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The Trypsin-Sensitive RVER Domain in the Capsid Proteins of Minute Virus of Mice Is Required for Efficient Cell Binding and Viral Infection but Not for Proteolytic Processing *in Vivo*

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Analysis of a series of mutations in the trypsin-sensitive RVER region of the amino terminal domain in the capsid proteins (VP1 and VP2) of the autonomous parvovirus, minute virus of mice (MVM), demonstrates that this sequence is not essential for proteolytic processing of VP2 into VP3 *in vivo*, but specific amino acids within this domain are important for viral infection. Analysis of the most deficient of these mutants, VP(Δ2842-2863), a 7-aa deletion of aa 159–165 in VP1 and 17–23 in VP2, has identified at least two steps in MVM infection in which this domain is important. VP(Δ2842–2863) was 3-fold defective in binding to murine $A9_{2L}$ cells and, when an equivalent amount of virus was bound to cells, additionally 10-fold deficient compared to wild-type in initiating a productive infection. However, in those cells effectively infected, VP(Δ2843–2863) replicated similar to wild-type. These results suggest that these seven amino acids constitute a region important for both binding and a subsequent step prior to the start of DNA replication such as viral uptake or transport to the nucleus. © 1993 Academic Press, Inc.

INTRODUCTION

Parvoviruses are small, nonenveloped, icosahedral viruses that contain a linear, single-stranded DNA genome. Parvoviruses that infect vertebrates are divided into two genera: dependoviruses, (the adeno-associated viruses (AAV)) which require coinfection of a helper virus such as an adenovirus or herpesvirus for efficient replication, and the autonomous parvoviruses, which replicate efficiently and produce progeny virions without the aid of a helper virus. Minute virus of mice (MVM) is a well-characterized member of a subgroup of closely related autonomous parvoviruses that includes Kilham's rat virus (KRV), H1, canine parvovirus (CPV), feline panleukopenia virus (FPV), and porcine parvovirus (PPV).

The genetic organization of MVM is typical of this subgroup (Cotmore and Tattersall, 1987) (Fig. 1A). Two nonstructural proteins, NS1 and NS2, which are required for viral DNA replication (Tullis *et al.*, 1988; Naeger *et al.*, 1990), are encoded by two overlapping reading frames in the left half of the viral genome (Cotmore and Tattersall, 1986). The structural proteins, VP1 and VP2, are encoded from a single large open reading frame in the right half of the viral genome (Labieniec-Pintel and Pintel, 1986). The relative amounts of VP1 and VP2 produced during infection depends on which splice donor is used to excise a small intron at map units 44 to 46 (Jongeneel *et al.*, 1986, Morgan and Ward, 1986; Clemens *et al.*, 1990). Selection of the first donor site results in the deletion of the VP1 initiation codon. VP2 is then translated from a second initiation codon further downstream in the same open reading frame. The entire primary sequence of VP2 is, therefore, shared by VP1. Typically, VP1 makes up only about one-fifth of the total capsid proteins found in both infected cells and purified virions (Tattersall *et al.*, 1976). The smaller capsid protein, VP2, is sufficient to form a capsid structure and encapsidate progeny DNA; however, the larger, minor capsid protein, VP1, is, by itself, unable to assemble into a capsid structure, although it is required to produce an infectious particle (Tullis, Burger, and Pintel, manuscript in preparation).

VP1 and VP2 have an unusual glycine-rich sequence that is predicted to form a coil structure (Garnier *et al.*, 1978). This glycine coil ends with the sequence GVGXSTG and is conserved among all of the vertebrate parvoviruses sequenced to date except B19 and AAV (see Fig. 1B). While MVM, the hamster parvovirus H1, CPV, FPV, and PPV all share 50–60% homology in the capsid gene, bovine parvovirus (BPV) and Aleution Disease virus (ADV) also retain this glycine coil structure even though they share less than 20% homology with MVM capsid proteins outside of this domain (Ranz *et al.*, 1989).

The structure of the CPV capsid has recently been determined by X-ray crystallography; however, the position of the first 37 aa of VP2, including the glycine coil sequence, was ambiguous (Tsao *et al.*, 1991). The first aa position determined was valine 38 (V^{38}) within the

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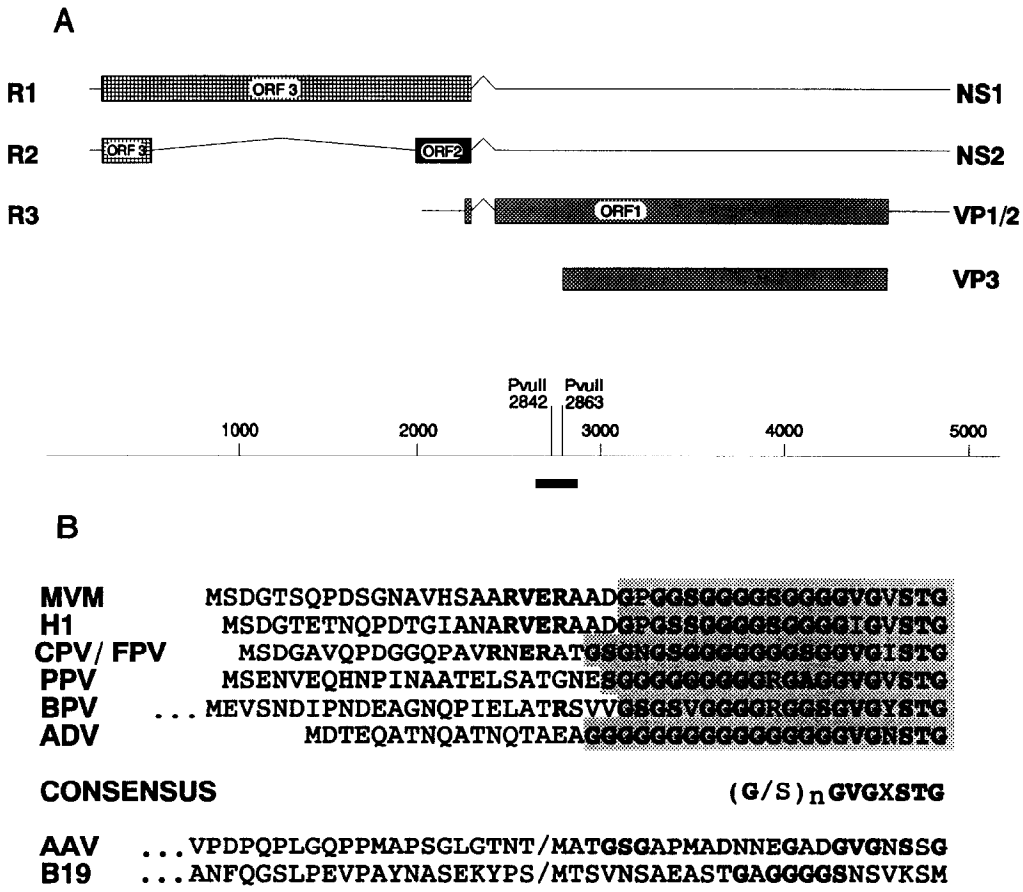

FIG. 1. (A) Diagram of the three major classes of MVM RNA transcripts and their respective coding sequences. The names of the RNAs are listed on the left side and the protein products they encode are listed on the right. VP3 is not a primary translation product but produced by proteolytic processing of VP2. The scale at the bottom corresponds to nucleotide numbers in the MVM_p genome. The black bar designates the area shown in B below. (B) Putative proteolytic processing site in the capsids of parvoviruses. The trypsin-accessible sequence, RXXR, in MVM, H1, CPV, and FPV is indicated in bold and the putative, hydrophobic glycine coil is highlighted in gray. The aa sequence shown corresponds to the N-terminus of MVM (Astell *et al.*, 1983), H1 (Rhode and Paradiso, 1983), canine parvovirus and feline panleukopenia virus (CPV and FPV; Parrish *et al.*, 1988), porcine parvovirus (PPV; Ranz *et al.*, 1989), and aleution disease virus (ADV; Bloom *et al.*, 1988). The region shown for bovine parvovirus (BPV) corresponds to the putative VP4 processing site (Chen *et al.*, 1986). The initiation sites for VP3 from adenovirus-associated virus (AAV; Srivastava *et al.*, 1983; Becerra *et al.*, 1985) and VP2 from the human parvovirus B19 (Shade *et al.*, 1986) are indicated by a slash.

conserved GVGXSTG sequence, which was found to reside on the interior of the capsid. Previous results, however, have suggested that the amino terminus of VP2 is exposed on the exterior of the capsid because it is susceptible to proteolytic cleavage *in vivo* to form a truncated form of VP2 called VP3. The cleavage of MVM VP2 to VP3 can be mimicked *in vitro* by digestion of full virions with trypsin which is thought to cleave VP2 at one of the two arginines found just prior to the glycine coil, as in the closely related virus, H1 (Fig. 1B) (Tattersall *et al.*, 1977; Paradiso *et al.*, 1984). These two arginines are conserved in all the MVM-like autonomous parvoviruses sequenced to date except PPV. Proteolytic mapping of VP3 suggests that the cleavage *in vivo* is not identical to that achieved by trypsin treatment but probably occurs within a few amino acids of arginine 22 (R^{22}) (Tattersall *et al.*, 1977). Although this same site occurs in VP1 (aa 164), VP1 remains resistant to trypsin digestion even under conditions in which up to 80% of the VP2 molecules have been cleaved, suggesting that the amino terminus of VP1 remains internalized (Tattersall *et al.*, 1977). In empty virions, both VP1 and VP2 are resistant to trypsin digestion.

In an attempt to determine if the trypsin-sensitive RVER sequence, within the VP2 to VP3 cleavage region at VP2 aa 19–22, is important for viral infectivity, we have constructed a series of mutations at that site. Our results indicate that the cleavage of VP2 to VP3 occurs just prior to the glycine coil but may not be sequence-specific. Surprisingly, mutations in this region, including a single amino acid substitution at arginine 22, had a dramatic effect on the ability of MVM to

form plaques after transfection of cloned DNA into mouse fibroblasts. The most deficient of these mutants, VP(Δ2842–2863), was threefold deficient in binding to cells. When equivalent amounts of mutant and wild-type virus were initially bound to the cells, VP(Δ2842–2863) still produced fewer infectious centers than wild-type MVM; however, DNA replication within those infectious centers was normal. These results suggest that this mutant has an additional defect prior to DNA replication such as nuclear transport or uncoating. In addition, this mutant appears to be defective in escaping from the cell late in infection of asynchronous cells. This deletion does not disrupt all capsid functions, however. VP(Δ2842–2863) was not detectably deficient in capsid assembly, encapsidation of progeny DNA, and egress from cells early in infection. These results suggest that this domain is important for specific steps in viral infection.

MATERIALS AND METHODS

Construction of the mutants

The mutants pVP(Δ2842–2863) and pVP(RSTL) were constructed by digesting plasmid pAB with *Pvu*II, religating, and transforming *Escherichia coli*-strain HB101. Plasmid pAB contains a subfragment of MVM (nucleotides (nts) 1086–5146) cloned into pML, a pBR322 derivative. Transformants were screened for either the loss of the 21-bp *Pvu*II fragment (corresponding to nts 2843–2864 in MVM) or its insertion in the reverse orientation by restriction enzyme digestion and DNA sequencing. The mutations were rebuilt into the full-length infectious clone (pMVM, Merchlinsky *et al.*, 1983; Tullis *et al.*, 1988) with an *Xho*I (nt 2072) to *Xba*I (nt 4342) fragment. Full-length clones were maintained in *E. coli*-strain JC8111 (Boissy and Astell, 1985). To create mutant pVP(RVEL), a C-to-A transversion at nt 2860 was introduced into MVM sequences (nts 1085–3519) inserted in the polylinker of M13mp19, by site-directed mutagenesis as previously described (Naeger *et al.*, 1990), and rebuilt into the infectious clone of MVM using an *Xho*I (2071) to *Bgl*II (3451) fragment. Similarly, a T to A transition at nt 2859 was introduced into the pVP(RSTL) mutant to create mutant pVP(RSTR).

To determine if the *Pvu*II deletion in VP(Δ2842–2863) was responsible for the aberrant migration of VP1 (see Fig. 3B), a 700-bp *Ban*II fragment (nts 2188–2897) was exchanged between the wild-type infectious clone and pVP(Δ2842–2863). As expected, VP1 generated from the clone containing the wild-type 700-bp *Ban*II fragment comigrated with wild-type VP1. Conversely, VP1 generated from the clone containing the 700-bp *Ban*II fragment from pVP(Δ2842–2863), migrated slower than wild-type VP1 (data not shown). The mutation responsible for this aberrant migration must, therefore, reside within this 700-bp fragment. Sequencing of the entire 700-bp region in pVP(Δ2842–2863) confirmed that the 21-bp *Pvu*II deletion was the only mutation within this region (data not shown).

Isolation of viral stocks

Mutant and wild-type MVM_p was obtained by transfecting murine $A9_{2L}$ cells (Littlefield, 1964) with full-length infectious clones by the $CaPO_4$ precipitate method (Labieniec-Pintel and Pintel, 1986). Monolayers of $A9_{2L}$ cells were scraped into cold phosphate-buffered saline (PBS) and pelleted in a tabletop microcentrifuge. The pellet was resuspended in TE 8.7 and virus released from the cells by five consecutive cycles of freezing and thawing. Some of the viral stocks used in these experiments were obtained from an additional round of infection on $A9_{2L}$ cells. In order to minimize the accumulation of additional secondary mutations during cell passage, no viral stocks were passaged more than one additional time after transfection from plasmid DNA. Full virions were isolated by centrifugation (250,000 *g*) through a sucrose gradient (10–40%) in TE 8.0.

Cell-binding and viral DNA replication assays

In order to standardize the amount of virus bound to an equivalent number of cells, $A9_{2L}$ cells were prelabeled for 24 hr with [^{3}H]thymidine (1 μCi/ml). Tritium-labeled $A9_{2L}$ cells were then seeded onto plastic tissue culture plates at 4×10^5 cells/60-mm plate. For the experiment shown in the inset in Fig. 5, the cells were synchronized by a combination of isoleucine deprivation for 48 hr and aphidicolin, 12 μg/ml, for 12 hr (Cotmore and Tattersall, 1987). In similar experiments, asynchronous cells were used with no obvious differences in the amount of virus bound. Monolayers of tritium-labeled $A9_{2L}$ cells were infected with VP(Δ2842–2863) or wild-type MVM_p for 10, 20, or 30 min at 37° in the presence of aphidicolin, 12 μg/ml. Dilutions of sucrose gradient purified wild-type or mutant virions were equilibrated for equivalent amounts of viral single-stranded DNA and aliquots of each viral inoculum were rechecked by Southern blot. For wild-type MVM_p the multiplicity of infection was estimated to be 0.1 PFU/cell. The amount of virus bound to the cells at various times was determined by rinsing the cells four times with cold PBS and then freezing the cells at −70° until all time points had been collected. Cell pellets were then thawed into lysis buffer (2% SDS, 0.15 *M* NaCl, 10 m*M* Tris–HCl, pH8, 1 m*M* EDTA, 0.25 mg/ml proteinase K) and lysed at 37° for 30 min and

65° for another 30 min. Cellular DNA was sheared by passing the samples through a 25-gauge needle. Each sample was assayed for incorporated ^3H by spotting small aliquots onto DE81 filters in quadruplicate (Maniatis *et al.,* 1982) and counting in 5 ml of Bio-Safe NA (Research Products International). Equivalent ^3H counts were loaded on a 1% agarose gel. Cellular DNA content was confirmed by staining the gel in ethidium bromide, 5 μg/ml, prior to Southern blotting. Cellular DNA was quantitated by photographing the gel with Polaroid type-55 film and scanning the negative with a laser densitometer (LKB, Sweden) (Naeger *et al.,* 1990). Preliminary experiments demonstrated that the results of this method were linear between 0.05 and 0.20 μg cellular DNA with a relative deviation of 15% (data not shown). Samples were readjusted for equivalent cellular DNA content if necessary. Viral DNA was detected by probing the Southern blot with a ^{32}P-labeled, MVM-specific probe generated with random primers (Pharmacia). Southerns were quantitated with a Molecular Dynamics PhosphorImager.

For DNA replication assays, $A9_{2L}$ cells were prelabeled with [^3H]thymidine and infected at 37° for 30 min as above except that no aphidicolin was used and that neuraminidase, 0.06 U/ml (Sigma Chemical, St. Louis, MO), was added to the media after 30 min to prevent reinfection by progeny virus. The input VP(Δ2842–2863) virus was adjusted to give an equivalent amount of virus bound as wild-type MVM_p under these conditions. Double-stranded replicative DNA and progeny single-stranded DNA present in the cells was assayed by Southern blots as above. The number of infected cells per plate was determined by transferring the cell monolayers to a nitrocellulose membrane and probing with a ^{32}P-labeled, MVM-specific probe (Cater and Pintel, 1992).

Export of progeny virus from the cells

$A9_{2L}$ cells (4×10^5 cells/60-mm plate) were infected at 37° for 1 hr with 0.1 infectious units/cell of either the wild-type MVM_p or VP(Δ2842–2863). Neuraminidase, 0.1 U/ml, was added to the media after 1 hr to prevent reinfection. At various times after infection, the cells were scraped into the media and SDS was added to a final concentration of 2% (w/v). Cells were isolated from duplicate samples by spinning the samples in a microfuge for 2 min. The resulting cell pellet was resuspended in lysis buffer containing 2% SDS. SDS (2% final concentration) was also added to the cleared media. All samples were lysed at 55° for 30 min and 65° for an additional 30 min. All samples were treated with proteinase K (0.25 mg/ml at 37° for 1 hr) prior agarose gel electrophoresis. The cellular DNA present in total (cells plus media) or cellular samples was visualized by ethidium staining and quantified as for the cell binding and viral DNA replication assays described above. The amount of progeny virus present in either the media or cells was assayed by Southern blot as above. The amount of cell-associated virus was calculated by comparing the amount of single-stranded DNA present in isolated cells versus the total (cells plus media) after normalizing for equivalent cellular DNA content if necessary. In some experiments the cells were prelabeled with [^3H]thymidine and synchronized as described above.

RESULTS

To determine if the RVER region of VP1 and VP2, just prior to the putative glycine coil structure, is required for parvovirus infection, we constructed a small, in-frame deletion at this site. This was accomplished by deleting a 21-bp *Pvu*II fragment (nts 2842–2863) from the infectious clone of MVM_p. This clone, pVP(Δ2842–2863), was severely deficient in plaque formation after transfection into $A9_{2L}$ cells (Table 1), even though DNA replication following transfection of $A9_{2L}$ cells was normal (Fig. 2). These results suggested that pVP(Δ2842–2863) was blocked at some step subsequent to DNA replication and/or upon reinfection. A mutant in which a single copy of this 21-bp *Pvu*II fragment was reinserted in the reverse orientation was also isolated. This clone, pVP(RSTL), in which only three amino acids in VP1 and VP2 are altered (see Table 1), had a phenotype similar to pVP(Δ2842–2863) following transfection of $A9_{2L}$ cells (Table 1).

To further address which of these three amino acid changes were responsible for the inability of pVP(RSTL) to form plaques following transfection, two additional mutants were constructed by site directed mutagenesis. First, L^{22} in pVP(RSTL) was restored to the wild-type R^{22} (pVP(RSTR), and second, R^{22} in wild-type MVM was changed to leucine (pVP(RVEL)) (see Table 1). Both of these mutants were defective in plaque formation after transfection (Table 1). The single substitution at R^{22} (pVP(RVEL)) was as deficient as pVP(RSTL) and the deletion mutant (pVP(Δ2842–2863)). The double substitution mutant (pVP(RSTR)), unlike the other mutants, was able to produce plaques; however, they were much smaller than wild-type MVM_p plaques and occurred at a lower frequency.

Normally, during a wild-type MVM_p infection, all progeny single-stranded DNA is encapsidated and sediments into a sucrose gradient within full virions (Cotmore and Tattersall, 1987). In the absence of an intact capsid structure, no single-stranded DNA can be detected (Rhode, 1976; Hermonat *et al.,* 1984; Tratschin

TABLE 1

MUTANTS IN THE TRYPSIN-SENSITIVE RVER REGION OF MVM ARE DEFICIENT IN PLAQUE FORMATION BY DNA TRANSFECTION AND AS A VIRUS

		Transfection		Infection		
Name	—AA seq—[a]	Plaques/μg[b]	Size	PFU/ml	Genomes/PFU[c]	Size
pMVM	—HSAARVERAADG—	40	Large	1.9×10^7	2.8×10^4	wt
pVP (Δ2843–2863)	—HS·····ADG—	0	—	0	—	—
pVP (RSTL)	—HSAARSTLAADG—	0	—	4.4×10^6	10.6×10^4	wt
pVP (RSTR)	—HSAARSTRAADG—	11	Small	1.7×10^7	1.7×10^4	Small
pVP (RVEL)	—HSAARVELAADG—	0	—	2.3×10^7	1.3×10^4	wt

[a] Sequence shown corresponds to aa 15–26 in VP2 and 157–168 in VP1.
[b] Represents the average of four experiments, each done in quadruplicate.
[c] The number of MVM genomes present in the inoculum was estimated by quantitating viral ss DNA on a Southern blot and comparing the samples to a MVM DNA marker of known concentration.

et al., 1984; Tullis, Burger, and Pintel, manuscript in preparation). Therefore, even though these mutants were deficient in forming plaques after transfection into mouse $A9_{2L}$ cells, it seemed likely that they produced progeny virions because they produced similar amounts of progeny single-stranded DNA as wild-type (Fig. 2). Indeed virus, which sedimented to a similar position in a sucrose gradient as wild-type MVM (data not shown), could be isolated from $A9_{2L}$ cells transfected with each of the mutant clones. In order to minimize the effect of secondary mutations which might accumulate during multiple rounds of reinfection, all viral stocks used in the experiments described below were obtained either directly from cells transfected with mutant clones or after a single passage on $A9_{2L}$ cells. In addition, the double-stranded replicative form DNA generated from VP(Δ2842–2863) was checked by restriction enzyme digestion for the presence of the *Pvu*II deletion.

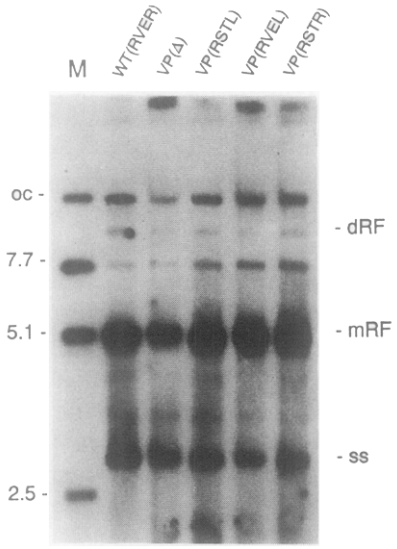

FIG. 2. Southern blot of MVM replicative DNA forms 48 hr following transfection into $A9_{2L}$ cells. Wild-type pMVM (WT(RVER)) and mutant clones are indicated at the top of the lanes. The position of dimer (dRF), monomer (mRF), and viral single-stranded DNA (ss) are indicated on the right. Markers are derived from pMVM digested with *Bam*HI to release MVM (5.1 kb) and vector sequences (2.5 kb) or linearized with *Xho*I (7.7 kb). Nicked, open circular pMVM is indicated by oc.

When these viral stocks were assayed by plaque assay, all the mutants were able to form plaques except for VP(Δ2842–2863) (Table 1). After normalizing for equivalent amounts of viral DNA present in the viral stocks, VP(RSTL) appeared to be four- to fivefold deficient in plaque formation compared to wild-type MVM_p; however, the plaques were wild-type in size. The double-substitution mutant, VP(RSTR), produced an equivalent number of plaques as did wild-type but they were notably smaller, similar to results obtained following transfection. In contrast, the single-substitution mutant, VP(RVEL), which was severely deficient in plaque formation by DNA transfection, was indistinguishable from wild-type by viral plaque assay. Similar results were obtained with a second, independent set of viral stocks that were obtained from a second set of transfections (data not shown).

The defect in infectivity of these mutants is not, as might have been expected, due to a block in the cleavage of VP2 to VP3. As with wild-type MVM_p, not all viral stocks of the mutants contained significant amounts of VP3; however, VP3 could be demonstrated for all the mutants (Fig. 3A). The cleavage of VP2 to VP3 in wild-type MVM_p must, therefore, either occur outside of the region defined by the pVP(Δ2842–2863) mutation, or the cleavage must be independent of the primary amino acid sequence. This latter interpretation is consistent with the results of our attempts to directly sequence the amino terminus of VP3. These experiments

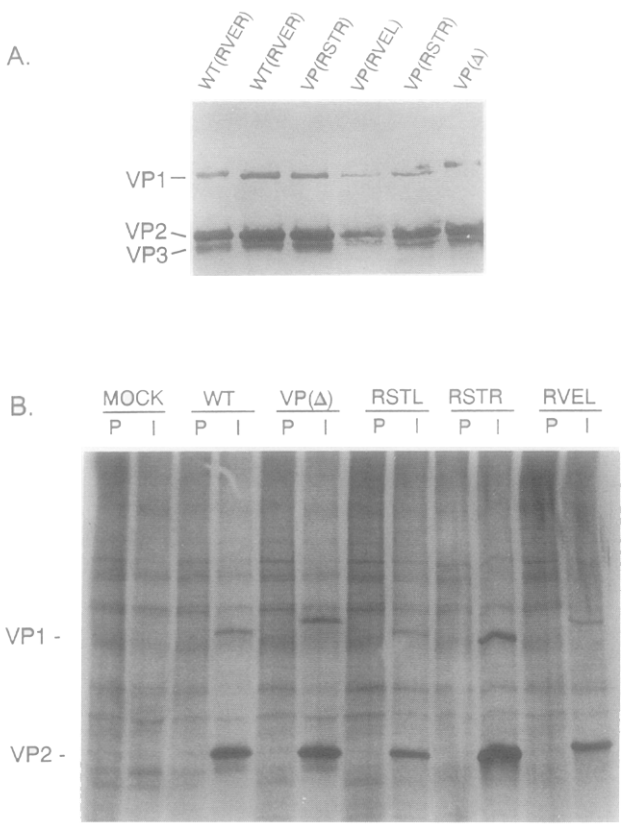

FIG. 3. (A) Western blot of MVM_p (WT(RVER)) and mutant viral proteins. Proteins were separated on a small SDS–polyacrylamide gel (10%; 8.5 by 7.5 cm), transferred to nitrocellulose, and probed with anti-MVM sera. (B) Immunoprecipitation of viral capsid proteins. MVM_p or mutant proteins were immunoprecipitated with either normal rabbit sera (P) or immune sera (I) 48 hr postinfection (p.i.) and separated on a SDS–polyacrylamide gel (10%) gel. Cells were labeled with [^{35}S]methionine and cysteine (^{35}S trans-label, New England Nuclear) 8 hr prior to immunoprecipitation.

suggested that the VP3 sequence starts at a heterogeneous site just upstream of the glycine coil (data not shown).

While surveying a number of mutant viral preparations for the presence of VP3 by Western blot analysis, it appeared that VP1 from several mutant samples migrated aberrantly. To more accurately size VP1 and VP2 from these mutants, capsid proteins were immunoprecipitated from cells that had been transfected with wild-type or mutant infectious clones. Under these conditions, VP2 from wild-type MVM and pVP(Δ2842–2863) appeared to comigrate, whereas the migration of VP1 from pVP(Δ2842–2863) appeared to be retarded relative to wild-type VP1 (Fig. 3B). Similarly, the mobilities of both VP1 and VP2 from pVP(RVEL) appear to be retarded (Fig. 3B). We do not know the nature of this aberrant migration at this time. However, the aberrant migration of pVP(Δ2842–2863) VP1 is a consequence of the *Pvu*II deletion at nts 2842–2863 and not due to a second mutation; VP1 from a second, independently constructed clone displayed a similar retarded migration (data not shown) which cosegregates with the *Pvu*II deletion in a reciprocal marker exchange experiment (see Materials and Methods).

Because we could propagate VP(Δ2842–2863) by infection even though it did not produce plaques, we wished to determine if the failure of this mutant to produce plaques was due to a defect in the lysis of cells or some point earlier in infection. To distinguish between these possibilities, monolayers of $A9_{2L}$ cells were infected with equivalent amounts of MVM_p or VP(Δ2842–2863), as determined by DNA content, and the infected cells were visualized, following transfer of the cell monolayers to nitrocellulose, by probing with an MVM-specific, ^{32}P-labeled probe. Two days after infection both wild-type MVM_p and VP(Δ2842–2863) produced foci of infection of similar intensity, although VP(Δ2842–2863) had generated approximately 13-fold fewer infectious centers than wild-type (Fig. 4B). Based on the number of viral genomes present in the inoculum (Fig. 4A), we estimate that an average of approximately 28,500 full particles of VP(Δ2842–2863) were required to produce a single infectious center compared to 3700 full particles per infectious center of wild-type MVM_p. By 7 days postinfection (p.i.) the MVM_p infection had spread to form large, distinct plaques. VP(Δ2842–2863), however, failed to spread and remained limited to small foci of infection, similar in size and intensity to those seen at 2 days p.i. This suggested that VP(Δ2842–2863) either failed to exit the initially infected cell or that the released virus was deficient in infecting the surrounding cells (Figs. 4C and 4D).

We, therefore, tested the ability of VP(Δ2842–2863) to bind to $A9_{2L}$ cells in a simple cell binding assay (Fig. 5). In this assay, monolayers of $A9_{2L}$ cells were overlayed for various lengths of time with either VP(Δ2842–2863) or wild-type sucrose gradient-purified full virions that had been equalized for DNA content. The cells were subsequently rinsed four times with PBS, lysed, and the amount of virus that remained bound was determined by Southern blot analysis. The cells were maintained in aphidicolin to prevent replication of viral DNA, so that all viral DNA detected by Southern blot was from the input virus. Under these conditions, VP(Δ2842–2863) was consistently approximately threefold deficient in binding to $A9_{2L}$ cells (Fig. 5). In preliminary experiments, VP(RSTR) was similarly deficient in binding to cells (data not shown). Binding of both mutant and wild-type virus was approximately 70% complete after 10 min at 37°, consistent with previous binding studies with MVM_p (Linser and Armentrout, 1978; Spalholz and Tattersall, 1983).

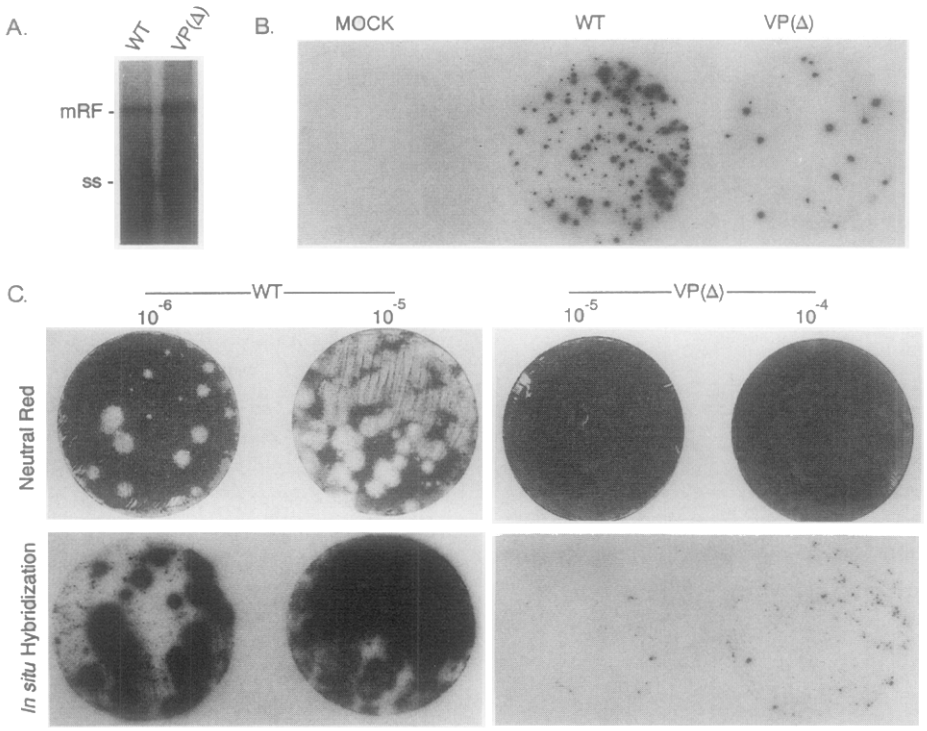

FIG. 4. Plaque assay of wild-type MVM_p and VP(Δ2842–2863). (A) Southern blot of viral DNA present in wild-type MVM_p and VP(Δ2842–2863) stocks used in this assay. The number of full particles in these stocks were estimated by quantifying, in triplicate, the viral single-stranded DNA with a Molecular Dynamics PhosphorImager. Wild-type MVM was 1.2×10^{11} copies/ml and VP(Δ2842–2863) was 1.7×10^{11} copies/ml, calculated by comparison with DNA markers of known concentration. Because these stocks are crude infected-cell lysates, unencapsidated viral replicative forms (mRF) are present but do not contribute to the infectivity of the viral stocks (LRB and GT, data not shown). (B) Autoradiograph of infected cell monolayers transferred to nitrocellulose 2 days p.i. and probed *in situ* with a ^{32}P-labeled MVM-specific probe. Monolayers of $A9_{2L}$ cells were infected with 10^{-5} dilutions of viral stocks shown in A. Only one of duplicate filters is shown. (C) Infected cell monolayers 7 days p.i. Upper row shows cell monolayers after staining with neutral red and transferring to nitrocellulose 7 days p.i. Bottom row is autoradiograph of filters shown directly above. Autoradiographs of WT and VP(Δ2842–2863) are from the same exposure and were hybridized with the same probe. The filters infected with 10^{-5} dilutions of viral stocks were lifted from duplicate plates of those shown in B.

Because the above experiment could not distinguish between binding to the cell surface and uptake of virus, and because a 3-fold deficiency in binding did not seem sufficient to explain the 13-fold reduction in infectivity we had observed, we attempted to determine if VP(Δ2842–2863) was additionally blocked prior to the initiation of viral DNA replication such as in viral entry or transport to the nucleus. Since these mutants are not deficient in viral DNA replication following transfection (Fig. 2), we decided to use the appearance of double-stranded viral DNA replicative forms as an assay for defects in steps earlier in infection. Mutants that are deficient in entry into the cells or nuclear transport, for example, would be delayed in the start of DNA replication, resulting in the accumulation of less replicative DNA at early times postinfection. For these assays, the amount of VP(Δ2842–2863) and wild-type MVM_p required to give equivalent binding to $A9_{2L}$ cells after 30 min at 37° was first determined as above. Neuraminidase, which presumably blocks viral infection by cleaving sialic acid residues on the viral receptor (Cotmore and Tattersall, 1987), was added to the culture media after 30 min to prevent reinfection. In experiments in which equivalent amounts of mutant and wild-type virus was bound to the cells after 30 min, VP(Δ2842–2863) accumulated significantly less replicative DNA forms than wild-type MVM_p at 18 and 24 hr p.i., suggesting that VP(Δ2842–2863) has an additional defect prior to the start of viral DNA replication (Fig. 6A). In three experiments, this defect has ranged from 2- to 15-fold. However, hybridization of a MVM-specific probe to infected cell monolayers from the same experiment, transferred to a nitrocellulose membrane 24 hr p.i., showed that even though there was a significant decrease in the number of infectious centers produced, the magnitude of DNA amplification within each center was similar to wild-type. These results suggest that the reduction in DNA-replicative forms was due to a reduction in the number of infections effectively initiated rather than to a reduction in DNA replication within these infected centers (Fig. 6B). Therefore, VP(Δ2842–2863) was significantly defec-

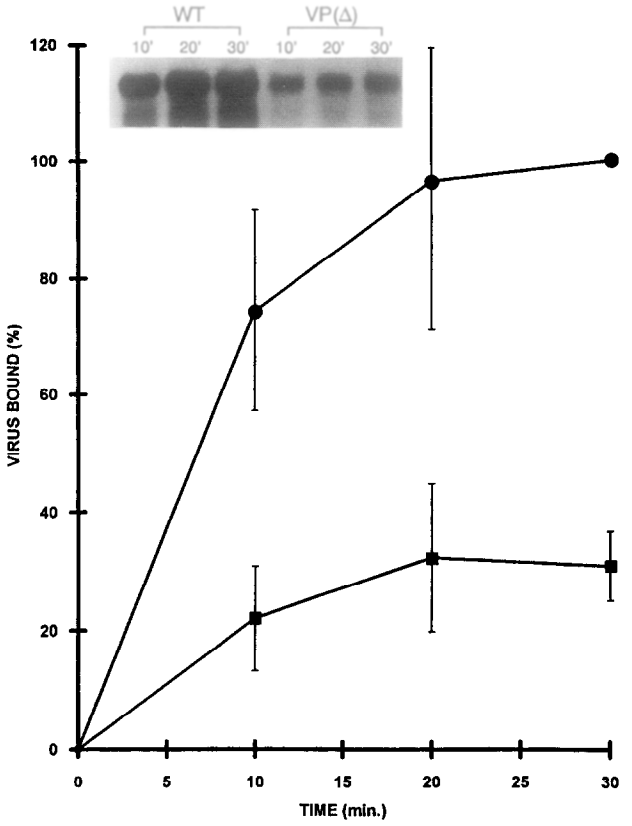

FIG. 5. Cell-binding assay. Monolayers of A_{2L} cells were overlayed with sucrose gradient-purified full particles of either MVM_p (●) or VP(Δ2842–2863) (■) at 37° in the presence of aphidicolin for various lengths of time after which the cells were rinsed four times in PBS, pelleted, and stored frozen on dry ice until all samples had been taken. Cell pellets were resuspended in isotonic buffer containing 2% SDS and the amount of virus bound was determined by Southern blot analysis. Error bars represent 95% confidence limits based on four independent experiments. All samples have been normalized relative to the amount of wild-type virus bound after 30 min at 37°. Inset shows autoradiograph from one experiment.

tive in initiating infection subsequent to its defect in binding.

Whether the defects observed in the initiation of infection were sufficient to fully account for the failure of VP(Δ2842–2863) to produce an efficient infection was unclear. To determine if this mutant was also defective in steps following DNA replication, such as encapsidation and egress from the cell, the following experiment was done. $A9_{2L}$ cells were infected with equivalent infectious center titers of MVM_p and VP(Δ2842–2863) as determined by *in situ* hybridization to infected cell monolayers (see Fig. 4), to assure that an approximately equivalent number of cells were infected with each virus. Neuraminidase was added to the media to prevent binding of progeny virions to the cells. At various times after infection, the cells were scraped into the media and either the total sample was lysed with SDS (2%) or the cells were spun out of the media and lysed separately. The presence of virus in either the cells or media was then analyzed by Southern blot (Fig. 7). Because the samples were equilibrated for equal DNA content, the percentage of progeny virions that remain cell associated can be quantitated by comparing the amount detected in isolated cells versus that found in total cells and media. An approximately equivalent volume of media was also loaded on the gels shown in Fig. 7 for visual purposes, but is not quantitative.

Normally, during a MVM infection most of the virus is found to be associated with the cells; however, when neuraminidase is added to the media, as in this experiment, most of the virus is found in the media (data not shown). At 18 hr p.i. approximately 20% of both wild-type MVM and VP(Δ2842–2863) was associated with the cells (Fig. 7A). However, at 39 hr after infection while only 20% of wild-type virus was cell-associated, almost 90% of the mutant virus was associated with the cells (Fig. 7B). The majority of monomeric replicative DNA, however, remained cell-associated at both 18 and 39 hr p.i., suggesting that general degradation of the cells had not occurred. We have observed a similar effect following infections initiated by DNA transfection (data not shown). These results suggested that, perhaps, export of the mutant virus was deficient late in infection. However, alternatively, the defect that VP(Δ2842–2863) displayed in release from the cells late in infection might be the result of an indirect defect in cytolysis rather than a failure of a specific export mechanism late in infection.

Because MVM requires cells to be in S-phase in order to replicate, infection of asynchronous cells, as in the previous experiment, is not necessarily representative of events within individual infected cells. Therefore, to better define the time during infection in which a reduction in free virus occurs, we repeated these experiments with highly synchronized cells. It has previously been reported that full virions are preferentially exported during a synchronized infection (Cotmore and Tattersall, 1989). Ninety-five percent of the full virions were found to be exported to the media at 6–25 hr after release into S-phase. In our experiments, greater than 90% of the full MVM_p virions were found in the media between 8 and 48 hr after release into S-phase, consistent with previous observations. However, in contrast to our results with asynchronous cells, a similar percentage of VP(Δ2842–2863) full virions were also released into the media at these times (data not shown). VP(Δ2842–2863), therefore, shows no defect in egress from cells in a synchronized infection. We do not know the reason for this apparent discrepancy at this time; however, both experiments suggest that

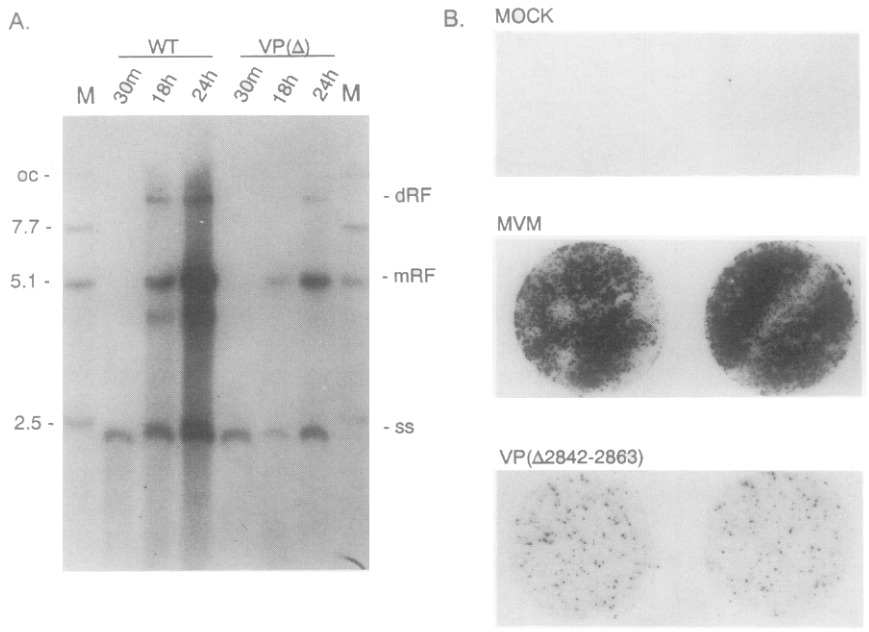

FIG. 6. Infectivity of MVM_p and VP(Δ2842–2863) after equilibrating for binding. (A) Southern blot of MVM replicative forms either immediately following infection (30 min, confirming equivalent binding) with sucrose gradient-purified full particles of wild-type MVM_p or VP(Δ2842–2863), or 18 or 24 hr later. Viral inputs were adjusted to give equivalent amount of virus bound after 30 min at 37°. Markers are the same as in Fig. 2. (B) *In situ* hybridization of infected cell monolayers transferred to nitrocellulose 24 hr p.i. Plates were replicates of those used in A.

VP(Δ2842–2863) is not defective in the encapsidation and specific export of progeny virions out of the cell. VP(Δ2842–2863) may in addition be defective in a nonspecific cytolytic effect later in infection. These experiments also demonstrate that, when equal numbers of infectious centers are examined, VP(Δ2842–2863) replicates similar to wild-type and produces an equivalent yield of progeny single-stranded DNA.

DISCUSSION

We have analyzed a set of mutants within the amino terminal region of VP1 and VP2 including a mutant with a deletion of aa 159–165 in VP1 and 17–23 in VP2. This domain has previously been shown to include the site at which VP2 is cleaved to form VP3. Our results indicate, however, that amino acids within this region,

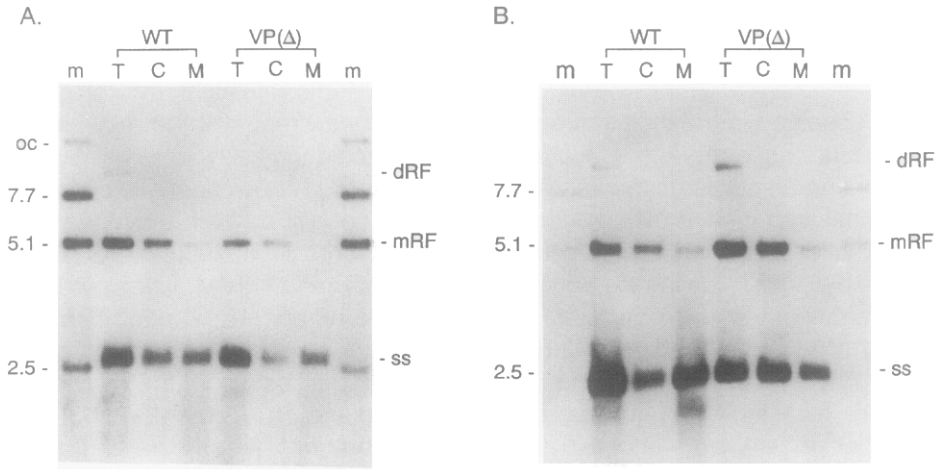

FIG. 7. Export of progeny virus from cells. $A9_{2L}$ cells were infected with equivalent titers of MVM_p (WT) and VP(Δ2842–2863) as determined by *in situ* plaque assay (Fig. 4). At either 18 hr p.i. (A) or 39 hr p.i. (B), total cells plus media (T) or isolated cells (C) and media (M) were lysed and viral DNA (ss) detected by Southern blot. An approximately equivalent number of cells have been loaded in T and C lanes as described under Materials and Methods. Markers (m) are the same as in Fig. 2.

including the trypsin-sensitive RVER site, are not essential for the processing of VP2 to VP3 *in vivo,* but specific amino acid residues within this motif are important for viral infectivity. A more detailed analysis of the deletion mutant, VP(Δ2842–2863), has identified at least two steps in the viral life cycle in which this RVER site may be important: cell binding and a step prior to the onset of DNA replication.

The position of the RVER motif and the putative glycine coil of VP1 and VP2 was not sufficiently ordered in CPV crystals to determine its position within the viral particle by X-ray crystallography (Tsao *et al.,* 1991); however, the amino terminus of VP2 is likely to be on the surface of full viral particles because the RVER sequence is accessible to trypsin digestion (Tattersall *et al.,* 1977; Paradiso *et al.,* 1984). Tsao *et al.* (1991) have proposed that the highly conserved glycine-rich sequence may allow the amino terminus of VP2 to reach the viral surface through a cylindrical hole on the 5′ axis of the virion. However, up to 80% of the VP2 molecules in MVM can be cleaved by trypsin (Tattersall *et al.,* 1977) and only 12 such holes exist per virion. Therefore, either each hole must accommodate at least four amino acid chains simultaneously, or cleavage of VP2 to VP3 is a dynamic process: as one amino acid chain is cleaved it falls back and another takes its place. It is interesting to note that in the only two examples of mammalian parvoviruses in which the glycine coil has not been retained, AAV and B19, the smallest capsid protein is produced by internal initiation rather than proteolytic processing (Becerra *et al.,* 1985; Shade *et al.,* 1986), suggesting that there may be a functional link between the two (Fig. 1B).

All of the mutants within this region, including VP(Δ2842–2863), could be cleaved to form VP3, demonstrating that the trypsin-sensitive RVER site is not essential for proteolytic cleavage of VP2 *in vivo.* Proteolytic cleavage of VP2 to VP3 in wild-type MVM_p may occur just outside of the region deleted in VP(Δ2842–2863) or the cleavage may not be sequence-specific. In the latter case, either multiple amino acid residues can be recognized by perhaps multiple proteases or the *in vivo* protease(s) recognizes a secondary structure rather than a primary amino acid sequence. The RVER sequence is, however, important for viral infectivity because amino acid substitutions within this motif inhibited plaque formation following DNA transfection even though viral DNA replication was normal. The severity of this defect was quite different when the infections were initiated with virus rather than DNA transfection. VP(RVEL), for example, produced no plaques by DNA transfection but wild-type plaques following viral infection. This most likely reflects a difference in the way the infections were initiated rather than a transfection-specific phenotype. The higher yield of virus initially obtained from an infection instead of a transfection, for example, may compensate for mutants slightly defective in infection.

Further analysis of the most deficient of these mutants, VP(Δ2842–2863), demonstrated that the RVER motif may be important in at least two steps in the initiation of infection: binding and an additional step prior to the start of DNA replication. Because this mutant affects both VP1 and VP2, we cannot distinguish the relative contribution of each to the mutant phenotype. The binding defect, however, is likely a VP2 effect, because virions lacking VP1 are not defective in binding to cells in this same assay (Tullis, Burger, and Pintel, manuscript in preparation). Because the amino terminus of VP2 is accessible to the viral surface, the simplest explanation is that this region facilitates adherence to the cell surface. However, this interpretation is not consistent with previous studies that showed that this domain can be physically removed from the viral particle by proteases without affecting the binding or infectivity of the virus (Clinton and Hayashi, 1976; Linser and Armentrout, 1978). In addition, empty particles, in which the amino terminus of VP2 is resistant to protease digestion, compete as efficiently as full particles for cellular binding sites (Linser and Armentrout, 1978). Taken together these results suggest that this region exerts a secondary effect on the receptor-binding site rather than participating directly in receptor binding.

In addition to its 3-fold defect in cell binding, VP(Δ2842–2863) had an additional defect at some point subsequent to binding to cells and prior to the initiation of DNA replication such as viral uptake or transport of the particle to the nucleus. Similar amounts of VP(Δ2842–2863) full virions (as measured by DNA content) produced approximately 10-fold fewer infectious centers as wild-type MVM_p. DNA replication within these infectious centers, however, attains wild-type levels suggesting, perhaps, that fewer VP(Δ2842–2863) virions, on the average, reach the nucleus where they can begin DNA replication. Those that do, however, replicate normally. Little is known about the uptake and nuclear transport of parvoviruses at this time. MVM was reported to be taken up by coated pits (Linser and Armentrout, 1978). CPV, on the other hand, appears to be internalized primarily in small, uncoated vesicles which fuse to form larger, endosome-like compartments (Basak and Turner, 1992). In the presence of the lysosomotropic reagents NH_4Cl and chloroquine, CPV accumulates in these large vesicles suggesting that CPV may require an acidic environment to escape from the endosomal compartment. In one model, proteolytic cleavage of VP1 or VP2 by

acid proteases at or near the RVER motif might unmask the hydrophobic glycine-rich sequence; subsequent insertion of this very hydrophobic domain into the endosomal membrane may be required for release of the virus from this compartment. VP(Δ2842–2863) may be defective at this putative step because the number of potential protease cleavage sites has been reduced. If so, then pretreatment of VP(Δ2842–2863) particles with a protease might relieve this defect. Because the mobility of VP1 from VP(Δ2842–2863) is retarded on SDS polyacrylamide gels, it may have an unusual post-translational modification which contributes to this effect. We are currently investigating the nature of this modification as well as the relative roles of VP1 and VP2 in this defect.

In addition to defects in the initiation of infection, VP(Δ2842–2863) was deficient in escaping from the cells late in an asynchronous infection. Because progeny VP(Δ2842–2863) virions were efficiently transported to the media early in the same infection and in highly synchronized infections, we suspect that this effect might be a nonspecific defect in some aspect of cytolysis rather than a defect in a specific transport mechanism. Preliminary cytotoxicity experiments, based on the clonogenicity of infected cells on plastic dishes, suggest that VP(Δ2842–2863) kills host cells as efficiently as wild-type MVM_p (L.B., unpublished observation). However, these experiments only measure the ability of the virus to kill cells and do not directly address the kinetics of cytolysis. VP(Δ2842–2863), for instance, might actually kill the cells earlier than would wild-type MVM_p thereby shutting down export of virus to the media; alternatively, VP(Δ2842–2863) may kill cells with similar kinetics as wild-type MVM_p but not lyse the plasma membrane sufficiently to release virions.

Analysis of these mutants suggests that the trypsin-sensitive RVER site is an important motif for viral infection even though this motif is not essential for the proteolytic cleavage of VP2 to VP3. Specific amino acids within this motif, particularly R^{22}, are important for MVM capsid function. A more detailed analysis of a 7-amino-acid deletion in this domain has identified at least two points in infection where this region may be important: cell binding and a subsequent step such as viral uptake and/or nuclear transport. This deletion, however, did not disrupt all capsid functions; this domain was not required for viral assembly, encapsidation of DNA, or egress from the cells suggesting that the RVER region of VP1 and VP2 plays a role at specific steps in a MVM infection. Further analysis of these mutants should help clarify the contribution of this motif to viral infection.

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