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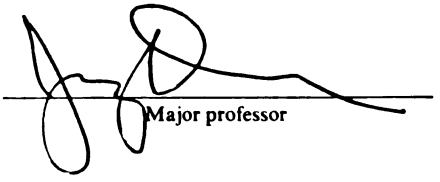
Identification of a host protein interacting with the Marek's disease virus SORF2 protein

presented by

Hsiao-Ching Liu

has been accepted towards fulfillment
of the requirements for

Ph.D degree in Genetics



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IDENTIFICATION OF A HOST PROTEIN INTERACTING WITH THE MAREK'S DISEASE VIRUS SORF2 PROTEIN

by

Hsiao-Ching Liu

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ABSTRACT

IDENTIFICATION OF A HOST PROTEIN INTERACTING WITH THE MAREK'S DISEASE VIRUS SORF2 PROTEIN

by

Hsiao-Ching Liu

Marek's disease virus (MDV) is a naturally occurring oncogenic avian herpesvirus that causes a lymphoproliferative disease in domestic chicken. The genome is a double stranded linear DNA molecule of approximately 180 kb. Only serotype 1 MDV has potential pathogenic characteristics, which rapidly induce lymphomatosis and mononuclear infiltration of various organs. Which gene(s) triggers MD oncogenesis and by what mechanism is still not understood. In an attempt to study the biological characteristics of MDV genes, a unique gene, SORF2, came to our attention. An interesting phenomenon was discovered in the MDV recombinant strain RM1, which contains a REV LTR insertion upstream of the SORF2 gene. The unregulated overexpression of SORF2 due to the LTR promoter was correlated with the loss of MDV oncogenicity. This finding prompted further investigation of SORF2. Prior to this study, it was not clear if SORF2 protein is naturally expressed upon infection in a wild type MDV. To investigate the expression of SORF2, polyclonal antibodies that specifically recognize SORF2 were generated by immunizing rabbit with purified SORF2 protein synthesized in *E.coli*. A 21kD polypeptide was precipitated with rabbit anti-SORF2 antibodies from MDV infected CEF cells. To directly test if SORF2 is expressed *in vivo*, experiments of histochemical staining in MDV infected tissues and induced tumors was performed. The results demonstrated that SORF2 is expressed in MDV infected tissues

and MD tumors. In a search of potential functional protein partner(s), SORF2 was used as the bait in yeast two-hybrid screening of a cDNA library constructed from splenic T cells. The chicken growth hormone structural peptide was found to specifically interact with SORF2 protein. To corroborate the interaction, the isolated growth hormone cDNA was used to synthesize corresponding polypeptides in *E. coli*, which then were assayed for their ability to interact with SORF2 *in vitro*. Co-immunoprecipitation of SORF2 and the interacting protein, growth hormone, using specific antibodies confirmed the biochemical evidence for the observed protein-protein interaction. To test the co-localization of both proteins in vitro and in vivo, IFA double staining examined with confocal microscopy and gold particles staining examined with transmission electron microscopy were employed. Results further suggested that these two proteins may interact with each other since the two co-localized in the cytoplasm. In an attempt to reveal the potential possibility of cGH to be involved in MD susceptibility, a genetic approach using 272 F2 chickens selected from White Leghorn inbred line 6 x line 7 for the association studies was performed. A direct association of MD incidence with the cGH gene was not found ($P > 0.05$).

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CHAPTER I

Introduction and Literature Review

Marek's Disease

Marek's Disease (MD) is a neurological and T cell lymphoma disease that is found in domestic chickens; it was first described by Josef Marek (Marek, 1907). The disease is characterized by a mononuclear infiltration of the peripheral nerves, gonads, iris, various viscera, muscles, and the skin. Partial or complete paralysis is the common symptom of MD as a result of the accumulation and proliferation of tumor cells in peripheral nerves. The relationship of this type of fowl paralysis to other leukotic diseases found in poultry was once unclear. MD was originally lumped among other leukotic diseases found in poultry under the term "avian leukosis complex" which was classified into three forms of lymphomatosis: neural, visceral, and ocular (Jungherr 1939; Jungherr and Hughes, 1965). Subsequently, based on pathological and field observations, both Campbell *et al.* (1956) and Biggs *et al.* (1961) reported that neural, visceral, and ocular form of fowl paralysis were distinct—both causally and pathologically—from lymphoid leukosis. Moreover, the successful experimental transmission of the disease was accomplished by inoculating day-old birds with blood or lymphoma cells from diseased birds supporting the evidence for the neural, visceral, and ocular forms of "avian lymphomatosis" (Sevoian and Chamberlain, 1962; Sevoian *et al.*, 1962; Biggs and Payne, 1963). Later, Biggs and Payne provided further evidence for disassociating MD from lymphoid leukosis with respect to their etiological agents (Biggs and Payne, 1963, 1967).

Unlike the avian leukosis virus, the causative agent of MD is highly cell-associated, infectivity is dramatically reduced when cells are killed or disrupted. In 1967, it was shown that MD is induced by an avian, oncogenic, cell-associated herpesvirus, which came to be referred to as the Marek's Disease Virus (MDV) (Churchill and Biggs, 1967; Nazerian and Burmester, 1968; Solomon *et al.*, 1968). This virus is quite distinct from the retroviruses that cause avian leukoses. Prior to the establishment and application of vaccines, MD generated tremendous economic losses to the poultry industry. In 1970, annual losses from MD were estimated to exceed $200 million in the United States alone. Since the 1970s, MD has been controlled fairly well through the use of vaccination. Although vaccination prevents the formation of lymphoma and other symptoms of MD, it does not, however, prevent infection, reduce replication and the spread of the virus (Edison *et al.*, 1971, Purchase and Okazaki, 1971). Moreover, even though commercially available vaccines are used worldwide to protect chickens against the disease, MD still remains a threat due to increasingly frequent outbreaks of highly virulent strains of the MDV combined with the incomplete immunity that is elicited by vaccination (Witter *et al.*, 1980; Schat *et al.*, 1981).

Considerable effort has been expended in an effort to develop safer and more effective vaccines through molecular approaches. Apart from its economic importance, MD is also of interest to biomedical researchers since it was the first herpes-virus-induced neoplastic disease to be controlled by vaccination. MD has become a preferred model, therefore, for the study of viral-induced oncogenesis and atherosclerosis.

The Marek's Disease Virus

Classification and Genome Structure

Herpesviruses are defined as eukaryotic viruses with single linear double-stranded DNA genomes ranging from 120 kb to 240 kb. Genomes are replicated and packaged into icosahedral capsids within the nuclei of infected cells. The capsids range in size from 100 to 200 nm in diameter and consist of 162 capsomers. The virion is then embedded in an amorphous protein, the tegument, surrounded by a protein-containing lipid envelope (Honess, 1984; Honess and Watson, 1977). More than 100 types of herpesviruses have been isolated; they are found within a wide range of vertebrates and each is usually highly restricted in its ability to infect other species, generally a very limited number. Based on biological properties, herpesviruses are classified into three subfamilies: alpha-, beta-, and gamma-herpesviruses (Roizman *et. al*, 1992). Although there is a remarkable variation in biological properties and genomic structure, all herpesviruses share two basic common features: (1) the lytic infection is characterized by a highly organized and complex cascade of gene expression: immediate early (IE) genes are expressed first, followed by early (E) genes and late (L) genes. (2) All herpesviruses are capable of establishing long term latency in specific cell types in immunocompetent hosts (Wagner, 1991).

The nuclear capsid of MDV is about 100 nm in diameter and contains 162 capsomers arranged in icosahedral symmetry (Nazerian and Burmester, 1968). The MDV genome is a double stranded DNA molecule of approximately 160-180 kb with a G/C content of 46-47% (Lee *et al.*, 1971; Cebrian *et al.*, 1982). The genome consists of two unique long and short DNA segments (UL and US), each flanked by terminal and

inverted repeats: terminal repeat long (TRL), inverted repeat long (IRL), inverted repeat short (IRS), and terminal repeat short (TRS) (Figure1). Like the herpesviruses Epstein–Barr virus (EBV) and Saimiri, Southern blot hybridization has also revealed the presence of repetitive internal sequences within the MDV genome at both termini (Cebrian *et al.*, 1982; Ross *et al.*, 1983; Hirai et. al., 1984). Variation in the number of the repeated sequences results in terminal heterogeneity.

MDV is primarily a cell-associated herpesvirus that has lymphotropic properties similar to those of gamma herpesviruses. Members of this group, such as EBV in humans, and MDV in chickens, generally have a restricted host range, and latency is often evident in lymphoid tissue. Viruses of this group are capable of transforming cells in natural hosts. MDV was re-classified, however, because its molecular structure and genomic organization more closely resemble alpha herpesviruses, such as Herpes Simplex Virus (HSV) and Varicella-Zoster Virus (VZV) (Buchmaster et al., 1988; Brunovskis and Velicer, 1995).

On the basis of agar gel precipitation analysis and viral antibody neutralization, three serotypes of MDV have been identified. Serotype 1 viruses include the oncogenic MDVs and their cell-culture-attenuated variants. Serotype 2 viruses include the naturally occurring non-oncogenic MDVs found in chickens. The non-oncogenic Turkey Herpesviruses (HVT) found are classified as serotype 3 (Bulow and Biggs, 1975). These strains share a similar A antigen (Ross *et al.*, 1973; Long *et al.*, 1975; Isfort *et al.*, 1986; Isfort *et al.*, 1987), but have group-specific B and C antigens that can be distinguished by serological methods (Bulow and Biggs, 1975). Tissue isolates from MD tumors can be used to infect a monolayer of avian fibroblast cells such as chicken embryo fibroblast

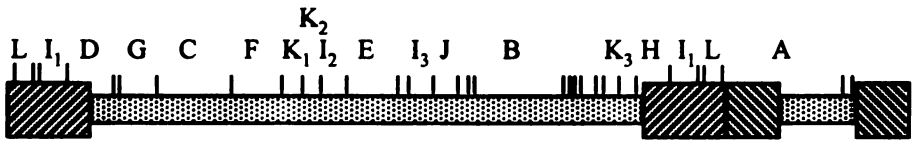
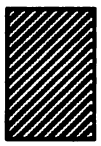
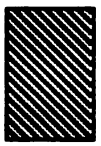


Figure 1. *Bam*HI restriction endonuclease map of serotype 1 MDV. The letters (A, B, C, etc.) represent unique fragments.



= long terminal repeat or inverted repeat long



= short terminal repeat or inverted repeat short

(CEF) or duck embryo fibroblast (DEF) cells (Churchill and Biggs, 1967; Nazerian *et al.*, 1968). The majority of virus particles produced in cell cultures, however, are non-enveloped. When cultivated in cell cultures, oncogenic serotype 1 viruses grow best in DEF cells or chicken kidney cell cultures, producing small plaques. Serotype 2 viruses grow best in CEF cells and produce medium plaques with some large syncytia. Serotype 3 viruses (HVT) grow best in CEF cells and produce large plaques. Although MDV infection in chickens is tightly cell-associated, it was found that MDV could replicate completely in the epithelial cells of feather follicles (Calnek *et al.*, 1970).

Serotype 1 isolates can be further categorized into very virulent plus (*vv*+ MDV), very virulent (*vv* MDV), virulent (*v* MDV), mild (*m* MDV), and attenuated strains based on their ability to promote disease in unvaccinated and vaccinated experimental chickens (Witter, 1997). 648A, a *vv*+ isolate, cannot be prevented through vaccination with bivalent vaccine. Md5, Md11, and RB1B strains are classified as *vv*MDV and are responsible for causing many of the outbreaks in vaccinated chickens, viruses in this class cannot be prevented in HVT vaccinated chickens. GA, HPRS-16, and JM strains are classified as *v*MDV and can cause a high incidence of MD in unvaccinated chickens. The CU2 strain belongs to the *m*MDV group. Serial passage *in vitro* results in the attenuation of virulent isolates (Churchill *et al.*, 1969; Rispens *et al.*, 1972; Witter, 1982). Since the 1970s, MD vaccines have been produced using non-pathogenic serotype 2 MDV strains (Rispens *et al.*, 1972), and serotype 3 MDV strains (Okazaki *et al.*, 1970), as well as cell culture-attenuated serotype 1 MDV strains (Churchill *et al.*, 1969; Witter, 1982; de Boer *et al.*, 1987).

Viral Attenuation

Attenuated strains of MDV have been produced by repeated passage of an oncogenic MDV strain through cell cultures and have been used successfully for vaccination against MD (Nazarian, 1970; Edison et. al, 1971). Attenuated MDVs are characterized by loss of tumorigenicity and pathogenicity, increased replication rate in cultured fibroblasts cells, and alterations in DNA structure. Comparison of viral DNA structures from pathogenic and nonpathogenic strains of MDV suggest that expansion of BamHI-D and –H fragments in nonpathogenic strains is strongly correlated with MDV attenuation (Silva and Witter, 1985; Fukuchi *et al.*, 1985). Maotani *et al.* (1986) reported that this expansion was due to the amplification of a 132 bp repeat sequence found within the BamHI-D and –H fragments. Later, it was discovered that the 132 bp repeats are part of a transcript containing an open reading frame (ORF) named *Bha* which exhibits a complex pattern of splicing in cells lytically infected with oncogenic MDV strains and in MDV transformed cell lines. The ORF encodes a transmembrane protein of 63 amino acids with limited homology in its N- terminus to a mouse T cell lymphoma TLM oncogene protein. An overexpressed Bha gene prolongs the growth of CEF cells and enables CEF cells to grow in very low serum concentrations (Peng *et al.*, 1993). This region is important because the number of copies of the 132 bp repeat increases following cell-culture attenuation of serotype 1 viruses, and, thus, can be used as a marker for indicating decreased tumorigenic capability (Silva, 1992). Moreover, it has been hypothesized that a crucial gene involved in oncogenicity could be disrupted by the expansion of the 132 bp repeat region (Ross *et al.*, 1983; Bradley *et al.*, 1989; Chen and Velicer, 1991). Besides the variation of the 132 bp repeat sequence, there is also a 200

bp deletion in the *Bam*HI-L fragment located in IRL and TRL regions in attenuated strains (Wilson and Coussens, 1991). Evidence from *in vivo* studies, however, indicates that the 200 bp deletion found in attenuated Md11 does not directly affect MDV oncogenicity.

One approach to identifying putative MD oncogene(s) is to study the mutation associated with viral attenuation. The classical approach is to inactivate the gene(s) and then compare the phenotypes of wild-type and mutant viruses. In general, a wild-type MDV gene can be replaced or disrupted by homologous recombination with a mutant version carried on a plasmid. This site-specific insertional mutagenesis approach is a powerful tool that serves to assign the function of a gene. Unfortunately, as MDV is a highly cell-associated virus, the virus spreads through cell to cell contact but is not released from the cell. Thus, the selection of the recombinant virus is an extremely inefficient process in MDV mutagenesis study. Therefore, alternative molecular approaches are needed in order to create attenuated MDV viruses.

Pathology of Marek's Disease

As stated previously, the MDV is a highly cell-associated herpesvirus. Usually, enveloped virions enter the cell by conventional absorption and penetration within one hour following infection. Natural transmission of MD occurs through direct contact between infected and uninfected chickens, or indirectly through dust-borne viral particles (Calnek et. al, 1970). Clinical signs, including gross lesions, generally start to appear approximately four to six weeks following infection.

MD is characterized by lymphoid infiltration of various peripheral nerves and visceral organs, most frequently the spleen and the gonads. An association of certain

MDV isolates with ocular lesions and blindness has also been noted. Nerve lesions, such as enlargement and gray-yellow discoloration, are the most common observations that have been made with respect to diseased chickens. There are at least two distinct pathological forms of MD that have been recognized. The classical type of MD predominantly attacks peripheral nerves and causes cytolytic infections. In contrast, the acute form of MD involves a form of lymphoproliferation that results in the formation of tumors in various organs (Calnek and Witter, 1997). Microscopic sections of MD lesions are characterized by the morphologic heterogeneity of neoplastic lymphoid cells. Various sizes of lymphocytes, plasma cells, and lymphoblasts have been observed in the peripheral nerves and/or visceral organs of diseased birds. Lymphomas are developed in one or more of the following organs: the spleen, the liver, the gonads, the kidney, the heart, the pancreas, and the proventriculus, as well as muscle and skin tissue.

The lymphoproliferative nature of MD lesions can be easily confused with that of lymphomas induced by avian leukosis virus (ALV) or reticuloendotheliosis virus (REV). MD lymphomas are of T-cell origin, whereas ALV and REV mainly cause B cell lymphomas. These lymphomas can be distinguished by immunocytochemical tests using antibodies specific to cell surface antigens of B and T lymphocytes. The molecular mechanism of MDV oncogenesis is quite different from that of ALV. It is hypothesized that MDV carries an oncogene, whereas the ALV transforms target cells by integration within or near a cellular oncogene such as c-*myc* causing the overexpression of its gene product which initiates oncogenesis.

Pathogenesis of Marek's Disease

By inoculating susceptible chickens with oncogenic MDV, four different phases of infection have been established: (1) early cytolytic infection of the lymphoid organs; (2) a period of latent infection; (3) late cytolytic infection; and (4) the transformation of T-lymphocytes. In genetically resistant hosts, only the early cytolytic infection followed by the establishment of lifelong latency has been observed.

The early cytolytic infection phase occurs 3 to 5 days following inoculation and mostly affects B-lymphocytes plus a few activated T-lymphocytes (Shek *et al.*, 1983; Calnek and Spencer, 1985). This productive-restrictive infection can provoke an acute inflammatory reaction resulting in macrophage and granulocyte infiltration (Payne and Roszkowski, 1973), which leads to local necrosis. Common consequences of these events are spleen degeneration and atrophy of the thymus. The severity of this early cytolytic phase, however, is related to the virulence of the virus.

The infection switches from a cytolytic infection of primarily B-lymphocytes to a latent infection of predominantly T-lymphocytes at 6-7 days following infection (Shek *et al.*, 1983). This switch is generally associated with the development of an immune response in the host. Latently infected T-cells can persist for the lifetime of the host. However, latent infection has also been observed in non-lymphoid cells, such as nonmyelinating Schwann cells and satellite cells in spinal ganglia (Pepose *et al.*, 1981). The mechanism of MDV persistence in T-cells is unknown.

Progression to a second cytolytic infection has only been observed in genetically susceptible birds at 2 to 3 weeks following inoculation. In this phase, affected tissues are not limited to lymphoid organs, but also include epithelial cells in various visceral

organs. Cell-free infectious viral particles produced from feather follicle epithelium can be observed at this stage (Calnek *et al.*, 1970). Focal necrosis and intranuclear inclusions also occur in the kidney, pancreas, blood vessels, peripheral nerves, and central nervous system (Payne, 1992).

Lymphoproliferation and the development of T-cell tumors represent the final stages of MD pathogenesis (Buscaglia and Calnek, 1988; Calnek and Witter, 1997). The composition of lymphomas, consisting of a mixture of neoplastic, inflammatory, and immune cells involve almost all visceral organs, skin, muscles, and nerves. This stage usually occurs at 4 to 6 weeks following inoculation.

Cell transformation is the most salient characteristic of oncogenic MDV strains. Although the virus initially causes a lytic infection in lymphoid cells, it persists in the natural host by establishing a life-long, latent infection of T-lymphocytes. There are no viral antigens that have been detected in latently infected cells. All three serotypes of MDV can cause latent infection in cells, but only serotype 1 MDV has pathogenic potential. Although the molecular basis of oncogenic transformation by MDV has not been established, it has been hypothesized that MDV contains a distinct oncogene(s) in contrast to chronic transforming RNA oncoviruses, which do not posses an oncogene but transform cells indirectly by activation of a cellular oncogene (hit-and-run mechanism). Schat *et al.* 1991 reported that cellular transformation by the MDV occurs only in a limited subset of activated T-cells. Because of the rapid onset of tumor formation and the polyclonal nature of the tumors, it is likely that MDV carries its own oncogene(s). The final pathogenesis of MD, however, is influenced by many factors, such as virus strain and the age, sex, and genetic background of the host (Calnek and Witter, 1997).

Virus-Cell Interaction

The MDV is most frequently contracted from the environment via the respiratory system (Payne, 1985). Once the MDV enters the host, three major virus-cell interactions have been recognized: (1) productive infection, (2) non-productive latent infection, and (3) transforming infection (Schat, 1985; Calnek and Witter, 1997). Productive infection occurs mainly in nonlymphocytes. Two types of productive infection have been observed: fully productive and productive-restrictive. Fully productive infection by the MDV has only been observed in feather follicle epithelium (Calnek *et al.*, 1970), which results in the development of large numbers of enveloped and fully infectious virions. Nevertheless, a productive-restrictive infection can occur in some lymphoid and epithelial cells in the host and in most cultured cells, where most of the virions produced are not enveloped and thus noninfectious. Cell to cell fusion, therefore, becomes the major virus spreading mechanism in productive-restrictive infections (Calnek and Witter, 1997). In productive infections, the number of replications of viral genome copies per cell can exceed 1200. *In vivo* productive infection normally leads to the formation of intranuclear inclusion bodies, cell destruction, and necrosis. In cultured fibroblast cells, polykaryocytosis is a major component of viral plaques, and it is commonly used as a marker in biological assays of virus infections.

Latent infection is not a productive type. There are very few copies (about 5) of the viral genome that have been observed in latently infected lymphocytes, predominantly in T cells, but also in some B cells. Viral gene expression is highly limited in latently infected cells. Most translation does not occur, and, normally, no virus

or tumor associated antigens can be detected (Calnek *et al.*, 1981; Sharma, 1981), although some genes may be transcribed.

A third type of interaction is transforming infection, only found in T lymphocytes transformed by virulent serotype 1 MDV. Transformed cells contain more copies (5-15) of the viral genome than latently infected cells (Ross, 1985), and there is more extensive viral gene expression, occasionally resulting in antigen production (Nakajima *et al.*, 1987; Nakajima *et al.*, 1989). The MDV's viral DNA in transformed cell lines is highly methylated, whereas methylation has not been detected in MDV DNA from productively infected cells (Kanamori *et al.*, 1987). To date, no viral antigens have been found to have any specific association with transformed cells. Nevertheless, an activated T-cell marker, Marek's associated tumor surface antigen (MATSA), is expressed at a higher level in transformed cells (McColl *et al.*, 1987). The majority of these transformed T-cells are activated T helper CD4+/CD8- cells. Nevertheless, other cell lines that have been established from experimentally induced lesions are CD4+/CD8+, and CD4-/CD8- T-cells (Schat *et al.*, 1991). Transformation is only known to occur in T cells. It is also believed that T cells are susceptible to MDV infection only after activation. The mechanism that leads from latency to transformation is not well understood. Yet, current evidence suggests that latent infection is a prerequisite to transformation.

The Regulation of Gene Expression

As with other herpesviruses, MDV gene expression in the lytic infected state is temporally regulated in a highly organized, cascade fashion (Maray *et al.*, 1988; Schat *et al.*, 1989). In general, MDV genes have been classified into three kinetic families: immediate-early genes (IE or α), early genes (E or β), and late (L or γ) genes, based on

the requirements for viral protein synthesis or DNA replication (Honess and Roizman, 1974).

Immediate-early Genes

IE genes are expressed immediately upon virus entry into host cells and do not require *de novo* viral protein synthesis. The expression of IE transcripts is controlled by enhancer elements located within their promoters. It has been suggested that a virion-associated transcriptional activator, VP16, mediates MDV IE gene expression (Boussaha *et al.*, 1996), which itself is produced late in infection (Campbell *et al.*, 1984). VP16 triggers the transcription of the IE genes in HSV, including infected cell peptides (or ICP) ICP0, ICP4, and ICP27. IE gene transcripts accumulate in the presence of metabolic inhibitors such as cycloheximide (CHX), a protein synthesis inhibitor. Several MDV IE genes have been identified which are homologous to HSV-1, including ICP4, ICP22, and ICP27 (Anderson *et al.*, 1992; Ren *et al.*, 1994; Hong and Coussens, 1994; Brunovskis and Velicer, 1995). An MDV specific 14 kDa phosphoprotein (pp14) encoded by a 1.4 or 1.6 Kb IE transcript has also been reported (Hong and Coussens, 1994). Studies have demonstrated that MDV pp14 is detected not only in serotype 1 infected cells, but can also be found in MDV serotype 1 - transformed lymphoma cell line, MSB-1 (Hong *et al.*, 1995). However, the basic function of pp14 is still not clear.

Early Genes

Early genes become activated following IE gene expression and are regulated by IE gene products. Most E gene products are involved in nucleotide precursor metabolism and are essential for viral DNA synthesis (Roizman and Sears, 1995). E gene products are identified by their accumulation in the presence of viral DNA synthesis inhibitors

such as phosphonoacetic acid (PAA). Several E genes in MDV have been identified, including thymidine kinase (TK) (Scott *et al.*, 1989), DNA polymerase (UL30) (Sui *et al.*, 1995), a unique phosphoprotein with 38 kDa molecular weight (pp38) (Cui *et al.*, 1990; Cui *et al.*, 1991; Chen *et al.*, 1992), and a MDV- specific gene, *meq*, which encodes a protein with high homology to the fos/jun family of oncogenes (Jones *et al.*, 1992). The pp38 has been shown to be abundantly expressed in the MDV MSB-1 tumor cell line (Cui *et al.*, 1991). It has been suggested that the biological function of pp38 is potentially related to MDV oncogenicity (Calnek and Witter, 1997). The expression of E genes is responsible for initiating viral DNA synthesis and subsequently for the induction of late gene expression.

Late Genes

Late gene expression requires both viral protein synthesis and viral DNA replication (Wagner, 1991). Late genes mainly encode structural proteins essential for virion, capsid, tegument, and envelop assembly (Roizman and Sears, 1995). Viral glycoproteins are primarily encoded by L genes whose functions include virus attachment, penetration, and cell-to-cell fusion (Roizman and Sears, 1995). Since the key feature of late gene expression is its requirement for activation by viral DNA replication, L gene transcription will be inhibited in the presence of DNA replication inhibitors. Based on their dependence on viral DNA replication, L genes can be grouped into γ1 and γ2 subfamilies. γ1 gene transcription occurs prior to the initiation of viral DNA synthesis, and is only minimally affected by inhibitors of DNA synthesis. γ2 genes are expressed late after infection and are not detectable in the presence of effective concentrations of inhibitors (Roizman and Sears, 1995).

L genes in MDV have also been identified, including glycoproteins gB (Ross *et al.*, 1989), gC (Isfort *et al.*, 1987; Coussens and Velicer, 1988), gD (Brunovskis and Velicer, 1995), gE (Brunovskis and Velicer, 1995), gH (Scott *et al.*, 1993), gI (Ross *et al.*, 1991), gK (Ren *et al.*, 1994), and gL (Yoshida *et al.*, 1994). Among these glycoproteins, a recombinant fowlpox virus (FPV) expressing the MDV gB has shown to provide some effective protection against MD in vaccinated chickens (Nazerian *et al.*, 1992).

Latency Associated Genes

The establishment of latency is a hallmark of herpesviruses. Latency is a reversible, non-productive infection stage involving a replication of a competent virus. The development of latency by a virus involves the evasion of the host's immune response system and the persistence of a viral genome in latently infected cells. This is relatively easy for neurotropic herpesviruses, such as HSV, where latency is established in non-dividing neuron cells. For lymphotropic herpesviruses, however, this is more difficult since the virus must latently infect dividing or mitotic cells, such as B or T cells. In this case, the virus requires a specialized origin of replication to ensure that its genome is retained in each daughter cell. The mechanism of MDV genome persistence in latently infected cells is unknown. However, it is clear that CD4+ T-helper cells are the primary targets of latent infection, although a few B cells may also be involved (Schat *et al.*, 1991).

Only a limited set of viral genes is expressed in latently infected cells. The majority of latency-associated transcripts of the MDV were mapped to the repeats flanking the unique, long region of the genome by Northern blot analysis (Tillotson *et al.*,

1988). The transcripts, however, are cell-type restricted. In the MSB1-transformed cell line (an expressing cell line) 29 transcripts have been mapped to different regions of the MDV (Becker *et al.*, 1988). Schat *et al.* (1988) reported 4 and 7 transcripts in HP1 (non-producing cell line) and CV41 (non-expressing cell line), respectively. Additionally, most transcripts found in MDV-lymphoblastoid cells are IE genes (Silver *et al.*, 1979; Schat *et al.*, 1989), suggesting that IE genes could play a significant role in the maintenance of latency.

Another important finding is that viral DNA is heavily methylated in these transformed cell lines. This phenomenon is presumably related to limited gene expression. In addition to the circular plasmid form in the latent stage, viral DNA has also been found to be integrated into the host genome in some cell lines (Hughes *et al.*, 1980; Kato and Hirai, 1985; Delechuse and Hammerschmidt, 1993). The genome sites for MDV integration are found to be random, although telomeres appear to be the preferential targets (Delechuse and Hammerschmidt, 1993). However, the viral integration is not found to be essential for viral transformation.

Immunoresponse to MDV Infection

The outcome of MDV infection is influenced by the host's gender, age, genetic makeup, and immune system response. Male birds are more resistant to MD than females (Purchase and Biggs, 1967; Cole, 1968) although the reason for this is not known. Usually older birds are more resistant to a virulent MDV infection (Witter *et al.*, 1973). Both humoral and cell-mediated immunity have been found to play a role in resistance to virus infection.

Humoral Immunity

Infection of MDV at cytolytic stage primary occur in B lymphocytes results in the release of viral antigens. Thus, virus-neutralizing antibodies can be detected within 1-2 weeks following MDV infection. These antibodies generally persist throughout the lifetime of the bird. Because bursectomized birds can survive a MDV infection, it is presumed that the humoral antibody response may not be essential for resistance to MD (Sharma and Witter, 1975). Maternal antibodies also reduce the level of MD development (Chubb and Churchill, 1968; Ball *et al.*, 1971).

To examine the specific viral antigens induced protective immunity, viral proteins expressed via fowlpox virus or baculovirus were used as vaccines followed by challenging birds with oncogenic MDV. Results indicated that only gB has partial effects for the induction of virus-neutralizing antibodies (Nazarian *et. al.*, 1992). Niikura *et al.* (1991) demonstrated that although gC can induce the development of gC specific antibodies, the antibodies does not have effect in protecting against MD development. Similar study in unique gene pp38 also demonstrated that it does not induce the production of protective antibodies (Nazarian *et al.*, 1992).

Cell-Mediated Immunity (CMI)

As a consequence of the production of viral antigens, T cells become activated and capable of being infected. Since the MDV has a cell-associated nature that CMI has been strongly suggested to be critical in controlling viral infection and resistance to MD development (Omar and Schat, 1996). However, due to the lack of *in vivo* evidence, this suggestion is inconclusive. The CMI responses are directed against viral antigens and include delayed hypersensitivity (Byerly and Dawe, 1972), *in vitro* cytotoxicity as

measured by plaque reduction, and antibody dependent, cellular cytotoxicity (Powell, 1976; Sharma and Coulson, 1977; Confer and Adldinger, 1980).

Interference of antigen presentation upon virus infection has been suggested to be one of the potential mechanisms incapacitating the CMI to MDV. However, due to the limitation that cultured primary cells do not express the MHC class I antigen or/and MDV viral antigen, there is no significant association has been made between any particular viral antigen and MHC down regulation. Recent studies using chemical transformed CEF cell line OU2 indicated that MDV early gene pp38 might be involved in the down regulation of MHC class I glycoproteins via targeting peptide transportation (Hunt *et al.*,1999). This result indicated that pp38 may have an association with the depression of immunity after viral infection. Typically, CMI has been tested by measuring the ability of mitogens to activate normal peripheral T-lymphocytes to undergo blast transformation and proliferation. Results indicated that older birds of resistant chicken lines have lower responses to mitogens (Schat *et al.*, 1978; Fredericksen and Gilmour, 1981), although the reciprocal response may occur at a young age (Fredericksen and Gilmour, 1983; Lee and Bacon, 1983).

Non-Specific Immunities

Macrophages are found to be playing a central role in the regulation of the MDV infected immune response. It may be involved in resistance by directly limiting virus replication (Higgins and Calnek, 1976; Haffer *et al.*, 1979), or by cooperating with antibodies (Kodama *et al.*, 1979; Lee, 1979). Gupta *et al.* (1989) demonstrated that the use of activated macrophages by injection of thioglycollate broth into the peritoneal cavity significantly reduced the incidence of MD in challenged birds. Moreover, *in vitro*

studies showed that activation of macrophages can inhibit viral DNA synthesis and cell proliferation in MD lymphoblastoid cell lines (Lee *et al.*, 1978; Sharma, 1980; Ozaki *et al.*, 1983). *In vivo* studies suggested that the activity of macrophage among different resistance chicken flocks is different (Dr. M. Qureshi, personnel communication). These evidences demonstrated that macrophage act as the first line of defense to MDV infection.

It is believed that cytokines are involved in regulating MDV gene expression (Volpini *et al.*, 1995). It has been reported that the level of interferon, an early response to MDV infection, is higher in resistant birds (Hong and Sevoian, 1971). Cytokines also appear to be different in birds infected by various MDV isolates (Sharma, 1989). Particular cytokines have been associated it is important with the development and maintenance of latency with the MDV (Buscaglia *et al.*, 1988; Volpini *et al.*, 1995).

Natural killer (NK) cells are cytotoxic for MD tumor cells and may be involved in genetic resistance to MD (Powell, 1985; Schat, 1987). There is evidence of increased NK cell activity after vaccination with the HVT or SB-1 viruses (Sharma, 1981; Heller and Schat, 1985). Moreover, the increasing levels of NK cells in regressive, but not progressive, tumors could also indicate that NK plays a role with respect to intratumoral immunity in tumor regression (Sharma, 1983).

Vaccines Against MD

A number of vaccines have been available since 1970 for controlling MD development in the field. These vaccines consist of attenuated serotype 1 isolates and serotype 2 or 3 viruses, administered alone or in combination. Recently, recombinant MD vaccines have also been developed that protect chickens against MD; however, they

are not as effective as hoped. The recombinant vaccines were constructed by inserting immunogenic genes of the MDV, such as gB, in fowl poxvirus or HVT (Nazerian *et al.*, 1992, Ross *et al.*, 1993). A number of MD vaccines are currently in use. HVT continues to be an important constituent of the most popular vaccines since it is easily made and economical in price. Vaccinated chickens become resistant to tumor development and other symptoms of MD caused by virulent MDV. As stated above, however, vaccination does not prevent the infection, replication, and spread of the virus (Edison *et al.*, 1971; Purchase *et al.*, 1972). The superinfecting oncogenic virus and the vaccine strains are still able to replicate, and the viral particles shed from feather follicles can still infect unprotected chickens.

The method for MD vaccine delivery is undergoing a rapid change. Vaccines were usually administered subcutaneously or intramuscularly in newly hatched chickens in the hatchery (Oei and Boer, 1986). Recently, *in ovo* embryo vaccination technology has been used in over 80% of commercial broilers in the United States. With this technology, vaccines are injected into eggs at 17-18 days of embryonation with the use of multiple-head injection machines that result in lower labor costs and greater precision of the vaccination (Calnek and Witter, 1997).

Humoral immune responses to tumor-associated antigens have not been detected in chickens following vaccination. Instead, the number of antibodies increases following hyperimmunization with lymphoma cells. Thus, vaccinal immunity appears to be based primarily on cellular responses to viral antigens supplemented by humoral anti-viral and cellular anti-tumor immune responses.

Vaccination efficiency is effected by several factors, including genetic makeup, age at challenge, stress, and possible infection by other immunosuppressive viruses (Calnek and Witter, 1997). Vaccine failures, however, result mainly from early exposure to and the emergence of new, more virulent MDV strains. The use of genetically resistant birds in ongoing research has come to be seen as an especially promising approach for controlling MD.

The Genetics of Host Resistance to Marek's Disease

Selection of genetically superior animals through the use of a planned breeding system has made it possible to influence the genetic makeup, breed (or line) of future generations. Those chickens that are resistant to MD are those that fail to develop characteristic MD symptoms upon exposure to oncogenic MDV. Genetic differences in resistance to MD paralysis have been reported for more than 60 years (Asmundson and Biely, 1932). Since then, several MD resistant inbred lines have been generated via selection and mating programs at the Avian Disease and Oncology Laboratory (ADOL). Of 15 lines developed, White Leghorn lines 6 and 7 proved to be the most interesting with respect to MD studies. When line 6 chicks are inoculated with the JM strain of MDV at 1 day of age, less than 3% of the birds develop MD symptoms. In contrast, a similar inoculation into line 7 birds resulted in a mortality rate over 85%. These lines are maintained at the ADOL and are over 99% inbred. The level of disease resistance as measured by mortality among F1 siblings of a cross between lines 6 and 7 is intermediate to the parents (~60%); however, the levels of resistance observed in an F2 population encompass a large spectrum instead of following one gene (two alleles) Mendelian

segregation manner. This indicates that there is more than one gene involved in MD resistance (Stone, 1975).

MHC Genetic Resistance

The major histocompatibility complex (MHC) is a group of closely linked loci that codes for highly polymorphic cell surface proteins as well as the proteins involved in the control and development of immune system responses (Klein, 1986). There are two types of molecules designated, Class I and Class II, respectively, that present intracellularly processed peptides to T lymphocytes. A distinct feature of many MHC molecules is their extensive polymorphism. Many MHC gene variants are known to be more or less associated with disease resistance. The chicken MHC, known as the B complex, is the best understood mechanism for the development of genetic resistance to MD. MHC-related resistance functions by reducing the MDV infection level during latent phase infection, also eliminating the second wave of cytolytic infection.

By measuring the frequency of specific blood groups, it has been observed that certain B alleles are associated with resistance or susceptibility. Chickens with the B21 allele have been found to be more resistant than those with other B haplotypes (Bacon, 1987; Bacon and Witter, 1992). Other studies have allowed for the relative ranking of the other B alleles: moderate resistance, B2, B6, B14; susceptibility, B1, B3, B5, B13, B15, B19, B27 (Longenecker and Mosmann, 1981). Recent studies indicate that B-haplotype influences vaccinal immunity and that some haplotypes develop better protection with vaccines of one serotype than with others (Bacon and Witter, 1994). Now, instead of directly testing lines for MD resistance, breeders have the advantage of being able to indirectly select favorable birds by using B blood group antigens. This

blood typing system has not been widely adopted, however, primarily because the MHC has pleiotropic effects. In other words, selecting for alleles that confer resistance to MD may be disadvantageous for selecting resistance to other diseases or production traits. Recent studies of a MHC-like, Rfp-Y haplotype indicated that it might significantly influence the outcome of infection with MDV (Wakenell *et al.*, 1986). However, a similar study using an experimental intercross between lines 6 and 7 indicated that the Rfp-Y genes do not influence MD resistance (Vallejo *et al.*, 1997). Thus, this suggests that Rfp-Y genes may have only a minor effect or that only certain alleles are involved in MDV resistance.

Non-MHC Genetic Resistance

Recent research suggest that the contribution of resistance to MD by non-MHC genes may be significantly larger than that of the MHC (Groot and Albers, 1992). Studies were done on three commercial White Leghorn lines with different MD resistance and MHC haplotypes. Two sire lines, heterozygous for MHC haplotype, from susceptible or resistant lines, were mated to dams, homozygous for MHC haplotype, from a moderately resistant line. 1359 chicks were produced, scored for MHC haplotype, and infected with MDV at one day of age. Results indicate that the sire line had a larger effect than either the maternal line or the parental MHC haplotype. This study suggests that a higher level of resistance to MD can be derived from non-MHC genes than from MHC alleles. To further identify non-MHC genes which have an influence on disease resistance, genetic markers were used to screen for linkage to phenotypic traits that are associated with MD resistance.

QTL Search and Genome Mapping

QTL and Genetic Markers

Animal breeders from around the world are working on developing genetic maps to help identify specific genes which effect economic traits. These genes are believed to act in combination to produce continuous or quantitative variations in a trait. These specific regions or genes are called quantitative trait loci (QTLs). The more we know about the underlying genetic variation of QTLs, the more we will be able to predict the responses that will have a major impact on the production, reproduction, and general health traits of our livestock. We may also be able to produce animals that are designed for specific environments and increase the level of inheritability of certain desirable traits. The identification of QTLs could also increase the accuracy of selection for existing traits and allow for the selection of new traits that are positively associated with reproduction and health characteristics (Rothschild, 1994).

Unfortunately, our knowledge of the identity of QTL genes, how many QTLs in combination are responsible for certain variations, as well as individual actions and interactions, is still very limited. Recently, genetic markers have been used to develop a better understanding the behavior of QTLs, using marker-QTL linkage. Tanksley *et al.* (1982) first reported the linkage between DNA-based markers and QTLs in plants. Two or more loci are defined as linked if we can observe the non-independent segregation of alleles at these loci (Botstein *et al.*, 1980). QTLs can be most easily detected in crosses between inbred lines with large phenotypic differences. This will provide better evidence for segregation as well as the fraction of QTLs in the F2 generation (Ellegren, 1993).

Genome Mapping

The purpose of gene mapping in breeding programs is to find genetic markers which are well spaced and which provide broad coverage. QTL detection depends on the density and polymorphism of DNA-based markers. The development of genetic maps will also provide the means necessary for a better understanding the evolutionary relationships between breeding lines. Two types of maps are commonly used: genetic maps and physical maps.

On a genetic map, the estimation of linkage distance between two markers is based on the frequency of recombination during the formation of gametes. Genes which co-segregate during meiosis are said to be linked, and a collection of linked genes on the same chromosome is then referred to as a "linkage group." The distance between these genetic markers is expressed in centiMorgans (cM). The measure of 1 cM is equal to a recombination rate of 1%. In chicken genome studies, it is estimated that 1cM is about equivalent to about 500 Kb. The current consensus concerning the chicken genetic map contains 1889 loci which describe 50 linkage groups (Groenen *et al.*, 1999). This map spans 3800 cM, which is considerably larger than previous estimates for the chicken genome (Levin *et al.*, 1994). The map contains 350 markers within expressed sequences, 201 of which represent identified genes sequences that have significant sequence identities with known genes.

In contrast, a physical map is a map that displays the position of genes on the chromosome, usually according to the length of DNA between them. The relation of genetic distance to physical distance is not uniform, due to the fact that recombination does not occur evenly along each chromosome or species. On a genetic map, marker

genes are used as "landmarks" which can then be used to follow the inheritance of sections of a chromosome; physical maps, on the other hand, mainly provide a direct measurement of the physical distance along a chromosome.

A large collection of good genetic markers is needed to map QTLs and genes that determine performance and reproduction traits as well as genetic diseases. In general, there are two types of markers that are used; they are designated as Type I and Type II. A type I marker is used within a certain coding region of a specific gene which can be identified, for example, by means of restriction fragment length polymorphism (RFLP) analysis (Fries *et al.*, 1993). Type II markers are anonymous markers that are usually used for non-coding regions of the genome, such as microsatellite markers. Although a lot of genetic markers have been described for both human or livestock genomes, many of the markers which were originally described are only contain two alleles and, therefore, are less useful in the analysis of disease resistant genes or QTLs (Nakamura *et al.*, 1987). Polyallelic markers, such as microsatellites, are most useful in mapping QTLs.

When QTLs have been successfully mapped, they could be employed in breeding programs through the use of "marker-assisted selection." Marker-assisted selection, selection for favorable QTLs via selection of linked markers, is based on the fact that QTLs may be located on marker-linked genomic regions (Soller *et al.*, 1976; Soller and Beckmann, 1983; Dentine, 1990; Lande and Thompson, 1990). Dentine (1994) suggests that the utilization of markers in selection will be most effective for operations with high selection intensities, multiple offspring from individual matings, and the potential of selecting individuals based on numerous progeny, as is the case with poultry and swine.

Dentine (1994) points out, however, that the use of linked markers instead of QTL alleles themselves might complicate selection, since recombination could occur between the markers and QTLs. Hence, we risk losing the QTLs and increasing the errors that are made in breeding strategies that stretch over several generations. The distance between related markers, therefore, will be key to identifying precise QTL positions.

The Current Status Of MD-QTL Research

It has been demonstrated that a component of MD resistance is associated with the MHC (B) locus, which encodes Class I and II MHC antigens (Calnek, 1985). But other loci must also be important since chicken line 6 (the resistant line) and line 7 (the susceptible line) share the same B locus yet show significantly different levels of sensitivity to MDV infection. Recently, 14 QTLs have been identified in F2 chickens from the intercross of inbred line 6 and line 7 that confer resistance to MD (Figure 2), through the use of biometrical analysis and microsatellite markers (Yonash *et al.*, 1999). Unfortunately, many of these QTLs were found in large intervals or linked only to a single marker. So as to better define the location of these QTLs, additional markers need to be mapped in these regions. The development of recombinant congenic strains (RCS), using lines 6 and 7, will provide a powerful tool for further refining the location of these QTLs.

Specific Aims

Since MDV is a herpesvirus that causes malignant T-cell lymphoma in chickens, increasingly effective management of the disease will require a better understanding of the molecular basis for viral tumorgenicity, which will in turn provide a valuable reference for safer vaccine development. **There are two specific goals in this project.**

One is to identify host protein(s) that interact with the MDV serotype 1 specific gene, short open reading frame 2 (SORF2) protein. The other is to further evaluate the potential association of SORF2-interacting protein with resistance to Marek's disease. Further exploring the interactions between novel virus-host proteins has great promise for advancing our current level of knowledge concerning the molecular biology of MDV.

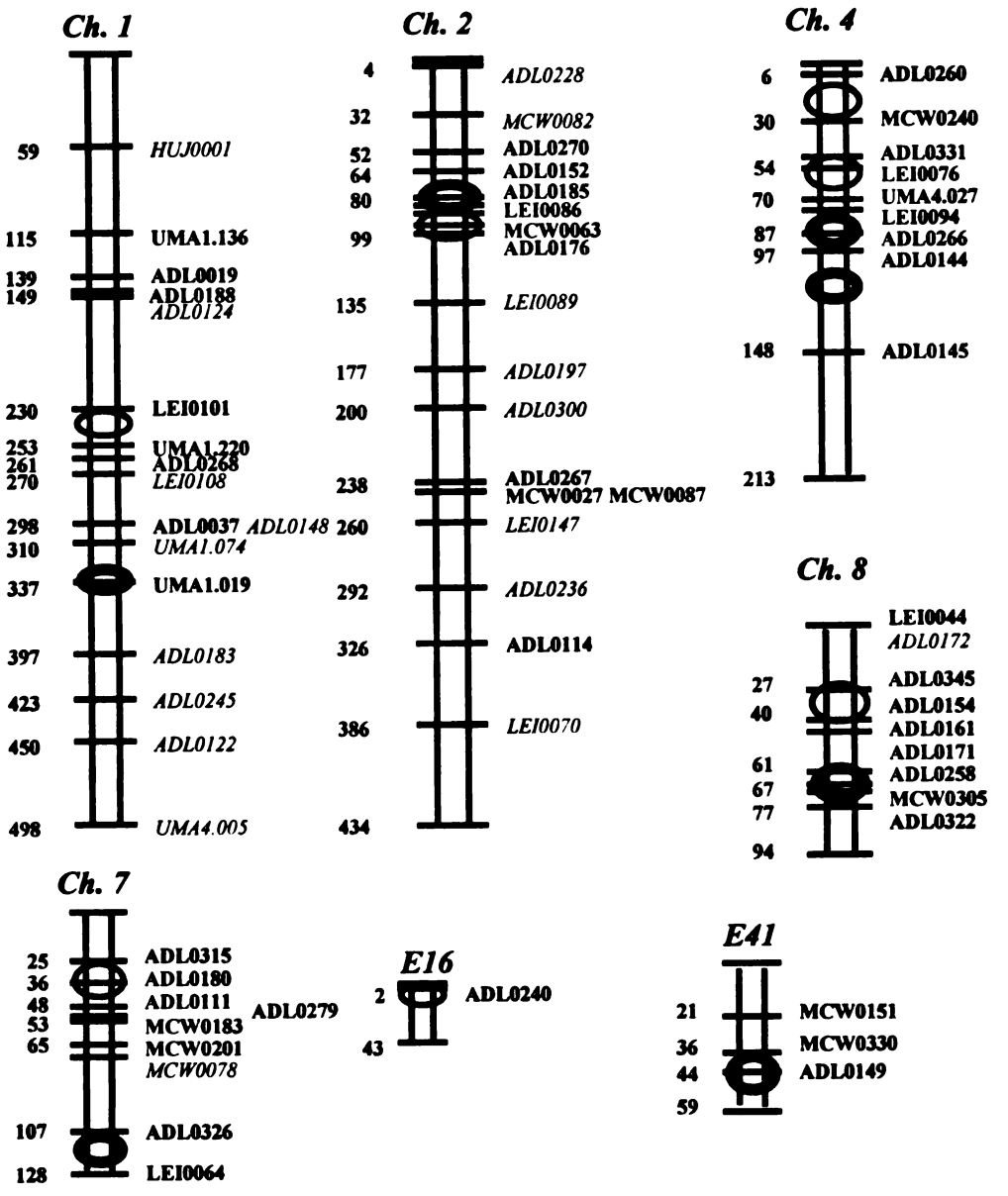


Figure 2. MD QTL on East Lansing chicken genetic map. Bold and unbold circles represent significant and suggestive QTL, respectively. QTL=quantitative trait loci.

CHAPTER II

Identification of Chicken Growth Hormone Interacting with MDV SORF2

Introduction

Marek's disease virus (MDV) is a naturally occurring oncogenic avian herpesvirus that causes lymphoproliferation and induces neoplastic disease in chickens. A key goal of research on Marek's disease is to identify the gene(s) in MDV that promote oncogenesis. One approach to accomplish this goal is to investigate the genes which function in viral replication, attenuation, latency, and transformation.

Current sequence information suggests that MDV is colinear with the HSV and VZV genome (Buckmaster *et al.*, 1988). A number of HSV-homologous genes have been identified and investigated. These genes include gB, gD, gE, gI, gH, ICP4, TK, VP16, and ICP27 (Ross *et al.*, 1991; Ren *et al.*, 1994; Brunovskis and Velicer, 1995; Koptidesova *et al.*, 1995; Boussaha *et al.*, 1996). However, there are also novel MDV genes including short open reading frame 1 (SORF1), 2 (SORF2), and 3 (SORF3) which are located within the MDV US region. The functions of these remain unknown.

The gene SORF2 is of special interest because a retrovirus LTR region integrated upstream of the SORF2 gene, which yielded attenuated oncogenicity in the MDV recombinant strain RM1 (Witter *et al.*, 1997). Isfort *et al.* (1992) have shown that co-infection of the retrovirus reticuloendotheliosis virus (REV), and MDV in cultured cells results in integration of the REV LTR into MDV genome. It was observed that the stable integration of the LTR predominantly occurred at the junctions of the unique and repeat regions (Jones *et al.*, 1993). In fact, sequence data of the junction of the UL, TRL, and IRL indicated a region of high homology to the LTR region of REV (Isfort *et al.*, 1992).

In addition, this is the region that shows significant heterogeneity among MDV strains. The incorporated LTR sequence may regulate the transcription of genes around the repeat regions that may lead to phenotypic changes of MDV. The RM1 clone is derived from the JM strain of MDV through REV insertional mutagenesis. It contains REV proviral LTR sequences inserted at the junction of the internal short repeat and unique short regions of the genome (Figure 3) presumably due to homologous recombination.

The RM1 clone appeared attenuated for oncogenicity but retained other *in vivo* properties of virulent viruses including thymic and bursal atrophy, early immunosuppression, early cytolytic infection followed by efficient replication, and contact spread. Those characteristics are normally absent in attenuated strains (Witter *et al.*, 1997). In addition, chickens vaccinated with RM1 clones were protected against challenge with virulent MDV, and levels of protection exceeded those of other attenuated serotype 1 vaccine virus (Witter *et al.*, 1997). Molecular analysis indicated that RM1 overexpressed a 3.2 Kb transcript initiated from the LTR promoter which extended across the coding sequences of SORF2, US1, and US10, terminating after the US10-proximal poly (A) signal in the US region (Jones *et al.*, 1996). There is no evidence to indicate that the 132 bp repeat region contributed to the attenuation of RM1 oncogenicity since expansion of the 132 bp repeat was not observed. No other gross alteration of the RM1 strain genome was detected when compared to that of the oncogenic JM parent virus. Genes expressed from the inserted LTR promoter might potentially be responsible for altered biological properties of RM1 MDV. This phenomenon prompted investigation into the biological role of SORF2 during MD oncogenesis. Since the SORF2 is an MDV unique gene and one of the overexpressed genes of RM1 strain. Detailed studies on the

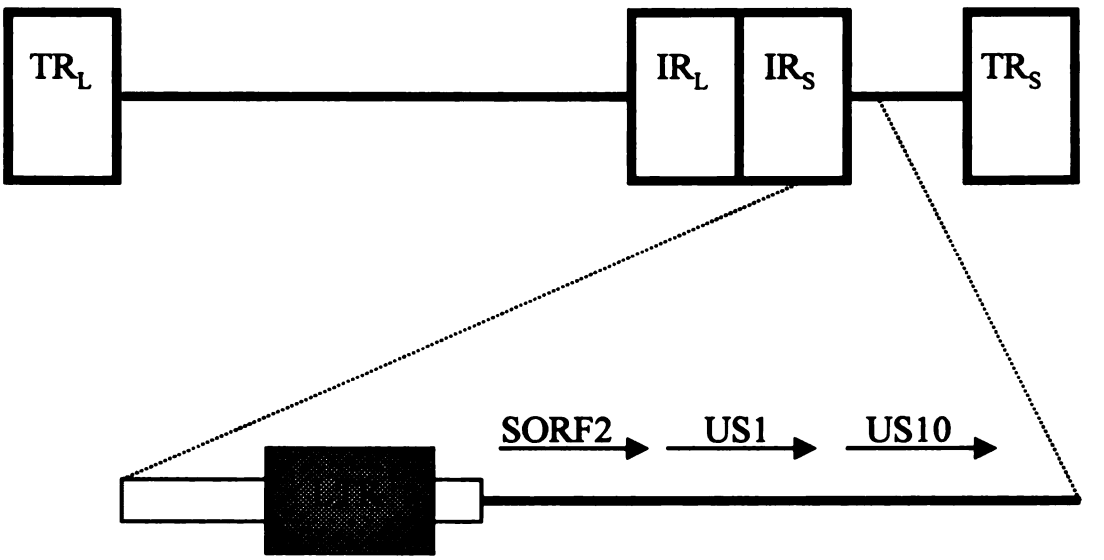


Figure 3. Schematic representation of REV LTR inserts in RM1 clone. REV=reticuloendotheliosis virus; LTR=long terminal repeat; SORF2=short open reading frame 2; US1=unique short 1; US10=unique short 10.

biological characteristics of RM1 and genes affected by LTR transcriptional activation are necessary for a better understanding of MDV oncogenesis.

SORF2 is a MDV serotype 1-unique gene presumably encoding a 179-amino-acid protein. However, the actual existence of the SORF2 gene product in a wild type virus infected cells has yet to be confirmed. A comparison of its predicted amino acid sequence with those of other herpesvirus genes demonstrates that SORF2 might contain three recognizable domains with homology to human cytomegalovirus US22, UL36, and human herpesvirus 6 EPLF3. The detailed function of these genes remains unknown. However, studies suggest an association with transactivation ability (Tomley *et al.*, 1988; Stasiak and Mocarski, 1992; Kashanchi *et al.*, 1994; Nicholas and Martin, 1994). Deletion experiments performed in the MDV US region demonstrated that the SORF2 gene is dispensable for growth in cell culture as well as for *in vivo* growth (Purcell *et al.*, 1994; Purcell *et al.*, 1995). In an attempt to delineate the role of SORF2 during MD oncogenesis, the yeast two-hybrid system was used to screen for host protein(s) interacting with the SORF2 protein. Identification of protein(s) interacting with SORF2 may provide a significant insight into the function of this novel gene.

The yeast two-hybrid system (Fields and Song, 1989) has been used successfully to detect protein-protein interactions of various species. The yeast two-hybrid system relies on the modular nature of eukaryotic site-specific transcriptional activators to generate a transcriptional signal from the interaction of a protein (as bait) fused to a DNA-binding domain with another protein (as prey) fused to a transcription activation domain. Interaction between the bait protein and a partner polypeptide encoded by a member of a cDNA library will be detected by activation of the selective marker gene

and the reporter gene in the appropriate yeast strain. It is a powerful genetic screening tool since the two-hybrid system detects protein-protein interactions occurring directly *in vivo*.

In this chapter of my thesis, I have shown that the SORF2 protein is expressed in infected cells both *in vitro* and *in vivo*. In search of potential functional protein partner(s) SORF2 was used as the bait in yeast two-hybrid screening. Chicken growth hormone as isolated from a cDNA library constructed from splenic T cells was found to interact specifically with SORF2 protein. In order to corroborate the interaction detected in the yeast two-hybrid system, the isolated growth hormone cDNA was used to synthesize corresponding polypeptides in *E. coli*, which then were assayed for their ability to interact with SORF2 *in vitro*. Co-immunoprecipitation of SORF2 and the interacting protein, growth hormone, using specific antibodies confirmed the biochemical evidence for the observed protein-protein interaction. Results gathered from both *in vivo* and *in vitro* co-localization assays also provided further evidence for our observation.

Materials and Methods

Cells and Virus Stock

Chicken embryo fibroblasts (CEF) cells were grown in Leibowitz-McCoy medium (GIBCO, BRL), supplemented with 4% calf serum (growth medium) or 1% calf serum (maintenance medium). Primary or secondary CEF cultures were prepared as described (Silva and Lee, 1984) and infected with the following cell-associated serotype 1 MDVs: pathogenic Md11, JM102, Md5, 648A, CU2, 584; cell cultured-attenuated JM102, Md11; naturally attenuated CVI988.

Sequence Comparison

Total cellular DNA was extracted from various MDV serotype 1 infected CEF cells by standard methods (Sambrook *et al.*, 1989). SORF2 specific primers (Operon Technologies) were designed to amplify the SORF2 gene from each strain by polymerase chain reaction (PCR). The primers were as followed: forward, 5' ATG CAG CGC CAA ACC GGA CAT, and reverse, 5' CTA ATG TAC TAG TTG CTC TAT. PCR was carried out on a MJ Research Thermal Cycler with the following cycles: 94°C, 2 min; 30 cycles of 30 sec at 94°C, 30 sec at annealing temperature 55°C and 30 sec at 72° followed with a 5 min extension at 72°C. Each PCR reaction mixture contained 25 ng of cellular DNA, 200 μM of each dNTPs, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 1.0 U Taq DNA polymerase (Life Technologies, Inc.). PCR products were cloned into the TA cloning vector for sequencing. Sequencing analysis was done by an ABI 377 automatic DNA sequencer. Sequence comparison was done using the DNASTAR program (DNASTAR, Inc.).

Generating Antibodies Specific to SORF2 Protein

In order to overexpress the SORF2 protein, the PCR product of SORF2 with restriction sites for *Bam*HI and *Hind* III incorporated into the 5' end of forward and reverse primers, respectively, was cloned into a pET28a expression vector (Novagen, Inc.) at its *Bam*HI and *Hind* III sites. A positive pET-SORF2 recombinant was then sequenced to confirm it contained the correct reading frame. The host strain *E. coli* BL21 used for cloning and expression was prepared by standard procedures (Sambrook *et al.*, 1989). The expression and purification of SORF2 recombinant protein were according to the manufacturer's procedure (Novagen, Inc.). Briefly, pET-SORF2 recombinant protein

expression was induced with 0.4 mM isopropylthio-β-D-galactopyranoside (IPTG) for 5 hours at 30°C with 250rpm shaking when OD_{600} reached 0.6-1.0 measured by spectrophotometer. The cells were harvested when OD_{600} reached 2.5-3.0 by centrifugation at 5000 xg for 5 min at 4°C. The pellet was washed with 50 ml phosphate buffered saline (PBS), and resuspended in 20 ml PBS. The cell suspension was sonicated, and the cell debris was removed by centrifugation. For purification of pET-SORF2 recombinant protein, a Ni^{2+}-affinity His-Bind Resin column was used to bind a stretch of 6 consecutive histidine residules expressed from the pET28a vector. The supernatant was applied to the Ni^{2+}-His column pre-equilibrated with the binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9) plus 6 M urea, the column was further washed with washing buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). The target protein was recovered by elution with 1 M imidazole. Following dialysis against water overnight, total proteins were purified by preparative sodium dodecyl sulfate (SDS)-8% polyacrylamide gels and used as antigen to prepare polyclonal antisera by immunizing rabbits (Bio Syntheses, Inc.).

Yeast Two-Hybrid Screening

Two-hybrid library screening is based on the MATCHMAKER LexA system (Clontech Laboratories, Inc). The yeast reporter host strain *Saccharomyces cerevisiae* EGY48 was grown in YPD (yeast extract/pepton/dextrose) or appropriate selection medium to maintain plasmids. Yeast transformation was done by the lithium acetate method as recommended by the manufacturer.

SORF2 was inserted in-frame into the pBD-Lex A vector downstream of the binding domain between its *Bam*HI and *Not*I restriction endonuclease multiple cloning

sites. The insert subcloned into pBD-LexA was derived from PCR amplification with the *Bam*HI, *Not*I nucleotide sequences sites incorporated into the 5' end of forward and reverse primers, respectively. A chicken cDNA library (kindly provided by Dr. Robin Morgan) obtained from splenic T cells was fused with the pB42AD vector. Plasmid p8op-lacZ was used for integrating the LacZ reporter gene into host strain EGY48 chromosome via transformation to create the EGY48 (p8op-lacZ) strain.

SORF2 in the pBD-LexA vector was used as a bait to screen the chicken spleen cDNA library in the yeast expression vector system. The protein-protein interaction screen was performed with a sequential transformation procedure. The yeast reporter strain, EGY48 (p8op-lacZ) was first transformed with the bait, pBD-SORF2. Then 100 μg of library plasmid DNA was introduced into the yeast strain expressing the pBD-SORF2 hybrid protein. Approximately 1.5×10^6 yeast transformants were selected on seventy 15 cm plates with synthetic dropout medium without histidine, tryptophan, and uridine (-His, -Trp, -Ura). After a 15-day incubation at 30°C, all clones were scraped and replated at high density on –Leu SD induction medium (SD/Gal/Raf//-His/-Leu/-Trp/-Ura) plus 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for an additional 15 days. The resulting blue colored transformants were streaked onto SD/-His/-Trp/-Ura synthetic medium plates for β-galactosidase (β-gal) assays. For the β-gal assay, yeast transformants were incubated for 4 days at 30°C, then transferred to Whatman #1 filter paper (Whatman, Inc.). The filter was immersed in liquid nitrogen for 15 sec, thawed at room temperature, and then placed on top of another Whatman #1 filter presoaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 2 mM $MgSO_4$, pH 7.0) with

0.27% β-mercaptoethanol and X-gal enzyme substrate at 0.75 mg/ml. The filters were incubated at 30°C for at least 2 hours or until blue color developed.

Cloning of Interacting Proteins

The plasmids from identified positive clones were isolated by standard procedures (Sambrook *et al.*, 1989), then transformed into *E. coli* KC8 competent cells by electroporation. Transformed cells were plated onto M9/-Trp plates (M9 medium contains 100mM Na_2HPO_4, 50mM KH_2PO_4, 50m M NaCl, 10 mM NH_4Cl, 20 mM $MgSO_4$, 1% glucose, and 10 mM $CaCl_2$, pH 7.4) as recommended by the manufacturer. Plasmids then were isolated and subjected to nucleotide sequencing using the pB42AD sequencing primer provided with the screening kit. Sequence data were used for homology searching with the BLAST program.

In vitro Binding Assay

In order to confirm the interaction detected from the yeast two-hybrid system, a glutathione S-transferase (GST) fusion protein of structural peptide of GH was generated by inserting it in frame into the pGEX-3X vector (Pharmacia Biotech, Inc.). The GST-GH plasmid was expressed in the *E. coli* BL21 DE3 strain (Gibco BRL), induced with IPTG, and a cell pellet was prepared as described previously. For purification of the GST-GH fusion protein, Triton X-100 was added into a 20 ml sonicated *E. coli* cell suspension (1% final concentration in PBS), and gently mixed for 30 min. The cell debris was removed by centrifuging at 12,000 g for 10 min at 4°C. One ml PBS-equilibrated 50% slurry of Glutathione Sepharose 4B (Pharmacia Biotech Inc.) was added to the supernatant and incubated for 30 min with gentle agitation at room temperature. The sepharose was washed with PBS and collected by centrifugation several times. The

GST-GH fusion protein (or GST protein alone as a negative control partner) was then used for the *in vitro* binding assay without eluting from the sepharose beads.

In vitro synthesis of protein was done by the Single Tube Protein System 3 (STP3) (Novagen). pET-SORF2 plasmid containing the entire SORF2 coding sequence was used as a template for a coupled T7-directed *in vitro* transcription-translation reaction according to the manufacturer's instructions. During the incubation with rabbit reticulocyte lysates, 40 μCi [^{35}S]-methionine (New England Nuclear, Life Science Products) was added to the mixture. Incubation was at 30°C for 30 min. The reaction product (2 μl) was analyzed by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), while 10 μl of the sample was used for the *in vitro* protein binding assay.

Both the GST protein control and the GST-GH fusion protein were incubated with [^{35}S]-methionine labeled pET-SORF2 protein translated *in vitro* at room temperature with mild agitation for 30 min, followed by several washes with PBS. The supernatants from each wash were collected for further analysis. Bound protein was eluted with elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0). All the samples were subjected to 8% SDS-PAGE analysis.

Immunoprecipitation and Co-Immunoprecipitation

MDV infected and mock infected CEF cells were labeled with 100 μCi [^{35}S]-methionine at 72 hours post infection for 4 hours. Then cultured cells were washed twice with cold PBS, and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, and 25 mM PMSF). Cytoplasm extract was obtained after centrifugation then incubated with normal rabbit serum and protein A-sepharose for 1 hr. Supernatants were obtained by centrifugation and then gently mixed with rabbit anti-

SORF2 antiserum or pre-absorbed anti-SORF2 antiserum and protein A-sepharose for 1 hr. Following serial washing with PBS, precipitates were resuspended in 2X electrophoresis sample buffer (10 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 10% β-mecaptoethanol, and 0.006% phenol red), boiled for 5 min and subjected to electrophoresis. The pre-absorbed antiserum was prepared by incubating anti-SORF2 antiserum with recombinant pET-SORF2 protein synthesized in *E.coli* at 4°C with gentle agitation overnight. For co-immunoprecipitation, GH cDNA was digested from pB42AD vector DNA and cloned into eukaryotic expression vector pcDNA3 using *Eco*RI and *Xho*I cloning sites. Cell lysates from the MDV infected or mock infected cells were obtained and prepared as described above except replacing RIPA buffer with a mild lysis buffer (containing 142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.5, 1 mM EGTA, 0.2% NP-40, and protease inhibitors). Cell lysates were incubated with *in vitro* translated structural GH peptide, synthesized from pcDNA3-GH with or without [^{35}S] labeling, and 10% (v/v) protein A sepharose. After addition of either rabbit anti-SORF2 polyclonal antibodies or mouse anti-GH monoclonal antibodies (provided by Dr. Luc Gerghman, University of Texas), immunocomplexes were precipitated with protein A-sepharose. Precipitates were washed extensively and analyzed by SDS-PAGE.

Transient Expression of truncated cGH

The pcDNA3-GH construct was prepared for transfecting into MDV infected or mock-infected CEF cells. Cells were seeded on coverslips carried by 35 mm culture plates. Transfection was carried out using M199 medium (Gibco, BRL) pH 7.2 and the calcium phosphate procedure. Briefly, the transfection mixture contained 3 μg plasmid DNA in 0.125 M calcium chloride solution, to which an equal amount of 2X Hepes

solution (140 mM NaCl, 1.5 mM Na_2PO_4, 50 mM Hepes pH 7.5) was gently added. The mixture was held at room temperature for 30 min before adding to culture plates. Cells were shocked with 15% glycerol solution for 5 min after 4 hr of incubation in transfection mixture. After removing the glycerol, cells were washed with PBS twice, then re-fed with a normal CEF culture medium.

Indirect Immunofluorescence Assay (IFA)

Samples taken from MDV infected cells, mock-infected cells, and pcDNA3-GH transfected cells were subjected to IFA studies. Cells were originally seeded on coverslips and fixed with ice cold acetone for 5 min and then air-dried. IFA was performed as follows: samples were incubated with anti-SORF2 antiserum, pre-absorbed anti-SORF2 antiserum, or anti-GH antiserum diluted 1:1000 in PBS for 30 min at room temperature. Cells were then washed with PBS for 15 min. The secondary antibodies, either goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugated IgG or goat anti-mouse Texas Red conjugated IgG (Kirkegaard & Perry Laboratories), were diluted 1:1000 in PBS and incubated with samples for an additional 30 min. The coverslips were then rinsed extensively and sealed with 50% glycerol in PBS. All the samples were viewed with the laser scanning confocal microscope (Carl Zeiss, Inc.) in the photographic facility, Michigan State University. The Adobe photoshop program (Adobe Systems, Inc.) was used to process the images.

Immunohistochemical Staining

Various MDV serotype 1 infected birds with observed tissue lesions, organ enlargement, or tumor development were obtained from the Avian Disease and Oncology Laboratory (ADOL). Immunostaining of tissues for SORF2 or cGH was performed using

the biotinylated horseradish peroxidase complex (ABC) system (Vector Laboratories). Sections were incubated sequentially with blocking serum, antibodies, and washing buffers according to the manufacturer's instruction. Briefly, unstained formalin-fixed, paraffin-embedded sections of tissues mounted on microscope slides were deparaffinized by immersion in xylene, followed by rehydration in graded ethanol solutions of decreasing concentration. The slides were then immersed in methanol containing 2.5% H_2O_2 for 10 min at room temperature to inactivate endogenous peroxidases. The slides were rinsed in PBS 3 times for 2 min each, followed by incubation in protease solution for 10 min and another rinse with PBS. To minimize nonspecific background staining, the sections were incubated in a serum blocking solution for 10 min. The sections were then stained with primary antibodies (either cGH or SORF2) diluted 1:1000 in PBS for 45 min, followed by rinsing with PBS, then incubated with biotinylated secondary antibodies (either biotinylated anti-rabbit IgG or anti-mouse IgG) for 10 min and further rinsing with PBS. Color development was done with a peroxidase system in which Nova Red was used as the chromogen. Negative controls consisted of staining non-infected tissues, as well as leaving out the primary antibody. The positive control consisted of staining sections of pituitary gland for cGH, as well as staining sections of RM1 infected-tissues for SORF2. Finally, slides were rinsed in distilled water and counterstained with hematoxylin for 2 min and examined microscopically.

Immunoelectron Microscopy Analysis

Immunogold labeling was carried out on ultrathin cryosections. Initially, neoplastic spleen tissue was fixed in formalin and put into a paraffin block. Routine histological stains were obtained from sections. The block then was deparaffinized and

placed in glutaraldehyde and embedded on Ni-grids (Electron Microscopy Science, Inc.). One micron thin sections were obtained using a LKB Ultramictrome, followed by sequential incubation with blocking serum, antibodies, and washing buffers, as suggested by the manufacturer. The secondary antibodies used for SORF2 were the goat anti-rabbit IgG conjugated with 40 nm gold particles, whereas for cGH were the goat anti-mouse IgG conjugated with 20 nm gold particles (Ted Pella, Inc.). Finally, the sections were stained with uranyl acetate and lead citrate and examined with a Philips 301 Electron Microscope at the Division of Pathology, Michigan State University. Sections of CEF cells were prepared by the same procedures and used as negative controls.

Results

The SORF2 Gene Encodes a Functional Polypeptide

In order to generate polyclonal antibodies specific to SORF2, the polypeptide corresponding to the full-length SORF2 protein was produced in *E. coli* as a histidine-tagged protein using the pET28a vector system (Novagen, Inc.). The polypeptide was purified using Ni^{2+}-chromatography and injected into rabbits to induce the production of anti-SORF2 antibodies. Rabbit antiserum were collected and used to monitor protein expression encoded by the SORF2 gene *in vitro* and *in vivo*. To test the specificity of rabbit anti-SORF2 polyclonal antibodies, immunoprecipitation analyses of MDV-infected CEF cells with the rabbit anti-SORF2 antibodies or anti-SORF2 antibodies pre-absorbed with purified SORF2 protein were performed. Results in Figure 4 indicated that a major band corresponding to a polypeptide with molecular weight 21 kDa was precipitated with anti-SORF2 polyclonal antibodies in MDV-infected CEFs. Presumably it was SORF2

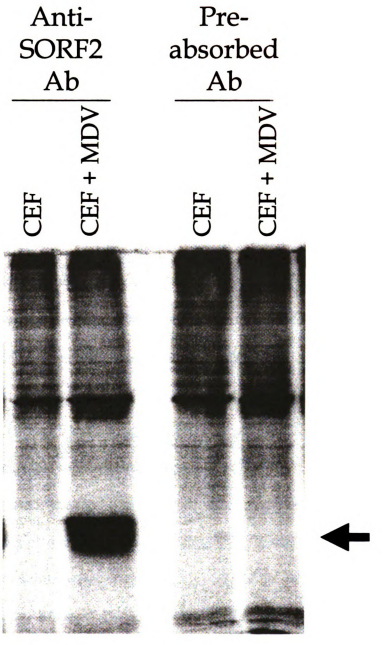


Figure 4. Immunoprecipitation analysis of MDV-infected CEF. Left two lanes=Rabbit anti-SORF2 polyclonal antibodies were used in an immunoprecipitation assay with MDV-infected CEF cells labeled with ^{35}S-methionine. A 21 kD polypeptide (indicated by the arrow) was preciptated and visualized by radioautography. Right two lanes=the same as before except the anti-SORF2 antibody was preabsorbed with recombinant SORF2 protein.

protein expressed upon MDV infection. To confirm this, immunoprecipitation analysis with the anti-SORF2 polyclonal antibodies pre-absorbed with purified recombinant pET-SORF2 protein was carried out. Results in Figure 4 shown that the 21kDa peptide was not detected in infected cells while using pre-absorbed antiserum. Immunofluorescent assay was performed in MDV infected cells using pre-absorbed antiserum indicated a negative result (data not shown) suggested that the obtained anti-SORF2 polyclonal antibodies were specific to SORF2. Taken together, we concluded that the major 21kDa peptide seen in the result of immunoprecipitation were mostly likely to be MDV SORF2 products.

The SORF2 gene is a unique MDV serotype 1 gene which is presumed to encode a 179 amino acid protein (≈21 kD). DNA sequence comparison among 9 different strains including Md11/14 (fourteen cell-culture passages), Md11/78, JM102/13, JM102/60, Md5/15, 648A/21, 584/14, and CVI988/20 did not indicate there was any variability in SORF2 sequence (Figure 5). This result clearly suggests that SORF2 is conserved among various strains and stable while passing in cultured cells.

In order to study the localization and expression pattern of SORF2 in MDV infected cells during plaque development, immunofluorescent labeling of SORF2 was performed at various time points after virus inoculation. Results were monitored by confocal microscopy. The confocal images in Figure 6 demonstrate that SORF2 was found in both the cytoplasm and nucleus at early stages post infection. However, it was localized only in the cytoplasm once plaques start appearing. This was an important observation since SORF2 was suggested to have homology to transactivation domains of other genes from herpesviruses. In theory, transactivation function would normally be

```
  1    ATGCAGCGCC AAACCGGACA TATGGAAGAC AAAAAGAGAA
 41    CCGGTTTGGA ATCGCAGGGG ACCGAGAATG CTTTTTCAGA
 81    TGGCAGAGAT GGCAAAGATG GATTGTTACA TGAAGGAATT
121    AATGAGCCCA TTTTGATTCC GTCTACCATC GCAGATCTCG
161    AGGGGATTCG TGAATTGGTC CGAAAATTCC GTGGTCGTCT
201    ACTGCCCTTT GAAAAGTGTC CCGATTTTTG TCTGAGAATT
241    GGGGGTTTGG AGGCCAGCTT TCATAAAGGG CAGGAGGAGC
281    TGTTAGAGTA TTGTGAAGCA CTTTATTTAC CACAACCTGT
321    TAAGATGGAA ATAGTAGGCA TTGTAGACGA TGTGCCATGT
361    CTGGCAACGG GGATGCAATT ACTCATTCTT GTTGCCGAGG
401    GGGGAGAGGT ATATGCCTAT GAAGAAGATA CTCTGCATAA
441    GTTAGCCACG AGTTTTTCCG AATTCCTTGA AATTGGAGTG
481    AAATCTTTAG GGAGGGAGGT TTACCATTGT GGAGAATATA
521    TAGAGCAAGT AGTACATTAG
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Figure 5. Sequence of the SORF2 gene.

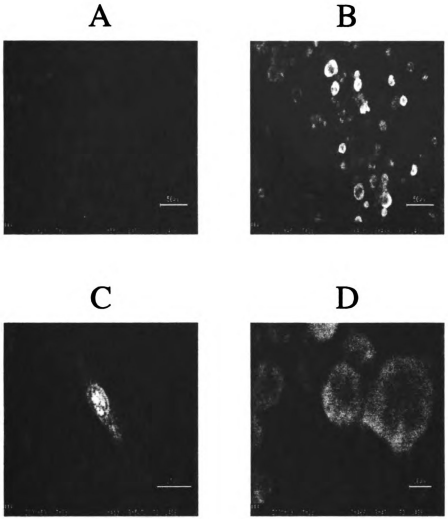

Figure 6. Localization characteristics of SORF2 on cultured CEF cells. A=mocked infected CEFs stained with SORF2 antibody. B=MDV-infected CEF cells stained with SORF2 antibody. C=Same as B except prior to MDV plaque formation. D=Same as B except at higher magnification of the MDV plaque.

observed in the nucleus. However, our finding suggested that at least late in infection, the bulk of MDV SORF2 remains outside of the nucleus.

To study further the possible expression of MDV SORF2 in infected tissues, histochemical examinations of neoplastic organs from a diseased chicken were compared to atrophied thymus from RM1 infected chickens, as well as tissues from negative birds. Moderate to strong immunolabeling for SORF2 was found among all tissues examined. The MDV-free bird tissues or omission of primary antibody both showed no positive staining for SORF2. As judged by anti-SORF2 staining distribution, vagus nerve and feather follicle (about 2% of cells expressed) exhibited fewer SORF2- expressing cells than MDV-infected spleen, thymus, and bursa (about 15% of cells expressed). Kidney and ovary showed intermediate level of expression. Sections obtained from RM1-infected thymus, as predicted, showed a higher level of staining even though a depletion of lymphoid cells was noticed. The observation of a high amount of SORF2 expression in RM1-infected thymus confirmed the earlier evidence that SORF2 mRNA was overexpressed in RM1 strain infections (Jones *et al.*, 1996). Tissues examined were taken at 4 weeks post inoculation, and example results are shown in Figure 7.

Microscopy of immunoreactive sections revealed the expressed SORF2 proteins were deposited mainly in the cytoplasm (Figure 7). These observations corroborated our earlier *in vitro* finding, namely, that SORF2 was expressed in cytoplasm of CEF cells. Taken together, we conclude that the SORF2 gene expresses a polypeptide corresponding to 21 kD in cultured cell CEFs, as well as in infected birds.

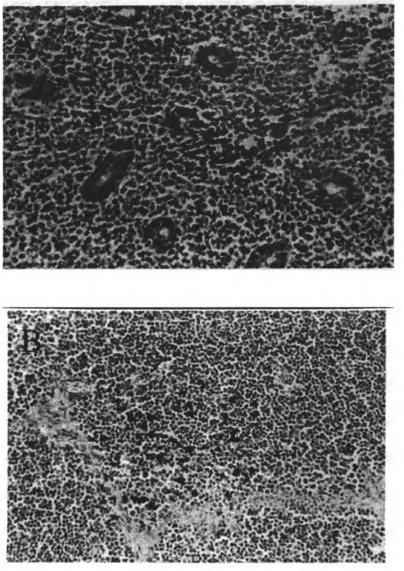

Figure 7. Immunohistochemical staining of SORF2 in neoplastic tissues. A=MDV-induced tumor from kidney. B=MDV-induced tumor from thymus. Magnification in both is 20X.

Identification of a Protein Homologue to Chicken Growth Hormone that Interacts with MDV SORF2 Protein

In a screen to identify functional polypeptides that interact with the MDV SORF2 protein, a chicken cDNA library derived from splenic T cells mRNA in the pB42AD yeast expression vector was employed. The results are summarized in Figure 8. Approximately 1.5 x 10^6 yeast transformants containing pBD-SORF2, the bait, transfected with library plasmid DNA were plated on SD/Gal/Raf/-Ura/-His/-Leu/-Trp plus X-gal plates. All transformants grown on this induction medium were LEU2 positive clones. After 15 d of incubation at 30°C, 7 LacZ positive colonies were isolated. Among these, only 2 were confirmed to be positive upon β-gal assay and were processed for further studies. After selecting against the pBD-SORF2 bait plasmid using M9/-Trp medium, the prey plasmids were amplified in *E. coli* cells and subjected to nucleotide sequence analysis. A database search and sequence alignment demonstrated in Figure 9 identified both sequences as corresponding to that of chicken growth hormone with an open reading frame coding for 216 amino acids (Tanaka *et al.*, 1992). These two independent clones were identical. Interestingly, both lacked the signal polypeptide and the first amino acid of the structural polypeptide. The start codon ATG from the signal peptide was retained on both clones (Figure 10) indicating that they might be artifact cDNA clones perhaps due to mRNA secondary structure caused the skipping of this missed region while coping the mRNA. However, this suggestion needs to be confirmed by further experiments.

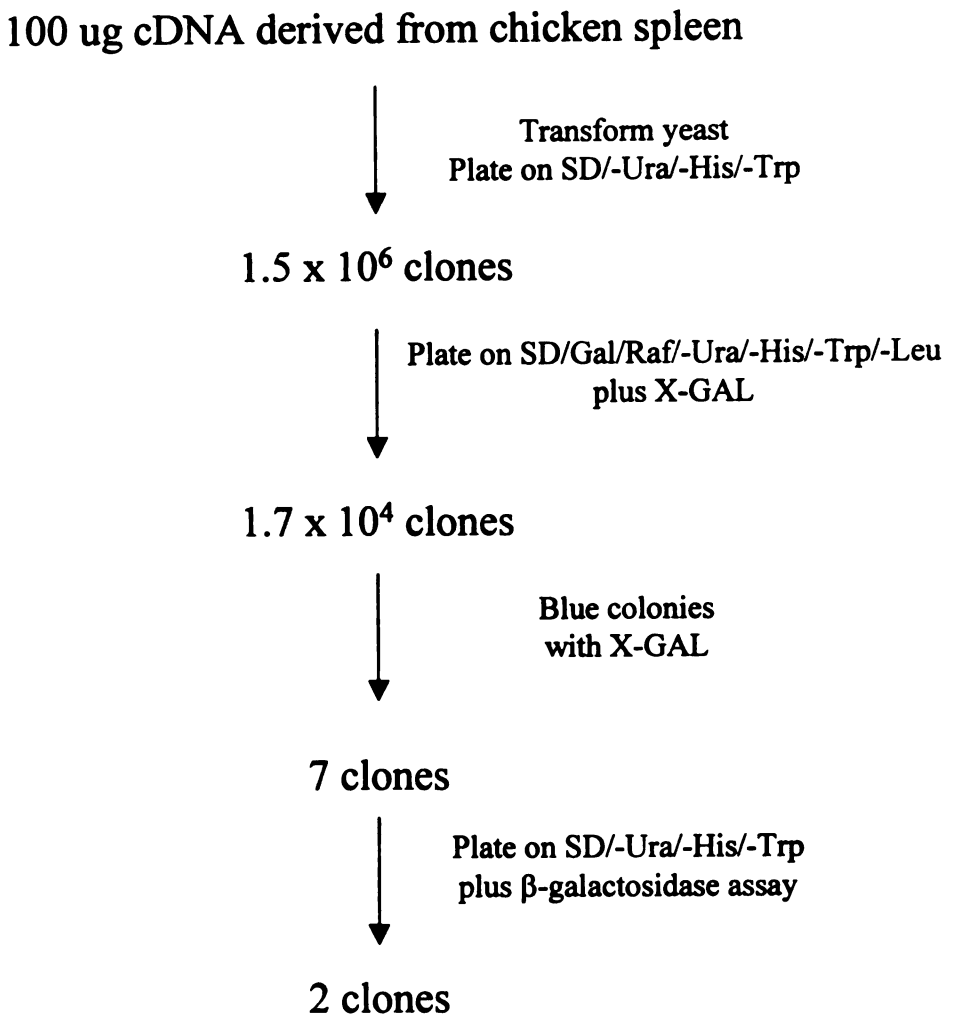

Figure 8. Summary of yeast two-hybrid library screen.

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  1  ggaggccgtt caagcaacac ctgagcaact CTCCCGGCAG GAATGgctcc
 51  aggctcgtgg ttttctcctc tcctcatcgc tgtggtcacg ctgggactgc
101  cgcaggaagc tgctgccacc TTCCCTGCCA TGCCCCTCTC CAACCTGTTT
151  GCCAACGCTG TGCTGAGGGC TCAGCACCTC CACCTCCTGG CTGCTGAGAC
201  ATACAAAGAG TTCGAACGCA CCTATATTCC GGAGGACCAG AGGTACACCA
251  ACAAAAACTC CCAGGCTGCG TTTTGTTACT CAGAAACCAT CCCAGCTCCC
301  ACGGGGAAGG ATGACGCCCA GCAGAAGTCA GACATGGAGC TGCCTCGGTT
351  TTCACTGGTT CTCATCCAGT CCTGGCTCAC CCCCGTGCAA TACCTAAGCA
401  AGGTGTTCAC GAACAACTTG GTTTTTGGCA CCTCAGACAG AGTGTTTGAG
451  AAACTAAAGG ACCTGGAAGA AGGGATCCAA GCCCTGATGA GGGAGCTGGA
501  GGACCGCAGC CCGCGGGGCC CGCAGCTCCT CAGACCCACC TACGACAAGT
551  TCGACATCCA CCTGCGCAAC GAGGACGCCC TGCTGAAGAA CTACGGCCTG
601  CTGTCCTGCT TCAAGAAGGA TCTGCACAAG GTGGAGACCT ACCTGAAGGT
651  GATGAAGTGC CGGCGCTTCG GAGAGAGCAA CTGCACCATC TGAGGCCCCG
701  TGCTGCGCCA TGGCTGACGG CCCTGTCCCC CCCCCCCCCT CCTCCCCGTC
751  ACCAAAAACA CGAGGAATAA ACCC
```

Figure 9. Sequence of the yeast two-hybrid clones. Comparison of the sequences for growth hormone (GH) clones recovered in the yeast two-hybrid screen to the published cDNA sequence. The bases in capitol letters are common to both sequences while those in small letters are found only in the published GH sequence. The ATG start codon at position 43 is boxed. The signal peptide, which is missing in the yeast two-hybrid clones, spans bases 46 to 120.

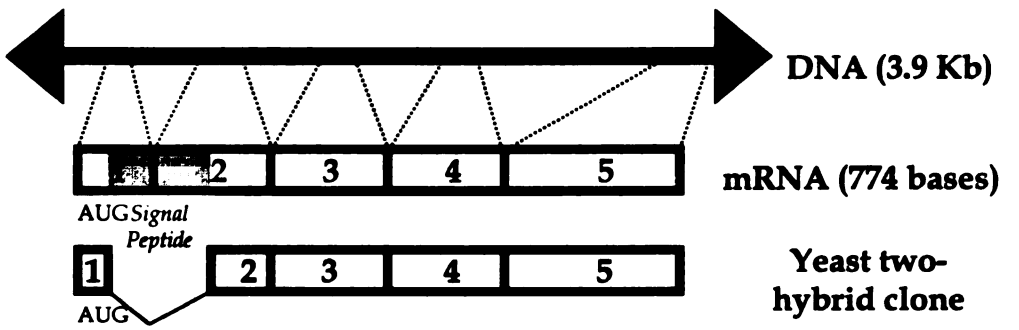

Figure 10. Schematic representation of cGH genome structure and cDNA clone obtained from chicken splenic yeast expression library screen. Each box represents an exon. The shaded region represents the signal peptide.

Interaction of the cGH with SORF2

Experiments were performed to confirm direct binding of cGH to SORF2 as suggested by results of yeast two-hybrid screening. An *in vitro* protein binding assay was performed using GST-fusion proteins. The GST-fusion protein, with or without the added structural GH polypeptide (designed as sGH) identified from yeast two-hybrid, was incubated with [^{35}S]-methionine-labeled SORF2 protein translated *in vitro*. After extensive washing, bound protein and [^{35}S]-SORF2 were eluted with 100 mM glutathione. Aliquots of the protein from the supernatants, washes and eluants were resolved on SDS-PAGE and exposured to X-ray film. The results shown in Figure 11 demonstrated that, while SORF2 protein was not retained by GST protein alone, SORF2 can be retained by GST-GH fusion protein presumably due to the presence of GH. This result is in agreement with the result of the yeast two-hybrid system assay and indicates that the interaction between SORF2 and sGH is a direct and specific protein-protein interaction without other intermediary factors (e.g., yeast proteins) involved.

The *in vitro* binding assay provided the biochemical evidence for the hypothesized SORF2-cGH interaction. To further characterize the possibility of interaction between native forms of the two proteins, co-immunoprecipitation using either SORF2-specific or cGH-specific antibodies was carried out. Previous evidence showed that cGH is not expressed in cultured CEF cells (data not shown), therefore, *in vitro* translated sGH protein was used in this study. The fact that SORF2 and sGH have similar molecular weights complicates the analysis. Alternative [^{35}S] labeling of SORF2 or sGH was used to overcome this problem. MDV-infected CEF cell lysates were mixed with [^{35}S]-labeled sGH translated *in vitro*. Proteins were precipitated with anti-SORF2

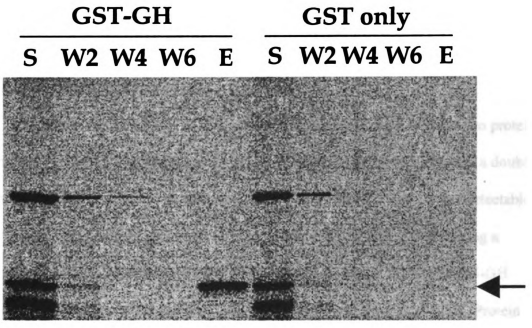

Figure 11. *In vitro* protein-protein interaction assay. Labeled SORF2 was added to beads bound with GST or GST fused to GH. S=supernatant or unbound material; W2, W4 and W6=wash two, four, and six, respectively; E=elution. The arrow indicates the location of SORF2.

polyclonal antibodies then subjected to SDS-PAGE analysis. Under the same precipitation conditions, [^{35}S]-labeled MDV infected cell lysates were incubated with *in vitro* translated unlabeled GH peptides, then precipitated with anti-GH monoclonal antibodies. Results (Figure 12) indicate that SORF2-specific antibodies were capable of co-precipitating sGH peptides only when the SORF2 proteins (from cell lysates) were present. Likewise, SORF2 proteins were co-precipitated by cGH-specific antibodies together with sGH. Both co-precipitations showed equal efficiency. Taken together, the above findings demonstrate that the observed protein interaction was specific and efficient *in vitro*. It thus seems likely SORF2 and sGH interact with each other *in vivo*.

Co-Localization of SORF2 and cGH

To validate the SORF2–cGH interaction, we examined whether these two proteins are capable of co-localizing within cells. Co-localization was determined using a double immunofluorescence staining approach. As described previously, cGH is not detectable in CEF cells. Therefore, transient expression of sGH was induced by introducing a eukaryotic expression construct pcDNA3-GH into cultured cells. The pcDNA3-GH plasmid DNA was transfected into MDV-infected CEFs 2 days post infection. Protein expression was detected by IFA assay at 24 hours after transfection. In order to localize the proteins, FITC-labeled goat anti-rabbit IgG conjugate and Texas Red-labeled goat anti-mouse IgG were used, followed by visualization with confocal microscropy. As noted previously, we observed that SORF2 was expressed in both the nucleus and cytoplasm before plaque formation. It was evident that transiently expressed sGH co-localized with SORF2 in the cytoplasm (Figure 13). Another observation was both SORF2 and sGH were found mainly in the cell cytoplasm after plaques appeared (data

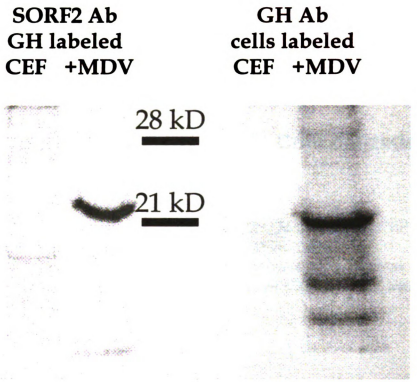

Figure 12. Co-immunoprecipitation analysis of SORF2 and cGH. Lysates from uninfected and MDV-infected cells were precipitated with antibody to SORF2 or GH. In the case of SORF2 antibody, *in vitro* labeled GH was added. For the GH antibody, unlabeled *in vitro* translated GH was added to labeled cell lysates.

SORF2 **GH** **Co-localization**

Figure 13. Co-localization of SORF2 and cGH. MDV-infected CEF cells were co-transfected with pcDNA3-GH plasmid. Protein expressions were detected by immunofluorescence assay at 24 hours after transfection. SORF2 and GH are labeled with FITC-labeled and Texas Red-labeled antibody, respectively.

not shown). This result suggested that SORF2 and variant GH, if it ever exist, or secreted structural GH which lacks the signal peptide are possible to interact if they present at the same biological space.

cGH is a hormone secreted primarily from the pituitary gland. Previously, it has been suggested that cGH may also be expressed in immune-responding tissues (reviewed by Harvey and Hull, 1997). To confirm this observation, histochemical staining of various normal and neoplastic tissues was performed. It was obvious that deposited cGH proteins were detected in MDV-infected neoplastic spleen, thymus, ovary, kidney, and liver, indicating that cGH is not solely a pituitary gland secreted protein and therefore could potentially interact with SORF2. Partial results are demonstrated in Figure 14. Positive staining for cGH in the bursa, spleen, thymus tissues (data not shown) of MDV negative birds was also observed.

To identify colocalization *in vivo*, double staining for cGH and SORF2 was performed by staining for GH antigen using 20-nm gold particles as markers followed by staining for SORF2 using 40-nm gold labels as markers. Samples were taken from MDV-infected neoplastic spleen since the cDNA library used for yeast two-hybrid screening was derived from spleen. The negative controls used included mock-infected CEFs as well as the omission of primary antibodies in neoplastic spleen during the staining process. Results were examined by transmission electron microscopy and are presented in Figure 15. Not surprisingly, cytoplasmic reactions were observed in MDV induced neoplastic spleen cells for cGH and SORF2, although staining was occasionally, but rarely, observed in the nucleus as well. These *in vivo* observations support the *in vitro* observations that cGH and SORF2 proteins may co-localize in the same subcellular

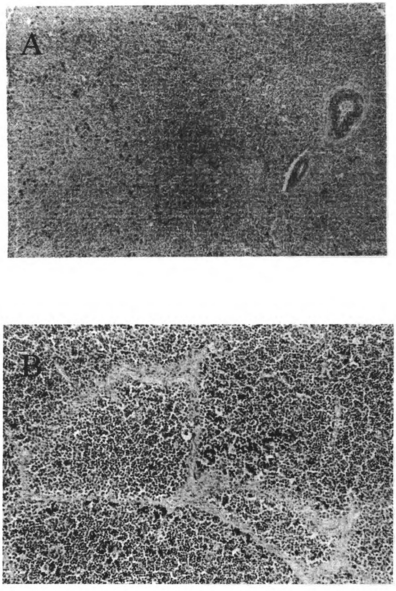

Figure 14. Immunohistochemical staining of cGH in neoplastic tissues. A=MDV-induced tumor from spleen. B=MDV-induced tumor from thymus. Magnification in both is 20X.

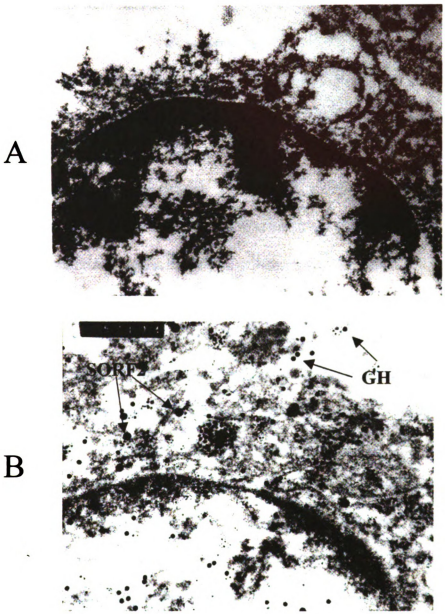

Figure 15. Immunoelectron microscopy of cGH and SORF2 in neoplastic spleen. A=system control where the SORF2 and GH antibodies were omitted. B=same as A except SORF2 and GH antibody were added. SORF2 and GH are labeled with 40 nm and 20 nm gold particles, respectively. The magnification is 57,000X.

compartments, but specific compartments could not be identified. Negative control staining confirmed the specificity of the labeling observed. This further confirmed the positive staining results were reliable.

Discussion

Previous studies suggested that overexpression of the SORF2 gene in a recombinant MDV clone, RM1, possibly results in the attenuation of viral oncogenicity (Jones *et al.*, 1996; Witter *et al.*, 1997). Prior to our studies, it was not clear whether SORF2 was expressed in a natural infection. Here, we have demonstrated that serotype 1 viruses contain a functional SORF2 gene encoding a 21 kD polypeptide.

Sequence comparison among various strains including oncogenic, cell culture attenuated, and naturally non-oncogenic strains indicated that there is no difference in SORF2 sequence among those strains tested. FA and immunohistochemical staining further confirmed that SORF2 is expressed in lytically infected culture cells and in infected or neoplastic tissues. Taken together, we propose that normal expression of SORF2 itself is not directly associated with oncogenicity since SORF2 protein is detected in attenuated strains as well. Moreover, the failure to detect SORF2 in FA assay of tumor derived cell lines MSB1, and RP1 (data not shown) suggested that SORF2 might be involved in viral replication or latency/activation. Jones *et al.* (1996) reported that the viral replication and/or spread is as least as rapid in RM1 as in the fully virulent JM parent virus. Apparently, overexpression of SORF2 does not impair the replicative ability of the virus. However, deletion of the gene results in decreased viral plaque-forming units (Dr. Mark Parcell, personal communication). Thus, it remains possible that SORF2 is involved in viral replication but is not essential.

Cytoplasmic localization of SORF2, however, could suggest an alternative hypothesis as to biological role. A yeast two-hybrid screen was utilized to identify proteins that might interact with SORF2. The cDNA library used was derived from splenic T cells activated by the mitogen concanavalin A to mimic an immune response stage after virus infection. Two clones of the chicken growth hormone gene were isolated by this method. Further experiments were done to examine the interactions of cGH with SORF2.

It was demonstrated that SORF2 protein could directly and specifically bind to a GST-GH fusion protein without other intermediary factors. In addition to *in vitro* binding evidence, co-immunoprecipitation was used to confirm this interaction. Results demonstrated that either sGH or SORF2 protein could be precipitated by antibody to the other protein when sGH protein translated *in vitro* was added to MDV-infected cell lysates. cGH is a pituitary hormone, and the cGH gene contains an open reading frame encoding a signal polypeptide (4 kD) which functions in protein translocation joined to a structural polypeptide (22 kD) involved in cGH signalling. The cDNA library used for the yeast expression system was constructed from splenic T cells. Might immune cells themselves be a source of GH? Recently, considerable effort has been expended in evaluating the interactions of the endocrine and the immune system. Numerous such interactions have been described between these two systems (reviewed by Auernhammer and Strasburger, 1995). Studies of human growth hormone (hGH) have shown it to play an important role in the cellular and humoral immune system. Moreover, hGH peptides have been shown to be synthesized and secreted by immunocompetent cells (Kao *et al.*, 1992; Lytras *et al.*, 1993), with hGH receptors also being present on these cells (Barnard

et al., 1985; Badolato *et al.*, 1994). Similarly, our studies in the chicken as well as those of other groups, demonstrated the detection of cGH cDNA in spleen, thymus, and bursa (Render *et al.*, 1995), indicating there is a possible association between GH and the immune response.

The cDNA clones isolated from the yeast two-hybrid screen encoded an altered form of cGH (Figure 8), lacking the signal polypeptide and the first amino acid of the structural polypeptide, indicating that the cDNA clones obtained derived from a 5'-truncated cDNA or arose from alternative initiation or splicing of cGH mRNA. The signal polypeptide of cGH consists of Exon 1 and part of Exon 2 (Figure 10). Sequence data from our clones lack the entire signal polypeptide, as would be required for the cGH polypeptide to be expressed intracellularly so that it could function in the two-hybrid assay. There were no consensus splice sites or obvious alternate promoters flanking the 5' end of the cGH coding sequence in the two clones. This finding suggests that the deduced cGH may be a product of a 5' truncated cDNA clone, which was selected by the requirements of the two-hybrid assay system. However, the observation of human GH mRNA derived by alternative splicing (McCarthy and Phillips, 1998) indicates that alternative splicing or initiation of transcription of a cGH mRNA cannot be ruled out.

One major characteristic of yeast two-hybrid screening is that the protein interaction must occur inside the nucleus. Any cGH cDNA in the two-hybrid library that retain a functional signal sequence would likely result in secretion of the hybrid protein and would therefore fail to be detected. The signal polypeptide functions in the secretion of cGH, and it is cleaved during the secretion process. To test if the cDNA library used contained complete cGH cDNA, PCR reactions were performed using primers upstream

of the ATG start codon (forward 5'-TGA GCA ACT CTC CCG GCA GGA) and downstream of the signal peptide sequence (reverse 5'-TGG GAG CTG GGA TGG TTT ATG). As predicted, two fragments of different sizes were amplified. Sequence analysis confirmed that one fragment represented the complete cGH mRNA form, the other fragment represented the altered form. This result confirmed that the complete cGH cDNA was present in the library, however, but did not interact with SORF2 in the yeast two-hybrid system as predicted. Whether the truncated cGH cDNA was generated in cDNA synthesis or derived from a different cGH mRNA isoform in the cell remains to be determined.

Independent immunohistochemical staining experiments demonstrated that cGH proteins exist in chicken immune cells (Render *et al.*, 1995). It is now well established that the activity and proliferation of lymphoid cells and lymphoid organs are stimulated by growth hormone (reviewed by Gelato, 1993; Weijent and Blalock, 1995). Moreover, cellular localization of growth hormone receptors and binding proteins in immune tissues has been described (Hull *et al.*, 1996). Our findings further support the association between GH and immune system. IFA co-localize truncated cGH and SORF2 *in vitro,* EM study using immune-gold particles co-localized both proteins *in vivo*. However, further elucidation of the biological function(s) of the interaction is essential to reveal the role of SORF2, if any, during MD oncogenisis.

An early observation indicated that rapid viral replication was observed in the RM1 strain (Witter *et al.*, 1997). T-cell activation is a prerequisite for MDV infection and cell transformation. The rapid replicative phenotype of RM1 strain, presumably due to unregulated viral gene expression, may limit the life span of infected lymphocytes

resulting in rapid cell death. The rapid cell death may limit resources available to the infecting virus, therefore diminishing the T cell activation response. During the normal viral cytolytic infection that SORF2 is possibly released from lytic cells. Thus, SORF2 and circulated GH are possible to interact if they happen to locate at the same place. Whether the RM1 caused phenomenon was due to the overexpression of SORF2 genes resulting undesired SORF2-cGH interaction remains unknown. Further study will be required to examine this hypothesis.

A number of questions concerning the role of MDV transformation in the natural host have yet to be addressed. The identification of viral gene products and the elucidation of their function will substantially strengthen our understanding of specific virus-host interaction. Cell transformation is one of the defining characteristics of oncogenic MDV. It consists of a complex and highly regulated process beginning with the specific recognition between viral and host proteins, which leads to tumor formation in certain organs. The identification of the SORF2-cGH binding might provide a valuable example of one such interaction.

CHAPTER III

The Association Between the Chicken Growth Hormone Gene and Resistance To Marek's Disease

Introduction

Marek's disease (MD) is a lymphoproliferative disease of chickens that affects peripheral nerves and most visceral organs. Diseased chickens may present varying severity of symptoms, ranging from chronic peripheral nerve neuropathy, characterized by demyelination and partial or complete paralysis, to an acute cancerous disease in which multiple visceral lymphomas can be observed (Payne, 1985; Calnek and Witter, 1997). The causative agent of MD is a cell-associated herpesvirus named Marek's disease virus (MDV). MDV can interact differently with different types of cells resulting in productive infection, latent infection, or cell transformation. To date, MD is fairly well controlled by vaccines. However, MD vaccines control rather than eliminate losses from MD because they do not block MDV infection and replication. It becomes obvious that genetic resistance is needed to complement vaccinal protection.

One major goal in studying genetic bases for resistance and immunity to Marek's disease is to identify and characterize physiologic factors that regulate or affect optimal immune system function, development, and regulation. A better understanding of the function and structure of the individual factors will further genetic improvement of chickens. The MHC, or B locus, of chickens is involved in the control of disease resistance and immune responsiveness. The MHC genes in chicken are organized in two genetically independent polymorphic gene clusters. The B complex and the recently

discovered Rfp-Y clusters both contain class I and class II genes which are believed to be involved in mediating cell-cell interactions and antigen presentation in the immune response (Miller *et al.*, 1996). The B complex and Rfp-Y genes are on the same microchromosome but are genetically unlinked (Fillon *et al.*, 1996; Miller *et al.*, 1996). Although the MHC is thought to play a central role in MD resistance; genetic studies indicate that the contribution of resistance to MD by non-MHC genes may also be significant (Groot and Albers, 1992; Yonash *et al.*, 1999).

Recently, advances in understanding chicken non-MHC loci involved in MD resistance have been made using ADOL inbred lines 6 and 7. Line 6 is very resistant to MD, but line 7 is very susceptible. They both share the generally resistant MHC B2 haplotype. Hence, it is believed that the response to MDV in line 6 and line 7 is governed by non-MHC susceptibility genes. Comparison studies of these two inbred lines have been focused on their virological and immunlogical characteristics related to resistance to MD.

Growth of MDV in cell cultures derived from either of the two lines of chicken is similar (Shama and Purchase, 1974), whereas adsorption of MDV to spleen cells as well as growth of the virus in chicken embryos or chickens is greater in line 7 than in line 6 (Lee *et al.*, 1981; Powell *et al.*, 1982). Gross pathological changes in the lymphoid organs during cytolytic infection are more severe and virus replication is greater in line 7 than in line 6 birds (Lee *et al.*, 1981). When line 6 chicks that have undergone thymectomy receive a transplantation of thymus fragments from line 7 birds, they show a decrease in resistance to MD in comparison with normal line 6. These results suggest that the lymphocytes of line 7 birds may be more susceptible to transformation (Powell *et al.*,

1982, 1986). Another possibility is that line 7 lymphocytes may have more receptors for MDV, or line 6 have fewer target cells (lymphoid cells) with the MDV receptor on their surface (Powell *et al.*, 1982). Moreover, studies also demonstrated that line 6 chickens have a higher cellular immunity against MDV-infected cells and tumor cells than line 7 chickens (Lee *et al.*, 1981). Given what we know so far, it is proposed that the mechanism of MD resistance observed in line 6 chickens is operating mainly at the level of target cells of viral infection, as well as the immune response to viral antigens and tumor antigens (Lee *et al.*, 1981).

Another differential immune response has been observed in macrophages. The function of macrophages as part of the immune system is complicated. They play important roles in immune monitoring, enhancing or inducing immune responses, and under certain conditions they also inhibit the immune response of the host. In MD resistance, macrophages on one hand inhibit the growth of virus or tumor cells but on the other hand, they lower the responsiveness of normal lymphocytes to mitogens. In general, there is no correlation between resistance to MD and various chicken lines with different macrophage activity except in lines 6 and 7. In vivo studies point out that the phagocytosis index of normal macrophages originating from line 6 is significant higher than of those from line 7. Upon infection, both lines develop increased phagocytosis and inhibition of MDV plaque formation; however, the effect is significantly higher in line 7. The number of target cells against MDV is also found to be more in line 7 (Gallatin and Longenecker, 1981). It is suspected that MDV itself or MDV related antigens may take part in the mechanism of macrophage activation in line 7 chickens (Powell *et al.*, 1983). Studies of macrophage-produced cytokines demonstrated that line 6 macrophages (LPS

stimulated culture supernatant fractions) produced greater TNF-like factor, and lower nitrite activity than line 7 macrophages (Dr. M. Qureshi, personal communication). This observation may help explain the basis of intrinsic immune response differences between these two lines.

The use of genetic markers coupled with a pedigreed reference population make it possible to identify genome regions associated with disease resistance. This genetic approach requires reference/resource families making use of diverse crosses to produce at least three generation families demonstrating a measurable variation in quantitative traits among F2 individuals. Great efforts have been made towards identifying quantitative trait loci (QTL) affecting MD susceptibility using co-segregation analysis in F2 intercross chickens bred from line 6 and line 7 (Vallejo *et al.*, 1997; Yonash *et al.*, 1999). This 6x7-reference family offers the opportunity to identify non-MHC QTLs affecting several components of MD susceptibility. Recent results from our studies suggest that chicken growth hormone (cGH) gene could be a candidate for MD QTL.

It is becoming more apparent that the endocrine system plays a significant role in the regulation of the immune system and cell transformation. Previous studies using a yeast two-hybrid analysis identified cGH as a cellular protein that specifically binds to the MDV SORF2 gene product. To further examine the relationship between the cGH locus and MD incidence, the 6x7 F2 cross was employed to test for co-segregation of the cGH gene and MD resistance.

Many studies demonstrate that cGH exerts a wide range of cell- and tissue-specific responses, including regulation of lipid, nitrogen and carbohydrate metabolism, as well as induction of cell growth and differentiation. These effects are mediated by the

binding of cGH to its cell surface receptor (GHR), activation of downstream cellular signal transduction events and altered expression of specific genes in target cells. Moreover, there is considerable evidence for the importance of GH in the maintenance, control, and modulation of the immune system (reviewed by Harvey and Hull, 1997). It is therefore possible that cGH may play an important role in MD oncogenesis, especially as it interacts with the MDV unique gene product SORF2.

Indeed, immune function is impaired in GH deficiency and can be restored by exogenous GH therapy (Khansari and Gustad, 1991; Corpas *et al.*, 1993; Johnson *et al.*, 1993). Immunostimulatory effects of GH include increased lymphocyte proliferation (Yoshida *et al.*, 1992; Murphy *et al.*, 1993) and the production of cytokines (Chandratilleke *et al.*, 1994; Kappel *et al.*, 1994), immunoglobulins (Kimata and Yoshida, 1994), and other immune factors (Murphy *et al.*, 1992). Immune cells and immune tissues thus are likely to be target-sites for GH action, especially as the GHR is structurally homologous with cytokine receptors (Wlodawer *et al.*, 1993). Hemopoietic and lymphoid actions of exogenous GH include increased thymic size, thymocyte proliferation and differentiation, proliferation of thymic epithelial cells and their secretion of thymulin, activation and proliferation of lymphocytes, increased production of cytokines, and the activation of monocytes, macrophages, phagocytosis, the generation of superoxide anions, and the intravascular migration of immune cells (Aurenhammer and Strasburger, 1995).

Immune tissues are not only GH target tissues, but also sites of GH synthesis. It is well established that GH is derived from the pituitary gland via systemic circulation. Classically, it has been considered to be an endocrine hormone with actions distant from

its site of production. Pituitary somatotrophs are primarily responsible for the synthesis of GH within the pituitary gland, although GH synthesis also occurs within prolactin and thyrotropin secreting cells (Harvey, 1995). It is suspected that a large portion of GH circulates through binding to a protein similar in structure to target-cell GHR. Free GH in the circulation degrades rapidly and only has a half-life of 20-30 minutes.

It is now known that GH gene expression is not only restricted to the pituitary gland. GH immunoreactivity is found widespread in many tissues, and mRNA analysis suggests almost ubiquitous expression of the GH gene or closely related genes. *In vivo* studies showed that GH mRNA is present in human and rat peripheral blood leukocytes and in the spleen, thymus, and bone marrow, in which about 10% of mononuclear cells are GH immunoreactive (Weigent *et al.*, 1988; Weigent and Blalock, 1991). The translation of the GH transcript in immune tissues is indicated by the incorporation of ^{3}H-labeled amino acids into immunoreactive GH. Studies in chicken GH immunoreactivity in the spleen, thymus, and bursa also detected GH concentrations at 10%, 2%, and 1%, respectively, of that in the pituitary gland (Render *et al.*, 1995). Furthermore, the Pit-1 gene, a regulatory factor for growth hormone, was originally thought to be a pituitary specific factor required for somatotroph proliferation and GH gene expression. It has been found in pituitary thyrotrophs and lactotrophs, the immune system, and the placenta. Most of these tissues also possess GHR that is responsive to GH stimulation. It is therefore believed that GH may have paracrine or autocrine actions within some of its sites of synthesis (reviewed by Harvey and Hull, 1997).

Stimulation of GH synthesis or release in immune tissues is likely to be closely regulated by autocrine, paracrine, or endocrine actions of GH-releasing hormone

(GHRH) produced in those cells (Guarcello *et al.*, 1991). The locally produced GH appears to exert an autocrine effect. This is supported by the study of insulin-like growth factor I (IGF-I) production. IGF-I is a mitogenic peptide structurally related to proinsulin and IGF-II which mediates many of the growth-promoting effects of GH in postnatal animals (Froesch *et al.*, 1985). There is compelling evidence that IGF-I production is regulated by both insulin and GH and that both hormones interact in vivo to maintain normal IGF-I concentration. Evidence has been presented that IGF-I production by leukocytes is reduced due to the lack of GH function by the presence of GH antibodies (Baxter *et al.*, 1991). Similarly, a block in GH synthesis by addition of GH antisense oligonucleotides reduced the proliferation of rat lymphocytes *in vitro* (Weigent *et al.*, 1991). These studies point out that some immune responses are mediated in an autocrine manner. However, the relative importance of endogenous and exogenous GH to lymphocyte functioning remains to be elucidated.

Studies showed that the production of GH in rat by leukocytes from spleen, thymus, and the peritoneum is increased during immune challenge, as provoked by intraperitoneal injections of lipopolysaccharide (LPS), and Freund's Complete Adjuvant (Baxter *et al.*, 1991). The expression of GH from myeloid cell lines is similarly increased in actively proliferating cells (Costoya *et al.*, 1996). It is also known that the GHR gene is expressed in immune tissues. Other studies showed that labeled GH and GHR immunoreactivity can be detected on the surface of circulating immune cells and immortalized lymphocyte cell lines (Arrenbrecht, 1974; Ban *et al.*, 1991; Badolato *et al.*, 1994). These receptors are present in subpopulations of B- and T-lymphocytes and natural killer cells and are most abundant in B cells of mammals. In contrast, in avians

the splenic, thymic, and bursal GHR/GH binding protein (GHBP) immunoreactivity is largely associated with macrophages and other large mononuclear nonlymphoid cells and is particularly present in the nucleus (Calduch-Giner *et al.*, 1995). In the chicken, it is found that GHR/GHBP immunoreactivity can be detected in thymic, medullary, antigen-presenting interdigitating, and ellipsoid-associated cells. In all cases, it is present in both nuclear and cytoplasmic compartments (Hull *et al.*, 1996). The finding of widespread distribution of GHR/GHBP immunoreactivity indirectly suggests a possible role for GH in regulating immune function.

Clearly, GH exerts some marked effects on immune responding tissues. It stimulates the proliferation of immunocompetent cells and modulates humoral and cellular immune function. As discussed in Chapter II, cGH has been found to be capable of interacting with MDV SORF2 protein. The importance of cGH in MD remains to be revealed. In this chapter, a candidate gene analysis was performed testing the cGH gene as a possible contributor to the variation in MD susceptibility among ADOL 6x7 intercross F2 birds.

Materials and Methods

Experimental Animals

A reference pedigree was established at the Avian Disease and Oncology Laboratory (ADOL), East Lansing for the purpose of genome mapping. It is comprised of 400+ backcross (BC) progeny using an inbred White Leghorn line and an inbred Red Jungle Fowl line as grandparents. Initially, one breed-crossed F1 male was backcrossed with several inbred White Leghorn females to generate BC progeny, of which 52 BC animals have been used for establishing genetic linkage groups and genome mapping.

For QTL identification purposes, inbred lines 6 and 7 were used as a grandparental generation to produce more than 300 fully pedigreed F2 intercross chickens. The F2 intercross chickens, along with grandparental lines and F1 control birds, were produced in five separate hatches (i.e., ~72 chickens per hatch) for the evaluation of MD susceptibility.

Assessment of MD Susceptibility

The F2 intercross chickens generated from 6x7 were randomly assigned into six isolators within each hatch (i.e., 16 birds per isolator). The rearing and monitoring of chickens was conducted using standard poultry research conditions. At 1 week of age, the chickens were challenged intra-abdominally with 2000 pfu (plaque formation unit) of MDV JM102 strain virus. The infected birds were died or survived for additional 10 weeks and then were sacrificed by CO_2 inhalation and necropsied. Nerves and other organs were examined for gross and/or microscopic MDV lesions and tumor development. Blood was obtained from each bird during the experiment and was used for DNA purification.

Several components of MD susceptibility were measured before termination and at termination. Viremia (VIR) is the MDV concentration at 2 weeks after MDV challenge (i.e., number of virus pfu per 10^6 peripheral blood cells; Witter *et al.*, 1969). Tissue (TIS) is the number of different tissue or organs showing gross tumors or lesions at necropsy, including vagus, brachial or sciatic nerves, heart, gonad, spleen, bursa, lung, and thymus viscera. Survival (SUR) is the number of days from the chicken MDV challenge to death. Disease (DIS) is the overall phenotypic assessment of each chicken

for MD susceptibility judged by either presence (noted as susceptible) or absence (noted as resistant) of any gross lesion. Tumor index (TUM) is an index developed using the following scoring system: 0=alive, healthy, absent of tumors; 1=single neural lesion; 2=single visceral lesion or multiple neural lesions or microscopic lesions; 3=multiple neural lesions; 4=single visceral plus multiple neural lesions or multiple visceral plus single neural lesions; 5=multiple visceral plus multiple neural lesion at necropsy or dead with gross lesions. MD index (MDI) is an empirical pooled index that was developed to assign weights to each MD trait based on consensus suggestions from experienced MD pathologists and poultry veterinarians. It was estimated using standardized MD data and the following expression:

$$MDI = \log_{10}\{[(VIR*15)+(TIS*25)+(DIS*25)+(SUR*10)+(TUM*25)]+100\}$$

RFLP Analysis

A 550 bp cGH fragment was excised from the pcDNA-GH plasmid using *BamH*I and *Hind*III restriction enzymes. The isolated fragment was random labeled with ^{32}P-dCTP using an Oligolabelling kit (Pharmacia, Inc.). The DNA samples were prepared either from blood cells or from -70°C frozen spleens. After digestion of 15 μg of each DNA sample with the restriction enzyme *Msp*I or *Sac*I in a 37°C water bath for 18 hours, the DNA fragments were separated via electrophoresis in 0.8% agarose gels and transferred onto charged nylon membranes. The blots were prehybridized in a mixture containing 10% dextran sulfate, 0.5% SDS, 50 mM Na_2HPO_4, 5X Denhart solution, and 0.5 M NaCl. Membranes were hybridized with the ^{32}P-labelled GH probe for 16-20 hours at 65°C. Final washes were at 65°C in 0.7 X SSC, 0.5% SDS for 15-20 min. Autoradiography was carried out overnight at -80°C using Kodak XAR-5 film and

Cronex Lighting Plus intensifying screens. Fragments were measured using a 1 Kb Ladder (Gibco, BRL) as standard.

For PCR-RFLP, primers for PCR were as follows: GH For 5'-ACC TGG AAG AAG GGA TCC AAG; GH Rev 5'-GGC CGT CGT GGA GCT GTG AGC. Each PCR mixture contained 50 ng of genomic DNA, 200 μM of each dNTP, 4 μl of 10 X PCR buffer, 1.5 mM $MgCl_2$, and 0.2 U Taq DNA polymerase (Life Technologies, Inc.). A total of 10 pmole of each primer was added to a final volume of 40 μl. PCR was carried out on a MJ Research Thermal Cycler with the following cycles: 94°C, 3 min; 30 cycles of 30 sec at 94°C, 30 sec at annealing temperature 55°C and 30 sec at 72°C followed with a 5 min extension at 72°C. A total of 20 μl of the amplified product was mixed with 2.5 μl 10 X digestion buffer A, 1 U *Sac*I restriction enzyme (Perkin Elmer, Inc.) in a final volume of 25μl reaction mixture. A one hour 37°C incubation was carried out followed by mixing reaction mixture with the loading buffer and separating on 1% agarose gels; results were visualized after ethidium bromide staining.

Linkage Analysis

The East Lansing genetic maps were developed previously by multipoint analysis of the genotypes on the 52 BC family using the program MAPMAKER/EXP, version 3.0 (Lander *et al.*, 1987; Lincoln *et al.*, 1992). The data gathered from the cGH genotyping was used to localize the cGH gene. Linkage groups were determined by the results of pairwise comparisons (two-point analysis) with a minimum LOD linkage (log10 of odds) score of 3.0 for statistical acceptance of linkage and with a recombination fraction of θ =0.32. Following this, three-point analyses were performed for each linkage group comparing three consecutive markers at a time. A LOD value of 3.0 was again used as

the linkage criteria for triples, while the multi-point analysis generated the most likely loci order and genetic distances expressed in centiMorgans.

Statistical Analysis

For association analysis, the components of MD susceptibility were assessed for significant deviation among 272 F2 generated from lines 6 x 7 with various cGH genotypes. The factors of hatch, isolator, sex, and weight of chicken were used as covariates to correct the measurements in a nested design using the GLM procedure (SAS 1988). A single point analysis using one-way ANOVA with each genotype class considered as a treatment was performed. Simple associations between the F2 progeny GH genotypes and MD susceptibility values were assessed using analysis of variance (ANOVA; SAS 1988). The tests of significance were conducted using the ANOVA F-test.

DNA Sequences Comparison

PCR primers were designed from the intron regions that will amplify fragment across each cGH exon. PCR reactions were carried out using inbred lines 6 and 7 DNA as templates separately. The reaction conditions were the same as described previously. Primers for each exon are as follows: Exon 1, For 5'-AAC CAG GCA GGA AAA TCA, Rev 5'-TCA GCC CAC CAC AGC ACA; Exon 2, For 5'-CAC TGC TCT CCA CCC TGT, Rev 5'-ATC ACC TCT CCT CCC CTA; Exon 3, 5'-CCG GGA AAG AGT GAG GAA, Rev, 5'-GCC AGC AGC CCC TCG CTC; Exon 4, 5'- GCC AGC AGC CCC TCG CTC Rev, 5'-CCC TCC TCC CTC CCC TTT. Each PCR product was cloned into TA cloning vectors then transformed into *E. coli* competent cells provided by the manufacturer (Invitrogen, Inc.). DNA was then isolated and subjected to DNA sequence

analysis using Dye terminator reactions (Perkins Elmer, Inc.) by 377 ABI automatic DNA sequencers. Sequence comparisons were carried out using the DNASTAR program.

Results

Mapping cGH Gene

In order to position the cGH gene on the East Lansing chicken genetic map, linkage analysis was carried out using 52 BC birds. DNA polymorphisms for the cGH gene were obtained from East Lansing reference family using RFLP analysis. Two alleles of 1.3 Kb and 1.1 Kb were detected using the restriction enzyme MspI. Allelic segregation analysis agreed with 1:1 Mendelian segregation of the two alleles. Partial data are shown in Figure 16. The genotypes of each of the individual birds were scored for linkage group identification, in which 1130 marker loci had been mapped previously. Linkage analyses demonstrated that the cGH gene belonged to a linkage group E59. The linear order of this linkage group is ACLY-LEI0016-ADO0376-GCT0022-ROS0315 (ROS0071)-COL1A1a-COL1A1b-cGH-LEI0350 (Figure 17). The length of the linkage group was estimated at 58.5cM (sex averaged), and the genetic distance between each locus was estimated as shown in Figure 17.

Association of cGH with MD Disease Resistance

In order to obtain individual genotypes for F2 population, inbred lines 6 and 7 were tested for DNA polymorphisms using the RFLP technique. DNA polymorphisms for the cGH gene were detected using restriction enzyme *Sac*I which gave rise to two alleles of 4 kb and 4.4 kb (Figure 18). In the original RFLP test, a 4kb fragment was denoted as the A allele observed in line 6, and a 4.4 kb fragment was denoted as the B allele obtained from line 7. Using the published cGH gene sequence (Tanaka *et al.*,

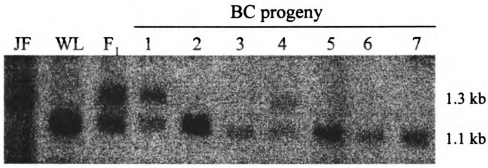

Figure 16. RFLP analysis of East Lansing reference family. The polymorphism was obtained using *Msp*I restriction enzyme and probed with cGH.

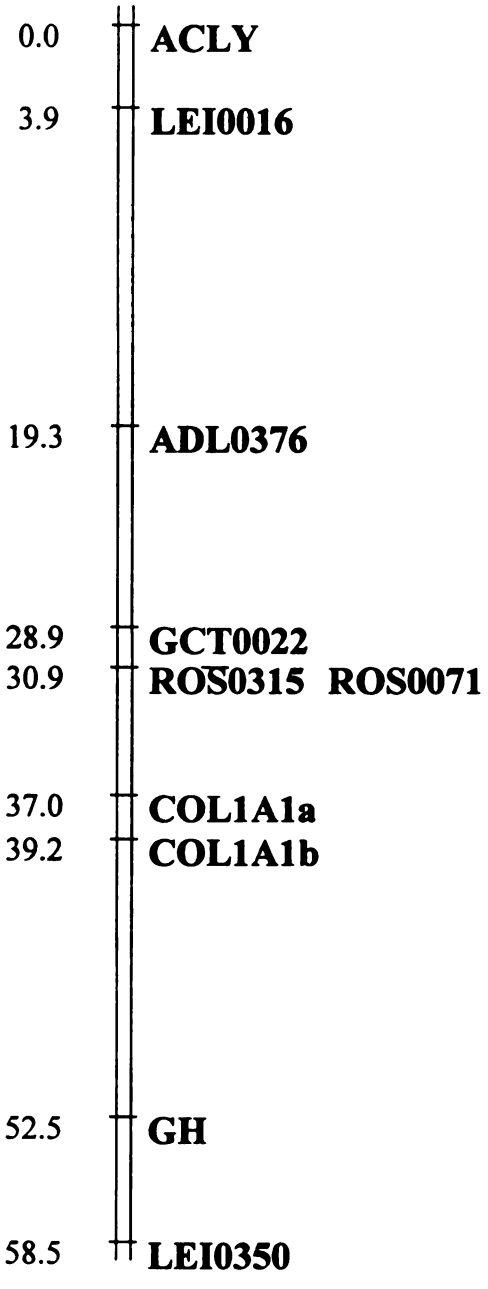

Figure 17. Genetic map of linkage group E59. Markers or genes are shown to the right while the distance in cM is on the left.

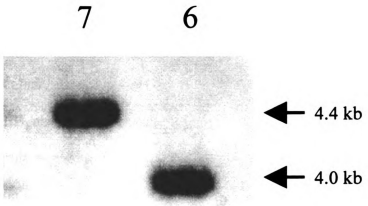

Figure 18. RFLP analysis of GH in parents of MD resource population. Polymorphism was obtained by using *Sac*I restriction enzyme and probed with cGH.

1992), a PCR technique was designed to amplify a 1Kb fragment of intron 4 which covers this predicted *Sac*I polymorphic site. All F2 birds were genotyped for the cGH polymorphism using the PCR-RFLP technique. The polymorphic restriction site for *Sac*I was identified at position 3633-3638. The amplified DNA digested with *Sac*I yields a single 1Kb fragment representing the allele from line 7, or produces two fragments of 360 and 640 bp representing the allele from line 6. Partial results of PCR-RFLP in intron 4 are presented in Figure 19. Allele frequencies in the F2 averaged 0.48 for the A allele and 0.53 for the B allele. Genotype frequencies were estimated as 0.24 for AA, 0.48 for AB, and 0.28 for BB, about the 1:2:1 ratio expected.

The cGH polymorphisms typed in the F2 6x7 intercross were used to assess the possible association with disease resistance to MD. The traits measured on the F2 birds were viremia at 2 weeks after MDV inoculation, number of tissues showing gross tumors or lesions at necropsy, number of days which birds survived from MDV infection to death, tumor index scores from 0 to 5, and MD index (MDI) after adjustment of VIR, TIS, DIS, SUR, and TUM measurements. No statistically significant association between the cGH polymorphic alleles and the variation in incidence traits among the F2 was revealed (Table 1). Maximum F ratios, standard errors and average mean of each genotypic class for each MD incidence were also presented in Table 1. The overall phenotypic assessment of MD susceptibility (DIS) for each individual bird did not indicate any statistical significance ($P>0.05$) after using chi-square analysis. Thus, we conclude that no major effect of the cGH locus on the disease incidence of MD could be detected in the White Leghorn line 6x7 intercross F2 population.

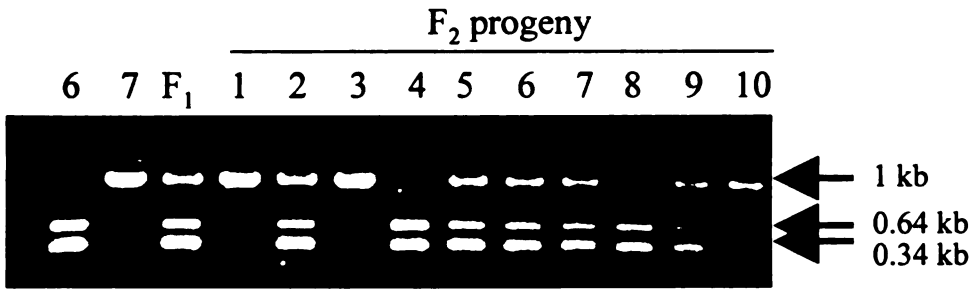

Figure 19. PCR-RFLP analysis of MD resource family. Polymorphism were obtained by digestion of PCR product with *Sac*I restriction enzyme followed by resolution with agarose gel electrophoresis.

Table1. Association analysis for cGH and MD incidence. Mean ± standard error for MD incidence according to cGH genotypes in lines 6 x 7 intercross F2. The F-statistic and p- values for tests of differences among genotypic classes are also given.

Traits[a]	Genotypes of cGH among F2				
	A/A (n=66)	A/B (n=128)	B/B (n=75)	F	P[b]
VIR	96.00± 11.23	102.21±8.00	83.44±10.37	1.03	0.36
TIS	1.23±0.16	1.27± 0.11	1.09±0.15	0.43	0.65
SUR	44.54±2.19	45.91±1.58	47.68±2.05	0.55	0.58
TUM	1 .05±0.13	1.05±0.09	0.93±0.12	0.31	0.73
MDI	1.65±0.09	1.64±0.07	1.55±0.08	0.45	0.65

[a]VIR= MDV plaque formation unit(pfu) in 1 x 10^6 peripheral white blood cells at 2 weeks post-challenge; TIS= number of different tissues with MD lesions in diseased chicken; SUR= number of days from chicken MDV challenge to death; TUM = MD tumor index; MDI = MD index.

[b]p-value≤ 0.05 and ≤ 0.01 are considered as suggestive and significant, respectively.

Exons Comparison between Line 6 and Line 7

PCR fragments covering each exon of cGH were cloned into TA cloning vectors, followed by DNA sequence analysis using 377 ABI automatic sequencers. Results of DNA sequence comparison indicated that no nucleotide substitution was found between these two inbred lines (data not shown).

Discussion

In an attempt to reveal the potential possibility of cGH to be involved in MD susceptibility, a genetic approach using 272 F2 chickens selected from inbred line 6 x line 7 for the association studies was performed. One of the major approaches of MD resistance research in chicken has been the "top-down" approach; that is, mapping and identification of QTLs through the use of linked molecular markers. Recent studies have successfully identified 14 QTLs involved in MD susceptibility using genetic markers (Yonash *et al.*, 1999).

Kuhnlein *et al.* (1997) reported that the cGH gene was found to be significantly associated with MD resistance in non-inbred selected White Leghorn strains. Moreover, previous studies demonstrated that cGH is capable of binding to MDV SORF2 protein. These results suggested that cGH might play a role in MD oncogenesis. cGH has been proven to have a wide variety of physiological effects on traits such as appetite control, growth, body composition, aging and reproduction (Byatt *et al.*, 1993; Copras *et al.*, 1993; Apa *et al.*, 1994; Vasilatos-Younken, 1995) as well as immune responsiveness (Marsh, 1992; Blulock, 1994; Kelley and Felton, 1995). In this study, the importance of the cGH gene as a candidate QTL was examined by investigating the possible co-

segregation of the cGH gene and variation in MD susceptibility among intercross F2 chickens.

First cGH was mapped to E59 in the East Lansing genetic map. Previous work on QTL identification found no QTL on E59 (Yonash *et al.*, 1999). However, due to the large intervals between each marker, single marker analysis was carried out using another population derived from the 6 x 7 intercross F2. Several components of MD traits based on MD susceptibility were tested for association with cGH alleles. A direct association of MD incidence with the cGH gene was not found in this particular population. Overall, preliminary analyses from this study showed no significant cGH allelic co-segregation with the genetic susceptibility to MD in 6x7 F2 White Leghorn chickens. However, we cannot exclude the possibility that other cGH alleles exist outside the lines 6 and 7 which could significantly affect MD resistance.

Digestion of the cGH PCR fragment reveals one polymorphic recognition site for *Sac*I at Intron 4. However, this polymorphic region is located in an intron. DNA sequence from each exon indicated that there is no nucleotide substitution detected between chicken lines 6 and 7. Results suggest that cGH is quite conserved among strains at the amino acids level.

Although there is no amino acid difference of cGH between lines 6 and 7, it is noticed that immune response organs, such as spleen, thymus, and bursa, have a greater weight (percent relative to body weights) in chickens from line 7. It has been reported that cGH is involved in cell proliferation of immune response organs (reviewed by Harvey and Hull, 1997). Thus, it is reasonable to suspect that the expression of cGH may be varied between these two lines in terms of amount or timing. Therefore, genes

involved in regulation of cGH, e.g., somatostatin, growth hormone releasing factor, growth hormone receptor, and cGH specific transcription factors, would be potential candidates to test for QTLs. Once polymorphisms at these genes locus are identified, combined genotypic analysis can be further used to define the basis of cGH effects in ADOL lines 6 and 7.

CHAPTER IV

Conclusion and Future Directions

Summary of Results

In an attempt to study the biological characteristics of MDV genes, a unique gene, SORF2, came to our attention. An interesting phenomenon was discovered in the MDV recombinant strain RM1 which was found to have a REV LTR insertion upstream of the SORF2 gene. The unregulated overexpression of SORF2 due to the LTR promoter resulted in the loss of MDV oncogenicity. This finding prompted further investigation of SORF2.

In this study we have shown that the unique MDV gene SORF2 is expressed in naturally infected culture cells and *in vivo*. In a screen to identify host proteins that interact with SORF2, a genetic approach using the yeast two-hybrid system was employed. The chicken growth hormone structural polypeptide was found to interact with SORF2. To test this interaction directly, *in vitro* protein binding assays and co-immunoprecipitations were used to demonstrate that these two proteins associated with one another. In addition, SORF2 and a truncated form of cGH lacking its signal sequence were shown to co-localize in cultured CEFs. Sections of neoplastic tissues induced by oncogenic MDV were examined using antibodies that specifically recognize either cGH or SORF2. Positive staining was observed for both proteins. Electron microscopy of neoplastic spleen sections using colloidal gold markers confirmed a common cytoplasmic localization for the two proteins. These results provided biological evidence that these two protein are capable of interacting with each other.

To further investigate if the cGH gene was associated with genetic disease resistance, analysis was carried out in an intercross 6x7 F_2 population. We did not detect any significant association between the polymorphic alleles of cGH and the variation of MD incidence among F_2 animals. To provide a precise location of the cGH gene on the chicken linkage map, segregation analysis was done using the East Lansing BC resource family. The cGH locus was tested for linkage against 1130 genetic markers previously scored in this pedigree. cGH was firmly assigned to the E59 linkage group.

In summary, we successfully identified and confirmed that cGH and SORF2 are capable of binding with each other. Further studies will be required to elucidate the biological meaning of this specific interaction.

Future Directions

It has been well studied that MDV infection in genetically susceptible chickens results in an extensive lymphoproliferation leading to the formation of gross lymphomas, while in resistant birds there is only limited lymphoproliferation and tumor formation is very rare. The cGH polypeptide is found to interact with viral SORF2 protein, moreover, it is involved in cell proliferation of immune response organs. It is important to reveal the meaning of this interaction. There are several immediate questions that need to be addressed.

Does MDV infection induce cGH alternative splicing?

The cDNA library used for yeast two-hybrid screen was constructed from mitogen activated splenic cells. As mentioned in chapter II an altered form of cGH was identified from this library by its interaction with SORF2 protein. It will be necessary to determine if this altered clone resulted from alternative splicing or initiation of

transcription or is just an artifact form generated during the synthesis of cDNA. To answer this, mRNA directly isolated from MDV infected splenic cells will be examined. There are two strategies that can be employed in this study: the primer extension assay and S1 nuclease protection assay. In the primer extension assay, a primer designed downstream of the suspected splice acceptor or transcription start site will be used to prime cDNA synthesis from the total mRNA pool. If two products of correct size are generated by extension of the primer, it might suggest an alternative splicing event occured. Similarly, the 3' isotope-labeled single strand DNA which complementary to the full length mRNA of GH will be hybridized to total mRNA. Following S1 nuclease digestion, if two different labeled DNA products are detected, this might also suggest that the altered form of cGH is a product of alternative splicing. If MDV are capable of inducing the production of the altered cGH, which will be retained inside the cell instead of secretion, it can be suggested that SORF2 –GH interaction may preserve a particular function, and it will be further evaluated.

Does this interaction have any effect on cell proliferation?

Studies showed that the administration of antiserum against bovine GH to mice results in decreased growth and large reductions in the weights of thymus and of spleen (Pierpauli and Sorkin, 1968; Pandian and Talwar, 1971). This effect can be reversed by the concomitant administration of GH (Pierpauli and Sorkin, 1968). Our studies indicate that both SORF2 and cGH proteins can be detected in immune cells. An important question is whether this interaction has any meaning in terms of cell proliferation.

Amino acid sequence alignment indicates that there is no similarity between SORF2 and the growth hormone receptor. Will SORF2-GH interaction interfere GH-

GHR interaction? To answer this, the chicken adenohypophyseal cells will be used for this study. Cultured adenohypophyseal cells were originated from pituitary gland that express cGH and have been used broadly for the study of interaction among the cGH and other factors. The SORF2 gene will be cloned into an eukaryotic expression vector then transfected into CEF cells. Protein SORF2 purified from cultured cells will be added to cultured adenohypophyseal cells. *In vitro* cell proliferation assays will be performed to examine the effect of interaction in cell proliferation.

Once these specific questions are answered, one can propose a possible test for the role of the SORF2-cGH interaction, if any, with respect to the biological role of SORF2 in MD pathogenesis. Then, further studies will evaluate whether the SORF2 acts as an antagonist to block the normal cGH signaling pathway. Other functions during viral oncogenesis via interaction with cGH are also possible.

REFERENCE LIST

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Anderson, A.S., A. Francesconi, and R.W.Morgan.1992. Complete nucleotide sequence of the Marek's disease virus ICP4 gene. Virology 189:657-667.

Apa, R., A. Lanzone, and F. Miceli. 1994. Growth hormone induces in vitro maturation of follicle- and cumulus-enclosed rat oocytes. Mol. Cell. Endocrinol. 106:207-212.

Arrenbrecht, S. 1974. Specific binding of growth hormone to thymocytes. Nature 15: 252-255.

Asmundson, V.S., and J. Biely. 1932. Inheritance and resistance to fowl paralysis (neuro-lymphomatosis gallinarum). I. Differences in susceptibility. Can. J. Res. 6:171-176.

Auernhammer, C.J., and C.J. Strasburger. 1995. Effects of growth hormone and insulin-like growth factor I on the immune system. Eur. J. Endocrinol. 133:635-645.

Bacon L. D. and R.L. Witter. 1992. Influence of turkey herpesvirus vaccination on the B-haplotype effect on Marek's disease resistance in 15.B-congenic chickens. Avian Dis. 36:378-385.

Bacon L. D. and R.L. Witter. 1994. Serotype specificity of B-haplotype influence on the relative efficacy of Marek's disease vaccines. Poult. Sci. 73:481-487.

Bacon, L.D. 1987. Influence of the major histocompatibility complex on disease resistance and productivity. Poult. Sci. 66:802-811.

Badolato, R., H.M. Bond, G. Valerio, A. Petrella, G. Morrone, and M.J. Waters. 1994. Differential expression of surface membrane growth hormone receptor on human peripheral blood lymphocytes by dual fluorochrome flow cytometry. J. Clin. Endocrinol. Metab. 79:984-990.

Ball, R.F., J.F. Hill, J. Lyman, and A. Wyatt. 1971. The resistance to Marek's disease of chicks from immunized breeders. Poult. Sci. 50:1084-1090.

Ban,E., M.C. Gagnerault, H. Jammes, M.C. Postel-Vinay, F. Haour, and M. Dardenne. 1991. Specific binding sites for growth hormone in cultured mouse thymic epithelial cells. Life Sci.48:2141-2148.

Barnard, R., P.G. Bundesen, D.B. Rylatt, and M.J. Waters. 1985. Evidence from the use of monoclonal antibody probes for structural heterogenity of the growth hormone receptor. Biochem. J. 231:459-468.

Baroni C.D., N. Fabris, and G. Bertoli. 1969. Effects of hormones on development and function of lymphoid tissues. Synergistic action of thyroxin and somatotropic hormone in pituitary dwarf mice. Immunology 17(2):303-14.

Baxter, J.B., J.E. Blalock, and D.A. Weigent. 1991. Characterization of immunoreactive insulin-like growth factor I from leukocytes and its regulation by growth hormone. Endocrinology 129:1727-1734.

Becker, Y., H. Levi, M. Moyal, I. Davidson, M. Malkinson, and T. Maray. 1988. Early and late MDV-1 RNA transcripts for regulatory and structural proteins in infected and transformed cells. In S. Kato, T. Horiuchi, T. Mikami, and K. Hirai (ed.), Advances in Marek's disease research. pp. 121-127.Japanese Assoc. on Marek's Dis., Osaka.

Biggs, P.M. 1961. A discussion on the classification of the avian leucosis complex and fowl paralysis. Brit. Vet. J. 117:326-334.

Biggs, P.M. and L.N. Payne. 1963. Transmission experiments with Marek's disease (fowl paralysis). Vet. Rec. 75:177-179.

Biggs, P.M. and L.N. Payne. 1967. Studies on Marek's disease. I. Experimental transmission. J. Nat. Cancer Inst. 39:267-280.

Blulock, J.E. 1994. The syntax of immune-neuroendocrine communication. Immunol. Today. 15:504-511.

de Boer, G. F., J. Pol. and H. Oei. 1987. Biological characteristics of Marek's disease vaccine CVI-988 clone. Clin. Vet. Q. 9: 16S-28S.

Botstein , D., R.L. White, M.H. Skolnick, and R.W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32:314-331.

Boussaha, M., W. Sun, R. Pichyaugura, S.J. Triezenberg, and P.M. Coussens. 1996. Marek's disease virus (MDV) UL48 (VP16) contains two multiple functional domains and transactivates both homologous and heterologous immediate early gene promoters. In: *Current Research on Marek's Disease*, Proceedings of the 5th International Symposium on Marek's Disease (ed. by R. F. Silva, H. H. Cheng, P. M. Coussens, L. F. Lee, and L. F. Velicer), pp.148-153. American Association of Avian Pathologists, Inc., Kennett Square, Pennsylvania.

Bradley, G., M. Hayashi, G. Lancy, A. Tanaka, and M. Monoyama. 1989. Structure of the Marek's disease virus BamHI-H gene family: genes of putative importance for tumor induction. J. Virol. 63:2534-2542.

Brunovskis, P., and L.F. Velicer. 1995. The Marek's disease virus (MDV) unique short region: alpha herpesvirus - homologous, fowlpox virus - homologous, and MDV-specific genes. Virology 206:324-338.

Buckmaster, A.E., S.D. Scott, M.J. Sanderson, M.E.-G. Boursnell, L.J.N. Ross, and M.M. Biggs. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implication for herpesvirus classification. J. Gen. Virol 69:2133-2142.

Buckmaster, A.E., S.D. Scott, M.J. Sanderson, M.E. Boursnell, N.L. Ross, and M.M. Binns. 1988. Gene sequence and mapping data from Marek's disease virus and herpesvirus of turkeys: implications for herpesvirus classification. J. Gen. Virol. 69:2033-2042.

Bulow, V.V., and P.M. Biggs. 1975. Precipitating antigens associated with Marek's disease viruses and a herpesvirus of turkeys. Avian Pathol. 4:133-146.

Bumstead, N. 1998. Genomic mapping of resistance to Marek's disease. Avian Pathol. 27: S78-S81.

Buscaglia, C. and B.W. Calnek. 1988. Maintenance of Marek's disease herpesvirus latency *in vitro* by a factor found in conditioned medium. J. Gen. Virol. 69:2809-2818.

Buscaglia, C., B.W. Calnek, and K.A. Schat. 1988. Effect of immunocompetence of the establishment and maintenance of latency with Marek's disease herpesvirus. J. Gen. Virol. 69:1067-1077.

Byatt, J.C., N.R. Staten, W.J. Salsgiver, J.C. Kostelec, and R.J. Collier. 1993. Stimulation of food intake and weight gain in mature female rats by bovine prolactin and bovine growth hormone. Am. J. Physiol. 264:E986-992.

Byerly, J.L. and D.L. Dawe. 1972. Delayed hypersensitivity reactions in Marek's disease virus-infected chickens. Am. J. Vet. Res. 33:2267-2273.

Calduch-Giner, J.A., A. Sitja-Bobadilla, P. Alvarez-Pellitero, and J. Perez-Sanchez. 1995. Evidence for a direct action of GH on hematopoietic cells of a marine fish, the gilthead sea bream (Sparus aurata). J. Endocrinol. 146:459-467.

Calnek, B.W. 1985. Genetic Resistance, p. 293-328. Payne, L.N. (Ed.) Marek's disease. Martinus Nijhoff, Boston.

Calnek, B.W. and L.L. Spencer. 1985. Pathogenesis of Marek's Disease, p. 374-390. Calnek, B.W. (ed). Proceedings of the international symposium on Marek's disease. American Association of Avian Pathologists, Kennett Square, PA.

Calnek, B.W. and R. L. Witter. 1997. Marek's disease, p. 367-413. Calnek, B.W., Barnes, H.J., Beard, C.W., McDougald, L.R., and Siaf, Y.M. (eds). Disease of Poultry. 10th ed. Iowa State University Press, Ames, Iowa, USA.

Calnek, B.W., H.K. Adlinger, and D.E. Kahn. 1970. Feather follicle epithelium: A source of enveloped and infectious cell-free herpesvirus from Marek's disease. Avian Dis. 14:219-233.

Calnek, B.W., W.R. Shek, and K.A. Schat. 1981. Latent infections with Marek's disease virus and turkey herpesvirus. J. Natl. Cancer Inst. 66:585-590.

Campbell, J.G. 1956. Leucosis and fowl paralysis compared and contrasted. Vet. Rec. 68:527.

Campbell, M.E., J. W. Palfreyman, and C.M. Preston. 1984. Identification of herpes simplex virus DNA sequence with encode a trans-acting polypeptide responsible for stimulation of immediate-early transcription. J. Mol. Biol. 180:1-19.

Cebrian, J., C. Kaschka-Dierich, N. Berthelot, and P. Scheldrick. 1982. Inverted repeat nucleotide sequences in the genome of Marek's disease virus and the herpesvirus of the turkey. Pro. Natl. Acad. Sci. USA 79:555-558.

Chandratilleke, D., C.G. Scanes, and J.A. Marsh. 1994. Effect of triiodothyronine and in vitro growth hormone on avian interleukin-2. Dev. Comp. Immunol. 18:353-362.

Chen, X., and L.F. Velicer. 1991. Multiple bidirectional initiations and terminations of transcription in Marek's disease virus long repeat regions. J. Virol. 65:2445-2451.

Chen, X.B., P.J. Sondermeijer, and L.F. Velicer. 1992. Identification of a unique Marek's disease virus gene which encodes a 38-kilodalton phsophoprotein and is expressed in both lytically infected cells and latently infected lymphoblastoid tumor cells. J. Virol. 66:85-94.

Chubb, R.C. and A.E. Churchill. 1968. Precipitating antibodies associated with Marek's disease. Vet Rec. 82:4-7.

Churchill, A.E., and P.M. Biggs. 1967. Agent of Marek's disease in tissue culture. Nature 215: 528-530.

Churchill, A.E., L.N. Payne, and R.C. Chabb. 1969. Immunization against Marek's disease using live attenuated virus. Nature 221:744-747

Cole, R.K. 1968. Studies on genetic resistance to Marek's disease. Avian Dis. 12:9-28.

Confer, A.W. and H.K. Adldinger. 1980. Cell-mediated immunity in Marek's disease: Cytoxic responses in resistant and suceptible chickens and relation to disease. Am. J. Vet. Res. 41:307-312.

Corpas, E., S.M. Harman, and M.R. Blackman.1993. Human growth hormone and human aging. Endocr. Rev. 14:20-39.

Costoya,A., A. Vidal, M. Garcia-Barros, M. Gondar, J.A.F. Tresguerres, and V. Arce. 1996. Expression of the human growth hormone normal gene (hGH-N) in proliferating and differentiated HL-60 cells. Exp. Cell Res. 228:164-167.

Coussens, P.M. and L.F. Velicer. 1988. Structure and complete nucleotide sequence of the Marek's disease herpesvirus gp57-65 gene. J. Virol. 62:2373-2379.

Cui, Z.Z., L.F. Lee, J.L. Liu, and H.J. Kung. 1991. Structural analysis and transcriptional mapping of the Marek's disease virus gene encoding pp38, an antigen associated with transformed cells. J. Virol. 65:6509-6515.

Cui, Z.Z., D. Yan, and L.F. Lee. 1990. Marek's disease virus gene clones encoding virus-specific phosphorylated polypeptides and serological characterization of fusion proteins. Virus Genes 3:309-322.

Dalecluse, H.-J, and W. Hammerschmidt. 1993. Status of Marek's disease virus in established lymphoma cell lines: Herpesvirus integration is common. J. Virol. 67: 82-92.

Darlington, C.D., and K. Mather. 1949. The Elements of Genetics. Allen & Unwin, London.

Dentine, M. R. 1990. Using molecular biology to improve the accuracy of selection. Proceedings of the 4th World Congress on Genetics Applied to Livestock Production, XIV:35-44, Edinburgh, Scotland.

Dentine, M. R. 1994. The State of QTL Research. Proceedings :Future Genetics for the Animal Industry. 1:27-34. St. Louis, MO.

Edison, C.S., O.J. Fletcher, S.H. Kleven, and D.P. Anderson. 1971. Detection of Marek's disease antigen in feather follicle epithelium of chickens vaccinated against Marek's disease. J. Natl. Cancer. Inst. 47:113-120.

Ellegren, H. 1993. Genome analysis with microsatellite markers. SLU/Repro, Uppsala, Sweden.

Fields S., and O. Song. 1989. A novel genetic system to detect protein-protein interactions. Nature 340:245-246.

Fillon, V., R. Zoorob, M. Yerle, C. Auffray, and A. Vignal.1996. Mapping of the genetically independent chicken major histocompatibility complexes B and Rfp-Y to the same microchromosome by two-color fluorescent *in situ* hybridization. Cytogenet. Cell Genet.75:7-9.

Foote, S., D. Vollrath, A. Hilton, and D.C. Page. 1992. The human Y chromosome : overlapping DNA clones spanning the euchromatic region. Science 258:60-66.

Fredericksen, T.L., and D.G. Gilmour. 1981. Chicken lymphocyte alloantigen genes and responsiveness of whole blood cells to concanavalin A. Fed. Proc. 40:977.

Fries, R., A. Eggen, and J.E. Womack. 1993. The bovine genome map. Mamm. Genome 4:405-428.

Froesch, E.R., C. Schmid, J. Schwander, and J. Zapf. 1985. Actions of insulin-like growth factors. Annu. Review Physiol. 47:443-467.

Fukuchi, K., A. Tanaka, L.W. Schierman, R. Witter, and M. Nonoyama. 1985. The structure of Marek's disease virus DNA: the presence of unique expression in nonpathogenic viral DNA. Proc. Natl. Acad. Sci. USA. 82:751-754.

Gallatin, W.M. and B.M. Longenecker. 1981. Genetic resistance to herpesvirus-induced lymphoma at the level of the target cell determined by the thymic microenvironment. Int. J. Cancer 27:373-380.

Gavora, J.S., and J.L. Spencer. 1979. Studies on genetic resistance of chickens to Marek's disease- a review . Comp. Immun. Microbiol. Infect. Dis. 2:359-371.

Gelato,M.C. 1993. Growth hormone insulin-like growth factor I and immune function. Trends Endocrinol. Metab. 4:106-110.

Groenen, M.A.M., H.H. Cheng, N. Bumstead, B. Benkel, E. Briles, D.W. Burt, T. Burke, L.B. Crittenden, J. Dodgson, J. Hillel, S. Lamont, F.A. Ponce de Leon, M. Soller, H. Takahashi, and A. Vignal. 1999. A consensus linkage map of the chicken genome. Animal Genet. (accepted).

Groot, A.J.C., and G.A.A. Albers. 1992. The effect of MHC on resistance to Marek's disease by White Leghorn crosses. Proc. World's Poultry Congress 1:185-188.

Guarcello V., D.A. Weigent, and J.E. Blalock. 1991.Growth hormone releasing hormone receptors on thymocytes and splenocytes from rats. Cell Immunol. 136:291-302.

Gupta, M.K., H.V. Chauhan, G.J. Gha, and K.K. Singh. 1989. The role of the reticuloendothelial system in the immunopathology of Marek's disease. Vet. Microbiol. 20:223-234.

Haffer, K., M. Sevoian, and M. Wilder. 1979. The role of the macrophages in Marek's disease: *In vitro* and *in vivo* studies. Intl. J. Cancer 23:648-656.

Haley, C. S., J.F.Brown, H.A. McQueen, S. Couperwhite, and A.L. Archibald. 1993. The pig gene mapping project (PiGMaP). Pig News and Information. 14:13N-14N.

Harper, M.E., and G. F. Saunders. 1981. Localization of single copy DNA sequences on G-banded human chromosomes by *in situ* hybridization. Chromosoma 83:431-439.

Harvey, S., and K.L. Hull. 1997. Review: Growth hormone. A paracrine growth factor? Endocrine 7:267-279.

Harvey, S.1995. In Growth Hormone, Harvey,S., Daughaday,W.H., and Scanes, C.G.(eds.).CRC Press; Boca Raton, FL, pp.39-78.

Heller, E.D., and K.A. Schat. 1985. Inhibition of natural killer activity in chickens by Marek's disease virus-transformed cell lines. In B.W. Calnek and J.L. Spencer (eds.), Proc. Int. Symp. Marek's Dis. pp. 286-294. Am. Assoc. Avian Pathol. Kennett Square, PA.

Higgins, D.A. and B.W. Calnek. 1976. Some effects of silical treatment on Marek's disease. Infect. Immun. 13:1054-1060.

Hirai, K., K. Ikuta, K. Maotani, and S. Kato. 1984. Evaluation of DNA homology of Marek's disease virus, herpesvirus of turkeys and Epstein-Barr Virus under varied stringent hybridization conditions. J. Biochem. 95:1215-1218.

Honess, R.W. 1984. Herpes simplex and 'The herpes complex': diverse observations and a unifying hypothesis. J. Gen. Virol. 65:2077-107.

Honess R.W, and B. Roizman. 1974. Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14:8-19.

Honess, R.W. and D.H. Watson. 1977. Unity and diversity in the herpes viruses. J. Gen. Virol. 37:15-37.

Hong, C.C. and M. Sevoian. 1971. Interferon production and host resistance to type II avian (Marek's) leukosis virus (JM strain). Appl. Microbiol. 22:818-820.

Hong, Y., and P.M. Coussens. 1994. Identification of an immediate-early gene in the Marek's disease virus long internal repeat region which encodes a unique 14-kilodalton polypeptide. J. Virol. 68:3593-3603.

Hong, Y., M. Frame, and P.M. Coussens. 1995. A 14-kDa immediate-early phosphoprotein is specifically expressed in cells infected with oncogenic Marek's disease virus strains and their attenuated derivatives. Virology 206:695-700.

Hughes, S.K., E. Stubblefield, K. Nazerian, and H.E. Varmus. 1980. DNA of a chicken herpesvirus is associated with at least two chromosomes in a chicken lymphoblastoid cell line. Virology 105:234-240.

Hull, K.L., A.Thiagarajah, and S. Harvey. 1996. Cellular localization of growth hormone receptors/binding proteins in immune tissues. Cell Tissue Res.286:69-80.

Hunt, H., B. Lupiani, B. Miller, M. Gimeno, L. Lee, and M. Parcells. 1999. Marek's disease virus down regulates surface expression of MHC(B complex) class I (BF) glycoproteins during active but not latent infection of chicken cells. (submitted to Virology).

Isfort, R., H.J. Kung, and L. Velicer. 1987. Identification of the gene coding Marek's disease herpesvirus A antigen. J. Virol. 61:2614-2620.

Isfort, R., D. Jones, R. Kost, R. Witter, and H.-J. Kung. 1992. Retroviral insertion into herpesvirus in vitro and in vivo. Proc. Natl. Acad. Sci. USA 89:991-995.

Isfort, R., R.A. Stringer, H.J. Kung, and L.F. Velicer. 1986. Synthesis, processing and secretion of the Marek's disease herpesvirus A antigen glycoprotein. J. Virol. 57:464-474.

Johnson, B.E., C.G. Scanes, D.B. King, and J.A. Marsh.1993. Effect of hypophysectomy and growth hormone on immune development in the domestic fowl. Dev. Comp. Immunol. 17:331-339.

Jones, D., R. Isfort, R. Witter, R. Lost, and H.J. Kung. 1993. Retroviral insertions into a herpesvirus are clustered at the junctions of the short repeat and short unique sequences. Proc. Natl. Acad. Sci. USA 90:3855-3859.

Jones, D., L. Lee, J.L. Liu, H.J. Kung, and J.K. Tillotson. 1992. Marek disease virus encodes a basic-leucine zipper gene resembling the fos/jun oncogenes that is highly expressed in lymphoblastoid tumors. Proc. Natl. Acad. Sci. USA 89:4042-4046.

Jones, D., P. Brunovskis, R. Witter, and H. Kung. 1996.Retroviral insertional activation in a herpesvirus:, transcriptional activation of Us genes by an integrated long terminal repeat in a Marek's disease virus clone. J. Virol. 70:2460-2467.

Jungherr, E.L. 1939. Neurolymphomatosis phasianorum. J. Am. Vet. Med. Assoc. 94:49-52.

Jungherr, E.L. and W.F. Hughes. 1965. The avian leukosis complex. In H.E. Biester and L.H. Schwarte (eds.), Disease of Poultry. 5th Ed., pp. 512-567. Iowa State Univ. Press, Ames.

Kanamori, A., K. Ikuta, G. Veda, S. Kato, and K. Hirai. 1987. Methylation of Marek's disease DNA in chicken T-lymphblastoid cell lines. J. Gen. Virol. 68:1485-1490.

Kao, T.L., D.V. Harbour, and W.J. Meyer III. 1992. Immunoreactive growth hormone production by cultured lymphocytes. Ann. NY Acad. Sci. 650:178-181.

Kappel, M., M.B. Hansen, M. Diamant, and B.K. Pedersen.1994. In vitro effects of human growth hormone on the proliferative responses and cytokine production of blood mononuclear cells. Horm. Metab. Res. 26:612-614.

Kashanchi, F., J. Thompson, M.R. Sadaie, J. Doniger, J. Duvall, J.N. Brady, and L.J. Rosenthal. 1994. Transcriptional activation of minimal HIV-1 promoter by ORF-1 protein expressed from the Sal1-L fragment of human herpesvirus 6. Virology 201:95-106.

Kato, S., and K. Hirai. 1985. Marek's disease virus. Adv. Virus Res. 30:225-277.

Kelly, S.M., and D.L. Felten. 1995. Experimental basis for neural-immune interactions. Physiological Rev. 75:77-106.

Khansari, D.N., and T. Gustad.1991. Effects of long-term, low-dose growth hormone therapy on immune function and life expectancy of mice. Mech. Ageing Dev. 57:87-100.

Kimata, H., and A. Yoshida. 1994. Differential effect of growth hormone and insulin-like growth factor I, insulin-like growth factor II, and insulin on Ig production and growth in human plasma cells. Blood 83:1569-1574.

Klein, J. 1986. Natural history of the major histocompatibility complex. John Wiley and Sons, New York.

Kodama, H., T. Mikami, M. Inoue, and H. Izawa. 1979. Inhibitory effects of macrophages against Marek's disease virus plaque formation in chicken kidney cell cultures. J. Natl. Cancer Inst. 63:1267-1271.

Koptidesova, D., J. Kopacek, V. Zelnik, N.L.J. Ross, S. Pastorekova, and J. Pastorek. 1995. Identification and characterization of a cDNA clone derived from the Marek's disease tumore cell line RPL1 encoding a homologue of α-transducing factor (VP16) of HSV-1. Arch. Virol. 140:355-362.

Kuhnlein, U., L. Ni, S. Weigend, J.S. Gavora, W. Fairfull, and D. Zadworny. 1997. DNA polymorphisms in the chicken growth hormone gene: response to selection for disease resistance and association with egg production. Anim. Genet. 28:116-123.

Lande, R., and R.Thompson. 1990. Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124:743-756.

Lander, E.S., P . Green, J. Abrahamson, A. Barlow, and M.J. Daly. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181.

Lee, L.F. 1979. Macrophage restriction of Marek's disease virus replication and lymphoma cell proliferation. J. Immunol. 123:1088-1091.

Lee, L.F., and L.D. Bacon. 1983. Ontogeny and line differences in the mitogenic response of chicken lymphocytes. Poultry Science 62: 579-584.

Lee, L.F., E.D. Bachenheimer, B. Roizman, P.G. Spear, B.R. Burmester, and K. Nazerian. 1971. Size and composition of Marek's disease virus deoxyribonucleic acid. J. Virol. 7:289-294.

Lee, L.F., J.M. Sharma, K. Nazerian, and R.L. Witter. 1978. Suppression of mitogen-induced proliferation of normal spleen cells by macrophages from chickens inoculated with Marek's disease virus. J. Immunol. 120:1554-1559.

Lee, L.F., P.C. Powell, M. Rennie, L.J.N. Ross, and L.N. Payne. 1981. Nature of genetic resistance to Marek's disease in chickens. J. Natl. Cancer Inst. 66:789-796.

Levin,I., I. Santangelo, H. Cheng, L.B. Crittenden, and J.B. Dodgson. 1994. An autosomal genetic linkage map of the chicken. J. Hered. 85: 73-78.

Lincoln, S.E., M. Daly, and E.S. Lander. 1992. Constructing Genetic Maps With MAPMAKER/EXP 3.0, Whitehead Institute Technical Report, Ed.3, Cambridge, MA.

Long, P.A., J.L. Clark, and L.E. Velicer. 1975. Marek's disease herpesviruses. II. Purification and further characterization of Marek's disease herpesvirus A antigen. J. Virol. 15:1192.

Longenecker, B.M., and T.R. Mosmann. 1981. Structure and properties of the major histocompatibility complex of the chicken. Speculations on the advantages and evolution of polymorphism. Immunogen. 13:1-23.

Lytras, A., N. Quan, M.E. Vrontakis, J.E. Shaw, P.A. Cattini, and H.G. Friesen. 1993. Growth hormone expression in human Burkitt lymphoma serum-free ramos cell line. Endocrinology 132:620-628.

Maotani, K., A. Kanamori, K. Ikuta, S. Veta, S. Kato, and K. Hirai. 1986. Amplification of a tandem direct repeat within reverted repeats of Marek's disease virus DNA during serial *in vitro* passage. J. Virol. 58:657-660.

Maray, T., M. Malkinson, and Y. Becker. 1988. RNA transcripts of Marek's disease virus (MDV) serotype 1 infected and transformed cells. Virus Genes 2:49-68.

Marek, J. 1907. Multiple Nervenentzuendung bei Huehnern. Deutsche Tieraerztliche Wochenschrift 15: 417.

Marsh, J.A. 1992. Neuroendocrine-immune interactions in the avian species – a review. Poult. Sci. Rev.4:129-167.

McColl, K., B.W. Calnek, W.V. Harris, K.A. Schat, and L.F. Lee. 1987. Expression of a putative tumor-associated antigen on normal versus Marek's disease virus-transformed lymphocytes. J. Nat. Cancer Inst. 79:991-1000.

McCarthy, E.M., and J.A.Phillips. 1998. Characterization of an intron splice enhancer that regulates alternative splicing of human GH pre-mRNA. Hum. Mol. Genet.7:1491-1496.

Miller, M.M.., R.M. Goto, R.L. Jr. Taylor, R. Zoorob, C. Auffray, R.W. Briles, W.E. Briles, and S.E. Bloom. 1996. Assignment of Rfp-Y to the chicken MHC/NOR microchromosome and evidence for high frequency recombination associated with the nucleolar organizer region. Proc. Nat. Acad. Sci. USA 93:3958-3962.

Murphy, W.J., S.K. Durum, and D.L. Longo. 1992. Human growth hormone promotes engraftment of murine or human T-cells in severe combined immunodeficient mice. Proc. Nat. Acad. Sci. USA 89:4481-4485.

Murphy, W.J., S.K. Durum, and D.L. Longo. 1993. Differential effects of growth hormone and prolactin on murine T-cell development and function. J. Exp. Med. 178 :231-236.

Nakajima, K., K. Ikulta, M. Naito, S. Ueda, S. Kato, and K. Hirai. 1987. Analysis of Marek's disease virus serotype-1 specific phosphorylated polypeptides in virus-infected cells and Marek's disease lymphoblastoid cells. J. Gen. Virol. 68:1379-1390.

Nakajima, K., T. Shibayama, M. Yokota, K. Ikuta, S. Kato, and K. Hirai. 1989. Identification of virus-specific polypeptides by monoclonal antibodies against serotype-2 Marek's disease virus. J. Gen. Virol. 70:2563-2572.

Nakamura, Y., M. Leppert, P. O'Connell, R. Wolff, T. Holm, M. Culver, C. Martin, E. Fujimoto, M. Hoff, E. Kumlin, and R. White. 1987. Variable number of tandem repeat (VNTR) markers for human gene mapping. Science. 235:1616-1622.

Nazerian, K. 1970. Attenuation of Marek's disease virus and study of its properties in two different cell cultures. J. Natl. Cancer Inst. 44:1257-1267.

Nazerian, K., and B.R. Burmester. 1968. Electron microscopy of a Herpesvirus associated with the agent of Marek's disease in cell culture. Cancer Res. 28:2454-2462.

Nazerian, K., L.F. Lee, N. Yanagida, and R. Ogawa. 1992. Protection against Marek's disease by a fowlpox virus recombinant expressing the glycoprotein B of Marek's disease virus. J. Virol. 66:1409-1413.

Nicholas, J., and M.E.E. Martin. 1994. Nucleotide sequence analysis of a 39.5-kilobase-pair region of the genome of human herpesvirus 6 encoding human cytomegalovirus immediate-early gene homologs and transactivating functions. J. Virol. 68:597-610.

Niikura, M., Y. Matsuura, M. Hattori, M. Onuma, and T. Mikami. 1991. Expression of the A antigen (gp57-65) of Marek's Disease virus by a recombinant baculovirus. J. Gen. Virol. 72:1099-1104.

Oei, H.L., and G.F. deBoer. 1986. Comparison of intrmuscular and subcutaneous administration of Marek's disease vaccine. Avian Pathol. 15:569-579.

Okazaki, W., H.G. Purchase, and B.R. Burmester. 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Disease 14:413-429.

Omar, A.R. and K.A. Schat. 1996. Syngeneic Marek's disease virus (MDV)-specific cell-mediated immune responses against immediate early, late, and unique MDV proteins. Virology 222:87-99.

Ozaki, K., H. Kodama, M. Onuma, H. Iqawa, and T. Mikami. 1983. In vitro suppression of proliferation of Marek's disease lymphoma cell line (MDCC-MSB1) by peritoneal exudate cells from chickens infected with MDV or HVT. Zentralbl. Veterinaermed. (B) 30:223-231.

Pandian, M.R. and G.P. Talwar. 1971. Effect of growth hormone on the metabolism of thymus and on the immune response against sheep erythrocytes. J. Exp Med. 134:1095-113.

Parcells, M.S., A.S. Anderson, and R.W. Morgan. 1995. Retention of oncogencity by a Marek's disease virus mutant lacking six unique short region genes. J. Virol. 69:7888-7898.

Parcells, M.S., A.S. Anderson, J.L. Cantello, and R.W. Morgan. 1994. Characterization of Marek's disease virus insertion and deletion mutants that lack US1 (ICP22 homolog), US10, and/or US2 and neighboring short-component open reading frames. J. Virol. 68:8239-8253.

Payne, L.N. 1985. Pathology, p. 43-75. Payne, L.N., (ed.). Marek's disease. Martinus Nijhoff, Boston.

Payne, L.N. 1992. Pathogenesis of Marek's Disease: recent development s. En G. de Boer and S.H.M. Jeurissen (eds). In Proc. 4th Int. Symp. Marek's Disease. Ponsen & Looijen, Wageningen, Amsterdam, The Netherlands, pp.189-194.

Payne, L.N. and J. Roszkowski. 1973. The presence of immunologically uncommitted bursa and thymus dependent lymphoid cells in the lymphomas of Marek's disease. Avian Pathol. 1:27-34.

Peng, F.Y., J. Donovan, S. Spector, A. Tanaka, and M. Nanoyama. 1993. Prolonged proliferation of primary chicken embryo fibroblasts transfected with cDNAs from the BamHI-H gene family of Marek's disease virus. Intl. J. Oncol. 3:587-591.

Pepose, J.S., J.G. Stevens, M.L. Cook, and P.W. Lampert. 1981. Marek's disease as a model for the Lan-dry-Guillain-Barre Syndrome: Latent viral infection in nonneuronal cells is accompanied by specific immune responses to peripheral nerve and myelin. Am. J. Pathol. 103:309-320.

Pierpaoli, W., and E. Sorkin. 1968. Hormones and immunologic capacity. I. Effects of heterologous antigrowth hormone (ASTH) antisera on thymus and peripheral lymphatic tissue in mice. Induction of a wasting syndrome. J. Immunol. 101:1036-1043.

Powell, P.C. 1976. Studies on Marek's disease lymphoma-derived cell lines. Biblio. Haemat. Biblioteca Haematologica J1 - Biblioteca Haematologica J2 - Biblioteca Haematologica 43:348-350.

Powell, P.C. 1985. Immunity. In L.N. Payne (ed.) Marek's disease. pp. 177-201. Martimus Nijhoff. Boston.

Powell, P.C., K.J. Hartley, B.M. Mastill, and M. Rennie. 1983. Studies on the role of macrophages in Marek's disease of the chicken. J. Reticul. Soc. 34:289-297.

Powell, P.C., L.F. Lee, B.M. Mastill, and M. Rennie. 1982. The mechanism of genetic resistance to Marek's disease in chickens. Int. J. Cancer 29:169-174.

Purchase, H.G., and P.M. Biggs. 1967. Characterization of five isolates of Marek's disease. Res. Vet. Sci. 8:440-449.

Purchase, H.G., and W. Okazaki. 1971. Effect of vaccination with herpesvirus of turkeys (HVT) on horizontal spread of Marek's disease herpesvirus. Avian. Dis. 15:391-397.

Purchase, H.G., W. Okazaki, and B.R. Burmester. 1972. The minimum protective dose of the herpesvirus turkeys vaccine against Marek's disease. Vet. Rec. 91:79-84.

Ren, D., L.F. Lee, and P.M. Coussens. 1994. Identification and characterization of Marek's disease virus genes homologous to ICP27 and glycoprotein K of herpes simplex virus-1. Virology 204:242-250.

Render,C.L., K.L. Hull, and S. Harvey. 1995. Expression of the growth hormone gene in immune tissue. Endocrine 3:729-735.

Rispens, B.H., H. van Vloten, N. Mastenbroek, H.J. Maas, and I.C. Hendrick. 1972. Control of Marek's disease virus strain (CVI988) and its use in laboratory vaccination trial. Avian Disease 16: 126-138.

Roizman, B., and A.E. Sears. 1995. Herpes simplex viruses and their replication, p. 2231-2296. Fields, B.N., Knipe, D.M., and Howley, P.M. (eds.). Virology. Lippincott-Raven, Philadelphia, New York.

Roizman, B., R.C. Desrosiers, B. Fleckenstein, C. Lopez, A.C. Minson, and M.J. Studdert. 1992. The Family Herpesviridae: an update. Arch. Virol. 123:425-49.

Ross, L.J. 1985. Molecular Biology of the Virus, p. 113-150. Payne, L.N. (ed.). Marek's Disease - Scientific Basis and Methods of Control. Martinus Nijhoff Publishing, Boston.

Ross, L.J. and M.M. Binns. 1991. Properties and evolutionary relationships of the Marek's disease virus homologous of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. J. Gen. Virol. 72:939-947.

Ross, L.J., M.M. Binns, P. Tyers, J. Pastorek, V. Zelnik, and S. Scott. 1993. Construction and properties of a turkey herpesvirus recombinant expressing the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 74:371-377.

Ross, L.J., M. Sanderson, S.D. Scott, M.M. Binns, T. Doel, and B. Milne. 1989. Nucleotide sequence and characterization of the Marek's disease virus homologue of glycoprotein B of herpes simplex virus. J. Gen. Virol. 70:1789-1804.

Ross, L.J.N, P.M. Biggs, and A.A. Newton. 1973. Purification and properties of the 'A' antigen associated with Marek's disease virus infections. J. Gen. Virol. 19:299.

Ross, L.J.N., B. Milne, and P.M. Biggs. 1983. Restriction endonuclease analysis of Marek's disease virus DNA homology between strains. J. Gen. Virol. 64:2785-2790.

Ross, L.J.N., M.M. Binns, and J. Pastorek. 1991. DNA sequence and organization of genes in a 5.5 kbp EcoRI fragment mapping in the short segment of Marek's virus (strain RB1B). J. Gen. Viol. 72:949-954.

Rothschild, M. F. 1994. Genome Mapping in Livestock: A Journey, Not a Destination. Proceeding of the Meeting of Future Genetics for the Animal Industry. 1:19-23. St. Louis, MO.

SAS Institute. 1988. SAS/STAT User's Guide, Ed.6.03. SAS Institute Inc., Cary, NC.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning, A Laboratory Manual (2nd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schat, K.A. 1985. Characteristics of the Virus. In L.N. Payne (ed.). Marek's disease. Developments in Veterinary Virology. Martinus Nijhoff, Boston, MA. pp.77-112.

Schat, K.A. 1987. Immunity in Marek's disease and other tumors. In A. Toivanen and P. Toivanen (eds.), Avian Immunology: Basis and Practice, pp.101-128. CRC Press, Boca Raton, FL.

Schat, K.A., B.W. Calnek, and J. Fabricant. 1981. Influence of oncogenicity of Marek's disease virus on evaluation of genetic resistance. Poult. Sci. 60:2559-2566.

Schat, K.A., A. Buckmaster, and L.J. Ross. 1989. Partial transcription map of Marek's disease herpesvirus in lytically infected cells and lymphoblastoid cell lines. Int. J. Cancer 44:101-109.

Schat, K.A., C.-L.H. Cheng, B.W. Calnek, and D. Char. 1991. Transformation of T-lymphocytes subsets by Marek's disease herpesvirus. J. Virol. 65:1408-1413.

Schat, K.A., R.D. Schultz, B.W. Calnek, J. Hilgers, and D.S. Yohn. 1978. Marek's disease: Effect of virus pathogenecity and genetic susceptibility on response of peripheral blood lymphocytes to concanavalin-A, p. 183-186. Bentvelzen, P. (ed.). Advances Comparative Leukosis Research. Elsevier, Amsterdam.

Scott, S.D., N.L. Ross, and M.M. Binns. 1989. Nucleotide and predicted amino acid sequences of the Marek's disease virus and turkey herpesvirus thymidine kinase genes; comparison with thymidine kinase genes of other herpesviruses. J. Gen. Virol. 70:3055-3065.

Scott, S.D., G.D. Smith, N.L. Ross, and M.M. Binns. 1993. Identification and sequence analysis of the homologues of the herpes simplex virus type 1 glycoprotein H in Marek's disease virus and the herpesvirus of turkeys. J. Gen. Virol. 74:1185-1190.

Sevoian, M. and D.M. Chamberlain. 1962. Avian lymphomatosis. II. Experimental reproduction of the ocular form. Vet. Med. 57:608-609.

Sevoian, M., D.M. Chamberlain, and F.T. Counter. 1962. Avian lymphomatosis. I. Experimental reproduction of the neural and visceral forms. Vet. Med. 57:500-501.

Shama, J.M. and H.G. Purchase. 1974. Replication of Marek's disease virus in cell cultures derived from genetically resistant chickens. Infect. Immun. 9:1092-1097.

Sharma, J.M. 1980. In vitro suppression of T-cell mitogenic response and tumor cell proliferation by spleen macrophages from normal chickens. Infect. Immun. 28:914-922.

Sharma, J.M. 1981. Fractionation of Marek's disease virus-induced lymphoma by velocity sedimentation and association of infectivity with cellular fractions with and without tumor antigen expression. Am. J. Vet. Res. 42:483-486.

Sharma, J.M. 1983. Presence of adherent cytotoxic cells and nonadherent natural killer cells in progressive and regressive Marek" disease tumors. Vet. Immunol. Immunopathol. 5:125-140.

Sharma, J.M., and B.D. Coulson. 1977. Cell-mediated cytotoxic response to cells bearing Marek's disease tumor-associated surface antigen in chickens infected with Marek's disease virus. J. Natl. Cancer Inst. 58:1647-1651.

Sharma, J.M., and R.L. Witter. 1975. The effect of B-cell immunosuppression on age-related resistance of chickens to Marek's disease. Cancer Res. 35:711-717.

Shek, W.R., B.W. Calnek, K.A. Schat, and C.-L.H. Chen. 1983. Characterization of Marek's disease virus-infected lymphocytes: Discrimination between cytolytically and latently infected cells. J. Natl. Cancer Inst. 70:485-491.

Silva, R.F, and R.L. Witter. 1985. Genomic expansion of Marek's disease virus DNA is associated with serial *in vitro* passage. J. Virol. 54:690-696.

Silva, R.F. 1992. Differentiation of pathogenic and nonpathogenic serotype 1 Marek's disease viruses (MDVs) by the polymerase chain reaction amplification of the tandem repeats within the MDV genome. Avian Dis. 36:521-528.

Soller, M, and J.S. Beckmann. 1983. Genetics polymorphism in variety identification and genetic improvement. Theoretical App. Genet. 67:25-33.

Soller, M., T. Brody. and A. Genizi. 1976. On the power of experimental designs for the detection of linkage between marker loci and quantitative loci in crosses between inbred lines. Theoretical App. Genet. 47:35.

Solomon, J.J., R.L. Witter, K. Nazerian, and B.R. Burmester. 1968. Studies on the etiology of Marek's Disease. I. Propagation of the agent in cell culture (32649). Proc. Soc. Exptl. Biol. Med. 127: 173-177.

Spencer, J.L., J.S. Gavora, A.A. Grunder, A. Robertson, and G.W. Speckmann. 1974. Immunization against Marek's disease: influence of strain of chickens, maternal antibody, and type of vaccine. Avian Dis. 18:33-44.

Stasiak, P.C., and E.S. Mocarski. 1992. Transactivation of the cytomegalovirus ICP36 gene promoter requires the α gene product TRS1 in addition to IE1 and IE2. J. Virol. 66:1050-1058.

Stone, H.A. 1975. Use of highly inbred chickens in research. USDA Technical Bulletin No. 1514.

Sui, D., P. Wu, H.J. Kung, and L.F. Lee. 1995. Identification and characterization of a Marek's disease virus gene encoding DNA polymerase. Virus Res. 36:269-278.

Tanaka, M., Y. Hosokawa, M. Watahiki, and K. Nakashima. 1992. Structure of the chicken growth hormone-encoding gene and its promoter region. Gene 112: 235-239.

Tanksley, S. D., H. Medina-Filho and C.M. Rick. 1982. Use of naturally occurring enzyme variation to detect and map genes controlling quantitative traits in an interspecific backcross of tomato. Heredity 49:11-25.

Tillotson, J.K., L.F. Lee, H.J. Kung, T. Horiuchi, T. Mikami, and K. Hirai. 1988. Accumulation of viral transcripts coding for a DNA binding protein in Marek's disease tumor cells, p. 128-134. Kato, S. (ed). Advances in Marek's disease research. Japanese Assoc. on Marek's Dis., Osaka.

Tomley, F., M. Binns, J. Campbell, and M. Boursnell. 1988. Sequence analysis of an 11.2 kilobase, near-terminal, BamHI fragment of fowlpox virus. J. Gen. Virol. 69:1025-1040.

Vallejo, R. V., L.D. Bacon, H.C. Liu, R.L. Witter, M.A. Groenen, J. Hillel, and H.H. Cheng. 1997. Genetic mapping of quantitative trait loci affecting susceptibility to Marek's disease virus induced tumors in F_2 intercross chickens. Genetics 148:349-360.

Vallejo, R. V., G.T.Pharr, H.C. Liu, H.H. Cheng, R.L. Witter, and L.D. Bacon. 1997 Non - association between *RFP-Y* MHC-like genes and susceptibility to Marek's disease virus induced tumors in 6_3 x 7_2 F_2 intercross chickens. Anim. Genet. 28: 331-337.

Vasilatos-Younken, R. 1995. Proposed mechanisms for the regulation of growth hormone action in poultry: metabolic effects. J. Nutrition 125:1783S-1789S.

deVicente, M.C., and S.D. Tanksley. 1993. QTL analysis of transgressive segregation in an interspecific tomato cross. Genetics 134:585-596.

Volpini, L.M., B.W. Calnek, M.J. Sekellick, and P.I. Marcus. 1995. Stages of Marek's disease virus latency defined by variable sensitivity to interferon modulation of viral antigen expression. Vet. Microbiol. 47:99-109.

Wagner, E.K. 1991. Herpesvirus transcription: general aspects. In Herpesvirus Transcription and its Regulation. (E.K. Wagner, ed.) CRC Press, Inc. Florida.

Wakenell, P.S., M.M. Miller, R.M. Goto, W.J. Gauderman, and W.E. Briles. 1996. Association between the Rfp-Y haplotype and the incidence of Marek's disease in chickens. Immunogenetics 44:242-245.

Weigent, D.A. and J.E. Blalock. 1991. The production of growth hormone by subpopulations of rat mononuclear leukocytes. Cell Immunol. 135;55-65

Weigent, D.A., and J.E. Blalock. 1995. Growth hormone effects on the immune system. In: Shiverick KT, Rosenbloom AL (eds.) Growth Hormone: Basic and Clinical Aspects. CRC Press. Boca Raton, pp141-152.

Weigent, D.A., J.E. Blalock, and R.D. LeBoeuf. 1991. An antisense oligonucleotide to growth hormone messenger ribonucleic acid inhibits lymphocyte proliferation. Endocrinology 128:2053-2057.

Weigent,D.A., J.B., Baxter, W.E. Wear, L.R. Smith, K.L. Bost, and J.E. Blalock. 1988. Production of immunoreactive growth hormone by mononuclear leukocytes. FASEB J. 2:2812-2818.

Wilson, M.R., and P.M. Coussens. 1991. Purification and characterization of infectious Marek's disease virus genome using pulsed field electrophoresis. Virology 185:673-680.

Witter, R.L. 1982. Protection by attenuated and polyvalent vaccines against highly virulent strains of Marek's disease virus. Avian Pathol. 11:49-62.

Witter, R.L. 1997. Avian tumor viruses: persistent and evolving pathogens. Acta Vet. Hungarica. 45:251-266.

Witter, R.L., D. Li, D. Jones, L.F. Lee, and H.-J. Kung. 1997. Retroviral insertional mutagenesis of a herpesvirus: a Marek's disease virus mutant attenuated for oncogenicity but not for immunosuppression or in vivo replication. Avian Dis. 41:407-421.

Witter, R.L., J.M. Sharma, and A.M. Fadly. 1980. Pathogenicity of variants Marek's disease isolates in vaccinated and unvaccinated chickens. Avian Dis. 24:210-232.

Witter, R.L., J.M.. Sharma, J.J.Solomon, and L.R. Champion. 1973. An age related resistance of chickens to Marek's disease: some preliminary observation. Avian Pathol. 2:43-54.

Wlodawer, A., A. Pavlovsky, and A. Gustchina. 1993. Hematopoietic cytokines – similarities and differences in the structures, with implications for receptor binding. Protein Sci. 2:1373-1382.

Yonash, N., L.D. Bacon, R.L. Witter, and H.H. Cheng. 1999. High resolution mapping and identification of new quantitative trait loci (QTL) affecting susceptibility to Marek's disease. Anim. Genet. 30:126-135.

Yoshida, A., C. Ishioka, H. Kimata, and H. Mikawa. 1992. Recombinant human growth hormone stimulates B-cell immunoglobulin synthesis and proliferation in serum-free medium. Acta Endocrinol. 126:524-529.

Yoshida, S., L.F. Lee, N. Yanagida, and K. Nazerian. 1994. Identification and characterization of Marek's disease virus gene homologous to glycoprotein L of herpes simplex virus. Virology 204:414-419.