Comparative Studies on Marek’s Disease Virus and Herpesvirus of Turkey DNAs

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SUMMARY

DNA of Marek’s disease virus (MDV) was compared to that of herpes virus of turkey (HVT). Centrifugation of the two virus DNAs in neutral glycerol and CsCl density gradients showed that the MDV genome was slightly larger than that of HVT and that the buoyant density (1.705 g/ml) of MDV DNA in CsCl gradients was slightly lower than that (1.707 g/ml) of HVT DNA. MDV and HVT DNAs were digested with either EcoRI or HindIII restriction endonuclease and analysed by 0.5% agarose gel electrophoresis. The cleavage patterns of HindIII or EcoRI DNA digests of two strains of these two viruses showed general similarities between the strains, but not between MDV and HVT. However, a few fragments of EcoRI or HindIII digests of MDV DNA co-migrated with those of HVT DNA. DNA–DNA reassociation kinetics and DNA–RNA hybridization between these two viruses indicated that MDV and HVT DNAs share detectable homology, although it is less than 5%. The DNA of a HVT variant, which has lost the ability to protect chickens from Marek’s disease, appeared similar to DNA of the vaccine strain in size and buoyant density and in its restriction endonuclease cleavage pattern.

INTRODUCTION

Marek’s disease virus (MDV) is an aetiological agent of Marek’s disease, a highly contagious malignant lymphoma of chickens. A herpes virus of turkey (HVT) was shown to be serologically related to MDV and has been used to protect chickens from challenge with MDV. However, multiple passages of HVT in culture were found to result in loss of the ability to protect chickens from the disease (Konobe et al. 1972; Witter, 1977).

In this work we have examined whether MDV and HVT share any common physical and genetic properties and whether the loss of activity in the vaccine HVT strain results from structural heterogeneity of the HVT genome. Many physicochemical studies on MDV and HVT DNAs have been reported (Lee et al. 1969, 1971, 1972; Bachenheimer et al. 1972; Kaaden & Dietzschold, 1972; Kaaden et al. 1977), but most of these studies were analytical, rather than comparative. In this work we have attempted to detect differences between MDV and HVT DNAs.

It has been reported that RNA complementary to HVT DNA, formed in vitro by Escherichia coli RNA polymerase, hybridized well with the DNA from MDV infected cells, indicating the presence of homologous DNA sequences in HVT and MDV (Nazerian et al. 1973). In the present work we have examined the extent of homology between the DNAs of MDV and HVT and the structural heterogeneities of these two viruses by DNA–DNA
reassociation kinetics and DNA–RNA hybridization, and examination of restriction endonuclease-cleavage patterns.

METHODS

Virus and cells. Two MDV strains, GA (Eidson & Schmittel, 1968) and C2 (Kato et al. 1970), and two HVT strains, 01 (Ono et al. 1974) and FC-126 (Witter et al. 1970), were propagated in primary chick embryo fibroblasts (CEF) and were used for analysis of DNAs. CEF was grown in minimum essential medium (MEM) containing 5% calf serum and 5% tryptose phosphate broth (TPB). The HVT-01 strain that has a history of more than 60 serial passages in culture and has lost the ability to protect chickens against Marek's disease, was designated HVT-HP. The HVT-01 strain used as a vaccine strain was named HVT-VA. The HVT-VA strain used here has been passaged six times in duck embryo fibroblasts (DEF), eight times in quail embryo fibroblasts (QEF) and five to seven times in CEF, while the HVT-HP strain used has been passaged six times in DEF, 58 times in QEF and 1 to 12 times in CEF.

Infection with cell-associated viruses. Primary cultures of CEF cells grown in roller bottles or plastic Petri dishes were inoculated with MDV or HVT infected CEF cells to give 10 to 15% of MDV- or HVT-specific antigen positive cells at the time of inoculation. From 2 h p.i. the infected cells were cultured in MEM containing 2% calf serum and 5% TPB at 37 °C.

Labelling of cells with 3H-thymidine and 32P. 3H-thymidine (10 μCi/ml, New England Nuclear Corp., Boston, U.S.A.) was added to the culture 2 h p.i. with cell-associated MDV or HVT. At 24 h p.i. 10 μCi/ml of 3H-thymidine was again added to the culture. For labelling virus DNA with 32P, the virus-infected cells at 3 h p.i. were incubated in phosphate-free MEM containing 2%, foetal calf serum and 20 μCi/ml of 32P (Japan Radioisotope Association, Tokyo) and at 24 h p.i. 20 μCi/ml of 32P were again added to the cultures. The cells were harvested when 80 to 100% of the cells showed c.p.e.

Isolation of detergent-treated virions. The MDV or HVT infected CEF cells were scraped off with a rubber policeman and suspended in RSB buffer (0.01 M-tris-HCl, pH 7.4, 0.01 M-NaCl, 0.0015 M-MgCl2) containing 1% Nonidet P-40 (NP40). The suspension was kept in ice for 30 min and then the cells were disrupted by pipetting them up and down several times and centrifuging at 1000 g for 10 min. The supernatant cytoplasmic fractions were placed on two layers of sucrose, consisting of equal vol. of 60% and 40% (w/w) sucrose in 0.1 M-tris-HCl, pH 7.4, 0.15 M-NaCl (virus buffer). The tubes were centrifuged in a Beckman SW41 rotor at 175000 g for 2 h or in a Beckman SW25.2 rotor at 60000 g for 2 h at 4 °C. The detergent-treated virus formed a band at the interface of the two layers of sucrose and was collected, diluted with the virus buffer and centrifuged in an SW41 rotor. The pellet was used for DNA extraction.

Purification of virus DNA by sedimentation in neutral glycerol gradients. The virus pellets were lysed in 0.1 M-tris-HCl, pH 9.0, 0.1 M-NaCl, 0.001 M-EDTA, 1% SDS at 37 °C overnight with 1 mg/ml of Pronase. The lysates were layered on linear gradients of 10 to 30% glycerol in 0.01 M-tris-HCl, pH 7.4, 0.01 M-EDTA, 1% Sarkosinate (Nikkol, provided by Nikko Chemicals Corp., Tokyo) and centrifuged in a Beckman SW41 rotor at 175000 g for 4 h at 18 °C. Fractions were collected from the bottom of the gradients and the radioactivity in each fraction precipitated by trichloroacetic acid (TCA) was determined to locate the virus DNA as shown in Fig. 1. The virus DNA was collected by alcohol precipitation, usually co-precipitated with λDNA.

Alkaline sucrose gradients. The 32P-labelled MDV or HVT DNA isolated from the neutral glycerol gradients was mixed with 3H-labelled T4 DNA and layered on a linear alkaline gradient of 10 to 30% sucrose containing 0.3 M-NaOH, 0.001 M-EDTA, 0.1% Sarkosinate.
The gradient was centrifuged at 175,000 g for 4 h in a Beckman SW41 rotor at 18 °C. Then fractions were collected from the bottom and the radioactivity of the DNA precipitated with TCA was determined.

CsCl density gradient equilibrium centrifugation. DNA in 3.5 ml of 0.001 M-EDTA was mixed with 4.3 g of solid CsCl and centrifuged at 105,400 g in a Hitachi RP40T-2 rotor for 68 h at 20 °C. The radioactivity of DNA precipitated with TCA was determined.

Detection of MDV and HVT sequences by DNA-RNA hybridization. The cell DNA used for hybridization was extracted from cells as described previously (Hirai et al. 1977). Single-stranded DNA immobilized on membrane filters was hybridized with MDV and HVT 3H-complementary RNA (cRNA) made in vitro by Escherichia coli DNA-dependent RNA polymerase as described previously (Hirai et al. 1977). The virus DNA used here as a template was purified from detergent-treated virions by velocity sedimentation in a neutral glycerol gradient and then isopycnic centrifugation in CsCl, as described above.

Nick translation. Labelling of virus DNA with 3H-TTP in vitro was done as described by Nonoyama & Pagano (1973). The sp. act. of MDV and HVT 3H-DNA were 4.8 × 10^6 and 3.9 × 10^6 ct/min/μg, respectively. The virus DNA was purified from the detergent-treated virions by velocity sedimentation in neutral glycerol gradient and then isopycnic centrifugation in CsCl, as described above.

DNA-DNA reassociation kinetics. 3H-labelled virus DNA was mixed with sonicated virus or cell DNA in 0.5 ml of 0.3 N-NaOH, boiled for 15 min and neutralized with HCl. Then 5 m-NaCl was added to a final concentration of 2 m in 1 ml of the mixture and samples were incubated at 67 °C. At suitable times during incubation, 0.1 ml samples were removed and stored at −20 °C. Later they were assayed by single-strand specific nuclease digestion (nuclease S1; Seikagaku Kogyo Co., Tokyo) to determine the amount of reassociated 3H-labelled virus DNA. The percentage of labelled double-stranded DNA was determined from the ratio of the amounts of TCA-precipitable label in the control and enzyme-digested samples. The following equation was used for analysis of the data: (C_0/C)^10^-42 = 1 + kC_0t, where C and C_0 are the concentrations of single-stranded 3H-virus DNA at times t and t=0, and k is the reassociation constant (Gubbins et al. 1977).

Restriction endonuclease digestion. Fifty μl of MDV or HVT DNA in 0.05 M-tris-HCl, pH 7.4, 0.05 M-NaCl, 0.006 M-MgCl_2, 0.006 M-2-mercaptoethanol, containing 1 to 2 μg DNA was digested with 10 to 20 units of EcoRI or HindIII restriction endonuclease (Miles Laboratories Inc., Elkhart, Indiana, U.S.A.) at 37 °C for 2.5 h. The reaction was stopped by adding 5 μl of 60% sucrose in 0.01 m-tris-HCl, pH 7.4, 0.01 m-EDTA containing 0.1% bromophenol blue.

Agarose gel electrophoresis. About 60 μl of the digested DNA was subjected to electrophoresis on a 0.5% horizontal agarose gel at 60 V for about 20 h at 22 °C as described by Lee et al. (1977). After electrophoresis of 32P-labelled DNA, the gels were dried under vacuum and autoradiographed with Sakura X-ray film.

RESULTS

Isolation of MDV and HVT DNA by velocity sedimentation in neutral glycerol gradients

The detergent-treated virions labelled with 3H-thymidine or 32P were isolated from the cytoplasm of cells infected with MDV or HVT as described in the Methods. DNA was extracted from the virions and the virus DNA was isolated by velocity sedimentation in neutral glycerol gradients using T4 DNA as a sedimentation marker (Fig. 1).

The HVT DNA from the variants HP and VA of HVT sedimented just behind T4 DNA (mean mol. wt. 110 × 10^6; Freifelder, 1970), while the MDV DNA from strains GA and C2 sedimented like T4 DNA. Therefore, the mol. wt. of MDV DNA was concluded to be
Fig. 1. Neutral glycerol gradient sedimentation of MDV and HVT DNAs. MDV or HVT infected CEF cells were labelled with 20 µCi/ml ^32^P or 10 µCi/ml ^3H^-thymidine at 3 and 24 h p.i. The detergent-treated virions were isolated from the cytoplasm of the infected cells when the cultures showed 80 to 100% c.p.e. The SDS-Pronase lysates containing MDV or HVT DNA were layered on a linear gradient of 10 to 30% glycerol containing 0.01 M-tris-HCl, pH 7.4, 0.01 M-EDTA, 1% Sarkosinate as described in the Methods. Centrifugation was carried out at 175,000 g and 18°C for 4 h in a Beckman SW41 rotor. Sedimentation was from right to left. In the case of co-sedimentation with T4 DNA, T4 phage virions, labelled with ^3H^-thymidine, were mixed with the SDS-Pronase lysate containing ^32^P-labelled MDV or HVT virions and incubated at 37°C overnight before they were layered on the gradients. (a), ^3H^-HVT-HP DNA; the arrow on the graph indicates the position of the T4 DNA marker, which was centrifuged in a separate tube. (b) • — •, ^32^P-HVT-VA; ○ — ○, ^3H^-T4 DNA; (c) • — •, ^32^P-MDV-C2 DNA; (d) • — •, ^32^P-MDV-GA DNA; ○ — ○, ^3H^-T4 DNA. The radioactivities of ^32^P and ^3H in the fractions from about 15 to the top fraction are not shown on the graphs of (b), (c) and (d).

about 1.1 × 10^8, which is similar to the value reported previously (1.03 × 10^6; Bachenheimer et al. 1972), and to be slightly larger than that of HVT DNA. The result was confirmed by co-sedimentation of ^3H^-HVT-VA and ^32^P-MDV-C2 DNAs in the same gradient (not shown). The position of HVT-HP in the gradient was identical with that of HVT-VA, indicating that the DNA of strain 01 did not change significantly in size during multiple passages in culture (Fig. 1a, b). The radioactive peaks in fraction 5 to 8 in Fig. 1 were collected to examine the properties of the virus DNA.
Fig. 2. CsCl density gradient equilibrium centrifugation of MDV and HVT DNAs. Equilibrium sedimentation in caesium chloride of MDV and HVT DNAs was performed in a Hitachi RPsOT-2 rotor for 68 h at 105,400 g and 20 °C. Fractions were collected from the bottom (left of each graph). The bottom 30 and top 55 fractions are not shown. ●—●, 32P-DNA; ○——○, 3H-DNA. (a) 32P-MDV-GA DNA and 3H-MDV-C2 DNA; (b) 32P-HVT-VA DNA and 3H-HVT-HP DNA; (c) 32P-MDV-GA DNA and 3H-HVT-VA DNA; (d) 32P-MDV-C2 DNA and 3H-HVT-VA DNA; (e) 32P-HVT-VA DNA and 3H-CEF cell DNA; (f) 32P-HSV-HF DNA and 3H-HVT-VA DNA.

Sedimentation of HVT DNA in an alkaline sucrose gradient

Lee et al. (1971) reported that MDV DNA is double-stranded with random single-stranded breaks. When 32P-HVT-VA DNA was co-sedimented with 3H-T4 DNA in an alkaline sucrose gradient (not shown), the HVT DNA gave a heterogeneous pattern between 26 and 65S while T4 DNA gave a single band of 73S (Studier, 1965). These results indicate that HVT DNA is double-stranded and contains single-strand breaks, like those found in MDV DNA (Lee et al. 1971).

Isopycnic centrifugation of MDV DNA with HVT DNA in CsCl solution

The buoyant density values of MDV and HVT DNAs in CsCl solution were reported to be within the narrow range of 1.705 to 1.707 g/ml (Lee et al. 1971, 1972; Bachenheimer et al. 1972; Kaaden et al. 1977). However, since these reported values were not obtained
Fig. 3. Restriction endonuclease-cleavage patterns of MDV and HVT DNAs. MDV or HVT DNA labelled with $^{32}$P was isolated by sedimentation in a neutral glycerol gradient and was digested with EcoRI (b and c) or HindIII (d and e), or EcoRI and Hind III (f and g), and the products were separated by electrophoresis in a 0.5% agarose gel. The gel was dried and autoradiographed. (a) The EcoRI-digests of $^{32}$P-HSV-I (HF) DNA were used to estimate the mol. wt. of the digestion products. The mol. wt. scale is plotted on the right side of this figure. (b) EcoRI-digest of MDV-C2 DNA. (c) EcoRI-digest of HVT-VA DNA. (d) HindIII-digest of MDV-C2 DNA. (e) HindIII-digest of HVT-VA DNA. (f) EcoRI and HindIII-digest of MDV-C2 DNA. (g) EcoRI and HindIII-digest of HVT-VA DNA. DNA bands were designated by number.

by co-centrifugation of the two virus DNAs in a CsCl density gradient, it is uncertain which DNA shows the higher buoyant density.

Therefore, we compared the densities of the DNAs of MDV (GA and C2) and HVT (VA and HP) by co-centrifugation of $^3$H- and $^{32}$P-labelled virus DNAs (Fig. 2). No
MDV and HVT DNAs

significant difference was found between the densities of MDV-GA and -C2 DNAs nor between those of HVT-VA and -HP DNAs (Fig. 2a, b). However, it is clear from Fig. 2(c) and (d) that MDV DNA is slightly less dense than HVT DNA; the buoyant densities of MDV and HVT DNAs were found to be 1.705 and 1.707 g/ml, respectively, when HSV (HF strain, 1.725 g/ml; Hirai, 1979) and CEF cell (1.7008 g/ml, Sober, 1970) DNAs were used as density markers (Fig. 2e, f).

Restriction endonuclease cleavage of MDV and HVT DNAs

The 32P-labelled virus DNA isolated by velocity sedimentation in neutral glycerol gradients (Fig. 1) was mixed with λDNA and digested with either EcoRI or HindIII restriction endonuclease and then subjected to electrophoresis in 0.5% agarose gels containing ethidium bromide. The gel was examined under u.v. light to confirm that digestion of λDNA was complete. The digestion patterns shown in Fig. 3 and 4 were obtained when the reaction was complete. The EcoRI-digests of 32P-HSV type I (HF strain) DNA were also subjected to electrophoresis in the same gel to estimate the mol. wt. of the digestion products (Fig. 36).

Comparison of the cleavage patterns of MDV and HVT DNAs

Fig. 3 shows autoradiographs of the cleavage patterns of MDV and HVT DNAs with either EcoRI or HindIII. All the digestion products ranged from 0.5 × 106 to 15 × 106 mol. wt., except for one large fragment with a mol. wt. of about 24 × 106 in HindIII-digested HVT DNAs. The visible bands on the autoradiograph are designated by number according to their mol. wt. (Fig. 3). Comparison of the cleavage patterns of MDV-C2 and HVT-VA DNAs with either EcoRI or HindIII revealed that most of the digestion products of MDV-C2 DNA did not co-migrate with those of HVT-VA DNA (Fig. 3). However, the two EcoRI fragment bands (no. 1 and 2) of MDV-C2 appear to be identical in mobility with the two bands (no. 2 and 3) of HVT DNA digestion products (Fig. 3b, c). Among the products of HindIII, five co-migrating bands were observed in the region corresponding to 1.5 × 106 to 1.9 × 106 mol. wt.: no. 10 to 14 for MDV-C2 and no. 17 to 21 for HVT-VA (Fig. 3d, e). When the virus DNA was digested with the two endonucleases, EcoRI and HindIII, it was also found that most of the fragments of MDV-C2 and HVT-VA DNA, especially those with mol. wt. of more than 2 × 106, did not co-migrate (Fig. 3f, g). These cleavage patterns were not changed by further addition of the restriction endonucleases.

The cleavage patterns of MDV and HVT DNAs showed the presence of strong and faint bands, as found in the cleavage products of HSV DNA (Fig. 36). The mol. wt. and molar amounts of DNA in each fragment band were estimated from this autoradiograph (Table 1). Both MDV and HVT DNA fragments produced by EcoRI were generated in various molar amounts. Major fragments were generated in 1 M or more than 2 M amounts, and minor fragments were generated in submolar amounts. Several fragments of HVT-VA produced by EcoRI (Band no. 7 to 9) were present in less than 0.1 M quantities. Total mol. wt. of both virus DNA fragments were determined from relative molar ratios and mol. wt. of these virus DNA fragments. The total mol. wt. for the EcoRI-digests of MDV-C2 and HVT-VA DNAs were approx. 108.

Comparison of the cleavage patterns of EcoRI and HindIII-digested DNAs of the two MDV strains C2 and GA

The patterns of the fragments of MDV DNA cleaved by EcoRI (Fig. 4a, b) and HindIII (Fig. 4c, d) show that those of MDV-C2 DNA contained almost all the fragments of MDV-GA obtained with EcoRI or HindIII. The unique DNA fragments of MDV-C2 are indicated by arrows in Fig. 4. A minor band with a mol. wt. of 7 × 106 among the EcoRI-products,
Table 1. Estimation of mol. wt. and approximate molar ratio of DNA fragments in EcoRI digests of MDV and HVT DNAs

<table>
<thead>
<tr>
<th>Band no.</th>
<th>MDV-C2</th>
<th>HVT-VA</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mol. wt.</td>
<td>Relative molar ratios</td>
</tr>
<tr>
<td>1</td>
<td>$1.0 \times 10^{-2}$</td>
<td>0.25</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>7.3</td>
<td>1.0</td>
</tr>
<tr>
<td>4</td>
<td>7.0</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td>6</td>
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<tr>
<td>25</td>
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* Mol. wt. was estimated using a standard curve of electrophoretic mobility in agarose gels. Mol. wt. was plotted on a logarithmic scale using the EcoRI fragments of HSV-1 (HF) DNA as a reference species (K. Hirai, unpublished data).

† Using a microdensitometer, the relative molar ratio was estimated from the percentage of the average area per peak virus and the mol. wt. In those instances where the relative molar ratio was clearly in excess of 1, the fragment was considered to have two (or more) components of equal mol. wt.

indicated by an arrow, is likely to be a partial digest. Therefore, there appears to be only one unique fragment in C2 with both enzymes.

Comparison of digestion products of DNAs of two HVT-01 strains, VA and HP

No obvious differences were detected in the cleavage patterns of HVT-VA and -HP DNAs with HindIII or HindIII and EcoRI (Fig. 4e, f, and h, i). However, it should be noticed that the fragments 2 and 3 to 5 in Fig. 4(f) are different from those in Fig. 4(e). These minor differences may reflect defective DNA. Comparison of the cleavage patterns of the DNAs of the FC-126 and 01 strains showed that most of the HindIII-cleavage fragments of HVT-01 DNA co-migrated with those of HVT-FC-126 DNA.

Hybridization of MDV and HVT 3H-cRNA with DNA from MDV or HVT-infected CEF cells

Nazerian et al. (1973) reported that cRNA to HVT DNA hybridized with DNA from MDV-infected cultures, indicating the presence of homologous DNA sequences in MDV and HVT DNAs. We have confirmed their results (Table 2). HVT 3H-cRNA hybridized well with DNA from HVT-infected CEF cells. MDV 3H-cRNA also hybridized with DNA from HVT-infected CEF cells. The radioactivities of 3H-cRNA hybridized with DNA from
virus-infected CEF cells were definitely higher than the values with DNA from control (uninfected) cells. Therefore, there is a slight degree of homology between MDV and HVT DNAs. However, the extent of homology between the DNAs of MDV and HVT cannot be calculated from these data, since we do not know whether a specific portion of these virus genomes is transcribed by *Escherichia coli* RNA polymerase.
Table 2. Hybridization of MDV-C2 and HVT-HP ³H-cRNA with DNA from control, MDV-C2 or HVT-HP infected CEF cells

<table>
<thead>
<tr>
<th>Immobilized DNA on filter</th>
<th>Amount (µg)</th>
<th>MDV ³H-cRNA* hybridized (ct/min)</th>
<th>HVT ³H-cRNA* hybridized (ct/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEF cells</td>
<td>5.0</td>
<td>306, 294†</td>
<td>256, 234</td>
</tr>
<tr>
<td>MDV-C2 infected cells</td>
<td>5.0</td>
<td>11981, 12746</td>
<td>651, 703</td>
</tr>
<tr>
<td>HVT-HP infected cells</td>
<td>5.0</td>
<td>1439, 1098</td>
<td>10227, 9859</td>
</tr>
</tbody>
</table>

* Two x 10⁶ ct/min in 1 ml of incubation mixture.
† Results of two independent experiments.

Fig. 5 Reassociation kinetics between MDV and HVT DNAs. 0.05 µg MDV or HVT DNA (4.8 x 10⁶ and 3.9 x 10⁶ ct/min/µg, respectively), prepared in vitro by nick translation, was mixed with sonicated unlabelled virus or cellular DNA. The DNA–DNA reassociation kinetics were examined as described in Methods. (a) ³H-labelled MDV-C2 DNA was mixed with the following DNAs: ○—○, 1 µg of MDV-C2 DNA and 1 mg of salmon sperm DNA; •—•, 1 µg HVT-HP DNA and 1 mg of salmon sperm DNA; △—△, 200 µg of uninfected CEF cell DNA and 800 µg of salmon sperm DNA. (b) ³H-labelled HVT-HP DNA was mixed with the following DNAs: •—•, 1 µg HVT-VA DNA and 1 mg of salmon sperm DNA; ○—○, 1 µg MDV-C2 DNA and 1 mg of salmon sperm DNA; △—△, 200 µg of uninfected CEF cell DNA and 800 µg of salmon sperm DNA.
Reassociation kinetics between MDV and HVT DNAs

The reassociation kinetics between MDV and HVT DNAs were examined to determine the extent of DNA sequence homology between these two viruses (Fig. 5). The reassociation of ³H-MDV-C2 DNA was not increased by the addition of HVT-DNA (Fig. 5a). The presence of unlabelled MDV-C2 did not accelerate the reassociation of ³H-HVT-HP DNA in the reaction, while the addition of unlabelled HVT-VA DNA to the reaction did accelerate the reassociation (Fig. 5b). These results indicate that MDV does not have detectable DNA homology with HVT. However, it must be mentioned that if the homology between the two virus DNA's is less than 5%, the initial reassociation could not be detected under the conditions used here. Since slight homology could be detected between the DNAs of MDV and HVT by DNA-RNA hybridization (Table 2), it appears that these two virus DNAs share detectable homology within 5%.

DISCUSSION

In this report we have examined the possible relationship between the genomes of two avian herpesvirus, MDV and HVT. The genome of MDV was found to be slightly larger than that of HVT and the mol. wt. of MDV DNA was estimated as about $110 \times 10^8$. The buoyant density (1.705 g/ml) of MDV DNA in a CsCl gradient was slightly less than that (1.707 g/ml) of HVT DNA, as reported previously (Lee et al. 1972). Sedimentation of HVT DNA in an alkaline sucrose gradient indicates that HVT DNA is a linear double-stranded molecule with single-strand breaks, as reported for MDV DNA (Lee et al. 1971) and other herpesviruses (Kieff et al. 1971; Nonoyama & Pagano, 1972; Iltis et al. 1977).

Electrophoresis of digestion products of MDV and HVT DNAs with HindIII and EcoRI revealed the presence of major and minor bands. Some of the fragments of both virus DNAs are present in greater than molar quantities or in submolar quantities. This may indicate the presence of repeated and inverted sequences in MDV and HVT DNA, as found in the HSV genome (Hayward et al. 1975; Clements et al. 1976). Molar ratio differences among fragments were also found in restriction endonuclease digests of the DNAs of cytomegalovirus (Kilpatrick et al. 1976), EBV (Sugden et al. 1976) and varicella-zoster virus (Oakes et al. 1977). However the MDV and HVT used for infection here were not plaque-purified isolates. In addition, the two viruses were passed as cell-associated virus without any information about multiplicity, because otherwise it was not possible to isolate any detectable virus DNA. Since the defective DNA of HSV, generated by virus passaged at a high m.o.i., has been shown to consist of tandem repetitions of a specific region of the HSV genome (Frenkel et al. 1975), the presence of major and minor bands, formed by digestion of MDV and HVT DNA with restriction endonuclease can be explained by either defective or repetitive virus DNA. The restriction endonuclease-cleavage patterns of MDV and HVT, respectively, of different strains were almost identical. Therefore, the patterns can be used for identification of these herpesvirus DNAs.

The difference in restriction endonuclease-cleavage patterns between two virus DNAs may not indicate the lack of DNA sequence homology between these two viruses. Therefore, the extent of homology between two virus DNAs was demonstrated by DNA-DNA reassociation kinetics and DNA-RNA hybridization of MDV and HVT (Fig. 5 and Table 2). These results indicate that these two virus DNAs share detectable homology within 5%. Since the mol. wt. of MDV DNA is about $10^8$, 2% of the virus genome, for example, is enough to code for two to three proteins with an average mol. wt. of $5 \times 10^4$. However, it is premature at present to speculate about whether these proteins are responsible for the success of vaccination with HVT against Marek's disease. The homologous sequences must be analysed by the Southern blotting hybridization technique (Southern, 1975) to examine which portion of the virus genome contains the homologous sequences.
When the vaccine strain, 01 of HVT, was passed more than 60 times in DEF, QEF and CEF, it was found to be deficient in the ability to protect chickens against Marek's disease (Konobe et al. 1972). The DNA of the HVT variant, HVT-HP, was not distinguishable from that of the original vaccine 01 strain, HVT-VA, in its sedimentation behaviour in neutral glycerol and alkaline sucrose gradients or in its buoyant density in a CsCl density gradient. Moreover, no significant difference was found in the restriction enzyme-cleavage patterns of the DNAs of the two HVT-01 variants, VA and HP. Therefore, it seems that the reason for the loss of activity of HVT-HP as a vaccine strain cannot be explained by the increase in amount of defective DNA in HVT DNA molecules during serial passage in an analogous way to the HSV DNA generated during undiluted passage. However, the minor difference in restriction enzyme-cleavage patterns in Fig. 4(e) and (f) may indicate that there are some differences in homogeneity between the two variants of HVT-01 strains. Witter (1977) has also reported that when passed about 70 times in CEF and DEF, HVT strain FC-126 lost its ability to protect chickens against Marek's disease. He showed that the FC-126 variant of HVT, as well as the attenuated JM strain of MDV, appeared to be temperature sensitive in replication at 41 °C and normal at 37 °C. He also suggested that this failure to replicate at 41 °C may be the explanation for the loss of protective ability of vaccines, since strong in vivo replicative ability may be necessary for induction of immunity to Marek's disease (Witter & Offenbecker, 1979). It is possible that the variation occurred as the result of a minor change in the virus genome, such as a point mutation, which could not be detected by the technique used in the present work. Possible genetic differences of the variants of HVT in cells infected with these viruses require further investigation.

During preparation of this manuscript, Dr Nonoyama kindly told us that his group are also studying the homology between MDV and HVT DNA, labelled in vitro by nick translation using *Escherichia coli* DNA polymerase (Lee et al. 1979). They have found no detectable homology between MDV and HVT DNA by DNA–DNA reassociation kinetics, but have detected 1 to 4% sequence homology between these two virus DNAs by the more sensitive Southern blotting hybridization technique (Southern, 1975). After submitting this manuscript, it was learned that Kaschka-Dierich et al. (1978) had reported experiments on the relatedness of MDV and HVT DNAs. They found no detectable homology between these two virus DNAs using DNA–RNA hybridization.

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MDV and HVT DNAs


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