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An Immunoblot Assay Reveals that Bacteriophage T4 Thymidylate Synthase and Dihydrofolate Reductase Are Not Virion Proteins

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Numerous reports describe the phage T4 enzymes thymidylate synthase and dihydrofolate reductase as structural components of the baseplate. However, Y. Wang and C. K. Mathews (J. Virol. 63:4736–4743, 1989) reported that antisera against the respective recombinant enzymes failed to neutralize phage infectivity, in contrast to previous results. Moreover, a deletion mutant lacking the genes for these two enzymes adsorbed normally to host cells. Since these findings tended to undermine the idea of the two enzymes as structural proteins, we developed a quantitative immunoblot assay to resolve the issue directly. Our results show that both enzymes are present only as minor contaminants (<0.05 copy per phage) and as such cannot be bona fide structural proteins.

In the early 1970s, several lines of evidence indicated that the T-even bacteriophage-encoded enzymes dihydrofolate reductase (DHFR) and thymidylate synthase (TS) play structural as well as metabolic roles in the biology of the viruses. Following the discovery (18) that T4 tail baseplates contain a pteridine compound identified as dihydropteroylhexaglutamate, Kozloff et al. (15, 19) detected DHFR activity in disrupted preparations of phage ghosts, and Mosher et al. (28) showed even more activity in 11^- phage particles (i.e., virions from phage carrying an unsuppressed gene 11 mutation). Antiserum to purified T4 DHFR was found to neutralize phage infectivity (27), and evidence suggested that the virion form of DHFR was the target for this activity.

Comparable evidence was developed for T4 TS; phage were neutralized by a polyclonal antiserum against purified T4 TS (4), and enzyme activity was detected in virions (14). Genetic evidence also supported structural roles for these two enzymes; crossing the T6 phage structural gene for either DHFR (25) or TS (4) into a T4 background altered the thermostability of the recombinant phage virion, indicating that the phage *frd* and *td* genes, encoding DHFR and TS, respectively, encode determinants of a physical property of the virion.

Although it was realized that these two enzyme proteins might represent contaminants adventitiously bound to phage particles, the localization of the proteins to the same substructure that contains a bound pteridine, plus evidence that the virion proteins were targets for antibody neutralization or heat inactivation, suggested that the proteins played a specific structural role.

However, several other observations were less supportive of such a role. First, the DHFR activity in 11^- phage particles suggested a stoichiometry of less than one enzyme molecule per virion (28). Second, phage that were reported to bear deletions extending through the *td* and/or *frd* genes were still neutralized by antisera against either TS or DHFR (4, 28). Third, the viability of these deletions suggested that the structural role played by these proteins was nonessential, although the mutant phage grew poorly. Fourth, in radiolabeling studies designed to identify phage structural proteins that are synthesized early in infection (as are DHFR and TS), electrophoretic analysis of phage particles and tails revealed no early-labeled proteins with molecular weights corresponding to that of DHFR or TS (28).

In early work, both DHFR and TS were purified from T4-infected bacteria. More recently, Wang and Mathews (36) prepared an antiserum against recombinant T4 DHFR, a preparation containing no other T4 proteins. This antiserum had no phage-neutralizing activity, although the 15-year-old antiserum against nonrecombinant T4 DHFR had retained this activity. These observations suggested that the phage-neutralizing activity in the old antiserum was antibody to a contaminating phage protein, not DHFR. Since the antiphage activity of the DHFR antiserum was key evidence supporting a structural role for DHFR, the more recent results prompted a reexamination of the evidence relative to both enzymes, using a battery of research techniques more incisive than those available two decades ago.

In order for either enzyme to be a virion structural protein, it must be present to the extent of at least one molecule per phage. Since the stoichiometry of most of the other virion proteins is well established, we sought to relate their known copy numbers to those of the enzymes as a way of determining the levels of DHFR and TS in phage and tails. The assay that we developed uses a combination of immunoblots probed with antisera against the recombinant enzymes and the scanning of Coomassie blue-stained gels to determine DHFR and TS copy numbers. We demonstrate that the sera are specific, that the enzymes are both synthesized only early after infection, that they do not undergo any modifications during productive infection, and that their stoichiometry is well below the minimal level of one copy per virion. The assay can be used to determine the level of minor proteins in a variety of biological structures.

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TABLE 1. Phage strains

T4 mutant	Amber or deletion mutant	Structure produced
34 × 37	A455 × N52	Fiberless phage
23	B17	Tails
10	B255	Heads
10 × 21	B255 × (E322 × N121)	Prehead
td × *frd*	del.9	TS^- and $DHFR^-$ phage

MATERIALS AND METHODS

Bacterial and phage strains. In the following experiments, *Escherichia coli* B^e was used as a nonpermissive host strain to prepare T4 particles and substructures in culture. *E. coli* CR63 was used to grow stocks of T4 amber mutants. *E. coli* S/6 was used as the nonpermissive plating indicator for amber mutations. *E. coli* B40 Su^+ was used as the permissive (or amber suppressor) plating indicator. The amber and deletion mutants of phage T4D used in these experiments are listed in Table 1.

The del (63-32) 9 deletion mutant (10) (referred to here as del.9) grew well in CR63, and we obtained stock titers in excess of 10^{12} phage per ml with the single-plaque multicycle technique. CR63 host cells were grown to 4×10^8 cells per ml, chilled, harvested, and resuspended in 0.1 culture volume in chilled M9A. A single 4- to 5-h plaque grown at 37°C was cored with a Pasteur pipette, blown into a 500-ml flask containing 200 ml of CR63 at 2×10^7 cells per ml in M9A (8), and incubated at 37°C on a rotary shaker at 250 rpm. The virus titer rises exponentially and catches up to the cell titer in about 2 h. The cells then become superinfected and will not lyse for several hours. The time when the culture is completely infected is noted by taking a small aliquot of the culture and vortexing it with a few drops of $CHCl_3$. Ten to 15 min after all the cells are infected, the sample will clear with the added $CHCl_3$. After 2 to 3 h of additional incubation, DNase was added to 1 μg/ml for 5 min, and the cells were harvested without chilling in 250-ml bottles in a preparative centrifuge at 8,000 × *g* for 6 min at 4°C. The pellet was resuspended in 10 ml of buffer (B^+ plus gelatin) (8) with 5 μg of DNase per ml and lysed with $CHCl_3$. The cell debris was removed by a 12,000 × *g* centrifugation for 10 min, and the stock was titered and stored over $CHCl_3$.

Reagents. Purified recombinant TS and DHFR and their corresponding polyclonal rabbit antisera were prepared by the Maley laboratory (TS) (22) and the Mathews laboratory (DHFR) (31).

Preparation of extracts of mutant-infected cells. A series of dilutions of B^e cells was grown overnight in M9A medium in a shaker at 37°C so that one of the cultures was still in log phase by the next morning. This culture was diluted 100-fold in prewarmed M9A medium and grown to 4×10^8 cells per ml at 37°C on a rotary shaker. The cells were chilled to 4°C, harvested by centrifugation at 8,000 × *g* at 4°C in a JA-14 rotor (Beckman, Palo Alto, Calif.) for 7 min, and then resuspended in 4°C M9A medium to a concentration of 4×10^9 cells per ml. Five minutes before infection, the cells were rapidly mixed 1:1 (vol/vol) with phage in 4°C M9A at a multiplicity of infection of 5 so that the final concentration of cells was 2×10^9 cells per ml and that of phage was 10^{10} PFU/ml. To initiate infection (0 min), the culture was warmed in a 50°C water bath for 1 min and quickly transferred to a 37°C shaker. Superinfection at the same multiplicity of infection added in 0.1 culture volume occurred at 7 min postinfection (p.i.). At 55 min p.i., 3 μg of DNase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml was added, and after a further 5 min, the culture was chilled on ice and harvested by centrifugation at 8,000 × *g* at 4°C for 10 min. The viscous pellet was resuspended in 0.01 culture volume of G buffer (250 potassium glutamate, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ [pH 7.5]) containing DNase (10 μg/ml). For tail structures (23^- extracts), the resuspended pellet was lysed by three cycles of freeze-thawing (liquid nitrogen and 30°C water bath). For preheads ($10^- \times 21^-$), heads (10^-), fiberless phage ($34^- \times 37^-$), and phage (del.9) extracts, the resuspended pellet was lysed with $CHCl_3$. These preparations were clarified by pelleting at 12,000 × *g* for 8 min in a microcentrifuge.

Isolation of T4 particles and substructures. Various viral structures were isolated on linear 5 to 45% sucrose gradients containing 1.5× M9A salts, 1 mM $MgSO_4$, and 1.3 mM phenylmethylsulfonyl fluoride. The gradients were formed by mixing equal volumes of degassed 5 and 45% sucrose solutions in SW41 tubes with a rotational gradient former (Gradient Master; BioComp, Fredericton, New Brunswick, Canada) by rotating tubes 81.5° about their long axis for 1 min 16 s at 21 rpm (9). To obtain the different T4 particles, 0.4- to 0.6-ml samples were centrifuged through sucrose gradients at 40,000 rpm and 15°C in a SW41 rotor in an L5-75 ultracentrifuge (Beckman) for 1,000 $\omega^2 t$ for phage and heads, 4,000 $\omega^2 t$ for preheads, and 5,000 $\omega^2 t$ for tails, respectively. Bands of phage structures were visualized by scattered light and removed from the gradients by using a piston fractionator (6).

SDS-PAGE. The procedure used was based on the method of Laemmli (21), with the following modifications. Samples from gradients were chilled and then precipitated with 0.1 volume of 100% (wt/vol) trichloroacetic acid. After being washed twice with 0.02 M Tris-HCl (pH 6.8) in 90% acetone, the pellets were air dried and stored at minus 20°C. Samples were prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by resuspending each pellet in 1.0 ml of Laemmli buffer and heating the pellets at 90°C in a water bath for a total of 10 min with vortexing at 2-min intervals.

The protein concentrations of TS and DHFR samples were determined by the method of Bradford (3), using hen egg lysozyme as a standard. The undiluted stock (1:1) solutions were 81 μg/ml for TS and 30 μg/ml for DHFR. In each case, 30 μl of each sample and the subsequent dilutions were run in each lane of the gel. Electrophoresis was performed in a stacking gel measuring 1 mm by 20 cm by 1.5 cm and a separating gel measuring 1 mm by 20 cm by 10 cm. Stacking and separating gels were 4 and 12.5% acrylamide, respectively. The separating gel contained 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. Gradient SDS-polyacrylamide gels were prepared as described above by using a 10 to 15% acrylamide gradient separating gel.

The gels were run at a constant voltage of 75 V for 1 h during stacking and for 4 to 5 h at 150 V until the dye reached the bottom. The gels were fixed in sulfosalicylic acid-trichloroacetic acid, stained for 24 h with 0.1% Coomassie blue R-250 in destain solution (4% acetic acid, 25% ethanol), and destained as described by Bjellgvist and Ek (2) or transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore, Bedford, Mass.) as described below.

Densitometric analysis. The fixed and stained gels were scanned with a GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, Calif.). The data were captured and analyzed by using Maxima 820 software (Waters, Milford, Mass.) connected to the densitometer. Peak areas were averaged from two or three scans.

Immunoblot analysis. Immunoblot analysis was carried out essentially as described by Towbin et al. (34) except that PVDF membranes were used instead of nitrocellulose membranes. The membranes were prepared for electroblotting according to the manufacturer's instructions. Briefly, they were wetted in absolute methanol for 1 min and then equilibrated in a transfer buffer (25 mM Tris-HCl, 192 mM glycine [pH 8.3]) and placed on the top of the separating gel as soon as electrophoresis was complete. The proteins were then transferred to the filter in a Bio-Rad liquid TransBlot Cell containing 4°C transfer buffer (15.6 mM Tris, 120 mM glycine [pH 8.3]) at a constant voltage of 90 V for 3 h. The temperature of the transfer buffer was kept lower than 14°C during electrotransfer. Once electrophoretic transfer was completed, the PVDF membrane was air dried at room temperature for 4 h and stored at 4°C.

The PVDF blots containing separated T4 proteins were wetted in absolute methanol for 1 min and then immersed in TBST (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 10 min. The membranes were then probed for 1 h at room temperature in a roller bottle apparatus (Navigator; BioComp) with rabbit antisera against TS and DHFR appropriately diluted in TBST (the dilutions of antisera against TS and DS were 1:2,000 and 1:3,000, respectively). The blots were washed three times for 15 min each with TBST and then incubated for another hour with alkaline phosphatase-conjugated anti-rabbit antibody (Bio/Can Scientific Inc., Mississauga, Ontario, Canada) diluted 1:5,000 in TBST. After three additional 15-min washes with TBST, the PVDF membranes were incubated with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP) substrate (Bio/Can Scientific Inc.) diluted in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$) until the desired color intensity was reached. The reaction was stopped by rinsing the membranes in deionized water; the membranes were air dried at room temperature and stored in the dark.

Preparation of [^{35}S]methionine-labeled lysates of mutants. B^e cells in M9A medium were infected and superinfected as described previously. [^{35}S]methionine (40 mCi/mmol) was added at 10 min p.i. to a final concentration of 5 μCi/ml. At 60 min p.i., the culture was harvested and lysed, and then labeled phage particles were isolated as described above for unlabeled lysates. Labeled samples were mixed with liquid scintillation cocktail (Ready Protein; Beckman), and the radioactivity was measured in a Beckman LS 6000 LL or LS 7000 scintillation counter.

RESULTS

Development of an immunoblot assay for viral protein copy number. The identification of viral proteins as bona fide structural parts of the virion is an important and recurring problem, especially as we seek to identify the role of minor virion components. Without regard to constraints of symmetry, we set as our minimum criterion for structural proteins a stoichiometry of one molecule per virion. As a corollary, ruling out a structural role for a protein requires the demonstration that it has a stoichiometry of substantially less than one.

We examined several methods that we hoped would provide the sensitivity required for this determination. Radioisotope labeling of viral proteins and quantitation following SDS-PAGE by autoradiography or direct scintillation counting of the blotted proteins was rejected because it required that we positively identify the proteins in an area of the gel crowded

with other more abundant virion species. Immunoblotting was more promising because it was targeted to the species in question, but possible cross-reaction with other virion species and sensitivity to the target proteins adventitiously bound to the virion were still potential problems. We felt, however, that these reservations could be overcome if we could develop a quantitative immunoblot assay that would place an upper limit on the stoichiometry of the proteins in question that was far short of the required level of one copy per virion.

The assay that we developed involves the following steps. (i) Diluted aliquots of the virus and the purified protein of interest are electrophoresed on the same gel and stained with Coomassie blue. The gel is then scanned with a densitometer, and the peak areas for the standards and one or more virion proteins whose copy number is known are determined. (ii) An identical gel is run, and the proteins are transblotted to a PVDF membrane. The blot is exposed to antiserum against the protein, and the bands are stained with second antibody and dye. (iii) One then visually examines the immunoblot for a band in the dilution series of pure protein that matches the intensity of the stained band in the one of the neighboring virion protein lanes. This establishes the total mass of the protein in the virion. (v) Working backwards using the peak areas from the scanned gel, one can then determine the copy number of the unknown protein in the virion.

In practical terms, the following procedure is followed. We choose one of the virion lanes in the Coomassie blue-stained gel that has the best band separation and staining characteristics for the virion proteins that will be used as standards. In our case, for example, we know that gene product 19 (gp19) is present in 144 copies per virion (1).

The peak area (A) of the known protein is the product of its molecular weight (MW), its copy number (Cn), and a constant (K), which is the response of the measuring system to a given mass of protein. Thus,

$$A = \mathrm{MW} \times \mathrm{Cn} \times K\,(1) \quad (1)$$

Solving for K,

$$K = \frac{A_1}{\mathrm{MW}_1 \times \mathrm{Cn}_1} \quad (2)$$

Looking across to the adjacent dilution series of the unknown protein, we choose a dilution with the same approximate band intensity and peak area as our known virion proteins. If we know the molecular weight of the unknown and its peak area, we can solve its copy number by using the following formula, where A_1, MW_1, and Cn_1 are the virion standard and A_2, MW_2, and Cn_2 are the unknowns.

$$K = \frac{A_1}{\mathrm{MW}_1 \times \mathrm{Cn}_1} = \frac{A_2}{\mathrm{MW}_2 \times \mathrm{Cn}_2} \quad (3)$$

Solving for Cn_2, the copy number of our unknown, we obtain

$$\mathrm{Cn}_2 = \frac{A_2 \times \mathrm{MW}_1 \times \mathrm{Cn}_1}{\mathrm{A}_1 \times \mathrm{MW}_2} \quad (4)$$

We now know the copy number of the unknown band in lane X of the dilution series. In other words, if a band of that intensity and peak area appeared in the virion, it would have this copy number.

We now focus our attention on the immunoblot. Assuming that the virion lane contains an immunostained band with the same mobility as our unknown (TS or DHFR), we compare the intensity of the virion band with the dilution series of TS or DHFR standards and select the dilution which is one step

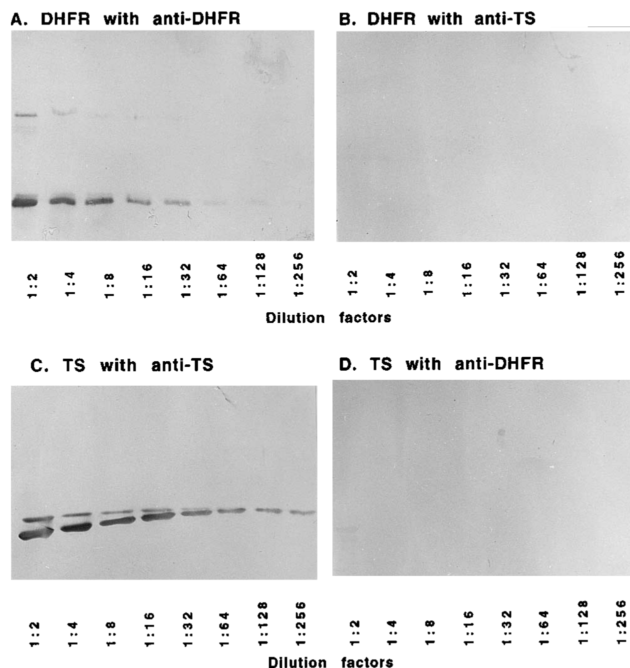

FIG. 1. Specificity and sensitivity of the anti-TS and anti-DHFR sera. TS and DHFR stock solutions were electrophoresed at the indicated dilutions and transblotted to a PVDF membrane. The blot was then probed with the indicated polyclonal antiserum and stained. (A) DHFR dilutions probed with anti-DHFR serum. (B) DHFR dilutions probed with anti-TS serum. (C) TS dilutions probed with anti-TS serum. (D) TS dilutions probed with anti-DHFR serum.

more intense than the staining in the virus band. Knowing the dilution factor between the unknown whose peak area and copy number were measured on the Coomassie blue-stained gel and the dilution of the unknown matching the virion in the immunoblot, one can calculate the real copy number in the virion, Cn_v. For example, if the (1:1) dilution in the Coomassie blue-stained gel has a copy number of 36 compared with the virion standard, and the (1:512) dilution on the immunostain gel matches the intensity of the same band in virions, then the copy number of the protein is 36/512 = 0.07. Thus,

$$\mathrm{Cn}_v = \frac{\mathrm{Cn}_2}{\mathrm{dilution}} \quad (5)$$

Establishing the specificity and sensitivity of the antisera. In the present example, we have used this procedure to calculate the copy numbers of TS and DHFR in phage T4 virions. We began by establishing that the enzyme standards were sufficiently pure and that the polyclonal antisera had the desired specificity. Figure 1 demonstrates that over a wide range of enzyme dilutions, the antisera do not cross-react with the nontarget enzyme. This specificity allowed us to combine the two antisera for many of our assays, permitting us to conserve reagents without sacrificing resolution. The double band at high TS concentrations in Fig. 1 was not observed on other blots (see Fig. 3) and probably arose through a shift in the gel during blotting.

In Fig. 2A, we use the combined antisera to probe gels of T4-infected cells at various times after infection to observe whether TS and DHFR behaved as early proteins and whether they underwent any modifications (cleavages or fusions) during the course of infection. By comparison with the del.9-infected cells, which lack both genes, one can observe that the enzymes

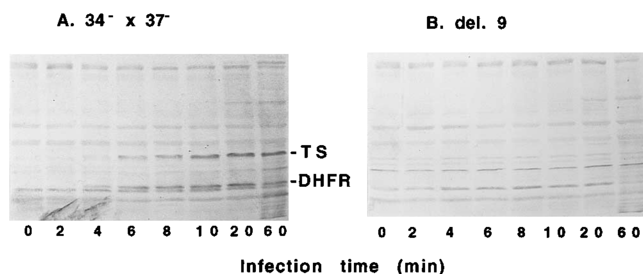

FIG. 2. Immunoblot analysis of TS and DHFR production during infection. B^e cells were infected at 37°C with $34^- \times 37^-$ (A) or del.9 (B) phage, and 0.5-ml samples were withdrawn at the indicated times. The cells were treated with EDTA (0.1 mM) and NaN_3 (1 mM) and lysed by the addition of 3.75% SDS on ice for 10 min. Forty-microliter aliquots were mixed with an equal volume of Laemmli buffer, boiled, and electrophoresed on a 10 to 15% gradient minigel. The gels were transferred to a PVDF membrane and probed with combined TS and DHFR antisera.

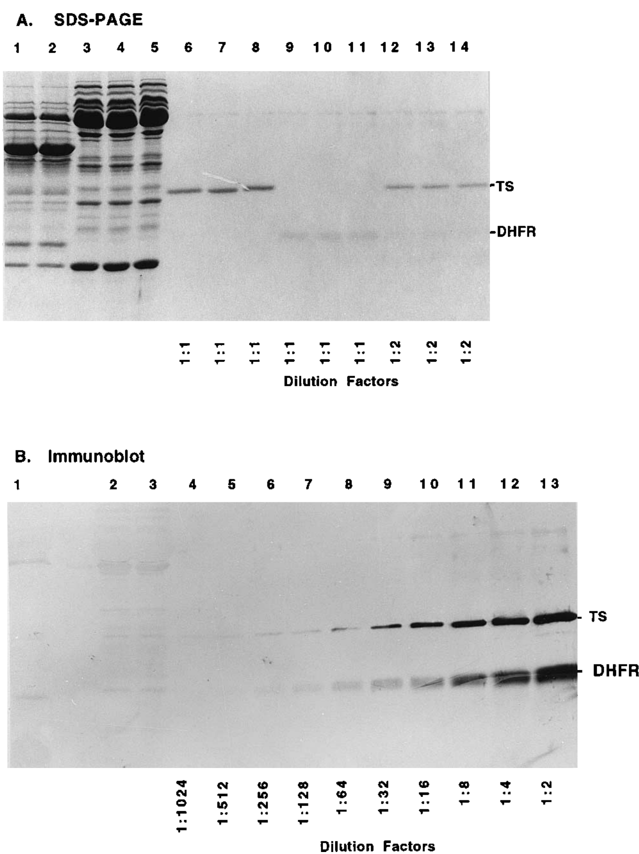

FIG. 3. The copy number assay. (A) Coomassie blue-stained gel. Lanes 1 and 2, $34^- \times 37^-$ fiberless phage; lanes 3 to 5, 23^- tails; lanes 6 to 8, undiluted TS stock; lanes 9 to 11, undiluted DHFR stock; lanes 12 to 14, a 1:1 mixture of the two enzymes (a 1:2 dilution). (B) Immunoblot. Lane 1, $34^- \times 37^-$ fiberless phage as in lane 1 of panel A; lanes 2 and 3, 23^- tails as in lanes 3 to 5 of panel A; lanes 4 to 13, twofold dilution series of the TS-DHFR mixture shown in lanes 12 to 14 of panel A. After transfer to a PVDF membrane, the membrane was probed with combined TS and DHFR antisera.

are detectable between 2 and 4 min p.i. and appear to reach maximal levels at 10 min at 37°C. No shifts are seen in intensity or mobility for the duration of infection, in agreement with findings of Mosher and Mathews (29). The absence of new phage-encoded proteins in the del.9 samples (Fig. 2B) demonstrates the specificity of the sera. The stained proteins near the positions of TS and DHFR are the homologous enzymes from the host *E. coli* (data not shown).

Determining the TS and DHFR copy numbers. Once we had established the specificity of our antisera, we then ran and developed the SDS-polyacrylamide gels for the copy number assay (Fig. 3). The Coomassie blue-stained gel is shown in Fig. 3A. Lanes 1 and 2 depict fiberless phage, while lanes 3 to 5 are replicates of gradient-purified tails. The remaining lanes are the standards for TS and DHFR at the indicated dilutions.

Figure 3B shows an immunoblot of the same fiberless phage and tails and a dilution series of the two enzymes probed with the combined antisera. Examination of the blot reveals that a band in the tail lanes migrating at about the same position as TS matches the intensity of the pure TS standard somewhere between the 1:512 and 1:256 dilutions. Although DHFR appears to have a comigrating band in the tail samples, close examination shows that the tail band (likely gp11) is slightly above DHFR. Thus, tails lack detectable levels of DHFR. Table 2 shows the copy numbers of the known phage proteins gp9 and gp19 and their areas obtained from the scan of the Coomassie blue-stained gel in Fig. 3A, lanes 3 to 5. Also shown are the areas of the TS and DHFR bands scanned from Fig. 3A, lanes 6 to 8 and 9 to 11, respectively. It is apparent from the immunoblot (Fig. 2B) that virtually all tail proteins cross-react with the antisera at the high concentrations used, and so the copy numbers that we obtained are almost certainly overestimates. For DHFR, the lack of a visible band on the immunoblots at the appropriate position puts its copy number in tails as less than the weakest visible immunoblot band in the dilution series (1:512), giving a maximal value of less than 0.01 copy per tail. The calculated value of 0.04 copy for TS is well below any reasonable threshold for a copy number of 1.

We were puzzled by the reproducible observation that fiberless phage contained significant amounts of what appeared to be DHFR (Table 2). This finding implies that either there is a cross-reacting species unique to heads, the enzyme is contaminating the phage preparations, or perhaps DHFR resides in the head rather than the baseplate.

We addressed the problem two ways. First, we examined the possibility that DHFR was cosedimenting with phage nonspecifically. Fiberless phage were isolated from a sucrose gradient along with areas of the gradient immediately above and below the band. The purified phage were dialyzed and rerun on a second gradient, and the same fractionation was carried out. If the enzyme was simply a contaminant, we should expect to see less of it in the second gradient (Fig. 4).

For reactive proteins with the mobility of TS, there was a dramatic reduction in the second gradient, with virtually no signal now present (Fig. 4, lanes 2 to 4). For DHFR, however, the signal in the second gradient remained and was found only in the phage and not in fractions immediately above or below (Fig. 4, lanes 2 to 4).

Since we had been using combined antisera for these blots, we used separate serum in an additional test to determine which one was the source of the signal. Figure 5 shows the two antisera used as probes of fiberless phage. While anti-TS sera shows a slight reaction at the position of gpIPIII*, most of the signal here arises from the anti-DHFR serum.

The issue was finally resolved by using a gradient gel to separate the cross-reacting species from DHFR prior to blotting. We also analyzed phage lacking TS or DHFR isolated from the del.9 deletion mutant of Homyk and Weil (10), as well as preheads containing uncleaved head proteins isolated from a $21^- \times 10^-$ infection. Figure 6A shows the autoradiograph of the labeled structures as isolated from gradients.

TABLE 2. Copy number calculations for TS and DHFR

Gene product	Mol wt[a]	Peak area (μV · s)		No. of copies/virion[b]	
		Tails	Phage	Tails	Phage
9	30,997	93,145 ± 9%	10,928 ± 5.3%	18	18
19	18,462	386,566 ± 11%	47,273 ± 5.6%	144	144
TS	33,073	55,215 ± 13%	55,215 ± 13%	0.04 ± 17% (gp19) 0.04 ± 16% (gp9)	0.19 ± 14% (gp19) 0.17 ± 22% (gp9)
DHFR	21,713	8.897 ± 21%	8.897 ± 21%	<0.01 (gp9, gp19)	0.38 ± 22% (gp19) 0.34 ± 22% (gp9)

[a] From Coombs and Arisaka (7).
[b] The value of 18 for gp9 derives from crystallographic studies of Strelkov et al. (33) on purified gp9 and from our laboratory (9a) on intact phage. For TS and DHFR, the copy numbers (means and standard deviations) have been calculated on the basis of gp19 or gp9. The values shown are upper limits and do not reflect the likelihood that the bands identified in tails and phage as TS or DHFR are not these species but rather cross-reacting phage proteins (see text). The values presented for tails and phage are based on the intensity of Coomassie blue and immunoblot staining of gp9 and gp19 in the tail and phage lanes in Fig. 3A and B compared with the TS and DHFR standards as described in the text.

Unlabeled DHFR and TS were added to odd-numbered lanes for the immunoblot and do not appear on the autoradiograph. In Fig. 6B, the immunoblot of the same gel clearly shows that the band observed in phage probed with anti-DHFR serum is the head protein IPIII*, the cleaved form of IPIII, and not DHFR. In samples containing IPIII*, there is a separate band just below the added DHFR. Interestingly, in uncleaved preheads this band is absent. The del.9 phage lacking TS and DHFR show the same immunoblotting pattern as the fiberless phage controls, indicating that all the immunoblot bands were cross-reacting species and that there is no DHFR or TS in phage or in tails. From these analyses, we conclude that TS and DHFR are not structural virion proteins.

DISCUSSION

The immunoblot copy number assay. We have developed a method for determining whether minor virion proteins are structural. It first establishes quantitative linkages between virion proteins of known copy number and the purified protein in question on Coomassie blue-stained gels. The process is then reversed on an immunoblot by comparing a dilution series of the immunostained pure protein with the staining intensity of the unknown species in purified virion structures. As can be seen in Fig. 3 and Table 2, the data obtained are sensitive to the presence of cross-reacting species observed when the gels are overloaded with sufficient sample to detect substoichiometric levels of the minor protein. Under these conditions, most viral proteins show some level of reactivity with the sera, but by calculating the copy number, one can determine whether a putative species exceeds the minimum required level of one copy per virion. In the examples presented here, the values we obtained for TS and DHFR are so low that they rule out any structural role for the enzymes in the virion, demonstrating the ability of the assay to discriminate between cross-reacting species and true structural proteins.

Whether the assay could have discriminated between minor species if the copy number had been closer to 1 or whether it could accurately measure copy numbers above that threshold is an open question. There are two potential sources of error that would need to be eliminated.

The first involves K (see equation 2). We have assumed that the sensitivity of our measurements is constant for a given mass of protein. The standard virion proteins, gp9 and gp19, gave ratios of peak areas and calculated masses that are well correlated (1:4.15 for peak area; 1:4.77 for total mass for gp9: gp19). However, the response to DHFR and TS was less well correlated. Loading a mass ratio of 1:2.7 (TS:DHFR) as measured by the Bradford (3) Coomassie blue assay gave a peak area ratio of 1:6.2, a twofold discrepancy. The discrepancy could arise from impurities in the enzyme preparations or from partial breakdown of the polypeptides during prolonged storage. Whereas the gel assay looks at only full-length polypeptides of the protein in question, the Bradford method measures all polypeptides in the solution.

There is also ample evidence of differential staining of different proteins and of nonlinear responses caused by time-dependent diffusion and protein-dye colloid formation in more concentrated areas of the band (30). Thus, in order to calculate

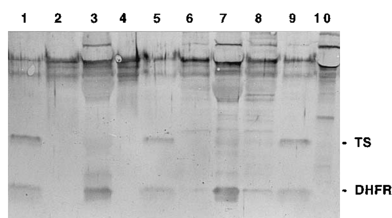

FIG. 4. Two-step purification of phage and its impact on the reaction to TS and DHFR antisera. Fiberless $34^- \times 37^-$ phage were purified through two serial sucrose gradients. Following the first gradient, the phage band was dialyzed and resedimented through a second identical 5 to 45% sucrose gradient. The fractions were precipitated with trichloroacetic acid, electrophoresed, and transblotted to a PVDF membrane, and the blot shown was probed with anti-TS and anti-DHFR sera as in Fig. 3. Lanes 1, 5, and 9, controls of 1:128-diluted enzymes as in Fig. 3; lanes 2 to 4, the second gradient of fiberless phage, sampling below, in, and above the phage band, respectively; lanes 6 to 8, the first gradient, sampling below, in, and above the band; lane 10, 23^- tails.

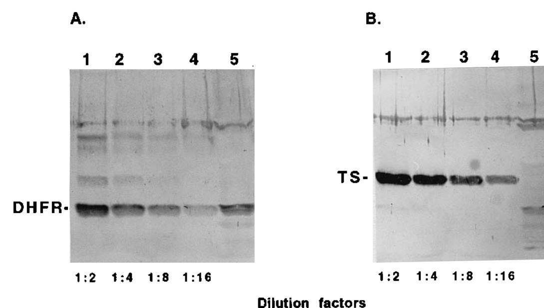

FIG. 5. Analysis of the immunoblot signal observed in phage. Immunoblots of purified enzymes and $34^- \times 37^-$ phage probed with separate anti-DHFR (A) and anti-TS (B) sera. Lanes 1 to 4 contain both TS and DHFR at the indicated dilutions as in Fig. 3; lanes 5 contain gradient-purified $34^- \times 37^-$ fiberless phage.

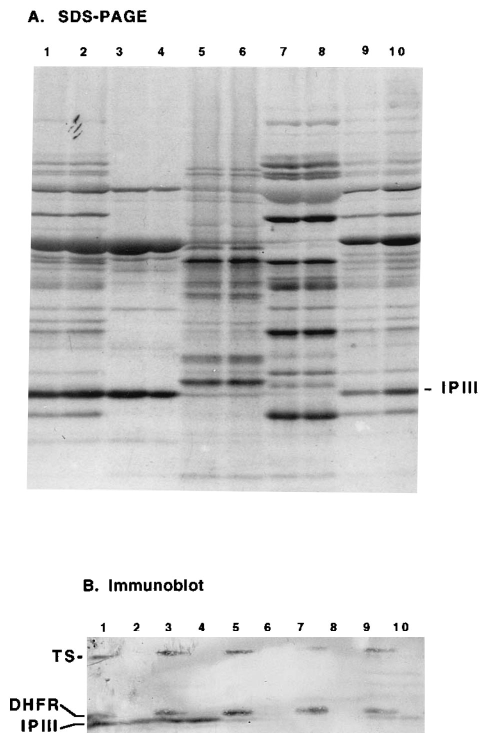

FIG. 6. IPIII* cross-reacts with the anti-DHFR serum. Various radiolabeled, gradient-purified phage structures were analyzed by gradient (10 to 15%) gel electrophoresis and transferred to a PVDF membrane for autoradiography and immunoblot staining. Odd-numbered lanes contain added TS and DHFR at a 1:64 dilution as controls. (A) Autoradiograph of the PVDF membrane; (B) immunoblot of the same gel in the area of interest. Lanes 1 and 2, $34^{-} \times 37^{-}$ fiberless phage; lanes 3 and 4, 10^{-} heads; lanes 5 and 6, $10^{-} \times 21^{-}$ preheads; lanes 7 and 8, 23^{-} tails; lanes 9 and 10, del.9 phage.

copy number with precision, one should ensure that each protein is electrophoretically pure and know the relative *K* value for each protein in the assay (30). We obtained a linear response for peak areas of scanned dilutions of TS ($r^2 = 0.997$) and a near-linear response for DHFR ($r^2 = 0.981$) that overlapped the areas of gp9 (not shown), so the determination of *K* for each protein is possible. The real difficulty in the determination of absolute copy numbers, however, is the absolute measure of protein mass for the virion standards and the unknown. The Coomassie blue-based Bio-Rad protein assay shows fivefold variation in the response to a fixed quantity of a variety of proteins, and similar problems have been documented for the Lowry assay (3, 23). The dry weight of the protein would seem to be the best measure but this is usually difficult to determine.

Increased precision could also be achieved by scanning the immunoblot with a reflectance scanner to quantify the stained bands to generate actual copy numbers rather than upper limits, as in the current assay.

Even without these improvements, however, the assay is capable of determining whether a protein of interest is present in substoichiometric amounts. In the current examples, the possible 2-fold error in the assay is completely overwhelmed by the 25- and 100-fold increases for TS and DHFR needed to reach the minimal copy number of 1.

Structural roles for TS and DHFR reconsidered. Considering their roles in phage metabolism, DHFR and TS are unlikely candidates for structural roles. Both are early proteins involved in nucleotide metabolism, whose synthesis ceases at 10 min p.i. as their early promoters shut down during the programmed shift to middle and then late protein synthesis (references 24 and 35 and this study). While there are examples of structural proteins which are expressed early (gp*alt* and gpIPIII), these head genes have late promoters to extend their synthesis and provide sufficient quantities for virion assembly (see references 5 and 32 for reviews). Thus, the TS and DHFR genes have been the only strictly early genes reported to have structural roles.

The genes for these enzymes are dispensable in phage infection since they duplicate the functions of the host enzymes that they closely resemble (26). Deletion mutants of these two genes are viable in the laboratory strains of *E. coli* (10), and so they, like many other T4 genes, are carried in the genome to extend the phage's host range in bacteria lacking compatible enzymes.

Despite these indications to the contrary, other evidence gathered over the years suggested that TS and DHFR were part of the T4 baseplate. The germinal finding that eventually led to their proposed structural role was that virions contained a pteridine compound, dihydropteroylhexaglutamate (16). The presence of this unusual molecule in the T4 baseplate provided the rational framework for the enzymes' presence there: one could envisage TS binding the polyglutamyl end of the folate and DHFR binding the pteroyl end and these links helping to organize the six wedges around the central hub (12).

There was also physical and genetic evidence that the enzymes were structural (see reference 13 for a review). Enzymatic activity was found to be associated with the virion and specifically with the baseplate, antisera against highly purified enzymes inactivated phage, and genetic chimeras of T4 containing only the T6 *frd* gene had the same virion heat inactivation kinetics as T6.

While space does not permit critical evaluation of each of these experiments, it can be said that they shared one potential problem: all measured the presence of the enzymes indirectly.

The availability of clones producing these two enzymes has made it possible to do a direct assay since we can now synthesize single virus proteins devoid of cross-reacting virion species and raise antisera against them. Wang and Mathews (36) first used these immunoglobulin reagents to show that phage were not inactivated by anti-TS and anti-DHFR sera and that the deletion mutants lacking the genes for these enzymes and wild-type virus adsorbed to cells with identical kinetics. We have extended the use of the reagents to determine the maximum copy number and show that neither enzyme is structural. For DHFR, our inability to detect any comigrating band in the immunoblots places its highest copy number in tails below 0.01 per phage. Initial results showing that phage and heads had a much higher copy number were clarified by gradient gel analysis that revealed that IPIII* cross-reacted with both antisera.

Both sera stained virtually all tail proteins to some extent at the high loading levels needed to maximize our sensitivity. Thus, it is not surprising that the anti-TS serum stained one of the many tail proteins that migrate in its molecular weight range (Fig. 3). However, the copy number of 0.04 per phage determined for TS shows the value of the assay in discriminating between structural and nonstructural proteins.

In the light of these findings, it is now possible to address the question of whether other minor proteins involved in baseplate assembly, such as gp51, gp26, and gp28, are also structural (12, 17, 20) or catalytic as originally proposed by Kikuchi and King (11) (see reference 7 for a full discussion). None have been directly detected in phage, and the evidence supporting their structural role is similar to the indirect evidence developed for TS and DHFR. We have cloned these genes and are currently

developing the required immunoreagents. The assay should be of use in this and other systems in which the role of minor proteins is under investigation.

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