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Mapping of the Fibrotropic and Lymphotropic Host Range Determinants of the Parvovirus Minute Virus of Mice

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The fibrotropic and lymphotropic strains of minute virus of mice are each unable to grow lytically in the differentiated host cell type of the other strain. To map the viral sequences responsible for the target cell specificities of the two strains, we constructed chimeric viral genomes in vitro from infectious genomic clones. The phenotypes of viral progeny derived from the chimeric genomes were tested by transfecting the plasmids into fibroblast monolayers and assaying plaque formation and by testing stocks of the recombinant viruses for cytotoxicity in fibroblast and lymphocyte cultures. Both the fibrotropic and lymphotropic determinants mapped to the same 237-nucleotide sequence within the coding region of the virus structural gene. A second sequence, near the viral promoter at map unit 38, was also shown to affect viral growth in fibroblast host cells profoundly.

The autonomous parvoviruses are a group of physically and chemically similar animal viruses with fetocidal and teratogenic potential. They can cause a variety of fetal and neonatal abnormalities by destroying specific differentiated cell populations that proliferate rapidly during the normal course of development (5, 17). Studies on the autonomous parvovirus growth cycle in cultured cells have provided a rationale for these in vivo observations. The predilection for rapidly dividing cell populations is due to a dependence on cellular functions expressed transiently during the S phase of the cell cycle and the inability of the virus to induce resting cells to enter the S phase. The observation that only certain proliferating cell populations are infected during fetal and neonatal development reflects the modulation of lytic parvovirus growth by developmentally regulated components operating at the cellular level (5, 17).

The two strains of minute virus of mice (MVM), called allotropic variants, are a well-characterized model system for analyzing the molecular basis of parvovirus differentiation-dependent target cell specificity. MVM(p), the prototype strain, productively infects mouse fibroblasts, whereas the immunosuppressive strain, MVM(i), productively infects mouse T lymphocytes (22) and suppresses a number of T-cell-mediated functions in vitro (6). Each virus strain is restricted for growth in the host cell type of the other. The two strains are serologically indistinguishable, and they bind to and compete for the same cell surface receptor (19). In both restrictive infections—MVM(p) into T lymphocytes and MVM(i) into fibroblasts—virus particles are internalized and viral DNA replication is significant but reduced (18, 19). Because of a block at the level of transcription initiation, the amount of viral RNA present in restrictive infections is reduced to a few percent of the level seen in productive infections (E. M. Gardiner, B. A. Spalholz, D. C. Ward, and P. Tattersall, manuscript in preparation). It appears, therefore, that the developmentally controlled cellular factors which interact differentially with the two MVM strains are necessary for establishment of the productive viral transcription program.

The encapsidated MVM genome is a 5-kilobase linear single-stranded DNA molecule with short terminal palindromes (5). After virus entry, the single-stranded genome is converted to a duplex replicative form (24), which is infectious when transfected into cultured cells (11). Infectious genomic clones of both MVM(p) and MVM(i) have been constructed (7, 11). The DNA sequences of the genomes of the two strains are 97% homologous, and the nucleotide differences between them, primarily single-base changes, are scattered throughout the genomes, as diagrammed in Fig. 1 (2, 16).

Transfections of the MVM(p) and MVM(i) genomic clones into cultured cells yield viral progeny with the fibrotropic and lymphotropic phenotypes, respectively (7). This confirms that the determinant of virus host range is encoded in the viral genome. The additional finding that it is possible to isolate, at low frequency, stable mutants of each variant which have either an extended or an altered host range (15; Tattersall, unpublished observations) supports this conclusion. The molecular nature of this genetic component, termed the allotropic determinant, which differs between the two MVM strains and specifies the productive host cell type for each virus, has not been directly addressed in previous studies.

The analysis of recombinant viruses is an approach which has been successfully used in a number of viral systems to map and characterize viral functions. By using this approach, Parrish and Carmichael have mapped the mutations responsible for a canine parvovirus (CPV) host range mutation to a region of the viral capsid gene (13). As the next step in our analysis of the MVM allotropic determinant(s), a series of recombinant viral genomes were generated by exchanging restriction fragments between infectious clones of the genomes of MVM(p) and MVM(i). The target cell specificities of the viral progeny generated from the chimeric genomes were determined by direct transfection of the clones for plaques in fibroblast monolayers and by infection of lymphocyte and fibroblast cultures with recombinant virus stocks.

MATERIALS AND METHODS

Bacterial strains. Ligation products were routinely transfected into *Escherichia coli* LE392. When unmethylated

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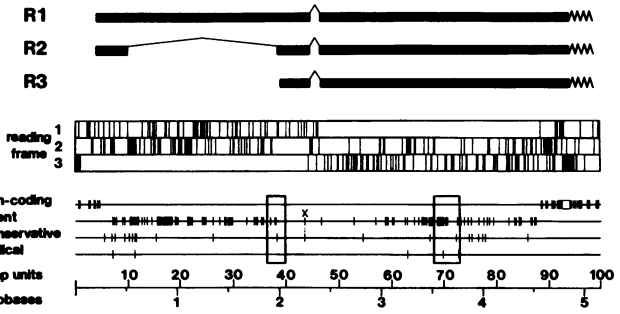


FIG. 1. Comparison of the genomes of MVM(i) and MVM(p). Genetic map of MVM showing the distribution of nucleotide differences between the allotropic variants MVM(p) and MVM(i). The viral genome is displayed with the 3′ end of virion single-stranded DNA, the negative strand with respect to transcription, on the left at 0 map units. At the top of the diagram the three major cytoplasmic transcripts R1, R2, and R3 are represented by solid blocks, with single wavy lines indicating their polyadenylated tails and single straight lines indicating the introns spliced out in the production of mature mRNA. Beneath the transcripts is a block diagram in which vertical bars represent translation termination codons; the diagram shows the regions of open reading frame in all three frames of the transcribed (or complementary) DNA strand. The horizontal line labeled noncoding uses vertical bars to indicate nucleotide differences between MVM(p) and MVM(i) in the noncoding region of the genome, while the three lines below it similarly indicate nucleotide changes in the coding sequences which are silent, conservative, or radical with respect to the encoded amino acid. The bar labeled X denotes a conservative change in the sequence of NS-1 which does not affect the sequence of NS-2. The boxed regions between map units 37 and 40 and map units 68 and 73 contribute significantly to virus phenotype, as described in the text.

plasmid DNA was needed for further chimera construction, plasmids were transferred into the *dam* strain GM33. Some clones were propagated in the *recBC sbcB recF* host strain JC8111 to minimize deletion of right-hand palindromic sequences (4).

Construction and genotypic characterization of recombinant genomic clones. Plasmids were constructed from pMM984, the infectious MVM(p) clone (11), from pMVMp, a derivative of pMM984 which has a *Cla*I site at the right-hand end of the viral insert, from the MVM(i) infectious clone pMVMi, and from subgenomic clones of the MVM(i) termini (7) by standard methods (10). These parental plasmids are diagrammed in Fig. 2. Figure 3 shows the maps of the recombinant genomic plasmids, and Table 1 lists the restriction fragments from which each was generated. At the top of Fig. 3 is a diagram of the polymorphic restriction sites which were used to distinguish MVM(i) and MVM(p) genomic segments. The particular polymorphic restriction sites which were used in checking the genotypes of each recombinant are indicated on the individual chimeric genomes. Also indicated at the right-hand ends of the maps is a difference in the lengths of the MVM(i) and MVM(p) genomes. This difference is due to the existence at map unit 92 of a tandem duplication in MVM(p) of a 65-base sequence present only once in MVM(i) (2, 16). This length difference was used as a genotypic marker in analysis of all the recombinant genomes.

The identities of the individual clones were carefully documented by restriction endonuclease and fragment length analysis. With knowledge of the origins of the restriction fragments used in chimera construction (Fig. 2 and

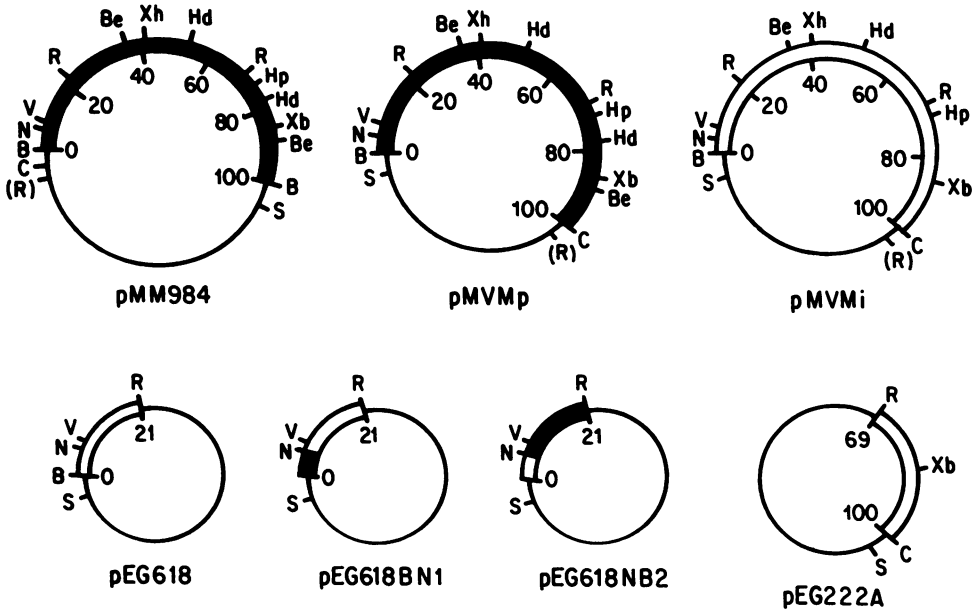


FIG. 2. Genomic and subgenomic plasmids used in chimeric MVM plasmid constructions. Solid blocks represent sequences derived from MVM(p), open blocks indicate MVM(i) sequences, and thin lines represent plasmid sequences. pMM984 is the MVM(p) infectious clone (11). Plasmids pEG222A, pEG618, pMVMp, and pMVMi have been described elsewhere (7); pEG618BN1 and pEG618NB2 were constructed by replacing the *SalI-NcoI* and *NcoI-EcoRI* fragments in pEG618, respectively, with the corresponding fragments from pMVMp. The vector in pMM984 is pBR322; all other clones possess pAT153 vector sequences (23). Restriction site abbreviations: B, *Bam*HI; Be, *Bst*EII; C, *Cla*I; D, *Dra*I; He, *Hae*II; Hd, *Hin*dIII; Hf, *Hin*fI; Hh, *Hha*I; Hp, *Hpa*I; N, *Nco*I; P, *Pst*I; R, *Eco*RI; S, *Sal*I; S3, *Sau*3AI; V, *Eco*RV; Xb, *Xba*I; Xh, *Xho*I; Xm, *Xmn*I. The *Sal*I site is in vector sequences.

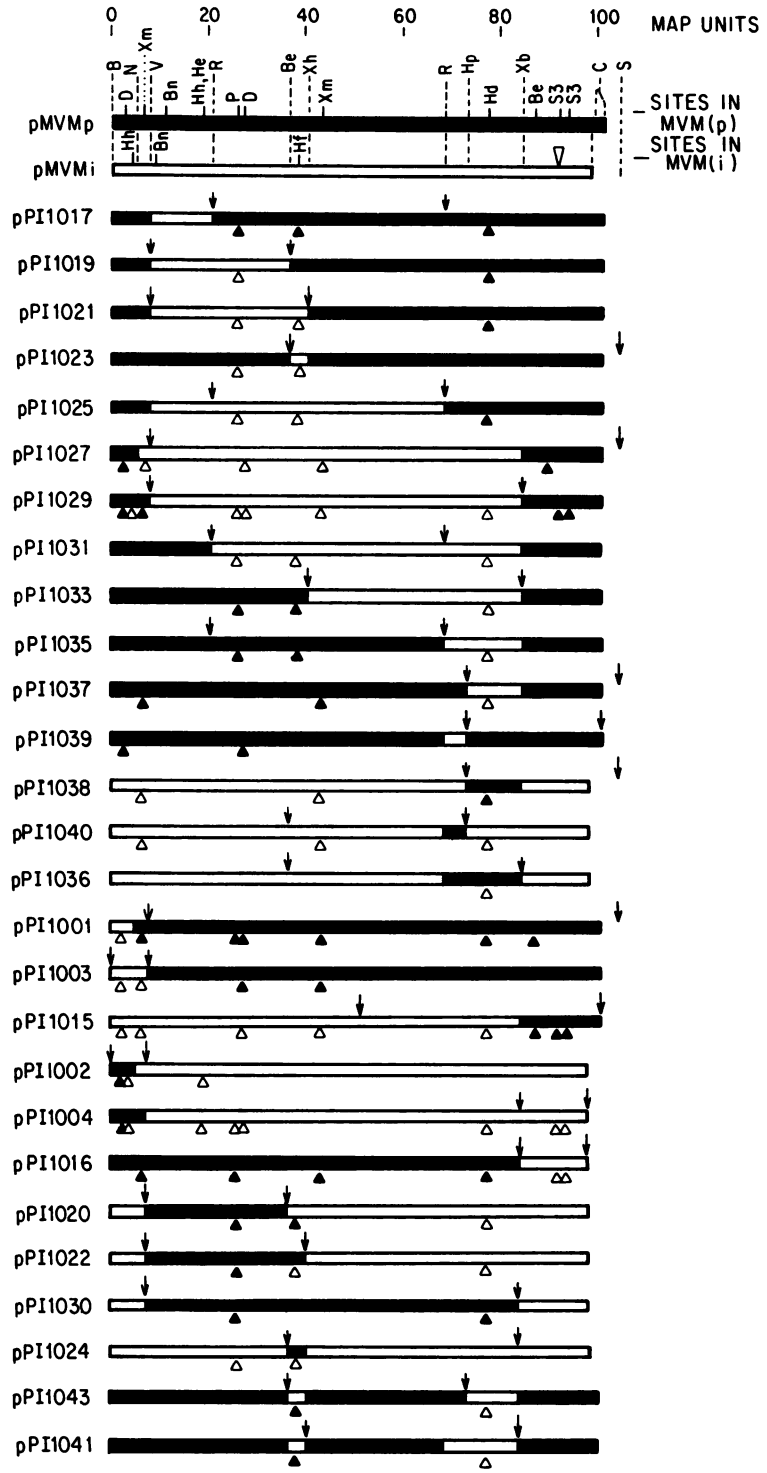


FIG. 3. Restriction sites used in construction and characterization of chimeric MVM plasmids. Chimeric genomes with MVM(p) sequences at the right-hand end are designated by odd numbers, with even numbers denoting recombinants with MVM(i) right-end sequences. Arrows above the chimeric genomes indicate the restriction sites that were used to construct the recombinants. Arrowheads below the genomes denote restriction site polymorphisms that were confirmed in identifying the individual chimeras. An open arrowhead denotes the absence of a site; a solid arrowhead indicates that the site is present. Shaded blocks denote MVM(p) sequences, and open blocks represent MVM(i), as in Fig. 2. The inverted triangle near the right end of the MVM(i) genome indicates the 65-nucleotide sequence which is present twice in MVM(p) but only once in MVM(i) (2, 16). Restriction enzyme symbols are explained in the legend to Fig. 2, plus Bn, *Bst*NI.

TABLE 1. Restriction fragments used in construction of chimeric viral plasmids[a]

Plasmid	Parent A	Fragment	Map units	Parent B	Fragment	Map units
pPI1001	pEG618BN1	*Sal*I-*Eco*RV	0–7	pMVMp	*Eco*RV-*Sal*I	7–100
pPI1002	pEG618NB2	*Bam*HI-*Eco*RV	0–7	pPI1004	*Eco*RV-*Bam*HI	7–100
pPI1003	pEG618	*Bam*HI-*Eco*RV	0–7	pMVMp	*Eco*RV-*Bam*HI	7–100
pPI1004	pPI1029A	*Cla*I-*Xba*I	0–84	pEG222A	*Xba*I-*Cla*I	84–100
pPI1015	pMVMi	*Cla*I-*Hin*dIII	0–52	pPI1029A	*Hin*dIII-*Cla*I	52–100
pPI1016	pMVMp	*Cla*I-*Xba*I	0–84	pMVMi	*Xba*I-*Cla*I	84–100
pPI1017	pMM984	*Eco*RI-*Eco*RI	21–68	pPI1021	*Eco*RI-*Eco*RI	0–21, 68–100
pPI1019	pMVMi	*Eco*RV-*Bst*EII	7–37	pMVMp	*Bst*EII-*Eco*RV	0–7, 37–100
pPI1020	pMVMp	*Eco*RV-*Bst*EII	7–37	pMVMi	*Bst*EII-*Eco*RV	0–7, 37–100
pPI1021	pMM984	*Xho*I-*Xba*I	40–84	pPI1029	*Xba*I-*Xho*I	0–40, 84–100
pPI1022	pMVMp	*Eco*RV-*Xho*I	7–40	pMVMi	*Xho*I-*Eco*RV	0–7, 40–100
pPI1023	pMVMp	*Sal*I-*Bst*EII	0–37	pPI1021	*Bst*EII-*Sal*I	37–100
pPI1024	pPI1033	*Bst*EII-*Xba*I	37–84	pMVMi	*Xba*I-*Bst*EII	0–37, 84–100
pPI1025	pMM984	*Eco*RI-*Eco*RI	21–68	pPI1021	*Eco*RI-*Eco*RI	0–21, 68–100
pPI1027	pEG618NB2	*Sal*I-*Eco*RV	0–7	pPI1029A	*Eco*RV-*Sal*I	7–100
pPI1029	MVM(i) RF	*Eco*RV-*Xba*I	7–84	pMM984	*Xba*I-*Eco*RV	0–7, 84–100
pPI1030	pMVMp	*Eco*RV-*Xba*I	7–84	pMVMi	*Xba*I-*Eco*RV	0–7, 84–100
pPI1031	pPI1029	*Eco*RI-*Eco*RI	21–68	pPI1033	*Eco*RI-*Eco*RI	0–21, 68–100
pPI1033	pMM984	*Eco*RV-*Xho*I	7–40	pPI1029A	*Xho*I-*Eco*RV	0–7, 40–100
pPI1035	pMM984	*Eco*RI-*Eco*RI	21–68	pPI1033	*Eco*RI-*Eco*RI	0–21, 68–100
pPI1036	pPI1025	*Bst*EII-*Xba*I	37–84	pMVMi	*Xba*I-*Bst*EII	0–37, 84–100
pPI1037	pMVMp	*Sal*I-*Hpa*I	0–73	pPI1035	*Hpa*I-*Sal*I	73–100
pPI1038	pMVMi	*Sal*I-*Hpa*I	0–73	pPI1016	*Hpa*I-*Sal*I	73–100
pPI1039	pPI1035	*Cla*I-*Hpa*I	0–73	pMVMp	*Hpa*I-*Cla*I	73–100
pPI1040	pPI1025	*Bst*EII-*Hpa*I	37–73	pMVMi	*Hpa*I-*Bst*EII	0–37, 73–100
pPI1041	pPI1023	*Xho*I-*Xba*I	40–84	pPI1035	*Xba*I-*Xho*I	0–40, 84–100
pPI1043	pPI1037	*Bst*EII-*Hpa*I	37–73	pPI1023	*Hpa*I-*Bst*EII	0–37, 73–100

[a] Parent B indicates the fragment which encodes the genomic right-hand end of a chimeric clone. Parent A encodes the remaining sequences.

Table 1), it was possible to confirm unequivocally the identities of all the recombinant genomes except pPI1037 and pPI1040. Because of a lack of diagnostic restriction site polymorphisms, each of these recombinants can be distinguished from only one of its parents. Fortunately, in each case the phenotype of the daughter chimeric virus differs significantly from that of the parent in question. Because of this observation and other consistent phenotypic data, and because rPI1040 DNA and RNA hybridize to oligonucleotide probes specific for the MVM(p) sequence from nucleotides 3575 to 3594 and not to the MVM(i) sequence (data not shown), we are confident of the identities of pPI1037 and pPI1040.

Here and throughout this report, nucleotide numbers are those of the MVM(p) sequence. The prefix pPI indicates a chimeric genomic plasmid, whereas rPI precedes the number of the corresponding virus stock.

Cell lines. A9 ouab^{r}11 cells are a mouse fibroblast line and S49 1TB2 is a T-cell lymphoma line; 324K is a simian virus 40-transformed human newborn kidney cell line in which both MVM strains form plaques. Hybl/11-iD5 is a subclone of a somatic cell hybrid between A9 ouab^{r}11 cells and the EL4 mouse T lymphoma line and is a productive host cell line for both MVM strains. Culture conditions and origins of cell lines have been described in detail elsewhere (7).

Virus strains. The cloning of MVM(i) by terminal dilution in lymphocyte cultures has been described (22), as has the cloned stock of the prototype strain MVM(p) (21). Standard virus stocks were prepared by low-multiplicity infection of the appropriate host cell, S49 1TB2 for MVM(i) and A9 ouab^{r}11 for MVM(p), as described elsewhere (22).

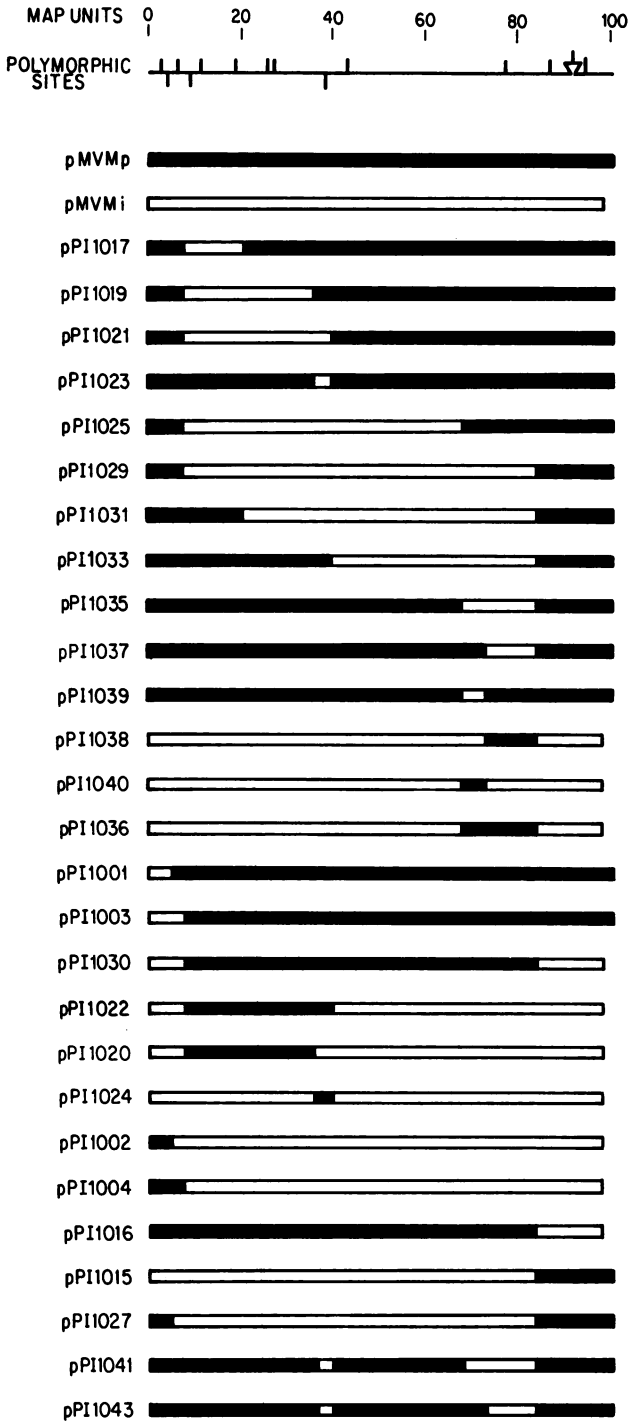
Infectivity assays. Calcium phosphate transfections for plaques were performed as previously described (11). Each plasmid preparation of every clone was shown to possess nondeleted sequences in the genomic right-hand palindrome (11), and in all experiments, uncut plasmids were transfected.

In the MTT cytotoxicity assay, cultures of A9 ouab^{r}11 or S49 1TB2 cells were infected at various multiplicities at an initial seeding density of 10^3 cells per well in 96-well microtiter plates. After 7 days of culture, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma Chemical Co.) dissolved in phosphate-buffered saline was added to a final concentration of 0.45 mg/ml, and the cultures were incubated for 2 h at 37°C. The medium was then removed from the wells, and acid isopropanol (0.04 N HCl in isopropanol) was added to solubilize the formazan product. After an hour at room temperature, the plates were read on a Dynatech MR600 microplate reader at a test wavelength of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99 (8, 12).

Preparation of chimeric virus stocks. Hybl/11-iD5 cells (5×10^5) were transfected with calcium phosphate coprecipitates containing 20 μg of MVM plasmid DNA. When transfected cultures showed significant cytopathic effect, lysates were prepared in TE8.7 (50 mM Tris, pH 8.7, 0.5 mM EDTA) and used to infect larger cultures. After three more infection cycles, cleared TE8.7 lysates were prepared, and titers were determined by standard methods.

RESULTS

Transfection efficiencies of chimeric viruses in 324K monolayers. All but three of the chimeric genomes were infectious in 324K cells, generating plaques after transfection into monolayers of these nonselective cells. The various efficiencies with which the recombinants formed plaques fell into no simple pattern (Fig. 4). Since the sequences of the two viral genomes, which differ at 163 nucleotides, have been naturally selected for infectivity in different cellular



	TRANSFECTION EFFICIENCY IN PFU/μG		PLAQUE MORPHOLOGY
	324 K	A9OUAB^{R}II	A9OUAB^{R}II
pMVMp	70	300	WILD TYPE
pMVMi	54	<0.05	N.P.
pPI1017	48	64	SMALL
pPI1019	17	51	SMALL
pPI1021	59	4	PINPOINT
pPI1023	49	4	PINPOINT
pPI1025	24	1	PINPOINT
pPI1029	9	<0.05	N.P.
pPI1031	2	<0.05	N.P.
pPI1033	7	<0.05	N.P.
pPI1035	11	<0.05	N.P.
pPI1037	6	80	WILD TYPE
pPI1039	<0.05	<0.05	N.P.
pPI1038	<0.05	<0.05	N.P.
pPI1040	20	91	PINPOINT
pPI1036	11	89	PINPOINT
pPI1001	>67	300	WILDTYPE
pPI1003	142	360	WILDTYPE
pPI1030	49	570	WILDTYPE
pPI1022	24	<0.05	N.P.
pPI1020	8	<0.05	N.P.
pPI1024	10	<0.02	N.P.
pPI1002	2	<0.05	N.P.
pPI1004	2	<0.05	N.P.
pPI1016	>53	27	WILDTYPE
pPI1015	18	<0.05	N.P.
pPI1027	8	<0.07	N.P.
pPI1041	<0.05	<0.05	N.P.
pPI1043	1	9	PINPOINT

FIG. 4. Transfection efficiencies of MVM plasmids. Monolayers of 324K or A9 ouab^{r}11 cells were transfected with calcium phosphate coprecipitates with up to 20 μg of uncut plasmid and overlaid with agarose-containing medium. Six days later, plaques were visualized by neutral red staining and counted. Except for pPI1003 and pPI1027, at least two independent clones of each construction were tested for infectivity and phenotype. Plaque morphology in 324K monolayers is less distinct than in A9 ouab^{r}11 or Hybl/11-iD5 monolayers and therefore was not scored for phenotype. NP, No plaques after application of 20 μg of uncut plasmid. Other symbols are as in Fig. 3.

environments, it is not surprising that exchanging sequences between the two strains generated viruses that were less or, in at least one case (pPI1003), more efficient than the parents from which they were derived.

The nonviable double recombinant plasmid pPI1041 combined two single reassortments which individually reduced virus plaquing ability compared with that of the parent clone pMVMp (Fig. 4). Replacement of the region in the MVM(p) genome around the promoter at map unit 38 with the MVM(i) sequence reduced the size of the transfection plaques, particularly in mouse fibroblasts (pPI1023), but also in Hybl/11-iD5 monolayers (data not shown). The second replace-

ment, of MVM(i) sequences for MVM(p) sequences in the coding portion of the capsid gene, significantly decreased the 324K transfection efficiency (pPI1035). The pairing of these two nonlethal switches in the pPI1041 genome appeared to create a chimeric virus with infectivity so reduced that it was unable to generate plaques in this assay. Whether this inability to rise above a threshold of plaque-forming activity was due to insufficient levels of gene expression, to structural alterations in viral gene products, or to both factors is not known.

Transfection of up to 20 μg of the nonviable single recombinant genomes pPI1038 and pPI1039 into 324K monolayers also failed to generate plaques. In addition, no detectable infectious virus was generated after they were transfected into cultures of S49 1TB2 or A9 ouab^{r}11 cells (data not shown). Extensive restriction endonuclease analysis of these two chimeras detected no obvious cloning artifacts to explain their nonviability, and transfections of independent isolates of each clone were consistently negative for infectivity in these assays.

It is not known why these two constructions did not yield viral progeny. It is possible that the juxtaposition of the MVM(i) polypeptide sequence to the left of the *Hpa*I site and the MVM(p) sequence to the right of the *Hpa*I site yielded chimeric capsid proteins that were sterically incompatible with the formation of infectious particles. A poliovirus intertypic recombinant involving an exchange of sequence in a capsid gene similarly failed to yield viable viral progeny (20). These authors postulated that the nonviability was due to amino acid changes which prevented correct assembly or functioning of the capsid.

Transfection efficiencies in mouse fibroblast monolayers. Although almost all of the chimeric genomes were infectious in 324K cells, a very different result was obtained when they were transfected into mouse fibroblast monolayers. The recombinants displayed a variety of plaque phenotypes and plaquing efficiencies in A9 ouab^{r}11 cells (Fig. 4). The results of these transfections identified two small sections of the genome that had particularly striking effects on plaque formation in mouse fibroblasts.

The first region of interest lay between the *Bst*EII site at nucleotide 1886 and the *Xho*I site at nucleotide 2072 (Fig. 3). In recombinants which form plaques in mouse fibroblasts, the presence of MVM(i) sequences in this position consistently resulted in the formation of "pinpoint" plaques (Fig. 4; note particularly recombinants pPI1021, pPI1023, and pPI1025). In two cases (recombinants pPI1023 and pPI1040), the sizes of plaques generated by virus stocks were tested, and in each case the recombinant virus particles generated pinpoint plaques in both A9 ouab^{r}11 and Hyb1/11-iD5 monolayers. Furthermore, stocks of nonfibrotropic chimeric viruses (rPI1035 and rPI1024) also generated tiny plaques in Hyb1/11-iD5, as did MVM(i) (data not shown).

The second region delineated by the data in Fig. 4 was a 237-nucleotide portion of the gene encoding the virus structural proteins. This sequence, defined by recombinants pPI1035, pPI1037, and pPI1040 and mapping between the *Eco*RI and *Hpa*I restriction sites at nucleotides 3522 and 3759, respectively, was necessary for expression of the fibrotropic phenotype in this assay. Chimeric genomes that had MVM(i) sequences here were incapable of generating plaques after transfection of up to 20 μg of plasmid into mouse fibroblasts, although they were infectious in the nonselective cell line 324K. More significantly, replacement of this region of MVM(i) with the corresponding sequence from MVM(p) created a chimera (pPI1040) that formed plaques efficiently in A9 ouab^{r}11 cells. No other region of MVM(p) when transferred into the MVM(i) background was capable of conferring the fibrotropic phenotype in this assay.

Cytotoxicities in mouse lymphocyte and fibroblast cultures. The ability of a virus to generate plaques in a cell monolayer is a fairly stringent test of its infectivity in that cell line. Unfortunately, we have been unable to develop a plaque assay specific for the lymphotropic MVM strain with the nonattaching T-lymphocyte line. We therefore used the MTT cytotoxicity assay to test for the lymphotropic phenotype. In this colorimetric assay, the extent of conversion of a yellow tetrazolium dye, MTT, to a purple formazan crystal directly correlates with the number of viable cells present in the culture (8, 12). In the T-lymphocyte line S49 1TB2 (Fig. 5A), infection with a virus preparation of rPI1035, which contains MVM(i) sequences only in the region of the fibrotropic determinant mapped above, killed lymphocytes almost as efficiently as MVM(i) did. Conversely, rPI1040 virus, which carries the fibrotropic determinant, had very little effect on T lymphocytes except at very high multiplicities. The phenotypes of the other two recombinant stocks tested were consistent with these observations. The ability to kill mouse T lymphocytes therefore mapped in this assay to the same region of the MVM genome as did the fibrotropic determinant mapped by plaque assay.

The MTT assay of the cytotoxicities of the recombinants in A9 ouab^{r}11 cultures (Fig. 5B) did not present such a clear picture of the fibrotropic determinant as did the plaque assay results reported in Fig. 4. The minimal fibrotropic recombinant identified by plaque assay, rPI1040, killed A9 cells more efficiently than its approximate reciprocal rPI1035 but less effectively than either rPI1024 or rPI1023, which killed the fibroblasts quite well. Recombinant rPI1040 carries the MVM(i) sequence throughout the body of the structural genes. Correlation in this experiment between fibroblast cytotoxicity and the presence or absence of any single region of the MVM(p) or MVM(i) genome was not apparent. Possible reasons for this result are discussed below.

DISCUSSION

MVM phenotypic assays. The ideal assay for MVM target cell specificity would test the ability of a particular virus strain to spread through and destroy a cell population. The plaque assay fulfills these criteria fairly well. Formation of a virus plaque is initiated by a single infected or transfected cell and results from several rounds of virus infection in a defined location in a cell monolayer. The presence of a plaque is therefore a concrete demonstration of multiple infectious cycles. By this test, only one portion of the MVM(p) genome, lying between nucleotides 3522 and 3759, is capable of imparting the fibrotropic phenotype.

Because the MTT assay measures virus infectivity as a function of inhibition of cell growth, data from this assay can be subject to more than one interpretation. A reduction in the number of live fibroblasts at one time point 7 days after infection, as measured here, may be the result of gradual destruction of the culture by multiple rounds of relatively inefficient virus infection. However, the same effect would be observed if the cell population was depleted in the initial infection by a restricted virus that cannot produce progeny but does express a small amount of cytotoxic product potent enough to kill a fraction of the original cell population, and therefore to register in the MTT assay.

This single MTT experiment does not identify what event(s) in the rPI1024 infection is more efficient than in the

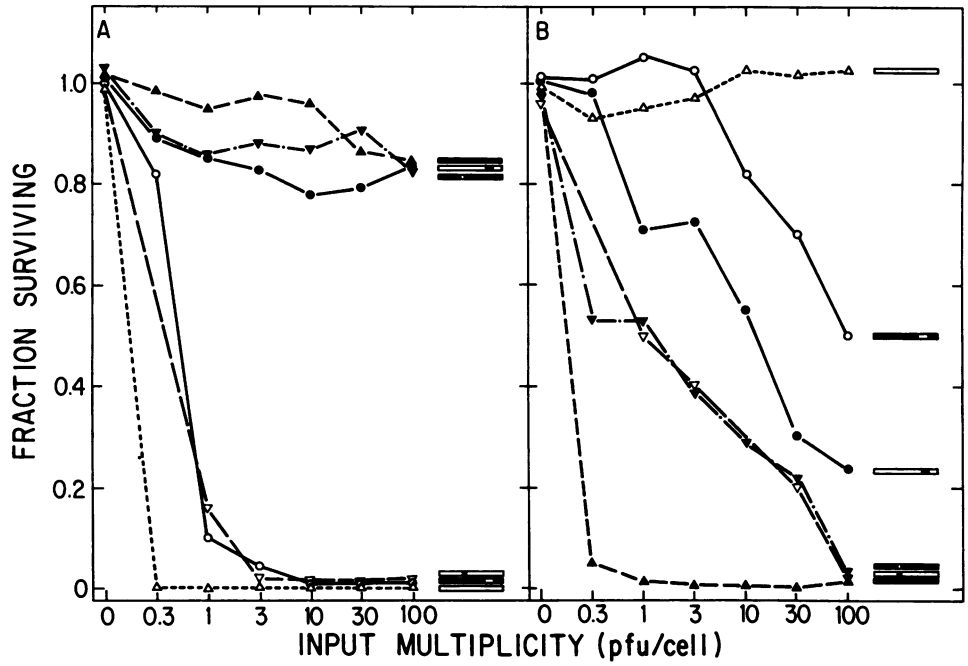


FIG. 5. MTT viral cytotoxicity assays in T lymphocytes and in fibroblasts. Cultures were infected at increasing multiplicities with wild-type and recombinant viruses and cultured for 7 days. The fraction of surviving cells was calculated by dividing the absorbance value obtained for infected cultures by the value for uninfected cultures. Symbols: ▲, MVM(p); △, MVM(i); ▼, rPI1023; ▽, rPI1024; ○, rPI1035; ●, rPI1040. Diagrams of the viral genomes are shown to the right of the curves to which they correspond. Solid and open blocks denote MVM(p) and MVM(i) sequences, respectively. (A) S49 1TB2 T lymphocytes; (B) A9 ouab^{r}11 fibroblasts.

parallel rPI1040 infection. These results clearly warrant further investigation because they suggest that the sequence of the MVM(p) P38 region significantly affects the ability of a virus to kill mouse fibroblasts even though it may not be able to form plaques on monolayers of these cells. Because the plaque assay measures multiple cycles of virus infectivity less ambiguously than does the MTT assay, however, the ability to form plaques in fibroblasts is the better indicator of the fibrotropic phenotype. We conclude, therefore, that the sequence which maps in the structural gene is the primary determinant of MVM(p) target cell specificity.

Logically, this reasoning should also be applied to the mapping of the lymphotropic determinant. Since it is not possible to measure plaque formation in the lymphocyte cell line, however, the less-informative assay must be used. Fortunately, the clearcut results of this experiment are entirely consistent with the coincidence of the two host range determinants.

MVM(i) and MVM(p) sequences in the P38 region. The sequence between nucleotides 1886 and 2072 is in a region which, because of the existence of transcriptional control and RNA-processing signals and overlapping reading frames, is very highly conserved between the two MVM strains. The 186-nucleotide fragment delineated here, enclosed by the left-hand box in Fig. 1, includes the P38 promoter, the R3 RNA cap site, the 3′ splice site for the large intron, and a portion of the colinear reading frames encoding the carboxy-terminal portions of the nonstructural proteins NS-1 and NS-2 (Fig. 6). There are only four nucleotide differences between the two MVM genomes in this sequence, and none of them fall within regions of homology to consensus transcriptional control sequences. Similarly, neither the R3 5′ end nor the 3′ splice site for R2 is affected by the changes. All four of the changes lie within the large intron and thus affect only the NS-1 coding sequence (Fig. 1). Of these, only the fourth change affects the encoded polypeptide sequence, replacing a leucine with a serine residue. In the absence of direct experimental analysis of the question, it is not possible to define which of the four nucleotide changes is (are) responsible for the reduction in plaque size or the behavior of rPI1024 in the MTT fibroblast cytotoxicity assay.

MVM(i) and MVM(p) sequences in the capsid region allotropic determinant. Because the plasmid pPI1039 failed to produce viable viral progeny, the minimum amount of MVM(i) in this tested region is that extending from the *Eco*RI site at nucleotide 3522 to the *Xba*I site at 4342. In the additional 583 nucleotides of MVM(i) which are carried in rPI1035, there are a further 17 single base changes between the two strains. Thus, rPI1035 alone does not provide unequivocal evidence that the ability to kill lymphocytes and the fibrotropic determinant map to the same 237-nucleotide fragment. However, the fact that rPI1040, which carries the MVM(i) sequence in the 583-base region between the *Hpa*I and *Xba*I sites (Fig. 2), did not kill S49 1TB2 cells strongly supports the conclusion that the lymphotropic determinant lies outside the 583-nucleotide region and within the same 237-base-pair region as the fibrotropic determinant (Fig. 2).

This region is boxed in Fig. 1, and the DNA sequence of these 237 nucleotides is shown in Fig. 7. There are 16 nucleotide differences between the two strains in this fragment, 3 of which alter the encoded capsid protein amino acid sequence. The three protein changes occur at nucleotides 3620, 3743, and 3756 and result in serine to proline, threonine to alanine, and glycine to glutamate conversions [(MVM(p) to MVM(i)], respectively. There is also a hypervariable stretch of 25 residues between nucleotides 3577 and 3601 in which eight changes occur, all silent with respect to the coding sequence because each occupies the third position of a codon triplet. Which of the 16 differences between the two

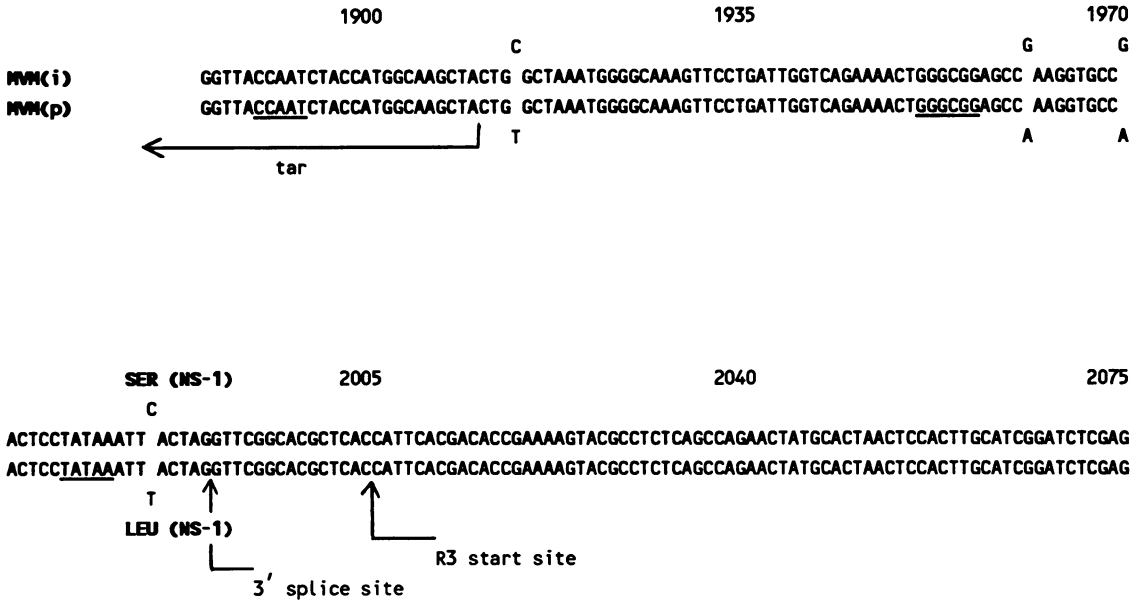


FIG. 6. DNA sequence of the P38 region. Sequences of the *Bst*EII-*Xho*I fragments from MVM(i) and MVM(p). Nucleotide differences are displaced above and below the lines, and one predicted amino acid difference between the viral strains is shown. The DNA sequence through this region of pMVMi has been confirmed in this laboratory and matches that reported by Sahli et al. (16) rather than that reported by Astell et al. (2). Consensus transcriptional control sequences are underlined, and the 3′ splice site (9), R3 cap site (3), and transactivation target region (tar), as determined for the closely related virus H-1 (14), are indicated.

strains is (are) responsible for the different host cell specificities which map to this region is not yet known, although similar work on CPV suggest that the threonine to alanine and glycine to glutamate changes at positions 3743 and 3756 may be coordinate and responsible for the host range difference between the two MVM strains.

The CPV host range mutation mentioned in the introduction maps to a sequence of the viral coat protein gene close

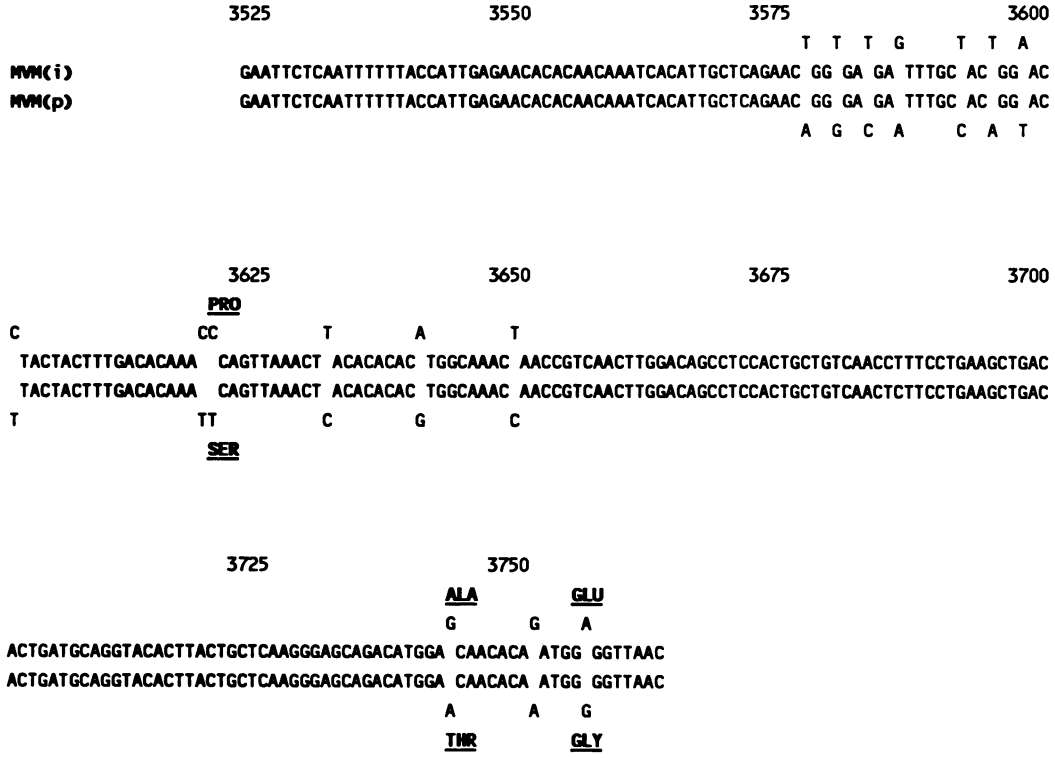


FIG. 7. DNA sequence of the region encoding the MVM fibrotropic determinant. Sequences of the *Eco*RI-*Hpa*I fragments from MVM(i) and MVM(p). Nucleotide differences are displaced above and below the lines, and amino acid differences are also indicated.

to the region encoding the MVM allotropic determinant and also results in two amino acid substitutions, threonine to isoleucine and alanine to aspartate (13). Interestingly, this sequence change can be detected by a monoclonal antibody that both neutralizes virus infectivity and inhibits erythrocyte hemagglutination, making it likely that the amino acid changes directly or indirectly alter an exposed portion of the virus capsid. As in the MVM restrictive interactions, infection by the CPV mutant is not blocked at the cell surface, and it is characterized by a diminished level of viral replicative form synthesis. Because viral RNA synthesis has not been studied in the CPV infection, however, it is not known whether the block is the same as that in the MVM restrictive infection.

Recombinant mapping studies and DNA sequence analysis of an extended host range mutant of MVM(i), *hr*101, selected by its ability to form plaques in fibroblasts, have also detected coordinate sequence changes at nucleotides 3743 and 3756, resulting in alanine to threonine and glutamate to alanine (MVM(i) to *hr*101) amino acid changes, respectively (R. Moir and P. Tattersall, unpublished data). These results, together with the observation that transfection of MVM(p) and MVM(i) genomic clones bypasses the block to lytic infection in restrictive virus-cell combinations (7), strongly suggest that the capsid protein plays a role in the target cell specificity of the virus. Since, for MVM, this is mediated after entry of the virus but before expression of the coat protein gene, this implies that the incoming capsid interacts with a developmentally regulated host cell factor to regulate viral gene expression.

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ADDENDUM

After submission of this manuscript, Antonietti et al. (1) reported that the MVM(i) lymphotropic determinant maps between map units 69 and 85.

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