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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/transformicity)

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ABSTRACT DNA of Marek disease virus (MDV) consists of two unique regions U₅ and U₆ flanked by long inverted repeat regions TR₅ and IR₅, and short inverted repeat regions TR₆ and IR₆, 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 BamHI D and H, located at the long inverted repeat regions TR₅ and IR₅, 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 BamHI 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 (3, 4). 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 (U₅, long unique region, and U₆, short unique region), both flanked by inverted repeats, long inverted repeat regions TR₅ (long terminal region) and IR₅ (long inverted region), or short inverted repeat regions TR₆ (short terminal region) and IR₆ (short inverted region) (Fig. 1). Direct repeats divide U₅ into U₅₁ and U₅₂ 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 (kb) 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 TR₅ and IR₅ in the attenuated strains of MDV (shown by double-headed arrows in Fig. 1).

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MATERIALS AND METHODS

Viruses 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 (SPAFA) 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/NaDodSO₄ 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 NaDodSO₄/proteinase K treatment (4). BamHI 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). ³²P-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 BamHI restriction pat-
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 BamHI digestion (4), the BamHI-B, -C, and -D bands could not be clearly resolved. In the case of JM102W* (Fig.

![Fig. 1. Detailed restriction maps of BamHI-D and -H and location of expanded region in MDV strain GA*. ↔ indicates location of expanded region.](image)

2, lane 2), BamHI-H and -I fragments, which contain 1 M I$_1$ and I$_2$ and 2 M I$_3$, converged to one band of 5.1 kbp. To determine whether this band was composed of BamHI-H, -I$_1$, -I$_2$, and -I$_3$ (4), each BamHI-H, -I$_1$, -I$_2$, or -I$_3$ probe was prepared to hybridize to the BamHI digest of JM102W* DNA. All probes were found hybridized to the 5.1-kbp band (data not shown). Thus, the BamHI-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 BamHI-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 BamHI-O$_2$ and -P in CVI 988 strains and slightly in Md5* and GA C1 21* (lanes 3, 4, and 7).

**BamHI-D and -H DNA Fragments in Pathogenic and Nonpathogenic Strains.** To further study the fate of BamHI-D and -H in nonpathogenic viral DNA, cloned BamHI-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 BamHI-D fragment belongs to TR$_L$ and a part of BamHI-H also is found in its counterpart, IR$_L$, BamHI-D probe can detect the region of interest in both BamHI-D and -H (Fig. 1). In pathogenic strains, BamHI-D and -H appeared as distinct bands (Fig. 3, lanes 1–4). Again, BamHI-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 BamHI-D and -H are expanded either by insertion or amplification of multiple repeat units as discussed below.

**Fine Restriction Maps of BamHI-D and -H and Location of the Expanded Regions.** To determine the precise location of the expansion, cloned BamHI-D and -H DNA fragments from GA* (4) were further mapped by use of several restriction enzymes. The TR$_L$-UL$_{11}$ junction region was determined to be located within 700 bp from the right end of BamHI-D/EcoRI subfragment b (D-EcoRI-b) and that of UL$_1$-IR$_L$ was between the left end of H-Pst I-a and the left end of H-EcoRI-a (Fig. 1). This was further confirmed by hybridization studies using H-EcoRI-c probe to hybridize to D-EcoRI-a and D-Pst I-b. No hybridization was evident between H-Pst I-b and BamHI-D subfragments (data not shown). Both
Pathogenic and nonpathogenic viral DNAs were double-digested with BamHI and Bgl I or with BamHI and Pst I and were hybridized with BamHI-D probe (Fig. 4). As shown in Fig. 4A, 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. 4A), thus identifying a region responsible for faster movement of BamHI-D fragments of these two viral strains. The subfragment patterns of nonpathogenic strains are shown in Fig. 4B. 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. 4B). Furthermore, as shown in Fig. 4B (lane 5), for hybridization to BamHI/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, BamHI/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 BamHI-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 BamHI-D and -H fragments, whereas BamHI-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 BamHI-D and -H affected the degree of tumorigenicity of MDV.

**DISCUSSION**

In this paper, we have reported that BamHI-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 BamHI fragments may be a common feature of nonpathogenic viral DNA. However, contrary to the expectation that
a deletion might have occurred in BamHI-D and -H, we have found that BamHI-D and -H fragments have been heterogeneously expanded in the DNA of nonpathogenic strains. We have precisely defined the expanded regions within 1.5 kbp of Bgl I/Prs I subfragments of BamHI-D and -H. The expansion was caused by addition of $\sim 150$-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 BamHI-D and -H and virus with BamHI-D and -H expanded. Tumor induction studies in chickens confirmed that the cloned virus with the expanded region (GA C1 19) was not efficient in inducing tumors, whereas virus with normal BamHI-D and -H fragments (GA C1 21*) efficiently caused tumors in chickens. These experiments taken together suggest that the regions in BamHI-D and -H may contain a gene responsible for tumor induction.

There is some possibility, however, that the expansion of BamHI-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 $TR_3$ and $IR_3$ (unpublished observations). Thus, this may imply that expansion of $TR_3$ and $IR_3$ is specific to the nonpathogenic strain; if so, a gene present in BamHI-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 BamHI-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 $U_1$ region and none of the inverted repeat regions is involved in transformation (22). The transforming region for equine herpesvirus-1 also resides in the $U_1$ region (23). Thus, the location of a potential oncogenic sequence in the $TR_3$ and $IR_3$ is a unique feature of MDV oncogenicity.

Among the DNAs of the pathogenic strains, mobilities of BamHI-D and -H fragments were different because of the increase in size of the subfragments $Pst$ I-b or $Bgl$ I-c of BamHI-D and $Pst$ I-a of BamHI-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 BamHI-D and -H regions, restriction enzyme patterns of pathogenic and nonpathogenic viral DNAs were almost identical. The merging of three different BamHI-I fragments (4) at the position of BamHI-H in JMI102W* DNA was observed in a repeat experiment because of the faster migration of BamHI-H to join BamHI-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 BamHI-D and -H fragments, as serial passage of pathogenic strains containing intact BamHI-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 C1 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 C1 19 and GA C1 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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