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IDENTIFICATION AND CHARACTERIZATION OF MAREK'S DISEASE VIRUS GENES PUTATIVELY RESPONSIBLE FOR IMMUNOPROTECTION AND ONCOGENICITY

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By

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF MAREK'S DISEASE VIRUS GENES PUTATIVELY RESPONSIBLE FOR IMMUNOPROTECTION AND ONCOGENICITY

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Identification of Marek's disease (MD) virus (MDV) genes responsible for oncogenicity and immunoprotection is essential to understand the mechanisms involved. Through DNA sequence and cDNA analyses, an open reading frame (pp38-ORF) encoding 290 amino acids was identified in BamHI-H. Antisera against trpE-pp38 fusion proteins immunoprecipitated 38- and 24-kDa polypeptides (designated pp38 and p24, respectively). Pulse-chase and immunoblot analyses showed no precursor-product relationship between pp38 and p24; and pp38 is the primary gene product. pp38 was detected in MSB-1 lymphoblastoid cells and was found to be phosphorylated, MDV serotype-1-specific, immunogenic in birds with MD, and one of the early classes of herpesvirus proteins. Among the major glycoproteins found in MDV-infected cells is the B antigen (gp100, gp60, gp49). While the B antigen gene was not unequivocally identified, MDV's gB homolog gene was identified by others. To determine whether they are the same, antisera against trpE-MDV-gB were prepared, which immunoprecipitated gp100, gp98, gp60, gp49, and pr88. Based on trpE-MDV-gB competition and blocking assays, it was concluded that they are the same and pr88 is the primary precursor polypeptide. MDV gB homolog processing appears to involve cotranslational glycosylation of pr88 to form gp98, which is processed to yield gp100, which is then cleaved to form gp60 and

gp49. Serial in vitro passage of virulent MDV results in amplification of 132-bp repeat in its genome's long repeat regions and loss of tumorigenicity which may result from failure of gene expression. Six cDNAs were isolated, mapping in the expanded regions of virulent low passage MDV whose 132-bp repeat number ranges between one and seven. cDNA sequence and S1 nuclease analyses revealed that four groups of transcripts are either initiated or terminated within or near the expanded regions at multiple sites in both directions. These RNAs, containing various copies of 132-bp repeat at either their 5' or 3' ends, were found to be 0.67-, 1.6-, 1.8-, and 3.1-kb. Furthermore, each 132-bp repeat contains promoter and polyadenylation consensus sequences in each direction. Together, these data suggest that the 132-bp repeat and its copy number are involved in transcriptional regulation and generation of four groups of transcripts potentially responsible for MDV tumorigenicity.

To My Mom and Dad, and My Late Grandmother

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Literature Review

1. Overview of Marek's Disease Virus

(i) The Virion

Marek's disease virus (MDV) has characteristics of a typical herpesvirus, which can be identified by double-stranded linear DNA in the core of the virion (15, 16, 55, 60, 76, 80, 84), an icosahedral capsid containing 162 capsomeres assembled in the nucleus (76), and an envelope derived from the nuclear membrane (74). The enveloped particles in the nucleus of infected cells measure 150-180 nm in diameter; those from the cytoplasm measure 250-280 nm (73, 74, 76, 77). The nucleocapsid measures about 100 nm (76).

(ii) The Genome

The MDV genome has characteristics of alphaherpesvirus genome structure (17, 33); a long unique region (U_L) flanked by a long terminal repeat (TR_L) and a long internal repeat (IR_L), and a short unique region (U_S) flanked by a short internal repeat (IR_S) and a short terminal repeat (TR_S). The density of MDV DNA was calculated to be 1.705 g/cm^3 from equilibrium centrifugation in neutral CsCl density gradient (60), indicating that MDV DNA has a base composition of 46-47% guanine plus cytosine. The molecular weight of MDV DNA was calculated to be 110 x 10^6 and 119 x 10^6, from the sedimentation values by Lee et al. (60), and Hirai et al. (43), respectively. The total sum of molecular weight of DNA fragments from digestion with EcoRI, HindIII, and SalI was calculated to be 100-110 x 10^6 (33), which is approximately equal to that obtained from the sedimentation value (43, 60).

(iii) Classification

Based on the relatedness of virus antigens (16, 84), oncogenic MDVs are classified as serotype 1; nononcogenic MDVs are classified as serotype 2; and herpesvirus of turkeys (HVT) is classified as serotype 3. Based on comparison of its biological properties, especially its lymphotropism, in relation to Epstein-Barr virus (EBV) (91), MDV is classified as a gammaherpesvirus. However, based on comparison of MDV's genome structure in relation to herpes simplex virus (HSV) (17, 33), the conservation and arrangement of genes and gene clusters in relation to HSV and varicella-zoster virus (VZV) (8, 94, 95), and the arrangement of the terminal sequence involved in packaging of the viral genome in relation to HSV (58), it would be more appropriate to classify MDV as an alphaherpesvirus. Furthermore, it is suggested (91) that delineation and evolutionary relatedness of genes responsible for biological properties should be used for both evolutionary relatedness and classification, which may be a more significant criterion than the arrangement and evolution of genes conserved throughout the family herpesviridae.

(iv) Clinical Disease

MDV was found to be the causative agent of Marek's disease (MD) in the late 1960's (22, 76, 77, 107), almost six decades after the first description of the disease (16, 23, 84, 85). Clinically, MD is recognized as three forms (16, 85): the classic form, the acute form, and transient paralysis. The classic form is usually neurologic and varies with the peripheral nerves affected; commonly, there is paralysis affecting the legs and wings, occasionally torticollis, and respiratory and alimentary disorders, accompanied by loss of weight. The acute form is a more virulent type of MD than the classic form, in which lymphomatosis of various visceral organs and tissue is common, and mortality

may reach 80% of a flock. Moreover, neural involvement and signs may not be evident, particular in old birds. Transient paralysis is an uncommon acute encephalitic expression of infection, apparently healthy birds suddenly develop various locomotor disorders, such as paralysis of legs, wings, and necks, or incoordination. Mortality is low, and symptoms usually disappear within 24-48 h.

(v) Antigenic and Genetic Relatedness with HVT

HVT is isolated from turkeys, and is naturally nonpathogenic to both turkeys and chickens (56). Due to its antigenic relatedness, especially of the A (gp57-65) and B (gp100, gp60, and gp49) antigens held in common with MDV (81, 82), HVT is the most commonly used vaccine virus to prevent MD in chickens in U. S. (119).

Genetically, various degree of DNA homology between MDV and HVT were reported. The earlier studies, using cRNA hybridization or DNA-DNA reassociation kinetics revealed no or minor sequence homology between MDV and HVT DNAs (54, 61). Later, Southern blot analysis was used (35, 46). Under high stringency conditions, only the MDV BamHI B fragment formed stable hybrids with a 3.9 kbp portion of the cloned HVT BamHI J fragment (46); the corresponding regions of MDV and HVT genomes were later found to encode MDV and HVT A antigens (gp57-65) (2, 25, 26), which were then found to be 67% identical in nucleotide sequence (26). Under less stringent conditions, which permit the detection of regions of homology with as much as 30-35% base mismatch, most of the MDV DNA fragments have their counterparts in HVT's DNA, with the extent of homology at nucleotide level to be 2-5% (46) or 70-80% (35). However, the precise extent of homology between MDV and HVT will not be resolved until the complete nucleotide sequences of both MDV and HVT are determined.

2. Glycoproteins

Glycoproteins are integral parts of the enveloped viruses, including herpesvirus. They have diverse and highly important functions throughout the virus replication cycle (39, 108). First, they are involved in the attachment (adsorption) of the virus to the cell and then penetration (fusion of virus envelope with cell membrane); those capacities of the glycoproteins determine which cell type the virus can infect. Second, they are required for formation of a fully infectious viral particle (virion) and may also be essential for proper exit of the virion from the cell. Third, they stimulate both humoral and cellular immune responses of the infected host that act in congregate to bring an end to the acute infection; capacities of that are the immunological basis for the potential use of glycoproteins as subunit or recombinant DNA-based vaccines. In the MDV system, glycoproteins have also been the focus of efforts to determine the mechanism of the protective immunity conferred by HVT against MDV in chickens (26, 50, 51, 52, 102, 106).

HSV, the most extensively studied alphaherpesvirus, encodes eight well characterized glycoproteins, possibly with more to be identified (92). These are glycoproteins B, C, D, E, G, H, I, and J (designated gB, gC, gD, gE, gG, gH, gI, and gJ, respectively). The genes encoding gB, gC, and gH were located in the U_L region while the other five were located in the U_S region. Of those eight HSV glycoprotein genes, six MDV homologs (gB, gC, gD, gE, gH, and gI) were conserved and collinear with HSV (2, 7, 8, 26, 94, 95, 96). Although five of the six MDV homolog genes (gB, gC, gD, gE, and gI) have been sequenced, only MDV gB (B antigen complex; gp100, gp60, and gp49) and gC (A antigen; gp57-65) homologs were well characterized at the antigen level. Therefore, only the MDV gB and gC homologs will be discussed further.

(1) A Antigen (gp57-65; the MDV gC homolog)

A antigen was characterized chemically and physically as having an isoelectric point of 6.68, sensitivity to trypsin, stability at pH 2 to 10, a sedimentation coefficient of 3.7 S, and a tendency to aggregate (64, 65). It was reported to be a glycoprotein (64, 82, 93), with an apparent molecular weight of 57,000 to 65,000 in its fully glycosylated form (36, 52) and therefore is also designated gp57-65 (25) based on current MDV protein nomenclature (99a). A combination of pulse-chase and cell-free translation studies have shown that A antigen is first synthesized as a precursor polypeptide with an apparent molecular weight of 44,000 (pr44) (25, 52).

The gene encoding MDV A antigen was identified and located in the 2.35-kbp PvuII-EcoRI subfragment of BamHI-B of MDV genome by Isfort et al. (50), by using Northern blot analysis and hybrid selection of its mRNA with cloned MDV DNA, cell-free translation of the mRNA, and immunoprecipitation of the polypeptide. The MDV A antigen gene was subsequently sequenced by Coussens and Velicer (25), and Binns and Ross (2); it represents the MDV homolog of HSV gC gene. Therefore, MDV A antigen is referred as the MDV gC homolog hereafter. Since HSV gC was well studied and its roles in the HSV replication cycle and immunity were well understood, some properties of the HSV gC may be predicted to apply to the MDV gC homolog. The calculated molecular weight of the MDV gC homolog is 56,000 with 27 amino acid putative signal peptide, and 53,000 without the signal peptide (2, 25). However, the molecular mass of the A antigen precursor polypeptide is 44,000 Da. Considering that 44,000 Da is only an estimate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and since other factors (such as surface charge of polypeptide) affect the migration of protein in SDS-PAGE, those two estimates are not really unreasonably divergent.

The gene encoding HVT gC homolog was also identified and sequenced (26). Its location in BamHI-K_1 and -M is collinear with that of the MDV gC homolog gene. The HVT gC homolog gene is 66% identical in nucleotides to MDV's, with great similarity in the 3' proximal two thirds of the genes. Furthermore, the predicted amino acid sequence of the HVT gC homolog gene is 77% homologous (81% when conservative amino acid substitutions are considered) to MDV's, with many potential common antigenic epitopes (26).

After cleavage of a small signal peptide, the polypeptide of the MDV gC homolog is glycosylated and secreted from the cell in a precisely programmed manner (52). While the MDV gC homolog is primarily secreted from infected cells, a small amount of it is also associated with the plasma membrane in a specific manner (48, 74). Although MDV gC homolog is one of the primary proteins recognized by immune chicken sera (ICS) from naturally infected birds, its precise role, if any, in the immunoprevention or pathogenesis of MD is unclear. It has been postulated that the extensive secretion of the antigen may play an immunoevasive role in the pathogenesis of MD (52), and therefore it is a candidate for causing the early-stage immunosuppresion that occurs after MDV infection (50) and that may be one of the key events that lead to neoplastic disease (84), especially in view of the observation that various virus particles, either infectious or noninfectious, inhibit mitogenic responses (115).

Long et al. (65) reported that the rabbit antisera monospecific to MDV gC homolog did not neutralize cell-free MDV; a similar result was also obtained for antibodies to HVT gC homolog (55). However, immunization of mice with purified HSV gC or HSV gC generated by recombinant vaccinia virus induces a neutralizing antibody response that protects against lethal HSV-1 challenge (88, 100, 116). HSV gC was reported to be an important antigen for HSV-1-specific memory cytotoxic T lymphocytes (CTL) (38), and acts as a receptor for C3b

component of complement (37). HSV gC was also reported to be involved in the process of virus adsorption process (66), and is suggested to be one of the virus proteins which may bind to cellular heparan sulphate. Although HSV gC is one of the most abundent viral surface antigens in infected cells (37), and MDV gC homolog is primarily secreted into cell culture media from infected cells (36, 52), these two antigens are well conserved (2, 26). With sequence analysis (2, 25) and large-scale expression (79) of MDV gC homolog gene by the baculovirus expression vector system, it is now possible to determine whether MDV gC homolog has similar functions as HSV gC.

(ii) B Antigen (gp100, gp60, and gp49; the MDV gB Homolog)

MDV B antigen was identified by Velicer et al. (114) as a glycoprotein that is found primarily in the infected cell lysate; can be eluted from concanavalin A by α-methylmannoside; is resistent to a pH of 2.0, 1 M urea, and 0.05% Brij; and has a pI of 4.54, a sedimentation coefficient of 4.4 S, and an apparent molecular weight of 58,000 or 69,000, as determined either by gel filtration on sephadex G-200 or from the sedimentation coefficient, respectively (114). With SDS-PAGE analysis, three glycoproteins with apparent molecular masses of 100, 60, and 49 kDa (101, 102) were found to be MDV B antigen, therefore were redesignated as B antigen complex (gp100, gp60, and gp49) (51, 101, 102). The common HVT B antigen complex also consists of three similar sized glycoproteins (20, 49, 101). Tunicamycin (TM) inhibition of N-linked glycosylation resulted in identification of various unglycosylated, or possibly O-linked glycosylated, precursor polypeptides: pr88/pr83 of MDV and pr90 of HVT (49), pr80/pr110/pr125 of MDV (29), or pr88 and pr44 of MDV and HVT (51, 106). In the latter report, it was hypothesized that pr44 dimerizes to form pr88 (106).

A preliminary report by Sithole et al. (105) suggested that the gene encoding the MDV B antigen complex was located in the BamHI-H fragment, which spans the junction of the U_L and IR_L regions of the MDV genome. Upon further analysis, Chen et al. (18) reported that a phosphoprotein pp38 was encoded in this region of BamHI-H; moreover, no open reading frames characteristic of glycoprotein genes were identified. Thus, the location of the gene encoding the MDV B antigen complex remained to be determined. Recently, Ross et al. (96) reported the identification of the gene encoding the MDV homolog of HSV glycoprotein B (gB) (MDV gB homolog gene), using Southern blot analysis with a DNA fragment containing HSV gB gene as a probe. By Western blot analysis with antipeptide sera generated against the synthetic oligopeptides predicted from the coding sequence of the MDV gB homolog, the MDV gB homolog was found to be composed of gp110, gp64, and gp48 (96). In the presence of TM, three smaller polypeptides (94, 90, and 84 kDa) were found to represent the unglycosylated, or possibly O-linked glycosylated, precursor polypeptides of MDV gB homolog, with an additional 48 kDa polypeptide suggested to be a truncated form of the former ones (96). Solely based on the estimated sizes of the three glycoproteins, they suggested that MDV gB homolog could be the MDV B antigen complex.

Chen and Velicer (20) expressed the MDV gB homolog gene as trpE fusion proteins (trpE-MDV-gB) and antisera against trpE-MDV-gB were prepared. Based on immunoprecipitation analyses with antibodies to trpE-MDV-gB, and the use of trpE-MDV-gB competition and blocking assays, the MDV B antigen complex was found to be the MDV gB homolog. Further, size comparison of the three glycoproteins of the B antigen complex with those detected with anti-peptide sera prepared based on the sequence of the newly discovered MDV gB homolog (96), are consistent with this finding (20). Finally

the glycoprotein processing pathway which will be discussed later is typical of those for gB homologs of other herpesviruses. Therefore, MDV B antigen complex is hereafter refered to the MDV gB homolog. Since HSV gB is extensively studied, and the roles of HSV gB in the virus replication cycle and immunity are well understood, some properties of HSV gB may be predicted to apply to MDV gB homolog.

Chen and Velicer (20) reported that, with TM inhibition, and trpE-MDV-gB competition and blocking assays, pr88 was found to be the primary unglycosylated (or possibly O-Linked glycosylated) precursor polypeptide of the MDV gB homolog gene. This interpretation is also supported by reports from studies of gB homolog proteins of other herpesviruses. In cases of equine herpesvirus (EHV) (68, 110), cytomegalovirus (CMV) (5, 6), VZV (39, 69), pseudorabies virus (PRV) (89, 117), and bovine herpesvirus (BHV) (32), where the mature gB homolog proteins are cleaved to form two smaller products, only one unglycosylated precursor polypeptide was observed; and no cleavage of the unglycosylated precursor polypeptide was reported.

In addition to gp100, gp60, and gp49, an additional glycoprotein, gp98, was detected with antisera against trpE-MDV-gB and found to be an intermediate of gp100 of the MDV gB homolog (20). This result is similar to a report by Sithole et al. (106) that a similar sized molecule was detected at 0 and 3 min chase intervals after a 5 min pulse in an earlier study of B antigen complex processing. With identification of gp98 as an intermediate between pr88 and gp100 (20), the MDV gB homolog processing pathway (20) appears similar to that observed for EHV (68, 110), CMV (5, 6), PRV (89, 117), and VZV (39, 69) gB homolog processing. It appears that the unglycosylated precursor polypeptide, pr88, which is seen only after TM inhibition, is cotranslationally glycosylated to form gp98 (20). gp98 is further processed to form gp100 (20),

possibly by sialylation. Then gp100 is cleaved to form gp60 and gp49 (20, 49, 102, 106), which can be blocked by monensin (a monovalent ionophore inhibiting transport of glycoproteins) (106). Based on the estimated molecular masses of approximately 44 kDa for the deglycosylated backbone polypeptides of both gp60 and gp49 on an SDS-polyacrylamide gel (106), and the calculated molecular masses of the predicted amino acid sequences of MDV gB homolog for the N-terminal and C-terminal halves being 47,723 and 47,930 Da (20, 96), respectively, the molecular masses of the amino acids for both gp60 and gp49 are approximately equal. Therefore, the size difference between gp60 and gp49 is likely due to the disproportionate extent of their glycosylation. Examination of potential N-linked glycosylation sites in the predicted amino acid sequence of MDV gB homolog (96) showed that eight are located in the N-terminal half (excluding one site in the signal peptide) and one is located in the C-terminal half. With each glycan contributing approximately 2.5 kDa (59) to a glycoprotein's apparent molecular mass, gp60 and gp49 would likely be derived from the N- and C-terminal halves of gp100, respectively (20).

In the MDV system a molecule called pr44 may have mistakenly been thought to be the primary precursor polypeptide of MDV gB homolog (B antigen complex) (51, 106), leading to an incorrect hypothesis that pr44 dimerizes to form pr88 (106), for the following three reasons. First, there was an early report that pr44 was the most prominant band seen after TM treatment, using the same antisera that immunoprecipitated gp100, gp60, and gp49 (102). Second, several proteins (including the 44 kDa protein previously called pr44) of that approximate size were apparently nonspecifically trapped in the earlier work (51, 106); as has been recently demonstrated by Chen and Velicer (20) during immunoprecipitation by the monoclonal antibody IAN86, RαPM, antisera against trpE-MDV-gB, and ICS. These nonspecifically trapped proteins seen in the

immunoprecipitates of MDV-infected cell lysates were also observed in the immunoprecipitates of mock-infected cell lysates at a relatively lower level (20, 106), suggesting that they may be cell proteins. Third, since gp100 of the MDV gB homolog is cleaved to form gp60 and gp49, the cleavage results in formation of the N- and C-terminal halves with the calculated molecular masses of their polypeptides being approximately equal, 47,723 and 47,930 Da, respectively (20, 96). The previous results, obtained by deglycosylation of gp60 and gp49 by endo-F and -H (106), indicated that the backbone polypeptides of gp60 and gp49 have similar estimated molecular masses (approximately 44 kDa), which are in good agreement with the calculated molecular masses for N- and C-terminal halves. Based on the above and other data, it was originally concluded that pr44 was the primary polypeptide, and it was further hypothesized that pr44 dimerizes to form pr88 (106). The results recently reported by Chen and Velicer (20) indicate that the previous hypothesis is not correct and that pr88 is the primary product of the MDV gB homolog gene.

When antisera against either the N- or C-terminal half of the MDV gB homolog were used (20), both gp60 and gp49 were detected in addition to gp100. The results suggest that gp49 is specifically associated with gp60 through a disulfide bond(s) which is(are) then broken on the reducing SDS-polyacrylamide gel, as was the case for the two cleaved forms of EHV and VZV gB homologs (68, 69).

It has been known for sometime that antibodies to the MDV gB homolog neutralize MDV in cell culture (49), and that affinity purified HVT gB homolog elicits partial protection against MD in chickens (83). It is not clear if this partial protection is because another antigen(s) is needed, or because of inadequate antigen, adjuvant or other factors. Identification of the gene encoding this important antigen will make it possible to use molecular biological

approaches to study immunoprotection against MD in more depth, including resolution of the questions presented above. MDV gB homolog was shown to be located on the surface of infected cell as well as in the cytoplasm and nucleus by immunofluorence and cell fractionation (49; unpublished result, X. Chen and L. F. Velicer). The MDV gB homolog could be a virion envelope component and therefore important for virus adsorption, as is the case for HSV gB (40, 41, 42), because virus neutralization is usually associated with structural glycoproteins of viruses.

Besides the known roles held in common between HSV gB and MDV gB homolog (protective immunity, and possibly in virus adsorption), HSV gB was found to play several other roles in the HSV replication cycle. HSV gB promotes virus entry (11, 12, 13, 40, 41, 42, 63) since single-amino-acid differences within the gB polypeptide alter the rate of entry (11, 12, 13, 41) and extent of virus-induced cell fusion (30, 41). Monoclonal antibodies to gB do not block cell fusion, but have been found to alter the rate of virus entry and to reduce plaque size, which indicates that gB affects the spread of virus (41). Mutations that increase the cell fusion function of gB are located in the cytoplsmic domain; and those that decrease the cell fusion function of gB are located in the external domain (11, 12, 13, 41). Since HSV gB is a HSV virion component, it is also involved in the virus envelopement (97). With identification of MDV gB homolog gene (96) which encodes B antigen complex (20), mutational analysis of MDV gB homolog will facilitate the identification of the roles of MDV gB homolog in the MDV replication cycle.

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3. Latency

When chickens are infected by MDV, the initial lytic infection occurs mainly in nonlymphocytes but occasionally in lymphocytes. Fully enveloped virions are produced in the feather follicle epithelial cells, which is the only cell where infectious virions can be isolated (16, 84, 85). Viral antigens are expressed in all the infected cells. At about 6-7 days postinfection, the infection switches to latency, coincident with the developement of immune response. Cell-mediated immunity (CMI) has been shown to be important in the switch (9), perhaps through a soluble mediator (10). While the lytically infected cells are nonlymphocytes, the latently infected cells are basically lymphocytes (14, 15, 87), although infected nonlymphocytes such as Schwann and satellite cells in spinal ganglia (86) were observed. Most of the latently infected lymphocytes are activated T cells which are $CD4^{+}CD8^{-}$ (99). Therefore, most of the latently infected T cells are helper T cells (99). To a limited extent, the infected lymphocytes are B cells (14, 15).

To understand the molecular mechanism of how latency is established and maintained, MDV transformed lymphoblastoid cell lines are used. The cell lines can be grouped into three types. Type 1 is the partial nonproducer cell lines, such as MSB-1 (1), which may contain up to about 2% spontaneously reactivated lytically infected cells. Type 2 is the nonproducer cell lines, such as MTK-1 (104, 111). MDV can be rescued when MTK-1 is co-cultivated with CEF cells or is inoculated into chickens. Type 3 is the true nonproducer cell lines, such as RP1 (78). MDV can not be rescued, even though this true nonproducer cell line is co-cultivated with CEF cells or is inoculated into chickens. Due to the above characteristics, and limited expression of MDV genome (15, 16, 55, 85), lymphoblastoid tumor cell lines have been used, but have not been universally accepted as an *in vitro* system for MDV latency.

The transformed lymphoblastoid cell lines contain about 5-15 copies of the MDV viral genome. However, the number of the viral genome copies varies in different cell lines and within the same cell line under different conditions, perhaps in relation to the proportion of the lytically infected cells in the population (14, 44, 75). The viral genomes in the transformed cell lines were shown to exist as closed circular DNA (44, 111), although it can not be excluded that viral DNA is integrated into cellular chromosome (45). The viral DNA in the cell lines is extensively methylated, whereas methylation was not detected in viral DNA in the lytically infected cells (53), which may regulate gene expression in the latently infected cells. It has been known that DNA methylation can regulate gene expression (62); the methylated gene is inactive, but the nonmethylated gene is active.

Since tumor cell lines are transformed and possibly are latently infected, genes expressed in the tumor cell lines may be responsible for latency and transformation. A limited degree of latent MDV genome transcription has been demonstrated by DNA-RNA reassociation kinetics in nonproducer cell lines, compared with the transcription from nearly the whole genome in the lytically infected cells (31). Recently, Northern blot analysis was used to detect transcripts in the tumor cell lines: 4 to 7 transcripts were reported by Schat et al. (98) in their partial transcriptional mapping of MDV; 32 transcripts clustered in the short and long repeat regions were reported by Suguya et al. (109); and 29 transcripts spread over almost the entire MDV genome were reported by Maray et al. (67).

Although many transcripts have been detected in the tumor cell lines, so far only one phosphoprotein, pp38, was detected (18, 47, 72). Silva and Lee (101) first reported the existence of three viral proteins of 41, 38, and 24 kDa (then designated p41, p38, and p24, respectively) that were detected only in

serotype-1 MDV (oncogenic MDV)-infected cells, but not in the nononcogenic serotype-2 MDV and nononcogenic serotype-3 HVT-infected fibroblast cells. p38 was the most prominant molecule. Later, Ikuta et al. (47) and Nakajima et al. (72) reported a similar group of MDV specific antigens of 43, 39, 36, and 24 kDa, that were also detected in the latently infected MSB-1 lymphoblastoid tumor cells, but only when the cells were treated with 5-iodo-2-deoxyuridine (IUdR). The latter workers (72) reported that these proteins were phosphorylated at serine residues. Furthermore, based on in vitro translation analysis, they suggested that the 24 kDa and 39/36 kDa (their newer terminology) phosphoproteins are translated from distinct mRNAs and are encoded from overlapping genes, or from separate regions with partial DNA homology, within the MDV genome (72). The MDV-specific phosphoproteins were also detected in tumor lesions of chickens with MD (71). Cui et al. (27) further reported identifying two λgt11 clones which may encode p41, p38, and p24 (101). Both clones were mapped to the BamHI-H fragment of the MDV genome. Five proteins (135, 41, 38, 24, 20 kDa), including p38, were detected by antisera against two MDV-lacZ fusion proteins (27). However, the nucleotide sequence in the two clones was not determined, and the structural relationship between these five proteins, if any, remained to be elucidated, and the entire gene(s) encoding the phosphoprotein(s) was not defined (27).

Chen et al. (18) reported that an open reading frame (subsequently designated pp38 ORF) which encodes a predicted polypeptide of 290 amino acids was identified in BamHI-H through cDNA and nucleotide sequencing analysis. By using trpE-pp38 fusion proteins, antisera against pp38 were prepared. Upon immunoprecipitation and SDS-PAGE analysis, a predominant virus-specific 38,000 Da phosphorylated polypeptide (designated pp38), and a minor 24,000 Da polypeptide (designated p24), were found. No precursor-

product relationship was found between pp38 and p24 by pulse-chase analysis, and only pp38 was detected by Western blot analysis with anti-pp38. Expression of the gene encoding pp38 is relatively insensitive to phosphonoacetic acid inhibition, suggesting that pp38 may belong to one of the early classes of herpesvirus proteins.

Previously (47, 72), pp38 was detected in the MSB-1 lymphoblastoid tumor cell line only when IUdR, a drug known to enhance overall gene expression in the latently infected lymphoblastoid tumor cell (31, 76), was used, implying that pp38 is not readily expressed in the latency stage. Chen, et al. (18) reported that an abundant level of pp38 was expressed and detected in the latently infected MSB-1 lymphoblastoid tumor cell line in absence of IUdR treatment. The fact that pp38 is abundantly expressed in a constitutive manner, not only in the lytically infected fibroblast cells, but also in the latently infected MSB-1 lymphoblastoid tumor cells, suggests that it may play a role in the establishment and maintenance of latency.

Although a viral gene(s) determine(s) whether the virus becomes latent, non-viral factors can also influence MDV latency. It has been reported (9) that host animal immunocompetence is required for the establishment and maintenance of latency. A cellular factor from activated chicken spleen cells was found to be able to maintain viral latency in vitro (10). The latency maintenance factor appears to have an molecular weight greater than 10,000 and to be inactivated by heating to 90^{o}C for 5 min, and is not gamma interferon or interleukin 2. However, gamma interferon may also have had some similar effect as latency maintenance factor (10).

4. Transformation and Oncogenicity

Following the MDV latent infection phase, a proliferative phase occurs, involving nonproductively infected lymphoid cells (mainly T helper cells) that may or may not progress to the point of lymphoma formation. For the progression of lymphoma, it is obvious that the nonproductively infected (possibly latently infected) T cell must be transformed either by viral oncogene(s) or by activated cellular oncogene(s). To identify the viral oncogene(s) or viral gene(s) which may activate cellular oncogene(s), comparative molecular analysis of oncogenic serotype 1 MDVs, either with their own attenuated nononcogenic counterparts or with nononcogenic serotype 3 HVT, was carried out.

Several investigators (24, 72) demonstrated that serial in vitro passage of virulent MDV in primary chick embryo fibroblast cells resulted in a loss of MDV tumorigenicity. The loss of tumorigenicity was found to correlate with an expansion in BamHI-D and -H, present in MDV genome TR_L and IR_L, respectively (34, 103, 112). Maotani et al. (70) reported that the expansion was due to the amplification of a 132-bp direct repeat sequence found within BamHI-D and -H. Since the amplification of this region was only detected in nonpathogenic strains of MDV which had a decreased tumorigenic capability, as determined by analysis of tumor induction in chickens (34), it was hypothesized that the oncogenic potential of MDV might be attributed to a gene(s) transcribed partly from the expanded regions of BamHI-D and -H.

Bradley et al. (3) reported that a putative group of 1.8-kb oncogenes are transcribed rightwardly from the expanded region of BamHI-H. The 1.8-kb messages are transcribed only from the pathogenic strains and are composed of two exons. Exon I has two species with the same 5' ends, a small one whose 3' end is mapped near the expanded region and a large one whose 3' end is

mapped within the expanded region. Exon II exists as five species with identical 3' ends, and with 5' ends mapping at various sites outside of the expanded region. Later, Bradley et al. (4) reported that the disappearance of the 1.8-kb transcripts, associated with attenuation and loss of tumorigenicity, is due to truncation of the 1.8-kb transcripts, resulting in the production of 0.4-kb transcripts.

Recently, Chen and Velicer (19) reported that, with combination of cDNA and S1 nuclease analyses, a gene family composed of four groups of transcripts that were either initiated or terminated in both rightward and leftward directions and at multiple sites within or near the expanded regions of the 132-bp direct repeat. Furthermore, each 132-bp repeat contains one TATA box and two polyadenylation consensus sequences in each direction. These RNAs contain a partial copy, or one or more full copies, of the 132-bp direct repeat, at either their 5' or 3' ends. Northern blot analysis showed that the majority of transcripts are 1.8 kb in size, while the minor species range in size from 0.67 to 3.1 kb. Together, these data suggest that the 132-bp direct repeat, and indirectly its copy number, are involved in the regulation of RNA initiation and termination, and therefore in the generation of four groups of transcripts that may be important in MDV tumorigenicity. Since the potential MDV oncogenes are bidirectionally transcribed from the expanded regions, groups 1 and 2 transcripts are complementary to those of groups 3 and 4, respectively. It has been hypothesized that the complementary transcripts, or antisense RNAs, may play a role in gene regulation. In the EBV system, antisense regulation seems to be involved in the regulation of six major nuclear antigens (90). Although it is unknown at this time if a protein product is produced, the existence of bidirectional transcription described by Chen and Velicer (19) makes it

reasonable to speculate that there may exist a similar pattern of antisense gene regulation for the potential MDV oncogene family.

The gene family in the expansion region of MDV IR_L and TR_L is not present in the HVT genome (34a,; X. Chen, and L. F. Velicer, unpublished results), further suggesting that the gene family may be responsible for MDV tumorigenicity. This hypothesis is again supported by a recent report of Kawamura et al. (57) that an oligonucleotide (part of group 3 transcripts that are complementary to group 1 transcripts of the gene family; 19) inhibits the proliferation of MD-derived MSB-1 lymphoblastoid cell line and colony formation in soft agar. Those results by Kawamura et al. (57) therefore support the hypothesis by Chen and Velicer (19) that antisense regulation may be involved in the transcriptional regulation of the gene family which is composed of four groups of transcripts. It is hypothesized (57) that the expression of the gene family is directly associated with the maintenance of the tumorigenic state of the transformed MD-derived lymphoblastoid cells.

Although serial in vitro passage results in the 132-bp expansion in the MDV TR_L and IR_L that is associated with attenuation and loss of oncogenicity, the overall length of MDV genome is not increased, instead is shortened, as reported by Wilson and Coussens (118). Their results indicate that the genome of attenuated high passage serotype 1 MDV strain JM may contain deletions totalling 15-kbp that are present in its low passage counterpart. Using contour-clamped homogeneous electric fields electrophoresis-purified cell-free MDV genomes from infected cells as hybridization probes, a 200-bp deletion was observed in the attenuated genomes of the very virulent MDV strain MD11. The deletion is located in the BamHI L fragment in the TR_L and IR_L of MDV genomes. At present, it is unclear if this 200-bp deletion is related to the

mutations which lead to loss of oncogenicity or pathogenicity in attenuated MD11.

Since pp38 is also expressed in the MD-derived lymphoblastoid cell line (18, 47) and is a gene unique to MDV serotype 1 (101), it is suggested that pp38 may be associated with MDV oncogenicity (72). However, based on the protein homology analysis (18), there is no significant homology between the predicted amino acid sequences of pp38 and any known oncogenes. In addition, pp38 was not found to be a nuclear DNA binding protein (28), although that is not a universal requirement to be an oncogene. Silva and Lee (31) reported that p38 (pp38) was detected in cells infected with the attenuated, avirulent serotype-1 Md11/75C MDV strain. Thus, if pp38 plays a role in MDV transformation two possibilities exist: (i) it was modified during attenuation, or (ii) it is necessary but insufficient and indirect, and attenuation also involves another gene(s). Whether pp38 is a unique MDV oncogene remains to be determined.

Tillotson et al. (113) reported identification of a gene which encodes a polypeptide with a leucine zipper motif. The gene was located in the EcoRI-Q (part of BamHI-I_2) fragment in the TR_L and IR_L regions of MDV genome, and is abundently expressed as mRNA in MSB-1 lymphoblastoid cell line. The predicted amino acid sequence of this MDV gene has a sequence 50% identical with that of the *jun* oncogene in the leucine zipper DNA binding region. Its exact function has yet to be elucidated, but in view of its similarity to other nuclear transcriptional activators, including the jun, myc, and fos oncogenes, it seems likely that it is a nuclear protein involved in DNA binding, with an activator function related to the oncogenic role played by the MDV virus in lymphocytes. Further efforts to identify the protein product, its location, and its role in transformation are needed to understand the mechanism by which MDV induces tumors.

REFERENCES

1. **Akiyama, Y., and S. Kato.** 1974. Two cell lines from lymphomas of Marek's disease. Biken J. 17:105-116.

2. **Binns, M. M., N. L. J. Ross.** 1989. Nucleotide sequence of the Marek's disease virus (MDV) RB-1B A antigen gene and identification of the MDV A antigen as the herpesvirus simplex virus-1 glycoprotein C homologue. Virus Res. 12:371-382.

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

4. **Bradley, G., G, Lancz, A. Tanaka, and M. Nonoyama.** 1989. Loss of Marek's disease virus tumorigenicity is associated with truncation of RNAs transcribed within BamHI-H. J. Virol. 63:4129-4135.

5. **Britt, W. J., and D. Auger.** 1986. Synthesis and processing of the envelope gp55-116 complex of human cytomegalovirus. J. Virol. 58:185-191.

6. **Britt, W. J., and D. Auger.** 1989. Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. J. Virol. 63:403-410.

7. **Brunovskis, P., and L. F. Velicer.** 1991. The complete DNA sequence of the Marek's disease virus (MDV) unique short region: presence of alphaherpesvirus-homologous, fowlpoxvirus-homologous, and MDV-specific genes. Submitted for publication.

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

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

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

11. **Bzik, D. J., B. A. Fox, N. A. DeLuca, and S. Person.** 1984. Nucleotide sequence of a region of the herpes simplex virus type 1 gB glycoprotein gene: mutations affecting rate of virus entry and cell fusion. Virology 137:185-190.

12. **Cai, W., B. Gu, and S. Person.** 1988. Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62:2596-2604.

13. **Cai, W., S. Person, C. Debroy, and B. Gu.** 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1: an analysis of linker insertion mutants. J. Mol. Biol. 201:575-588.

14. **Calnek, B. W., K. A. Schat, L. J. N. Ross, W. R. Shek, and C.-L. H. Chen.** 1984. Further characterization of Marek's disease virus-infected lymphocytes. I. In vivo infection. Int. J. Cancer 33:289-298.

15. **Calnek, B. W.** 1986. Marek's disease - a model for herpesvirus oncology. Crit. Rev. Microbiol. 12:293-320.

16. **Calnek, B. W., and R. F. Witter.** 1991. Marek's disease, p. 342-385. *In* B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, Jr. (ed), Disease of poultry. Iowa State University Press, Ames, Iowa.

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

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

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

20. **Chen, X., and L. F. Velicer.** 1991. The Marek's disease virus B antigen complex (gp100, gp60, gp49) is the homolog of the herpes simplex virus glycoprotein B (gB). Submitted for publication.

21. **Chubb, R. C., and A. E. Churchill.** 1969. Precipitating antibodies associated with Marek's disease. Vet. Res. 83:4-7.

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

23. **Churchill, A. E., and P. M. Biggs.** 1968. Herpes-type virus isolated in cell culture from tumors of chickens with Marek's disease. II. Studies in vivo. J. Nat. Cancer Inst. 41:951-956.

24. **Churchill, A. E., R. C. Chubb, and W. Baxendale.** 1969. The attenuation, with loss of antigenicity, of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. J. gen. Virol. 4:557-563.

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

26. **Coussens, P. M., M. R. Wilson, H. Camp, H. Roehl, R. J. Isfort, and L. F. Velicer.** 1990. Characterization of the gene encoding herpesvirus of turkeys gp57-65: comparison to Marek's disease virus gp57-65 and herpes simplex virus glycoprotein C. Virus Genes 3:211-227.

27. **Cui, 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.

28. **Cui, Z., L. F. Lee, J.-L. Liu, and H.-J. Kung.** 1991. Structural analysis and transcriptional mapping of the Marek's disease virus gene pp38, encoding an antigen associated with transformed cells. J. Virol. 65:6509-6516.

29. **Davidson, I., M. Malkinson, and Y. Becker.** 1988. Marek's disease virus serotype-1 antigens A and B and their unglycosylated precursors detected by Western blot analysis of infected cells. Virus Genes 2:5-18.

30. **DeLuca, N. A., D. J. Bzik, V. C. Bond, S. Person, and W. Snipes.** 1982. Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion, and production of glycoprotein gB (VP7). Virology 122:411-423.

31. **Dunn, K., and K. Nazerian.** 1977. Induction of Marek's disease virus antigens by IdUrd in a chicken lymphoblastoid cell line. J. gen. Virol. 34:413-419.

32. **Fitzpatrick, D. R., T. Zamb, M. D. Parker, S. D. L. Hurk, L. A. Babiuk, and M. J. P. Lawman.** 1988. Expression of bovine herpesvirus 1 glycoproteins gI and gIII in transfected murine cells. J. Virol. 62:4239-4248.

33. **Fukuchi, K., M. Sudo, Y.-B. Lee, A. Tanaka, and M. Nonoyama.** 1984. Structure of Marek's disease virus DNA: Detailed restriction enzyme map. J. Virol. 51:102-109.

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

34a. **Fukuchi, K., M. Sudo, A. Tanaka, and M. Nonoyama.** 1984. Map location of homologous regions between Marek's disease virus and herpesvirus of turkey and the absence of detectable homology in the putative tumor-inducing gene. J. Virol. 53:994-997.

35. **Gibbs, C. P., K. Nazerian, L. F. Velicer, and H.-J. Kung.** 1983. Extensive homology exists between Marek's disease herpesvirus and its vaccine virus, herpesvirus of turkeys. Proc. Natl. Acad. Sci. USA 81:3365-3369.

36. **Glaubiger, C., K. Nazerian, and L. F. Velicer.** 1983. Marek's disease herpesvirus. IV. Molecular characterization of Marek's disease herpesvirus A antigen. J. Virol. 45:1228-1234.

37. **Glorioso, J., C. H. Schroder, G. Kumel, M. Szczesiul, and M. Levine.** 1984. Immunogenicity of herpes simplex virus glycoprotein gC and gB and their role in protective immunity. J. Virol. 50:805-812.

38. **Glorioso, J., U. Kees, G. Kumel, H. Kirchner, and P. H. Krammer.** 1985. Identification of herpes simplex virus type 1 (HSV-1) glycoprotein gC as

the immunodominant antigen for the HSV-1-specific memory cytotoxic T lymphocytes. J. Immunol. 135:575-582.

39. **Grose, C.** 1990. Glycoproteins encoded by Varicella-Zoster virus: biosynthesis, phosphorylation, and intracellular trafficking. Annu. Rev. Microbiol. 44:59-80.

40. **Highlander, S. L., W. Cai, S. Person, T. C. Holland, M. Levine, and J. C. Glorioso.** 1988. Monoclonal antibodies define a domain on herpes simplex virus glycoprotein B involved in virus penetration. J. Virol. 62:1881-1888.

41. **Highlander, S. L., D. J. Dorney, P. J. Gage, T. C. Holland, W. Cai, S. Person, T. C. Holland, M. Levine, and J. C. Glorioso.** 1989. Identification of mar mutations in herpes simplex virus type 1 glycoprotein B which alter antigenic structure and function in virus penetration. J. Virol. 63:730-738.

42. **Highlander, S. L., W. F. Goins, S. Person, T. C. Holland, M. Levine, and J. C. Glorioso.** 1991. Oligomer formation of the gB glycoprotein of herpes simplex virus type 1. J. Virol. 65:4275-4283.

43. **Hirai, K., K. Ikuta, and S. Kato.** 1979. Comparative studies on Marek's disease virus and herpesvirus of turkey DNAs. J. gen Virol. 45:119-131.

44. **Hirai, K., K. Ikuta, N. Kitamoto, and S. Kato.** 1981. Latency of herpesvirus of turkey and Marek's disease virus genomes in a chicken T-lymphoblastoid cell line. J. gen. Virol. 53:133-143.

45. **Hirai, K., K. Maotani, K. Ikuta, H. Yasue, M. Ishibashi, and S. Kato.** 1986. Chromosomal sites for Marek's disease virus DNA in two chicken lymphoblastoid cell lines MDCC-MSB1 and MDCC-RP1. Virology 152:256-261.

46. **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.

47. **Ikuta, K., K. Nakajima, M. Naito, S. H. Ann, S. Ueda, S. Kato, and K. Hirai.** 1985. Identification of Marek's disease virus-specific antigens in Marek's disease lymphoblastoid cell lines using monoclonal antibody against virus-specific phosphorylated polypeptides. Int. J. Cancer 35:257-264.

48. **Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1983. Monoclonal antibodies reactive with the surface and secreted glycoproteins of Marek's disease virus and herpesvirus of turkeys. J. gen. Virol. 64:2597-2610.

49. **Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1984. Processing of glycoprotein gB related to neutralization of Marek's disease virus and herpesvirus of turkeys. Microbiol. Immunol. 28:923-933.

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

51. **Isfort, R. J., L. Sithole, H-J. Kung, and L. F. Velicer.** 1986. Molecular characterization of Marek's disease herpesvirus B antigen. J. Virol. 59:411-419.

52. **Isfort, R. J., 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.

53. **Kanamori, A., Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1987. Methylation of Marek's disease virus DNA in chicken T-lymphoblastoid cell lines. J. gen. Virol. 68:1485-1490.

54. **Kaschka-Dierich, C., G. W. Bornkamm, and R. Thomssen.** 1979. No homology detectable between Marek's disease virus (MDV) and herpesvirus of the turkey (HVT) DNA. Med. Microbiol. Immunol. 165:223-239.

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

56. **Kawamura, H., D. J. King, Jr., and D. P. Anderson.** 1969. A herpesvirus isolated from kidney cell culture of normal turkeys. Avian Dis. 13:853-863.

57. **Kawamura, H., M. Hayashi, T. Furuichi, M. Nonoyama, E. Isogai, and S. Namioka.** 1991. The inhibitory effects of oligonucleotides, complementary to Marek's disease virus mRNA transcribed from the BamHI-H region, on the proliferation of transformed lymphoblastoid cells, MDCC-MSB1. J. gen. Virol. 72:1105-1111.

58. **Kishi, M., G. Bradley, J. Jessip, A. Tanaka, and M. Nonoyama.** 1991. Inverted repeat regions of Marek's disease virus DNA possess a structure similar to that of the a sequence of herpes simplex virus DNA and contain host cell telomere sequences. J. Virol. 65:2791-2797

59. **Klenk, H. D., and R. Rott.** 1980. Co-translational and post-translational processing of viral glycoproteins. Current Topic in Microbiol. and Immunol. 90:19-48.

60. **Lee, L. F., E. D. Kieff, S. L. 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.

61. **Lee, Y.-S., A. Tanaka, S. Silver, M. Smith, and M. Nonoyama.** 1979. Minor DNA homology between herpesvirus of turkey and Marek's disease virus? Virology 93:277-280.

62. **Lewin, B.** 1987. Genes. 3rd edition. John Wiley & Sons. New York.

63. **Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology 114:149-160.

64. **Long, A. P., P. Kaveh-Yamini, and L. F. Velicer.** 1975. Marek's disease herpesviruses. I. Production and preliminary characterization of Marek's disease herpesvirus A antigen. J. Virol. 15:1182-1191.

65. **Long, A. P., J. L. Clark, and L. F. Velicer.** 1975. Marek's disease herpesviruses. II. Purification and further characterization of Marek's disease herpesvirus A antigen. J. Virol. 15:1192-1201.

66. **Lycke, E., M. Johansson, B. Svennerholm, and U. Lindahl.** 1991. Binding of herpes simplex virus to cellular heparan sulphate, an initial step in the adsorption process. J. gen. Virol. 72:1131-1137.

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

68. **Meridith, D. M., J.-M. Stocks, G. R. Whitaker, I. W. Halliburton, B. W. Snowden, and R. A. Killington.** 1989. Identification of the gB homologues of equine herpesvirus types 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. J. gen. Virol. 70:1161-1172.

69. **Montalvo, E. A., and C. Grose.** 1987. Assembly and processing of the disulfide-linked Varicella-zoster virus glycoprotein gpII (140). J. Virol. 61:2877-2884.

70. **Maotani, K., K. Kanamori, K. Ikuta, S. Ueda, S. Kato, and K. Hirai.** 1986. Amplification of a tandem direct repeat within inverted repeats of Marek's disease virus DNA during serial in vitro passage. J. Virol. 58:657-659.

71. **Naito, M., K. Nakajima, N. Iwa, K. Ono, I. Yoshida, T. Konobe, K. Ikuta, S. Ueda, S. Kato, and K. Hirai.** 1986. Demonstration of a Marek's disease virus-specific antigen in tumor lesions of chickens with Marek's disease using monoclonal antibody against a virus phosphorylated protein. Avian Pathol. 15:503-510.

72. **Nakajima, K., K. Ikuta, 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. 68:1379-1389.

73. **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.

74. **Nazerian, K.** 1973. Marek's disease. A neoplastic disease of chickens caused by a herpesvirus. Adv. Cancer Res. 17:279-315.

75. **Nazerian, K., and L. F. Lee.** 1976. Selective inhibition by phosphonoacetic acid of MDV DNA replication in a lymphoblastoid cell line. Virology 74:188-193.

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

77. **Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmester.** 1968. Studies on the etiology of Marek's disease. II Finding of a herpesvirus in cell culture. Proc. Soc. Exp. Biol. Med. 127:177-182.

78. **Nazerian, K., E. A. Stephens, J. M. Sharma, L. F. Lee, M. Gailitis, and R. L. Witter.** 1977. A nonproducer T lymphoblastoid cell line from Marek's disease transplantable tumor (JMV). Avian Dis. 21:69-76.

79. **Nilkura, 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.

80. **Nonoyama, M.** 1982. The molecular biology of Marek's disease herpesvirus. p. 333-346. *In* B. Roizman (Ed.), The Herpesviruses, Vol. 1, Plenum, New York.

81. **Okazaki, W., W. G. Purchase, and B. R. Burmester.** 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys (HVT). Avian Dis. 16:108-125.

82. **Onuma, M., T. Mikami, and T. T. A. Hayshi.** 1974. Properties of common antigen associated with Marek's disease herpesvirus and turkey herpesvirus infections. J. Natl. Cancer Inst. 52:805-813.

83. **Ono, K., M. Takashima, T. Ishikawa, M. Hayashi, I. Yoshida, T. Konobe, K. Ikuta, K. Nakajima, S. Ueda, S. Kato, and K. Hirai.** 1984. Partial protection against Marek's disease in chickens immunized with glycoproteins gB purified from turkey-herpesvirus-infected cells by affinity chromatography coupled with monoclonal antibodies. Avian Dis. 29:533-539.

84. **Payne, L. N.** 1982. Biology of Marek's disease virus and the herpesvirus of turkeys. p. 347-431. *In* B. Roizman (Ed.), The Herpesviruses, Vol. 1, Plenum, New York.

85. **Payne, L. N.** 1985. Pathology, p. 43-75. In L. N. Payne (ed.), Marek's Disease. Martinus Nijhoff Publishing, Boston.

86. **Pepose, J. S., J. G. Stevens, M. L. Cook, and P. W. Lampert.** 1981. Marek's disease virus as a model for the Landry-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.

87. **Powell, P. C., L. N. Payne, J. A. Frazier, and M. Rennie.** 1974. T lymphoblastoid cell lines from Marek's disease lymphoma. Nature. 251:79-80

88. **Roberts, P. L., B. E. Duncan, T. J. G. Raybould, and D. H. Watson.** 1985. Purification of herpes simplex virus glycoproteins B and C using monoclonal antibodies and their ability to protect mice against lethal challenge. J. gen. Virol. 66:1073-1085.

89. **Robbins, A. K., D. J. Dorney, M. W. Wathen, M. E. Whealy, C. Gold, R. J. Watson, L. E. Holland, S. D. Weed, M. Levine, J. C. Glorioso, and L. W.**

Enquist. 1987. The pseudorabies virus gII gene is closely related to the gB glycoprotein gene of herpes simplex virus. J. Virol. 61:2691-2701.

90. **Rogers, R. P., and S. H. Speck.** 1990. Bidirectional transcription of the Epstein-Barr virus major internal repeat. J. Virol. 64:2426-2429.

91. **Roizman, B.** 1990. Herpesviridae: a brief introduction, p. 1787-1793. *In* B. N. Fields and D. M. Knipe (ed.), Fields Virology. 2nd edition. Raven Press, New York.

92. **Roizman, B., and A. Sears.** 1990. Herpes simplex viruses and their replication, p. 1795-1841. *In* B. N. Fields and D. M. Knipe (ed.), Fields Virology. 2nd edition. Raven Press, New York.

93. **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. 18:291-304.

94. **Ross. L. J. N., and M. M. Binns.** 1991. Properties and evolutionary relationships of Marek's disease virus homologues of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. J. gen. Virol. 72:939-947.

95. **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 unique segment of Marek's disease virus (strain RB1B). J. gen. Virol. 72:949-954.

96. **Ross, L. J. N., 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.

97. **Sarmiento, M., and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. IV. Conformation of the virion glycoprotein designated VP7(B2). J. Virol. 29:1159-1167.

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

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

99a. **Schat, K. A., G. F. de Boer, K. Hirai, L. F. Lee, K. Nakajima, R. F. Silva, and L. F. Velicer.** 1988. A proposed nomenclature for the protein of Marek's disease herpesvirus, p. 438-443. In S. Kato, T. Horiuchi, T. Mikami, and K. Hirai. (ed.), Advances in the Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

100. **Schrier, R. D., L. I. Pizer, and J. W. Moorhead.** 1983. Type-specific delayed hypersensitivity and protective immunity induced by isolated herpes simplex virus glycoprotein. J. Immunol. 130:1413-1418.

101. **Silva, R. F., and L. F. Lee.** 1984. Monoclonal antibody-mediated immunoprecipitation of proteins from cells infected with Marek's disease virus or turkey herpesvirus. Virology 136:307-320.

102. **Silva, R. F., and L. F. Lee.** 1985. Isolation and partial characterization of three glycoproteins common to Marek's disease virus and turkey herpesvirus-infected cells, p. 101-110. *In* B. W. Calnek and J. L. Spencer (ed.), Proceedings of the International Symposium on Marek's Disease. American Association of Avian Pathologists, Inc., Kennet Square, Pa.

103. **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.

104. **Silver, S., A. Tanaka, and M. Nonoyama.** 1979. Transcription of the Marek's disease virus genome in a nonproductive chicken lymphoblastoid cell line. Virology 93:127-133.

105. **Sithole, L, P. M. Coussens, and L. F. Velicer.** 1988. Identification of Marek's disease herpesvirus B antigen's precursor polypeptide and the gene encoding it, p. 148-154. In Kato, S., T. Horiuchi, T. Mikami, and K. Hirai. (ed.), Advances in Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

106. **Sithole, L, L. F. Lee, and L. F. Velicer.** 1988. Synthesis and processing of the Marek's disease herpesvirus B antigen glycoprotein complex. J. Virol. 62:4270-4279.

107. **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. Proc. Soc. Exp. Biol. Med. 127:173-177.

108. **Spear, P. G.** 1985. Glycoproteins specified by herpes simplex virus, p. 315-356. In B. Roizman (ed.), the herpesviruses, vol. 3. Plenum press, New York.

109. **Sugaya, K., G. Bradley, M. Nonoyama, and A. Tanaka.** 1990. Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. J. Virol. 64:5773-5782.

110. **Sullivan, D. C., G. P. Allen, and D. J. O'Callaghan.** 1989. Synthesis and processing of equine herpesvirus type 1 glycoprotein 14. Virology 173:638-646.

111. **Tanaka, A., S. Silver, and M. Nonoyama.** 1978. Biochemical evidence of the nonintegrated status of Marek's disease virus DNA in virus-transformed lymphoblastoid cells of chickens. Virology 88:19-24.

112. **Tanaka, A., Y.-S. Lee, and M. Nonoyama.** 1980. Heterogeneous population of virus DNA in serially passaged Marek's disease virus preparation. Virology 103:510-513.

113. **Tillotson, J. K., L. F. Lee, and H.-J. Kung.** 1988. Accumulation of viral transcripts coding for a DNA binding protein in Marek's disease tumor

cells, p. 128-134. In Kato, S., T. Horiuchi, T. Mikami, and K. Hirai. (eds.), Advances in the Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

114. **Velicer, L. F., D. R. Yager, and J. L. Clark.** 1978. Marek's disease herpesviruses. III. Purification and characterization of Marek's disease herpesvirus B antigen. J. Virol. 27:205-217.

115. **Wainberg, M. A., B. Beiss, and E. Isreal.** 1980. Virus-mediated abrogation of chicken lymphocyte responsiveness to mitogenic stimulus. Avian Dis. 24:580.

116. **Weir, J. P., M. Bennett, E. M. Allen, K. L. Elkins, S. Martin, and B. T. Rouse.** 1989. Recombinant vaccinia virus expressing the herpes simplex virus type 1 glycoprotein C protect mice against herpes simplex virus challenge. J. gen. Virol. 70:2587-2594.

117. **Whealy, M. E., A. K. Robbins, and L. W. Enquist.** 1990. The export pathway of the pseudorabies virus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. J. Virol. 64:1946-1955.

118. **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.

119. **Witter, R. L.** 1985. Principles of vaccination, p. 203-250. In L. N. Payne (ed.), Marek's Disease. Martinus Nijhoff Publishing, Boston.

Chapter II

Multiple Bidirectional Initiations and Terminations of Transcription in the Marek's Disease Virus Long Repeat Regions

Xinbin Chen and Leland F. Velicer. 1991. J. Virol. 65:2445-2451.

ABSTRACT

Marek's disease (MD) is an oncogenic disease of chickens caused by Marek's disease virus (MDV). Serial in vitro passage of pathogenic MDV results in amplification of a 132-bp direct repeat in the MDV genome's TR_L and IR_L repeat regions and loss of tumorigenicity. This led to the hypothesis that upon such expansion, one or more tumor-inducing genes fail to be expressed. In this report, a group of cDNAs mapping in the expanded regions were isolated from a pathogenic MDV strain in which the 132-bp direct repeat number was found to range between one and seven. Partial cDNA sequencing and S1 nuclease analysis revealed that the corresponding transcripts are either initiated or terminated within or near the expanded regions at multiple sites in both rightward and leftward directions. Furthermore, each 132-bp repeat contains one TATA box and two polyadenylation consensus sequences in each direction. These RNAs contain a partial copy or one or more full copies of the 132-bp direct repeat at either their 5' or 3' ends. Northern blot analysis showed that the majority of transcripts are 1.8 kb in size, while the minor species range in size from 0.67 to 3.1 kb in size. Together, these data raise the possibility that the 132-bp direct repeat, and indirectly its copy number, may be involved in the regulation of transcriptional initiation and termination and therefore in the generation of four groups of transcripts from the TR_L and IR_L, although this remains to be determined.

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by a herpesvirus, Marek's disease virus (MDV), which results in T-cell lymphomas and peripheral nerve demyelination (3, 9). Marek's disease was one of the first neoplastic diseases found to be caused by herpesviruses. This disease was a major cause of economic loss to the poultry industry until the early 1970's, when a live vaccine was developed from the antigenically related yet apathogenic herpesvirus of turkey (17). Consequently Marek's disease become the first naturally occurring lymphomatous disorder to be effectively controlled by vaccination.

Several investigators (4, 16) demonstrated that serial in vitro passage of virulent MDV in primary chick embryo fibroblast cells resulted in a loss of MDV tumorigenicity. The loss of tumorigenicity was found to correlate with an expansion in BamHI-D and -H, present in MDV genome long terminal repeat (TR_L) and long internal repeat (IR_L) respectively (6, 21). Maotani et al. (14) reported that the expansion was due to the amplification of a 132-bp direct repeat sequence found within BamHI-D and -H. Since the amplification of this region was only detected in nonpathogenic strains of MDV which had decreased tumorigenic capability, as determined by analysis of tumor induction in chickens (6), it was hypothesized that the oncogenic potential of MDV might be attributed to a gene(s) transcribed partly from the expanded regions of BamHI-D and -H.

Bradley et al. (1) reported that a gene family, hypothesized to be directly associated with MDV's tumorigenic potential, is transcribed rightwardly from the expanded region of BamHI-H to yield 1.8-kb RNAs. The 1.8-kb messages are transcribed only from the pathogenic strains and are composed of two exons. Exon I has two species with the same 5' ends, a small one whose 3' end maps

near the expanded region and a large one whose 3' end maps within the expanded region. Exon II exists as five species with identical 3' ends and with 5' ends mapping at various sites outside of the expanded region. Later, Bradley et al. (2) reported that the disappearance of the 1.8-kb transcripts, associated with attenuation and loss of tumorigenicity, is due to truncation of the 1.8-kb transcripts, resulting in the production of 0.4-kb transcripts.

A previous report (1) and our own observations show that both BamHI-D and -H can exist in multiple forms in MDV genomes. In the very pathogenic strain RB1B, BamHI-D and -H each have three forms, with one to three copies of 132-bp direct repeat (1). In contrast, the attenuated strain CVI-988 contains from 1 to over 30 copies of the 132-bp direct repeat (1). For the GA strain used in this study, recently obtained as cell-free virus from extracts of feather tips from birds with symptoms of Marek's disease, BamHI-D and -H were found to contain one to seven copies of 132-bp direct repeat. Therefore, because the forms of BamHI-D and -H found in MDV genomes vary in degree of attenuation, RNAs transcribed from the expanded regions of BamHI-D and -H will differ within each pathogenic or attenuated MDV strain as well as between pathogenic and attenuated strains. As a result, probes generated from single BamHI-D or -H fragments out of their entire populations cannot be used for accurately mapping of the transcripts from the expanded regions by S1 nuclease protection analysis alone, and subsequently the results can not be correctly interpreted.

In this report cDNA analysis and S1 nuclease protection assay were used together and the presence of multiple forms of BamHI-D and -H in MDV genomes was taken into consideration for data interpretation. We found that four groups of transcripts can be initiated or terminated in both rightward and leftward directions and at multiple sites within or near the expanded regions of 132-bp direct repeat.

MATERIALS AND METHODS

Cells and viruses. The preparation, propagation, and infection of duck embryo fibroblast (DEF) cells with MDV were performed as described previously by Glaubiger et al. (7) and more recently by Isfort et al. (8). The MDV GA strain was used in this study at cell culture passage level 6 following isolation of cell-free virus from feather tips (20) obtained from infected birds showing symptoms of Marek's disease. The virus was prepared in this manner to permit preparation of stocks (in DEF cells) of recently demonstrated pathogenicity for further use. The virus used had previously been through 25 passages in cell culture and was still highly pathogenic when put into birds (as stated above), since 100% of the infected chickens developed Marek's disease. The six additional passages are the minimum necessary to expand the originally low-titer cell-free virus to the level of cell-associated MDV necessary to conduct biochemical studies.

Isolation of cellular DNA, restriction enzyme digestion, gel electrophoresis, and Southern blot analysis. Total cellular DNA was isolated from uninfected and MDV-infected DEF cells by standard methods (13). Restriction enzymes (Boehringer Mannheim Biochemicals) were used according to the manufacturer's specifications. The DNA digests were electrophoresed through agarose and transfered to nitrocellulose filter (13). Probes were generated by nick-translation of cloned viral DNA (13). Hybridization of the ^{32}P-labeled probe to the nitrocellulose-bound restriction fragments resulting from Southern blotting was performed by the method of Maniatis et al. (13).

Isolation of cellular RNA and purification of poly(A)$^+$ RNA. Total cellular RNA was isolated from mock-infected and MDV-infected DEF cells essentially as described before (13). Briefly, cells were lysed in 4 M guanidinium isothiocyanate. DNA was sheared by repeated passage of the cell lysates

through an 18 gauge syringe. The final preparation was loaded on a cushion of 5.7 M CsCl in 0.1 M EDTA. Centrifugation was carried out at 174,000 x g for 16 h. RNA pellets were solubilized in 100 mM Tris-HCl (pH 8.0)-10 mM EDTA and extracted twice with phenol-chloroform (1:1) and once with chloroform. Following precipitation with ethanol, RNA was solubilized in water and stored at -70°C. Purification of polyadenylated [poly(A)$^+$] RNA was carried out as by the method of Maniatis et al. (13).

RNA gel electrophoresis and Northern blot analysis. Electrophoresis of RNA was performed essentially as described before (22). Following electrophoresis, Northern (RNA) blotting was performed by transfer of formaldehyde-denatured RNA to nitrocellulose by the method outlined by Maniatis et al. (13). Nick translation and hybridization with ^{32}P-labeled probes were performed as described above for Southern blot analysis. Transcript size determinations were based on comparison with a Bethesda Research Laboratories (BRL) RNA ladder run in parallel.

Construction and screening of cDNA library. A cDNA synthesis kit from BRL was used according to the manufacturer's specifications. cDNAs were trimmed by S1 nuclease and then made blunt-ended via the fill-in reaction catalyzed by the Klenow fragment of *Escherichia coli* polymerase I. The blunt-ended cDNA was directly cloned into the SmaI site of calf intestine alkaline phosphatase-treated pUC18. An in situ DNA hybridization method was used to screen the cDNA library by Maniatis et al. (13). Briefly, E. coli DH5α cells were transformed, and the recombinant colonies were replicated onto a nitrocellulose filter. The colonies on the filter were lysed with 10% SDS, denatured with 0.5 M NaOH-1.5 M NaCl, and neutralized with 0.5 M Tris-HCl (pH 8.0)-1.5 M NaCl. After the filter was baked for 2 h at 80°C in a vacuum oven, the filter was

hybridized to ^{32}P-labeled probes and washed as in the Southern blot analysis above.

S1 nuclease protection studies. S1 nuclease protection studies were carried out essentially as described before (13), with the probes described in the text. To generate the single-strand labeled probes 1, 2, and 3, appropiate restriction endonuclease digestions of double strand-labeled fragments were performed, followed by agarose gel purification of desired single-strand labeled subfragments. To generate the single-strand labeled probe 4 labeled at its 3' end, the 1.6-kbp AvaI-PstI fragment was 3' end labeled at AvaI site via the fill-in reaction catalyzed by the klenow fragment of *E. coli* polymerase I. The radiolabeled DNAs from a DNA sequencing reaction were used as size markers. Hybridizations were performed at 46^{o}C because of the G+C contents of the DNA probes.

DNA sequencing. Initial cDNA sequencing was carried out by the pUC sequencing system since cDNA was cloned in pUC18. Further sequencing of cDNA was carried out by the M13 sequencing system. The dideoxy chain termination method of Sanger et al. (19) was used for M13 sequencing, along with the Sequenase enzyme (United States Biochemical Corp.) in place of the Klenow fragment. The basic method used for pUC sequencing was the same as for M13 sequencing with the following exceptions. Miniprep DNA was generated by the boiling method (13) and denatured by NaOH. Both forward and reverse 17-mer universal primers were used to sequence both ends of each cDNA.

RESULTS

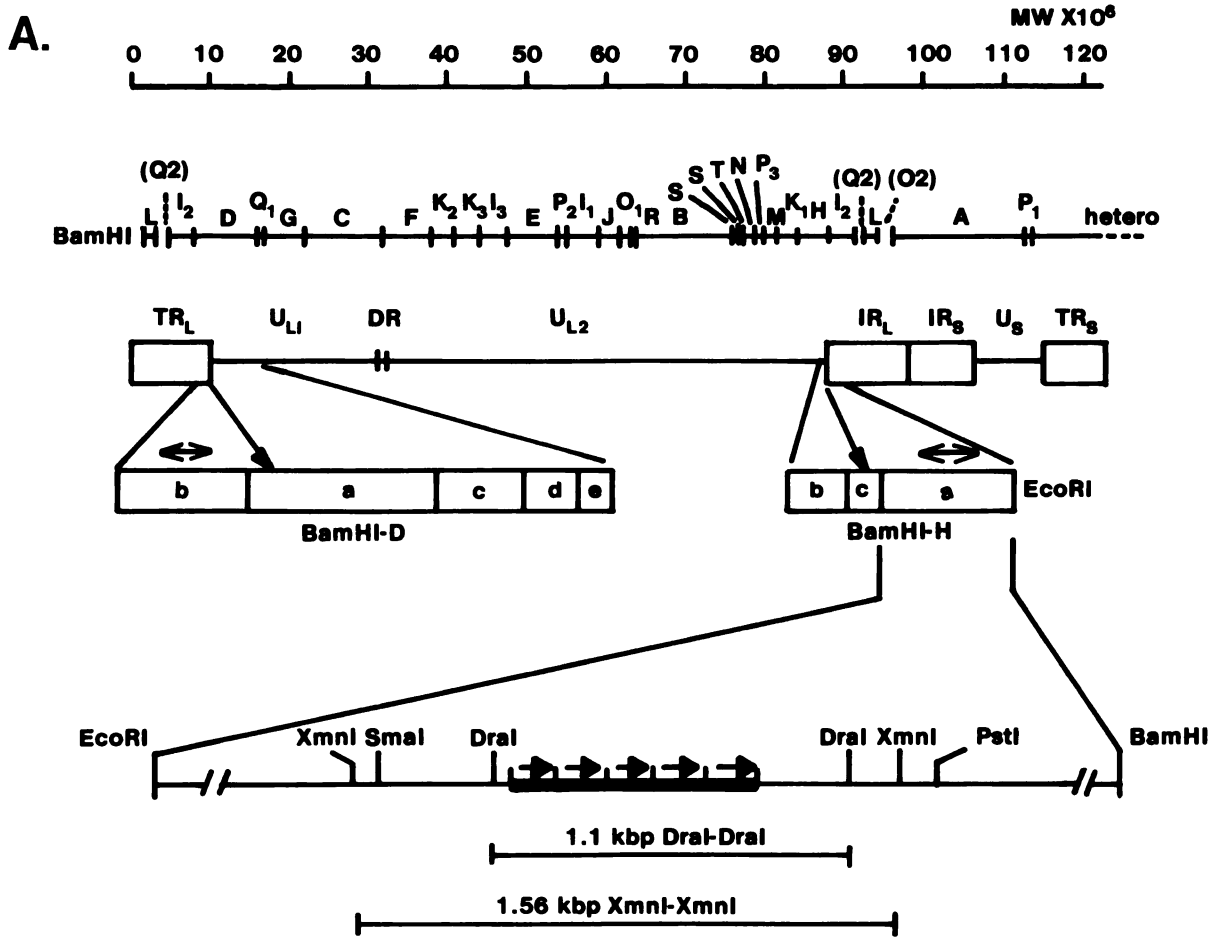
The Number of 132-bp direct repeat in BamHI-D and -H of the MDV GA strain. A 1.1-kbp DraI-DraI fragment containing five copies of the 132-bp direct repeat was used as the probe (Fig. 1A). For the BamHI-D and -H fragments containing one copy of the 132-bp direct repeat, the hybridized DraI-DraI fragment would be 594-bp in size, while the XmnI-XmnI fragment would be 1,028-bp in size, on the basis of the published sequence of the EcoRI-a subfragment of BamHI-H (1). By determining the sizes and number of DraI-DraI or XmnI-XmnI fragments that hybridized with the probe, the GA strain of MDV used in this study was found to contain between one and seven copies of the 132-bp direct repeat (Fig. 1B).

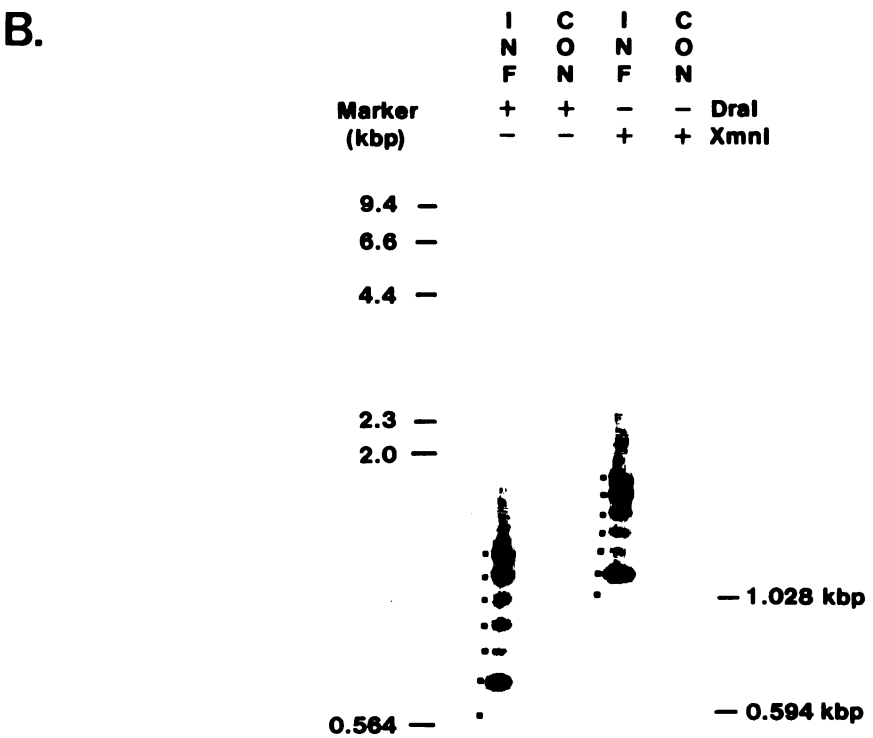
Isolation and localization of cDNAs. The 3.2-kbp EcoRI a subfragment of BamHI-H was used to probe the cDNA library in pUC18. Sixteen of 24 clones identified by in situ hybridization contained cDNA inserts. Of these 16 cDNA clones, 6 hybridized to the EcoRI-a subfragment of BamHI-H (cDNA 1, 3, 4, 6, 11, and 15) (data not shown). When cDNA 3, 11, and 15 were used as probes for Southern blot analysis, they were determined to map partly within or near the expanded regions of BamHI-D and -H (data not shown).

Partial cDNA sequencing analysis. Alignments of six cDNAs with the published sequence of the EcoRI-a subfragment of BamHI-H (1) are shown in Fig. 2. The message for cDNA 3, containing 2 copies of the 132-bp direct repeat at its 5' end, was initiated within the expanded region in a leftward direction. In contrast, the messages for cDNAs 1 and 11, which also initiate within the expanded region, were transcribed in rightward direction and contained either one or two copies of the 132-bp direct repeat at their 5' ends, respectively. While the messages for cDNAs 4, 6, and 15 were found to be transcribed leftwardly toward the expanded region, only cDNA 4 terminated

FIG. 1. Southern blot hybridization to detect the copy number of the 132-bp direct repeat in the BamHI-D and -H fragments of MDV GA strain used in this study. (A) Schematic representation of the MDV genome. The 120-MDa genome of MDV is shown with its BamHI sites (5), along with the EcoRI restriction maps of BamHI-D and -H (6). The single-headed arrows indicate the location of the junction regions of the TR_L and IR_L with the long unique regions UL_1 and UL_2, respectively. The double-headed arrows locate the two regions containing the sequences which undergo amplification upon repeated passage in vitro. The direct repeat (DR), short internal repeat (IR_S), short terminal repeat (TR_S), and short unique (U_S) regions are shown. The location of the probe, a 1.1-kbp DraI-DraI fragment with five copies of the 132-bp direct repeat, was obtained from EcoRI-a subfragment of BamHI-H. The size of XmnI-XmnI subfragment with five copies of the 132-bp direct repeat is 1.56-kbp. The heavy line in the enlarged EcoRI-a subfragment represents the expanded region, with each arrow above it representing one copy of the 132-bp direct repeat. (B) Total cellular DNA from MDV-infected (INF lanes) or mock-infected (CON lanes) DEF cells were digested with DraI or XmnI and electrophoresed at 10 μg per lane. Solid squares indicate positions of the fragments containing direct repeat. In the two INF lanes, the lowest square identify fragments with a single 132-bp direct repeat within the fragments, and the other six squares in each lane represent those fragments with two through seven repeats.

Fig.1.


A.
MW X10⁶
0 10 20 30 40 50 60 70 80 90 100 110 120
(Q2)
L I2 D Q1 G C F K2 K3 I3 E P2 I1 J O1 R B S T N P3 M K1 H I2 L (Q2) (O2) A P1 hetero
BamHI H
TRL ULI DR UL2 IRL IRS US TRS
b a c d e
BamHI-D
b c a EcoRI
BamHI-H
EcoRI XmnI SmaI DraI DraI XmnI PstI BamHI
1.1 kbp DraI-DraI
1.56 kbp XmnI-XmnI


B.
INF CON INF CON
Marker + + − − DraI
(kbp) − − + + XmnI
9.4
6.6
4.4
2.3
2.0
0.564
1.028 kbp
0.594 kbp

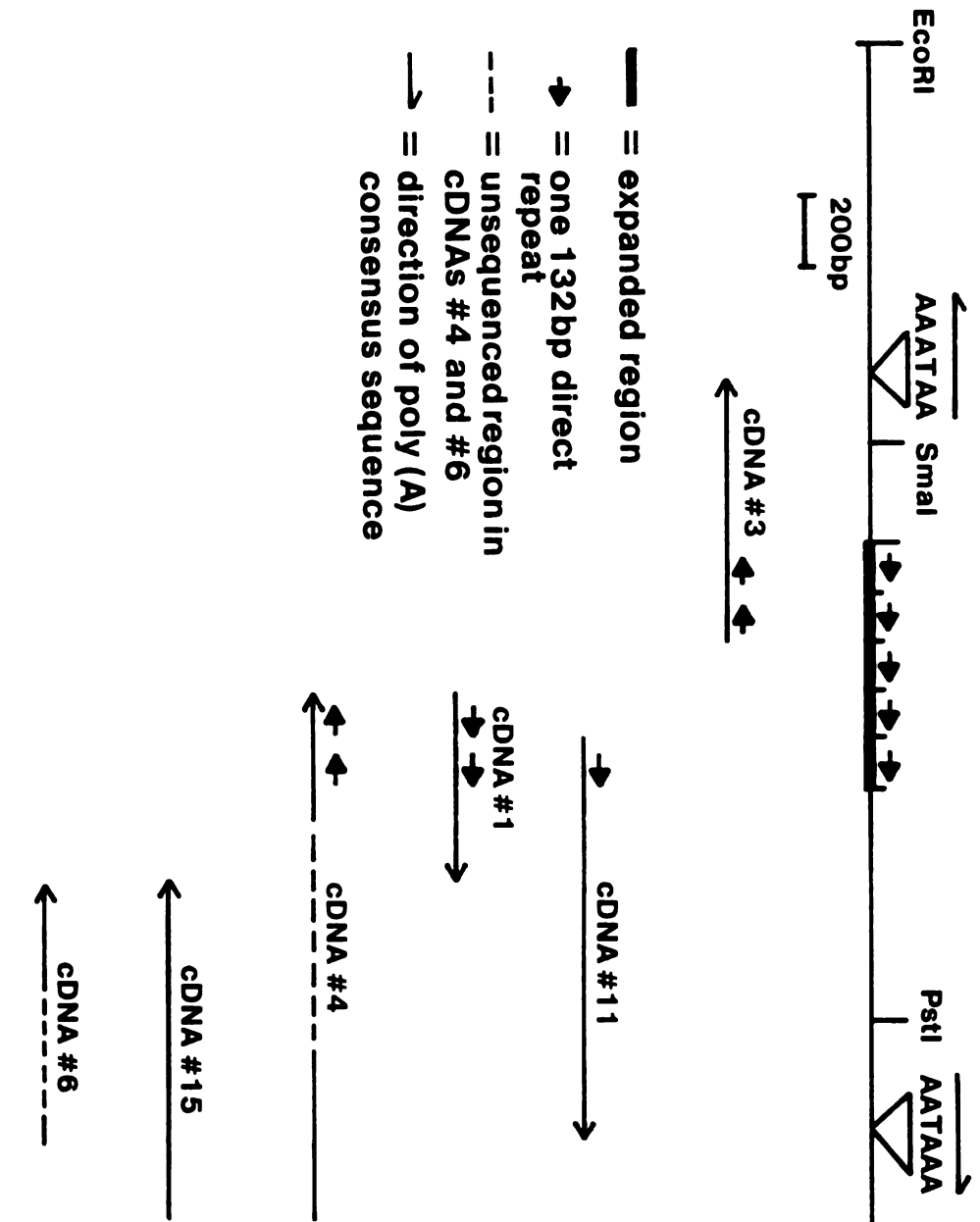
within that region. Furthermore cDNA 4 contained two copies of the 132-bp direct repeat at its 3' end; in contrast, cDNAs 6 and 15 terminated about 150-bp downstream of the expanded region.

Detection of transcripts from within or near the expanded regions of BamHI-D and -H. A 1.6-kbp SmaI-PstI fragment probe (Fig. 2), which spans the expanded region of BamHI-H, hybridized to a major 1.8-kb transcript, with minor components detected at 1.6 to 3.1 kb (Fig. 3A; short exposure data are not shown). The detection of a major transcript at 1.8 kb was similar to a finding in a previous report (1). To determine the respective transcripts of the isolated cDNAs, they were used as probes for Northern blot analysis. cDNA 3 (mapped at the left side of the expanded region) hybridized to two major (0.67 and 1.8 kb) transcripts (Fig. 3B), with minor components detected at 1.6 and 3.1 kb. cDNAs 1, 4, 11, and 15 (mapped at right side of the expanded region) hybridized to a major 1.8-kb transcript (Fig. 3C, D, E and F), with minor components detected at 1.6 and 3.1 kb (see below).

Since these transcripts vary in abundance, various exposure times were required to visualize all of them. Therefore, representative data were included in Fig. 3 to demonstrate the existence of each transcript. For example, Fig. 3A includes data from a longer exposure, so the 3.1-kb transcript can be seen. However, the 1.6-kb and 1.8-kb transcripts were more clearly seen on a short exposure (data not shown). For Fig. 3B, an exposure time was selected to best demonstrate the 0.67-kb transcript, since it was found only with this probe, but the transcript at 3.1-kb was readily apparent on longer exposures (data not shown). For Fig. 3C to F, short exposures were selected to best demonstrate the 1.6-kb and 1.8-kb transcripts, but the 3.1-kb transcript was apparent on longer exposure (data not shown).

FIG. 2. Mapping of cDNAs on the EcoRI-a subfragment of BamHI-H. Each heavy arrow represents one 132-bp direct repeat. Dashed lines represent unsequenced regions in cDNAs 4 and 6. The heavy line indicates the location of the expanded region. Longer arrows above the map represents the direction of transcription of the polyadenylation consensus sequence.

Fig. 2.


EcoRI
AATAA
SmaI
PstI
AATAAA
BamHI
200bp
cDNA #3
= expanded region
= one 132bp direct repeat
= unsequenced region in cDNAs #4 and #6
= direction of poly (A) consensus sequence
cDNA #11
cDNA #1
cDNA #4
cDNA #15
cDNA #6

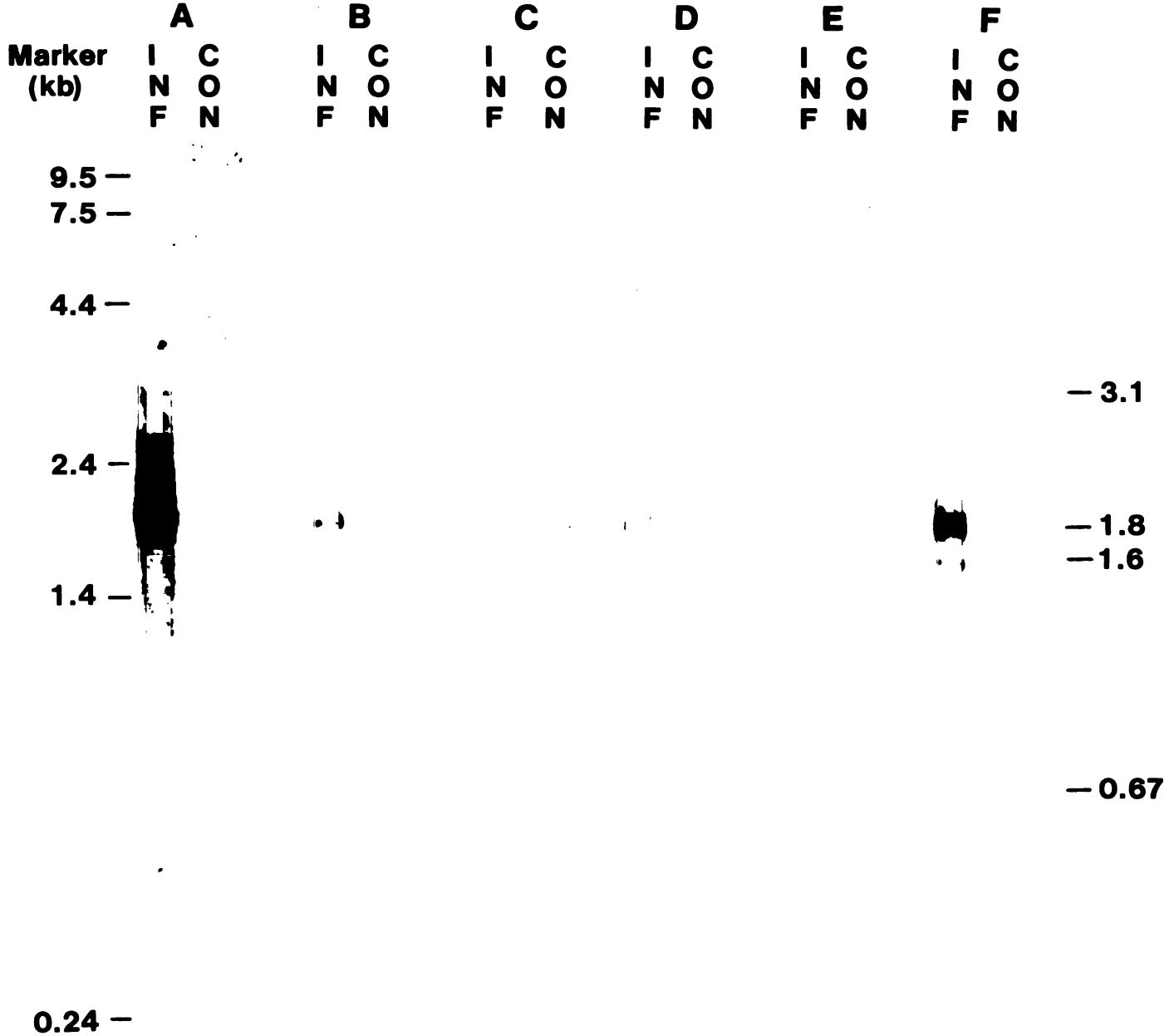
S1 nuclease mapping of transcripts from partly within or near the expanded regions of BamHI-D and -H. Since the expanded regions of BamHI-D and -H are the same (sequence alignment between the expanded regions of BamHI-D and -H shows 98% identity; data not shown), only probes from the EcoRI-a subfragment of BamHI-H were used. All the probes used have five copies of the 132-bp direct repeat. Figure 4A shows the probes used as well as the sites in the probes where S1 nuclease cuts.

To map the 5' ends of transcripts which initiate rightwardly within the expanded region of BamHI-H, probe 1, a 760-bp DraI-BanI fragment 5' end labeled at its BanI site by [γ-^{32}P]ATP, was used. Seven major RNA-DNA hybrids (from 100 bases to 600 bases) were protected from S1 nuclease digestion, indicating that the respective transcripts are heterogeneous and contain between one and five copies of the 132-bp direct repeat at their 5' ends (Fig. 4B). Since there are five copies of the 132-bp direct repeat in the probe used, one can not determine whether some of the transcripts might contain more than five copies of the 132 base direct repeat at their 5' ends.

To map 5' ends of transcripts which initiate leftwardly within the expanded region of BamHI-H, probe 2, a 760-bp DraI-BanI fragment 5' end labeled at its DraI site by [γ-^{32}P]ATP, was used. Two RNA-DNA hybrids (150 bases and 280 bases) were protected, indicating that the respective transcripts contained one or two copies of the 132-bp direct repeat at their 5' ends (Fig. 4C).

To map the 3' ends of transcripts which terminate leftwardly within the expanded region of BamHI-H, probe 3, a 1.0-kbp SmaI-BanI fragment 3' end labeled at its BanI site by [α-^{32}P]dCTP, was used. Ten RNA-DNA hybrids (from 170 bases to 750 bases) were protected, indicating that the respective transcripts contained between one and five copies of the 132-bp direct repeat at

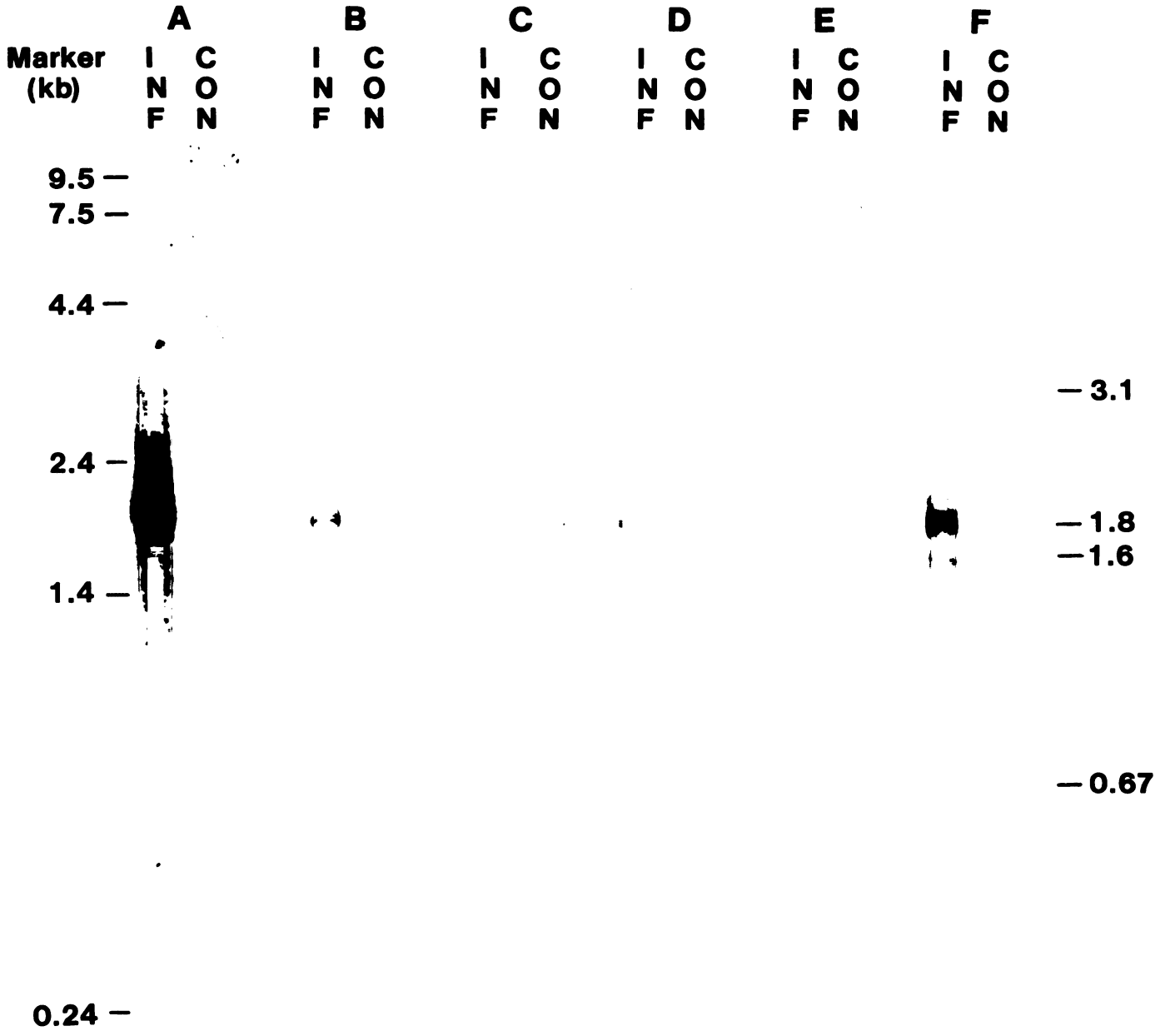
FIG. 3. Northern blot hybridization to detect transcripts from the expanded regions. poly(A)$^+$ RNA (0.5 μg) was used per lane. (A) Probe: 1.6-kbp SmaI-PstI subfragment which spans the expanded region of BamHI-H. (B) Probe: cDNA 3. (C) Probe: cDNA 1. (D) Probe: cDNA 4. (E) Probe: cDNA 11. (F) Probe: cDNA 15. INF, RNA from DEF cells infected with MDV. CON, RNA from uninfected DEF cells. The precise location of the probes is shown in Fig. 2.



40

Fig. 3.

FIG. 3. Northern blot hybridization to detect transcripts from the expanded regions. poly(A)$^+$ RNA (0.5 μg) was used per lane. (A) Probe: 1.6-kbp SmaI-PstI subfragment which spans the expanded region of BamHI-H. (B) Probe: cDNA 3. (C) Probe: cDNA 1. (D) Probe: cDNA 4. (E) Probe: cDNA 11. (F) Probe: cDNA 15. INF, RNA from DEF cells infected with MDV. CON, RNA from uninfected DEF cells. The precise location of the probes is shown in Fig. 2.



46

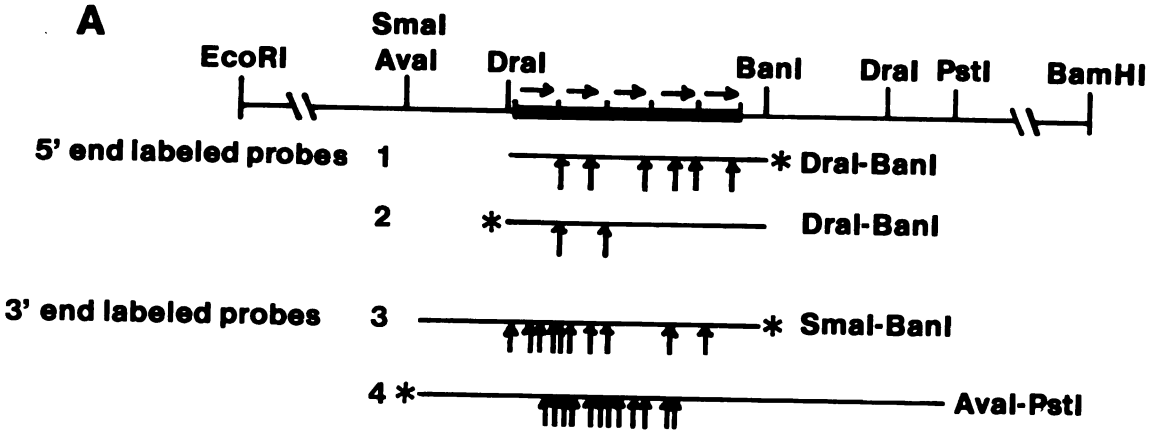
Fig. 3.

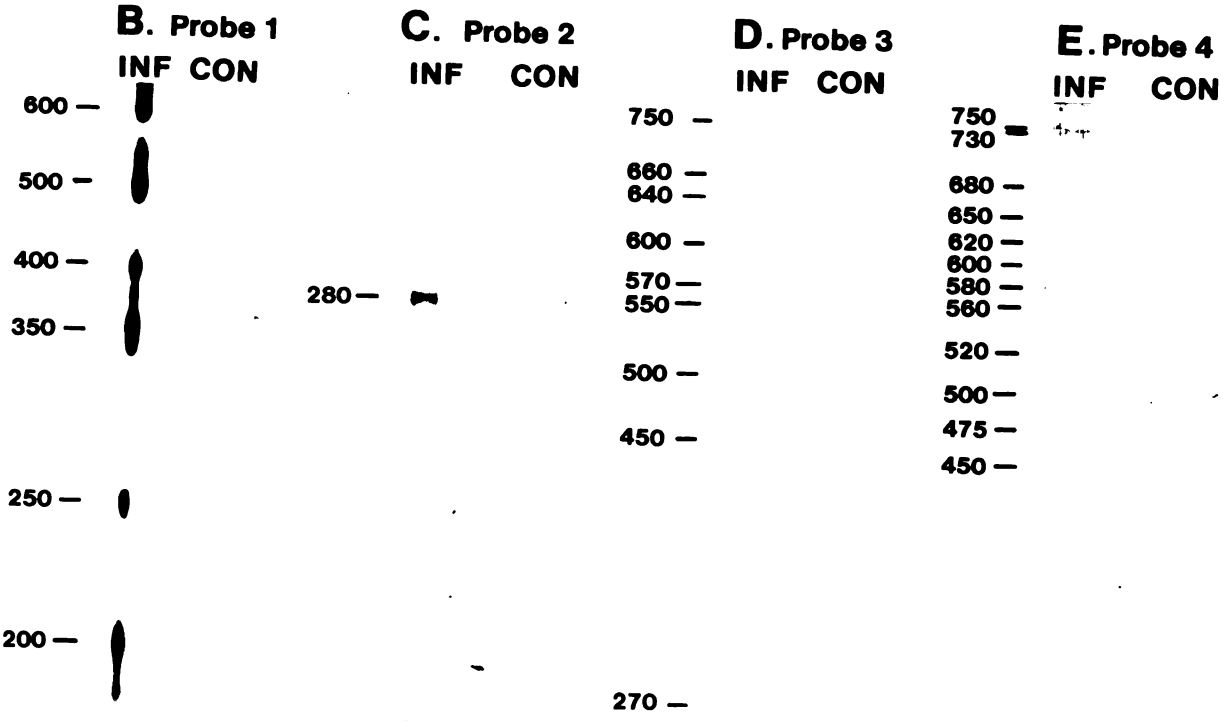
their 3' ends (Fig. 4D). For the same reason given for the probe 1, some of the transcripts might contain more than five copies of the 132-bp direct repeat at their 3' ends.

To map 3' ends of transcripts which terminate rightwardly within the expanded region of BamHI-H, probe 4, a 1.6-kbp AvaI-PstI fragment 3' end labeled at its AvaI site by [α-32p]dCTP, was used. Twelve RNA-DNA hybrids (from 450 bases to 750 bases) were protected, indicating that the respective transcripts contained one to four copies of 132 base direct repeat at their 3' ends (Fig. 4E).

FIG. 4. S1 nuclease protection assay to determine the 5' and 3' termini of the four groups of mRNA transcribed partly from the expanded region. (A) Schematic of the EcoRI-a subfragment of BamHI-H, with location of the probes (1 to 4) and the sites digested by S1 nuclease. The heavy line represents the expanded region, with each horizontal arrow representing one 132-bp direct repeat. Vertical arrows below each probe represent S1 nuclease digestion sites. (B, C, D, E) Results of S1 nuclease assays with the probes indicated for each panel. The sizes (in nucleotides) of the protected fragments were calculated from the positions of radiolabeled DNA from a DNA sequencing reaction run in parallel as markers (not shown). Lanes: INF, total cellular RNA from MDV-infected DEF cells; CON, total cellular RNA from uninfected DEF cells. Thirty micrograms of total RNA was used per lane.

FIG. 4.


A
SmaI
EcoRI
AvaI
DraI
BanI
DraI PstI
BamHI
5' end labeled probes
1
* DraI-BanI
2
* DraI-BanI
3' end labeled probes
3
* SmaI-BanI
4 *
AvaI-PstI


B. Probe 1
INF CON
600
500
400
350
250
200
100
C. Probe 2
INF CON
280
150
D. Probe 3
INF CON
750
660
640
600
570
550
500
450
270
170
E. Probe 4
INF CON
750
730
680
650
620
600
580
560
520
500
475
450

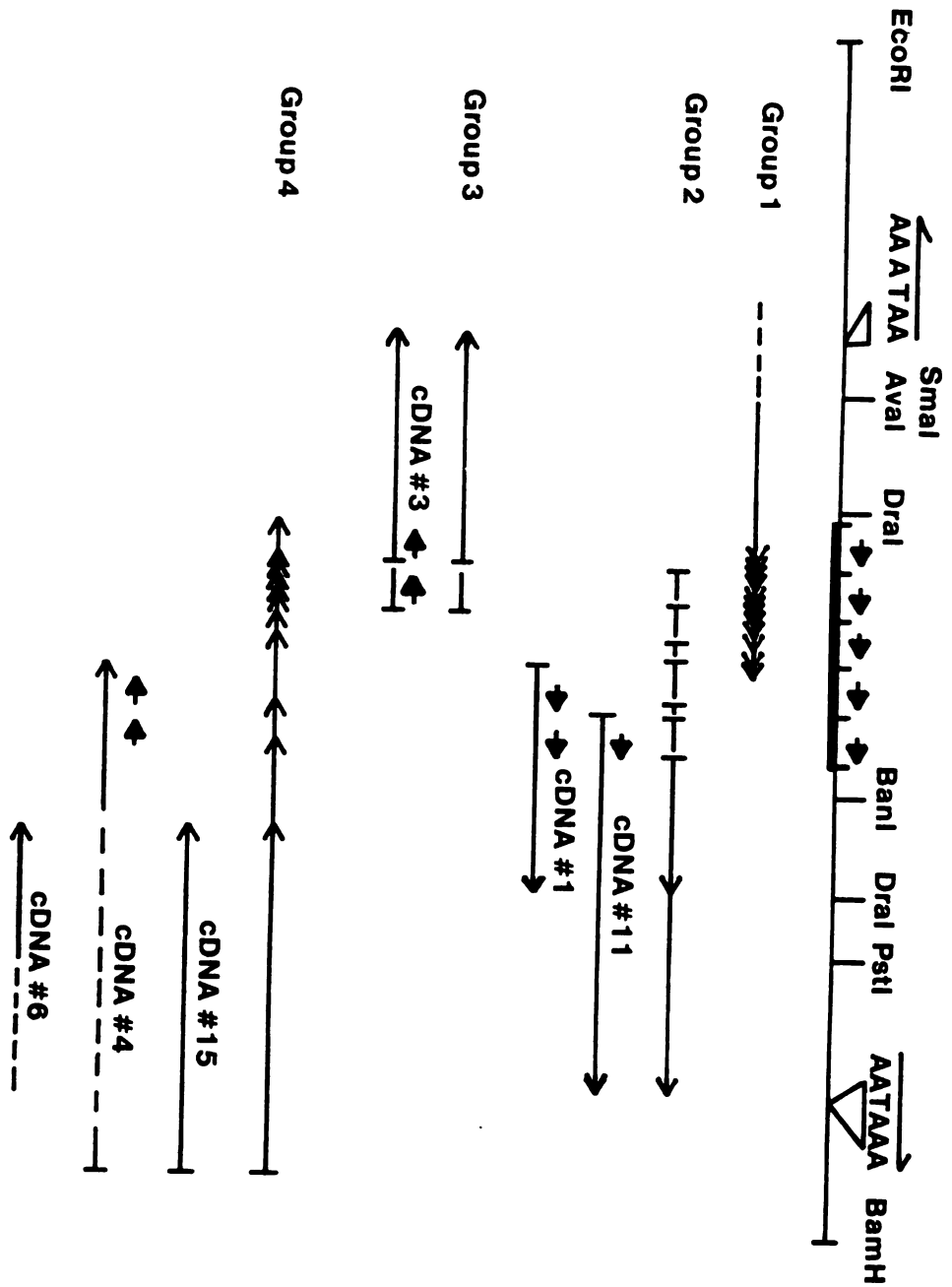
DISCUSSION

Our results, from both cDNA analysis and S1 nuclease protection studies, consistently indicate that transcription can either be initiated or terminated within or near the expanded regions of BamHI-D and -H at multiple sites and in both rightward and leftward directions. Based on the directions of transcription, and therefore whether they are initiated from or transcribed toward the expanded regions, the transcripts were categorized into four groups. Figure 5 shows the precise positions of the four groups of transcripts, mapped according to the S1 nuclease protection and cDNA sequence alignment. Due to the multiple transcriptional initiations and terminations and presence of multiple BamHI-D and -H fragments containing different numbers of 132-bp direct repeat (Fig. 1), RNAs with variable sizes may be transcribed as demonstrated by Northern blot analysis (Fig. 3).

Previously, Bradley et al. (1) reported the results of S1 nuclease protection studies indicating that a gene family, hypothesized to be directly associated with the tumorigenic potential of MDV and transcribed to yield 1.8-kb RNA, is composed of two groups of exons that are transcribed rightwardly from the expanded region of BamHI-H. Their observations are contradicted by the results of this report (summarized above), which were generated via cDNA sequencing combined with S1 nuclease protection analysis with conventional radiolabeled probes. Possibly the differences result from major variations in the methods used: (i) reliance on sequence analysis of cDNAs, in the study reported here, and (ii) use of S1 nuclease protection studies dependent on "cold probes" and probe generation methods that make it difficult to determine the direction or limits of transcription, in the report of Bradley et al. (1). Not only does our S1 nuclease protection analysis avoid such interpretation problems; this analysis is further confirmed by our use of cDNA analysis. While the

FIG. 5. Precise positions of the four groups of transcripts in the EcoRI-a subfragment of BamHI-H, mapped by S1 nuclease protection assay and cDNA sequence alignment with the published sequence of EcoRI-a subfragment (1), along with the assignment of the cDNAs from Fig. 2 to the four groups of transcripts, respectively. Vertical bars mark multiple initiation sites, with each bar representing one possible initiation site; arrowheads on the lines mark multiple termination sites, with each arrowhead representing one possible termination site. Dashed lines in group 1 RNA transcript and in cDNAs 4 and 6 are the undetermined regions. The heavy line at the top is the expanded region. The heavy arrows above the expanded region and above the cDNAs each represent one 132-bp direct repeat. Longer arrows above the map represent the direction of the polyadenylation consensus sequence.

Fig. 5.


EcoRI
AATAA
SmaI
AvaI
DraI
BanI
DraI
PstI
AATAAA
BamHI
Group 1
Group 2
Group 3
Group 4
cDNA #11
cDNA #1
cDNA #3
cDNA #15
cDNA #4
cDNA #6

specific results reported here differ from those reported by others (1), the general suggestion that a gene family hypothesized to be directly associated with the tumorigenic potential of MDV is involved (1) deserves further consideration. Therefore, in agreement with that suggestion, and to emphasize potential significance and future research directions, the four groups of transcripts reported here will also be referred to as originating from a gene family (as described above) for discussion purposes. However, future studies will be required to identify the specific gene or genes that are actually responsible for MDV's tumorigenic potential.

In this study, a 0.67-kb RNA transcript hybridized only with cDNA 3 (Fig. 3B), indicating that it is transcribed on the left side of the BamHI-H expanded region without the inclusion of any 132-bp direct repeat sequence. It is possible that this 0.67-kb transcript represents the 0.4-kb truncated RNA transcript of the 1.8-kb RNA family reported by Bradley et al. (2). The size variation could be explained by the difference in size markers used. In contrast to their work, this study used the RNA ladder containing markers extending below 0.67 and 0.4 kb (Fig. 3), making possible a more precise size determination. If such an interpretation is correct, the small transcript found in this study may result from a small subpopulation of virus already having undergone genomic amplification and attenuation. This was the explanation proposed by Bradley et al. (2) for the low level of their small transcript found in the preparations of viruses known to be pathogenic. If their hypothesis is correct, the finding of relatively minor amounts of the small transcript in this study in relation to the abundant 1.8 kb transcript (Fig. 3B), strongly suggests that most of the virus in the preparation used was not yet attenuated. Further experiments, needed to confirm that the two small transcripts found in both studies are actually the same and to independently confirm the correlation of its presence with attenuation, are

beyond the scope of this preliminary study, which serves to clarify a different issue.

Analysis of the nucleotide sequence of the 132-bp direct repeat at both rightward and leftward directions revealed several potential TATA box and polyadenylation signal consensus sequences (1, 14; unpublished data). There is one TATA box each in both the rightward (TTATTAAAT) and leftward (TTTAATAA) directions, and two polyadenylation signal consensus sequences (15) each in the rightward (AATAAG, ATTAAA) and leftward (CATAAA, AATAAG) directions. By correlating these consensus sequences for RNA initiation and polyadenylation with our results from S1 nuclease protection assay and cDNA analysis, it appears that these potential initiation and polyadenylation signals are likely to generate the diversity of the four groups of transcripts (Fig. 5). The concept that the 132-bp direct repeat serves as a bidirectional promoter region is not surprising, considering the observation of a similar phenomenon in the Epstein-Barr virus system (12). Their transfection experiments, which involved the use of the chloramphenicol acetyltransferase (CAT) reporter gene, demonstrated the presence of a bidirectional latent promoter region at the right-hand end of the Epstein-Barr virus genome, expressing the latent membrane protein (LMP) gene leftward and the terminal protein 2 (TP2) gene rightward (12).

Since the four groups of transcripts are bidirectionally transcribed from the expanded regions, group 1 transcripts are complementary to group 3 transcripts and group 2 transcripts are complementary to group 4 transcripts. It has been hypothesized that the complementary transcripts, or antisense RNAs, may play a role in gene regulation. Rogers and Speck (18) reported a bidirectional transcription of the Epstein-Barr virus major internal repeat whose rightward transcripts encode the six known viral nuclear antigens. They further

suggest that the leftward transcription may antagonize the expression of those nuclear antigen messages by formation of RNA duplexes. In an experimental system with the thymidine kinase gene, thymidine kinase activity is stably reduced by the regulation of antisense RNA through formation of duplex RNA-RNA in the nucleus (10). It has also been reported (11) that an antisense mRNA can direct the covalent modification of the transcript encoding fibroblast growth factor in Xenopus oocytes. It is important to note that this latter result occurs in a natural system. Although we do not know at this time whether a protein product is produced, the existence of bidirectional transcription described in this report makes it reasonable to speculate that a similar pattern of antisense gene regulation may exist for the four groups of MDV transcripts.

In summary, the work reported here, showing both rightward and leftward transcription from multiple sites within or near the expanded regions, raises questions concerning the results of the transcriptional analysis by Bradley et al. (1), in which experimental design precluded determining the direction of transcription. Also, from our cDNA analysis and S1 nuclease protection studies, it now appears that the gene family hypothesized to be directly associated with the tumorigenic potential of MDV (1) is composed of four groups of transcripts (this report) rather than two groups of exons (1).

ACKNOWLEDGMENTS

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REFERENCES

1. **Bradley, G., M. Hayashi, G. Lancz, A. Tanaka, and M. Nonoyama.** 1989. Structure of the Marek's disease virus BamHI-H gene family: genes of putative importance for tumor induction. J. Virol. **63**:2534-2542.
2. **Bradley, G., G, Lancz, A. Tanaka, and M. Nonoyama.** 1989. Loss of Marek's disease virus tumorigenicity is associated with truncation of RNAs transcribed within BamHI-H. J. Virol. **63**:4129-4135.
3. **B. W. Calnek.** 1985. Marek's disease - a model for herpesvirus oncology. Crit. Rev. Microbiol. **12**:293-320.
4. **Churchill, A. E., R. C. Chubb, and W. Baxendale.** 1969. The activation, with loss of antigenicity of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. J. Gen. Virol. **4**:557-564.
5. **Fukuchi, K., M. Sudo, Y.-B. Lee, A. Tanaka, and M. Nonoyama.** 1984. Structure of Marek's disease virus DNA: Detailed restriction enzyme map. J. Virol. **51**:102-109.
6. **Fukuchi, K., A. Tanaka, L. W. Schierman, R. L. Witter, and M. Nonoyama.** 1985. The structure of Marek's disease virus DNA: the presence of unique expansion in nonpathogenic viral DNA. Proc. Natl. Acad. Sci. USA **82**:751-754.
7. **Glaubiger, C., K. Nazerian, and L. F. Velicer.** 1983. Marek's disease herpesvirus. IV. Molecular characterization of Marek's disease herpesvirus A antigen. J. Virol. **45**:1228-1234.
8. **Isfort, R. J., H.-J. Kung, and L. F. Velicer.** 1987. Identification of the gene encoding Marek's disease herpesvirus A antigen. J. Virol. **61**:2614-2620.
9. **Kato, S., and K. Hirai.** 1985. Marek's disease virus. Adv. Virus Res. **30**:225-277.

10. **Kim, S. K., and B. J. Wold.** 1985. Stable reduction of thymidine kinase activity in cells expressing high levels of anti-sense RNA. Cell **42**:129-138.

11. **Kimelman, D., and M. W. Kirschner.** 1989. An antisense mRNA directs the covalent modification of the transcript encoding fibroblast growth factor in Xenopus oocytes. Cell **59**:687-696.

12. **Laux, G., A. Economou, and P. J. Farrell.** 1989. The terminal protein gene 2 of Epstein-Barr virus is transcribed from a bidirectional latent promoter region. J. Gen. Virol. **70**:3079-3084.

13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

14. **Maotani, K., K. Kanamori, K. Ikuta, S. Ueda, S. Kato, and K. Hirai.** 1986. Amplification of a tandem direct repeat within inverted repeats of Marek's disease virus DNA during serial in vitro passage. J. Virol. **58**:657-659.

15. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1983. Inhibition of RNA cleavage but not polyadenylation by a point mutation in mRNA 3' consensus sequence AAUAAA. Science **305**:600-605.

16. **Nazarian, 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.

17. **Okazaki, W., W. G. Purchase, and B. R. Burmester.** 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys (HVT). Avian Dis. **16**:108-125.

18. **Rogers, R. P., and S. H. Speck.** 1990. Bidirectional transcription of the Epstein-Barr virus major internal repeat. J. Virol. **64**:2426-2429.

19. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463-5467.

20. **Sharma, J. M., and H. A. Stone.** 1972. Genetic resistance to Marek's disease. Delineation of the response of genetically resistant chickens to Marek's disease virus infection. Avian Dis. **16**:894-906.

21. **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.

22. **Stuart, P., M. Ito, C. Stewart, and S. E. Conrad.** 1985. Induction of cellular thymidine kinase occurs at the mRNA level. Mol. Cell. Biol. **5**:1490-1497.

Chapter III

Identification of a Unique Marek's Disease Virus Gene Which Encodes a 38 Kilodalton Phosphoprotein and Is Expressed in both Lytically Infected Cells and Latently Infected Lymphoblastoid Tumor Cells

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1992. J. Virol. 66:85-94.

Dr. P. J. A. Sondermeijer provided 2.6-kbp nucleotide sequence of BamHI-H and helped writing this manuscript.

ABSTRACT

The Identification of unique Marek's disease (MD) virus (MDV) antigens expressed not only in lytically infected cells but also in latently infected MD lymphoblastoid tumor cell lines is important in understanding the molecular mechanisms of latency and transformation by MDV, an oncogenic lymphotropic herpesvirus of chickens. Through cDNA and nucleotide sequence analysis, an open reading frame (designated the pp38 ORF) which encodes a predicted polypeptide of 290 amino acids was identified in BamHI-H. Demonstration that the pp38 ORF spans the junction of the long MDV unique and long internal repeat regions (MDV has an alphaherpesvirus genome structure) precludes the presence of the gene encoding the B antigen complex (gp100, gp60, and gp49) in the same region of BamHI-H, where it was originally thought to exist. Duplication of the complete pp38 ORF was not observed in BamHI-D, but part of it (encoding 45 amino acids) was found in the long terminal repeat region of the fragment. By use of trpE-pp38 fusion proteins, antisera against pp38 were prepared. By immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, a predominant virus-specific 38,000 Da polypeptide (designated pp38) and a minor 24,000 Da polypeptide (designated p24), were found. No precursor-product relationship was found between pp38 and p24 by pulse-chase analysis, and only pp38 was detected by Western blot analysis with antiserum to pp38. pp38 was found to be phosphorylated and present in oncogenic serotype-1 but not in nononcogenic serotype-3, MDV-infected cells. Expression of the gene encoding pp38 was relatively insensitive to phosphonoacetic acid inhibition, suggesting that pp38 may belong to one of the early classes of herpesvirus proteins. pp38 was also detected in the latently infected MSB-1 lymphoblastoid tumor cell line. The detection of antibody against pp38 in immune chicken sera indicates that pp38 is an immunogen in

birds with MD. Most of the properties described here for a protein detected by methods based on finding the ORF first are identical to those of a 38 kDa phosphoprotein reported by others, suggesting that they are the same. Collectively, the data reported here provides (i) more definitive information on the complete ORF of another MDV gene and the protein that it encodes, (ii) clarification of the gene content within a specific region of the MDV genome, and (iii) the molecular means to conduct further studies to determine whether pp38 plays a role in MDV latency and transformation.

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by cell-associated MD virus (MDV) and is characterized principally by T-cell lymphomas and peripheral nerve demyelination (4). The T-cell lymphomas occurs after the establishment of latency in the MDV-infected chickens, raising questions regarding the viral gene(s) expressed to cause these two phenomena. Presumably one or more viral genes are responsible for the establishment and maintenance of latency and for tumor induction. Whether these events are the result of the same gene or separate ones is unknown. Identification of an MDV-specific antigen(s) expressed not only in MDV-infected fibroblast cells but also in latently infected and transformed MD lymphoblastoid tumor cell lines and MD tumors is an important first step toward an understanding of the mechanisms of latency and transformation by MDV.

In lytically infected cells, there is extensive gene expression over the entire MDV genome (19, 29). In latently infected lymphoblastoid tumor cell lines derived from MD tumors, 4 to 7 transcripts were reported by Schat et al. (29) in their partial transcriptional mapping of MDV, 32 transcripts clustered in the short and long repeat regions were reported by Sugaya et al. (33), and 29 transcripts spread over almost the entire MDV genome were reported by Maray et al. (19). Very few of the transcripts detectable in both lytically and latently infected cells were further characterized. Relevent to this study was the report of a 1.9-kb immediate early (IE) transcript localized to BamHI-H (29).

Silva and Lee (31) first reported the existence of three viral proteins of 41, 38, and 24 kDa (then designated p41, p38, and p24, respectively) detected in serotype-1 MDV (oncogenic MDV)-infected cells but not in fibroblast cells infected with nononcogenic serotype 2 MDV and nononcogenic serotype 3 herpesvirus of turkeys (HVT). p38 was the most prominant molecule. Later,

Ikuta et al. (13) and Nakajima et al. (22) reported a similar group of MDV specific antigens of 43, 39, 36, and 24 kDa also detected in latently infected MSB-1 lymphoblastoid tumor cells, but only when the cells were treated with 5-iodo-2-deoxyuridine (IUdR). The latter workers reported that these proteins were phosphorylated at serine residues. Furthermore, on the basis of in vitro translation analysis, they suggested that the 24-kDa and 39- or 36-kDa (their newer terminology) phosphoproteins were translated from distinct mRNAs and encoded from overlapping genes or from separate regions with partial DNA homology within the MDV genome (22). The MDV-specific phosphoproteins were also detected in tumor lesions of chickens with MD (21). Cui et al. (6) further reported the identification of two λgt11 clones which may encode p41, p38, and p24 (31). Both clones were mapped to the BamHI-H fragment of the MDV genome. Five proteins (135, 41, 38, 24, and 20 kDa), including p38, were detected by antisera against two MDV-lacZ fusion proteins (6). However, the nucleotide sequences of the two clones were not determined, the structural relationship among the five proteins, if any, remained to be elucidated, and the entire gene(s) encoding the phosphoprotein(s) was not defined (6).

In this study, an open reading frame (ORF) was identified in the BamHI-H fragment of the MDV genome through cDNA and nucleotide sequence analyses. The predicted protein product of the ORF was detected in both lytically and latently infected cells and was found to be 38 kDa, phosphorylated, not detected in HVT-infected cells, and immunogenic in birds with MD.

MATERIALS AND METHODS

Cells and viruses. The preparation, propagation, and infection of duck embryo fibroblast (DEF) cells with MDV and HVT were performed as described previously (5). The MSB-1 cell line (1) was used as a representative MD lymphoma-derived cell line and was cultured in RPMI 1640 supplemented with 10% fetal calf serum at 37^0C in a humidified atmosphere of 5% CO_2 in air. At the time at which the MSB-1 cell line was used, its infection with MDV was latent, as shown by a lack of the MDV A antigen (gp57-65), the MDV B antigen complex (gp100, gp60, and gp49) and MDV p79. The MDV GA strain used in this study was at cell culture passage 6 following the isolation of cell-free virus from feather tips obtained from infected birds showing symptoms of MD. The HVT FC-126 vaccine strain virus at cell culture passage 13 was used in this study.

Antisera. The immune chicken sera (ICS) were convalescent-phase sera obtained from chickens with MD as a result of natural infection. ICS contain antibodies against the MDV A antigen (gp57-65), the MDV B antigen complex (gp100, gp60, and gp49), MDV p79 and other MDV proteins that remain to be characterized.

Construction and screening of the cDNA library. A cDNA synthesis kit from Bethesda Research Laboratories (BRL) was used in accordance with the manufacturer's specifications. The newly synthesized cDNAs were made blunt-ended via the fill-in reaction catalyzed by the Klenow fragment of *Escherichia coli* polymerase I. The blunt-ended cDNAs were directly cloned into the SmaI site of calf intestine alkaline phosphatase-treated pUC18. An in situ DNA hybridization method was used to screen the cDNA library and was carried out essentially as described previously (5).

DNA sequencing and nucleotide sequence analysis. The initial cDNA sequencing was carried out with the pUC sequencing system as previously described (5). Sequencing of genomic DNA and further sequencing of cDNA were carried out with the M13 sequencing system as previously reported (28). The BamHI-H fragment used in this study is from the BamHI library of the MDV GA strain (kindly provided by M. Nonoyama). Genepro software (Riverside Scientific Enterprises) was used to perform the ORF analysis. For protein homology analysis, a search of the National Biochemical Research Foundation-Protein database (Release 21.0, 6/89) was made with the Genetics Computer Group program FASTA from University of Wisconsin.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated from mock- and MDV-infected DEF cells essentially as described previously (5). Purification of poly(A)$^+$ RNA was carried out as described previously (27). RNA was size fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and was transferred to a nitrocellulose filter. The Northern blot was hybridized (probes are described in Figure legends) and washed as described previously (5). Transcript size determinations were based on a comparison with a BRL RNA ladder run in parallel.

S1 nuclease analysis. S1 nuclease analysis was carried out essentially as described previously (27) with the probes described in the text. The radiolabeled DNAs from a DNA sequencing reaction were used as size markers. Hybridizations were performed at 46°C, a temperature chosen because of the G+C contents of the MDV DNA probes.

Expression of trpE fusion proteins. The vector system used to express the MDV ORF in E. coli consists of a group of plasmids (pATH vectors) encoding approximately 37 kDa of the bacterial trpE ORF under the control of the inducible trp operon promoter (17). A polylinker with multiple cloning sites

at the 3' end of the trpE ORF allows in-frame insertion of a foreign ORF. Five DNA fragments from the MDV ORF were inserted into various trpE pATH vectors, and their respective trpE fusion proteins were generated. The precise locations of these DNA fragments and the sizes of their respective trpE fusion proteins are detailed in Results.

Antibody production. Fusion proteins were purified by electrophoresis in preparative 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were stained with Coomassie brilliant blue to identify the positions of the fusion proteins. The areas of the gels containing the fusion proteins were excised and emulsified in complete Freund's adjuvant (Difco Laboratories). Initial immunization of 2-kg New Zealand White female rabbits was done with 200 to 500 μg of protein. Rabbits were boosted after 3 weeks with 100 to 200 μg of protein in incomplete Freund's adjuvant, and sera were collected 7 to 10 days later. Subsequent booster immunizations were done approximately 2 weeks apart as needed to obtain detectable and usable titers.

Radiolabeling of proteins. Mock- and MDV-infected DEF cells were labeled with [^{35}S]methionine (specific activity, 1,000 μCi/mmol, ICN) at 48 h postinfection for 4 h by a standard method as previously described (10). MSB-1 cells (5 x 10^6 cells per 60-mm plate) were labeled with 100 μCi of [^{35}S]methionine per plate for 4 h. Similar method was also used for ^{32}P-labeling of DEF cells, with following exceptions. Phosphate-free Dulbecco modified Eagle medium was used for 1 h before and during the 4 h labeling and 500 μCi of ^{32}Pi (carrier-free, Amersham Corp.) was used per 60-mm plate. Pulse-chase labeling was done by method already established for the MDV system (10), with following minor modifications. Preincubation without methionine was for 1 h, pulse-labeling was done by incubation with 250 μCi of [^{35}S]methionine in 1 ml of medium per 60-mm plate for 5 or 15 min, and chases

were done in the presence of unlabeled methionine for 0, 5, 15 or 30 min. For labeling in the presence of phosphonoacetic acid (PAA) (Sigma) at concentration of 200 μg per ml (23) or 2 μg of tunicamycin (Calbiochem-Behring Corp.) per ml, cells were preincubated with PAA for 24 h or with tunicamycin for 1 h and treated at same concentration during the 4-h labeling period. After labeling was complete, the culture medium was collected, cells were washed three times with sterile phosphate-buffered saline, and ice-cold detergent buffer was added to lyse the cells (10). The culture medium samples and lysates were clarified by centrifugation as previously reported (10).

Immunoprecipitation and SDS-PAGE analyses. Immunoprecipitation analysis was carried out as previously described (10). The immunoprecipitates were washed, suspended in sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Protein markers (BRL) were used as molecular weight standards. Molecular sizes were calculated by interpolation between standard proteins by the method of Weber and Osborn (34). Fluorography was carried out as previously described (2). 10% SDS-polyacrylamide gels were used unless indicated otherwise. Anti-pp38, an antiserum against one of the trpE fusion proteins of the MDV ORF was used unless indicated otherwise.

Western blot analysis. Western blot analysis was carried out essentially as described previously (27). ^{35}S-labeled infected cell lysates were used for immunoprecipitation analysis with anti-pp38, and the immunoprecipitates were run on a 10% SDS-polyacrylamide gel. Proteins were blotted onto a nitrocellulose filter. The filter was probed with anti-pp38 at a 1:400 dilution. Horseradish peroxidase conjugated goat anti-rabbit immunoglobulin G (Sigma) was used as the second antibody. Bound second antibody was detected by subsequent incubation in the presence of the substrate solution (4-chloro-1-naphthol) (Sigma).

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been given GenBank accession number M73484.

RESULTS

Isolation and localization of cDNA 2 and cDNA 7. The MDV alphaherpesvirus genome structure and the locations of BamHI-D and -H within the MDV genome (3) are presented in Fig. 1A. The 917-bp EcoRI c subfragment of BamHI-H (Fig. 1B) was used to probe the MDV cDNA library in pUC18. Two cDNAs were identified by in situ hybridization and designated cDNA 7 (297-bp) (Fig. 1C) and cDNA 2 (1,092-bp) (Fig. 1D). Both cDNAs were completely sequenced. Alignment of the two cDNA sequences with the sequence of BamHI-H (see below) revealed the precise locations of the two cDNAs (Fig. 1C and 1D). The 3' end of cDNA 2, presumably the 3' end of its mRNA, was located in the EcoRI b subfragment 567 bases to the left of an EcoRI restriction site designated EcoRI site-2, which separates the EcoRI b and c subfragments of BamHI-H. The sequence alignment also revealed that cDNA 7 is a part of cDNA 2 (Fig. 1C and 1D), and that the direction of transcription for their mRNA is from right to left in relation to the MDV genome (Fig. 1E).

Detection of the transcript for cDNA 2. Since cDNA 7 is a part of cDNA 2, only the latter was used as a probe for Northern blot analysis. A major 1.95-kb transcript was detected along with two minor components at 1.3- and 0.8-kb (Fig. 2A, data from a long exposure are presented to demonstrate the existence of the latter two). When the EcoRI c subfragment was used as a probe (Fig. 1B), only the 1.95-kb mRNA was detected (Fig. 2B), even upon prolonged exposure (data not shown). Since 525-bp of cDNA 2 is located in the EcoRI c subfragment (Fig. 1D) and only the 1.95-kb mRNA was detected by both probes (cDNA 2 and EcoRI c subfragment), the 1.95-kb major transcript (Fig. 1E) is the mRNA from which cDNA 2 is generated.

Defining the 5' end of the 1.95-kb mRNA by S1 nuclease analysis. On the basis of the location of 1,092-bp cDNA 2 (Fig. 1D) and the size (1.95-kb) of the

FIG. 1. Organizational summary of the MDV alphaherpesvirus genome structure (3), restriction enzyme map of BamHI-H, locations of the two cDNAs, and locations of the mRNA and coding region for the pp38 gene. (A) Schematic representation of the MDV genome. The MDV genome consists of a U_L region, flanked by a TR_L and an IR_L, and a short unique region (U_S), flanked by a short inverted repeat (IR_S) and a short terminal repeat (TR_S). D and H represent the locations of the BamHI-D and -H fragments in the MDV genome, respectively. (B) Restriction enzyme map of BamHI-H. The EcoRI a, b, and c subfragments of BamHI-H are labeled as such. (C) Location of cDNA 7. (D) Location of cDNA 2. (E) Location and direction of transcription of the 1.95-kb mRNA. (F) Location of the pp38 ORF.

Fig. 1.

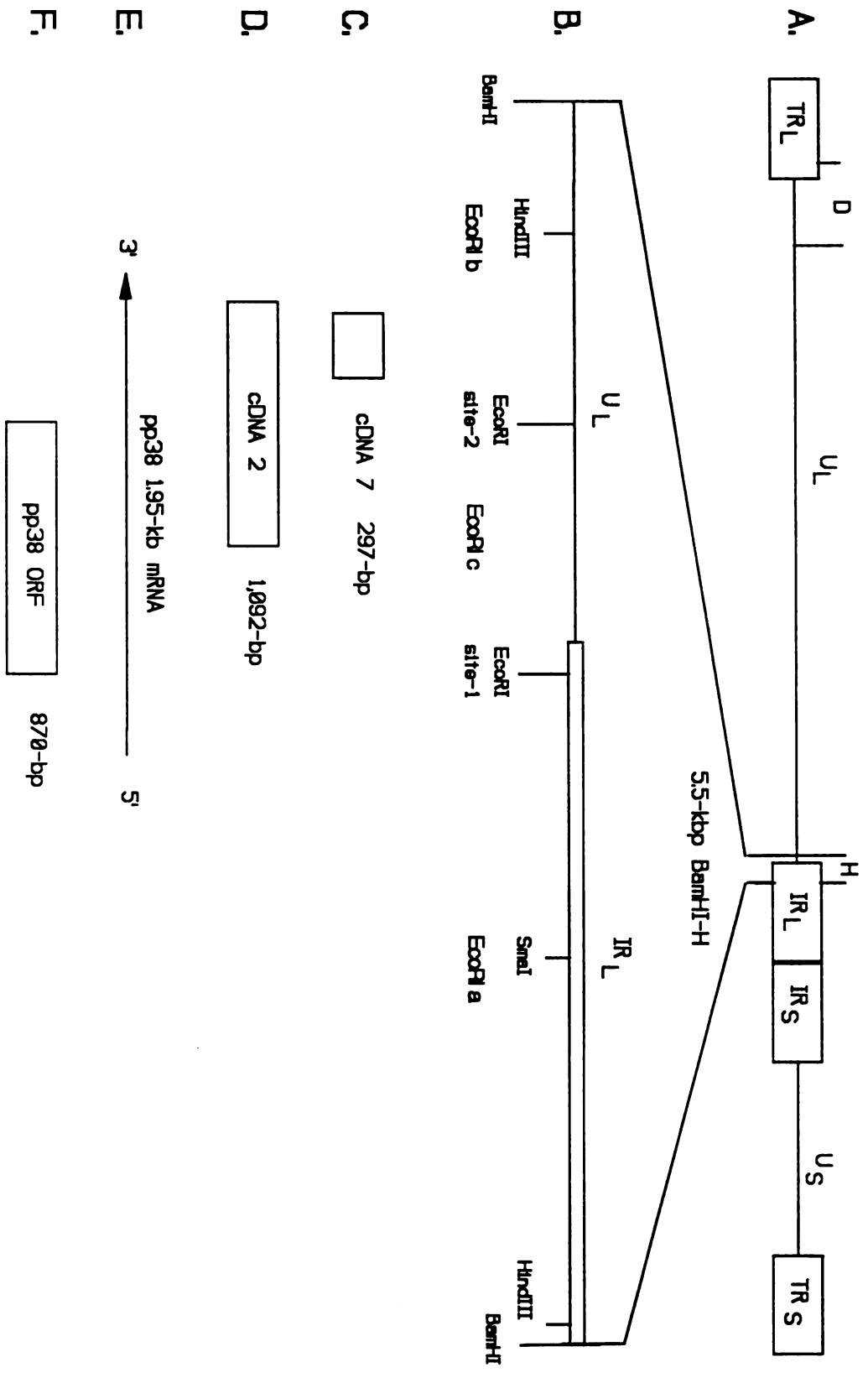

A.
TRL
D
UL
H
IRL
IRS
US
TRS
5.5-kbp BamHI-H
B.
BamHI
HindIII
EcoRI b
EcoRI site-2
EcoRI c
EcoRI site-1
SmaI
EcoRI a
HindIII
BamHI
UL
IRL
C.
cDNA 7 297-bp
D.
cDNA 2 1,092-bp
E.
3'
5'
pp38 1.95-kb mRNA
F.
pp38 ORF
870-bp

FIG. 2. Northern blot hybridization for the detection of the transcript of cDNA 2. (A) cDNA 2 was used as the probe. (B) EcoRI c subfragment of BamHI-H was used as the probe. A total of 0.5 μg of poly(A)$^+$ RNA was used per lane. INF, RNA from MDV-infected cells. CON, RNA from mock-infected cells.

Fig. 2.

transcript, the 5' end of the mRNA was predicted to be located adjacent to an EcoRI restriction enzyme site, designated EcoRI site-1, which separates the EcoRI c and a subfragments of BamHI-H (Fig. 1B). Two different probes were used to determine more precisely the 5' end of the 1.95-kb transcript.

A 1.87-kbp EcoRI-SmaI subfragment resulting from partial digestion of BamHI-H (Fig. 1B) was 5' end labeled. Following its digestion by EcoRI, a 917-bp EcoRI-EcoRI (EcoRI c) subfragment was 5' end labeled only at EcoRI site-2. The results of an S1 nuclease assay indicated that this 917-bp EcoRI c subfragment was fully protected (data not shown), suggesting that the 5' end of the 1.95-kb transcript is located to the right of EcoRI site-1 in the EcoRI a subfragment. To further map the 5' end, an EcoRI a subfragment was 5' end labeled. Following its digestion by SmaI, a 950-bp EcoRI-SmaI subfragment (Fig. 1B) was 5' end labeled only at EcoRI site-1. A 370-bp RNA-DNA hybrid was protected from S1 nuclease digestion (Fig. 3), indicating that the 5' end of the 1.95-kb mRNA is located in the EcoRI a subfragment 370 bases to the right of the EcoRI site-1. These results indicate that the transcript extends from its 5' end, 370-bp to the right of the EcoRI site-1, through the 917-bp EcoRI c subfragment, as determined both by S1 analysis, to its 3' end, 567-bp to the left of the EcoRI site-2 as determined by cDNA analysis. Therefore the size of the mRNA is 1.85-kb, not including the poly(A) tail, which presumably contributes to the 1.95-kb of the actual transcript.

Nucleotide sequencing and ORF analysis. Both cDNA 2 and its corresponding region on BamHI-H were completely sequenced in both directions. After sequence alignment, only 2 nucleotides were found to differ between cDNA 2 and the genomic DNA (Fig. 4): a C in cDNA 2 compared with an A in the genomic DNA at position 1,045, the third position of a codon within a predicted ORF, resulting in no predicted amino acid change; and a T in cDNA

FIG. 3. S1 nuclease protection assay for the determination of the 5' end of the 1.95-kb mRNA. A 950-bp EcoRI-SmaI subfragment of BamHI-H (Fig. 1B) was used as the probe. The sizes (in nucleotides) of the protected fragment was calculated from the positions of radiolabeled DNAs from a DNA sequencing reaction run in parallel as a marker (not shown). Lanes: PROBE, undigested probe control; CON, total cellular RNA from uninfected DEF cells; INF, total cellular RNA from MDV-infected DEF cells. Thirty micrograms total RNA was used per lane.

Fig. 3.

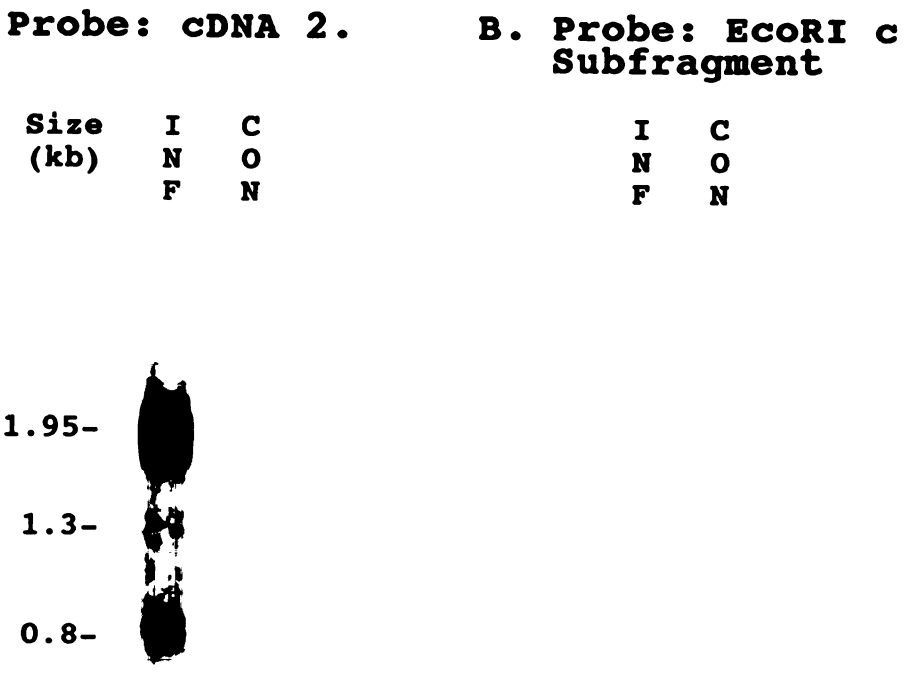
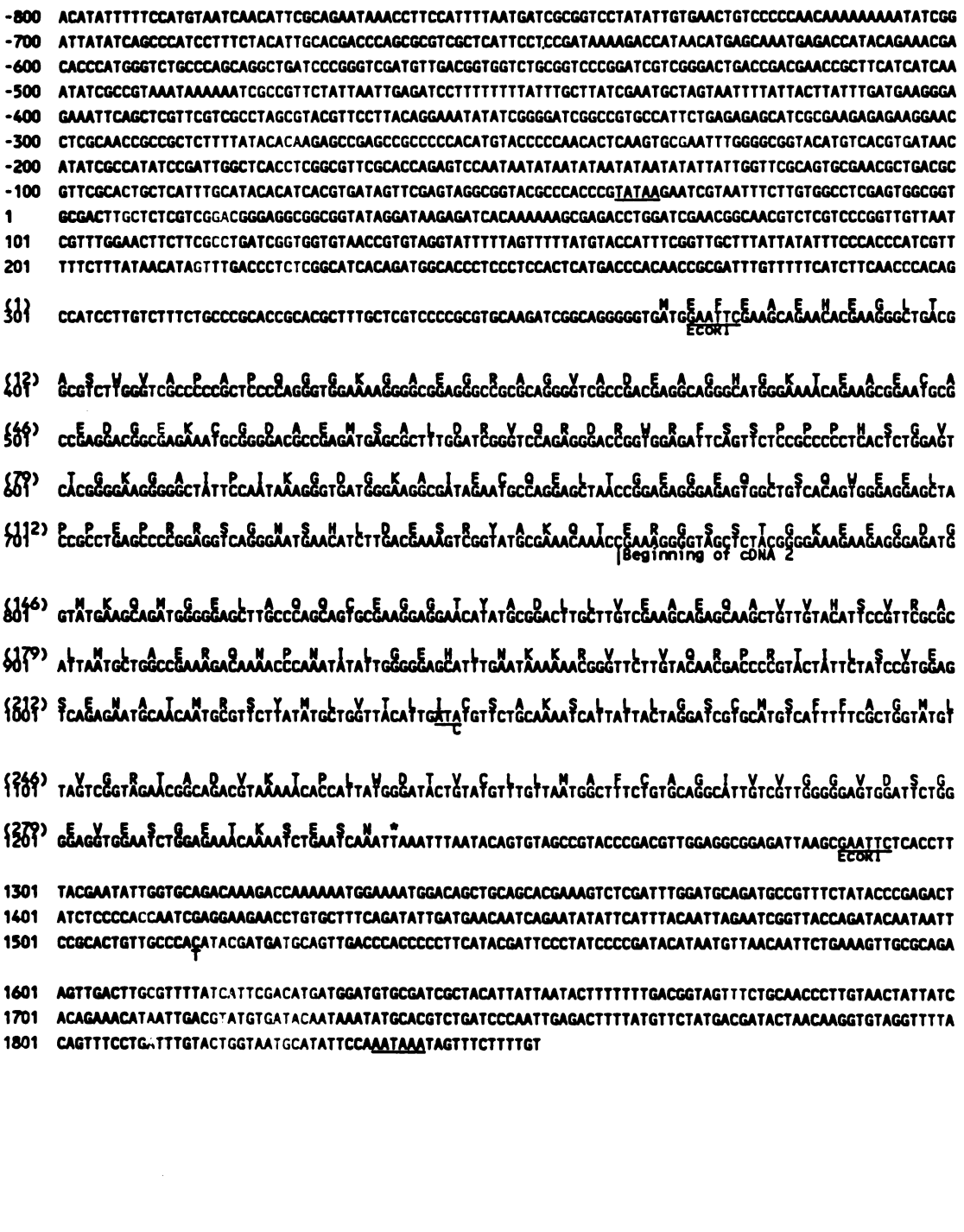

A. Probe: cDNA 2.
B. Probe: EcoRI c Subfragment
Size (kb)
I N F
C O N
I N F
C O N
1.95-
1.3-
0.8-

FIG. 4. Nucleotide sequence of the pp38 gene and analysis of its ORF. The 5' end of the mRNA determined by S1 nuclease analysis is numbered as nucleotide 1. The predicted amino acid sequence of the ORF is shown by the single-letter code above the nucleotides with amino acid numbers in parentheses. The potential TATA box and AATAAA consensus sequence for polyadenylation are underlined. The two EcoRI restriction enzyme sites are underlined and labeled as such. The vertical bar followed by "Beginning of cDNA 2" represents the start of cDNA 2. The two nucleotide differences found between genomic DNA and cDNA 2 are underlined at nucleotide positions 1,045 and 1,516.

Fig. 4.


-800 ACATATTTTTCCATGTAATCAACATTCGCAGAATAAACCTTCCATTTTAATGATCGCGGTCCTATATTGTGAACTGTCCCCCAACAAAAAAAAATATCGG
-700 ATTATATCAGCCCATCCTTTCTACATTGCACGACCCAGCGCGTCGCTCATTCCTCCGATAAAAGACCATAACATGAGCAAATGAGACCATACAGAAACGA
-600 CACCCATGGGTCTGCCCAGCAGGCTGATCCCGGGTCGATGTTGACGGTGGTCTGCGGTCCCGGATCGTCGGGACTGACCGACGAACCGCTTCATCATCAA
-500 ATATCGCCGTAAATAAAAAATCGCCGTTCTATTAATTGAGATCCTTTTTTTTATTTGCTTATCGAATGCTAGTAATTTTATTACTTATTTGATGAAGGGA
-400 GAAATTCAGCTCGTTCGTCGCCTAGCGTACGTTCCTTACAGGAAATATATCGGGGATCGGCCGTGCCATTCTGAGAGAGCATCGCGAAGAGAGAAGGAAC
-300 CTCGCAACCGCCGCTCTTTTATACACAAGAGCCGAGCCGCCCCCACATGTACCCCCAACACTCAAGTGCGAATTTGGGGCGGTACATGTCACGTGATAAC
-200 ATATCGCCATATCCGATTGGCTCACCTCGGCGTTCGCACCAGAGTCCAATAATATAATATAATATATTATTGGTTCGCAGTGCGAACGCTGACGC
-100 GTTCGCACTGCTCATTTGCATACACATCACGTGATAGTTCGAGTAGGCGGTACGCCCACCCGTATAAGAATCGTAATTTCTTGTGGCCTCGAGTGGCGGT
1 GCGACTTGCTCTCGTCGGACGGGAGGCGGCGGTATAGGATAAGAGATCACAAAAAAGCGAGACCTGGATCGAACGGCAACGTCTCGTCCCGGTTGTTAAT
101 CGTTTGGAACTTCTTCGCCTGATCGGTGGTGTAACCGTGTAGGTATTTTTAGTTTTTATGTACCATTTCGGTTGCTTTATTATATTTCCCACCCATCGTT
201 TTTCTTTATAACATAGTTTGACCCTCTCGGCATCACAGATGGCACCCTCCCTCCACTCATGACCCACAACCGCGATTTGTTTTTCATCTTCAACCCACAG
(1) M E F E A E H E G L T
301 CCATCCTTGTCTTTCTGCCCGCACCGCACGCTTTGCTCGTCCCCGCGTGCAAGATCGGCAGGGGGTGATGGAATTCGAAGCAGAACACGAAGGGCTGACG
ECORI
(13) A S V V A P A P Q G G K G A E G R A G V A D E A G H G K T E A E C A
401 GCGTCTTGGGTCGCCCCCGCTCCCCAGGGTGGAAAAGGGGCGGAGGGCCGCGCAGGGGTCGCCGACGAGGCAGGGCATGGGAAAACAGAAGCGGAATGCG
(46) E D G E K C G D A E M S A L D R V Q R D R V R F S S P P P H S G V
501 CCGAGGACGGCGAGAAATGCGGGGACGCCGAGATGAGCGCTTTGGATCGGGTCCAGAGGGACCGGTGGAGATTCAGTTCTCCGCCCCCTCACTCTGGAGT
(79) T G K G A I P I K G D G K A I E C Q E L T G E G E Q L S Q V E E L
601 CACGGGGAAGGGGGCTATTCCAATAAAGGGTGATGGGAAGGCGATAGAATGCCAGGAGCTAACCGGAGAGGGAGAGTGGCTGTCACAGTGGGAGGAGCTA
(112) P P E P R R S G N S H L D E S R Y A K Q T E R G S S T G K E E G D G
701 CCGCCTGAGCCCCGGAGGTCAGGGAATGAACATCTTGACGAAAGTCGGTATGCGAAACAAACCGAAAGGGGTAGCTCTACGGGGAAAGAAGAGGGAGATG
Beginning of cDNA 2
(146) M K Q M G E L A Q Q C E G G T Y A D L L V E A E Q A V V H S V R A
801 GTATGAAGCAGATGGGGGAGCTTGCCCAGCAGTGCGAAGGAGGAACATATGCGGACTTGCTTGTCGAAGCAGAGCAAGCTGTTGTACATTCCGTTCGCGC
(179) L M L A E R Q N P N I L G E H L N K K R V L V Q R P R T I L S V E
901 ATTAATGCTGGCCGAAAGACAAAACCCAAATATATTGGGGGAGCATTTGAATAAAAAACGGGTTCTTGTACAACGACCCCGTACTATTCTATCCGTGGAG
(212) S E N A T M R S Y M L V T L I C S A K S L L L G S C M S F F A G M L
1001 TCAGAGAATGCAACAATGCGTTCTTATATGCTGGTTACATTGATATGTTCTGCAAAATCATTATTACTAGGATCGTGCATGTCATTTTTCGCTGGTATGT
C
(246) V G R I A D V K T P L V D T V C L L M A F C A G I V V G G V D S G
1101 TAGTCGGTAGAACGGCAGACGTAAAACACCATTATGGGATACTGTATGTTTGTTAATGGCTTTCTGTGCAGGCATTGTCGTTGGGGGAGTGGATTCTGG
(279) E V E S G E T K S E S N *
1201 GGAGGTGGAATCTGGAGAAACAAAATCTGAATCAAATTAAATTTAATACAGTGTAGCCGTACCCGACGTTGGAGGCGGAGATTAAGCGAATTCTCACCTT
ECORI
1301 TACGAATATTGGTGCAGACAAAGACCAAAAAATGGAAAATGGACAGCTGCAGCACGAAAGTCTCGATTTGGATGCAGATGCCGTTTCTATACCCGAGACT
1401 ATCTCCCCACCAATCGAGGAAGAACCTGTGCTTTCAGATATTGATGAACAATCAGAATATATTCATTTACAATTAGAATCGGTTACCAGATACAATAATT
1501 CCGCACTGTTGCCCACATACGATGATGCAGTTGACCCACCCCCTTCATACGATTCCCTATCCCCGATACATAATGTTAACAATTCTGAAAGTTGCGCAGA
T
1601 AGTTGACTTGCGTTTTATCATTCGACATGATGGATGTGCGATCGCTACATTATTAATACTTTTTTTGACGGTAGTTTCTGCAACCCTTGTAACTATTATC
1701 ACAGAAACATAATTGACGTATGTGATACAATAAATATGCACGTCTGATCCCAATTGAGACTTTTATGTTCTATGACGATACTAACAAGGTGTAGGTTTTA
1801 CAGTTTCCTGTTTTGTACTGGTAATGCATATTCCAAATAAATAGTTTCTTTTGT

2 compared with a C in the genomic DNA at position 1,816, which is in the 3' noncoding region of a predicted ORF.

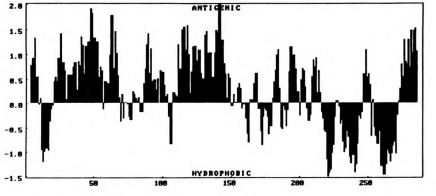
Analysis of the DNA sequences of cDNA 2 and BamHI-H (Fig. 4) resulted in identification of an ORF, designated pp38 ORF (Fig. 1F), which encodes a predicted polypeptide of 290 amino acids. The first in frame ATG initiation codon of the pp38 ORF is located just 3 nucleotides upstream of the EcoRI site-1 (Fig. 4). A 367 base 5' untranslated leader sequence and a 617 base 3' untranslated sequence were found in the 1.95-kb mRNA (Fig. 4). A potential TATA consensus sequence is located 38-bp upstream of the mRNA initiation site (Fig. 4). An AATAAA motif for polyadenylation was found at position 1,836, 13-bp before the start of the poly(A) tail in cDNA 2 (Fig. 4); this position is within the general range of 10 to 30 bases upstream of the 3' end before the addition of poly(A) tail (20). The calculated molecular weight of the predicted 290 amino acid polypeptide is 31,169. Only one potential N-linked glycosylation site was found at predicted amino acid position 214 (Fig. 4). Upon hydropathy analysis, the carboxy terminal end of the predicted polypeptide was found to contain two hydrophobic potential transmembrane domains (data not shown). No signal peptide was found. As determined by GenBank protein homology analysis, the predicted 290 amino acid sequence lacks any significant homology to any other known protein sequences, including those of other herpesviruses and oncoproteins.

Generation of fusion proteins and production of antibodies against them. The locations of the potential epitopes predicted from the antigenicity plot are presented in Fig. 5A. Five DNA fragments (Fig. 5B, fragments 1, 2, 3, 4, and 5) from the pp38 ORF coding region were cloned into various pATH vectors, and five respective trpE fusion proteins were generated (Fig. 5C, lanes 1, 2, 3, 4 and 5). Because of the initial low expression of the fragment 3 fusion protein (Fig.

FIG. 8. Antigenicity profile of the pp38 ORF and generation of trpE fusion proteins. (A) Antigenicity plot of the predicted amino acid sequence encoded by the ORF. The antigenicity plot was obtained with the Hopp-Woods program (12). (B) Positions and sizes of DNA fragments 1, 2, 3, 4, and 5 used for the generation of trpE fusion proteins. (C) SDS-PAGE analysis of fusion proteins on a 7.5% SDS-polyacrylamide gel. Lanes: C, bacterial strain RR1 protein; T, 37 kDa trpE protein; 1, 2, 3, 4, and 5, trpE fusion proteins of fragments 1, 2, 3, 4, and 5, respectively; 3A, an additional trpE fusion protein of fragment 3. Fusion proteins are indicated by dots. The gel was stained with Coomassie brilliant blue.

Fig. 5.

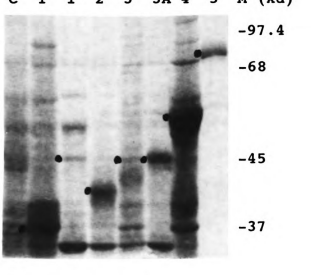
A. The Hopp and Woods Antigenicity Profile



B. The Locations and Sizes of DNA Fragments Used to Generate TrpE Fusion Proteins

1. from amino acid 2 to 62
2. from amino acid 65 to 114
3. from amino acid 116 to 182
4. from amino acid 133 to 290
5. from amino acid 2 to 290

C. SDS-PAGE Analysis of TrpE Fusion Proteins



5C, lane 3), an additional fusion protein of fragment 3 was produced (Fig. 5C, lane 3A). Antibodies against these six fusion proteins were produced in rabbits as described in Materials and Methods, and the sera were designated antisera 1, 2, 3, 3A, 4, and 5, respectively.

Identification of pp38 as the only gene product of the pp38 ORF. Immunoprecipitation analysis of culture media and lysates from mock- and MDV-infected DEF cells was done with all six antisera produced against the pp38 ORF trpE fusion proteins. No MDV-specific protein was immunoprecipitated from the culture media by the six antisera (data not shown). However, one predominant 38 kDa protein (designated pp38 on the basis of phosphorylation analysis, see below) and a minor 24 kDa protein (designated p24) were found in immunoprecipitates from the MDV-infected cell lysates by five of the six antisera (Fig. 6, lanes 1, 3, 3A, 4, and 5). The antiserum against the fragment 2 fusion protein (Fig. 6, lane 2) was negative for these two proteins, supporting the prediction by the Hopp-Woods program (12) that a major portion of fragment 2 is poorly antigenic (Fig. 5A). Antibody titration analysis indicated that antiserum 5 contained the highest antibody titer among the five positive antisera (data not shown). Therefore, only antiserum 5, designated anti-pp38 hereafter, was used in the subsequent immunoprecipitations.

Pulse-chase labeling analysis was performed to determine whether there was any precursor-product relationship between pp38 and p24. Both mock- and MDV-infected DEF cells were pulse labeled for 15 min and chased for 0, 5, and 15 min. Lysates from labeled cells were subjected to immunoprecipitation analysis with anti-pp38 (Fig. 7A). There seemed to be no apparent relationship between pp38 and p24. pp38 was partly degraded during the first 5 min of the chase and then remained stable. However, no concomitant increase in p24 was

FIG. 6. Identification of pp38 as the protein product of the pp38 ORF. Lanes: C, lysate from mock-infected cells subjected to immunoprecipitation with antiserum 5 (anti-pp38); 1 to 5, lysates from MDV-infected cells subjected to immunoprecipitation with antisera 1 to 5, respectively. pp38 and p24 represent 38- and 24-kDa proteins found in immunoprecipitates with antisera 1, 3, 3A, 4, and 5, respectively.

Fig. 6.

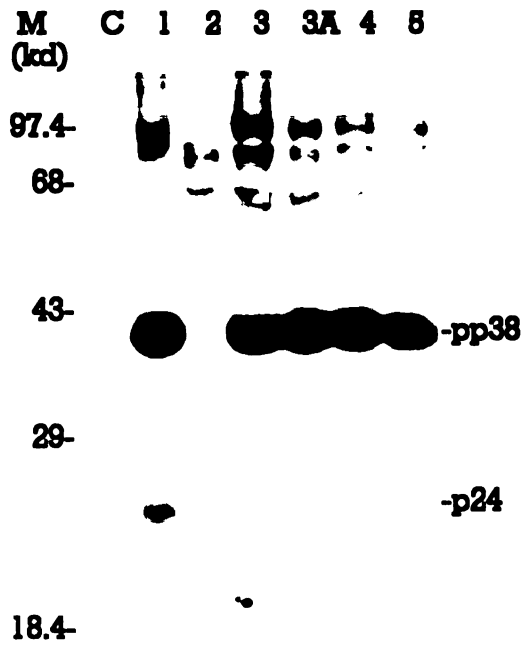
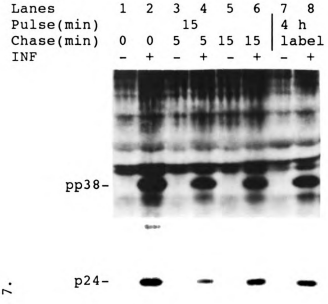


FIG. 7. Identification of pp38 as the only gene product of the pp38 ORF (A) Kinetics of MDV pp38 processing, as determined by pulse-chase analysis. A 15 min pulse was followed by chases of various times shown above each lane. Controls were labeled for 4 h. INF, MDV-infected cell lysates. Lanes: -, uninfected; +, infected. (B) Western blot analysis of pp38 with anti-pp38. Lanes: INF, anti-pp38 immunoprecipitate from an MDV-infected cell lysate; CON, anti-pp38 immunoprecipitate from a mock-infected cell lysate. (C) Autoradiographic analysis of the filter used in the Western blot analysis. p79, pp38, and p24 represent 79-, 38-, and 24-kDa proteins, respectively.

A. Kinetics of pp38 Processing



B. Western Blot Analysis of pp38

C. Autoradiographic Analysis of the Filter Used in Western Blot Analysis

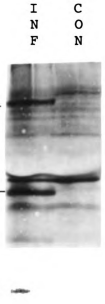


Fig. 7.

observed. Similar results were obtained when 5 min of pulse labeling along with chases for 0, 5, 15, and 30 min were used (data not shown).

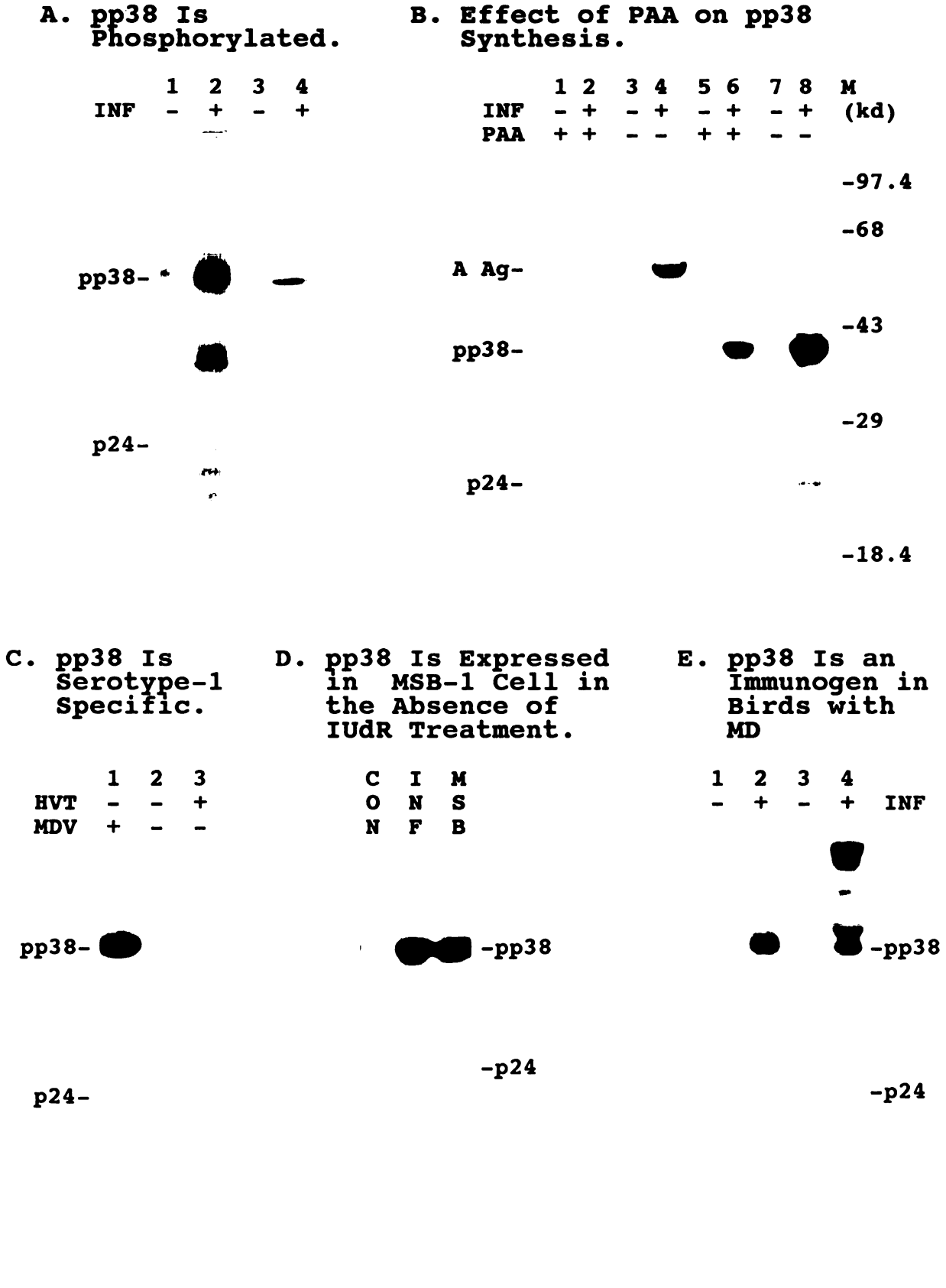
Western blot analysis was conducted to determine whether both pp38 and p24 are protein products of the pp38 ORF. Only pp38 was observed in MDV-infected cell lysates when the nitrocellulose filter was probed with anti-pp38 (Fig. 7B, lane INF). As determined by autoradiographic analysis of the filter used for the Western blot, the amount of p24 transferred to the filter was quite substantive (Fig. 7C). Note also that, p79, a MDV-specific protein that always tends to be nonspecifically trapped during immunoprecipitation (Fig. 7C) served as an internal negative control. Despite the abundance of both p24 and p79 on the filter, neither was detected by anti-pp38 on the Western blot.

Characterization of pp38. To determine whether the 38-kDa protein identified in this study is phosphorylated, we immunoprecipitated lysates from ^{32}P-labeled mock- and MDV-infected DEF cells with anti-pp38 (Fig. 8A, lanes 1 and 2). Lysates from ^{35}S-labeled mock- and MDV-infected DEF cells were used as controls (Fig. 8A, lanes 3 and 4). The results of SDS-PAGE analysis showed that pp38 is phosphorylated (Fig. 8A, lane 2). An additional phosphorylated protein was found at the 31 kDa position in infected cells (Fig. 8A, lane 2), but it seems to be a cell protein since a similar protein was slightly labeled in the control lane (Fig. 8A, lane 1). There was little, if any, ^{32}P-labeled protein at the 24 kDa position in either control or infected cell lysates (Fig. 8A, lanes 1 and 2).

Generally, PAA-insensitive herpesvirus genes tend to encode one of the early classes (IE or early) of herpesvirus proteins (9). To determine whether the expression of the gene encoding pp38 is sensitive to PAA treatment, we immunoprecipitated lysates from treated and untreated cells with anti-pp38 (Fig. 8B, lanes 5, 6, 7, and 8). As positive late protein controls (Fig. 8B, lanes 1, 2, 3,

FIG. 8. Characterization of pp38. (A) pp38 is a phosphoprotein. Lanes: 1 and 2, lysates from ^{32}P-labeled mock- or MDV-infected cells, respectively; 3 and 4, lysates from ^{35}S-labeled mock- or MDV-infected cells, respectively. (B) Effect of PAA on the synthesis of pp38. Lanes: 1 and 2, culture media from ^{35}S-labeled, PAA-treated, mock- or MDV-infected cells; 3 and 4, culture media from ^{35}S-labeled, untreated, mock- or MDV-infected cells; 5 and 6, lysates from ^{35}S-labeled, PAA-treated, mock- or MDV-infected cells; 7 and 8, lysates from ^{35}S-labeled, untreated, mock- or MDV-infected cells. ICS, which contain antibodies to PAA sensitive A antigen (gp57-65), were used for lanes 1 to 4. Anti-pp38 was used for lanes 5 to 8. (C) pp38 is a serotype 1 specific antigen. Lanes: 1, lysate from ^{35}S-labeled MDV-infected cells; 2, lysate from ^{35}S-labeled mock-infected cells; 3, lysate from ^{35}S-labeled HVT-infected cells. (D) pp38 is expressed in latently infected lymphoblastoid tumor cells. Lanes: CON, lysate from ^{35}S-labeled mock-infected cells; INF, lysate from ^{35}S-labeled MDV-infected cells; MSB, lysate from ^{35}S-labeled MSB-1 lymphoblastoid tumor cell line. (E) ICS contain antibodies reactive with pp38. Lanes: 1 and 3, lysates from mock-infected cells; 2 and 4, lysates from MDV-infected cells. The antisera used were anti-pp38 for lanes 1 and 2 and ICS for lanes 3 and 4.

Fig. 8.


A. pp38 Is Phosphorylated.
1 2 3 4
INF - + - +
pp38-
p24-
B. Effect of PAA on pp38 Synthesis.
1 2 3 4 5 6 7 8 M
INF - + - + - + - + (kd)
PAA + + - - + + - -
-97.4
-68
A Ag-
-43
pp38-
-29
p24-
-18.4
C. pp38 Is Serotype-1 Specific.
1 2 3
HVT - - +
MDV + - -
pp38-
p24-
D. pp38 Is Expressed in MSB-1 Cell in the Absence of IUdR Treatment.
C I M
O N S
N F B
-pp38
-p24
E. pp38 Is an Immunogen in Birds with MD
1 2 3 4
- + - + INF
-pp38
-p24

and 4), the corresponding culture media from treated and untreated DEF cells were immunoprecipitated with immune chicken sera (ICS) to determine the amount of gp57-65 (A antigen), an MDV homolog of herpes simplex virus gC, which was known to be predominantly present in the media (10) and which is encoded by a gene that is sensitive to PAA treatment (14, 33a). The results of SDS-PAGE analysis showed that the gene encoding pp38 is relatively insensitive to PAA treatment (Fig. 8B, compare lane 6 with lane 8), while the gene encoding the A antigen is sensitive to PAA treatment (Fig. 8B, compare lane 2 with lane 4).

A preliminary Southern blot analysis suggested that no gene homologous to the MDV pp38 ORF is present in HVT (data not shown). However, since the HVT counterpart of an MDV antigen often shares a common epitope(s) (10, 16), immunoprecipitation analysis of mock- and HVT-infected DEF cell lysates, along with an MDV-infected cell lysate as a positive control, was performed with anti-pp38 to further determine whether there is an HVT homolog. No HVT homolog of the MDV pp38 was found (Fig. 8C, lane 3).

To directly demonstrate whether a gene related to the pp38 gene identified in this study is expressed in the latently infected and transformed MSB-1 lymphoblastoid tumor cell line, we performed immunoprecipitation analysis of a lysate from ^{35}S-labeled MSB-1 cells (Fig. 8D, lane MSB), along with lysates from ^{35}S-labeled mock- and MDV-infected DEF cells (Fig. 8D, lanes CON and INF) with anti-pp38. pp38 was expressed in the MSB-1 tumor cell line in the absence of induction by IUdR (Fig. 8D, lane MSB).

To determine whether pp38 is an immunogen in birds with MD, we used ICS for immunoprecipitation and SDS-PAGE analysis of lysates from mock- and MDV-infected DEF cells. ICS were found to contain antibodies reactive with pp38 (Fig. 8E, lane 4). This result was also confirmed by Western blot analysis,

in which ICS detected both the trpE-pp38 fusion protein and pp38 in an MDV-infected DEF cell lysate (data not shown).

DISCUSSION

In this study, the use of cDNA analysis to detect the entire gene encoding pp38 was done as part of laboratory's general MDV gene identification program and, more specifically, resulted from an attempt to further locate the gene encoding the MDV B antigen (gp100, gp60, and gp49), which was originally thought to be located in the same region of the genome (32). This identification of the complete gene encoding pp38 and the subsequent analysis of its nucleotide sequence facilitated many experiments and observations that confirmed and greatly extended our understanding of this important MDV antigen. Of central importance was the ability to prepare a highly specific antibody reactive against pp38 (anti-pp38) by using the gene nucleotide sequence and predicted amino acid sequence data in a fusion protein approach to antibody production. On the basis of analysis of the pp38 gene nucleotide sequence data and the single protein truly immunoprecipitable by anti-pp38, the following points can be summarized. Despite the early reports of multiple phosphoproteins immunoprecipitable with monoclonal (6, 13, 22, 31) and polyclonal (6) antibodies, it appears from this study that pp38 is the primary gene product. Furthermore, it appears that p24 is not a processing or degradation product of pp38. Through the use of anti-pp38, it was possible to determine that the product of the gene described in this study is phosphorylated, serotype 1 specific (with respect to serotype 3), and expressed both in lytically infected fibroblast cells and in latently infected and transformed lymphoblastoid tumor cells, thereby confirming the reported properties of pp38 from a gene-based perspective. Also this is the first report that pp38 is an immunogen in birds with MD. Finally, by finding the complete gene encoding pp38, it was possible to determine that the gene encoding the B antigen complex is not located in the same region of the genome, as was previously

thought to be the case. When appropriate, the points summarized above will be discussed below to further contribute to our understanding of the molecular biology of MDV.

To ensure that the pp38 identified in this study is the same as the 38 kDa phosphoprotein reported by others (6, 13, 31), we characterize the properties of pp38. Most of the properties of pp38 described here, such as its phosphorylation and failure to be detected in HVT-infected cells, are identical to those of the 38-kDa phosphoprotein, suggesting that these proteins are the same. The approach used to identify the gene encoding pp38 in this study was based on finding the ORF first and then determining its product, an approach entirely different from that of Cui et al. (6). In addition, nucleotide sequence analysis of the complete gene found by their approach (18a) resulted in an ORF nucleotide sequence identical to that of the pp38 ORF reported here.

The term pp38 used in this study is derived from that which was first used by Cui et al. (6) and which was apparently based on the original discovery of a 38 kDa polypeptide as one of a group of three (31) and is consistent with the proposed MDV protein nomenclature (30). Furthermore, the size estimate of 38 kDa in this study (Fig. 6) is in full agreement with the published size (6, 31). Nakajima et al. (22) used the term pp39/36, possibly as an extension of their earlier report of four polypeptides (13). However, the band more recently identified as pp39/36 (22) seems to be a single polypeptide, not a doublet, and most likely is the pp38 described by Cui et al. (6) and in present report. Of importance are the observations that pp38 (pp39/36) is always the most prominent (8 to 10-fold more) polypeptide of the group of phosphoproteins (6, 13, 22, 31) and that a larger polypeptide (41 or 43 kd) was not detected in this study. The protein data of this study (discussed in more depth below) all point to pp38 as the sole phosphoprotein among the groups reported by others.

Identification and sequence analysis of the gene encoding pp38 in this study provide further support for continuing the present nomenclature and are expected to provide clarity as MDV protein nomenclature evolves to accommodate gene identification (30).

The facts that pp38 is expressed in the MSB-1 lymphoblastoid tumor cell line (Fig. 8D) and tumor lesions of MDV-infected chickens (21) and that convalescent-phase sera from chickens with MD are capable of immunoprecipitating pp38 from MDV-infected DEF cells (Fig. 9, lane 4) fulfills the early criteria for a tumor antigen as originally set forth for polyomavirus large T antigen (11). Therefore, on the basis of these criteria, pp38 can appropriately also be referred to as an MDV tumor antigen. Later, the polyomavirus large T antigen was found to be an oncogene (9). Nakajima et al. (22) suggested that the group of phosphoproteins may be associated with MDV oncogenicity. However, on the basis of the protein sequence homology analysis in this study, there is no significant homology between the predicted amino acid sequences of the pp38 gene and that of any known oncogenes. In addition, pp38 was not found to be a nuclear DNA binding protein (18a), although that is not a universal requirement for an oncogene. Silva and Lee (31) reported that p38 was detected in cells infected with the attenuated, avirulent serotype 1 Md11/75C MDV strain. Thus, if pp38 plays a role in MDV transformation two possibilities exist: (i) it was modified during attenuation or (ii) it is necessary but insufficient and indirect, and attenuation involves another gene(s). Whether pp38 is a unique MDV oncogene remains to be determined.

On the basis of their initial analysis of λgt11 clones, Cui et al. (6) suggested that duplicate copies of the pp38 gene may exist in BamHI-D and -H, which contain MDV long terminal repeat (TR_L) and long internal repeat (IR_L) sequences, respectively. Since the 5' end of the pp38 ORF identified in this

study is located in IR_L, duplication of this part of the ORF in TR_L region was considered a possibility. A 2.7-kbp DNA fragment which spans the long unique (U_L) and TR_L in BamHI-D was sequenced (data not shown). Duplication of the complete pp38 ORF in BamHI-D was not observed, but part of it (encoding 45 amino acids) was found because BamHI-D contains TR_L.

Previously, Sithole et al. (32) suggested that the gene for the MDV B antigen complex (gp100, gp60, and gp49) is located in BamHI-H, spanning the IR_L-U_L junction. Finding the pp38 ORF spanning the junction precludes finding the other gene in the same BamHI-H region. The gene encoding the MDV B antigen complex has recently been identified, and it is not located in BamHI-H. The MDV homolog of the herpes simplex virus gB gene, previously reported to be located in BamHI-I_3 and -K_3 by Ross et al. (26), encodes glycoprotein gp100, gp60, and gp49 (5a), previously identified as MDV B antigen complex (16).

In Northern blot analysis, a 1.95-kb transcript for the pp38 gene was identified with both cDNA 2 and the EcoRI c subfragment of BamHI-H as probes (Fig. 2A and 2B). Considering that a 100-200 base poly(A) tail is usually present in mRNA (20), the size (1.95-kb) of the transcript identified is consistent with the size of 1.85 kb as determined by S1 nuclease analysis (Fig. 3). Two minor transcripts of 1.3 and 0.8 kb were detected by the cDNA 2 probe; they were not detected by the EcoRI c subfragment probe. These results indicate that the two minor mRNAs are transcribed from the EcoRI b subfragment, an observation that is similar to previous ones (3). Since the coding region of pp38 was located almost entirely (all but 3 nucleotides) in the EcoRI c subfragment, no common amino acid sequence is expected between the pp38 gene and the genes that are transcribed to yield the 1.3 and 0.8 kb transcripts, should they encode any protein.

The calculated molecular weight of the polypeptide predicted for the pp38 gene is 31,169, a molecular weight which appears smaller than the apparent molecular weight of pp38 in SDS-PAGE. Three factors may contribute to the discrepancy. Extensive phosphorylation of pp38 (Fig. 8A) changes the secondary structure and surface charges and subsequently slows the mobility of the protein (7). Strong hydrophilic domains predicted by hydrophilicity analysis (data not shown) and further suggested by antibody production against the trpE fusion proteins may affect the mobility of pp38 (18). N-linked glycosylation is an unlikely explanation for this size discrepancy, since there was no size change as a result of tunicamycin treatment (data not shown).

Nakajima et al. (22) reported that p24 is an MDV gene product and suggested that p24 either shares an overlapping DNA sequence with the pp38 gene (their p39/36) or is translated from a separate region with partial DNA homology to that of pp38 (their p39/36). In SDS-PAGE analysis, both pp38 and p24 were found in immunoprecipitates formed with anti-pp38 in this study, with the former being much more abundant. However, in this study pulse-chase analysis suggested and Western blotting confirmed that only pp38 is the gene product of the pp38 ORF. This conclusion is further supported by the observation that, among the multiple phosphoproteins, only pp38 was immunoprecipitated from a cell-free translation product by ICS (32a). These data indicate that no common epitope exists between pp38 and p24, despite the fact that these proteins are present in the same immunoprecipitates formed with anti-pp38. The nucleotide sequence analysis done in this study showed that there is no ORF overlapping the pp38 ORF which could potentially encode p24. Although one-third of the nucleotide sequence of the 1.95-kb transcript of the pp38 gene is located in the IR_L region of BamHI-H, only 15% of the nucleotide sequence of the ORF of the pp38 gene (the amino acid coding region) is in this

IR_L region. While the same sequence was found in the TR_L region of BamHI-D, as expected, the small size of the sequence (45 amino acids) precludes its encoding p24. Several questions need to be determined for future experiments: (i) whether or not p24 forms a specific complex with pp38 or is nonspecifically trapped during immunoprecipitation and (ii) whether p24 is MDV specific or cell specific.

Cui et al. (6) reported that pp38 and four other proteins (135, 41, 24, and 20 kDa) were immunoprecipitated by antisera against two MDV-lacZ fusion proteins. Since the pp38 gene reported here is located in the same region (Fig. 1) as their two λgt11 clones (6), it is possible that the four other proteins are not encoded in that region of BamHI-H. Since the lacZ system was used in their study (6), as opposed to the trpE system that was used in this study, it is possible that antibodies against the epitopes of the larger lacZ protein cross-react with some viral proteins. However, it is more likely that the four other polypeptides were nonspecifically trapped during immunoprecipitation.

The fact that pp38 gene expression is relatively insensitive to PAA treatment suggests that pp38 could be one of the early classes of herpesvirus proteins. Furthermore, such an interpretation of the data is consistent with a previous report (29) which showed that an unidentified 1.9-kb IE gene transcript was detected in the BamHI-H. While the precise location of this unidentified IE gene remains to be clarified in relation to the location of the pp38 gene, the distinct possibility exists that these genes are the same. Many IE genes in other herpesviruses tend to regulate the early or late gene expression (9). However, since the amount of pp38 was partially reduced when PAA was used, it is also possible that the gene encoding pp38 is a leaky late gene. Whether the pp38 gene is actually an IE, early, or leaky late gene and whether it has a regulatory function remain to be determined.

ICS were found to contain antibodies capable of immunoprecipitating pp38 (Fig. 8E, lane 4), indicating that pp38 is an immunogen in birds with MD. This result is different from that previously reported (31). ICS also contain antibodies against the B antigen complex (16), which were found to elicit virus neutralization (15) and immunoprotection against MDV (24). Whether pp38, as an immunogen in birds with MD, is capable of eliciting virus neutralization and immunoprotection against MDV remains to be determined.

The finding in this study that pp38 is expressed in the MSB-1 cell line confirms previous observations (6, 13). However, IUdR, a drug known to enhance overall gene expression in the latently infected MSB-1 lymphoblastoid tumor cell line (8), was used in the previous studies (6, 13) to detect pp38, implying that pp38 is not readily expressed in the latency stage. In this study, no IUdR was used and an abundant level of pp38 was expressed and detected in the latently infected MSB-1 lymphoblastoid tumor cell line (Fig. 8D). The fact that pp38 is abundantly expressed in a constitutive manner not only in lytically infected fibroblast cells, but also in latently infected MSB-1 lymphoblastoid tumor cells suggests that it may play a role in the establishment and maintenance of latency.

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REFERENCES

1. **Akiyama, Y., and S. Kato.** 1974. Two cell lines from lymphomas of Marek's disease. Biken J. 17:105-116.

2. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.

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

4. **Calnek, B. K.** 1985. Marek's disease - a model for herpesvirus oncology. Crit. Rev. Microbiol. 12:293-320.

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

5a. **Chen, X., and L. F. Velicer.** 1991. The Marek's disease virus B antigen complex (gp100, gp60, gp49) is the homolog of the herpes simplex virus glycoprotein B (gB). Summitted for publication.

6. **Cui, 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.

7. **DeCaprio, J. A., J. W. Ludlow, D. Lynch, Y. Furukawa, J. Griffin, H. Piwnica-Worms, C.-M. Huang, and D. M. Livingston.** 1989. The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58:1085-1095.

8. **Dunn, K., and K. Nazerian.** 1977. Induction of Marek's disease virus antigens by IdUrd in a chicken lymphoblastoid cell line. J. gen. Virol. 34:413-419.

9. **Fields, B. N., D. M. Knipe, R. M. Chanock, M. S. Hersch, J. L. Melnick, T. P. Monath, and B. Roizman.** 1990. Herpes simplex virus and its replication, p. 1795-1841. B. Roizman, and A. Sears. Fields Virology. 2nd edition. Raven Press, New York

10. **Glaubiger, C., K. Nazerian, and L. F. Velicer.** 1983. Marek's disease herpesvirus. IV. Molecular characterization of Marek's disease herpesvirus A antigen. J. Virol. 45:1228-1234.

11. **Habel, K.** 1965. Specific complement-fixing antigens in polyoma tumors and transformed cells. Virology 25:55-61.

12. **Hopp, T. P., and K. R. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequence. Proc. Natl. Acad. Sci. USA 78:3824-3828.

13. **Ikuta, K., K. Nakajima, M. Naito, S. H. Ann, S. Ueda, S. Kato, and K. Hirai.** 1985. Identification of Marek's disease virus-specific antigens in Marek's disease lymphoblastoid cell lines using monoclonal antibody against virus-specific phosphorylated polypeptides. Int. J. Cancer 35:257-264.

14. **Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1983. Monoclonal antibodies reactive with the surface and secreted glycoproteins of Marek's disease virus and herpesvirus of turkeys. J. gen. Virol. 64:2597-2610.

15. **Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1984. Processing of glycoprotein gB related to neutralization of Marek's disease virus and herpesvirus of turkeys. Microbiol. Immunol. 28:923-933.

16. **Isfort, R. J., I. Sithole, H.-J. Kung, and L. F. Velicer.** 1986. Molecular characterization of Marek's disease herpesvirus B antigen. J. Virol. 59:411-419.

17. **Konopka, J. B., R. L. Davis, S. M. Watanabe, A. S. Ponticelli, L. Schiff-Maker, N. Rosenberg, and O. N. Witte.** 1984. Only site-directed antibodies

reactive with the highly conserved src-homologous region of the v-abl protein neutralize kinase activity. J. Virol. 51:223-232.

18. **Lahijani, R. S., E. W. Ottesen, J. D. Adlish, and S. C. S. Jeor.** 1991. Characterization of a human Cytomegalovirus 1.6-kilobase late mRNA and identification of its putative protein product. J. Virol. 65:373-381.

18a. **Lee, L. F.** personal communication.

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

20. **Montell, C., E. F. Fisher, M. H. Caruthers, and A. J. Berk.** 1983. Inhibition of RNA cleavage but not polyadenylation by a point mutation in mRNA 3' consensus sequence AAUAAA. Science 305:600-605.

21. **Naito, M., K. Nakajima, N. Iwa, K. Ono, I. Yoshida, T. Konobe, K. Ikuta, S. Ueda, S. Kato, and K. Hirai.** 1986. Demonstration of a Marek's disease virus-specific antigen in tumor lesions of chickens with Marek's disease using monoclonal antibody against a virus phosphorylated protein. Avian Pathol. 15:503-510.

22. **Nakajima, K., K. Ikuta, 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. 68:1379-1389.

23. **Nazerian, K., and L. F. Lee.** 1976. Selective inhibition by phosphonoacetic acid of MDV DNA replication in a lymphoblastoid line. Virology 74:188-193.

24. **Ono, K., M. Takashima, T. Ishikawa, M. Hayashi, I. Yoshida, T. Konobe, K. Ikuta, K. Nakajima, S. Ueda, S. Kato, and K. Hirai.** 1984. Partial protection against Marek's disease in chickens immunized with glycoproteins gB

purified from turkey-herpesvirus-infected cells by affinity chromatography coupled with monoclonal antibodies. Avian Dis. 29:533-539.

25. **Payne, L. N.** 1985. Marek's disease - scientific basis and methods of control. Martinus Nijhoff Publishing, Boston.

26. **Ross, L. J. N., 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.

27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

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

30. **Schat, K. A., G. F. de Boer, K. Hirai, L. F. Lee, K. Nakajima, R. F. Silva, and L. F. Velicer.** 1988. A proposed nomenclature for the protein of Marek's disease herpesvirus, p. 438-443. In S. Kato, T. Horiuchi, T. Mikami, and K. Hirai. (ed.), Advances in the Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

31. **Silva, R. F., and L. F. Lee.** 1984. Monoclonal antibody-mediated immunoprecipitation of proteins from cells infected with Marek's disease virus or turkey herpesvirus. Virology 136:307-320.

32. **Sithole, I., P. M. Coussens, and L. F. Velicer.** 1988. Identification of Marek's disease herpesvirus B antigen's precursor polypeptide and the gene encoding it, p. 148-154. In S. Kato, T. Horiuchi, T. Mikami, and K.

Hirai. (ed.), Advances in the Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

32a **Stringer, R. A., X. Chen, and L. F. Velicer.** Unpublished data.

33. **Sugaya, K., G. Bradley, M. Nonoyama, and A. Tanaka.** 1990. Latent transcripts of Marek's disease virus are clustered in the short and long repeat regions. J. Virol. 64:5773-5782.

33a. **Velicer, L. F.** Unpublished data.

34. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

Chapter IV

The Marek's Disease Virus B Antigen Complex (gp100, gp60, gp49) Is the Homolog of the Herpes Simplex Virus Glycoprotein B (gB)

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ABSTRACT

Marek's disease (MD) is caused by Marek's disease virus (MDV), and is prevented by immunization with an antigenically related, but apathogenic, herpesvirus of turkey (HVT). Among the major glycoproteins found in MDV- and HVT-infected cells is the B antigen complex (gp100, gp60 and gp49), detected by immunoprecipitation and SDS-PAGE analysis with antisera previously shown to be reactive with B antigen in immunodiffusion analysis. However, the gene encoding this important MDV antigen complex was not unequivocally identified. Recently, an MDV homolog of the gene encoding herpes simplex virus (HSV) glycoprotein B (gB) was identified and sequenced (Ross, L. J. N., M. Sanderson, S. D. Scott, M. M. Binns, T. Doel, and B. Milne. J. gen. Virol. 70:1789-1804. 1989). To determine if the MDV gB homolog gene might encode the B antigen, antisera were prepared against trpE fusion proteins of the MDV gB homolog (trpE-MDV-gB). These antisera immunoprecipitated gp100, gp60, gp49 and pr88. Based on size comparison, trpE-MDV-gB competition and blocking assays, it was concluded that: 1) the MDV homolog of HSV gB gene encodes MDV B antigen, and 2) pr88 was found to be the only precursor polypeptide, while pr44, previously thought to dimerize to form pr88, is a protein that is nonspecifically trapped during immunoprecipitation. The antisera against trpE-MDV-gB also contained antibody reactive with the HVT B antigen, consistent with the known antigenic relatedness between the MDV and HVT B antigens. A new MDV glycoprotein, gp98, was also detected by the antisera. Results from pulse-chase analysis suggest that gp98 is a glycoprotein processing intermediate. The MDV gB homolog processing pathway appears to involve cotranslational glycosylation of pr88 to form gp98, which is further processed to yield gp100, which is then cleaved to form gp60 and gp49. This

processing pathway is consistent with those of other gB homologs, further supporting the gene identification described above.

INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease of chickens caused by cell-associated Marek's disease herpesvirus (MDV), and is characterized principally by T-cell lymphomas and peripheral nerve demyelination (4, 24). The disease has been effectively controlled by vaccination with apathogenic but antigenically related herpesvirus of turkey (HVT).

To determine the mechanism of immunity conferred by HVT, efforts have focused on antigens found in common between MDV and HVT, particularly the A and B antigens (9, 10, 11, 15, 16, 17, 18, 29, 32). The genes encoding the common MDV and HVT A antigens (gp57-65) have been identified and characterized; these represent the MDV/HVT homologs of herpes simplex virus (HSV) glycoprotein C (gC) (9, 10).

The MDV and HVT B antigens were characterized as a complex of three glycoproteins, gp100, gp60 and gp49 (16, 17, 29, 32). Tunicamycin (TM) inhibition of N-linked glycosylation resulted in identification of various unglycosylated, or possibly O-linked glycosylated, precursor polypeptides: pr88/pr83 of MDV and pr90 of HVT (16), pr80/pr110/pr125 of MDV (11), or pr88 and pr44 of MDV and HVT (17, 32). In the latter report, it was hypothesized that pr44 dimerizes to form pr88 (32). HVT B antigen complex prepared by immune affinity purification was also shown to elicit partial protective immunity against MDV in chickens (23), and antibodies against B antigen are able to neutralize the infectivity of MDV in cell cultures (16).

A preliminary report by Sithole et al. (31) suggested that the gene encoding MDV B antigen was located in the BamHI-H fragment, which spans the junction of the unique long (U_L) and inverted repeat long (IR_L) regions of the MDV genome. Upon further analysis, Chen et al. (5) reported that a

phosphoprotein pp38 was encoded in this region of BamHI-H; moreover, no open reading frames characteristic of glycoprotein genes were identified. Thus, the location of the gene encoding MDV B antigen remained to be determined.

Ross et al. (26) reported the identification of the gene encoding the MDV homolog of HSV glycoprotein (gB) (MDV gB homolog gene). By Western blot analysis, the MDV gB homolog was found to be composed of gp110, gp64 and gp48 (26). In the presence of TM, three smaller polypeptides (94, 90 and 84 kDa) were found to represent the unglycosylated, or possibly O-linked glycosylated, precursor polypeptides, with a 48 kDa polypeptide being a truncated form of the former ones (26). Solely based on the estimated sizes of the three glycoproteins, they suggested that MDV gB homolog could be the MDV B antigen complex. However, despite the fact that antisera against the B antigen complex neutralize MDV infectivity (16), their anti-peptide sera against MDV gB homolog failed to neutralize MDV infectivity (26), an observation which is not consistent with their above suggestion. It was suggested (26) that this failure to neutralize MDV could be explained if their anti-peptide sera reacted predominantly with the primary structure of the antigen, and therefore may be unable to react with epitopes on the intact virion.

This study provides conclusive evidence that the MDV B antigen complex is in fact the MDV homolog of HSV gB. In addition, it was also learned that pr88 is the actual unglycosylated (or possibly O-linked glycosylated) MDV gB primary precursor polypeptide; and that MDV gB homolog processing has a similar pattern as to those of equine herpesvirus (EHV), cytomegalovirus (CMV), pseudorabies virus (PRV) and varicella-zoster virus (VZV).

MATERIALS AND METHODS

Cells and viruses. The preparation, propagation and infection of duck embryo fibroblast (DEF) cells with MDV or HVT was performed as described previously (6, 13). The MDV GA strain used in this study was at cell culture passage level 6 following isolation of cell-free virus from feather tips obtained from infected birds with MD. The HVT FC-126 strain of vaccine virus at cell culture passage level 13 was used in this study.

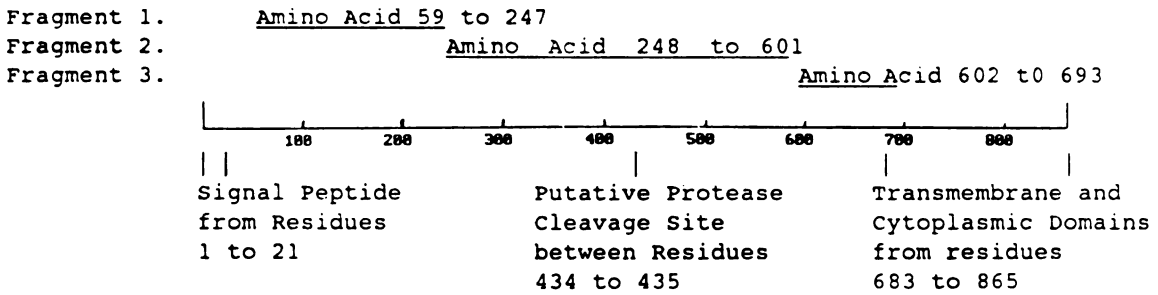
Antisera. The preparation and characterization of rabbit antibody against MDV-infected DEF cell plasma membrane (RαPM) has been described previously (17, 32). RαPM and the monoclonal antibody IAN86 are immunoreactive with the MDV B antigen complex (17, 29, 32).

Generation of trpE fusion proteins of MDV gB homolog (trpE-MDV-gB). The vector system used to express the MDV gB homolog gene's open reading frame (ORF) in *Escherichia coli* consists of a group of plasmids (pATH vectors) encoding approximately 37 kDa of the bacterial trpE ORF under control of the inducible trp operon promoter (20). A polylinker with multiple cloning sites at the 3' end of the trpE ORF allows in-frame insertion of foreign ORFs. Three DNA fragments, designated as fragments 1, 2 and 3 (Fig. 1), from the MDV gB homolog gene's coding region (26), were purified and cloned into various trpE expression vectors according to the method by Sambrook et al. (27), and their respective trpE-MDV-gB fusion proteins were generated.

Fusion protein antibody production. Fusion proteins were purified by electrophoresis in preparative 7.5% SDS-polyacrylamide gels. The gels were stained with Coomassie brilliant blue to identify the positions of the fusion proteins. The fusion proteins were excised and emulsified in complete Freund's adjuvant (Difco Laboratories). Initial immunization of 2.0 kg New Zealand White female rabbits was with 200 to 500 μg of proteins. Rabbits were boosted after 3

FIG. 1. The locations and sizes of DNA fragments used to generate trpE fusion proteins. Fragment 1, a 566-bp XbaI-EcoRV subfragment of BamHI-I_3, whose trpE fusion protein was used to generate antiserum Z43. Fragment 2, an 1,058-bp EcoRV-BamHI subfragment of BamHI-I_3, whose trpE fusion protein was used to generate antiserum Z45. Fragment 3, a 277-bp BamHI-HaeIII subfragment of BamHI-K_3, whose trpE fusion protein was used to generate antiserum Z42. The locations of the signal peptide, putative protease cleavage site, and transmembrane and cytoplasmic domains were reported by Ross et al. (26).

Fig. 1.


Fragment 1. Amino Acid 59 to 247
Fragment 2. Amino Acid 248 to 601
Fragment 3. Amino Acid 602 t0 693
100
200
300
400
500
600
700
800
Signal Peptide from Residues 1 to 21
Putative Protease Cleavage Site between Residues 434 to 435
Transmembrane and Cytoplasmic Domains from residues 683 to 865

weeks with 100 to 200 μg of proteins in incomplete Freund's adjuvant, and sera were collected 7 to 10 days later. Subsequent booster immunizations were approximately 2 weeks apart, as needed to obtain detectable and usable titers. The antisera against the trpE fusion proteins of fragments 1, 2 and 3 (Fig. 1) were designated as Z43, Z45, and Z42, respectively.

Radiolabeling of proteins and preparation of lysates. Mock-, HVT- and MDV-infected DEF cells were labeled with [^{35}S]methionine (specific activity, 1,000 μCi/mmol, ICN) at 48 h postinfection for 4 h, as previously described (13). Pulse-chase labeling was performed as previously described (18, 32), with the following modifications. Cells were preincubated without methionine for 1 h; pulse labeling was done by incubation with 250 μCi of [^{35}S]methionine per 60 mm plate in 1.0 ml media for 5 min; and chases were done in the presence of unlabeled methionine for 0, 5, 10, 20, 40, 80, 160, and 320 min. For labeling in presence of 2.0 μg/ml tunicamycin (TM) (Calbiochem-Behring Corp.), cells were preincubated with TM for 1 h, and treated at same concentration during the 4 h labeling period. After labeling was complete, cells were washed three times with ice-cold phosphate-buffered saline, followed by the addition of ice-cold detergent buffer (0.01 M NaH_2PO_4-Na_2HPO_4, pH 7.5, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate) (36) to lyse cells. The lysates were clarified by centrifugation as previously reported (36).

Immunoprecipitation and SDS-PAGE analysis. Immunoprecipitation analysis was carried out as previously described (13). The immunoprecipitates were washed, suspended in sample buffer, and analyzed by sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis (SDS-PAGE). Protein markers (BRL) were used as molecular mass standards. Molecular sizes were calculated by interpolation between standard proteins by the method of Weber and Osborn (34). Fluorography was carried out as previously described (1).

RESULTS

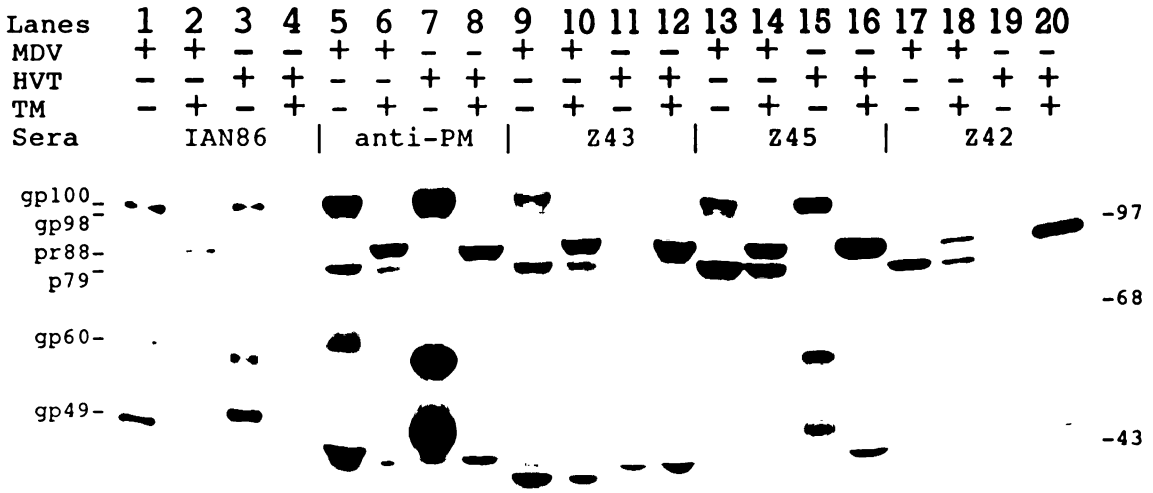
Identification of MDV and HVT gB homolog gene products. The antisera against trpE-MDV-gB (Z43, Z45 and Z42), along with the monoclonal antibody, IAN86, and polyclonal antiserum, RαPM, were used for immunoprecipitation analysis of lysates from both MDV- and HVT-infected DEF cells labeled with [^{35}S]methionine in the absence or presence of TM.

In the absence of TM, gp100, gp60 and gp49 were detected by IAN86 and RαPM in both MDV- and HVT-infected cell lysates, as expected (Fig. 2, lanes 1, 3, 5, and 7). Note that for HVT, the gp60 and gp49 counterparts migrate slightly different from MDV's (Fig. 2, compare lane 1 with lane 3). However, established MDV terminology (29) is used here for simplicity and ease of identification. Also note that the IgG heavy chain of polyclonal RαPM tends to distort the migration of gp49 (Fig. 2, compare lanes 5 and 7 with lanes 1 and 3), a result of the volume of this lower titer serum needed for immunoprecipitation. For MDV-infected DEF cells, while little, if any, glycosylated MDV-specific proteins were detected by Z42 (Fig. 2, lane 17); gp100, and an additional new MDV glycoprotein, designated gp98 (immediately below gp100), were detected by Z43 and Z45 (Fig. 2, lanes 9 and 13). However little, if any, gp60 and gp49 were detected by Z43 and Z45 (Fig. 2, lanes 9 and 13). In contrast, in HVT-infected DEF cells, gp60 and gp49 were detected only by Z45 (Fig. 2, lane 15), while gp100 was detected by Z43, Z45 and Z42 (Fig. 2, lanes 11, 15 and 19).

In the presence of TM, virus-specific pr88 was detected by all five antisera used (Fig. 2, Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20). Note that the apparent molecular weights for the precursor polypeptides of HVT and MDV are approximately equal (Fig. 2, lanes 2, 6, 10, 14, and 18 compared with lanes

FIG. 2. Identification of MDV and HVT gB homolog gene products. Lysates from MDV- or HVT-infected DEF cells labeled in the absence or presence of TM (see Materials and Methods) were immunoprecipitated by IAN86, anti-PM (RαPM), Z43, Z45, and Z42 as indicated above each lane. IAN86 and anti-PM (RαPM) were used as positive controls, which were known to be able to immunoprecipitate the MDV B antigen complex (gp100, gp60, gp49) (lanes 1, 3, 5 and 7) and its precursor polypeptide pr88 (lanes 2, 4, 6 and 8). gp98, a protein immediately below gp100 in lanes 9 and 13, is detected only in the immunoprecipitates of MDV-infected DEF cell lysates with antisera Z43 and Z45. Note that p79, a band below pr88, is an MDV-HVT-specific protein with a tendency to be nonspecifically trapped, and therefore is present in all lanes, except lanes 1 to 4 where the monoclonal antibody IAN86 was used. Virus-specific proteins are indicated to the left. Molecular mass standards are indicated to the right.

Fig. 2.

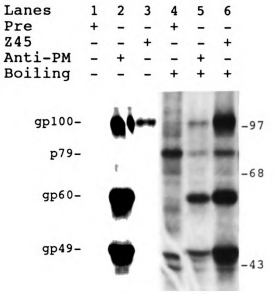

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
MDV + + - - + + - - + + - - + + - - + + - -
HVT - - + + - - + + - - + + - - + + - - + +
TM - + - + - + - + - + - + - + - + - + - +
Sera IAN86 | anti-PM | Z43 | Z45 | Z42
gp100-
gp98-
pr88-
p79-
gp60-
gp49-
-97
-68
-43

4, 8, 12, 16, and 20, respectively). Based on size comparison, it appears that the pr88 immunoprecipitated by the antisera against trpE-MDV-gB is the same pr88 immunoprecipitated by IAN86 and RαPM, suggesting that the MDV B antigen complex is the MDV gB homolog. The band that often appears below pr88 is p79, an MDV-HVT-specific protein with a tendency to be nonspecifically trapped (17). While its presence is nonspecific (in respect to the sera used) (17, unpublished results) and unintended, p79 often serves as a useful internal marker within the same gel lane; its presence below pr88 in all but the monoclonal antibody lanes (Fig. 2, lanes 1 to 4) serves to strengthen the size comparison refered to above. Previously, a polypeptide called pr44 was thought to be the precursor polypeptide for the B antigen complex (17, 32). However, in this experiment there is no virus-specific pr44 immunoprecipitated by antisera against trpE-MDV-gB (Fig. 2, lanes 10, 12, 14, 16, 18, and 20), as confirmed by subsequent fusion protein competition analysis. Although there is a band at approximately the 44 kDa position (the top band of the doublet) (Fig. 2, lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20; upon longer exposure two light bands were visible at lanes 2 and 4 where IAN86 was used), it now appears that it, probably what was previously termed pr44 (17, 32), is only one of the several nonspecific bands of that approximate size that are trapped during immunoprecipitation. That pr88 is the only precursor polypeptide for MDV gB homolog, is further confirmed by fusion protein competition analysis (see below).

Consistent with the above suggestion that the MDV B antigen complex is the MDV gB homolog, antisera against trpE-MDV-gB should recognize gp60 and gp49. To enhance the detection of these MDV gB homolog glycoproteins, boiling of MDV-infected cell lysates prior to immunoprecipitation was used to disrupt protein secondary structure; with the intent that boiling might result in a

FIG. 3. Boiling of MDV-infected cell lysates to enhance the detection of MDV gB homolog by Z45. Boiled (lanes 4, 5, 6) or unboiled (lanes 1, 2, 3) MDV-infected cell lysates were immunoprecipitated by preimmune serum (pre), Z45 and anti-PM (RαPM) as indicated above each lane. Virus-specific proteins are indicated to the left. Molecular mass standards are indicated to the right.

Fig. 3.


Lanes 1 2 3 4 5 6
Pre + - - + - -
Z45 - - + - - +
Anti-PM - + - - + -
Boiling - - - + + +
gp100-
p79-
gp60-
gp49-
-97
-68
-43

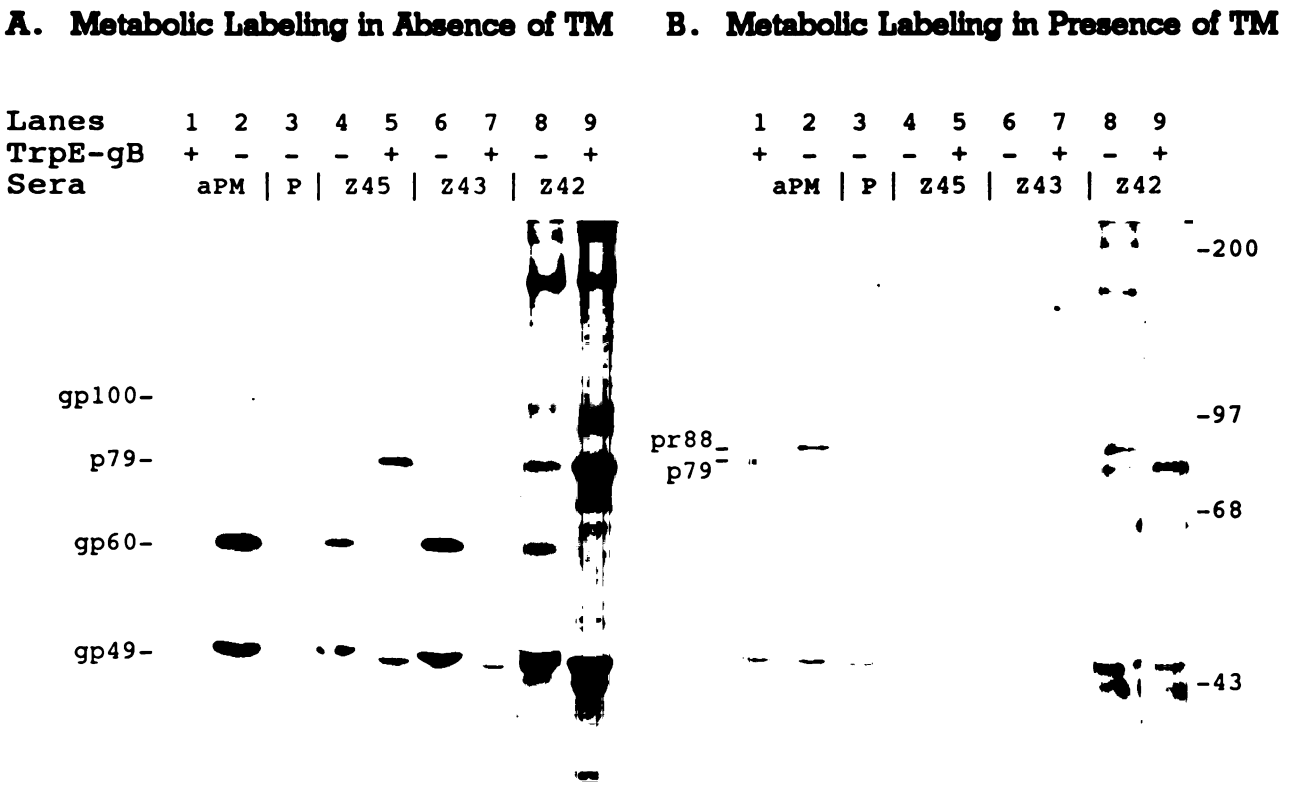
protein's hidden continuous epitopes becoming available for recognition by antisera against trpE-MDV-gB. No MDV-specific proteins were detected by preimmune sera, except for the nonspecifically trapped p79 (Fig. 3, lanes 1 and 3), whether the lysate was unboiled or boiled. In the Z45 immunoprecipitate of the boiled MDV-infected cell lysate (Fig. 3, lane 6), not only was more gp100 detected (Fig. 3, lane 6 compared to lane 3), but also gp60 and gp49 were now detected; the latter two were only minimally detectable in the immunoprecipitate of unboiled MDV-infected cell lysate (Fig. 3, lane 3 compared with lane 6), suggesting that the antisera against trpE-MDV-gB are mainly reactive with continuous epitopes. In contrast, much less gp100, gp60 and gp49 were detected by RαPM when the lysate was boiled (Fig. 3, lane 2 compared with lane 5), suggesting that the majority of antibodies in the RαPM sera are reactive with conformational (discontinuous) epitopes that were disrupted by boiling.

Fusion protein competition analysis of MDV-infected cell lysates. To further assess the specificity of the antisera against trpE-MDV-gB, and to confirm that MDV B antigen is a MDV gB homolog, a competition assay was performed in which fragment 1, 2 and 3 fusion proteins were separately added to each of three boiled lysates prior to addition of their respective antisera, as indicated above each lane (Fig. 4A and 4B, lanes 5, 7 and 9) while all three fusion proteins were added to one boiled lysate when RαPM was used (Fig. 4A and 4B, lane 1).

In the absence of TM, no virus-specific proteins were detected in MDV-infected cell lysate when preimmune sera was used as a control (Fig. 4A, lane 3). When RαPM and Z45 were used, trpE-MDV-gB competition resulted in gp100, gp60 and gp49 no longer being detected (Fig. 4A, compare lanes 2 and 4 with lanes 1 and 5, respectively), further supporting the earlier suggestion (based on size comparison) that the MDV B antigen complex is a MDV gB

FIG. 4. Fusion protein competition analysis of MDV-infected cell lysates. (A) MDV-infected cell lysates were boiled prior to immunoprecipitation. Fragment 1, 2 and 3 fusion proteins were added separately to each of three boiled lysates prior to addition of their respective antisera as indicated above each lane (lanes 5, 7 and 9). All three fusion proteins were added to one boiled lysate when anti-PM (RaPM) was used (lane 1). An identical amount of phosphate lysis buffer was added to the controls (lanes 2, 3, 4, 6 and 8). (B) All of the assay conditions were the same as in panel A except that the lysates were from MDV-infected DEF cells labeled in the presence of TM. The virus specific proteins are indicated to the left at each panel. Molecular mass standards are indicated to the right of panel B. P, representing preimmune sera.

Fig. 4.


A. Metabolic Labeling in Absence of TM
B. Metabolic Labeling in Presence of TM
Lanes 1 2 3 4 5 6 7 8 9
TrpE-gB + - - - + - + - +
Sera aPM | P | Z45 | Z43 | Z42
gp100-
p79-
gp60-
gp49-
pr88-
p79-
-200
-97
-68
-43

homolog. When Z43 was used, gp100, gp60 and gp49 were detected in the boiled MDV-infected cell lysate (Fig. 4A, lane 6); the latter two were not detectable in the immunoprecipitate of unboiled MDV-infected cell lysate (Fig. 2, lane 9). TrpE-MDV-gB competition resulted in gp100, gp60 and gp49 becoming undetectable (Fig. 4A, compare lane 6 with lane 7). While little, if any, gp100, gp60 and gp49 were detected by Z42 in unboiled lysate (Fig. 2, lane 17), all three were detectable when the lysate was boiled (Fig. 4A, lane 8). TrpE-MDV-gB competition resulted in gp100, gp60 and gp49 becoming undetectable (Fig. 4A, compare lane 8 with lane 9). Note that nonspecific trapping was increased, most notably of p79, when trpE-MDV-gB was used in the competition assay (Fig. 4A, compare lanes 4, 6, and 8 with lanes 5, 7, and 9, respectively). Results similar to those that occured with trpE-MDV-gB competition analysis were obtained by immunoprecipitation analysis of MDV-infected cell lysates by antisera (RαPM and Z45) that had been preincubated with trpE-MDV-gB (designated as trpE-MDV-gB blocking assay) (data not shown).

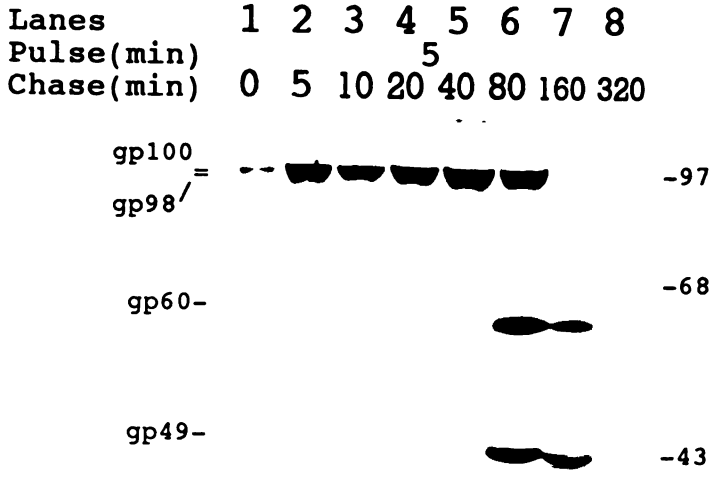
In the presence of TM, pr88 was not detected by preimmune sera (Fig. 4B, lane 3). While pr88 was detected in the immunoprecipitates of the boiled lysates when RαPM, Z45, Z43 and Z42 were used, it became undetectable as a result of trpE-MDV-gB competition (Fig. 4B, compare lanes 2, 4, 6, and 8 with lanes 1, 5, 7, and 9, respectively). A similar pattern (as in Fig. 4A) of increased nonspecific trapping was observed, especially of p79, when trpE-MDV-gB was used in the competition assay. Results similar to those that occured with trpE-MDV-gB competition assay were also obtained by a trpE-MDV-gB blocking assay similar to that described above for Fig. 4A (data not shown). Together, these results are consistent with the notion that pr88 is the primary precursor

polypeptide of MDV gB homolog; this further supports the above conclusion that the MDV B antigen complex is the homolog of HSV gB.

Kinetics of MDV gB homolog glycoprotein processing as determined by pulse-chase analysis. For EHV (21, 33), CMV (2, 3), PRV (25, 35), and VZV (14, 22), a glycoprotein intermediate of gB homolog initially appears, which is slightly smaller in molecular mass than that of the mature glycoprotein. Upon further processing, the glycoprotein intermediate is converted to the mature glycoprotein, which is subsequently cleaved to form two smaller cleavage products. Since there is a 98 kDa glycoprotein detected by antisera Z43 and Z45 (Fig. 2, lanes 9 and 13), as well as two smaller cleavage products gp60 and gp49 (16, 32), a similar processing pathway appeared to be involved in MDV gB homolog processing. To determine if the 98 kDa glycoprotein is an intermediate in the processing of pr88 to gp100, a 5 min pulse was done and was followed by various chase periods (Fig. 5). Since RαPM does not recognize gp98 as well as Z45 does, but does recognize gp60 and gp49 when the lysate is not boiled (Z45 does not), a pool of these two antisera were used. Upon immunoprecipitation and SDS-PAGE analysis, the amount of gp98 gradually decreased, being easily detectable only with a 0 min chase (Fig. 5, lane 1) and barely detectable with a 5 min chase (Fig. 5, lane 2). Coincident with this decrease, the amount of gp100 gradually increased (Fig. 5, lanes 1 to 5). After a 20 min chase, cleavage of gp100 to form gp60 and gp49 began to take place, with most of this processing occuring between 40 and 80 min of chase (Fig. 5, lanes 5 and 6; upon longer exposure, gp60 and gp49 were visible in lane 5). As this processing continued gp100 was reduced to very low levels (Fig. 5, lanes 7 and 8, still seen in four-fold longer exposures of lane 8). Processing of gp60 and gp49 began to occur after a 80 min chase, with most of protein processing occuring between 160 and 320 min chases (Fig. 5, lanes 7

FIG. 8. Kinetics of MDV gB homolog processing as determined by pulse-chase analysis. A 5 min pulse labeling was followed by chases of various times shown above each lane. Immunoprecipitation analysis was done with a pool of antisera Z45 and anti-PM (RαPM). The virus specific proteins are indicated to the left. Molecular mass standards are indicated to the right.

Fig. 5.


Lanes 1 2 3 4 5 6 7 8
Pulse(min) 5
Chase(min) 0 5 10 20 40 80 160 320
gp100
gp98
-97
gp60-
-68
gp49-
-43

and 8). In summary, these data show that the 98 kDa glycoprotein is the intermediate between pr88 and gp100, which is then cleaved to yield gp60 and gp49.

DISCUSSION

This study provides several lines of evidence that the MDV B antigen complex (gp100, gp60, gp49) is encoded by the MDV gB homolog gene. This conclusion is based on immunoprecipitation analyses with antibodies to trpE-MDV-gB, and the use of trpE-MDV-gB competition and blocking assays. Further, size comparison of these three glycoproteins with those detected with anti-peptide sera prepared based on the sequence of the newly discovered MDV homolog of HSV gB, are consistent with this finding. Finally the glycoprotein processing pathway reported in this study is typical of those for gB homologs of other herpesviruses. This conclusion eliminates, not only the uncertainty that previously existed concerning the identity and location of the gene encoding the MDV B antigen complex, but also current confusion regarding the nomenclature of the MDV B antigen complex.

The three glycoproteins (gp100, gp60, gp49) first seen on SDS-PAGE (29) were previously shown to be the MDV B antigen complex (17) through use of antisera that was originally defined as having anti-B reactivity by immunodiffusion analysis, the historical basis for naming several antigens A, B and C (7, 8) found in common between MDV and HVT. Thus, the use of the letter B in MDV glycoprotein nomenclature predates the first use of gB terminology in the HSV and other herpesvirus systems. Prior to the evidence reported in this paper, confusion sometimes arose when it was then erroneously assumed, based on the use of the letter B, that the MDV B antigen was a homolog of HSV gB. Compounding the MDV B antigen nomenclature confusion was the frequent use of the term gB by some MDV workers, as still another way to refer to the glycoprotein B antigen, not because of the evidence of homology to HSV gB. Their tentative nomenclature choice was to use gA and gB, for the secreted (A antigen) and virus neutralization-related (B antigen) glycoproteins

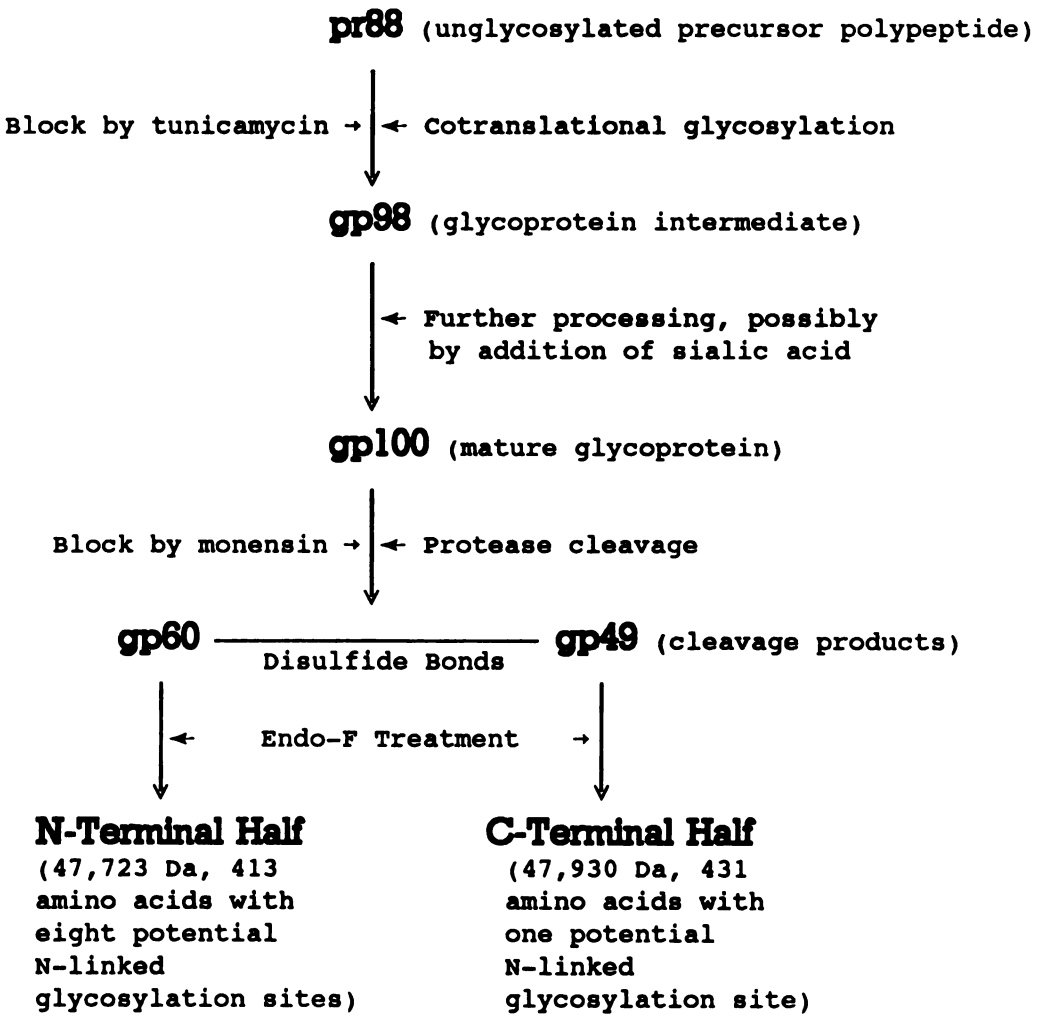
(15, 16). Now that homology of the MDV B antigen with HSV gB has been shown experimentally in the study reported here, it is suggested that the gene encoding it be designated as the MDV homolog of the HSV gB gene (MDV gB homolog gene), and that the glycoproteins (gp100, gp60, gp49) be designated as the MDV homolog of the HSV gB (MDV gB homolog). For current report, the tentative use of the abbreviation MDV gB seems appropriate, following the precedent of Ross et al. (26); but that may be reconsidered as alphaherpesviruses and MDV/HVT glycoprotein nomenclature develops.

Results from TM inhibition and immunoprecipitation analysis (Fig. 2, lanes 10, 14 and 18), trpE-MDV-gB competition assay (Fig. 4B) and trpE-MDV-gB blocking assay (data not shown) clearly demonstrate that pr88 is the primary unglycosylated (or possibly O-Linked glycosylated) precursor polypeptide of the gp100, gp60 and gp49 complex. This interpretation is also supported by reports from studies of gB homolog proteins of other herpesviruses. In cases of EHV (21, 33), CMV (2, 3), VZV (14, 22), PRV (25, 35), and bovine herpesvirus (BHV) (12), where the mature gB homolog proteins are cleaved to form two smaller products, only one unglycosylated precursor polypeptide is observed; and no cleavage of the unglycosylated precursor polypeptide was reported. The appearence of a gp98 intermediate in this study (Fig. 2, lanes 9 and 13; Fig. 5, lane 1) is consistent with the appearence of a similar sized molecule at 0 and 3 min chase intervals after a 5 min pulse in an earlier study of B antigen processing (32).

With identification of gp98 as an intermediate between pr88 and gp100 (Fig. 5), MDV gB homolog processing pathway appears similar to that observed for EHV (21, 33), CMV (2, 3), PRV (25, 35) and VZV (14, 22) gB homolog processing. The MDV gB homolog processing pathway is outlined in Fig. 6, and is based on a combination of the conclusions of this study and part of those

FIG. 6. MDV gB homolog processing pathway.

Fig. 6.


pr88 (unglycosylated precursor polypeptide)
Block by tunicamycin →
← Cotranslational glycosylation
gp98 (glycoprotein intermediate)
← Further processing, possibly by addition of sialic acid
gp100 (mature glycoprotein)
Block by monensin →
← Protease cleavage
gp60
Disulfide Bonds
gp49 (cleavage products)
← Endo-F Treatment →
N-Terminal Half
(47,723 Da, 413 amino acids with eight potential N-linked glycosylation sites)
C-Terminal Half
(47,930 Da, 431 amino acids with one potential N-linked glycosylation site)

of the previous study on processing of the B antigen complex (32). It is clear that the unglycosylated precursor polypeptide, pr88, which is seen only after TM inhibition, is cotranslationally glycosylated to form gp98 (Fig. 5, lane 1). N-linked glycosylation can be blocked by TM to generate the pr88 primary precursor polypeptide (Fig. 2, compare lane 1 with lane 2; 32). gp98 is further processed to form gp100 (Fig. 5), possibly by sialylation. Then gp100 is cleaved to form gp60 and gp49 (Fig. 5; 32), which can be blocked by monensin (a monovalent ionophore inhibiting transport of glycoprotein) (32). Based on the estimated molecular masses of approximately 44 kDa for the deglycosylated backbone polypeptides of both gp60 and gp49 on SDS-PAGE (32), and the calculated molecular masses of the predicted amino acid sequences of MDV gB homolog for the N-terminal half (413 amino acids, excluding 21 amino acids for signal peptide, based on the locations of the putative signal peptide and protease cleavage site; 26) and C-terminal half (431 amino acids) being 47,723 and 47,930 Da, respectively, the molecular masses of the amino acids for both gp60 and gp49 are approximately equal. Therefore, the size difference between gp60 and gp49 is likely due to the disproportionate extent of their glycosylation. Examination of potential N-linked glycosylation sites in the predicted amino acid sequence of MDV gB homolog (26) indicates that eight are located in the N-terminal half (excluding one site in the signal peptide) and one is located in the C-terminal half. With each glycan contributing approximately 2.5 kDa (19) to a glycoprotein's apparent molecular weight, gp60 and gp49 would likely be derived from the N- and C-terminal halves of gp100, respectively (Fig. 6).

In the MDV system a molecule called pr44 may have mistakenly been thought to be the primary precursor polypeptide of MDV gB homolog (B antigen complex) (17, 32), with a hypothesis that pr44 dimerizes to form pr88 (32), for the following three reasons. First, there was an early report that pr44 was the

most prominant band seen after TM treatment, using the same antisera that immunoprecipitated gp100, gp60, and gp49 (30). Second, several proteins (including the 44 kDa protein previously called pr44) of that approximate size were apparently nonspecifically trapped in the earlier work (17, 32); as has become readily apparent in this study during immunoprecipitation by the monoclonal antibody IAN86 (Fig. 2, lanes 1 to 4; the light bands at 44 kDa position were visible upon longer exposure), RαPM (Fig. 2, lanes 5 to 8), antisera against trpE-MDV-gB (Fig. 2, lanes 9 to 20) and immune chicken sera (data not shown) in this study. These nonspecifically trapped proteins seen in the immunoprecipitates of MDV-infected cell lysates were also observed in the immunoprecipitates of mock-infected cell lysates at a relatively lower level (32, unpublished results), suggesting that they may be cell proteins. Third, since gp100 of the MDV gB homolog is cleaved to form gp60 and gp49, the cleavage resulted in formation of the N- and C-terminal halves with the calculated molecular masses of their polypeptides being approximately equal, 47,723 and 47,930 Da, respectively (Fig. 6). The previous results, obtained by deglycosylation of gp60 and gp49 by endo-F and -H (32), indicated that the backbone polypeptides of gp60 and gp49 have similar estimated molecular masses (approximately 44 kDa), which are in good agreement with the calculated molecular masses for N- and C-terminal halves. Based on the above and other data, it was concluded that pr44 was the primary polypeptide, and further hypothesized that pr44 dimerizes to form pr88 (32). The results reported here indicate that the previous hypothesis is not correct and that pr88 is the primary product of the MDV gB homolog gene.

The established nomenclature of this laboratory, of pr88 for the primary gene product, is used in this report for consistency with published work first establishing (17) and then using (32) this designation. That size was based on

the 88 kDa estimate of both Ikuta et al. (16) and this laboratory (17). Based on their analysis of the MDV gB homolog gene, Ross et al. (26) calculated that the unglycosylated primary gene product is 95.5 kDa, excluding the signal peptide, and in their gels a size of 94 kDa was observed. Discrepancy with the original estimate of 88 kDa (16, 17) used to establish the nomenclature adopted by this laboratory (17) is likely due to the variation inherent in SDS-PAGE analysis, especially between laboratories. Compounding factors could be a change (by the manufacturer) in the reported size of the phosphorylase B marker from 92.5 kDa (17) to 97 kDa (26, Fig. 2), and the use of the 116 kDa b-galactosidase marker (26); which may have resulted in a SDS-PAGE-based estimate (94 kDa) much closer to that derived from sequence data (26). Possibly the term pr88 will need to be revised, especially as new MDV nomenclature evolves to accommodate sizes more precisely determined by calculations based on open reading frames shown to encode the polypeptides (28). In that event, the term pr95.5 is suggested based on the calculated size of the predicted amino acid sequence (26), rather than the SDS-PAGE derived size (16, 17). Possibly knowledge of function and/or virion location will be included in such new nomenclature.

Since fragments 1 and 3 (their trpE fusion proteins were used to prepare antisera Z43 and Z42, respectively) are from regions located at either N- or C-terminal parts of the putative cleavage site of the predicted MDV gB homolog amino acid sequence (Fig. 1), both Z43 and Z42 should recognize gp100, and one of the cleaved forms of gp100 (either gp49 or gp60), but not both of the cleaved forms, especially when the lysates were boiled. However, gp100, gp60 and gp49 were detected by both Z43 and Z42 (Fig. 4A, lanes 6 and 8, respectively). There is no significant amino acid homology found between the N- and C-terminal halves. Probably, gp49 is specifically associated with gp60

through a disulfide bond(s) which is(are) then dissolved on the reducing SDS-PAGE, as was the case for the two cleaved forms of EHV and VZV gB homologs (21, 22).

The fact that Z45 recognizes the undenatured gp60 and gp49 of HVT, but not those of MDV (Fig. 2, compare lane 13 with lane 15), suggests significant differences in the manner in which HVT's epitopes are displayed compared to MDV's. The failure of Z43, Z45 and Z42 to detect either gp100 (Fig. 2, lane 17) or the cleaved forms of gp100 (gp60 and gp49) (Fig. 2, lanes 9, 13 and 17), except upon denaturation (Fig. 4A, lanes 4, 6 and 8), is likely due to glycosylation and/or creation of secondary structures in the cleavage products that made certain epitopes inaccessible for recognition by antisera against trpE-MDV-gB. A similar phenomenon was reported by Whealy et al. (35); boiling of PRV-infected cell lysates increases the apparent reactivity of the mature 92 kDa protein of gII (PRV gB homolog) with 282 antiserum (raised against a denatured *E. Coli*-produced Cro-gII fusion protein).

The conclusion that the MDV B antigen complex is encoded by MDV's homolog of the HSV gB gene will facilitate future studies on the immunobiology of MD. It has been known for sometime that antibodies to MDV gB homolog neutralize MDV in cell culture (16), and that affinity purified HVT gB homolog elicits partial protection against MD in chickens (23). It is not clear if this partial protection is because another antigen(s) is needed, or because of inadequate antigen, adjuvant or other factors. Identification of the gene encoding this important antigen will make it possible to use molecular biological approaches to study immunoprotection against MD in more depth, including resolution of the questions presented above.

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REFERENCES

1. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. 46:83-88.
2. **Britt, W. J., and D. Auger.** 1986. Synthesis and processing of the envelope gp55-116 complex of human cytomegalovirus. J. Virol. 58:185-191.
3. **Britt, W. J., and D. Auger.** 1989. Processing of the gp55-116 envelope glycoprotein complex (gB) of human cytomegalovirus. J. Virol. 63:403-410.
4. **Calnek, B. K.** 1985. Marek's disease - a model for herpesvirus oncology. Crit. Rev. Microbiol. 12:293-320.
5. **Chen, X., P. J. A. Sondermeijer, and L. F. Velicer.** 1992. Identification of a unique Marek's disease virus gene which encodes a 38 kd phosphoprotein and is expressed in both lytically infected cells and in latently infected lymphoblastoid tumor cells. J. Virol. 66:85-94.
6. **Chen, X., and L. F. Velicer.** 1991. Multiple bidirectional initiations and terminations of transcription in the Marek's disease virus long repeat regions. J. Virol. 65:2445-2451.
7. **Chubb, R. C., and A. E. Churchill.** 1969. Precipitating antibodies associated with Marek's disease. Vet. Res. 83:4-7.
8. **Churchill, A. E., R. C. Chubb, and W. Baxendale.** 1969. The attenuation, with loss of antigenicity, of the herpes-type virus of Marek's disease (strain HPRS-16) on passage in cell culture. J. gen. Virol. 4:557-563.
9. **Coussens, P. M., and L. F. Velicer.** 1988. Structure and complete nucleotide sequence of the Marek's disease herpesvirus gp57-65. J. Virol. 62:2373-2379.
10. **Coussens, P. M., M. R. Wilson, H. Camp, H. Roehl, R. J. Isfort, and L. F. Velicer.** 1990. Characterization of the gene encoding herpesvirus of

turkeys gp57-65: comparison to Marek's disease virus gp57-65 and herpes simplex virus glycoprotein C. Virus Genes 3:211-227.

11. **Davidson, I., M. Malkinson, and Y. Becker.** 1988. Marek's disease virus serotype-1 antigens A and B and their unglycosylated precursors detected by Western blot analysis of infected cells. Virus Genes 2:5-18.

12. **Fitzpatrick, D. R., T. Zamb, M. D. Parker, S. D. L. Hurk, L. A. Babiuk, and M. J. P. Lawman.** 1988. Expression of bovine herpesvirus 1 glycoproteins gI and gIII in transfected murine cells. J. Virol. 62:4239-4248.

13. **Glaubiger, C., K. Nazerian, and L. F. Velicer.** 1983. Marek's disease herpesvirus. IV. Molecular characterization of Marek's disease herpesvirus A antigen. J. Virol. 45:1228-1234.

14. **Grose, C.** 1990. Glycoproteins encoded by Varicella-Zoster virus: biosynthesis, phosphorylation, and intracellular trafficking. Annu. Rev. Microbiol. 44:59-80.

15. **Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1983. Monoclonal antibodies reactive with the surface and secreted glycoproteins of Marek's disease virus and herpesvirus of turkeys. J. gen. Virol. 64:2597-2610.

16. **Ikuta, K., S. Ueda, S. Kato, and K. Hirai.** 1984. Processing of glycoprotein gB related to neutralization of Marek's disease virus and herpesvirus of turkeys. Microbiol. Immunol. 28:923-933.

17. **Isfort, R. J., I. Sithole, H.-J. Kung, and L. F. Velicer.** 1986. Molecular characterization of Marek's disease herpesvirus B antigen. J. Virol. 59:411-419.

18. **Isfort, R. J., 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.

19. **Klenk, H. D., and R. Rott.** 1980. Co-translational and post-translational processing of viral glycoproteins. Current Topic in Microbiol. and Immunol. 90:19-48.

20. **Konopka, J. B., R. L. Davis, S. M. Watanabe, A. S. Ponticelli, L. Schiff-Maker, N. Rosenberg, and O. N. Witte.** 1984. Only site-directed antibodies reactive with the highly conserved src-homologous region of the v-abl protein neutralize kinase activity. J. Virol. 51:223-232.

21. **Meridith, D. M., J.-M. Stocks, G. R. Whitaker, L. W. Halliburton, B. W. Snowden, and R. A. Killington.** 1989. Identification of the gB homologues of equine herpesvirus types 1 and 4 as disulphide-linked heterodimers and their characterization using monoclonal antibodies. J. gen. Virol. 70:1161-1172.

22. **Montalvo, E. A., and C. Grose.** 1987. Assembly and processing of the disulfide-linked Varicella-zoster virus glycoprotein gpII (140). J. Virol. 61:2877-2884.

23. **Ono, K., M. Takashima, T. Ishikawa, M. Hayashi, I. Yoshida, T. Konobe, K. Ikuta, K. Nakajima, S. Ueda, S. Kato, and K. Hirai.** 1984. Partial protection against Marek's disease in chickens immunized with glycoproteins gB purified from turkey-herpesvirus-infected cells by affinity chromatography coupled with monoclonal antibodies. Avian Dis. 29:533-539.

24. **Payne, L. N.** 1985. Marek's Disease - Scientific Basis and Methods of Control. Martinus Nijhoff Publishing, Boston.

25. **Robbins, A. K., D. J. Dorney, M. W. Wathen, M. E. Whealy, C. Gold, R. J. Watson, L. E. Holland, S. D. Weed, M. Levine, J. C. Glorioso, and L. W. Enquist.** 1987. The pseudorabies virus gII gene is closely related to the gB glycoprotein gene of herpes simplex virus. J. Virol. 61:2691-2701.

26. **Ross, L. J. N., 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.

27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

28. **Schat, K. A., G. F. de Boer, K. Hirai, L. F. Lee, K. Nakajima, R. F. Silva, and L. F. Velicer.** 1988. A proposed nomenclature for the protein of Marek's disease herpesvirus, p. 438-443. In S. Kato, T. Horiuchi, T. Mikami, and K. Hirai. (ed.), Advances in the Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

29. **Silva, R. F., and L. F. Lee.** 1984. Monoclonal antibody-mediated immunoprecipitation of proteins from cells infected with Marek's disease virus or turkey herpesvirus. Virology 136:307-320.

30. **Silva, R. F., and L. F. Lee.** 1985. Isolation and partial characterization of three glycoproteins common to Marek's disease virus and turkey herpesvirus-infected cells, p. 101-110. *In* B. W. Calnek and J. L. Spencer (ed.), Proceedings of the International Symposium on Marek's Disease. American Association of Avian Pathologists, Inc., Kennet Square, Pa.

31. **Sithole, L, P. M. Coussens, and L. F. Velicer.** 1988. Identification of Marek's disease herpesvirus B antigen's precursor polypeptide and the gene encoding it, p. 148-154. In S. Kato, T. Horiuchi, T. Mikami, and K. Hirai. (ed.), Advances in the Marek's disease research. Japanese association on Marek's disease, Osaka, Japan.

32. **Sithole, L, L. F. Lee, and L. F. Velicer. 1988.** Synthesis and processing of the Marek's disease herpesvirus B antigen glycoprotein complex. J. Virol. 62:4270-4279.

33. **Sullivan, D. C., G. P. Allen, and D. J. O'Callaghan. 1989.** Synthesis and processing of equine herpesvirus type 1 glycoprotein 14. Virology 173:638-646.

34. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl-sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

35. **Whealy, M. E., A. K. Robbins, and L. W. Enquist.** 1990. The export pathway of the pseudorabies virus gB homolog gII involves oligomer formation in the endoplasmic reticulum and protease processing in the Golgi apparatus. J. Virol. 64:1946-1955.

36. **Witte, O. N., and D. F. Wirth.** 1979. Structure of the murine leukemia virus envelope glycoprotein precursor. J. Virol. 29:735-743.

Chapter V

Summary and Conclusions

The major focus of the thesis research was to confirm our laboratory's previous preliminary identification of the gene encoding the MDV B antigen complex (gp100, gp60, and gp49) in BamHI-H, to locate the expected duplicate copy of the gene in BamHI-D (MDV has an alphaherpesvirus genome structure: both BamHI-D and -H contain part of long repeat regions), and to identify the HVT homolog of the MDV B antigen gene. Preliminary nucleotide sequence analysis suggested the presence of a spliced gene in BamHI-H, resulting in cDNA analysis. No open reading frame characteristic of a glycoprotein gene was identified in either BamHI-D or -H by DNA sequence and cDNA analyses; and no significant DNA homology was found between BamHI-D or -H and the equivalent parts of HVT's genome by Southern blot analysis. Instead, as a result of the cDNA analysis a gene encoding pp38 was identified in BamHI-H, and a gene family composed of four groups of transcripts were mapped in both BamHI-D and -H. Southern blot analysis showed that both the gene encoding pp38 and the gene family composed of four groups of transcripts are not present in HVT, suggesting that these genes are unique to MDV. The gene encoding the B antigen complex was not found in BamHI-D and -H, where it was previously thought to exist. However, identification of the gene encoding the MDV homolog of herpes simplex virus (HSV) glycoprotein B (gB) was reported by others. Therefore, determining whether the B antigen complex is the MDV gB homolog seemed to be the logical next choice in the effort to identify the gene encoding the MDV B antigen complex.

To determine whether the MDV gB homolog gene might encode the B antigen complex, antisera were prepared against trpE fusion proteins of the MDV gB homolog (trpE-MDV-gB). These antisera immunoprecipitated gp100,

gp98, gp60, gp49, and pr88. Based on size comparison, and trpE-MDV-gB competition and blocking assays, it was concluded that the MDV gB homolog is the B antigen complex and pr88 is the precursor polypeptide, while pr44, previously thought to dimerize to form pr88, is a protein nonspecifically trapped during immunoprecipitation. The MDV gB homolog processing was found to be consistent with those of other herpesvirus gB homologs; and appears to involve cotranslational glycosylation of pr88 to form gp98, which is further processed to yield gp100, which is then cleaved to form gp60 and gp49.

The conclusion that the B antigen complex is the MDV gB homolog indicates that the previously hypothesized dimerization of pr44 to form pr88 of the B antigen complex is incorrect, clarifies the uncertainty regarding the location of the gene encoding the B antigen complex, and will facilitate future studies on the immunobiology of MD. It has been known for sometime that antibodies to MDV gB homolog neutralize MDV in cell culture and that affinity-purified HVT gB homolog elicits partial protection against MD in birds. It is not clear if this partial protection is because another antigen(s) is needed, or because of inadequate antigen, adjuvant or other factors. Identification of the gene encoding this important antigen will make it possible to use molecular biological approaches to study immunoprotection against MD in more depth, including resolution of the questions presented above.

Through DNA sequence and cDNA analyses, an open reading frame (designated pp38 ORF) encoding 290 amino acids was identified in BamHI-H and is not duplicated in BamHI-D. Using trpE-pp38 fusion proteins, antisera against pp38 were prepared. With immunoprecipitation and SDS-PAGE analyses, two polypeptides (38- and 24-kDa) (designated pp38 and p24, respectively) were found. No precursor-product relationship was found between pp38 and p24 by pulse-chase analysis, and only pp38 was detected by

immunoblot analysis with anti-pp38. pp38 was found to be phosphorylated, MDV serotype-1-specific, an immunogen in birds with MD, belong to one of the early classes of herpesvirus proteins, and it was detected in MSB-1 lymphoblastoid cells in absence of IUdR treatment.

Some of the properties predicted for a protein detected by molecular methods, based on finding the ORF first, are identical to those of a 38-kDa phosphoprotein reported by others, suggesting that they are the same. However, many new pp38 properties were identified, which greatly extend our understanding of pp38. The identification of pp38 as the sole gene product of the pp38 ORF clarified the status of two other components, p24 and p41, of the previously identified group of three proteins that included pp38. They are not part of the gene product of the pp38 ORF, and they may specifically or nonspecifically associate with pp38, respectively. Detection of pp38 in the MSB-1 lymphoblastoid cell line in absence of IUdR treatment indicates that pp38 is constitutively expressed in the transformed and latently infected tumor cell line, suggesting that pp38 may play a role in MDV latency and maintenance of the transformed state of the tumor cell line. Absence of significant amino acid homology of pp38 to any known oncogenes, and expression of pp38 in attenuated nononcogenic serotype-1 MDV (reported by others) suggests that if pp38 plays a role in MDV transformation, two possibilities exist: (i) it is modified during attenuation or (ii) it is necessary but insufficient and indirect, and attenuation involves another gene(s). Identification of pp38 as one of the early classes of herpesvirus proteins suggests that pp38 may have a regulatory function, as do other herpesvirus early proteins. The finding that immune chicken sera (ICS) contain antibodies against pp38 indicates that pp38 is an immunogen in birds with MD. Since pp38 is an immunogen and constitutively expressed in the MSB-1 tumor cell line, and since inactivated MSB-1 tumor cells

were found by others to induce anti-tumor immunity in birds, it is possible that pp38 may play a role in MDV tumorigenicity and may be used as recombinant DNA derived vaccine to prevent tumor formation.

The gene family composed of four groups of transcripts identified with cDNA and S1 nuclease analyses were mapped in the expansion regions of BamHI-D and -H of MDV genome, and are either initiated or terminated within or near the expanded regions at multiple sites in both rightward and leftward directions. These RNAs, containing various copies of the 132-bp repeat at either their 5' or 3' ends, were found to be 0.67-, 1.6-, 1.8-, and 3.1-kb. Since the MDV genome expansion in the regions of BamHI-D and -H is correlated with the loss of the MDV tumorigenicity, the gene family transcribed from the expanded regions may play a role in MDV tumorigenicity. The finding that the gene family is composed of four groups of transcripts clarifies the erroneous published data, in which the gene family was thought to be composed of two groups of exons. Because of the bidirectional transcriptions, groups 1 and 2 transcripts are complementary to those of groups 3 and 4, respectively. Therefore, antisense regulation may be involved in the formation and functions of the gene family. DNA sequence analysis revealed that the transcripts can be initiated or terminated within the 132-bp direct repeat and contain various copies of 132-bp repeat, and each 132-bp repeat contains one TATA box and two polyadenylation consensus sequences in each direction. Taken together, these data suggest that the 132-bp repeat, and indirectly its copy number, may be involved in transcriptional regulation and therefore in the generation of four groups of transcripts potentially responsible for MDV tumorigenicity, although this remains to be demonstrated.