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A. The Search for a Chicken Major Histocompatibility Complex II Alpha Gene
B. Transformation-Related Viral Transcripts in Marek's Disease Virus-Transformed Cell Lines

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A. THE SEARCH FOR A CHICKEN MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II ALPHA GENE

B. TRANSFORMATION-RELATED VIRAL TRANSCRIPTS IN MAREK'S DISEASE VIRUS-TRANSFORMED CELL LINES

By

Joanne Kivela Tillotson

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ABSTRACT

A. THE SEARCH FOR A CHICKEN MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II ALPHA GENE.

B. TRANSFORMATION-RELATED VIRAL TRANSCRIPTS IN MAREK'S DISEASE VIRUS-TRANSFORMED CELL LINES.

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A. Because the chicken major histocompatibility complex (MHC) has been implicated in disease resistance, studies of the genes were initiated using molecular cloning techniques. Since the RP-9 B-lymphoblastoid cell line is positive for surface MHC Ia antigens, it was used a source for the isolation of cDNA clones which hybridized at low stringency with a human DQ alpha cDNA. Sequence analysis of these cDNA clones, however, revealed that they did not code for MHC proteins, but did represent multiple, tandemly-repeated copies of a 10 base pair sequence of chicken highly repetitive DNA. It was concluded that there is no DQ alpha gene expressed in the chicken which contains regions that are even 70% identical to the human DQ alpha gene.

B. Historically, the avian disease of most economic impartance to the poultry industry has been Marek's disease, a T-lymphoblastoid tumor induced by a herpesvirus, Marek's disease virus (MDV). Although the disease has been well characterized and vaccines developed, little is known about the mechanism of viral transformation. Toward this end, established tumor cell lines were studied to determine their transcription of RNAs from various regions of the viral genome. A

region of the viral genome from the repeats flanking the long unique sequences was identified which is transcribed in all tumors and tumor cell lines tested, and at higher levels than in productively infected cells. These RNA transcripts appear to be spliced to a region of DNA which may be altered in the tumor cells, compared to its configuration in DNA obtained from productively infected cells. Further data will be required to define the relationship between the transformed state, the viral transcripts, and the DNA configuration.

This dissertation is dedicated to

Mark

and Robin;

their patience and love have supported

me throughout these studies.

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Chapter A: The search for a chicken major histocompatibility complex Class II alpha gene.

The major histocompatibility complex (MHC) is a cluster of genes which are involved in controlling a number of critical immune functions of vertebrates. The majority of the known genes within the MHC are included in the groups referred to as Class I and Class II. These classes code for highly polymorphic, cell-surface proteins, and are members of the immunoglobulin supergene family (Steinmetz and Hood, 1983.) The MHC antigens consist both of domains which are characteristically "variable" or polymorphic, and also constant domains; it is the constant domains which have strong sequence homology to the immunoglobulins (Hood et al, 1985.) Each domain is usually encoded by a single exon, and follows the general size and structural characteristics of the immunoglobulin family, including the conserved locations of the disulfide bonds (Flavell et al, 1986.)

The Class I and II antigens are transmembrane proteins which must be recognized in conjunction with foreign antigens in order for the T cell receptor to recognize the antigen and mount an effective immune response (Malissen, 1986.) Cytotoxic T lymphocytes (CTL) normally lyse cells expressing a "foreign" antigen on the cell surface, in an attempt to destroy tumor or virus-infected cells, but they can only recognize that antigen in the context of the appropriate MHC Class I molecule (Zinkernagel and Doherty, 1979.) Apparently, some CTLs can also recognize a "foreign" MHC Class I antigen and attack any cell expressing that antigen. This strong reaction is responsible for the rejection of tissue transplants (Zaleski et al, 1983,) and Class I antigens are also

the traditional transplantation antigens which are responsible for the general name "major histocompatibility complex."

On the other hand, helper T lymphocytes can stimulate B cells to produce antibody to a foreign antigen only if that antigen is recognized by the helper T cell in the context of the correct Class II MHC antigen (Flavell et al, 1986.) These antigens are normally expressed only by antigen-presenting cells such as monocytes and macrophages, and by B cells (Kaufman et al, 1984,) but their presence can be induced on other cell types by many factors, including interferon and probably some growth factors (Flavell et al, 1986.)

Both the human (HLA) and the mouse (H-2) major histocompatibility complexes have been extensively studied. In both, there are at least three Class I loci expressed, and two or three distinct Class II protein products (each heterodimers encoded by two MHC genes.) All of these antigens exhibit considerable polymorphism--as many as 50-60 alleles being known for some Class I loci, and 25-40 alleles for some of the Class II loci (Zaleski et al, 1983.) This polymorphism is one of the most unique characteristics of the MHC. The specific arrangement of alleles at each locus is collectively known as the haplotype; with multiple loci and many alleles available at each locus, the number of haplotypes possible within a species is huge. This large number of haplotypes apparently confers selective advantage upon species in their ability to recognize foreign antigens and effectively resist disease-causing organisms (Longenecker and Mosmann, 1981.) This polymorphism is apparantly maintained by a combination of mechanisms (Quddus et al,

1986): pseudogenes exist in the MHC which have a high rate of mutation and appear to be a reservoir of genetic material used in gene conversion mechanism, which is widely recognized to occur in the MHC (Arden and Klein, 1982; Weiss et al, 1983; Klein, 1984; Miyada et al, 1985; Jaulin et al, 1985), recombinational hot spots occur at various places in the MHC (Steinmetz et al, 1986; Kobori et al, 1986), and unequal crossing over has apparently occured in some species, but its role is still not well understood (Steinmetz and Hood, 1983.)

The major histocompatibility complex of the chicken, the B-complex, like the mammalian MHC, is composed of a series of genes which control certain immune functions. It is located on the microchromosome which also contains the ribosomal RNA gene cluster and the nucleolar organizer region (Bloom and Bacon, 1985; Muscarella et al, 1985.) The B-complex codes for at least three antigens, B-F, B-L, and B-G, which have been characterized and compared with both the structure and function of the well-studied mammalian MHC gene products (Figure A1; Hala et al, 1981a; Toivanen and Toivanen, 1983.) The B-G antigen does not have a mammalian homolog (Hala et al, 1981b): its unique nature may reflect its presence on erythrocytes, which are nucleated in avian species. The B-F and B-L antigens appear to represent the chicken MHC Class I and II molecules, not only in structure (Ziegler and Pink, 1975,) but also in tissue distribution and in function (Hala, 1981a). Like the mammalian MHC, the genes are clustered, but appear to undergo less frequent recombination in the chicken (Koch et al, 1983; Skjodt et al, 1985.) Thus, the

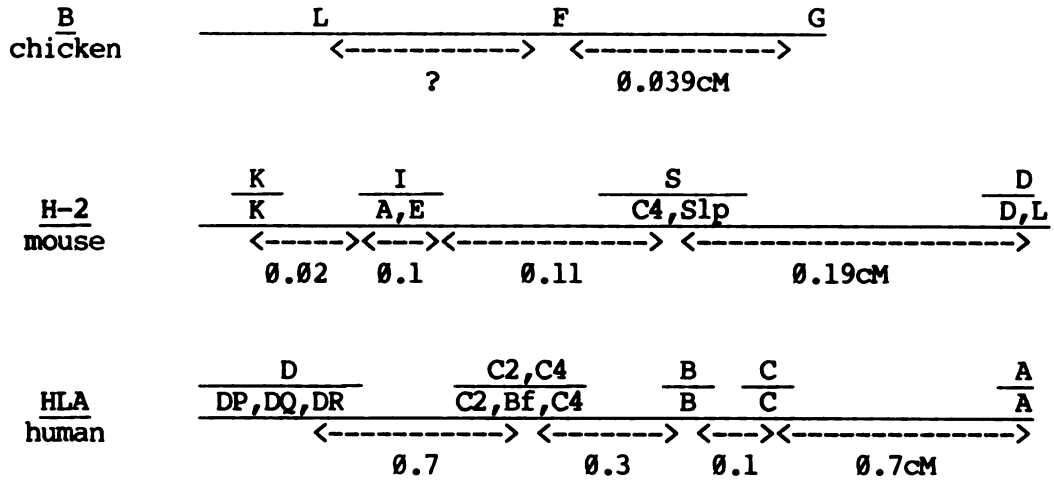

Figure A1: Genetic maps of avian, murine, and human MHC.

Recombinational distances between loci are shown; multiple antigens coded within each locus are shown for mouse and human (adapted from Hala et al, 1981a.)

Class	Chicken	Mouse	Human	Cellular distribution	Molecular composition
I	F	K,D,L	A,B,C	All nucleated cells	about 40kD, associates with beta-2-microglobulin
II	L	A,E	DR,DQ, DP	B cells, monocytes macrophages	heterodimer, each about 30kd
III	?	Ss,Slp	Bf,C2,C4	secreted complement components	
IV	G	?	?	erythrocytes	heterodimer, 31 and 42 kD

Figure A2: Characterization of MHC subloci and molecular composition.

complex genetic maps constructed for the murine and human MHCs have not been duplicated in the chicken (see Figure A2,) and assignment of the MHC functions to subregions or specific antigens has assumed parallelism between mammalian and avian MHC structure-function relationships. Hala, 1981a.)

More precise understanding of both the individual antigens and their functions would be beneficial in studies of immune function in various avian diseases (Bacon, 1987.) For example, obese strain (OS) chickens provide a useful model for studying human autoimmune thyroiditis, and an MHC influence has been shown (Bacon et al, 1974; Bacon et al, 1977.) Whether information obtained from this model will also be applicable to other autoimmune disorders has yet to be shown. Connections between the MHC and susceptibility to coccidiosis (Ruff and Bacon, 1984; Clare et al, 1985) and fowl cholera (Lamont et al, 1987) have been postulated.

Some of the most striking disease correlations have investigated the role of the MHC in virus-induced neoplasms. Marek's disease is an economically important T-cell lymphoma caused by a herpesvirus. Resistance to lymphoma development (but not virus infection) is associated with certain B-haplotypes (Briles et al, 1977; Bacon et al, 1981, Briles et al, 1983; Bacon et al, 1983.) Rous sarcoma virus is a laboratory retrovirus which contains the src oncogene and causes tumor development at the site of injection within two weeks, regardless of B-type. However, the B-haplotype governs the fate of the tumor, with complete regression occurring in some haplotypes, but continued tumor growth in other B-types until host death occurs (Bacon et al, 1981;

Plachy and Benda, 1981, Bacon et al, 1983, Plachy et al, 1984.) The long latency tumors developing as a result of ALV infection and subsequent oncogene activation by promotor insertion vary in type depending upon the cell type and the identity of the activated oncogene. There is a B-haplotype influence upon the virus spread (Bacon et al, 1987), and perhaps also upon the tumor development or progression (Bacon et al, 1981; Bacon et al, 1983.)

None of these disease correlation studies has been able to fully understand mechanisms by which the MHC influence is mediated, or to attribute disease resistance functions to either the Class I or II subregions of the B-complex because of the lack of knowledge about the molecules in the chicken MHC and the scarcity of recombinant haplotypes. Strong linkage disequilibrium of the chicken MHC genes is indicated by the existence in field strains of chickens of haplotypes which consistently pair the same sets of alleles at each locus within the haplotype; that is, L2 is always seen with F2 and G2, etc. This is confirmed by numerous studies which have attempted to identify MHC recombinants within large groups of birds which have been typed serologically. A few recombinants have been identified separating B-G from the other two loci, but the B-F and B-L have not been seen to recombine in these attempts. Thus, an estimated genetic distance between B-G and B-F/L is 0.039-0.048 centimorgan, and the distance between B-F and B-L is less than that (Hala et al, 1981b; Koch et al, 1983; Skjodt et al, 1985.) Thus, the frequency of crossing over between Class I and Class II regions in the chicken is at least one and probably

two orders of magnitude lower than in the human and mouse MHC (Skjodt et al, 1985.)

Therefore, a molecular approach to learn more about the chicken MHC would not only contribute to understanding of evolutionary relationships of MHC molecules, but also would lay the foundations for continued study of the immunological components of these diseases, which are either of economic importance to the poultry industry, or are animal models which may prove extremely valuable in the understanding of specific human diseases.

PROTEINS OF THE CHICKEN MHC

In 1947, Briles et al (1948) described a group of erythrocyte antigens in the chicken. With the subsequent description of the mouse H-2 complex as the major histocompatibility complex (Gorer et al, 1948,) the chicken Ea-B antigens were identified as the chicken MHC (Briles, 1950) because of their similar characteristics. A standardized nomenclature for the B-complex has been adopted (Briles and Briles, 1982; Briles et al, 1982.)

B-F antigens are found on virtually all somatic cells, are highly polymorphic, and consist of a 40-42 kD MHC-encoded transmembrane alpha-chain which associates non-covalently on the cell surface with beta-2-microglobulin (Zeigler and Pink, 1975.) These characteristics identify it as the homolog of the mammalian Class I antigens, such as the murine H-2-K, -D, and -L and the human HLA-A, -B, and -C proteins. Although three different, but related, polymorphic Class I antigens occur in both

of these mammalian MHCs, the chicken may have only one Class I locus. Immune precipitation of antigens and subsequent two dimensional gel analysis have shown a 40-42 kD peptide, non-covalently joined to beta-2-microglobulin, but have not provided clear evidence for more than one locus (Kubo et al, 1977.) Limited N-terminal microsequencing of immunoprecipitated protein shows that B-F is clearly homologous to the mammalian Class I antigens; the sequence homogeneity suggests that a single locus is expressed in each line (Vittetta et al, 1977; Huser et al, 1978.) It may well be that the chicken has only a single Class I gene, and therefore is considerably less complex than the mammalian species in this major histocompatibility complex locus. However, in one case, sequential immunoprecipitation by separate antisera have indicated the possibility of two separate Class I antigens (Bisati and Brogen, 1980.)

B-L antigens are the chicken Class II proteins. They are polymorphic antigens found on some cells of the monocyte/macrophage lineage, as well as on B-cells and activated T-cells (Ewert and Cooper, 1978; Ewert et al, 1984; Hala et al, 1984.) The proteins expressed on the cell surface consist of a 33-34 kD non-polymorphic alpha-chain and a variable beta chain; this dimer is bound noncovalently with an invariant chain intracellularly, before its expression on the cell surface (Crone et al, 1981a; Guillemot et al, 1986.) The size of the beta chain has been reported at 27 kD (Ewert et al, 1984) or 32-36 kD (Guillemot et al, 1986); No amino acid sequence data is available, but antisera of varying reactivities have indicated the possibility that two distinct B-L molecules may exist on some cells (Crone et al, 1981b.) This is in

agreement with the two-dimensional gel data of Guillemot et al (1986), who found a single alpha chain, but two distinct beta chains. Therefore, although it appears that the Class II region of the chicken is less complex than the homologous human region, it may be more similar to the mouse in complexity.

B-G is the third antigen mapped to the chicken MHC. It is found only on erythrocytes, consists of two chains of 31 and 42 kD, does not associate with beta-2-microglobulin, and is highly polymorphic (Hala, 1981a.) There is no mammalian homolog which has been described.

FUNCTIONS OF THE CHICKEN MHC

Because recombinants have been identified which separate the B-G region from the remainder of the MHC, functional testing of the role of the B-G antigen has been possible. Such experiments have revealed that it is not involved in the well-known MHC characteristics such as allograft rejection, GVH reaction, immune response to specific antigens, resistance to Marek's disease, or regression of RSV induced tumors (Briles et al, 1983; Vainio et al, 1984; Plachy et al, 1984.) B-G does appear to have an adjuvant effect, however, in that injection of erythrocytes expressing both new F and G antigens produces high titers of both anti-F and anti-G antibodies. But if only the F antigen differs, few or no chickens produce detectable anti-F antisera; if only

G differs, all chickens seem capable of responding with antibody production to G (Hala et al, 1981b).

The B-F region has been shown to regulate allograft rejection (Hala et al, 1981a) and GVH reaction, which can be blocked by specific anti-F antisera (Simonsen et al, 1977, Vilhelmova et al 1977.) These funcions are homologous to the functions determined by mammalian Class I antigens, and help to confirm the identity of B-F as the true Class I region of the chicken MHC.

The B-L region is assumed to play a role in immune response similar to the control exerted by the mammalian Class II antigens (Hala et al, 1981a.) In one study, B-L control of T-B cell interactions was studied using H.B19 chickens which have been serologically typed as B-F19, B-G19, and B-L12. Only the B-L antigen identity was significant in controlling the adoptive transfer of bursal cells (Vainio et al, 1984.) It has been assumed that the high/low response to the synthetic polypeptide GAT as studied by Pevzner et al (1978) would be controlled by this region, and preliminary work with antisera produced by reciprocal immunizations showed that the antigens targeted by the antisera were of the correct size for B-L antigens (Birkemeyer and Nordskog, 1982; Pevzner et al, 1978,) but to date this has not been confirmed by other investigators (S. Lamont, personal communication.) Whether this Ir-GAT gene actually codes for an MHC antigen is still uncertain, but there does appear to be a linkage between Ir-GAT and Rous

regression. (Gebriel et al, 1979; Lee and Nordskog, 1981; Gebriel and Nordskog, 1983.)

Thus, the major functions attributed to the mammalian MHC are also present in the chicken MHC. There is some evidence that certain of these functions are associated with the homologous proteins, and there is no evidence to date that the remaining functions are not correlated with the homologous proteins.

Therefore, it is a reasonable assumption that the chicken MHC is highly analagous in both structure and function with the mammalian MHC, although its apparent decreased complexity makes it a useful model for studying correlations of the MHC with various questions of disease resistance and immune function. Therefore, further molecular and functional characterization of the MHC is highly desirable. A first step in this characterization could be the cloning of various regions of the MHC to facilitate comparison of the genes at a molecular level by restriction fragment length polymorphism analysis, and to deduce the protein sequences for further comparison with known mammalian proteins.

CLONING OF MAMMALIAN MHC GENES

The decade of the 80's has seen considerable progress in the elucidation of mammalian major histocompatibility genes, with a variety of laboratories using a variety of methodologies for isolation and identification of the MHC genes. Early efforts at understanding the structure of the Class I antigens included the laborious task of protein

sequencing (Coligan et al, 1981, Maloy et al, 1981, Nathenson et al, 1981, Ezquerra et al, 1985), and more recently have included X-ray crystallography and determination of three-dimensional structure (Bjorkman et al, 1985.) The information gained from sequencing has proven immensely valuable in subsequent cloning efforts, both in confirmation of the gene identity after DNA sequencing and in synthesis of oligonucleotides to be used in the cloning strategies. For example, Sood et al (1981) used a synthetic mixture of 11-mers, derived from a reported conserved HLA 3'-peptide sequence, in a primer extension experiment to produce a specific 30mer using reverse transcriptase, ddATP, and enriched mRNA. After sequencing it, they were able to use this specific 30-mer as a probe to select specific HLA clones from a cDNA library (Sood et al, 1981.) These clones were then used by others at lower stringency to select clones from mouse cDNA and genomic libraries (Steinmetz et al, 1981a, Steinmetz et al, 1981b, Steinmetz et al, 1982.)

Early cloning and characterization also depended heavily upon the use of highly specific antisera. For example, Ploegh et al (1980) and Kvist et al (1981) constructed human and mouse cDNA libraries respectively, both from poly A+ RNA which had been size fractionated, then selected fractions which could be translated in vitro to produce immunoprecipitable products of the correct size. After constructing libraries, the plasmid DNAs were used to hybrid select mRNA which was tested in in vitro translation and immunoprecipitation. The HLA-B7 clone (Ploegh et al, 1980) provided sequence information which later proved useful in further cloning attempts (Sood et al, 1981.) Kvist's

pH-2d-1 clone was used by others to clone additional mouse (Bregegere et al, 1981) and human Class I sequences (Jordan et al, 1981; Malissen et al, 1982a; Malissen et al, 1982b.)

A number of groups, using various procedures, reported the cloning of class II antigen genes beginning in 1982. Again, the procedures depended heavily upon good, specific antibodies. In the approach taken by Wake et al (1982) and Long et al (1982), a cDNA clone bank was constructed from B-lymphoblastoid mRNA enriched for specific DR mRNA as assayed by injection into Xenopus laevis oocytes, and immunoprecipitation of the translated products. Individual clones were then identified which were able to hybrid select mRNA which could be translated to give the correct, immunoprecipitable products. These DR alpha and DR beta clones were later used to identify additional human genomic genes (Gorski et al, 1984; Tonnelle et al, 1985) as well as in cloning the entire mouse Ia region (Steinmetz et al, 1982.) Another approach requiring specific antibodies was taken by Korman et al (1982a) in cloning DR alpha cDNA by immunoprecipitation of polysomes to yield the specific mRNA used for library construction and for 32P-cDNA probe preparation. The identity of clones was confirmed by hybrid selection of mRNA, in vitro translation and immunoprecipitation of products. This clone was later used to select genomic clones, identify the exon/intron organization, and confirm the protein sequence (Korman et al, 1982b; Lee et al, 1982,; Schamboeck et al, 1983.) It was also used by Auffray (1982) in differential 5'- and 3'-hybridization to detect related, but not homologous clones. A third approach to cloning the DR alpha chain used a combination of methods including mRNA enrichment by selecting

membrane-bound polysomes to prepare RNA for the cDNA cloning, and used a synthetic oligonucleotide probe prepared after primer extension experiments identified the correct nucleotide sequence out of the possibilities deduced from the available amino acid sequence (Stetler et al, 1982.) Stetler's human DR alpha cDNA clone was then used to identify mouse E and A alpha cDNA and genomic clones (Benoist et al, 1983; Mathis et al, 1983.) A similar primer extension/oligonucleotide probe strategy was employed to identify both DR alpha and DR beta clones (Das et al, 1983a; Das et al, 1983b; Bell et al, 1985.) A final methodology used by Davis et al (1984) was successful in locating the mouse A alpha gene. Cosmids coding for the entire Ia region (Steinmetz et al, 1982) were probed with B-cell 32P-cDNA after it had been hybridized with T-cell mRNA to remove common sequences. Similar subtracted cDNA was also cloned.

In most of the examples given here, the identity of the Class II genes was confirmed by immunoprecipitation of the protein coded for by the clones. However, particularly for some of the Class I genes, identity of the genes has been established by transfecting L cells, or B-cell lines, and detecting a new specificity with antibodies (Goodenow et al, 1982a; Goodenow et al, 1982b; Goodenow et al, 1983; Zuniga et al, 1983.) The cloning and transfection techniques have enabled researchers to dissect functions of MHC gene products in new and productive ways

(Woodward et al, 1982; Reiss et al, 1983; Malissen et al, 1984; Shimizu et al, 1986; Vogel et al, 1986.)

Even more elegant functional expression of both Class I and Class II antigens have been studied in transgenic mice (Bieberich et al, 1986; LeMeur et al, 1985; Yamamura et al, 1985.) The technology is available and the desire is certainly present for similar functional assays of genes related to disease resistance, including MHC genes, in the transgenic chicken (Crittenden, 1986; Salter et al, 1987.)

MATERIALS AND METHODS

Cell line: LSCC-RP-9 is a B-lymphoblastoid cell line transformed with RAV-2 virus (Okazaki et al, 1980). Cells were maintained in Leibowitz-McCoy medium with 10% bovine fetal serum, 20% chicken serum, 5% tryptose phosphate broth, and 0.1% fungizone, at 41 C with 5% CO_2.

RNA preparation: RP-9 cells were washed with sterile PBS and collected by centrifugation at 100 x g for 5 minutes. A cell pellet containing 10^8 cells was lysed by pipetting up and down in 8 ml 6M guanidinium isothiocyanate, containing 5mM sodium citrate (pH 7.0), 0.1M beta-mercaptoethanol, and 0.5% sodium lauryl sarcosinate. Cesium chloride was added to 2.4M and the solution was layered over a 3 ml cushion of 5.7M CsCl in an SW41 tube. This was centrifuged at 30,000 rpm at 20 C for 24 hr. The supernatant was carefully poured off and the walls of the tube were wiped to remove as much liquid as possible. The RNA pellet was resuspended in 400 ul water, NaCl added to 100mM, and 2,5 volumes of absolute ethanol added. The RNA was precipitated at -80C overnight or longer, and collected by centrifugation for 15 min in an Eppendorf microcentrifuge. The RNA was pelleted, washed carefully with 70% ethanol, dried, and resuspended in water immediately prior to use.

Polyadenylated RNA was selected on a column of oligo d(T) cellulose. Pelleted and dried RNA was dissolved in water containing 10mM vanadyl ribonucleoside complexes and heated to 65C for 5 minutes. An equal volume of 2X RNA loading buffer was added, and the cooled mixture was applied to a 1 ml column of oligo d(T) cellulose (Collaborative

Research, Inc., Lexington, MA) which had been washed thoroughly in succession with 0.1M NaOH, 0.005M EDTA, water, and 1X loading buffer. The column was washed with 1X loading buffer until the OD_{260} of the effluent was 0 (about 10 ml). The poly (A)+ RNA was eluted with several ml of 10mM Tris (pH 7.5), 1mM EDTA, 0.05% SDS, and the concentration was estimated by OD_{260} absorbance. Sodium chloride was added to 0.1M and 2.5 volumes of absolute ethanol was added before incubation overnight at -80C.

Plasmid DNA isolation: pDS12 was the generous gift of J. Silver (Chang et al, 1983,) and includes the entire coding sequence of a human Class II (DQ) alpha chain. pRAV-2 DNA, used as a control plasmid, was the gift of E.J. Smith. Plasmids were grown in E. coli strains HB101 or DH5 at 37C with shaking in LB broth, plus the appropriate drugs. Plasmids using the vectors pBR322 or pBR328 were grown to OD_{600} of 0.5-0.6, chloramphenicol was added to 150ug/ml to amplify yield, and incubation continued for a further 12-16 hr. Plasmids using the vectors pGEM3 or pGEM4 were allowed to grow under the same conditions for 20 hr, with no amplification step.

The cells were pelleted at 2500xg for 5 min and the media removed. Cells were resuspended in 1% original volume of GET buffer (usually 5 ml, 50mM glucose, 25mM Tris, pH 8.0, 10mM EDTA), and placed in a clean tube on ice. Ten ml of 1% SDS, 0.2M NaOH, was added, mixed gently, and left on ice for 5 min. 7.5 ml of cold potassium acetate (5M acetate, 3M potassium) was added with a few quick inversions of the tube, and incubated on ice for 5-10 min. After centrifugation for 10 min at

15000xg at 4C, the supernatant was removed to a new tube an mixed with 0.6 volume isopropanol. After 10 min at room temperature, the DNA was pelleted by centrifugation at 15000xg for 10 min. The pellet was drained very well and resuspended in 5 ml TE. 50ul heat-treated RNase (10mg/ml RNaseA, 10kU/ml RNaseT_1) was added, and the sample was incubated at 37C for 30 min. After addition of NaCl to 0.5M, the sample was thoroughly extracted with an equal volume of phenol:chloroform (1:1), and the aqueous phase was precipitated with two volumes of ethanol for at least 1 hr at -20C. The pelleted DNA was recovered after centrifugation at 15000xg for 10 min. The DNA was dissolved in 5 ml TE, NACl was added to 1.5M and 0.25 volumes of 30% PEG8000 in 1.5M NaCl was added. After mixing, the solution was incubated on ice for 1 hr, and centrifuged at 4C at 15000xg for 15 min. After draining thoroughly, the pellet was rinsed gently with 70% ethanol and dried.

The pellet was resuspended in TE and the concentration estimated from absorbance readings at 260 nm.

Genomic DNA isolation: Red blood cells (RBC) were obtained from 15I_5 congenic lines of White Leghorn chickens developed and maintained at the Regional Poultry Research Laboratory in East Lansing (Bacon et al, 1987b). Approximately 0.2 ml packed RBCs were resuspended in 10 ml NET buffer (100mM NaCl, 1mM EDTA, 50 mM Tris, pH 7.5). Pronase was added to 0.5mg/ml and SDS added to 0.5%, and the mixture was incubated at 37C overnight. 10ml phenol:chloroform (1:1) was added and mixed gently by inversion. After centrifugation to separate the phases, the aqueous phase was removed to a new tube using a large bore pipet to minimize

shearing of the DNA. This extraction was repeated several times until the interface was clear. The sample was then extracted one time with chloroform. RNaseA was added to 50ug/ml and the sample was incubated for 1 hr at 37C. The sample was extracted again with phenol:chloroform (1:1), and then with chloroform. The aqueous phase was then dialyzed exhaustively against TE, and the DNA concentration was determined by measurement of absorbance at 260 and 280 nm.

Bacteriophage DNA isolation: Minipreps were grown by infecting 100ul of overnight E. coli culture with 1/20 of a resuspended plaque from a plaque purified preparation. This was incubated 12-15 hr at 37C after plating in 3 ml 0.75% agarose on LB agarose plates. The top agarose was scraped off into 5 ml SM (100mM NaCl, 10mM $MgSO_4$, 5mM Tris pH 7.5) plus 100ul chloroform, and incubated at 4C overnight to elute the phage.

Debris was pelleted by centrifugation at 12000xg for 15 min, and the supernatant containing the phage was placed in a new tube. NaCl was added to 1M, and polyethylene glycol (MW=6000) was added to 10%. After all solids were dissolved, the solution was cooled to 4C on ice, and incubated on ice for at least 1 hr.The precipitated phage particles were collected by centrifugation at 15000xg for 30 min, and the tube was drained well. The pellet was resuspended in 400ul, transferred to an Eppendorf tube, and extracted once with chloroform. EDTA was added to 20mM, proteinase K to 50ug/ml, and SDS to 0.5%, and the mixture was incubated at 65C for 1 hr. The mixture was extracted 2 times with phenol and 2 times with chloroform, and 2 volumes ethanol was added to precipitate the DNA. 25% of the preparation was used for a restriction

digestion: if the concentration was too high or low, another sample was run with the corrected amount.

RNA electrophoresis and Northern blotting: Gels were prepared consisting of 1% agarose, 2.2M formaldehyde, and 1x gel buffer (200mM MOPS, 50mM sodium acetate[pH 7.0], and 10mM EDTA.) RNA samples containing 20ug total RNA, 50% formamide, 2.2M formaldehyde, and 1x gel buffer were heated to 60C for 5 min and cooled on ice. Before loading on the gel, 1/4 volume of 50% glycerol containing 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol was added to each sample. RNA was subjected to electrophoresis at 40 volts, 80 milliamps, for 16 hr. Lanes containing RNA markers were cut off, stained with 1 ug/ml ethidium bromide in 0.1M beta-mercaptoethanol, washed in distilled water, and photographed. The remaining lanes were soaked in 20xSSC for 30-60 min and transferred to nitrocellulose with 20xSSC by passive transfer overnight. The dried filter was baked at 80C for 2 hr before hybridization.

DNA electrophoresis and Southern blotting: DNA was digested in the enzyme buffer recommended by the vendor, in a volume appropriate for the size of the electrophoresis well, usually 20-100ul. Samples included 15ug genomic DNA, 1-2ug phage DNA or 0.5-1ug plasmid DNA. Digestion was carried out for 2-18 hr at the recommended temperature, using 1-2U enzyme/ug DNA. One tenth volume of 20% Ficoll, 10mM EDTA, 0.5%

bromophenol blue, 0.5% xylene cyanol was added to stop the reaction and prepare the sample for loading on the gel.

Agarose gel electrophoresis was carried out in TBE buffer (0.089M Tris, 0.089 M boric acid, 1mM EDTA), with the gel concentration, voltage, and time varying depending upon the size of fragments to be separated. Genomic DNA was electrophoresed through a 18x15cm gel containing 0.8% agarose at 35 volts for 16 hr.

After electrophoresis, the gel was removed to a shallow dish containing 0.1ug/ml ethidium bromide, and soaked at room temperature for 30 min with occasional shaking. The gel was photographed under ultraviolet illumination to identify the location of bands. The DNA was denatured by soaking the gel in 0.5M NaOH, 1.5M NaCl for 45-60 min, and then neutralized by soaking for 45-60 min in 0.5M Tris, pH 7.5, 1.5M NaCl. The DNA was passively transferred to nitrocellulose filters (S&S, Keene, NH) using 10xSSC, for 16 hr (cloned DNA) or 24-48 hr (genomic DNA.) The filters were baked at 80C for 2 hr.

Probe radiolabelling: All DNA to be used for low stringency hybridization probes was first digested with restriction enzymes and the specific DNA insert was gel purified. After staining with ethidium bromide and visualizing bands under UV light, the appropriate band was cut out with a razor blade, placed in a small piece of dialysis tubing with 2-5 ml TBE, and electroeluted at 50-80 volts for 2-4 hours, until the DNA was all in solution. The DNA was then purified on an Elutip-d

column (S&S, Keene, NH), according to manufacturer's instructions, and concentrated by ethanol precipitation.

For nick translation, 100-500 ng DNA was added to a tube containing 50-100uCi ^{32}P-alpha-dCTP (NEN, >800uCI/mMole) in 50mMTris, pH 7.2, 10mM $MgSO_4$, 0.1mM DTT, 50ug/ml bovine serum albumin (BSA), 20uM dATP, 20uM dGTP, and 20uM dTTP. To this mixture was added 5U E. coli DNA polymerase I and 0.5ul of 10^{-4}mg/ml DNaseI. After mixing, the reaction was incubated at 14C for 1.5 hr. Separation of probe from unincorporated nucleotides was accomplished by either multiple ethanol precipitations or passage over Sephadex G-50. Incorporation was usually in the range of 25-65%, yielding probes labelled to a specific activity of 10^7 to 10^8dpm/ug.

Hybridizations: All low stringency hybridizations were prepared in 20% formamide, 5xSSPE (1xSSPE=0.18M NaCl,10mM sodium phosphate, pH 7.4, 1mM EDTA), 2x Denhardt's (1x=0.02% each Ficoll, polyvinylpyrrolidone, and BSA), and 100ug/ml denatured, sheared herring sperm DNA. Filters were pre-hybridized in this solution for 1-15 hours at 25C, and then incubated in the same solution plus $5x10^6$-$5x10^7$ dpm nick-translated probe for 20-48 hours at 25C. Filters were then washed twice with 2xSSC at 25C for 15-30 min each, and then 2 times in 2xSSC at 56C for 15 min each. "Very" low stringency was the same conditions except the final washes were done at 50C. "Moderate" stringency was the same conditions except the final washes were done at 65C. "High" stringency was the

same conditions except that the final washes were done in 0.2xSSC at 65C.

cDNA library construction: Poly A+ RNA was used as a template for cDNA synthesis using reverse transcriptase and RNaseH, using the reagent purchased from Amersham Corp. Briefly, 5ug RNA was mixed with 100U AMV reverse transcriptase, in a 50 ul volume which included 100mM Tris, pH 8.3, 10mM $MgCl_2$,140 mM KCl, 100 ug/ml oligo dT_{18}, 0.5U.ul RNasin, 1mM dATP, 1mM dCTP, 1mM dGTP, 1mM dTTP, and 20uCi ^{32}P-alpha-dCTP. The reaction was allowed to continue at 42C for 45-90 minutes. Second strand synthesis was then initiated by addition of 5U RNaseH and 115 U E. coli polymerase I, in a total volume of 250ul, containing 0.1M HEPES, pH 6.9, 10 mM $MgCl_2$, 2.5mM DTT, 70mM KCl, and 1mM of each dNTP. This reaction was incubated at 14C for 2 hours; then the reaction was heated to 70C for 10 min to stop further enzyme activity, and cooled on ice. T4-DNA polymerase (10 U) was added to repair single-stranded ends, and the reaction was incubated at 37C for 15min. Further enzyme activity was stopped by addition of EDTA and SDS to 10mM and 0.1%. The cDNA was then extracted with phenol:chloroform (1:1) and then chloroform, and concentrated by ethanol precipitation.

In order to block any internal EcoR1 sites from cleavage during the cloning process, the cDNA was treated with 10U EcoR1 methylase in 100mM Tris, pH 8.0, 10mM EDTA, 400ug/ml BSA, and 80uM S-adenosyl methionine for 1 hour at 37C. After ethanol precipitation, the cDNA was mixed with lug phosphorylated 8-mer EcoR1 linkers (GGAATTCC) and 1U T4-DNA ligase in 70mM Tris, pH 7.6, 10 mM $MgCl_2$, 5mM DTT, and 50um ATP. The ligation

was allowed to proceed for 16 hours at 12C; the cDNA was digested with 50U EcoR1 for 2 hours at 37C, and excess linkers were removed by multiple spermine precipitations, after extractions with phenol:chloroform, and chloroform. The spermine precipitations involved adding 360ul TE, 40ul DMSO and 10ul 100mM spermine-HCl, pH 6.8, and then freezing briefly in dry ice, followed by slow thawing on ice and centrifugation for 30 min at 4C. The pellet was washed 3 times with 75% ethanol and dried.

The precipitated cDNA was taken up in 1x ligation buffer (30mM Tris, pH 7.5, 30 mM NaCl, 4mM $MgCl_2$, 0.5mM ATP, 2mM DTT, 100ug/ml BSA), and 5U T4-DNA ligase and 0.5ug dephosphorylated lambda gt10 arms were added. Ligation was allowed to continue at 12C for 16 hours. Phage DNA was then packaged in an in vitro packaging extract (Promega Biotec) by incubating in the freshly thawed mixture for 2 hours at 22C.

To titer the recombinant lambda phage produced in this library, serial 10-fold dilutions were made and 100ul of each dilution was added to 200ul overnight cultures of C600 or C600hfl strains of E. coli. After allowing 15 min at 37C for the phage to attach to the bacteria, 3 ml molten top (0.75%) agar was added to each sample. The samples were mixed gently and poured onto 100mm plates containing 25 ml 1.5% agar in LB broth. After the top agar had hardened, the plates were inverted and incubated for 12-16 hours at 37C. Plaques were counted on both C600 and C600hfl; gt10 wild-type will grow lytically on C600, but forms a lysogen on C600hfl. When recombinants are produced at the EcoR1 site,

insertional mutagenesis prevents lysogeny on C600hfl, and the recombinant phage grow lytically on this strain as well.

DNA sequencing: DNA sequencing was performed by the dideoxynucleotide method, directly on the double-stranded plasmids, using primers which were complementary to the SP6 and T7 promotor regions flanking the polylinker cloning site of pGEM plasmids (Promega Biotec.) The purified plasmid template DNA was linearized using PvuII, which cuts only in the vector. After phenol:chloroform extraction and ethanol precipitation, 1ug of recombinant plasmid and 30ng of appropriate promotor primer were mixed in a 10ul volume, containing 10mM Tris, pH 7.5 and 5mM NaCl. The mixture was heated in a boiling water bath for 3 min, then chilled quickly in ice water for 5-15 min. To the annealed template mix, 4ul [^{32}P]-alpha-dATP (DuPont, 800Ci/mmole, 10mCi/ml) was added and mixed; 3ul of this mixture was added to tubes for each of the four reactions, containing 4ul including 1.25 U Klenow enzyme in 34 mM Tris,pH 8.3, 6mM $MgCl_2$, 5mM DTT, 50mM NaCl, 250 mM each dGTP, dTTP, and dCTP, and one of the following: 100uM ddCTP, 3.6uM ddATP, 200uM ddTTP, or 50uM ddGTP. The tubes were mixed and incubated at 37C for 15 min; then 1 ul chase solution was added (containing 2mM concentration of each dATP, dCTP, dGTP, and dTTP in the same buffer) and the tubes were incubated at 37C an additional 15 min. The reactions were stopped by adding 5ul of a solution containing 98% formamide, 10mM EDTA, 0.3% xylene cyanole FF and 0.3% bromophenol blue. The reactions were heated to 70C for 3 min

immediately before loading 2.5 ul of each reaction onto adjacent wells of a sequencing gel.

Sequencing gels were 6% polyacrylamide (Bis:acrylamide = 1:20), and 8M urea in 1xTBE buffer; the gel size was 30x40x0.04 cm. Gels were pre-run in an IBI sequencing apparatus at 70-75 watts for 30-60 min before loading samples, and were run at the same power settings for about 2 hr (until the bromophenol blue reached the bottom of the gel.) Usually a second loading of samples was applied to the gel and electrophoresis was carried out for an additional two hours. Occasionally, a third application was also done. When the bromophenol blue of the final loading neared the bottom of the gel, the power was stopped and the gel removed from the apparatus. The gel was fixed by soaking in 5% acetic acid/5% methanol, transferred to Whatman 3MM paper, dried, and exposed to X-ray film.

RESULTS

In order to test whether mammalian cDNAs would hybridize to homologous regions of the chicken genome, digests of DNA from White Leghorn $15I_5$ B-congenic chickens were Southern blotted and probed with a variety of cloned cDNAs. Figure A3 is an autoradiogram of DS12-probed genomic blots, and shows the hybridization of DS12 to chicken genomic DNA digested with PstI. A major band of 4.55 kb is seen in all lanes, with both larger and smaller minor bands also seen in several lanes.

Northern blots of total RNA isolated from the Ia+ cell line, RP-9, are shown in Figure A4. A single RNA of about 1.95 kb, which hybridized to the DS12 probe is seen in lane 2, and marked with an asterisk. Lane 1 shows an autoradiogram of the same RNA blot after hybridization with pR1.9, a clone consisting of single copy chicken sequences which are not transcribed in RP-9 cells (unpublished observations); the background due to the low stringency hybridization conditions showed up in the rRNA bands of both lanes, and was clearly different from the specific hybridization of DS12 to the 1.95 kb RNA.

In order to clone cDNA of this 1.95 kb RNA in RP-9 cells, we isolated poly A+ RNA from RP-9 cells, used reverse transcriptase to make double stranded cDNA, added linkers, and cloned into the EcoR1 site of lambda gt11. After *in vitro* packaging, the numbers of total and recombinant phage were titered on C600 and C600hf1 strains of E. coli, and the library was amplified before further screening. There were 25000 recombinant phage, which was 53% of the total library.

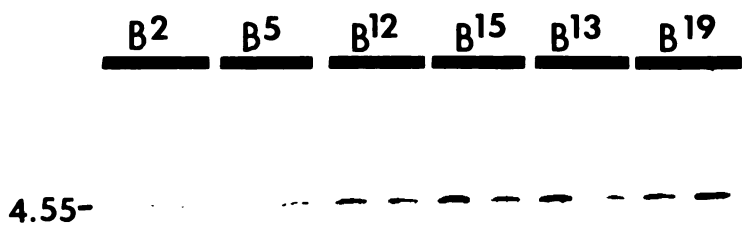

Figure A3: Southern blot of genomic DNA from congenic chickens. DNA from chickens of the labelled haplotypes, was digested with PstI and hybridized with nick translated pDS12 insert DNA at low stringency. The major band seen in all lanes is 4.55 kb.

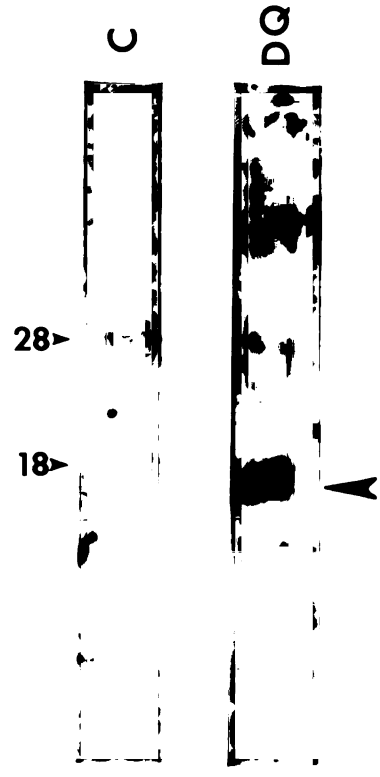

Figure A4: Northern blot of RP-9 total RNA hybridized with nick-translated pDS12 insert DNA (DQ). Position of the 28S and 18S ribosomal RNA is shown, determined from low stringency hybridization with a control plasmid (C)..

The amplified library was plated on six 150 mm plates, with 50000 total plaques per plate. Duplicate filters were pulled from each plate and processed according to the protocol of Benton and Davis (1977.) These filters were hybridized at low stringency with nick-translated DS12 insert DNA. The 32 regions which included any plaques whose autoradiogram signals were clearly above background on both filters were picked and eluted into 1 ml SM + 50ul $CHCl_3$ at 4C overnight. Each of these phage stocks was diluted and aliquots of each dilution plated on 100 mm plates. Triplicate filters were processed as before, with two hybrized to DS12 and the third to pRAV-2. A sample of filters processed from one plate (10A) is shown in Figure A5; panel A shows very low stringency washing, with all plaques exhibiting similar high signals; panel B shows the same filter after "normal" low stringency washes; panel C shows the duplicate to B, with arrowheads pointing out some of the positive duplicated signals; panel D shows a third filter from the same plate hybridized with pRAV-2, and not showing positive signals with the same plaques.

Out of the 32 plaques picked from the primary screening, 20 remained positive through the secondary screening and plaque purification procedures. These isolates are listed in Figure A6 along with details of characterization of their cDNA inserts.

All 20 clones were grown as plate lysates and minipreps of the gt11 DNA were prepared, digested with EcoR1, and electrophoresed to analyze the cDNA inserts. Only 15 of the clones resulted in preparations of

Figure A5: Secondary screening filters, after picking positive plaque # 10A on initial screening of the RP-9 cDNA library. Filter A was washed at very low stringency; Panel B shows the same filter after rewashing at normal low stringency; Filter C is the duplicate filter, washed as in Panel B: All three were hybridized with nick-translated pDS12 insert DNA. Filter D was hybridized with the control pRAV-2 plasmid probe. Arrows in panels B and C point to plaques which are positive on the duplicate filters.

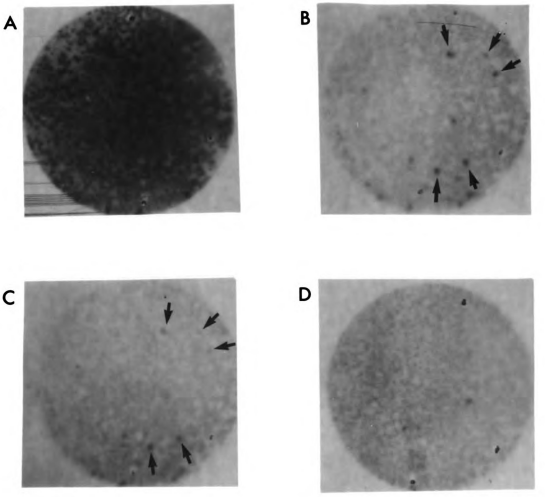

Figure A5:

Clone	secondary screen		insert DNA			Subclone
	DS	pRAV2	size	DS	p38	obtained?
	hybridization			hybridization		
1Ax	+	+	2800bp	++	-	-
3A	+	-	?	-	+	-
4A	+	-	450	-	-	-
7A	+	-	ND			
9A	+	-	375	+	+	-
10A	+	-	1500	++	++	p38
11A	+	-	800	++	++	-
12A	+	-	400	+	+	-
13A	+	-	ND			
14A	+	-	ND			
17A	+	-	400	-	-	-
18A	+	-	375	++	++	p16
21A	+	-	400	+	+	-
22A	+	-	400	+	+	-
23A	+	-	400	+	+	-
25A	+	-	ND			
26A	+	-	400	+	+	-
27A	+	-	ND			
28A	+	-	175	-	-	-
29A	+	-	?	-	-	-

Figure A6: Characteristics of recombinant clones from RP9 cDNA.

Figure A7: Southern blots of DNA from recombinant clones. Panel A shows the results of a Southern blot of the lambda DNA isolated from eight of the recombinant clones isolated, after probing with the nick-translated pDS12 insert DNA.. Panel B shows the same filter after removing the probe and rehybridization with nick-translated p38 plasmid DNA, and washing at high stringency.

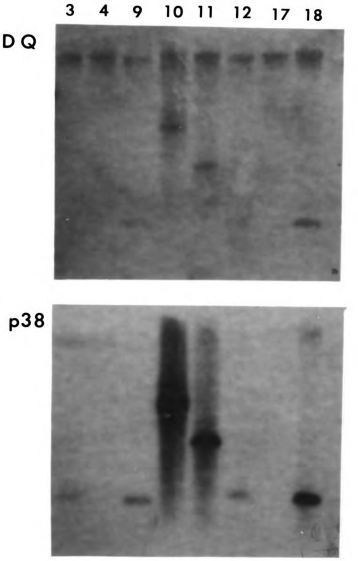

Figure A7:

adequate DNA for restriction analysis. Two of these produced vector bands, but no visible insert bands. This could have been due to the small quantity of DNA digested, the very small size of the insert, or one of the EcoRl sites could be missing at the ends of the insert. The visible inserts ranged from 175 to 2800 bp, with nine of them being around 400 bp. These fragments were transferred to nitrocellulose and hybridized with DSl2 to ensure that the positive signal detected in the phage screening was due to hybridization of the probe to the insert DNA (Figure A7a). Ten of the 15 clones had detectable inserts hybridizing with DSl2. Three of the inserts detectable with ethidium bromide were not recognized by hybridization with DSl2; there was no hybridization seen with either of the clones which had no detectable inserts by ethidium bromide staining.

The DNAs from several of the clones which showed the most positive signals were digested with EcoRl, mixed with EcoRl-cut and phosphatased pGEM-4 DNA, ligated, transformed into competent DH5 bacteria, and selected on LB plates containing 25ug/ml ampicillin. For two of the clones, # 10A and 18A, subclones of the cDNA were obtained, designated p38 and pl6. Since 10A has the longest cDNA insert hybridizing specifically with DSl2, additional subcloning was not deemed necessary at this time.

In order to examine the relationships between the various clones, nick-translated p38 was used to hybridize at high stringency to the same filter seen in figure A7a, after the DSl2 had been removed. All of the inserts previously positive with the DSl2 probe were also positive with

p38 (Figure A7b.) In addition, one of the inserts (3A) which had not been detected by either ethidium bromide or DS12 hybridization, was positive using p38; we assume that the quantity of DNA in this fragment was too small to be seen with the first two techniques, but was adequate for high stringency hybridization with an homologous probe. Thus, there was no evidence that we had isolated any DS12-hybridizing clones which did not hybridize to p38; p38 was therefore assumed to include most of the sequence of the mRNA hybridizing to DS12.

In order to determine which portion or portions of DS12 were homologous to the cDNA clones which had been isolated, the DS12 plasmid was digested with various enzymes and the separated fragments were Southern blotted and probed with p38. The enzymes used did not cut within the vector, and the pertinent restriction sites with the DS12 insert are shown in Figure A8a, along with sizes of the major fragments. Figure A8b shows the autoradiograms after probing these fragments with p38. The large ApaI fragment ,which includes the entire coding region for the mature polypeptide chain, does hybridize to p38, indicating that the region of homology is within the DS12 coding region. The StuI and SacI sites, when double digested with ApaI remove the cytoplasmic and transmembrane regions, respectively. The ApaI-StuI fragment retained the ability to hybridize, but this was entirely removed in the ApaI-SacI fragment. This indicates that the entire low stringency hybridization involves this 90 bp SacI-StuI fragment, which encodes primarily the transmembrane region.

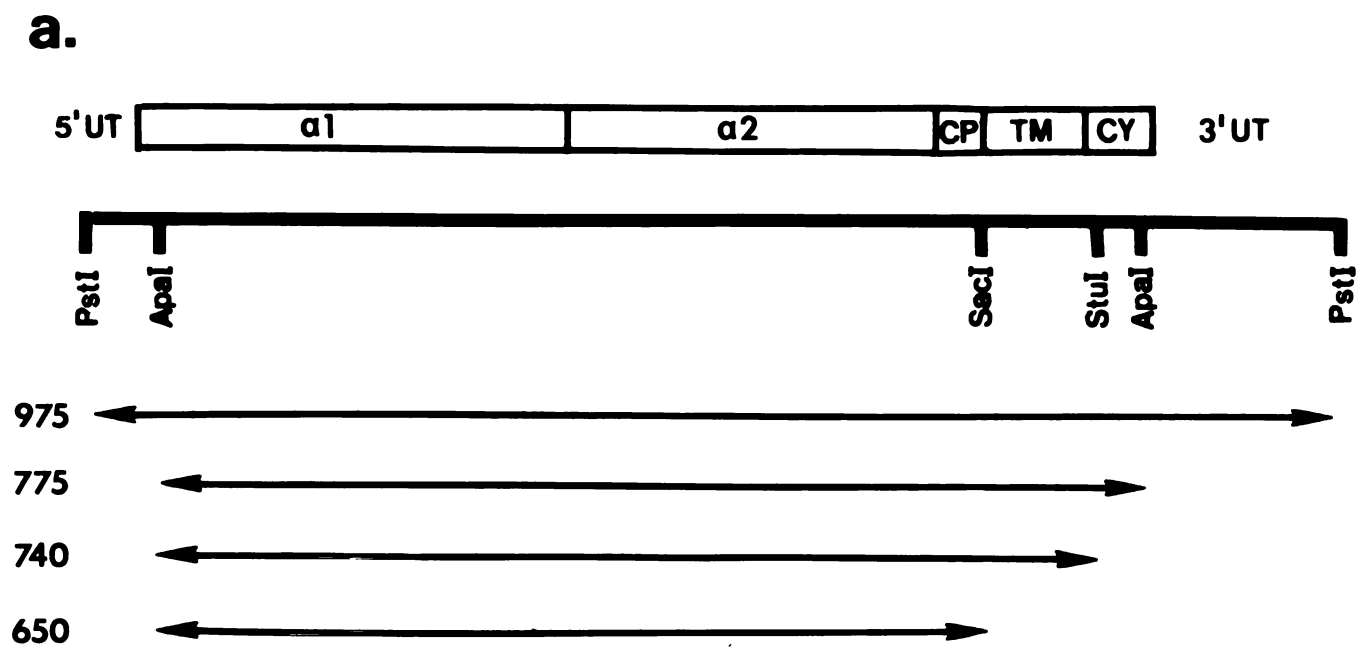

Figure A8: Mapping of the p38 homology region within pDS12 cDNA. Panel a shows a partial restriction map for the human cDNA clone, pDS12, and indicates the expected sizes of fragments after digestion with the indicated enzymes.

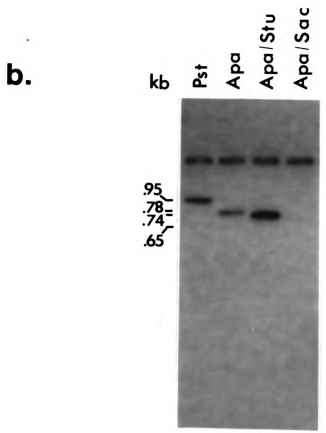

Figure A8: Mapping of the homology region within pDS12 cDNA. Panel b shows the results of a Southern blot of fragments from pDS12, probed with the p38 chicken cDNA clone.

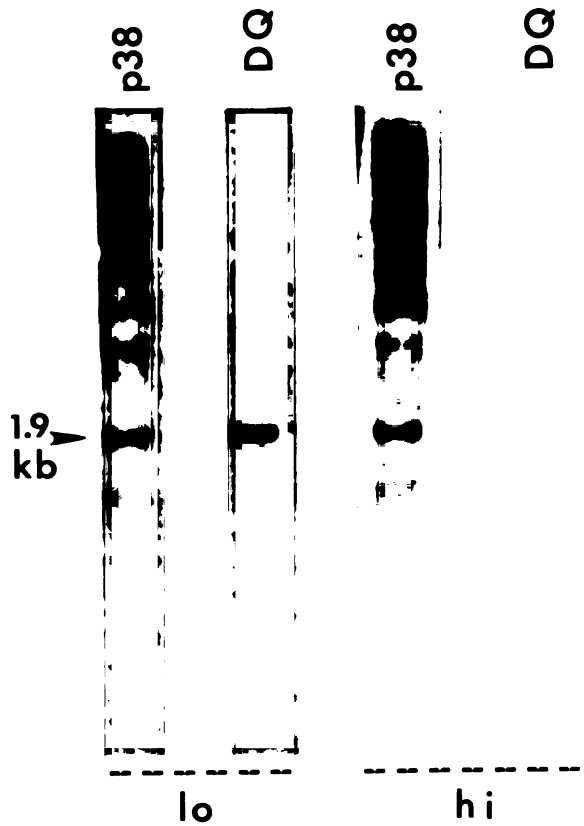

Figure A9: Northern blot of RP-9 total RNA hybridized with either pDS12 or p38 cDNA. Filters were washed at low or high stringency, as designated.

In order to ascertain whether the p38 cDNA represented the expected 1.95 kb RNA originally seen with the DS12, we ran Northern blots of RP-9 RNA as in Figure A4, and probed adjacent lanes with nick-translated p38 and DS12. The autoradiogram is shown in Figure A9. At low stringency, both probes hybridized to the 1.95 kb RNA, although p38 also recognized a small amount of larger molecular weight RNA. After high stringency washes, the DS12 probe washed off, while the p38 remaind hybridized, indicating extensive homology with the RNA. Thus, p38 represents most of the 1.95 kb RNA which hybridized with DS12.

At this time sequence analysis was begun on both the p16 and p38 clones. The sequencing strategy is shown in Figure A10; this was simplified by the availability of primers homologous to the SP6 and T7 promotors which flank the polylinker in the pGEM4 plasmid. Therefore, dideoxy sequencing could be carried out directly on the double-stranded plasmid DNA. To facilitate sequencing of the internal portion of p38, two subclones were constructed in pGEM4, utilizing the SacI site in the middle of the insert. The six areas sequenced are shown in Figure A11(a-f), with an arrow indicating the primer, strand, and direction of sequencing.

These sequences were compared with the Genbank data files, using the Bionet system program Align. The most significant correlation found is shown in Figure A12; it aligns a region of p16 with the human DQ chain transmembrane region. This introduces one gap of two nucleotides and matches 29 of 34 nucleotides, for approximately 85% homology over this region. This homology would be adequate to account for the

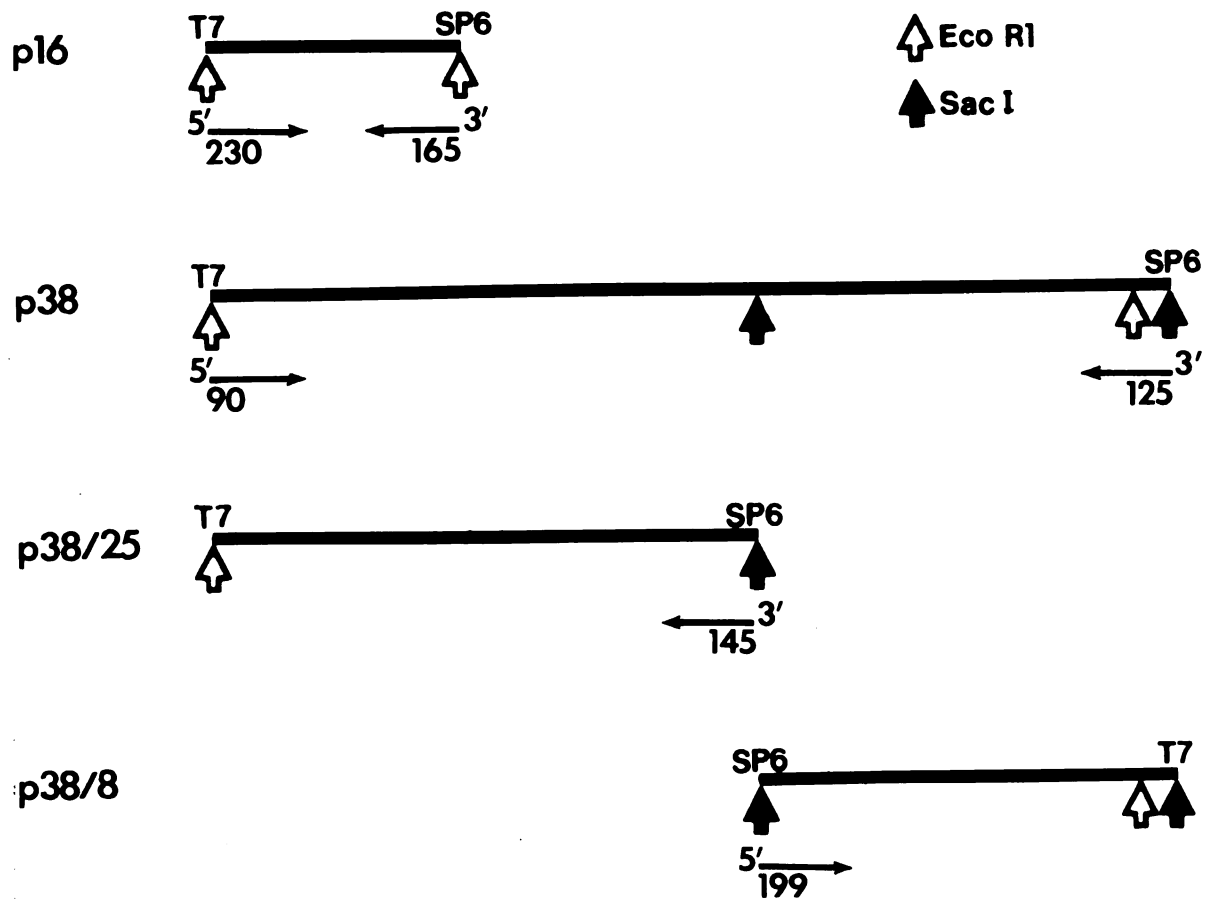

Figure A1Ø: Partial restriction map of the recombinant subclones. Sequencing strategy is indicated by the arrows, along with the number of nucleotides for which the sequence was read.

Figure A11 (a-f): Partial sequences of p16 and p38 deduced using the dideoxynucleotide method. Sequencing strategy and location of promotor primer regions are shown in Figure A10. Arrows show direction and origin of sequencing reactions. The p38 internal SacI site and the polyadenylation signal are underlined.

```
5'-C A C T A  G C G C C  C C A C T  G C G C C  C C A C T
3'-G T G A T  C G C G G  G G T G A  C G C G G  G G T G A

   G C G C C  C C A C A  G C G C C  C A C A G  C G A C C
   C G C G G  G G T G T  C G C G G  G T G T C  G C T G G

   C A C A G  A G C C C  C A T A G  C A A C C  C A T T G
   G T G T C  T C G G G  G T A T C  G T T G G  G T A A C

   A G C C C  C A T G G  A G T T C  C A T A T  C A C T C
   T C G G G  G T A C C  T C A A G  G T A T A  G T G A G

   C A G A G  C G C C C  C A T A G  C G C C C  A C A C C
   G T C T C  G C G G G  G T A T C  G C G G G  T G T G G

   C C G C C  C C A T A  G C G G C  C C A T T  G A G C C
   G G C G G  G G T A T  C G C C G  G G T A A  C T C G G

   C C A C A  G T G C C  C C A T G  A A C C C  A C A G A
   G G T G T  C A C G G  G G T A C  T T G G G  T G T C T

   T C A T C  C C A T A  G C A C A  A T T G T  G C C C A
   A G T A G  G G T A T  C G T G T  T A A C A  C G G G T

   T A G C G  C C A C A  C C G C C  C A T G A  G C A C A
   A T C G C  G G T G T  G G C G G  G T A C T  C G T G T

   T G G C C-3'
   A C C G G-5'
```

Figure All(a):pl6-T7 sequence

```
5'-C C C A A  T G A C C  A C A T G  T G C C C  A T A G C
3'-G G G T T  A C T G G  T G T A C  A C G G G  T A T C G

   G C C C C  A C A C C  C C G C C  C C A T A  G C G C C
   C G G G G  T G T G G  G G C G G  G G T A T  C G C G G

   C C A C T  G A G C C  C C A C A  G T G C C  C C A C T
   G G T G A  C T C G G  G G T G T  C A C G G  G G T G A

   G A A G C  C C A C A  G A C C A  C C C C A  T A G C T
   C T T C G  G G T G T  C T G G T  G G G G T  A T C G A

   C C A C A  T T G T G  C C C C A  T A G C G  C C C C A
   G G T G T  A A C A C  G G G G T  A T C G C  G G G G T

   T T T A T  T G C C C  C T A A A  A G C C G  G G G C G
   A A A T A  A C G G G  G A T T T  T C G G C  C C C G C

   C T A T G  G C G G-3'
   G A T A C  C G C C-5'
```

Figure All(b): pl6-SP6 sequence

```
5'-C G T A G  C G A C C  C A T G G  A G C C C  C A C A C
3'-G C A T C  G C T G G  G T A C C  T C G G G  G T G T G

   A G C G C  C C C A C  A G T G A  C C C A C  T G A G A
   T C G C G  G G G T G  T C A C T  G G G T G  A C T C T

   C C C A T  A G C G A  C C C A T  C G G A G  C C C C A
   G G G T A  T C G C T  G G G T A  G C C T C  G G G G T

   C T G A G  C C C C A  C A G C G  A C C C-3'
   G A C T C  G G G G T  G T C G C  T G G G-5'
```

Figure All(c): p38-T7 sequence

```
5'-A G C C T  A T G A G  C C A C A  N A G T G  C G C A G
3'-T C G G A  T A C T C  G G T G T  N T C A C  G C G T C

   A G C G A  C C C A T  A G C G A  C C C A G  T G G A G
   T C G C T  G G G T A  T C G C T  G G G T C  A C C T C

   C C G A C  A C A G C  G A C A C  A T G G A  G C C C C
   G G C T G  T G T C G  C T G T G  T A C C T  C G G G G

   A C T T G  A G C C C  C A T A G  C G A C C  C A C T G
   T G A A C  T C G G G  G T A T C  G C T G G  G T G A C

   A G C C C  C A T A G  C G A C C  C A T G G  A G C C C
   T C G G G  G T A T C  G C T G G  G T A C C  T C G G G

   C A T T G  A G C C C  C A C G G  A G C T C-3'
   G T A A C  T C G G G  G T G C C  T C G A G-5'
```

Figure All(d): p38/25-SP6 sequence

```
5'-GAGCT CACAC CACGC CCCAT AGAGA
3'-CTCGA GTGTG GTGCG GGGTA TCTCT

   CCCAC GGAGT CCCAC AGCGA CCCAC
   GGGTG CCTCA GGGTG TCGCT GGGTG

   ACCTA CCCAT GGAGC CCCAA TGCAG
   TGGAT GGGTA CCTCG GGGTT ACGTC

   CCCAC TATAG AGCAC CCCAC ANNNC
   GGGTG ATATC TCGTG GGGTG TNNNG

   ACCAA CGCGA CCCAT GGAGC CCCAT
   TGGTT GCGCT GGGTA CCTCG GGGTA

   CTGAG CCCCA CAGAA GTGCA CCCAT
   GACTC GGGGT GTCTT CACGT GGGTA

   AGCGA CCCAG TGGAG CCCAA CCTGA
   TCGCT GGGTC ACCTC GGGTT GGACT

   GCCCA TAGCA GCCCA TTGAG CCC-3'
   CGGGT ATCGT CGGGT AACTC GGG-5'
```

Figure All(e): p38/8-SP6 sequence

```
5'-C A C A G  T G C C C  C A C T G  A A G C C  A C A G C
3'-G T G T C  A C G G G  G T G A C  T T C G G  T G T C G

   C A C C C  C A T A G  C T C C A  C A T G T  G C C C C
   G T G G G  G T A T C  G A G G T  G T A C A  C G G G G

   A T A C G  C C C C A  T A G C G  C C C C G  G C T T T
   T A T G C  G G G G T  A T C G C  G G G G C  C G A A A

   T A G G G  G C A A T  A A A T G  G G T G C  G G T C C
   A T C C C  C G T T A  T T T A C  C C A C G  C C A G G

   C G A C G  T G C A T  C G C A15-3'
   G C T G C  A C G T A  G C G T15-5'
```

Figure All(f): p38-SP6 sequence

```
pDS12--GAG CTC ACA GAG ACT GTG GTC TGT GCC CTG GGG TTG TCT GTG GGC CTC
       * * * *     * *              *  **   ** *** * * *** *** ***  **
pl6SP6-GGG CGC TAT GGG GCA CAA TGT GGA GCT ATG GGG TGG TCT GTG GGC TTC

  GTG GGC ATT GTG GTG GGG ACC GTC TTG ATC ATC CGA GGC CTG
^^  * *** * * *** * *     *   *     *  *       *  **   **
AGTGG GGC ACT GTG GGG CTC AGT GGG GCG CTA TGG GGC GGG GTG
```

Figure A12: Region of homology between pl6 and pDS12.

The nucleotide sequence of the pDS12 SacI-StuI fragment is taken from Chang et al, 1983. The pl6 sequence is aligned according to the best fit found by the BioNet Align program (between the open arrows.) The restriction sites are underlined; asterisks represent identical nucleotides; ^ represent insertions to maximize the homology.

hybridization seen between these two cDNAs, especially since 65% of the matching nucleotides are Gs and Cs.

Further significant regions were not found by comparing these sequences with MHC genes, or with any sequences present in the Genbank files.

An interesting aspect of the sequence data, however is obvious from inspection of the sequences determined. Nearly all regions sequenced are quite conserved repeats of a 10bp 'consensus' sequence, shown in Figure A13 along with the percentage of repeats having that nucleotide in that position. Figure A14(a-e) shows all of the sequenced regions aligned to show the repeat homology. It is evident that there are deviations from the consensus, including variations in the number of nucleotides as well as their arrangements. However, the origin of this DNA as duplications of a 10bp repeat seems obvious.

In order to determine whether these sequenced portions could code for an unidentified protein, all forward reading frames were translated, as shown in Figure A15. There is no open reading frame in any region sequenced.

A final blotting experiment was carried out to determine the genomic structure of the DNA transcribed into the RNA represented by p38. Figure A16 shows the autoradiograms of B-congenic DNAs cut with EcoR1(a) or BamHI(b). A large number of bands was seen in each blot, indicating multiple DNA sequences in the genome.

A	C/T	A	G	A/C	G	C	C	C	C
90%	C-38% T-44%	54%	81%	A-36% C-48%	76%	66%	88%	91%	96%

FIGURE A13: Consensus sequence of the 10bp repeats found in p38 and p16 cDNAs. Percentage of all repeats which contain consensus base at the given location is indicated on the second line.

Figure A14 (a-f): Sequences that were shown in Figure A11, aligned to maximize their identity with the repeat consensus sequence shown in Figure A13.

C A C T A G C G C C C C

A C T G C G C C C C

A C T G C G C C C C

A C A G C G C C C

A C A G C G A C C C

A C A G A G C C C C

A T A G C A A C C C

A T T G A G C C C C

A T G G A G T T C C

A T A T C A C T C C

A G A G C G C C C C

A T A G C G C C C

A C A C C C C G C C C C

A T A G C G G C C C

A T T G A G C C C C

A C A G T G C C C C

A T G A A C C C A C

A G A T C A T C C C

A T A G C A C

A A T T G T G C C C

A T A G C G C C A C

A C C G C C C A T

G A G C A C A T G G C C

Figure A14(a): p16-T7 sequence

C C C A A T G A C C

A C A T G T G C C C

A T A G C G C C C C

A C A C C C C G C C C C

A T A G C G C C C C

A C T G A G C C C C

A C A G T G C C C C

A C T G A A G C C C

A C A G A C C A C C C C

A T A G C T C C A C

A T T G T G C C C C

A T A G C G C C C C

A T T T A T T G C C C C

T A A A A G C C

G G G G C G C T A T

G G C G G

Figure A14b: p16-SP6 sequence

```
                              C
      G T A     G C G  A C C C
      A T G     G A G  C C C C
A C   A C A     G C G  C C C C
      A C A     G T G  A C C C
      A C T     G A G  A C C C
      A T A     G C G  A C C C
      A T C  G  G A G  C C C C
      A C T     G A G  C C C C
      A C A     G C G  A C C C
```

Figure A14(c): p38-SP6 sequence

```
                    A G C C
T A T       G A G  C C A C
A N A       G T G  C G C
A G A       G C G  A C C C
A T A       G C G  A C C C
A G T       G G A  G C C G
A C A       C A G  C G A C
A C A  T G  G A G  C C C C
A C T  T    G A G  C C C C
A T A       G C G  A C C C
A C T       G A G  C C C C
A T A       G C G  A C C C
A T G       G A G  C C C C
A T T       G A G  C C C C
A C G       G A G  C T C
```

Figure A14(d): p38/25-SP6 sequence

			GAG		CTCC
	ACA		CCA	CG	CCCC
	ATA		GAG		ACCC
	ACG		GAG		TCCC
	ACA		GCG		ACCC
	ACA		CCT		ACCC
	ATG		GAG		CCCC
	AAT		GCA		GCCC
ACT	ATA		GAG	CA	CCCC
	ACA		NNN		CACC
	AAC		GCG		ACCC
	ATG		GAG		CCCC
	ATC	T	GAG		CCCC
	ACA	GAA	GTG	CA	CCC
	ATA		GCG		ACCC
	AGT	G	GAG		CCC
	AAC		CTG	A	GCCC
	ATA		GCA		GCCC
	ATT		GAG		CCC

Figure A14(e): p38/8-SP6 sequence

C

A C A G T G C C C C

A C T G A A G C C A

C A G C C A C C C C

A T A G C T C C A C

A T G T G C C C C

A T A C G C C C C

A T A G C G C C C C

G G C T T T T A G G G G C

A A T A A A T G G G

T G C G G T C C C

G A C G T G C A T C

G C A_{15}

Figure A14(f): p38-SP6 sequence

Figure A15 (a-f): Translation if three reading frames for each of the sequenced regions. Dots signify stop codons. Blanks indicate areas where the translation cannot be determined due to nucleotide sequencing ambiguities.

Figure A15 (a): p16-T7 translation

```
                                    27                                  54
CAC TAG CGC CCC ACT GCG CCC CAC TGC GCC CCA CAG CGC CCA CAG CGA CCC ACA
His  .  Arg Pro Thr Ala Pro His Cys Ala Pro Gln Arg Pro Gln Arg Pro Thr
 Thr Ser Ala Pro Leu Arg Pro Thr Ala Pro His Ser Ala His Ser Asp Pro Gln
  Leu Ala Pro His Cys Ala Pro Leu Arg Pro Thr Ala Pro Thr Ala Thr His Arg

                                    81                                 108
GAG CCC CAT AGC AAC CCA TTG AGC CCC ATG GAG TTC CAT ATC ACT CCA GAG CGC
Glu Pro His Ser Asn Pro Leu Ser Pro MET Glu Phe His Ile Thr Pro Glu Arg
 Ser Pro Ile Ala Thr His  .  Ala Pro Trp Ser Ser Ile Ser Leu Gln Ser Ala
  Ala Pro  .  Gln Pro Ile Glu Pro His Gly Val Pro Tyr His Ser Arg Ala Pro

                                   135                                 162
CCC ATA GCG CCC ACA CCC CGC CCC ATA GCG GCC CAT TGA GCC CCA CAG TGC CCC
Pro Ile Ala Pro Thr Pro Arg Pro Ile Ala Ala His  .  Ala Pro Gln Cys Pro
 Pro  .  Arg Pro His Pro Ala Pro  .  Arg Pro Ile Glu Pro His Ser Ala
  His Ser Ala His Thr Pro Pro His Ser Gly Pro Leu Ser Pro Thr Val Pro
```

Figure A15 (b): p16-SP6 translation

```
                                    27                                  54
GCC ATA GCG CCC CGG CTT TTA GGG GCA ATA AAT GGG GCG CTA TGG GGC ACA ATG
Ala Ile Ala Pro Arg Leu Leu Gly Ala Ile Asn Gly Ala Leu Trp Gly Thr MET
 Pro  .  Arg Pro Gly Phe  .  Gly Gln  .  MET Gly Arg Tyr Gly Ala Gln Cys
  His Ser Ala Pro Ala Phe Arg Gly Asn Lys Trp Gly Ala MET Gly His Asn Val

                                    81                                 108
TGG AGC TAT GGG GTG GTC TGT GGG CTT CAG TGG GGC ACT GTG GGG CTC AGT GGG
Trp Ser Tyr Gly Val Val Cys Gly Leu Gln Trp Gly Thr Val Gly Leu Ser Gly
 Gly Ala MET Gly Trp Ser Val Gly Phe Ser Gly Ala Leu Trp Gly Ser Val Gly
  Glu Leu Trp Gly Gly Leu Trp Ala Ser Val Gly His Cys Gly Ala Gln Trp Gly

                                   135
GCG CTA TGG GGC GGG GTG TGG GGC GCT ATG GGC ACA TGT GGT CAT TGG G
Ala Leu Trp Gly Gly Val Trp Gly Ala MET Gly Thr Cys Gly His Trp
 Arg Tyr Gly Ala Gly Cys Gly Ala Leu Trp Ala His Val Val Ile Gly
  Ala MET Gly Arg Gly Val Gly Arg Tyr Gly His MET Trp Ser Leu
```

Figure A15 (c): p38-T7 translation

```
                                    27                                  54
CGT AGC GAC CCA TGG AGC CCC ACA CAG CGC CCC ACA GTG ACC CAC TGA GAC CCA
Arg Ser Asp Pro Trp Ser Pro Thr Gln Arg Pro Thr Val Thr His  .  Asp Pro
 Val Ala Thr His Gly Ala Pro His Ser Ala Pro Gln  .  Pro Thr Glu Thr His
   .  Arg Pro MET Glu Pro His Thr Ala Pro His Ser Asp Pro Leu Arg Pro Ile

                                    81
TAG CGA CCC ATC GGA GCC CCA CTG AGC CCC ACA GCG ACC C
 .  Arg Pro Ile Gly Ala Pro Leu Ser Pro Thr Ala Thr
 Ser Asp Pro Ser Glu Pro His  .  Ala Pro Gln Arg Pro
  Ala Thr His Arg Ser Pro Thr Glu Pro His Ser Asp
```

Figure A15 (d): internal p38 sequence, 5" portion - translation

```
                                    27                                  54
AGC CTA TGA GCC ACA NAG TGC GCA GAG CGA CCC ATA GCG ACC CAG TGG AGC CGA
Ser Leu  .  Ala Thr     Cys Ala Glu Arg Pro Ile Ala Thr Gln Trp Ser Arg
 Ala Tyr Glu Pro     Ser Ala Gln Ser Asp Pro  .  Arg Pro Ser Gly Ala Asp
  Pro MET Ser His     Val Arg Arg Ala Thr His Ser Asp Pro Val Glu Pro Thr


                                    81                                 108
CAC AGC GAC ACA TGG AGC CCC ACT TGA GCC CCA TAG CGA CCC ACT GAG CCC CAT
His Ser Asp Thr Trp Ser Pro Thr  .  Ala Pro  .  Arg Pro Thr Glu Pro His
 Thr Ala Thr His Gly Ala Pro Leu Glu Pro His Ser Asp Pro Leu Ser Pro Ile
  Gln Arg His MET Glu Pro His Leu Ser Pro Ile Ala Thr His  .  Ala Pro  .


                                   135                                 162
AGC GAC CCA TGG AGC CCC ATT GAG CCC CAC GGA GCT C
Ser Asp Pro Trp Ser Pro Ile Glu Pro His Gly Ala
 Ala Thr His Gly Ala Pro Leu Ser Pro Thr Glu Leu
  Arg Pro MET Glu Pro His  .  Ala Pro Arg Ser Ser
```

Figure A15 (e): internal p38 sequence, 3' portion

```
                                                                  162
                                                GA GCT CCA CAC CAN CGC CCC ATA
                                                   Ala Pro His     Arg Pro Ile
                                                Glu Leu His Thr     Ala Pro  .
                                                 Ser Ser Thr Pro     Pro His Arg

                                    189                                   216
GAG ACC CAC GGA GTC CCA CAG CGA CCC ACA CCT ACC CAT GGA GCC CCA ATG CAG
Glu Thr His Gly Val Pro Gln Arg Pro Thr Pro Thr His Gly Ala Pro MET Gln
 Arg Pro Thr Glu Ser His Ser Asp Pro His Leu Pro MET Glu Pro Gln Cys Ser
  Asp Pro Arg Ser Pro Thr Ala Thr His Thr Tyr Pro Trp Ser Pro Asn Ala Ala

                                    243                                   270
CCC ACT ATA GAG CAC CCC ACA NNN NCA CCA NAC GCG ACC CAT GGA GCC CCA TCT
Pro Thr Ile Glu His Pro Thr         Pro     Ala Thr His Gly Ala Pro Ser
 Pro Leu  .  Ser Thr Pro          His     Thr Arg Pro MET Glu Pro His Leu
  His Tyr Arg Ala Pro His           Thr     Arg Asp Pro Trp Ser Pro Ile  .

                                    297                                   324
GAG CCC CAC AGA AGT GCA CCC ATA GCG ACC CAG TGG AGC CCA ACC TGA GCC CAT
Glu Pro His Arg Ser Ala Pro Ile Ala Thr Gln Trp Ser Pro Thr  .  Ala His
 Ser Pro Thr Glu Val His Pro  .  Arg Pro Ser Gly Ala Gln Pro Glu Pro Ile
  Ala Pro Gln Lys Cys Thr His Ser Asp Pro Val Glu Pro Asn Leu Ser Pro  .

                                    351
AGC AGC CCA TTG AG
Ser Ser Pro Leu
 Ala Ala His  .
  Gln Pro Ile Glu
```

Figure A15 (f): p38-SP6 translation

```
                                    27                                  54
CAC AGT GCC CCA CTG AAG CCA CAG CCA CCC CAT AGC TCC ACA TGT GCC CCA TAC
His Ser Ala Pro Leu Lys Pro Gln Pro Pro His Ser Ser Thr Cys Ala Pro Tyr
 Thr Val Pro His  .  Ser His Ser His Pro Ile Ala Pro His Val Pro His Thr
  Gln Cys Pro Thr Glu Ala Thr Ala Thr Pro  .  Leu His MET Cys Pro Ile Arg

                                    81                                  108
GCC CCA TAG CGC CCC GGC TTT TAG GGG CAA TAA ATG GGT GCG GTC CCG ACG TGC
Ala Pro  .  Arg Pro Gly Phe  .  Gly Gln  .  MET Gly Ala Val Pro Thr Cys
 Pro His Ser Ala Pro Ala Phe Arg Gly Asn Lys Trp Val Arg Ser Arg Arg Ala
  Pro Ile Ala Pro Arg Leu Leu Gly Ala Ile Asn Gly Cys Gly Pro Asp Val His

                                   135
ATC GCA AAA AAA AAA AAA AA
Ile Ala Lys Lys Lys Lys
 Ser Gln Lys Lys Lys Lys
  Arg Lys Lys Lys Lys Lys
```

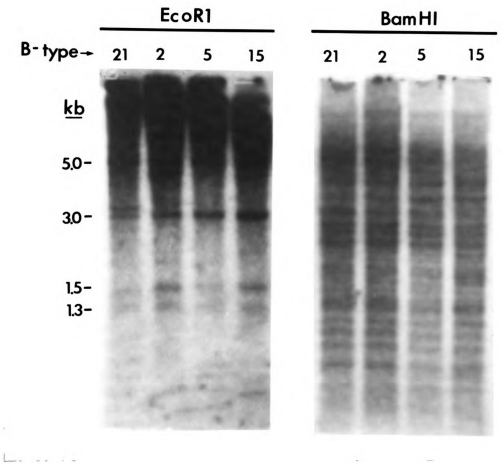

Figure A16: Genomic Southern blots of four different haplotype DNAs probed with nick translated p38. Panel A shows EcoR1 digestion; panel B shows BamHI digestion.

DISCUSSION

Once the decision was made to begin cloning genes of the MHC of the chicken, a number of possible approaches were evaluated. 1) Use of synthetic oligonucleotide probes was not possible due to lack of protein amino acid sequence data. In addition, the difficulty in obtaining adequate quantities of purified protein and lack of expertise in this area precluded an attempt on our part to obtain this sequence data prior to the cloning attempts. 2) Several approaches require the use of highly specific antibodies to screen expression libraries or to recognize in vitro translation products from hybridization-selected mRNAs. Although we have access to some excellent serologic typing reagents (Bacon et al, 1987b), these allo-antisera recognize the polymorphic determinants on the native cell-surface molecules which are glycosylated hetero-dimers. The antisera titers against the individual polypeptide chains produced in bacteria or in vitro is too low to be very useful for cloning purposes. 3) Since the structure and function of the chicken MHC proteins appears highly homologous to the mammalian proteins, it is possible that low stringency hybridization using the mammalian genes would be able to identify the homologous chicken genes. This approach has proven successful in cloning human Class I genes (using mouse Class I cDNA) and in cloning mouse Class II genes (using human Class II cDNA). Although it is very likely that chicken MHC genes would be more divergent from either mouse or human than the two mammalian species would be from each other, several lines of evidence led us to believe this approach might work. First, highly conserved genes, such as the precursors of oncogenes, had been detected in a

variety of species using low stringency hybridization conditions. Second, the degree of similarity between conserved MHC gene segments in mouse and human was as high as 68 - 76% (Auffray et al, 1984,) leading us to believe that even the greater divergence of the avian species might still allow for low stringency hybridization. Third, other chicken genes which had been cloned had diverged from their mammalian counterparts to a small enough degree that low stringency detection between them might be possible. For example, the chicken lambda light chain immunoglobulin gene had been cloned and was 73% and 54% identical to the human and mouse homologs, while the identity between mouse and human was only 41-51% (Reynaud et al, 1983.) This encouraged us to believe that chicken MHC genes might be picked up at low stringency using a mammalian homologous probe.

Consequently, we obtained several mammalian cDNA clones and tested their hybridization at low stringency to restriction enzyme-digested chicken genomic DNA (Figure A3). A single major band appeared in each lane, with some variations between haplotypes in the minor bands. However, we were concerned about the possibility of not being able to identify the location of less conserved or more polymorphic exons, even if we were successful in cloning the most conserved exon. We therefore felt that obtaining a cDNA clone would not only enhance our possibility for successful cloning, but also would yield additional information concerning the divergence of less conserved regions.

Since cDNA clones would need to be obtained from cells which were actively synthesizing the protein and we wanted a reproducible source

for the mRNA, we chose to look at RNA from RP-9 cells. RP-9 is a chicken B-lymphoblastoid cell line cultured from a transplantable tumor which originated in a $15_1 \times 7_2$ chicken infected with RAV-2. It has been shown to be positive for cell surface "Ia," using a specific anti-chicken monoclonal antibody (C-L. Chen, personal communication.) Since this is the equivalent of the human Class II MHC proteins, we examined the ability of the DS`12 cDNA clone to hybridize with RNA from RP-9 cells. As shown in Figure A4, a 1.95 kb RNA was detected under low stringency conditions. We therefore proceeded with construction of an RP-9 cDNA library in lambda gtlØ.

Initially, 2ØØ,ØØØ lambda gtlØ recombinant clones were screened with both DS`12 and pRAV-2 as probes. At high stringency, approximately Ø.14% of the clones hybridized with the pRAV-2 probe, which was within the expected range, since these cells release infectious virus and therefore are producing messages for the structural proteins of the virus. Using very low stringency conditions for the DS`12 probe, twenty plaques were considered to be positive on duplicate filters and were picked for further plaque purification. 75% of these were confirmed to be positive during the secondary screenings. However, it is estimated that those plaques picked after the low stringency primary screening represented only 1Ø-2Ø% of the similarly positive plaques. This low percentage arose because of the extremely low stringency used in screening the plaques; many regions of the filters contained sufficient background to prevent ability to discern positive plaques on the filter. Only those plaques detected on duplicate filters were picked for analysis. If the estimate of 1Ø-2Ø% is correct, then there were

actually 100-200 positives out of the original 200,000 plaques screened, or 0.05-0.1% of the mRNA is represented in the DS`12-positive fraction. This appears to be similar to the 0.3% abundance found in a human lymphocyte cDNA library by Erlich et al (1984).

Secondary screening and plaque purification yielded fifteen clones which remained positive throughout the procedure. Phage DNA was isolated from these clones, the EcoRI fragments were transferred to nitrocellulose, and the filters were hybridized with the DS`12 probe. Thirteen of the fifteen clones exhibited ethidium bromide-detectable inserts ranging in size from 175 to 1500 bp. Nine of the inserts were detectable on autoradiograms after the hybridization with DS`12 probe, as shown in Figure A7a. Subcloning of two of these inserts (10A and 18A) into the plasmid vector pGEM4 gave the recombinants designated p38 and p16, which allowed further mapping, sequencing, and hybridization analyses. After removing the ^{32}P-labelled probe from the filter shown in Figure A7a, this filter was rehybridized with the nick-translated insert from p38. The results shown in Figure A7b indicate that all of the gt10 inserts which could be detected by DS12 at low stringency could also be detected by p38 at high stringency, indicating that this set of clones consists largely (if not exclusively) of various cDNA copies of mRNA transcribed from a single gene or members of a closely related gene family. Since the initial subcloning attempts yielded a plasmid containing the largest of these closely related inserts, further subcloning did not appear to be necessary at this time. All further studies described here were conducted using the p38 and p16 subclones.

In order to determine which portion of the human cDNA was responsible for the hybridization to these clones, DS`12 was restricted with various enzymes and the nitrocellulose containing the fragments was hybridized with nick-translated p38. As shown in Figure A8b, all fragments which hybridized contain the small SacI--StuI fragment which spans the transmembrane portion of the DS alpha polypeptide. Hybridization using the p16 insert or either EcoRI--SacI fragment of p38 as the probe showed identical hybridization patterns to the pattern shown in Figure A8b (data not shown).

When Northern blots of RP-9 RNA hybridized with the nick-translated p38 clone were compared with an identical blot hybridized with the DS`12 probe, it was determined that both probes identified a band of identical size, although the DS12 band could only be seen under conditions of low stringency. Also, p38 picked up several less abundant bands of higher molecular weight, which may be partially processed precursors of the 1.95 kb band present in the total cellular RNA. Thus, the 1500 bases of p38 represent the major portion of the only RNA sequence which hybridizes to DS12.

Sequence analysis was begun on both p38 and p16, according to the strategy shown in Figure A10, and the deduced sequences are shown in Figure A11(a-f). Analysis of these sequences and their comparison with other published sequences leads to several conclusions. First, a 35 bp portion of p16 is >80% identical with the transmembrane portion of DS`12 (Figure A12.) This probably is adequate to account for the hybridization seen at low stringency. Second, p38 contains a

polyadenylation signal (AATAAA) 25 bp upstream of the 15 bp poly (A) stretch at one end of the clone. Assuming that no recombination events have occurred during cloning, this identifies the "coding" strand for the entire length of the clone. Third, all areas sequenced appear to consist primarily of highly conserved repeats of a 1Ø bp sequence Figures A13-A14.) Assuming that this repeating structure is the same on both clones, the portion of the p16 sequence which is homologous to the DS`12 transmembrane coding sequence is found on the p16 "non-coding" strand, indicating that it is insignificant to any protein structure which may be coded by this RP-9 RNA. Fourth, no long open reading frames have been located on the coding strand within those portions sequenced (Figure A15,) indicating that if a protein is coded for by this mRNA, it is not as large as an MHC Class II peptide chain appears to be. Fifth, no significant homology exists between the sequenced portions of this cDNA and DS12 or any other published mammalian MHC sequence, aside from the previously-mentioned transmembrane portion.

This leads to the question of the source of this unusual, repeat-containing, poly A+ RNA. Our first attempt to answer this was to look at its hybridization to genomic blots of normal chickens. As shown in Figure A16, when used as a probe, the p38 plasmid detected a large number of genomic fragments using several enzymes, such as BamHl or HindIII, indicating that it represents a repetitive sequence present many times in the genome. However, it was noticed that EcoRI digestion yielded many fewer bands, perhaps indicating that many of the repeats are clustered whithin these large EcoRI fragments. Thus, p38 apparently

represents a short, moderately repetitive sequence which is transcribed in RP-9 cells into a specific, large, polyadenylated RNA.

We have considered the possibility that p38 represents a cloning artifact, and that it may be misleading to base conclusions on the structure and sequence of p38. However, the size of p38 is reasonable to have come from the 1.95 kb RNA detected in Northern blots at low stringency with DS12 and at high stringency with p38. Although the polyadenylation signal lies within the only sequence which does not clearly fit the repeat structure, it probably represents the true 3' end of the RNA; since the cDNA was made from oligo dT cellulose-selected RNA, and was made using an oligo dT primer, the original cDNA most likely was polyadenylated, in order to be represented multiple times in the library.

If this is truely a polyadenylated RNA transcribed from repetitive DNA, why is it present in RP-9 cells? Transcriptional activation due to promotor insertion is known to occur as a result of retroviral integration. We do not know whether this has occurred in this cell line, but it could be tested by cloning the junction fragments of the inserted provirus(es) and looking for adjacent repeats of the type found in p38. It is also possible that the transcription of this RNA is common to some or all transformed cells. Numerous reports have documented the presence of highly repetitive sequences in poly A+ RNA of transformed cells or during embryonic development (Scott et al, 1983; Murphy et al, 1983, Kramerov et al, 1982; Miyahara et al, 1985; Yamamoto et al, 1983,) although the presence of repetitive elements is

frequently restricted to the 3'-untranslated portion, where it may affect mRNA stability (Croce, 1987.) Of course, it is possible that the 5'-400 nucleotides which are missing from this clone may contain coding sequences and that the entire 1500 bp of the cDNA is contained within the 3'-untranslated region of the 1950 bp mRNA. However, regardless of whether there are 150 or 190 copies of the repeats, the reason for this very large copy number of the repeat in this transcript is unknown.

Most of the very short, tandem repeats in highly repetitive portions of DNA have been shown to be present at telomere and centromeres of chromosomes (Jelenik and Schmid, 1982) where their function is unknown. In the newt, one such repetitive sequence is also present interspersed among histone genes, and is transcribed in lampbrush chromosomes when the polymerase fails to recognize the correct termination signal for the histone gene, and continues transcription through the adjacent repeat sequences (Diaz et al, 1981.)

Now that we know the 1.95 kb message does not code for an MHC Class II alpha chain, what conclusions can be reached about the chicken MHC from this study? This hybridization strategy should have picked up a DQ alpha homolog expressed in B cells of the chicken, if that gene maintained at least 65-70% homology over a substantial portion of the region coding for a single domain. This estimate is based upon the actual G-C content of pDS12, and wash stringency conditions of 2xSSC at 56C, which allows at least 30% mismatch in hybridization. The actual homology detectable in the Northern blots is lower than this, due to increased stability of RNA-DNA hybrids.

This means that either the DQ alpha homolog lacks adequate similarity to be detected by hybridization, or that there is no DQ alpha homolog expressed in the chicken. The latter explanation would fit with the data of Guillemot et al (1986), who found only a single, non-polymorphic alpha chain expressed on splenic lymphocytes of several B-haplotypes and on RP-9 cells. Thus, the single alpha chain expressed on these cells is likely to be the homolog of the human DR alpha or the mouse E alpha, which are both non-polymorphic.

The data described here lead to the following conclusions: 1) there may not be a DQ alpha homolog in the chicken, recognizing the possibility of fewer genetic loci and expressed proteins means less complexity in the chicken Class II MHC than the mammalian Ia regions, and 2) if there is a DQ alpha homolog in the chicken, it may not be expressed (Guillemot et al, 1986), and certainly diverges sufficiently to interfere with the success of hybridization analyses such as that described here..

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Chapter B: Transformation-related viral transcripts in Marek's Disease Virus-transformed cell lines.

Marek's disease (MD) is a malignant lymphoma of chickens, first described by Josef Marek (1907) as a polyneuritis causing paralysis. It was recognized as a viral disease, differentiated from the retroviral-induced lymphomas, as the result of work by Biggs (1961), Campbell (1956), Biggs and Payne (1963.) It has been among the most economically important diseases in commercial chicken flocks, especially in the late 1950s and 1960s, when its losses in the United States reached $0.5 million per day (Calnek and Witter, 1984.)

Marek's disease is a highly contagious disease, caused by a herpes virus (Marek's disease virus, MDV) which is usually strictly cell-associated (Nazerian, 1980). However, latent infection persists for the life of the bird and periodically erupts into productive infection in the feather follicle epithelium, releasing mature infectious virions into the environment when feathers are molted or dead cells are shed (Calnek, 1986.) Thus, the virus persists in all flocks. Losses have decreased tremendously, however, as a result of the introduction in 1969 and 1970 of vaccines composed of either attenuated MDV strains (Churchhill et al, 1969) or the antigenically-related, nonpathogenic herpesvirus of turkeys (HVT), (Okazaki et al, 1970.) Thus, MDV is the first tumor virus for which a safe and effective vaccine has been developed, and is in routine use.

Although economic losses have declined due to the vaccine, MDV and its lymphoma continue to be studied in several areas. The mechanisms of viral transformation, genetic resistance, and vaccinal immunity are all being studied, both in an effort to better understand Marek's disease,

and as a model for other herpesvirus oncology (Nonoyama, 1982; Calnek, 1986.) MD is an excellent model system because it is a naturally occuring disease for which the appropriate genetic strains of both host and virus are available and well characterized. In addition, vaccine development work continues. Very virulent forms of the virus have emerged in the field, against which the vaccine was less effective (Witter, 1983; Witter, 1985), and new approaches, such as development of bivalent vaccines (Witter, 1982), have been required in the past, and may be necessary again in the future.

Thus, the virus, its pathogenicity, the host's immunity to it, and vaccines have all been studied extensively, making Marek's disease one of the best understood of all the herpes virus-induced neoplastic diseases (Calnek, 1986.) However, even with the well-understood descriptive biology of the tumors, the molecular mechanisms responsible for the transformation remain elusive.

BIOLOGY OF MAREK'S DISEASE

MDV usually infects the host via the respiratory tract when the bird occupies a contaminated environment. Little or no virus infection can be detected in the cells of the respiratory passages, and means of travel to the primary lymphoid organs has not been established. Early cytolytic infection occurs in the spleen, bursa of Fabricius, and thymus. The viral internal antigens (VIA) are first detectable in a few cells at 1-2 days post infection. Maximum infection occurs between 3 and 5 days post infection, and the cytolytic infection is largely over

by 7 days post infection (Calnek et al, 1984a.) This stage can be largely prevented by embryonic bursectomy (Schat et al, 1980); the cells which are cytolytically infected at this stage have been shown by staining with appropriate antisera to be primarily B cells (Shek et al, 1983). However, a small proportion (usually less than 10%) of these cells are T cells (Calnek et al, 1984a.) As a result of the cytolytic infection, both the bursa and thymus show significant atrophy.

As the number of cytolytically infected cells drops off during days 6-7 post infection, latently infected cells appear in the spleen and peripheral blood. These cells appear to be primarily activated T cells, bearing both T cell markers and Ia antigen, although about 2-4% of latently infected cells are B cells (Shek et al, 1983, Calnek et al, 1984a.) The viral genome is present in only a few copies per cell (Calnek et al, 1984a) and VIA are not detected (Calnek et al, 1981b.) However, VIA expression can be induced in these cells by 24-48 hour cultivation in vitro (Calnek et al, 1981a.) The amount of transcription or protein expression from other viral genes is unknown. These lymphocytes remain latently infected for the life of the bird (Witter et al, 1971.)

It is not known whether other cell types may also harbor latent infections. However, after the second or third week post infection, foci of cytolytic infection appear in epithelial tissues, such as kidney, adrenal gland, and feather follicle epithelium. These infections result in necrosis and local inflammatory response with infiltration of mononuclear cells. The feather follicle epithelium is

the only tissue known to support a fully productive infection, releasing enveloped, infectious virus to the environment.

During this same period, beginning in the second or third week post infection, another wave of cytolytic infection begins in lymphocytes in the central lymphoid organs. It is not known whether it is primarily B or T cells which undergo cytolytic infection at this time.

A prominent feature of Marek's disease is the induction of immunosuppression (Lee, 1984.) A transient immunosuppression follows the initial wave of cytolytic infection in the central lymphoid organs. Although this may be partly due to the atrophy of both bursa and thymus following the cytolytic infection, both humoral and cell-mediated immune response to MDV are detectable by about 7 days post infection (Sharma and Coulson, 1977; Sharma et al, 1978; Confer et al, 1980). Therefore, the atrophy is not sufficiently complete to account for the total suppression seen. At this time, about 6-7 days post infection, a transient decrease in in vitro mitogen responsiveness (Lee et al, 1978; Liu and Lee, 1983b) has been attributed to the presence of suppressor macrophages (Theis, 1977.) However, by about three weeks post infection, a "permanant" immunosuppression develops at the same time as the second wave of cytolytic infection in genetically susceptible chickens. This immunosuppression includes depressed antibody responses (Jokowski et al, 1973), delayed skin graft rejection (Purchase et al, 1968) and impaired PHA response (Lee et al, 1978.)

The early transient immunosuppression, early cytolytic infection, and persistence of latently infected T cells occur in all chickens infected by MDV, regardless of the genetic resistance of the host chicken or the degree of pathogenicity attributed to the virus strain (Confer and Adldinger, 1980.) However, beginning at about three weeks post infection, the "permanent" immunosuppression and the second wave of cytolytically infected lymphocytes occur only in those chickens undergoing full-blown disease. It may be that there is a cause-effect relationship between the later cytolytic infection and the permanent immunosuppression (Calnek, 1986) but this has not been proven. It is also possible that the immunosuppression is due to the effect of soluble suppressor factors, which have been associated with the spleens of immunosuppressed chickens (Theis, 1977.)

A final (and probably the most characteristic) facet of Marek's disease is the neoplastic transformation of lymphocytes and the development of gross lymphomas. These tumors contain not only the transformed cells, but also a variety of other lymphoid cells, including T and B cells and macrophages (Payne and Roszkowski, 1973.) The exact time of cellular transformation is unknown, but microscopic lesions can be detected as early as two weeks post infection, and gross lymphomas of organs such as gonads, spleen, and nerves are seen after about three to four weeks (Payne, 1982.) Tumor cell lines have been established which can form tumors when injected into susceptible birds (Akiyama and Kato, 1974; Nazerian et al, 1977; Calnek et al, 1978;) and these cells are characterized as activated T cells, possessing both T cell markers and Ia (Nazerian and Sharma, 1975; Ross et al 1977; Schat et al, 1982.)

Numerous other antigens have been detected on the MD cell lines, but the most carefully studied of these has been MATSA, Marek's disease tumor-associated surface antigen (Witter et al, 1975.) Distribution of MATSA has been investigated using a variety of antisera--it is usually present on a majority of cells within the cultured MD cell lines, on a minority of cells from an MD tumor, and on a small percent of normal spleen cells (Witter et al, 1975.) It is not present on latently infected lymphocytes (Calnek et al, 1981b.) MATSA is probably host-gene encoded, as it varies among species or strains of host (Elmubarak et al, 1981; Ross et al, 1982; Powell et al, 1984.) It is also present on some cells infected with non-transforming strains of MDV (Schat and Calnek, 1978.) MATSA-bearing cells have been found in the lymphoid organs as early as 5-7 days post infection (Murthy and Calnek, 1978), and in the gonad at 16 days post infection (Powell and Rennie, 1985.) This indicates that either transformed cells, or "pre-tumor" cells are present in the organs at these times.

Although most tumor cells support a small amount of partially productive infection, very limited viral gene transcription and expression has been detected. Silver et al (1979) found only 12-14% of the viral genome was transcribed in a non-producer MD cell line, MKT-1, compared with transcription from nearly 50% of the genome in productively infected cells. Calnek et al (1981) found a very low level of expression of VIA and viral membrane antigens in a survey of 31 MD cell lines.

VACCINES

MDV strains fall into three serological groups; serotype I includes all pathogenic strains, and the tissue-culture attenuated strains derived from them. Serotype II strains are naturally occuring, non-pathogenic strains which infect chickens in the field. Serotype III is the herpesvirus of turkeys (HVT), which is normally found non-pathogenically infecting turkey flocks, but also is apathogenic when it infects chickens. Although there is some antigenic cross reactivity between these serotypes, they can easily be distinguished using various serologic reagents, including monoclonal antibodies (Lee et al, 1983.)

Vaccines have been developed using all three serotypes; attenuated serotype I or naturally non-pathogenic serotypes II and III. Most vaccines rapidly induce a cell-associated viremia, but do not produce a cytolytic infection of lymphocytes, as does the pathogenic MDV (Witter, 1985.) The majority of infected cells do not bear B cell or macrophage markers, although both latently-infected B and T lymphocytes may be present. The cell types infected by different serotypes may not be the same (Calnek et al, 1981b; Witter, 1985.) A transient immunosuppression, detected as a depression in the PHA response of T cells, has been noted at about 7 days post vaccination, but the response is quickly recovered (Lee et al, 1978.) The vaccination immune response involves both antibody and cell-mediated responses to viral antigens, but probably does not include anti-MATSA antibodies. Protection against MD is related more to cell-mediated than antibody response, and is directed more to viral than tumor antigens (Witter, 1985.)

GENETIC RESISTANCE TO MD

If a chicken is infected with an MDV strain other than a very virulent one, susceptibility or resistance of the host strain of chicken often mediates the outcome of the MDV infection (Schat et al, 1981,) which proceeds through the early stages of infection described above regardless of the genetic background. There are at least two genetic loci responsible for resistance, probably both are involved in regulating immune response (Lee et al, 1981,) although there is evidence that ability of MDV to infect T lymphocytes is also controlled by a locus which affects the incidence or severity of MD (Powell et al, 1982.).

A major host gene influence on MD succeptibility is the major histocompatibility complex, the B-locus; this probably involves an active rejection of the tumor cells (Pazderka et al, 1975; Longenecker et al, 1976; Briles et al, 1977; Briles et al, 1983.) Pevzner et al (1981) have also observed a linkage between Marek's resistance and response to GAT, which would be an immune response gene, probably within the B-complex. Another locus possibly involved in MD susceptibility is the Ly-4 locus, controlling a T cell antigen (Fredericksen et al, 1977.)

In general, the effects of genetic resistance and vaccination are additive, and selection of resistant strains is important, in an effective vaccination program (Witter, 1985.) However, in at least one experiment involving B-congenic chickens, some B-haplotypes conferred

increased vaccination response, and were ranked as more resistant than other haplotypes after vaccination, even though they were more susceptible before vaccination (Bacon, 1987.)

OTHER HERPESVIRUSES

Other animal herpesviruses have been described; some of them also appear to be oncogenic. Luecke's frog herpesvirus was the first oncogenic herpesvirus to be described (Granoff, 1982); it causes a renal adenocarcinoma in 1-9% of frogs in high incidence areas. The biology has not been completely characterized, but a unique temperature dependence of cytolytic activity exists. At warm temperatures, the virus is latent and tumors develop, at cold temperatures during hibernation, a percentage of tumor cells develop cytolytic infections (Sharma, 1976; Granoff, 1982.)

Other viruses have been associated with oncogenicity in the natural hosts, including Herpes sylvilagus in cottontail rabbits (Hinze and Chipman, 1971,) Herpes papio in baboons (Falk, 1980,) Herpesvirus saimiri in primates (Falk, 1980,) and Epstein-Barr and Herpes simplex type 2 in humans (zur Hausen, 1980; Nahmias and Norrild, 1980.)

Most studies of these viruses in regard to mechanism of transformation has used *in vitro* transformation assays. For example, hamster cells can be transformed constitutively by a small (about 6%) portion of the Equine herpesvirus type I genome, which integrates into the cellular genome (O'Callaghan et al, 1983.) Similar experiments with herpes

simplex and cytomegalovirus have yielded inconsistent, puzzling results with apparently different regions being involved in unknown functions related to transformation (Bishop, 1985.) A 2 kb region of Herpesvirus saimiri is required for transformation of T cells in culture, and for oncogenicity in primates, but it is not known whether this region is directly responsible for transformation (Desrosiers, 1985.)

Another applicable in vitro study of interest is the finding that an immediate early gene product of pseudorabies virus (and a similar protein from Herpes simplex virus) could substitute for the activity of the adenovirus Ela product (Ben-Porat and Kaplan, 1982; Kingston et al, 1985.) However, that the gene products are not totally homologous is shown by the fact that Ela cannot substitute for the herpesvirus gene products. Nevertheless, the parallelism is intriguing.

Herpes simplex is the herpesvirus for which the most molecular genetic information is available. HSV-1 DNA consists of a long unique region flanked by inverted repeats, which is joined to a short unique region, also flanked by inverted repeats. The long and short regions can invert with respect to each other, and all four possible configurations exist in equimolar amounts (Roizman, 1979.) The entire genome has been cloned (Goldin et al, 1981; Post et al, 198Ø) and sequencing is in progress (MCGeoch et al, 1985; Rixon and McGeoch, 1985.)

A considerable amount is known about specific gene transcription in HSV during productive infection (for review, see Wagner, 1982;) a very brief synopsis will be given here. HSV-1 mRNA is produced in the nucleus, is

capped and polyadenylated, and most is colinear with its gene, i.e., there is very little splicing that occurs in HSV mRNA. Overlapping mRNA families are common. The mechanisms for generation of these families include multiple promotors, inefficient termination at a polyadenylation site, an additional promotor within a gene initiating transcription of mRNA for a shorter peptide product, and in a few cases, differential splicing patterns. Whether these gene families encode a single peptide, closely related peptides, or non-related peptides appears to vary, as all three possibilities have been identified in HSV.

Immediate early (alpha) genes are transcribed prior to viral genome activity, and have been mapped in or near the repeat sequences flanking both unique regions. At least one of these, ICP-4, appears to be necessary throughout infection, and codes for a protein which is autoregulatory, in analagy to the T-antigens of papova viruses. The beta genes are expressed after viral protein synthesis begins, and the gamma gene products appear following viral genome replication. Several of the cell surface glycoproteins are encoded within the short unique region.

Specific regions of the HSV genome involved in transformation have not been clearly defined; in vitro transformation has yielded varying results (Bishop, 1985,) studies of cervical carcinomas induced by HSV-2 may implicate a 35-38 kD peptide, but its gene is not yet defined (Gilman et al, 1980.)

HSV gene expression in lytically-infected cells is interrupted in neuronal latency, and viral transcripts are undetectable in Northern blots (Puga et al, 1978.) However, more recent studies have indicated the continued expression of the ICP-0 gene in latently-infected mouse trigeminal ganglia, using various methods (Puga and Notkins, 1987; Deatley et al, 1987.)

The potential importance of some immediate early genes in not only lytic infection, but also latency, is echoed by their potential role in tumor-induction. Both adenoviruses and papovaviruses contain genes which have been involved in trans-activation of oncogenes--the Ela, Elb of adenoviruses, and the large T-antigen of polyoma and SV-40 (Kingston et al, 1985.) There are immediate early gene products of both pseudorabies and HSV which appear to have similar activities in vitro (Ben-Porat and Kaplan, 1982; Bishop, 1985.) This leads naturally to the possibility of herpesvirus immediate early gene products also playing a role in tumor induction.

MOLECULAR BIOLOGY STUDIES OF MDV

Serotype I MDV has a genome which consists of linear, double-stranded DNA of approximately 120×10^6 daltons (Lee et al, 1971,) or about 180 kb. The genome structure appears similar to that of Herpes simplex virus (HSV) with both long unique and short unique regions, each of which is flanked by inverted repeats (Cebrian et al, 1982,) although there is no evidence for isomers representing different arrangements between the

long and short unique sequences. Electron microscope comparison measurements indicated that the serotype III (HVT) genome is about the same total size as HSV DNA, with serotype I being about 15% larger.

The use of molecular cloning technology has enabled researchers to look carefully at structural characteristics of the viral genome. Fukuchi et al (1984) have cloned nearly all of the viral genome in BamHI fragments. Gibbs et al (1984) cloned a sizable number of the EcoRl fragments. The composite restriction enzyme map (adapted from Silva and Witter, 1985) which shows the location of these fragments is shown in Figure Bl. Dr. Nonoyama and Dr. Kung have kindly made these clones available to other researchers for further studies.

Restriction enzyme digest comparisons of DNAs from different strains has shown very similar patterns within strains of a given serotype, but totally different patterns between serotypes (Hirai et al, 1979; Ross et al, 1983; J. Carter, personal communication.) This comparison is in agreement with monoclonal antibody data on proteins in which certain antibodies can differentiate between serotypes, but very little difference is seen among strains within a serotype (Lee et al, 1983; Silva and Lee, 1984; Ikuta et al, 1983.) However, most antibody preparations recognize common determinants between serotypes, and the vaccines are apparently effective due to common epitopes between serotypes. Therefore, DNA hybridization studies have been carried out to further investigate the extent of homology between DNAs of the various serotypes. Initial hybridization studies indicated very little homology, less than 5% (Lee et al, 1979; Hirai et al, 1979.) However,

using very low stringency conditions, it has been determined that one can detect at least 70-80% similarity between HVT and serotype I MDV, over 90-95% of the genome (Gibbs et al, 1984; Fukuchi et al, 1985a; Hirai et al, 1984.)

Fruitful comparisons have also been made between the DNA of pathogenic serotype I strains and the attenuated strains derived from them (Hirai et al, 1981b; Ross et al, 1983; Fukuchi et al, 1985b; Silva and Witter, 1985; Maotani et al, 1986.) In particular, all groups found alterations within a region from the inverted repeats flanking the long unique region, and located within the cloned fragments BamHI-D, BamHI-H, and EcoRl-F. It was shown that the loss of this fragment in restriction analysis was correlated with the number of passages in tissue culture, and with loss of pathogenicity (Silva and Witter, 1985.) The altered region has been cloned from high passage DNA, and the specific area has been identified and sequenced (Maotani et al, 1986.) The region common to BamHI-H, BamHI-D, and EcoRl-F contains a 132bp repeated segment which apparently becomes duplicated a large number of times. This fragment does not contain an open reading frame, but does contain short inverted repeats which could indicate secondary structures of importance in regulation of gene expression.

In addition to expansion of this region, H-J. Kung and collaborators (personal communication) have detected regions within the same fragments which show homology by hybridization with the long terminal repeats of reticuloendotheliosis virus (REV.) Since REV is capable of inducing, in susceptible chickens, T cell lymphomas which appear quite similar to the

MDV lymphomas (Witter et al, 1986,) the sequence similarities within this region may also indicate that it is involved in significant regulatory functions.

Preliminary evidence that this region may play a significant regulatory role was discussed at the Herpesvirus Workshop in England (1986). Transcription of a specific 2 kb message beginning about 600 nt downstream from this expansion region of BamHI-H has been postulated to occur in CEF infected with pathogenic MDV strains, but not with high-passage, attenuated serotype I strains. This is distinct from a 1.8 kb mRNA, which is supposed to begin several kilobases upstream of the expanded region and is transcribed from the opposite strand.

Since most of the molecular biological studies have been done on in vitro infected fibroblasts, the status of viral DNA and its transcription in tumor cells is still largely unknown. Nazerian and Lee (1974) looked at the number of genomes present in the MSB-1 tumor cell line, which does support a limited amount of viral replication, and virus-specific antigens are detectable in 1-2% of the cells. They estimated an average of 60-90 genomes per cell. Previous estimates for MDV genomes in in vivo tumors was 3-15 per cell (Nazerian et al, 1973), but this estimate is probably too low because of the presence of non-tumor cells in the tumor. Whether transformation requires the integration of MDV DNA into the genome is not clear; there are reports of at least some of the MDV DNA being integrated in tumor cell lines (Kaschka-Dierich et al, 1979; Hughes et al, 1980,) although most is probably episomal (Tanaka et al, 1978.)

Specific efforts to detect transcription from the MDV genome in tumors or in cell lines derived from tumors has not been reported to date.

Efforts have begun, however, to identify and study the genes for immunogenic proteins produced by MDV infection. P. Coussins and L.Velicer (personal communication) have identified, mapped, and sequenced the gene for the A antigen from both MDV and HVT. It is located within the BamHI-B fragment of serotype I, and contains an open reading frame which apparently codes for a secreted glycoprotein of the expected size. The amino acid sequence homology between the proteins of the two serotypes is quite strong, but the nucleotide sequence contains many changes in silent or conservative positions. I. Sithole and L. Velicer are also making considerable progress in identifying and sequencing the gene for the B antigen (personal communication.)

MATERIALS AND METHODS

Many of the methods used have already been described. Only those which were unique to this project will be described here.

Cell lines: A synopsis of cell lines used and their characteristics is listed in Figure B3. The MSB-1 cell line was developed by Akiyama and Kato (1974.) The RP-1 cell line was developed from a transplantable tumor, JMV, which had been passaged in birds an unknown number of times before adaptation to tissue culture (Nazerian et al, 1977.) RP-4 was also developed from a transplantable tumor, induced by GA strain of MDV in a B^2 homozygous bird (Nazerian et al, 1978.) RP-19 is a turkey MDV-GA-induced cell line, which apparently is of immunoglobulin-secreting B cell origin (Elmubarak et al, 1981.) RP-13 is a B lymphoblastoid cell line, induced by REV (Nazerian et al, 1982.) RP-9 has been descibed previously; it is an ALV-induced B lymphoblastoid cell line.

All cell lines were grown in Liebowitz-McCoy media (1:1; GIBCO Laboratories, Grand Island NY) supplemented with 20% chicken serum, 10% bovine fetal serum, 5% tryptose phosphate broth, 2mM glutamine, 1mM sodium pyruvate, and 0.01mM 2-mercaptoethanol. They were grown at 41C in 5% CO_2.

Infected fibroblasts: Duck embryo fibroblasts (DEF) were prepared from 13 day embryos, and grown at 37C in Liebowitz-McCoy media supplemented with 4% calf serum. After reaching confluency, they were maintained in the same media with 1% calf serum.

Virus stocks used were cells infected with the following strains: GA (Eidson and Schmittle, 1968,) Md11 (Witter, 1983,) and Md11/75C (Witter and Lee, 1984; Silva and Witter, 1985.) Multiplicity of infection is difficult to determine with cell-associated virus, but the cultures were infected by adding one infected cell to about 3-6 uninfected fibroblasts. Usually, cells were harvested when the cultures displayed extensive cytopathic effects, although for one experiment, the RNA was purified from cultures only 8 hours post-infection in order to see transcripts from immediate early viral genes.

RESULTS

A series of cloned DNA fragments representing a large portion of the Marek's disease virus genome was selected from the BamHI (Fukuchi et al, 1984) and the EcoRl (Gibbs et al, 1984) clones available to us. A genomic map showing the locations of the cloned fragments used in this study is dagrammed in Figure Bl. The plasmid DNA was purified, the inserts were cut out with either EcoRl or BamHI, and the fragments separated in an agarose gel to verify the presence of an insert of the correct size (Figure B2.) The plasmid insert sizes and vectors are listed in Table Bl.

For the survey experiment, total RNA was isolated from three MD-induced lymphoblastoid cell lines using the method of centrifuging guanidinium isothiocyanate extracts through a 5.7M CsCl cushion. For each probe used, 15ug total RNA from each line was separated by electrophoresis through agarose gels containing formaldehyde, in adjacent lanes, and the RNA was blotted to nitrocellulose.

In order to crudely differentiate between early and late viral gene expression from Md11/75C MDV, a confluent culture of duck embryo fibroblasts (DEF) was infected using a multiplicity of infection (moi) of one infected cell per four DEF. RNA was harvested as before at 8 hours (for "early" transcripts) and at 48 hours (for "late" transcripts), when the cells exhibited substantial cytopathic effect. Because of the cell-associated nature of the virus, it is difficult to obtain cultures in which the cells are synchronized in the time of

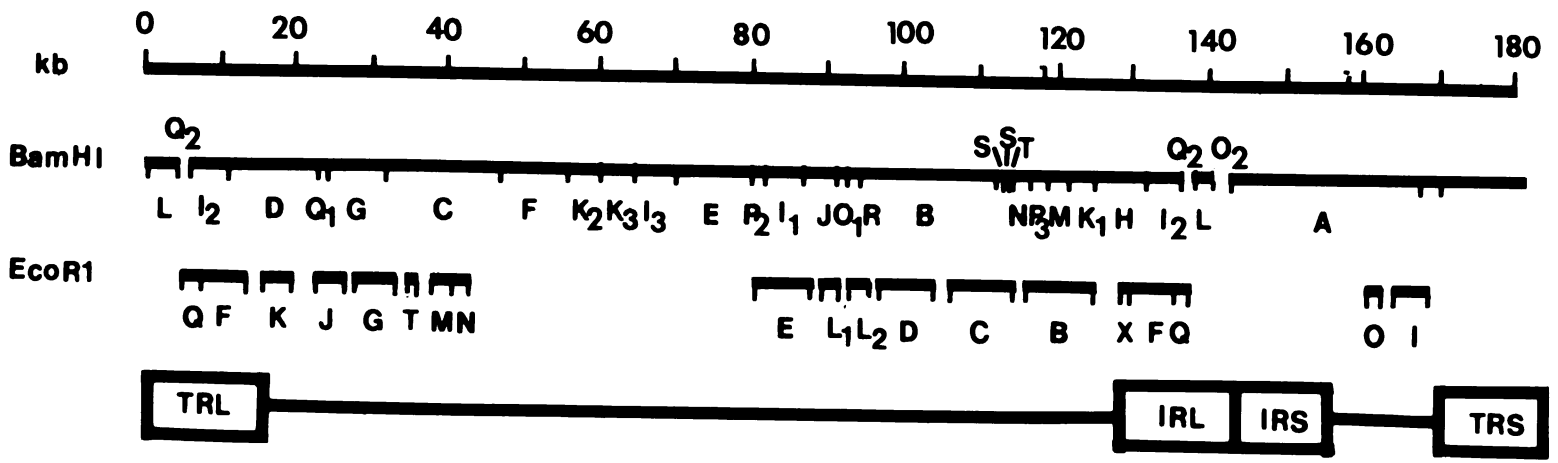

Figure B1: Restriction map of Marek's disease virus. The general genome organization and location of BamHI and EcoR1 clones are shown (adapted from Fukuchi et al, 1984; Silva and Witter, 1985.)

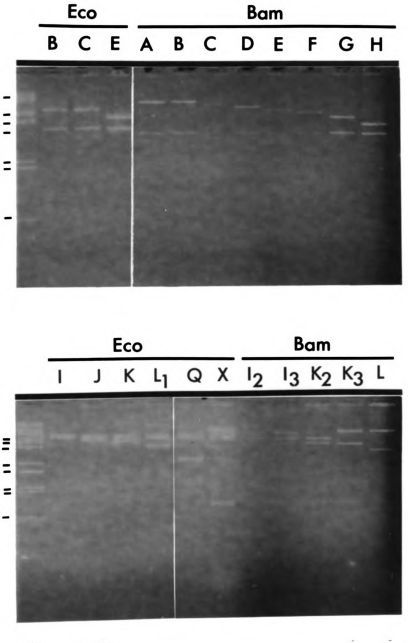

Figure B: Photograph of ethidium bromide stained gel showing BamHI or EcoRl digested plasmid clones.

Cell line	Species	Transforming Virus	Immunofluorescent reactions				Reference
			anti B cell	anti T cell	Rabbit anti MATSA	Monoclonal anti MATSA	
MSB1	chicken	MDV-BC1	-	+	95%	95%	Akiyama and Kato, 1974
RP1	chicken	MDV-JM	-	+	75	95	Nazerian et al, 1977
RP4	chicken	MDV-GA	-	+	85	85	Nazerian et al, 1978
RP9	chicken	ALV-RAV2	+	-	Ø	Ø	Okazaki et al, 1980
RP13	chicken	REV-CS	+	-	Ø	25	Nazerian et al, 1982
RP19	turkey	MDV-GA	+	-	35	<5	Elmubarak et al, 1981

Figure B 3: MDV tumor cell lines used in transcription survey.

Clone	insert	vector	Amp^r	Tet^r	Cam^r
EcoRl-B	12.5	pBR328	+	+	-
-C	11.8	"	+	+	-
-E	7.8	"	+	+	-
-F	6.9	"	+	+	-
-I	5.15	"	+	+	-
-J	4.4	"	+	+	-
-K	4.1	"	+	+	-
-Ll	3.35	"	+	+	-
-Q	2.2	"	+	+	-
-X+F	.9+6.9	"	+	+	-
BamHI-A	23.5	pACYC184	-	-	+
-B	18.5	"	-	-	+
-C	15.Ø	"	-	-	+
-D	11.5	"	-	-	+
-E	9.7	"	-	-	+
-F	8.9	"	-	-	+
-G	7.1	pBR322	+	-	-
-H	5.5	"	+	-	-
-I2	5.2	pACYC184	+/-	-	+
-I3	5.2	"	-	-	+
-K2	3.6	pBR322	+	-	-
-K3	3.6	pHC79	+	-	-
-L	3.Ø	"	+	-	-

Table Bl: MDV genomic clones used in transcription survey.

infection. Therefore, immediately after infection in this system, the infected cells which are added will be producing late gene products, while the newly-infected cells begin producing early gene transcripts. As a result, the "early" and "late" designations for the RNA obtained from these cultures only refer to the probable enrichment of the RNA for either early or late gene expression. Approximately 15ug of the early and late MD11/75C-infected DEF total RNA was electrophoresed and blotted in the same manner as the cell line RNA.

The total plasmid DNAs of the 23 BamHI and EcoR1 clones listed in Table B1 were individually nick-translated and hybridized at high stringency with filters containing the three cell line RNAs and the two infected cell RNAs. After washing in Ø.1xSSC at 65C, all filters were exposed to X-ray film with intensifying screens for 4, 18, and 48 hours. The most useful exposure of each was photographed, and is shown in Figures B4, B5, and B6, together with a map of the probe location on the genome. Major RNA bands seen in the lanes are marked by dots; the calculated sizes of these transcripts are tabulated in Table B2. Because each gel was run separately, and some of the molecular weight markers were not clearly visible, the sizes are all approximate, and in some cases, it may be misleading to directly compare the sizes obtained with different probes.

Further studies were undertaken to better understand the transcriptional pattern of genes located in the repeats flanking the long unique region. RNA blots were prepared as before with the following differences. Additional cell lines were used, to provide more information and better

Figure B4: Northern blots of RNA from cell lines and infected cells, probed with nick-translated DNA from the short region of the genome. 1=RP1, 2=RP4, 3=MSB1, 4=48hr culture of Md11/75C-infected DEF, 5=8hr culture of MD11/75C-infected DEF. Dots indicate major RNA transcript, and sizes are listed in Table B2.

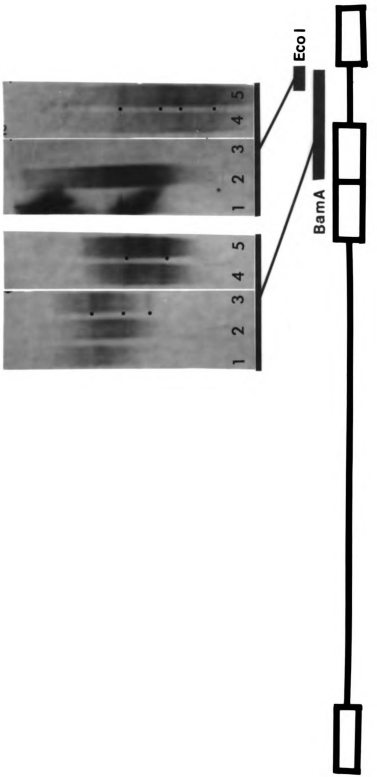

Figure B4:

Figure B5: Northern blots probed with DNA from the long unique region. RNA is the same as in Figure B4.

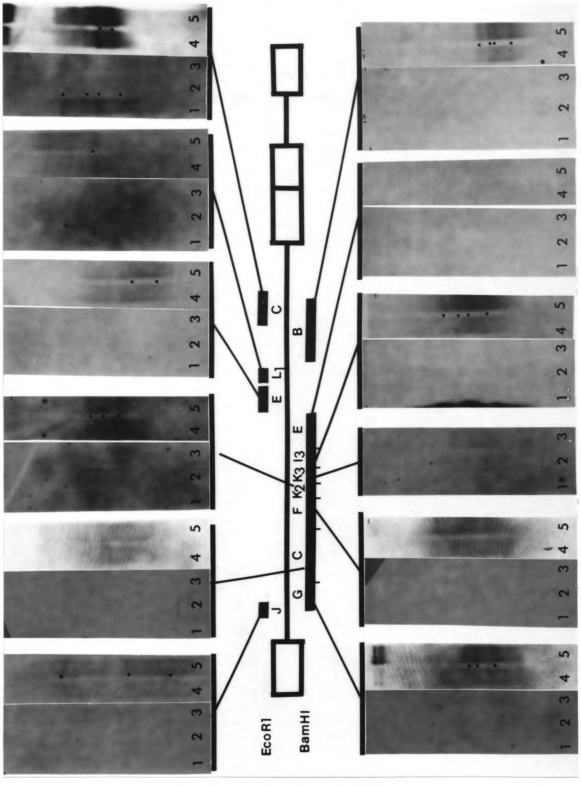

Figure B5:

Figure B6: Northern blots probed with DNA from the repeats flanking the long unique region. RNA is the same as in Figure B4.

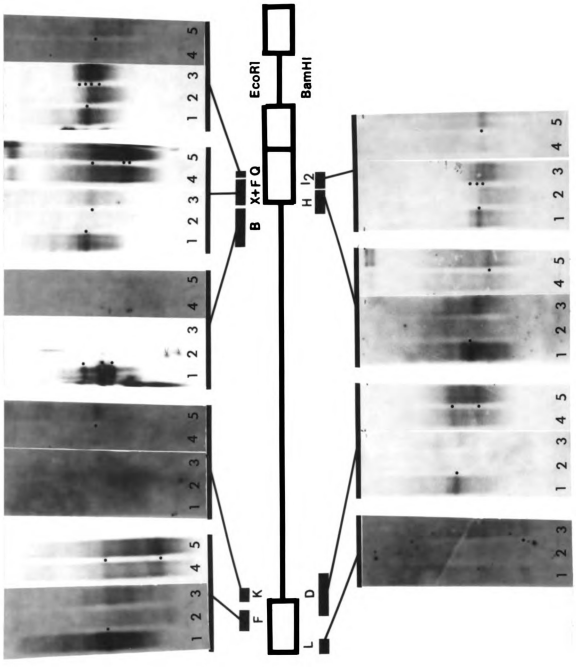
EcoRI
BamHI
L
F
K
D
B
X+F Q
H 1/2
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3

Figure B6:

Table B2: MDV transcripts identified in survey.

	Cell lines				infected cells		
Probe	size	RP-1	RP-4	MSB-1	size	48hr	8hr
1. BamHI-A	4.25	+	+	+	4.25	+	+
	1.9	+	+	+	1.9	+	+
	Ø.7	+	+	+			
2.EcoRl-I	?	-	+	-	6.3	-	+
					3.45	-	+
					2.8	+	+
					1.5	-	+
3. EcoRl-J	-	-	-	-	>7.5	-	+
					3.7	+/-	+
					1.5	+/-	+
4. BamHI-G	-	-	-	-	3.1	-	++
					2.6	+	++
					1.8	+	+
5. BamHI-C	-	-	-	-	?	+/-	+/-
6. BamHI-F	-	-	-	-	?	+	+
7. BamHI-K2	-	-	-	-	4.Ø	+/-	+/-
					2.8	+/-	+/-
					1.8	+	+
8. BamHI-K3	-	-	-	-		ND	ND
9. BamHI-I3	-	-	-	-	5.2	-	+
					3.7	+	+
					2.8	+	++
					1.8	+	+
1Ø. BamHI-E	-	-	-	-	-	-	-
11. EcoRl-E	-	-	-	-	3.Ø	+	+
					1.4	+	+
12. EcoRl-Ll	-	-	-	-	3.7	+	+
13. BamHI-B	-	-	-	-	3.6	+	+
					2.8	+	+
					2.5	+	+
					1.7	+	+
14. EcoRl-C	5.8	+	-	-	2.6	+	+
	3.5	+	-	-	1.8	+	+
	2.6	+	-	-			
	1.4	+	-	-			

Table B2: continued

15. EcoRl-F	2.3	++	-	+/-	3.6	+	+
					1.1	+	++
16. EcoRl-K	-	-	-	-	3.3	-	++
17. EcoRl-B	8.1	+	-	-	-	-	-
	5.2	++	-	-			
	4.2	++	-	-			
18. EcoRl-X+F	2.5	++	-	-	3.6	+	+
	2.1	-	-	+	1.6	++	++
					1.5	++	++
19. EcoRl-Q	3.0	-	-	+	2.05	+/-	+
	2.4	-	-	+			
	2.3	++	+/-	-			
	2.0	+/-	+	++			
	1.6	+/-	+/-	+			
20. BamHI-L	0.4	+/-	?	+/-	ND		
21. BamHI-D	2.3	+	-	?	3.6	+	+
					1.8	+	+
22. BamHI-H	2.2	+	?	?	1.75	+/-	+
23. BamHI-I2	2.2	+/-	-	+/-	1.4	+	++
	1.9	-	-	+			
	1.8	++	+/-	+			
	1.6	-	-	+/-			

ND=not done -,+/-, +, ++ = arbitrary designations comparing presence of bands within a single probe only

Figure B7: Northern blots of RNA from various tumor cell lines probed with cloned DNA which maps to the inverted repeats flanking the long unique region of the MDV genome.

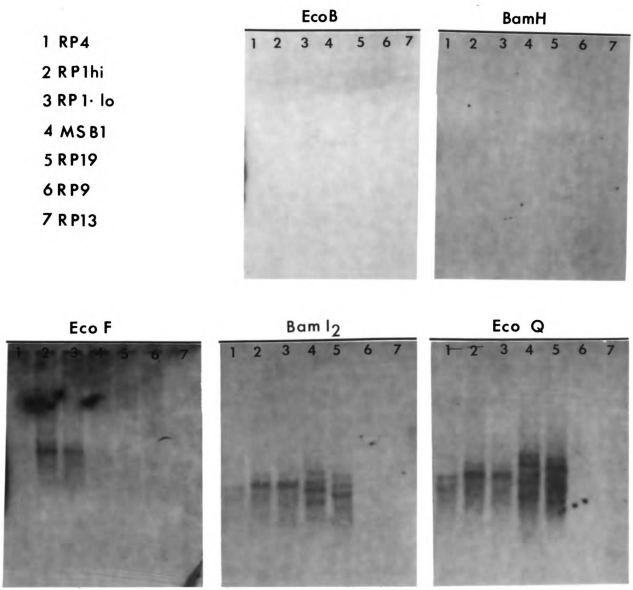
1 RP4
2 RP1hi
3 RP1· lo
4 MSB1
5 RP19
6 RP9
7 RP13
EcoB
1 2 3 4 5 6 7
BamH
1 2 3 4 5 6 7
Eco F
1 2 3 4 5 6 7
Bam I2
1 2 3 4 5 6 7
Eco Q
1 2 3 4 5 6 7

Figure B7:

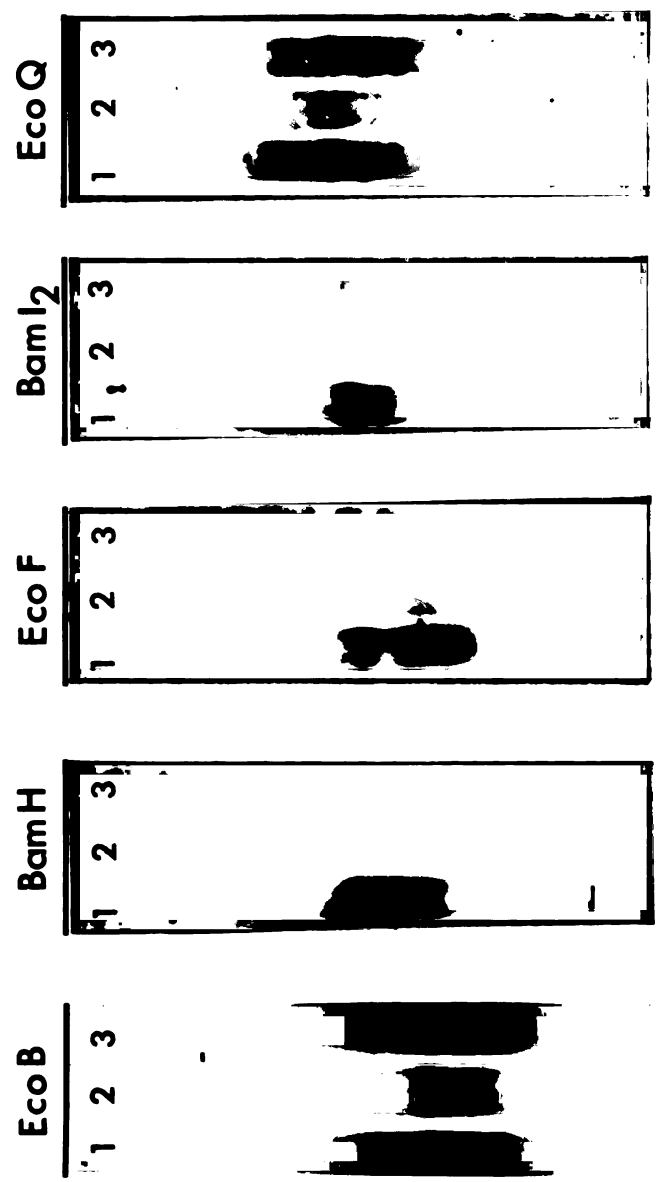

Figure B8: Northern blots of RNA from DEF infected with MDV virus, probed with the same probes as in Figure B7. 1-GA, 2-Md11, 3-Md11/75C

Table B3: Transcript sizes detected with probes specific to the long repeat regions of MDV.

	Tumor cell lines				MDV infected DEF		
Probe	RP1	RP4	MSB	RP19	GA	Md11	Md11/75C
EcoR1-B	-	-	-	-	5.1	5.1	5.1
					3.1	3.1	3.1
					2.0	2.0	2.0
BamHI-H	+/-	-	-	-	4.4	4.4	-
					1.5-2.3	1.5-2.3	?
					1.2-1.3	1.1-1.3	1.1-1.3
EcoR1-F	2.1	-	-	-	4.4	4.4	-
					1.5-2.3	1.5-2.3	?
					1.1-1.3	1.1-1.3	1.1-1.3
					.6-.7	.6-.7	.6-.7
BamHI-I2			3.3	3.0	4.8	4.8	-
	2.7		2.8	2.6	3.7	3.7	-
	2.2	2.1	2.3	2.1	2.2	2.2	2.2
	1.6	1.7	2.0	1.7	1.5	1.5	1.5
			1.5	1.3			
EcoR1-Q			3.4	3.2	4.8	4.8	4.8
	2.8		2.8	2.6	3.0	3.0	3.0
	2.3	2.3	2.3	2.1	2.5	2.5	2.5
		1.8	2.0	1.8			
	1.6	1.5	1.5				

controls. RNA was not obtained from infected cells at "early" times, but both pathogenic and attenuated serotype I viruses were grown in infected DEF. Probes used from the repeat regions included EcoRl-B, BamHI-H, BamHI-I2, EcoRl-F, and EcoRl-Q. The results are shown in Figures B7 and B8, and the sizes of the transcripts marked by dots are shown in Table B3. The Eco Rl-B transcription pattern was quite different from that previously obtained during the initial survey experiment. At that time, transcripts were detected only in RP-1 cells, and not in other cell lines or infected cells. The data in Figures B7 and B8 indicate substantial transcription in all infected DEF, but not in any cell lines. The reason for this discrepancy in not known.

In order to better understand the genomic clones used in probing transcription from this region, additional restriction mapping of some of the genomic clones was done; the composite map is shown in Figure B9a. The EcoRl-B fragment had not previously been located next to the EcoRl-X fragment, and the order of some of the BamHI fragments within the EcoRl-B insert has been changed from that published (Fukuchi et al, 1984.) In order to confirm that the EcoRl-X fragment cloned adjacent to EcoRl-F and EcoRl-B is the same as that seen in the BamHI-H clone, these three clones were digested with EcoRl, and the fragments were separated on agarose gels, transferred to nitrocellulose, and hybridized with BamHI-H. As seen is Figure Bl0, the 900bp fragment digested from all three of these clones hybridized to the BamHI-H probe, indicating their identity. The clones BamHI-H, EcoRl-Q, and EcoRl-B were digested with both EcoRl and BamHI, and the EcoRl-BamHI fragments were gel purified to produce the probes 1-5 marked on the map in Figure B9b. Probe 6 is a

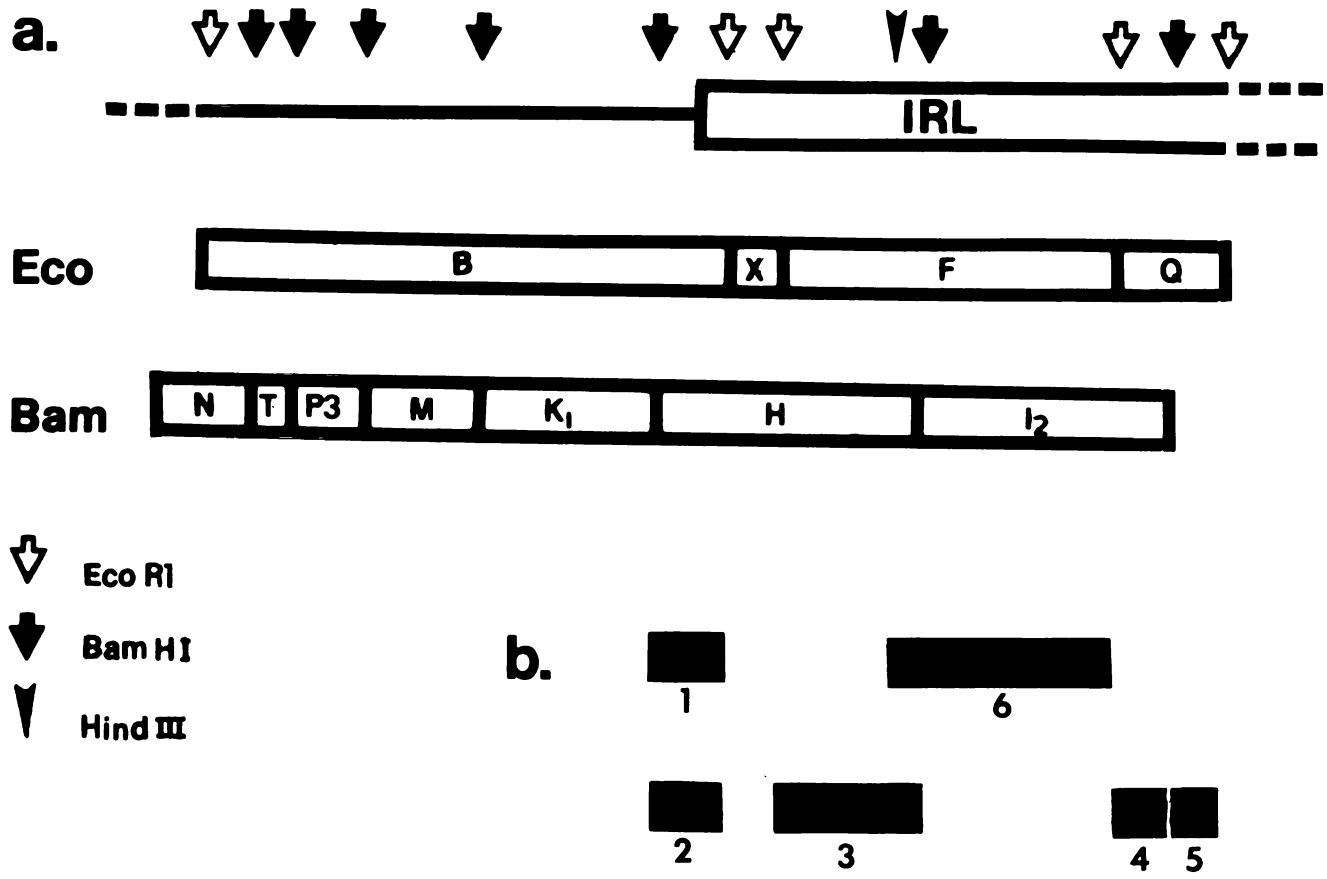

Figure B9: a. Map of MDV region at right end of long unique segment, including most of inverted repeat sequences. BamHI and EcoRl fragments are indicated. B. DNA fragments used for probes; 1-5 were gel purified from EcoRl or BamHI clones. 6 is a HindIII-EcoRl subclone of EcoRl-F (provided by R. Silva.)

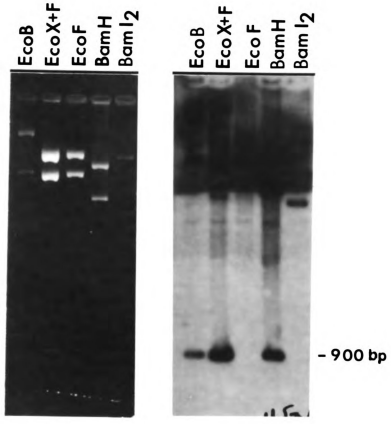

Figure Bl0: Identity of EcoRl-X fragment found in different genomic clones. The five genomic clones near the junction between the right end of the long unique region and the repeat sequence were digested with EcoRl and Southern blotted. On the left is the ethidium bromide stained gel; on the right is the corresponding autoradiogram after the filter was hybridized with nick-translated BamHI-H DNA. The location of the 900bp EcoRl-X fragment which is common to the BamHI-H, EcoRl-F+X, and EcoRl-B clones is indicated.

Figure B11: Northern blots of RP1 and MSB1 RNA probed with subclone DNA fragments (indicated in Figure B9b.) RNAs are: lane 1 - RP1, lane 2 - RP4, lane 3 - MSB1, lane 4 - 48hr Md11/75C infected DEF, and lane 5 - 8 hr Md11/75C infected DEF.

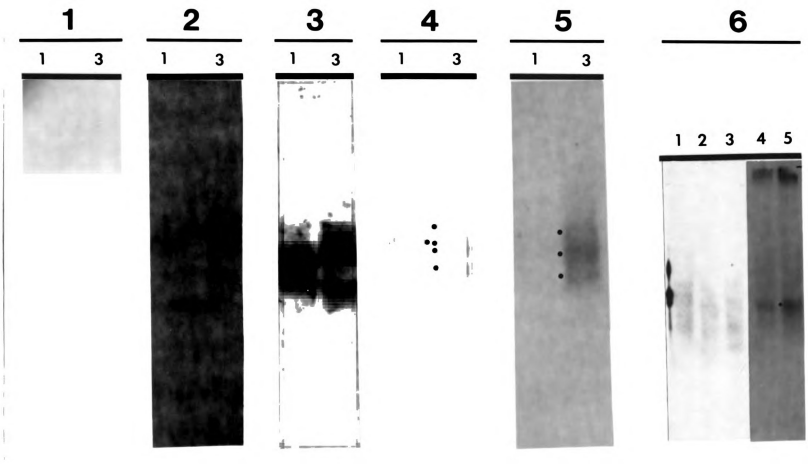

Figure B11:

total plasmid containing a HindIII-EcoRl fragment, which is the right half of EcoRl-F; it overlaps BamHI-H by only a few hundred nucleotides (between the HindIII and BamHI sites. These probes were used to hybridize to Northern blots of RP-1 and MSB-1 RNA, as shown in Figure B11. EcoRl-B/1600 (probe 1) and BamHI-H/1600 (probe 2) represent the same DNA fragment and do not detect any transcription in either cell line; BamHI-H/2900 (probe 3) apparently detects all the transcripts from these cell lines that hybridize with Bam HI-H, and most of the transcripts from EcoRl-F. Probe 6 detects a few small, faint transcripts in the cell lines, but apparently hybridizes to a discrete RNA found in the Md11/75C at both early and late times. EcoRl-Q/1200 (probe 4) hybridizes with all transcripts detected by the entire EcoRl-Q fragment; while EcoRl-Q/1000 (probe 5) detects a few transcripts in MSB-1 cells, but not in RP-1 cells.

Since the number and sizes of EcoRl-Q region transcripts varies among the cell lines, we extracted total high molecular weight DNA from the cell lines, digested it with EcoRl or BamHI, and hybridized the resulting blots with probes to detect the EcoRl-Q fragment. Figure B12 shows DNA from five cell lines digested with EcoRl and probed with the BamHI-I2 plasmid. In all lanes, the EcoRl-F fragment appears the expected size. However, the size of the EcoRl-Q fragment varies, and seems to disappear in the high-passage RP-1 cell line; multiple high molecular weight bands appear in this line. Another similar experiment is shown in Figure B13: probing with EcoRl-Q shows that the high molecular weight bands in high-passage RP-1 contain sequences derived from the EcoRl-Q region; again, the EcoRl-Q fragment is nearly missing

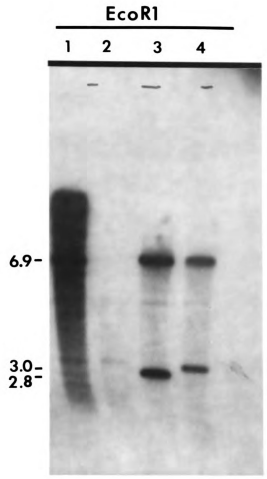

Figure B12: Tumor cell line Southern blots, probed with BamHI-I2. EcoR1 digested total genomic DNA extracted from the cell lines RP1-high passage (lane 1), RP1-low passage (lane 2), RP4 (lane 3), and MSB1 (lane 4). The blot was probed with the BamHI-I2 clone.

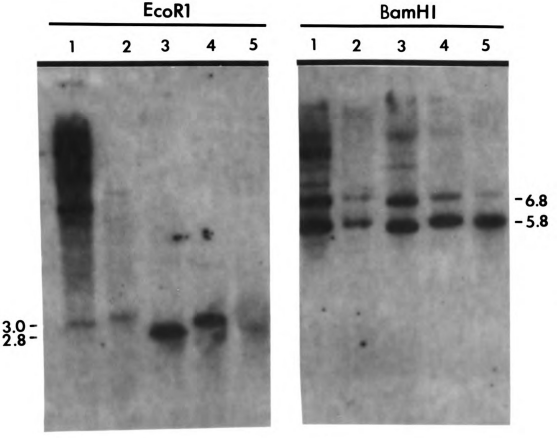

Figure B13: Genomic Blot of cell line DNA digested with either EcoR1 or BamHI and probed with nick-translated EcoR1-Q DNA. 1-RP1 high passage. 2-RP1 low passage. 3-RP4. 4-MSB1. 5-RP19.

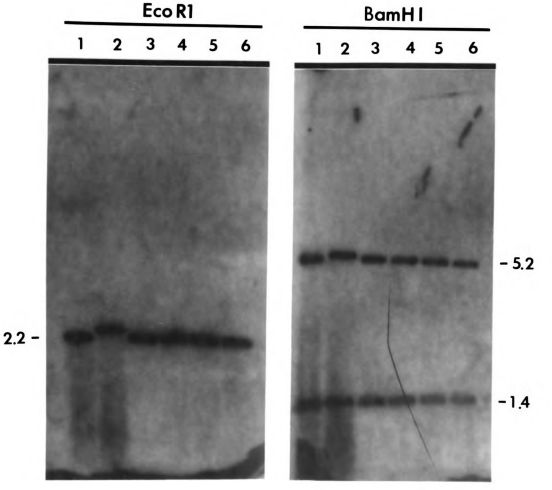

Figure B14: Southern blot of MDV-infected cell DNA, digested with either EcoR1 or BamHI, and probed with nick-translated EcoR1-Q DNA. 1- Md11. 2- CVI988. 3- Md11/75C. 4- Md11/75C/R2. 5- Md11/75C/R5. 6- Md11/75C/R8.

in the high passage RP-1 cells, and varies in size among the other cell lines shown. The BamHI-digested DNA lanes show the expected BamHI-I2 fragment at 5.2kb, but also show an unexpected 6.2kb fragment of unknown origin which hybridizes to the EcoRl-Q probe. According to the BamHI map as published, the adjacent BamHI fragment should be about 1.4kb. This size band was not seen on this filter.

Figure Bl4 shows that the expected sizes of fragments were seen for both EcoRl and BamHI digestions of infected DEF DNA.

Since the MD tumor cell lines are either highly selected tumor cells, or tumor cells which have undergone *in vitro* mutation which allows continued growth in tissue culture, we wanted to test whether the increased transcription from the EcoRl-Q region, as seen in the cell lines, was reflected in gross tumors *in vivo*. Six tumors were isolated from the internal organs of chickens in which lymphomas were induced by Md5, a very virulent strain of MDV. The tumors were immediately frozen in liquid nitrogen, and stored at -8ØC until use. The frozen tissues were smashed to a fine powder and, before thawing, were added to the guanidinium isothiocyanate solution for RNA extraction. At least 2Øug total RNA was applied to each lane of a formaldehyde gel; after Northern blotting, filters were hybridized with either EcoRl-F or EcoRl-Q. The EcoRl-F region was expressed in all tumors as a smear of RNA from about 2.Ø-2.5 kb (Data not shown.) EcoRl-Q transcripts of 3.2 kb were detected in all tumors (Figure Bl5.)

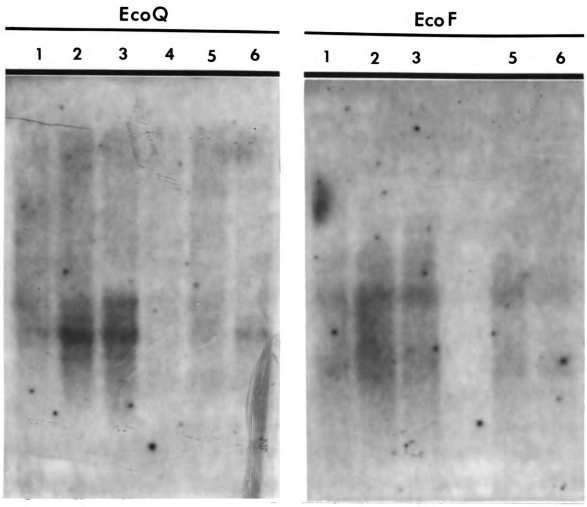

Figure B15: Northern blots of RNA extracted from in vivo tumors induced by MDV, and probed with either EcoRl-Q or EcoRl-F.

DISCUSSION

Since MD has been shown to be caused by a herpesvirus and can be prevented by vaccines, much of the research to date has centered on the biology of the cytolytic infection and the antigenic relationships between the pathogenic serotype I strains and vaccine strains of MDV. Several studies have looked at antigens specific to tumor cells, primarily targeted to diagnostic or vaccine applications. Very little information exists concerning the small amount of viral expression in tumors or the mechanism of transformation.

This project was undertaken specifically to survey the transcription of the genome among several MD cell lines, with the assumption that gene expression required for maintenence of transformation could be recognized as common patterns of transcription among all cell lines, which can develop into tumors when injected into syngeneic chickens.

The numbers of MD tumor cell lines now available and the cloning of the MDV genome, makes possible a survey of viral transcription in these cell lines. Possible functions of any transcripts common to all cell lines could be related to induction of transformation, maintenence of transformation, latency, or various aspects of the usual cytolytic infection. The latter class could presumably be identified by comparison with transcripts seen in the cytolytically-infected fibroblasts.

We therefore chose cloned fragments of the MDV DNA which represent almost the entire genome, prepared DNA, and checked sizes of inserts to be sure that it was in agreement with that predicted by the map. A list of these fragments, with their vectors and sizes is compiled in Figure B1, and confirmation of the sizes is shown in the photograph in Figure B2. The only anomaly noted was the presence in the EcoR1-B clone of an additional, small EcoR1 fragment. The existence of this fragment apparently had been noted in previous work (R. Silva, personal communication,) but its identity had not been established. It was decided to use the clone in the survey, and to investigate the origin of the small fragment further, if interesting transcription was related to this segment of the genome.

Three MD tumor cell lines were chosen for the initial survey. MSB-1, RP-1, and RP-4 are all MDV-transformed cells exhibiting T cell markers and MATSA. Characteristics and origins of the cell lines are shown in Figure B3.

Figures B4,B5, and B6 show the Northern blots produced from the survey using probes covering most of the MDV genome. The most surprising overall result from this survey was the amount of transcription seen in the RP-1 cell line. Since no virus can be rescued from these cells, unlike most other MD tumor cell lines, and no viral antigens are detectable (Nazerian et al, 1977,) it was believed that this cell line might represent a true latently-infected cell,. and therefore would exhibit transcription of only very limited portions of the genome. The

data presented here do not support that prediction--the transcription is at least as active in RP-1 cells as in the other cell lines studied.

Transcription from the short unique region, and its inverted repeats, is detected by the BamHI-A clone, shown in Figure B4. There are multiple transcripts encoded within this 23 kb fragment, some are common to both cytolytically infected cells and the tumor cell lines; several also may be transcripts of early genes, judging by presence only at early times of infected-cell harvesting. This correlates well with predictions based upon the genes present in the homologous region of other herpesviruses, such as HSV and pseudorabies (Petrovskis et al, 1986; Wagner, 1982.) The short unique region of these viruses codes for the structural surface glycoproteins, and some transcription would be expected in any cell from which virus can be rescued. In addition, in HSV it has been shown that most of the early genes are present in the inverted repeats flanking both unique regions. The data presented here are consistent with a similar set of genes being present in MDV.

Transcription from the large unique region includes many transcripts present in cytolytic infections, as well as a few present in the tumor cell lines, as seen in Figure B5. Large areas of the genome did not detect any transcription, such as BamHI-C and BamHI-E. The overlapping BamHI-G and EcoRl-J both detect considerable transcription in infected DEF, but not in tumor cell lines. A similar pattern of transcription in cytolytically infected cells, but not tumor cell lines, is seen with fragments EcoRl-E, EcoRl-L1, and BamHI-B. BamHI-B is known to be the fragment which encodes the A antigen (P. Coussens, personal

communication), but it is not known whether any of these transcripts represents that gene. A contrasting pattern was detected, however, with EcoRl-C, with transcripts present in both tumor cell lines and infected fibroblasts.

The regions which were transcribed most heavily in the tumor cell lines were the areas in and near the repeats which flank the long unique region (Figure B6.) Overlapping EcoRl and BamHI clones were used in this region; the fragments detected "families" of transcripts in both tumor cells and infected DEF. Several very interesting regions were noted. EcoRl-K, which is included within the unique portion of BamHI-D, detects a transcript which apparently is limited to very early expression; it was seen in cultures 8 hours after infection, but not at all in later cultures. At the other end of the long unique region, the EcoRl-B fragment detects large quantities of transcription in RP-1 cells, but not in other cell lines or in infected DEF. This pattern of gene expression could be consistent with a function involved in maintenence of latency, but further experiments have not confirmed this result (see Figure B7-B8.) BamHI-H and EcoRl-F detected a common "family" of transcripts; BamHI-I2 and EcoRl-Q detected another "family." Both families were transcribed in the cell lines; the EcoRl-F family was transcribed at much higher levels than the EcoRl-Q family in the infected DEF, and the EcoRl-Q family appeared to be transcribed at higher levels in the tumor cell lines..

Because of these results showing high levels of transcription, as well as the interesting pathogenicity comparisons of the DNA expansions in

these repeats flanking the long unique region, it was decided to look more closely at their transcription. In order to do this, additional mapping studies of the clones in this region were undertaken. Figure B8a shows the resulting map of the overlapping clones, EcoRl-B, BamHI-H, EcoRl-F, BamHI-I2, and EcoRl-Q. Inclusion of the EcoRl-B clone was due partly to its interesting transcription in RP-1 cells and partly to the presence of the additional, small EcoRl fragment in the clone. A small EcoRl fragment had also been found in an EcoRl clone which included the MDV-F fragment, and had been mapped adjacent to EcoRl-F because of hybridization studies with BamHI-H. Similar hybridization including the small fragment from the EcoRl-B clone is shown in Figure B1Ø; the EcoRl-X fragment derived from all three clones is identical. This raised the possibility that EcoRl-B might overlap with the unique portion of BamHI-H; further mapping has indicated that this is the case (see Figure B8a.) Thus, we have the repeat region on contiguous EcoRl clones, as well as BamHI clones (from the unique region through BamHI-I2 and EcoRl-Q.) This allows additional deductions to be made about the transcription detected by these relatively large cloned fragments. For example, since EcoRl-B did not detect any transcription in cytolytically infected cells, the region of BamHI-H which produces the "family" of transcripts must not include the unique region in common with EcoRl-B. This is particularly significant due to the possibility raised at the Herpes workshop (Leeds, England, 1986) that there may be a 1.8 kb message specifically transcribed within the unique portion of BamHI-H, from both pathogenic and attenuated viruses. Our data does not support that conclusion.

To more carefully define transcription from the repeat region, additional cell lines were included. RP-13 and RP-9 are chicken B-lymphoblastoid cell lines induced by retroviruses, RP-19 is a turkey B cell MDV-induced tumor cell line, and RP-1(hi) is a much higher passage level of the RP-1 line used previously, and is no longer capable of tumor growth when injected into birds (Nazerian et al, 1984.) In addition, DEF were infected with three serotype I viruses, GA and Md11 (patogenic viruses) and Md11/75C (high-passage, tissue culture attenuated virus.) Northern blots of RNA from these cells were hybridized to the nick translated genomic clones as shown in Figures B7 and B8. Although the EcoRl-F 2.3 kb transcript was seen in RP-1 RNA, this experiment did not pick up the small amount of MSB-1 transcription seen previously, and did not detect transcription in any cell lines using the BamHI-H probe. In contrast, both BamHI-H and EcoRl-F hybridized to relatively large amounts of 2.2 kb and smaller RNAs in the infected DEF RNA.

The EcoRl-Q and BamHI-I2 fragments detected similar sizes of RNA transcripts (see Table B3 for sizes.) There was transcription of this family in all MD cell lines tested, although the number and sizes of the transcripts varied. There was also substantial transcription in all the infected DEF cultures, with a predominant 2.2 kb transcript with BamHI-I2, and less abundant transcripts of 2.5 and 3.Ø seen with EcoRl-Q. The BamHI-I2 2.2kb transcript could be the same as the largest transcript seen with EcoRl-F or BamHI-H and probably would obscure the visualization of the 2.5 and 3.Ø kb transcripts seen with EcoRl-Q, if they were present.

The identity and function of the transcripts from these repeat regions are unknown at this time. However, several characteristics may be described. The proposed 2 kb message seen only upon infection with pathogenic MDV strains, may be the same as our 2.2 kb transcript seen in DEF infected with GA or MD11. However, the proposed start site is near the right end of BamHI-H with the coding region extending into the BamHI-I2 fragment. Our 2.2 kb RNA is detected in infected cells with BamHI-H, EcoRl-F, and probably with BamHI-I2 probes, and so could fit this proposed location. If this transcript is important in transformation, it is only in the initiation phase, as it is not seen in any of the cell lines tested here. In our study, there were no MD cell line transcripts detected with both the BamHI-H and BamHI-I2 probes, i.e., no transcripts include sequences from both sides of the BamHI site. That leads to the question, where do these tumor-specific transcripts map? Several RNAs were probed with DNA from a subclone of EcoRl-F, designated pHE391, which contains the sequences from the HindIII site about 1.2 kb downstream of the expanded region to the right end of the EcoRl-F clone. While this picked up a 1.6-1.8 kb transcript in infected fibroblasts, it did not recognize any of the major bands in the tumor cell lines. Likewise, EcoRl-B does not pick up the 2.3 kb RP-1 transcript or any other cell line RNA, indicating that none of these is coded to the left of the EcoRl-X fragment. This leaves about 2.7 kb of genome which is common to BamHI-H and EcoRl-F, within which the 2.3 kb RP-1 tumor transcript appears to be coded. This also includes the DNA region which expands upon attenuation. The expanded region is not

transcribed in infected DEFs (Maotani et al, 1986,) but has not been used to probe the RP-1 RNA.

The EcoRl-Q family expressed in tumor cells is of interest because of the increased quantity as well as the diversity of sizes transcribed in the tumor cell lines. We know from the data shown in Figure B11 that this family does not include transcription of the DNA which lies between BamHI-H and EcoRl-Q. All of the transcripts are detected by the BamHI-I2 fragment, so they must all include at least part of the 1.2 kb fragment which is common to the two genomic clones. In order to further clarify the relationship of these transcripts, the EcoRl-Q clone was digested with EcoRl and BamHI, and the two insert pieces were gel purified to use for probes. The results shown in Figure 11 indicate that although the larger 1.2 kb fragment does hybridize to all expected RNA sizes, the other fragment only hybridizes to a few RNAs in MSB-1 cells. These transcripts are considerably larger than the 1.2 kb region which has been used to detect them; some may continue into the adjacent portion of EcoRl-Q, but some appear to be spliced further to the right. Unfortunately, no clones are available of the region to the right of EcoRl-Q, up to the BamHI-L fragment. The BamHI-L fragment does identify some transcription in RP-1 and MSB-1 cells, but their amount and size has not been accurately determined, and the fragment has not been tested as a probe against infected cell RNA. This probable splicing of the messages transcribed from the EcoRl-Q region represents the only splicing known so far in MDV transcription, and it is not known whether this phenomenon is limited to tumor cells, or whether it occurs also in productively infected cells, either in vivo or in vitro.

The Southern blotting experiments with the tumor cell line DNAs and EcoRl-Q probes indicate that there are DNA anomalies present which may be associated with the increased expression and/or the splicing of these messages detected by EcoRl-Q probes. Although the probe detects the expected fragments predicted by the published maps in DNA extracted from productively infected DEF, the same probes detect unusual sizes of fragments in the viral DNA of tumor cell lines. Although other possibilities cannot yet be ruled out by the data, it may be that DNA rearrangements have occurred in the viral DNA of tumor cells (either the cause or the result of transformation) which have altered the expression of mRNA transcribed from this portion of the viral genome. The nature of this rearrangement has not yet been identified, and source of the additional DNA which is now close to the EcoRl-Q-derived DNA has not been identified.

The data described in this chapter lead to several conclusions about transcription from the EcoRl-Q region of the MDV genome. 1) EcoRl-Q transcription occurs at higher levels in tumor cell lines than in infected cells. 2) EcoRl-Q transcription has been found in all tumor cell lines and all tumors tested. 3) EcoRl-Q transcription in the tumor cell lines appears to result in spliced messages. 4) EcoRl-Q detects alterations in the DNA of tumor cell lines, which map to the right of the EcoRl-Q fragment.

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