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Characterization of an Immunosuppressive Parvovirus Related to the Minute Virus of Mice

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We have characterized an immunosuppressive parvovirus related to the minute virus of mice (MVM). The parvovirus, MVM(i), grew efficiently on the murine lymphoma cell line EL-4 and not on the A-9 strain of L-cells which is a host for the prototype MVM. MVM(i) was immunosuppressive for allogeneic mixed leukocyte cultures, inhibiting the generation of cytolytic T lymphocytes. MVM had no effect on mixed leukocyte cultures. MVM and MVM(i) particles were similar in buoyant density, sedimentation rate, appearance in the electron microscope, and polypeptide composition. We present restriction enzyme maps of the DNAs of MVM and MVM(i) which show that they are closely related. Out of 109 restriction endonuclease cleavage sites (representing together about 10% of the nucleotide sequence), 86 sites were shared by MVM and MVM(i), whereas 22 sites were absent from one of the two viruses. MVM(i) DNA had an apparent deletion of about 60 nucleotides relative to MVM, located near the 5′ terminus of viral DNA.

The minute virus of mice (MVM) is a well-characterized member of the parvovirus group (28). The parvoviruses are small icoshedral viruses containing a single-stranded DNA molecule about 5 kilobases long with short hairpin structures at each end (2). The only virus-specified proteins identified so far are the structural proteins of the virions (25, 26). Autonomous parvoviruses require growing cells for their replication (24). MVM is normally grown on A-9, a murine fibroblast cell line (24). Evidence that a virus related to MVM was present in the culture fluid of a subline of the murine lymphoma EL-4(G-) was reported by Bonnard et al. (3). This agent was found to be immunosuppressive for allogeneic mixed leukocyte cultures.

Using virus purified from an EL-4(G-) cell supernatant by terminal dilution, we have isolated a parvovirus related to MVM which inhibits the generation of cytolytic T lymphocytes in mixed leukocyte cultures (H. Engers et al., manuscript in preparation). In contrast, the prototype MVM has not been found to inhibit mixed leukocyte cultures (3; Engers et al., in preparation). MVM and the variant MVM(i) are distinct in their specificity for the differentiated state of the host cell and in other biological properties. An interesting question is: what differences in the viral genomes account for these biological differences? We have compared the DNAs of MVM and MVM(i) by restriction endonuclease digestion and by heteroduplex analysis. The DNAs of MVM and MVM(i) are similar, but not identical.

MATERIALS AND METHODS

Materials. We obtained analytical grade phenol from E. Merck AG, Darmstadt, W. Germany. The phenol was stabilized with 0.1% 8-hydroxyquinoline, saturated with either water or 0.5 M NaCl and stored at 4°C. Agarose, either normal (no. A-6877, type II) or with a low gelling temperature (no. A-4018, type VII) was from Sigma Chemical Co. Restriction enzymes were from either New England Biolabs or Bethesda Research Laboratories, Inc. The enzymes were used as suggested by the suppliers. T4 polynucleotide kinase was purchased from P. L. Biochemicals. Pronase was obtained from Calbiochem, and S1 nuclease and bacterial alkaline phosphatase were kind gifts from U. Schibler and O. Hagenbüchle (this Institute), respectively. [α-^{32}P]dGTP (400 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) were from the Radiochemical Centre, Amersham, England.

Mixed leukocyte cultures and assay of cytolytic activity. Cytolytic T lymphocytes (C57BL/6 responding against DBA/2) were generated in mixed leukocyte cultures by methods previously described (5, 9). Various dilutions of virus (0.2 ml in Eagle medium) were added to the mixed leukocyte cultures (4 ml) upon initiation of culture. After 5 days the cytolytic activities were assayed using 10^4 ^{51}Cr-labeled DBA/2 tumor target cells (5, 9) in a 3-h assay.

Preparation of virus and viral DNA. MVM was grown on the A-9 variant of mouse L-cells (12). The initial virus inoculum was kindly provided by P. Tattersall (Yale University, New Haven, Conn.). MVM(i) was grown on EL-4, a lymphoma cell line (19). The

original MVM(i) virus was a gift of G. Bonnard (National Institutes of Health, Bethesda, Md.) and was subsequently purified by growth from terminal dilution by P. Tattersall. Three days after cells were infected at high multiplicity, MVM and MVM(i) virus particles were isolated on CsCl density gradients by the method of Tattersall et al. (25). Virus was lysed with 0.5% sodium dodecyl sulfate at 37°C for 1 h in the presence or absence of 0.5 mg of pronase (see below) per ml. This was followed by extraction with water-saturated phenol and ethanol precipitation of the DNA.

Isolation of RF viral DNA. Replicative-form (RF) DNAs were purified by a modified Hirt extraction (11). The volumes below are expressed per 52.8-cm^2 (8.2-cm-diameter) petri dish. We routinely processed 50 to 100 dishes. Medium was removed, and 1 ml of 10 mM Tris-hydrochloride–10 mM EDTA (pH 7.4)–0.6% sodium dodecyl sulfate was added. After 20 min the lysed cells were harvested into 40-ml Sorvall centrifuge tubes (25 dishes per tube). Pronase (20 mg/ml, 0.025 ml) was added, gently mixed, and allowed to digest for 2 h at 37°C. (The pronase was predigested at 37°C for 2 h in 20 mM Tris-hydrochloride–10 mM EDTA [pH 7.5].) Then 0.25 ml of 5 M NaCl was added, and the solution was gently mixed, left on ice for 1 h, mixed again, and kept on ice overnight. The tubes were centrifuged in a Sorvall SS-34 fixed-angle rotor at 15,000 rpm for 40 min at 4°C. The supernatant was removed, and DNA was precipitated with ethanol without prior extraction with phenol. The ethanol precipitate was vacuum dried and suspended in 0.1 ml of 50 mM Tris-hydrochloride–5 mM EDTA (pH 8.5). To this 0.001 ml of RNase A (10 mg/ml; Sigma) preheated at 70°C for 30 min to inactivate DNases was added and incubated for 30 min at 37°C. Control experiments indicated that this concentration of RNase A (100 μg/ml) did not nick purified, supercoiled pBR322 DNA. An additional 0.1 ml of 50 mM Tris-hydrochloride–5 mM EDTA (pH 8.5) was added, and the solution was heated for 5 min at 100°C and quick-cooled on ice. NaCl was added to a concentration of 0.5 M, and the solution was extracted with 2 volumes of phenol saturated with 0.5 M NaCl for 2 min. The mixture was centrifuged in a Sorvall HB4 swinging bucket rotor at 10,000 rpm for 10 min at 4°C. The aqueous phase was carefully recovered, avoiding the interphase containing the single-stranded DNA, and reextracted with chloroform and centrifuged as above. This aqueous phase was precipitated with 3 volumes of ethanol at −70°C for 15 min and centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was suspended in 0.05 ml of 10 mM Tris-hydrochloride–1 mM EDTA (pH 7.5), and 0.005 ml of this solution (which represents one-tenth of a petri dish) of DNA was applied per slot on preparative 1% agarose gels (see below and Fig. 5). The viral DNAs were essentially free of cellular DNA.

Sucrose density gradient centrifugation of virions. Virions were labeled in vivo with either [^{35}S]methionine in the case of MVM, or [^{3}H]methionine in the case of MVM(i). Full and empty capsids were purified in CsCl by the method of Tattersall et al. (25). Full and empty capsids from both MVM and MVM(i) were mixed and centrifuged in an SW65 rotor at 40,000 rpm for 40 min at 20°C in 5 to 30% (wt/vol) sucrose gradients containing 50 mM Tris-hydrochloride–0.5 mM EDTA (pH 8.7). Fractions were collected from the bottom of the tube, and the amount of ^{3}H and ^{35}S in each fraction was determined.

Electron microscopy. Purified virions were concentrated by pelleting in an ultracentrifuge, suspended, and adsorbed to carbon-coated grids. The samples were negatively stained with phosphotungstate and photographed in a Philips electron microscope 400 (7).

Further purification of viral RF DNAs by electrophoresis in low-gelling-temperature agarose gels. DNA to be 5′ end labeled was further purified by a newly developed method for preparative gel electrophoresis. Heat-treated RF DNA (prepared as above, also see Fig. 5) was electrophoresed in 1% low-gelling-temperature agarose gels (200 by 140 by 3 mm) in 40 mM Tris-acetate–2 mM EDTA (pH 7.8) for 4 h at 100 V (best results were obtained when the agarose solution was cooled to 37°C before pouring the gel). The monomer RF band (see Fig. 5) was visualized either by ethidium bromide staining (2 μg/ml, 15 min) or by UV fluorescence inhibition on a PEI cellulose F plate (Merck), both with a 358-nm-wavelength lamp. Agarose slices containing the DNA were transferred to heat-resistant plastic tubes to which 0.1 volume of 5 M NaCl–0.1 M EDTA were added. The tubes were heated at 68°C for 5 to 10 min until the gel had melted. The tubes were blended for 5 s in a Vortex mixer, and the tube contents were extracted for 2 min with 2 volumes of phenol saturated with 0.5 M NaCl. The extraction mixture was centrifuged for 2 min at 10,000 rpm in the HB4 rotor at 4°C. The aqueous phase was carefully removed, avoiding the interphase containing the agarose and other contaminants. The aqueous phase was reextracted twice with 0.5 M NaCl-saturated phenol at room temperature. The final aqueous phase was extracted with 2 volumes of diethylether and centrifuged as above. The ether phase was removed, and the aqueous phase was precipitated either with 2.5 volumes of ethanol at −2O°C for 2 h or with 3 volumes of ethanol at −70°C for 30 min. With this method about 90% of the viral DNA was recovered and did not contain inhibitors of the enzymes used in these experiments. We have successfully used this method with agarose concentrations between 0.7 and 3% and with DNAs of 0.3 to 48 kilobase pairs (kbp).

Restriction enzyme site mapping by partial digestion of 5′ end-labeled RF DNA. We were not able to label efficiently the 5′ termini of MVM or MVM(i) DNA by using polynucleotide kinase and the procedure of Maxam and Gilbert (13). The 5′ termini have been reported to contain covalently attached protein (17). This protein might account for our inability to label the 5′ termini by using polynucleotide kinase even after pronase digestion. The DNAs were therefore digested with *Msp*I (98 map units [MU]) or *Xba*I (83 MU) which cut once near the MVM 5′ end (see Fig. 9 and 10). DNAs were treated with bacterial alkaline phosphatase and then labeled with [γ-^{32}P]-ATP and polynucleotide kinase (13). The DNA preparations thus contained a large fragment and a very short one, each being strongly labeled at only one end. The 5′ end-labeled fragments were then used directly

for restriction enzyme cleavage site mapping by partial digestion as described by Smith and Birnstiel (20). To increase the resolution, especially in the left half of the genome, the three *Eco*RI-generated DNA fragments (see Fig. 10) were purified by preparative gel electrophoresis, and restriction sites within each were mapped as described above. This was done with all restriction enzymes examined, except *Hin*dIII and *Xho*I. Fragments B (69 to 100 MU) and C (0 to 23 MU) were labeled at only one end for the reasons mentioned above; fragment A (23 to 69 MU) labeled at one end was prepared by removing a short DNA fragment from the other end by *Bgl*II (67.5 MU) digestion after end labeling. The restriction maps obtained were checked by comparison with the patterns of DNA fragments produced by complete digestion with the respective restriction enzyme.

Mapping of the position of the deletion in MVM(i) by using S1 nuclease. *Xba*I-digested MVM RF DNA was 5′ end labeled with ^{32}P (see above) and then digested with *Msp*I to remove the terminal hairpin structure (see Fig. 9). The DNA was denatured and mixed with a 10-fold excess (0.2 μg/ml) of single-stranded DNA from MVM(i) virus particles and hybridized for 2 h at 68°C in 40 μl of 0.6 M NaCl–10 mM Tris-hydrochloride–2 mM EDTA (pH 7.5). The solution was overlayered with paraffin oil to prevent evaporation. After hybridization, 10 volumes of cold S1 nuclease reaction buffer with 20 μg of denatured salmon sperm DNA per ml was added, and single-stranded DNA was digested as described by Berk and Sharp (1). The paraffin oil was removed by extraction with ether, and DNA was precipitated with 10 μg of tRNA carrier and 3 volumes of ethanol at −70°C. The recovered nucleic acids were electrophoresed in a 2.5% agarose gel at 100 V for 5 h. The gel was dried onto Whatman DE 81 paper and autoradiographed at −70°C with Dupont Cronex intensifying screens.

RESULTS

Inhibition of the generation of cytolytic T lymphocytes by MVM(i). MVM was propagated on A-9 fibroblasts, and MVM(i) was propagated on EL-4 lymphoma cells (see above). Under the standard conditions of infection utilized, neither virus grew efficiently on the normal host cell of the other. Another clear difference between MVM and MVM(i) was the effect on allogeneic mixed leukocyte cultures. In cultures containing spleen cells from C57BL/6 mice responding against X-irradiated (2,000 rads) spleen cells from DBA/2 mice, cytolytic T cells directed against DBA/2 alloantigens are generated (9). The cytolytic activity of T cells was detected by using a ^{51}Cr release assay (5, 9). Addition of MVM(i) to mixed leukocyte cultures at initiation of culture inhibited the appearance of the cytolytic T cells (Fig. 1). The inhibitory effect was detectable with virus-containing infected cell culture fluid diluted as much as 2,000-fold. In contrast, addition of an analogous undiluted suspension of MVM had no detectable effect on the generation of cytolytic T lymphocytes. With this experiment we demonstrate that the immunosuppressive agent reported by Bonnard et al. (3) is indeed different from MVM. A more detailed characterization of the inhibition of T cell functions by MVM(i) will be published elsewhere (Engers et al., in preparation).

Comparison of viral particles. MVM was labeled with [^{35}S]methionine, and MVM(i) was labeled with [^{3}H]methionine. Each virus was purified by banding in CsCl (see above), and the full particles and empty capsids were collected. Virus particles of MVM and MVM(i) were mixed together and sedimented through a sucrose gradient. The same was done with the empty capsids of MVM and MVM(i). Figure 2 shows that the virus particles of MVM and MVM(i) sedimented at the same rate; this was also true for the empty capsids. MVM and

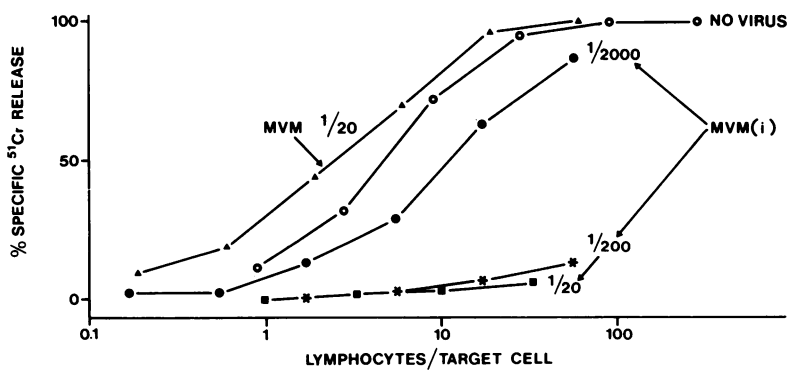

FIG. 1. *Inhibition of the generation of cytolytic T lymphocytes by MVM(i). Mixed leukocyte cultures were prepared in the absence of virus, or with various dilutions of MVM(i), or with concentrated MVM. After 5 days the cytolytic activities were assayed by measuring the percentage of specific ^{51}Cr release from labeled P-815 target cells at various lymphocyte/target cell ratios as described in the text.*

MVM(i) also had the same density in CsCl gradients (data not shown). MVM and MVM(i) virions were stained with phosphotungstate and examined by electron microscopy (Fig. 3). Roughly spherical particles, both full and empty, with a diameter of 19 nm were seen. The MVM(i) particles were not distinguishable from those of MVM (7). MVM particles have previously been shown to contain three interrelated capsid polypeptides (83, 66, and 62 kilodaltons) (25). We found that the major capsid proteins of MVM(i) comigrated with those of MVM on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). In summary, the physical properties of MVM and MVM(i) have so far proved indistinguishable.

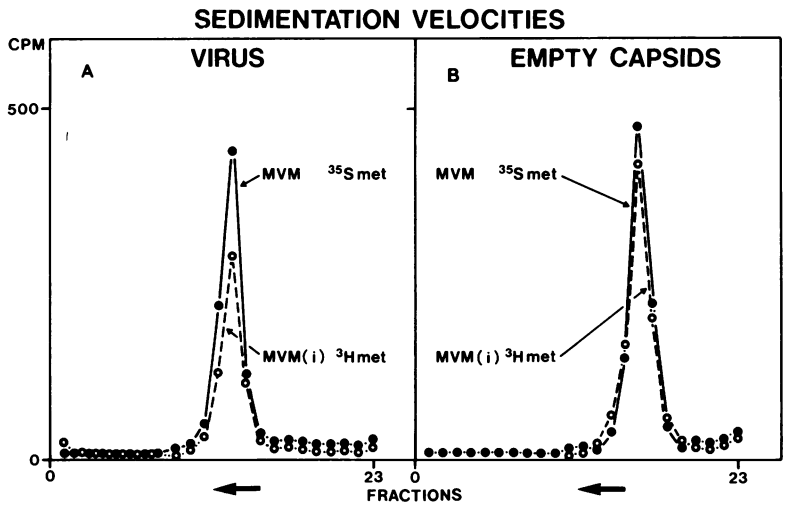

FIG. 2. *Comparison of CsCl-purified MVM ([^{35}S]methionine labeled) and MVM(i) ([^{3}H]methionine labeled) full particles and empty capsids by centrifugation in 5 to 30% sucrose gradients. Full particles (A) from MVM and MVM(i) were mixed and then centrifuged in a SW65 rotor at 40,000 rpm for 40 min at 20°C. The same was done with the empty capsids of MVM and MVM(i) (B). The arrows indicate the direction of sedimentation.*

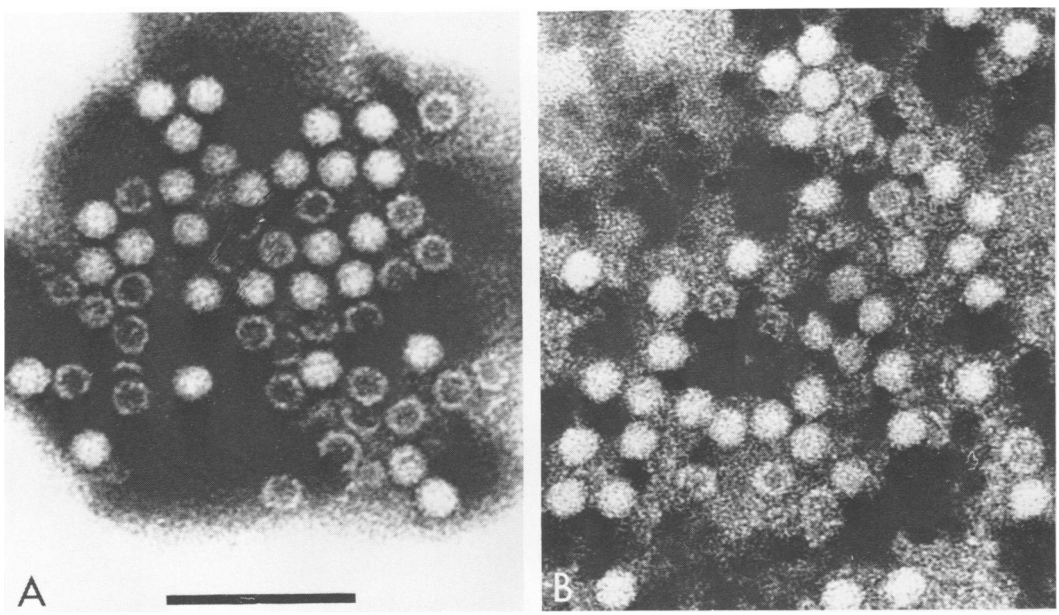

FIG. 3. *Visualization of full and empty particles of MVM (A) and MVM(i) (B) in the electron microscope. Bar, 0.1 μm.*

Method for extraction of RF MVM DNA from infected cells. The double-stranded RF MVM and MVM(i) DNAs were extracted by a modification of the method of Hirt (11). The supernatant fraction contained most of the RF DNA of monomer and dimer length present in the cells together with contaminating cellular DNA (Fig. 4, lanes b and d). The DNA was then heated at 100°C and quick-cooled. Viral RF DNA renatured spontaneously due to its hairpin structure (30), whereas cellular DNA remained essentially single stranded and was removed by extraction with phenol in the presence of 0.5 M NaCl (8). Lanes c and e of Fig. 4 show MVM and MVM(i) RF DNAs prepared in this way and then electrophoresed in a 1% low-gelling-temperature agarose gel. The RF monomer DNA, which was the predominant band seen, had an apparent length of about 5 kbp for both MVM and MVM(i). Dimer RF DNA of about 10 kbp and, in some preparations, DNA species of between 2 and 4 kbp were also seen (Fig. 4, lanes d and e). The latter species hybridized with an *Eco*RI-A fragment (23 to 69 MU) of MVM cloned in *Escherichia coli*, but their structure has not been further studied (data not shown).

DNAs of MVM and MVM(i) have different lengths. We estimated the total length of the MVM genome by measuring the lengths of restriction fragments produced from RF DNA by *Xba*I, *Eco*RI, and *Msp*I and by gel electrophoresis under both neutral and alkaline conditions. The denaturing alkaline gels were used to determine the lengths of end fragments, which contain base-paired hairpin structures at neutral pH (30). The values obtained by this method and from alkaline gel electrophoresis of DNA from virus particles (see below) were both 4.9 ± 0.1 kbp for the fully extended nonhairpin form of MVM. This confirms the earlier estimates made by Bourguignon et al. (4) and Faust and Ward (10).

DNA was extracted from MVM and MVM(i) virus particles with 0.5% sodium dodecyl sulfate in the presence or absence of pronase (0.5 mg/ml). It was then electrophoresed through a 1.5% agarose gel at pH 7.8 (Fig. 5). MVM(i) DNA migrated slightly faster than did MVM DNA. The difference in migration was not due to tightly bound protein since it was unaffected by pronase treatment of the DNAs, nor was it due to a difference in DNA secondary structure because we found that the DNAs migrated with slightly different mobilities in alkaline gels (14) or after denaturing with glyoxal (15) (data not shown). The length difference between MVM and MVM(i) double-stranded RF DNAs was not resolved by electrophoresis at neutral pH (Fig. 4). Only after digestion with restriction enzymes were we able to detect the length difference between the RF DNAs. For example, RF DNAs were digested with enzymes *Xba*I and *Msp*I and electrophoresed in a 2% agarose gel (Fig. 6, left panel). The DNA fragment extending from the *Xba*I site (83 MU) to the *Msp*I site (98 MU, see Fig. 10) had a length of 0.70 kbp in MVM and 0.64 kbp in MVM(i). The results reported below

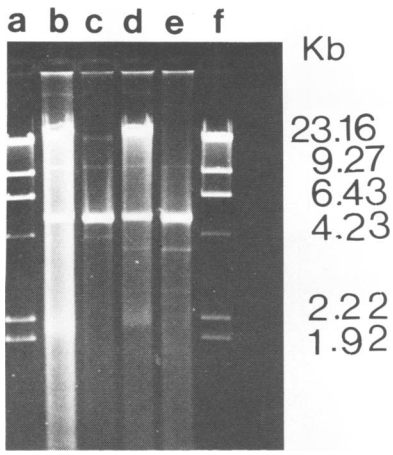

FIG. 4. *Purification of MVM and MVM(i) RF DNAs from Hirt supernatants by heat treatment and phenol extraction in 0.5 M NaCl. The samples were electrophoresed in a 1% low-gelling-temperature agarose gel for 4 h at 100 V. (a and f) Bacteriophage λ DNA HindIII markers (16); (b) supernatant containing MVM(i) DNA; (c) same sample as in (b) after heat treatment and phenol extraction in 0.5 M NaCl; (d) supernatant containing MVM DNA; and (e) same sample as in (d) after heat treatment and phenol extraction in 0.5 M NaCl.*

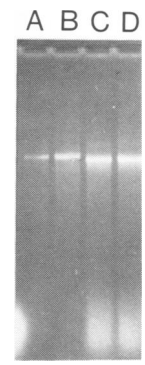

FIG. 5. *Electrophoresis in a 1.5% agarose gel at pH 7.8 of single-stranded DNAs from MVM (A and B) and MVM(i) (C and D) virus particles after extraction in 0.5% sodium dodecyl sulfate at 37°C in 1 h in either the presence (B and D) or absence (A and C) of 0.5 mg of pronase per ml.*

indicate that the DNA of MVM(i) is missing 60 bases at 93 MU when compared with that of MVM; we will call this a deletion in MVM(i).

The length difference was also apparent on electrophoresis of partial restriction enzyme digests of RF DNAs labeled at one end. Monomer RF DNAs isolated from agarose gels were digested with *Msp*I, and the 5′ termini were labeled with ^{32}P (see above). The labeled DNAs were partially digested with *Alu*I and electrophoresed through a 1.2% agarose gel, and the labeled DNA bands were detected by autoradiography (Fig. 6, right panel). With this technique (20) a band was seen corresponding to each *Alu*I site in the DNA. The shortest labeled fragment represented the *Alu*I site (86 MU) which was closest to the *Msp*I site (98 MU) site, and the successively longer fragments represented *Alu*I sites at increasing distances from it. Each band of MVM(i) DNA migrated slightly faster than the corresponding band of MVM DNA. The smaller *Msp*I fragment (98 to 100 MU) is not seen in Fig. 6. This result indicates that the deletion is located between the *Msp*I site (98 MU) and the closest *Alu*I site (86 MU). This is consistent with the result in Fig. 6 (left panel). The two experiments locate the deletion to within 0.6 kbp to the left of the *Msp*I site.

The site of the deletion was mapped more precisely by making heteroduplexes between MVM and MVM(i) DNAs and then cleaving these in the non-base-paired regions with the single strand-specific nuclease S1 (20). MVM RF DNA was cut with *Xba*I (83 MU), and the 5′ termini were labeled with ^{32}P and analyzed by gel electrophoresis (Fig. 7). The upper band is the DNA fragment from 0 to 83 MU. The two lower bands correspond to the two forms of the viral 5′-terminal region of RF DNA (30). The hairpin duplex migrated faster than the extended nonhairpin form (Fig. 7B). After digestion with *Msp*I (98 MU) the two lower bands were seen as a single band (Fig. 7C). This DNA was then hybridized with an excess of unlabeled single-stranded DNA from MVM(i) virions. The mixture was digested with S1 nuclease, and DNA was recovered by ethanol precipitation and electrophoresed (Fig. 7D). A band appeared which was not previously seen (Fig. 7D, arrow). It had a length of 0.46 ± 0.02 kbp, which represents the distance from the *Xba*I site to the

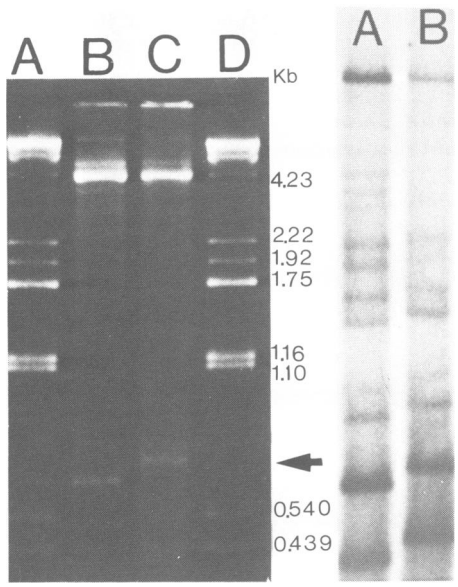

FIG. 6. *Left panel: location of the deletion in MVM(i). MVM and MVM(i) RF DNAs were digested with XbaI and MspI and electrophoresed through a 2% agarose gel at 100 V for 5 h. (A and D) Size markers of λ HindIII and simian virus 40 HindIII; (B) XbaI plus MspI digest of MVM(i) RF DNA; (C) XbaI plus MspI digest of MVM RF DNA. The arrow indicates the XbaI-MspI fragment, which is 60 base pairs shorter in MVM(i). Right panel: deletion in MVM(i) as shown by 5′ end labeling and partial restriction enzyme digestion. Agarose gel-purified RF DNAs were digested with MspI, labeled at their 5′ ends with ^{32}P, and then partially digested with AluI. They were electrophoresed through a 1.2% agarose gel for 4.5 h at 100 V. The gel was dried and autoradiographed. (A) MVM(i); (B) MVM.*

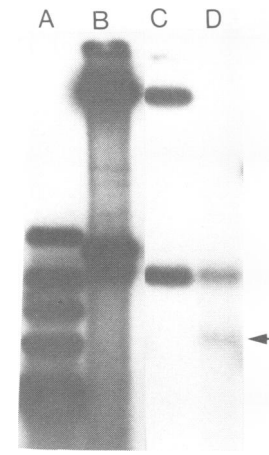

FIG. 7. *Heteroduplex and S1 nuclease mapping of the deletion of MVM(i). The samples were prepared as described in the text and electrophoresed through a 2.5% agarose gel. (A) pBR322 AluI markers; their lengths in base pairs from the top are: 910, 659 to 655, 521, 403, 281, and 257 (22). (B) MVM RF DNA digested with XbaI then 5′ end labeled with ^{32}P. (C) DNA as in lane (B) further digested with MspI. (D) DNA as in lane (C) after hybridization to an excess of MVM(i) virion DNA and S1 nuclease digestion. The arrow indicates the new band.*

deletion. The low intensity of this band may be due to incomplete digestion by S1 nuclease. Annealing of homologous complementary strands of MVM or MVM(i) followed by S1 nuclease digestion did not result in the appearance of a new band. This experiment was repeated by using unlabeled DNAs; the bands in the gel were detected by Southern transfer (21) and hybridization with the radioactive *Xba*I small fragment. A 0.46-kbp DNA was again detected (data not shown), which demonstrates that this band was derived from the smaller *Xba*I fragment.

In analogous experiments with MVM RF DNA end labeled at the *Msp*I site, we have endeavored to measure the distance from the *Msp*I site to the deletion. These experiments proved more difficult: short DNA fragments formed during the S1 nuclease digestion tended to obscure specific DNA bands less than 200 base pairs long. Nevertheless, we reproducibly observed a new specific band created by S1 nuclease digestion of MVM-MVM(i) heteroduplexes labeled at the *Msp*I site (data not shown). The band corresponded to a DNA fragment about 150 ± 20 base pairs, and we provisionally concluded that this is the distance from the *Msp*I site to the S1 nuclease-sensitive region in the heteroduplexes. In agreement with this conclusion, further restriction enzyme mapping experiments (see below) showed that the deletion is located between the *Mbo*II sites at 89.6 and 93.5 MU (see Fig. 10).

Mapping of the cleavage sites of several restriction enzymes on MVM and MVM(i) DNAs. The cleavage sites of a series of restriction enzymes were mapped with respect to the *Msp*I, *Xba*I, and *Eco*RI sites by the method of Smith and Birnstiel (20) (see above). An example of *Hin*dIII partial digestion of RF DNA labeled at the *Xba*I site is shown in Fig. 8. In this case a *Hin*dIII cleavage site present in MVM (Fig. 8B) was absent in MVM(i) (Fig. 8A). The missing site was known to be to the left of the *Xba*I site (see Fig. 10) because the smaller *Xba*I-generated fragment has no *Hin*dIII site in either virus. In an analogous way, the cleavage sites for the restriction enzymes shown in Fig. 9 and 10 were mapped. Of a total of 109 sites, 22 (20%) were present in only one of the two strains, MVM or MVM(i). Seventeen of these differences were in three clusters at 9.4 to 12.0, 30 to 40, and 72 to 80 MU.

A cleavage site in a terminal hairpin region of RF RNA would be expected to be present twice in the extended nonhairpin form. This was observed in the case of *Hha*I (Fig. 10). However, only one *Msp*I site was detected at about 98 MU when RF DNA was digested with *Xba*I, 5′ end labeled, and then partially digested with *Msp*I

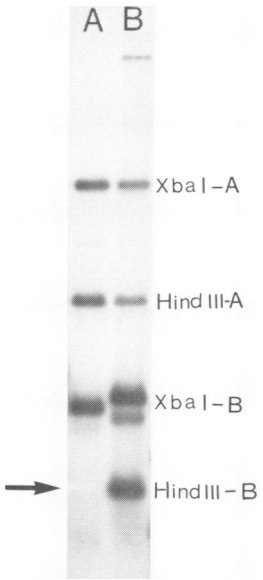

FIG. 8. *Restriction enzyme site mapping by partial digestion of 5′ end-labeled DNA. MVM(i) (lane A) and MVM (lane B) RF DNAs were digested with XbaI and then labeled at their 5′ ends with ^{32}P. Two fragments, XbaI-A and -B, were formed. This mixture was partially digested with HindIII and electrophoresed for 4.5 h at 100 V. After the partial digestion two new fragments, HindIII-A and -B, were seen in the case of MVM. Both were products of the XbaI-A fragment. However, with MVM(i) (lane A) the HindIII-B band was not detected (arrow). The band of XbaI-B from both viruses is a doublet due to the presence of hairpin and extended ends (see the text).*

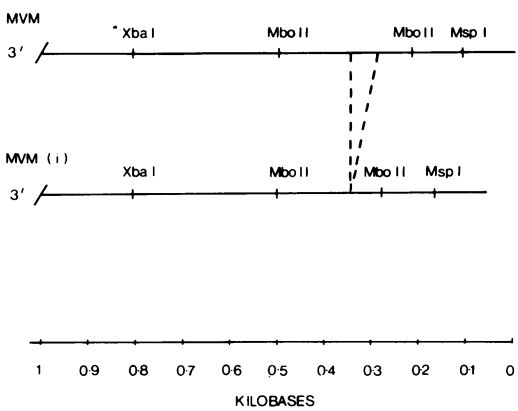

FIG. 9. *Map of the 5′ ends of MVM and MVM(i) DNAs showing the deletion in MVM(i).*

(Fig. 10). This suggests that the *Msp*I site or sites may be near the closed end of the hairpin loop. Two other observations are consistent with this idea. First, when MVM RF DNA was digested to completion with *Msp*I and then 5′ end

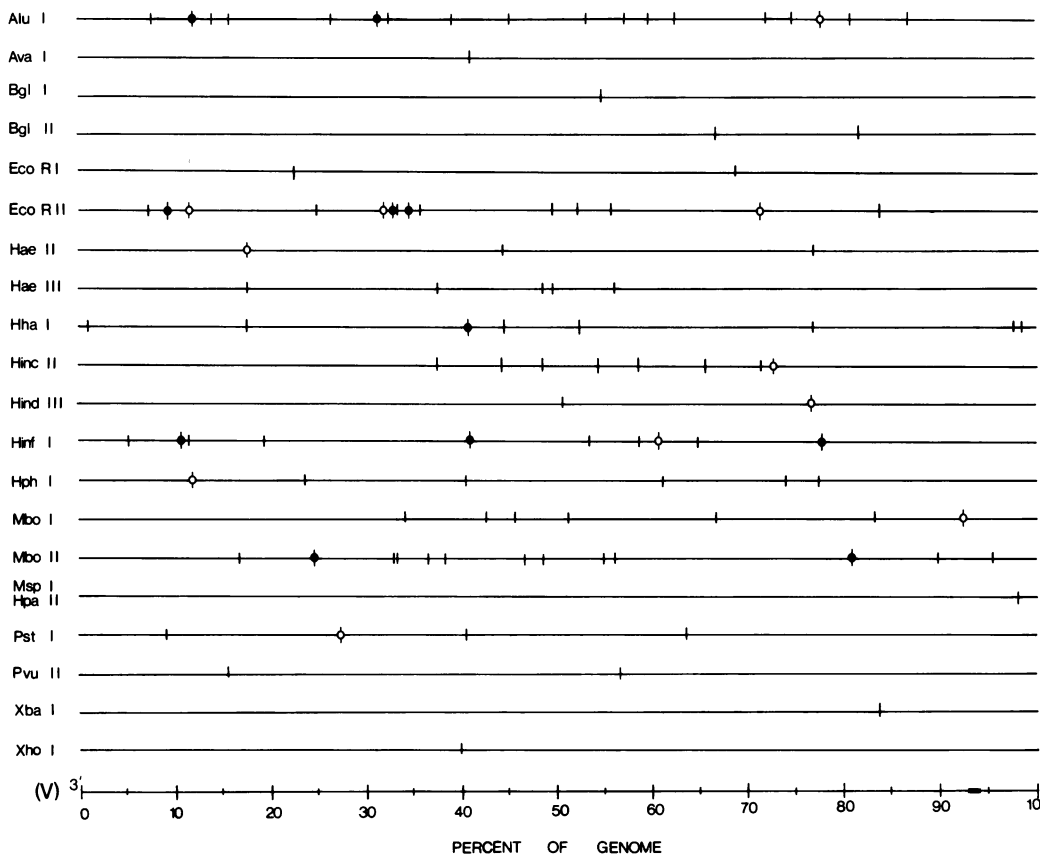

FIG. 10. *Comparative restriction maps of MVM and MVM(i). The map represents single-stranded virion DNA (hairpin structures denatured). Restriction sites were derived from the digestion of double-stranded RF DNA. The scale gives the map units (percent of the genome) of MVM starting at the 3' end as conventional for parvoviruses. Vertical lines depict sites present in both MVM and MVM(i). Open circles are sites only in MVM, and filled circles are sites only in MVM(i). The bar at 93 MU on the percentage of genome scale shows the region deleted in MVM(i). Restriction enzymes which do not digest either DNA are: BamHI, KpnI, SmaI, SalI, and SacI.*

labeled and electrophoresed in a 3% agarose gel at alkaline pH, only two bands, corresponding to the fragments 0 to 98 MU and 98 to 100 MU, were detected (data not shown). This gel could detect a DNA fragment of 20 nucleotides. Second, *Msp*I digestion of the extended and hairpin forms of *Xba*I small fragment of RF DNA (84 to 100 MU) gave, in agarose gels at neutral pH, a single band at the same position as the hairpin form (Fig. 7).

DISCUSSION

We have isolated and characterized the immunosuppressive agent described by Bonnard et al. (3) and shown that it is a new parvovirus closely related to MVM. The two viruses differ in their host cell specificity and in the ability to suppress T lymphocyte functions in vitro. In an attempt to understand the molecular basis of these differences the genome structures of MVM and MVM(i) were compared by restriction enzyme analysis. Out of a total of 109 restriction enzyme sites examined, 87 appeared to be common for MVM and MVM(i) within the resolution of the methods applied, whereas 22 were unique to one virus or the other. These 109 restriction sites represent approximately 10% of the nucleotide sequence of the viral genomes. MVM(i) had an apparent deletion relative to MVM, about 60 base pairs long and beginning about 0.3 kbp from the 5' end of virion DNA (Fig. 9 and 10). Since we have mapped this deletion to a region not much larger than its own length, it is likely to involve a continuous stretch of DNA. DNA sequencing will be required, however, to confirm this. We conclude from these

results that the genome organization and nucleotide sequence are similar in the two viruses, and that MVM and MVM(i) are probably derived from a common ancestor by a small number of mutations.

Previously, 5 of the 21 restriction maps of MVM have been published (6, 10). Four of these have been confirmed in this paper. For the fifth, *Hin*fI, a portion of the map presented here differs from that previously published by Faust and Ward (10). These authors detected two additional sites at 86.5 and 94.2 MU. We are confident that these sites are not present in our strain of MVM. In independent experiments, complete *Hin*fI digestion of DNA end labeled at the *Msp*I (98 MU), *Xba*I (83 MU), or *Eco*RI (69 MU) sites located the closest *Hin*fI site at 64 MU. Faust and Ward (10) mentioned that an additional 100-base-pair *Hin*fI fragment was not mapped definitively, but was known to lie between positions 17.8 and 94.2 MU. This fragment probably represents the two sites mapped by us at 62 and 64 MU.

We expect that there are other differences between the DNAs of MVM and MVM(i) in addition to those already found. The distinct biological properties of the two viruses may result from one (or more) of the differences we detected or from one or more that we did not. Almost the entire length of the MVM genome apparently serves as template for an initial RNA transcript (23). The major cytoplasmic polyadenylic acid-containing RNA represents this transcript from which 30% has been spliced beginning about 450 base pairs from the RNA 5′ end. This species is probably the mRNA coding for the virion polypeptides (23). Thus, the sequence differences we have observed between MVM and MVM(i) are within a transcribed part of the MVM genome. Several restriction site differences are grouped in the region corresponding to the proposed RNA splice junctions around 9 and 38 MU (23). None of the three major virion polypeptides (83, 66, and 62 kilodaltons) showed a difference when the proteins of MVM and MVM(i) were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (P. Beard and K. Nyfeler, unpublished data). We have repeatedly observed a fourth polypeptide, from MVM and MVM(i) virions, which migrated between the 66- and 62-kilodalton polypeptides. This polypeptide from MVM(i) did migrate slightly faster than that from MVM. Further work is required to determine whether this polypeptide is virus coded.

MVM and MVM(i) may differ in a DNA sequence which interacts with host cell components during viral DNA replication. The deletion in MVM(i) is close to the 5′ end of virion DNA, where replication of the double-stranded RF DNA probably initiates (27). It is interesting to note that Rhode (18), working with the autonomous parvovirus H1, has observed a variant RF DNA species with additional DNA at a location analogous to that of the deletion in MVM(i).

The significance of the differences between MVM and MVM(i) DNAs may be understood when we know in detail at which stage of virus replication MVM and MVM(i) are blocked in their respective nonpermissive cells. The isolation of additional MVM variants with an altered specificity for host cell differentiation type may allow one to pinpoint which structural features are important in determining this specificity. MVM(i) does not produce wide-spread cytopathic effect in mixed leukocyte cultures (Engers et al., in preparation). Since MVM(i) replicates in these cultures it is likely that a subpopulation of responding cells is specifically susceptible to infection by the virus. This would be a simple explanation for the immunosuppressive activity of MVM(i) observed in vitro.

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