Restriction Endonuclease Analysis of Marek's Disease Virus DNA and Homology between Strains

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SUMMARY

The restriction endonuclease patterns of viral DNA obtained from serotypes 1 and 2 strains of Marek's disease virus have been compared and homology between the strains examined by hybridization. The results have shown that HPRS 16 (serotype 1) DNA has a structure similar to its attenuated variant HPRS 16/att except for a few fragments that are present only in the virulent strain. Evidence was obtained which suggested that these restriction fragments contained repeat sequences and that insertion of heterogeneous DNA occurred at these sites during attenuation of HPRS 16. The restriction enzyme patterns of HPRS 24 (serotype 2) differed substantially from those of HPRS 16 and HPRS 16/att and reassociation experiments showed that HPRS 24 shares less than 10% homology with either HPRS 16 or herpesvirus of turkeys (serotype 3).

Strains of Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) have been divided into three types on serological grounds (Von Billow & Biggs, 1975a, b). Type 1 comprises pathogenic strains of MDV such as HPRS 16, GA and JM and their attenuated variants. Type 2 includes the naturally occurring non-pathogenic strain HPRS 24 and type 3 includes HVT. HPRS 24 differs from viruses of types 1 and 3 in having a lower growth rate in vitro and in forming smaller plaques in chicken kidney cells (Biggs & Milne, 1972). Hirai et al. (1981) have recently reported a change in restriction endonuclease patterns of the serotype 1 strains GA and JM during attenuation. The purpose of this study was to compare the patterns obtained with HPRS 16, HPRS 16/att and HPRS 24 and in particular to study the homology between these strains, since little is known about the genetic relatedness of serotypes 1 and 2.

The virus strains used were as follows: HPRS 16 (Purchase & Biggs, 1967), HPRS 16/att (Churchill et al., 1969), HVT strain FC126 (Witter et al., 1970) and HPRS 24 (Biggs & Milne, 1972). All virus strains were propagated in chick embryo fibroblasts (CEF) as cell-associated virus. HPRS 16 had been plaque-purified using virus derived from feather follicles of infected birds and was used within six passages in CEF. When inoculated into susceptible chickens, typical Marek's disease lesions were produced.

The pathogenicity of HPRS 24 was investigated by inoculating Rhode Island Red chickens (HPRS RIR) with 1000 p.f.u. intra-abdominally. A score was given for lesions in peripheral nerves and in visceral organs and tissues as described previously (Biggs & Milne, 1972). In this system, lesions apparent to the naked eye were given a score of 5. Those chicks with no gross lesions were examined histologically and scores from 1 to 4 were given according to the severity of the lymphoid infiltration in the gonads and in the brachial, sciatic and coeliac plexuses. The scores for each chick in a group were added and a mean calculated. At 42 days after infection, 0/10 birds showed gross MD lesions in the group inoculated with HPRS 24 compared with 10/10 in the case of HPRS 16. Some birds infected with HPRS 24 showed microscopic lesions and scores for neural and visceral lesions were 0·8 and 0·1 respectively. Corresponding scores for birds infected with HPRS 16 were 5 and 4·5. Although these results showed that HPRS 24 was non-pathogenic, depressed growth was noted in long-term experiments.

To obtain labelled virus DNA, infected cells were cultured in the presence of [32P]-
Fig. 1. Restriction endonuclease patterns of HPRS 16, HPRS 16/att and HPRS 24 DNAs labelled with [32P]orthophosphate. Samples containing 5000 to 8000 d/min (<20 ng DNA) were electrophoresed in 0.8% agarose gels. Fragments are labelled alphabetically and fragment sizes are indicated in kilobase pairs. Arrows show fragments in HPRS 16 that are not found in digests of HPRS 16/att. Asterisks show probable terminal fragments. Note the marked differences in the patterns obtained with HPRS 24 and HPRS 16.

orthophosphate (Amersham International) as described by Hirai et al. (1979) and high mol. wt. DNA was extracted from purified virions as described by Lee et al. (1982). Labelled viral DNA was digested with restriction endonuclease EcoRI, BamHI and SmaI (Bethesda Research Laboratories) as described by the manufacturers and the digests subjected to agarose gel electrophoresis as described previously (Ross et al., 1981). Unlabelled bacteriophage λ DNA was added to all digestion reaction mixtures to confirm that digestion was complete and to provide markers for estimation of mol. wt.

Fig. 1 shows that the restriction patterns obtained with HPR 16 and HPR 16/att were similar using all three enzymes for digestion except for the presence of some fragments in HPR 16 that were missing in HPR 16/att. These extra fragments were EcoRI F (6-8 kbp), SmaI F (11 kbp) and BamHI D (12 kbp). Other differences noted in preparations of HPR 16/att were the presence of broader fragments D and E in EcoRI digests, smears between bands A and E in SmaI digests and a broad band below C but larger in size than D in BamHI digests suggesting heterogeneity. Changes in molarity of some bands were also observed. The sum of fragment sizes in digests of HPR 16, HPR 16/att and HPR 24 ranged from 104 to 121 kbp, 98 to 108 kbp, and 110 to 124 kbp respectively. These are clearly underestimates, since the molarity of many bands appears to be greater than unity and differences in molarity have not been taken into account in calculating the sum of fragment sizes. Interestingly, the patterns obtained with HPR 24 differed substantially from those of HPR 16 and HPR 16/att using all three enzymes for digestion, indicating that HPR 24 has a distinct evolutionary history. We are currently attempting to construct models for these strains and have obtained evidence that BamHI
fragments J and L and SmaI fragments I and L of HPRS 24 are probably terminal, since densitometry showed that the counts associated with these bands were reduced by 58 to 65% by pretreatment of viral DNA with exonuclease III (Bethesda Research Laboratories) compared to a reduction of 20 to 30% for other fragments. We have also obtained evidence that BamHI A fragment is near to one end of the genome of HPRS 16/att as has been reported for the GA strain (Lee et al., 1982).

To determine the relationship between the strains, purified viral DNA obtained from virions of HPRS 16/att were labelled with $^{32}$P (10$^9$/d/min/μg) by nick translation and hybridized to digests of homologous and heterologous DNAs according to the Southern blotting procedure (Southern, 1975) as described previously (Ross et al., 1981). There was considerable homology between HPRS 16 and HPRS 16/att (Fig. 2), but only weak hybridization with a few fragments in digests of HPRS 24. Since this probe also hybridizes weakly with two fragments in digests of cellular DNA (Fig. 2), the cross-reactions observed between HPRS 16 and HPRS 24 are attributable at least in part to cell DNA. However, BamHI fragments B, F and G of HPRS 24 appear to cross-react specifically with HPRS 16. Conversely, a probe made with HPRS 24 DNA hybridized strongly with all fragments in digests of HPRS 24 but weakly and with only three or four fragments in digests of HPRS 16 (not shown). These results show that there is little homology between HPRS 16 and HPRS 24.
Reassociation experiments in liquid conditions were carried out to determine the percentage homology between the three serotypes. $^{32}$P-labelled viral DNA was hybridized to an excess of unlabelled DNA mainly as described by Hirai et al. (1979) except that, for this experiment, unlabelled viral DNA was obtained from infected CEF cultures by Hirt extraction and the Hirt supernatant was used as a source of viral DNA. These preparations contain high concentrations of viral DNA but also contain cellular DNA. The results of hybridization summarized in Table 1 show that approximately 60 to 70% of the radioactivity in HPRS 16/att and HPRS 24 DNAs reassociated with their respective homologous DNAs within 20 h, reaching 75 to 80% by 144 h. However, only 6% approximately of the counts of HPRS 16/att DNA formed S1 nuclease-resistant hybrids with HPRS 24 or HVT DNA. We conclude that there is little homology ($<10\%$) between the three serotypes and that the results support the separation of HPRS 16, HPRS 24 and HVT into three distinct types.

To examine further the relationship between HPRS 16 and HPRS 16/att, individual cloned DNA fragments were labelled by nick translation and hybridized to digests of viral DNAs obtained from purified virions using the Southern blotting procedure. Of the cloned DNAs available, **BamHI** $H$ (5-4 kbp) (derived from the genomic library of the GA strain, a gift from Dr M. Nonoyama, Showa University, Florida, U.S.A.) was of special interest since it cross-hybridized (Fig. 2) with the restriction endonuclease fragments **EcoRI** $C$ (13 kbp) and **F** (6-8 kbp); **BamHI** $H$ (5-4 kbp) and **D** (12 kbp); **SmaI** $C$ (19 kbp), **F** (11 kbp) and **I** (5-5 kbp) of HPRS 16, some of which were lacking in digests of HPRS 16/att (see Fig. 1). Furthermore, the fact that **BamHI** $H$ hybridized to **BamHI** $D$ suggested that these fragments contain repeat sequences. Since the **BamHI** $H$ probe prepared from the GA strain is identical in size to **BamHI** $H$ of HPRS 16 (5-4 kbp), it is unlikely that the probe used could span **BamHI** $H$ and **D** fragments in HPRS 16 since this probe also detects **SmaI** fragments $C$, $F$ and $I$, all of which are larger than 5-4 kbp. It is therefore highly probable that **BamHI** $H$ and **D** fragments of HPRS 16 contain reiterated sequences.

When **BamHI** $H$ probe was hybridized to **BamHI** digests of HPRS 16/att, discrete bands corresponding to **BamHI** $H$ and **BamHI** $D$ were not observed (Fig. 2). Instead, **BamHI** $D$ was larger in size and more diffuse. **BamHI** $H$ fragment was not present and seemed to have been replaced by a smear 7 to 12 kbp in size. An additional band (3-3 kbp) which hybridized weakly to **BamHI** $H$ was also observed. These results suggested that **BamHI** $H$ and **D** fragments of HPRS 16 became modified during attenuation. The precise mechanism of the modification is not yet clear, although the results suggest that insertion of heterogeneous sequences might have occurred.

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**Table 1. Reassociation of HPRS 16/att and HPRS 24 DNA in the presence of homologous and heterologous DNA**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>HPRS 24</th>
<th>HPRS 16</th>
<th>HVT</th>
<th>CEF</th>
<th>HPRS 24</th>
<th>HPRS 16</th>
<th>HVT</th>
<th>CEF</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>76†</td>
<td>77</td>
<td>61</td>
<td>77</td>
<td>100</td>
<td>162</td>
<td>150</td>
<td>130</td>
</tr>
<tr>
<td>8</td>
<td>648 (1.5)</td>
<td>6738 (62)</td>
<td>540 (0.4)</td>
<td>501</td>
<td>5020 (46)</td>
<td>430 (0.2)</td>
<td>420 (0.2)</td>
<td>400</td>
</tr>
<tr>
<td>20</td>
<td>873 (2.3)</td>
<td>7632 (70)</td>
<td>725 (0.8)</td>
<td>645</td>
<td>6400 (58)</td>
<td>660 (0.6)</td>
<td>640 (0.4)</td>
<td>600</td>
</tr>
<tr>
<td>70</td>
<td>1389 (5)</td>
<td>8500 (75)</td>
<td>1387 (5)</td>
<td>889</td>
<td>8492 (73)</td>
<td>1800 (6)</td>
<td>1780 (5-8)</td>
<td>1200</td>
</tr>
<tr>
<td>144</td>
<td>1510 (5-6)</td>
<td>8956 (80)</td>
<td>1450 (5)</td>
<td>950</td>
<td>8800 (75)</td>
<td>1920 (6-2)</td>
<td>1900 (6)</td>
<td>1300</td>
</tr>
</tbody>
</table>

* Viral DNA extracted from purified virions was labelled with $^{32}$P by nick translation and used as probe. The sp. act. ranged from $5 \times 10^7$ to $10^8$ d/min/$\mu$g. The reassociation mixture contained labelled HPRS 16/att or HPRS 24 DNA (8 ng/ml and 5 ng/ml respectively), 100 $\mu$g/ml of unlabelled DNA derived from the Hirt supernatant of infected or uninfected CEF, and salmon sperm DNA (1 mg/ml). The mixture was sonicated, denatured with alkali, neutralized, boiled, quenched and incubated at 70 °C in the presence of 1 M-NaCl. At the times indicated, aliquots containing 10000 ct/min were removed and assayed for S1 nuclease resistance.

† S1 nuclease-resistant ct/min (mean of two estimations, each counted for 10 min).

‡ Percentage reassociated, corrected for hybridization to CEF DNA: $[(Ct/min of sample - ct/min of CEF)/10000] \times 100$. 
Dot blot hybridization of labelled HPRS 24 DNA to cloned DNAs derived from serotype 1 viruses (results not shown) have demonstrated that cloned DNAs sharing the greatest degree of homology with HPRS 24 were as follows: BamHI fragments I_3 (5·1 kbp), J (4·3 kbp), M (2·4 kbp), P (1·1 kbp) derived from the genomic library of the GA strain and EcoRI fragments C (11 kbp), E (7·5 kbp), I (5·5 kbp) and L (4 kbp) derived from the genomic library of HPRS 16/att (Ross et al., 1981). Minor homology with BamHI D and H was also noted. Since HPRS 24 is serologically related to HPRS 16, it is possible that some of these sequences code for common antigenic determinants.

The restriction enzyme patterns obtained with HPRS 16 are similar to those reported for the GA and JM strains of serotype 1 and we have shown that loss of some fragments also occurs during attenuation of HPRS 16 as has been found for the JM strain (Hirai et al., 1981). We have shown further that although extra fragments are resolved in digests of HPRS 16 compared to HPRS 16/att, sequences homologous to these fragments are nonetheless present in digests of HPRS 16/att and evidence was obtained which suggested that regions of viral DNA containing repeat sequences (BamHI D and H) became modified by insertion of heterogeneous DNA. Since a definitive map of MDV DNA is not available (Lee et al., 1982), we do not know whether the modifications occurred in terminal or internal repeats or both. Whether these changes relate directly or indirectly to loss of virulence or only to the loss of 'A' antigen (Churchill et al., 1969) remains to be determined. However, it is of interest that BamHI D and H fragments are preserved in the Marek's disease lymphoid cell line MDCC 2 (Fig. 2) despite continuous culture of this line in vitro and that virus re-isolated from this line retained the capacity to synthesize the 'A' antigen as found previously (Ross et al., 1977).

Our results have shown that restriction enzyme analysis is capable of differentiating between serotypes of MDV and between virulent and attenuated variants of HPRS 16. This approach is probably preferable to the analysis of immunoprecipitates which could not distinguish between virulent and attenuated variants of serotype 1 virus (Zaane et al., 1982). Analysis of additional strains of serotypes 1 and 2 is in progress.

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REFERENCES


Short communication


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