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Genetic analyses of putative conformation switching and cross-species inhibitory domains in *Microviridae* external scaffolding proteins

April D. Burch[1] and Bentley A. Fane*

Department of Veterinary Sciences and Microbiology, University of Arizona, Tucson, AZ 85721, USA

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Abstract

Putative conformational switching and inhibitory regions in the *Microviridae* external scaffolding protein were investigated. Substitutions for glycine 61, hypothesized to promote a postdimerization conformational switch, have dominant lethal phenotypes. In previous studies, chimeric α3/ϕX174 proteins for structures α-helix 1 and loop 6/α-helix 7 inhibited ϕX174 morphogenesis when expressed from high copy number plasmids. To determine if inhibition was due to overexpression, chimeric genes were constructed into the ϕX174 genome. In coinfections with wild-type, protein ratios would be 1:1. The helix 1 chimera has a recessive lethal phenotype; thus, overexpression confers inhibition. In single infections, the mutant cannot form procapsids, suggesting that helix 1 mediates the initial recognition of structural proteins. The lethal chimeric helix 7 protein has a dominant phenotype. Alone, the mutant forms defective procapsids, suggesting a later morphogenetic defect. The results of second-site genetic analyses indicate that the capsid-external scaffolding protein interface is larger than revealed in the crystal structure.

Introduction

The proper assembly of viral proteins and nucleic acids into a biologically active virion involves numerous and diverse macromolecular interactions. While structural proteins must correctly interact with other structural proteins, proper morphogenesis is equally dependent upon interactions between structural and scaffolding proteins, which promote the efficiency and fidelity of particle formation (King and Casjens, 1974; Marvik et al., 1995; Prevelige et al., 1993).

Microvirus assembly (Fig. 1) is dependent on two scaffolding proteins, and internal and external species (Hayashi et al., 1988). After the formation of 9S coat protein pentamers, the internal scaffolding protein binds to the underside of the pentamer. This induces conformational changes which prevent 9S particle aggregation and allows further interactions with both the major spike and the external scaffolding proteins (Siden and Hayashi, 1974; Fane and Hayashi, 1991; Burch et al., 1999; Dokland et al., 1997, 1999). Twelve of these intermediates are then organized into the procapsid by 240 copies of the external scaffolding (or D) protein, which forms a lattice around the immature viral particle (Fujisawa and Hayashi, 1977; Fane et al., 1993; Dokland et al., 1997, 1999). After procapsid formation, single-stranded DNA is concurrently synthesized and packaged (Hayashi et al., 1988).

The D protein participates in many interactions during the assembly process. The crystal structure of the ϕX174 procapsid reveals four D proteins (D1, D2, D3, and D4) associated with one underlying coat protein (Fig. 2). The subunits are arranged as two similar, but not identical, asymmetric dimers, D1D2 and D3D4 (Dokland et al., 1997, 1999). The "canonical monomer" in the crystal structure is composed of seven α-helices separated by loop regions, but there is considerable structural variation between the subunits, which bears no resemblance to quasiequivalence. Due to this unique arrangement, each D protein makes a unique

* Corresponding author. Department of Veterinary Sciences and Microbiology, Building 90, University of Arizona, Tucson, AZ 85721. Fax: +1-520-626-6366.

E-mail address: bfane@u.arizona.edu (B.A. Fane).

[1] Current address: Department of Microbiology, University of Connecticut Health Center, 263 Farmington, Ave. MC2305, Farmington, CT 06030.

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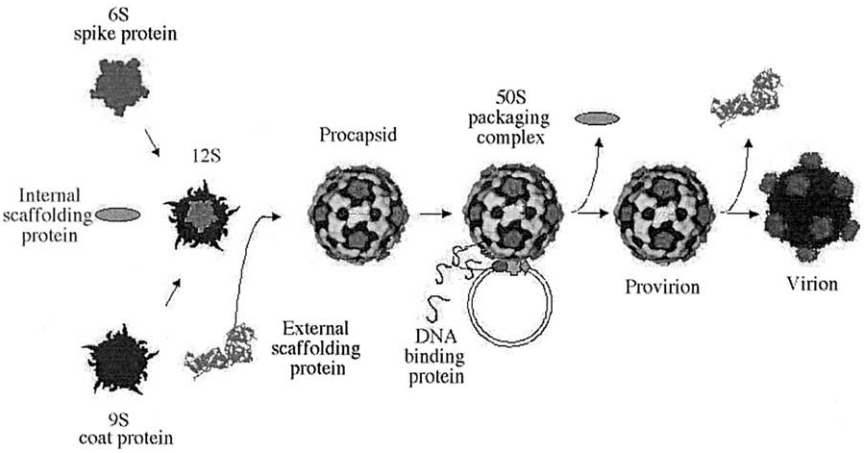

Fig. 1. The morphogenetic pathway of the ϕX174-like phages.

set of contacts with neighboring subunits and the major spike and capsid proteins. Therefore, mutations may only affect the functions of one subunit. However, if the mutated protein retains enough function to allow its incorporation into the morphogenetic pathway, it can act as an assembly inhibitor (Burch and Fane, 2000), demonstrating how

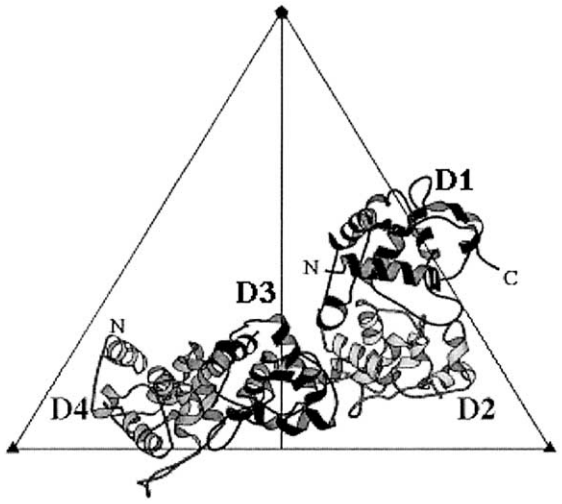

Fig. 2. The four D proteins associated with each asymmetric unit and the primary sequences of the ϕX174 and α3 external scaffolding proteins.

Table 1
Dominance assays

	Wild-type	*G61V*	*G61D*	*cdah1*	*cdal6*[a]	*cdah7*	*ChiD*^{R}
wild-type	1.0[b]	6.0×10^{-2}	6.0×10^{-6}	1.1	0.3	7.0×10^{-2}	
G61V		6.0×10^{-3}					
G61D			5.0×10^{-4}				
cdah1				2.0×10^{-3}	6.0×10^{-2}	6.0×10^{-3}	
cdal6					4.0×10^{-2}	2.0×10^{-2}	
cdah7						8.0×10^{-3}	
						4.0×10^{-2}[c]	0.6[c]
ChiD^R							1.0[c]

[a] *cda16* experiments conducted at 20°C.
[b] For wild-type bursts, 1.0 = 75 ϕ/cell at 33°C and 30 ϕ/cell at 20°C. Values were normalized according to the temperature of the experiment.
[c] In these experiments values were normalized to the *ChiD*^{R} control infection, 1.0 = 17 ϕ/cell.

closely related scaffolding proteins could be developed into antiviral agents.

The results of both structural and genetic analyses have identified at least four putative inhibitory domains and/or critical amino acid residues. For example, the crystal structure of the ϕX174 procapsid indicates that the unique D protein arrangement is mediated, in part, by glycine residue 61. One monomer in each dimer is bent 30° at this site. The kink may be needed to switch the second monomer into a nonsticky conformation. Without this flexibility, D proteins might assemble into an indefinitely growing helical bundle or some other equally abominable structure. If mutations at this site allow the protein to fold into only one conformation, the mutant protein should still interact with other subunits, conferring a dominant lethal phenotype. This hypothesis was tested genetically and the results are presented in this article.

Other inhibitory domains were identified by the use of cloned chimeric scaffolding proteins between bacteriophages ϕX174 and α3, which acted as morphogenesis inhibitors (Burch and Fane, 2000). The percentage identities between the external scaffolding and coat proteins of these phages are 73.7 and 72.2, respectively (Kodaira et al., 1992; Sanger et al., 1978). The primary sequences constituting helices 2–6 are strongly conserved between bacteriophages ϕX174 and α3 (Fig. 2). These structures mediate the vast majority of intra- and interdimer contacts. However, the sequences forming helix 1, loop 6, and helix 7 have diverged.

The chimeric proteins used in previous studies were constructed by interchanging the first helices of the bacteriophage ϕX174 and α3 D proteins. Strong inhibition correlated with the identity of the first helix, suggesting that it serves as a vehicle for the incorporation of chimeras into the procapsid. It may also imply that it mediates the first coat-scaffolding or coat–spike protein interaction. Once incorporated, foreign amino acid sequences, either loop 6 and/or helix 7, block the association of the procapsid with the DNA packaging machinery. Accordingly, a resistance phenotype is conferred by a mutation in protein A, a component of the packaging apparatus. However, when overexpressing proteins from inducible plasmids, it is not possible to rigorously control intracellular protein ratios. Artificially high concentrations of the cloned inhibitory protein could drive reactions that may not readily occur in a typical coinfection. In addition, if related proteins are going to be adapted as possible antiviral therapies, means for determining the efficacy of the inhibitor must take into account the inhibitory: wild-type protein ratio in the infected cell. To determine this, chimeric genes were constructed directly into the phage genome. These strains were also used to characterize the particles formed by the chimeric scaffolding proteins and second-site genetic analyses.

Results

Characterization of mutants with amino acid substitutions for glycine 61

The G61 codon could not be fully randomized. The codon overlaps with the gene E start codon in an overlapping reading frame. More specifically, the G of the ATG start codon overlaps with the first G of the glycine 61 codon. Hence, the following amino acid substitutions for G61 were possible without abrogating the expression of gene E: glutamic acid (GAA, GAG), aspartic acid (GAC, GAT), alanine (GCN), and valine (GTN). After mutagenesis, mutants were recovered in cells overexpressing the wild-type protein. All possible substitutions, G $\rightarrow$ V, D, E, and A, were recovered. With the exception of the *G61A* mutation, all substitutions conferred a lethal phenotype. *G61A* can form pin-prick plaques at temperatures above 33°C. Unlike amber mutants, rescue by overexpression of the wild-type D protein is poor, suggesting a dominant lethal phenotype. Only stocks of two mutants, *G61V* and *G61D,* could be produced with high enough titers (10^8) and low enough reversion frequencies ($<10^{-2}$) to conduct inhibition assays. As seen in Table 1, the mutations are dominant. The burst size in coinfected cells is over an order of magnitude lower than the wild-type control. This result is consistent with the structural data which predicts the formation of unproductive

dimers. The characteristics of the glycine 61 mutants proved too refractory to conduct further experiments. Stocks could not be grown to high enough titers and/or reversion frequencies lower than 1×10^{-2}.

The function of helix 1 of the external scaffolding protein as defined by chimeric proteins encoded from phage genomes

The results of inhibition assays with cloned chimeric proteins demonstrated that the foreign helix 1 structure could inhibit ϕX174 morphogenesis (Burch and Fane, 2000). To determine whether inhibition was due to overexpression of the inhibitory protein, the chimeric gene was constructed directly into the ϕX174 genome. The mutant *cdah1* (chimeric D alpha3 helix 1) has a lethal phenotype. In coinfections with wild-type, in which mutant:wild-type protein ratios would be approximately 1:1, it is recessive (Table 1). Therefore, the inhibition observed in experiments with cloned genes was most likely conferred by artificially high intracellular concentrations of the inhibitory protein.

The morphogenetic pathway was analyzed in cells infected with *cdah1* mutant. Phage-related particles were isolated as described under Materials and methods and analyzed by sucrose gradient sedimentation. For the analyses involving mutants, exogenous *am*(*E*) virions were added to the gradients to serve as a virion marker (114S). After fractionation, the protein concentration of each fraction was determined by OD_{280} and titered to determine the location of the marker virion (Fig. 3). In cells infected with the *cdah1* mutant, no large particles, virions (114S), or procapsids (108S) were detected.

This result is consistent with the mutant's recessive phenotype. Presumably, in coinfected cells both homogeneous and heterogeneous dimers would form. D protein dimerization involves amino acids found between helices 2 and 6 (Dokland et al., 1997, 1999), which are unaltered in the chimeric proteins. Since the *cdah1* mutant is unable to build procapsids in single infections, in coinfected cells, the chimeric protein is either entirely excluded from the lattice or from certain locations within it.

The functions of helix 7 and loop 6 of the external scaffolding protein as defined by chimeric proteins encoded from phage genomes

The results of earlier experiments demonstrated that sequences in the C-terminus of the α3 protein inhibited ϕX174 morphogenesis (Burch and Fane, 2000). However, the cloned chimeric genes contained foreign sequences for both loop 6 and helix 7. To determine which structure conferred inhibition, chimeric D genes containing only one foreign sequence were constructed directly into the ϕX174 genome. The *cdah7* (chimeric D alpha3 helix 7) mutant has a lethal phenotype. The *cdal6* mutant (chimeric D alpha3 loop6), on the other hand, is cold-sensitive (*cs*). As seen in Table 1, only the *cdah7* phenotype is dominant. The burst size in coinfected cells is an order of magnitude lower than the wild-type control. In contrast, the *cs cdal6* phenotype is recessive. These results indicate that helix 7 is both necessary and sufficient for inhibition and at a wild-type: chimeric protein ratio of 1:1. In Burch and Fane (2000), a mutation in the DNA packaging protein (*ChiD*^{R}), which conferred resistance to the inhibitory effects of an inducible loop 6/helix 7 chimeric protein, was isolated. In coinfections of *ChiD*^{R} and helix 7 mutants, progeny were produced (Table 1). These data suggest that helix 7 may directly interact with the packaging machinery. Alternatively, it may alter the placement or topography of the D protein lattice in a manner that inhibits this interaction.

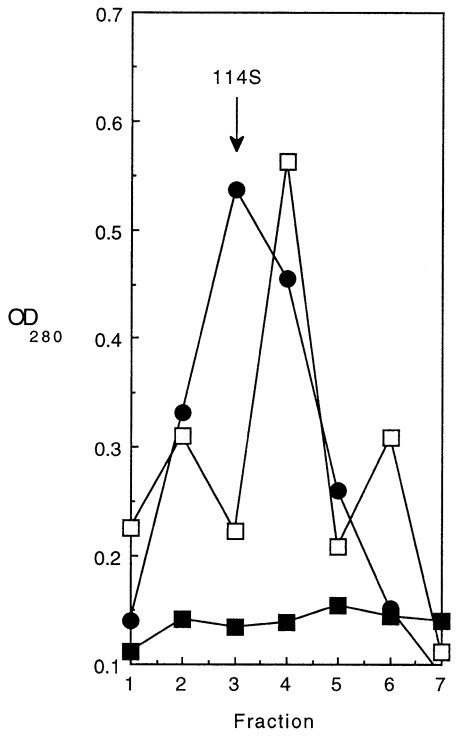

Fig. 3. Particles formed in cells infected with wild-type, *cdah1*, and *cdah7*. Gradients were aligned by the position of infectious *am*(*E*)*W4* marker virions (114S). Symbols: closed circles, wild-type; closed and open squares, *cdah1* and *cdah7* mutants, respectively.

In cells infected with *cdah7*, particles with S values slightly smaller than 114S (marker virion) were detected, indicating the presence of procapsids (108S). However, incorporation of the chimeric protein arrests morphogenesis before DNA packaging. This result is consistent with the dominant phenotype. Whether the foreign helix fails to interact with the underlying coat protein, but can still be placed in the D4 position, or makes a detrimental interaction remains to be determined. The *cs* phenotype of *cdal6* mutant proved too weak and leaky to conduct similar analyses.

Table 2
Capsid protein suppressors of the cold-sensitive *cda16* phenotype

Amino acid	Substitution
187	thr → ile
144	thr → ala
200	ala → ser
204	thr → ile
205	asp → asn
316	asp → val

Reversion analyses

Reversion analyses were conducted with all the chimeric phages. Despite using multiple temperatures and several independently grown stocks, no second-site revertants for *cdah1* or *cdah7* mutants were obtained. If second-site suppressors exist for either of these foreign sequences, they occur at a frequency $<10^{-8}$. On the other hand, cs^{+} revertants of *cdal6* were readily isolated and characterized.

Both intragenic and extragenic substitutions were identified. The intragenic substitution changes the central amino acid of the seven-residue loop from glutamic to aspartic acid. This residue in the D4 subunit mediates the only known loop 6–coat protein contact (Dokland et al., 1997, 1999). The extragenic suppressors mapped to gene F (Table 2), which encodes the major coat protein. To verify that the identified substitution was responsible for the phenotype, three suppressing coat protein genes were cloned into a Topo 2.1 vector and assayed for the ability to rescue via recombination. Rescue occurred at frequencies in the 10^{-4} range, 1.5 orders of magnitude above reversion frequency.

The extragenic suppressors confer amino acid substitutions on the outer surface of the capsid protein (Fig. 4). The majority of these substitutions would affect D4 interactions, mapping to residues directly adjacent to or one amino acid away from known contact sites (Dokland et al., 1999). However, their location is not limited to the vicinity of D4, indicating that the D4 position can be adjusted by the position of neighboring subunits. These suppressors may elucidate interactions lost in the X-ray model due to particle maturation within the crystal, which includes a radial collapse of capsid proteins away from the external scaffolding lattice (Dokland et al., 1999).

Discussion

The results of previous structural and genetic studies have suggested that regions and/or amino acid residues in the ϕX174 external scaffolding protein mediate distinct processes during procapsid morphogenesis. While structural studies can be used to formulate working hypotheses regarding key residues, further genetic and biochemical analyses are instrumental in testing them. Furthermore, previous genetic studies have utilized cloned chimeric proteins that inhibit morphogenesis. However, the overexpression of these proteins could drive reactions which may not normally

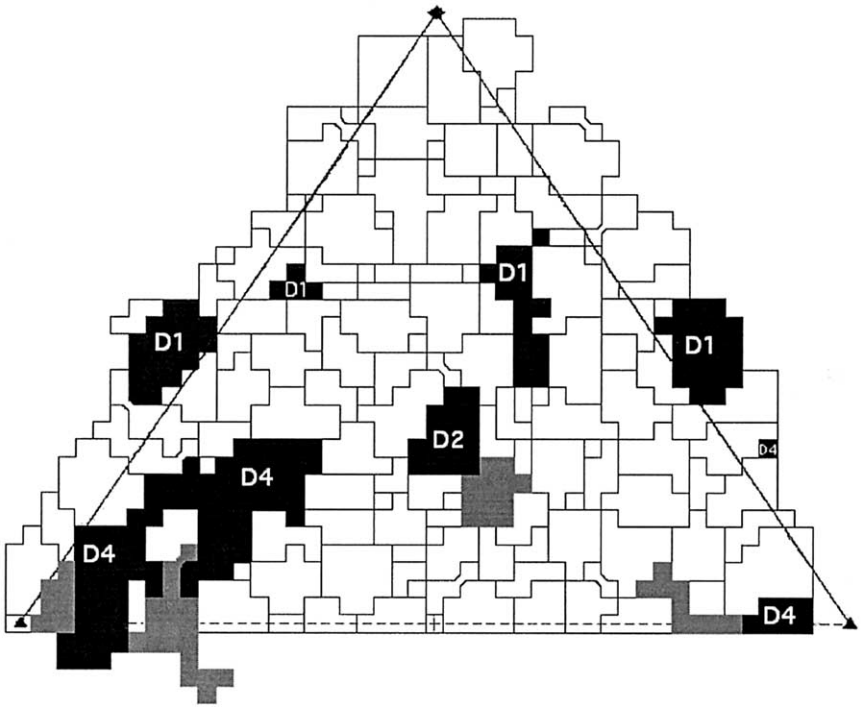

Fig. 4. The location of coat protein residues that contact the external scaffolding proteins. Black regions indicate contacts known from the atomic structure. Grey regions indicate regions identified from genetic analyses. The contacted D protein subunits are indicated as D1–D4. Twofold related D4-coat contacts are depicted twice; on the left side of the figure they are below the asymmetric unit.

occur during an infection process. To circumvent this, chimeric genes were constructed into the ϕX174 genome. Therefore, transcriptional and translational control should mimic the processes found in a wild-type infection.

Dimerization of scaffolding protein subunits

Structural data suggest that the assembly active state of protein D is a dimer (Dokland et al., 1997, 1999). There are two asymmetric dimers associated with each viral coat protein (Fig. 2). One monomer in each dimer is bent 30° at the site of glycine residue 61. Without this kink, D proteins might assemble into an indefinitely growing helical bundle or prevent the formation of the asymmetric tetrameric arrangement. Missense substitutions at this site confer dominant lethal phenotypes, which is consistent with a mutant protein that can interact with wild-type protein and essentially remove them from the morphogenetic pathway. *Microviridae* genome structure also alludes to the importance of this residue. Gene E, which encodes the host cell lysis function (Hutchison and Sinsheimer, 1966; Young and Young, 1982), resides in an overlapping reading frame with gene D (Sanger et al., 1978). Although genetic and evolutionary analyses indicate that the E protein is fairly tolerant of substitutions (Maratea et al., 1985), the G61 codon overlaps the gene E start codon in all ϕX174-like genomes (Godson et al., 1978; Kodaira et al., 1992; Sanger et al., 1978).

Coat protein recognition

Structural and genetic data suggest that helix 1 interacts with the major spike and capsid proteins in the D1 and D4 subunits, respectively (Dokland et al., 1997, 1999; Fane et al., 1993). Two lines of evidence suggest that helix 1 may mediate the initial interactions with the viral coat protein, acting as substrate specificity domain. The *cdah1* mutant is unable to build procapsids in single infections. In coinfected cells, the mutant is recessive, indicating that it is either entirely excluded from the lattice or from certain locations within it. If the foreign helix 1 is unable to interact with the major spike and/or coat proteins, it may be unable to occupy the D1 and/or D4 positions, respectively. However, this would not exclude it from the D2 and/or D3 locations.

In experiments conducted with a cloned chimeric protein, the protein inhibited wild-type ϕX174 morphogenesis (Burch and Fane, 2000). Since this result contrasts the coinfection data, the observed inhibition was most likely due to the overexpression of the chimeric protein. If related scaffolding proteins are to be adapted as possible antiviral therapies, means for determining the efficacy of the inhibitor must take into account the inhibitory:wild-type protein ratio in the infected cell.

Interactions with the DNA packaging machinery

In the crystal structure, helix 7 only interacts with the major capsid protein and only in the D4 position. The ability of the mutant protein to form procapsids suggests that it is neither excluded from the lattice nor any position within it. However, these procapsids cannot be filled, indicating that the external scaffolding lattice blocks the association of the DNA packaging machinery. In coinfections, the lethal *cdah7* phenotype is dominant, indicating that overexpression of the chimeric protein obtained in earlier analyses (Burch and Fane, 2000) does not substantially contribute to the mechanism of inhibition. Mutations in protein A, a component of the DNA packaging machinery, rescues procapsid formed in coinfected cells but not procapsids formed with the chimeric protein alone. Hence the altered A protein allows the packaging machinery to interact only with lattices composed of both wild-type and chimeric proteins.

In the D4 subunit, helix 7 burrows into the capsid protein (Dokland et al., 1997, 1999). Numerous interactions are made by the terminal residues, which have diverged between ϕX174 and α3. Considering the extensive interactions, double mutational events may be required to achieve a conformation that produces an external lattice capable of interacting with the packaging machinery. In addition, it may not be possible to recover intragenic helix 7 substitutions due to sequence overlap with the Shine–Delgarno sequence of the downstream gene, an evolutionary constraint similar to that found with the glycine 61 codon. Similar to *cdah7*, second-site revertants were not recovered for *cdah1*. Structural and genetic data suggest that helix 1 interacts with the major spike and capsid proteins in the D1 and D4 subunits, respectively (Dokland et al., 1997, 1999; Fane et al., 1993). Hence, multiple mutational events may be required.

The external scaffolding–coat protein interface

In contrast with the foreign helix 1 and helix 7 chimeric proteins, chimeric loop 6 proteins confer only weak cold-sensitive phenotypes, which proved too refractory for biochemical analyses of the assembly pathway. However, the *cdal6* was the only mutant for which second-site suppressors could be isolated. The location of these suppressors (Fig. 4) suggests that the coat–external scaffolding protein interface is more extensive than revealed in the crystal structure. This is particularly apparent with the D4 subunit. This result is not entirely surprising. Comparisons between cryoimage reconstructions and crystal models of *Microviridae* procapsids (Ilag et al., 1995; Dokland et al., 1997, 1999) suggest that during crystallization particles matured, a process that includes a radial inward collapse of the major capsid protein.

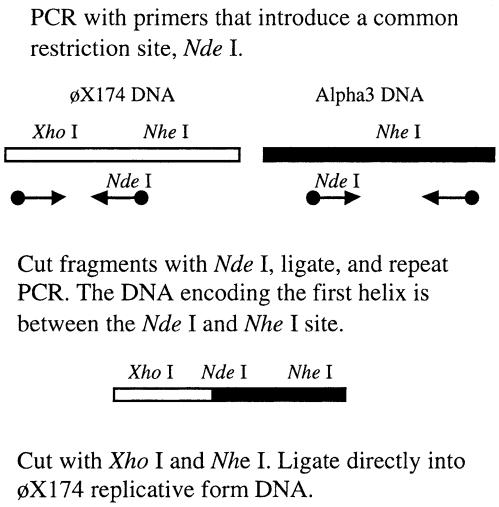

Fig. 5. PCR-mediated technique for the generation of chimeric genes within the viral genomes.

Materials and methods

Phage plating, media, burst experiments, stock preparation, generation of RF DNA, and DNA isolation

The reagents, media, buffers, and protocols for burst experiments, single-stranded DNA isolation, and RF DNA isolation have been previously described (Fane and Hayashi, 1991; Burch et al., 1999).

Bacterial strains and plasmids

The *Escherichia coli* C strains C122 (*sup*°) and BAF30 *recA* have been previously described (Burch et al., 1999; Fane and Hayashi, 1991). The construction pϕXDJ, a complementing clone of the ϕX174 external scaffolding and DNA binding proteins, is described in Burch and Fane (2000). The construction of the complementing clone containing only the D gene is described in Hayashi et al. (1989). The host slyD mutation confers resistance to E-protein mediated lysis (Roof et al., 1994).

Phage mutants

All mutations were placed into a ϕX174 genome modified with unique *Nhe*I and *Pvu*I restriction sites (Burch and Fane, 2000). The *helix 1* mutant (*cdah1*) was generated by constructing the chimeric gene in vitro and then by placing it into the ϕX174 genome. A schematic of the protocol is presented in Fig. 5. It can be easily adapted to making chimeric genes in any system. If the restriction enzymes can be heat inactivated and the various enzyme buffers are compatible, after the purification of the initial PCR products, all subsequent steps can be performed without intermediate purifications. PCR primers, which introduced a silent *Nde*I restriction site upstream from the helix 1 sequence, were designed to anneal to the beginning of the ϕX174 and α3 D genes. After amplification the two fragments were purified, cut with *Nde*I, and ligated. An aliquot of the ligation reaction was used in a second PCR reaction to amplify the ligated chimeric fragment. The chimeric DNA was then digested with the *Nhe*I and *Xho*I, purified, ligated into ϕX174 RF DNA cut with the same enzymes, transfected into BAF30 pϕXDJ, and incubated at 33°C until plaques appeared. Progeny were stabbed into three indicator lawns seeded with C122, incubated at 24, 33, and 42°C, and one seeded with BAF30 pϕXDJ lawn and incubated at 33°C. The mutant was identified by its complementation-dependent phenotype. An identical protocol was used to generate the *cdah7* mutant with the exception that primers introduced a unique *Hind*III site, and the chimeric gene was placed in RF DNA using *Nhe*I and *Pvu*I. All mutants are complemented by plasmids containing only the D gene. The loop 6 chimera, *cdal6*, was generated by oligonucleotide-mediated mutagenesis (Fane et al., 1993). Mutagenized DNA was transfected into BAF30 pϕXDJ cells and incubated at 33°C until plaques appeared. Progeny were stabbed into the indicator lawns described above. The mutant was first identified by a *cs* phenotype. Two independently isolated mutants were sequenced and verified.

The G61 mutants were also generated by oligonucleotide-mediated mutagenesis with randomized primers designed to encode the four possible changes. After recovery in BAF30 pϕXDJ, mutants were stabbed into indicator lawns, as described above, and identified by complementation-dependent phenotypes. Differences in plaque morphologies on BAF30 pϕXDJ and leaky *cs* phenotypes on C122 were used to distinguish between possible substitutions. All mutants were verified by sequencing.

Detection of virion and procapsid from infected cells

Two hundred milliliters of *slyD* cells were infected with wild-type, *cdah1*, and *cdah7* at a multiplicity of infection of 3.0 and incubated for 4 h. Cells were concentrated, resuspended in 8.0 ml BE buffer (Fane and Hayashi, 1991), and lysed and extracts were prepared as described in Hafenstein and Fane (2002). Extracts were layered atop CsCl gradients and spun as described in Fane and Hayashi (1991). Material with densities between and including virion (1.4 gm/cm^3) and soluble protein (1.3 gm/cm^3) was pooled. Soluble protein bands were present in all gradients; procapsid and virion bands were clearly visible in extracts in which they were formed. After dialysis, 200 μl was loaded atop 5–30% sucrose gradients, spun, and fractionated as described in Ekechukwu et al. (1995). Material was detected by taking OD_{280} readings of 1/10 dilutions of each fraction. The position of infectious virion or marker *am*(*E*)*W4* phage was determined by titering each fraction.

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