasma cell.

3:1.6 MHC antigen expression at the cell surface

All tumour cell lines were stained with mAb recognizing non-polymorphic determinants that are locus rather than haplotype specific. The mAb W6/32 recognizes all human HLA A, B and C Class I haplotypes, while L243 recognizes Class II HLA-DR. These mAb have been described elsewhere (section 2:3.1). The results obtained in one of two similar experiments are discussed.

As in the case of the murine tumours, all the human tumours expressed MHC Class I antigens at varying levels (Figure 3:1.3). The range of mean fluorescence intensities was much greater (180.3 to 1020.4, 6-fold variation) than in the case of the murine tumours.

DR antigen expression paralleled that of murine I-A antigens (Figure 3:1.3). The three tumours representing immature and mature B lymphocyte stages (RAJI, RAMOS, RPMI 7666) expressed high levels of DR antigens (range of mean fluorescence intensities 303.4 to 493, 1.6-fold variation), while the

plasmacytoma (HS-Sultan) expressed 9- to 15-fold less DR (mean fluorescence intensity 33.5). The mean fluorescence intensity of staining of HS-Sultan with L243 (33.5) was only slightly higher than with GAMIg (20.7) suggesting that DR expression was very low.

3:1.7 Expression of DR α mRNA

Fifteen μ g of total cellular RNA prepared from each of the four human B cell tumours was analysed by Northern blotting. Figure 3:1.4 shows the results of hybridization with a ^{32} P labelled nucleic acid probe for the DR α gene. All of the tumours were found to express DR α gene transcripts, amounts of which roughly corresponded to the relative amount of DR expression at the cell surface.

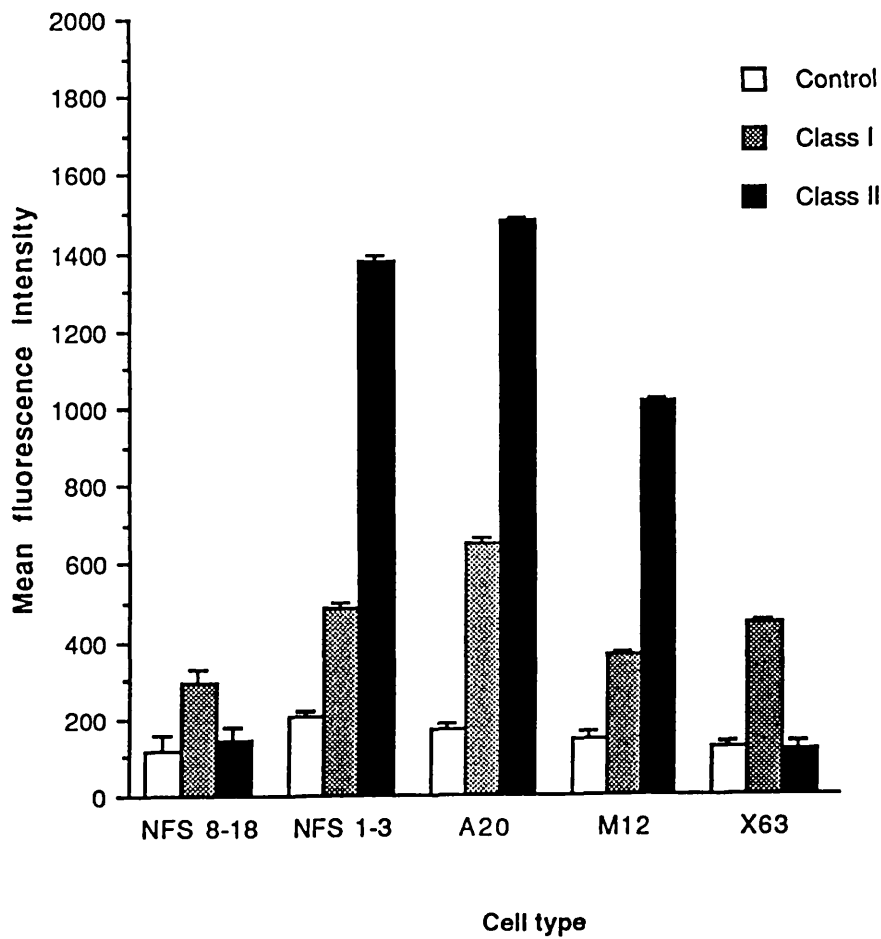
The human plasmacytoma HS-Sultan expressed small amounts of DR antigen and DR α mRNA, in contrast to the murine plasmacytoma X63. This observation may be the result of low levels of DR α gene transcription, which is consistent with the conclusion that Class II gene expression is controlled transcriptionally in the B lymphocyte lineage. However post-transcriptional regulatory mechanisms cannot be excluded, a point which is discussed in later sections.

LEGEND TO FIGURE 3:1.1

Single cell suspensions were stained with mAb to the MHC antigens by indirect immunofluorescence and analysed on the FACS as described in sections 2:3.2 and 2:3.3. Briefly, ten thousand cells were analysed per sample, and appropriate gates were set to exclude non-viable cells and cell debris. The mean fluorescence intensity ($\pm$ standard deviation) of staining with each mAb was calculated using the B/DIS Consort computer programme : these values are depicted. All results depicted in this Figure were obtained during a single FACS analysis with identical machine settings.

This figure shows the results obtained with one of three similar experiments. NFS 8-18 and NFS 1-3 were stained with the mAb K9-18 (anti-D^q, Class I) and K22-8.7 (anti-I-A^{S^q}, Class II). All other cells were stained with HB75 (anti-D^d, Class I) and HB3 (anti-I-A^d, Class II). Negative control staining was with fluorochrome conjugated GAMIg alone (Control).

MHC antigen expression by murine B lymphoid tumours



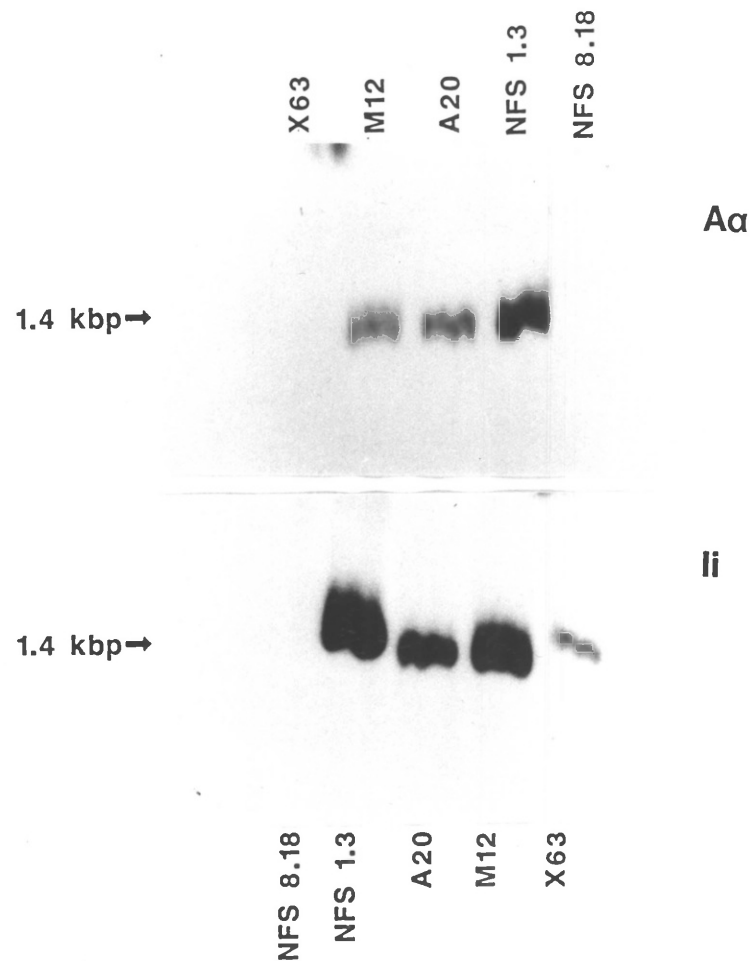
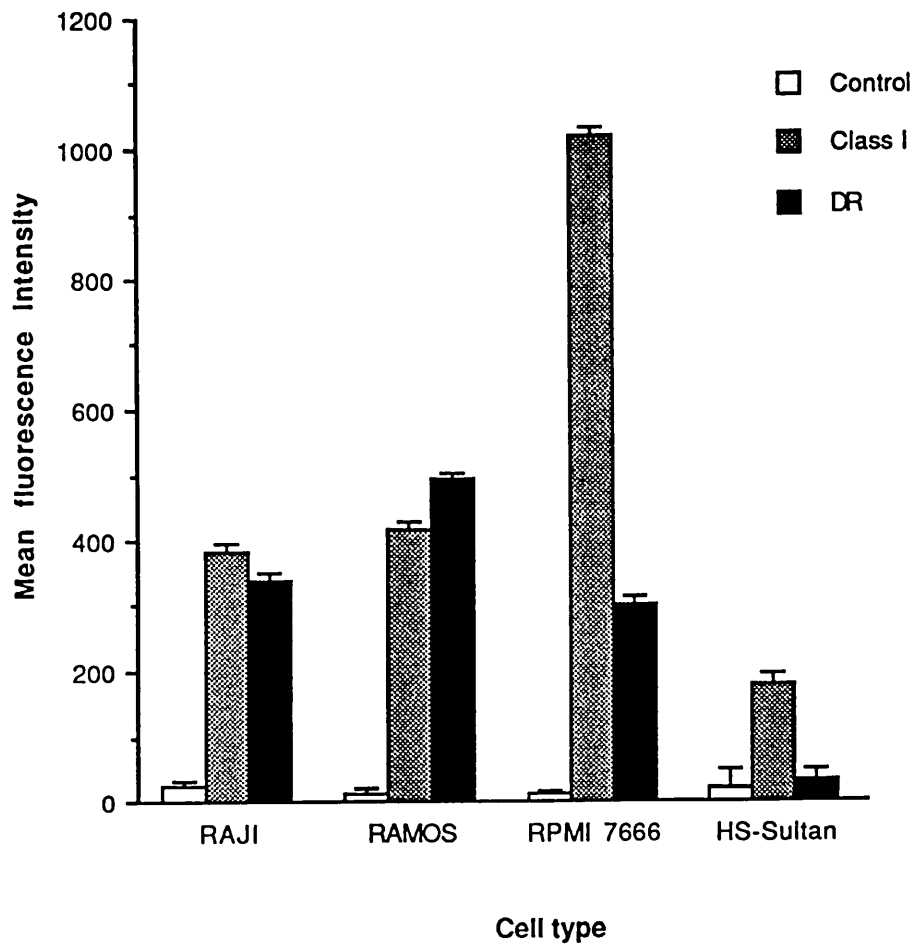


FIGURE 3:1.2

EXPRESSION OF A α AND Ii GENE TRANSCRIPTS BY MURINE TUMOURS

A Northern blot containing 15 μ g. of total cellular RNA in each track was hybridized with a ^{32} P-labelled probe for the A α gene and autoradiographed. The blot was stripped free of radioactivity before being re-probed with an Ii gene probe. The Ii autoradiograph was inadvertently photographed back-to-front, which is the reason for inversion of the track pattern.

MHC antigen expression by human B lymphoid tumours**FIGURE 3:1.3**

Human tumour cells were analysed as described in Figure 3:1.1. The cells were stained with the mAb W6/32 (anti-HLA A,B,C, Class I) and L243 (anti-HLA DR, Class II). Negative control staining was with fluorochrome conjugated GAMIg (Control).

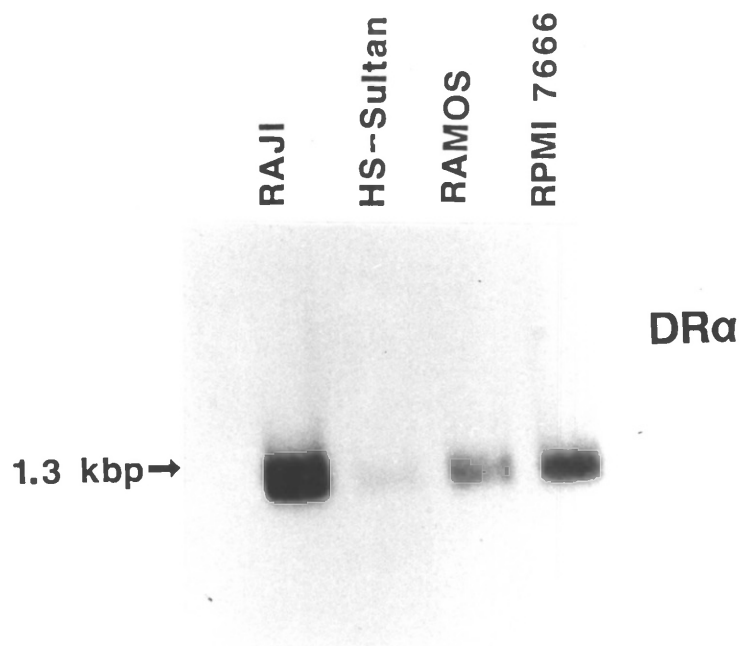


FIGURE 3:1.4

EXPRESSION OF DR α GENE TRANSCRIPTS BY HUMAN TUMOURS

Each track contains 15 μ g of total cellular RNA. Analysis was carried out as described in Figure 3:1.2.

Cell surface phenotypes of stages of B cell differentiation

Differentiation marker	Pre-B cell	Immature B lymphocyte	Mature B lymphocyte	Immuno-blast	Plasma cell
BP-220	+	+	+	+	+/-
slg	-	+	+	+/-	+/-
Ig secretion	-	-	-	+	+
C-receptor	-	-	+	+	+/-
PC-1	-	-	-	+/-	+

TABLE 3:1.1

(Adapted from the data of Kim et al., 1979; Lanier et al., 1981 and Davidson et al., 1984)

3:2 THE MURINE PLASMACYTOMA NS1 EXPRESSES MHC CLASS I BUT NOT CLASS II ANTIGENS

3:2.1 Introduction

The preceding section suggests that at least two plasmacytoma cell lines, murine and human, suppress MHC Class II gene expression, but intriguingly leaves the mechanism of suppression open to question. In order to develop an experimental system in which to investigate this mechanism, MHC gene product expression in the murine plasmacytoma cell line NS1 was studied in detail. The results obtained in this approach are the focus of the remainder of the thesis.

P3-NS1/Ag4.1 (NS1) is a nonsecreting murine plasmacytoma cell line, which like X63, is derived from the MOPC-21 tumour of BALB/c mice (Kohler et al., 1976). I analysed these cells by indirect immunofluorescence and immunoprecipitation to determine if MHC antigens are expressed at the cell surface and/or intracellularly. Except where otherwise specified, the immunofluorescence assays discussed are the results of one of at least three similar experiments.

3:2.2 MHC antigen expression at the cell surface

A panel of mAb, all of the same IgG2a class, were used in indirect immunofluorescence assays. Figure 3:2.1 shows the mean fluorescence intensity of staining with mAb against the H-2^d subloci - T19.191 (anti-D^d, L^d), HB75 (anti-D^d), HB3 (anti-I-A^d) and 13/4 (anti-I-E^d). In comparison, mAb against H-2^k subloci - H100-5 (anti-K^k) and TIB92 (anti-I-A^k) - were used as negative controls. The results clearly show that NS1 cells express MHC Class I antigens at the cell surface.

What of Class II ? The mean fluorescence intensity of staining with mAb against I-A and I-E (10.89 and 11.53, mAb 13/4 and HB3 respectively) was slightly greater than with a negative control mAb (8.83, mAb TIB92) or GAMIg alone (8.39). These

differences were not found to be statistically significant by the Student's T-test, as the standard deviations were between 10 and 15% of the mean. The differences were, however, consistent and reproducible.

These results (which are similar to those obtained with the human plasmacytoma HS-Sultan) might indicate that NS1 cells express low levels of MHC Class II antigens. Three analyses were performed to test this further.

First, comparison to normal BALB/c strain splenic cells (Figures 3:2.1, 3:2.2) showed that NS1 cells stained about 60% as brightly with anti-Class I (mean fluorescence intensity 61.5 versus 106.1 with mAb HB75) but less than 6% as brightly with anti-Class II (11.53 versus 184.3 with mAb HB3). In all the indirect immunofluorescence experiments performed, saturating concentrations of both first-layer mAb and second-layer fluorochrome conjugates were used. Under these conditions the mean fluorescence intensity of stained cells is directly proportional to the amount of mAb bound, which is in turn proportional to the number of antigen molecules expressed at the cell surface. Hence differences in mean fluorescence intensity must represent differences in the number of antigen molecules expressed. Comparison with splenic cells therefore indicates that MHC Class II expression, if any, by NS1 must be extremely low. NS1 cells expressed only 6% of the average number of MHC Class II molecules found in an unfractionated splenic cell population that includes a substantial proportion (30 to 50%) of T lymphocytes that do not express Class II. In contrast, NS1 expressed 60% of the number of Class I molecules, although these are found on all splenic cells.

Second, the fluorescence histograms obtained by staining NS1 cells with anti-I-A^d and (as negative control) anti-I-A^k (Figure 3:2.3) were compared using the Kolmogorov-Smirnov statistical test, which determines the probability that two histograms are significantly different (Young, 1977). No significant difference was found with 99% confidence limits.

Third, two other mAb which react with I-A and I-E antigens of several mouse haplotypes (17.227, TIB154) were used to stain

NS1 cells. They failed to show the very slight positive staining observed with HB3 (Figure 3:2.4).

A single cell sorting experiment was performed to exclude the possibility that a very small proportion of NS1 cells did express MHC Class II antigens. Five million cells stained with HB3 were enriched for the most intensely fluorescent 10% using the FACS. These "bright" cells were then re-cultured for 4 days, and re-analysed for MHC antigen expression with unsorted NS1 cells for comparison. There was no difference in the staining pattern (data not shown). This makes it very unlikely that a subpopulation of NS1 cells expressing MHC Class II antigens exists. This is further supported by the results of the Kolmogorov-Smirnov statistical test (Figure 3:2.3) : no statistical evidence was found for the presence of such a subpopulation.

Taken together these results indicate that NS1 cells do not express MHC Class II antigens in detectable amounts at the cell surface. Work on inbred mouse strains that lack I-E antigen expression demonstrates that E α or E β polypeptides may be present intracellularly despite the lack of cell surface expression. Jones and co-workers (1978b) have shown that mice of the H-2^b and H-2^s haplotypes synthesize E β but not E α polypeptide. The E β polypeptide is found in the cytoplasm but is not inserted into the cell membrane in the absence of E α . In contrast in the H-2^{ap5} haplotype E α polypeptide is found in the cytoplasm and inserted into the membrane, despite the absence of E β synthesis.

It is conceivable that the lack of cell surface antigen expression in NS1 is due to defects in the expression of one or the other constituent polypeptide chains. This possibility was tested by biosynthetic labelling and immunoprecipitation of NS1 cell extracts.

3:2.3 Expression of MHC antigens intracellularly

NS1 and A20 cells were biosynthetically labelled with ³H-leucine, ³H-lysine and ³H-serine, and cell extracts

immunoprecipitated with a polyclonal rabbit antiserum against murine I-A^d/I-E^d antigens as described elsewhere. Figure 3:2.5 shows that the antiserum precipitates radio-labelled proteins with molecular weights of approximately 34, 30 and 28 kD from extracts of A20 but not NS1 cells. These correspond respectively to the α , Ii and β chains of the murine MHC Class II antigens.

Ii chain was not precipitated from NS1 extracts. It is possible that this protein is not synthesized. However, the polyclonal rabbit antiserum was raised by immunisation with MHC Class II antigens purified by affinity chromatography using a mAb that recognizes α and β chains (TIB120, Bhattacharya et al., 1981). The antiserum might not therefore precipitate Ii chain in the absence of α and β chains.

These results show that none of the MHC Class II polypeptide chains are present intracellularly in NS1 cells, implying that selective defects in their expression are not the cause of the failure to express MHC Class II.

MHC antigen expression by NS1

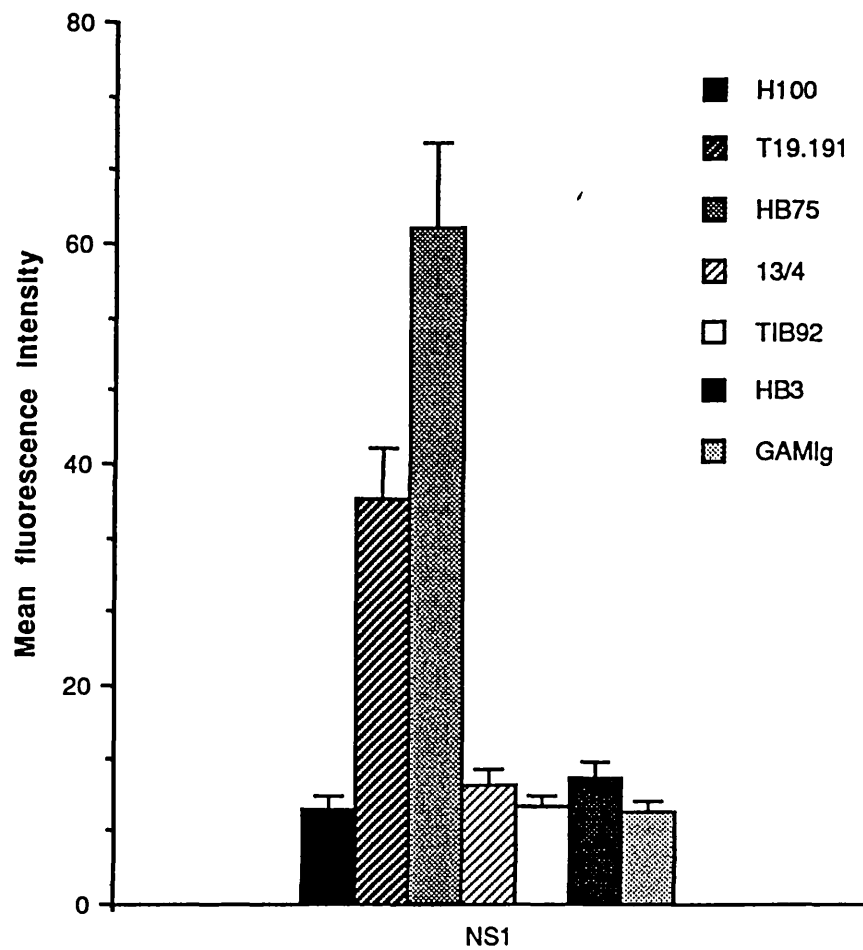


FIGURE 3:2.1

NS1 cells were analysed as described in Figure 3:1.1.

The mAb used were : H100 (anti- K^k), T19.191 (anti- D^d , L^d), HB75 (anti- D^d), 13/4 (anti- $I-E^dk$), TIB92 (anti- $I-A^k$) and HB3 (anti- $I-A^d$). All mAb are of the IgG2a class. Negative control staining is with fluorochrome conjugated GAMIg alone.

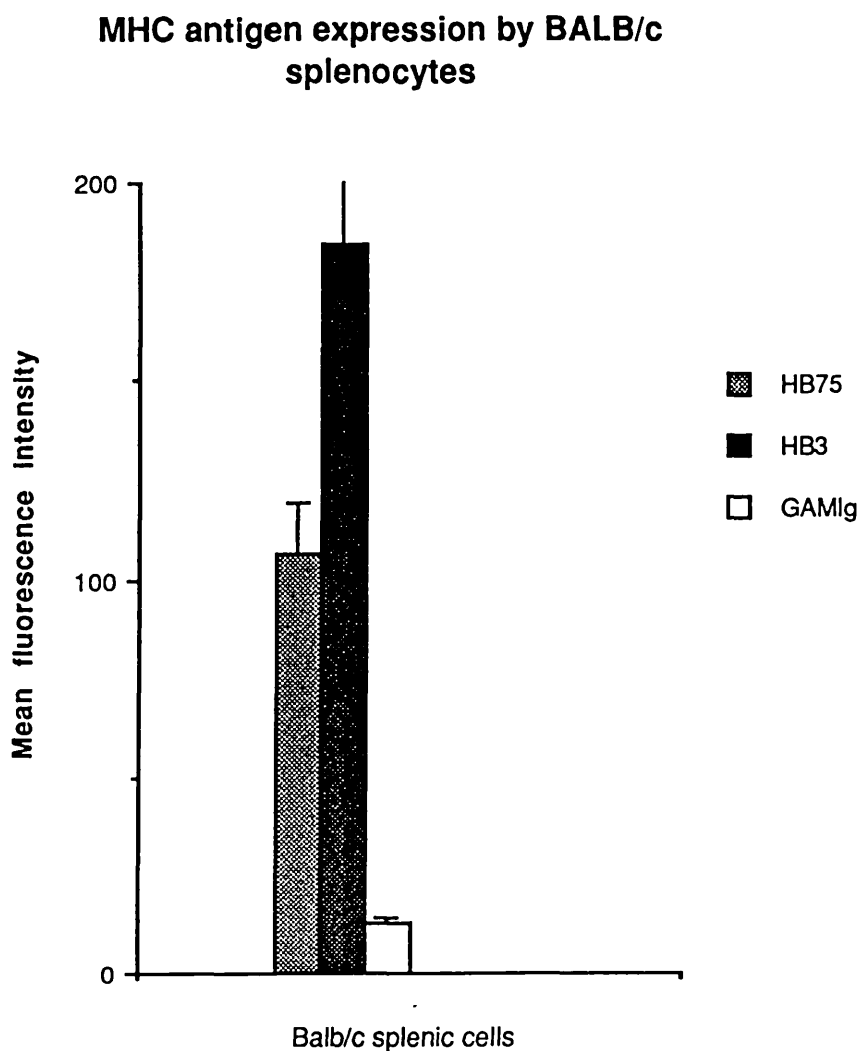


FIGURE 3:2.2

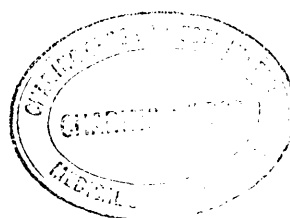
Analyses were performed on freshly isolated, unfractionated splenic cell suspensions as described in Figure 3:1.1, except that no gates were set so that all splenic subpopulations were included. Mab used were HB75 (anti-D^d) and HB3 (anti-I-A^d). Negative control staining was with fluorochrome conjugated GAMIg alone. This figure shows the results of one of four experiments performed at the same time as the one shown in Figure 3:2.1.

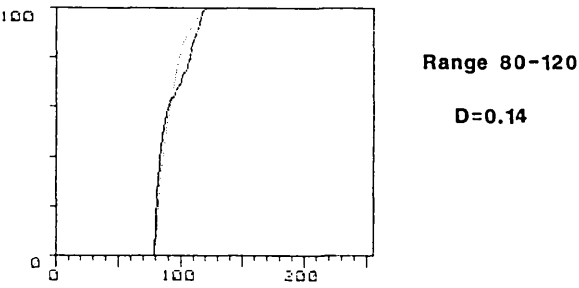
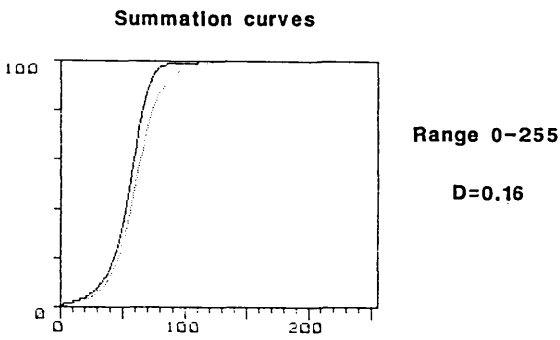
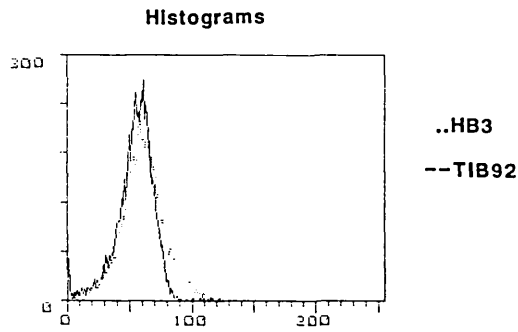
FIGURE 3:2.3ANALYSIS OF FLUORESCENCE HISTOGRAMS BY THE KOLMOGOROV-SMIRNOV
TEST

Fluorescence histograms obtained by staining single cell suspensions of NS1 with the mAb HB3 (anti-I-A^d) and the negative control mAb TIB92 (anti-I-A^k) were calculated using the B/DIS Consort computer programme : they are shown at the top of the figure. The samples were not gated, so that all 10 000 cells were included in the analyses. The Kolmogorov-Smirnov (K-S) test was performed to compare these histograms using the same computer programme. Horizontal axis = fluorescence intensity (channel numbers). Vertical axis = relative cell number.

The lower half of the figure shows the results of the K-S test. The graphs shown are cumulative frequency distributions (summation curves) for fluorescence intensity. The first test was performed using the entire fluorescence distribution (from 0 to 255 channels). The value computed for the K-S statistic, D, was 0.16. This value was compared to the standard probability distribution for the K-S statistic given in mathematical tables (Sokal and Rohlf, 1981b), adjusted for 10 000 events. A value of 0.20 or greater indicates that there is greater than 99% probability that the two histograms are significantly different. In this case, they are not.

Any divergence between the summation curves suggests the presence of a subpopulation of cells with different (ie. greater or smaller) fluorescence intensities. A small divergence between channel numbers 80 and 120 was observed. A second K-S test was therefore performed using the fluorescence distribution between these channels (80 and 120) as a more stringent analysis for the presence of a positively stained subpopulation of NS1 cells. Again the computed K-S statistic, D (=0.14), was less than the critical value (0.20); indicating that there was no significant difference between the histograms.





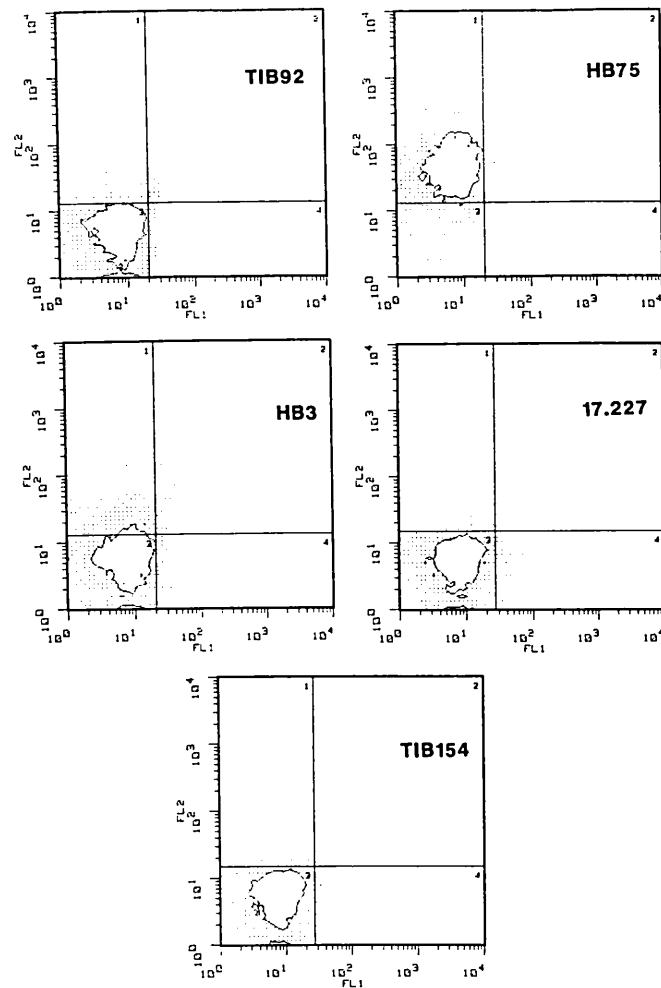


FIGURE 3:2.4

ANTI-CLASS II mAb DO NOT STAIN NS1 CELLS

Immunofluorescence staining and FACS analysis were carried out as described in Figure 3:1.1, except that the results are shown as fluorescence contour graphs. The quadrant markers indicate control negative staining with GAMIg alone. HB75 (anti-D^d) brightly stains all NS1 cells. In contrast, HB3 (anti-I-A^d), 17.227 (anti-I-A^d, I-E^d) and TIB154 (pan-anti-I-A) do not. The slight positivity found with HB3 (see text) is not seen with the other anti-Class II mAb. The results shown were obtained in one of three similar experiments.

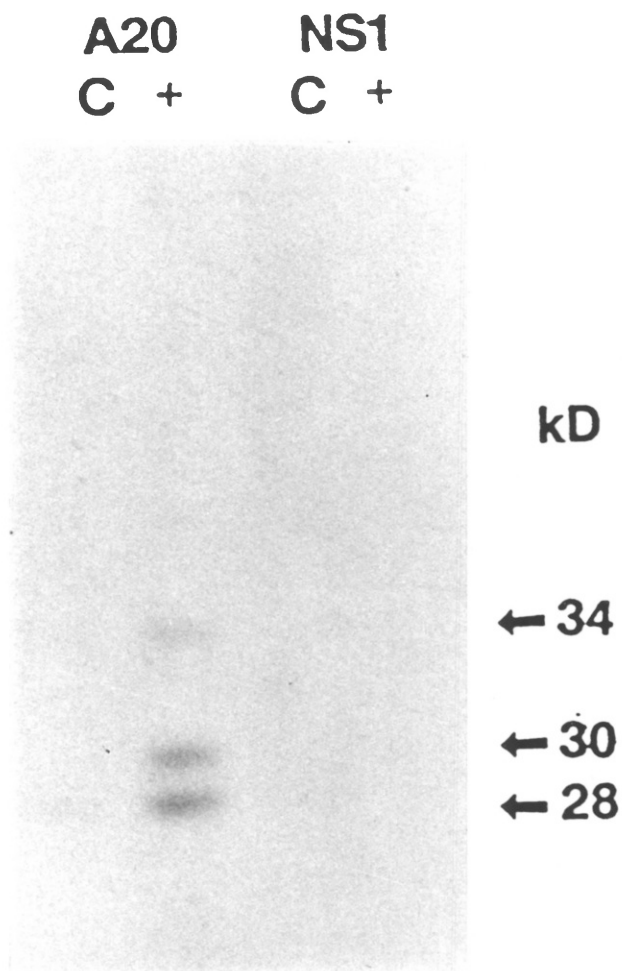


FIGURE 3:2.5

IMMUNOPRECIPITATION OF LABELLED CELL EXTRACTS

Cell extracts from 5×10^6 A20 and NS1 cells biosynthetically labelled with ^3H amino acids (leucine, lysine, serine) were immunoprecipitated with a polyclonal rabbit antiserum against murine Class II antigens. Immunoprecipitated proteins were denatured by boiling in the presence of dithiothreitol/SDS and resolved by SDS-PAGE using the Laemmli system (10% gel). The dried gel was autoradiographed with fluorographic enhancement.

The rabbit anti-Class II serum (+) precipitates 34, 30 and 28 kD bands from A20 but not NS1 extracts. Negative control immunoprecipitations (C) are with normal rabbit serum.

3:3 NS1 ACTIVELY SUPPRESSES MHC CLASS II EXPRESSION BY A SPECIFIC MECHANISM

3:3.1 Introduction

NS1 cells do not express MHC Class II antigens in detectable amounts either intracellularly or at the cell surface, although the closely related MHC Class I antigens are expressed. What is the explanation for this observation ?

The most interesting explanation is that a specific regulatory mechanism suppresses MHC Class II gene expression. Current knowledge of the regulation of these genes has been summarized earlier (section 1:3). It is apparent that certain sequence-specific DNA-binding regulatory proteins are necessary for the initiation of MHC Class II gene transcription; and that quantitative and qualitative alterations in these proteins cause the non-expressing phenotype of the B lymphocyte mutants developed by several groups (Accolla et al., 1985; Salter et al., 1985; Peterlin et al., 1987). Mechanisms that actively suppress Class II gene expression have not been identified in these mutants. However the existence of such mechanisms is suggested by the fact that at least certain DNA sequence elements of the Class II gene promoters have a "negative" effect : deleting them causes an enhancement of transcription (Boss and Strominger, 1986; Thanos et al., 1988). These sequences might serve as targets for the binding of "negative" regulatory proteins.

Alternative, trivial explanations are also possible. NS1 is a tumour cell line that has been maintained for many generations in vitro. Chromosomal changes such as mutations, rearrangements or deletions of the MHC Class II structural genes or their 5' regulatory sequences might also account for the specific defect in Class II expression. Such a defect (a 650 bp deletion in the E α promoter region) causes the lack of E α gene expression in the I-E deficient H-2^b mouse haplotype (Mathis et al., 1983a).

I used somatic cell hybridization experiments to distinguish between these possibilities. This approach was simplified by the fact that NS1 lacks the enzyme hypoxanthine guanosine phosphoribosyl transferase (HGPRT) (Kohler et al., 1976). This enzyme is encoded by an X-linked and therefore hemizygous gene (Shows and Brown, 1975), which makes recessive HGPRT- mutants extremely stable. HGPRT activity is essential in the salvage pathway for purine and pyrimidine synthesis, and hence HGPRT- NS1 cells are eliminated by culture in hypoxanthine, aminopterin, thymidine (HAT) medium. Somatic cell hybridization with HGPRT+ (ie., normal) cells rescues NS1 hybrids from HAT medium sensitivity.

Figure 3:3.1 shows the strategy adopted for somatic cell hybridization experiments. NS1 cells were fused with a murine splenocyte population, which includes a large number of MHC Class II expressing cells (see Figure 3:2.2), prepared from mice of three different H-2 haplotypes. Hybrid cell lines were selected in HAT medium, and analysed for MHC antigen expression 16-25 days after fusion.

3:3.2 Hybrid cell lines are polyploid in comparison to parent cells

As an additional measure to ensure that the HAT-resistant cell lines isolated from hybridization experiments were in fact hybrids, their nuclear DNA content was determined by propidium iodide (PI) staining and FACS analysis. The intensity of fluorescence after PI staining is directly proportional to nuclear ploidy (Taylor, 1980). Figure 3:3.2 shows the results with one representative hybrid : the fluorescence peak corresponding to DNA content in the G_0/G_1 phase of the cell cycle is shifted to the right in comparison to the parent cells. The hybrid is indeed polyploid.

3:3.3 NS1 x splenic cell hybrids express MHC Class I but not Class II antigens

Sixteen uncloned hybrid cell lines obtained 16-25 days after NS1 x splenic B cell fusions were analysed for MHC antigen expression by indirect immunofluorescence. Uncloned cell lines were used in order to ensure that results were typical of the products of somatic cell hybridization.

The results obtained are presented in Figures 3:3.3, 3:3.4 and 3:3.5. Each figure shows the mean fluorescence intensities of staining with mAb in a single experiment. Six hybrid cell lines (two from each of the three fusions) were re-analysed 30-50 and 60-90 days after fusion; with similar results. Standard deviations were 10 to 15 % of the mean fluorescence intensities. Average 12.5% error bars are shown rather than individual standard deviations.

A number of mAb were used in these experiments. CBA (H-2^k) x NS1 (H-2^d) hybrids were stained with the mAb T19.191 (anti-D/L^d), H100-5 (anti-K/D^k), HB3 (anti-I-A^d), TIB92 (anti-I-A^k) and 13/4 (anti-I-E^{d,k}). SJL (H-2^s) hybrids were stained with HB75 (anti-D^d), T17.5 (anti-D^{s,b}), HB3 (anti-I-A^d) and K25-8.7 (anti-I-A^{s,q}). BALB/c hybrids, where both parents were of the same (H-2^d) haplotype, were stained with HB75 (anti-D^d), HB3 (anti-I-A^d) and 13/4 (anti-I-E^{d,k}).

Of the seven CBA hybrids (Figure 3:3.3), one (H7) did not bind mAb against Class I or Class II antigens. The remaining 6 were Class I positive (four for the d haplotype, one for the k haplotype and one (H1) for both parental haplotypes), but Class II negative. H6 did bind anti-K/D^k slightly. However it was termed positive for Class I^d alone as there was no significant difference between anti-K/D^k binding (mean fluorescence intensity 24.9) and negative control GAMIg binding (18.13).

Four additional CBA x NS1 hybrids were analysed by Dr E.J. Culbert (ICI Plc., Alderley Park, Cheshire) in a parallel experiment using the same mAb. They were all found to be positive for Class I of a single parental haplotype, but not for Class II (data not shown).

The five SJL hybrids (Figure 3:3.4) were similar, although more (2/5) were positive for Class I of both parental haplotypes than in the case of the CBA hybrids (1/10). No hybrid was

positive for Class I^s alone - the three hybrids which expressed a single parental Class I haplotype expressed Class I^d. Since the H-2^s haplotype is I-E deficient, the hybrids were not stained with anti-I-E mAb.

The four BALB/c hybrids (Figure 3:3.5) were of the same Class I positive, Class II negative phenotype.

A summary of the results obtained in these experiments is presented in Table 3:3.1. This includes the four CBA hybrids analysed by Dr Culbert.

3:3.5 NS1 actively suppresses MHC Class II gene expression by an active, specific mechanism

Three facts indicate that the cell lines isolated from NS1 x splenic lymphoid cell hybridizations are true hybrids. The cell lines are HAT-resistant, they are polyploid with respect to the parent cells and many of them express MHC Class I antigens of both parental haplotypes.

The murine splenic lymphoid population contains a large number of B lymphocytes that express MHC Class II antigens at high levels (see Figure 3:2.1). Polyethylene glycol-mediated somatic cell hybridizations between NS1 and splenic cells from immunised mice have been performed extensively for the production of mAb secreting hybridomas (reviewed by Yelton et al., 1980) and this experience suggests that a large number of NS1 x B lymphocyte hybrids are produced. Although between 30 and 50% of the splenic cell population are T lymphocytes (Hudson and Hay, 1980) which do not express Class II, NS1 has not been successfully used for the production of stable T lymphocyte hybrids; which appear to be formed only when T lymphoid fusion partners such as EL4 are used (Taniguchi and Miller, 1978). It is therefore highly probable that the uncloned hybrid cell lines obtained 16-25 days after NS1 x splenic cell hybridizations consist mostly, if not completely, of B lymphocyte hybrids.

If alterations of the MHC Class II structural genes or their regulatory sequences were responsible for the non-expressing phenotype of NS1, it would be expected that the hybrid cells

obtained would express the Class II genes of the normal parental B cell haplotype. Hybridization would also be expected to correct any deficiency of "permissive" regulatory factors required for Class II gene expression by NS1, and result in the expression of MHC Class II antigens of both parental haplotypes. However the hybridization experiments consistently yielded hybrids that did not express either parental Class II, although MHC Class I derived from both parental haplotypes was expressed.

These results are clearly incompatible with both the above hypotheses, and must imply that NS1 cells suppress MHC Class II gene expression by a specific mechanism.

These results further support the conclusion that selective defects in the expression of one or other constituent chains of MHC Class II antigens are not responsible for the lack of Class II expression (see Section 3:2.3). If this were true, such a defect would be resolved by fusion with Class II expressing splenic cells.

3:3.6 K3 x A20 hybrids express MHC Class I but not Class II antigens.

To further validate these results K3, a cloned NS1 x B cell hybridoma, was fused to the A20 lymphoma and the resulting hybrids were analysed for MHC expression. Figure 3:3.6 shows that K3 had the typical MHC Class I positive, Class II negative hybrid phenotype. A20, in contrast, expressed MHC Class II at high levels (Figure 3:1.1).

The hybrids were stained with mAb against the Class I and Class II loci of the H-2^d haplotype (HB75, HB3). Figure 3:3.6 shows that all of the hybrids express MHC Class I antigens, while none express Class II.

These results further strengthen the conclusions reached in the previous section, and argue that a phenotypically dominant, active, specific mechanism is responsible for the suppression of MHC Class II gene expression in NS1.

3:3.7 Hybrids of X63 express MHC Class I but not Class II antigens

Does the murine plasmacytoma X63, which like NS1 does not express MHC Class II (Figure 3:1.1), suppress Class II expression in a similar manner ?

An X63 x splenic B cell hybrid, Sp2/0, and two Sp2/0 hybrids (HB124 and HB125) were studied by radioimmunoassay to partially answer this question. Sp2/0, a nonsecreting murine myeloma cell line, is a cloned hybrid derived from the fusion of X63 with BALB/c strain splenic cells (Schulman et al., 1978). HB124 and HB125 are cloned hybridoma cell lines produced by fusing Sp2/0 with BALB/c strain splenic cells (Schroer et al., 1983).

All these cell lines are of the H-2^d haplotype. Hence the mAb HB75 (anti-D^d) and HB3 (anti-I-A^d) were used, with a murine mAb to ricin β chain as negative control (provided by Dr E.J. Culbert). Figure 3:3.7 shows the results obtained as means of duplicate experiments. It is apparent that, like NS1 hybrids, X63 hybrids also fail to express MHC Class II antigens. Class I expression seems normal.

These experiments are not, of course, definitive proof that the MHC Class II negative phenotype of X63 is phenotypically dominant. Cloned hybrid cells were used, and they are not necessarily representative of the other products of fusion. Nevertheless the similar results obtained with NS1 fusions provide compelling circumstantial evidence that X63 also suppresses MHC Class II by an active, specific mechanism.

STRATEGY FOR SOMATIC CELL HYBRIDIZATION EXPERIMENTS

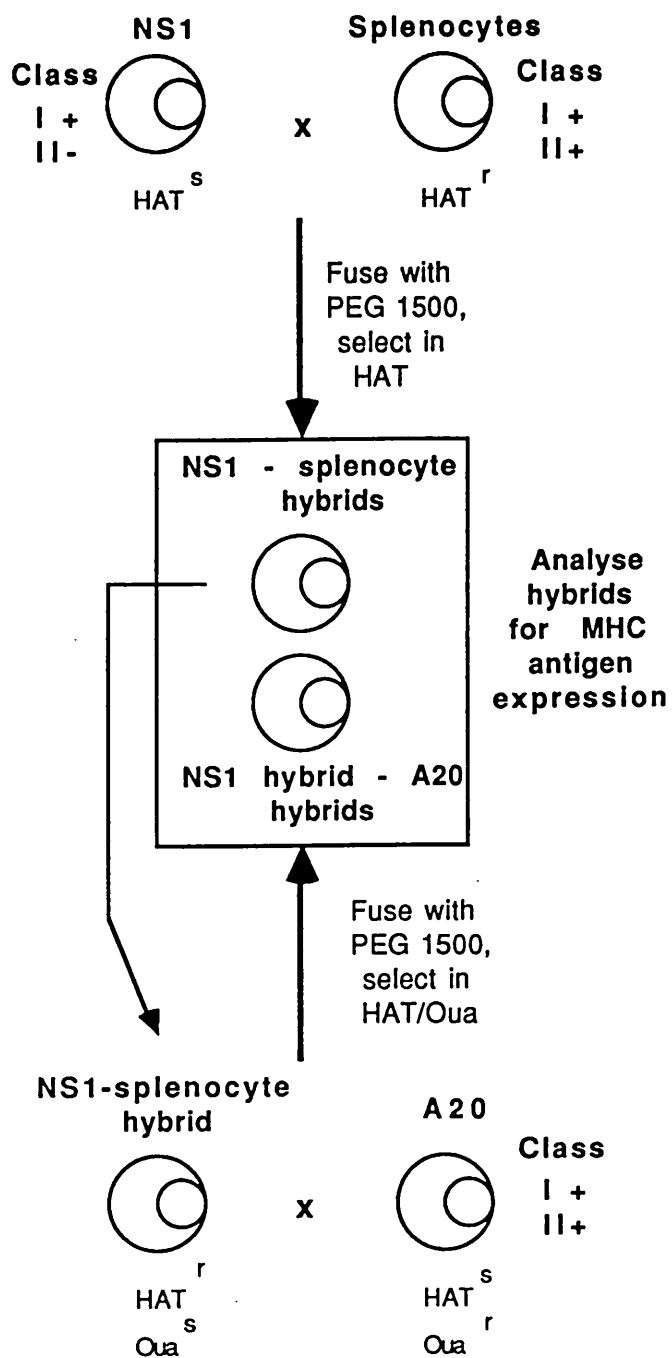


FIGURE 3.3.1

STRATEGY FOR SOMATIC CELL FUSION EXPERIMENTS

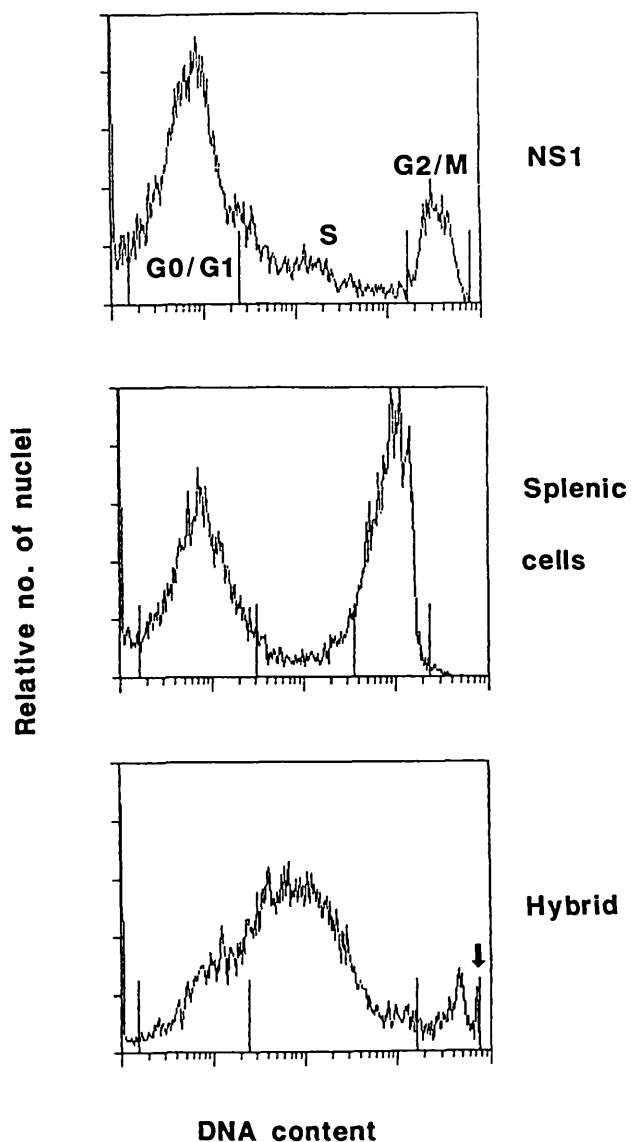


FIGURE 3:3.2

HYBRIDS ARE POLYPLOID WITH RESPECT TO PARENT CELLS

Cell nuclei from asynchronous cell cultures were stained with propidium iodide, and analysed on the FACS as described in section 2:2.4. The G_0/G_1 peak marked for NS1 and splenic parental cell types represents a near-diploid number of nuclei. This peak is clearly shifted to the right in the case of a representative hybrid. More than one G_0/G_1 and G_2/M peak (arrow) are present, indicating that more than one hybrid cell population exists.

NS1 x CBA hybrids express Class I but not Class II

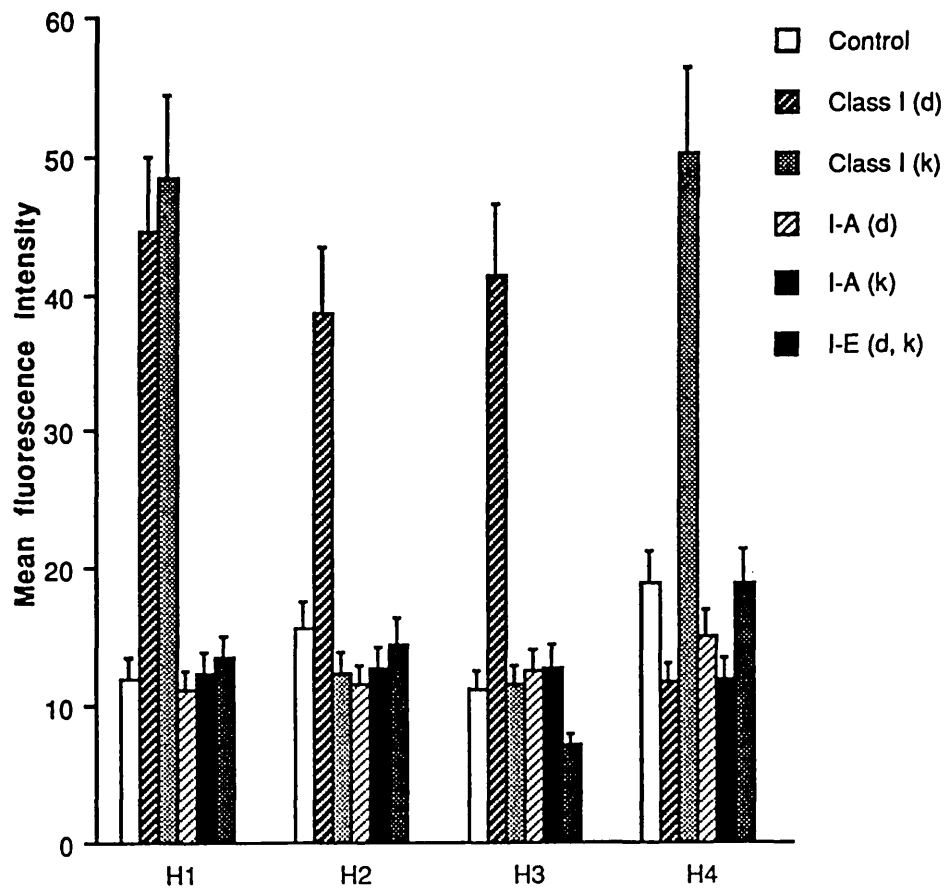
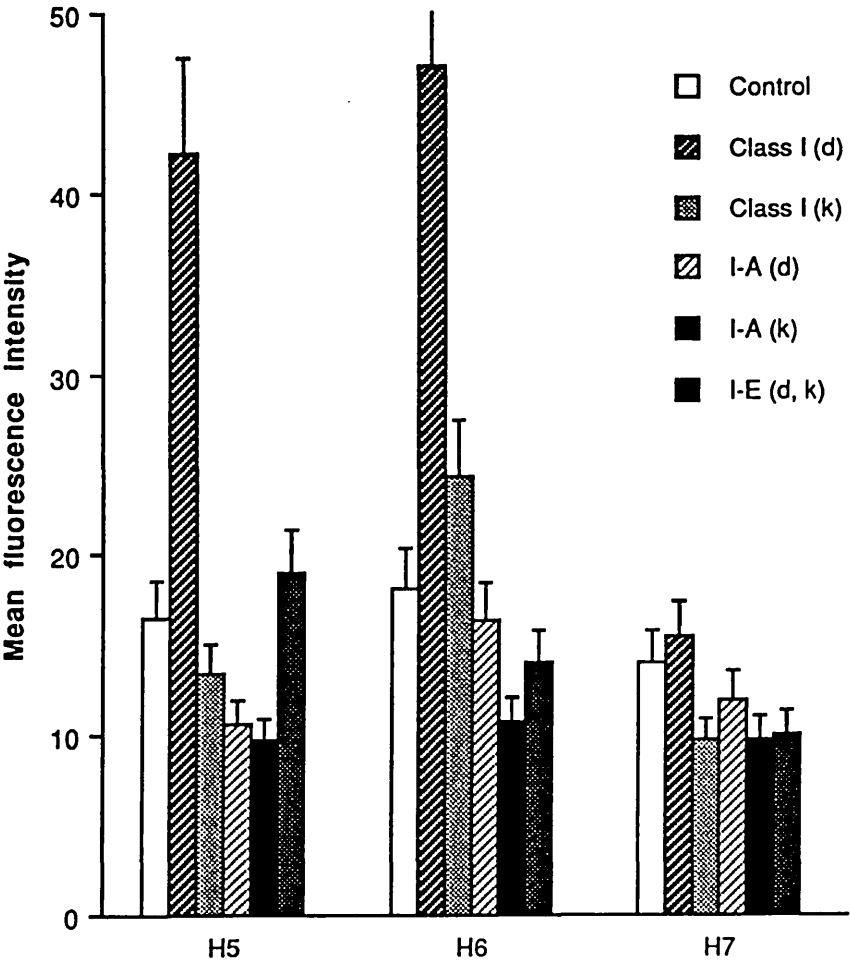


FIGURE 3:3.3

Single cell suspensions were stained with mAb to the MHC antigens by indirect immunofluorescence and analysed on the FACS as described in Figure 3:1.1.

Seven CBA splenic cell x NS1 hybrids (H1-H7, on two pages) were stained with the mAb HB75 (anti- D^d , Class I (d)), H100-5 (anti- K/D^k , Class I (k)), HB3 (anti- $I-A^d$, I-A (d)), TIB92 (anti- $I-A^k$, I-A (k)) and 13/4 (anti- $I-E^{dk}$, I-E (d,k)). Negative control staining was with fluorochrome conjugated GAMIg alone (Control).



NS1 x SJL hybrids express Class I but not Class II

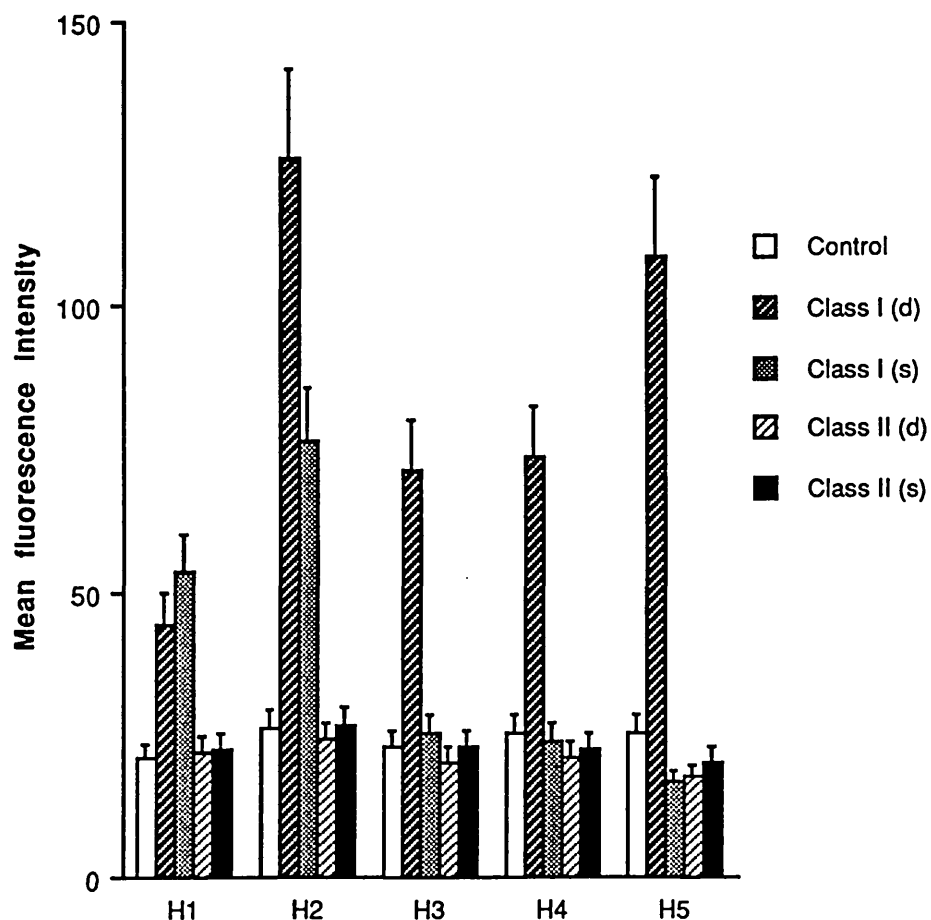
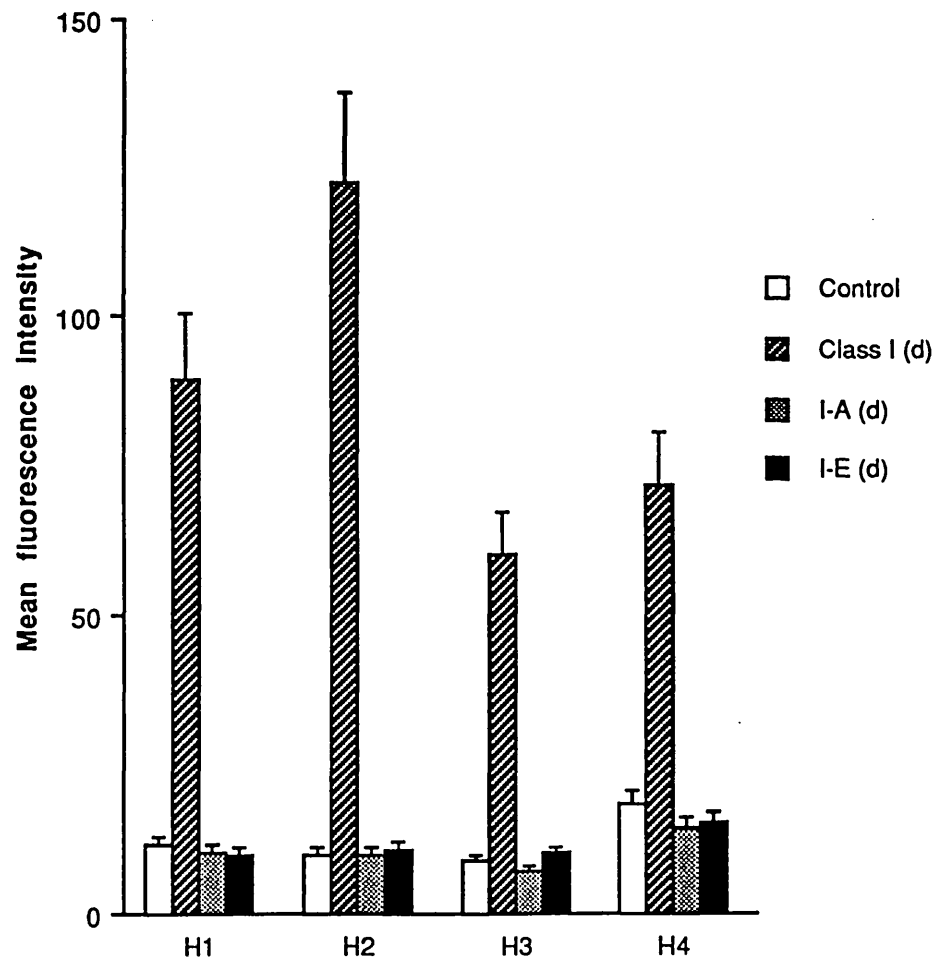


FIGURE 3:3.4

Five SJL splenic B cell x NS1 hybrids were stained with the mAb HB75 (Class I (d)), T17.5 (anti-D^S, Class I (s)), HB3 (Class II (d)) and K25-8.7 (anti-I-A^{Sq}, Class II (s)) as described in Figure 3:1.1.

NS1 x BALB/c hybrids express Class I but not Class II**FIGURE 3:3.5**

Four BALB/c splenic B cell x NS1 hybrids were stained with the mAb HB75 (Class I (d)), HB3 (I-A (d)) and 13/4 (I-E (d)) as described in Figure 3:1.1.

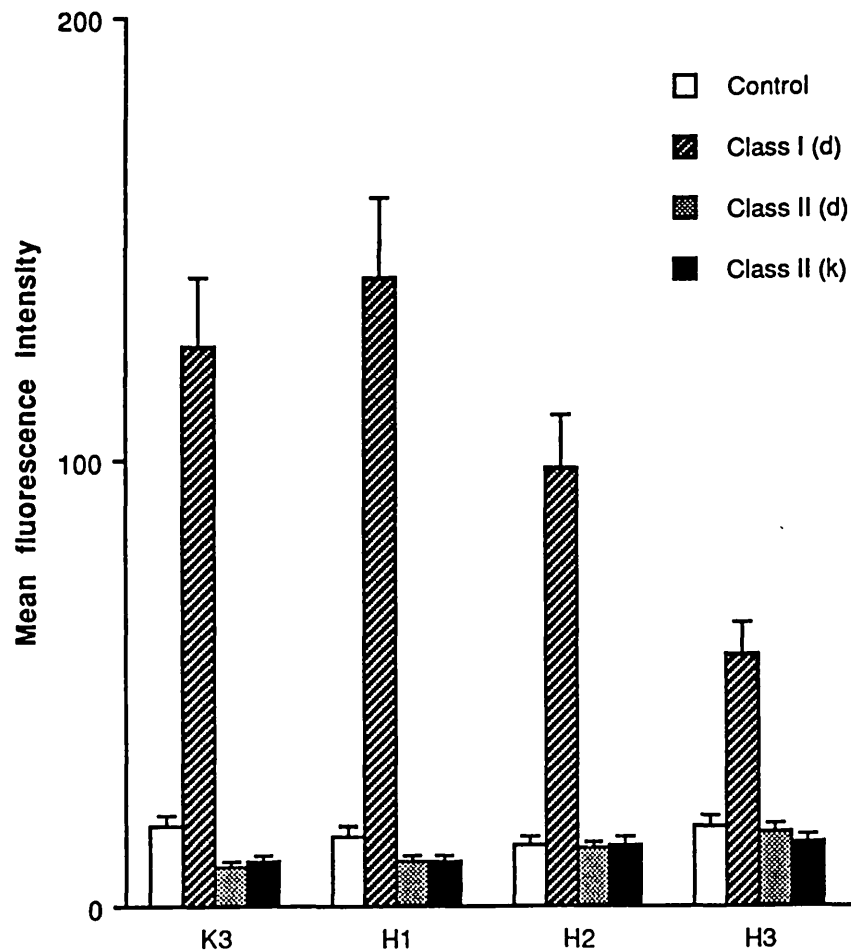
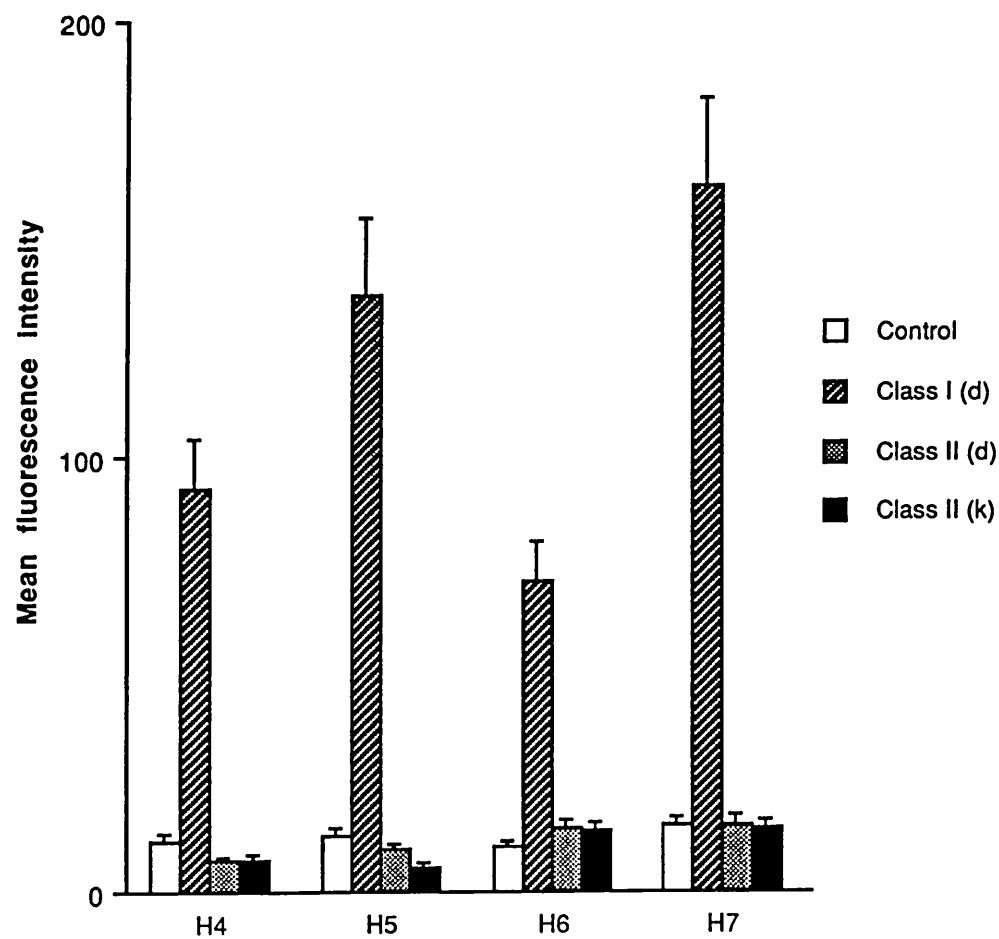
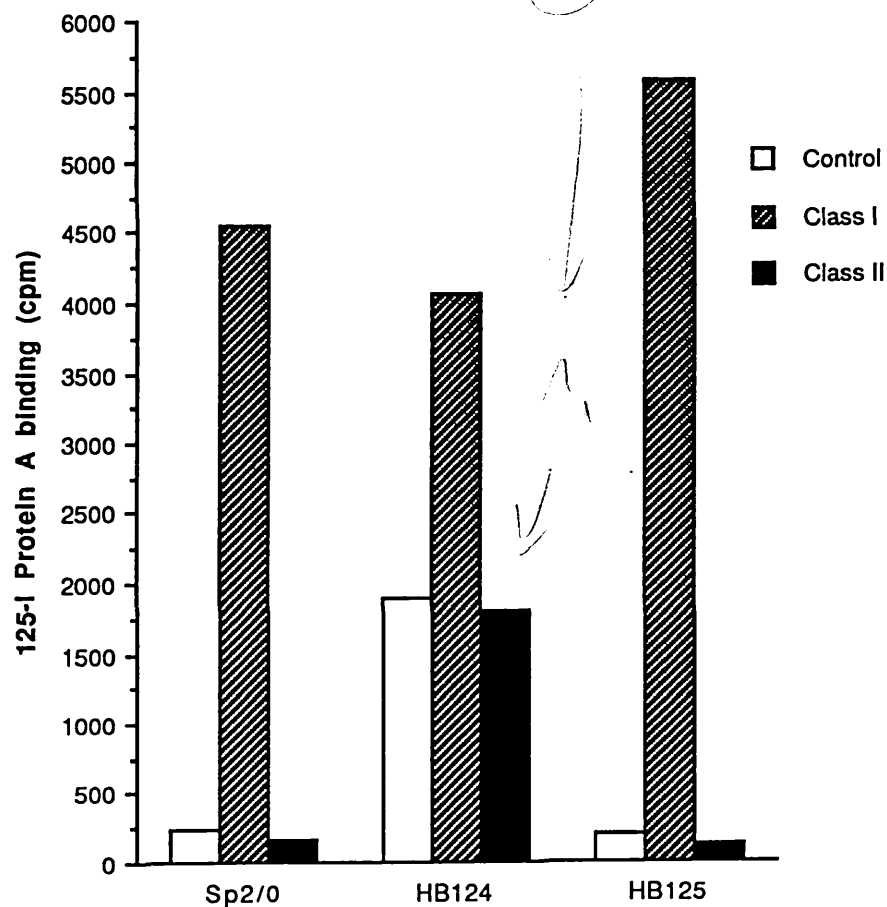
A20 x K3 hybrids express Class I but not Class II

FIGURE 3:3.6

K3, and seven K3 x A20 hybrids (H1-H7, on two pages) were stained with the mAb HB75 (Class I (d)), HB3 (Class II (d)) and TIB92 (Class II (k)) as described in Figure 3:1.1. The results for staining of A20 with the same mAb are depicted in Figure 3:1.1, but cannot be directly compared because the FACS analyses were not performed with identical machine settings.



Sp2/0 and hybrids express Class I but not Class II**FIGURE 3:3.7**

Single cell suspensions of Sp2/0 and its hybrids, HB124 and HB125, were analysed by radioimmunoassay, using 50 000 cpm of ^{125}I -Protein A as the second layer, as described in section 2:3.4. First layer mAb used were HB75 (Class I) and HB3 (Class II). Murine mAb to the β chain of ricin was used as a negative control (Control). Results are expressed as means of duplicate experiments. SDs were $\leq 20\%$ of the mean.

TABLE 3:3.1

MHC ANTIGEN PHENOTYPES OF NS1 X SPLENOCYTE HYBRIDS¹

Fusion (No. of hybrids)	Percentage of hybrids expressing:		
	one parental Class I	both parental Class I	either parental Class II
NS1 x CBA (11 ²)	82	9	0
NS1 x SJL (5)	60	40	0
NS1 x BALB/c (4)	100	3	0

¹ Four of the 11 NS1 x CBA hybrids were prepared and tested by Dr E. J. Culbert, ICI Plc., Alderley Park, Cheshire.

² One hybrid expressed neither MHC Class I or Class II antigens.

³ Both parents are of the same haplotype, H-2^d.

3:4 MHC CLASS II GENE TRANSCRIPTION IN NS1 CELLS

3:4.1 Introduction

As discussed in section 1:3.3, cells that lack Class II antigen expression fail to transcribe the Class II genes in most instances. In the studies presented here, the Class II negative phenotype of the murine plasmacytoma X63 is apparently due to the lack of gene transcription (see section 3:1). The active, specific suppressive mechanism responsible for the failure of NS1 cells to express MHC Class II antigens might accordingly be expected to operate at the level of gene transcription. I therefore studied MHC Class II gene transcription in these cells.

Biochemical analyses of the α and β polypeptide chains of the I-A and I-E antigens have generally shown that the β chains are more polymorphic than the α chains (Cullen et al., 1976; Freed, 1981; Klein and Figueroa, 1981). More recently, comparison of the nucleotide sequences for cloned allelic E α genes shows very few differences (McNicholas et al., 1982; Mathis et al., 1983b). Although allelic A α chains are more variable (Benoist et al., 1983) the variation is only of the order of 5-7%. Hence nucleic acid probes derived from the α chain genes of one murine H-2 haplotype will hybridize with those of most other haplotypes even under conditions of high stringency. For these reasons, transcription of the A α and E α genes was studied. There is compelling evidence that the murine α and β chain genes are co-ordinately expressed and regulated (discussed in section 1:3.1), suggesting that the results obtained are equally valid for the β chain genes.

The expression of mRNA for the non-polymorphic MHC Class II Ii gene was also studied because Ii antigen appears to be co-ordinately expressed with I-A and I-E antigens during B lymphocyte development (Koch and Harris, 1984), although it does not map to the MHC locus (Koch et al., 1982a; Day and Jones, 1983). It has been speculated that the Ii peptide is important

in the assembly and transport of α - β heterodimers to the cell membrane (Koch et al., 1982b); although it has more recently been shown that Ii is not necessary for the expression of murine (Miller and Germain, 1986) or human (Sekaly et al., 1986) Class II antigens at the cell surface.

3:4.2 NS1 cells express correctly-sized RNA for the A α , E α and Ii genes

RNA extracted from the nuclear and cytoplasmic fractions of NS1 cells was analysed by Northern blotting as described previously, and hybridized with ^{32} P-labelled probes for the MHC Class II genes. Figure 3:4.1 shows the results obtained with one Northern blot, which was stripped free of radioactivity between successive hybridizations.

NS1 cells express mRNA for the A α , E α and Ii genes. The respective transcript sizes of 1.4 kbp, 1.2 kbp and 1.4 kbp correspond to those found in MHC Class II antigen expressing A20 cells (compare Figures 3:1.1 (A α , Ii) and 3:4.4 (E α)). Similar transcript sizes have been reported in the literature (Davis et al., 1984; Mathis et al., 1983a; Singer et al., 1984). Transcripts were found in both the nuclear and cytoplasmic RNA fractions, although levels were higher in the cytoplasm.

The presence of Class II gene transcripts in NS1 cells was surprising, given that most Class II regulation is believed to be transcriptional. The transcripts do, however, provide further strong evidence that the Class II genes are structurally intact in NS1 cells and that lack of expression is therefore due to a specific regulatory mechanism.

Transcripts of the Ii gene are also found, although translation has not been demonstrated (section 3:2.3). However the failure to find I-A and I-E antigens intracellularly argues that lack of Ii chain is not the primary cause of the non-expressing phenotype of NS1. In addition there is good evidence that Ii chain expression is not a strict pre-requisite for the expression of I-A and I-E antigens at the cell surface (Miller and Germain, 1986).

3:4.3 E α gene transcripts are poly-A tailed

Total cellular RNA extracted from NS1 cells was enriched for poly-A⁺ species by affinity chromatography on oligo-dT conjugated paper as described in section 2:6.3. Poly-A⁺ RNA was analysed by Northern blotting, and hybridized with a ³²P-labelled probe for the E α gene. Figure 3:4.2 shows that 1.2 kbp E α transcripts are found in the poly-A⁺ enriched RNA fraction, indicating that E α transcripts are poly-A tailed. Taken together with the fact that correctly-sized transcripts are present in both the nucleus and cytoplasm, this must provide presumptive evidence that post-transcriptional RNA splicing and nuclear-cytoplasmic transport proceed normally.

3:4.4 Comparison of RNA levels with those in splenocytes and A20 cells

The level of RNA expression in NS1 cells was compared with that in freshly isolated, unfractionated splenic lymphoid cells prepared from BALB/c mice by RNA slot blot analysis. The procedure, and the precautions taken to ensure that it was quantitative, have been discussed in 2:9.6. Briefly, RNA slot blots were hybridized with probes for the A α , E α and Ii genes, and the autoradiographs scanned on a densitometer to quantitate the amount of radioactivity bound in each slot. The blots were then stripped free of radioactivity and re-probed with the 7B6 probe which detects an RNA species the expression of which does not vary during the cell cycle (Kaczmarek et al., 1985). The 7B6 autoradiographs were scanned, and the amount of MHC Class II gene-specific RNA standardised with respect to 7B6 in order to eliminate any differences in the total amount of RNA loaded into each slot.

Figure 3:4.3 demonstrates the results which are summarized numerically in Table 3:4.1. The amounts of MHC Class II RNA expressed in NS1 cells was at least equivalent to that found in unfractionated splenic lymphoid cells when compared in this way.

It must be noted that the unfractionated splenic cell population includes MHC Class II expressing cells (macrophages, dendritic cells and B lymphocytes) as well as non-expressing cells (T lymphocytes) in roughly equal proportions. Hence EQ RNA expression was also compared with that in A20 cells in a similar manner, using the Northern blot shown in Figure 3:4.4. This analysis showed that NS1 cells expressed approximately a fifth the amount of EQ RNA in A20 cells.

Could low levels of RNA expression be responsible for the failure to detect Class II antigen expression in NS1 ? The data on expression presented previously (section 3:2.2) suggests that this is not the case. Indirect immunofluorescence analysis of the unfractionated splenic cell population shows levels of Class II antigen expression (mean fluorescence intensity 184.3) nearly 20 times higher than in NS1 (mean fluorescence intensity 11.53), although RNA levels are approximately equal. This argument is not conclusive, however, because different subpopulations of splenic cells express Class II at different levels.

An alternative approach was therefore also used. Potent inducers of Class II antigen expression like IFN γ are known to upregulate mRNA levels several-fold (Nakamura et al., 1984; Rosa et al., 1984). Does treatment with IFN γ lead to the induction of detectable levels of Class II antigen expression in NS1 cells ? This would be expected to be the case if low levels of mRNA are the cause of the failure to detect expression.

3:4.5 IFN γ does not induce Class II antigen expression although it enhances Class II mRNA levels in NS1 cells

NS1 cells were treated with 10, 100, 500 and 1000 u/ml of recombinant murine IFN γ (the kind gift of Dr G. Adolf, Ernst-Boehringer Institute, Vienna, Austria) and analysed for MHC antigen expression every 24 hours for 72 hours by indirect immunofluorescence using mAb against D^d (HB75) and I-A^d (HB3). The dose of 100 u/ml was found to have the maximal effect on Class I antigen expression. The smaller dose of 10 u/ml had less effect, while doses of 500 and 1000 u/ml did not affect MHC

antigen expression but had an anti-proliferative effect.

The results obtained with 100 u/ml IFN γ are shown in Figure 3:4.5. Class I expression was enhanced approximately 1.5-fold with a peak at 24 hours (mean fluorescence intensity 46.45 in comparison to 33.63 for untreated cells). However there was no induction of Class II antigen expression.

In contrast, treatment with 100 units/ml of IFN γ enhanced both Class I and Class II antigen expression in A20 cells (peak expression at 48 hours with 2-fold enhancement of Class I and 2.2-fold enhancement of Class II) (Figure 3:4.6). A smaller dose of 50 units/ml induced Class II expression in the BALB/c derived macrophage cell line WEHI-3 (peak expression at 72 hours with 1.5-fold enhancement of Class I and 4-fold enhancement of Class II) (Figure 3:4.6). These results confirm that the IFN γ preparation used was biologically active.

Although there was no induction of Class II antigen expression, treatment of NS1 cells with 100 units/ml of IFN γ enhanced the levels of mRNA for the A α , E α and Ii chain genes 16 hours later (Figure 3:4.7). This surprising result provides some insight into the mechanism of Class II gene regulation in NS1 cells.

First, there is much evidence that IFN γ activates Class II gene transcription (reviewed in Sullivan et al., 1987), indicating that NS1 cells retain the capacity to enhance Class II gene transcription although uninduced cells have only low levels of Class II mRNA. Second, no Class II antigens were detected despite enhanced mRNA levels. This suggests that a post-transcriptional block to expression probably exists and that failure to detect Class II antigen expression is unlikely to be merely due to low mRNA levels in uninduced cells. These results provide suggestive, but not conclusive, evidence that a post-transcriptional regulatory mechanism prevents the expression of Class II antigens in NS1 cells.

3:4.6 Preliminary results : enhancement of Class II gene transcription by the protein synthesis inhibitor CHX ?

The effects of the protein synthesis inhibitor cycloheximide (CHX) were also studied. Treatment of NS1 cells with 10 µg/ml of CHX for 120 minutes resulted in an enhancement of E α mRNA levels (Figure 3:4.7). Pre-treatment with 100 units/ml IFN γ for 12 hours followed by similar CHX treatment resulted in a superinduction of E α mRNA levels over those with IFN γ alone (Figure 3:4.7). In both instances increases were seen in the nuclear as well as the cytoplasmic RNA fraction.

The induction or suppression of MHC Class II gene transcription is believed to be mediated by the interaction of regulatory proteins with DNA sequence elements. These DNA binding proteins are likely to be turned over very rapidly to enable rapid changes in the level of gene expression; and the most interesting non-trivial explanation for the observed effect of CHX is that it inhibits the synthesis of such a short-lived "negative" regulatory protein.

CHX might also exert its effects post-transcriptionally, by stabilising Class II gene transcripts. However this possibility is unlikely for two reasons. First, enhancement of E α transcript levels is seen in the nucleus as well as the cytoplasm suggesting that de novo synthesis is occurring; and second, preliminary results show that the half-life of cytoplasmic E α transcripts in CHX treated NS1 cells is not prolonged in comparison to untreated controls (data not shown).

It is conceivable that CHX synergises with IFN γ (which may increase Class II gene transcription by displacement of a repressor protein from the 'IFN γ response element' of the promoter (Boss and Strominger, 1986; Thanos et al., 1988)) by inhibition of repressor protein synthesis. Further experiments are in progress to examine this possibility.

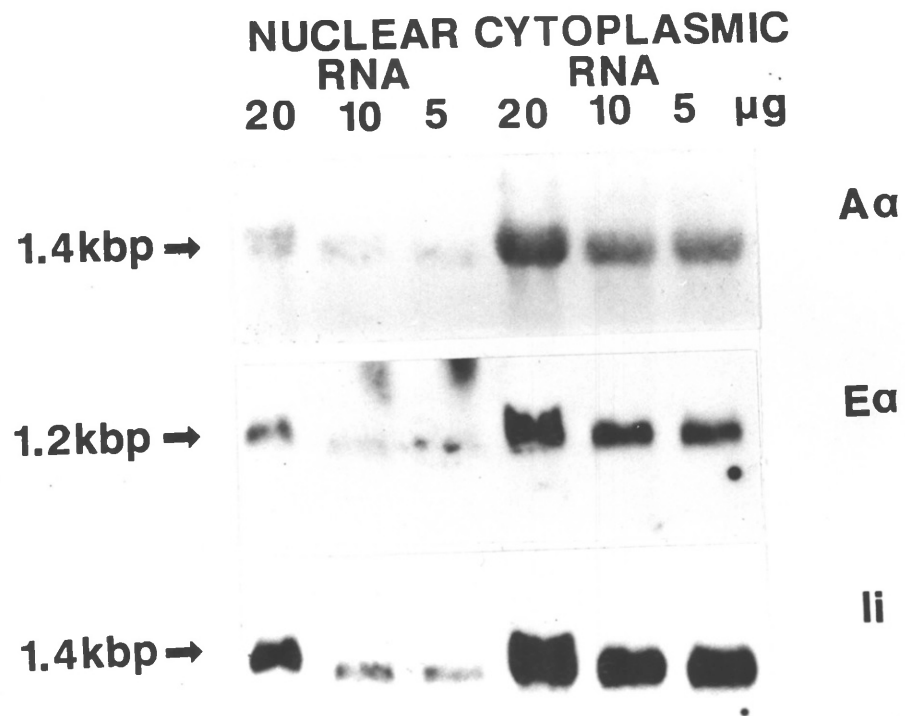


FIGURE 3:4.1

NS1 CELLS EXPRESS RNA FOR THE MHC CLASS II GENES

Nuclear and cytoplasmic RNA fractions prepared from NS1 cells were analysed by Northern blotting as described in section 2:7.2. Blots were hybridized with probes for the A α , E α (pCA39) and Ii chain genes radio-labelled to activities $> 10^8$ cpm/ μ g by the random oligo-priming method. Figures show the amount of RNA loaded in each lane (in μ g).

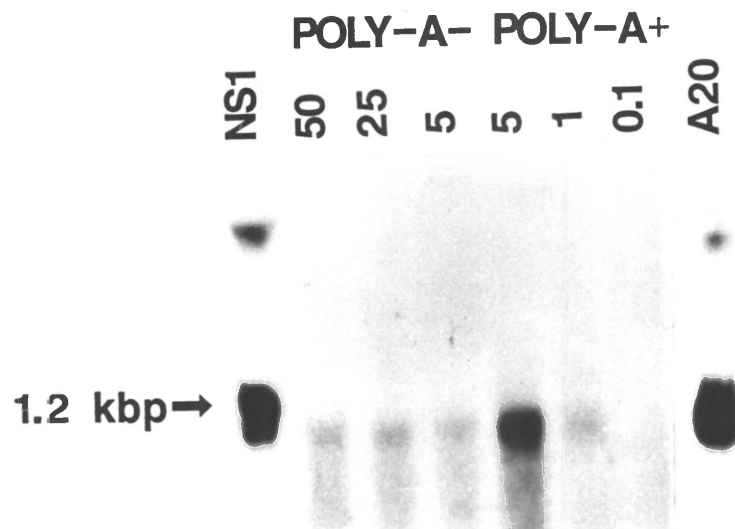


FIGURE 3:4.2

EX GENE TRANSCRIPTS ARE POLY-A TAILED

Total cellular RNA extracted from NS1 cells was enriched for poly-A⁺ species by affinity chromatography on oligo-dT conjugated paper and analysed by Northern blotting as described in section 2:7.2. The blot was hybridized with the EX cDNA probe pSPEAC₁ radiolabelled to an activity of $>10^8$ cpm/ μ g by SP6 transcription. Lanes contain the indicated amount of RNA.

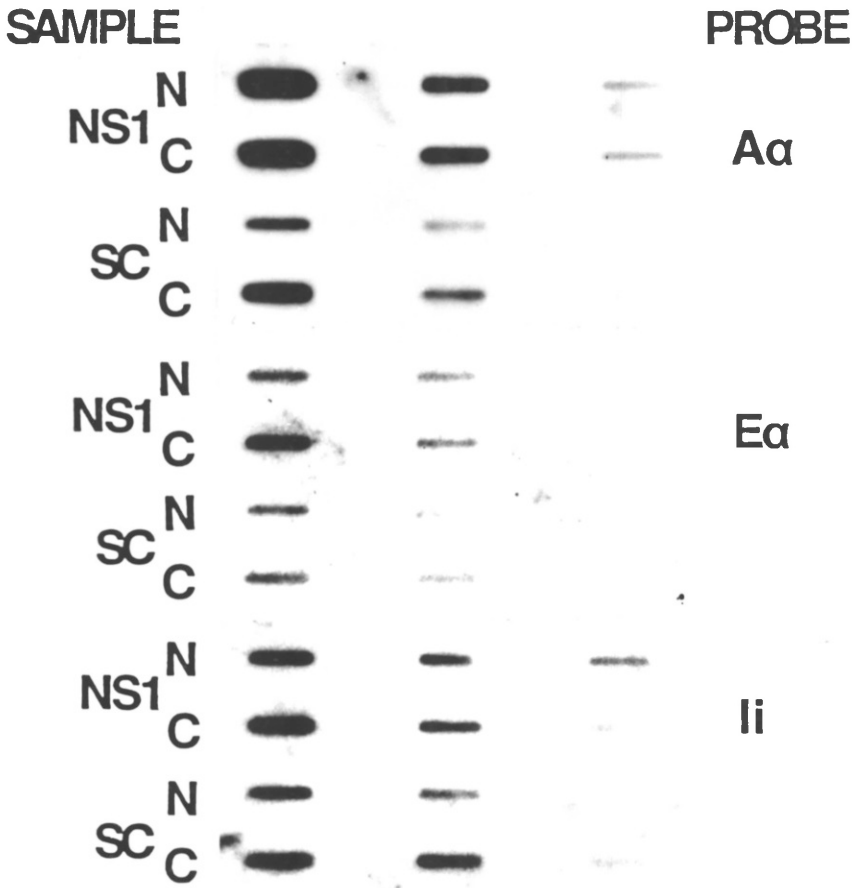
Although A20 total cellular RNA was analysed on the same blot, a shorter (2 days vs. 5 days) autoradiographic exposure is shown.

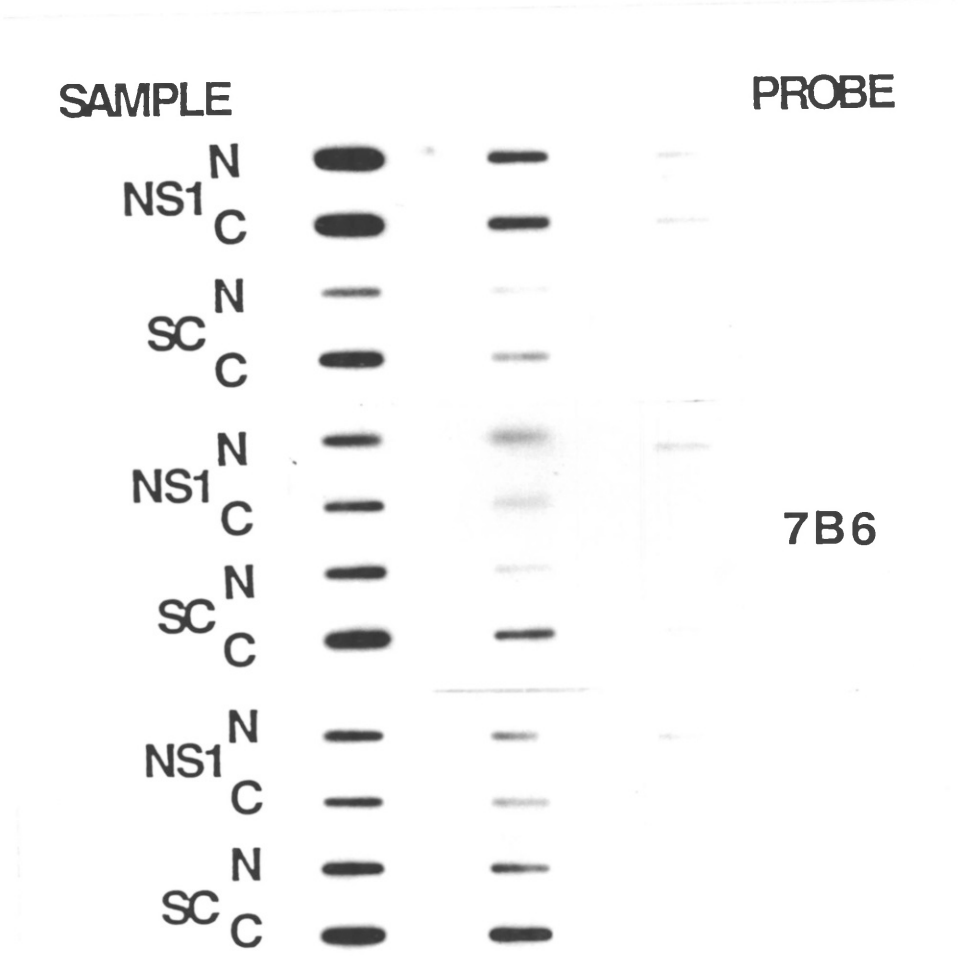
FIGURE 3:4.3

COMPARISON OF RNA LEVELS WITH THOSE IN SPLENOCYTES

RNA prepared from the nuclear (N) and cytoplasmic (C) fractions of NS1 cells and unfractionated BALB/c splenocytes (SC) was analysed by RNA slot blotting as described in section 2:7.3. Columns contain 5, 1 and 0.2 µg of RNA respectively. Blots were hybridized with radiolabelled probes for the A α , E α (pCA39) and Ii genes as shown. In order to quantitate RNA expression the filters were stripped free of radioactivity and re-hybridized with the 7B6 probe (next page), which detects an RNA species that is invariant during the cell cycle, before quantitation by densitometry.

The results obtained are summarized numerically in Table 3:4.1.





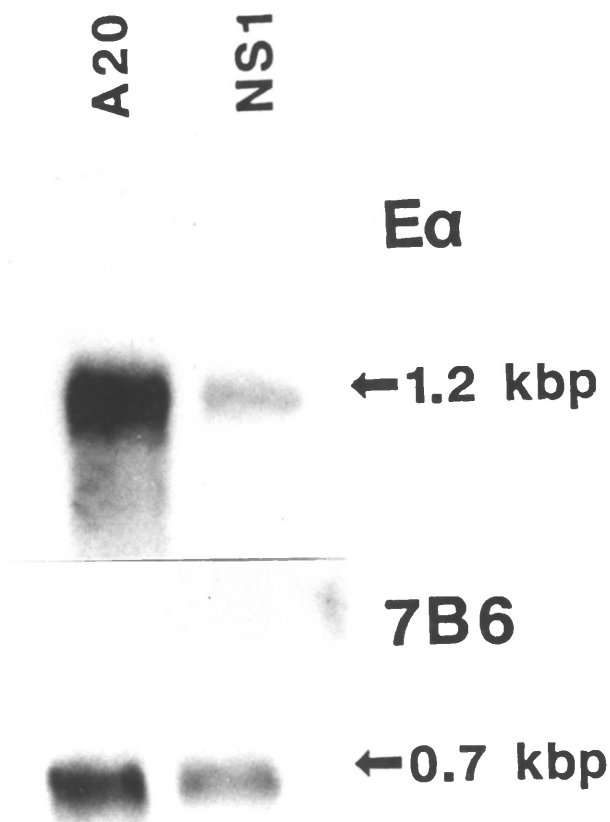


FIGURE 3:4.4

COMPARISON OF RNA LEVELS WITH THOSE IN A20 CELLS

Total cellular RNA prepared from NS1 and A20 cells was analysed by Northern blotting as described in section 2:7.2. The blot was hybridized with a radio-labelled probe for the E α gene (pCA39) and autoradiographed. The filter was stripped free of radioactivity and re-hybridized with the 7B6 probe before quantitation by densitometry. Densitometric readings were taken with the instrument's measuring slit placed perpendicular to the centre of the band being quantitated. The results obtained are summarized numerically in Table 3:4.1.

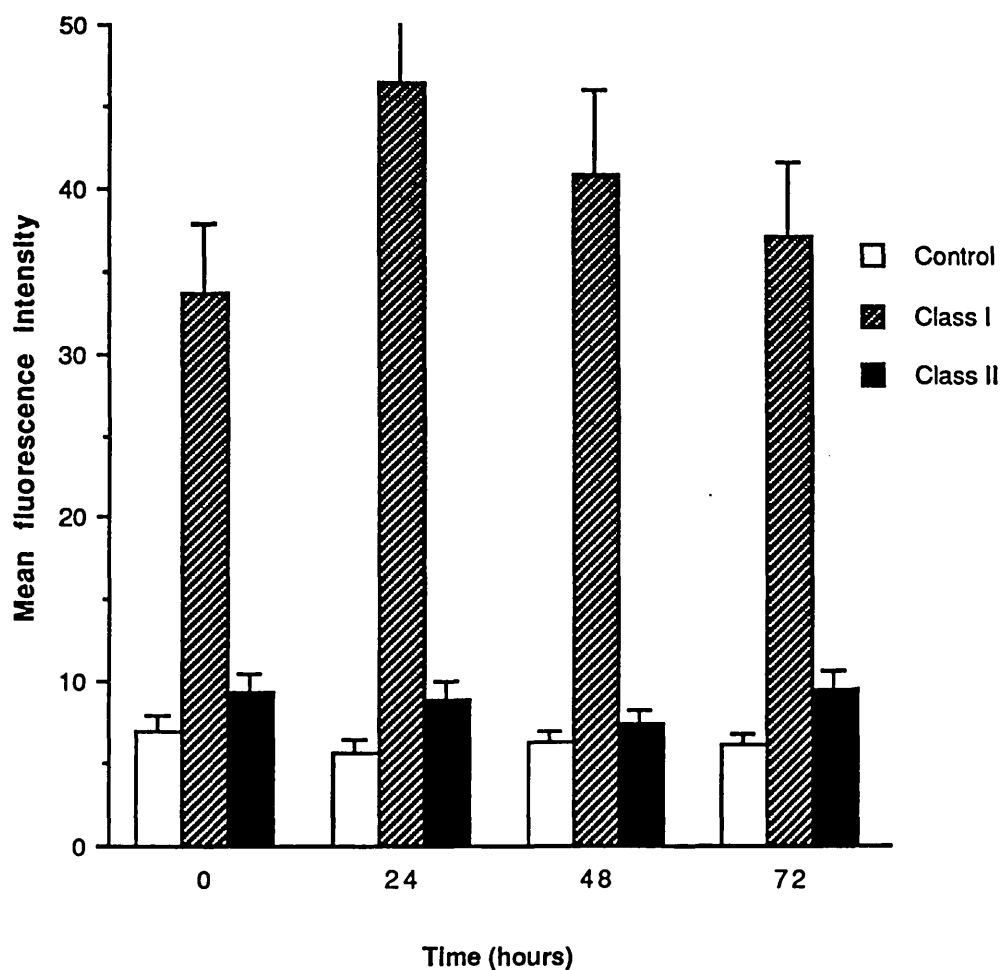
IFN gamma does not induce Class II on NS1 cells

FIGURE 3:4.5

NS1 cells treated with 100 u/ml recombinant murine IFN γ were analysed for MHC antigen expression every 24 hours for 72 hours. Indirect immunofluorescence staining and FACS analysis were performed as described in Figure 3:1.1. The figure shows the results of one of two similar experiments.

Cells were stained with the mAb HB75 (anti-D^d, Class I) and HB3 (anti-I-A^d, Class II). Control staining was with fluorochrome conjugated GAMIg alone (Control).

IFN gamma enhances Class I and Class II expression on A20 cells

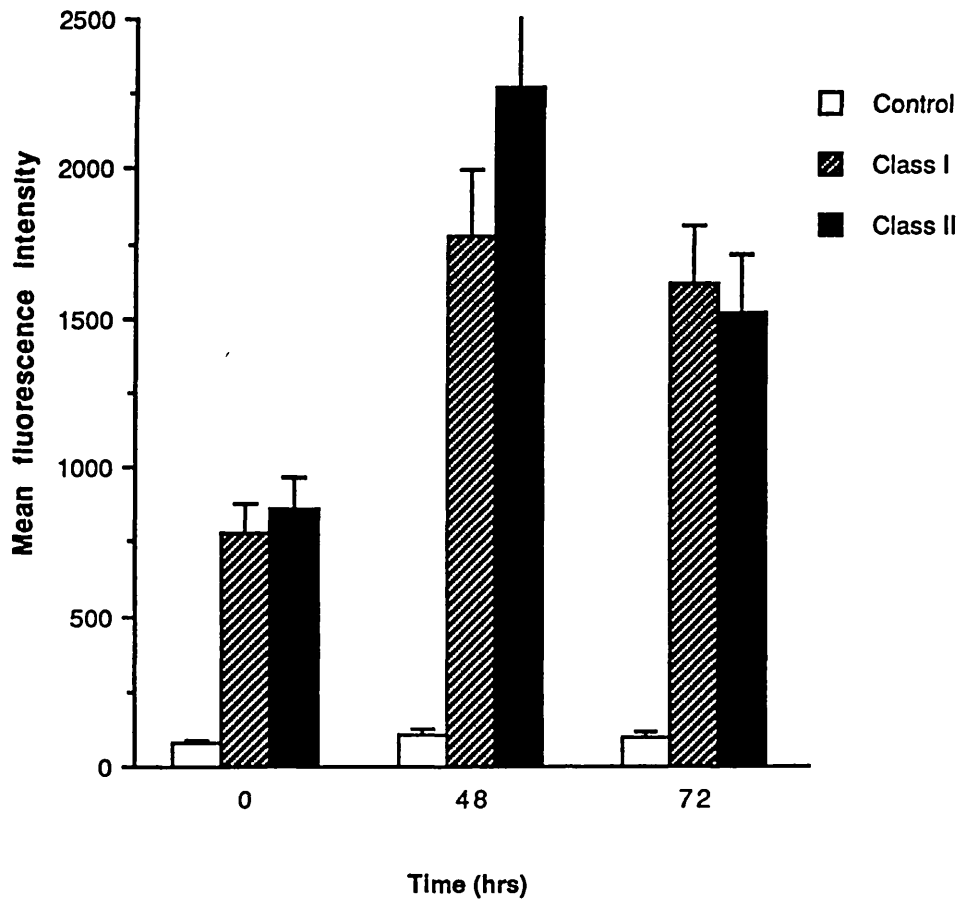
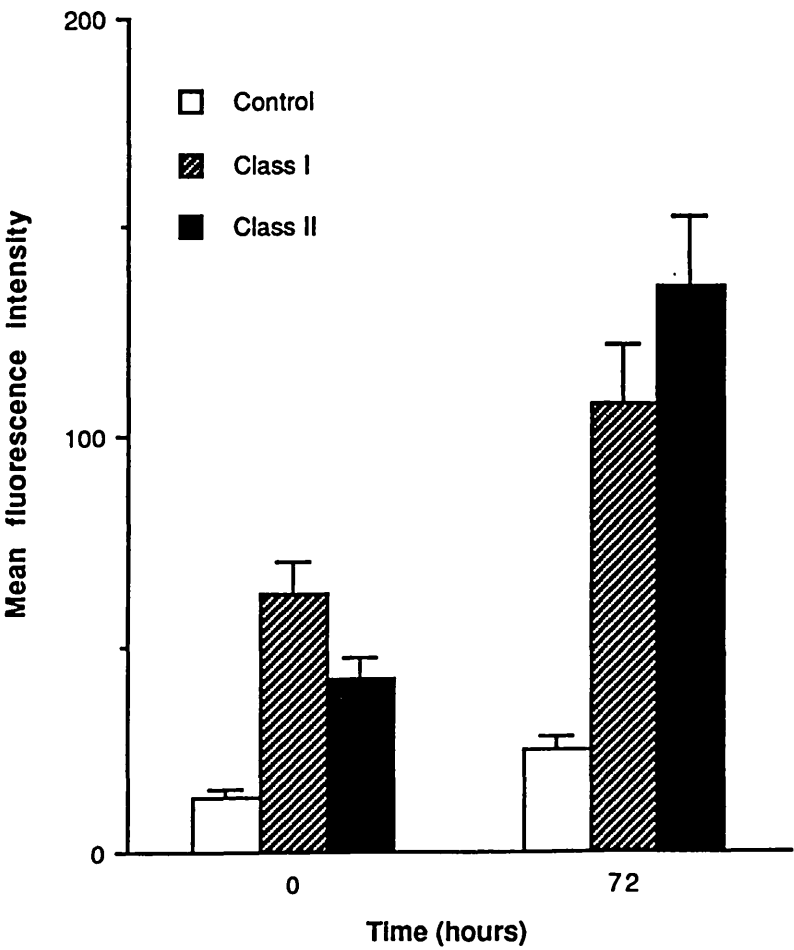


FIGURE 3:4.6

A20 cells treated with 100 u/ml, and WEHI-3 cells treated with 50 u/ml (next page), of recombinant murine IFN γ were analysed for MHC antigen expression as described in Figure 3:1.1. This figure shows the results of one of two similar experiments.

Cells were stained with the mAb HB75 (anti-D^d, Class I) and HB3 (anti-I-A^d, Class II). Control staining was with fluorochrome conjugated GAMIg alone (Control).

**IFN gamma induces Class II expression
on WEHI-3 cells**



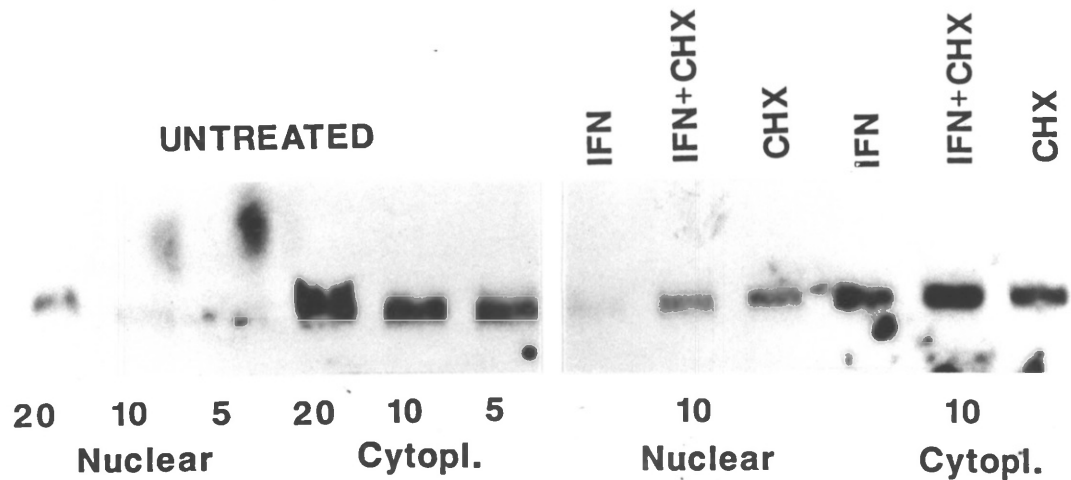


FIGURE 3:4.7

IFN γ AND CHX ENHANCE LEVELS OF mRNA FOR THE E α GENE IN NS1 CELLS

This figure shows a Northern blot of RNA from the nuclear and cytoplasmic (cytopl.) fractions of untreated NS1 cells; or cells treated with 100 u/ml IFN γ for 12 hours (IFN), 10 μ g/ml cycloheximide for 120 minutes (CHX), or 100 u/ml IFN γ for 12 hours followed by 10 μ g/ml CHX for 120 minutes (IFN+CHX). Numbers refer to the amount of RNA loaded (μ g). The blot was hybridized with an E α probe (pCA39). The first half of the figure showing untreated cells is the same as shown in Figure 3:4.1, all 12 lanes are from the same Northern blot.

TABLE 3:4.1

QUANTITATION OF mRNA EXPRESSION BY NS1 CELLS

Probe	Relative mRNA expression :			
	Nuclear fraction		Cytoplasmic fraction	
	NS1	Splenocytes	NS1	Splenocytes
A α	1	1.30	1	0.76
E α	1	0.81	1	0.58
Ii	1	1.42	1	1.38
E α	NS1 (Total RNA)		A20 (Total RNA)	
	1		4.74	

This table summarizes numerically the results obtained by densitometric scanning of the slot and Northern blots shown in Figures 3:4.3 and 3:4.4. Two calculations were performed : the first, to obtain the intensity of hybridization per unit amount of RNA in order to correct for errors due to inaccurate loading of the tracks or slots; and the second, to compare these "corrected" hybridization signals.

The "corrected" intensity of the hybridization signal per unit amount of RNA loaded into each track or slot on the blots was first determined with respect to the 7B6 probe (which detects an RNA species that is invariant in the cell cycle) as described in section 2:9.6. The "corrected" hybridization signals obtained with splenocytes and A20 cells were then expressed as a ratio relative to the NS1 hybridization signal which was taken to be 1. These ratios, which are shown in the Table, represent the relative level of mRNA expression in the different cell types in comparison to NS1.

3:5 NS1 CELLS REGULATE MHC CLASS II GENE EXPRESSION POST-
TRANSCRIPTIONALLY ; PREPARATION AND ANALYSIS OF DR4/Dw15
GENE TRANSFECTANTS

3:5.1 Introduction

The experiments presented in the preceding section provide circumstantial evidence that NS1 cells suppress MHC Class II gene expression at the post-transcriptional level, but fall just short of proving the contention. The Class II mRNA expressed by NS1 cells appears to be correctly-sized and normally processed, but it is found at lower levels than in Class II expressing A20 cells (although levels are at least equivalent to those in unfractionated splenic cells). There are two possible explanations for this data : first, that the level of mRNA expressed is too small for the synthesis of detectable amounts of protein (ie., transcriptional regulation); or second, that protein synthesis is blocked by a post-transcriptional mechanism that operates subsequent to mRNA processing and transport.

An approach was designed to differentiate between these possibilities and to permit analysis of the mechanism of regulation in further detail. A construct containing 8 tandem copies each of the cDNAs encoding the complete sequence of the α and β chains of a human Class II gene (DR4/Dw15) under the control of individual SV40 promoters and the 72 bp repeat enhancer sequence (Benoist and Chambon, 1981; Gruss et al., 1981) was transfected into NS1 and A20 cells, and expression of the transfected gene was analysed. Dr H. Ikeda (Imperial Cancer Research Fund, London) kindly provided this construct, R/RW, which has been described in detail elsewhere (section 2:10.1 and Ikeda et al., 1988).

The coding region of the DR α gene cDNA is closely homologous to that of the murine E α gene (Lee et al., 1982; Das et al., 1983; Mathis et al., 1983b) and putative post-transcriptional regulatory regions in the 5' untranslated sequence are highly conserved (Figure 3.5.1). Thus it is plausible that this gene

will be regulated in a similar manner to the endogenous E α gene. However, sufficient sequence differences exist that mAb and nucleic acid probes specific to DR do not cross-react with I-E.

Thus transfection of the DR gene construct would permit transcriptional and post-transcriptional regulatory mechanisms to be clearly distinguished. The SV40 promoter and enhancer are active in most eukaryotic cell types, and would therefore not be subject to the same constraints as regulate MHC Class II gene transcription. In addition, relatively high levels of mRNA are stably expressed when the construct is transfected into murine L cells (Ikeda et al., 1988). If Class II gene regulation operates post-transcriptionally in NS1, the transfected DR gene will not be expressed. However if regulation is transcriptional, detectable amounts of DR protein ought to be synthesized. This approach permits the key issue raised by the results of the experiments presented earlier to be addressed directly.

3:5.2 DR4 antigens are expressed in transfected A20 cells

A20 cells were transfected with the R/RW gene construct, and stable transfectant lines were selected by growth in Hygromycin B as described. Nine stable, Hygromycin B resistant lines were obtained. They were analysed for the expression of I-A^d and DR4 by indirect immunofluorescence on the FACS on two occasions, 35 and 45 days after transfection. The results of both analyses were similar : Figure 3:5.2 shows the results obtained 35 days after transfection.

The mAb HU.43 (Bodmer et al., 1984) was used to detect DR4 antigen expression. It is specific for a determinant common to the DR1, 4, 7 and 9 haplotypes; and does not stain untransfected A20 cells (Figure 3:5.2). Six of the nine A20 transfectants expressed the DR4 antigen. The mean fluorescence intensity of staining of these positive transfectants varied over a 4-fold range (25.2 to 100.3), and might therefore be attributable to cell cycle dependency (see section 3:1.3). This possibility was supported by the fact that similar variability

was found in the expression of I-A^d (range 45.7 to 154.5); however there was no correlation between expression of the two antigens. The transfectants expressed the Class I antigen D^d at levels similar to those in untransfected A20 cells (data not shown).

3:5.3 DR4 antigens are expressed in transfected P815 cells

As a control to ensure that the R/RW expression construct was able to direct the synthesis of DR antigens in murine cells that do not express MHC Class II, P815 mastocytoma cells which had been transfected with R/RW by Dr. H. Ikeda were analysed by indirect immunofluorescence. Transfectants were selected for Hygromycin B resistance essentially as described earlier.

Figure 3:5.3 shows that untransfected P815 cells did not express the murine Class II antigen I-A^d, although the Class I antigen D^d was expressed. Untransfected cells did not bind anti-DR4 mAb. Transfected P815 cells expressed DR4 at high levels, showing that the R/RW construct can direct the synthesis of DR4 antigens in murine cells that do not themselves express endogenous MHC Class II. Expression of murine Class I and Class II antigens in the transfectants was similar to that in untransfected P815 cells.

3:5.4 DR4 antigens are not expressed in transfected NS1 cells

In contrast to A20 and P815, none of the 7 stably Hygromycin B resistant NS1 cell lines obtained expressed DR4 antigens (Figure 3:5.4). Expression of the murine Class I (D^d) and Class II (I-A^d) antigens was similar in untransfected cells and transfectants. The staining patterns observed were consistent at 35 and 45 days after transfection.

In order to verify that transfected NS1 cells did not express a modified DR4 antigen not recognized by the mAb HU.43, they were also stained with the mAb L243 (anti-DR non-polymorphic determinant; Lampson and Levy, 1980) and HU.48 (anti-DR4, 7; Bodmer et al., 1984). Staining with these mAb was

negative (data not shown).

One additional NS1 transfectant not shown in Figure 3:5.4, NS1/DR13, could only be obtained in sufficient numbers for analysis 47 days and 54 days after transfection. This cell line was found to express DR4 at very low levels (mean fluorescence intensity 31.5 with HU.43 in comparison to 15.54 with GAMIg alone) on day 47 but was DR4 negative (mean fluorescence intensity 19.2 with HU.43 in comparison to 16.7 with GAMIg alone) on day 54. This cell line is being studied further.

3:5.5 Intracellular DR antigen expression

Are DR antigens present intra-cellularly, even though they are not expressed at the cell surface ? This remains a possibility in spite of the evidence presented in section 3:2.2 that there is no murine Class II polypeptide synthesis in NS1 cells. Three NS1 transfectants and one A20 transfectant which had been kept in continuous culture for 90 days after transfection were pre-fixed with 1% paraformaldehyde for 30 minutes at 4⁰ C in order to permeabilize the cells, before analysis by indirect immunofluorescence in the usual way.

As shown in Figure 3:5.5, the mAb HU.43 did not stain permeabilized NS1 transfectants (NS1/DR11, DR14, DR43); although a mAb against D^d did. In contrast, HU.43 stained the permeabilized A20 transfectant (A20/DR5) more intensely than it did non-permeabilized cells. This indicates that DR antigens are present intra-cellularly in A20 but not NS1 transfectants. Intracellular synthesis of DR polypeptide chains is being analysed by biosynthetic labelling and immunoprecipitation to verify this.

3:5.6 NS1 and A20 transfectants have integrated the DR4 gene

In the expression construct R/RW, the Hygromycin B resistance gene and the DR α and β chain genes are physically separate; and controlled by independent promoters (see Figure 2:6). It is therefore conceivable that recombinational or

deletional events during or after gene transfection might result in Hygromycin B resistant cells that have not integrated copies of the DR genes. A20 and NS1 transfectants were therefore analysed to verify DR gene integration.

The DR α chain gene in R/RW was cloned into an EcoRI site. Therefore genomic DNA prepared from A20 and NS1 transfectants was digested to completion with EcoRI, and analysed by Southern blotting. Figures 3:5.6 and 3:5.7 show the results of hybridization to a radiolabelled DR α probe. As expected, the characteristic 1.2 kbp band corresponding to the cloned DR α chain was found in six of the seven NS1 transfectants. Four of these clearly contain multiple copies of the insert, while two contain two or fewer copies. The internal quantitative standard in the autoradiographs was provided by the additional 8.3 and 2.9 kbp bands that are present in both untransfected and transfected cells : these are EcoRI fragments of the murine EQ gene which cross-hybridize with the DR α probe (Mathis et al., 1983b). The EQ gene is, of course, present in two copies in NS1 and A20 cells.

Four DR4 antigen expressing and 2 non-expressing A20 cell lines are shown in Figure 3:5.7. Of the 4 expressing lines, two had the characteristic 1.2 kbp EcoRI fragment, while two others had instead a 4 kbp band of the same intensity. This larger fragment may represent a partial EcoRI digest; however this observation was reproducible when the experiment was repeated. The two DR4 antigen non-expressing A20 transfectants did not appear to have integrated the DR α gene, since their hybridization pattern was identical to that of untransfected A20 cells.

The supposition made is that the DR4 genes integrated into NS1 cells are transcriptionally active because the SV40 promoter/enhancer sequences are known to be active in most eukaryotic cell types. However, local influences related to the site of integration in the genome might also play a role : for example, genes integrated into an inactive region of the genome may not be transcribed. Given that the site of integration is random; the fact that none of the seven NS1 transfectants

expressed DR4 (in contrast to 6 of 9 A20 transfectants) must tend to exclude this as a relevant factor. Nevertheless, transfectants were analysed for DR α gene mRNA expression.

3:5.7 Preliminary results : NS1 transfectants express DR α gene mRNA

In order to verify that the radiolabelled DR α probe did not cross-hybridize to the EQ gene, with which it is highly homologous, it was hybridized to total cellular RNA prepared from A20 (which expresses high levels of EQ gene mRNA (see section 3:4.2)) and one A20 transfectant, A20/DR5. The DR α probe identified two characteristic mRNA species of size 1.4 and 1.2 kbp in A20/DR5, but did not cross-hybridize to any messenger species in untransfected A20 cells (Figure 3:5.8). The larger (1.4 kbp) DR α mRNA species may reflect the presence of additional start sites or splicing sites in the transfected gene, and is commonly found in transfectants (Ikeda et al., 1988; and personal communication). Similar results were obtained with the P815 transfectant line, P815/DR (Figure 3:5.8).

Figure 3:5.9 shows the results of RNA slot blot analysis of the seven NS1 transfectants. They all expressed mRNA for the DR α gene, albeit at slightly varying levels, suggesting that the transfected genes are transcribed.

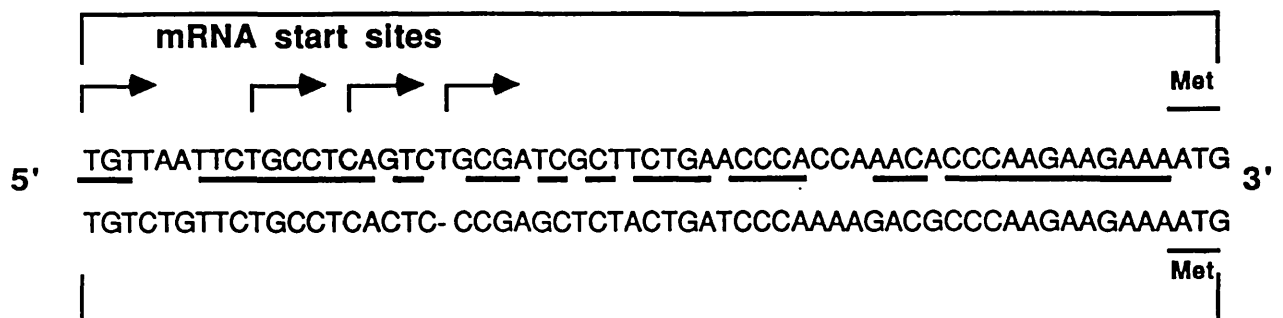
Several important questions still need to be answered. Is the level of DR α mRNA expression similar in NS1 and A20 transfectants ? Is the mRNA poly-A tailed ? Is it found in both the nucleus and cytoplasm ? Is the stability (that is, half-life) of the mRNA equal in both A20 and NS1 ? Further experiments to study mRNA synthesis in detail are in progress.

Taken together these data imply that NS1 cells suppress MHC Class II gene expression post-transcriptionally; since the SV40 promoter-enhancer controlled human Class II gene DR4 was not expressed when transfected into these cells, although it was

expressed at high levels when transfected into both Class II positive A20 cells and Class II negative P815 cells. Complete verification of this hypothesis requires two further demonstrations : first, that the rate of DR4 gene transcription in transfected A20 and NS1 cells is similar; and second, that non-Class II genes controlled by the same SV40 sequences can be expressed when transfected into NS1 cells. Experiments are in progress that address these points.

**THE 5' UNTRANSLATED mRNA SEQUENCES OF THE
E alpha AND DR alpha GENES ARE HOMOLOGOUS**

E alpha (Mathis et al., 1983b)



DR alpha (Das et al., 1983)

OVERALL SEQUENCE HOMOLOGY = 77.6%

FIGURE 3:5.1

Sequences of cDNA clones of the two genes were obtained from Mathis et al., 1983b and Das et al., 1983 respectively. Alignment was from the ATG codons. One base pair was added to the DR alpha sequence for alignment, and is indicated by a -. The mRNA start sites indicated are for the EQ gene (Mathis et al., 1983b).

A20 transfectants express DR antigens

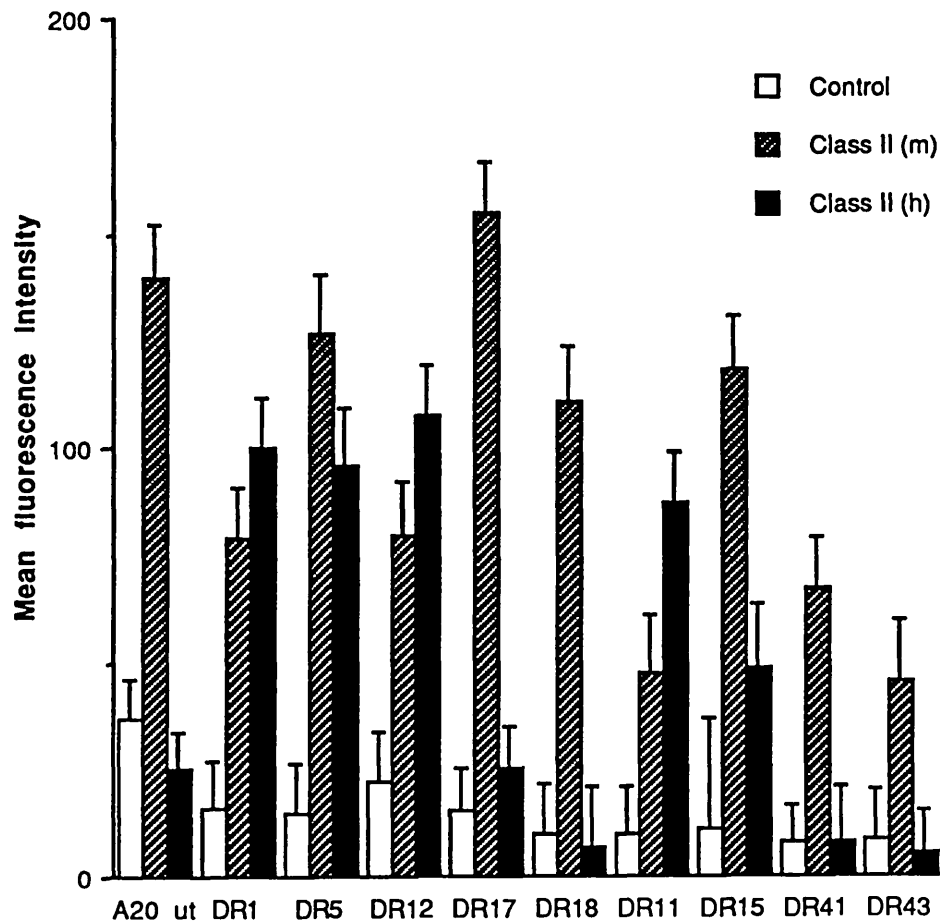


FIGURE 3:5.2

A20 cells stably transfected with the DR4 expression construct R/RW were analysed for MHC antigen expression by indirect immunofluorescence and FACS analysis 35 days after transfection as described in Figure 3:2.1. The mAb used were HB3 (anti-I-A^d, Class II (m)) and HU.43 (anti-DR4, Class II (h)). Control negative staining was with fluorochrome conjugated GAMIg alone (Control).

A20 ut refers to untransfected A20 cells, while the other designations (DR1, DR5, etc.) refer to the different transfectant cell lines.

P815 transfectants express DR antigens

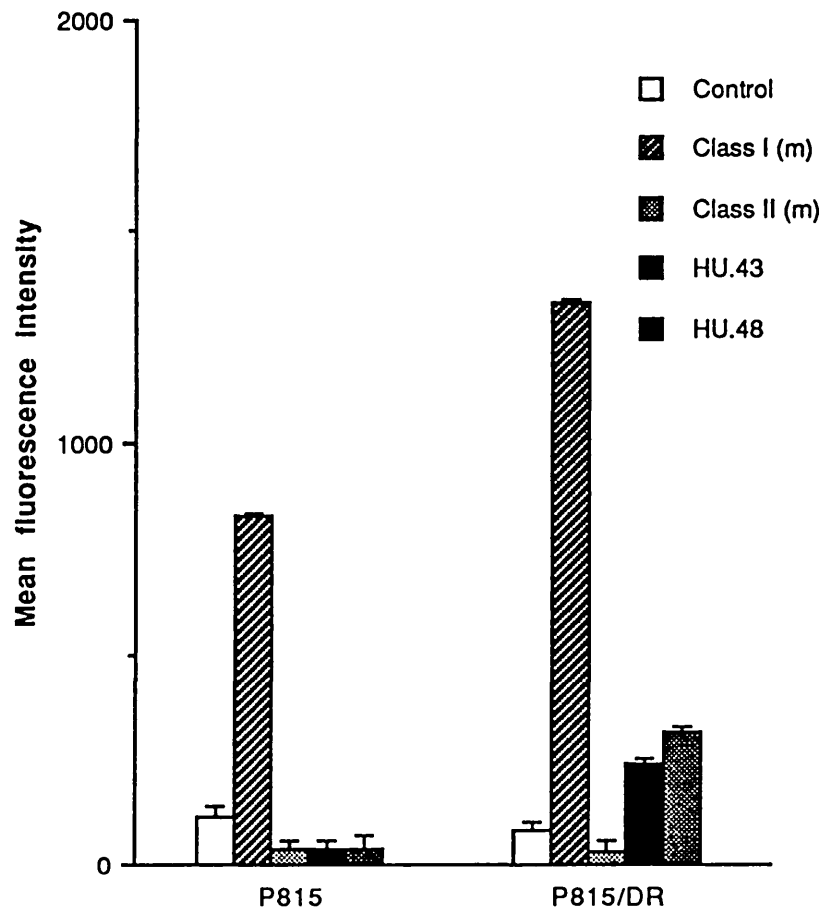


FIGURE 3:5.3

P815 mouse mastocytoma cells stably transfected with the DR4 expression construct R/RW (kindly provided by Dr. H. Ikeda, ICRF, London) were analysed for MHC antigen expression by indirect immunofluorescence and FACS analysis as described in Figure 3:1.1. The mAb used were HB75 (anti-D^d, Class I (m)), HB3 (anti-I-A^d, Class II (m)), HU.43 (anti-DR1,4,7,9) and HU.48 (anti-DR4,5). Control negative staining was with fluorochrome conjugated GAMIg alone (Control).

P815 refers to untransfected cells, while P815/DR refers to the transfectant cell line.

NS1 transfectants do not express DR antigens

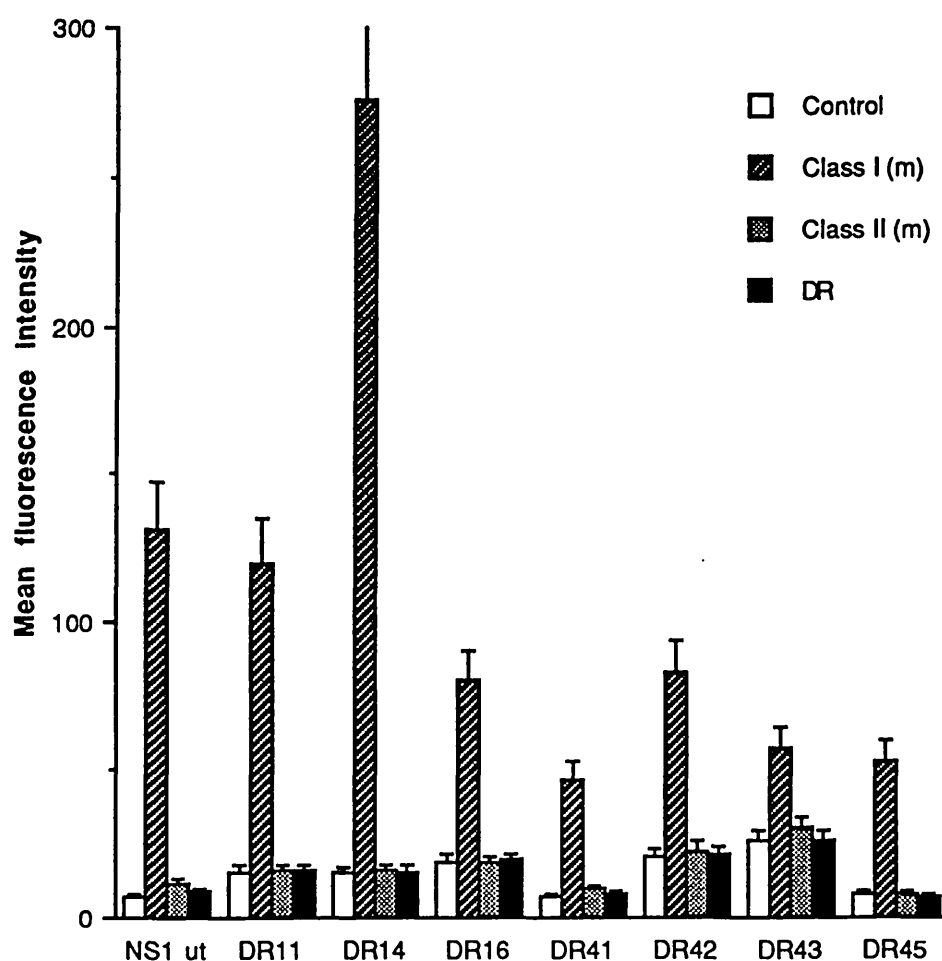


FIGURE 3:5.4

NS1 cells stably transfected with the DR4 expression construct R/RW were analysed for MHC antigen expression by indirect immunofluorescence and FACS analysis 35 days after transfection as described in Figure 3:1.1. The mAb used were HB75 (anti-D^d, Class I (m)), HB3 (anti-I-A^d, Class II (m)) and HU.43 (anti-DR4, DR). Control negative staining was with fluorochrome conjugated GAMIg alone (Control).

NS1 ut refers to untransfected NS1 cells, while the other designations (DR11, DR14, etc.) refer to the different transfectant cell lines.

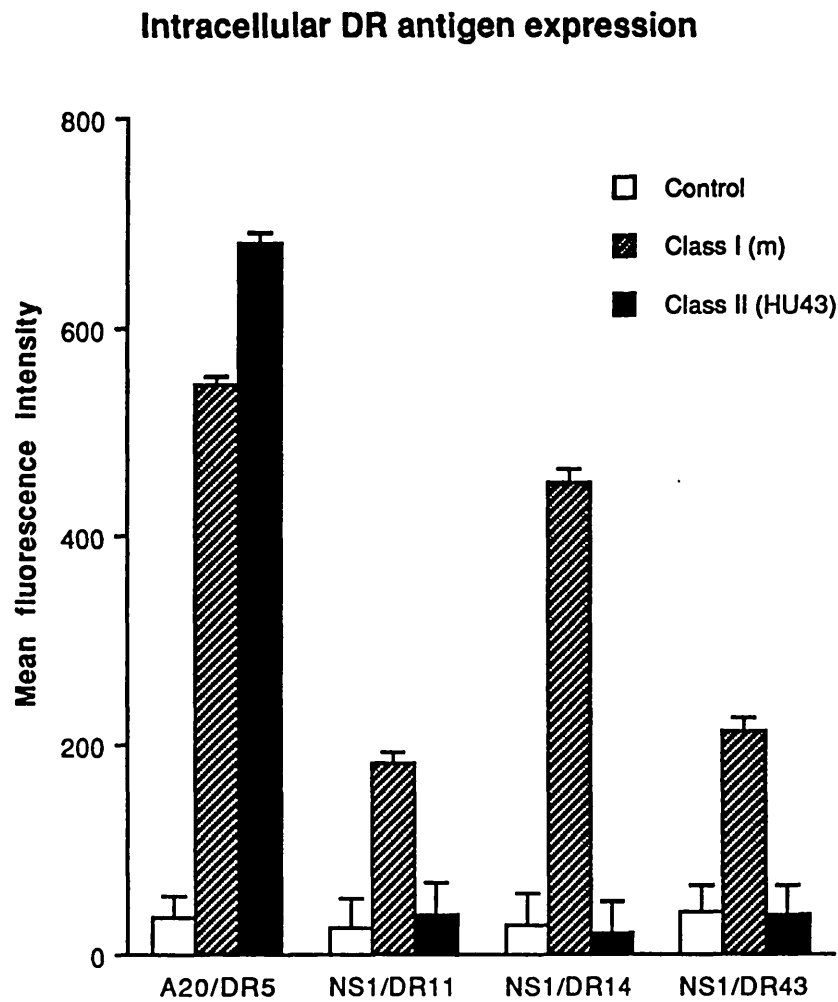


FIGURE 3:5.5

Four different transfectant lines - one of A20 and the other three of NS1 - were permeabilized by fixation in 1% paraformaldehyde before indirect immunofluorescence staining and FACS analysis in the usual way. The mAb used were HB75 (anti-D^d, Class I (m)) and HU.43 (anti-DR4, Class II (HU.43)). Control negative staining was with fluorochrome conjugated GAMIg alone.

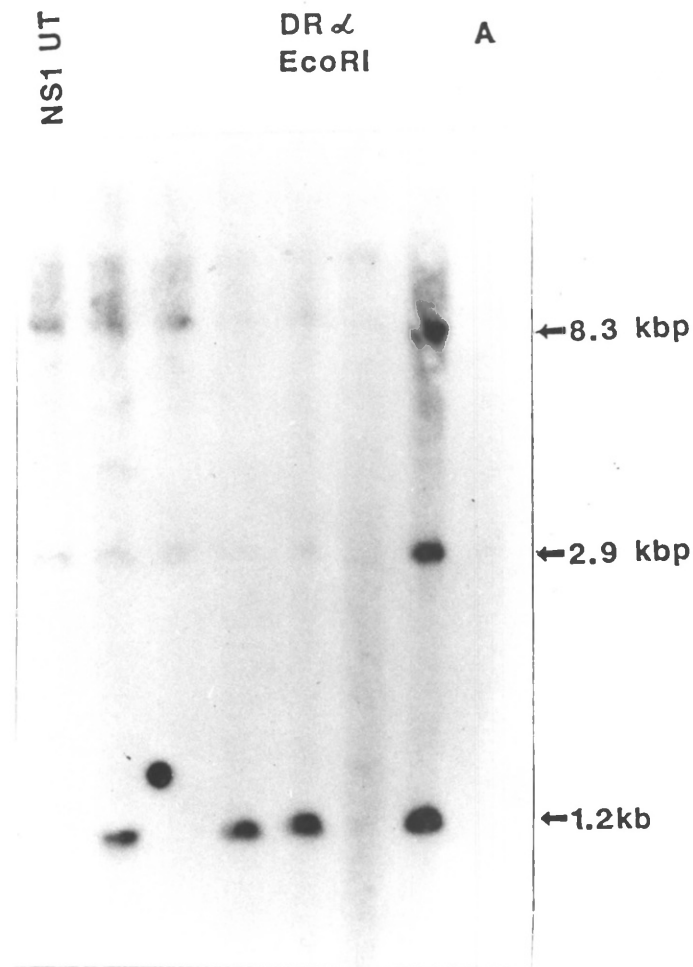


FIGURE 3:5.6

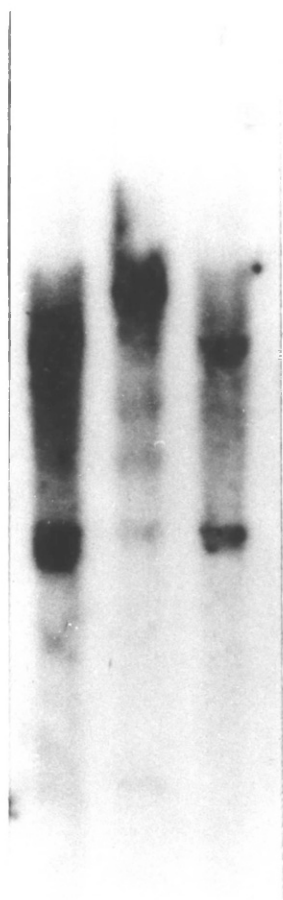
NS1 TRANSFECTANTS HAVE INTEGRATED THE DR α GENE

Genomic DNA prepared from untransfected NS1 cells (NS1 UT) and NS1 transfectants was completely digested with EcoRI and analysed by Southern blotting as described in section 2:7.1. Blots were hybridized to a DR α probe. A. shows the results with six transfectants (as seen in Figure 3:5.4, except DR41). Each track contains 10 μ g of DNA. Four clearly contain multiple copies of the DR α gene. The two transfectants which were negative in A. were re-analysed using 20 μ g of DNA per track as shown in B. (next page). They contain two or fewer copies. Bands apart from the 1.2 kbp band may represent partial digests.

DR α
EcoRI

NS1 UT

B



←1.2kb

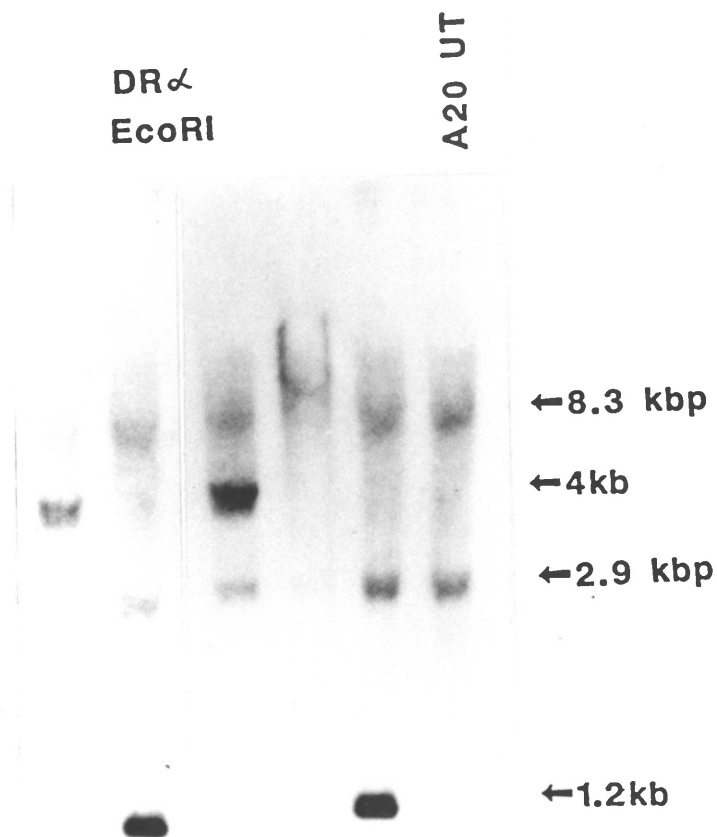


FIGURE 3:5.7

A20 TRANSFECTANTS HAVE INTEGRATED THE DR α GENE

Genomic DNA from untransfected A20 cells (A20 UT) and five A20 transfectants (four positive, one negative for DR4 antigen expression; see Figure 3:5.2) was analysed by Southern blotting. Each track contains 10 μ g of DNA. Two of the four DR4 positive transfectants (A20/DR5, DR11) had the characteristic 1.2 kbp band, two others (DR12, DR17) had a 4 kbp band that may represent a partial digest. The DR4 negative transfectant (DR43) had no DR α integration.

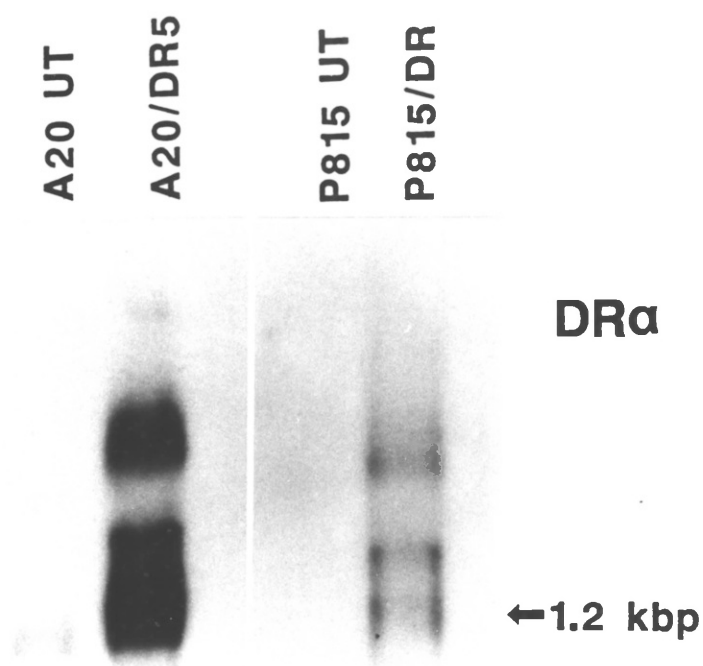


FIGURE 3:5.8

A20 AND P815 TRANSFECTANTS EXPRESS DRα mRNA

Total cellular RNA from one A20 transfectant (A20/DR5) and the P815 transfectant (P815/DR) lines was analysed by Northern blotting as described in section 2:7.2. UT refers to untransfected parent cells. Each track contains 25 µg of RNA.

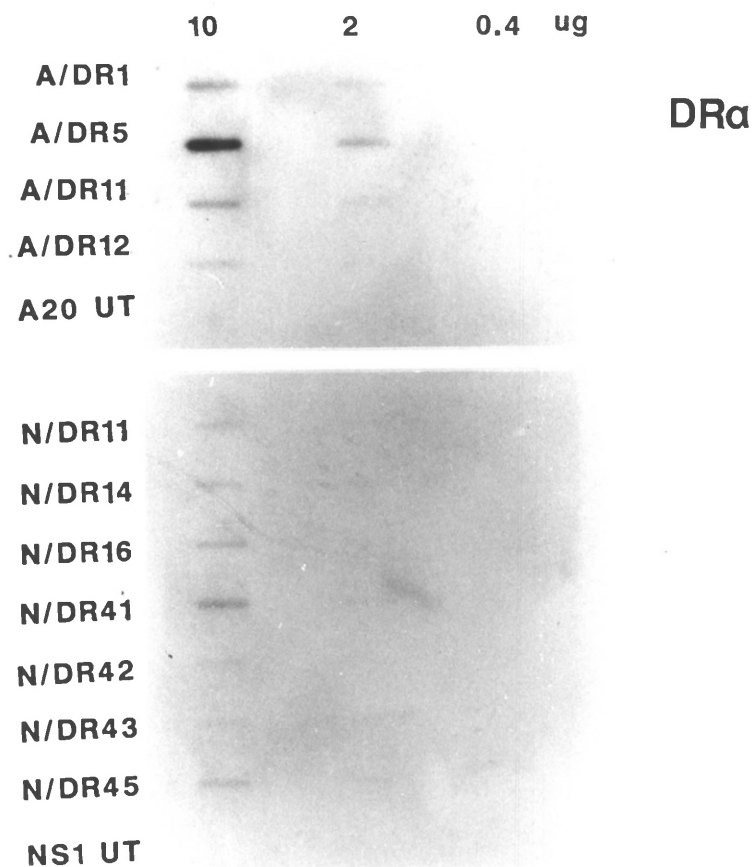


FIGURE 3:5.9

NS1 TRANSFECTANTS EXPRESS DRα mRNA

Total cellular RNA prepared from untransfected parent cells (UT) and A20 and NS1 transfectants was analysed by RNA slot blotting as described in section 2:7.3. Columns contain the indicated amounts of RNA in μg.

CHAPTER 4

GENERAL DISCUSSION

4 GENERAL DISCUSSION

4:1 Summary of main findings and conclusions (Figure 4.1)

A series of murine and human tumour cell lines representing cells 'frozen' in various B lymphocytic differentiation stages was used as a model for the analysis of Class II gene regulation in the B lymphocyte lineage : the Class II antigen phenotypes of these lines corresponded to those of the normal cell types they represent. In these cells, the expression of Class II antigens was correlated with the presence and amount of mRNA for the Class II genes. Messenger RNA was not present in Class II antigen non-expressing cell lines, while cell lines that expressed Class II antigens also expressed mRNA in amounts that corresponded to the level of antigen expression. Thus these tumour cell lines regulate Class II expression at the level of gene transcription.

The mechanism of suppression of Class II gene expression in a BALB/c strain derived plasmacytoma, NS1, was studied in further detail. NS1 cells did not express Class II antigens, or their constituent polypeptide chains, either at the cell surface or intracellularly. The closely related Class I antigens were, however, expressed. Twenty uncloned hybrid cell lines obtained by the fusion of NS1 with Class II expressing splenic B lymphocytes prepared from CBA, SJL and BALB/c strain mice all expressed Class I antigens of either or both parental haplotypes; however none expressed Class II antigens of either haplotype. These results were further validated using one such (NS1 x splenic B lymphocyte) hybrid cell clone, K3. When K3 was fused to the Class II expressing B lymphoma A20, the seven resulting hybrid cell lines were again Class I positive but Class II negative. These results indicate that NS1 cells suppress Class II gene expression by an active, highly specific mechanism.

Evidence was also found that another murine plasmacytoma,

X63, suppressed Class II gene expression by a similar mechanism. One X63 x splenic B lymphocyte hybrid clone, Sp2/0, and two Sp2/0 x splenic B lymphocyte hybrid clones, were found to express Class I but not Class II antigens. This demonstration was less rigorous than in the case of NS1 as only a small number of cloned, rather than uncloned, hybrids were tested.

X63 cells, which expressed neither Class II antigens or mRNA, were similar to other B cell tumours in that they regulated Class II gene expression at the level of transcription. It was therefore surprising that NS1 cells expressed correctly-sized, poly-A tailed transcripts for the Class II genes A α , E α and the associated Ii chain gene in both the nucleus and cytoplasm. The amounts of RNA were equivalent to those in an unfractionated splenocyte population, and approximately one-fifth those in A20 cells. A known inducer of Class II gene transcription, IFN γ , enhanced mRNA levels but did not result in Class II antigen expression. These results imply that NS1 cells suppress Class II gene expression by a novel post-transcriptional regulatory mechanism operating subsequent to RNA processing and nuclear-cytoplasmic transport.

Further strong evidence for post-transcriptional Class II gene regulation was found. NS1 cells were transfected with an expression construct containing 8 copies each of the complete human DR α and DR β gene cDNA sequences under the control of the strong viral SV40 early promoter/enhancer sequences which are transcriptionally active in most eukaryotic cells. None of the seven NS1 transfectants which had integrated the construct expressed DR antigens intracellularly or at the cell surface, although preliminary evidence for DR α gene mRNA expression was found. In contrast, DR antigens were expressed by six of nine transfectants of the Class II expressing A20 cell line; as well as by transfectants of Class II non-expressing P815 cells.

Two important conclusions can be drawn from these results : first, that plasma cells suppress Class II expression by an

active, specific mechanism; and second, that this suppression may operate either transcriptionally or by a novel post-transcriptional mechanism. These conclusions will be examined from two points of view : in relation to the mechanisms that regulate the expression of other genes in plasma cells, and in the context of Class II gene regulation. The major issues that will be discussed are summarized in Figure 4.2.

4:2 The expression of several membrane molecules is down-regulated in plasma cells

In the course of differentiation from resting B cells, plasma cells down-regulate membrane-bound sIg expression while upregulating the capacity to secrete Ig molecules (Alt et al., 1980) and the expression of PC-1 antigen (Anderson et al., 1984). They simultaneously down-regulate the expression of several other integral membrane proteins, including Class II antigens, complement receptors, F_c receptors and the recently described BP-1 antigen (Cooper et al., 1986). This is clearly of physiological relevance as plasma cells represent a terminal, short-lived stage in B cell differentiation and have no important function apart from Ig secretion. A reasonable hypothesis is that these effects are the result of implementation of a genetic differentiation programme. Is there a common mechanism involved ?

There may be. The results presented here indicate that a specific, phenotypically dominant mechanism suppresses Class II expression in plasma cells. A similar mechanism is responsible for the suppression of BP-1 antigen expression : BP-1 expression in pre-B cell tumours is extinguished by fusion with plasmacytomas (Cooper et al., 1986). The mechanism(s) that regulate Ig synthesis may also be phenotypically dominant. Fusion of a sIgM⁺, μ_M and μ_S mRNA expressing B cell lymphoma with the plasmacytoma NS1 results in a sIg⁻, μ_M mRNA⁻ hybrid (Sitia et al., 1987).

4:3 Regulation of Class II gene expression in plasma cells :
transcriptional or post-transcriptional ?

Other workers have now confirmed that plasmacytomas suppress Class II gene expression by an active, specific mechanism that is phenotypically dominant in hybrids using a similar approach of somatic cell hybridization (Latron et al., 1988). They found that the Class II negative phenotype of the murine plasmacytoma P3-U was dominant in hybrids with the human B lymphoblastoid cell line RAJI, and that the hybrids had no human Class II gene transcripts. It must, however, be noted that low levels of mRNA (one-seventh to one-eighth the level in M12, a tumour very similar to A20 (Kim et al., 1979)) for the murine Class II genes were found in P3-U as well as in P3-U hybrids : these results are similar to those obtained with NS1 in this thesis. Latron and co-workers interpret these findings to mean that suppression acts at the level of transcription or accumulation of Class II specific mRNA.

It is certainly true that certain plasmacytomas (X63, as demonstrated here; and a variant of NS1 (Mathis et al., 1983b)) do not express mRNA for the Class II genes : regulation in these cases is unequivocally at the level of gene transcription. However at least two other plasmacytomas (NS1, as presented here; and P3-U) do express similar, low levels of Class II specific transcripts. It is possible to reconcile these findings by hypothesizing that all four plasmacytomas suppress Class II gene transcription, but that in NS1 and P3-U suppression is not total.

However, two observations indicate that Class II gene expression is, additionally, regulated post-transcriptionally in NS1 cells : first, IFN γ upregulates Class II-specific mRNA levels without inducing surface expression of Class II antigens; and more conclusively, DR antigens are not expressed when SV40 early promoter-enhancer controlled DR α/β gene cDNA sequences are

transfected into NS1 cells, despite preliminary evidence that the DRQ gene is transcribed. This suggests that at least in NS1, and perhaps by extension P3-U, regulation operates at two levels : transcriptional as well as post-transcriptional. This possibility is not unprecedented, multiple mechanisms regulate the expression of Ig genes in B cells (reviewed in Cook et al., 1987).

It would be of interest if plasmacytomas that suppress Class II gene expression at the transcriptional level, such as X63, also possessed the (in this case, redundant) capacity to suppress at the post-transcriptional level. Experiments are in progress to determine if the DR gene construct R/RW will direct the synthesis of DR antigens when transfected into X63 cells.

4:4 The mechanism of post-transcriptional regulation in NS1

The exact post-transcriptional level at which Class II gene expression is regulated in NS1 cells is not yet clear. Synthesis of the murine Class II polypeptide chains is not demonstrable by biosynthetic labelling and immunoprecipitation with a polyclonal rabbit antiserum against I-A and I-E antigens of a wide range of haplotypes. Also, no intra-cellular DR antigens are detected in NS1 transfectants by immunofluorescent staining using an anti-DR1, 4, 7, 9 mAb. Do these results mean that Class II gene translation is suppressed ?

This is not necessarily the case. The antiserum and mAb used to detect Class II antigens are known to recognize denatured antigens as well as intact membrane bound antigens : however this does not exclude the possibility that newly synthesized immature polypeptide chains are present, but not bound by these reagents. Alternatively, rapid intracellular degradation may prevent detection. Regulation may therefore also be post-translational.

Translational control is known to occur in several systems. Unfertilized eggs of the surf clam, Spisula solidissima,

translate certain mRNAs much less efficiently than do fertilized eggs (Rosenthal et al., 1980). Mammalian cells which have been heat-shocked increase transcription and favour translation of heat-shock genes while inhibiting the rate of translation of other mRNAs (Kruger and Benecke, 1981; Ballinger and Pardue, 1983). In at least two prokaryotic systems - the E. coli ribosomal protein operons, and R17 phage-coat protein and its RNA - translational control is mediated by the site-specific binding of proteins to a common double-stranded loop secondary structure formed by the RNA, which represses translation (Starzyck, 1987).

Post-translational control also occurs. Adenoviruses of all subgenera apart from A down-regulate HLA Class I antigen expression in infected cells by manufacturing an early glycoprotein, E19, which binds to nascent Class I antigens in the endoplasmic reticulum and impedes their transport to the cell surface by impairing terminal glycosylation (Andersson et al., 1985; Paabo et al., 1986). Structural mutations have also been reported to impede intracellular transport of membrane proteins including murine Class I (Miyazaki et al., 1986), and more recently, Class II antigens (Griffith et al., 1988). These mutations are believed to alter the proper conformation of nascently synthesized polypeptide chains which are essential for normal transport and maturation. Also, specific sequences in the protein may serve as signals for intracellular transport mechanisms (Fitting and Kabat, 1982; Coleman et al., 1985; Munro and Pelham, 1987).

It is important to note that several post-transcriptional mechanisms are known to regulate Ig gene expression in plasma cells. The characteristic increase in Ig secretion with a concomitant decrease in sIg that occurs when B cells differentiate into plasma cells is due to several mechanisms : a reduction in the ratio of μ_M to μ_S mRNA (Alt et al., 1980; Lamson and Koshland, 1984), reduced translational efficiency of μ_M mRNA and impaired transport/enhanced degradation of μ_M

polypeptide in the endoplasmic reticulum (Sitia et al., 1987). The latter two mechanisms, at least, could be involved in Class II regulation in NS1 cells.

4:5 Approaches to determining how post-transcriptional suppression of Class II gene expression works

Additional experiments are still necessary to prove that the NS1 transfectants regulate DR gene expression in a similar manner to the endogenous Class II genes : these have been mentioned earlier (section 3:5.6). Experiments to show that DR transcripts are poly-A tailed and are present in the same amounts as in A20 transfectants are already underway, and additional experiments to study DR mRNA synthesis in detail will be performed.

Once the identity of DR and endogenous Class II gene regulation is demonstrated, it will become possible to take advantage of the fact that many mAb recognizing both intact and denatured DR antigen (for example, Bodmer et al., 1984) are available. Pulse-chase and subcellular fractionation experiments to study the kinetics of DR polypeptide synthesis, intracellular transport, processing and assembly will become possible.

These experiments will help to fix the level of post-transcriptional regulation more accurately. The availability of the DR gene transfectants will make it feasible to perform deletion-substitution analysis of putative regulatory sequences in the DR gene cDNA, focusing on those identified by comparison of the DR α /E α sequences and what is known about sequence requirements for various post-transcriptional regulatory mechanisms.

This work promises to yield interesting information on the mechanisms involved in the regulation of Class II gene expression and cell differentiation in the B lymphocyte lineage.

SUMMARY OF CONCLUSIONS

1. A number of murine and human B cell tumour lines that correspond to different stages of differentiation regulate Class II gene expression at the level of transcription.
2. The plasmacytoma NS1 expresses Class I but not Class II antigens, or constituent polypeptides, either at the cell surface or intracellularly.
3. NS1 suppresses Class II gene expression by an active, specific mechanism. There is limited evidence that a similar mechanism suppresses Class II expression in another plasmacytoma, X63.
4. NS1 cells express correctly-sized, poly-A tailed Class II gene specific transcripts in amounts equivalent to those found in unfractionated splenocytes, and one-fifth of those found in the B lymphoma A20. This suggests that suppression of Class II expression is mediated by a post-transcriptional mechanism. that operates subsequent to RNA processing. In contrast X63 does not express Class II gene transcripts.
5. DR antigens are not expressed when DR alpha/beta gene cDNAs controlled by SV40 promoter/enhancer sequences are transfected into NS1 cells; although there is preliminary evidence that the genes are transcribed. This provides further strong evidence that a novel post-transcriptional mechanism suppresses Class II gene expression in NS1 cells. Further studies to characterise this mechanism are in progress.

FIGURE 4.1

PERSPECTIVES

1. Is the down-regulation of several integral membrane molecules with concomitant enhancement of Ig secretion in plasma cells part of a differentiation programme mediated by common molecular mechanism(s) ?
2. Do the mechanisms that suppress Class II expression in plasma cells operate transcriptionally as well as post-transcriptionally ?
3. How does post-transcriptional suppression of Class II gene expression work in NS1 cells ?

FIGURE 4.2

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APPENDIX

Ashok R. Venkitaraman⁺,
Eric J. Culbert[○] and
Marc Feldmann

Immunology Unit, The Charing Cross
Sunley Research Centre, London and
Immunoregulation Section, Corporate
Biosciences Group, Imperial Chemical
Industries Plc.[○], Cheshire

A phenotypically dominant regulatory mechanism suppresses major histocompatibility complex class II gene expression in a murine plasmacytoma

The expression of major histocompatibility complex (MHC) class II antigens is down-regulated when B cells differentiate into plasma cells. We have studied the mechanism of down-regulation of MHC class II expression in a BALB/c strain-derived murine plasmacytoma cell line, NS1. NS1 cells express MHC class I antigens but not MHC class II antigens. We tested 20 uncloned hybrid cell lines obtained from the fusion of NS1 cells with MHC class II-expressing splenic B cells prepared from CBA, SJL or BALB/c mice. All the hybrid cell lines expressed MHC class I antigens of either or both parental haplotypes but did not express MHC class II. One NS1 × splenic B cell hybrid clone, K3, was used to further validate these results; K3 cells expressed MHC class I but not MHC class II antigens. K3 was fused to the MHC class II-expressing B lymphoma A20, and the seven resulting hybrid cell lines were again found to express MHC class I but not MHC class II antigens. Since NS1 is a subclone of the P3-X63Ag8 murine plasmacytoma, we also tested one P3-X63Ag8 × splenic B cell hybrid, Sp2/0, and two Sp2/0 × splenic B cell hybrids. All were found to express the appropriate MHC class I antigens but did not express MHC class II. Thus, our results suggest that the NS1 plasmacytoma suppresses MHC class II expression by a phenotypically dominant regulatory mechanism. We found that NS1 cells express correctly sized mRNA for the MHC class II genes A α , E α and the invariant chain. The co-expression of MHC class I protein and I-A and I-E region gene transcripts provides strong evidence that the MHC gene cluster is structurally intact, and that lack of class II expression is due to a genetic regulatory mechanism. The amounts of class II mRNA expressed by NS1 cells were at least equivalent to those found in splenic lymphocytes. Therefore, this regulation must operate post-transcriptionally.

1 Introduction

The major histocompatibility complex (MHC) encodes two major groups of cell-surface proteins, the MHC class I and MHC class II antigens [1]. The MHC class I antigens are expressed in most nucleated cell types and appear to function as target restriction molecules for cytotoxic T lymphocytes. In contrast, the MHC class II antigens have a highly restricted distribution, being found primarily on cells of the immune system: macrophages, dendritic cells, B lymphocytes and thymic epithelial cells.

In the B cell lineage, MHC class II expression is related to the stage of differentiation. Thus, murine pre-B cells are MHC class II negative, mature B cells express MHC class II at high levels and plasma cells are either MHC class II negative or express at very low levels [2]. The B cell lineage thus provides a unique model for the study of MHC class II regulation.

Most data on the mechanism of MHC class II regulation by B cells has come from work on nonexpressing null variants

derived from MHC class II-positive Epstein-Barr virus-transformed human B cell lines [3]. This work, and that of other groups [4], indicates that MHC class II gene transcription requires the presence of one or more trans-acting genetic elements that have the effect of “permitting” expression. Presumably they encode proteins that bind to specific sequences in the gene promoter. MHC class II-negative mutants lack this “permissive” trans-acting element, which can be restored by somatic cell fusion with normal human or murine B cells. Segregation analysis of a series of human-mouse fusions has allowed the assignment of one such trans-acting element to murine chromosome 16 [5].

Lack of MHC class II expression by plasma cells could therefore be due to lack of a “permissive” element or alternatively to the presence of a “suppressive” one. There is evidence that target sequences for such “suppressive” regulatory elements may exist. Studies on a human class II DQ β gene construct transfected into fibroblasts [6] indicate that the deletion of an upstream flanking sequence increases transcription of the gene. These upstream sequences are highly conserved between human and murine class II genes [7]. We hence studied an MHC class II nonexpressing cell line, the murine plasmacytoma NS1, in order to detect negative regulatory elements, if any, that suppress MHC class II expression.

We report here that NS1 cells suppress the expression of MHC class II but not class I proteins by a phenotypically dominant regulatory mechanism. Surprisingly, correctly sized mRNA for the class II genes A α , E α and the invariant chain (Ii) was present in at least normal amounts. Thus, the mechanism of this suppression is post-transcriptional rather than transcriptional.

[I 6103]

⁺ Research Fellow of the Lady Tata Memorial Trust for Research on Leukemia, London.

Correspondence: Ashok R. Venkitaraman, Charing Cross Sunley Research Centre, Lurgan Avenue, London W68LW, GB

Abbreviations: MHC: Major histocompatibility complex A α : I-A α chain E α : I-E α chain Ii: Invariant chain HAT: Hypoxanthine, aminopterin, thymidine HT: Hypoxanthine, thymidine mAb: Monoclonal antibody(ies)

2 Materials and methods

2.1 Cells

P3-NS1/1A1g4.1 (NS1) is a murine plasmacytoma cell line cloned from P3-X63Ag8, which is derived from the mineral oil-induced MOPC-21 plasmacytoma of the BALB/c mouse strain [8]. NS1 cells are sensitive to hypoxanthine, aminopterin, thymidine (HAT) selective medium. The NS1 cells used in these experiments were originally obtained from the American Type Culture collection (ATCC, Bethesda, MD) and maintained in our laboratory.

Single-cell suspensions of splenic lymphoid cells were prepared by teasing organs through sterile nylon mesh, followed by thorough washing. Red blood cells were removed by treatment with Tris-ammonium chloride lysis buffer.

A20 is a murine B cell lymphoma cell line derived from another mineral oil tumor of BALB/c mice [9]. A HAT-sensitive, ouabain-resistant mutant of this cell line kindly provided by Dr. B. Chain was used in these experiments.

K3 a cloned hybrid cell line derived from a NS1 × CBA strain splenic cell fusion was provided by Dr. A. Feinstein. These cells are resistant to HAT selective medium.

Sp2/0-Ag14 (Sp2/0), a nonsecreting murine myeloma, is a cloned hybrid cell line derived from the fusion of P3-X63Ag8 with BALB/c strain splenic cells [10]. Sp2/0 is HAT sensitive and is used as a fusion partner for producing murine hybridomas. Two cloned hybridoma cell lines from a Sp2/0 × BALB/c strain splenic cell fusion, HB124 and HB125 [11], were also obtained from the ATCC.

All cells were maintained in complete growth medium (RPMI 1640 containing 25 mM HEPES and 2 mM L-glutamine (Gibco Europe, Uxbridge, Middlesex, GB) supplemented with 10% fetal calf serum (Gibco).

2.2 Somatic cell fusion

NS1 × splenic cells: approximately 10^7 NS1 cells from a culture in the log phase of growth were fused with approximately 10^8 splenic lymphocytes. The cells were washed free of serum, and pelleted together at $500 \times g$. All supernatant fluid was carefully aspirated and 1 ml of 50% polyethylene glycol [$M_r = 1500$; Sigma Chemical Co., Poole, Dorset, GB) in RPMI 1640 (v/v)] warmed to 37°C was slowly added. The cells were gently resuspended and incubated for 6 min at 37°C . Twenty-five ml of complete growth medium with 20% fetal calf serum warmed to 37°C was then gently added to the cell suspension. The fusion mixture was plated out at 5×10^5 – 10×10^5 cells/ml in 24-well culture plates. After 24 h incubation at 37°C , growth medium was removed and HAT selective medium (complete growth medium containing $100 \mu\text{M}$ hypoxanthine, $16 \mu\text{M}$ aminopterin and 100 nM thymidine; Sigma Chemical Co.) was added. The cells were maintained in HAT selective medium until the growth of HAT-resistant hybrid cells was obvious (usually 8–12 days after fusion). The hybrid cell lines were then grown for 3–4 days in HT selective medium (HAT medium without aminopterin), and finally expanded in complete growth medium. Aliquots of

these uncloned hybrid cell lines were preserved in liquid nitrogen 15–25 days after fusion.

A20 × K3: fusions were performed in the manner described above except that the selective medium used was HAT medium containing 10 mM ouabain (Sigma Chemical Co.).

2.3 Monoclonal antibodies (mAb)

The following mAb against the murine MHC antigens were obtained from Prof. G. Hämmerling: H100-5.28 (anti- K^k, D^k), K9-18 (anti- K^d, D^d), T19.191 (anti- K^d, L^d), 13/4 (Anti- E^k, E^d). In addition the following were obtained from the ATCC: HB3 (anti- A^d), HB6 (anti- E^k), TIB92 (anti- A^k) and HB75 (anti- D^d).

2.4 Fluorescence-activated cytometry

The expression of cell-surface MHC molecules was assayed by fluorescence-activated cytometry on the FACS cell sorter (Becton Dickinson Immunocytometry Systems, Mountain View, CA) using standard techniques [12]. Single-cell suspensions were stained with mAb to the murine MHC antigens followed by fluorescein-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). Cells stained with fluorescein-labeled goat anti-mouse IgG alone were used as negative controls in each experiment.

2.5 Radioimmunoassay

The expression of cell-surface MHC molecules was also tested by radioimmunoassay. Single-cell suspensions were incubated with mAb to the MHC antigens for 1 h at 4°C , washed, and 50 000 cpm of ^{125}I -labeled protein A (Amersham Corp., Amersham, GB) was added. After a further hour of incubation, the cell suspensions were washed and the bound radioactivity was counted on a Minigamma γ -counter (LKB Instruments, Croydon, Surrey, GB). Murine mAb to the β chain of ricin was used as the negative control in these assays.

2.6 RNA preparation and analysis

Total cellular RNA was prepared by cell lysis in 4 M guanidinium isothiocyanate, 0.1 M 2-mercaptoethanol followed by CsCl density gradient ultracentrifugation [13]. Nuclear RNA fractions were separated by lysing cells gently in 0.5% Nonidet-P40 (NP40), followed by centrifugation to separate nuclei. The nuclear pellet was washed, lysed in guanidinium isothiocyanate and RNA isolated as before. The cytoplasmic fraction of the NP40 lysate was used to prepare cytoplasmic RNA.

Five to twenty μg of purified RNA was separated by electrophoresis in a 1% denaturing agarose gel, blotted onto nitrocellulose membrane filters (Schleicher and Schuell, Dassel, FRG) and hybridized with nucleic acid probes as described below. RNA slot blots were prepared on nitrocellulose membrane filters using a Schleicher and Schuell Minifold apparatus.

2.7 Nucleic acid probes and hybridization

Nucleic acid probes for the murine class II genes A α (pCDN17), E α (pCA39) were obtained from Dr. B. Malissen, Centre d'Immunologie, Marseille, France, and for the Ii chain (pIi-1) from Dr. N. Koch, German Cancer Center, Heidelberg, FRG. pCDN17 contains a 1050-bp cDNA insert comprising the entire A α -coding sequence [14]. pCA39 contains a 3.4-kbp genomic fragment with the E α chain α 1, α 2, TM and CY domain exons [15]. pIi-1 contains a 920-bp cDNA insert comprising the entire Ii-coding sequence together with an approximately 450 bp 3' noncoding region [16]. These probes were labeled to a specific activity of 10^8 to 10^9 cpm/ μ g of DNA with ^{32} P-labeled dCTP (Amersham) by the random oligo-priming method. Filter hybridizations were performed using 10^6 cpm/ml of labeled probe in 50% formamide, $5 \times$ SSC, $2.5 \times$ Denhardt's solution, 50 mM phosphate buffer and 500 μ g/ml spermidine at 43°C for 18–24 h. The filters were washed to a stringency of $0.1 \times$ SSC at 50°C for 30 min and exposed to X-ray film (NIF Rx, Fuji Photo Film Co., Tokyo, Japan) at -70°C .

3 Results

3.1 NS1 cells do not express MHC class II antigens

Surface MHC antigen expression on NS1 cells was determined by fluorescence-activated cytometry. As shown in Fig. 1, NS1 cells bind antibody against K ^{d} but not K ^{k} molecules. The level of anti-A ^{d} and anti-E ^{k} ,E ^{d} binding was consistently slightly greater than the level of anti-A ^{k} and fluoresceinated anti-murine IgG binding. This might reflect extremely low levels of MHC class II expression by NS1 cells. As shown in Fig. 2a, the level of MHC class I expression by NS1 is approximately half that of uninduced splenic cells. In contrast the level of MHC class II expression, if any, by NS1 is less than 1/200th that of splenic cells. These results indicate that NS1 cells express MHC class I but little or no MHC class II antigens.

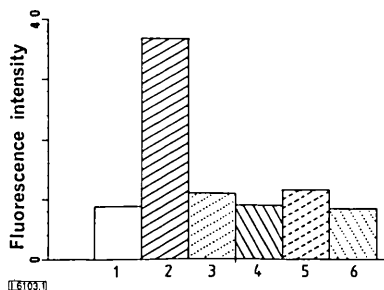


Figure 1. NS1 cells express MHC class I but not MHC class II antigens. NS1 cells were stained with mAb to K ^{k} (H100) (Bar 1), K ^{d} (T19.191) (Bar 2), E ^{k} ,E ^{d} (13/4) (Bar 3), A ^{k} (TIB92) (Bar 4) and A ^{d} (HB3) (Bar 5) and analyzed by fluorescence-activated cytometry. Bar 6 shows the negative control (fluorescein-conjugated goat anti-mouse Ig). A total of 10000 cells were analyzed per sample, and appropriate gates were set to exclude dead cells and cellular debris. A histogram of fluorescence intensity to relative cell number was made, and the mean intensity calculated using the B/DIS Consort 30 computer program. Fluorescence intensities are expressed in channel numbers. Standard deviations were $<10\%$ of the mean and are not indicated.

Table 1. MHC antigen expression by NS1 $\times$ splenic B cell hybrids

Fusion (No. of hybrids)		Percentage of hybrids expressing		
		one parental class I	both parental class I	either parental class II
NS1 $\times$ CBA	(11) ^a	82	9	0
NS1 $\times$ SJL	(5)	60	40	0
NS1 $\times$ BALB/c	(4)	100	^b	0

a) One hybrid expressed no MHC class I or MHC class II.

b) Both parents are of the same haplotype.

3.2 NS1 $\times$ splenic B cell hybrids do not express MHC class II antigens

Uncloned hybrid cell lines obtained from the fusion of NS1 cells with splenic B cells were analyzed for surface MHC antigen expression (Table 1). Hybrids obtained with CBA (H-2 ^{k} haplotype), SJL (H-2 ^{s} haplotype), and BALB/c (H-2 ^{d} haplotype) spleen cells expressed MHC class I antigens of

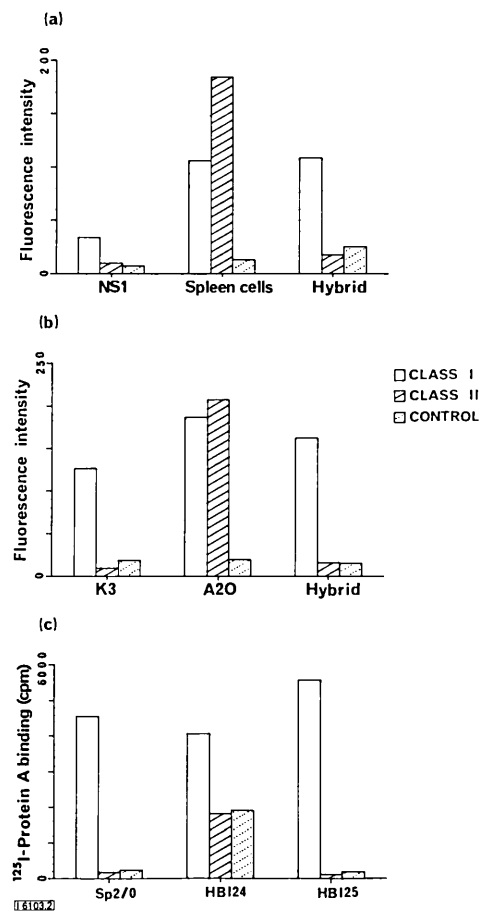


Figure 2. NS1 $\times$ splenic cell hybrids. K3 $\times$ A20 hybrids and Sp2/0 hybrids express MHC class I but not MHC class II antigens. The results obtained with one representative uncloned NS1 $\times$ SJL strain splenic cell hybrid line are shown in (a), (b) shows one representative K3 $\times$ A20 hybrid line. Assays were performed as described in Fig. 1. For (c) cells were stained with mAb and analyzed by radioimmunoassay as described in Sect. 2.5. Results are expressed as means of duplicate experiments.

both parental haplotypes. However, none of them expressed MHC class II antigens of either parental haplotype. Fig. 2a shows the results obtained with one representative hybrid.

In order to ensure that the cells being screened were hybrid cells, we measured the DNA content of asynchronous cultures by propidium iodide staining and fluorescence-activated cytometry using a standard procedure [17]. We found that the fluorescence peak corresponding to DNA content in the G₀/G₁ cell cycle stage is shifted to the right in hybrid cell lines in comparison to the parent cells, indicating that the hybrids are indeed polyploid (data not shown). We also tested Sp2/0 and two Sp2/0 × splenic cell hybrids for MHC expression. As shown in Fig. 2c, all of these cell lines expressed MHC class I but not MHC class II antigens.

3.3 K3 × A20 hybrids do not express MHC class II antigens

One NS1 × splenic B cell hybrid, K3, was used to further test the validity of these results. K3 has an MHC class I-positive, MHC class II-negative phenotype (Fig. 2b). We analyzed hybrids of K3 with the MHC class II-expressing B lymphoma A20 (Fig. 2b) for surface MHC antigen expression. Of the seven uncloned hybrid cell lines obtained in this experiment, all expressed the appropriate MHC class I antigens, but lacked MHC class II antigen expression. Results for one representative hybrid are shown in Fig. 2b.

3.4 NS1 expresses mRNA specific for the MHC class II genes

Northern blot analysis of nuclear and cytoplasmic RNA fractions from NS1 cells (Fig. 3) revealed the expression of mRNA specific for the MHC class II genes A α , E α and the Ii chain. Transcripts were expressed in both the nuclear and cytoplasmic fractions, although levels were higher in the cytoplasm. The level of RNA expression in NS1 cells was compared with that in unfractionated splenic cells prepared from BALB/c mice using the slot blot technique. After hybridization with probes for the class II genes (Fig. 4) the autoradiographs were scanned with a Joyce-Loebl (Gateshead, Tyne-

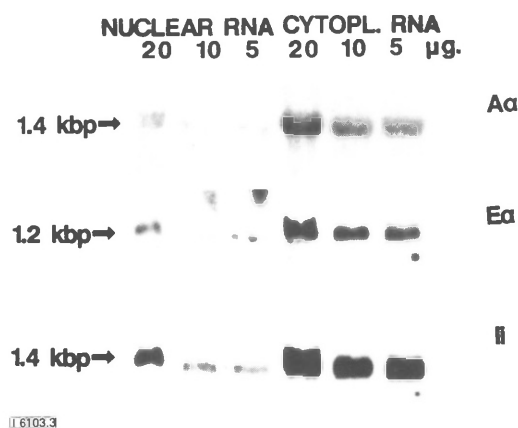


Figure 3. NS1 cells express RNA specific for the A α , E α and invariant chains. Nuclear and cytoplasmic RNA fractions prepared from NS1 cells were size-separated on a denaturing 1% agarose gel, blotted onto nitrocellulose membranes and probed with ³²P-labeled A α , E α and Ii chain probes. Lanes contain the indicated amount of RNA.

and-Wear, GB) Chromoscan 3 densitometer to quantitate the amount of radioactivity in each slot. The slot blots were then stripped free of radioactivity, reprobed with 7B6 (Fig. 5, a probe which has been shown to detect an RNA species that does not fluctuate during the cell cycle [18]), and scanned. The amount of class II-specific RNA in each slot was then standardized with respect to 7B6 in order to eliminate any differences in the total amount of RNA in each slot. The level of expression of MHC class II RNA in NS1 cells was at least equivalent to that in splenic cells when quantitated in this way (data not shown).

4 Discussion

NS1 cells express little or no MHC class II antigen at the cell surface. This might be due to a genetic regulatory mechanism,

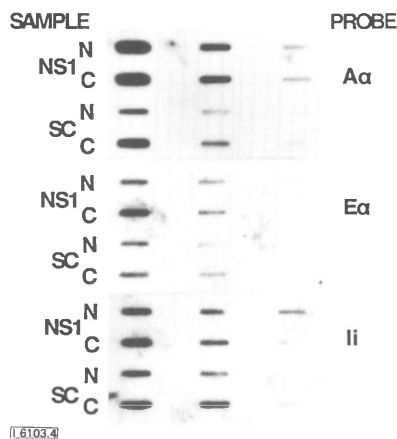


Figure 4. NS1 cells express RNA for the class II genes A α , E α and invariant chain. Slot blots were made using 5, 1 and 0.2 μ g of RNA in the respective columns. The rows contain RNA prepared from NS1 nuclei (N), NS1 cytoplasm (C), splenic cell (SC) nuclei and splenic cell cytoplasm. These slot blots were probed with ³²P-labeled A α , E α and Ii probes and the amount of radioactivity quantitated by scanning the autoradiographs on a densitometer.

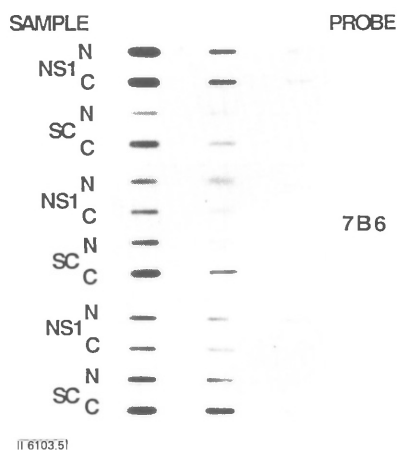


Figure 5. Control hybridizations with 7B6 probe. Slot blots shown in Fig. 4 were stripped of radioactivity, reprobed with the 7B6 probe (which detects an RNA species that does not vary during the cell cycle), and the amount of radioactivity quantitated by scanning the autoradiographs on a densitometer. The amount of RNA in each slot was standardized using the intensity of the 7B6 hybridization signal (see text).

such as the lack of a “permissive” regulatory element or the presence of a “suppressive” one. Alternatively, it might be the result of mutations or deletions in the MHC class II structural genes or promoters, since NS1 is a tumor cell that has been maintained for many generations *in vitro*. We fused NS1 cells with splenic cells of several different H-2 haplotypes to distinguish these possibilities. Splenic cells contain a large number of B lymphocytes that express MHC class II antigens at high levels (Fig. 2a), and NS1 is believed to form stable hybrids mainly with such cells. If lack of MHC class II expression by NS1 was simply due to alterations in its genes or promoters, it would be expected that the hybrid cells obtained *would* express MHC class II antigens derived from the normal parental cell. If lack of expression was due to the lack of a “permissive” regulatory element, this deficiency would be corrected by fusion with an expressing cell type, and the hybrid cells obtained *would* be expected to express MHC class II derived from *both* parental cells.

However, fusion of NS1 cells with splenic cells prepared from mice of different H-2 haplotypes consistently yielded hybrids that did not express MHC class II derived from *either* parental cell, although MHC class I derived from *both* parental cells was expressed. This implies that NS1 actively suppresses MHC class II expression by a phenotypically dominant genetic regulatory mechanism. The regulatory mechanism is specific for the class II genes as the hybrid cells obtained from such fusions did express MHC class I.

We further tested the validity of this conclusion by fusing a cloned NS1 $\times$ splenic B-cell hybrid (K3) with a MHC class II-expressing B lymphoma cell clone (A20). Again, all the uncloned hybrid cell lines obtained were MHC class II negative, but did express the appropriate MHC class I antigens.

Since NS1 cells are derived from the P3-X63Ag8 myeloma, we tested Sp2/0, a P3-X63Ag8 $\times$ BALB/c splenic cell hybrid, for MHC expression. Like NS1 hybrids, Sp2/0 expressed MHC class I but not MHC class II antigens. Further, two hybrids of Sp2/0 with splenic B cells were also MHC class I positive but MHC class II negative. These results suggest that the P3-X63Ag8 cell line suppresses MHC class II expression in a similar manner to NS1.

Thus, these results clearly indicate that NS1 suppresses MHC class II gene product expression by a specific genetic regulatory mechanism. We proceeded to study this mechanism at the molecular level.

Surprisingly, NS1 cells were found to express mRNA specific for the MHC class II genes A α , E α and the Ii chain. The transcripts were of the same sizes as those found in class II-expressing cell types. The presence of these transcripts provides further strong evidence that the class II genes are structurally intact in NS1 cells, and that the lack of expression of class II antigens is due to a regulatory mechanism. The absence of restriction fragment length polymorphisms between the MHC class II genes of NS1 and splenic cells derived from BALB/c mice (Venkitaraman, unpublished data) supports this conclusion.

Our results also show that the amount of mRNA for the A α , E α and Ii genes found in NS1 cells is not very different from the amount in MHC class II antigen expressing splenic cells. This indicates that NS1 suppresses class II expression by a

post-transcriptional mechanism, in contrast to previous studies on the regulation of the murine MHC class II genes [19] which show that most regulatory factors affect the rate of gene transcription.

Two interesting parallels exist. First, plasmacytomas regulate Ig gene expression by a post-transcriptional mechanism. It has been found that the amount of Ig heavy and light chain mRNA in the cytoplasm of plasmacytomas is typically 50 times higher than in B cell lymphomas, although the rate of gene transcription is not changed (J. O. Mason and M. S. Neuberger, personal communication). Thus, this mechanism enhances rather than suppresses gene product expression. Second, the expression of several B lymphocyte surface proteins including complement receptors, Fc receptors and the BP-1 antigen is suppressed when they differentiate into plasma cells. BP-1 antigen expression in pre-B cell tumors has recently been shown to be extinguished by somatic cell fusion with plasmacytomas [20], showing that it is suppressed by a phenotypically dominant mechanism. It is not known at what level of gene expression this mechanism operates.

MHC class II-expressing cells can activate antigen-specific T lymphocytes, and this activation can be completely blocked by mAb to the appropriate MHC class II molecules. Thus, MHC class II expression is crucial to antigen presentation, the first step in any immune response. Interferon- γ or other lymphokines can induce aberrant expression of MHC class II antigens on epithelial cells, which is believed to be a factor in the induction of autoimmune disease [21]. Maintenance of the restricted tissue distribution of expression of the MHC class II gene must therefore be of central importance to normal immune function. Our work identifies a phenotypically dominant regulatory element that suppresses class II gene product expression in a plasmacytoma. Similar regulatory elements may be important in maintaining the tissue specificity of MHC class II gene expression in other cell types.

We thank Drs. B. Chain, A. Feinstein, G. Hämmerling, B. Malissen and N. Koch for supplying cells and reagents. We are grateful to Dr. M. Kahan for FACS analysis, and to Prof. G. Dawes and C. Hewitt for comments on the manuscript.

Received April 9, 1987; in revised form July 14, 1987.

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