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The structure of Marek disease virus DNA: The presence of unique expansion in nonpathogenic viral DNA

(restriction enzyme pattern/cloned DNA/direct repeat/tumorigenicity)

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ABSTRACT **DNA of Marek disease virus (MDV) consists of two unique regions U_L and U_S flanked by long inverted repeat regions TR_L and IR_L, and short inverted repeat regions TR_S and IR_S, respectively, similar to herpes simplex virus DNA. Comparison of restriction patterns between pathogenic and nonpathogenic MDV DNA was made to identify a region of viral DNA different between these two types of MDV, as it may be responsible for the tumorigenicity of MDV in chickens. The results indicated that *Bam*HI-D and -H, located at the long inverted repeat regions TR_L and IR_L, were specifically expanded in nonpathogenic viral DNA. The location of the expanded region has been determined within 1.5 kilobase pairs of the *Bgl* I/*Pst* I fragment of *Bam*HI-D and -H, close to the junction between the inverted repeat and the unique region. The possibility that a gene responsible for tumor induction may be disrupted by such expansion has been discussed.**

Marek disease virus (MDV) causes lymphoproliferative disease in chickens. Pathogenic and apathogenic viral strains have been isolated in various laboratories (1). Attenuated strains of MDV have also been isolated by serial passages of virus in tissue culture (2, 3). Prior infection by these nonpathogenic viral strains can protect chickens from Marek disease induced by MDV infection (1, 3). We have recently completed the physical maps of the restriction enzyme fragments of MDV DNA (4), which indicate that the basic structure of MDV DNA resembles that of herpes simplex virus (HSV)-1 and -2 (5); i.e., it consists of two unique regions (U_L, long unique region, and U_S, short unique region), both flanked by inverted repeats, long inverted repeat regions TR_L (long terminal region) and IR_L (long inverted region), or short inverted repeat regions TR_S (short terminal region) and IR_S (short inverted region) (Fig. 1). Direct repeats divide U_L into U_{L1} and U_{L2} of MDV DNA. Generation of defective interfering particles in preparation of herpesvirus passaged at high multiplicity is a well established phenomenon (6–9). More pertinent to this report is the attenuation of oncogenic herpesvirus, herpesvirus saimiri, and accompanied alteration of viral DNA (10, 11). The generation of the attenuated strain of herpesvirus saimiri by serial propagation in Vero cells caused deletion of 2.3 kilobase pairs (kbp) of DNA at the left junction between low density and high density DNA with concomitant loss of virus oncogenicity. In contrast, this paper describes the acquisition of unique expansion by 150-base-pair (bp) repeat units within the TR_L and IR_L in the attenuated strains of MDV (shown by double-headed arrows in Fig. 1).

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MATERIALS AND METHODS

Virus Strains. Pathogenic strains used for this study were GA* (12), JM102W* (13), and Md5* (13) (* indicates pathogenic strain), and nonpathogenic strains were attenuated GA and JM102W/40D, and natural nonpathogenic strain CVI 988 (14). Attenuated GA and JM102W/40D were artificially made by repeated passage in cell culture as described (3). GA C1 19 and GA C1 21* were plaque-purified from oncogenic GA*.

Cellular, Viral, and Cloned DNA. Eleven-day-old chicken embryo fibroblasts prepared from specific pathogen-free chicken embryo (SPAFAS) were infected with MDV strain GA*, Md5*, or CVI 988, and duck embryo fibroblasts were infected with MDV strain JM102W* in Eagle's minimal essential medium containing 2% fetal calf serum at a ratio of 10 parts chicken embryo fibroblast to 1 part virus-infected cells.

Cells were harvested when 80% of cells showed a cytopathic effect. Total cellular DNA was extracted with Pronase/$NaDodSO_4$ followed by phenol extraction. Viral DNA was isolated as described (4). Briefly, intact nucleocapsids were extracted from infected cells by Nonidet P-40 treatment, and viral DNA was purified through a 10%–30% (vol/vol) glycerol gradient by centrifugation after $NaDodSO_4$/proteinase K treatment (4). *Bam*HI restriction enzyme fragments of MDV DNA were cloned into bacterial plasmids as described (4).

Gel Electrophoresis and Hybridization. Restriction enzymes were purchased from Bethesda Research Laboratories. Digested DNA fragments were separated by 0.6% agarose gel electrophoresis and subsequently transferred to nitrocellulose filter paper by the method of Southern (15). ^{32}P-labeled probes for the hybridization experiments were prepared by nick-translation (16).

Inoculation of Chicken with MDV. Two-week-old chickens, line GB-1 (17), were inoculated with 1500 focus-forming units of plaque-purified GA C1 19 or 500 focus-forming units of GA C1 21*.

The experiment was terminated 35 days after inoculation.

RESULTS

Restriction Patterns of Pathogenic and Nonpathogenic Viral DNA. Since not all the viral strains were easily grown in tissue culture to obtain sufficient amounts of viral DNA from purified virus, infected cell DNA was used as a viral DNA source for the study. Fig. 2 shows the *Bam*HI restriction pat-

Abbreviations: kbp, kilobase pair(s); TR_L, long terminal region; TR_S, short terminal region; IR_L, long inverted region; IR_S, short inverted region; U_L, long unique region; U_S, short unique region; MDV, Marek disease virus; HSV, herpes simplex virus.

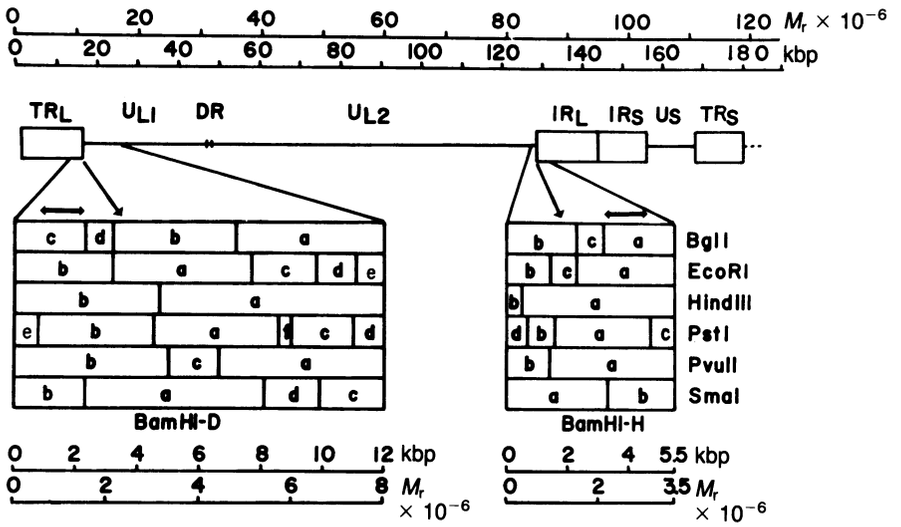

FIG. 1. Detailed restriction maps of *Bam*HI-D and -H and location of expanded region in MDV strain GA*. ↔ indicates location of expanded region.

terns of these viral DNAs as obtained by Southern blot hybridization of infected cell DNA with ^{32}P-labeled MDV strain GA* DNA. Pathogenic strains GA*, JM102W*, and Md5*, whose pathogenicity was confirmed, and cloned strain GA C1 21*, which was found to be pathogenic as described later, had identical distinctive restriction patterns. Since MDV DNA is known to have terminal heterogeneity (4), which was indicated by a smeared region of 12–20 kbp upon *Bam*HI digestion (4), the *Bam*HI-B, -C, and -D bands could not be clearly resolved. In the case of JM102W* (Fig. 2, lane 2), *Bam*HI-H and -I fragments, which contain 1 M I_1 and I_3 and 2 M I_2, converged to one band of 5.1 kbp. To determine whether this band was composed of *Bam*HI-H, -I_1, -I_2, and -I_3 (4), each *Bam*HI-H, -I_1, -I_2, or -I_3 probe was prepared to hybridize to the *Bam*HI digest of JM102W* DNA. All probes were found hybridized to the 5.1-kbp band (data not shown). Thus, the *Bam*HI-H fragments of JM102W* as well as Md5* (Fig. 2, lanes 2 and 3) migrated slightly faster than those of GA* strains (lanes 1 and 4). On the other hand, the restriction patterns of attenuated GA, JM102W/40D, CVI 988, and cloned strain GA C1 19, which did not induce tumors in chickens in a preliminary experiment, did not show distinct *Bam*HI-D and -H bands (lanes 5–8). This is in agreement with a previous observation of Hirai *et al.* (18, 19). The remainders of the bands were identical for both pathogenic and nonpathogenic viral DNA, except for an extra band observed between *Bam*HI-O_2 and -P in CVI 988 strains and slightly in Md5* and GA C1 21* (lanes 3, 4, and 7).

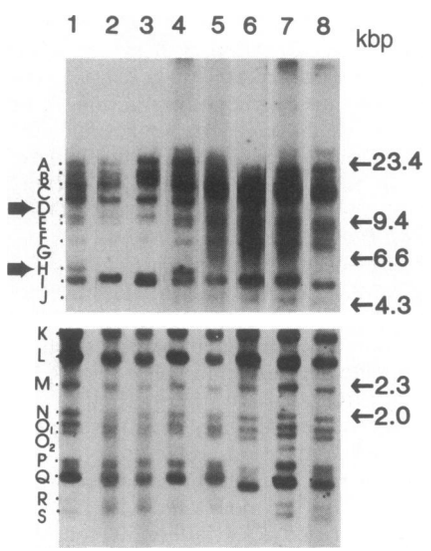

FIG. 2. *Bam*HI restriction patterns of pathogenic and nonpathogenic viral DNAs. Five micrograms of infected cellular DNA was digested with *Bam*HI and the resulting DNA fragments were electrophoresed through a 0.6% agarose gel. After transferring onto nitrocellulose filter paper, the DNA fragments were hybridized with ^{32}P-labeled nick-translated total viral DNA. Lanes: 1, GA*; 2, JM102W*; 3, Md5*; 4, GA C1 21*; 5, GA att; 6, JM102W/40D; 7, CVI 988; 8, GA C1 19. Size marker, *Hin*dIII-digested λ DNA. Bands A–J were obtained by short exposure; K–S were obtained by longer exposure. Each dot indicates A–S from the top. Heavy arrows indicate *Bam*HI-D and -H, which are missing in nonpathogenic viral DNA. Southern blot hybridization in this experiment tended to show stronger intensity for multimolar bands, such as I, K, and L, than expected from size and molar ratio. Multimolar ratio fragments are as follows: *Bam*HI-I with 4 M; K, P, and Q with 3 M; L and S with 2 M (4).

BamHI-D and -H DNA Fragments in Pathogenic and Nonpathogenic Strains. To further study the fate of *Bam*HI-D and -H in nonpathogenic viral DNA, cloned *Bam*HI-D probe (4) labeled with ^{32}P was used to hybridize to digests of the viral DNAs as described in the above study. Since a part of *Bam*HI-D fragment belongs to TR_L and a part of *Bam*HI-H also is found in its counterpart, IR_L, *Bam*HI-D probe can detect the region of interest in both *Bam*HI-D and -H (Fig. 1). In pathogenic strains, *Bam*HI-D and -H appeared as distinct bands (Fig. 3, lanes 1–4). Again, *Bam*HI-H of JM102W* and Md5* moved slightly faster than those of the other two strains. In nonpathogenic strains, these two fragments appeared as larger smeared regions (lanes 5–8), which will be termed "expansion," as certain regions of *Bam*HI-D and -H are expanded by either insertion or amplification of multiple repeat units as discussed below.

Fine Restriction Maps of *Bam*HI-D and -H and Location of the Expanded Regions. To determine the precise location of the expansion, cloned *Bam*HI-D and -H DNA fragments from GA* (4) were further mapped by use of several restriction enzymes. The TR_L–U_{L1} junction region was determined to be located within 700 bp from the right end of *Bam*HI-D/*Eco*RI subfragment b (D-*Eco*RI-b) and that of U_{L2}–IR_L was between the left end of H-*Pst* I-a and the left end of H-*Eco*RI-a (Fig. 1). This was further confirmed by hybridization studies using H-*Eco*RI-c probe to hybridize to D-*Eco*RI-a and D-*Pst* I-b. No hybridization was evident between H-*Pst* I-b and *Bam*HI-D subfragments (data not shown). Both

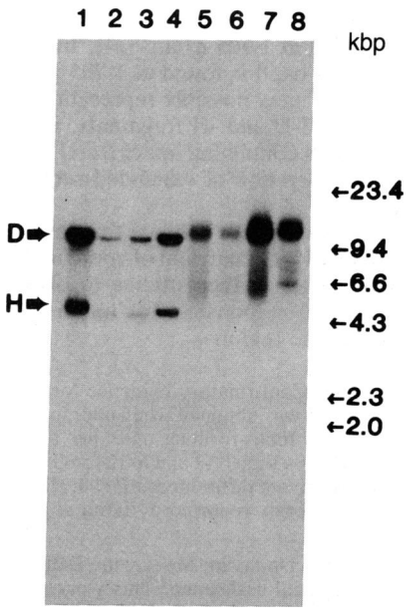

FIG. 3. *Bam*HI-D and -H fragments in pathogenic and nonpathogenic viral DNAs. Five micrograms of infected cellular DNA was digested with *Bam*HI, electrophoresed through a 0.6% agarose gel, and transferred onto nitrocellulose filter paper. DNA fragments bound to nitrocellulose paper were hybridized with ^{32}P-labeled nick-translated plasmid that contains *Bam*HI-D. Lanes: 1, GA*; 2, JM102W*; 3, Md5*; 4, GA C1 21*; 5, GA att; 6, JM102W/40D; 7, CVI 988; 8, GA C1 19. Size marker, *Hin*dIII-digested λ DNA.

pathogenic and nonpathogenic viral DNAs were double-digested with *Bam*HI and *Bgl* I or with *Bam*HI and *Pst* I and were hybridized with *Bam*HI-D probe (Fig. 4). As shown in Fig. 4*A*, all subfragments were observed as distinct bands in pathogenic strains. In the case of JM102W* and Md5*, D-*Pst* I-b, H-*Pst* I-a, and D-*Bgl* I-c migrated faster than those of GA* (Fig. 4*A*), thus identifying a region responsible for faster moving *Bam*HI-H fragment of these two viral strains. The subfragment patterns of nonpathogenic strains are shown in Fig. 4*B*. In this case, D-*Pst* I-a, -c, -d, -e, and D-*Bgl* I-a, -b, and -d were observed to be distinct bands. However, D-*Pst* I-b, H-*Pst* I-a, and D-*Bgl* I-c bands disappeared in the nonpathogenic strains and were replaced by smeared regions (Fig. 4*B*). Furthermore, as shown in Fig. 4*B* (lane 5), for hybridization to *Bam*HI/*Bgl* I digest of CVI 988 DNA, this smeared region contained ≈150-bp multiple repeats. Since resolution of 150-bp repeats is difficult to obtain in this type of gel, *Bam*HI/*Bgl* I digests of JM102W/40D and GA C1 19 showed multiple repeats only faintly (Fig. 4, lanes 3 and 7). Thus, the DNA has been rearranged by expansion of this fragment with multiple repeats, and this expansion is located within 1.5 kbp of the *Bgl* I/*Pst* I subfragments of *Bam*HI-D and -H.

Infection of Chickens. A preliminary study was conducted to test the tumorigenicity of GA C1 19 and C1 21*, which were plaque-purified from the pathogenic strain of GA*. As discussed, GA C1 21* contained intact *Bam*HI-D and -H fragments, whereas *Bam*HI-D and -H of GA C1 19 were expanded. Four of eight chickens infected with GA C1 21* developed Marek disease lymphoma within 35 days of inoculation, while none of eight chickens inoculated with GA C1 19 developed the disease.

These chickens, however, developed the antibody titers, and infectious viruses were isolated from them. Although, because of the small group of chickens used, this study did not conclude that the GA C1 19 is not tumorigenic, it indicates that the expansions of *Bam*HI-D and -H affected the degree of tumorigenicity of MDV.

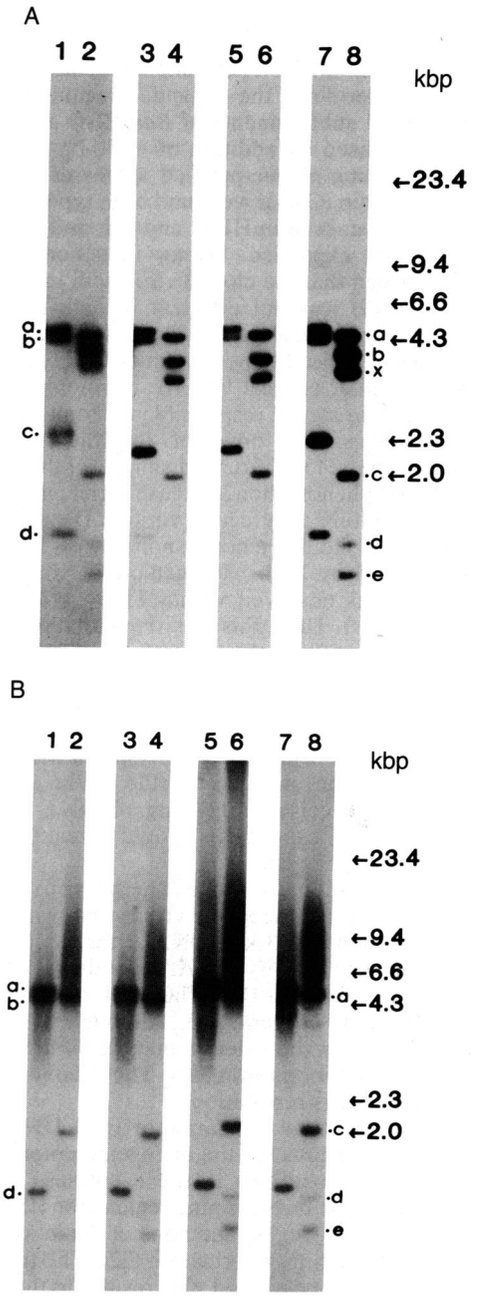

FIG. 4. Determination of expanded region in *Bam*HI-D and -H. Five micrograms of infected cellular DNA was digested with *Bam*HI/*Bgl* I or *Bam*HI/*Pst* I, electrophoresed through a 0.6% agarose gel, and transferred onto nitrocellulose filter paper. DNA fragments were hybridized with ^{32}P-labeled nick-translated plasmid that contains *Bam*HI-D. Bands were labeled according to the detailed map of *Bam*HI-D (see Fig. 1). (*A*) Lanes: 1 and 2, GA*; 3 and 4, JM102W*; 5 and 6, Md5*; 7 and 8, GA C1 21*. (*B*) Lanes: 1 and 2, GA att; 3 and 4, JM102W/40D; 5 and 6, CVI 988; 7 and 8, GA C1 19. *Bam*HI/*Bgl* I double-digestion was performed in lanes 1, 3, 5, and 7 in both *A* and *B*; *Bam*HI/*Pst* I double-digestion was done in lanes 2, 4, 6, and 8 in both *A* and *B*. Size marker, *Hin*dIII-digested λ DNA. ×, *Bam*HI-H/*Pst* I subfragment a.

DISCUSSION

In this paper, we have reported that *Bam*HI-D and -H fragments consistently disappeared in DNA of nonpathogenic strains of MDV. This is in agreement with the observations of Hirai *et al.* (18, 19). Thus, the disappearance of these two *Bam*HI fragments may be a common feature of nonpathogenic viral DNA. However, contrary to the expectation that

a deletion might have occurred in *Bam*HI-D and -H, we have found that *Bam*HI-D and -H fragments have been heterogeneously expanded in the DNA of nonpathogenic strains. We have precisely determined the expanded regions within 1.5 kbp of *Bgl* I/*Pst* I subfragments of *Bam*HI-D and -H. The expansion was caused by addition of ≈150-bp multiple repeats. Upon isolating plaque-purified clones of MDV from our pathogenic strain (GA*), we found both types of virus—i.e., virus with intact *Bam*HI-D and -H and virus with *Bam*HI-D and -H expanded. Tumor induction studies in chickens confirmed that the cloned virus with the expanded region (GA Cl 19) was not efficient in inducing tumors, whereas virus with normal *Bam*HI-D and -H fragments (GA Cl 21*) efficiently caused tumors in chickens. These experiments taken together suggest that the regions in *Bam*HI-D and -H may contain a gene responsible for tumor induction. There is some possibility, however, that the expansion of *Bam*HI-D and -H and loss of tumorigenicity of the virus may be a coincidental phenomenon, because serial passage of the virus in tissue culture may cause various DNA rearrangements (6–8, 20). It should be noted, nonetheless, that similar expansion, carried by both pathogenic and nonpathogenic strains of MDV, is observed within TR_S and IR_S (unpublished observations). Thus, this may imply that expansion of TR_L and IR_L is specific to the nonpathogenic strain; if so, a gene present in *Bam*HI-D and -H may be responsible for tumor induction, and the expansion of the sequence within the gene may have caused inactivation of the gene. Since we can obtain a cloned virus with the expanded *Bam*HI-D and -H by single plaque isolation, this gene should not be essential for viral replication. Precise sequencing of DNA along with mRNA mapping in these regions should reveal the cause of the inactivation of the gene.

A similar observation was made in herpesvirus saimiri (10, 11). Attenuation of the oncogenic virus strain was accompanied by the deletion of 2.3-kbp DNA at the left junction between low and high density DNA. Low density DNA is a unique region of infectious DNA flanked by highly repetitive DNA of high G+C content (21). Although it is not confirmed, a gene within the deletion may be directly responsible for tumor induction in primates. The deleted region was not required for virus replication.

Despite the structural similarity of viral DNA between MDV and HSV, regions for the oncogenic potential appear to be in different locations in these viruses. All the sequences identified as transforming regions for HSV-1 and -2 are located in the U_L region and none of the inverted repeat regions is involved in transformation (22). The transforming region for equine herpesvirus-1 also resides in the U_L region (23). Thus, the location of a potential oncogenic sequence in the TR_L and IR_L is a unique feature of MDV oncogenicity.

Among the DNAs of the pathogenic strains, mobilities of *Bam*HI-D and -H fragments were different because of the increase in size of the subfragments *Pst* I-b or *Bgl* I-c of *Bam*HI-D and *Pst* I-a of *Bam*HI-H. It has not been determined whether this difference in size may be due to insertion of the same repeat unit as observed in nonpathogenic viral DNA. Except for the expansion of *Bam*HI-D and -H regions, restriction enzyme patterns of pathogenic and nonpathogenic viral DNAs were almost identical. The merging of three different *Bam*HI-I fragments (4) at the position of *Bam*HI-H in JM102W* DNA was observed in a repeat experiment because of the faster migration of *Bam*HI-H to join *Bam*HI-I.

We have reported previously the heterogeneity of viral DNA populations in serially passaged viral preparations upon CsCl centrifugation, with one strain banding at 1.700 g/cm^3 and the other at 1.705 g/cm^3 (24). In plaque-purified viral DNA, a single band is found at 1.705 g/cm^3. The viral DNA at 1.700 g/cm^3 may possibly represent DNA having an expansion of *Bam*HI-D and -H fragments, as serial passage of pathogenic strains containing intact *Bam*HI-D and -H generally results in generation of expanded sequences in these same regions.

Although the present data indicate that loss of tumorigenicity is accompanied by expansion of specific regions of viral DNA, we must await further studies to obtain direct evidence that a gene(s) responsible for tumor induction is indeed located in those regions.

Note Added in Proof. Confirmatory evidence for the nononcogenicity of GA Cl 19 MDV was obtained when additional GB-1 chickens were inoculated (1000 focus-forming units per bird). Incidence of MD lesions after 49 days was 0/12 and 6/12 for GA Cl 19 and GA Cl 21, respectively (significant difference at $P < 0.02$). A similar expansion also has been recently reported by Ikuta *et al.* (25).

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