Two Amino Acid Substitutions within the Capsid Are Coordinately Required for Acquisition of Fibrotropism by the Lymphotropic Strain of Minute Virus of Mice

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Nucleotide changes at both codons 317 and 321 in the VP2 capsid gene of the immunosuppressive strain of the murine parvovirus minute virus of mice, MVM(i), are required to create a virus capable of growing in A9 fibroblasts. This double mutant virus, ILB1, has growth characteristics very similar to those of the prototype fibrotropic strain MVM(p) in both single- and multiple-round infections of fibroblasts and is about 100-fold better at infecting fibroblasts than MVM(i). When only one nucleotide position is changed, either in codon 317 (as in ILB2) or in codon 321 (as in ILB3), the resulting viruses are less than twice as efficient as their parent MVM(i) at infecting fibroblasts. In the restrictive infection of A9 cells by the single mutants and MVM(i), gene expression and DNA replication were markedly reduced compared with ILB1 infection of the same cells or compared with infections of permissive hybrid cells by each of the viruses. This suggests that restriction acts predominantly at an early step in the infection. Since the phenotypes of ILB2 and ILB3 are essentially indistinguishable in restrictive infections, it is most likely that the individual loci affect the same step in the viral life cycle. The dramatic increase in fibroblast infectivity shown by ILB1 indicates a synergistic interaction between these two amino acid residues in the same rate-limiting process in fibroblast infection.

Minute virus of mice (MVM), an autonomous parvovirus, is a small, nonenveloped icosahedral virus which contains a single-stranded DNA genome approximately 5 kb in length (7). Parvoviruses require cellular functions which are expressed transiently during the S phase of the cell cycle (25). Since they cannot induce resting cells to enter S phase (30), they require host cells to be actively proliferating. Although MVM is capable of replicating without the aid of a helper virus, its growth is dependent on the differentiated state of the host cell (17, 31). Distinct strains of the same parvovirus serotype show different pathological effects in vivo (5, 14, 20, 21, 25) and host cell type specificities in tissue culture (23, 25). This suggests that viral tropism involves an interaction between a mutable viral component and a differentiation-specific host cell factor(s).

Two allotropic strains of MVM provide a useful model system for studying the molecular mechanism of parvoviral tropism. MVM(p), the prototype strain, grows in fibroblast cells. In contrast, the immunosuppressive strain MVM(i) productively infects erythropoietic progenitors (23) and T lymphocytes (32) and is capable of suppressing a number of T-cell-mediated functions in vitro (10). These two strains are serologically indistinguishable and have 97% sequence identity, but are reciprocally restricted for growth in each other's host cell type. Hybrid cell lines which are fusions between T-lymphocyte lines and a fibroblast line are permissive for both viruses, suggesting that each restrictive host cell is lacking a factor(s) necessary for productive infection by the restricted virus (32). Restriction for growth is not due to a difference in cell surface receptors, as both viruses compete efficiently for and bind to the same receptor on each cell type (27). The viral fibrotropic determinant has been defined by the use of intertypic recombinants between MVM(p) and

MVM(i) (1, 12), and it maps to the *EcoRI-HpaI* fragment within the capsid gene in the region common to VP1 and VP2 (Fig. 1). This 236-bp fragment constitutes only 4% of the viral genome but contains almost 10% of the 160 single-nucleotide differences between MVM(i) and MVM(p) (2, 3). Thirteen of these changes are silent, and the remaining two alter the coding sequence of the capsid gene at amino acid residues 317 and 321 in the VP2 polypeptide (4, 12).

An extended host range variant of MVM(i), *hr101*, was found to contain a number of coding changes from its parent. By analyzing intratypic recombinants between MVM(i) and *hr101*, the fibrotropic determinant of the mutant was also mapped to this small region of the capsid gene (4). In fact, the same two residues were changed at amino acid positions 317 and 321 in *hr101*, and these two changes were found to be necessary for the extended-host-range phenotype. The mutation at position 317 changes the alanine in MVM(i) to a threonine in *hr101*, the same amino acid found at this position in MVM(p). However, the amino acid at position 321 in *hr101* is an alanine, rather than the glutamic acid found in MVM(i) or the glycine in MVM(p).

Our primary interest was to determine whether either or both changes at amino acids 317 and 321 in MVM(i) were necessary for acquisition of the fibrotropic phenotype. We also wished to determine whether, if both were required, each change affected a different step in the viral life cycle. The infectious clone of MVM(i) was engineered by site-directed mutagenesis to contain either of the two coding changes individually or in combination. Viral stocks were generated for each of the constructs and used to compare various parameters of virus and cell growth in multiple-round infections of the A9 fibroblast cell line. In addition, we compared, under single-step conditions, macromolecular synthesis in hybrid and fibroblast cell lines infected with each virus, in order to characterize the block(s) in restrictive infection.

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FIG. 1. Nucleotide sequence of the *EcoRI*-*HpaI* fragment of MVM. All silent nucleotide differences between the MVM(i) and MVM(p) genomes (2-4) in this fragment are indicated, as are the two coding changes with their respective amino acid differences. The sequence of the oligonucleotides used for the site-directed mutagenesis is underlined, and the nucleotide(s) and amino acid(s) changed from the MVM(i) sequence in each of the mutants are shown. Also shown is the region of the genome encoding the VP1-specific coat protein sequence (▨) and the capsid gene sequence common to both VP1 and VP2 (▩).

MATERIALS AND METHODS

Cell lines. A9 ouab^r11 cells (called A9 cells throughout this article) are a ouabain-resistant derivative of the HGPRT⁻ cell line A9 (32), and 324K is a clone of simian virus 40-transformed human newborn kidney cells (32). Hyb1/11-iD5 (called iD5 cells throughout) is a subclone of the somatic cell hybrid line Hyb1/11, which was formed by fusion of A9 ouab^r11 cells with EL4 cells as described previously (32), and is a productive host for both MVM(i) and MVM(p). All cell cultures were maintained as described previously (32).

Site-directed mutagenesis. ILB1, ILB2, and ILB3 were constructed by site-directed mutagenesis of the *EcoRI*-*XbaI* fragment of MVM(i) cloned into a phagemid vector. The single-stranded template was synthesized in a *dut ung Escherichia coli* strain, and the mutation was introduced by oligonucleotide-directed mutagenesis (15) with the adaptations described previously (4). The mutagenic oligonucleotides were 35 nucleotides in length and covered the region from nucleotides 3732 to 3766 in the MVM(i) genome, as shown in Fig. 1. After being sequenced to confirm the change(s), the mutated fragment was reconstructed into the infectious MVM(i) clone. The mutation for ILB1 was amplified by polymerase chain reaction from the original isolate and recloned into pMVMi because the original isolate contained an insertion mutation separate from those introduced by site-directed mutagenesis. Supercoiled plasmid DNA was

purified on cesium chloride gradients by standard methods (16).

Sequencing of plasmid constructs and viral stocks. All sequencing was performed with modified T7 DNA polymerase (Sequenase; USB, Cleveland, Ohio) according to the instructions of the manufacturer. The sequence of the mutated *EcoRI*-*XbaI* fragment was confirmed in each of the infectious clones between nucleotides 3380 and 4350. The substrate DNA was alkaline lysis miniprep DNA or cesium gradient-purified plasmid DNA (approximately 3 µg per sequencing reaction mix). The single-stranded product of asymmetric polymerase chain reaction, synthesized from viral stock as described previously (4), was also sequenced to confirm that the specific mutation was maintained in each of the viruses recovered.

Generation of viral stocks and determination of viral titers. Viral stocks were generated by transfection of infectious clone DNA (pMVMi, pMVMp, pILB1, pILB2, and pILB3) into iD5 cells as described previously (4). This first-passage virus was titered on 324K and A9 cells (32), and 324K cell titers were used to determine multiplicities of infection (MOIs) in all experiments.

Growth curve results. Asynchronous A9 cells were infected at an MOI of 8 and harvested at 4, 24, 48, 72, 96, and 120 h postinfection (p.i.). Samples were counted in a hemacytometer, and trypan blue exclusion was used to determine

viable and dead cell numbers. Supernatant and cell pellets from each time point were analyzed by plaque assay to determine virus production as described previously (32).

Parasynchronized infections. A9 and iD5 cells were incubated for approximately 40 h in isoleucine-minus medium. Cells were infected with each virus at an MOI of 1 and changed to Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 100 μ g of neuraminidase (type V from *Clostridium perfringens*; Sigma Chemical Co., St. Louis, Mo.) per ml at 3.5 h p.i. in order to prevent second-round infection (31). Samples were harvested simultaneously for RNA, DNA, and protein analysis at 24 h p.i. Parallel infections of 50- μ l cultures on Teflon-coated microscope slides (Cel-Line Associates, Inc., Newfield, N.J.) were fixed at 4.5 and 24 h p.i. as described previously (8).

Immunofluorescence of parasynchronized cells. The immunofluorescence assay was performed as described previously, using a rabbit polyclonal antiserum to MVM capsids (32) or a rabbit polyclonal antiserum to a bacterially expressed fusion protein of the common N terminus of the nonstructural polypeptides (7). Both antibodies were used at a 1:50 dilution, and the secondary antibody in both cases was a 1:50 dilution of fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG) antibody (Tago, Inc., Burlingame, Calif.).

RNA isolation and RNase protection assays. Total cellular RNA was isolated by a modification (11) of the guanidinium isothiocyanate procedure (16). RNase protection assays were performed as described previously (11), except a high-specific-activity SP6 RNA transcript was synthesized by using 13.8 μ M [α - 32 P]GTP, 50 μ M GTP, and 500 μ M each ATP, CTP, and TTP. The templates for the P4 region riboprobe synthesis were pIP465 and pPP465, which transcribe MVM(i)- and MVM(p)-specific probes, respectively, from nucleotides 58 to 762 of the MVM genome (11). The templates for the P38 region riboprobe synthesis were pIP3864 and pPP3864, which synthesize MVM(i)- and MVM(p)-specific transcripts, respectively, from nucleotides 1885 to 2072 (11). Approximately 1.5×10^6 cpm of the P4- or P38-specific riboprobe was hybridized with 5 μ g of purified cellular RNA, and digestions with 400 U of RNase T₁ (Sigma) were carried out at 30°C for 60 min for the P4 protections and at 34°C for 55 min for the P38 protections. Protected fragments were separated on 6% (P4) or 8% (P38) polyacrylamide-urea sequencing gels.

DNA purification and isolation. Total cell DNA was prepared from approximately 10^6 infected or mock-infected cells washed in phosphate-buffered saline and resuspended in 20 mM Tris-150 mM NaCl-10 mM EDTA (pH 7.5). Proteinase K and sodium dodecyl sulfate (SDS) were added to final concentrations of 200 μ g/ml and 1%, respectively, and samples were incubated at 37°C overnight. The DNA was extracted with phenol-chloroform and precipitated with ethanol. Following digestion with 100 μ g of RNase A per ml, 5×10^4 cell equivalents of DNA were loaded per well on a 1.4% agarose gel. Southern transfer (26) was carried out with Hybond N (Amersham Corp., Arlington Heights, Ill.). The DNA was linked to the nylon by UV irradiation and prehybridized, and hybridization was performed with an MVM negative-strand-specific riboprobe (specific activity, approximately 10^8 cpm/ μ g RNA), which detects both viral replicative duplexes and single-strand virion DNA, as described previously (11).

Detection of proteins. Cell extracts were fractionated by electrophoresis on linear gradient 10 to 20% polyacrylamide-SDS gels (Jule, Inc., New Haven, Conn.) for detecting NS-1

TABLE 1. Titers of viral stocks generated in Hyb1/11-iD5 cells

Virus stock	Titer (PFU/ml)		
	324K	A9	Ratio, 324K/A9
ILB1	3.3×10^9	1.3×10^8	25.4
ILB2	3.8×10^7	6.3×10^2	6.0×10^4
ILB3	1.6×10^7	1.3×10^3	1.2×10^4
MVM(i)	1.3×10^8	<10	$>1.3 \times 10^7$
MVM(p)	1.45×10^{10}	3.65×10^9	4.0

and NS-2 or 10% polyacrylamide-SDS gels for detecting VP1 and VP2. Western immunoblots were performed as described previously (18) with either a 1:1,000 dilution of a rabbit antibody specific for the common N terminus of NS-1 and NS-2 (7) or a 1:600 dilution of a mouse polyclonal antiserum raised against disrupted MVM virions, which detects both VP1 and VP2. 125 I-protein A (Amersham Corp.) was used to detect bound immunoglobulin.

RESULTS

Productive and restrictive infections of the site-directed mutant viruses. Figure 1 shows the mutagenic oligonucleotides used for site-directed mutagenesis of the infectious clone of MVM(i) at amino acid positions 317 and 321 in the capsid genes of the viruses. ILB1 contains both MVM(p) amino acids at these two positions, and ILB2 and ILB3 individually contain either of these two amino acid changes within the MVM(i) clone. Transfected MVM(i) DNA is not restricted in fibroblast cells during the first cycle of infection (11), so determination of phenotype by direct transfection of the clones was not possible. Because the construct phenotypes were unknown, infectious clones were transfected into a nonselective host, iD5 cells, to generate viral stocks for use in examining the restrictive phenotype. All of the mutants produced virus stocks, and typical 324K and A9 cell plaque assay results for these are shown in Table 1. The plaque size of each virus on 324K cells differs (data not shown), and only MVM(p) and the double mutant ILB1 were capable of plaquing efficiently on A9 monolayers. However, ILB1 generated much smaller plaques and was approximately sixfold less efficient than MVM(p) in the A9 fibroblast plaque assay, relative to titers determined on 324K monolayers (Table 1).

Various parameters of virus and host cell growth were examined for 5 days following infection of asynchronous A9 fibroblast cells (Fig. 2). In this multiple-round infection, only the double mutant ILB1 and wild-type MVM(p) decreased cell viability and the rate of cell growth compared with the mock-infected control. The ILB1 infection showed slower kinetics than the MVM(p) infection in A9 fibroblasts, particularly in the rate of cell killing, as shown in Fig. 2B. When viral yield was examined, ILB1 and MVM(p) showed similar curves for virus production (Fig. 2A), with ILB1 consistently lower than MVM(p) in yield by 2- to 10-fold. In contrast, MVM(i) and the two single mutants, ILB2 and ILB3, yielded 100-fold less infectious virus. In addition, the cell viability and growth rate of A9 cultures infected with ILB2 and ILB3 essentially paralleled those of the mock-infected culture over the 5-day period examined. These results indicate that ILB1 is not as efficient at replicating in a multiple-round infection of A9 fibroblasts as MVM(p), but it is at least 100-fold better than ILB2, ILB3, or MVM(i) in producing virus.

FIG. 2. Growth analysis in A9 fibroblasts. Asynchronous A9 fibroblast cells were infected at an MOI of 8 with ILB1 (○), ILB2 (●), ILB3 (▽), MVM(i) (▼), or MVM(p) (□) or mock infected (■). Samples were harvested at 4, 24, 48, 72, 96, and 120 h p.i. (A) Virus yield for all samples is indicated as the sum of cell-associated and medium virus, as determined by plaque assay on 324K cells. (B) Percent viable cells was determined by dividing the number of live cells by the total cell number (live plus dead) of the sample and multiplying by 100. (C) Total cell number for each sample was determined by counting the number of live and dead cells.

Viral infections of A9 fibroblast cells and nonselective iD5 cells were examined by immunofluorescence with a primary antibody directed against MVM capsids (Fig. 3). A similar proportion of the hybrid cells (iD5) were infected by each of the viruses, as detected by this antibody or an anti-nonstructural protein antibody (NS). Infection of A9 fibroblast cells by ILB1 and MVM(p) yielded a similar fraction of antigen-positive cells. In contrast, infection of A9 cells with equal multiplicities of ILB2, ILB3, and MVM(i) produced approximately 20% of the number of antigen-positive cells seen with ILB1 and MVM(p) as detected by either the anti-NS or anticapsid antibody (Table 2). The similarity of cell response to infection by ILB2, ILB3, and MVM(i) suggested that all three viruses were restricted at an early point(s) in the first round of infection in A9 fibroblasts.

Biochemical parameters of single-round infections. Single-round infections of A9 and iD5 cells were examined at the molecular level to determine whether the block in the replication cycles of ILB2, ILB3, and MVM(i) in A9 cells was at the same or different stages of the growth cycle of each virus. These restrictive infections were compared directly with the productive infection of A9 cells by ILB1 and MVM(p) and of hybrid iD5 cells by all five viruses. In all cases, neuraminidase was added to the culture medium 4 h after infection to ensure single-cycle growth conditions. Cells were harvested at 24 h p.i., and steady-state levels of replication, transcription, protein synthesis, and virus production were determined.

(i) Accumulation of viral DNA. When viral DNA replication was analyzed by Southern blotting, major differences were seen between iD5 and A9 infections. In hybrid iD5 cells (Fig. 4A), all the viruses synthesized similar levels of replicative-form (RF) and single-stranded progeny DNA. In contrast, infections of fibroblast cells (Fig. 4B) by ILB2, ILB3, and MVM(i) showed drastically reduced levels of all

forms of viral DNA compared with ILB1 or MVM(p) fibroblast infections. MVM(i) DNA replication was further reduced compared with that of ILB2 or ILB3 in A9 cells when a longer exposure of the blot was examined. On long exposures of the blots, MVM DNA was not detectable under identical probing conditions in DNA samples harvested at 4 h p.i. from either host cell infected with any of the virus stocks (data not shown). This indicates that the single-strand progeny DNA detected on longer exposures of the Southern blot shown in Fig. 4 represents newly synthesized DNA. ILB1 and MVM(p) had similar levels of replicative and single-strand forms of viral DNA in the A9 infection. Single-strand synthesis by ILB3 is reduced in the permissive infection compared with MVM(i) and may be an intrinsic property of this virus independent of host cell type.

(ii) Accumulation of viral transcripts. The accumulation of R1, R2, and R3 mRNAs was determined by RNase protection either with a probe which detected P4 promoter-driven transcripts R1 and R2 (Fig. 5A and B) or with one which also detected the P38 promoter-driven transcript R3 (Fig. 5C and D) (11). In the nonselective iD5 hybrid cell infections (Fig. 5A and C), accumulation of transcripts arising from both promoters was within twofold for each of the MVM(i)-derived viruses [MVM(i), ILB1, ILB2, and ILB3] and for MVM(p). In these cells, the overall levels of P4 promoter-driven transcripts were similar between the MVM(i)-like viruses and MVM(p), but the R1:R2 ratios were notably different. R1 is the predominant P4 transcript of the MVM(i)-derived viruses, whereas this ratio is consistently reversed for MVM(p). In A9 fibroblasts (Fig. 5B), the levels of all three transcripts in cells infected with ILB3, ILB2, and MVM(i) were considerably reduced compared with those found in MVM(p)- and ILB1-infected cells. Relative levels of MVM(p) and ILB1 transcripts in the fibroblast infection were not quantitatively very different from those found for these two viruses in the iD5 infection.

(iii) Accumulation of viral proteins. Accumulation of viral proteins was studied by Western blotting with antibody against the two nonstructural protein classes, NS-1 and NS-2 (Fig. 6A and B) or an anticapsid antibody to detect VP1 and VP2 (Fig. 6C and D). A9 cells restrictively infected with MVM(i), ILB2, and ILB3 accumulated dramatically less viral protein than is seen in the productive infection of these cells by ILB1 and MVM(p) (Fig. 6B and D). Since NS-2 and VP1 are low-abundance proteins in the productive infection by MVM(i), ILB2, and ILB3, it is not surprising that, in the restrictive infection, they are not detectable within the limits of sensitivity of the assay. Infection of A9 cells with MVM(i) induced slightly less viral protein accumulation than infection with either ILB2 or ILB3, but this difference was at most twofold. As Fig. 6A and C indicate, in the permissive infections of hybrid iD5 cells with MVM(i), MVM(p), and each of the site-directed mutants, the levels of NS-1, NS-2, and capsid proteins were very similar. Infection of hybrid iD5 cells with MVM(p) consistently induced less NS-1 and more NS-2 than was found in iD5 cells infected with each of the MVM(i)-like viruses. Since the NS-1 and NS-2 proteins are products of the R1 and R2 transcripts, respectively, this difference in polypeptide accumulation probably reflects the difference in the ratios of these two transcript classes observed in Fig. 5A.

(iv) Accumulation of infectious virus. The infectious virus yield from both the iD5 and A9 fibroblast parasynchronized single-round infections was determined by plaque assay of supernatant and cell-associated virus in 324K cells (Table 3). In A9 fibroblasts, MVM(i), ILB3, and ILB2 synthesized only

FIG. 3. Immunofluorescence of infected parasynchronized Hyb1/11-iD5 or A9 fibroblasts. Cells were infected at an MOI of 1 and fixed at 24 h p.i., and MVM capsid was detected with a rabbit polyclonal serum and fluorescein-conjugated goat anti-rabbit IgG. Pictures were taken with a 20× Fluor objective and Kodak Gold 400 ASA film with a 7.5-s exposure time. Panels A through F show cells infected with ILB1, ILB2, ILB3, MVM(i), MVM(p), and a mock inoculum, respectively.

TABLE 2. Immunofluorescence analysis of A9 fibroblast infections^a

Virus	% Positive cells (% of control)	
	NS	Capsid
ILB1 (control)	22.4 (100)	14.0 (100)
ILB2	3.9 (17.4)	3.0 (21.4)
ILB3	4.6 (20.5)	2.5 (17.9)
MVM(i)	2.9 (12.9)	2.3 (16.4)

^a Parasynchronized A9 cells were fixed at 24 h p.i. and stained as described in Materials and Methods.

a small fraction of the virus they produced in the hybrid cells, whereas ILB1 and MVM(p) produced similar levels of infectious virus in both cell types. These results are consistent with the 24-h viral yield in the asynchronous, multiple-round infection of A9 cells shown in Fig. 2A.

DISCUSSION

Two mutations are required for acquisition of fibrotropism. Previous studies with intertypic recombinants between MVM(p) and MVM(i) mapped the region controlling target cell specificity to the small region of the coat protein gene

FIG. 4. Analysis of viral DNA replication. A total of 3.5×10^4 cell equivalents of purified total cell DNA harvested 24 h after a parasynchronized infection of (A) iD5 or (B) A9 cells were fractionated by electrophoresis on a 1.2% agarose gel and transferred to a nylon membrane. DNA samples were loaded as follows: lane 1, mock infection; lane 2, MVM(p); lane 3, ILB1; lane 4, ILB3; lane 5, ILB2; lane 6, MVM(i). The various replicative forms (RF), dimer RF (dRF) and monomer RF (mRF), as well as newly synthesized single-strand (ss) DNA are indicated.

FIG. 5. Analysis of transcription in parasynchronized iD5 and A9 infections. A 5- μ g amount of total cellular RNA (iD5 in panels A and C; A9 in panels B and D) was hybridized to a P4 RNA probe (A and B) or a P38 RNA probe (C and D) under conditions of probe excess as described in Materials and Methods. Lane 1 of all panels was protected fragments generated when MVM(p) RNA was hybridized to an MVM(p)-specific RNA probe; an MVM(i)-specific RNA probe was hybridized to RNA from cells infected with MVM(p) (lane 2), ILB1 (lane 3), ILB3 (lane 4), ILB2 (lane 5), MVM(i) (lane 6), or mock infected (lane 7). The MVM(p) probe control was used to indicate the level of MVM(p) RNA transcripts without RNase T₁ cleavages resulting from sequence differences between the MVM(i) probe and the MVM(p) RNA (lane 2, smaller fragments generated). P4 products were analyzed on a 6% polyacrylamide-urea gel, and P38 products were analyzed on an 8% polyacrylamide-urea gel. The location of undigested probe is indicated for P4, as are the protected RNA fragments R1, R2, and R3. Sizes of the protected fragments are correct, as determined by using DNA markers run on the same gel (data not shown).

shown in Fig. 1 (1, 12). Here, 15 base changes occur between the two parental viruses, of which only two, at VP2 codons 317 and 321, would change the coat protein sequence. Acquisition of fibrotropism by an extended-host-range mutant of MVM(i), *hr101*, was recently mapped to the same small region, in which *hr101* and MVM(i) differ by two single-base substitutions (4). Significantly, these mutations occur at the same nucleotides in codons 317 and 321 which are different between MVM(p) and MVM(i) and cause similar coding changes. Codon 317 specifies Ala in MVM(i) and Thr in MVM(p) and *hr101*, whereas codon 321 specifies Glu in MVM(i), Gly in MVM(p), and Ala in *hr101*.

The experiments reported in this article show that both of these nucleotides in the capsid gene of MVM(i) must be

FIG. 6. Western blot analysis of nonstructural and capsid proteins. Extracts from 3.2×10^4 iD5 cells (A and C) or 6.4×10^4 A9 cells (B and D) were run on a 10 to 20% linear gradient polyacrylamide-SDS (A and B) or a 10% polyacrylamide-SDS (C and D) gel and analyzed by Western blot. MVM proteins were detected with rabbit anti-N terminus polyclonal antiserum (A and B) or mouse anticapsid polyclonal antiserum (C and D) and then with ^{125}I -protein A. Extracts were from MVM(p)-infected cells (lane 1), ILB1-infected cells (lane 2), ILB3-infected cells (lane 3), ILB2-infected cells (lane 4), MVM(i)-infected cells (lane 5), or a mock-infected control (lane 6). The identities of MVM polypeptides are indicated.

changed to create a fibrotropic virus. This virus, ILB1, has growth characteristics very similar to those of MVM(p) and is about 100-fold better at infecting fibroblasts than MVM(i). However, when only one nucleotide position is changed, either in codon 317 (as in ILB2) or in codon 321 (as in ILB3), the resulting viruses are less than twice as efficient as their parent MVM(i) at infecting fibroblasts. In A9 cells, all of the parameters of infection we studied showed severe restriction for either of these single mutants compared with ILB1 infection of the same cells or compared with infections of hybrid cells by each of the viruses. Because the phenotypes of the single-change mutants were indistinguishable in the restrictive infection, the individual loci appear to affect the same step(s) in the viral life cycle. The dramatic increase in fibroblast infectivity shown by ILB1 implies a synergistic interaction between these two loci in the same rate-limiting process in fibroblast infection.

We have also found that both changes at these nucleotide positions are required for the extended-host-range phenotype of *hr101*. The previously described site-directed double mutant ILB4 (4) has the same nucleotide sequence as *hr101*

at VP2 codons 317 and 321 in an MVM(i) background and behaves like ILB1 in the parasynchronized infection of fibroblast cells. We have constructed a further site-directed single mutant, ILB5, which contains only the codon 321 mutation of *hr101*. This mutant is also incapable of efficiently infecting fibroblast cells (data not shown). ILB5 has a phenotype indistinguishable from that reported here for ILB2, which contains the codon 317 single mutation of *hr101* alone.

Since two changes are required for the fibrotropic phenotype, we would expect the single mutants which already encode one or the other of the two necessary mutations to yield fibrotropic variants at a much higher frequency than their parent MVM(i). In support of this hypothesis, we have observed that all virus stocks of the single mutants have low but measurable plaque titers on A9 monolayers. In contrast, it has been particularly difficult to demonstrate or isolate virus capable of plaquing on A9 cells in any equivalent MVM(i) viral stock. At present we do not know the relative contributions of leakiness and mutation to plaques formed by ILB2 and ILB3 on A9 monolayers, but it is likely that many of them represent viruses containing a further mutation. The genetic instability of ILB2 and ILB3 could also be due to a form of selective pressure unrelated to tropism, resulting from changes in the physical stability of the virion induced by each single mutation. If either single change produced a somewhat less stable virion than that of MVM(i) or ILB1, selective pressure for reversion or for acquisition of the coordinate, or equivalent, amino acid change would be significant. In the analyses reported here, we have tried to minimize the effects of such second-site mutants by using low-multiplicity infections (1 PFU/cell) with first-passage virus stocks which gave titers at least 10,000-fold lower on A9 fibroblasts than on 324K monolayers.

Interestingly, there are differences between MVM(p) and the MVM(i)-derived viruses even in permissive infections. The relative proportions of R1 and R2 transcripts differ between MVM(i)-derived viruses and MVM(p), and these differences are reflected in the levels to which the NS-1 and NS-2 proteins encoded by these two transcripts accumulate. Because the R1 and R2 transcripts are formed by differential splicing of the same R1 precursor transcript (22), these results indicate a difference in transcript processing between an MVM(i) infection and an MVM(p) infection. This difference in transcript and protein accumulation is also seen between MVM(p) and ILB1 infections of fibroblasts, even though both viruses efficiently infect these cells, and thus is not involved in determining tropism.

The results presented in Table 3 show that the difference in virus yield between MVM(p) and ILB1 is less than twofold in a single-round infection of parasynchronized fibroblast cells. The 10-fold difference between MVM(p) and ILB1 virus yields found in multiple-round infections of asynchronous fibroblasts (Fig. 2A) is presumably a result of one or more of the other 158 nucleotide differences between these two viral genomes. Thus, only the two changes at codons 317 and 321 are required to dramatically increase the efficiency with which MVM(i)-like viruses replicate in fibroblasts, whereas any or all of the other nucleotide changes may merely serve to optimize viral growth in the productive host cell type. Because ILB1 and MVM(i) are genetically identical except for these minimal changes necessary to confer fibrotropism, they provide an excellent system for further study of the mechanism of MVM(i) restriction in fibroblasts in the absence of complicating secondary factors.

TABLE 3. Viral yield from single-round parasynchronized infections^a

Virus	Yield (PFU/ml)	
	Hyb1/11-iD5	A9
MVM(p)	2.17×10^8	3.97×10^7
ILB1	2.46×10^8	2.37×10^7
ILB2	5.3×10^6	2.95×10^4
ILB3	6.1×10^7	3.84×10^5
MVM(i)	4.19×10^7	1.35×10^5

^a Total yield of cell-associated and supernatant virus from 24-h infections of parasynchronized Hyb1/11-iD5 or A9 cells was determined on 324K cells.

Role of the coat protein in determining parvoviral tropism. Experiments reported from our laboratory indicated that replication of transfected MVM(i) DNA was not restricted in the first round of a fibroblast infection, suggesting that the capsid itself might mediate tropism (11). In support of this hypothesis, the fibrotropic determinant was mapped to a 237-bp fragment in the coat protein gene by using intertypic recombinants constructed between the fibrotropic and lymphotropic viruses. However, because this fragment contained 13 silent changes as well as the two coding changes, a *cis*-acting sequence element which influenced tropism could not be ruled out. The data presented in this article indicate that the silent changes between MVM(i) and MVM(p) do not, in fact, play any major role in determining tropism. ILB1 contains MVM(i) nucleotides at each of the silent-change positions in this region and replicates to titers within twofold of those of MVM(p) in a parasynchronized fibroblast infection. Of particular significance is the group of three closely spaced changes between MVM(p) and MVM(i) which span codons 317 to 321, as shown in Fig. 1, of which the outer two control tropism. Given the evidence for a direct role for the coat protein, and the fact that *hr101* also switches tropism through changing coding but not silent nucleotide positions, it seems unlikely that this nucleotide stretch acts as a DNA element to determine target cell specificity.

There are a number of other viral systems in which tropism appears to be controlled by the coat protein, yet restriction appears to act at an intracellular stage in the virus life cycle. Hamster polyomavirus can be propagated by successive virion-mediated productive cycles in GD36 hamster cells but not in several other hamster and murine cell lines. This restriction can be circumvented by transfection of viral DNA, whereupon a burst of virus production ensues. The spread of virus through the culture is arrested in the subsequent round of infection by the apparent failure of an essential postpenetration step (9). Restriction of human immunodeficiency virus type 1 in certain T-cell lines can be circumvented by transfection of proviral DNA (6). In the case of human immunodeficiency virus type 1, the determinant controlling host cell tropism maps to the *env* coding sequence but appears to act after receptor binding and prior to reverse transcription (6, 18, 24, 28, 29).

While our results give some new insight into the virion sequence requirements for the acquisition of fibrotropism, they do not directly address the mechanism underlying parvoviral target cell specificity or the interactions involved. At virus doses which induced similar fractions of antigen-positive nuclei in hybrid cells infected with each of the viruses, the fraction of such positive nuclei in restrictive interactions was considerably depressed. This suggests that the majority of cells are restricted at an early step in infection, prior to the expression of nonstructural proteins and ensuing viral DNA replication. Because MVM(i) and MVM(p) compete for the same receptor on A9 fibroblast cells (27), the restriction of MVM(i), ILB2, and ILB3 replication in these cells presumably must occur after adsorption. The antigen-positive cells in restrictive infections appear to be as positive as their equivalents in productive infections, and the fraction of positive cells in restrictive infections is enough to account for most of the macromolecular synthesis observed. However, the 5- to 10-fold decrease in these parameters of infection in the restrictive population is not enough to explain the 100-fold decrease in infectious virus yield observed. The possibility remains that restriction occurs at both an early and a late step, the latter being at the

point of mature infectious virus production. Interestingly, our results imply that both of these putative steps must involve the two amino acids encoded in positions 317 and 321 in VP2.

In a closely related parvovirus serotype, comprising the feline and canine parvoviruses, the virus's ability to infect canine versus feline cells also maps to a small region of the capsid gene. This region contains three silent and four coding changes between the feline and canine parvoviruses (19). The MVM coat protein gene is very similar in primary amino acid sequence to that of the feline and canine parvoviruses, allowing an extensive and meaningful line-up of their VP2 proteins. This allows an approximate projection of the MVM VP2 sequence onto the three-dimensional structure of the canine parvovirus virion, recently determined by Tsao and colleagues (33). This type of comparison predicts that MVM VP2 amino acid position 321 will be on the surface of the particle, and amino acid 317 should be near the surface, both of them on a loop encircling the threefold axis of symmetry of the particle. Thus, it is quite feasible that these residues might interact directly with a host factor(s) at an early stage in infection. Such an interaction might catalyze an essential early step, such as entry into the cell, transport through the cytoplasm, entry into or localization in the nucleus, or uncoating of the virion DNA. Some of these processes could involve specific interactions with host factors common to both early and late steps in virus growth.

Future experiments will compare MVM(i) with ILB1 in order to define the level at which the restrictive block to MVM(i) infection of fibroblasts occurs. Such analyses should be more precise than previous results obtained with MVM(i) and MVM(p), which differ at 158 additional nucleotides throughout the genome (12, 27).

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