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Extensive homology exists between Marek disease herpesvirus and its vaccine virus, herpesvirus of turkeys

(malignant lymphoma/genomic library/DNA homology/hybridization stringency)

CAROL P. GIBBS*†, KEYVAN NAZERIAN‡§, LELAND F. VELICER*, AND HSING-JIEN KUNG†

*Department of Microbiology and Public Health, and †Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824; and ‡U.S. Department of Agriculture Regional Poultry Research Laboratory, East Lansing, MI 48823

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ABSTRACT **Marek disease is a lymphomatous disease of chickens caused by infection of a herpesvirus, Marek disease virus (MDV). Marek disease is the only neoplastic disease for which a successful vaccine has been developed. The vaccine virus, herpesvirus of turkeys (HVT), is non-oncogenic in chickens. Despite the strong antigenic relationship between these viruses, previous studies showed that the two viral DNAs share little or no homology. Using less stringent hybridization conditions and methods that greatly improve the reassociation kinetics, we have reexamined the sequence homology between MDV and HVT DNA. We report here that HVT and MDV are far more closely related than previously reported. The homology between these two viral DNAs ranges between 70% and 80% at the nucleotide level and appears to extend over 90–95% of the respective genomes. Under the low stringency conditions used, MDV DNA fails to cross-hybridize with DNA from feline rhinotracheitis virus, an antigenically unrelated herpesvirus with a G-C content identical to that of MDV and HVT.**

Marek disease virus (MDV) is a herpesvirus of chickens that is the cause of a naturally occurring malignant lymphoma of T-cell origin. MDV is an extremely contagious virus that is spread horizontally and has been responsible for major economic losses. The biology of this virus has recently been reviewed by Calnek (1) and Nazerian (2).

An antigenically related virus, herpesvirus of turkeys (HVT), has been used as an effective vaccine. This naturally occurring turkey virus is apathogenic for both turkeys and chickens but elicits an immune response in chickens that protects against subsequent tumor development by MDV (1, 2). Thus, MDV is the first tumor virus for which a safe and effective vaccine has been developed and is routinely used.

We have been interested in studying antigens common to MDV and HVT, particularly those involved in the protective immune response. Several cross-reactive antigens have been identified and partially characterized (refs. 3–8; R. F. Silva and L. F. Lee, personal communication). The virus-specific protein profiles of cells infected with these two viruses, as analyzed by gel electrophoresis, appear quite similar. Nevertheless, earlier reports indicated that MDV and HVT share little (1–5%) (9, 10) or no (11) homology at the DNA level. This minor degree of homology seemed rather puzzling in light of the facts that a number of immunologically cross-reactive proteins are shared between the two viruses and HVT effectively protects chickens against MDV-induced lymphoma.

In the present study, highly sensitive methods were used to reexamine the degree of homology between the two viral genomes. Hybridization conditions used previously had been quite stringent, only allowing the detection of highly stable hybrids, and they were carried out to a C_0t value much lower than that required for completion of the hybridization reaction. Using relaxed hybridization conditions in conjunction with an enhanced reaction rate, we are now able to demonstrate a high degree of homology between MDV and HVT, extending over ≈90–95% of the respective viral genomes. These data suggest that the two viruses are closely related and can readily explain the similar protein profiles and close antigenic relationship observed for MDV and HVT.

MATERIALS AND METHODS

Cell Cultures and Viruses. MDV and HVT were propagated in duck embryo fibroblast (DEF) and chicken embryo fibroblast (CEF) cultures, respectively. Cloned purified GA (12) strain of MDV is highly pathogenic for chickens and causes lymphoid tumor in a variety of visceral organs. FC-126 isolate of HVT (13) is apathogenic for turkeys and chickens and protects well against GA-induced lymphomas.

Crude supernatant fluid from heavily infected roller bottle cultures was removed and centrifuged at 10,000 rpm for 10 min to remove cell debris. Viral nucleocapsids in the clarified supernatant fluids were then precipitated by addition of 50% polyethylene glycol (14), dissolved in buffer (0.15 M NaCl/20 mM Tris·HCl, pH 7.5), and purified twice by density gradient centrifugation in a 12–52% sucrose gradient in buffer at 27,000 rpm in an SW27 rotor of a Beckman 65B ultracentrifuge. The nucleocapsid band was collected and examined by electron microscopy for the presence of viral nucleocapsids.

Viral DNA. The nucleocapsids were adjusted to 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA, incubated with 50 μg of DNase (Sigma) per ml and 50 μg of RNase (Sigma) per ml at room temperature for 2 hr, disrupted by the addition of one-half of the volume of 3% Sarkosyl/75 mM Tris·HCl, pH 9.0/50 mM EDTA, and incubated at 55°C for 10 min. Pronase (Sigma) was added to 1 mg/ml and the samples were incubated at 37°C for 2 hr. DNA was extracted twice with phenol and once with chloroform and was precipitated with ethanol.

Cellular DNA. Total cellular DNA was isolated from uninfected DEF cultures and from cultures heavily infected with either MDV or HVT. Cells were suspended in phosphate-buffered-saline and incubated at 37°C for 60 min in 1% $NaDodSO_4$/0.5 mg of Pronase per ml (Sigma). DNA was extracted twice with phenol, extracted once with chloroform, and dialyzed against 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA.

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Abbreviations: MDV, Marek disease virus; HVT, herpesvirus of turkeys; FRV, feline rhinotracheitis virus; CEF, chicken embryo fibroblast(s); DEF, duck embryo fibroblast(s); kb, kilobase(s).

§To whom correspondence should be addressed.

Digestion, Electrophoresis, and Transfer of DNA. Restriction enzymes obtained from BRL and Promega BioTec were used according to the manufacturer's specifications. The DNA digests were electrophoresed through agarose and transferred to nitrocellulose (15).

Nick-Translation. Viral DNAs were nick-translated by using standard protocols (16). Labeled DNA was purified by chromatography through a Sepharose G-50 column.

Plasmid Library Construction. MDV viral DNA isolated from the purified nucleocapsids was digested with EcoRI and ligated to EcoRI-digested pBR328 that had been treated with calf intestine alkaline phosphatase (Boehringer Mannheim). HB101 host bacteria were transformed (16) and colonies with the correct drug resistance were selected. Clones containing MDV inserts were identified by *in situ* colony hybridization with nick-translated MDV viral DNA as probe. The MDV probe was prepared with DNA isolated from nucleocapsids obtained from the supernatant fluid of infected CEF to minimize the selection of clones with inserts derived from DEF DNA contaminating the nucleocapsid suspension. Approximately 45% of the colonies were positive for MDV sequences.

***In Situ* Colony Hybridization.** Sterile nitrocellulose filters were placed on minimal agar plates containing ampicillin and tetracycline. Colonies were picked with toothpicks and spotted onto duplicate filters. To serve as a hybridization control, bacteria harboring the pBR328 vector were spotted onto each filter. Plates were incubated at 37°C until colonies were ≈2 mm in diameter. DNA was liberated from the bacteria and bound to the nitrocellulose by standard protocols (16).

λ Screening. The MSB-I phage genomic library (a gift of J. Casey, Louisiana State University) was screened by using standard protocols (16). To unambiguously identify the MDV clones, two probes were used: the first was nick-translated MDV viral DNA and the second consisted of a pool of MDV-positive plasmid clones. A total of 14 clones containing MDV inserts was obtained and confirmed by Southern blot analysis.

Hybridizations and Washings. Hybridizations were performed under either conventional or relaxed conditions. Conventional conditions were used for colony screening with the MDV probe and for screening of the MSB-I phage library. Only relaxed conditions were used for cross-hybridizations. To increase the rate of association, dextran sulfate was incorporated into all hybridization buffers (17). Also, the reactions were performed in minimal volumes. These modifications allowed hybridizations to reach to $3\times$ $C_0t_{1/2}$ within 3–4 days.

Conventional hybridization conditions were 50% formamide, 5× concentrated Denhardt's solution (100× concentrated Denhardt's solution is 2% each in Ficoll, polyvinylpyrrolidone, and bovine serum albumin), 5× concentrated SSPE buffer (20× concentrated SSPE buffer is 3.6 M NaCl/200 mM NaH_2PO_4, pH 7.4/20 mM EDTA, pH 7.4), 0.1% $NaDodSO_4$, 100 μg of sheared, single-stranded salmon sperm DNA (Sigma) per ml, 100 μg of yeast RNA (Sigma) per ml, 10% dextran sulfate (Pharmacia), and incubation at 42°C.

The washing procedure consisted of three-10 min washes in 0.3 M NaCl/0.03 M sodium citrate, pH 7.0/0.1% $NaDodSO_4$ at room temperature followed by washing in 0.15 M NaCl/0.015 M sodium citrate/0.1% $NaDodSO_4$ at 68°C for 60 min.

Low stringency hybridizations were performed in a buffer consisting of 1 M NaCl, 10 mM Tris·HCl (pH 7.4), 25% formamide, 100 μg of sheared, single-stranded salmon sperm DNA per ml, 100 μg of yeast RNA per ml, and 10% dextran sulfate at 42°C. Washings were with 30 mM NaCl/3 mM sodium citrate/0.2% $NaDodSO_4$ at 36°C (low stringency), 50°C (medium stringency), or 64°C (high stringency). These conditions were calculated to allow 30%, 20%, or 10% base mismatching, respectively, according to the following formulas (18): $T_m = 81.5 + 16.6 (\log M) + 0.41 (\%\ G\text{-}C) - 0.72 (\%\ F)$ and $\%\ mm = (T_m - T_H)/1.4$, in which T_m = melting temperature, M = monovalent salt molarity, % G-C = percent guanine plus cytosine, % F = percent formamide, TH = hybridization temperature, and % mm = percent base mismatch.

RESULTS

Cross-Hybridization Between Viral DNAs. Under conventional hybridization conditions, reactions involving a DNA species with a complexity as high as the MDV or HVT genome [150–180 kilobases (kb)] follow a relatively slow reassociation kinetics: usually a lengthy incubation period is needed to complete such a reaction (16). Therefore, conditions were desired that would improve the kinetics as well as increase the sensitivity of the hybridizations. Conditions with different stringencies were tested, including dextran sulfate, a compound effective in enhancing hybridization kinetics by 10-fold (17), in the reaction mixture. As described below, these modifications improved the detection of moderately stable hybrids between the MDV and HVT genomes.

Initially, hybridization patterns of MDV viral DNA to the MDV or HVT genomes present in infected DEF cells, which carry multiple copies of the respective viral genome, were examined. A preparation of DNA from uninfected DEF was included as a negative control. The experimental conditions would allow the detection of hybrids with 30% or less base mismatch (low stringency) and enable the reaction to proceed to a value of $3\times$ $C_0t_{1/2}$ (16). Total cellular DNA was digested with *Eco*RI or *Hin*dIII, separated by agarose gel electrophoresis, and transferred to nitrocellulose paper. MDV viral DNA was nick-translated and used as a hybridization probe at low stringency. As shown in Fig. 1*A*, discrete bands corresponding to the MDV (lanes 1 and 2) or HVT (lanes 3 and 4) genome could readily be detected in DNA from the virus-infected cells. The banding patterns correspond to the restriction fragments in ethidium bromide-stained digests of viral DNAs (11) and are characteristic for the respective genome. Virtually all of the HVT fragments can be detected with the MDV probe, indicating that homologous sequences exist over a substantial portion of the genome. In contrast, only a faint smear was observed in DNA from the uninfected DEF (lanes 5 and 6), presumably due to cross-hybridization with contaminating CEF DNA sequences present in the probe: under more stringent conditions (see below), this smear was not detected (Fig. 1 *B* and *C*, lanes 5 and 6). These control experiments attest to the specificity of hybridization under our conditions.

To study the degree of homology between the MDV and HVT genomes, the filters of Fig. 1*A* were rewashed to allow 20% base mismatch (medium stringency). This resulted in an overall decrease in intensity of the HVT bands (Fig. 1*B*, lanes 3 and 4) relative to the intensity of the MDV bands (lanes 1 and 2). The filters were then washed under the high stringency conditions designed to allow 10% base mismatch (Fig. 1*C*). Bands in the HVT lanes (lanes 3 and 4) were extremely faint, whereas bands in the MDV lanes (lanes 1 and 2) remained intense. These data indicate that MDV and HVT hybrids are not stable under conditions that allow <10–20% base mismatch. The approximate overall degree of homology between these two genomes is therefore estimated to be between 70% and 80%.

The reciprocal cross-hybridization was also performed: nick-translated HVT viral DNA was hybridized to a Southern blot containing *Bam*HI-digested MDV viral DNA (Fig. 2). Results were in complete accord with those observed in Fig. 1. Under low stringency (lane A), strong cross-hybrid-

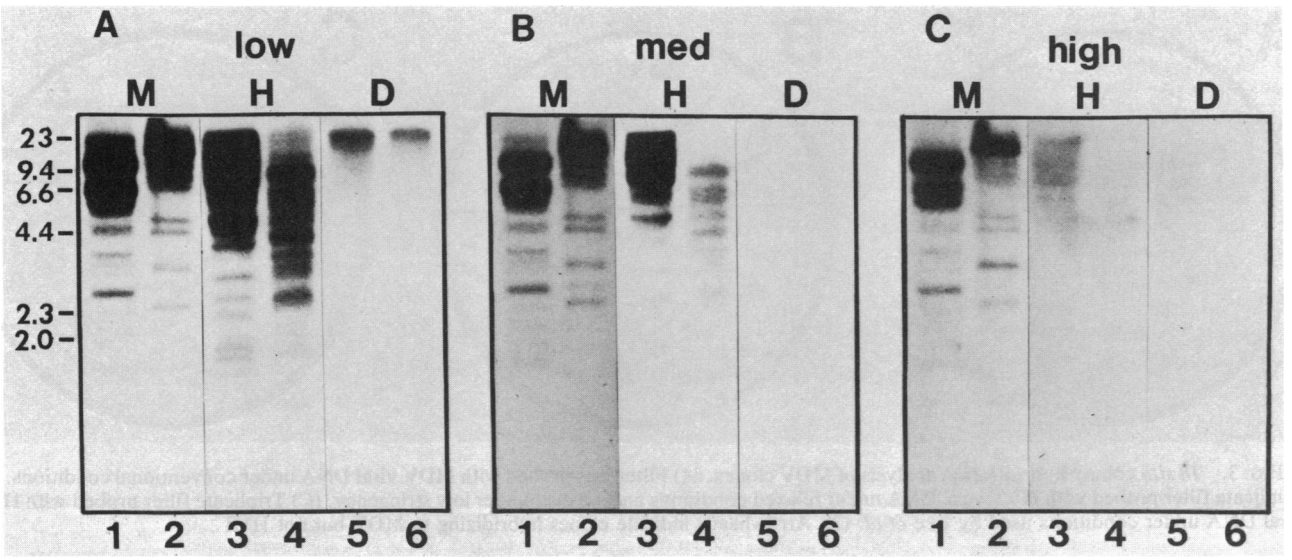

FIG. 1. Southern blots of total cellular DNA isolated from viral-infected or uninfected DEF probed with MDV viral DNA under relaxed conditions. M, MDV-infected DEF; H, HVT-infected DEF; D, uninfected DEF; DNAs were digested with *Eco*RI (lanes 1, 3, and 5) or *Hin*dIII (lanes 2, 4, and 6). Blots were washed under low stringency (*A*), medium stringency (*B*), or high stringency (*C*) conditions. The molecular size standards (kb) are derived from a *Hin*dIII digest of λ.

ization between HVT and MDV DNA was detected. Upon washing at medium stringency, the bands became fainter (lane B); with further washing under high stringency, bands could barely be detected (lane C). These data substantiate the cross-hybridization results of Fig. 1 and provide additional evidence for the extensive homology between HVT and MDV.

In this experiment, a negative control was also included to demonstrate the hybridization specificity. FRV is a herpesvirus with a G-C content identical to that of MDV (2, 19). FRV viral DNA was nick-translated and hybridized to a Southern blot of *Bam*HI-digested MDV viral DNA under the low stringency conditions used in Fig. 2, lane A (Fig. 2, lane D). No bands were apparent, even after long exposures. In contrast, when the same FRV probe was hybridized to digests of FRV viral DNA, the characteristic FRV bands were readily detected (Fig. 2, lane E). These results demonstrate the hybridization conditions used are indeed specific for homologous sequences.

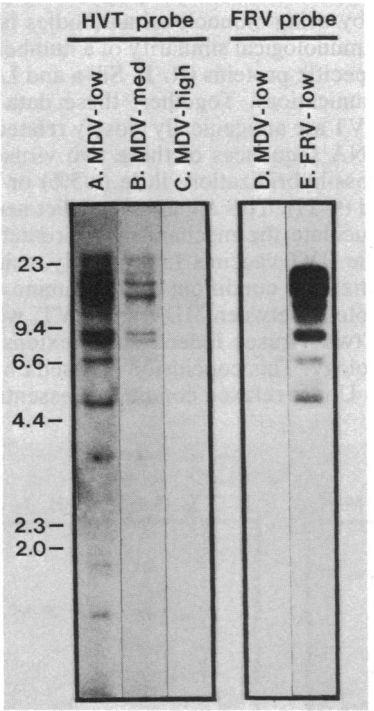

FIG. 2. Lanes A–C, *Bam*HI-digested MDV viral DNA probed with HVT viral DNA under relaxed conditions. The Southern blot was successively washed under the low, medium, and high stringency conditions used in Fig. 1. Lane D, *Bam*HI-digested MDV viral DNA, and lane E, *Eco*RI-digested feline rhinotracheitis virus (FRV) viral DNA probed with FRV viral DNA under the low stringency conditions. The molecular size standards (kb) are derived from a *Hin*dIII digest of λ.

Hybridization Studies Using Cloned MDV DNA Fragments. The above hybridization studies utilized viral genomic DNAs isolated from purified nucleocapsids and from infect ed cells. To firmly establish the apparent homology between MDV and HVT, individual cloned MDV DNA fragments were used as hybridization substrates. Two MDV genomic libraries were developed: a plasmid library was constructed by insertion of *Eco*RI fragments of MDV viral DNA into the pBR328 vector, and a phage library was obtained by ligation of *Mbo* I partially digested MSB-I cell DNA into the λL47 vector (16). MSB-I (20) is a chicken lymphoblastoid cell line derived from a splenic lymphoma of an MDV-infected chicken, with an average of 50 copies of the MDV genome per cell (14). Hybridization of HVT viral DNA to MDV clones isolated from these two genomic libraries confirmed the extensive homology between MDV and HVT. The studies with the plasmid library are described in more detail below.

In situ *colony hybridization*. MDV EcoRI fragments were ligated to the plasmid vector pBR328 and transformed into the host bacteria HB101. Initially, the colonies obtained after transformation were screened by *in situ* colony hybridization to an MDV viral DNA probe allowing identification of those plasmids bearing MDV inserts. Typical hybridization results are shown in Fig. 3*A*. Duplicate filters were hybridized to the HVT viral DNA probe under relaxed conditions. The HVT probe hybridized to the great majority of the MDV-positive clones (Fig. 3*B*); however, a few exceptions (indicated by arrowheads) were noted. More than 300 colonies were positive with the MDV probe: ≈95% of these clones were positive with the HVT probe. In no case did a clone hybridize to the HVT probe but not to the MDV probe. These data are consistent with the extensive homology be-

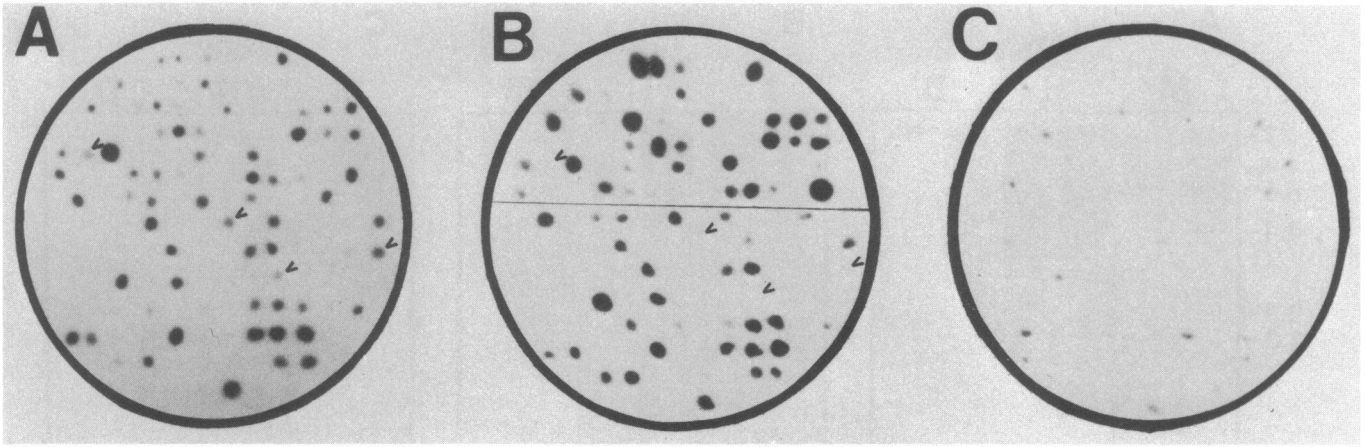

FIG. 3. *In situ* colony hybridization analysis of MDV clones. (*A*) Filter was probed with MDV viral DNA under conventional conditions. (*B*) Duplicate filter probed with HVT viral DNA under relaxed conditions and washed under low stringency. (*C*) Triplicate filter probed with HVT viral DNA under conditions used by Lee *et al.* (7). Arrowheads indicate clones hybridizing to MDV but not HVT.

tween the two viral genomes observed in Southern hybridizations.

In situ colony hybridization was also repeated with the HVT probe by using the more stringent conditions described by Lee *et al.* (9) (Fig. 3*C*). Hybridization signals were extremely weak, with a very low signal-to-noise ratio, and only a few clones scored positive. Nevertheless, those that did give a positive signal were also positive under our more sensitive conditions. The differences in the sensitivity of hybridization conditions help to account for the discrepancy between our principal conclusion and those of previous workers.

Southern hybridization. Individual MDV DNA fragments purified from the MDV-positive clones by *Eco*RI digestion and Southern blot hybridization were analyzed. A minimum of 25 different size classes was obtained, with inserts ranging from 0.3 to 15 kb. Fourteen representative clones are shown in Fig. 4*A* (clones L and M each contain 2 inserts). The DNA was blotted and hybridized to nick-translated MDV viral DNA (Fig. 4*B*). As expected, all inserts hybridized to this probe, confirming the MDV origin of the clones. Hybridization to the smaller band in lane M is not obvious in the photograph but can be seen in the original autoradiogram. When a duplicate blot was hybridized to the HVT probe under low stringency (30% base mismatch), 13 of the 16 inserts were positive (Fig. 4*C*). The pBR328 vector bands did not hybridize, indicating the specificity of the hybridizations. A large number of clones were analyzed; ≈90% of the inserts cross-hybridize with HVT DNA. These data clearly show that the general homology between HVT and MDV extends over a large portion of the genome. However, the presence of fragments that do not hybridize with HVT indicates that divergent regions do exist.

DISCUSSION

Marek disease, a herpesvirus-induced malignant lymphoma of chickens, is of great economic importance in the poultry industry and is also the only neoplastic disease for which an effective vaccine has been developed for routine use. The HVT vaccine is capable of eliciting a strong immune response that protects chickens against lymphoma induced by subsequent MDV infection. Also, two-dimensional gel analysis (6, 7) reveals that many of the MDV and HVT viral proteins are structurally indistinguishable and share common antigenic determinants. Recent analyses of proteins in infected cells by using monoclonal antibodies further demonstrate the immunological similarity of a number of MDV and HVT viral-specific proteins (R. F. Silva and L. F. Lee, personal communication). Together, these data indicate that MDV and HVT are antigenically closely related. In contrast, when the DNA sequences of these two viruses were compared by cross-hybridization, little (≤5%) or no homology was detected (9–11). This apparent conflict needed to be resolved to elucidate the mechanism of protective immunity elicited by the HVT vaccine. In this study, using highly sensitive hybridization conditions to reexamine the DNA sequence homology between MDV and HVT, we demonstrated that the two viruses indeed share extensive DNA sequence homology. This conclusion is based on the following evidence: (*i*) Under relaxed conditions, essentially all of the

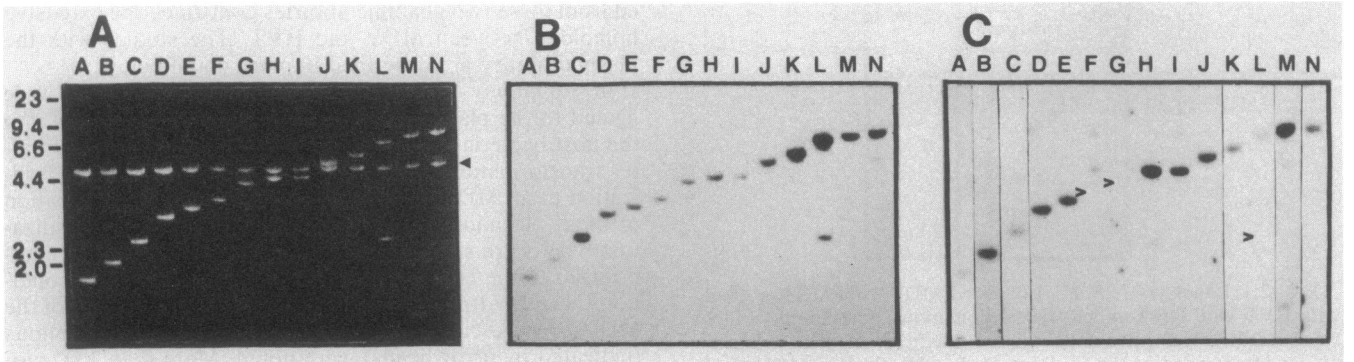

FIG. 4. Southern hybridization analysis of MDV clones. Plasmids containing MDV inserts were digested with *Eco*RI and electrophoresed through 0.7% agarose. (*A*) Ethidium bromide staining pattern. (*B*) Hybridization to MDV viral DNA. (*C*) Hybridization to HVT viral DNA under relaxed conditions. Clones L and M contain two inserts. The pBR328 vector band is indicated by ◂; inserts that do not hybridize to HVT are indicated by >. The molecular size standards (kb) are derived from a *Hin*dIII digest of λ.

restriction fragments of HVT viral DNA can be detected by radiolabeled MDV viral DNA (Fig. 1). The converse is also true: most, if not all, of the restriction fragments of MDV viral DNA hybridize to radiolabeled HVT viral DNA (Fig. 2); (*ii*) 95% of the MDV-positive clones in the plasmid library scored positive with the HVT DNA probe (Fig. 3); (*iii*) Southern analysis of the DNA inserts isolated from MDV clones revealed that >90% of clone-purified MDV fragments cross-hybridize with HVT DNA (Fig. 4; unpublished data).

The above data clearly show that MDV and HVT are closely related. Sequence homology extending over 90–95% of the viral genome can be readily detected under hybridization conditions that allow 30% mismatch. When the incubation conditions are more stringent, allowing only 10% or 20% mismatch, most of the MDV/HVT hybrids dissociate. Our calculations indicate that a 20–30% sequence divergence extending throughout the viral genomes exists between MDV and HVT DNAs. This pattern of divergence indicates that MDV and HVT originate from a common progenitor virus but have undergone independent evolutionary changes.

The lack of cross-hybridization between MDV and FRV, an antigenically unrelated herpesvirus with a G-C content identical to that of MDV and HVT, clearly demonstrates the specificity of the hybridization conditions. Since no cross-hybridization between MDV and FRV could be detected under the low stringency conditions used, the bands detected in the MDV/HVT cross-hybridizations are not the result of nonspecific hybridization under relaxed conditions.

Comparison of the sensitivity of our hybridization conditions with those used by Lee *et al.* (9), revealed a striking difference (Fig. 3), possibly influenced by two factors. First, our low stringency conditions detect hybrids with 30% mismatch, whereas their conditions only detect base pairs with <20% mismatch, and, as shown by us, most MDV/HVT hybrids are unstable under 20% mismatch conditions. Second, the use of dextran sulfate during hybridization enhances the DNA reassociation rate at least 10-fold. This allows the hybridization reaction to proceed to completion within 2–3 days, thereby minimizing the degradation of hybridization probes due to prolonged incubation. These improved hybridization conditions should be readily applicable to the analysis of the genetic relationships between other serotypes of MDV.

The data described in this report provide biochemical evidence that MDV and HVT are genetically and evolutionarily related, although significant divergence has apparently occurred between these viruses. The base changes appear to be scattered throughout the genomes because the restriction enzyme digestion patterns of these viruses are quite different. Nevertheless, based on our results, 70–80% of the nucleotide sequences are conserved. These results can readily account for the close immunological relationship between these viruses and the similarity of the virus-specific protein patterns in infected cells. This enhances our understanding of the efficacy of the HVT vaccine: the strong protective immunity may involve a multitude of antigenically related virus-specific proteins, far more than the few previously indicated from the initial reports of very limited genetic homology.

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1. Calnek, B. W. (1980) in *Oncogenic Herpesviruses*, ed. Rapp, F. (CRC, Boca Raton, FL), pp. 103–143.
2. Nazerian, K. (1980) in *Viral Oncology*, ed. Klein, G. (Raven, New York), pp. 665–682.
3. Glaubiger, C., Nazerian, K. & Velicer, L. F. (1983) *J. Virol.* **45**, 1228–1234.
4. Long, P. A., Clark, J. L. & Velicer, L. F. (1975) *J. Virol.* **15**, 1192–1201.
5. Ikuta, K., Ueda, S., Kato, S. & Hirai, K. (1983) *J. Gen. Virol.* **64**, 961–965.
6. Van Zaane, D., Brinkhoff, J. M. A., Westenbrink, F. & Gielkens, A. L. J. (1982) *Virology* **121**, 116–132.
7. Van Zaane, D., Brinkhoff, J. M. A. & Gielkens, A. L. J. (1982) *Virology* **121**, 133–146.
8. Velicer, L. F., Yager, D. R. & Clark, J. L. (1978) *J. Virol.* **27**, 205–217.
9. Lee, Y.-S., Tanaka, A., Silver, S., Smith, M. & Nonoyama, M. (1979) *Virology* **93**, 277–280.
10. Hirai, K., Ikuta, K. & Kato, S. (1979) *J. Gen. Virol.* **45**, 119–131.
11. Kaschka-Dierich, C., Bornkamm, G. W. & Thomssen, R. (1979) *Med. Microbiol. Immunol.* **165**, 223–239.
12. Purchase, H. G. (1969) *J. Virol.* **3**, 557–565.
13. Witter, R. L., Nazerian, K., Purchase, H. G. & Burgoyne, G. H. (1970) *Am. J. Vet. Res.* **31**, 525–538.
14. Nazerian, K. & Lee, L. F. (1974) *J. Gen. Virol.* **25**, 317–321.
15. Southern, E. (1975) *J. Mol. Biol.* **98**, 503–517.
16. Maniatis, T., Fritsch, E. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
17. Wahl, G. M., Stern, M. & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3683–3687.
18. Howley, P. M., Israel, M. A., Law, M.-F. & Martin, M. A. (1979) *J. Biol. Chem.* **254**, 4876–4883.
19. Wildy, P., Field, H. J. & Nash, A. A. (1982) in *The Society for General Microbiology, Symposium 33: Viral Persistence*, eds. Mahy, B. W., Minson, A. C. & Darby, G. K. (Cambridge Univ. Press, United Kingdom, pp. 136–137.
20. Akiyama, Y. & Kato, S. (1973) *Biken's J.* **16**, 177–179.