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Efficient Complementation by Chimeric *Microviridae* Internal Scaffolding Proteins Is a Function of the COOH-Terminus of the Encoded Protein

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Microviridae morphogenesis is dependent on two scaffolding proteins, an internal and external species. Both structural and genetic analyses suggest that the COOH-terminus of the internal protein is critical for coat protein recognition and specificity. To test this hypothesis, chimeric internal scaffolding genes between *Microviridae* members ϕX174, G4, and α3 were constructed and the proteins expressed *in vivo.* All of the chimeric proteins were functional in complementation assays. However, the efficient complementation was observed only when the viral coat protein and COOH-terminus of internal scaffolding were of the same origin. Genes with 5′ deletions of the ϕX174 internal scaffolding gene were also constructed and expressed *in vivo.* Proteins lacking the first 10 amino acids, which self-associate across the twofold axes of symmetry in the atomic structure, efficiently complement ϕX174 *am(B)* mutants at temperatures above 24°C. These results suggest that internal scaffolding protein self-associations across the twofold axes of symmetry are required only at lower temperatures.

INTRODUCTION

The proper assembly of proteins and nucleic acids into biologically active virions involves numerous and diverse macromolecular interactions. While structural proteins must correctly interact, proper morphogenesis is equally dependent on scaffolding proteins, which are transiently associated with nascent protein complexes during virion assembly (Casjens and Hendrix, 1988; Hayashi *et al.,* 1988; King *et al.,* 1980).

Microviridae morphogenesis (Fig. 1) is dependent on two scaffolding proteins, an internal and external species, proteins B and D, respectively. The first intermediates in the pathway are the 9S and 6S particles, respective pentamers of viral coat and spike proteins. In cells infected with temperature-sensitive internal scaffolding mutants, these particles accumulate at restrictive temperatures but can be chased into larger structures following temperature shifts (Ekechukwu and Fane, 1995). Five B proteins bind to the underside of a 9S particle. This probably triggers conformational changes on the particle's upper surface (Fane and Hayashi, 1991; Dokland *et al.,* 1997, 1999), allowing subsequent interactions with spike and external scaffolding proteins, and preventing the premature associations of 9S particles into 55–60S aggregates (Siden and Hayashi, 1974).

The atomic structures of the internal scaffolding and coat proteins are depicted in Fig. 2. Density could be ascribed to only approximately one half, the COOH-terminus, of the internal scaffolding protein. The amino-terminus, which is mostly unordered, is not depicted in Fig. 2. The only region in the amino-terminus to which density could be ascribed consists of the first 10 amino acids. This region of the protein appears to make scaffolding-scaffolding contacts across the twofold axes of symmetry within the procapsid. The lack of ordered density within the amino-terminus of the protein suggests that the internal scaffolding protein may be inherently flexible. Genetic data support this hypothesis. The *Microviridae* internal scaffolding proteins for ϕX174, G4, and α3 can cross-function, despite divergence as great as 70% in primary structure (Burch *et al.,* 1999). There was one instance in which cross-complementation was not observed. The ϕX174 protein is incapable of complementing G4 *am(B)* mutants. Morphogenesis is blocked before the first B-mediated step in the pathway. Two G4 *ϕXB-utilizer* mutants that productively utilize the ϕX174 B protein have been isolated and characterized. Both confer substitutions in regions of the G4 coat protein that most likely contact the COOH half of the B-protein.

To further investigate the importance of the COOH-termini interactions, chimeric ϕX174/G4 B genes have been generated and assayed for their ability to direct morphogenesis. The results of these experiments support the hypothesis that the COOH terminus is primarily responsible for mediating coat protein recognition. This may be a general property of internal scaffolding proteins. Similar results have been obtained both with Herpesviruses and P22 scaffolding proteins (Parker *et al.,*

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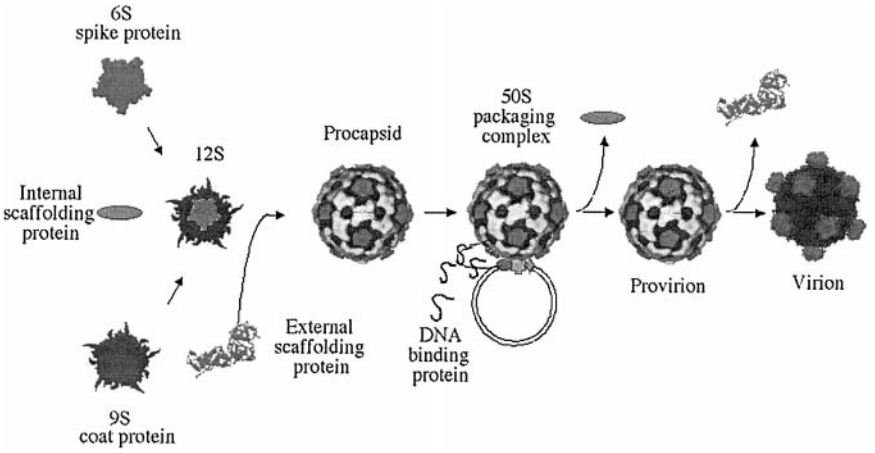

FIG. 1. The *Microviridae* morphogenetic pathway.

1998; Preston *et al.,* 1997; Tuma *et al.,* 1998). In addition, ϕX174 genes with 5′ deletions were also constructed. The first 10 amino acids of the protein, which appear to mediate interactions across the twofold axes of symmetry, is required only at lower temperatures.

RESULTS AND DISCUSSION

The generation and characterization of chimeric B genes

The atomic structure of the internal scaffolding protein (Dokland *et al.,* 1997, 1999) was used as a guide in determining the junction points within the chimeric genes (Fig. 2). The most logical junction appeared to be between amino acids 60 and 70. This region of the protein contains no secondary structure and bridges the structurally defined COOH terminus with the less-defined NH_2-terminus density. The chimeric genes were constructed using identically placed *Sac*I sites as described under Materials and Methods. This produces junctions in the chimeric proteins between amino acids 63 and 64. Figure 3 contains a restriction digest of the ϕXG4 and G4ϕX constructs.

The chimeric genes were assayed for their ability to complement *am(B)* mutants of bacteriophage G4, α3, and ϕX174 (Table 1). When the COOH-terminus of the chimeric scaffolding protein is from the same origin as the viral coat protein, complementation is efficient, yielding plating efficiencies of 1.0. For example, both the G4ϕX and α3ϕX chimeric genes efficiently complement the ϕX174 *am(B)* mutant. Like the wild-type ϕX174 B protein, neither chimera complemented the *G4 am(B)* mutant. On the other hand, the ϕXG4 B protein complements the G4 *am(B)* mutant, demonstrating that the inability of the wild-type ϕX174 B protein to interact with the G4 coat protein (Burch *et al.,* 1999) was a function of COOH-terminus recognition, or lack thereof.

Burch *et al.* (1999) demonstrated that wild-type internal scaffolding proteins of ϕX174, G4, and α3 were cross-functional. While the complementation behavior of the chimeric ϕXG4 and α3ϕX proteins is broad, it is not always as efficient as cross-complementation by wild-type proteins. For example, complementation of the ϕX174 and α3 *am(B)* mutants by the ϕXG4 B gene is down a factor of 10 and 100, respectively; however, these values are still several orders of magnitude above the negative control. Complementation by the G4ϕX protein is narrow. While its inability to complement the G4 *am(B)* mutant was expected, no activity above background was detected in experiments with *α3 am(B)*. The decreased complementation efficiencies of some of the chimeric proteins suggest that the two termini may not function as totally separate and independent domains. There are eight specific contacts made between the scaffolding

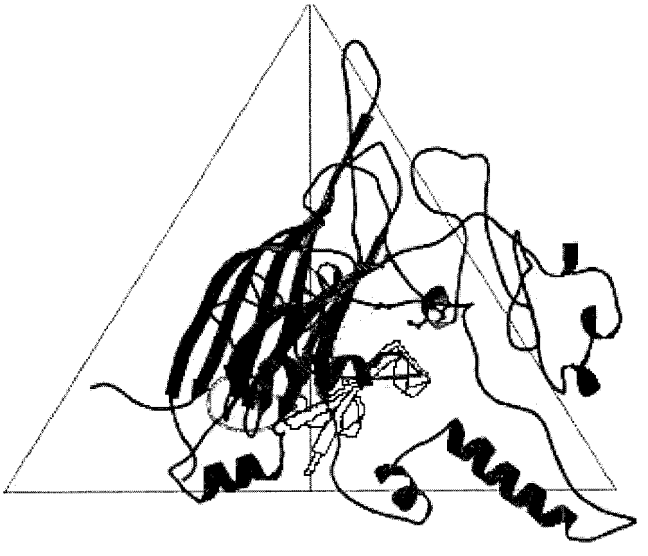

FIG. 2. The atomic structure of the ϕX174 coat and internal scaffolding protein. The viral coat protein is depicted in black; the internal scaffolding protein, in white.

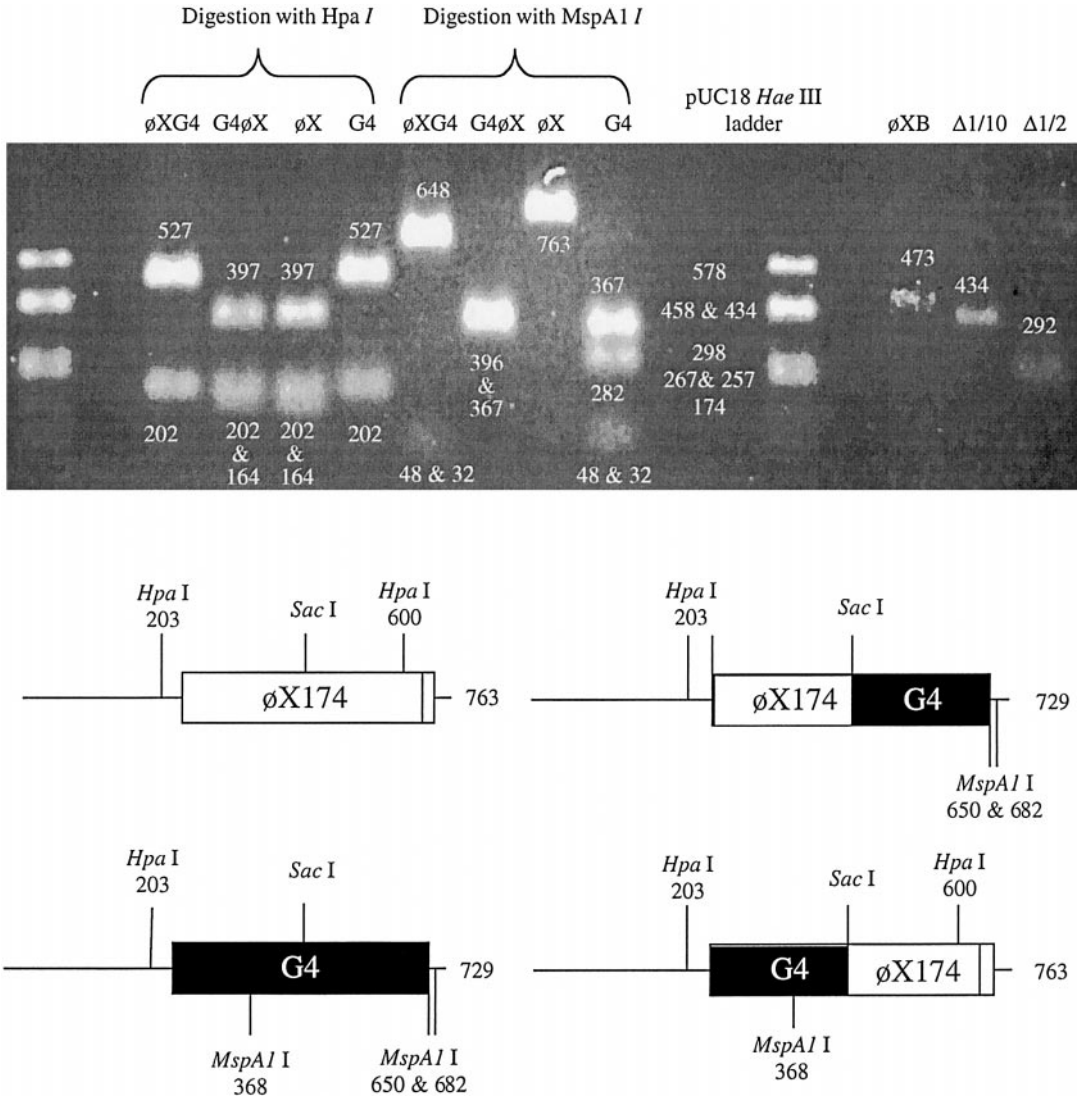

FIG. 3. Restriction digests of the chimeric genes and 5′-deleted clones. The chimeric and wild-type genes were amplified. Fragments were then purified and digested with either *Hpa*I or *Msp*AI. Genetic maps of the constructs are given in the figure. Clones of the 5′-deleted genes were digested with restriction enzymes that cut directly at the 5′ end of the gene, *Avr*II or *Nco*I, and *Xho*I, which cuts 112 nucleotides downstream from the end of the gene. The sizes of the restriction fragments are given within the figure.

and coat proteins (Dokland *et al.,* 1997, 1999). The involved amino acids are located in the COOH-terminus of the B protein, and are conserved in ϕX174, G4, and α3. While these specific contacts are critical, nonspecific contacts, which may be mediated by residues located in the unordered amino-terminal half of the protein, cannot be disregarded. However, a different type of genetic analysis will be required to elucidate these nonspecific scaffolding-coat contacts or interfaces.

Expression of the cloned chimeric genes appears to have no effect on wild-type plating efficiency or plaque size (data not shown). In cells infected with wild-type viruses, two species of scaffolding protein would be present, the indigenous and the chimeric species. Either the indigenous protein binds more effectively to viral coat proteins than the chimeric protein, or a complete complement of indigenous proteins is not required for the production of infectious virions. The characterization of the protein content of viral procapsids formed in the presence of two proteins should directly address these alternatives.

The complementation behavior of the chimeric genes and the isolation of mutations that affect foreign scaffolding protein utilization (Burch *et al.,* 1999) suggest that the COOH-termini of the internal scaffolding proteins play a critical role in viral coat protein recognition and specificity. This may be a general property of internal scaffolding proteins. Similar results have been obtained in studies with Herpesviruses and P22 scaffolding proteins (Parker *et al.,* 1998; Tuma *et al.,* 1998). The scaffolding proteins of VSZ and HSV-1 can cross-complement, forming procapsid-like structures if the COOH-termini of these proteins

TABLE 1

Complementation Efficiency of Chimeric B Genes[a]

	BAF30 (*recA*) with				
Virus	pϕXG4B	pG4ϕXB	pα3ϕXB	BAF8 (*supF*)	C122 (*sup*°)
ϕX174 *amB*	0.1	1.0	1.0	1.0	10^{-6}
G4 *amB*	1.0	10^{-5}	10^{-5}	1.0	10^{-5}
α3 *amB*	10^{-2}	10^{-4}	0.2[b]	1.0	10^{-4}

[a] Assay titer/titer on BAF8 (*supF*) at 33°C.

[b] Complementation efficiency calculated at 42°C. At 33°C, the α3ϕXB gene complements α3 *amB* mutant with an efficiency near 1.0. However, plaque size is very small. At 42°C, plaques have a pinprick morphology.

are interchanged (Preston *et al.,* 1997). Baudet-Miller *et al.* (1996) characterized small COOH-terminal domains in the HSV-1 and CMV scaffolding proteins, which govern both capsid protein interactions and specificity. Studies with fragments of the P22 scaffolding protein demonstrate that the COOH-terminal half of the protein is both necessary and sufficient for icosahedral particle formation (Parker *et al.,* 1998; Tuma *et al.,* 1998). The last 11 residues of the protein are particularly critical for function. Similarly, in the atomic structure of the ϕX174 procapsid, the last 10 amino acids of the internal scaffolding protein mediate the majority of the close-range interactions with the viral coat protein (Dokland *et al.,* 1997, 1999). Although the *Herpesviridae* and P22 scaffolding proteins are more complex than the *Microviridae* proteins, mediating the incorporation of minor capsid proteins (Bazinet and King, 1988); do not exhibit a strict stoichiometric relationship with the major capsid protein; and exhibit less icosahedral symmetry (Zhou *et al.,* 1998; Thuman-Commike *et al.,* 1996), these proteins appear to recognize coat proteins via similar mechanisms.

The generation and characterization of ϕX174 B genes with 5′ deletions

Two ϕX174 B genes with 5′ deletions were generated as described under Materials and Methods. The deleted genes were constructed using the atomic structure of the ϕX174 B protein as a guide. The Δ1/2 gene contains only the last 60 codons. This region encodes the amino acids that appear as ordered density in the atomic structure. Although the majority of the NH_2-terminus is unordered, density could be ascribed to the first 10 amino acids. These residues are located directly at the twofold axis of symmetry and may be responsible for contacts across this axis. If this small region is critical in B-B contacts, it may be in the same location in each asymmetric unit, perhaps explaining why it is the only well-ordered density seen in the amino half of the protein. The Δ1/10 gene lacks the codons that encode this region of the protein. Restriction digests of the plasmids containing the 5′ deletion and full-length genes are depicted in Fig. 3.

The deleted genes were assayed for their ability to complement a ϕX174 *am(B)* mutant and to inhibit wild-type ϕX174 morphogenesis. As can be seen in Table 2, the Δ1/2 gene was unable to complement at any temperature. The expression of the deleted proteins does not appear to inhibit wild-type ϕX174 morphogenesis. Either the full-length protein more effectively binds the viral coat protein, or a complete complement of full-length proteins is not required for morphogenesis.

The Δ1/10 gene was capable of complementing the ϕX174 *am(B)* mutant. However, complementation is temperature dependent. At 24°C, plating efficiency drops 2 orders of magnitude. Plating efficiency by the full-length gene is also reduced at this temperature, but only by a factor of 4. This result suggests that B-B interactions

TABLE 2

Complementation Efficiency of 5′-Deleted B Genes[a]

	BAF30 (*recA*) with					
	pϕXB		pΔ1/10			
Virus	24°	33°	24°	33°	pΔ1/2	C122 (*sup*°)
ϕX174 *amB*	0.3	1.0	10^{-2}	1.0	10^{-6}	10^{-6}
ϕX174 *wt*	0.4	1.0	0.4	1.0	1.0	1.0

[a] Assay titer/titer on BAF30 pϕXB at 33°C.

across the twofold axes of symmetry may be required only at lower temperatures. The external scaffolding protein, protein D, also makes contacts across the two- and threefold axes of symmetry. Previous genetic analyses suggest that the formation of D-D contacts across the threefold axes of symmetry may be somewhat cold-sensitive (Ekechukwu and Fane, 1995). In addition, cold-sensitive substitutions at glutamic acid residues 102 and 112, which mediate the twofold-related contacts between D subunits, have been isolated (unpublished results). Therefore, the first nine amino acids of the B protein may be required only at lower temperatures, at which external scaffolding protein interactions are somewhat inhibited.

MATERIALS AND METHODS

Plating assays, burst experiments, stock preparation, and DNA isolation

Reagents, media, buffers, and protocols are described in Fane and Hayashi (1991).

Bacterial phage strains and cloning of the ϕX174, G4, and α3 B genes

The phage mutants, *E. coli* C strains, and the cloning of the B genes are described in Burch *et al.* (1999).

Generation of chimeric genes

The chimeric B genes were constructed by placing a unique *Sac*I site within the clone ϕX174 sequence at the same location as the unique *Sac*I site in G4 sequence. The cloned genes were digested with *Sac*I and *Nco*I. The *Nco*I site is located upstream of the cloned gene in the MCS in pSE420. Fragment and vector purification and ligation protocols are described in Burch *et al.* (1999). The α3ϕX chimeric B gene was constructed by amplifying the 5′ end of the cloned α3 gene with a mutagenic primer designed to create a *Sac*I site at the 3′ end of the fragment and a primer which annealed to the 5′ end of the MCS. The fragment and cloned ϕX174 B gene were digested with *Sac*I and *Nco*I, and the digested α3 fragment was ligated to the cut vector.

Generation of ϕX174 genes with 5′ deletion

The 3′ end of the ϕX174 B gene was amplified using mutagenic primers designed to introduce *Nco*I sites, which contain ATG start codons, in the 5′ end of the fragments and a primer annealing to the 3′ end of the MCS. Fragments were digested with *Nco*I and *Xho*I, and cloned into pSE420 digested with the same enzymes. An *Xho*I site is found downstream from gene B.

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