The Expression of N-Terminal Deletion DNA Pilot Proteins Inhibits the Early Stages of φX174 Replication

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The φX174 DNA pilot protein H contains four predicted C-terminal coiled-coil domains. The region of the gene encoding these structures was cloned, expressed in vivo, and found to strongly inhibit wild-type replication. DNA and protein synthesis was investigated in the absence of de novo H protein synthesis and in wild-type-infected cells expressing the inhibitory proteins (ΔH). The expression of the ΔH proteins interfered with early stages of DNA replication, which did not require de novo H protein synthesis, suggesting that the inhibitory proteins interfere with the wild-type H protein that enters the cell with the penetrating DNA. As transcription and protein synthesis are dependent on DNA replication in positive single-stranded DNA life cycles, viral protein synthesis was also reduced. However, unlike DNA synthesis, efficient viral protein synthesis required de novo H protein synthesis, a novel function for this protein. A single amino acid change in the C terminus of protein H was both necessary and sufficient to confer resistance to the inhibitory ΔH proteins, restoring both DNA and protein synthesis to wild-type levels. ΔH proteins derived from the resistant mutant did not inhibit wild-type or resistant mutant replication. The inhibitory effects of the ΔH proteins were lessened by the coexpression of the internal scaffolding protein, which may suppress H-H protein interactions. While coexpression relieved the block in DNA biosynthesis, viral protein synthesis remained suppressed. These data indicate that protein H’s role in DNA replication and stimulating viral protein synthesis can be uncoupled.

Although the atomic structure of protein H remains to be elucidated, the results of bioinformatic analyses predict an N-terminal transmembrane (23) and several coiled-coil domains (16) in the C terminus (Fig. 1). During penetration, H protein is ejected from the capsid along with the penetrating DNA (14). Initially, protein H and the incoming DNA are associated with the outer membrane (13) at sites of cell wall adhesion (2, 3). From there, the single-stranded DNA (ssDNA) is delivered to the cytoplasmic membrane, the site of DNA synthesis (2). The transmembrane domain most likely mediates this DNA-piloting function. Although naked ssDNA can be transfected, replication efficiency is enhanced if H protein is included in the reaction (12). This suggests that the protein may stimulate stage I DNA synthesis: the conversion of the ssDNA genome to a double-stranded replicative-form (RF) molecule, either directly or indirectly by piloting the penetrating DNA to the proper site of replication. Due to the positive polarity of the genome, this occurs without de novo viral protein synthesis. During stage II DNA synthesis, the RF DNA is amplified. This continues until the viral protein C and procapsids accumulate, which signals the switch to stage III DNA synthesis, the concurrent synthesis and packaging of ssDNA genomes.

While coiled-coil domains are known to mediate protein oligomerization (1, 10, 15) and the purified H protein coiled-coil domains do form oligomers in solutions (J. Nardozzi and G. Cingolani, personal communication), H protein oligomers have yet to be observed during the φX174 life cycle. However, past studies of this low-copy protein have focused on its association with assembly intermediates. The protein appears as a monomer in early assembly intermediates (6), and its incorporation into these intermediates appears to be mediated by the internal scaffolding protein (5, 20). Due to icosahedral averaging, the 10 to 12 copies of the H protein in the virion could not be resolved in the X-ray structure (18, 19). Therefore, it is not known whether H-H protein interactions could occur within the capsid or during DNA penetration.

To determine whether the oligomerizing coiled-coil domains could interact with wild-type H proteins, cloned N-terminal deletion genes (ΔH) were expressed in vivo and assayed for possible effects on wild-type φX174 replication. The ΔH proteins acted as potent inhibitors. The inhibitory mechanism was characterized, and a mutant resistant to the expression of the coiled-coil domains was isolated.

MATERIALS AND METHODS

Phage plating, media, buffers, and stock preparation. The reagents, media, buffers, and protocols used in this study have been previously described (7). The φX174 mutant resistant to the expression of the inhibitory H proteins, φX174ΔHφV286L., was selected by plating wild-type φX174 on cells expressing the ΔH142 protein.

Escherichia coli C and φX174 strains. E. coli C strains C122 (supO), BAF8 (supF), and BAF30 (recA) have been previously described (7.8). The host supD mutation in E. coli C900 confers resistance to E-protein-mediated lysis (21). The amber H, am(H)Q26, mutant was constructed by oligonucleotide-mediated mutagenesis (9). The mutant oligonucleotide was designed to introduce an amber mutation into codon 26 of gene H, which encodes the amino acid glutamine. The mutant was isolated in BAF5 (supE). The genotype of the strain was verified by direct sequence analysis. The am(H)L258 mutant is the am(H)N1 mutant used in earlier studies (22). The name was changed to reflect the location of the amber mutation.

Construction of plasmids expressing H proteins with N-terminal deletions. To construct the 5′ deletion genes, the φX174 H gene was amplified by PCR. The...
RESULTS

Expression of the C terminus of the minor spike protein inhibits wild-type plaque formation. Using the location of the predicted coiled-coil domains as a guide (Fig. 1), 5' -terminal deletion genes with start codons at positions 142, 186, 238, 277, and 299 were constructed as described in Materials and Methods. The proteins were expressed in vivo and assayed for the ability to inhibit wild-type φX174 plaque formation (Table 1). The expression of the ΔH142, ΔH186, ΔH238, and ΔH277 proteins inhibited wild-type plaque formation. The severity of inhibition appears to be a function of the size of the expressed protein. The expression of ΔH299 had no effect on wild-type plating efficiency.

Effects of de novo H protein synthesis and the expression of the inhibitory ΔH proteins on viral protein synthesis. To determine the mechanism by which the C-terminal fragments inhibit wild-type morphogenesis, the infection products synthesized in amber H mutant-infected cells and wild-type-infected cells expressing the inhibitory H protein were analyzed (data not shown). However, it was difficult to detect assembly intermediates. The recovery of viral proteins in both the pellet and soluble fractions appeared to be abnormally low compared to that in the wild-type control infection, suggesting a reduction in viral protein synthesis. To determine whether de novo H protein synthesis or the expression of the ΔH proteins affected the overall level of viral protein synthesis, whole-cell lysates were examined by SDS-PAGE. As shown in Fig. 2, the overall viral protein synthesis is decreased in amber H mutant infection cells (Fig. 2B, lanes 3 and 4) and in wild-type-infected cells expressing the ΔH142 protein (Fig. 2A, lane 4) compared to that in the wild-type control infected cells (Fig. 2A, lanes 3, and B, lane 2). Using ImageJ software, the relative levels of coat protein F synthesis was determined by comparing its band intensity and area with that of a host cell protein. The level of coat protein synthesis in amber H infections and in cells expressing the inhibitory protein was found to be approximately 20% of that of the wild-type control.

The ΔH proteins inhibit early stages of DNA synthesis, which does not require de novo H protein synthesis. There are several mechanisms by which the H protein could lead to elevations in viral protein synthesis. It could stimulate transcription, translation, or viral DNA synthesis. As φX174 is a positive ssDNA virus, transcription is dependent on the

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**TABLE 1.** Wild-type and φX174 ΔHV286La plating efficiencies in cells expressing N-terminal ΔH proteins

<table>
<thead>
<tr>
<th>Expression protein</th>
<th>Plating efficiencya</th>
</tr>
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<tbody>
<tr>
<td>Wild-type φX174</td>
<td>φX174 ΔHV286La</td>
</tr>
<tr>
<td>None</td>
<td>1.0</td>
</tr>
<tr>
<td>ΔH142</td>
<td>&lt;10^-6</td>
</tr>
<tr>
<td>ΔH186</td>
<td>&lt;10^-6</td>
</tr>
<tr>
<td>ΔH238</td>
<td>10^-2</td>
</tr>
<tr>
<td>ΔH277</td>
<td>10^-2</td>
</tr>
<tr>
<td>ΔH299</td>
<td>1.2</td>
</tr>
<tr>
<td>ΔH142+ Bc</td>
<td>0.05</td>
</tr>
<tr>
<td>ΔH142V286Ld</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a A φX174 mutant resistant to the expression of ΔH proteins. The mutation confers a V-λL substitution at amino acid 286.

b Plating efficiency was determined as the titer on cells expressing ΔH proteins divided by the titer on cells expressing no exogenous proteins.

c N-terminal H deletion proteins (ΔH): the number following ΔH is the number of amino acids deleted from the N terminus.

d The induced plasmid contains two genes, the ΔH142 gene immediately followed by the internal scaffolding protein B gene.

e The expressed protein contains the V286L mutation, which confers resistance to the expression of the ΔH proteins.
The ΔH proteins on DNA synthesis were examined in amber H mutant-infected cells and wild-type-infected cells expressing the inhibitory proteins, respectively. In these experiments, plasmid DNA served as an internal standard to which RF DNA levels could be compared. Because both the RF and plasmid DNAs are circular and similar in size, the extracted DNA was digested with SspI. This enzyme cuts RF DNA once, yielding a 5,386-bp linear fragment. There are multiple SspI sites in the plasmid DNA. The large 5,324-bp digestion product was used as an internal standard to assess the efficiency of viral DNA synthesis.

As shown in Fig. 3, the level of RF DNA recovered was significantly lower in cells expressing the ΔH142 protein (lane 5) than in control infected cells, in which the gene for ΔH142 was not induced (lane 4). Using ImageJ software, the relative amounts of RF and plasmid DNAs were determined by comparing band intensities and areas. RF DNA recovered from cells expressing the cloned gene was approximately 5% of that obtained without expression.

The timing of ΔH142 gene induction affected RF recovery. If the inducer was added at the time of infection (lane 1), as opposed to inducing the gene 20 min before infection (lane 5), RF levels were reduced to only 20% of the control level (lane 4). In contrast, RF DNA synthesis does not appear to be inhibited in amber H-infected cells (lane 6). These results indicate that de novo H protein synthesis is not required to stimulate the early stages of DNA synthesis and suggest that the inhibitory proteins interfere with the wild-type protein’s DNA-piloting function by interacting with the H protein entering the cell with the penetrating DNA.

A mutation in gene H confers resistance to the expression of the inhibitory fragments. To further investigate the inhibitory mechanism conferred by the ΔH142 proteins, resistance mutants were selected by plating wild-type φX174 phage on cells expressing the ΔH142 protein. To ensure a wide range of synthesis of the negative DNA strand, which occurs during stage I DNA synthesis. Afterwards, the double-stranded RF DNA is amplified, producing more templates for transcription (11).

The effects of de novo H protein synthesis and expression of

FIG. 3. Relative recovery of plasmid and viral RF DNAs in cells expressing the inhibitory ΔH142 protein. Plasmid and viral RF DNAs were isolated from infected cells and digested with SspI as described in the text. Lanes: 1, wild-type (WT)-infected cells expressing (+) the ΔH142 protein (ΔH) with inducer added at the time of infection (0); 2, digested RF DNA; 3, digested plasmid DNA (P); 4, wild-type-infected cells without induction of the inhibitory gene (−); 5, wild-type-infected cells expressing the ΔH142 protein (+) with inducer added 20 min before infection (−20); 6, amber H-infected cells (amH); 7, wild-type-infected cells coexpressing the internal scaffolding and inhibitory ΔH142 proteins (ΔHB); 8, wild-type-infected cells without induction of the internal scaffolding and inhibitory ΔH142 genes; 9, wild-type-infected cells expressing the ΔH142V286L protein, which was derived from the resistant mutant (ΔH9); 10, wild-type-infected cells without induction of the ΔH142V286L gene; 11, φX174ΔH11002. (RR, resistant mutant)-infected cells expressing the ΔH142 protein; 12, φX174ΔH11002V286L-infected cells without induction of the ΔH142 gene; 13, digested plasmid DNA; 14, digested RF DNA.
resistant mutant isolation, five independent wild-type φX174 stocks were used in the selection (17); however, only one mutant, φX174ΔH142V286L, was repeatedly recovered. The mutation, which confers a V→L substitution at amino acid 286 of the H protein, elevates plating efficiencies on cells expressing the inhibitory proteins (Table 1). To determine whether the identified mutation was both necessary and sufficient to confer the resistance phenotype, recombination rescue experiments were performed. Wild-type phage was passaged through cells harboring pΔH142 or pΔH142V286L, the construct derived from the V286L resistant mutant. The cloned genes were not induced. Recombination between the wild-type genome and the cloned φX174 DNA sequences would occur in both infections. However, only recombination events that transfer the V286L mutation to the viral genome should produce the resistance phenotype, which was assayed by plating on cells expressing the ΔH142 protein. The resistant progeny frequencies (resistant phage/total progeny) from the pΔH142/H9004/H9278 competition-based model, the ΔH142 protein appears to have no inhibitory effects, eliminating models in which the primary target is stage I DNA synthesis. The recovery of RF DNA is affected by the timing of inhibitory gene induction. Preinduction of the gene results in much lower recovery of RF DNA. In addition, RF DNA recovery is not affected by de novo viral protein synthesis. The mechanism of inhibition most likely involves a nonproductive association between the ΔH and incoming wild-type proteins. A single amino acid change in protein H is both necessary and sufficient to confer resistance, restoring viral protein and DNA synthesis to nearly wild-type levels. A ΔH protein derived from this mutant appears to have no inhibitory effects, eliminating models in which the resistant protein outcompetes the inhibitory protein for some critical interaction(s) with some host or phage component(s) or is less prone to associate with inhibitory proteins. To distinguish between these two models, the effects of a deletion protein derived from the resistance mutant, ΔH142V286L, on wild-type and φX174ΔH142V286L replication were examined. In a competition-based model, the ΔH142V286L protein should be more inhibitory than the ΔH142 protein. As shown in Table 1, expression of the ΔH142V286L protein does not inhibit wild-type or φX174ΔH142V286L plaque formation. Moreover, the expression of this protein appears to have little or no effect on viral protein and RF DNA synthesis (Fig. 2A, lane 6, and 3, lanes 9 and 10, respectively). These data suggest that the V286L protein is less prone to associate with inhibitory proteins. To coexpress the internal scaffolding protein lessens the inhibitory effects of the ΔH proteins. The above data suggest that expression of the ΔH proteins leads to the removal of full-length H into nonfunctional protein complexes. During assembly, H protein appears to be monomeric when it is incorporated into the early 12S* assembly intermediate, in a reaction that is mediated by internal scaffolding protein B (5, 20). In addition to one copy of the H protein, the 12S* intermediate contains five copies of the major coat, spike, and internal scaffolding proteins. As the B protein may prevent H-H protein interactions, the effects of coexpressing the internal scaffolding and ΔH142 proteins were examined. As shown in Table 1, plating efficiency was raised 4 orders of magnitude in cells coexpressing the ΔH142 and B proteins (pΔH142+B). While the coexpression of the B protein did not appear to raise viral protein synthesis to wild-type levels (Fig. 2A, lanes 4 and 5), it did appear to alleviate the block in DNA biosynthesis (Fig. 3, lanes 7 and 8).

**DISCUSSION**

The results of bioinformatic analyses predict the presence of several coiled-coil domains in the C terminus of the protein (Fig. 1). The ability of coiled-coil domains to mediate protein oligomerization is very well documented (1, 10, 15), and the purified H protein coiled-coil domains do form oligomers in solutions (J. Nardozzi and G. Cingolani, personal communication). To determine whether the expression of the predicted coiled-coil domains could inhibit φX174 replication, a series of N-terminal deletion genes were constructed, expressed in vivo, and assayed for the ability to inhibit φX174 replication. Expression of the proteins containing the predicted coiled-coil domains strongly inhibited φX174 plaque formation. The severity of inhibition correlated with the size of the expressed protein.

At the onset of infection, protein H pilots incoming DNA to the outer membrane (13) at sites of cell wall adhesion (2, 3). This reaction is most likely mediated by the predicted N-terminal transmembrane helix. From there, the ssDNA is delivered to the cytoplasmic membrane, the site of DNA synthesis (2). Microvirus DNA synthesis occurs in three distinct stages (11). During stage I DNA synthesis, the infecting ssDNA is converted into a double-stranded RF molecule. Protein H increases naked ssDNA transfection efficiencies (12, 14), suggesting that the incoming protein may facilitate stage I DNA synthesis. Due to the positive polarity of the genome, this occurs without de novo viral protein synthesis. During stage II DNA synthesis, the RF DNA is amplified. This continues until the viral protein C and procapsids accumulate, which signals the switch to stage III DNA synthesis, the concurrent synthesis and packaging of ssDNA genomes.

The expression of the inhibitory ΔH proteins leads to a reduction in viral DNA synthesis. Two observations suggest that the primary target is stage I DNA synthesis. The recovery of RF DNA is affected by the timing of inhibitory gene induction. Preinduction of the gene results in much lower recovery of RF DNA. In addition, RF DNA recovery is not affected by de novo H protein synthesis. The mechanism of inhibition most likely involves a nonproductive association between the ΔH and incoming wild-type proteins. A single amino acid change in protein H is both necessary and sufficient to confer resistance, restoring viral protein and DNA synthesis to nearly wild-type levels. A ΔH protein derived from this mutant appears to have no inhibitory effects, eliminating models in which the resistant protein outcompetes the inhibitory protein for some critical interaction with a host or viral component. Moreover, these data suggest that the V286L protein is less prone to associate with the inhibitory ΔH proteins.

**Protein H may affect several stages in the viral life cycle. While the level of RF production in amber H mutant-infected cells appears to be unaltered, the level of viral protein synthesis is reduced, suggesting that de novo H protein synthesis is required to stimulate viral protein synthesis.** The mechanism by which this is accomplished remains to be elucidated. H protein may act directly as a transcription factor. Alternatively,
it may direct replicating DNA to locations within the cell where it has access to host cell transcription machinery.

The effects of the inhibitory proteins could well be pleiotropic. Besides inhibiting RF DNA synthesis, they could associate with de novo synthesized wild-type H protein, inhibiting viral protein synthesis or assembly by reducing the pool of H protein monomers. However, in a positive ssDNA viral life cycle, transcription is dependent on DNA synthesis. Thus, these possible secondary effects would be obscured by the reduction of RF DNA. The coexpression of the internal scaffolding protein may somewhat uncouple these pleiotropic effects. While it does not appear to return viral protein synthesis to wild-type levels, it relieves the inhibition of DNA synthesis and allows virion production, which would require the incorporation of the monomeric H protein during assembly.

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REFERENCES


