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New Late Gene, *dar*, Involved in the Replication of Bacteriophage T4 DNA

II. Overproduction of DNA Binding Protein (Gene 32 Protein) and Further Characterization

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We have previously shown that the arrested DNA synthesis of mutants defective in T4 phage gene 59 can be reversed by a mutation in *dar*. In this paper, we have examined the effect of the *dar* mutation on the kinetics of gene 32 protein (DNA binding protein) synthesis, DNA packaging, progeny formation, and several other processes. Several lines of evidence are presented showing that the regulation of synthesis of gene 32 protein is abnormal in *dar*1-infected cells. In these cells, gene 32 protein, an early protein, is also expressed late in the infectious cycle. Our data also indicate that the packaging of DNA into T4 phage heads is delayed in *dar* mutant-infected cells, and this in turn results in a 6- to 8-min delay in intracellular progeny formation, although the synthesis of late proteins appears to be normal, as shown by gel electrophoresis. We have also studied the phenotypes of the double mutant *dar-am*C5 (gene 59). The increased sensitivity to hydroxyurea caused by a mutation in the *dar* gene can be alleviated by a second mutation in gene 59, but an increased sensitivity to UV irradiation caused by a mutation in gene 59 cannot be alleviated by a second mutation in the *dar* gene. Therefore, the double mutant still exhibits abnormalities in the repair of UV lesions.

Virus-coded proteins are synthesized immediately after the infection of *Escherichia coli* with T4 phage (10). These proteins can be divided into early, quasilate, and late according to their function and time of expression during the infectious cycle (5, 10, 28, 29). Evidence indicates that their expression is well controlled; early and quasilate genes are transcribed in a counterclockwise direction, whereas late genes are transcribed in a clockwise direction (13).

Gene 32 protein is an early protein that has been isolated by Alberts and Frey (1). They have shown that this protein preferentially and cooperatively binds to single-stranded DNA (1). The importance of the gene 32 protein in replication, UV repair, and recombination of DNA has also been shown (1, 35); however, the precise mechanism by which synthesis of this protein is regulated remains obscure, although autoregulation has been reported (18, 31).

A mutation in gene 59 of phage T4 results in arrested DNA synthesis soon after initiation (38). We have isolated suppressors of the gene 59-defective mutant, referring to them as *dar* (DNA-arrested restoration) (36). *dar*, a late mutant, specifically restores arrested DNA synthesis caused by a mutation in gene 59, but the biological function of *dar* and its biochemical role in DNA synthesis are still unknown. In this paper, we report our findings pertaining to the expression of the *dar* mutant, especially those relating to regulation of gene 32 protein synthesis, mechanisms of delay in progeny formation, and other related phenotypes.

MATERIALS AND METHODS

Bacteria and bacteriophage. *E. coli* K strain CR63, which carries *su-1*$^+$, was used as a permissive host for T4 amber (*am*) mutants. *E. coli* B strain Tr201, a low-thymine-requiring strain, obtained from G. R. Greenberg, was used as a nonpermissive host for T4 amber mutants in [^{3}H]thymine incorporation experiments. *E. coli* BO21 was used as a nonpermissive host in the determination of burst size and in the preparation of ^{14}C-labeled T4 proteins.

Bacteriophage T4D was used as the wild type in all experiments. T4 mutants used, all of which were derived from T4D, are listed in Table 1. The gene 59 mutant (*am*C5), purified by backcrossing five times with the wild type and used to isolate new *dar* mutants, was originally obtained from R. S. Edgar. Five *dar* mutants (*dar*1 to *dar*5) were isolated in our laboratory. Mutants *dar*1 and *dar*2 have been described previously (36). The other new *dar* mutants

TABLE 1. *T4 mutants used*

Mutant	Gene	Mutant phenotype or known product
*am*C5	59	DNA arrested synthesis
*am*N82	44	No DNA synthesis
*am*N122	42	dCMP hydroxymethylase
*am*B17	23	Major head component
*am*N131	26	Base plate formation blocked
ym	*y*	UV sensitive, recombination defective
v	*v*	UV sensitive, endonuclease V
*dar*1	*dar*	Restoration of DNA arrest phenotype and burst size of gene 59 mutants
*dar*2	*dar*	
*dar*3	*dar*	
*dar*4	*dar*	
*dar*5	*dar*	

were recently isolated using the same procedure from *am*C5 (gene 59) stocks described above. All *dar* mutants have been backcrossed with the wild type five times. Complementation tests in the presence of 20 mM hydroxyurea (HU) (*dar* mutants are sensitive to Hu) suggest that they are located in one gene. Late amber mutants were provided by W. B. Wood. Mutants *y* and *v* were obtained from K. Ebisuzaki. Mutant *y* has been reported to contain other silent mutations (14). To remove these silent mutations, we backcrossed *y* with wild-type phage twice and obtained *ym* thereby (14).

Media. 1 × C medium (35) was used in [^{3}H]thymine incorporation experiments. The medium containing thymine was named 1 × CT$^+$; 1 × CT$^-$ indicates that no thymine was present. 3 × D medium (36) was used in preparing bacteriophage lysate. M9 medium was used in the preparation of ^{14}C-labeled T4 proteins. One liter of M9 medium contained: 7 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 24.7 mg of $MgSO_4$, 14.7 mg of $CaCl_2$, and 4 g of glucose.

Measurement of the kinetics of DNA synthesis. The procedure for measurement of DNA synthesis kinetics has been described previously (39).

SDS-polyacrylamide slab gel electrophoresis. An overnight culture of *E. coli* BO21 was diluted 100-fold with M9 medium and grown to a cell density of 5 × 10^8 cells per ml (optical density at 590 nm = 0.8). Cells were infected with T4 phage (multiplicity of infection = 6) in the presence of DL-tryptophan (20 μg/ml). At various times, samples of infected cells were mixed with a ^{14}C-labeled amino acid mixture for the desired period. The ^{14}C-labeled amino acid incorporation was terminated by adding 5 volumes of prechilled 2% Casamino Acids (Difco). The cell pellet was collected by centrifugation, washed once with 50 mM Tris-hydrochloride (pH 6.8), and resuspended in 0.1 ml of sample buffer (0.0625 M Tris-hydrochloride, pH 6.8, 2% sodium dodecyl sulfate [SDS], 10% glycerol, and 5% 2-mercaptoethanol). The protein subunits were completely dissociated by immersing the mixture in boiling water for 1.5 min. The sample buffer was made fresh every week and stored in a refrigerator. The electrophoresis was carried out as described by O'Farrell et al. (29). The buffer systems used in making the gel and in electrophoresis were the same as those reported by Laemmli (19). In cases of autoradiogram scanning, the buffer systems described by Maizel (25) were used to obtain a total recovery of input radioactivity in densitometric tracing. The Laemmli buffer system gives a better resolution, but the proteins of molecular weight <10,000 will result in a large band at the bottom of the gel and usually cannot be traced by densitometry with proper sensitivity.

The identification of T4 proteins on the autoradiograms was based on the studies of O'Farrell et al. (28, 29) and Vanderslice and Yegian (34).

DNA-agarose column chromatography. *E. coli* BO21 cells were grown in M9 medium at 30°C to an optical density at 590 nm of 0.8 (5 × 10^8 cells per ml). DL-Tryptophan was added to a final concentration of 20 μg/ml. Cells were infected with T4 phage (multiplicity of infection = 5) at 30°C and labeled from 10 to 25 min after infection with a ^{14}C-amino acid mixture (0.5 μCi/ml). The labeling was stopped by pouring the infected cells into 2 volumes of a prechilled buffer solution containing 10% sucrose, 10 mM Tris-hydrochloride (pH 7.5), 5 mM EDTA, and 2% Casamino Acids. The infected cells were harvested by centrifugation, washed once with the same buffer without Casamino Acids, and resuspended in 0.5 ml of a sucrose-Tris buffer (25% sucrose, 10 mM Tris-hydrochloride, pH 7.5, and 5 mM EDTA). A 0.5-ml sample of lysozyme solution (1 mg/ml) was added, and the mixture was incubated at 25°C for 15 min. An equal volume of lysis solution, containing 2% Triton X-100 and 10 mM Tris-hydrochloride (pH 7.5), was added to lyse the cells, and the mixture was maintained at 0°C for 1 h. One milliliter of 5 M NaCl solution was added to the lysate, and the mixture was stirred gently for 10 min. The lysate was centrifuged at 100,000 × *g* for 1 h to remove cell debris and DNA. The supernatant was dialyzed extensively at 0°C against a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 0.05 M NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol, the buffer used for preparing the DNA-agarose column. The DNA-agarose column was made as described by Schaller et al. (32). After the dialyzed supernatant was absorbed by the column, 0.1, 0.2, 0.4, 0.8, and 2 M NaCl solutions, containing 10 mM Tris-hydrochloride (pH 7.5), 0.5 mM EDTA, 1 mM dithiothreitol, and 5% glycerol, were used to elute the proteins. Each fraction was dialyzed against distilled water before further treatment.

Analysis on alkaline sucrose density gradients. The treatment of infected cells, lysing procedure, and conditions for sedimentation have been reported previously (39). The lysing procedure described by Miller et al. (26) was used.

UV irradiation. Procedures for UV irradiation have been described in a previous report (39).

Spot test for HU sensitivity. The procedure for the spot test for HU sensitivity reported by Karam and Bowles (17) was used.

RESULTS

Effect of the *dar* mutation on the kinetics of progeny formation. The one-step and intracellular growth curves of *dar*1 are shown in Fig. 1. Both one-step and intracellular growth of

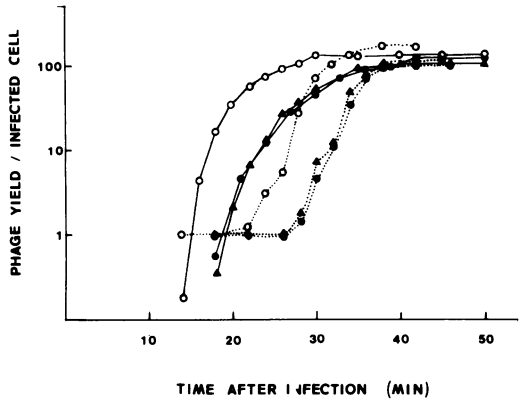

FIG. 1. *One-step growth and intracellular growth curves of T4D (○), dar1 (●), and dar1-amC5 (▲). E. coli BO21 cells in log phase (5×10^8 cells per ml) were infected with T4 phage (multiplicity of infection = 0.5) at 37°C in 1 × CT⁻ medium with aeration. At 5 min after infection, the infected culture was diluted 10^4-fold in the same medium. At various times, 0.1 ml of the diluted culture was taken, and phage yield per infected cell was determined. In the one-step growth experiment (dotted line), the infected cells were plated on E. coli BB at 37°C without lysis for phage yield determination. In the intracellular growth experiment (solid line), the infected cells were lysed with chloroform before determining phage yield.*

the *dar*1 mutant, compared to that of the wild type, were delayed for about 6 to 8 min at 37°C. Similar results were observed in *dar*1-*am*C5 and in *dar* and *dar*2-*am*C5 (data not shown), indicating that the *dar* mutation delays both intracellular progeny formation and one-step growth and that the introduction of a gene 59 mutation into *dar* mutants does not eliminate this delay.

A delay in progeny formation can generally be ascribed to a factor involved in either DNA synthesis or morphogenesis, or both. Since the initiation of DNA synthesis in *dar* mutant-infected cells coincides with that of cells infected with wild-type phage (36), the abnormality in early gene expression is not likely. Therefore, the effects of the *dar* mutation on late gene expression were studied.

Effect of the *dar* mutation on gene expression. (i) Protein synthesis in T4 *dar*1-infected cells. The synthesis of T4-induced proteins can be monitored by polyacrylamide gel electrophoresis in the presence of SDS (25, 28, 29, 34). It is possible to solve the time course of gene expression by comparing the autoradiograms of samples prepared in various short labeling intervals. The results of such experiments are shown in Fig. 2. In general, the sequence of gene expression in the *dar*1 mutant is

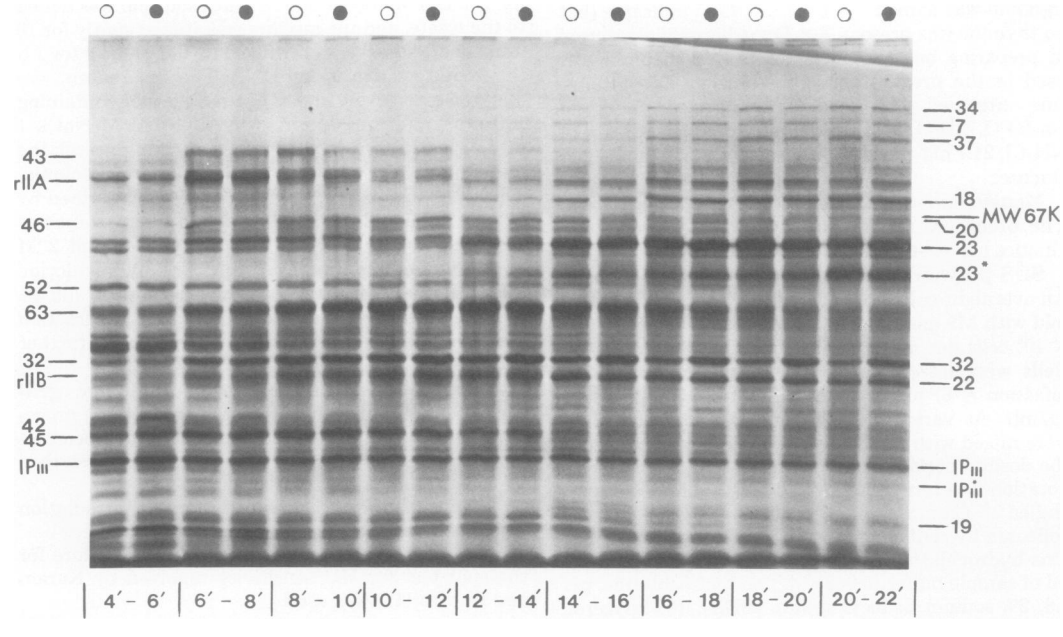

FIG. 2. *Autoradiogram showing patterns of protein synthesis of wild-type T4D and the dar1 mutant. E. coli BO21 cells in log phase (5×10^8 cells per ml) were infected with wild-type T4D (○) and dar1 (●) (multiplicity of infection = 6) in M9 medium at 30°C. At the indicated times after infection, 2-ml portions of the infected cultures were labeled with a L-^{14}C-amino acid mixture for 2 min. Lysates were prepared and subjected to electrophoresis on a 10% SDS-acrylamide gel.*

normal, the pattern of proteins synthesized in *dar*1-infected cells being identical to that of the wild type. For both, the shut-off of early protein synthesis and the initiation of late protein synthesis occurred about 12 min after infection at 30°C (e.g., early proteins P52, P43, P42, and P45 and late proteins P19, P22, P23, P34, P7, and P37), a result consistent with the report of O'Farrell and Gold (28).

The proteolytic cleavage of several late proteins involved in head assembly at steps preceding prohead III, such as P22, P23 to P23*, and IP III to IP III* (15, 19, 20, 30), appeared to be normal in *dar*1-infected cells (Fig. 2), indicating that the delay in progeny formation follows the formation of prohead III. This result is more evident in the chase experiment described below.

Abnormal synthesis of only one or a few late proteins required for structural assembly could delay progeny formation. The results obtained from autoradiograms such as Fig. 2 allow for the possibility that the *dar* mutation causes an abnormality in the structure or expression of minor components that cannot be detected in the autoradiogram. This possibility has been tested by another approach. Chloramphenicol (100 μg/ml) was added to the infected cells at various times after infection, blocking further gene expression. Subsequently, the infected cells were incubated for 60 min and then lysed by chloroform. The burst size in each sample was determined. The kinetics of phage production found in *dar*1-infected cells was indistinguishable from that of the wild-type control (data not shown), strongly indicating that the delay in intracellular progeny formation and one-step growth was not caused by a delay in gene expression.

The autoradiogram shown in Fig. 2 also indicates that at late times in *dar*1-infected cells (i) the gene 32 protein is continuously produced and (ii) the band for a 67,000-molecular-weight protein is induced. These two observations were further analyzed, and the *dar*1-induced, 67,000-dalton protein turned out to be a gene fusion product resulting from the *dar*1 mutation (Wu and Yeh, unpublished data).

(ii) Prolonged synthesis of gene 32 protein (P32). A 7.5% polyacrylamide gel was run in a phosphate buffer system (25) to obtain total recovery of input radioactivity. The autoradiograms were cut and scanned with a Gilford gel scanner connected to a Beckman model DU spectrophotometer. The amount of protein is represented as a percentage of the total recovery calculated from scanned patterns (see legend to Fig. 4). A plot of this percentage versus time after infection (Fig. 3) indicates that gene 32

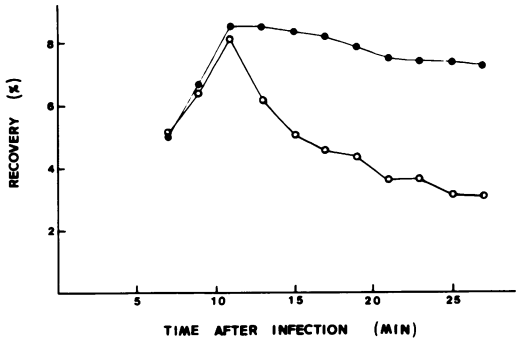

FIG. 3. *Prolonged synthesis of gene 32 protein in dar1-infected cells. E. coli BO21 cells in log phase (5 × 10^8 cells per ml) were infected with T4 phage (multiplicity of infection = 5) at 30°C in M9 medium. At various times, a sample of the infected culture was labeled with a L-^{14}C-amino acid mixture for 2 min. ^{14}C-labeled T4 proteins were analyzed by 7.5% SDS-polyacrylamide gels using the phosphate buffer system. The resulting autoradiograms were scanned with a Gilford scanner connected to a Beckman model DU spectrophotometer (550 nm). The peak corresponding to gene 32 protein was cut out from the scans and weighed. The percentage of gene 32 protein was calculated. Symbols: (●) dar1; (○) T4D.*

protein rapidly increased after infection, reaching a maximum at 12 min in both T4D and *dar*1. After this point, the rate of synthesis tapered off in T4D but not in *dar*1. Moreover, the percentage ratio of *dar*1 to T4D for any protein band can serve as a parameter for the overproduction of that protein. The results of such calculations are shown in Fig. 4A (for early proteins) and Fig. 4B and C (for late proteins). The ratios of the P32 band of *dar*1 to that of the wild type reached 1 and 2 at 12 and 22 min, respectively (Fig. 4A), signifying that *dar*-infected cells overproduce P32 (about twofold) after 12 min of infection at 30°C. Other proteins tested were not significantly affected by the *dar*1 mutation.

The prolonged and excessive P32 synthesis described above was identified by the intensity of the band corresponding to P32 on autoradiograms (28). Alberts and Frey have reported that gene 32 protein strongly binds to single-stranded DNA (1). More convincing evidence for the excessive formation of gene 32 protein might be that obtained from experimentation based on this characteristic. In one such experiment, infected cells were labeled with a ^{14}C-amino acid mixture from 10 to 25 min after infection at 30°C. A protein extract was prepared by the gentle lysing procedure described in Materials and Methods. Most of the DNA was removed by this treatment. The radioactive proteins were loaded onto a DNA-agarose column

prepared by using the method of Schaller et al. (32). Single-stranded calf thymus DNA was used and eluted stepwise with 0.1, 0.2, 0.4, 0.8, and 2 M NaCl buffer solutions (1 mM dithiothreitol-0.5 mM EDTA-5% glycerol-10 mM Tris-hydrochloride, pH 7.5). Each fraction was extensively dialyzed against distilled water and concentrated by lyophilization. The recovery of radioactivity in each fraction was calculated (Table 2). Fractions eluted with higher concentrations of NaCl buffer (>0.4 M NaCl) were more radioactive in the *dar*1 preparation than in that of the wild-type control, the difference being about twofold in the last fraction eluted with 2 M NaCl buffer. Proteins in each fraction were examined by 10% SDS-polyacrylamide slab gel electrophoresis. The autoradiogram (Fig. 5) showed that most of the protein eluted at higher

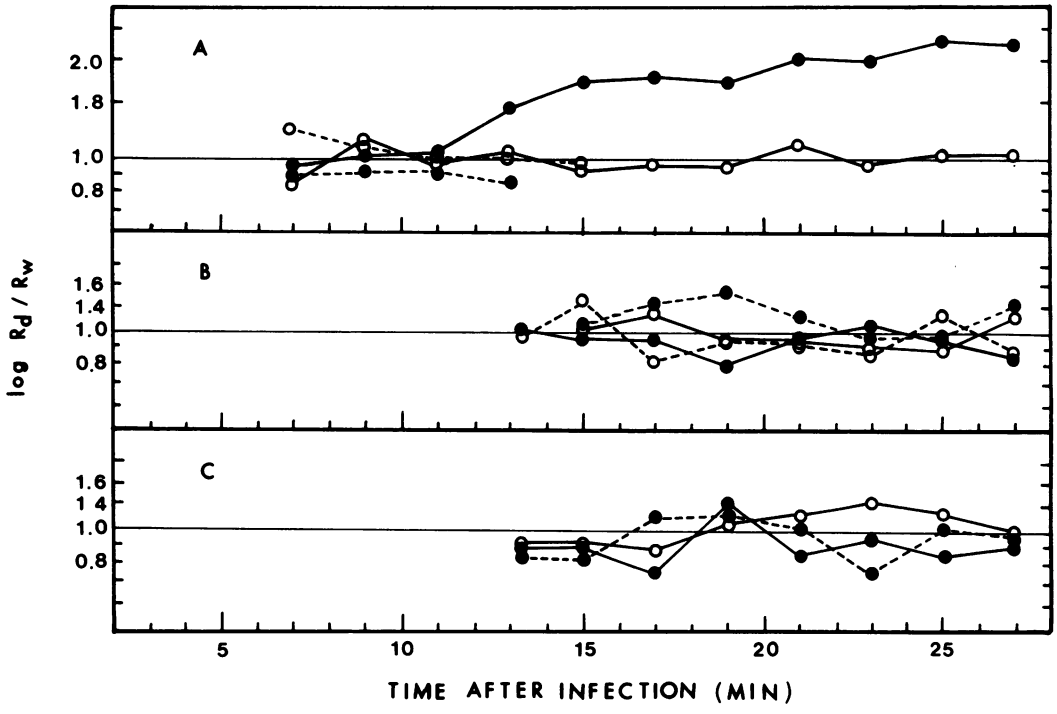

FIG. 4. *Comparison of the relative amounts of some T4 proteins between the dar1 mutant and wild-type T4D. The conditions of infection, electrophoresis, and scanning of autoradiograms were the same as those described in Fig. 3. The peak corresponding to a particular protein was cut out from the scan paper, and each peak of paper was weighed. The percentage of that protein band was calculated from the ratio of the weight of that peak to the total weight of all peaks. The ratio of R_d to R_w on a log scale is plotted versus time after infection, where R_d is the percentage of a particular protein in the dar1 mutant and R_w is the same protein in wild-type T4D. The precision of this calculation is ±5%. (A) Relative amount of some early proteins:* (●——●) *P32;* (○——○) *PIP_{III};* (○- - -○) *P42-P52;* (●- - -●) *P43. (B) Relative amount of some late proteins with lower molecular weight (<55,000):* (●——●) *P23;* (●- - -●) *P23*-P24;* (○——○) *P19;* (○- - -○) *P13-P22. (C) Relative amount of some late proteins with higher molecular weight (>100,000):* (○——○) *P7;* (●——●) *P37;* (●- - -●) *P34.*

TABLE 2. *Recovery of ^{14}C-labeled T4 proteins after DNA-agarose chromatography*

Concn of NaCl in eluting solution[a] (M)	T4D		*dar*1		Ratio of % recovery (*dar*1/T4D)
	cpm	% Recovery	cpm	% Recovery	
Total input	14,500,000	100	16,000,000	100	1
0.1 (wash)	11,600,000	80.5	12,500,000	78.0	0.97
0.2	1,000,000	7.25	1,170,000	7.3	1.0
0.4	306,000	2.1	350,000	2.2	1.0
0.8	421,000	2.9	673,000	4.2	1.4
2.0	231,000	1.6	478,000	3.0	1.9

[a] The values reported apply to the stepwise application of NaCl solution.

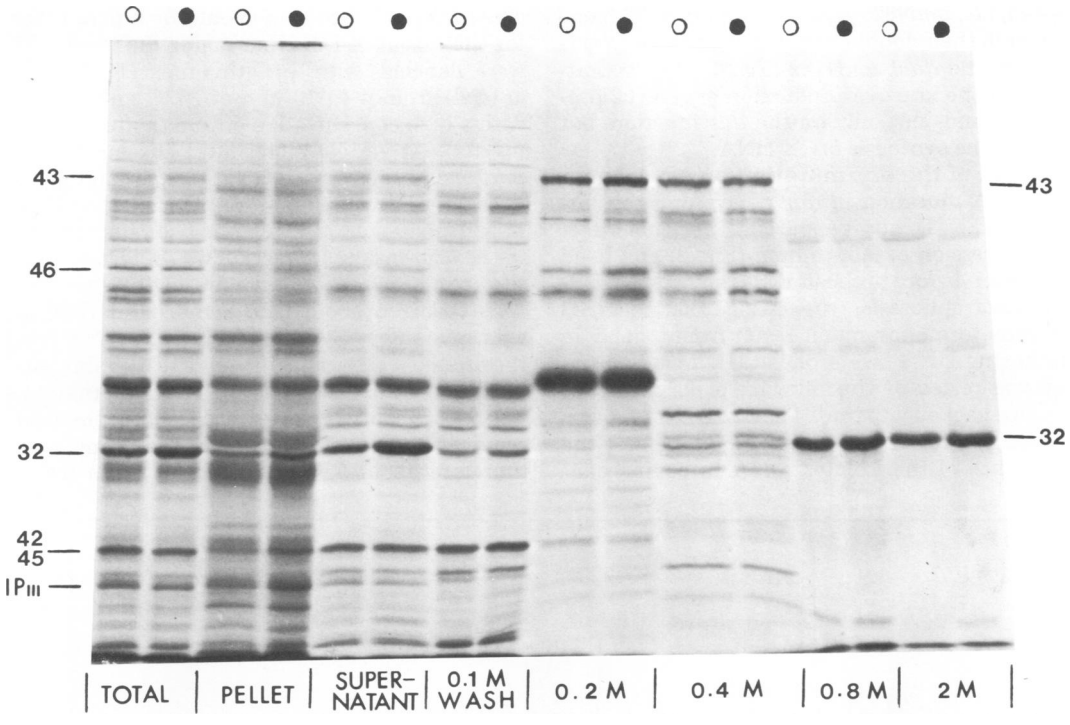

FIG. 5. *Autoradiogram showing protein patterns of fractions eluted from a DNA-agarose column. A ^{14}C-labeled extract was prepared and fractionated by DNA-agarose column as described in the text. The fractions were separated on a polyacrylamide slab gel (10%) containing SDS. Symbols:* (○) *T4D;* (●) *dar1.*

concentrations of NaCl buffer (>0.4 M) was located in the P32 band. This result strongly indicates that at 30°C the synthesis of gene 32 protein in *dar*1-infected cells is prolonged such that late in infection it is about twice that of the wild type.

The possibility that the late overproduction of gene 32 protein results from a decrease in turnover instead of an increase in synthesis was tested by the following experiment. Infected cells were labeled with ^{14}C-amino acids at either early (from 4 to 8 min at 30°C) or late (from 14 to 18 min) times and then chased with a high concentration of cold amino acid mixture (2% Casamino Acids). At various intervals after chasing, a sample of the infected culture was taken and the cells were lysed. The protein pattern was analyzed by 10% SDS-gel electrophoresis. Again, gene 32 protein exceeded the normal level in samples labeled late in infection (data not shown). Because it did not significantly degrade after 15 min of chasing in either early or late samples, turnover of gene 32 protein cannot account for the excessive amount of protein found in *dar*1-infected cells. Moreover, this experiment also demonstrated that the proteolytic cleavage of P22, P23 to P23*, and IP III to IP III* in *dar*1-infected cells reflected that of T4D (data not shown), a result consistent with that described in Fig. 2.

Other *dar* mutants, such as *dar*2, *dar*3, *dar*4, and *dar*5, also caused a prolonged synthesis of the gene 32 protein, but this phenomenon was not observed in dar^+ revertants isolated from a *dar*1 stock (data not shown), indicating that the overproduction of gene 32 is caused by the *dar* mutation. Furthermore, a double mutant of *dar* and gene 59 has been checked under the same experimental conditions, and it has been found that the introduction of a gene 59 mutation into the *dar* mutant does not affect the overproduction of gene 32 protein by the *dar* mutation (data not shown).

Whether or not the *dar* mutation is the only requirement for the overproduction of gene 32 protein described above is answered by the following experiment. Both DNA synthesis and late protein expression are blocked by DO-type (DNA zero) mutants (5, 10, 21). Since *dar* is a late gene (36), DO mutants should suppress *dar*-protein expression and cause an overproduction of gene 32 protein if *dar* protein is the only factor involved in the overproduction. This was not observed, nowever, in two DO mutants

tested, i.e., *am*N82 (gene 44) and *am*N122 (gene 42), or in their double mutants of *dar*1, i.e., *dar*1-*am*N82 and *dar*1-*am*N122 (Fig. 6), demonstrating that the late overproduction of gene 32 protein depends not only on the *dar* function, but also on the synthesis of T4 DNA.

Effect of the *dar* mutation on DNA packaging. A mutation in *dar* delays progeny formation (Fig. 1), but it apparently does not affect the expression of late protein (Fig. 2) and head formation before prohead III, as shown by SDS-gel electrophoresis, suggesting that delayed progeny formation might result from a delay in packaging DNA into prohead III. This hypothesis was tested by the following experiment.

Once packed into the T4 heads, DNA becomes insensitive to DNase digestion. Therefore, the resistance of the isolated DNA to bovine pancreas DNase I digestion should be a parameter for the extent of DNA packaging. Infected cells were labeled with [^{14}C]thymine (1 μCi[2.24 μg]/ml) from 4 to 10 min at 37°C and chased with a high concentration of cold thymidine (1.5 mg/ml). At various times after chasing, 0.5-ml portions of infected cells were taken and lysed in the presence of 10 mM $MgSO_4$, and the lysate was incubated at 30°C for 2 h. The radioactivity in the acid-insoluble fraction was determined as shown in Fig. 7. DNA packaging in *dar*1-infected cells occurred about 6 to 8 min later than in wild-type infections. Only 58% of the DNA synthesized at early times (from 4 to 10 min) was packaged into the heads of the *dar*1 mutant, whereas 75% of DNA was packaged in the wild type. The relative efficiency of the former was therefore about 75% of the wild type, a result

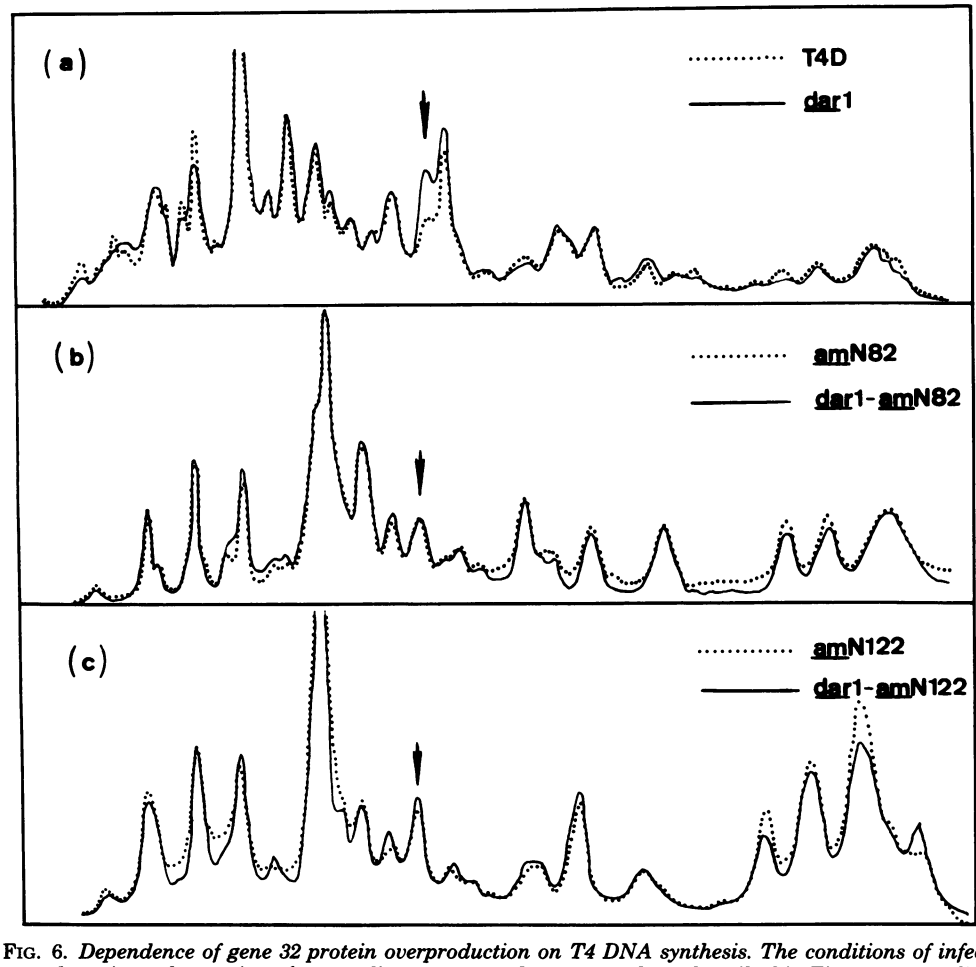

FIG. 6. *Dependence of gene 32 protein overproduction on T4 DNA synthesis. The conditions of infection, electrophoresis, and scanning of autoradiograms were the same as those described in Fig. 3 except 10% SDS-polyacrylamide gels were used in electrophoresis. The infected cells were labeled with a* L-^{14}C*-amino acid mixture from 18 to 20 min at 30°C. amN82, gene 44; amN122, gene 42. Arrows indicate the position of gene 32 protein.*

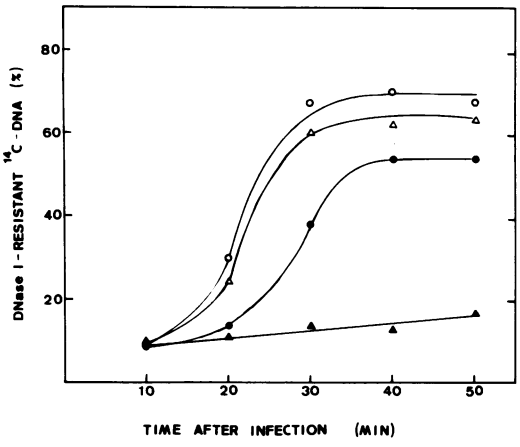

FIG. 7. *Kinetics of DNA packaging into T4 heads. Intracellular DNA in phage-infected cells (E. coli Tr201, 5 × 10⁸ cells per ml; multiplicity of infection = 5) was labeled with [¹⁴C]thymine (1 μCi [2.24 μg]/ml) from 4 to 10 min at 37°C and then chased with a high concentration of cold thymine (1.5 mg/ml). At various times after infection, 0.5-ml portions of the cell suspension were lysed with chloroform. Bovine pancreas DNase I (500 μg/ml) was added to the lysate in the presence of 10 mM $MgSO_4$, and the lysate was incubated at 30°C for 2 h. Trichloroacetic acid-insoluble radioactivity was determined. The radioactivities in samples without treatment with DNase I were taken to be 100%. Symbols: (○) T4D; (●) dar1; (△) amN131 (gene 26); (▲) amB17 (gene 23).*

consistent with the observed burst size (see Table 3). Two other mutants, *am*B17 and *am*N131, were also tested. Mutant *am*B17, defective in gene 23 (major head protein), blocked head assembly at the first step (prohead I) and showed a very low level of protected DNA. Mutant *am*N131, defective in gene 26 (a component of the base plate in tail formation), formed an intact head and therefore had a packaging efficiency similar to that of the wild type. From this result and others previously described, it is clear that the delayed progeny formation in *dar* mutants results from delayed DNA packaging.

Effect of *dar* on UV sensitivity of the gene 59 mutation. Wu and Yeh (39) have shown that gene 59-defective mutants are highly sensitive to UV light. A mutation in *dar* restored progeny formation to normal (36), but in a survival-UV dose experiment it did not suppress the UV sensitivity of the gene 59 mutation (data not shown). The survival curves of a gene 59 mutant (*am*C5) and double mutant of *dar* and gene 59 (*dar*1-*am*C5) were essentially the same as those of two UV-sensitive mutants, *y*m (a purified mutant derived from the original *y*) and *v*. The survival curve of the *dar* mutant (*dar*1) in either *E. coli* BB or *E. coli* CR63 was similar to that of the wild-type control.

The relative slopes of various phage strains tested in *E. coli* BO21 were: T4D, 0.67; *dar*1, 0.72; *y*m, 1.00; *v*, 1.19; *am*C5, 1.11; *dar*1-*am*C5, 0.99. Mutant *y*m appeared to be an amber mutant. When tested in the amber suppressor strain *E. coli* CR63, it was not as sensitive to UV irradiation, a result consistent with the original report (6).

The above conclusions were confirmed by the following experiment. Because the time-dependent change in sedimentation rate of single-stranded DNA in alkaline sucrose gradients reflects the extent of repair in UV-damaged DNA, it can be used as a parameter for UV sensitivity. Infected cells were labeled with [³H]thymine (30 μCi[1 μg]/ml) from 4 to 10 min after infection at 33°C, treated with UV irradiation (700 ergs/mm²), and chased with a medium containing a high concentration of cold thymine (2 mg/ml). At various intervals after chasing, intracellular DNA was extracted at an alkaline pH (0.4 N NaOH) and sedimented in alkaline sucrose gradients. The results of this experiment are shown in Fig. 8. After chasing for 40 min, UV-damaged DNA in the *dar*1 preparation was repaired and cosedimented with standard T4 DNA (73S), although the rate of repair in *dar*1 was slower than that in wild-type T4D. However, after a long period of chasing, the fragmented DNA in the *dar*1-*am*C5 preparation remained fragmented, consistent with a lack of effect of *dar*1 on the UV sensitivity of *am*C5.

Effect of the gene 59 mutation on HU sensitivity of *dar*. A mutation of *dar* sensitizes phage T4 to HU (36). The introduction of a gene 59 mutation (*am*C5) into *dar*, however, increases the survival of *dar* in the presence of HU. All double mutants of *dar* and gene 59 made almost clear spots on a bacterial lawn in the presence of HU. Their average burst size was about 6 in the presence of 20 mM HU, in contrast to an average of about 0.2 obtained from *dar* mutants, a 30-fold increase (Table 3). This result indicates that the gene 59 mutant suppresses the HU sensitivity of the *dar* mutation. Moreover, in the absence of HU, the burst size of *dar* mutants varied from 167 (*dar*3) to 94 (*dar*4) (data not shown). The variation in burst size may reflect the degree of biological defect in the *dar* function, implying that each strain is mutated at different sites in gene *dar*, or it could be due to differences in the stocks.

Effect of *dar* on DNA synthesis of a gene 46-defective mutant. We have reported that *dar* does not suppress other DNA arrest mutations such as genes 46 and 47 (36), as evidenced by the growth of tested phage in a nonpermissive

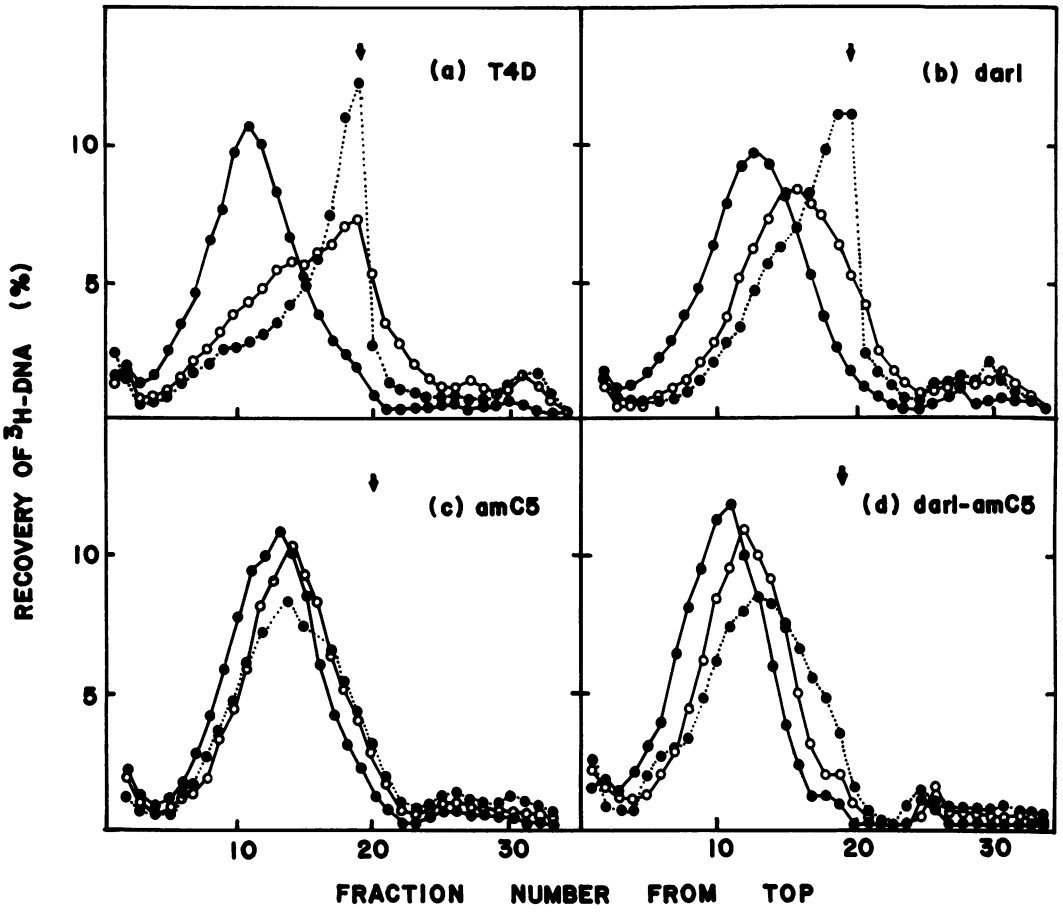

FIG. 8. *Sedimentation profiles of UV-irradiated T4 DNA determined by alkaline sucrose gradient centrifugation. E. coli B Tr201 cells in log phase (5 × 10⁸ cells per ml) were infected with T4 phage (multiplicity of infection = 5) at 33°C in 1 × CT⁻ medium with aeration. The infected cells were labeled with [³H]thymine (30 μCi[1 μg]ml) from 4 to 10 min, followed by UV irradiation (700 ergs/mm²), and then chased in the dark with the same medium containing a high concentration of cold thymine (2 mg/ml). At various intervals after chasing, a portion of the sample was taken and cellular DNA was extracted with alkali (0.4 N NaOH) and sedimented in linear 5 to 20% alkaline sucrose gradients. (a) T4D DNA; (b) darl DNA; (c) amC5 DNA; (d) darl-amC5 DNA. Symbols: (●——●) chased for 1 min; (○——○) chased for 20 min; (●·····●) chased for 40 min. Arrows indicate the sedimentation position of single-stranded mature T4 phage DNA (73S).*

TABLE 3. *Suppression of HU sensitivity of dar mutants by an extragenic mutation in gene 59*

Phage	Burst size[a]	
	No HU	HU (20 mM)[b]
T4D (wild type)	198	23
*dar*1	145	0.21
*dar*1-*am*C5	122	7

[a] Phage produced per infected cell at 37°C in 60 min in *E. coli* BO21.

[b] HU was added before infection.

host. The burst size of a double mutant of *dar* and gene 46 (*dar*1-*am*N130) was similar to that of a single mutant of gene 46 (*am*N130) under nonpermissive conditions (Table 4), indicating that *dar* does not suppress the gene 46 mutation in terms of progeny formation. However, the arrest of DNA synthesis seen in the gene 46 mutant was shown to be suppressed by *dar* (Fig. 9). DNA synthesis in *am*N130-infected cells was arrested 10 min after infection (although with a low rate of continuing synthesis). The introduction of an extragenic mutation into *dar* restored DNA synthesis to the wild-type level. Both mutants, *dar*1-*am*N130 and *dar*1-*am*N130-*am*C5, had the same phenotype (Fig. 9).

DISCUSSION

A 6- to 8-min delay in progeny formation was observed in *dar* mutant-infected cells as revealed by one-step and intracellular growth ex-

TABLE 4. *Effect of dar mutation on gene 46 mutant for phage production*

Phage	Burst size[a]	
	CR63	BO21
T4D	214 (100)[b]	227 (100)
*am*N130 (gene 46)	160 (74.8)	6 (2.6)
*dar*1-*am*N130	117 (54.7)	8 (3.5)

[a] Phage yield per infected cell at 37°C in 50 min. The numbers presented are mean values of two experiments.

[b] Numbers in parentheses are the relative burst sizes; the burst size of the wild type (T4D) is standardized as 100%.

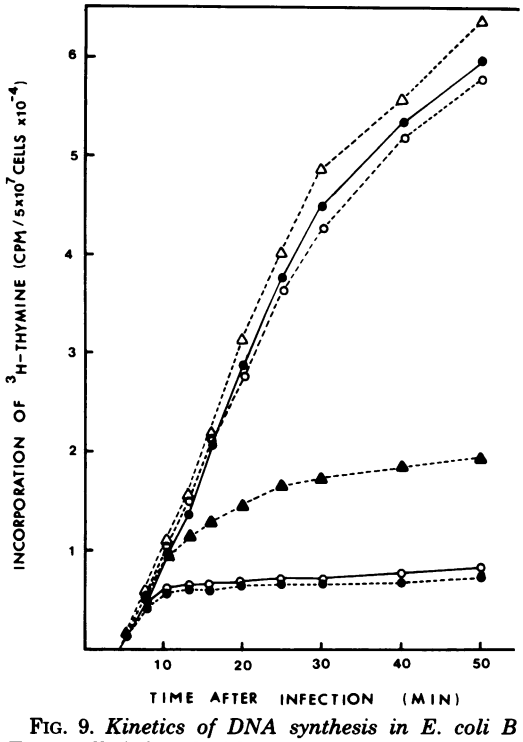

FIG. 9. *Kinetics of DNA synthesis in E. coli B Tr201 cells infected with amN130 (gene 46) or dar1-amN130. E. coli B Tr201 cells in log phase (5 × 10^8 cells per ml) were infected with T4 phage (multiplicity of infection = 5) at 37°C in 1 × CT$^-$ medium with aeration. At 3 min after infection, [^{3}H]thymine (10 μCi[2 μg]/ml) was added to the infection culture. At various intervals, 0.1 ml of the infected culture was taken and trichloroacetic acid-insoluble radioactivity was determined. Symbols: (●——●) T4D; (○——○) amC5; (▲- - -▲) amN130; (△- - -△) dar1-amN130; (○- - -○) dar1-amN130-amC5; (●---●) amN130-amC5.*

periments (Fig. 1). Three lines of evidence support the hypothesis which couples this delay to the DNA packaging step. (i) The late gene expression appears to be normal with respect to the wild type (Fig. 2), suggesting that late structural proteins do not retard assembly and thus ruling out the involvement of tail and tail fiber assembly. (ii) The proteolytic cleavages of P22, P23 to P23*, and IP III to IP III* occurring in *dar* mutants reflect those in wild-type T4D (Fig. 2), indicating that the step involved in delay coincides with or follows prohead III synthesis. (iii) The 6- to 8-min delay in the appearance of DNA I-resistant particles in *dar*1-infected cells (Fig. 7) indicates that DNA packaging is the step involved.

The process of DNA packaging involves two elements: (i) protein factors—the machinery for packaging, and (ii) DNA factors—the intact structure of DNA. A defect in either one would cause unsuccessful packaging. Since the expression of late proteins is not affected in *dar* mutants (Fig. 2 and 4), the delay in DNA packaging must be due to the abnormal DNA structures, which are revealed by velocity sedimentation in neutral and alkaline sucrose gradients as shown in the accompanying paper (37).

In *dar*-infected cells, compared to the wild type, three kinds of abnormalities are observed in DNA replication. (i) There are unusually rapidly sedimenting intermediates (800S). (iii) When examined under alkaline conditions, there is less single-stranded DNA exceeding 1 phage unit. (iii) The rate of repair of DNA intermediates is slower. Which abnormality causes the delay in DNA packaging? Since 200S intermediates were observed in the *dar*-gene 59 double mutant (see 37), which still showed a delay in progeny formation (Fig. 1), it is unlikely that the packaging delay results from the unusual molecular size of *dar* DNA intermediates. Fewer single-stranded DNA molecules exceeding 1 phage unit in size are secondary to the slow repair rate of DNA fragments, as described in the accompanying paper (37). Thus, the DNA packaging delay must be due to the slow repair. In other words, the presence of more gaps or nicks on *dar* DNA intermediates delays DNA packaging and subsequent progeny formation.

The "headful" hypothesis of T4 phage has been proposed by Streisinger et al. (33) to explain the increase in terminal redundancy caused by deletion mutations. According to this model of phage morphogenesis, packaging proceeds by filling the empty heads with concatemeric DNA molecules and cleaving these molecules afterwards. Because of this, the length of the mature T4 DNA molecule should be dictated by the capacity of the head. That petite phage particles contain short genomes (9, 27) and giant phage contain long ones support this hypothesis (4). From the recent experiments of Luftig et al. (24), Frankel et al. (11), and Luftig and Ganz (22) comes a mechanism by which the headful model may operate. It is postulated that the

energy needed to synthesize DNA provides the driving force for packaging a full DNA equivalent into capsid intermediate structures, at a site distinct from the replication point. The fact that on-going DNA synthesis is a direct requirement for DNA packaging supports this postulate (23). Since the *dar* mutant does not affect the formation of preformed heads before DNA packaging, as determined by the normal cleavage of some late proteins at steps before prohead III formation (Fig. 2), the proposed model for *dar* mutant DNA packaging is reconciled with the headful hypothesis but further requires a prepackaged DNA structure cleared of gaps or nicks.

How DNA structure affects the DNA packaging has yet to be determined. It has been reported that the lethality of gene 30 (DNA ligase) can be suppressed by the introduction of an extragenic mutation in *rII* (3, 16), although viability of the resulting phages is but 10% (3). Progeny particles formed in this system contained breaks in their DNA as judged from alkaline sucrose gradient centrifugation. Apparently these breaks are gaps, for they could not be repaired by DNA ligase alone in vitro, whereas a combination of DNA ligase and DNA polymerase significantly repaired them (8). The progeny particles formed in *dar* mutant-infected cells have been examined by the same procedures, but no such phenomenon occurred. Possibly the defective structure of *dar* DNA intermediates contains gaps because of the overproduction of gene 32 protein (see below). Still it is not clear why this gap-containing DNA cannot be packaged into *dar* phage particles. Possibly the gaps existing on *dar* DNA intermediates are extended single-stranded regions, or perhaps the function of *rII* is to examine DNA structure as packaging proceeds. In fact, populations of *rII* mutant phage grown under conditions of rapid lysis include particles containing short DNA fragments (7), suggesting that encapsulation of DNA with gaps in gene 30 and *rII* double-defective mutants may derive from a "maturation defect" caused by the *rII* mutation. Further experimentation is required, however, and *dar* mutants provide a good system for such studies.

Under normal conditions (in T4 wild type), the synthesis of gene 32 protein (P32) rapidly increases after infection, peaks with the appearance of late proteins, and then gradually decreases to a low constant level (Fig. 3 and 4). In contrast, the synthesis of P32 does not drop late in *dar* mutant infection, as shown by SDS-gel electrophoresis (Fig. 2 and 3) and by its binding activity to single-stranded calf thymus DNA (Table 2, Fig. 5). At 30°C production of P32 at late times for *dar* mutants is about twofold that of the T4 wild type (Fig. 3 and Table 2).

It has been reported that regulation of synthesis of P32 occurs by an autoregulatory mechanism: P32 is a repressor of its own synthesis (12, 18), and the regulation is at the level of translation (31). Therefore, there are two systems by which the production of P32 can be augmented: (i) in infections of *E. coli* by T4 phage carrying amber or temperature-sensitive mutations in gene 32, the altered P32 is biologically inactive for its autoregulation under nonpermissive conditions and, thus, is synthesized at greatly elevated rates; (ii) the synthesis of P32 is also elevated by a reduction of free P32 in the intracellular pool through its binding to abnormally extended single-stranded DNA regions (gaps) which result from mutations in genes other than gene 32, thereby turning on the synthesis by feedback. Since the overproduction of P32 in *dar* mutant-infected cells depends on DNA synthesis (Fig. 6), the second system would account for this overproduction. This interpretation implies that more gaps occur in *dar* DNA intermediates, an implication strongly supported by studies of *dar* DNA intermediates analyzed in alkaline gradients (see 37).

Gene 55 mutants overproduce P32 late in infection too (18). Since gene 55 controls late gene expression and thereby *dar*, a late gene, this overproduction could derive from the loss of the *dar* function. Mutations in other late genes so far investigated did not induce the overproduction of gene 32 protein (18).

The observation that gene 59 mutants suppress the HU sensitivity of *dar* mutants (Table 3) suggests that the gene 59 product functions as an endonuclease. A mutation in *dar* causes HU sensitivity in progeny formation, as determined by burst sizes in the presence of various concentrations of HU (36), but the effect of the *dar* mutation on host DNA degradation cannot account for its HU sensitivity (36). From velocity sedimentation studies of DNA replicative intermediates under both alkaline and neutral conditions, it has been shown that HU not only causes more nicks or gaps, but also causes double-stranded cuts on the DNA intermediates of *dar* mutants (unpublished data), implying that HU somehow activates an endonucleolytic activity which attacks the DNA intermediates of *dar*-defective phage. This reaction eventually blocks progeny formation due to the production of structurally abnormal DNA, which interferes with DNA packaging. Gene 59 seems to control this endonucleolytic activity so that a mutation of this gene suppresses the HU sensitivity of the *dar* mutant.

That the gene 59 mutation partially inhibited the DNA degradation of DNA ligase-defective mutants also supports the proposed endonucleolytic function of gene 59 protein (unpublished data). DNA synthesized before 10 min at 37°C is largely degraded (to the extent of 85%) if the phage is devoid of gene 30 function. However, the introduction of the gene 59 mutation into a gene 30-defective mutant reduced the extent of degradation by 50%, indicating that at least a portion of the degraded DNA (about 35%) in gene 30 mutant-infected cells is contributed by gene 59 function. This result suggests that the function of gene 59 involves nucleolytic activity, but it does not identify this function as either an exonuclease or an endonuclease. It has been reported by Beguin, though (2), that the primary contribution in T4 DNA degradation caused by gene 30 mutants results first from endonucleolytic attack, as examined by alkaline sucrose gradients.

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