Retrovirus D/New England and Its Relation to Mason-Pfizer Monkey Virus

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Seventeen isolates of retrovirus D/New England have been obtained from three species of macaques at the New England Regional Primate Research Center. Seven of the isolates were obtained from macaques who subsequently died with the macaque immunodeficiency syndrome; other isolates were obtained from macaques with less severe or other forms of illness. Attempts to isolate type D retrovirus from peripheral lymphocytes of 97 apparently healthy macaques have not been successful. Cloned DNA was prepared from Hirt supernatants of cells infected with one of these isolates (D/New England 398). By restriction endonuclease analysis, cloned pD398 DNA represented full-length viral DNA with one long terminal repeat. A detailed restriction endonuclease map of pD398 was derived and compared with a map of the cloned Mason-Pfizer monkey virus genome. Forty-six percent (13 of 28) of restriction endonuclease sites were found to be conserved when these related viruses were compared. Five of the D/New England isolates, including those from three different macaque species, were examined for strain variability by restriction endonuclease typing. Comparison of over 30 restriction endonuclease sites has not distinguished any of these D/New England isolates. It thus appears that a single strain of type D retrovirus is infecting three different species of macaques in the New England colony. Markedly reduced cross-hybridization was observed between cloned pD398 and Mason-Pfizer monkey virus DNAs at high stringency; this reduced cross-hybridization was localized to the *pol-env* regions of the genome. Only very weak hybridization of D/New England DNA to cloned squirrel monkey type D retrovirus DNA could be detected even at low-stringency conditions. What role type D retrovirus plays in the immunodeficiency syndrome of macaques remains to be determined.

An immunodeficiency syndrome of macaque monkeys has been described at three Primate Research Centers (PRC) in the United States (13, 20, 28). Affected animals develop opportunistic infections and profound loss of T-cell blastogenic responsiveness. These macaques also develop lymphadenopathy, neutropenia, anemia, and finally a wasting syndrome. At the New England colony and possibly at Davis as well, lymphoproliferative disorders are associated with this syndrome (12, 17, 18, 27); in the Seattle colony, an unusual retroperitoneal fibromatosis is associated with the disease (28).

In 1984, we reported the isolation of a type D retrovirus from macaques of the New England Regional Primate Research Center (NERPRC) colony (4) and isolation of type D retrovirus was also described by investigators working with the Davis and Seattle PRC colonies (23, 28). However, attempts to define the role of type D retroviruses in the macaque immunodeficiency syndrome have not yielded conclusive results at this time (10, 19, 23). The genetic information for type D retrovirus has been detected in retroperitoneal fibromatosis tissues (28).

Here, we describe the isolation of type D retrovirus from peripheral blood lymphocytes of 17 ill macaques and present the results of cloning and characterization of D/New England DNA. The relatedness of the D/New England genome to that of Mason-Pfizer monkey virus (MPMV), the original macaque type D retrovirus, is also described.

MATERIALS AND METHODS

Cells and viruses. In all cases except that of *Macaca mulatta* 225-81 type D retrovirus isolation was achieved by cocultivation of peripheral blood lymphocytes (PBL) with Raji cells (American Type Culture Collection) (5). Although *M. mulatta* 225-81 was markedly immunodeficient for a prolonged period of time and had opportunistic infections, several attempts to isolate type D retrovirus from PBL and plasma by cocultivation with Raji cells were not successful. Explant cultures of thymus, lung, and lymph node established at the time of death of *M. mulatta* 225-81, however, did yield type D retrovirus. Isolates were determined to be type D retrovirus by the characteristic formation of syncytia in Raji cells, by electron microscopy, and by DNA analysis of selected isolates (5). Filtered saliva, urine, and feces from some of the animals whose PBL yielded D retrovirus also yielded D retrovirus upon cocultivation with Raji cells. Other cell types found to support the replication of D/New England included human lung, macaque fetal lung, macaque foreskin, Vero, mink lung, bat lung, and canine thymus.

Preparation of replicative intermediate DNA. A growing culture of Raji cells was infected with cell-free supernatant virus at 0.1 to 1.0 syncytium-forming units of virus per cell. After 24 to 48 h, the cells were pelleted and suspended in 8 ml of cold 10 mM Tris-hydrochloride-1 mM EDTA (pH 8.0) per 2×10^8 cells. This suspension was transferred to an SW40 ultracentrifuge tube on ice, and 0.6 ml of 10% sodium dodecyl sulfate was added. The tube was mixed gently by inversion, 2.0 ml of 5 M NaCl was added, and the sample

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TABLE 1. Type D retrovirus isolations from the NERPRC colony^a

Isolate no.	Species	Physical signs	Immunological status	Necropsy findings
184-80	<i>M. cyclopis</i>	LAD	D	CMV, P, LPD
167-76	<i>M. cyclopis</i>	LAD, Di	D	RF, LD, IA
225-81	<i>M. mulatta</i>	LAD	D	Ca, Cr, T, CMV
99-83	<i>M. mulatta</i>	LAD	D	LPD, T
398-80	<i>M. cyclopis</i>		N	CMV, LPD, Th, LD
134-79	<i>M. cyclopis</i>		N	LD, Th, LPD, Noma
67-82	<i>M. cyclopis</i>		N	LD, LPD, RF, P
331-81	<i>M. mulatta</i>	LAD, Bite wounds	NT	Staphylococcal infection
107-83	<i>M. cyclopis</i>	F, LAD, R	NT	Still alive (330 DPI)
91-83	<i>M. cyclopis</i>	F, LAD, Di	N	Still alive (330 DPI)
112-83	<i>M. cyclopis</i>	F, LAD, R	N	Still alive (330 DPI)
427-76	<i>M. cyclopis</i>	LAD	N	Still alive (200 DPI)
103-82	<i>M. mulatta</i>	F, LAD, R	NT	Still alive (510 DPI)
101-82	<i>M. mulatta</i>	F, LAD, R	NT	Still alive (510 DPI)
138-79	<i>M. fascicularis</i>	Sp	NT	Still alive (330 DPI)
114-83	<i>M. fascicularis</i>	LAD	NT	Still alive (350 DPI)
214-83	<i>M. fascicularis</i>	LAD	NT	Still alive (180 DPI)

^a Abbreviations: CMV, cytomegalovirus infection; F, fever; P, paramyxovirus; LAD, lymphadenopathy; Di, diarrhea; Ca, candida; LPD, lymphoproliferative disorder; R, rash; Cr, cryptosporidia; LD, lymphocytic depletion; Sp, splenomegaly; T, trichomonads; IA, intestinal amyloidosis; Th, thymic atrophy; RF, retroperitoneal fibromatosis; D, markedly decreased or absent T-cell blastogenic responsiveness; N, normal or near normal T-cell blastogenic responsiveness; NT, not tested; DPI, days postisolation of virus.

was mixed again by inversion. After a 2-h incubation on ice, the sample was centrifuged at 35,000 rpm for 2 h at 4°C. The supernatant was then removed and extracted directly with phenol and then chloroform (5, 15). The nucleic acids were precipitated by the addition of 2 volumes of absolute ethanol. The precipitate was washed, dried, and suspended in 10 mM Tris-hydrochloride–1 mM EDTA.

Molecular cloning. Previous results had indicated that circular replicative intermediate DNA of D/New England strain 398 was cut once by *Bam*HI, yielding DNA of about 8 kilobase pairs (kbp) (5). Replicative intermediate DNA prepared as described above from D398-infected Raji cells was cut with *Bam*HI, and the digested DNA was layered over a

5 to 20% sucrose gradient in 10 mM Tris-hydrochloride–1 mM EDTA–100 mM NaCl (pH 8.0). After centrifugation at 29,000 rpm for 16 h at 4°C in an SW40 rotor, fractions were collected from a hole punctured in the bottom of the tube. DNA of around 8 kbp contained within a single fraction was precipitated with ethanol, washed, dried, and suspended in 10 mM Tris-hydrochloride–1 mM EDTA. A portion of this was ligated with *Bam*HI-cleaved pBR322 DNA and introduced into *Escherichia coli* HB101. Bacterial colonies were screened by Grunstein-Hogness colony hybridization (11) with a fragment of cloned Mason-Pfizer virus DNA (C. Barker, J. Wills, J. Bradac, and E. Hunter, Virology, in press). A single positive colony was expanded, and plasmid

FIG. 1. Restriction endonuclease maps of D/New England and MPMV DNA. The size and location of LTRs were determined as described in the text. The left-to-right orientation is 5' to 3' (Barker et al., in press). Abbreviations: H, *Hpa*I; Hd, *Hind*III; B, *Bam*HI; E, *Eco*RI; P, *Pst*I; I, *Bgl*II; II, *Bgl*III; X, *Pvu*I (*Xor*II); K, *Kpn*I; T, *Bst*EII; S, *Sst*I; V, *Pvu*II; Sp, *Sph*I.

DNA was prepared as previously described (6, 14). Restriction endonuclease maps were derived by a strategy of single and double digests, proceeding from the simple to the more complex.

A full-length clone of MPMV DNA (pMPMV-H4) containing one long terminal repeat (LTR) was obtained in a similar manner. Replicative intermediate DNA from infected cells was cut with *Sst*I and inserted into the phosphatased *Sst*I site of lambda gtWES B as described elsewhere (C. Barker and E. Hunter, manuscript in preparation). The plasmid pMPMV-H4 was derived by subcloning the insert from lambda gtWES B into the *Sst*I site of pJC2, a derivative of pAT153 containing an *Sst*I site in place of the *Eco*RI site. The pJC2 plasmid was kindly provided by John Jenkins. Derivation of restriction endonuclease maps was achieved similarly (Barker et al., in press).

The circularly permuted cloned genomes in pD398 and pMPMV-H4 were recloned via the *Sph*I site within the LTR region to facilitate restriction enzyme site comparisons. The viral DNA inserts of pD398 and pMPMV-H4 were excised with *Bam*HI and *Sst*I and isolated from agarose gels. Each DNA was then ligated with itself by using T4 DNA ligase. The ligated DNA was then cut with *Sph*I, and DNA of 7.8 kbp was isolated from agarose gels. DNA was then ligated into the *Sph*I site of pAT153. Plasmid DNAs from colonies with appropriately restructured viral DNA inserts designated pMPMV-H4Sp and pD398-Sp were used for comparative analyses (see Fig. 2). These *Sph*I clones contain nonpermuted viral genomes colinear with the viral RNA genome (see Fig. 1).

Cloned DNAs for gibbon ape leukemia virus (9) and for squirrel monkey type D retrovirus (2) were kindly provided by Edward Gelmann and Stuart Aaronson.

Cloned DNA pD398 was tested for infectivity by the following procedure. pD398 was cut with *Bam*HI to excise the insert, and the DNA fragments were then ligated by T4 DNA ligase. Up to 2 µg of ligated DNA was transfected into macaque fibroblast cells, Vero cells, and SV80 cells by the Ca^{2+} precipitation procedure with salmon sperm DNA as carrier; supernatants were monitored over the course of 2 months for reverse transcriptase activity and syncytium-forming ability on Raji cells. Cloned DNA pD398 does not appear to be infectious.

Gel electrophoresis, Southern transfer, and hybridization. Gel electrophoresis and Southern transfer of DNA were performed as described previously (6, 26). For Southern transfer hybridizations (see Fig. 3, 4, and 5), aqueous hybridization in $4\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 67°C was used, followed by rinsing at 67°C in $2\times$ SSC–0.1% sodium dodecyl sulfate. For hybridization at various stringencies (see Fig. 6 and 7), the procedure of Wahl et al. (30) was used; rinsing of the filter was performed in $2\times$ SSC at the temperature and percent formamide used in the hybridization.

Size-fractionated DNA was purified from agarose gels by electroelution.

RESULTS

Virus isolation. Over an 18-month period, type D retrovirus was isolated from 17 different macaques of the NERPRC colony (Table 1). Isolates were obtained from *M. mulatta* (rhesus monkey), *Macaca fascicularis* (crab eating macaque or cynomolgus monkey), and *Macaca cyclopis* (Taiwanese rock macaque). Although fewer *M. cyclopis* (<50) are housed at NERPRC than the other two macaque species (>400),

FIG. 2. Demonstration of size and restriction site conservation. pD398-Sp and pMPMV-H4-Sp DNAs were cut with the indicated restriction endonucleases and electrophoresed through agarose gels. The gel was soaked in ethidium bromide stain and photographed over UV light.

most isolates were obtained from *M. cyclopis*. All isolates were obtained from ill macaques. Eight of the 17 animals have died subsequent to virus isolation. Four of these animals, *M. cyclopis* 184-80 and 167-76 and *M. mulatta* 225-81 and 99-83, clearly had clinical, pathological, and immunological evidence of the macaque immunodeficiency syndrome as previously described (20). The clinical histories of three of these animals have been described previously (5, 20, 22). Although *M. cyclopis* 398-80, 134-79, and 67-82 had nearly normal T-cell blastogenic responsiveness at the time of testing, necropsy findings were consistent with the immunodeficiency syndrome (18). Other animals had less severe and poorly definable diseases, and all but one of these animals are still alive. Whether some of these had less severe forms of the macaque immunodeficiency syndrome or whether they had unrelated medical problems is not clear at this time. It is also possible that clinical signs and virus recovery among these animals were simply due to primary infection with the virus. Attempts were made to isolate type D retrovirus after recovery from illness in only two cases (*M. mulatta* 101-82 and 103-82); these attempts were not successful. Attempts to isolate type D retrovirus from 97 apparently healthy macaques by using the same procedures also failed to yield virus.

FIG. 3. Identification of linear and circular forms of replicative intermediate DNA, location of LTRs, and demonstration that pD398 has one LTR. Cloned pD398 DNA, unfractionated replicative intermediate (Hirt supernatant) DNA, or purified linear or circular replicative intermediate DNA from D398-infected cells was electrophoresed through agarose gels after digestion with the indicated restriction endonuclease or with no enzyme treatment. DNA was transferred to nitrocellulose by the procedure of Southern (26) and hybridized with a ^{32}P -labeled 7.8-kbp fragment insert of pD398. Sizes were determined relative to lambda DNA $\times$ *Hind*III markers.

Cloning and mapping. The first type D retrovirus isolate, MPMV, was derived from a *M. mulatta* mammary carcinoma (4). Since our isolates were also derived from macaques it was important that they be compared to this prototype type D retrovirus. Cloned DNA was prepared from replicative intermediate Hirt supernatant DNA of D/New England strain 398 (D398) and compared to a similar MPMV clone; both appeared to be full-length clones containing one LTR (see below). Restriction endonuclease maps were derived independently for these cloned DNAs (Fig. 1). It is clear from inspection of the restriction endonuclease maps that D398 and MPMV are related viruses. Thirteen of 28 restriction endonuclease sites (46%) are conserved when pD398 and pMPMV are compared. The regions of greatest conservation include the *gag* region, the extreme 3' end of the genome and the LTR regions. There is a striking lack of

conservation of restriction endonuclease sites in the *pol* region; a series of eight restriction endonuclease sites are not conserved in this central portion of the genome.

The pD398- and pMPMV-H4-cloned DNAs both contained 7.8 kbp of viral DNA and they contained a single LTR (see below). The apparent alignment of conserved sites in pD398 and pMPMV-H4 was verified by comigration of appropriate DNA fragments in adjacent lanes of a gel following single or double digestions. Size differences were not detected in any region along the genomes by comparison of the sizes of restriction endonuclease fragments generated from conserved sites (e.g., *Kpn*I to *Pst*I, *Pst*I to *Hpa*I, *Hind*III to *Hind*III, and *Hind*III to *Hind*III partial). This was even more apparent when restriction endonuclease analysis was performed with cloned MPMV and D398 DNAs reoriented as inserts from the conserved LTR *Sph*I sites (Fig. 2; other data not shown).

Comparison of cloned DNA to replicative intermediate DNA. Analysis of cells infected by a variety of retroviruses has revealed circular and linear forms of viral replicative intermediate DNA (for an example, see reference 24). The linear form consists of full-length LTR to LTR double-stranded DNA copied from the genomic RNA. Double-stranded circular DNA can be found with one and two LTRs (25). After electrophoresis of replicative intermediate DNA from D398-infected cells through agarose gels, a band of viral DNA at 8.1 kbp was observed, the size expected for linear replicative intermediate DNA (Fig. 3). A doublet of viral DNA was also observed where linear DNA standards of 5.0 to 5.5 kbp migrated; this is where supercoiled circular

TABLE 2. Viral DNA fragments produced by restriction endonuclease digestion of replicative intermediate and cloned DNA^a

Enzyme digestion	Viral DNA fragments (kbp)		
	pD398	Supercoil	Linear
<i>Bam</i> HI	7.8	7.8, 8.1	2.8, 5.3
<i>Bam</i> HI plus <i>Eco</i> RI	2.6, 5.2	2.6, 5.2, 5.5	2.6, 2.8, 2.8
<i>Bam</i> HI plus <i>Kpn</i> I	2.8, 5.0	2.8, 5.0, 5.3	2.8, 2.8, 2.6
<i>Bam</i> HI plus <i>Pst</i> I	1.4, 2.1, 4.3	1.4, 2.1, 2.45, 4.3	1.1, 1.35, 1.4, 4.3

^a Results are taken from Fig. 2 and 3 and other data not shown.

replicative intermediate DNA containing one and two LTRs would be expected to run in this gel system.

Presumed circular and linear DNA forms were eluted from the 5.0- to 5.5-kbp and 8.1-kbp regions of an agarose gel. Analyses of these purified DNAs indicated that they were greater than 90% pure (Fig. 3). Analysis of these gel-fractionated DNAs by restriction endonuclease digestion, gel electrophoresis, and Southern transfer hybridization indicated (i) that the linear and circular DNA fractions yielded viral DNA fragments consistent with the maps shown in Fig. 1 and the structure of linear and circular DNA forms described above, (ii) that the distance from a given restriction endonuclease site to the ends of the LTRs could be determined from analysis of linear DNA, (iii) that pD398 DNA contains one LTR, and (iv) that the differences in size between circular forms with one and two LTRs is about 350 base pairs; i.e., the size of the LTR is 350 base pairs.

FIG. 4. pD398 is a full-length clone with one LTR. Replicative intermediate DNA, containing circular and linear forms from D398-infected cells, and pD398 DNA were cut with the indicated restriction endonucleases, electrophoresed through agarose gels, transferred to nitrocellulose, and hybridized to a ^{32}P -labeled 7.8-kbp fragment insert of pD398. Sizes were determined relative to lambda DNA $\times$ HindIII markers.

FIG. 5. D/New England strain variability. Replicative intermediate DNA containing circular and linear forms from infected cells was cut with the indicated restriction endonuclease and analyzed by gel electrophoresis and Southern transfer hybridization as described in the legends to Fig. 3 and 4.

These points are illustrated by the following results. Although *EcoRI* and *BamHI* linearized the circular DNA from D398-infected cells to DNA of 7.8 and 8.1 kbp, both *EcoRI* and *BamHI* cut the linear DNA into two fragments, indicating the distance to the ends of the linear DNA molecules. Similarly, cutting with *PstI* revealed sites 1.1 and 1.35 kbp from the ends of linear DNA (Fig. 3; Table 2). In the circular DNA, these ends were joined (via one and two LTRs) yielding *PstI* fragments of 2.10 and 2.45 kbp. pD398 DNA cloned as a 7.8-kbp *BamHI* insert contained the 2.1-kbp *PstI* fragment but not the 2.45-kbp *PstI* fragment, indicating the presence of a single LTR. The difference in size between these *PstI* fragments is also the size of a single LTR, 0.35 kbp. The location of the LTRs in D398 as determined by these procedures agrees with the location of the MPMV LTRs determined independently by different procedures (Barker et al., in press). The size of the MPMV LTRs determined by more accurate measurements is 350 base pairs (Barker et al., in press).

Comparison of restriction endonuclease cleavage patterns of pD398 with replicative intermediate DNA has not revealed any deletions in the cloned DNA under conditions where a 100-base-pair deletion should have easily been detected (Fig. 4). A summary of the restriction endonuclease

FIG. 6. Reduced cross-hybridization between D/New England and MPMV at high stringency. Viral DNA inserts (7.4 to 8.4 kbp) of cloned DNA were excised with the appropriate restriction endonuclease and analyzed by gel electrophoresis and Southern transfer hybridization under various degrees of stringency to a ^{32}P -labeled 7.8-kbp fragment insert of pD398. Hybridization took place in 30 to 60% formamide at 49°C in $6\times$ SSC as described in Wahl et al. (30). DNA (restriction endonuclease): pGaLV (*HindIII*); pSMRV-D (*EcoRI*); pMPMV-U1 (*SstI*); pD398 (*BamHI*); pT7.4 from *Herpesvirus saimiri* (*TaqI*). Standards are lambda DNA $\times$ *HindIII*.

fragments shown in Fig. 3 and 4 is given in Table 2. We have not found any discrepancies in restriction endonuclease sites between pD398 and replicative intermediate DNA.

Strain variability. Replicative intermediate Hirt supernatant DNA from MPMV-infected Raji cells as well as from cells infected with five different strains of D/New England were compared by examination of fragmentation patterns with *PstI*, *HindIII*, *HpaII*, *TaqI*, *AccI*, and a number of single-cut restriction endonucleases. The results of *PstI* and *HpaII* digestion of MPMV and three strains of D/New England (398, 114, and 225) DNA are shown in Fig. 5. Although D/New England isolates were easily distinguished from MPMV, these enzymes were not able to differentiate the D/New England isolates. After examination of over 30 restriction endonuclease sites, we have been unable to distinguish the D/New England isolates. Similar results were obtained with D/New England isolates 184 and 331.

Degree of homology along the D/New England and MPMV genomes. DNAs of MPMV, D398, gibbon ape leukemia virus, squirrel monkey type D (SMRV-D) and *Herpesvirus saimiri* (pT7.4) were excised from cloned plasmid DNAs and electrophoresed through agarose gels. DNA fragments were transferred to nitrocellulose and hybridized to a ^{32}P -labeled 7.8-kbp insert of pD398 under various degrees of stringency (Fig. 6). Hybridization in $6\times$ SSC at 49°C in 30 or 40% formamide revealed strong cross-homology between D/New England 398 and MPMV DNAs. However, when the formamide concentration was raised to 60%, considerably less hybridization of MPMV DNA was observed. No significant hybridization was observed between D/New England and gibbon ape leukemia virus or *Herpesvirus saimiri* pT7.4, even under conditions of low stringency ($6\times$ SSC, 39°C , 30% formamide). Cross-hybridization between D/New England and SMRV-D was very weak even under these low-strin-

FIG. 7. Reduced MPMV-D/New England cross-hybridization at high stringency localizes to *pol-env* regions. pMPMV-H4 cloned DNA was cut with the indicated restriction endonucleases and analyzed by gel electrophoresis and Southern transfer hybridization to a ^{32}P -labeled 7.8-kbp insert of pD398 in 60% formamide at 49°C as described in the legend to Fig. 6. MPMV viral DNA fragments which gave weak or no hybridization are shown schematically in Fig. 8.

gency conditions; about 100 times more SMRV-D than D398 DNA was required to produce equal intensity on the autoradiogram when ^{32}P -labeled pD398 DNA was used as the probe (data not shown). Previous studies have demonstrated cross-hybridizing sequences between MPMV and SMRV-D along the length of the genome, with the strongest homologies localized to the *pol* region (E. Hunter, C. Barker, J. Bradac, S. Chatterjee, R. Desrosiers, and J. Wills, in P. Furmanski, J. Hager, and M. Rich, ed., *RNA tumor viruses, oncogenes, human cancer and AIDS: on the frontiers of understanding*, in press).

To localize the regions of weakest homologies between D/New England and MPMV, fragments of MPMV DNA were Southern blotted and hybridized to D/New England DNA at very high stringency (Fig. 7). pMPMV-H4 DNA was first cut with *SstI* to excise the viral DNA, ethanol precipitated, and then cut with *PvuII*, *PstI*, *PvuII* plus *PstI*, or *HindIII*. Some fragments gave no detectable hybridization under the high-stringency conditions; these include the

1.8-kbp *PvuII* fragment, the 1.5-kbp *PvuII-PstI* fragment, and the 2.8-kbp *HindIII* fragment. Thus a contiguous stretch of 3.6 kbp in the *pol-env* region did not hybridize. The 2.3-kbp *HindIII* fragment gave very weak hybridization under these conditions, indicating an intermediate level of homology in the 1.5 kbp leftward from the 3.6-kbp region of weakest homology. The strongest homology was observed with fragments derived from the *gag* region. A schematic summary of these results is shown in Fig. 8. We did not examine the LTR region of MPMV separately, and, therefore, we do not know the level of homology in this 350-base-pair region.

DISCUSSION

Cloned DNA representing the complete genome of retrovirus D/New England has been prepared and characterized. This cloned DNA will be useful for further investigation of endemic disease in the New England colony as well as for comparison of D/New England with type D retrovirus isolates from other colonies. By comparison of pD398-cloned DNA with viral DNA in infected cells, we have shown the cloned DNA to represent permuted full-length replicative intermediate DNA with one LTR. We have demonstrated the relatedness of D/New England to MPMV by comparison of restriction endonuclease maps and by cross-hybridization of cloned DNAs. About 50% of restriction endonuclease sites are not conserved. These differences appear analogous to the considerable variation observed in gibbon ape leukemia virus isolates obtained from different colonies (9, 29). The weakest homology between D/New England and MPMV is localized to the presumed *pol-env* regions, and the strongest homology is localized to the presumed *gag* region. Fifty percent conservation of restriction endonuclease sites represents about 92% overall sequence homology, but the sequence homology would be expected to be higher in the *gag* region and lower in the *pol* and *env* regions. It will be important to determine how the macaque type D isolates from the other U.S. PRC colonies will compare, especially in view of the unique disease of the Seattle colony (28) and the greatest antigenic dissimilarity of type D retrovirus isolated from this Seattle colony (L. Arthur, J. Bess, R. Gilden, R. Benveniste, K. Stromberg, and P. Marx, manuscript in preparation).

A region of greatest divergence mapping to the *env* region is not surprising; however, the degree of divergence in the *pol* regions of MPMV and D398 was unexpected. *pol* sequences generally seem to be rather conserved (3), and in fact, the region of greatest homology between MPMV and SMRV-D maps to the *pol* region (Hunter et al., in press). These results raise questions as to the mechanism by which this divergence arose. Large amounts of endogenous sequences in primates homologous to D retrovirus genetic information have been described (1; Barker et al., in press; Hunter et al., in press). Whether some of the variation between D/New England and MPMV resulted from recombination with these endogenous sequences remains to be determined.

The lack of variation among individual macaque type D retrovirus isolates obtained from the New England colony is surprising when one considers the strain variation that can occur among viruses. The variation among gibbon ape leukemia virus and macaque type D isolates described above can be cited, as can the variation among other viruses. For example, any two isolates of *Herpesvirus saimiri* from the NERPRC squirrel monkey colony can be easily distin-

FIG. 8. Relative hybridization efficiency of MPMV DNA fragments to D398 DNA at very high stringency. The data were derived from the results shown in Fig. 7. The hybridization efficiency of the LTRs was not separately tested.

guished by using the same restriction endonuclease typing procedures that have not distinguished the five D/New England isolates that we have examined in greatest detail (7). The lack of variability is even more surprising when we consider that these isolates were obtained from three different species of macaques, *M. cyclopis*, *M. mulatta*, and *M. fascicularis*. It thus appears that a single strain of type D retrovirus is infecting three different species of macaques in the NERPRC colony. This suggests that introduction of the virus into the colony as a single event led to the observed infections in all three species.

Most D/New England isolates were obtained from *M. cyclopis*, despite the fact that *M. cyclopis* make up only a small fraction of the NERPRC macaque colony. It is notable that the T4/T8 ratios of juvenile and adult *M. cyclopis* are somewhat depressed when compared with those of juvenile and adult *M. mulatta* at the NERPRC (20). *M. cyclopis* also have a higher incidence of the macaque immunodeficiency syndrome than do other macaque species (20), and type D retrovirus was readily isolated from macaques with this syndrome. However, we must caution that the ability to isolate type D retrovirus from macaques with the immunodeficiency syndrome but not from healthy macaques does not provide evidence for a causative role for this virus in the disease. It is possible that a state of immunodeficiency allows reactivation or opportunistic replication of latent type D retrovirus. A causative role for D/New England in the macaque immunodeficiency syndrome would be suggested if evidence of prior infection was confined to the diseased monkey population, but we do not yet know the extent of type D retrovirus infection among the healthy, immunocompetent macaques of the NERPRC colony. Fine and Arthur, however, have described the presence of antibodies against the major core protein (p26) of MPMV among apparently healthy macaques of U.S. Primate Research Center colonies (8). Finally, experimental infection of juvenile macaques with D/New England has not induced the immunodeficiency syndrome (19).

Attempts to demonstrate type D retrovirus in 62 human acquired immune deficiency syndrome samples by a variety of procedures have been uniformly negative (E. Gelmann, B. Fleckenstein, R. Ruger, M. D. Daniel, N. Letvin, and R. Desrosiers, unpublished data).

It is important to study the immunodeficiency syndrome

of macaques and to identify the causative agent, since successful maintenance of macaques in captivity will depend upon the control of this disease. Additional benefit can be expected if the causative agent of the macaque disease is related to the virus that causes acquired immune deficiency syndrome in humans. The similarities between the human and macaque disease have been pointed out previously (21). If related viruses cause these two diseases, various approaches to vaccination and therapeutic intervention could be studied in a meaningful manner. In this regard, comparison of cloned D/New England DNA with HTLV-I, HTLV-II, and HTLV-III DNA has not revealed significant levels of homology even when low-stringency conditions are used (F. Wong-Staal, C. Butler, and R. Desrosiers, unpublished data). However, Homma et al. have noted that at least some of the macaques with immunodeficiency and lymphoproliferative disorders have serological evidence of prior infection with a virus related to HTLV (16). Continued investigation is needed to determine what role type D retrovirus plays in the macaque disease and how applicable the study of the macaque disease will be to human acquired immune deficiency syndrome.

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