Functional relevance of amino acid residues involved in interactions with ordered nucleic acid in a spherical virus*

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Running title: Mutational analysis of a viral DNA-capsid interface
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In the spherical virion of the parvovirus minute virus of mice, several amino acid side chains of the capsid were previously found to be involved in interactions with the viral single-stranded DNA molecule. We have individually truncated by mutation to alanine many (ten) of these side chains, and analyzed the effects on capsid assembly, stability and conformation, viral DNA encapsidation and virion infectivity. Mutation of residues Y270, D273 or D474 led to a drastic reduction in infectivity. Mutant Y270A was defective in capsid assembly; mutant D273A formed stable capsids, but it was essentially unable to encapsidate the viral DNA or to externalize the N-terminus of the capsid protein VP2, a connected conformational event. Mutation of residues Y270, D273 or D474 led to a drastic reduction in infectivity. Mutation of residues D58, W60, N183, T267 or K471 led to a moderate reduction in infectivity. None of these mutations had an effect on capsid assembly or stability, or on the DNA encapsidation process. However, those five mutant virions were substantially less stable than the parental virion in thermal inactivation assays. The results with this model spherical virus indicate that several capsid residues that are found to be involved in polar interactions or multiple hydrophobic contacts with the viral DNA molecule contribute to preserving the active conformation of the infectious viral particle. Their effect appears to be mediated by the noncovalent interactions they establish with the viral DNA. In addition, at least one acidic residue at each DNA-binding region is needed for DNA packaging.

Many fundamental biological processes involve protein-nucleic acid recognition. Double-stranded DNA-protein interactions have been extensively studied, but single-stranded (ss) DNA- or RNA-protein interactions are much less characterized. In particular, very little is known about the molecular interactions between the nucleic acid genome of ssDNA or RNA virus and its protein shell, or the functional role of those...
interactions. Such knowledge may be essential for a better understanding of the life cycle of viruses, and for the design of drugs aimed at disrupting nucleic acid-protein interfaces.

The structure of a helical virus, tobacco mosaic virus, has revealed defined interactions between repeating sets of three nucleotides in its ssRNA genome and each capsid subunit (reviewed in 1). In the crystal structures of spherical viruses (reviewed in 2-6) a large part, or all, of the nucleic acid component is invisible, because it is randomly oriented within the viral particles that form the crystal. Fortunately, in a few icosahedral viruses some segments of the nucleic acid molecule (about 10%-60% of the total) are arranged with the same symmetry as the capsid, and could be visualized. The structural analysis (reviewed in 4, 7-10) has revealed that the visible capsid-nucleic acid interfaces generally include a limited number of amino acids and some 7 to 30 nucleotides that in several, but not in all cases, form intramolecular double-helical segments. The tertiary structure acquired by the nucleic acid within the virion may be at least partly independent of the nucleotide sequence (11). Consistent with this observation, a capsid-binding nucleotide motif is not repeated exactly along the sequence, and thus the modelled nucleic acid stretch represents an average of the true individual sequences bound. Accordingly, many of the interactions detected in viral nucleic acid-capsid interfaces are van der Waals and polar interactions with the non-specific phosphate and pentose moieties, but more specific interactions with the bases are also observed. Ionic interactions between some phosphates and basic side chains protruding from the internal surface of the capsid, or located in disordered N-terminal (Nt) arms of capsid proteins occur in several ssRNA plant viruses (12-17) and nodaviruses (18-20), and in the ssDNA bacteriophage ΦX174 (21-23). Such interactions were not observed in the ssRNA bean pod mottle virus (24) or in ssDNA paroviruses (25-30), but many nucleic acid-capsid van der Waals contacts and hydrogen bonds were detected in the latter (25-30). A high-resolution structural model of the ssRNA satellite tobacco mosaic virus showed that binding of the RNA occurs mainly through an intricate network of direct and water-mediated hydrogen bonds (15). Specific and non-specific capsid protein-oligonucleotide interactions were also analyzed in detail in artificial complexes formed by a recombinant capsid of the bacteriophage MS2 and identical copies of a unique viral ssRNA fragment (OR) involved in translational repression and initiation of capsid assembly (31, 32). This allowed a structural interpretation of mutational analyses on the formation of a biologically relevant complex between OR and a MS2 capsid protein dimer (32-37).

Several studies have provided evidence for a functional role of the viral nucleic acid and its interactions with viral proteins in the assembly, conformation, stability and/or disassembly of spherical virions (for some examples see 4, 5, 9, 38-47). However, and partly due to the limited structural information available, mutational analyses to dissect the individual role(s) of specific capsid side
chains that interact with the nucleic acid molecule are scarce (e.g. 41, 46, 47). Also, no single-residue scanning of capsid side chains involved in major interactions at the viral nucleic acid-capsid interface had, to our knowledge, been described.

The parvoviruses canine parvovirus (CPV) and minute virus of mice (MVM) constitute good models for structure-function studies of residues involved in viral nucleic acid-capsid interactions, because of the structural simplicity of the virion and the relatively well-defined viral DNA-capsid interfaces (25-30). The icosahedral T=1 capsid of MVM (29, 30) or CPV (25-28) is formed by 60 protein subunits that are contributed by three non-identical polypeptides, which are derived from a single gene and show identical fold and core sequence. VP2 is the major capsid component, VP1 includes the VP2 sequence plus a Nt extension, and VP3 results from the cleavage of the Nt of some VP2 subunits (48, 49). The DNA recognition site is essentially equivalent in CPV and MVM (strain i), and the major ordered ssDNA stretch (11 nucleotides) adopts a very similar conformation in both viruses (25, 26, 30). This DNA segment interacts through multiple hydrophobic contacts and/or hydrogen bonds mainly with 7-9 side chains of the capsid of either CPV or MVM (25, 26, 30; Table I). Most of these side chains are chemically and sterically identical or similar in both viruses (50), and participate in similar interactions with the DNA. For MVMi, a few shorter DNA stretches, spatially close to the major ordered DNA segment, could also be modelled (29, 30). We have individually truncated to alanine many of those residues of the MVM capsid that were previously (26, 30) found to be more involved in interactions with ordered nucleic acid in the structural model of the virion. The individual effects of the mutations on virus infectivity, viral particle assembly, conformational stability, and DNA packaging have been analyzed.

MATERIALS AND METHODS

Recombinant plasmids and mutagenesis - Site-directed mutagenesis of the VP1/VP2 gene of MVM (strain p) was carried out using the QuickChange system (Stratagene), on recombinant plasmid pSVtk-VP1/2, and the mutant sequences were confirmed by automated DNA sequencing. The mutations were introduced by subcloning in the MVMp infectious clone pMM984, originally provided by P. Tattersall (51), and modified to include the VP2 sequence of the MVMp variant used in our laboratories (52). Two HindIII restriction sites, or unique sites XbaI and EcoRI in the VP2 gene were respectively used for mutations located upstream or downstream of the codon corresponding to amino acid residue 402. The mutant infectious clones were subjected to restriction analysis, and the presence of the mutations and the entire sequence inserted were confirmed by DNA sequencing. The concentration of purified DNA was estimated first by agarose gel electrophoresis, and quantitated by ultraviolet absorbance at 260 nm.

Electroporation of mammalian cells, immunofluorescence and infectivity assays - For mutational analyses,
mammalian NB324K cells at a concentration of 2x10^7 cells/ml were electroporated as described (53, 54) with equal, non-saturating amounts (10 µg) of the infectious recombinant plasmid carrying the appropriate mutations, using in each experiment the nonmutated infectious plasmid as a control. Virions were recovered at 72 h from transfected monolayers and titrated in standard plaque-formation assays (55). The results were normalized by quantitation, using SDS-PAGE (10% polyacrylamide) and immunoblotting with anti-MVM antiserum, of the amount of viral capsid proteins present in extracts of the transfected cells (56).

Immunofluorescence assays were as described (56), with minor modifications. The primary antibodies used were: i) a polyclonal antibody that recognises the MVM capsid proteins (VPs) (52); ii) a monoclonal antibody (B7) that recognises a discontinuous epitope specific of the assembled capsid (57); and iii) a polyclonal antibody elicited against the VP2 Nt, that recognises virions that have encapsidated the genome, and concomitantly externalized the VP2 Nt segment (56).

Purification of virions and empty capsids - Natural infections with MVMp or transfection with its infectious recombinant plasmid yield a large proportion of empty capsids mixed with a minor proportion of infectious virions (49). Partially purified mixtures of empty capsids and infectious virions were obtained essentially as described (56, 58) by centrifugation of the clarified cell extracts in sucrose gradients, followed by extensive dialysis against phosphate-buffered saline (PBS). Empty capsids and infectious virions were purified by centrifugation in cesium chloride (CsCl) density gradients (see below), and dialyzed against PBS. The capsid and virion preparations were found essentially free of contaminants by SDS-PAGE, and the concentration was estimated by densitometry of stained gels and/or UV spectrophotometry (59).

Conformational stability of MVM empty capsids and virions followed by spectrofluorimetry - A Varian Cary Eclipse luminescence spectrophotometer equipped with a computer-operated Peltier temperature control unit was used. Empty capsids or purified virions were added to a 2x10mm cell and irradiated with UV light (excitation wavelength 295 nm). The temperature was continuously increased from 25ºC to 85ºC (or 90ºC) at a constant rate of 1ºC/min, and the intrinsic tryptophan fluorescence at an emission wavelength of 330 nm was determined at 1 min. intervals (59, 60). During some experiments small aliquots were taken for the determination of the infectious virus titers (see below). The changes in fluorescence were fitted to sigmoidal, cooperative transitions using the program Kaleidagraph (Abelbeck Software), which allowed a determination of the half-transition temperature (T_m) (59, 60).

Thermal inactivation of MVM virions followed by infectivity - Two procedures were used: i) Virion preparations were subjected to a thermal gradient as described above (see also 59, 60), and the infectious viruses remaining at different temperatures were determined by titration of aliquots taken from the heated sample. 
ii) The kinetics of virus inactivation was determined as follows (61). Virus preparations were diluted in PBS to a concentration of about 1500 plaque-forming units/ml. 100 µl aliquots in thin-walled PCR tubes were incubated at a constant temperature (70ºC) for different amounts of time, and the remaining virus titers were determined in plaque assays. Non-mutated viral particles obtained in parallel transfection experiments were included in each heat-inactivation experiment as a positive control. The use of culture medium (DMEM) supplemented with fetal calf serum instead of PBS did not significantly affect the results.

Analysis of DNA encapsidation - NB324K cells (1.2x10^7 cells) were transfected by electroporation with 40 µg of the purified MVM plasmids, and cultured for 48 hours in DMEM containing 10% fetal calf serum. The monolayers were washed in PBS and harvested in 20 mM Tris pH 7.5, 2 mM EDTA, 0.2% SDS (TES buffer) and completely homogenized by freeze-thawing three times, and the cell extracts used for purification of MVM particles as described (56). Approximately 20 fractions were collected from the CsCl gradients, and the amount of VPs and of viral DNA were estimated by slot-dot (Hoeffer) analysis. Samples diluted ten times in PBS were bound under vacuum to nitrocellulose filters (Schleicher & Schull) soaked in the same buffer, and left at room temperature until dry. The amount of bound VP (capsid) was estimated immunochemically with a polyclonal anti-MVM capsid serum (52), using an enhanced chemiluminescence method (ECL, Amersham). To estimate the amount of viral DNA, the samples bound to membranes were denatured in 0.4N NaOH, 1.5M NaCl for 5 min. at room temperature, neutralized by incubation with 0.5M Tris-HCl (pH 7.5), 1.5M NaCl during 5 min., and bound to the membrane by heating at 80 ºC for 2 hours. Filters were hybridized under high stringency as described (51) using as a probe a 2.4 kpb EcoRI restriction fragment of the MVM genome (nucleotides 1105-3547, ref. 62) labeled by random priming with digoxigenin, and incubated with a solution of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP, Roche).

Molecular graphics and structural analyses - A Silicon Graphics workstation, the PDB coordinates of the immunosuppresive (i) strain of MVM (1mvm; 29, 30) and of CPV (4dpv; 27) and the programs InsightII (Biosym Technologies), RasMol (63) and Whatif (64) were used. The capsid of the MVM prototypic strain (p) used in this study is 98% identical to the immunosuppresive (i) strain (62), with a few non-conserved capsid residues located in surface-exposed loops, far away from the DNA-capsid interface (30).

RESULTS

Selection for mutational analysis of amino acid residues at the parvovirus nucleic acid-capsid interface - Based on analyses of the three-dimensional structures of the MVMi and CPV virions (25-30), ten residues of the MVM capsid were chosen for mutagenesis to alanine (Table 1 and Fig.1). Each mutation would
disrupt any interaction of the truncated side chain (beyond Cβ) with the DNA, while minimizing the possibility of altering the conformation of the polypeptide backbone (65). Some of those side chains are additionally involved in interactions with neighbouring amino acid residues, which would also be disrupted by the mutation; this has been also contemplated in our experimental approach (see below).

The interactions with the major ordered DNA stretch of residues N183, T267 and N491 in the MVMi structural model are similar to those of the equivalent, conserved residues N180, T263 and N492 in the refined CPV model. The sterically similar replacements of D58 and Y270 in MVM by N56 and F266 in CPV do not disrupt the major interactions of these residues with the DNA (Table I). Residues W60 and N491 in MVM appear to be involved in major interactions with two neighbouring, shorter DNA stretches that could not be clearly visualized in the CPV structure (30). However, these residues are conserved in CPV (W58 and N492), and their interactions could be also conserved. Finally, residues K278, K471, D273 and D474 in MVM, and their equivalents R274, K472, D269 and D475 in CPV are either identical or similar, and all but D269 of CPV contain the charged groups closest to visible DNA phosphates and could be involved in either attractive or repulsive electrostatic interactions with the DNA molecule. In brief, the ten residues of the MVM capsid chosen for mutation include many of those involved in major interactions with the viral nucleic acid, and most of those residues and their interactions with the DNA are conserved between MVM and CPV.

Several residues involved in DNA-capsid interactions are important for the infectivity of MVMp - The chosen mutations were individually introduced in an infectious DNA clone of MVMp, susceptible mammalian cells were transfected with quantitated, equivalent amounts of the mutant infectious DNA clones and of the non-mutated control, and the virus yields at 72 h after transfection were determined by titration. The entire experiment was repeated using a new set of infectious DNA preparations that were quantitated and electroporated in a completely independent way. The relative efficiency of the transfection process was determined as described in Materials and Methods. The normalized relative viral titers were not significantly different from those obtained in the first experiment. The results (Table II) revealed that the infectivity of mutants K278A or N491A was similar to that of the non-mutated control or only slightly reduced, that of mutants D58A, W60A, N183A, T267A, K471A was clearly reduced by about one order of magnitude, and that of mutants Y270A, D273A and D474A was drastically reduced by 3-4 orders of magnitude. The experiments described in the following paragraphs were carried out with the aim of identifying the step(s) in the viral life cycle that could be responsible for the reduced infectivity of those mutants.

Expression of VPs, nuclear transport and capsid assembly - In situ immunofluorescence assays with cells transfected with equal amounts of plasmids were carried out. VP expression
was detected at 48 h after transfection with an antibody that recognises the VPs (even if non-assembled; 56). All of the mutants and the wild-type expressed similar amounts of capsid proteins (Fig. 2A and Table II). In addition, the presence of intense nuclear fluorescence (Fig. 2A and not shown) revealed that all of these mutant VPs were able to translocate into the nucleus.

Capsid assembly within the nucleus was detected with monoclonal antibody B7 that recognises a capsid-specific, discontinuous epitope located on the virion external surface, at the vertex of the three-fold spikes (57), and far away from the residues mutated (which are located at the internal capsid surface, and closer to the two-fold axes). All mutants except Y270A were able to assemble into capsids at levels comparable to the non-mutated control (Fig. 2A). Most of the Y270A-transfected cells that showed positive nuclear reactivity with the anti-VP antibody showed only a very weak reactivity with the anti-capsid antibody, relative to the non-mutated control (Fig. 2A). This result is consistent with previous observations of uncoupling between nuclear transport and capsid assembly (54), and on the nature of the subviral oligomers that translocate across the nuclear membrane (Riolobos et al., in preparation). Extracts from cells transfected with mutant Y270A, either of two mutants (W60A and D273A) that were positive with the anti-capsid antibody and the non-mutated control, were subjected to sedimentation in CsCl gradients, and the amount of MVM capsids produced was estimated by quantitation of the hemagglutination (HA) activity present in the density band corresponding to empty capsids. The nonmutated control, W60A and D273A particles were produced at normal levels (>6400 HA units per 10^7 transfected cells), while Y270A yielded only 100-300 HA units per 10^7 transfected cells (not shown). This confirmed that mutant Y270A is defective in capsid assembly.

Externalization of the VP2 Nt and packaging of the viral DNA - The maturation within the nucleus of MVM virions with an encapsidating genome triggers the externalization of the Nt of VP2 (48), an event that is required for infectivity, as the externalized peptide segment allows the traffic of the particle outward of the nucleus (56). Thus, DNA-containing virions can be specifically detected in cells by in situ immunofluorescence with an antibody specific for epitopes located at the VP2 Nt (56). All mutants except D273A and Y270A reacted strongly with this antibody (Fig. 2B and Table II), indicating that they were able to efficiently externalize the VP2 Nt and to encapsidate the viral genome. As described above, Y270A yielded very low amounts of assembled capsids, and the reactivity with the anti-VP2 Nt antibody was proportionally reduced (Fig. 2B). Remarkably, the D273A mutant, which showed normal reactivity with the anti-VP and anti-capsid antibodies (Figs. 2A), showed an extremely reduced reactivity with the anti-VP2 Nt antibody (Fig. 2B).

It could be argued that mutation D273A may prevent the externalization of the VP2 Nt but not DNA encapsidation. We considered this possibility unlikely, because the two events have been
correlated (48, 56), and the mutations are located far away from the pores at the capsid five-fold axes where the Nt are externalized. However, to analyze this issue further, mutants D273A, Y270A, W60A and the nonmutated control were subjected to density gradient centrifugation analysis. As shown in Fig. 3, for the nonmutated control and mutant W60A a large amount of viral DNA was detected in the fractions with a density corresponding to virions, while viral DNA was detected at low levels in the equivalent Y270A fractions, and was barely detectable in the D273A fractions. This result is entirely consistent with that of the immunofluorescence analysis (Fig. 2), and indicates that Y270A maintains a DNA-encapsidation capacity proportional to the low amounts of capsid assembled, while D273A does form normal amounts of empty capsids but has a severely impaired ability to encapsidate the viral genome.

Analysis of the stability of the empty capsid - Most of the side chains involved in interactions with the viral DNA and analyzed in this study either do not interact with neighboring capsid residues, or establish with the latter a few van der Waals contacts only (Table III). However, the side chains of Y270 and W60 are each involved in multiple hydrophobic intrasubunit contacts and a hydrogen bond, and K278 and D474 interact with each other through an intersubunit salt bridge (Table II). Truncation of some of these side chains could potentially affect the stability of the viral particles, not by disruption of DNA-capsid interactions, but because of the disruption of intracapsid interactions.

Thus, the contribution of the mutated residues to the stability of the protein shell was estimated using empty capsids, as follows.

The thermal stability of purified natural empty capsids of MVMp (containing both VP2 and VP1) was determined first, by following the intrinsic fluorescence of the particles in controlled thermal gradients, as previously described for VP2-only empty capsids (virus-like particles or VLPs) of MVMp (59, 60). Superimposed with the linear decrease in fluorescence due to thermal quenching, two transitions were observed. The first transition yielded a $T_m = 42 \pm 3 \, ^\circ C$ (Fig. 4A), and the second transition a $T_m = 76.0 \pm 0.2 \, ^\circ C$ (Fig. 4B). Comparison with detailed analyses of the structurally very similar viral-like particles (VLPs) of MVMp (59) indicates that these two transitions correspond to a conformational rearrangement of the capsid associated with externalization of the VP2 Nt, and to capsid dissociation, respectively. Partially purified nonmutated empty capsids (containing a very small fraction of virions, that did not significantly contribute to the variation in the fluorescence signal) were then assayed as described above for purified empty capsids. The first transition occurred at a similar temperature and the second transition, corresponding to dissociation of the capsid, yielded the same $T_m$, within error (Table II). This result validated the use of partially purified capsids to determine capsid stability in this type of assay. Finally, the thermostability of partially purified mutant empty capsids was compared with that of the nonmutated control, using the
same assay. The eight mutant capsids tested showed evidence of a first conformational transition that occurred at a similar temperature in all cases, and dissociated with a $T_m$ indistinguishable (within error) from that of the non-mutated control (Fig. 4C and Table II).

**Analysis of the conformational stability of the virion** - The thermal stability of the nonmutated MVMp virion at neutral pH was analyzed first. The purified virions were exposed to a controlled thermal gradient, and the variation in their intrinsic fluorescence was determined. The virions exhibited a small, but detectable and reproducible transition at a temperature of about 54 ºC (Fig. 5A). This transition did not correspond to the conformational transition observed for VLPs (59) or empty capsids at a substantially lower temperature (see above), which is consistent with the fact that in the virions the VP2 Nt is already externalized (48, 56). Instead, the transition detected in the virion may be related to the externalization of the VP1 Nt, which has been shown to occur at a similar temperature (66). The transition corresponding to dissociation of the virion (66; Fig. 5B) yielded a $T_m$ of 77 ºC, which is very similar to that found for VLPs or empty capsids using the same approach (59). Also, no significant difference in stability between DNA-full virions and empty capsids was found when the hemagglutination activity of heated purified particles was compared at neutral pH (N.Valle and J.M.A., to be described elsewhere).

Even though the virion particle is disrupted only at high temperatures, its infectivity may be slightly reduced before capsid dissociation occurs (66, 67). Thus, during the thermal gradient experiments using purified nonmutated virions, aliquots were taken at different temperatures, and the remaining infectivity was determined in plaque assays. The results suggest that infectivity may be lost in two steps (Fig. 5C). The first step involved a moderate (approximately fivefold) reduction in infectivity at about 50-55 ºC, a temperature that was approximately coincident with that of the first transition observed by fluorescence (see above). The second step involved a drastic reduction of infectivity (several orders of magnitude), that started at about 70ºC and led to a nearly complete loss of infectivity at about 80ºC, temperatures that corresponded approximately to those of dissociation of the virion, empty capsids and VLPs (see above and refs. 59, 66).

The small virion:empty capsid ratio obtained from infections of cell lines with MVM (49), and the reduced infectivity of most mutants analyzed, made it difficult to obtain the relatively large amounts of each of the purified mutant virions that are needed for the above analysis. However, the conformational stability of mutant infectious virions could be compared by following the reduction of infectivity in thermal inactivation kinetic assays, which required only a small amount of partially purified virions (61). The results of these assays are shown in Fig. 6 for some representative variants, and summarized in Table II for all of the mutants analyzed. The stability of mutants D273A, Y270A and, possibly, N491A was not
substantially different to that of the nonmutated control. In contrast, the stability of most mutants, namely D58A, W60A, N183A, T267A, K278A, K471A and D474A was clearly reduced (Table II). To summarize, no mutation tested affected the thermal stability of the empty capsid, but most of them affected the thermal sensitivity of the infectious virion.

**DISCUSSION**

Two aspects related to the experimental approach used in this work may require some discussion. Firstly, the error in atomic positions in the MVM crystal structure (29, 30) in the absence of a complete refinement, and the necessary averaging of the true DNA sequences bound to symmetry-related sites in the capsid, introduce some uncertainty in the amino acid residues and interactions that participate in the DNA-capsid interface as defined in Table I. However, several evidences indicate that major DNA-capsid interactions (hydrogen bonds and multiple hydrophobic contacts) which involve the capsid residues analyzed in this study do occur in the MVM virion: i) the conformations of the major visible DNA stretch in the MVMi and the CPV models are extremely similar (25, 26, 30); ii) for many of the visible nucleotides the electron density is distinctive for purine or pyrimidine, and in some cases for individual base type (26, 30); iii) a complete refinement of the CPV structure did not change in essence the major DNA-capsid interactions observed or the residues involved (25-27); iv) the capsid residues involved in most interactions with ordered DNA are either conserved or conservatively substituted in both viruses (50); and v) most of the interactions between these residues and the DNA are similar in both the MVMi and the refined CPV structural models (26, 30).

Secondly, the use of empty capsids to analyze the contribution to virion stability of interactions between capsid amino acid residues assumes that these interactions are similar in both types of viral particles. The structure of the CPV (68) or the MVM capsid (29, 58) is nearly identical in both the empty particle and in the DNA-full virion. For CPV, a comparison of the refined structures of the virion and the empty capsid showed some local differences in the conformation of several residues in the DNA-binding region (68). However, a detailed contact analysis using the same structural models indicated that the residues equivalent to those of MVM we subjected to mutational analysis are involved in similar interactions in both the virion and the empty capsid: The side chains of Y270 and W60 in MVM, and the equivalent F266 and W58 in CPV, participate in multiple hydrophobic contacts with neighboring amino acid residues in the virion; in the empty capsid of CPV, nearly all of these contacts are preserved (further contacts were observed). K278 and D474 in MVM and the equivalent R274 and D475 in CPV are linked in the virion by an intersubunit salt bridge, and this salt bridge is also preserved in the empty capsid of CPV.

The results obtained, together with the considerations just discussed, allow us to propose defined structural roles for most of the residues that were found to be
involved in major interactions with the viral DNA, that can explain the observed functional involvement of these residues in the virus life cycle.

**Residues with moderate effects on infectivity** - Truncation to Ala of D58, W60, N183, T267, K278, K471 or N491 did not significantly affect capsid protein expression, assembly into capsids inside the nucleus, or externalization of the VP2 Nt. This latter fact indicates that those mutants are also able to encapsidate the DNA, as confirmed by direct encapsidation analysis using mutant W60A. The side chains of W60 and K278, but not the others, not only may interact with the DNA, but are also involved in multiple interactions with other capsid residues as well. However, none of those seven mutations had any significant effect on the stability of the capsid itself. In contrast, all of those mutations (with the possible exception of N491A, which had a lesser inhibitory effect on infectivity) caused a substantial reduction in the thermal sensitivity of the infectious virion. These results indicate that the interactions of the mutated residues with the DNA, and not the interactions with other capsid residues, have a role in preserving the particle conformation needed for the infectivity of the virus particle. The aromatic ring of W60 is oriented perpendicularly to the ring of a neighboring purine base, which allows extensive van der Waals contacts and favorable dipole interactions. D58, and N183 and N491 may be hydrogen-bonded respectively to a purine base and to the sugar-phosphate backbone. T267 is involved in several van der Waals contacts. K278 and K471 showed no contacts with the DNA, but they were relatively close to DNA phosphates, and could contribute to medium-range electrostatic interactions.

The results also showed that, even though no neutralization of the charge of most DNA phosphates has been detected, the thermal stability of the MVM virion is not reduced relative to that of the empty capsid at neutral pH. We propose that the manyfold repeated hydrophobic and electrostatic interactions between the viral DNA and D58, W60, N183, T267, K278 and K471, and perhaps other residues involved in capsid protein-viral DNA interactions (Table I) that have not been analyzed in the present study, may help to counteract the potential destabilization caused by repulsion between non-neutralized DNA phosphates in the virion. Truncation of the side chain of any of these residues except K278 led to a reduction of infectivity by about one order of magnitude. The individual contribution to virion stability and infectivity of each of those few residues involved in major interactions between the viral DNA and each capsid subunit is, thus, relatively moderate. However their combined effect, if additive as expected, could be dramatically important.

Mutation K278A also reduced virion stability, but not infectivity. One possibility to explain this anomalous result (under study) is that the reduction in stability could be compensated by another, favorable effect of the same mutation on another step of the viral cycle.

**Residues with drastic effects on infectivity** - Truncation of Y270, D273 and D474 did not affect capsid protein
expression or nuclear transport. However, contrary to the mutations discussed above, for these three mutants the reduction of infectivity was dramatic, and it does not appear to be caused by a reduction in the thermal stability of the infectious virion.

Mutant Y270A is clearly defective in capsid assembly. Y270 is involved in multiple interactions not only with the DNA, but also with neighboring capsid residues. Thus, the most likely explanation for the inefficient assembly of this mutant, and the dramatic reduction in viral yield, is the removal of some critical intracapsid interactions.

The D474A mutation did not prevent capsid assembly, and capsid stability was not reduced either. The D474A virion did show a reduced stability, but similar or higher reductions in stability were observed for many mutants whose infectivity (viral yield) was reduced by only ten-fold. Thus, the drastic reduction in infectivity of D474A may be related mainly to another, as yet unidentified step in the viral cycle.

Finally, the D273A mutation did not prevent capsid assembly, but it severely impaired the externalization of the Nt of VP2 and the encapsidation of the viral DNA, which explains its drastic effect on the infectivity. D273 is very close to a DNA phosphate, but not to any acidic or basic amino acid residue (the closest basic residue is K494 at a distance of 4.7 Å), and it makes no visible contacts with the DNA or with other amino acid residues. One possibility is that the charge distribution at the capsid internal surface, that may be significantly altered if 60 negative charges are removed by the D273A mutation, may be critical for DNA packaging. In addition, the D273A phenotype provides further support to the previous suggestion that encapsidation of the MVM genome occurs in empty capsids previously assembled inside the cell nucleus (56).

To conclude, the results with this model spherical virus indicate that several amino acid residues per capsid subunit that are involved in interactions with the viral nucleic acid molecule are important for virus infectivity, because of their role in preserving the integrity of the infectious virus particle. As most of these residues are not involved in substantial intracapsid interactions, and none of them contributes to the stability of the capsid itself, their effect may be directly mediated by the multiple non-covalent interactions they establish with the viral DNA. In addition, at least one acidic residue at each DNA-binding region is needed for DNA packaging.

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FOOTNOTES

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1 The abbreviations used are: ss, single-stranded; CPV, canine parvovirus; MVM, minute virus of mice; MVMi, MVMp, MVM strains i or p, respectively; HA, hemagglutination; PBS, phosphate-buffered saline; VLP, virus-like particle; VP, viral capsid protein.
FIGURE LEGENDS

**FIG.1.** Amino acid residues of the MVM virion chosen for mutational analysis. The amino acid residues are colour-coded according to atom (green, C; blue, N; red, O) and labelled. The ordered ssDNA stretches A, B and C (Table I) in the virion are shown in yellow, cyan and violet, respectively. The PDB coordinates 1mvm (29, 30) were used.

**FIG.2.** *In situ* immunofluorescence of mammalian cells transfected with nonmutated or with some mutant infectious plasmids of MVMp. A and B correspond to the results obtained in two different double-labelling experiments, using specific antibodies to detect expression of viral proteins (VPs) assembled capsids (*Capsid*) or externalization of the Nt of VP2 (*2Nt*) (see text). wt and mock indicates transfection with the nonmutated control plasmid or with no viral DNA; all other pictures correspond to transfection with the indicated mutant plasmid.

**FIG.3.** Analysis of DNA encapsidation. Mammalian cells were transfected with infectious non mutated (wt) or mutant (W60A, Y270A or D273A) plasmids of MVMp. The assembled empty capsids (buoyant density 1.32 g/ml) and ssDNA-full virions (buoyant density 1.39-1.41 g/ml) present in the cellular homogenates were separated according to density by CsCl centrifugation (1.38 g/ml average density). A, samples were obtained from 1.2x10^6 transfected cells; the amount of viral particles in the fractions corresponding to empty capsids (fractions 3-7) and virions (fractions 9-14) were estimated by slot-immunoblot assay. VLPs of MVMp, purified and quantitated as described (56) were used for calibration, and bovine serum albumin was used as a negative control. B, samples were obtained from 1x10^7 transfected cells; the amount of viral DNA in the fractions corresponding to full virions were estimated by DNA hybridization. Different amounts of the MVMp infectious plasmid, quantitated by absorbance at 259 nm and ethidium bromide staining of agarose gels, were used for calibration. Exposure was for 40 h using an intensifying screen.

**FIG.4.** Conformational change and dissociation of the MVMp empty capsid probed by intrinsic Trp fluorescence. A, B, relative fluorescence intensity (in arbitrary units, a.u.) emitted at 330 nm as a function of temperature. For reasons of scale and clarity, the complete curve obtained in the same experiment has been split into two parts corresponding to low (A) and high (B) temperature ranges, where the transition corresponding to a conformational change without dissociation, or the transition corresponding to capsid dissociation, respectively occur (59). C, Dissociation of nonmutated empty capsid (circles) and some mutants (T267A, triangles; N491A, inverted triangles; D474A, diamonds) followed by emission fluorescence, as indicated for (B). a.u. are arbitrary units. For clarity of representation, the curves have been vertically offset. The experimental data were fitted to sigmoidal transitions (continuous lines).
FIG. 5. Conformational stability of MVMp virion. A, B, relative fluorescence intensity (in arbitrary units, a.u.) emitted at 330 nm by purified MVMp virions as a function of temperature at neutral pH (pH=7). For reasons of scale and clarity, the complete curve obtained in the same experiment has been split into two parts corresponding to low (A) and high (B) temperature ranges. The experimental data were fitted to a sigmoidal transitions (continuous lines). C, Relative infectivity of purified MVMp virions as a function of temperature at pH=7. The titer at each temperature was obtained using several dilutions of the sample. No infectious virions were detected at the three highest temperatures tested. The experiment was repeated, and similar results were obtained.

FIG. 6. Thermal inactivation of nonmutated and two representative mutant infectious virions of MVMp as a function of time. Aliquots of the virus preparations were incubated at 70 ºC for the periods of time indicated, and the titers determined were normalized using the titer obtained in the absence of heating (t=0). Black bars, nonmutated virion; grey bars, mutant K471A; white bars, mutant N183A. Titers were determined in triplicate. The standard deviation was in each case about 15% of the average, except for t=99 min. In the latter case the number of plaque-forming units was very low, leading to somewhat larger errors.
### Table I. DNA-protein interactions observed in the structural models of MVMi and CPV

| ssDNA stretch in MVM | MVM side | Non-covalent interactions with DNA | Distance to closest phosphate (Å) | CPV side | Non-covalent interactions with DNA | Distance to closest phosphate (Å) | Conservation, or conservative mutation |
|---------------------|----------|----------------------------------|----------------------------------|----------|----------------------------------|----------------------------------|-------------------------------------|
| A                   | H52      | vdW, hc                          | 5.0                              | E50      | (vdW)                            |                                  | yes                                 |
|                     | R54      | (vdW)                            |                                  | K52      | (vdW)                            |                                  | yes                                 |
|                     | F55      | (vdW)                            |                                  | F53      | (vdW)                            |                                  | yes                                 |
|                     | D58*     | hbo, (vdW)                       |                                  | N56      | hbo                             |                                  | yes                                 |
|                     | Q145     | hbo, (vdW)                       |                                  | F141     | (vdW)                            |                                  | yes                                 |
|                     | V180     | (vdW)                            |                                  | Q143     | hbo                             |                                  | yes                                 |
|                     | N183*    | hbo, vdW                         |                                  | L177     | vdW                             |                                  | yes                                 |
|                     | I185     | (vdW)                            |                                  | N180     | hbo, vdW                        |                                  | yes                                 |
|                     | (F247)   |                                  |                                  | T182     | (vdW)                            |                                  | yes                                 |
|                     | T267*    | vdW,                             |                                  | Y244     | hbo, (vdW)                       |                                  | yes                                 |
|                     | Y270*    | vdW, hc                          |                                  | Y266     | vdW, hc                         |                                  | yes                                 |
|                     | Y271     | (vdW)                            |                                  | F267     | (vdW)                            |                                  | yes                                 |
|                     | N491*    | (vdW)                            |                                  | N492     | vdW                             |                                  | yes                                 |
|                     | N492     | vdW                              |                                  | N493     | hbo, vdW                        |                                  | yes                                 |
|                     | T541     | (vdW)                            |                                  | H543     | vdW, hc                         |                                  | yes                                 |
|                     | (N585)   |                                  |                                  | K582     | hbo, (vdW)                       | 4.8                              |                                     |
|                     | K278*    |                                  | 5.9                              | R274     | 5.2                             |                                  | yes                                 |
|                     | K471*    |                                  | 6.2                              | K472     | 5.1                             |                                  | yes                                 |
|                     | D474*    | (vdW)                            | 3.1                              | D475     | 4.1                             |                                  | yes                                 |
|                     | W60*     | vdW, hc                          |                                  | (W58)    |                                  |                                  |                                     |
|                     | Q137     | hbo, vdW                         |                                  | (E135)   |                                  |                                  |                                     |
|                     | N139     | (vdW)                            |                                  | (H137)   |                                  |                                  |                                     |
|                     | P276     | vdW                              |                                  | (P272)   |                                  |                                  |                                     |
|                     | K490     |                                  | 6.0                              | (Q491)   |                                  |                                  |                                     |
|                     | D273*    | (vdW)                            | 2.8                              | (D269)   |                                  |                                  | yes                                 |
|                     | N491*    | hbo                              |                                  | (N492)   |                                  |                                  | yes                                 |

### Footnotes to Table I

1 Contact analysis was carried out using the program Whatif (64) and the PDB files 1mvm (29, 30) and 4dpv (26, 27), including symmetry-related subunits for completeness. The MVM and CPV residues in each row occupy equivalent positions in the aligned sequence and in the three-dimensional structure. Three DNA stretches of 11, 4 and 1 nucleotides (denoted here as A, B, C, respectively) were included in the atomic coordinates of MVMi (29, 30), but only the equivalent to stretch A was included in both the unrefined (25) and the refined (26, 27) atomic coordinates of CPV. Residues in parentheses are those not seen to participate in interactions with the visible DNA stretches in either MVM or CPV. Conservation of sequence and conformation is consistent with the possibility that the CPV residues in parentheses could interact with the equivalent to DNA stretches B and C, even though these regions could not be modelled in the crystal structure of CPV.
The MVM residues chosen for mutagenesis are denoted by an asterisk.

Only the interactions of the side chain (beyond Cβ) are considered. hbo, hydrogen bond (cutoff distance 3.8 Å); vDW, multiple (more than 10) van der Waals contacts; hc, multiple (more than 5) carbon-carbon ("hydrophobic") van der Waals contacts; (vdW), few (less than 5) van der Waals contacts. For van der Waals contacts the cutoff distance was set 0.5 Å longer than the sum of the van der Waals radii of the two atoms considered. The CPV residues listed as involved in hydrogen bonds or multiple hydrophobic contacts are essentially coincident with those in the analysis by Chapman and Rossmann (26).

Distances are from the charged atom.

Residue conservation between MVM and CPV. A substitution is considered to be conservative if it could preserve most of the interactions with the DNA.
Table II. Effects of the truncation of capsid side chains involved in interactions with the nucleic acid in the MVM virion

| Virus       | Relative infectivity\(^1\) | Viral protein\(^2\) | Capsid\(^2\) | Externalization of VP2 Nt\(^2\) | \(t_{80}\) of inactivation of virion\(^3\) (min) | Relative \(t_{80}\) \(^4\) | \(T_m\) of dissociation of empty capsid\(^5\) (ºC) |
|-------------|-----------------------------|---------------------|-------------|---------------------------------|---------------------------------------------|-----------------------------|---------------------------------------------|
| nonmutated  | 1 ++ ++ ++ ++               | 34 ± 13             | 1           | 75.8 ± 0.8                      |
| D58A        | 0.14 ± 0.07 ++ ++ ++        | 12 ± 4              | 0.35        | 76.4 ± 0.3                      |
| W60A        | 0.15 ± 0.05 ++ ++ ++        | 11 ± 9              | 0.32        | 74.8 ± 0.3                      |
| N183A       | 0.07 ± 0.06 ++ ++ ++        | 4                   | 0.12        | 75.2 ± 0.3                      |
| T267A       | 0.15 ± 0.12 ++ ++ ++        | 15                  | 0.44        | 76.6 ± 0.1                      |
| Y270A       | 0.0002 ± 0.0006 ++ +/- +/-  | 75                  | 2.2         | ND                             |
| K278A       | 1.6 ± 1.1 ++ ++ ++          | 3 ± 1               | 0.09        | 75.2 ± 0.1                      |
| K471A       | 0.11 ± 0.03 ++ ++ ++        | 9                   | 0.26        | 75.3 ± 0.2                      |
| D474A       | 0.003 ± 0.0005 ++ ++ ++     | 11 ± 2              | 0.32        | 75.3 ± 0.2                      |
| N491A       | 0.33 ± 0.24 ++ ++ ++        | 18 ± 12             | 0.53        | 75.7 ± 0.1                      |

Footnotes to Table II

\(^1\) For each mutant, relative infectivity was obtained by dividing its average titer (from duplicate or triplicate determinations) by the average titer obtained for the non-mutated control in the same experiment, using equal amounts of each infectious DNA. For each mutant the indicated relative infectivity is the average ± standard deviation obtained in two or three completely independent transfection experiments done with two different DNA preparations (see text).

\(^2\) VP expression, capsid assembly and externalization of the VP2 Nt were determined in \textit{in situ} immunofluorescence experiments using antibodies specific for viral proteins, assembled capsids or the Nt segment of VP2, respectively. Reactivity is indicated as ++, similar to that of the nonmutated control; +/-, severely reduced, or -, indetectable.

\(^3\) \(t_{80}\) is the incubation time at 70ºC needed to inactivate 80% of the infectious virions. Virus partially purified through a sucrose cushion were used, except for Y270A and D273A, which could not be purified due to their very low infectivity. For these two mutants the data were obtained with non-purified virus. In each case the appropriate non-mutated MVM control was
included. The $t_{80}$ value and error given for the non-mutated virus are the average of five independent experiments using partially purified preparations. Use of non-purified non-mutated virus in five independent experiments yielded an average value of $t_{80} = 37 \pm 12$ min, the same (within error) obtained with partially purified virus. For all viruses titrations were carried out in duplicate or triplicate; in general, the standard deviation values were about 15% of the average value. In addition, for six mutants the $t_{80}$ values were obtained in two independent experiments; in these cases the average $\pm$ deviation of the two experimental values is indicated. Comparison of the estimated inactivation rate constants obtained by fitting of the kinetic curves to an exponential decay process did not modify the conclusions reached when $t_{80}$ values were compared.

4 Relative values for $t_{80}$ were obtained by dividing the value obtained for the mutant by the average $t_{80}$ value obtained for the non-mutated control in the same set of experiments.

5 Obtained by following the intrinsic Trp fluorescence in thermal gradients. For the non-mutated empty capsid the average value $\pm$ standard deviation obtained in four independent experiments are given. For the mutant capsids the experimental $T_m$ value $\pm$ mathematical fitting error are given. ND, not determined. The negligible amounts of capsids obtained for Y270A prevented its use in this assay.
Table III. Intracapsid interactions in the MVMi virion of the amino acid side chains analyzed in this study\(^1\).

| amino acid side chain | intracapsid interactions                  |
|-----------------------|------------------------------------------|
| D58                   | hboD58mc, vdW, hc intrachain             |
| W60                   |                                          |
| N183                  |                                          |
| T267                  |                                          |
| Y270                  | hboN492mc, vdW, hc intrachain           |
| D273                  |                                          |
| K278                  | sbD474sc, (vdW) inter & intrachain      |
| K471                  | (vdW) intrachain                        |
| D474                  | sbK278sc, (vdW) interchain              |
| N491                  | (vdW) intrachain                        |

\(^1\) The analysis was carried out using the program Whatif (64) and the PDB file 1mvm (29, 30). Intersubunit interactions were analyzed using pairs of symmetry-related subunits. Only the interactions of the side chain (beyond C\(\beta\)) are considered. Cutoff distances and abbreviations are as in Table I. Additional abbreviations are: mc, main chain; sc, side chain; sb, salt bridge.
Fig. 5: Graphs showing changes in fluorescence intensity at 330 nm and relative infectivity with temperature.

**A** Fluorescence intensity at 330 nm (a.u.) vs. Temperature (°C)

**B** Fluorescence intensity at 330 nm (a.u.) vs. Temperature (°C)

**C** Relative infectivity vs. Temperature (°C)
Functional relevance of amino acid residues involved in interactions with ordered nucleic acid in a spherical virus
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