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Controlled Conformational Transitions in the MVM Virion Expose the VP1 N-Terminus and Viral Genome without Particle Disassembly

Susan F. Cotmore,* Anthony M. D'Abramo, Jr.,* Christine M. Ticknor,† and Peter Tattersall*,†,[1]

*Department of Laboratory Medicine and †Department of Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510

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Antisera were raised against peptides corresponding to the N-termini of capsid proteins VP1 and VP2 from the parvovirus minute virus of mice. Epitopes in the 142-amino-acid VP1-specific region were not accessible in the great majority of newly released viral particles, and sera directed against them failed to neutralize virus directly or deplete stocks of infectious virions. However, brief exposure to temperatures of 45°C or more induced a conformational transition in a population of full virions, but not in empty viral particles, in which VP1-specific sequences became externally accessible. In contrast, the VP2 N-terminus was antibody-accessible in all full, but not empty, particles without prior treatment. An electrophoretic mobility shift assay, in which particles were heat-treated and/or preincubated with antibodies prior to electrophoresis, confirmed this pattern of epitope accessibility, showing that the heat-induced conformational transition produces a retarded form of virion that can be supershifted by incubation with VP1-specific sera. The proportion of virions undergoing transition increased with temperature, but at all temperatures up to 70°C viral particles retained structure-specific antigenic determinants and remained essentially intact, without shedding individual polypeptide species or subunits. However, despite the apparent integrity of its protective coat, the genome became accessible to externally applied enzymes in an increasing proportion of virions through this temperature range, suggesting that the conformational transitions that expose VP1 likely also allow access to the genome. Heating particles to 80°C or above finally induced disassembly to polypeptide monomers.

INTRODUCTION

Parvoviruses have small (5 kb) single-stranded DNA genomes encapsidated in approximately spherical, 280-Å-diameter protein capsids in which 60 copies of the coat protein are related by $T = 1$ icosahedral symmetry (Agbandje *et al.,* 1995). Virion proteins are encoded from a single gene, with alternative splicing allowing the synthesis of two primary translation products, as described in the legend to Fig. 1. VP2, a 64-kDa polypeptide comprising 587 amino acids, is the predominant species in the empty capsid and newly released virion. VP1, an 83-kDa polypeptide present at approximately 10 copies per particle, contains all of the VP2 sequence and, as shown in Fig. 1, has an additional, basically charged, 142-amino-acid N-terminal extension, derived by initiating translation from an upstream methionine present only in a minor mRNA species (Cotmore and Tattersall, 1987). A third capsid polypeptide, VP3, can be generated in mature virions, but not in empty capsids, by proteolytic cleavage of VP2 molecules approximately 25 amino acids in from their N-termini. Although this same proteolytic site is present in VP1 molecules, it is not accessible to proteolytic cleavage, suggesting that equivalent sequences in these peptides are disposed differently in the particle structure (Tattersall *et al.,* 1977).

The three-dimensional structures of viral particles from canine parvovirus (CPV), feline parvovirus, and minute virus of mice (MVM) have been determined to atomic resolution by X-ray crystallography (Tsao *et al.,* 1991; Agbandje *et al.,* 1993; Wu and Rossmann, 1993; Xie and Chapman, 1996; Llamas-Saiz *et al.,* 1997; Agbandje-McKenna *et al.,* 1998). For MVM, the atomic model identifies the position of 547 amino acids from the carboxy-termini of each capsid polypeptide chain, together with approximately 34% of the single-stranded DNA genome, which also displays icosahedral symmetry. However, while most of the protein sequence common to all three capsid polypeptides can be distinguished in the crystal structure, this order is lost at residue 40 in VP2, so that the locations of the N-terminal peptides of VP1 and VP2 (181 and 39 amino acids, respectively) remain uncertain. In the crystal structure, cylindrical projections encircled by deep (15 Å) canyon-like depressions surround each of the 12 five-fold symmetry axes, creating the second-most prominent topological feature on the virion surface. These projections, which are created by juxtaposed antiparallel β-ribbons from each of the five-fold related polypeptides, contain pores with a minimum diameter of ~8 Å that penetrate through the capsid shell to the

[1] To whom reprint requests should be addressed. Fax: (203) 688-7340. E-mail: Peter.Tattersall@Yale.edu.

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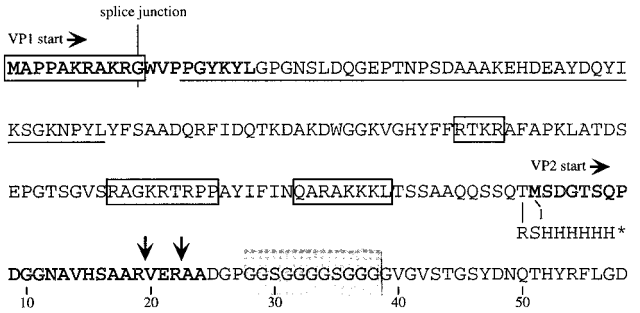

FIG. 1. Diagram of the N-termini of VP1 and VP2. The predicted amino acid sequences of VP1 and VP2 are shown in single-letter code. VP1 is made from a minor viral mRNA species that is alternatively spliced, at the junction indicated, so as to include the VP1 methionine start and a short (11 amino acid) exon contiguous with 131 amino acids of VP1-specific sequence and the whole of the VP2 sequence (only 58 residues shown). Most viral mRNAs are spliced to omit the VP1 start codon and the upstream exon, in which case translation starts at the VP2 N-terminal methionine labeled 1. Regions predicted to be nuclear localization signals by Tullis *et al.* (1993) are boxed. Sequences used to raise anti-peptide antibodies are shown in boldface. The highly conserved sequence present in almost all parvoviral VP1-specific regions is underlined. The polyglycine region modeled into the density running through the five-fold pore is shaded. Indicated beneath the N-terminus of VP2 is the $(his)_6$-tag sequence engineered at the C-terminus of the *E. coli*-expressed VP1-specific peptide described in the text.

particle interior. VP2 residue 40 lies on the inside of the capsid shell, at the base of these pores, and in mature virions, but not in empty viral particles, the channels contain additional, weak, X-ray-dense material. Reanalysis of the CPV structure following refinement to 2.9 Å (Xie and Chapman, 1996) supports the idea that this weak density represents peptide sequences from the N-termini of approximately 13% of the capsid polypeptides, which must therefore project to the particle exterior through the pore, while the N-termini of the remaining 87% remain on the inside of the capsid. Subsequent modeling of the MVM structure likewise indicated that the first peptide that is differentiated from its five-fold symmetry related neighbors, a highly conserved glycine-rich sequence (VP2 residue 28-GGSGGGGSGGG-38), denoted by the dashed underline in Fig. 1, can be modeled into the weak density found along this channel, while residues 36-GGG-38 from nonexternalized capsid proteins can be modeled extending into the interior of the particle (Agbandje-McKenna *et al.,* 1998). Since each pore can accommodate only one N-terminal peptide, a maximum of one in five of the N-termini can, theoretically, be externalized, but it is not clear whether these peptides belong to VP1 or VP2 molecules since the identified sequences are common to both. However, the observation that VP2 can be cleaved by externally applied trypsin (Tattersall *et al.,* 1977) suggests that at least some of the externalized sequences belong to this species. In the present paper we use antisera directed against the amino-terminal sequences of VP1 and VP2 to explore their accessibility in intact MVM virions and empty capsid particles.

Although parvovirus virions are known to be exceptionally rugged and resistant to inactivation (Berns *et al.,* 1995), we know little about the strategies they employ to traverse their host cell membranes and cytosol in order to deliver their genomes to the nuclear replication machinery. Genetic analysis suggests that the VP1-specific peptides of MVM, while not required for particle assembly, genome encapsidation, or the release of virions from their initial host cell, are essential for some step in the subsequent infectious process, acting at an as yet unidentified point following receptor binding and virus internalization in subsequent host cells (Tullis *et al.,* 1993; Maxwell *et al.,* 1995). The VP1-specific region contains candidate karyopherin-α recognition sequences that could serve as nuclear localization signals and a sequence of about 40 amino acids (MVM VP1 residues 15–58) which likely fulfills some critical biological function since it is highly conserved throughout the *Parvoviridae* (Chen *et al.,* 1986) with the sole exception of Aleutian disease virus of mink (Bloom *et al.,* 1988). In other viral families nuclear translocation of the incoming genome is invariably mediated by one or more of the virion polypeptides that possess the necessary cellular recognition signals and remain physically attached to the genome following particle disassembly (Greber *et al.,* 1994). Since the VP1-specific peptide contains such sequences and has been shown to mediate translocation of newly synthesized VP1 molecules to the nucleus (Lombardo *et al.,* 1997), it seems a likely candidate for such a trafficking vehicle. However, MVM virions are so small (280 Å diameter) that they could, theoretically, pass through the nuclear pore without prior disassembly. It was therefore of interest to ask whether VP1-specific sequences are exposed at the surface of newly released virions, and, if not, to determine whether they can be rendered accessible in the intact particle or whether they become accessible only following virion disassembly and separation of the individual polypeptides or subunits.

RESULTS

The VP1-specific region is sequestered in the great majority of newly released full or empty MVM particles

Newly released ^{35}S-labeled full and empty MVM particles were obtained from the medium of synchronized, MVM-infected A9 cells cultured in the presence of neuraminidase and harvested approximately 30 h after release into S-phase. Since neuraminidase destroys the A9 receptor for MVM, these newly released particles had neither bound to cell surface receptors on the remaining host cells nor, subsequently, been taken up by those cells. Such preparations are therefore significantly differ-

ent from the great majority of parvovirus stocks described in the literature that are routinely obtained from infected cell pellets after repeated rounds of infection and so contain mixed populations of particles including some that have yet to be released by their parental cell, others that have been released but have bound to the surface receptors of new target cells, and others that have just penetrated new host cells. Since cell lysis is still very limited in synchronized cells at this time in infection, full virions isolated under these conditions contained predominantly VP1 and VP2 polypeptides, showing little evidence of the proteolytic cleavage that later generates VP3 polypeptides from their VP2 precursors.

As expected, sera directed against the entire VP1-specific region of MVM expressed as a histidine-tagged peptide in bacteria (anti-VP1) or against a synthetic peptide corresponding to its N-terminal 19 amino acids (anti-MAPPA) reacted exclusively with the capsid protein VP1 when used to immunoprecipitate extensively denatured, ^{35}S-labeled forms of the viral capsid (Fig. 2A, lanes 4 and 5). Under the same conditions, a control serum directed against the "allopeptide" sequence that is present in all capsid polypeptides reacted with both VP1 and VP2 (Fig. 2A, lane 6). In contrast, rabbit anti-capsid sera PN1 and PN2, raised by repeated immunization with small amounts of intact capsids, reacted predominantly with structural determinants in the capsid, immunoprecipitating denatured capsid polypeptides poorly (Fig. 2A, lane 2) but reacting efficiently with native capsids (Fig. 2A, lane 7). A group of neutralizing monoclonal anti-capsid antibodies, illustrated here by MAb D10, similarly failed to precipitate denatured polypeptides (Fig. 2A, lane 3). The sera used in the following analyses can therefore be grouped into two categories: (a) anti-peptide sera that recognize linear epitopes and (b) anti-structural determinant sera that react with secondary, tertiary, or quaternary structures present only in the assembled particle.

When assayed under nondenaturing conditions, neither full nor empty MVM particles could be effectively immunoprecipitated with anti-VP1 or anti-MAPPA sera (Figs. 2B and 2C, lanes 4 and 5), and while a significant proportion of empty particles could be precipitated with anti-allopeptide sera (28% of the total population in the experiment shown in Fig. 2B, lane 6), this peptide was also masked in the great majority of full particles (Fig. 2C, lane 6). Thus, contrary to expectations, none of these sequences were accessible in most newly released MVM virions.

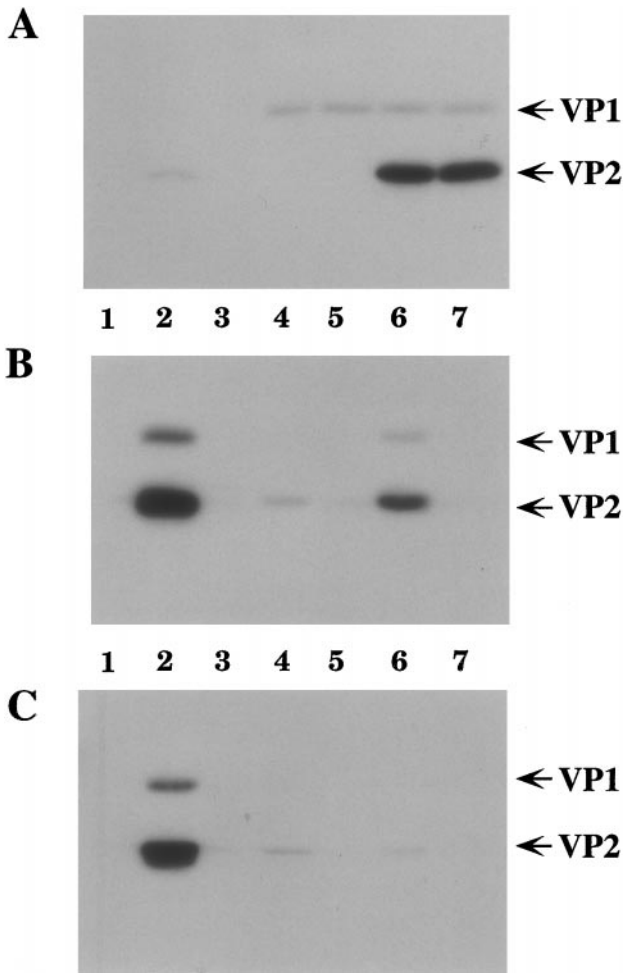

FIG. 2. Antibody specificity against denatured capsid proteins and intact particles. Fully denatured ^{35}S-labeled capsid polypeptides (A) reacted efficiently with antiallopeptide sera (lane 6) but were not immunoprecipitated with prebleed serum (lane 1) from a rabbit subsequently immunized with the entire VP1-specific sequence, while immune serum from the same rabbit specifically immunoprecipitated the VP1 polypeptide (lane 4), as did anti-MAPPA sera directed against the N-terminal 19 amino acids of this polypeptide (lane 5). In contrast, a neutralizing anti-capsid monoclonal antibody, MAb D10, was unable to react with denatured particles (lane 3), and anti-capsid serum PN1 precipitated just trace amounts of VP2 from this mixture (lane 2), but could react efficiently with native empty particles (lane 7). Native ^{35}S-labeled empty particles (B) or virions (C) were quantitatively precipitated with PN1 (B and C, lane 2), effectively removing all labeled proteins from the unbound supernatant (B and C, lanes 1), but were not precipitated with prebleed serum (B and C, lane 3) or anti-MAPPA (B and C, lane 5). Sera directed against the entire VP1-specific region reacted with just a few particles of each type (B and C, lane 4), while anti-allopeptide sera reacted poorly with virions (C, lane 6) but recognized approximately 26% of empty particles (B, lane 6).

VP1-specific epitopes become accessible in full virions, but not in empty particles, following exposure to heat

In many virus groups, virion structure has been shown to be metastable, so that exposure to an external energy source, which experimentally often takes the form of heat, induces them to undergo limited conformational transitions believed to mimic structural shifts seen in the infectious virion during the process of cell entry (Meyer *et al.,* 1992; Curry *et al.,* 1996). To ask whether MVM virions were similarly metastable, we heated virions and empty particles under physiological buffer conditions at a range of different temperatures for 10 min before im-

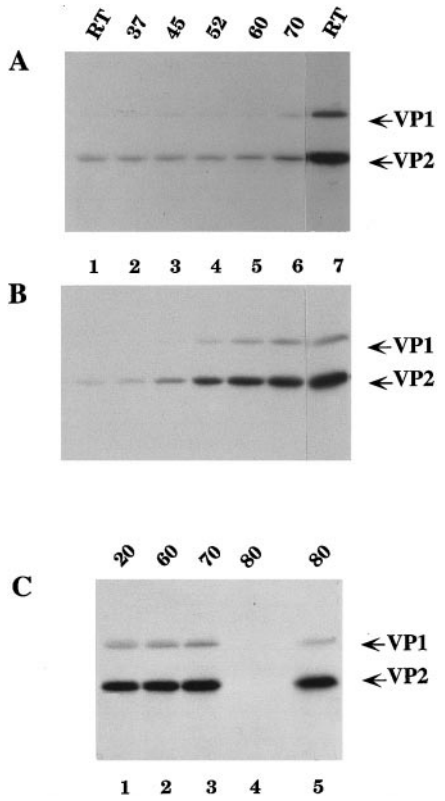

FIG. 3. Effect of heating particles on their reactivity with anti-VP1 or MAb D10. Native ^{35}S-labeled empty particle (A) and virions (B) were incubated for 10 min at the various temperatures indicated above Panel A and then immunoprecipitated with antisera to the VP1-specific region (lanes 1 through 6). Samples preincubated at room temperature (RT) and then quantitatively immunoprecipitated with PN1 anti-whole-capsid serum are shown in lanes 7. In C, native ^{35}S-labeled virions were preincubated for 10 min at 20°C (lane 1), 60°C (lane 2), 70°C (lane 3), and 80°C (lane 4), before being immunoprecipitated with the neutralizing monoclonal antibody D10. Samples preincubated at 80°C could still be quantitatively precipitated with anti-allopeptide sera (lane 5).

munoprecipitating them with anti-VP1 serum. This treatment had little effect on the proportion of empty particles that could be precipitated (Fig. 3A), but heating to comparable temperatures produced a progressive increase in the number of full virions that could subsequently be precipitated with anti-VP1 (Fig. 3B). In the experiment shown in Fig. 3B, 6% of virions were precipitated with anti-VP1 following preincubation at room temperature or 37°C, 14% were precipitated with anti-VP1 after heating to 45°C, 29% after heating to 52°C, 55% after heating to 60°C, and 67% after heating to 70°C. Similar values were obtained in separate studies, although the exact proportions varied somewhat from experiment to experiment.

While in this experiment samples were heated for 10 min, time course studies carried out at several temperatures in this range suggested that the transition routinely occurred within 3 min, so that the proportion of particles affected was determined predominantly by the temperature, rather than the duration, of each incubation (data not shown). Throughout this initial range of temperatures, up to 70°C, VP2 was coprecipitated in normal capsid proportions with the VP1 polypeptides, suggesting that the particles remained essentially intact.

Preincubation at temperatures up to 70°C had little effect upon the reactivity of MVM particles with the neutralizing monoclonal antibody D10 (Fig. 3C) or with polyclonal anti-capsid serum PN1 (data not shown), indicating that despite the conformational changes that led to VP1 exposure, a variety of structural epitopes remained ordered. However, at higher temperatures such reactivity was rapidly lost. This was most apparent in the example shown in Fig. 3C, where all reactivity with the monoclonal antibody D10 was lost following heating to 80°C, even though the remaining peptides could be quantitatively precipitated with anti-allopeptide serum (Fig. 3C, compare lanes 4 and 5). Reactivity with polyclonal serum PN1 was lost more gradually, with approximately 20% remaining after treatment at 80°C, but rapidly diminishing thereafter (data not shown). Thus reactivity with antibodies directed against structural determinants is preserved up to 70°C but is lost at higher temperatures, indicating that, above this temperature, the particle undergoes a major structural reorganization.

Sera directed against the N-termini of VP1 have little effect on viral infectivity in culture

Since VP1-specific sera failed to react with the great majority of newly released virions, we might not expect them to neutralize viral infectivity. However, the particle to infectivity ratio of MVMp when used to infect A9 cells in culture is approximately 300:1 (Tattersall, 1972), and thus it remained possible that the small proportion of non-heat-treated full particles that appeared to precipitate with anti-VP1 sera (~7% in Fig. 3B) could represent a highly infectious subset, accounting for most of the infectivity in the viral stock. To explore this possibility we first assessed the ability of each serum to neutralize MVM infections in cell culture using standard laboratory stocks of virus. Under assay conditions described under Materials and Methods, anti-capsid sera PN1 and PN2 neutralized infection totally at all dilutions from 10^{-1} to 10^{-6} and had a partial effect at 10^{-7}, while anti-VP1 serum, anti-MAPPA serum, and prebleed samples from the same rabbits failed to have any influence on virus infectivity at all dilutions tested (from 10^{-1} to 10^{-10}).

These results also indicate that particles with VP1-specific residues exposed do not account for a major proportion of the infectious virus in standard laboratory stocks or that antibodies directed against these epitopes are not neutralizing. We addressed this question using the more extensively characterized, newly released viral preparations described above. Immunodepletion experiments were carried out in which these stocks were first

TABLE 1

Immunodepletion with Anti-VP1-Specific Sera Does Not Reduce Virus Titers

Antibody used	Remaining titer (PFU/ml) Experiment 1	Experiment 2
None	2×10^7	3.5×10^7
Nonimmune serum	8.8×10^6	2.9×10^7
Anti-VP1	9.5×10^6	3.4×10^7
Anti-MAPPA	ND	3.5×10^7
Anti-capsid PN1	$<2.5\times10^4$	$<2.5\times10^4$

Note. ND, not determined.

cleared of all subpopulations reacting with a particular antiserum, by adding that serum plus an immunoadsorbant, and the titer of the remaining virus was then compared to samples similarly cleared with prebleed serum. Results from such experiments, shown in Table 1, indicate that the small proportion of virions that have VP1-specific epitopes exposed at their surface do not constitute a highly infectious subpopulation of virus and that particles in which these determinants remain sequestered are infectious.

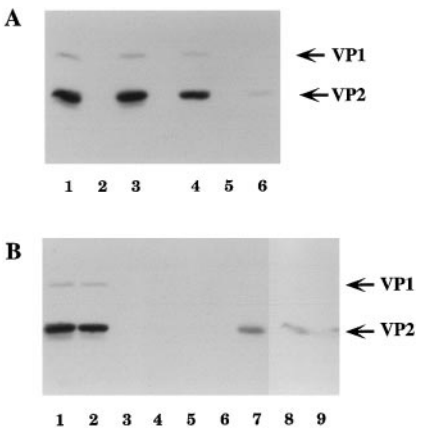

FIG. 4. N-terminal sequences of VP2 are exposed at the surface of all virions, but on few empty particles. (A) Native ^{35}S-labeled virions (lanes 1–3) and empty particles (lanes 4–6) were quantitatively immunoprecipitated with PN1 (lanes 1 and 4). Prebleed serum (from a rabbit subsequently immunized with the MSDGT oligopeptide corresponding to the N-terminal 25 amino acids of VP2) failed to react with either type of particle (lanes 2 and 5), but immune serum from the same rabbit precipitated virions efficiently (lane 3), while failing to react with most empty particles (lane 6). (B) Native ^{35}S-labeled virions were preincubated for 10 min at 37 or 60°C before being quantitatively immunoprecipitated with sera directed against the N-terminus of VP2 (lanes 1 and 2, respectively). Unbound material from such precipitations did not contain any labeled species that could subsequently be precipitated with PN1 (lanes 3 and 4, respectively). Equivalent unbound samples lacked any material that reacted with sera directed against the VP1-specific region (lanes 5 and 6, respectively). The proportion of native ^{35}S-labeled virions that could be precipitated directly with anti-VP1-specific sera following preincubation at 60°C is shown in lane 7. Native ^{35}S-labeled empty particles reacted poorly with sera directed against the N-terminus of VP2 following preincubation at 37 or 60°C (lanes 8 and 9, respectively).

TABLE 2

Heat Inactivation of Viral Infectivity Correlates with the Proportion of Virions in Which VP1-Specific Sequences Are Exposed

Pretreatment	% VP2 precipitated with anti-VP1[a]	Infectivity Remaining PFU	Inactivation
RT	5.0%	7.0×10^6	—
37°C	3.9%	5.0×10^6	29%
45°C	8.6%	5.8×10^6	17%
52°C	30%	4.1×10^6	41%
60°C	73%	2.5×10^6	64%
70°C	74%	1.4×10^6	80%
80°C	23%[a]	1.7×10^5	98%
90°C	13%[a]	$<2.5\times10^4$	>99.6%

[a] Amount of VP1 precipitated by anti-VP1 was between 94 and 97% in each case.

VP2 N-termini are exposed in all full MVM virions, but are sequestered in empty particles

Rabbit anti-MSDGT, raised against a synthetic peptide corresponding to the N-terminal 25 residues of VP2, immunoprecipitated all full MVM virions, reacting as efficiently as the rabbit anti-whole-capsid serum PN1, when analyzed under nondenaturing conditions (Fig. 4A, compare lanes 1 and 3). Under the same conditions, however, this serum immunoprecipitated empty viral particles with very low efficiency (Fig. 4A, lane 6), compared to PN1 (Fig. 4A, lane 4).

Exposure to 37 or 60°C for 10 min had little effect on virion reactivity with anti-MSDGT (Fig. 4B, lanes 1 and 2). Immunoprecipitation with anti-MSDGT removed essentially all of the full particles in the first round, so that none remained in the unbound material to be scavenged either with PN1 (Fig. 4B, lanes 3 and 4) or, in parallel samples, with anti-VP1 (Fig. 4B, lanes 5 and 6). For comparison, the increased proportion of full particles that became susceptible to precipitation with anti-VP1 following heat treatment at 60°C is shown in Fig. 4B, lane 7. Identical treatment of empty capsids at 37 or 60°C had no effect on their nonreactivity with anti-MSDGT (Fig. 4B, lanes 8 and 9). Thus, unlike the VP1 N-terminus, N-termini of VP2 are available at the surface of all newly released MVM virions without any temperature-induced transition(s), but neither VP1 nor VP2 N-terminal sequences are accessible on the surface of empty capsids or can be made so by heating. This suggests that it is the presence of the viral DNA inside virions that provides the force underlying both the constitutive protrusion of VP2 N-termini and the heat-triggered expulsion of the VP1-specific region.

Viral infectivity titers drop as VP1-specific sequences become exposed

To assess whether or not virions that had undergone heat-induced conformational shifts were infectious, we carried out the experiment shown in Table 2, in which ^{35}S-labeled virions were heated to various temperatures for 10 min in tissue culture medium containing 10% fetal bovine serum before being split into two aliquots to be used for immunoprecipitation with anti-VP1 and for infectivity assays. Despite the inherent variability of parvoviral infectivity assays, it is apparent from Table 2 that heating at temperatures up to 52°C had little effect on the infectivity of this virus stock. However, upon heating at higher temperatures, up to 70°C, the infectious titer dropped approximately in proportion to the increase in virions with exposed VP1-specific sequences. This suggests that virions that have exposed VP1 N-termini are probably no longer infectious, at least when applied to the outside of the cell.

Heated virions with VP1 epitopes exposed are otherwise intact

Since immunoprecipitation is a somewhat protracted procedure, we wished to use a more direct and rapid technique to monitor particle structure and antibody interactions. To this end, we developed an electrophoretic mobility shift assay (EMSA), using ^{35}S-labeled particles. Even though crystallographic studies show that intact virions and empty viral particles have essentially the same size and surface structure (Llamas-Saiz *et al.,* 1997; Agbandje-McKenna *et al.,* 1998), virions migrated substantially faster when electrophoresed through low-percentage agarose gels (Fig. 5A, compare lanes 1 and 2). This likely reflects a difference in net surface charge between the two structures, but precise measurements of this were not attempted. Despite this difference, when either type of particle was preincubated with antibodies directed against surface epitopes, its migration was effectively retarded, and the extent to which it was retarded appeared to reflect the number of antibody molecules bound. This is shown for empty particles in Fig. 5A, where tissue-culture medium from a control hybridoma supernatant failed to influence particle mobility at a dilution of 1:25 (Fig. 5A, lane 3), while supernatant from the anti-capsid hybridoma MAb D10 shifted all particles to the top of the gel at dilutions of 1:25, 1:50, and 1:100 (Fig. 5A, lanes 4–6, respectively). This monoclonal antibody is potentially able to bind one antigen-combining site to each of the 60 asymmetric units in the capsid and only at dilutions of 1:200 and 1:400 allowed some particles to enter the gel, migrating as a new band that was still retarded relative to the input particles (Fig. 5A, lanes 7 and 8). The stoichiometry of antibodies to particles in this band has not been determined, but the high dilution required to obtain this shift suggests that relatively few

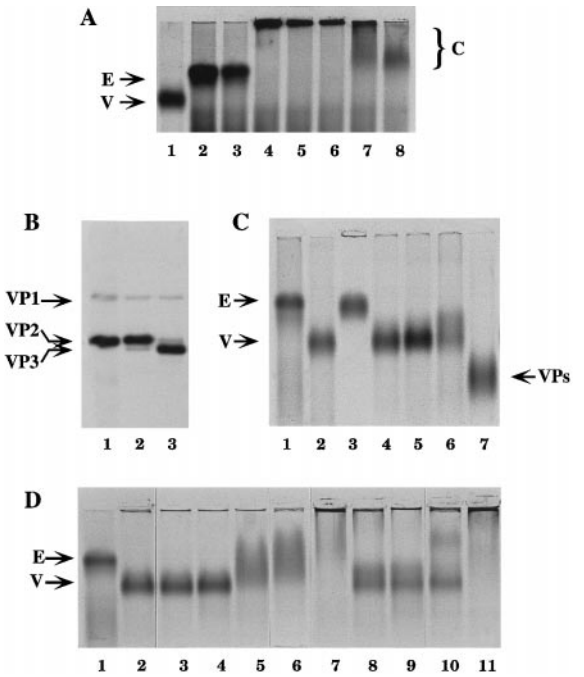

FIG. 5. Electrophoretic mobility shift assay analysis. (A) Native ^{35}S-labeled virions (lane 1) and empty viral particles (lanes 2–8) were electrophoresed through native 1% agarose gels before (lanes 1 and 2) and after incubation for 30 min with a 1:25 dilution of control tissue culture medium (lane 3) or the following dilutions of medium from the anti-capsid hybridoma MAb D10: 1:25 (lane 4), 1:50 (lane 5), 1:100 (lane 6), 1:200 (lane 7), or 1:400 (lane 8). E, empty particles; V, virions; C, particle:antibody complexes. (B) Discontinuous SDS–polyacrylamide gel showing ^{35}S-labeled capsid proteins from untreated empty particles (lane 1) and virions before (lane 2) and after (lane 3) treatment with trypsin, as described under Materials and Methods. (C) Untreated native ^{35}S-labeled empty particles (lane 1) and virions before (lane 2) and after (lane 3) treatment with trypsin were electrophoresed through native 1% agarose gels. Undigested ^{35}S-labeled virions are also shown following 10 min of preincubation at 37°C (lane 4), 45°C (lane 5), 65°C (lane 6), and 85°C (lane 7). E, empty particles; V, virions; VP, polypeptide subunits. (D) Untreated native ^{35}S-labeled empty particles (lane 1) and virions (lanes 2–11) were electrophoresed through native 1% agarose gels before (lane 2) and after incubation with antisera directed against the following determinants: VP1-specific region (1:100 dilution, lane 3); prebleed serum from a rabbit subsequently immunized with the N-terminal peptide of VP2 (1:100 dilution, lane 4); immune serum from the same rabbit (1:100 dilution, lane 5; 1:50 dilution, lane 6); 1:100 dilution of culture medium from MAb D10 (lane 7). Native ^{35}S-labeled virions were also preincubated for 10 min at 60°C and electrophoresed before (lane 8) or after incubation with the following sera: prebleed serum from a rabbit subsequently immunized with the VP1-specific region (1:100 dilution, lane 9); immune serum from the same rabbit (1:100 dilution, lane 10); anti-capsid MAb D10 (1:100 dilution, lane 11). E, empty particles; V, virions.

antibodies were bound to each particle that entered the gel and that this technique might be useful for isolating specific virus:antibody complexes.

Both the empty particles and the full virions used in these studies were made up predominantly of VP2 molecules (Fig. 5B, lanes 1 and 2), but when the latter were exposed to trypsin *in vitro,* most of the VP2 chains were cleaved to VP3-like polypeptides by removal of the ex-

posed N-terminal peptide (Fig. 5B, lane 3). Attempts to cleave the few remaining VP2 molecules by prolonged exposure to trypsin have not been successful, suggesting that the residual N-termini are not normally exposed at the virion surface (Tattersall *et al.,* 1977). When the electrophoretic mobilities of such populations were compared (Fig. 5C, lanes 1–3), it became apparent that removing the exposed N-termini of VP2 significantly reduced the migration rate of virions, so that they now comigrated with empty particles (Fig. 5C, compare lanes 1 and 3).

The mobility of full particles could also be influenced, albeit less dramatically, by heating. The effects of heating full particles to 37, 45, 65, and 85°C are shown in Fig. 5C, lanes 4–7, respectively. Each particle population migrated as a rather broad band, which presumably reflects charge heterogeneity within it, since DNA fragments electrophoresed in the same gel migrated as sharp bands (data not shown). After incubation at 60°C, however, a new and quite distinctly lower mobility form of the virion was apparent (Fig. 5C, lane 6). This form likely represents particles that have one or more of their highly basic VP1-specific peptides exposed at the particle surface as a result of the heat-induced conformational shift (see below). After being heated to 85°C, labeled proteins migrated much more rapidly than intact particles, suggesting that they had fallen apart into subunits or free polypeptides (Fig. 5C, lane 7).

We next used this EMSA approach to explore further the effects of sera directed against the N-termini of VP1 and VP2 on virion populations before and after heat treatment. While the migration of full particles was not influenced by incubation with anti-VP1-specific serum (Fig. 5D, lane 3) or with preimmune serum from a rabbit subsequently immunized with the VP2 N-terminal MSDGT peptide (Fig. 5D, lane 4), particle migration was somewhat retarded by incubation with anti-MSDGT immune serum (Fig. 5D, lane 5). However, the antibody: particle complexes generated with this serum entered the gel and migrated only slightly slower than input virions. By analogy with the results shown in Fig. 5A, therefore, it seems likely that substantially fewer than 60 antibody molecules were bound to each virion in this case. That this was a function of capsid structure rather than antibody concentration was indicated by the fact that adding twice as much serum had little additional effect on the distribution of retarded particles (Fig. 5D, lane 6). In contrast, most virions failed to enter the gel after incubation with a 1:100 dilution of the anti-capsid monoclonal antibody (Fig. 5D, lane 7), suggesting that in this case many more antibodies were bound to each particle.

After virions were heated to 60°C, a subpopulation of particles that had undergone a conformational transition and thus migrated more slowly than the rest of the input was once again apparent (Fig. 5D, lane 8), and while

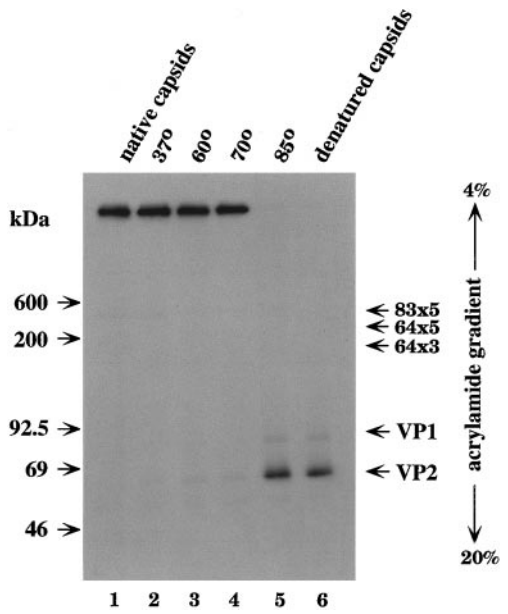

FIG. 6. Gradient polyacrylamide gel analysis of virion structural integrity following heat treatment. Untreated ^{35}S-labeled virions, incubated with 1% SDS at 37°C for 30 min before electrophoresis through gradient polyacrylamide gels in the presence of 0.1% SDS, migrated as large structures near the top of the gradient (lane 1). Equivalent samples were exposed to similar treatment after preincubation for 10 min at the following temperatures under physiological buffer conditions: 37°C (lane 2), 60°C (lane 3), 70°C (lane 4), and 85°C (lane 5). Samples boiled in 1% SDS for 5 min prior to electrophoresis are shown in lane 6. Apparent MWs are indicated along the left margin, and the theoretical positions of VP1 pentamers (83 kDa ×5), VP2 pentamers (64 kDa ×5), and VP2 trimers (64 kDa ×3) are indicated on the right.

incubation with a preimmune serum had no influence on the mobility of either particle type in this heated sample (Fig. 5D, lane 9), incubation with anti-VP1-specific immune serum caused the new, heat-induced species to be further retarded (Fig. 5D, lane 10) while having no influence on the mobility of the nonmodified form. Thus, heating induces a conformational shift in virion structure that reduces its electrophoretic mobility and causes VP1-specific sequences to become accessible at the particle surface. Since the retarded band generated with anti-VP1-specific serum migrated well within the gel, we presume that relatively few antibodies were bound in each complex. In contrast, antibodies directed against the intact capsid shifted essentially the entire particle population to the gel interface even after heat treatment (Fig. 5D, lane 11).

Particles remain intact when heated to 70°C, but rapidly dissociate into monomeric polypeptides at higher temperatures

To assess the integrity of the particle following heating we exposed virions to a range of different temperatures

under physiological buffer conditions, bound SDS to them at 37°C, and then electrophoresed them through gradient polyacrylamide gels without further heating. If native particles were exposed to this treatment (Fig. 6, lane 1), they migrated as large structures near the top of the gradient, well above the highest molecular weight marker (thyroglobulin, MW 600 kDa), but if denatured by boiling in SDS sample buffer prior to electrophoresis, they dissociated into monomeric VP1 and VP2 polypeptides (Fig. 6, lane 6). Experimental samples that had been exposed to temperatures of 37, 60, or 70°C for 10 min, prior to the addition of SDS, comigrated near the top of the gel with the native virions, showing no evidence of disintegration into intermediate trimer or pentamer subunits or any evidence that VP1 was preferentially released from the particles by this treatment (Fig. 6, lanes 2–4). However, when heated to 85°C particles dissociated totally into monomeric VP1 and VP2 polypeptides (Fig. 6, lane 5) that comigrated with viral polypeptides released from the virion by boiling in sample buffer (Fig. 5, lane 6). This confirms the previous observations that at temperatures up to 70°C virion polypeptides remain predominantly associated as intact particles, while at higher temperatures both structural epitopes and the ability to coprecipitate VP2 with anti-VP1 are rapidly lost as the particle disintegrates into its component polypeptides.

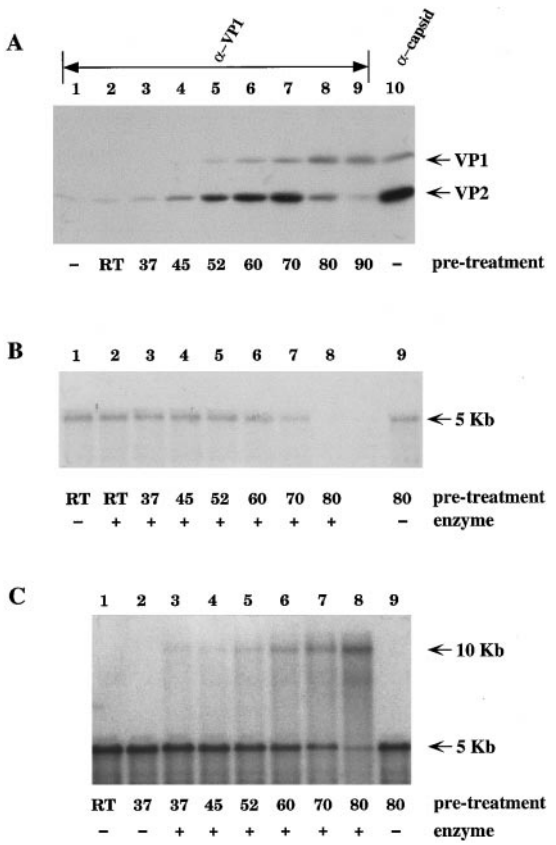

FIG. 7. VP1 and the viral genome become concomitantly accessible at the virion surface upon heating. Virions with ^{35}S-labeled capsid proteins were mixed with virions labeled with ^{32}P in their DNA. Aliquots of this mixture were analyzed by gel electrophoresis as described under Materials and Methods, after exposure to the indicated temperatures, and subsequently immunoprecipitated with sera against the VP1-specific region (A), digested with nuclease P1 (B), or incubated with Sequenase in the presence of unlabeled dNTPs (C). For comparison, untreated aliquots were also precipitated with PN1 (A, lane 10) or incubated without the modifying enzyme before or after exposure to heat (B, lanes 1 and 9; C, lanes 1, 2, and 9).

Viral DNA becomes progressively exposed at temperatures above 52°C

To determine whether viral DNA was still protected from the external environment in particles that had undergone the heat-induced conformational shift, we mixed virions that had ^{35}S-labeled capsid proteins with others that had ^{32}P-labeled DNA and exposed samples of the mixture to a variety of temperatures. Individual samples were then divided into three equal aliquots, one of which was immunoprecipitated with anti-VP1 serum, a second digested with P1 nuclease, while the remaining aliquot was incubated with Sequenase and unlabeled deoxynucleotides in order to replicate any exposed viral DNA. The last assay, in particular, measures the availability of the genomic 3′ end, which, in its hairpin configuration, will prime synthesis of a DNA strand complementary to packaged DNA, creating full-length duplex monomer forms of the viral genome.

Most, if not all, purified virion preparations appear to be contaminated with some unpackaged genomic DNA, presumably released by particles that become denatured during purification or during subsequent dilution using plastic tips and tubes in this experiment (see Materials and Methods). However, the proportion of such free DNA in the virions assayed here was quite low (Fig. 7B, compare lanes 1 and 2, and Fig. 7C, compare lanes 2 and 3). After heating to 37 or 45°C there was little, if any, increase in the susceptibility of viral DNA to P1 nuclease or Sequenase, suggesting that the genome remained essentially sequestered. However, at higher temperatures the genome became increasingly exposed to these enzymes, so that after being heated to 70°C most of the viral DNA was susceptible to P1 nuclease, although a somewhat lower proportion served as a polymerase substrate. Throughout this temperature range, VP1:VP2 ratios in the anti-VP1 precipitates suggested that particles had not disintegrated, but rather that the conformational shifts induced by heating led to exposure of both the viral genome and, as previously demonstrated, the VP1-specific sequences. This indicates that, energetically speaking, it is significantly easier to expose the MVM genome in the context of its intact capsid shell than it is to induce that capsid to dissociate into polypeptide subunits.

DISCUSSION

Since X-ray crystallographic analysis has not provided precise structural information concerning the disposition of the N-terminal regions of VP1 and VP2 within the MVM particle, we have used peptide-specific antibodies to show that while the N-termini of some VP2 molecules are exposed at the surface of the newly released virion, the entire N-terminal 142-amino-acid peptide specific to VP1 molecules is sequestered within it. This observation is somewhat unexpected because the analogous 227-amino-acid, VP1-specific peptide from parvovirus B19 has been shown to be surface accessible both in recombinant capsids, expressed in insect cells from baculovirus vectors, and in authentic serum-derived virus, where antisera directed against VP1 sequences could be used to neutralize viral infectivity *in vitro* (Rosenfeld *et al.,* 1992; Anderson *et al.,* 1995). However, MVM and B19 belong to different genera in the subfamily *Parvovirinae* and are genetically and structurally quite different from each other (Berns *et al.,* 1995). While, to date, the X-ray structure of B19 has been determined only for recombinant empty particles at low resolution (8 Å), their surface topology does appear quite distinct. In particular, the five-fold cylinders, which in MVM, CPV, and FPV virions appear to be hollow and modulate surface exposure of the VP N-termini, are closed in B19 (Agbandje *et al.,* 1994). Whether this apparent structural difference between the genera translates into different mechanisms of cell entry, intracellular trafficking, or uncoating of the genome remains to be explored.

Our data suggest that the glycine-rich sequence modeled into the cylindrical pore at the five-fold symmetry axis of the MVM particle is derived exclusively from VP2 molecules and that similar sequences from VP1 remain inside the capsid shell in close proximity to the DNA. In accord with the crystallographic data, neither of these N-terminal sequences is exposed at the surface of empty viral particles. In the crystal structure the pore is relatively narrow (<8 Å), so that it can accommodate only a single, unstructured peptide chain composed of amino acids that lack bulky side groups, such as the glycine/serine-rich sequence found between VP2 residues 28 and 38 (GGSGGGGSGGG) in MVM. As a result, a maximum of 12 VP2 N-termini would be expected to project from the particle surface at any one time.

Our electrophoretic mobility shift data support the idea that exposure of VP2 N-termini is limited, since immune complexes generated using anti-VP2 sera have much higher mobilities than similar complexes generated using antibodies that potentially recognize 60 epitopes on the particle surface. Similarly, Cortes *et al.* (1993) were unable to decorate virions with gold particles tagged to monoclonal antibodies directed against the N-terminus of CPV VP2 molecules, presumably because there were relatively few epitopes available to label, even though these antibodies were able to neutralize viral infectivity *in vitro.* However, when incubated with trypsin at 37°C, most of the VP2 molecules in newly released MVM virions can be cleaved at a position immediately N-terminal to the glycine-rich tract [see Fig. 5B and Tattersall *et al.* (1977)]. This suggests that even at 37°C there must be considerable flexibility in the capsid structure, allowing the pores to open and accommodate multiple polypeptide chains at one time or, at least, to allow the bulky side groups of N-terminal peptides from successive VP2 molecules to be threaded through the pore after the first molecule has been cleaved and its glycine chain retracted. Previously we have shown that in newly released MVM virions 24 nucleotides from the 5′ end of the single-stranded DNA genome also remain exposed to the external environment at the particle surface (Cotmore and Tattersall, 1989), and although we do not know exactly where this chain penetrates the capsid shell, flexibility around the five-fold pores would definitely be required to accommodate such a large structure by this route. However, the cylindrical projections that surround these pores appear to have few restraints that would seriously impede their opening, and although energy would be required to break existing interchain bonds, studies with poliovirus suggest that at 37°C the capsids of nonenveloped viruses can be substantially more flexible than their crystal structures indicate (Li *et al.,* 1994). Thus, while the three-fold axes of MVM are spanned by complex, interwoven polypeptide chains, the five-fold cylinders are composed of antiparallel β-ribbons from five different polypeptide chains that do not interdigitate around the upper part of the channel. Intersubunit contacts in the cylinders might, therefore, be broken and the individual peptide loops move outward into the space made available by the surrounding canyon, to facilitate the egress of larger structures.

It is tempting to speculate that heating particles to 45°C or above might induce even more extreme flexing of the five-fold cylinders, resulting in the conformational transitions described in this paper that render VP1-specific sequences surface-accessible and ultimately allow egress of the genome. Like many viruses, MVM must assemble a rugged protective virion in its parental host cell, which can protect its genome from the external environment and conditions encountered in the entry portals of its subsequent host cell, but which can then traffic its genome to the nucleus and open or disassemble in such a way as to render the genome accessible for replication by host enzymes. To this end, mature virions from many viral groups are known to be metastable, undergoing conformational transitions required for successive steps in the infectious process in response to specific stimuli encountered during entry [reviewed in Greber *et al.* (1994)]. In some cases such transitions can also be induced experimentally by exposure to elevated temperatures (Curry *et al.,* 1996), and it was our hope that

we would be able to recapitulate the heat-induced MVM conformational transitions described here using experimental conditions that the virus would be likely to encounter during the early stages of entry. Thus, for example, entry of both CPV and MVM can be blocked by drugs that prevent lowering of the endosomal pH (Basak and Turner, 1992; J. Gerlach and P. Tattersall, unpublished results), suggesting that these viruses pass through an obligate acidic environment during entry. However, exposure to acidic conditions alone (pH 4.5–6.0 for 30 min) failed to render the N-termini of VP1 surface accessible (data not shown). Likewise, exposure to the protease trypsin effectively cleaved most VP2 molecules to VP3, as occurs during entry, but failed to induce exposure of VP1-specific sequences. Moreover, exposure to a buffer consisting of 1 mM EGTA, 10 mM DTT, 50 mM Tris–HCl, pH 8.0, at 37°C for 1 h followed by addition of KCl to 850 mM and continued incubation for 30 min, recreating the conditions of calcium depletion and ionic shock used by Dean and colleagues to bring about the release of VP1 molecules from SV40 particles (Dean *et al.,* 1995), had no effect on exposure of the MVM VP1 N-terminal epitopes (data not shown). It should also be noted that incubation in 1% SDS at 37°C for 30 min failed to render these sequences accessible (data not shown), suggesting that a more specific stimulus is required to induce the MVM conformational shifts we describe.

It is thus of interest that when intact full MVM virions are incubated in the type of HeLa cell-free S100 extracts we routinely use to study viral DNA replication *in vitro* (Christensen *et al.,* 1997), a significant proportion of the particles (~30%) undergo a structural alteration that renders their genomes accessible to the cellular replication machinery (Cotmore *et al.,* 1998). This transition leaves the particle essentially intact, as evidenced by the fact that they can be immunoprecipitated with various structure-specific antibodies. During the process, the VP1-specific sequences are exposed and the newly replicated genome remains tightly associated with the now empty particle. Although this would represent a novel mechanism, disassembly of alphaviruses is known to be mediated by a specific cellular uncoating factor(s) associated with ribosomes (Wengler *et al.,* 1996). Previsani *et al.* (1997) have previously reported that extracts of EL4 lymphocytes contain a factor that can destabilize the virions of MVMi, an MVM serotype that replicates productively in murine lymphocytes, but not those of MVMp, the fibrotropic virus used in the studies reported here. Exactly which component of the replication extract induces this transition remains uncertain, but we are optimistic that the electrophoretic mobility shift assay described here will facilitate identification of the modulating factors and the conditions required for their operation.

MATERIALS AND METHODS

Cells, viruses, and isotopic-labeling protocols

The prototype strain of MVM (MVMp) was grown in the murine L-cell derivative A9 ouab^{r}11 as previously described (Tattersall and Bratton, 1983). Cells were synchronized using a double-block procedure described elsewhere (Cotmore and Tattersall, 1987). Briefly, cells were allowed to accumulate in G_0 by withholding isoleucine for 48 h and then were released from the isoleucine block while simultaneously being exposed to 10 μg/ml aphidicolin. MVM virions (10 PFU/cell) were added to the cells along with the polymerase inhibitor and were able to penetrate cells and deliver their genomes to the nuclei in the 20 h before the inhibitor was finally removed. All times of infection referred to here initiate at the point when the aphidicolin was finally removed and the cells became free to enter the S-phase of the cell cycle.

Infected cells were labeled with Trans ^{35}S-label (ICN Radiochemicals, Irvine, CA) at 0.2 mCi/ml in Dulbecco's modified Eagle's minimal medium containing 1/100 of the normal concentration of unlabeled methionine and cysteine and 5% dialyzed fetal calf serum or with [^{32}P]orthophosphate at 0.5 mCi/ml in medium containing 1/10 the normal concentration of unlabeled phosphate and 5% dialyzed fetal calf serum. Label was added 6 h after the cells were released into S-phase and removed 7 h later ($t = +13$), to be replaced with medium containing *Clostridium perfringens* neuraminidase (Sigma Chemical Co., St. Louis, MO) at 0.1 mg/ml. Neuraminidase destroys the A9 cell surface receptor used by MVM (Cotmore and Tattersall, 1989) and thus prevents newly released virus from adhering to or reentering host cells. Newly released virus was harvested by collecting the culture medium 12 h later ($t = 25$) and cleared of cellular debris by centrifugation at 15,000 *g* for 30 min at 4°C. Cleared supernatant was layered, in 5.5-ml amounts, onto 1 ml 60% sucrose on top of 4 ml CsCl (density 1.40), both in TE8.7 (0.5 mM EDTA, 50 mM Tris–HCl, pH 8.7) containing 1% fetal bovine serum, and centrifuged at 30,000 rpm for 25 h at 4°C in a Beckman SW41 rotor. Fractions were collected by aspiration from the bottom of the tube and analyzed for virus by hemagglutination assay, radiolabel profile, and gel electrophoresis. Peak fractions of full virions and empty capsids were pooled separately and dialyzed against three changes of 1000 vol of TE8.7 in the cold.

Infectivity and virus neutralization assays

MVM stocks were assayed for infectivity and antibody-mediated neutralization by plaque assay on 324K monolayers as described elsewhere (Tattersall and Bratton, 1983). For the immunodepletion experiments, samples were incubated with test and control sera as described below for immunoprecipitation, and residual viral titers were determined by plaque assay.

Construction and bacterial expression of a histidine-tagged form of the VP1-specific region

A DNA sequence encoding the N-terminal 141 amino acids of the 142 amino acids that make up the VP1-specific region of MVMp was cloned, with a 6-residue histidine tag at its carboxy-terminus, into a bacterial expression vector, pQE-60 (Qiagen, Chatsworth, CA), which allowed it to be expressed to high levels under the control of *lac* promoter/operator sequences. The VP1 protein encoded by this plasmid, p$(His)_6$-tag-VP1, is made up of an N-terminal 11-amino-acid exon (MVMp nucleotides 2286–2316) spliced to a second exon starting at nucleotide 2399 and extending to nucleotide 2790 in MVM, as shown in Fig. 1. Polymerase chain reaction primers were used to insert an *Nco*I site at the amino-terminus of VP1 and a *Bgl*II site at the downstream end of the VP1-specific region above, thus allowing it to be inserted in-frame, at its C-terminus, with the histidine tag sequence provided by the vector.

Very few transformants were isolated when p$(His)_6$-tag-VP1 was transformed into bacterial hosts, such as JM109, that failed to repress fully expression of the cloned sequence. Six of these transformants examined by DNA sequencing contained different single-point mutations scattered throughout the VP1 sequence, five of which influenced the predicted coding sequence. Interestingly, all attempts to recombine these plasmids to obtain wild-type transformants failed, suggesting that even low-level expression of the wild-type sequence might be toxic. The relief of such an extreme negative effect by single point mutations suggests that it is elicited by the VP1-specific peptide through a particular biological mechanism, which currently remains unknown. To circumvent this problem, plasmids were transformed into bacterial strain M15 containing pREP4, an F′ plasmid that expresses the *lac i* gene (Qiagen, Chatsworth, CA). pREP4 can be maintained under constant kanamycin selection, ensuring tight down-regulation of protein expression from the *lac* promoter in the absence of activator. Using this approach, transformants containing the wild-type VP1-specific sequence were obtained with close to normal frequency. The presence of a correct copy of the VP1-specific region in the final plasmid was confirmed by DNA sequencing.

VP1-$(His)_6$ peptide expression was induced by the addition of 2 mM isopropyl-β-D-thiogalactoside (Research Products International, Prospect, IL), and cells were harvested 4 h later. Since most of the induced peptide was insoluble, bacterial pellets were extracted with guanidinium hydrochloride, and the peptide was partially purified by chromatography on nickel–agarose according to protocols supplied by the manufacturer (Qiagen). Fractions containing VP1-$(His)_6$ were precipitated with ethanol and further purified by electrophoresis through SDS–PAGE gels. The VP1-specific peptide was excised and used to immunize a rabbit by a standard protocol employing Freund's adjuvant.

Anti-peptide antibody production

Three oligopeptides were synthesized to enable the production of specific polyclonal antibodies for these studies. "MAPPA" is a 20mer peptide, NH_2-A P P A K R A K R G W V P P G Y K Y L [C-COOH], that recapitulates the sequence of the 19 amino-terminal amino acids of MVMp VP1. "MSDGT" is a 25mer peptide with the sequence NH_2-M S D G T S Q P D G G N A V H S A A R V E R A A [C-COOH], which corresponds to residues 1 through 24 of the VP2 sequence. An 18mer oligopeptide denoted allopeptide represents the sequence between amino acid residues 311 and 327 in the MVMp VP2 sequence, namely, NH_2-Q G S R H G T T Q M G V N W V S K [C-COOH]. This spans an allotropic determinant within the MVMp coat, specifically comprising amino acids T317 and G321, which will switch the host range phenotype of the virus from lymphotropic to fibrotropic when coordinately substituted for A317 and E321 in the coat of MVMi (Ball-Goodrich and Tattersall, 1992).

In each case, an additional carboxy-terminal cysteine residue was added to each oligopeptide, to allow efficient conjugation to keyhole limpet hemocyanin. This step was accomplished using a conjugation kit (Pierce Chemical Corp., Rockford, IL) as directed by the supplier. Antibodies were raised in rabbits by repeated injection of the conjugate at multiple sites according to an approved protocol employing Freund's adjuvant.

Antibody purification

Sera directed against the MAPPA peptide showed reactivity against MVM particles that could not be inhibited by addition of the immunizing peptide. To overcome this problem, the peptide was conjugated to Reacti-Gel-6X (Pierce Chemical Corp.) according to instructions provided by the manufacturer, and the antibodies were purified by affinity-chromatography (Harlow and Lane, 1988).

Heat treatment, immunoprecipitation, and enzyme reactions

Purified virus particles are readily denatured by extreme dilution into protein-free medium. To avoid such effects, viral particles were first diluted into 10 vol of tissue culture medium containing 10% fetal calf serum and incubated for 15 min at room temperature before being heated to the specified temperature for 3–10 min as indicated.

For immunoprecipitation, 70 vol of buffer A (0.01 M Tris–HCl, pH 8.0, 0.15 M NaCl, 0.002 M EDTA) containing 1% Nonidet-P40 was added, and samples were captured on formalin-fixed *Staphylococcus aureus* (Boehringer

Mannheim, Mannheim, Germany) as previously described (Cotmore and Tattersall, 1986).

For digestion with P1 nuclease (Calbiochem–Novabiochem Corp., La Jolla, CA) heat-treated samples (in 10 vol of medium plus 10% fetal calf serum) were incubated at 37°C for 20 min with 0.125 units of enzyme (1 μl at 0.25 mg/ml, made up in 0.3 M sodium acetate, pH 5.2, 200 μM ZnCl), and reactions were stopped with EDTA. Samples were incubated with proteinase K (50 μg/ml) in the presence of 0.5% SDS prior to electrophoresis through neutral agarose gels.

For second-strand synthesis, heat-treated samples were incubated with 0.25 μl of Sequenase (U.S. Biochemical, Cleveland, OH) in the presence of all four unlabeled deoxynucleotides (250 μM), for 45 min at 37°C using conditions specified by the manufacturer. Reactions were stopped and samples were proteolysed as described for P1 nuclease, and samples were electrophoresed through denaturing, alkaline agarose gels.

Gel electrophoresis

Unless specified to the contrary, proteins were analyzed by electrophoresis through discontinuous polyacrylamide gels (7.5% acrylamide, 0.2% bis-acrylamide) in the presence of SDS according to the procedure of Laemmli (1970), and gels were incubated in 1 M salicylic acid (adjusted to pH 7.5 with NaOH) prior to drying and exposure to film. Gradient gels (4–20%) contained 0.1% SDS and were prepared with an acrylamide:bis-acrylamide ratio of 60:1.

Neutral or alkaline agarose gels were prepared according to standard procedures (Maniatis *et al.,* 1982). Samples for electrophoretic mobility shift assays (15 μl) generally contained buffer A (without detergent) and were incubated for 30 min at room temperature prior to the addition of 1/10 vol of sample buffer (to give 0.3% bromphenol blue and xylene cyanol, 50 mM Tris–HCl, 20 mM EDTA, 0.2% sarkosyl, 30% glycerol, pH 7.5) and electrophoresis through 1% neutral agarose gels in Tris–acetate buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.5). In experiments where some samples were heat-treated, all samples were initially preincubated with 10% fetal bovine serum at room temperature as described above. Samples (15 μl) to be digested with trypsin (2 μg) were incubated in 100 mM NaCl, 100 mM sodium acetate, 5 mM DTT, pH 7.5, for 1 h at 37°C. After electrophoresis, gels were fixed in 7% trichloracetic acid, neutralized, partially dehydrated by blotting on paper towels, infused with 1 M salicylic acid–NaOH, pH 7.5 (for ^{35}S-labeled samples), dried, and exposed to film. Quantitation of bands was accomplished on a PhosphorImager SI using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA).

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