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Autoregulation of the Bacteriophage P22 Scaffolding Protein Gene

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During the formation of each bacteriophage P22 head, about 250 molecules of the product of gene 8, scaffolding protein, coassemble with and dictate correct assembly of the coat protein into a proper shell structure. At approximately the time that DNA is inserted inside the coat protein shell, all of the scaffolding protein molecules leave the structure. They remain active and participate in several subsequent rounds of shell assembly. Previous work has shown that scaffolding protein gene expression is affected by the head assembly process and has generated the hypothesis that unassembled scaffolding protein negatively modulates the expression of its own gene but that it lacks this activity when complexed with coat protein in proheads. To test this model, a P22 restriction fragment containing the scaffolding and coat protein genes was cloned under control of the *lac* promoter. These cloned genes were then expressed in an in vitro DNA-dependent transcription-translation reaction. The addition of purified scaffolding protein to this reaction resulted in reduced scaffolding protein synthesis relative to coat and tail protein synthesis to an extent and at a protein concentration that was consistent with the observed reduction in vivo. We conclude that scaffolding protein synthesis is autoregulated and that scaffolding protein is the only phage-coded protein required for this process. In addition, these experiments provide additional evidence that this autoregulation is posttranscriptional.

The molecular biology of P22, a temperate bacteriophage of *Salmonella typhimurium*, has been extensively studied (see Susskind and Botstein [36] for review). All known genes involved in P22 morphogenesis are located in a single transcription unit, the late operon. The product of gene 23 is required for expression of these genes and is thought to permit late gene expression by allowing RNA polymerase to read through transcription termination sites distal to the late promoter (3, 13, 18). That these P22 morphogenic genes lie in a single operon without internal promoters is implied from studies with the polar insertion element Tn*1* (40) and from measurements of the lag time in the functional decay of mRNA's after the addition of rifampin (6).

The proteins coded by the late operon are required for phage assembly and cell lysis (36). During the assembly of P22, as of other double-stranded DNA phages, a protein particle called a prohead is formed, DNA is packaged into this structure, and finally the tail is added (4, 11, 42). The P22 prohead is formed by the copolymerization of about 420 molecules of gp5 (gp*X* refers to the protein product of gene *X* where *X* is any gene of P22), the coat protein, with 200 to 300 molecules of gp8, the scaffolding protein, along with 10 to 30 molecules each of gp1, gp7, gp16, and gp20 (4, 25, 33). Scaffolding protein is required for normal polymerization of the coat protein into prohead structures and assembles as an internal component of proheads (12, 15, 25).

The prohead is filled with DNA in a complex reaction that includes recognition of the DNA substrate, cleavage of the DNA to an appropriate length (20, 38), expansion of the coat protein shell (12), and the exit of all scaffolding protein molecules from the structure. These scaffolding protein molecules may then be used to form new proheads, with each molecule recycling an average of five times during a wild-type, lysis-defective infection (23). Thus, scaffolding protein has the unusual property of being required in large quantities per prohead formed and being tightly associated with this structure, yet its role in phage assembly is catalytic. Gp2 and gp3, which are not present in the prohead or completed phage, and gp1, an integral prohead protein, are also required for the DNA packaging reaction (8, 28, 32).

Although the scaffolding protein gene is located in the middle of the late operon, it is not always coordinately regulated with the other late genes. Scaffolding protein synthesis is turned down earlier in the infection than is the synthesis of other late proteins. In infections with phage defective in genes 1, 2, or 3, proheads accumulate, and scaffolding protein is overproduced about fourfold relative to a wild-type infection (23, 24). The model has been proposed that unassembled scaffolding protein depresses the synthesis of additional scaffolding protein but that scaffolding protein incorporated in proheads lacks this ability (24). In this paper we present data to directly demonstrate autoregulation by unassembled scaffolding protein and additional evidence that this regulation is posttranscriptional.

MATERIALS AND METHODS

Bacterial strains and plasmids. CA7027, an *Escherichia coli* strain that is HfrH, Δ*lacU169*, B1$^-$, was obtained from J. S. Parkinson. *S. typhimurium sup*$^+$ and SupE isogenic strains DB7000 and DB7004 (41) and P22 strains were obtained from D. Botstein. Strain HB101 and strain HB101 carrying pBR322 were from D. Carroll. Plasmid pP22-508 was from the collection of E. Jackson (34). Plasmid pOP203-2 was the gift of F. Fuller (14), and plasmid pPB13 was from P. Berget (2).

Scaffolding protein purification. Scaffolding protein was purified from extracts of DB7000 infected with P22 *c*1-7 5$^-$*am*N114 9$^-$*am*N110 13$^-$*am*H101. Coat protein is not produced in these infections, so that scaffolding protein is unassembled and need not be dissociated from proheads. The 13$^-$ mutation was included to block cell lysis, and the *c*1-7 mutation prevented lysogeny. The assay at each step was the appearance of a band at 39,000 kilodaltons (43) in Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gels. We estimate that the final purity was greater than 95%. This is probably a conservative estimate, since low-molecular-weight bands appear to be scaffolding protein degradation products. The details of this purification

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have been reported elsewhere (E. Wyckoff, Ph.D. thesis, University of Utah, Salt Lake City, Utah, 1984).

Construction of pOP854B. P22 DNA containing the scaffolding protein gene was cloned so that P22 genes were expressed from the *lacUV5* promoter of the plasmid vector pOP203-2 (14). Transcription from this promoter proceeds through a small region of lambda DNA containing a unique *Pst*I site. P22 *Pst*I fragment D is about 3,540 base pairs long and contains genes 8, 5, and 4 and the sequences for the amino portion of gp10. This fragment has been cloned into the *Pst*I site of pBR322 to give pP22-508 (34). *Pst*I fragment D was gel purified (37) from *Pst*I digests of pP22-508 and joined with *Pst*I-cleaved pOP203-2 by using T4 DNA ligase (gift of W. M. Huang) in 60-mM Tris-hydrochloride (pH 7.5)–11 mM $MgCl_2$–1 mM dithiothreitol–1 mM ATP. After incubation at 12.5°C for 15 h, strain HB101 was transformed with this mixture (9). Plasmids containing inserts were identified by screening tetracycline-resistant colonies for an increase in plasmid size by the method of Barnes (1). The insert size was checked in *Pst*I digests, and the orientation of the insert was determined by the *Eco*RI restriction pattern, in plasmids prepared by the method of Klein et al. (26).

Marker rescue. *Salmonella typhimurium* strains transformed with the appropriate plasmids were grown to stationary phase. The plating efficiencies of various P22 strains were determined on the plasmid-carrying host strains as previously described (10).

Preparation of S-30 extract. The S-30 extract was prepared by a modification of the procedure of Greenblatt and Miller (19). An overnight culture of *E. coli* strain CA7027 was diluted 1 to 10 into 8 liters of medium containing the following (per liter): 5.6 g of KH_2PO_4 (anhydrous), 28.9 g of $K_2HPO_4 \cdot 3H_2O$, 10 g of yeast extract, 10 μg of thiamine, and 40 ml of 25% glucose; the culture was grown in this medium for 4.5 h at 37°C. Cells were quickly chilled on ice and harvested by centrifugation at 8,000 rpm for 20 min in a Sorvall GS-3 rotor. The pellet was suspended in 250 ml of buffer A (19) containing 10 mM Tris-acetate (pH 7.8), 14 mM magnesium acetate, 60 mM potassium acetate, and 0.1 mM dithiothreitol and was centrifuged at 8,000 rpm in a Sorvall GSA rotor for 20 min. The pellet was washed with 150 ml of buffer A, and the wet cell paste was frozen at −70°C. Frozen cells were weighed and ground with twice their weight of alumina in a mortar and pestle. Buffer A was added to a concentration of 1.5 ml per gram of wet cells, and the extract was centrifuged twice at 17,000 rpm in a Beckman type 50 Ti rotor for 30 min. The volume of the final supernatant was measured and the following were added per ml: 100 μl of 1 M Tris-acetate (pH 7.8), 20 μl of 0.14 M magnesium acetate, 8 μl of 100 μM ATP, 120 μl of 75 mM trisodium phosphoenol pyruvate, 5 μl of 200 mM dithiothreitol, 10 μl of 1 mM stock of 20 amino acids, 5 μg of pyruvate kinase (Sigma Chemical Co.), and 44.8 μl of water. This mixture was incubated in the dark for 80 min at 37°C and dialyzed against two changes of buffer A. Samples were quick-frozen in a dry ice-ethanol bath and stored at −70°C. Each sample was thawed immediately before use.

DNA-dependent in vitro translations. In vitro transcription-translation reactions were carried out essentially by the method of Zubay (44), as modified by Greenblatt and Miller (19). Each 25-μl reaction contained 44 mM Tris-acetate (pH 8.2), 1.4 mM dithiothreitol, 55 mM potassium acetate, 0.22 mM of each amino acid, except methionine, 0.55 mM each of CTP, GTP, and UTP, 2.2 mM ATP, 21 mM trisodium phosphoenolpyruvate, 2.5 μg of tRNA (Sigma Chemical Co.), 0.625 μg of folinic acid, 14.7 mM magnesium acetate, 7.4 mM calcium acetate, 1.25 μg of plasmid DNA, 5 to 10 μCi of [^{35}S]methionine (Amersham Corp.), and S-30 to a final protein concentration of 4 mg/ml. Reactions were incubated 1 h at 37°C with gentle shaking. The pH of the triphosphates was adjusted to between 6 and 7 with KOH. Some reactions contained purified scaffolding protein which had been dialyzed against 100 mM Tris-hydrochloride (pH 7.8)–1 mM dithiothreitol. Where appropriate this was diluted with buffer from outside the dialysis tubing in the final dialysis flask. Bovine serum albumin (Pentex), when added, was dissolved in 10 mM Tris-hydrochloride (pH 7.8) and heated at 55°C for 45 min.

Analysis of in vitro translation products. The [^{35}S]methionine-labeled products of the DNA-dependent in vitro translation were analyzed on sodium dodecyl sulfate-polyacrylamide gels and quantitated by densitometry as previously described (24). To maximize consistency of the film exposure for the densitometric tracing, an equal number of trichloroacetic acid-insoluble counts and an equal amount of cold purified scaffolding protein were added to each lane of the gel.

RESULTS

Construction and characterization of pOP854B. The plasmid pOP203-2 is a pMB9 derivative containing the *lacUV5* promoter which directs transcription towards a short piece of lambda DNA containing a unique *Pst*I site (14). P22 *Pst*I fragment D containing genes 8, 5, and 4 was gel purified from *Pst*I-cleaved pP22-508 (34) and cloned in the *Pst*I site of pOP203-2, as described in the text. *Pst*I digests of both pOP854B and pOP854E had bands which comigrated with P22 *Pst*I fragment D (Fig. 1). The orientation of *Pst*I fragment D inserts was determined from the *Eco*RI digestion of the plasmids. pOP854B had *Eco*RI fragments of 6,300 and 3,000 base pairs (Fig. 1), the size expected when the insert is in the correct orientation for transcription of the P22 genes from the *lac* promoter. Restriction maps of pOP203-2 and pOP854-B are shown in Fig. 2.

Phage carrying amber mutations in genes 8, 5, or 4 plated as efficiently on nonsuppressing strains containing this plasmid as on amber-suppressing strains, as shown in Table 1, which is consistent with the idea that these mutations can be complemented by protein expressed from these plasmids. The synthesis of proteins that comigrate with scaffolding and coat proteins in sodium dodecyl sulfate-polyacrylamide gels correlated with the presence of pOP854B in vivo. These bands were not observed in extracts from cells containing pOP203-2 or the plasmid with the insert in the opposite orientation (data not shown). Synthesis of these proteins was strongly stimulated by the addition of the lactose operon inducer isopropyl-β-D-thiogalactopyranoside to the culture, indicating that these genes are transcribed primarily from the *lac* promoter. Furthermore, synthesis of these proteins was inhibited in vitro (see below) by the addition of purified lactose repressor (gift of G. Herrick). This effect is specific, since synthesis is not inhibited by the addition of lactose repressor together with isopropyl-β-D-thiogalactopyranoside (data not shown).

In vitro synthesis of scaffolding protein. A prediction of the model that unassembled scaffolding protein modulates its own synthesis is that the addition of purified unassembled scaffolding protein should depress scaffolding protein synthesis in vitro. To test this prediction we used pOP854B as template in DNA-dependent in vitro transcription-translation reactions carried out in *E. coli* extracts (19, 44). As an additional control, the plasmid pPB13, which contains the

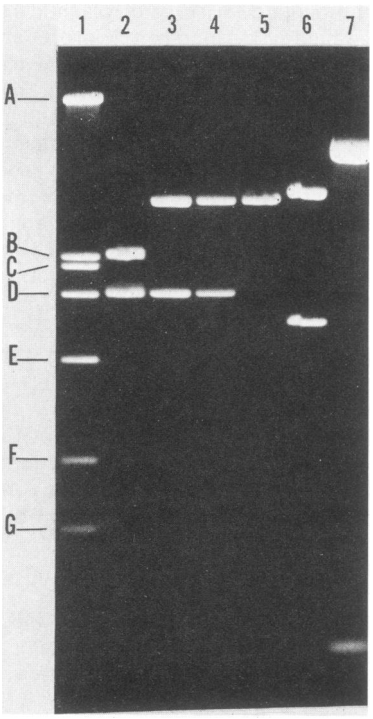

FIG. 1. Restriction enzyme analysis of plasmids used in the construction of pOP854B and verification of pOP854B structure. *Pst*I digests of P22 DNA (lane 1), pP22-508 (lane 2), pOP854B (lane 3), pOP854E (lane 4), and pOP203-2 (lane 5) and *Eco*RI digests of pOP854B (lane 6) and pOP854E (lane 7) were analyzed on 1% agarose gels stained with ethidium bromide.

P22 tail gene, gene 9, expressed from two tandem *lacUV5* promoters (2), was included as template in this reaction. Proteins of the same molecular weight as coat, scaffolding, and tail proteins were synthesized in this reaction. Prohead assembly naive scaffolding protein, purified as described above, was added to this reaction as described in the legend to Fig. 3. A summary of the densitometer tracing of this gel is shown in Fig. 4. With increasing concentrations of purified scaffolding protein, there were decreasing levels of total protein synthesis. The reason for this is unknown. However, the synthesis of scaffolding protein was lowered to a greater extent and at a lower concentration of added scaffolding protein than was the synthesis of coat and tail proteins. These data indicate a specific negative effect of scaffolding protein on its own synthesis.

TABLE 1. Plating efficiency of P22 amber mutations of plasmid-containing hosts

Host	Titer of the following phage strain on the host/titer on strain DB7004			
	8⁻*am*N123	5⁻*am*N114	4⁻*am*H1368	1⁻*am*N10
DB7004	1.0	1.0	1.0	1.0
DB7000	2.6×10^{-6}	1.3×10^{-6}	9.3×10^{-6}	4.8×10^{-6}
DB7000/ pOP203-2	2.2×10^{-6}	1.7×10^{-6}	7.8×10^{-6}	5.8×10^{-6}
DB7000/ pOP854B	9.3×10^{-1}	6.0×10^{-1}	8.9×10^{-1}	6.2×10^{-6}

Since added scaffolding protein significantly increased the total protein concentration in the protein synthesizing mixture, the effect of an increased protein concentration per se was tested. Bovine serum albumin was added to the in vitro translation reaction at the same concentrations as was scaffolding protein in the legend to Fig. 3. Added bovine serum albumin had no effect on the synthesis of scaffolding protein or on protein synthesis in general (data not shown).

DISCUSSION

Our previous in vivo studies have indicated that unassembled scaffolding protein depresses the synthesis of additional scaffolding protein but lacks this activity when it is

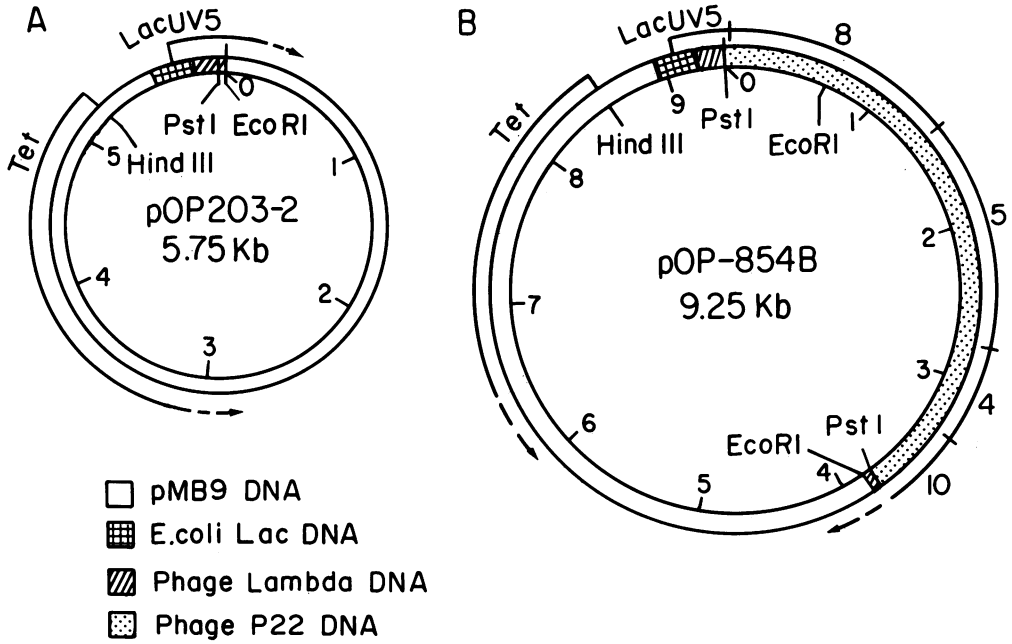

FIG. 2. Structure of the plasmids pOP203-2 and pOP854B. The source of the DNA fragments and the locations of various restriction endonuclease cleavage sites for plasmids pOP203-2 (A) and pOP854B (B) are shown. The small numbers around the outside are kilobase pairs.

complexed with the coat protein in the prohead (7, 24). In these in vitro studies, we show that the addition of unassembled scaffolding protein specifically depresses scaffolding protein synthesis in a manner consistent with the in vivo results. At a final concentration of added scaffolding protein of 1.76 mg/ml, the rate of synthesis of the coat, tail, and scaffolding proteins is lowered in this reaction by factors of 2.3, 2.2, and 17.4, respectively. We do not know if the activity that lowers the coat and tail protein synthesis is the scaffolding protein itself or a nondialyzable contaminant in the scaffolding protein preparation, but it has similar effects on coat and tail protein synthesis at all concentrations tested. The final change in the ratio of coat or tail protein to scaffolding protein synthesized is about eight to one.

The 17-fold depression in scaffolding protein synthesis is a minimal estimate. At high levels of added scaffolding protein, the scaffolding protein peak was difficult to distinguish from background in the densitometer trace. We measured the total area under the scaffolding protein peak for these lanes to give a maximum estimate of the scaffolding protein synthesized. We conclude that the purified unassembled scaffolding protein specifically depresses scaffolding protein synthesis in vitro and that scaffolding protein is autoregulatory.

In these experiments we are measuring the amount of protein accumulated during the translation reaction. Although we cannot rigorously eliminate the possibility that degradation is in someway involved, we believe this is unlikely for the following reasons. (i) This is an extremely stable protein in vivo. It does not detectably turn over during the course of a P22 infection. (ii) We have never observed any bands that appear to be candidates for degradation products. Such a band would be one not observed in the absence of template in Fig. 3 (lane 2), and if degradation were to affect the result of this experiment, it would have to increase with the concentration of added scaffolding protein. In this dark exposure of this gel, we should observe such a band if it represents a sizable proportion of the scaffolding protein synthesized.

The concentration of scaffolding protein required to observe depression of scaffolding protein synthesis is comparable to the concentration of unassembled scaffolding protein in vivo. In a P22 lysis-defective infection, ca. 500 phage and proheads are produced per infected cell. Each phage is made from ca. 400 molecules of coat protein (5). About 1/10 as many scaffolding protein molecules as coat protein molecules are synthesized, so ca. 2×10^4 scaffolding protein molecules are made per infected cell. In infections defective in coat protein synthesis, unassembled scaffolding protein is synthesized at about half the wild-type level (24), so there are ca. 10^4 molecules per cell. *E. coli* has an internal volume of ca. 0.6×10^{-15} liters (22), and the molecular weight of scaffolding protein is 39,000 (43). Thus, in infections defective in coat protein synthesis, scaffolding protein accumulates to a concentration of about 1.1 mg/ml, a value quite comparable to that required for modulation in vitro.

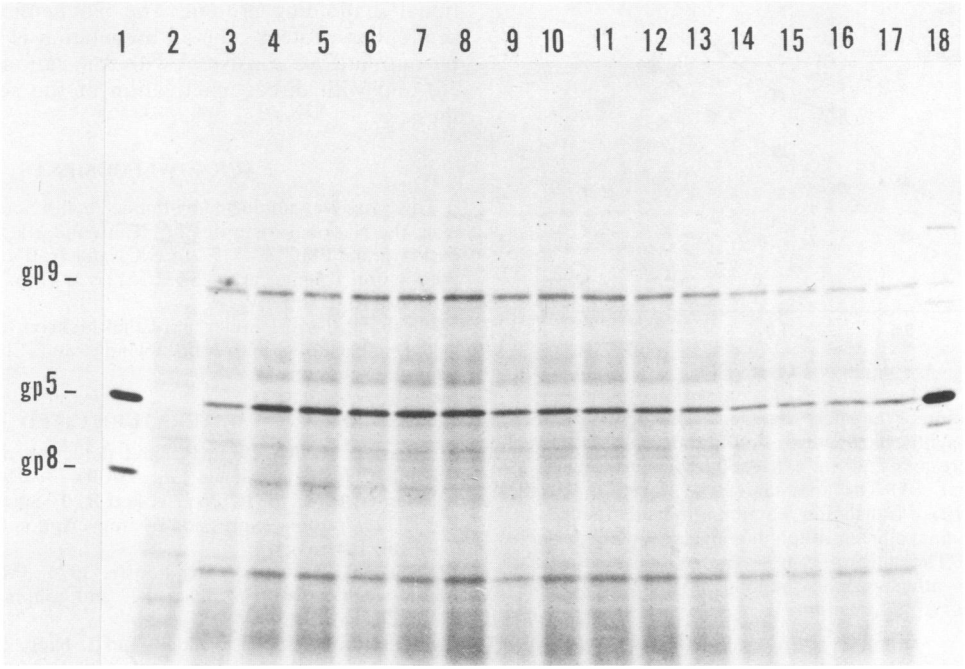

FIG. 3. Effect of added scaffolding protein on scaffolding protein synthesis in vitro. The [^{35}S]methionine-labeled products of in vitro translation were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by autoradiography. Molecular weight standards are [^{35}S]methionine-labeled proheads (lane 1) and phage (lane 18). The DNA templates were the plasmids pOP854B and pPB13, except for the no-DNA control (lane 2). Each reaction was performed in duplicate with final scaffolding protein concentrations (mg/ml) of 0 (lanes 4 and 5), 0.5 (lanes 6 and 7), 0.75 (lanes 8 and 9), 1.0 (lanes 10 and 11), 1.25 (lanes 12 and 13), 1.5 (lanes 14 and 15), and 1.76 (lanes 3, 16, and 17). The scaffolding protein band is somewhat broadened in this gel due to the high concentration of purified scaffolding protein in each lane. To make this effect the same in every lane, purified scaffolding protein was added to each sample after the translation, so that each had the same final concentration of scaffolding protein. The plasmid-specific band below scaffolding protein on the gel is encoded by pPB13, the tail gene clone, so it is not related to coat or scaffolding protein.

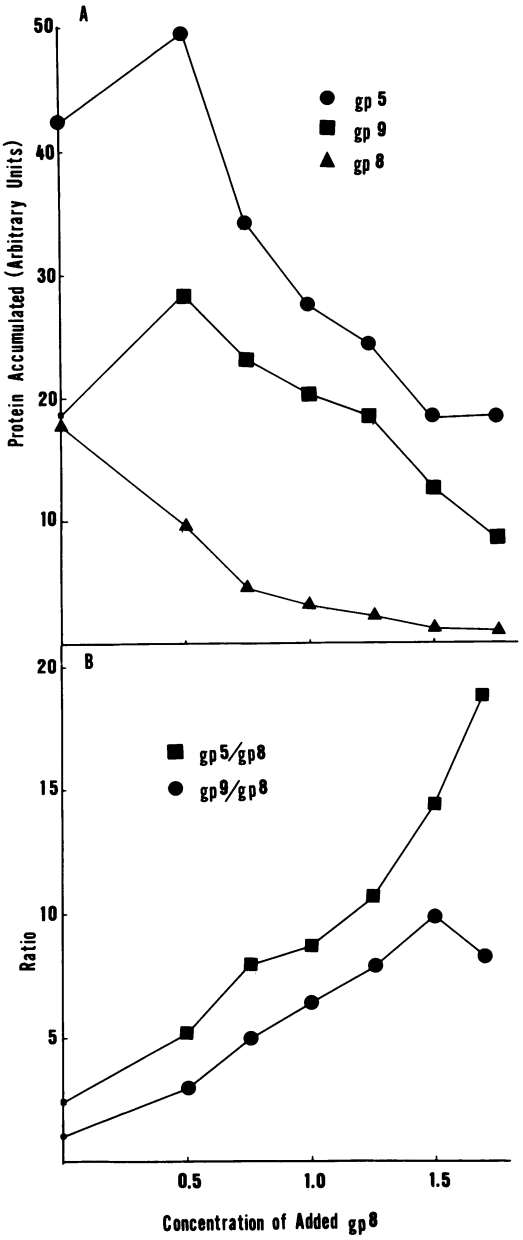

FIG. 4. Quantitation of the effect of added scaffolding protein on scaffolding protein synthesis in vitro. Autoradiograms of the gel shown in Fig. 5 were analyzed on a Joyce-Loebl densitometer as described in the text. (A) The area under the trace for specific proteins per microliter of translation reaction mix loaded on the gel is plotted versus the final concentration of added scaffolding protein in the reaction. (B) The ratio of coat or tail protein synthesis to scaffolding protein synthesis versus the final concentration of scaffolding protein added to the reaction.

Scaffolding protein is the only P22-coded protein added to the cell-free translation reaction. The coat protein and probably gp4 are also sythesized in vitro; however, their concentrations are extremely low compared with the in vivo levels. Although we cannot rigorously eliminate the possibility that these proteins are in some way involved, we conclude that scaffolding protein is almost certainly the only P22-coded protein required for scaffolding protein regulation. The S-30 extract used in this cell-free system was prepared from *E. coli* rather than *S. typhimurium*, the normal host of P22. Thus, if host factors are required for this autoregulation, they must also be present in *E. coli*.

The cloned P22 restriction fragment is the only template for scaffolding protein synthesis in the reaction. This fragment must contain all of the P22 DNA sequences required for autoregulation of the scaffolding protein gene. Although several lines of evidence indicate that the late promoter is the only promoter from which the scaffolding protein gene is transcribed during phage infection (6, 40), it is not necessary for autoregulation of the scaffolding protein gene. This together with the observation that regulation involves an alteration of the functional stability of the scaffolding protein message (6) is strong evidence that scaffolding protein regulation is posttranscriptional.

Autoregulation can fine tune the synthesis of a protein so that a sufficient amount, but not a large excess, is made (17). This has been observed in procaryotes at the transcriptional level, for example, the lambda cI gene (21) and the *E. coli lexA* gene (30), and at the posttranscriptional level, such as T4 gene 32 (27, 39) and *E. coli* ribosomal protein genes (29, 31). The normal function of these posttranscriptional autoregulatory proteins involves binding to nucleic acids. When the normal binding sites are saturated, any additional protein is thought to bind to the mRNA for that protein and inhibit its translation. At this time there is no evidence that scaffolding protein binds to nucleic acid during its normal function in head assembly. However, scaffolding protein gene autoregulation is analogous to these autoregulatory systems in that scaffolding protein molecules interact with the coat protein to form proheads, and any excess unassembled scaffolding protein modulates synthesis of additional scaffolding protein. The biochemical mechanism of scaffolding protein gene autoregulation is unknown, but so far our data are consistent with translational repression (16, 35) and with direct inactivation of the scaffolding protein message.

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