Uncoupling the functions of a multifunctional protein: The isolation of a DNA pilot protein mutant that affects particle morphogenesis

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Defective øX174 H protein-mediated DNA piloting indirectly influences the entire viral lifecycle. Faulty piloting can mask the H protein's other functions or inefficient penetration may be used to explain defects in post-piloting phenomena. For example, optional synthesis of other viral proteins requires de novo H protein biosynthesis. As low protein concentrations affect morphogenesis, protein H's assembly functions remain obscure. An H protein mutant was isolated that allowed morphogenetic effects to be characterized independent of its other functions. The mutant protein aggregates assembly intermediates. Although excess internal scaffolding protein restores capsid assembly, the resulting mutant H protein-containing particles are less infectious. In addition, nonviable phenotypes of am(H) mutants in Su+ hosts, which insert non-wild-type amino acids, do not always correlate with a lack of missense protein function. Phenotypes are highly influenced by host and phage physiology. This phenomenon was unique to am(H) mutants, not observed with amber mutants in other genes.

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Introduction

While the øX174 H protein's role as a DNA piloting protein was defined over 30 years ago (Jazwinski et al., 1975a; Jazwinski et al., 1975c), a full array of its functions remains to be elucidated. At the onset of infection, protein H pilots incoming DNA to cell wall adhesion regions on the outer membrane (Azuma et al., 1980; Bayer and Starkey, 1972; Jazwinski et al., 1975b). This reaction is most likely mediated by a predicted N-terminal trans-membrane helix (Tusnady and Simon, 2001). However, this has yet to be experimentally demonstrated. From its position associated with the outer membrane, protein H and the single-stranded (ss) DNA genome is delivered to the cytoplasmic membrane, the site of DNA synthesis (Azuma et al., 1980). During stage I DNA synthesis, the infecting ssDNA is converted into a double stranded replicative form (RF) molecule. Due to the positive polarity of the genome, this occurs without de novo viral protein synthesis. Although the second stage of DNA synthesis, the amplification of the RF molecule, occurs after viral protein synthesis, de novo H protein synthesis is not required for this reaction (Ruboyianes et al., 2009).

Inefficient penetration will affect the entire viral lifecycle. Thus, piloting defects may have masked the identification of other functions in past genetic and biochemical analyses (Spindler and Hayashi, 1979) or inefficient penetration may have been used to explain defects in post-piloting phenomena, such as viral DNA or protein biosynthesis. As assembly requires critical concentrations of structural and scaffolding proteins, suboptimal viral protein levels could affect particle morphogenesis (Fane and Prevelige, 2003; Prevelige et al., 1988; Uchiyama et al., 2007). In order to evaluate post-DNA piloting functions, processes must be uncoupled. It was recently shown that the optimal synthesis of other viral proteins requires de novo H protein biosynthesis, a post-penetration and DNA replication phenomenon (Ruboyianes et al., 2009). Wild-type (Su+) cells infected with permissively synthesized amH virions display normal stage I and stage II DNA synthesis, but viral protein levels were dramatically reduced. Thus, to accurately determine whether a mutant H protein affects assembly, at least two H protein-mediated processes, DNA piloting and the stimulation of viral protein synthesis, cannot be affected or means to circumvent these functions must be employed.

During assembly, the internal scaffolding protein B facilitates the incorporation of one H protein into pentameric assembly intermediates (Chen et al., 2007; Cherwa et al., 2008; Novak and Fane, 2004). The association of 12 of these pentamers produces a T = 1 virion that should have one H protein at each vertex; however, icosahedral averaging obscured the protein's direct visualization in atomic structures (Dokland et al., 1999; Dokland et al., 1997; McKenna et al., 1996; McKenna et al., 1994; McKenna et al., 1992). In the absence of a bona fide structure, bioinformatic analyses have provided some structural insights (Fig. 1). The protein contains a predicted N-terminal trans-membrane helix (Tusnady and Simon, 2001), which most likely functions during penetration, and several C-terminal coiled-coil domains (Lupas, 1996). Although coiled-coil domains are known to mediate protein oligomerization (Alfadhl et al., 2002; Harbury et al., 1993; Li et al., 2001), H protein oligomers have yet to be observed during the øX174 lifecycle.
However, purified coiled-coil domains do form oligomers in solution (J. Nardozzi and G. Cingolani, personal communication). In a previous study, the coiled-coil domains were expressed in øX174 sensitive cells. Expression inhibited the piloting function of the wild-type H protein that enters the cell with the infecting genome (Ruboyianes et al., 2009). Overexpression of the internal scaffolding protein lessened the detrimental effects, which led to a hypothesis that protein B may moderate H protein oligomerization, perhaps keeping a subset of the protein in a monomeric state for assembly.

In this initial genetic analysis, preexisting am(H) strains were assayed for defective phenotypes in various informational suppressing (Su+) hosts that insert non-wild-type amino acids during protein synthesis. The results of the analyses indicate that am(H) phenotypes in the SupD host are not a direct function of the synthesized missense proteins, as they are in SupE and SupF strains, but are highly influenced by host cell and phage physiology. This appears to be a unique characteristic of am(H) mutants, and was not observed with amber mutations in the other genes. One amber mutant produced a lethal phenotype in the SupF host. The corresponding missense mutation was placed directly into the genome of each coiled-coil domain prediction (Lupas, 1996) for the wild-type protein is given. The probability amino acids predicted to be involved in secondary structure formation. The probability of each coiled-coil domain prediction (Lupas, 1996) for the wild-type protein is given atop the schematic, the probability prediction resulting from an E→Y substitution in the fourth domain is given below the schematic.

Results

The phenotype of am(H) mutants in SupD cells is primarily governed by general aspects of host cell and phage physiology, not the resulting missense protein

The plaque forming ability of am(H) mutant strains was examined on SupD, SupE, and SupF hosts, which respectively insert serine, glutamine and tyrosine during protein synthesis. All of the am(H) mutants formed plaques on the SupE cell line (Table 1), and with the exception of am(H)E258, which is discussed below, formed plaques on the SupF host (data not shown). In contrast, all of the am(H) mutants appear to be restricted on the SupD host at 30 °C (Table 1: column SupD(b)). However, burst sizes (phage/cell produced in a single round of infection) were also measured at the restrictive 30 °C temperature as described in Materials and methods. Progeny production was assayed by titration on the permissive SupE host at 37 °C. As can be seen in Table 2, the am(H) mutants produced bursts in the SupD host at 30 °C. Moreover, a serine codon was placed directly in the am(H)E258 genome at the site of the amber mutation. The phenotype of the resulting missense mutant was indistinguishable from wild-type in plating, attachment, assembly, and eclipse assays (data not shown).

While burst experiments utilize cells in early exponential phase, standard plaque assays employ overnight cell cultures. Cells enter exponential phase after plates are incubated. To determine whether SupD cells must be in exponential phase to suppress am(H) mutants at lower temperatures, plating assays were repeated using early exponential phase SupD cells. As can be seen in Table 1 (column SupDEXP), viability was restored. This phenomenon appears to be confined to SupD cells, the growth phase of SupE or SupF cells did not appear to affect am(H) plating efficiencies or plaque morphologies. Strains with amber mutations in the genes encoding the other virion and procapsid proteins were also assayed with stationary and early exponential phase SupD cells (Table 1). The growth phase of the plating cells affected neither plating efficiency nor plaque morphology. Thus, this phenomenon appears to be unique to am(H) mutants, even though it is the least abundant protein in virions and procapsids by a factor of 5 compared to the coat F, major spike G, internal scaffolding B and DNA binding J proteins or a factor of 20 compared to the external scaffolding D protein. This unique phenotype, which is exquisitely sensitive to host cell physiology, most likely reflects general aspects of øX174 biology (see Discussion).

The lethal phenotype of am(H)E258 in SupF cells is a function of the resulting missense protein

The am(H)E258 mutation resides within the region of the gene encoding the fourth coiled-coil domain (Fig. 1). Theoretically, neither E→Q nor E→S substitutions; which would result from growth in SupE and SupD cells, respectively; alter the probability of coil formation (Lupas, 1996). However, the E→Y results in a much higher probability of coil

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The H protein with the E→Y substitution at position 258 removes assembly intermediates from the morphogenetic pathway

In order to biochemically characterize the defects conferred by the E258Y substitution, a tyrosine codon was placed directly into the am(H)E258 genome. The resulting mutant, lethal(H)E258Y, had an absolute lethal phenotype, but could be propagated in cells expressing an exogenous wild-type H gene. To ensure that all viral stocks were isogenic, the amber codon was also replaced with a glutamate codon. This strain served as the wild-type control in these studies. The particles produced in lethal(H)E258Y and wild-type infected cells were examined. Lysis resistant cells (slyD) were infected at an MOI of 5.0 with a pre-attachment step as described in Materials and methods. Infections were incubated for 3 h at 33 °C. am(H) mutants grown in Su cell lines produce overall lower viral protein levels. To determine whether the lethal(H)E258Y mutant had a similar effect on viral protein synthesis, an aliquot from each infection was used to generate a whole cell lysate for SDS-PAGE analysis. The rest of the infected cells were concentrated, lysed, and soluble material was subjected to rate-zonal sedimentation. Following centrifugation, samples were separated into approximately fifty 0.1 ml fractions. Particles, regardless of infectivity, were detected by UV spectroscopy at 280 nm. As can be seen in the sedimentation profiles in Fig. 2, a large virion peak (114S) was evident in the extracts of wild-type infected cells (●). In contrast no assembled particles were detected from prepared extracts of lethal(H)E258Y infected cells (○). In addition to virions, which sediment at 114S, these experiments are capable of detecting provirions (132S), procapsids (70S) and degraded procapsids (105S).

The inability to detect assembled particles in extracts of lethal(H)E258Y infected cells (Fig. 2: ○) was not due to suboptimal viral protein levels. Whole cell lysates were prepared from wild-type and lethal(H)E258Y infected cells and examined by SDS-PAGE. No significant differences in viral protein levels were observed between samples (Fig. 3A). The presence of small pentameric assembly intermediates, 125S, 95S and 65S particles, was examined by analyzing gradient fractions by SDS-PAGE. The 95S particle is a coat protein pentamer with five internal scaffolding proteins, whereas the 65S particle is a pentamer of the major spike protein G. The 95S and 65S particles join to form the 125S particle, which also contains protein H. As can be seen in Fig. 4, the only soluble viral assembly intermediate that could be detected within the gradient was the 65S particle. The vast majority of the viral coat protein was found in the pellet fraction. These data suggest that the lethal H protein most likely removes a coat protein-containing intermediate producing an insoluble off pathway byproduct, which makes it difficult to determine whether the lethal H protein is actually incorporated into an assembly intermediate. To address this question, fractions that normally contain the 125S assembly intermediate were concentrated 10-fold before electrophoresis. The subsequent gel was then silver stained to detect proteins. Typically concentration and silver staining are not required (Cherwa and Fane, 2009; Cherwa et al., 2008; Uchiyama et al., 2009). As seen in Fig. 3B, it was possible to detect this H protein-containing particle from lethal(H)E258Y infected cells.

Table 3

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Host of isolation</th>
<th>Host cell genotype</th>
<th>SupE</th>
<th>SupD</th>
<th>SupF</th>
<th>Su</th>
</tr>
</thead>
<tbody>
<tr>
<td>am(H)E258</td>
<td>SupD0</td>
<td>1.0</td>
<td>10^{-5}</td>
<td>10^{-6}</td>
<td>10^{-6}</td>
<td>10^{-6}</td>
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<tr>
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<td>1.0</td>
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<td>10^{-6}</td>
<td>10^{-6}</td>
</tr>
<tr>
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<td>10^{-6}</td>
<td>10^{-6}</td>
<td>10^{-6}</td>
</tr>
<tr>
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<td>10^{-6}</td>
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<tr>
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<tr>
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<td>10^{-4}</td>
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<tr>
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<td>10^{-6}</td>
<td>10^{-6}</td>
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<tr>
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<td>1.0</td>
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<td>10^{-6}</td>
<td>10^{-6}</td>
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<tr>
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<td>SupD0</td>
<td>0.9</td>
<td>1.0</td>
<td>10^{-7}</td>
<td>10^{-7}</td>
<td>10^{-7}</td>
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<tr>
<td>su(H)-G T714M</td>
<td>SupD0</td>
<td>0.9</td>
<td>1.0</td>
<td>10^{-7}</td>
<td>10^{-7}</td>
<td>10^{-7}</td>
</tr>
<tr>
<td>su(H)-G C09F</td>
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<td>0.9</td>
<td>1.0</td>
<td>10^{-7}</td>
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</tr>
<tr>
<td>su(H)-G P00S</td>
<td>SupD0</td>
<td>0.6</td>
<td>1.0</td>
<td>10^{-7}</td>
<td>10^{-7}</td>
<td>10^{-7}</td>
</tr>
<tr>
<td>su(H)-H Q247H</td>
<td>SupF0</td>
<td>0.9</td>
<td>10^{-5}</td>
<td>1.0</td>
<td>10^{-5}</td>
<td>10^{-5}</td>
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<tr>
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<td>SupF0</td>
<td>0.7</td>
<td>10^{-5}</td>
<td>1.0</td>
<td>10^{-5}</td>
<td>10^{-5}</td>
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<tr>
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<td>SupF0</td>
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<td>10^{-5}</td>
<td>1.0</td>
<td>10^{-5}</td>
<td>10^{-5}</td>
</tr>
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</table>

* All suppressor strains contain the am(H)E258 mutation. Nomenclature reflects the location and amino acid substitution of the suppressor mutation. The letter after the “-” indicates the gene in which the suppressor resides. The letters surrounding the numbers respectively indicates the wild-type amino acid, the position in the protein, and the amino acid substitution. Thus, su(H)-F P119S: a suppressor of an H defect in gene F that confers a P→S substitution at amino acid 119. Two suppressor mutations, su(H)-F Q327am and su(H)-F Q405am, created a second amber mutation.

a Overnight cells were used in these experiments.

Over expression of the internal scaffolding protein returns lethal(H)E258Y protein-containing assembly intermediates to the assembly pathway

The øX174 internal scaffolding protein B is known to facilitate protein H incorporation during morphogenesis (Chen et al., 2007). Moreover, the
co-expression of gene B lessens the lethal effects associated with the expression of cloned H protein coiled-coil domains, which form oligomers in solutions (Ruboyianes et al., 2009). This suggests that protein B may keep protein H in a monomeric state for assembly and/or prevent its nonproductive association with other viral proteins. To further test this hypothesis, the effect of exogenous B gene expression on lethal(H)E258Y morphology was determined by analyzing infected cell extracts by rate zonal sedimentation (Fig. 2: ●). Particles with an S value similar to virions were readily detected. The differential yield between the wild-type and lethal(H)E258Y infections may reflect the efficiency of B protein-mediated rescue; however, differences could have arisen during sample preparation.

The lethal(H)E258Y particles were characterized for infectivity and molecular content (Table 4). The OD260/OD280 ratio of the lethal(H)E258Y particles was 1.43, which did not differ significantly from 1.42 value obtained for the wild-type control. SDS-PAGE analysis of the peak revealed that the E258Y protein was incorporated into these particles (Fig. 3C). The gel was scanned, the image digitized and band intensities calculated with Image J software. The relative ratios of the coat and major spike proteins to the DNA pilot protein were calculated and the values obtained for the lethal(H)E258Y particles did not differ significantly from wild-type. Neither the external nor internal scaffolding proteins were present. Thus, the lethal(H)E258Y particles are virion-like. However, they are significantly less infectious. Their specific infectivity was 9.3 × 10^4 pfu/OD280, more than two orders of magnitude below the wild-type control value of 2.4 × 10^6 pfu/OD280.

Second-site genetic analyses

Second-site genetic analyses were conducted with am(H)E258 in both SupD and SupF cells. In these selections overnight cultures were used in plating assays. Second-site revertants, which had gained the ability to grow on the restrictive Su+ host, were distinguished from am− revertants by the retention of the amber phenotype: the inability to grow on the wild-type Su− host. As the defects observed in SupD and SupF cells appear to be fundamentally different, it was hypothesized that allele specific suppressors, in regard to the identity of the amino acid at the E258 site, would be isolated. Five independently grown am(H)E258 stocks were used in the selections.

Twenty-three genetically unique suppressors were isolated on SupD cells. Six of the suppressors mapped to gene G, which encodes the major spike protein. The remaining 17 mutations conferred substitutions in the coat protein. The mutations map throughout the two genes and do not appear to cluster in the quaternary or tertiary structures. The su/am(H) strain growth characteristics, in regard to their ability to form plaques on different amber suppressing hosts, are summarized in Table 3. There were no major phenotypic differences between the various strains isolated on the SupD host; therefore, only the data for a subset of the mutants are presented.

As predicted, the suppressors were only active in the SupD host, displaying no activity in SupF cells on the level of plaque formation (Table 3). If the defect in SupD cells is related to inefficient H protein synthesis, the mechanism of suppression may operate by restoring a balance of interacting components, as first defined in the classic studies of Floor and Sternberg (Floor, 1970; Sternberg, 1976). Two amber mutations were isolated as suppressors, su(H)-F Q372am and su(H)-F Q405am, which is consistent with this mechanism. Recombination rescue experiments were performed with one of the isolated suppressors, su(H)-G T41M, to demonstrate that it was both necessary and sufficient to confer the suppressing phenotype. A portion of the mutant or wild-type G gene was cloned and the resulting plasmids introduced into SupD cells. am(H)E258Y or am(H)Q26 plaque assays were then performed with stationary phase cells. Both mutants plated with an efficiency of approximately 10−3 in SupD cells containing the plasmid with the su(H)-G T41M sequence, whereas plating efficiency in cells harboring the plasmid with the corresponding wild-type sequence was less than 10−2. These data indicate that the identified suppressor is necessary and sufficient to confer the observed phenotype. The ability to rescue the am(H)Q26 mutant indicates that the suppressor is not allele specific, capable of restoring phage growth to genetically distinct nonviable H mutants in the SupD host in standard plating assays.

When the selection was performed with SupF cells, a different set of suppressors was isolated. Unlike the extragenic mutations identified above, all suppressors were intragenic, conferring substitutions near the site of the original mutation. Only three genetically unique suppressors were isolated. They are only active in the SupF host, displaying no activity in SupD cells (Table 3). Due to their close proximity to the original mutation, it was not feasible to conduct recombination rescue experiments as “necessary and sufficient” assays. Therefore the entire

![Fig. 3.](Image 35x109 to 285x741)

![Fig. 4.](Image 35x566 to 285x741)

<table>
<thead>
<tr>
<th>Table 4</th>
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<tr>
<td>Characteristic of the lethal(H)E258Y virion-like particles.</td>
</tr>
<tr>
<td>Relative ratio</td>
</tr>
<tr>
<td>OD260/OD280</td>
</tr>
<tr>
<td>Protein F/protein H</td>
</tr>
<tr>
<td>Protein G/protein H</td>
</tr>
<tr>
<td>Specific infectivity (pfu/OD280)</td>
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</tbody>
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Discussion

The DNA pilot protein H is the most poorly understood and understudied of the øX174 structural and scaffolding proteins. Mutant phenotypes are often complex and can exhibit a large degree of variation, which most likely reflects the protein’s multifunctional nature (Ruboyianes et al., 2009). Until the full range of a protein’s function is known, genetic data will be difficult to interpret. Results will be strongly influenced by the molecular defect conferred by the mutation, which may specifically affect a subset of the protein’s functions. Moreover, defects can be epistatic; a mutant H protein’s inability to pilot DNA and/or stimulate viral protein synthesis may mask its effect on particle morphogenesis. Finally, host cell contributions to mutant phase phenotypes cannot always be predicted, and therefore cannot be ignored. The results of the initial genetic analysis of gene H reported here have defined a general host cell phenomenon specifically associated with am[H] mutants and a H defective phenotype that specifically affects its proper incorporation during assembly.

While the phenotype of an amber mutant in different informational suppressing Su+ strains is usually a consequence of the missense protein produced, other factors may contribute to phenotype. None of the am(H) mutants in our collection could form plaques on SupD cells in standard plating assays, which utilize overnight cultures. However, the am(H) mutants produced progeny in SupD liquid culture infections and formed plaques when plating assays were conducted with exponential phase plating cells. Moreover, when an E→S codon was placed directly at the E258 site, the resulting mutant had a wild-type phenotype. These data suggest that SupD cells must be in exponential phase for informational suppression of gene H mutations to be efficient enough to form progeny. In plaque assays using stationary cells, the first round or rounds of growth would most likely occur in log phase cells. Subsequent infections, which would lead to plaque formation, would occur in exponential phase cells. This growth phase-dependent phenomenon in SupD cells appears to be unique to am[H] mutants, not observed with amber mutants in other genes, even though protein H is the least abundant of the virion and procapsid proteins, by a factor of 20 when compared to the external scaffolding protein and by a factor of 5 when compared to the internal scaffolding and other structural proteins.

The gene H transcript is the least abundant transcript of the structural and scaffolding proteins. When it is present, it is always the last sequence at the end of the polycistronic message that contains six or nine other genes (Hayashi and Fujimura, 1976). The paucity of H transcripts, low informational suppressor efficiency, and a lag phase host may result in H protein levels that are too meager to support the productive initial infection required for plaque formation. Moreover, inefficient informational suppression of the am(H) gene could also affect the level of other viral proteins. The second-site suppressors that restore am(H)E258 plaque formation in SupD cells may act by equalizing the balance between interacting components: protein H and the structural proteins found in early assembly intermediates, proteins F and G. The isolation of gene F and G amber mutations as second-site suppressors is consistent with this hypothesis. Informational suppression could lower the relative amounts of these proteins, thus equalizing the concentration of the components required to synthesize assembly-competent 12S+ particles. Similarly, missense mutations in proteins G and F may lower the concentration of functional or folded proteins. Indeed, many of these suppressors exhibit secondary temperature-sensitive and small plaques to other Su+ suppressors. These data further evidence that the initial defect and subsequent second-site suppression mechanism involves protein levels as opposed to protein functions. Considering the complex interplay between the many factors that could be affecting phenotype, extrapolating beyond this point may not be merited.

In contrast, the lethal phenotype of am(H)E258 in SupF cells appears to be a direct consequence of the E→Y substitution in the protein. When a tyrosine codon was placed directly at the E258 site, the resulting mutant had a lethal phenotype. The lethal(H)E258Y protein does not appear to affect the level of other viral proteins, as is observed in the absence of de novo H protein synthesis (Ruboyianes et al., 2009). Thus, the H protein-related function in stimulating viral protein synthesis has remained intact and the mutant protein’s effect on assembly could be examined directly. With the exception of 65 particles, pentamers of the major spike protein, very few particles appeared to be soluble in extracts of lethal(H)E258Y infected cells. Instead, the vast majority of the viral proteins appeared to be in the pellet fraction, suggesting that the lethal(H)E258Y protein was removing most of the early assembly intermediates. However, it was possible to obtain small quantities of 12S+ particles from extracts of lethal(H)E258Y infected cells and demonstrate that the mutant H protein could be incorporated into this particle. In a previous study, the H protein coiled-coil regions were cloned and the truncated gene expressed in øX174 sensitive cells. Expression interfered with the DNA piloting functions of the infecting H proteins (Ruboyianes et al., 2009). Overexpression of the internal scaffolding protein B gene lessened the dominant lethal effects of the coiled-coil domain. Presumably excess internal scaffolding protein prevented the coiled-coil domains from interacting with other viral components at the onset of the infection. The over-expression of the B gene appeared to rescue lethal(H)E258Y-containing assembly intermediates, allowing the formation of H protein-containing, virion-like particles. However, these particles are significantly less infectious. Thus, either the lethal(H)E258Y is in itself unable to pilot DNA or its incorrect incorporation prevents this function. The latter possibility seems more likely as single amino acid changes elsewhere in protein H can rescue the lethality associated with the E258Y substitution. While it is unknown whether the wild-type H protein plays a direct role in morphogenesis, the lethal(H)E258Y represents the first example of a mutant H protein actively affecting assembly.

Materials and methods

Phage plating, media, buffers, and stock preparation, attachment, eclipse and burst size assays

Media, buffers, plating and liquid culture stock preparations (Fane and Hayashi, 1991) and protocols for attachments and eclipse assays (Cherwa et al., 2009) have been previously described. For burst size assays, cells were grown to a concentration of 1.0 x 10^6 cells/ml, as determined by OD_660. Cells were concentrated, washed, and resuspended in iced HFB buffer supplemented with 10 mM MgCl_2 and 5.0 mM CaCl_2 and infected at a multiplicity of infection of 6.0; 3.0 of each phage in co-infections. Phage were pre-attached to cells by incubating the samples at 16 °C for 30 min and unattached phage were removed by centrifugation. Pellets were resuspended in pre-warmed TK media supplemented with 10 mM MgCl_2 and 5.0 mM CaCl_2. Infections were incubated for 40 min and titered. Burst size was calculated by dividing the phage titer with the uninfected cell titer.

Bacterial strain and plasmids

The Escherichia coli C strains C122 (Su+), BAF5 (SupE), BAF7 (SupD), BAF8 (SupF), and the plasmid used to overexpress the internal scaffolding protein B gene have been described previously (Fane and Hayashi, 1991; Novak and Fane, 2004). The C900 strain contains the host slyD mutation, which confers resistance to E protein-mediated lysis (Roof et al., 1997). Gene H was cloned by amplifying the gene with an upstream primer that introduces a Bgl II site before the gene’s ribosome binding site and a
downstream primer that introduces an Xho I site after the stop codon. The fragment was digested with Bgl II and Xho I and cloned into pSE420 (Invitrogen). Gene expression is under lac promoter control.

αX174 mutants and second-site reversion analyses

The am(H) mutants am(H)E258 and am(H)Q89 were originally described as amN1 and amH257, respectively (Spindler and Hayashi, 1979). The names used here reflect the location of the mutations in the gene: E258, an amber mutation in glutamic acid codon 258. The (am(H)Q26 and E2S8S mutants were generated by site-directed mutagenesis following previously published protocols (Fane and Hayashi, 1991; Fane et al., 1993). am(H)E258 DNA was used as the template to generate the latter strain. Second-site revertants were obtained by plating am(H)E258 on restrictive Su+ hosts. Phage from the resulting plaques was stabbed into lawns seeded with Su− and Su+ hosts. Second-site revertants were distinguished from am− revertants by the retention of the amber phenotype, and confirmed by a direct DNA sequence analysis.

To ensure all viral strains were isogenic, the wild-type strain used in these studies was generated by recombination rescue. am(H)E258 was passed through cells harboring a clone of the αX174 H gene. Wild-type recombinants appeared at a frequency of approximately 10−3, in these studies was generated by recombination rescue. am(H)E258 DNA sequence analysis.

10 mM MgCl2. Phage were added to a calculated multiplicity of infection of three orders of magnitude higher than the reversion frequency of 5.0 and allowed to pre-attach by incubating for 30 min at 16 °C. Following pre-attachment, infected cells were grown to a concentration of 1.0×108 cells/ml. Cells were concentrated, washed twice with HF buffer, and resuspended in 10 ml HF buffer supplemented with 5.0 mM CaCl2 and 10 mM MgCl2. Phage were added to a calculated multiplicity of infection of 5.0 and allowed to pre-attach by incubating for 30 min at 16 °C. Infections were initiated by the addition of 100 ml TK media supplemented with 5.0 mM CaCl2 and 10 mM MgCl2 pre-warmed to 33 °C. Infections were incubated for 4 h. Extract preparation, rate zonal sedimentation and protein electrophoresis protocols are identical to those previously published (Uchiyama and Fane, 2005).

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References


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