Article No. jmbi.1998.2450 available online at http://www.idealibrary.com on IDEAL J. Mol. Biol. (1999) 286, 95–104

JMB

# Cross-functional Analysis of the *Microviridae* Internal Scaffolding Protein

**April D. Burch, Josephine Ta and Bentley A. Fane***

*Department of Veterinary Science and Microbiology Building 90, University of Arizona, Tucson, AZ 85721 USA*

The assembly of the viral structural proteins into infectious virions is often mediated by scaffolding proteins. These proteins are transiently associated with morphogenetic intermediates but not found in the mature particle. The genes encoding three *Microviridae* (øX174, G4 and α3) internal scaffolding proteins (B proteins) have been cloned, expressed *in vivo* and assayed for the ability to complement null mutations of different *Microviridae* species. Despite divergence as great as 70% in amino acid sequence over the aligned length, cross-complementation was observed, indicating that these proteins are capable of directing the assembly of foreign structural proteins into infectious particles. These results suggest that the *Microviridae* internal scaffolding proteins may be inherently flexible. There was one condition in which a B protein could not cross-function. The øX174 B protein cannot productively direct the assembly of the G4 capsid at temperatures above 21 °C. Under these conditions, assembly is arrested early in the morphogenetic pathway, before the first B protein mediated reaction. Two G4 mutants, which can productively utilize the øX174 B protein at elevated temperatures, were isolated. Both mutations confer amino acid substitutions in the viral coat protein but differ in their relative abilities to utilize the foreign scaffolding protein. The more efficient substitution is located in a region where coat-scaffolding interactions have been observed in the atomic structure and may emphasize the importance of interactions in this region.



*Corresponding author

*Keywords:* scaffolding proteins; viral assembly; øX174

## Introduction

Scaffolding proteins, which have been identified in several viral systems (King & Casjens, 1974; Siden & Hayashi, 1974; Rixon, 1993; Leibowitz & Horowitz, 1975), are transiently associated with morphogenetic intermediates but not found in the mature particle. Scaffolding proteins may be inherently flexible, having to assume one structure that mediates capsid protein assembly and another one that allows for dissociation and/or extrusion from the assembled complex. The *Microviridae* system (canonical members: øX174, G4 and α3) is an ideal system in which to investigate the morphogenetic functions of scaffolding proteins. The atomic structures of the øX174 virion and a morphogenetic intermediate containing a full complement of both the internal and external scaffolding proteins have been solved (McKenna *et al*., 1992, 1994; Dokland *et al*., 1997). Therefore, the results of genetic and biochemical analyses can be interpreted within a structural context.

The *Microviridae* assembly pathway is illustrated in Figure 1 (for a review, see Hayashi *et al*., 1988). The first detectable morphogenetic intermediates are the 9 S and 6 S particles, respective pentamers of the viral coat and spike proteins. In a reaction mediated by the internal scaffolding (or B) protein, these intermediates associate, forming the 12 S particle. Although the procapsid crystal structure suggests that the 12 S particle may be the product of an off pathway reaction, this particle does exhibit properties associated with true morphogenetic intermediates: the ability to be chased into mature structures in pulse-chase experiments (Tonegawa & Hayashi, 1970). With the aid of the external scaffolding (or D) protein, twelve 12 S particles are organized into the procapsid. The DNA binding protein enters the morphogenetic pathway during the packaging reaction, perhaps mediating the extrusion of the internal scaffold. The highly aro-

Present address: J. Ta, University of Arkansas Medical School, Little Rock, AR, USA.

E-mail address of the corresponding author: bfane@u.arizona.edu

0022-2836/99/060095–10 $30.00/0

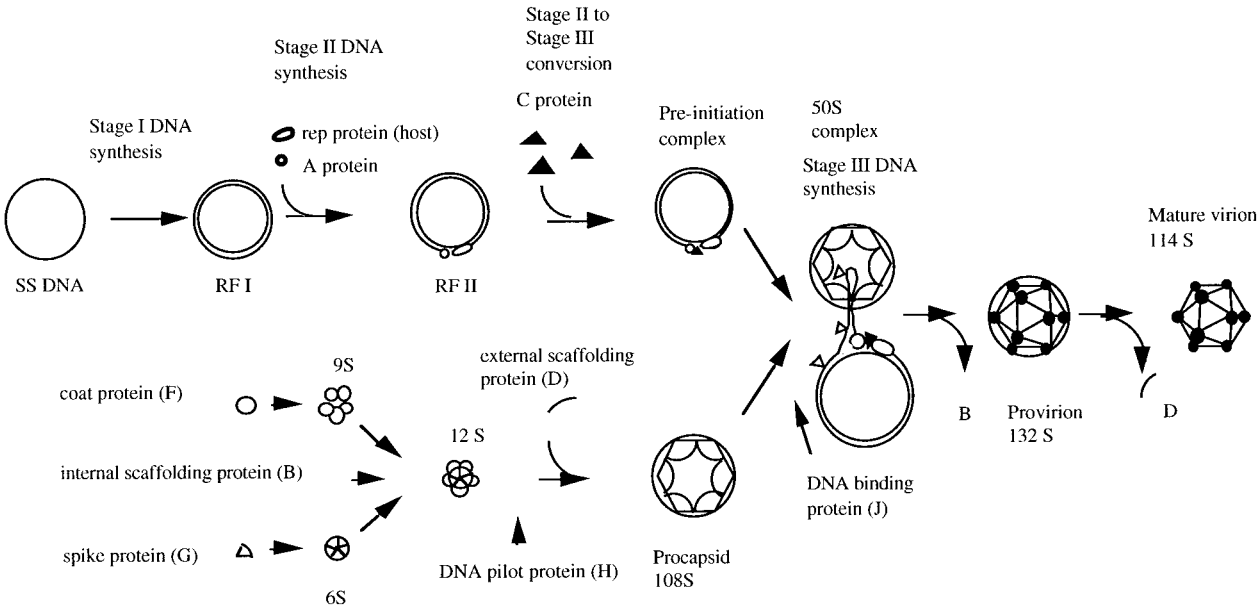


**Figure 1.** The øX174 morphogenetic pathway.

matic COOH termini of both proteins are known to interact with a common cleft within the viral coat protein (McKenna *et al*., 1992, 1994; Dokland *et al*., 1997). Maturation is complete upon the dissociation of the external scaffolding protein.

The atomic structure of the øX174 procapsid demonstrates that the external scaffolding (protein D) is inherently flexible, assuming at least three non-quasi-equivalent conformations. These conformations are determined by the subunit's location within the lattice relative to coat, spike, and other D proteins. The internal scaffolding (or B) protein, on the other hand, appears to occupy the same general location in each asymmetric unit. While portions of the protein are readily distinguished within the crystal structure, much of the density is unordered, suggesting that interactions with the overlying coat protein can be both variable and flexible. To further investigate the flexibility of scaffolding protein interactions the B genes of øX174, G4 and α3 have been expressed *in vivo* and assayed for the ability to cross-complement. As can be seen in Table 1 and Figure 2, the amino acid sequences of these proteins exhibit a high degree of divergence. Here we report that *Microviridae* scaffolding proteins with 70% divergent primary structures can productively direct the assembly of viral coat proteins in a non-species-specific manner.

**Table 1.** Per cent divergence between the *Microviridae* coat and scaffolding proteins

| Protein | α3/øX | α3/G4 | øX/G4 |
|---|---|---|---|
| B protein | 70 | 68 | 44 |
| F protein | 28 | 36 | 34 |

Taken from Kodaira *et al*. (1992).

## Results

### Cross-functional analysis of *Microviridae* internal scaffolding proteins

The cloned øX174, G4, and α3 B genes were assayed for the ability to complement various *am(B)* mutants in both plating and burst assays at 33 °C. The results of these experiments are in Table 2. With one exception (see below), the various *am(B)* mutants plate with comparable efficiencies regardless of the origin of the inner scaffolding protein, indicating that the B proteins can cross-complement despite divergence as large as 70% at the amino acid level. Although efficiencies were comparable, plaque morphologies varied. Plaques formed on hosts harboring a clone of the indigenous gene were generally larger than plaques formed *via* cross-complementation. Therefore to determine (1) a quantitative measure of cross-complementation and (2) if variations in plaque morphologies were significant, burst experiments were also performed.

For burst experiments, BAF30 (*recA*) cells harboring various B clones were grown to a concentration of $1.0 \times 10^8$ cells/ml and infected with *am(B)* mutants at an m.o.i. (multiplicity of infection) of 5.0. In these assays, burst sizes produced in cells harboring a clone of the indigenous gene and in the *sup*° host (C122) serve as standards by which the efficiency of cross-complementation can be assessed. In general, complementation by the indigenous gene appears to be more efficient than cross-complementation, producing larger bursts. For example, α3 *am(B)Q18* produced a burst of 63 ø/cell in BAF30 pα3B, but bursts of only 16.3 and 15.5 ø/cell in BAF30 pøXB and BAF30 pG4B, respectively. øX174 *am(B)Q18* yielded a burst of 24.8 ø/cell when complemented by its own B gene,

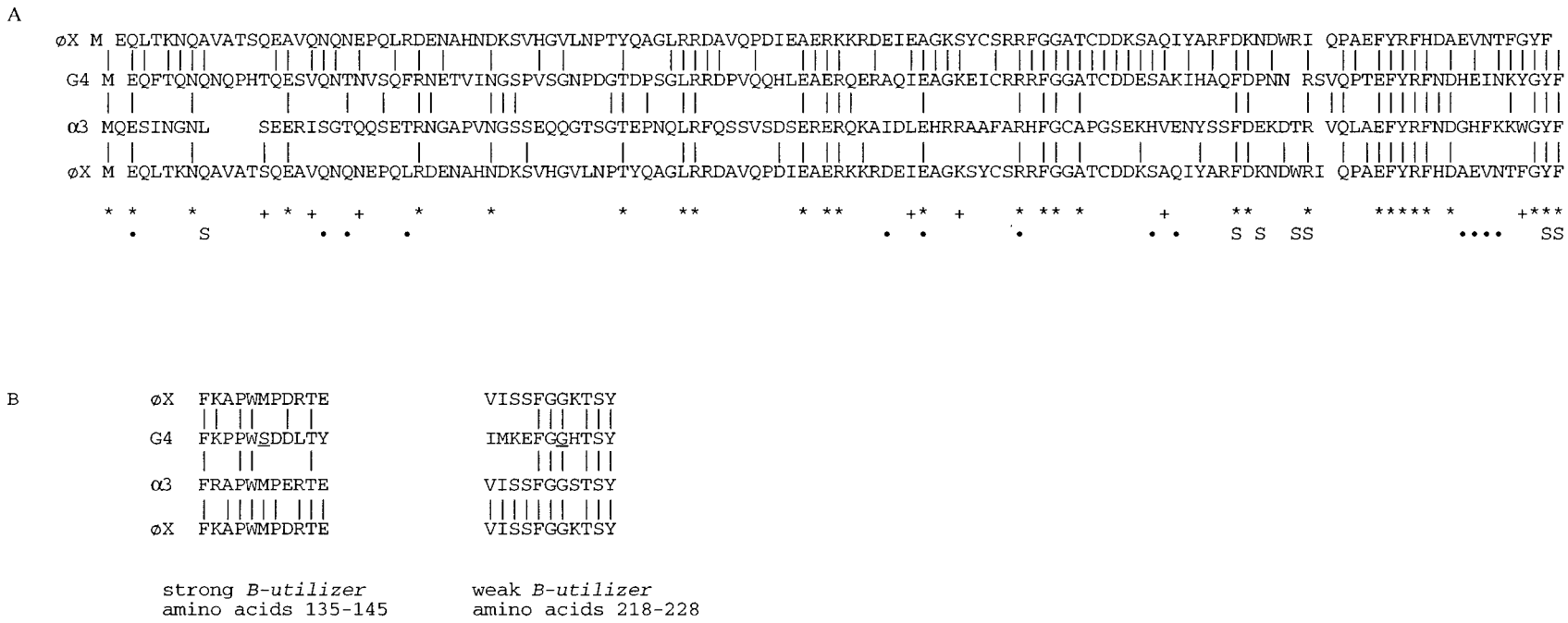


**Figure 2.** (a) Sequence alignment of the øX174, G4 and α3 B proteins. The øX174 ,G4 and α3 sequences were taken from Sanger *et al*. (1978), Godson *et al*. (1978) and Kodaira *et al*. (1992), respectively. Strictly conserved residues in all three proteins are marked by an asterisk. The plus (+) symbol represents a conservative variation in the amino acid. Circles indicate those residues that have been shown to tolerate variation by mutational analyses in øX174. Citations for each position are as follows: Fane & Hayashi (1991) for positions 18 and 20; Dalphin *et al*. (1992) for positions 68 and 76; Bull *et al*. (1997) for positions 64, 112, 114, 115; Weisbeek (1986) for positions 2, 68, 112, 113; W. D. Krill (personal communication) for position 89; B. A. Fane (unpublished results) for positions 25 and 87. The capital letter S denotes amino acids which interact with the viral coat protein in the atomic structure of the øX174 procapsid (Dokland *et al*., 1997). (b) Local sequence alignment of the viral coat proteins in the regions of the *B*-utilizer mutations. The amino acids altered by the *B*-utilizer mutations are underlined.

**Table 2.** Plating and burst experiments

| Mutant | E.O.P.[a] on BAF 30 with: | | | | Burst[b] in BAF 30 with: | | | |
|---|---|---|---|---|---|---|---|---|
| | pøXB | pG4B | pα3B | C122 (*sup°*) | pøXB | pG4B | pα3B | C122 (*sup°*) |
| øX *am*(*B*) | 1.0 | 1.5 | 0.4 | $10^{-6}$ | 24.8 | 19.2 | 6.8 | 0.02 |
| G4 *am*(*B*) | $10^{-7}$ | 1.0 | 0.6 | $10^{-7}$ | 0.09 | 150 | 93 | 0.01 |
| α3 *am*(*B*) | 0.7 | 0.9 | 1.0 | $10^{-4}$ | 16.3 | 15.5 | 63 | 0.3 |

[a] Efficiency of plating. Assay titer/titer on BAF5 (*supE*).
[b] Progeny virion/infected cell.

but only 6.8 ø/cell when complemented by the α3 gene. This preference for the indigenous scaffolding protein, however, was not always observed. Complementation of the øX174 mutant by the G4 and øX174 genes were comparable, 24.8 ø/cell *versus* 19.2 ø/cell.

There was one instance in which cross-complementation was not observed. The cloned øX174 B gene is unable to complement the G4 *am(B)Q18* mutant at temperatures between 33 and 42 °C. In burst experiments performed at 33 °C, the burst produced by the G4 mutant in BAF30 pøXB was only 0.09 ø/cell, which did not significantly differ from that obtained in the *sup°* host (0.01 ø/cell). These low bursts reflect the titers of unabsorbed virions determined ten minutes post infection. To determine whether the øX174 B protein was capable of inhibiting G4 morphogenesis, burst experiments were performed with wild-type G4 in the presence and absence of the inducer IPTG. Induction of the cloned øX174 B gene had no effect on yields, 165 ø/cell *versus* 173 ø/cell, respectively. These results indicate that the øX174 B protein either does not interact with G4 structural proteins or interacts productively if the indigenous protein is present. Plating assays were conducted with wild-type G4 at all temperatures. Expression of the foreign scaffolding appears to have no effect on wild-type plating efficiency. Failure to cross-complement, however, was found to be temperature-dependent. In plating assays conducted at 21 °C, the G4 *am(B)Q18* mutant plated with equal efficiencies on BAF30 pG4B and BAF30 pøXB. Burst experiments were also conducted at this temperature. Again complementation by the indigenous gene (40 ø/cell ) appears to be more efficient than cross-complementation (13 ø/cell). Failure to cross-complement, however, was found to be temperature-dependent. In plating assays conducted at 21 °C, the G4 *am(B)Q18* mutant plated with equal efficiencies on BAF30 pG4B and BAF30 pøXB. Burst experiments were also conducted at this temperature. Again complementation by the indigenous gene (40 ø/cell ) appears to be more efficient than cross-complementation (13 ø/cell). Low temperature complementation may reflect an overall change in scaffolding protein flexibility which enhances the coat-scaffold or two-fold scaffold-scaffold interactions. However, the experiments in this paper do not directly address this supposition.

### *In vivo* analysis of the *G4 am(B)Q18* morphogenetic pathway in the presence of the øX174 B protein

To further investigate the inability of the øX174 B protein to direct G4 morphogenesis, cells harboring clones of either the G4 or øX174 B genes were infected with G4 *am(B)Q18/am(E)W4* or wild-type G4. The *am(E)W4* mutation prevents cell lysis but does not affect virion morphogenesis. Radioactive extracts were prepared as described by Ekechukwu *et al*. (1995) and analyzed by sucrose gradient sedimentation. The sedimentation profiles from these experiments are presented in Figure 3. In extracts prepared from BAF30 pøXB cells infected with G4 *am(B)Q18/am(E)W4*, no large assembly intermediates or virions (>70 S) were detected (Figure 3(a): □). Large particles, however, were present in extracts made from BAF30 pG4B infected cells infected with this mutant (Figure 3(a): ○). The infectivity of each fraction was determined by titering. Infectivity co-sediments with the large peak at 114 S, indicating the presence of virions.

The sedimentation profiles of smaller assembly intermediates (<20 S) are presented in Figure 3(b). In extracts prepared from infected BAF30 pøXB and BAF30 pG4B, counts sediment at both 9 S and 6 S, the respective position of coat and spike protein pentamers (Tonegawa & Hayashi, 1970). Presumably, the counts sedimenting at these positions in the profiles reflect the presence of these particles. In the permissive infection (BAF30 pG4B), counts sediment at 12 S and 18 S. Formation of both particles is dependent on productive internal scaffolding-coat protein interactions (Tonegawa & Hayashi, 1970; Ekechukwu & Fane, 1995; B.A.F., unpublished results). These particles appear to be absent in the extracts prepared from BAF30 pøXB infected cells, suggesting that the øX174 B protein is incapable of mediating the earliest B-dependent steps in morphogenesis. The 12 S particle is composed of five copies each of the coat and spike proteins (Tonegawa & Hayashi, 1970). The 18 S particle is composed of a coat pentamer, a spike pentamer and, depending on isolation conditions, B and D proteins as well (B.A.F., unpublished results). Because of particle instability and the inability to trap large quantities of this particle by genetic mutation, the exact nature of the 18 S particle remains obscure. However, the atomic structure of the øX174 procapsid suggests that the 18 S

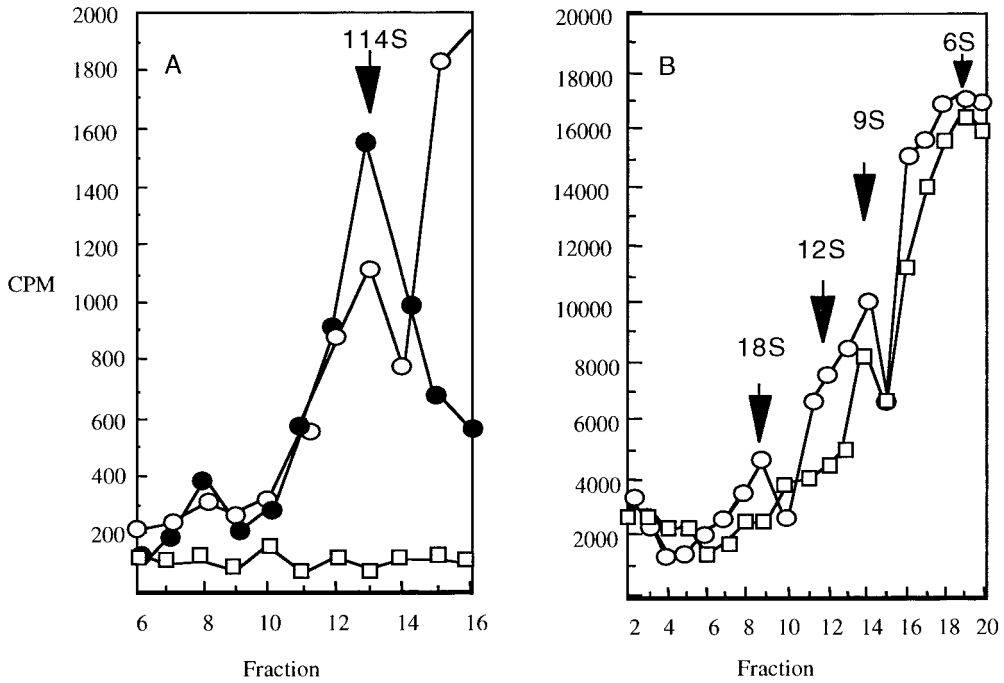


**Figure 3.** Characterization of the G4 *amB* and wt G4 morphogenetic pathways in the presence of the øX174 B protein. (a) High molecular mass particles. (b) Low molecular mass particles. (○) G4 *amB* infected cells harboring a clone of the G4 B gene. (●) wt G4 infected cells harboring a clone the øX174 B gene. (□) G4 *amB* infected cells harboring a clone of the øX174 B gene.

particle may be more representative of a true assembly intermediate than the 12 S particle, which may result from the degradation of the 18 S entity.

## Second-site reversion analysis

To isolate G4 mutants that have gained the ability to use the øX174 B protein (G4 *øXB*-utilizers), a second-site genetic analysis was conducted. G4 *am(B)Q18* was plated on BAF30 pøXB at 33 °C or 37 °C. Three types of reversion events were anticipated: (1) same-site events ($am \rightarrow am^+$), (2) extragenic events allowing the use of either the øX174 or G4 proteins (*øXB*-utilizers), and (3) extragenic events permitting only the use of the øX174 protein (*øXB*-demanders). To distinguish between these three events, revertant plaques were stabbed into three indicator lawns: C122 (*sup°*), BAF5 (*supE*), and BAF30 pøXB. *øXB*-utilizers would maintain the amber phenotype, failing to grow on C122 (*sup°*), but propagating on BAF5 (*supE*) and BAF30 pøXB. *øXB*-demanders, however, would only propagate on BAF30 pøXB. The frequencies of $am^+$ and *B-utilizers* revertants were $6.0 \times 10^{-6}$ and $4.0 \times 10^{-6}$, respectively. No *øXB*-demanders were recovered.

Based on plaque morphologies, two distinct phenotypes were distinguished: strong and weak. DNA was extracted and the coat protein gene was sequenced from two isolates of each phenotype and the parental strain. Both strong-*øXB*-utilizer mutations conferred serine → phenylalanine substitutions at position 140. The two weak-*øXB*-utilizer mutations conferred glycine → arginine substitutions at position 223. A mutagenic oligonucleotide designed to introduce the strong *B*-utilizer mutation into the *am(B)Q18* background and a non-mutagenic primer were used in rescue experiments to verify the identity of the strong mutation. After *in vitro* DNA synthesis, mutagenized and unmutagenized DNA was transfected into BAF5 (*supE*). A total of 200 plaques from each transfection were stabbed into indicator lawns seeded with C122 (*sup°*), BAF5 (*supE*), and BAF30 pøXB. Rescue was defined by two criteria: retention of the *am* phenotype and the ability to propagate in BAF30 pøXB. From the transfections with mutagenized DNA, 7.0 % rescue (14/200) was observed, whereas, less than 0.5 % rescue (0/200) was obtained from the control transfection.

To further characterize the øXB-utilizer mutations, additional burst experiments were performed. As can be seen in Table 3, the øX174 B protein can mediate the assembly of the mutant G4 coat proteins. Relative burst sizes, furthermore, are consistent with plating phenotypes. The strong *øXB*-utilizer mutant produced a significantly higher burst in BAF30 pøXB (58.6 ø/cell) than the weak *B*-utilizer (3.0 ø/cell). Although small, the burst produced by the weak *B*-utilizer mutant is significantly higher than the burst produced by the parental mutant (0.09 ø/cell), and reflects a titer fivefold higher than unabsorbed phage determined ten minutes post infection. The *B*-utilizer mutants also differ in their ability to utilize the α3 protein. While the parental mutant produced a burst of

**Table 3.** Cross-complementation of G4 *øXB*-utilizer mutants

| Mutant | Bursts in BAF 30 with: pøXB | pG4B | pα3B | C122 (*sup°*) |
|---|---|---|---|---|
| G4 *am(B)* | 0.09 | 150 | 93 | 0.01 |
| *am(B)/øXB-ut-S* | 58.6 | 136 | 158 | 0.06 |
| *am(B)/øXB-ut-W* | 3.0 | 83.6 | 3.4 | 0.13 |

93 ø/cell in BAF30 pα3B, the weak *øXB*-utilizer produced a burst of only 3.4 ø/cell.

## Discussion

### Cross-functional analyses of internal scaffolding proteins

Cross-species complementation and genetic reassortment studies have been used to demonstrate functional homology between viral proteins (Mettenleiter & Spear, 1994; Lamb & Krug, 1996). While these assays are ideal for proteins that interact minimally with other viral proteins, these techniques may not be applicable to proteins that interact in multimeric complexes. The genetic characterization of viable hybrid viral genomes, for example, suggests that genes encoding interacting proteins are concurrently transferred during recombination (Botstein, 1980). Scaffolding proteins, however, may be a notable exception. Despite vast divergence in primary structure, the *Microviridae* internal scaffolding proteins can direct the assembly of foreign coat proteins. There is, however, a preference for the indigenous scaffold. A similar phenomenon has also been documented with the scaffolding proteins of various *Herpesviruses* (Haanes *et al*., 1995; Preston *et al*., 1997).

Our results differ from a previously published report (Kodaira *et al*. , 1992), which suggests that the *Microviridae* internal scaffolding proteins are not cross-functional. This disparity can best be explained by examining the methodologies used in the two studies. Previously, the ability to cross-function was assayed in co-infections with viruses of different species. In co-infected cells, cross-complementation could have been masked by a vast range of non-productive, inter-species protein-protein interactions. Plasmid-based complementation experiments circumvent this problem and may represent a more specific assay of a scaffolding protein's ability to cross-function.

Having now been documented in two diverse viral families (Haanes *et al*., 1995; Preston *et al*., 1997), the cross-function phenomenon suggests that internal scaffolding proteins, in general, may be inherently flexible or undergo substrate-directed folding. While inherent flexibility invokes non-specific mechanisms, specific interactions are also occurring, most notably in the COOH terminus. The VZV and HSV-1 scaffolding proteins cross-function only when the COOH terminal regions of the proteins are interchanged. The COOH termini of the *Microviridae* B proteins are more strongly conserved than the rest of the protein. In addition, the atomic structure of the øX174 procapsid reveals that the majority of the amino acids known to interact with the viral coat protein are in this region. However, the $NH_2$ terminus probably interacts with both the coat protein and $NH_2$ terminus of a 2-fold related B protein *via* non-specific interactions as seen with molecular chaperones, which interact with substrates *via* non-specific mechanisms and most likely have flexible domains (Ellis & van der Vies, 1991; Horwich, 1995; Hartl & Martin, 1995). Considering the dynamics of viral assembly, some inherent flexibility is probably required. Internal scaffolding proteins must first assume a structure that directs the assembly of pentameric intermediates into a rigid capsid. Afterwards, these proteins must assume an alternate structure, one that allows for extrusion from an internal location. In P22 and øX174, this structure must be compact enough to exit through 20-30 Å pores (Ilag *et al*., 1995; Prasad *et al*., 1993). The atomic structure of the øX174 procapsid, furthermore, demonstrates that the external scaffolding (protein D) can assume at least three different conformations.

### Evolution of viral genome structure

Overlapping reading frames increase the amount of genetic information encoded in small viral genomes (Sanger *et al*., 1978; Kodaira *et al*., 1992; Godson *et al*., 1978; Sonigo *et al*., 1985; Griffin & Ito, 1993; Kobayashi & Koike, 1984). Gene B resides entirely within the reading frame of gene A and A*, and overlaps with gene K. While both the A* and K proteins are unessential for viral propagation (Colasanti & Denhardt, 1987; Tessman *et al*., 1980), the A protein plays a critical role in genome replication and must interact with many host cell proteins. øX174 replication, for example, is sensitive to host cell *rep* alleles (Tessman & Peterson, 1976). Host-range interactions between these two genes have been documented (Ekechukwu *et al*., 1995). In addition, mutations in gene A may play a critical role in adapting øX174 to propagate in *Samonella typhimurium* (Bull *et al*., 1997). The unessential A* and K genes are unlikely to hinder the ability of gene A to co-evolve with its host. Furthermore it is unlikely that the essential B gene will constrain the evolution of gene A; the results of the cross-functional analysis demonstrates that these proteins are inherently tolerant of extensive amino acid variations.

### Structure-function analysis of the *Microviridae* B proteins

Sequence comparisons of proteins with homologous structures and genetic analyses indicate that many regions within a protein can tolerate considerable variation without affecting function

(Miller *et al.*, 1979; Dikerson & Geis, 1983; Krebs *et al.*, 1983; Bashford *et al.*, 1987; Fane & King, 1987; McKenna *et al.*, 1992, 1996; Chapmann & Rossmann, 1993; Jennings & Fane, 1997). This is most likely the case for the *Microviridae* B proteins as well. These methodologies in combination with structural information and cross-functional analyses can be used to further understand protein-protein interactions within large multimeric complexes, such as viral procapsids.

As can be seen in Figure 2(a), the residues in the COOH termini are the most strongly conserved between the sequences. In addition, the vast majority of amino acids known to interact with the viral coat protein are in this region as well. This highly aromatic terminus fits into a cleft formed between α-helix 2 and the β-barrel of the coat protein (McKenna *et al.*, 1996; Dokland *et al.*, 1997). The strong-utilizer mutation described here confers a Ser → Phe substitution in this cleft (Figure 4) and most likely reflects the importance of these contacts and the involvement of aromatic amino acids. Tolerated substitutions have been documented in this region of the protein. Interestingly, they cluster in an unconserved area (Bull *et al.*, 1997; Weisbeek, 1986). If the conserved amino acid residues are critical for function, mutations at these sites may not have been recovered by classical mutagenesis. An amino acid sequence comparison of the viral coat proteins in the regions identified by the *B*-utilizer mutations may offer an explanation for the ability of the two most divergent B proteins (α3 and øX174) to cross-function. As depicted in Figure 2(b), these regions within the α3 and øX174 coat proteins are more highly conserved than the sequences found within the G4 coat protein. Sequence comparisons, however, do not fully explain all of the cross-functional data. Primarily, they offer no insights into the inability of the øX174 B protein to complement the G4 mutant while the more divergent α3 protein can cross-complement. Elucidating the atomic structures of the G4, α3, and hybrid procapsids containing foreign scaffolding proteins may be required to more fully understand the cross-functional phenomenon.

It is possible that the viral coat protein plays a critical role in determining the conformation of the internal scaffolding. The *Microviridae* coat proteins exhibit much less amino acid sequence divergence. The r.m.s. deviation between the $C^{\alpha}$ backbones of the øX174 and G4 coat proteins is only 0.36 Å (McKenna *et al.*, 1996). Initially, the internal scaffolding protein might interact with a coat protein in one specific region, perhaps in the above-mentioned cleft. Afterwards, the scaffolding adapts its structure to the coat protein. In the øX174 procapsid structure, the $NH_2$ terminus of the B protein, which resides along the 2-fold axis of symmetry, is disordered. Considering the high degree of sequence variation in the amino termini, it is unlikely that coat-scaffolding interactions in this region are governed primarily by specific side-chains. The sequence variation, cross-functional phenomenon and unordered density suggest that contacts made by this part of the protein are more variable than those found at the COOH terminus, and perhaps not as crucial. The weak *B*-utilizer substitution resides in close proximity to this disordered density and may explain the comparatively weak phenotype.

The atomic structure of the viral procapsid suggests that the B protein may have two domains: the COOH terminus, which interacts more extensively and specifically with the viral coat protein and the $NH_2$ terminus, which may primarily interact with other B proteins across the 2-fold axis of symmetry. However, the existence of *B*-utilizer mutations in two regions of the coat protein, contacting opposite ends of the internal scaffold, suggests that the two termini do not function as totally separate and independent domains. Altering the contacts made by one end of the protein can affect those made by the other. Recent experiments conducted with a chimeric B protein support this assertion. This chimeric protein is comprised of the $NH_2$ terminus of G4 and the COOH terminus of øX174. The chimeric scaffolding protein efficiently supports øX174 morphogenesis, but fails to direct the assembly of wild-type G4, the *B*-utilizer strains or α3 (unpublished data).

## Materials and Methods

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

The reagents, media, buffers, and protocols are described by Fane & Hayashi (1991).

### Bacterial strains

The *Escherichia coli* C strains C122 (*sup*°) and BAF5 (*supE*) are described by Fane & Hayashi (1991). BAF30 is a *recA* derivative of C122 (Fane *et al.*, 1992).

### Phage mutants

øX174 *am (B)Q18* has been described (Fane & Hayashi, 1991). The G4 *am (B)Q18* and α3 *am(B)Q18* mutants were generated by oligonucleotide-mediated mutagenesis as described by Fane *et al.* (1993). Mutagenized DNA was transfected into BAF5 (*supE*). Progeny were stabbed into two indicator lawns: C122 (*sup*°) and BAF5 (*supE*). Putative amber mutants were identified by the inability to propagate on the *sup*° host. To construct the G4 *am(B)Q18/am(E)W4* double mutant, the *am(E)W4* mutation was introduced in the *am(B)Q18* background. After transfection into BAF5 (*supE*), mutagenized progeny were stabbed into three indicator lawns: BAF30 pG4B, BAF5 (*supE*), and C122(*sup*°). Putative double mutants were identified by the inability to propagate on BAF30 pG4B and C122 (*sup*°). In order to clone the α3 B gene (see below), an α3 *am(A) Sac*$I^-$/*Xba*$I^+$ mutant was generated by two rounds of site-directed mutagenesis. Beginning with α3 *am(B)Q18* DNA, the first round of mutagenesis concurrently eliminated the amber mutation and an internal *Sac*I site. The second round of mutagen-

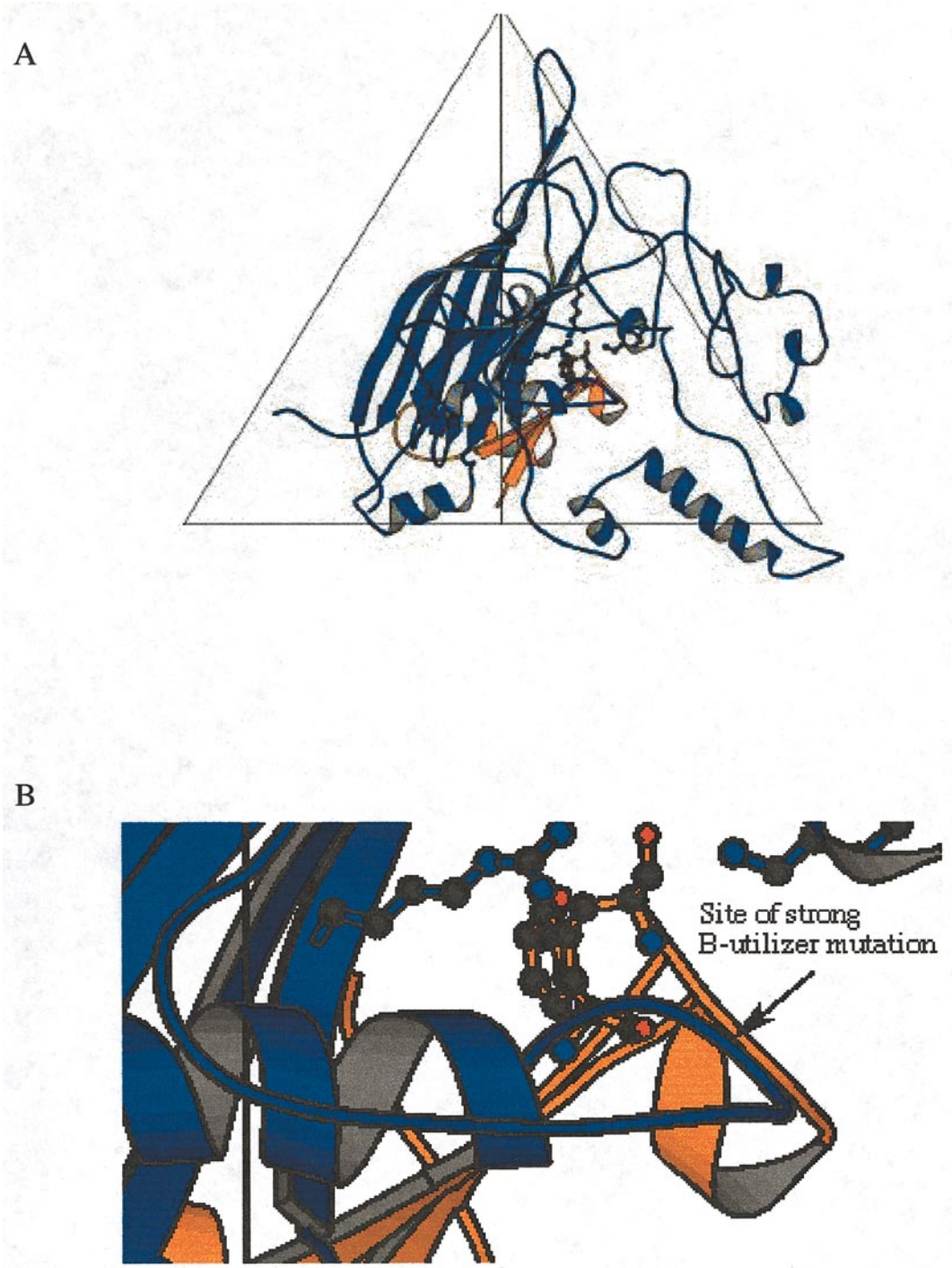


**Figure 4.** (a) The øX174 viral coat and internal scaffolding protein. (b) The internal scaffolding protein binding cleft. The coat protein is depicted in blue, the internal scaffolding in orange. Figure courtesy of Dr T. Dokland and Dr M. G. Rossmann.

esis concurrently created an amber mutation in codon 449 of gene A and created an *Xba*I site immediately before gene B. The genotypes of all new strains were verified by a DNA sequence analysis.

### Cloning the *Microviridae* B genes

The G4 B gene was cloned into pSE420 by digesting both plasmid and RF (replicative form) DNA with *Avr*II and *Pst*I. The øX174 B gene was subcloned out of a pBR322 derivative (Hayashi & Hayashi, 1985) and into pSE420 using *Eco*RI and *Bam*HI. The α3 B gene was cloned by digesting α3 *am(A)* *Sac*I$^{-}$/*Xba*I$^{+}$ RF DNA and pSE420 DNA with *Xba*I and *Sac*I. Standard conditions were used for digestion and ligation reactions (Sambrook *et al.*, 1985). Clones were verified by restriction digestion and the ability to complement *am(B)* mutants. All clones are under lactose induction.

### Generation of RF DNA

RF DNA was produced as described by Fujisawa & Hayashi (1976) with the following variations that have been found to increase yields. A 100 ml of cells was grown in TKG media (1.0 % (w/v) tryptone, 0.5 % (w/v) KCl, 0.5 % (v/v) glycerol) to a concentration of $5 \times 10^8$ cells/ml and harvested by centrifugation. The pellet was washed twice with HFB buffer and resuspended in 10.0 ml of HFB with 10 mM $MgCl_2$/5.0 mM $CaCl_2$. Phage were pre-absorbed (m.o.i. = 5) for 20 minutes at 37 °C. At 0 minutes, the mixtures were poured into 100 ml of TKG media, pre-warmed to 30 °C. At 8.5 minutes, chloramphenicol was added to 20 μg/ml. At 90 minutes, the cells were harvested and washed with 15 ml of 10 mM Tris (pH 8), 100 mM NaCl, 1.0 mM EDTA. Timing of chloramphenicol treatment and temperature are critical for optimal yields. RF DNA was then extracted as described by Hayashi & Hayashi (1985).

### Isolation of G4 *øXB*-utilizers

To isolate G4 mutants that could productively utilize the øX174 B protein during assembly (G4 *øXB*-utilizers), $1.0 \times 10^8$ plaque-forming units of G4 *am(B)Q18* were plated on BAF30 pøXB and incubated at 33 °C. Revertant plaques were stabbed into two indicator lawns: C122 (*sup°*) and BAF 5 (*supE*). Putative mutants were identified by the retention of the amber phenotype and B gene complementation dependent growth. The retention of the parental amber mutation was also verified by a direct sequence analysis.

### Preparation of radioactive lysates and sucrose gradient sedimentation analyses

The generation of radioactive lysates and centrifugation conditions were described by Ekechukwu *et al.* (1995), except [$^{35}$S]methionine and cysteine were used to label.

## Acknowledgments

We thank Dr M. G. Rossmann, Dr G. Mosig and S. Hafenstein, for discussion, Dr M. G. Rossmann and Dr T. Dokland for Figure 4, W. D. Krill for communicating unpublished results and Mrs Mavis Carroll for technical support. J.T. was supported, in part, by a undergraduate research fellowship from the American Society for Microbiology. This work was supported by a National Science Foundation grant to B.A.F.

## References

Bashford, D., Chothia, C. & Lesk, A. M. (1987). Determinants of a protein fold: unique features of the globin amino acid sequences. *J. Mol. Biol.* **196**, 199-216.

Botstein, D. (1980). A theory of modular evolution for bacteriophages. *Ann. NY Acad. Sci.* **354**, 484-490.

Bull, J. J., Badgett, M. R., Wichman, H. A., Hueselsenbeck, J. P., Hillis, D. M., Gulati, A., Ho, C. & Molineux, I. J. (1997). Exceptional evolution in a virus. *Genetics,* **147**, 1497-1507.

Chapman, M. S. & Rossmann, M. G. (1993). Structure, sequence and function correlations among parvoviruses. *Virology,* **194**, 491-508.

Colasanti, J. & Denhardt, D. T. (1987). Mechanism of replication of bacteriophage øX174. XXII. Site-specific mutagenesis of the A* gene reveals that A* protein is not essential for øX174 DNA replication. *J. Mol. Biol.* **197**, 47-54.

Dalphin, M. E., Fane, B. A., Skidmore, M. O. & Hayashi, M. (1992). Proteolysis of the bacteriophage øX174 prohead accessory protein gpB by *Escherichia coli* ompT protease is not essential for phage maturation *in vivo*. *J. Bacteriol.* **174**, 2402-2406.

Dikerson, R. E. & Geis, I. (1983). *Hemoglobin: Structure, Function, Evolution and Pathology*. Benjamin Cummings, Menlo Park, CA.

Dokland, T., McKenna, R., Ilag, L. L., Bowen, B. R., Incardona, N. L., Fane, B. A. & Rossmann, M. G. (1997). Structure of a viral assembly intermediate with molecular scaffolding. *Nature,* **389**, 308-313.

Ekechukwu, M. C. & Fane, B. A. (1995). Characterization of the morphogenetic defects conferred by cold-sensitive prohead accessory and scaffolding proteins of øX174. *J. Bacteriol.* **177**, 829-830.

Ekechukwu, M. C., Oberste, D. J. & Fane, B. A. (1995). Host and øX174 mutations which affect the morphogenesis or stabilization of the 50S complex, a single stranded DNA synthesizing intermediate. *Genetics,* **140**, 1167-1174.

Ellis, R. J. & van der Vies, S. M. (1991) Molecular chaperones. *Annu. Rev. Biochem.* **60**, 321-347.

Fane, B. A. & Hayashi, M. (1991). Second-site suppressors of a cold-sensitive prohead accessory protein of bacteriophage øX174. *Genetics,* **128**, 663-671.

Fane, B. A. & King, J. (1987). Identification of sites influencing the folding and subunit assembly of the P22 Tailspike polypeptide chain using nonsense mutations. *Genetics,* **117**, 157-171.

Fane, B. A., Head, S. & Hayashi, M. (1992). The functional relationship between the J proteins of bacteriophages øX174 and G4 during phage morphogenesis. *J. Bacteriol.* **174**, 2717-2719.

Fane, B. A., Shien, S. & Hayashi, M. (1993). Second-site suppressors of a cold sensitive external scaffolding protein of bacteriophage øX174. *Genetics,* **134**, 1003-1011.

Fujisawa, H. & Hayashi, M. (1976). Viral DNA-synthesizing intermediate complex isolated during assembly of bacteriophage øX174. *J. Virol.* **19**, 409-415.

Godson, G. N., Barrell, B. G., Standen, R. & Fiddes, J. C. (1978). Nucleotide sequence of bacteriophage G4 DNA. *Nature,* **276**, 236-247.

Griffin, B. E. & Ito, Y. (1993). DNA viruses; polyoma. In *Genetic Maps: Locus Maps of Complex Genomes* (O'Brien, S. J., ed.), pp. 105-1.113, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Haanes, E. J., Thomsen, D. R., Martin, S., Homa, F. L. & Lowery, D. E. (1995). The bovine herpesvirus 1 maturational proteinase and scaffold proteins can substitute for the homologous herpes simplex virus type 1 proteins in the formation of hybrid type B capsids. *J. Virol.* **69**, 7375-7379.

Hartl, F. U. & Martin, J. (1995). Molecular chaperones in cellular protein folding. *Curr. Opin. Struct. Biol.* **5**, 92-102.

Hayashi, M. & Hayashi, M. N. (1985). Cloned DNA sequences that determine mRNA stability of bacteriophage øX174 *in vivo* are functional. *Nucl. Acids Res.* **13**, 5937-5942.

Hayashi, M., Aoyama, A., Richardson, D. L. & Hayashi, M. N. (1988). Biology of the bacteriophage øX174. In *The Bacteriophages* (Calendar, R., ed.), vol. 2, pp. 1-71, Plenum Publishing Corporation, New York, NY.

Horwich, A. L. (1995). Molecular chaperones: resurrection or destruction? *Curr. Biol.* **5**, 455-458.

Ilag, L. L., Olson, N. H., Dokland, T., Music, C. L., Cheng, R. H., Bowen, Z., McKenna, R., Rossmann, M. G., Baker, T. S. & Incardonna, N. L. (1995). DNA packaging intermediates of bacteriophage ϕX174. *Structure,* **3**, 353-363.

Jennings, B. & Fane, B. A. (1997). Genetic analysis of the øX174 DNA binding protein. *Virology,* **227**, 370-377.

King, J. & Casjens, S. (1974). Catalytic head assembling protein in virus morphogenesis. *Nature,* **251**, 112-119.

Kobayashi, M. & Koike, K. (1984). Complete nucleotide sequence of hepatitis B virus DNA of subtype and its conserved gene organization. *Gene,* **30**, 227-232.

Kodaira, K., Nakano, K., Okada, S. & Taketo, A. (1992). Nucleotide sequence of the genome of bacteriophage α3: interrelationship of the genome structure and the gene products with those of the phages øX174, G4 and øK. *Biochem. Biophys. Acta,* **1130**, 277-288.

Krebs, H., Schmid, F. X. & Jaenicke, R. (1983). Folding of homologous proteins. The refolding of different ribonucleases is independent of sequence variation, proline content and glycosylation. *J. Mol. Biol.* **169**, 619-635.

Lamb, R. A. & Krug, R. M. (1996). *Orthomyxoviridae:* the viruses and their replication. In *Fundamental Virology* (Fields, B. N., Knipe, D. M. & Howley, P. M., eds), pp. 605-647, Lippincott-Raven Publishers, Philadeplhia, PA.

Leibowitz, J. & Horowitz, M. S. (1975). Synthesis and assembly of adenovirus polypeptides. III. Reversible inhibition of hexon assembly in adenovirus type 5 temperature-sensitive mutants. *Virology,* **66**, 10-24.

McKenna, R., Xia Willingmann, P., Ilag, L. L., Krishnaswamy, S., Rossmann, M. G., Olson, N. H., Baker, T. S. & Incardonna, N. L. (1992). Atomic structure of single-stranded DNA bacteriophage øX174 and its functional implications. *Nature,* **355**, 137-143.

McKenna, R., Ilag, L. L. & Rossmann, M. G. (1994). Analysis of the single-stranded DNA bacteriophage øX174 at a resolution of 3.0 Å. *J. Mol. Biol.* **237**, 517-543.

McKenna, R., Bowen, B. R., Ilag, L. L., Rossmann, M. G. & Fane, B. A. (1996). The atomic structure of the degraded procapsid particle of bacteriophage G4: induced structural changes in the presence of calcium ions and functional implications. *J. Mol. Biol.* **256**, 736-750.

Mettenleiter, T. C. & Spear, P. G. (1994). Glycoprotein gB (gII) of pseudorabies virus can functionally substitute for glycoprotein gB in herpes simplex virus type 1. *J. Virol.* **68**, 500-504.

Miller, J. H., Coulondre, C., Hofer, M., Schmeissner, U., Sommer, H., Schmits, A. & Lu, P. (1979). Genetic studies of the *lac* repressor. IX. Generation of altered proteins by the generation of nonsense mutations. *J. Mol. Biol.* **131**, 191-222.

Prasad, B. V. V., Prevelige, P. E., Marietta, E., Chen, R. O., Thomas, D., King, J. & Chui, W. (1993). Three-dimensional transformation of capsids associated with genome packaging in a bacterial virus. *J. Mol. Biol.* **231**, 65-74.

Preston, V. G., Kennard, J., Rixon, F. J., Logan, A. J., Mansfield, R. W. & McDougall, I. M. (1997). Efficient herpes simplex virus type 1 (HSV-1) capsid formation directed by the varicella-zoster virus scaffolding protein requires the carboxy-terminal sequences from the HSV-1 homologue. *J. Gen. Virol.* **78**, 1633-1646.

Rixon, F. J. (1993). Structure and assembly of herpes viruses. *Semin. Virol.* **4**, 135-144.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., Coulson, A. R., Friedmann, C. T., Air, G. M., Barrell, B. G., Brown, N. L., Fiddes, J. C., Hutchison, C. A., III, Slocombe, P. M. & Smith, M. (1978). The nucleotide sequence of bacteriophage øX174. *J. Mol. Biol.* **125**, 225-246.

Siden, E. J. & Hayashi, M. (1974). Role of gene *B* product in bacteriophage øX174 development. *J. Mol. Biol.* **89**, 1-16.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, O., Retzel, E., Tiollais, P., Haase, A. & Wain-Hobson, S. (1985). Nucleotide sequence of the Visna lentivirus: relationship to the AIDS virus. *Cell,* **42**, 369-382.

Tessman, E. S. & Peterson, S. K. (1976). Bacterial *rep*$^-$ mutations that block development of small DNA bacteriophages late in infection. *J. Virol.* **20**, 400-412.

Tessman, E. S., Tessman, I. & Pollock, T. J. (1980). Gene *K* of bacteriophage phi X174 codes for a nonessential protein. *J. Virol.* **33**, 557-560.

Tonegawa, S. & Hayashi, M. (1970). Intermediates in the assembly of øX174. *J. Mol. Biol.* **48**, 219-242.

Weisbeek, P. (1986). Genetic and restriction map of bacteriophage øX174. DNA viruses. In *Genetic Maps: Locus Maps of Complex Genomes* (O'Brien, S. J., ed.), pp. 1.79-1.83, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

***Edited by M. F. Moody***

*(Received 20 July 1998; received in revised form 25 November 1998; accepted 30 November 1998)*