A Thermolabile Mutant of Adenovirus 5 Resulting from a Substitution Mutation in the Protein VIII Gene

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The mutant adenoviruses H5sub304 and H5RI⁺ were isolated sequentially from adenovirus 5 wild type by selection for the loss of *EcoRI* restriction endonuclease sites by Jones and Shenk (Cell 13:181-188, 1978). sub304 lacks the site at 84.0 map units (m.u.), and RI⁺ lacks both that and the site at 75.9 m.u. A set of derivatives of RI⁺ that lack the site at 75.9 m.u. accumulated virus more slowly at 38.8 or 39.5°C than those with the site present, as measured by low-multiplicity passage or single-step replication cycles, respectively. Since the *EcoRI* site at 75.9 m.u. is predicted to lie in the gene encoding the precursor to virion polypeptide VIII (pVIII), the failure to accumulate virus rapidly could lie either in some step in processing and assembly of virions or in an increased virion thermolability. The latter possibility was shown to be the case, as all strains mutated at the *EcoRI* 75.9 m.u. site were extremely thermolabile in vitro, even at 37°C. CsCl equilibrium density centrifugation of heated crude stocks of RI⁺ and sub304 demonstrated that loss of infectivity in RI⁺ was accompanied by physical disruption of virions. Polyacrylamide gel electrophoresis of infected cell extracts or of purified virions showed that pVIII of RI⁺ had an apparent molecular weight that was slightly greater than that of sub304, and mature RI⁺ and sub304 virions displayed polypeptide VIIs which appeared to be of identical molecular weights. Nucleotide sequence analysis of RI⁺ demonstrated that it contained a 9-base-pair (bp) substitution for 6 bp found in sub304, leading to a loss of the *EcoRI* site and a predicted insertion of a single amino acid. Comparison of the sequence of sub304 with the published sequence of adenovirus 2 revealed two changes, a single transversion at bp 1,722 and a bp deletion at 1,749, leading to the loss of a *TaqI* site. The predicted reading frame change would lead to a stop codon at bp 1,885. This raises the question of whether adenovirus 2 and adenovirus 5 use the same reading frame for pVIII.

The adenovirus virion is a naked icosahedron containing at least 10 different polypeptide species whose contributions to the architecture of the virus have been broadly determined (18). As with many icosahedral viruses, the capsid is stable to a variety of environmental treatments, such as repeated freezing and thawing and alterations in pH and temperature. However, with the exception of protein IX, whose absence leads to the production of thermally unstable virions (3), the roles of the various structural proteins in maintaining virion stability are unknown. The data in the present paper document an important role of the hexon-associated internal protein VIII (5) in the maintenance of virion stability.

In the course of isolating spontaneous mutations lacking *EcoRI* restriction endonuclease sites, Jones and Shenk (10) discovered a strain of adenovirus 5 (Ad5) whose genome lacked the normal *EcoRI* site at 84.0 map units (m.u.). The genome of this virus, termed sub304, contains a substitution of foreign DNA between 83.2 and 85.1 m.u. in early region 3. Its replication characteristics are indistinguishable from Ad5 wild type, but it forms larger, clearer plaques on HeLa cells (unpublished data). This virus was subjected to further selection, and a strain termed RI⁺, which lacked the *EcoRI* site at 75.9 m.u., was obtained. Replication of RI⁺ at 37°C was also reported to be indistinguishable from wild type.

However, during the course of marker rescue experiments with *ts* derivatives of RI⁺ (F. C. Volkert, Ph.D. thesis, Columbia University, New York, N.Y., 1983), it was noticed that it was difficult to propagate some *ts*⁺ recombinants at the restrictive temperature of 38.8°C. Since the mutated *EcoRI* site at 75.9 m.u. was suspected of lying in the open reading frame encoding the precursor to protein VIII (pVIII), the observed phenotype could have arisen from a defect in the processing of pVIII to VIII (1, 9, 16) during the assembly of virions or the stability of the virions once formed. The data to be presented show that RI⁺ virions are indeed thermolabile in vitro, that the mutation consists of a substitution leading to a net increase of 3 base pairs, and that pVIII is slightly larger than the equivalent polypeptide in sub304. We can infer therefore that protein VIII plays an important role in virion structural stability.

MATERIALS AND METHODS

Virological methods. These have been described in detail in previous publications (2). KB cells were used for propagation of virus and titration by fluorescent focus assay (17, 20). HeLa cells were employed for plaque assay by using methods described previously (12, 22).

Viral strains. The wild-type strain of Ad5 has been described previously (4). H5sub304 and H5RI⁺ were derived from this wild-type strain by sequential selection for the loss of the *EcoRI* sites at 84.0, and at 84.0 and 75.9 m.u., respectively (10). The recombinants designated F1 and F4 were isolated after a series of marker rescues and infection crosses (Volkert, Ph.D. thesis). They both lack the *XbaI* sites at 28.8 and 78.5 m.u. and have the same substitution in early region 3 as sub304 but differ in that F1 also lacks the

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FIG. 1. Growth curves of *sub304* (●), *F4* (▲), *RI*⁻ (○), *F1* (△), and *dl309 ts10* (□) at 39.5°C in KB monolayer cultures. Cells were infected with 20 PFU per cell. Samples were withdrawn at various times, and yields were measured by fluorescent focus assay at 32°C. FFU, Focus-forming units.

EcoRI site at 75.9 m.u. *dl309* has been described by Jones and Shenk (11). It was derived from *sub304* by selection for the loss of the *XbaI* restriction cleavage sites at 78.5 and 28.8 m.u. The derivative *dl309 ts10* was obtained by marker transfer of *H5 ts10* into a *dl309* background as described previously (21).

Measurement of heat stability. Crude viral stocks were diluted 1:10 in 0.01 M phosphate-buffered (pH 7.5) saline or infecting fluid and then dispensed into samples of 0.1 ml per tube. The suspensions were incubated in a water bath at the appropriate temperature. Samples were withdrawn at various times and diluted immediately with ice-cold infecting fluid. The residual infectivity was titrated by fluorescent focus assay at 32°C.

Analysis of particles inactivated in vitro. KB cells infected with either *H5sub304* or *H5RI*⁻ were labeled with [³⁵S]methionine as follows. A total of 5×10^7 cells in 175-cm² culture flasks were washed with Hanks balanced salt solution 38 h after infection at 32°C and labeled for 5 h in low-methionine (5 mg/liter) medium containing [³⁵S]methionine at a final concentration of 5 μ Ci/ml. At the end of the labeling period, 40 ml of Dulbecco modified Eagle medium containing 2% serum and additional glutamine (0.6 mg/ml, final concentration) was added and incubated at 32°C until complete cytopathic effects (CPE) appeared. The cells were harvested and washed with ice-cold phosphate-buffered saline and then frozen and thawed at 32°C six times. The samples then were centrifuged at 2,000 rpm for 20 min to remove cellular debris. The supernatant was separated into two equal parts; one was heated at 45°C for 40 min and the second was kept on ice. The samples then were extracted with Freon once, layered on preformed linear CsCl density gradients (1.2 to 1.4 g/ml), and centrifuged at 22,500 rpm for 16 h in an SW41 rotor. After centrifugation, 20-drop fractions were collected. A sample of each fraction was spotted onto a 3-mm-diameter

filter pad (Whatman), the proteins were precipitated with trichloroacetic acid, and the pad was processed. The filters were dried, and radioactivity was measured in a liquid scintillation spectrometer. The refractive indices of the samples were also determined.

Polyacrylamide gel electrophoresis. Infected cell pellets were suspended in phosphate-buffered saline to a concentration of 10^7 cells per ml. The bands of purified virus from CsCl gradients were dialyzed overnight at 4°C against 10 mM Tris-hydrochloride–1 mM EDTA. Samples were processed as described previously (2). The polyacrylamide system of Anderson et al. (1) was employed for gel electrophoresis.

DNA sequence analysis. The *Bgl*II E fragments (69.0 to 77.9 m.u.) from *H5sub304* and *H5RI*⁻ were cloned into the *Bgl*II site of plasmid pKC7 (19), located in the kanamycin resistance gene. Plasmid DNAs from kanamycin-sensitive strains were screened for the presence of the *Bgl*II E fragment and then restricted with *Xma*I. The adenovirus-specific fragments extending from 75.8 to 76.3 m.u. were cloned into plasmid pDM2 (kindly provided by Donald Mills) at its unique *Sma*I site without any screening. Propagation of the *Sma*I-containing derivatives was performed in *dam Escherichia coli* GM33 (13). Plasmid DNAs were linearized with *Xho*I, 5' end labeled with polynucleotide kinase and [³²P]ATP, and then digested with *Bst*E II. Fragments containing the *Sma*I inserts were isolated from low-melting agarose and sequenced by the chemical method of Maxam and Gilbert (14). Since the inserts from the two viral strains were in opposite orientations, sequence data from both strands in the area of interest were obtained.

RESULTS

Initial genetic observations. In the course of experiments designed to examine linkage relationships in marker rescue, it was observed that *ts*⁺ recombinants from rescues employing *H5ts10* derivatives of *RI*⁻ and *dl309* fell into two classes. Plaque isolates passaged on KB cell monolayers at 38.8°C produced complete CPE in 1 week, or alternatively, by 2 weeks the infected cell monolayers were beginning to show only the first signs of CPE. On analysis of the viral DNAs by *Eco*RI restriction enzyme digestion, it was found that the DNAs from the 89 isolates with rapid production of CPE contained the *Eco*RI site at 75.9 m.u., whereas the genomes of the 43 viruses showing delayed CPE were lacking that restriction site. Thus, the lack of the *Eco*RI site was tightly linked to the phenotype of delayed CPE. In keeping with the hypothesis presented above, experiments were devised to determine whether the underlying mutation thought to lie in the gene for the precursor to virion polypeptide VIII caused a change in the processing of the mature protein VIII, which in turn affected the stability of the newly formed virions.

Viral replication at 39.5°C. The cytological observations described in the previous section suggested that the net accumulation of virus was delayed at 38.8°C. To examine this more closely, KB cells in replicate monolayer cultures were infected with *sub304*, *RI*⁻, two *ts*⁺ derivatives (*F1* and *F4*) from the *ts10* marker rescues, or one of the marker-rescued parents, *dl309 ts10*. Incubations were performed at the more restrictive temperature of 39.5°C to facilitate comparison of the truly *ts* virus and the two sets of *ts*⁺ virus. The cells from individual dishes were harvested at intervals, and viral infectivities were measured by fluorescent focus assay at 32°C. The final accumulation of infectious virus was reduced for both *RI*⁻ and the recombinant *F1* virus, both of which lack the *Eco*RI site at 75.9 m.u., as compared with the yields from *sub304* and *F4* infections (Fig. 1). As expected,

the *ts10* derivative produced very little virus (<0.01 focus-forming units per cell). In contrast, all five strains produced final yields at 32°C that were within threefold of each other, although the eclipse periods for RI^r and F1 were somewhat extended (data not shown). The quantitative results are consistent with the cytological observations at 38.8°C described in the previous section, because it was hypothesized that each round of multiplication would yield fewer infectious viral particles in EcoRI^r -infected than in EcoRI^s -infected cultures, with a consequent delay in the appearance of CPE.

In vitro thermal stability of virions. The results presented above show clearly that the net accumulation of infectious virus was reduced at 38.8 or 39.5°C if the genome lacked the *EcoRI* site at 75.9 m.u. This phenotype could arise from defects in the assembly or processing of the mutated pVIII polypeptide, or from a lack of virion stability of the newly formed virus, which might contain mutant protein VIII molecules. To test the latter possibility, crude stocks of *sub304*, RI^r , and F1 were subjected to thermal treatment at a variety of temperatures. Figure 2 illustrates the decreases

FIG. 2. Heat stability of *sub304*, RI^r , and F1 at 37°C . Crude viral stocks were diluted 1:10 in phosphate-buffered saline and then dispensed into samples of 0.1 ml per tube. The viral suspensions were incubated in a water bath. Samples were withdrawn at various times and diluted immediately in ice-cold infecting fluid. The residual infectivity was titrated by fluorescent focus assay at 32°C .

FIG. 3. CsCl gradients of heat-inactivated *sub304* and RI^r . A total of 5×10^7 cells, infected with 20 PFU per cell, were labeled with [^{35}S]methionine, extracted as described in the text, and separated into two equal parts; one was heated at 45°C for 40 min, and the other was kept on ice. The samples were extracted with Freon once, layered on preformed linear CsCl density gradients (1.2 to 1.4 g/ml), and centrifuged in an SW41 rotor for 16 h at $22,500$ rpm and 4°C . FFU, Focus-forming units.

in infectivity after inactivation at 37°C . It is apparent that both RI^r and F1 were extremely thermolabile, since the infectivities of both viruses decreased by 5 orders of magnitude in 3 h, whereas *sub304* retained 50% of its infectivity, as did Ad5 wild type and recombinant F4 in a separate experiment (data not shown). In all tests performed, a variety of different viruses lacking the *EcoRI* site at 75.9 m.u. exhibited this extreme thermolability, strongly suggesting that the phenotype was caused by the mutation at this site. Furthermore, it seems likely that a major reason for the failure to accumulate normal yields of virus at elevated temperatures resulted from the thermolability of the virions.

Physical basis for thermal inactivation. To determine whether thermal inactivation of RI^r virions results from disruption of viral particles, crude stocks of *sub304* and RI^r labeled with [^{35}S]methionine were heated at 45°C for 40 min and then gently extracted once with Freon (see above). The CsCl gradients of heat-inactivated viruses (Fig. 3) showed that for heated *sub304* (Fig. 3C) and unheated *sub304* (Fig. 3A) almost equivalent proportions of radioactive counts were found in a band with a buoyant density of 1.33 g/ml. This band corresponds to the buoyant density of complete virions. Also, both bands from unheated and heated *sub304* had approximately the same infectivity. However, for heated RI^r (Fig. 3D) the band with a buoyant density of 1.33 g/ml disappeared, and the infectivity of heated RI^r had decreased by almost 99.99% as compared with unheated RI^r . These

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses of the polypeptides specified by viruses containing or lacking the *EcoRI* site at 75.9 m.u. Infected cell extracts were prepared from KB monolayer cultures infected with wild type, *sub304*, *RI'*, and *dl309*. The contents of ca. 5×10^4 cells were loaded on a 17.5% polyacrylamide gel, and the processed gel was exposed to Kodak X-Omat film. Molecular weight standards were run in a parallel lane, and their positions are indicated.

findings indicate that heating actually disrupted the majority of *RI'* virions, whereas virions of *sub304* were relatively heat stable. This implies that the loss of infectivity of *RI'* upon heating results from particle disruption rather than from some subtle change in virion morphology.

Mutational change in *RI'*. The preceding sections have shown that the absence of the *EcoRI* site at 75.9 m.u. is associated with a profound loss of virion thermostability, which in all probability accounts for the lower yields of virus obtained at 38.8 or 39.5°C. Sequence data in the closely related serotype Ad2 (6, 8) show that this *EcoRI* site lies within an open reading frame appropriate to encode polypeptide pVIII, the message of which hybridizes to this region (15). Therefore, it seems reasonable to propose that the phenotypic consequences of the H5*RI'* mutation result from an alteration to pVIII. Previous investigations (10) demonstrated that the mutational change in *RI'* was not a major deletion or insertion but left open the question of whether it was a point mutation. To determine whether the mutation effected a gross alteration in the gene product, the sizes of both the intracellular polypeptide pVIII and the mature virion VIII were examined.

KB cells were infected with *RI'*, *sub304*, *dl309*, or Ad5 wild type, incubated for 24 h at 32°C, and then labeled with [35 S]methionine for 4 h. Cells were harvested, and samples were prepared for polyacrylamide gel electrophoresis. The only distinction between the polypeptides expressed by *RI'* and the other viruses (Fig. 4) is noted in a band migrating at an apparent molecular weight of ca. 26,000, a size compatible with that reported for pVIII (1). In *RI'*, this band

migrates slightly more slowly, suggesting that either the precursor has an extra amino acid(s) or is differently modified after translation. However, when polypeptides from purified virions were examined (Fig. 5), all strains of virus contained mature VIII molecules of indistinguishable mobility. These results might suggest that the modification in pVIII is removed during proteolytic cleavage, so that the final product is apparently of wild-type molecular weight. However, this immediately raises the question as to why the mature virions are thermolabile, a point which will be considered again below.

The polypeptide data suggested that the pVIII molecules synthesized by *RI'* were of higher molecular weight. To determine the nature of the mutation causing this change, the sequences surrounding the *EcoRI* site at 75.9 m.u. were sequenced in both *RI'* and the parent from which it was derived, *sub304*. From each virus, an *SmaI* fragment extending from 75.8 to 76.3 m.u. (nucleotide 1706 to 1936) was cloned into pDM2 and sequenced as described above. The interpretation of the sequencing gels is presented in Fig. 6, in which the sequence of *RI'* is compared with that of *sub304* and with that of Ad2 from previously published sources (6, 8). Three conclusions can be drawn. (i) *RI'* contains a substitution mutation of 9 base pairs, beginning at the fifth base pair of the *EcoRI* recognition sequence, for 6 base pairs in *sub304*. Thus, the genome of *RI'*, as expected, has lost the *EcoRI* recognition site at 75.9 m.u. Furthermore, the substitution is in frame and should lead to the insertion of a single amino acid in polypeptide pVIII. (ii) There is a single-base-pair deletion in the *sub304* and *RI'* sequences compared with the Ad2 sequence. This change would be expected to remove a *TaqI* site, present in the Ad2 genome, from the genome of *sub304*; restriction analysis of both plasmid and viral DNA from *sub304* and Ad2 has shown that this difference exists (data not shown). (iii) There is a single AT to CG transversion at nucleotide 1722 in the Ad5 sequences compared with the Ad2 sequence.

FIG. 5. Virion polypeptides specified by wild type, *RI'*, and *sub304*. Viruses were purified through two CsCl gradients, dialyzed against TE buffer disrupted in gel sample buffer, and analyzed on a 17.5% polyacrylamide gel that was stained with Coomassie brilliant blue and dried under vacuum.

DISCUSSION

The mutant with which this paper is primarily concerned, RI^r, was isolated as having a spontaneously *Eco*RI-resistant genome (10) and was initially reported to have growth characteristics indistinguishable from wild-type virus. The first indication that this might not be the case came from two observations. Firstly, in marker rescue experiments employing derivatives of RI^r, viral isolates from plaques obtained at 38.8°C fell into two categories upon blind passage at 38.8°C in KB cells: those yielding complete CPE in 1 week and those that required 2 weeks for the initiation of CPE. Subsequent genome analysis revealed that all viral isolates lacking the *Eco*RI site at 75.9 m.u. accumulated virus more slowly than their RI^r counterparts (Volkert, Ph.D. thesis). Secondly, although the plaquing ratio at 38.8 to 32°C of RI^r was similar to that of *sub*304, the immediate parent of RI^r that contains an *Eco*RI site at 75.9 m.u., the morphology of the 38.8°C plaques of RI^r was smaller and hazier than those of *sub*304. These observations, coupled with the fact that sequence analysis (6, 8) and mRNA mapping (15) in the closely related Ad2 serotype place the *Eco*RI site at 75.9 within the coding sequences for pVIII, suggested that the phenotype of RI^r could arise from a partially temperature-sensitive replication defect, from a virion structural alteration that led to virion thermolability, or from both.

The single-step growth curves in Fig. 1 show that at 39.5°C viral accumulation is markedly lower for viruses lacking the *Eco*RI site at 75.9 m.u. This explains the cytopathic observations of the blind passages of viral plaque isolates referred to above but does not distinguish between a replicative or a structural defect. Direct *in vitro* tests for virion thermolability (Fig. 2) indicated that even at the physiological temperature of 37°C, RI^r and the derivative F1 were much more thermolabile than *sub*304. In all strains tested, the absence of the *Eco*RI site at 75.9 m.u. correlated with such thermolability (data not shown). *In vitro* inactivation of crude stocks of [³⁵S]methionine-labeled virus was examined on equilibrium density CsCl gradients before and after thermal inactivation (Fig. 3), and the loss of infectivity of the RI^r sample can be attributed to the physical disruption of particles (Fig. 3D). In passing, it should be pointed out that in both *sub*304 and RI^r heated samples (Fig. 3C and D) there was the appearance of a prominent band whose equilibrium buoyant density corresponded to that of incomplete particles (1.29 g/ml). Whether these arose from breakdown of the full virion particles or from *in vitro* formation of aggregates from the large pool of soluble antigens is not clear. If the former explanation is correct, however, it implies that a significant proportion of the *sub*304 particles banding at 1.33 g/ml are both noninfectious and thermolabile, since no detectable loss of infectivity was observed.

The map position of the *Eco*RI site at 75.9 m.u. places it within the coding sequences of pVIII in the Ad2 serotype, and given the extremely close correspondence between Ad2 and Ad5 at the DNA sequence level, it seems reasonable to assume that the mutation in RI^r also falls within pVIII. However, paradoxical results were obtained when tests were carried out to determine whether the mutation had consequences for the molecular weights of pVIII or mature VIII polypeptides from infected cells and purified virions (Fig. 4 and 5). The molecular weight of pVIII was apparently slightly larger in RI^r than in *sub*304, yet the mature proteins VIII had identical mobilities in this gel system. This could be explained if the changed sequence causing an alteration in mobility of pVIII were cleaved off during processing so that

FIG. 6. Sequence analysis of RI^r and *sub*304. (a) The genome of Ad2 between the *Sma*I sites at 75.8 and 76.3 m.u. is displayed, and those of *sub*304 and RI^r are shown below. (b) The DNA sequence of Ad2 is shown in detail between base pairs 1,739 and 1,751 and compared with those of *sub*304 and RI^r. Apart from the differences shown, the Ad2 and Ad5 sequences are identical between the two *Sma*I sites. Base-pair numbering begins at the Ad2 *Eco*RI site at 71.2 m.u. (7), the 5'A of the site being no. 1. The sequences were taken from Galibert et al. and Hérisse et al. (6, 8). The shaded area shows the area of difference between RI^r and *sub*304.

VIII was of wild-type size. However, an explanation would then be required for virion thermolability. Furthermore, DNA sequence analysis (6, 8) and partial peptide analysis of mature VIII (C. W. Anderson and E. A. Oosterom-Dragon, personal communication) indicate that in Ad2 the *Eco*RI site lies in the portion of pVIII retained in the mature, processed protein.

To try to resolve this paradox, DNA sequence analysis of the region surrounding the *Eco*RI site was performed in both RI^r and *sub*304. The data (Fig. 6) show that RI^r has a 9-base-pair substitution for a 6-base-pair sequence in *sub*304. This substitution leads to the net insertion of a single amino acid and the loss of the *Eco*RI site. It seems unlikely that a single amino acid addition could lead to an observable alteration in the mobility of pVIII in a denaturing gel system designed to reflect molecular weights. Moreover, the alteration in mobility would have to occur in pVIII and not in VIII, despite the fact that both contain the same extra amino acid. It remains to be determined whether these apparent anomalies reflect another aspect of the disparities, in sodium dodecyl sulfate gel systems, between observed and calculated molecular weights commonly found among the adenovirus polypeptides. Despite these uncertainties, however, the insertion of an amino acid in a critical position in mature protein VIII is certainly compatible with the likelihood that it altered the protein structure such that it could not serve its usual function in the viral capsid, which in turn led to the observed virion thermolability of RI^r. Thus, it may be concluded that protein VIII serves a critical structural role in the stability of the adenovirus virion.

When the sequence of *sub*304 was compared with the published sequence of Ad2, two changes were observed (Fig. 6), one a transversion at base pair 1,722 and the other the deletion of base pair 1,749. The latter change removes a *Taq*I site, specific to Ad2, from the Ad5 genome (data not shown) and would have the effect of altering the reading frame proposed for pVIII in Ad2 so that a stop codon would

be encountered beginning at base pair 1,885. These data, extending from the *Sma*I site at base pair 1,706 to the *Sma*I site at base pair 1,936, show no evidence for a compensating mutation to restore the reading frame or to eliminate the stop codon. Further sequence analysis will be required to resolve this apparent difference between Ad5 and Ad2. Despite these uncertainties, it seems reasonable to conclude that the mutation in RI⁺ leads to an alteration in the primary amino acid sequence of both pVIII and VIII with the phenotypic consequences described in this paper.

Genetic data have now confirmed the importance of both protein IX (3) and protein VIII for the structural stability of the virion. In the former case, virions completely lacking protein IX are produced in cells infected with *dl*313, a mutant in which the sequence for the 5' end of the gene encoding IX is deleted (3). The consequence of this lack of protein IX is a marked virion thermolability. Other still unmapped mutations which confer alterations to the stability of the virion have also been described previously (23). Thus, functions of two of the smaller capsid proteins (i.e., proteins VIII and IX) have been more clearly defined, and similar genetic approaches will afford the opportunity to obtain insights into the roles of other as yet poorly characterized viral proteins in viral replication and structure.

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