Regulation of Gene 32 Expression during Bacteriophage T4 Infection of *Escherichia coli*

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The gene 32 protein of the bacteriophage T4 plays an important role in genetic recombination, DNA repair, and DNA replication; the protein functions in these processes by virtue of a strong binding capacity for single-stranded DNA. During infections of *Escherichia coli* by bacteriophage carrying amber or temperature-sensitive mutations in gene 32, the altered gene 32 protein (that is, the amber fragment or the missense polypeptide) is synthesized at greatly elevated rates. During infections by phages that are mutant in other genes (and wild type in gene 32), gene 32 expression is coupled to the quantity of single-stranded DNA produced during the infection. The data are consistent with a model in which the gene 32 protein binds preferentially to all available single-stranded DNA. When all available single-stranded DNA is complexed with gene 32 protein, free gene 32 protein represses its own synthesis. The high level expression of altered gene 32 proteins (amber fragments or missense polypeptides) is a direct consequence of the proposed autoregulation.

The gene 32 protein is an essential function of the bacteriophage T4, required both for DNA synthesis and genetic recombination (1, 2). P32 binds to single-stranded DNA in vitro, the binding is strongly cooperative (3, 4). Furthermore, P32 stimulates cell-free DNA synthesis under the direction of the T4-encoded DNA polymerase (5). The function of P32 is required throughout infection (3, 4).

In this paper, we show that regulation of the rate of synthesis of P32 occurs by an autoregulatory mechanism; P32 is a repressor of its own synthesis. In addition, we demonstrate that single-stranded DNA, by titrating P32, probably causes gene 32 derepression. In the accompanying paper, we demonstrate that the repression occurs at the level of translation (6). Many of the observations reported here have been reported previously by Krisch et al. (7). Our data in general confirm and extend their findings.

**MATERIALS AND METHODS**

**Bacteriophage Strains**—Mutants were generously provided as follows: temperature-sensitive gene 32 mutants from Bruce Alberts, amber gene 32 mutants from Bruce Alberts (University of California Medical School, San Francisco) and Peter Snustad (University of Minnesota, Minneapolis), and all other mutants from the collections of Charles Yegian (University of Colorado, Boulder), Dick Epstein (Université de Genève, Switzerland), Harris Bernstein (University of Arizona, Tucson), and Bill Wood (California Institute of Technology, Pasadena). Phage stocks were prepared as previously described (8).

**Conditions of Infections**—All infections were carried out at 30°C at a cell density of 3 x 10⁸ cells per ml and a phage multiplicity of about 8 (unless otherwise indicated in the legends). The medium was M9 plus tryptophan as previously described (8). Host bacteria were either *Escherichia coli* AS19, *E. coli* B, or *E. coli* B str (str is streptomycin-resistant—the strain was provided by Jim Karam). The infections described in Table II were performed in ultraviolet-irradiated cells; the irradiation was as previously described (8, 9).

Mixed *¹³C*-amino-acids were used to label proteins under the conditions described in the legends. The *¹³C*-amino-acids (with or without unlabelledcasamino acids) were added such that radioactive amino acids were incorporated linearly into protein during the labeling period. Labeling was terminated by the addition of excess casamino acids (1 mg per ml of infected cells), and the cultures were immediately put on ice. Following centrifugation, the pellets of cells were resuspended in 25 μl of 1 M NaOH and 100 μl of SDS-sample buffer; the samples were heated to bring about lysis (8, 9).

[methyl-*³H*]Thymidine was used to measure the accumulation of radioactive DNA; the concentration was 5 μCi/ml (specific activity equal to 0.2 μCi/μg). Labeling was terminated by dilution of the infected cells into an equal volume of 1 N NaOH. After overnight incubation at room temperature, incorporation into DNA was determined as trichloroacetic acid-insoluble material retained on glass fiber discs.

**SDS-Acrylamide Gel Electrophoresis of Proteins**—The procedure for electrophoresis has been described (6, 10, 11). The per cent acrylamide used in the separating gels is noted in the legends. All gels were stained with Coomassie blue, and the samples were heated to bring about lysis (8, 9).

The amount of P32 synthesized during a particular pulse of radioactive amino acids was obtained by measuring the peak weights from scans of the appropriate protein bands of the autoradiograms prepared as described previously (8).

The peaks have not been normalized to amino acid incorporation; equal quantities of infected cells were electrophoresed and quantified for each experiment. Thus data presented within any single figure reflect...
the absolute rates of P32 expression for the various infections (if we assume that amino acid pools do not change during the course of the experiments). In some experiments, phage strains included gene 44 or rIIH mutations (or both) since the protein products of these genes can interfere with densitometer analysis of P32 (8).

The molecular weights of the three protein fragments produced by infection with amber mutations in gene 32 were determined from a comparison of their mobilities with that of the wild type gene 32 protein and other specific T4 proteins whose molecular weights have been determined (8). The fragments were: amHL618, 17,500; amH18, 13,000; and amA453, about 10,000. These molecular weights are consistent with the published map position of the three ambers (12). Data for "P32" expression during gene 32 amber mutant infections have been corrected for the molecular weight difference between the fragment and wild type P32 (35,000 (3, 4)).

A double fragment is produced during infection with the gene 32 amber mutation HL618 (see Fig. 1). Neither band is derived from the other, since neither moves to the position of the other after a prolonged chase period. The less intense band on the autoradiogram might be a fragment produced by a translational reinitiation event at a site slightly distal to the amber codon (13). The sum of the molecular weights for these two bands is 36,000, in close agreement with the molecular weight of P32. Two independent isolates of HL618 (E315 and NG364) also show both fragments. The more intense, lower molecular weight band, has been used to quantify the amount of HL618 overproduction at higher rates than the temperature-sensitive polypeptides, at least at 30°. The maximal rate of "P32" synthesis in the temperature-sensitive mutants is about 25-fold the normal rate of P32 synthesis, whereas synthesis of the amber fragments is about 40-fold the rate of P32 synthesis. After 25 min of infection, the amber and temperature-sensitive polypeptides are present at 15 and 8 times the amount of wild type P32, respectively.

II. Overproduction of Wild Type P32

Infections with Mutants Altered in DNA Synthesis

P32 synthesis and DNA accumulation were measured during infections with mutants altered in many of the known prerplicative T4 genes that affect DNA synthesis (Table I). At least two amber mutations in each gene were tested. The amounts of P32 and DNA synthesized during each infection are expressed relative to the wild type amounts.

The genes may be categorized according to their effect on P32 synthesis: mutations in genes 46 and 47 cause underproduction of P32 and mutations in genes 30, 56, 41, and 61 cause overproduction of P32. Furthermore, specific alleles in four genes (43, 62, 39, and 52) give overproduction of P32 which can be completely or largely eliminated by using a streptomycin-resistant host.

RESULTS

I. Overproduction of Altered Gene 32 Proteins

Infections with three or four and two temperature-sensitive gene 32 mutants yield altered P32 ("P32") at increased rates (Fig. 1). The six gel patterns displayed in Fig. 1 are indistinguishable except for the overproduction of "P32"; mutations in gene 32 alter only gene 32 expression. Each phage strain includes a gene 45 amber mutation to prevent DNA replication in those infections that produce some gene 32 activity (1). The two temperature-sensitive mutants overproduce "P32" at the permissive temperature of 30°; these altered proteins are overproduced at temperatures from 20-43° (6) and see below.

The kinetics of overproduction of gene 32 mutant proteins are shown in Fig. 2; again, the experiment was performed in a gene 45- genetic background. The data are expressed as a ratio of "P32" to P32 synthesis; the rate of P32 synthesis is obtained from the control (i.e. 45-32+) infection. (In the 45-32- infection, the absolute rate of P32 synthesis diminishes by a factor of 2 during the course of the experiment (see below)). In all cases, the rates of "P32" overproduction increase throughout infection. The three amber polypeptides are synthesized at the rate of gene 32 overproduction, at least at 30°. The maximal rate of "P32" synthesis in the temperature-sensitive mutants is about 25-fold the normal rate of P32 synthesis, whereas synthesis of the amber fragments is about 40-fold the rate of P32 synthesis. After 25 min of infection, the amber and temperature-sensitive polypeptides are present at 15 and 8 times the amount of wild type P32, respectively.

![Fig. 2. Kinetics of "P32" synthesis. Bacteria (AS19) were infected at 30° with the mutants described in Fig. 1. The cultures were labeled with "C-aminoc acids (1 μCi/ml) for 2 min at various times. Radioactive proteins were analyzed on SDS-acrylamide gels. The data are plotted as the number of moles of "P32" synthesized in each pulse relative to the amount of P32 synthesized in the control infection (amE10, which is 45-). The symbols are: O, amE10-tsP7; O, amE10-tl171; □ amE10-amA453; △, amE10-amHL618; ▲, amE10-amH18; ▲, amE10-amHL618.](http://www.jbc.org/DownloadedFrom)
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TABLE I

P32 synthesis during infections by T4 mutants with altered DNA synthesis

The infected cells were labeled from 30 to 40 min after infection with [32P]amino-acids (1 pCi/ml; 20 µg of casamino acids/ml). The amount of P32 synthesized in each infection was determined. The data are expressed as the amount of P32 synthesized in each infection relative to the wild type level. DNA synthesis was measured as [3H]thymidine incorporation from 10 to 35 min postinfection; these data are expressed as the amount relative to the wild type level.

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<th>Host: Bat P32 synthesis</th>
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resistant host to reduce translation through the amber codon (16).

Infections with Maturation-defective Mutants

The products of genes 33 and 55 are not required for DNA synthesis (1, 17), but are required for late transcription (17-19). Infections with the double mutant 33-55 give P32 overproduction (Fig. 3); each single mutant also gives P32 overproduction. In addition, cells infected by 33-55 phage do not synthesize late proteins.

Fig. 4 illustrates the kinetics of P32 synthesis during infections with mutants that overproduce wild type P32. The data are expressed as the amount of P32 synthesis relative to that in cells infected by a gene 45 mutant (as in Fig. 2). Cells infected by 61- and 33-55 phage overproduce P32 throughout infection. Cells infected by 30-, 56, or 41 phage overproduce P32 to a lesser extent and do not continuously increase their rate of overproduction. All these infections synthesize less P32 than do cells infected with gene 32 mutants (see Fig. 2).

State of Intracellular DNA Controls Amount of Wild Type P32 Synthesis

Elimination of DNA Synthesis—Five abortive infections (gene 30-, 56-, 41-, 61-, or 33-55) give P32 overproduction; each abortive infection also shows substantial accumulation of newly replicated T4 DNA (see Table I). In each case, P32 overproduction is diminished by eliminating DNA synthesis (Fig. 5). Elimination of DNA replication and P32 overproduction in these abortive infections may be accomplished with mutations in genes other than gene 45 (7). As shown in Fig. 1, “P32” overproduction is not abolished by the addition of a gene 45 mutation to the gene 32 mutations.

Elimination of Exonuclease Function—Gene 46 mutant infections are deficient in an exonuclease function (20-22). The
Gene 32 Expression during T4 Infection of E. coli

Time After Infection (min)

FIG. 3. Overproduction of wild type P32 during 33-55- infection. Bacteria (AS19) were infected at 30° with wild type or amN134-amBL292 (33-55-) phage and labeled from 30 to 40 min after infection with 14C-amino-acids (1 μCi/ml; 20 μg of casamino acids/ml). The autoradiogram is from a 7.5% SDS-acrylamide gel.

intracellular DNA of such infections contains few, if any, single-stranded regions (20, 21, 23). Cells infected with gene 46 mutants replicate T4 DNA normally until about 15 min postinfection, at which time DNA synthesis arrests (24). Four infections that yield P32 overproduction (30-, 56-, 61-, and 33-55-) fail to do so in a 46- background, even though inclusion of the gene 46 mutation does not eliminate DNA synthesis (Fig. 5). In fact, the 46 56- infection accumulates more DNA than the 56- infection; presumably unstable deoxycytosine-containing DNA synthesized in gene 56 mutant infections is stabilized by the absence of the gene 46 exonuclease function (25). We note that P32 overproduction in 41- infections is not diminished by inclusion of a gene 46 mutation; evidently single-stranded DNA accumulates directly in 41- infections.

Several experiments have been carried out with 46-32- double amber mutants; the absence of the exonuclease function never diminishes the level of "P32" expression.

High Multiplicity Infections with Phages Unable to Undergo Replication—P32 synthesis was measured during infections with various mutants that might accumulate different amounts of single-stranded DNA in the absence of DNA replication; the infections were carried out at a multiplicity of 16 to increase the amount of parental DNA (Table II). Mutants lacking functional T4 polynucleotide ligase (encoded by gene 30) synthesize the highest amount of P32. Parental DNA in a 30-45- infection accumulates single-stranded regions (21, 23) whereas DNA in a 45-46- infection contains predominantly nicks (20, 21, 23). This experiment has been repeated several times; modest differences in the rate of P32 synthesis similar to those in Table II are always obtained.

Inhibition of DNA Replication during P32 Overproduction—Since DNA synthesis is required for P32 overproduction in cells infected with 33-55- phage (above), we interrupted DNA synthesis in 33-55- infected cells and measured subsequent P32 synthesis. The phages used were each 33-55- and included, as required, temperature-sensitive mutations in gene 43 (DNA polymerase) or gene 30 (DNA ligase), or both. An autoradiogram of infections with these mutants labeled at 30° and also following a shift to 42° is shown (Fig. 6). At 30°, a permissive temperature for each temperature-sensitive mutant, DNA synthesis is high (6) and P32 is overproduced. DNA synthesis stops rapidly upon transfer of cells infected by the gene 43 ts to the restrictive temperature (6, 26); P32 overproduction in the 33-55-43' infection is reduced following temperature shift-up. However, the inclusion of a DNA-ligase temperature-sensitive mutation in the 33-55-43' phage elimi-
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E. coli mutants that are temperature-sensitive in gene 32 are also quadruple mutants, and hence they do not overproduce gene 32 amber products when infected with a wild-type phage. The decrease in gene 32 expression is due to the activity of the amber suppressor encoded by the amber gene of the phage. This activity is further supported by the observation that the recombination between wild-type and amber mutant phages has an additional effect on gene 32 expression. The recombination event occurs at a higher rate in cells infected with the quadruple mutant than in cells infected with the wild-type phage. The reduction in gene 32 synthesis following temperature shift-up is due to the inactivation of the amber suppressor gene.

The state of the DNA, as well as the rate of DNA replication, can affect gene 32 expression. Detailed kinetics of gene 32 expression are presented in Figs. 7 and 8. P32 synthesis is first detectable at 4 to 5 min postinfection; until about 7 min postinfection, expression of gene 32 does not depend on the genotype of the infecting phage (Fig. 7). Seven minutes after infection, phages that are 45-32 amber or temperature-sensitive in gene 32 begin to overproduce “P32” relative to a 45-32 control; at 30°, a permissive temperature for the ts allele shown, the missense protein is synthesized at a lower rate than is the gene 32 amber fragment.

Table II

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<th>Genotype</th>
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The ability of parental DNA to affect P32 synthesis

Ultradet-irradiated bacteria (AS19) were infected with the following phage at a multiplicity of 16: amXC30-amE10 (30 45), amE10 (45), or amE10-amB23 (45 46). The infected cultures were labeled with 14C-amino-acids (1 μCi/ml; 10 μg of casamino acids/ml) from 25 to 35 min after infection. The data for P32 synthesis and total protein synthesis are expressed relative to the values obtained in the 45° infection.

From 7 until about 12 min postinfection, the rate of P32 synthesis increases in wild type and 33-35 infected cells. The increase measured during this period does not occur in infections by 32 phage deficient in DNA replication (45°) or exonuclease function (46°) (Fig. 8). At about 12 min postinfection, P32 synthesis in wild-type infected cells decreases; however, P23 synthesis in 33-35 infected cells continues to increase.

*Unpublished experiments from our laboratory.
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IV. Gene 32 Expression in Cells Mixedly Infected with Wild Type and Gene 32 Mutant Phage

The results presented thus far have been concerned with gene 32 expression in cells infected with either gene 32 mutants (I and III of this section) or phages carrying mutations that alter the amount or the state of intracellular DNA (II and III of this section) or phages carrying mutations that alter the amount or the state of intracellular DNA (II and III of this section). Here we examine the effects of gene 32 mutant alleles on wild type P32 synthesis in mixedly infected cells (Figs. 9 to 11).

Two phages were constructed, 32' rIIA' rIIB' and 45' rIIB'. Each phage was 45' to prevent DNA synthesis and rIIB' to preclude quantification problems during subsequent gel analyses of P32 synthesis. By mixedly infecting with various ratios of these two phages, both P32 and PrIIA synthesis can be measured as a function of gene dosage; PrIIA synthesis serves as the internal control for the fraction of 32' rIIA' genomes added. Infected cultures were labeled with radioactive amino acids both 8 to 10 min and 23 to 25 min postinfection. The rate of P32 synthesis for a given rate of PrIIA synthesis is shown (Fig. 9). The dashed line represents the expected data if both PrIIA and P32 are synthesized at a rate dependent only on the fraction of 32' rIIA' genomes added; in fact, gene 32 expression occurs at a high rate relative to rIIA expression. Regulation of gene 32 expression compensates for the diminishing fraction of 32' rIIA' genomes. As one would expect from the kinetics of gene 32 overexpression (Figs. 2, 4, 7, and 8), the compensation is more successful during the later pulse.

P32 and "P32" (i.e. the gene 32 amber fragment) were

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Fig. 7. Detailed kinetics of P32 versus "P32" synthesis. Three multiple mutant phage strains were constructed: amElO-amN82 deletion r638 (45 44 rIIB'), and two quadruple mutants carrying, in addition, amHL18 (32') or tsL171 (22'). Infected bacteria (AS19) were labeled with 14C-amino-acids (2 μCi/ml) for 1 min at various times after infection. The rates of P32 and "P32" synthesis are plotted at the midpoint of each labeling interval. The symbols are: O, 32'; A, 32'; O, 32'. Note that 30', the temperature of this experiment, is permissive for the gene 92 mutant tsL171.

Fig. 8. Detailed kinetics of P32 synthesis for underproducing and overproducing conditions. P32 (and "P32") synthesis was measured as described under "Materials and Methods" and the legends to Figs. 2, 4, and 7. The symbols are: A, amN134-amHL292 (32-55'); O, wild type T4; O, amElO (45'); and A, amB271 (46'). In addition, "P32" synthesis is shown (O) for infection by amE10-amHL618 (45 32 ).

Fig. 9. Compensation for diminishing gene 32 dosage. Bacteria (AS19) were infected with amEl0-deletion r638 (45' rIIB') and/or amE10deletion r1231 amHL618 (45' rIIB' rIIA' 32') phage at multiplicities of infection of 18 to 25. Aliquots from nine separate infections (32 rIIA', 32' rIIA', and seven mixed infections) were labeled with 14C-amino-acids (1 μCi/ml) from 8 to 10 and 23 to 25 min postinfection. The amount of P32 and PrIIA measured for the infection by amE10-deletion r638 (i.e. 32' rIIA') alone was defined as one; the quantities of P32 and PrIIA obtained from the mixed infections have been normalized to this control value. PrIIA is not synthesized late in infection; thus the amounts of P32 synthesized in each infection from 8 to 10 and from 23 to 25 min are plotted as a function of the amounts of rIIA synthesized from 8 to 10 min during each infection. The symbols are: O, P32 synthesis 8 to 10 min postinfection; O, P32 synthesis 23 to 25 min postinfection.
Gene 32 Expression during T4 Infection of E. coli

FIG. 10. Gene 32 expression during gene dosage experiments: compensation by coordinate regulation of wild type and 32- genomes. Data are taken from the 23- to 25-min labelings in the experiment described in Fig. 9. The quantities of "P32" (the gene 32 amber fragment produced in infections that included amE10-deletion 638- amHL618 phage), corrected for molecular weight, were determined. The molar quantities of "P32" are expressed relative to the amount of P32 synthesized in the amE10-deletion 638 (i.e. 32*) infection (taken to be 1.0 as in Fig. 9). The relative amounts of P32 (O) and "P32" (○) are plotted as a function of the amE10-deletion 638 (i.e. 32*) allele frequency. Also shown is the fraction of total gene 32 expression which is wild type P32 (i.e. P32*, see text); the values obtained for P32* agree with those predicted (- - -) for coordinate expression of wild type and amber gene 32 in proportion of the input allele frequency.

The Direct Relationship between P32 Expression and Amount of Single-stranded Intracellular DNA

The fate of parental and newly replicated T4 DNA during infection must be very complicated. We present (Fig. 12) a simplified metabolic chart for T4 DNA so that our discussion may originate from a set of shared assumptions. The gene products that are known to act on intracellular T4 DNA are shown; obviously many other proteins alter the state of the DNA. The synthesis of P32 has been measured during infections by mutants altered in each of the genes shown in Fig. 12. The results demonstrate unequivocally that there is a direct correlation between P32 synthesis and the quantity of single-stranded intracellular DNA. The results obtained with individual mutants have been interpreted within this framework.

Gene 30 Mutants—The T4-encoded DNA ligase (gene 30) seals single-stranded nicks; in the absence of P30, about 3-fold excess single-stranded interruptions are found in T4 DNA (23). These single-stranded interruptions are gaps, not nicks; the average gap is 300 to 400 nucleotides in length (23). Ligase competes with exonuclease function for those parental DNA nicks caused by endonuclease(s) (host or phage-encoded) or for those nicks in progeny DNA that arise through discontinuous replication (31). P32 synthesis is increased in infected cells that have elevated quantities of single-stranded DNA due to gene 30 mutations.

Gene 46/47 Mutants—Extracts of cells infected with gene 46 or 47 mutants have less exonuclease activity than extracts of T4+ infected cells (22). Intracellular T4 DNA of cells infected with gene 46 or 47 mutants contains a few single-stranded regions (20, 23). In addition, host DNA breakdown is abortive in 46- and 47- infections (32). We show P46 and P47 as proteins that increase exonuclease function; in fact, the direct protein products of genes 46 and 47 may be exonucleases. P46 and P47 increase the extension of nicks into gaps; diminished exonuclease function leads to decreased P32 synthesis.

Gene 41 Mutants—Oishi (33) has characterized the DNA synthesized in cells infected with gene 41 mutants; only low levels of DNA accumulate, but all of the DNA appears to be short and single-stranded; furthermore, Alberts and his collaborators have found P41 to be part of the replication apparatus (34). Single-stranded DNA probably is a short lived intermediate in 41+ infections and accumulates during abortive 41- infections. P32 synthesis is elevated in 41- infections.

Gene 56 Mutants—The protein product of gene 56 is a quantified and plotted as a function of the fraction of 32- phage used during each infection (Fig. 10). P32 expression remains essentially constant until the fraction of wild type phage falls below 0.2, whereas "P32" expression rises continually as the fraction of 32- genomes increases. Thus, total gene 32 expression (P32 + "P32") rises as well. The fraction of the total gene 32 expression that is wild type P32 (defined as P32*/P32/P32 + "P32") reflects exactly the fraction of wild type genomes in the input phage. Mechanistically, this result suggests that the regulating mechanism can act trans and does not distinguish between the 32+ and 32- alleles.

These direct measurements of compensation during gene dosage experiments suggest that the phage burst will be relatively indifferent to increasing amounts of 32- phages used during mixed infections; for fractions of wild type phage in the range 1.0 to about 0.1 this is the case (Fig. 11). Although this result is in agreement with the direct measurements of P32 expression (Figs. 9 and 10), it is in apparent contradiction to previously published gene dosage experiments involving gene 32 (12, 28, 29).

DISCUSSION

The Direct Relationship between P32 Expression and Amount of Single-stranded Intracellular DNA

The fate of parental and newly replicated T4 DNA during infection must be very complicated. We present (Fig. 12) a simplified metabolic chart for T4 DNA so that our discussion may originate from a set of shared assumptions. The gene products that are known to act on intracellular T4 DNA are shown; obviously many other proteins alter the state of the DNA. The synthesis of P32 has been measured during infections by mutants altered in each of the genes shown in Fig. 12. The results demonstrate unequivocally that there is a direct correlation between P32 synthesis and the quantity of single-stranded intracellular DNA. The results obtained with individual mutants have been interpreted within this framework.

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Gene 56 Mutants—The protein product of gene 56 is a
Gene 32 Expression during T4 Infection of E. coli

In destroying cytosine-containing T4 DNA (32); 46-56- infections responsible for P32 overproduction. P46 and P47 play a role in the concurrent accumulation of single-stranded DNA. Musting cytosine in place of hydroxymethylcytosine is rapid (32); dCTP (HM-dCTP) and dCTP are substrates for DNA synthesis (25); the T4 DNA polymerase, P43, will utilize either precursor in vivo (36). However, degradation of T4 DNA containing cytosine in place of hydroxymethylcytosine is rapid (32); the concurrent accumulation of single-stranded DNA must be responsible for P32 overproduction. P46 and P47 play a role in destroying cytosine-containing T4 DNA (32); 46-56- infections accumulate more T4 DNA than 56- infections but do not overproduce P32 (see Fig. 5).

Gene 1 and 42 Mutants—The products of genes 1 (deoxynucleotide kinase) and 42 (dCMP hydroxymethylase), like P46, influence the composition of the deoxynucleotide pools (37). Unlike 56- infections, 1- and 42- infections neither synthesize DNA nor overproduce P32 (Table I). P1 is required for phosphorylation of hydroxymethyl dCMP and also is used to increase the rate of phosphorylation of dGMP and dTMP (38); 1- infections have no dCTP or hydroxymethyl dCTP and severely reduced levels of dGTP and dTTP (37). Cells infected by gene 42 mutants have neither hydroxymethyl dCTP nor dCTP present in their deoxynucleotide pools (37). Thus, one would expect 42-56- infections (in which dCTP could survive) to be incorporated into unstable, P46-sensitive DNA to synthesize some DNA and overproduce P32; on the other hand, 1-56- infections might contain inadequate levels of dGTP and dTTP for DNA replication and hence not overproduce P32. P52 expression has been measured in each double mutant; the 42-56- infection does give P32 overproduction, whereas the 1-56- infection does not (39).

Gene 61 Mutants—Infections with gene 61 mutants (which have a DNA-delay phenotype (40)) overproduce P32 (Table I). The number of single-stranded breaks per genome length of DNA is abnormally large in 61- infections (41); furthermore, Hamlett and Berger (42) have shown that at 40 min postinfection, 61- infections contain at least 5 times more single-stranded DNA than is present in a wild type infection. The behavior of the other DNA delay genes is more complicated (see Table I and Ref. 7).

Other, less direct, arguments suggest that single-stranded DNA allows P32 derepression. Infections with a leaky gene 43 amber mutant (43) and also with a leaky gene 62 amber mutant (E1140) give P32 overproduction and some DNA synthesis (Table I). When the host is streptomycin-resistant (16), these phage allow no DNA replication and no P32 overproduction (Table I). Since P43 and P62 are components of the replication apparatus (34), the effect on DNA synthesis is expected; the surprising result is that weak suppression of these two ambers, and the concomitant slight synthesis of DNA, gives P32 overproduction (relative to both a true DNA negative infection and a wild type infection). Thus, under some conditions low levels of DNA replication caused by diminution of the quantity of a specific replication complex protein, may yield increased quantities of single-stranded intracellular DNA. The components of the replication apparatus should be balanced to give proper DNA synthesis.

What can be said about P32 overproduction in 33-55- infections? The protein products of genes 33 (18, 19, 43) and 55 (18, 19, 44) have been identified; these proteins alter the transcriptional specificity of RNA polymerase such that late RNA synthesis can occur (45). The rate of DNA synthesis in a 33 55- infection is at least as high as in a wild type infection. P32 expression is low in 33 55- 45- and 33 55- 46- infections; the effect of the gene 45 mutation is in keeping with the requirement for replication to provide potential P32 binding sites, and the effect of the gene 46 mutation suggests strongly that single-stranded DNA constitutes those sites (see Fig. 5). Since 33 55- infections do not yield any late proteins (see Fig. 3), a simple explanation of P32 derepression during 33 55- infections is that in wild type infections a late protein(s) eliminates single-stranded regions of T4 DNA. This putative late protein (P51) could be either a DNA-ligase, an anti-exonuclease, or a DNA-repair protein (see Fig. 12). Several facts suggest that late proteins cause DNA maturation into intact,
double helices; such DNA, ready for packaging, probably does not support further replication. Intracellular T4 DNA has many interruptions which are not present in the mature phage (46). Mutations in pre-replicative genes that give DNA arrest phenotypes are suppressible by both 33-55- and specific mutations in late genes (47, 48). In addition, Wu and Geiduschek (39) have measured gene 32 expression at very late times after infections with several replication-deficient mutants. Of the classic DNA-negative mutants (43-, 62-, 44-, 1-, 45-), all but 45- allow some replication-uncoupled late transcription and translation (39). As one would predict, a 45- infection (which allows no late gene expression and would contain no P32) allows more P32 synthesis at very late times than replication-deficient infections (43-, 62-, 44-, 1-) in which replication-uncoupled late gene expression occurs (39, 49). The encapsulation of T4 DNA into phage particles must occur subsequent to DNA maturation; packaging mutants (defective in genes 13, 16, 22, 23, and 24) synthesize wild type levels of P32.2

In summary, gene 32 mutant infections overproduce their respective amber fragment or missense polypeptide. Overproduction of altered gene 32 protein is not dependent on DNA replication nor inhibited by exonuclease deficiency. By contrast, during infections with wild type phage, P32 is synthesized at low levels. By altering the genetic constitution of the infecting phage (and thereby raising or lowering the quantities of intracellular single-stranded DNA), P32 synthesis can be derepressed or further repressed. These observations, in conjunction with the observed binding in vitro of P32 to single-stranded nucleic acids (3, 4, 50, 51) and the experiments reported in the following paper (6), lead to the model shown in Fig. 13. P32 can bind, in vivo, to single-stranded DNA; if none is available, the unbound P32 represses its own synthesis. Single-stranded regions of either parental or newly replicated DNA are positive regulators of P32 synthesis; any component of infected cells that alters the quantity or state of intracellular DNA can regulate P32 synthesis positively or negatively.

Gene 32 expression may be divided into three stages. During stage I, prior to DNA synthesis, gene 32 expression is uniform for all infections. The intracellular P32 concentration is probably low and no repression can occur. During stage II, both the onset of DNA synthesis and late gene expression, newly replicated DNA is responsible for continued derepression of P32 synthesis. The level of P32 synthesis in a wild type infection during stage II is nearly as high as that of "P32" in 32- infected cells; thus, by definition, wild type gene 32 expression is derepressed. Stage II might be the only time during infection when DNA replication is limited by the availability of gene 32 protein. The rate of P32 synthesis in infections that are 32+ and negative for DNA replication (such as 45-) does not markedly increase during stage II, presumably because repression occurs.

During stage III, subsequent to the onset of late gene expression, P32 synthesis in a wild type infection diminishes to the rate observed in infections which are replication-deficient (see Fig. 8). We think that the diminished rate of P32 synthesis in wild type infections results from a late protein(s) (P32) as above, we postulate that P32 aids in the maturation of DNA.

The three stages of P32 synthesis are diagrammed in Fig. 14; in fact, Fig. 14 is an abstraction of the data shown in Fig. 8. We have described T4 phages of various genotypes with four parameters: R, Dn, S, and Pn. These parameters are defined as follows: R is the repressor function of P32 (R- is 32-); Dn is the DNA replication function (Dn+ is capable of wild type levels of DNA synthesis), S is a DNA metabolism function (S+ is capable of generating wild type levels of single-stranded DNA), and Pn is the late protein(s) which matures DNA (Pn+ eliminates single-stranded DNA at wild type levels). P32 synthesis must respond to the total quantity of single-stranded intracellular DNA; that quantity is a complex function of each of the four parameters used (for example, R- is deficient in DNA replication, Dn- is deficient in late gene expression and hence has reduced quantities of Pn), S- is deficient in late DNA replication (e.g. infections), and Pn deficiency (e.g. 33-55- infections) may increase the rate of DNA replication). Thus, it is difficult to design experiments in which one parameter is systematically varied without influencing the others (see also Fig. 13).

Given the four parameters (above), 16 combinations (or "genotypes") are possible (Table III). Eight combinations are R+, i.e. have no repressor function, and hence generate maximal levels of gene 32 expression at all times. The eight other combinations are shown in Table III. The relative rates of P32 synthesis for examples of each combination have been
Role of P32 during T4 Infection: Replication, Recombination, and Repair

The DNA of T4 phage particles contains no single strand interruptions (46), yet, during T4 infection, several states through which the phage DNA passes must involve nicks and gaps. The role of P32 during T4 infection is most likely a direct function of its capacity to completely "cover" single-stranded regions of DNA which constitute, for the most part, a small fraction of the total DNA. Initial processes of repair, replication, and recombination probably each generate gaps in the DNA; these gaps must be filled by P32 to facilitate those processes as well as to prevent wanton nucleolytic attack at those gaps.

Wu and Yeh (52) have studied repair processes during T4 infection; their data show that P32 is involved in the repair process, and that P32 also prevents nucleolytic attack on the DNA. There are several T4 mutants unable to efficiently repair DNA; those mutants lacking an excision nuclease would be expected, as in the cases of gene 46 and 47 mutants, to underproduce P32. Mutants deficient in gap filling itself might be P32 overproducers.

The direct involvement of P32 in replication has been demonstrated. Under nonpermissive conditions, mutants defective in gene 32 do not synthesize any DNA (1). P32 stimulates P43-directed DNA synthesis in vitro (5). Because of Snustad's gene dosage experiments (discussed more fully below), P32 frequently is thought to be the rate-limiting component of the replication apparatus (53). Since we find that P32 can be synthesized at much greater than the wild type rates, we do not think of P32 as a rate-limiting component in vivo. Rather, we propose that P32 is synthesized as needed to fill gaps created during DNA replication. These gaps could result from the process of discontinuous replication (31), by the excision of RNA primers from the 5' termini of newly replicated DNA (54), or, perhaps, from the very first events used to generate origins of replication (55). With the development of cell-free systems in which the T4 replication apparatus functions, the biochemical role of P32 may be elucidated (34).

Perhaps P32 will facilitate the entrance of other replication components into replication origins and subsequently travel at that fork as an integral (and protective) component of that fork; this notion is, in fact, merely a restatement of the "glamorous" P32 model proposed by Alberts (56).

The role of P32 in recombination has been documented rather exhaustively; P32 is required both in vivo (2) and in vitro (57, 59). Most models for recombination at the molecular level involve either gapped DNA structures attacking a double helix (59) or complementary gapped DNA structures interacting with each other (60). Since the renaturation of single-stranded DNA can be facilitated in vitro by P32 (3, 4), the obligate role of P32 during recombination is plausible. Recombination during T4 infection must be stimulated by proteins which facilitate accumulation of single-stranded DNA and inhibited by proteins which facilitate DNA repair. Those mutant infections which give abnormal rates of P32 synthesis also exhibit abnormal levels of recombination (Table IV). Mutants which give increased P32 synthesis (and, presumably, gapped DNA) show increased recombination. Infections with

### Table III

**Gene 32 expression as function of four parameters**

<table>
<thead>
<tr>
<th>&quot;Genotype&quot;</th>
<th>Example</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. R D₄⁺ S P₅⁺</td>
<td>32-...-...</td>
<td>Fully derepressed for &quot;P32&quot; synthesis at all times after infection.</td>
</tr>
<tr>
<td>B. R D₄⁺ S P₅⁺</td>
<td>T₄⁺</td>
<td>Yields P32 synthesis at nearly the fully derepressed rate; no late gene expression occurs (39).</td>
</tr>
<tr>
<td>C. R D₄⁺ S P₅⁺</td>
<td>33-55⁻</td>
<td>Yields 12-fold less P32 at late times than 33-55⁺; after about 25 min P32 synthesis is comparable to the wild type rate (B).</td>
</tr>
<tr>
<td>D. R D₄⁺ S P₅⁺</td>
<td>46 33 55</td>
<td>Yields reduced P32 at early times because of replication defect (34); at late times no late transcription occurs (39), and hence no P₅⁺ accumulates.</td>
</tr>
<tr>
<td>E. R D₄⁺ S P₅⁺</td>
<td>45⁻</td>
<td>Yields reduced P32 because of replication defect and exonuclease deficiency; complete absence of late transcription (hence P₅⁺) prevents full repression of P32 synthesis.</td>
</tr>
<tr>
<td>F. R D₄⁺ S P₅⁺</td>
<td>46 45⁻</td>
<td>Yields reduced P32 because of replication defect and exonuclease deficiency; complete absence of late transcription (hence P₅⁺) prevents full repression of P32 synthesis.</td>
</tr>
<tr>
<td>G. R D₄⁺ S P₅⁺</td>
<td>43⁻</td>
<td>Similar to E, except that limited late transcription occurs (39); P₅⁺ can accumulate to a small extent, and hence at late times less P32 synthesis occurs than in E.</td>
</tr>
<tr>
<td>H. R D₄⁺ S P₅⁺</td>
<td>46 43⁻</td>
<td>Similar to G, except for the exonuclease deficiency. This phage yields less P32 at late times than any phage except I.</td>
</tr>
<tr>
<td>I. R D₄⁺ S P₅⁺</td>
<td>46⁺</td>
<td>This phage becomes nearly D₄⁺ at late times; late transcription occurs at nearly normal levels. The resulting &quot;genotype&quot; is nearly R D₄⁺ S P₅⁺, and the level of P₅⁺ is high. This phage yields less P32 than H, presumably because P₅⁺ is low in H.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time postinfection</th>
<th>Quantities of P32 synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>(C,B) &gt; (E,G) &gt; (D,F,H,I)</td>
</tr>
<tr>
<td>25</td>
<td>C &gt; &gt; (B,D,E,G) &gt; (F,H) &gt; I</td>
</tr>
<tr>
<td>40</td>
<td>C &gt; &gt; &gt; (B,D,E) &gt; (F,G) &gt; H &gt; I</td>
</tr>
</tbody>
</table>

measured at several times after infection (this paper and Refs. 7 and 39); these hierarchies of P32 synthesis are given as well.

The differences in P32 synthesis are very large; at 30 min postinfection P32 can be synthesized at a hundredfold higher rate in R D₄⁺ S P₅⁺ than R D₄⁺ S P₅⁺ infections (e.g. in 33-55⁻ infections as opposed to 46⁻ infections.² ³)
TABLE IV
Recombination frequency and gene 32 expression

<table>
<thead>
<tr>
<th>Gene (X)</th>
<th>Gene 32 expression</th>
<th>Recombination frequency</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.18</td>
<td>0.13</td>
<td>61</td>
</tr>
<tr>
<td>47</td>
<td>0.18</td>
<td>0.11</td>
<td>62</td>
</tr>
<tr>
<td>52</td>
<td>1.4</td>
<td>2.2</td>
<td>63</td>
</tr>
<tr>
<td>41</td>
<td>2.1</td>
<td>1.9</td>
<td>61</td>
</tr>
<tr>
<td>30</td>
<td>2.6</td>
<td>1.6</td>
<td>61</td>
</tr>
<tr>
<td>56</td>
<td>3.2</td>
<td>2.1</td>
<td>61</td>
</tr>
<tr>
<td>39</td>
<td>3.9</td>
<td>2.8</td>
<td>63</td>
</tr>
<tr>
<td>61</td>
<td>13.0</td>
<td>7.9</td>
<td>42</td>
</tr>
</tbody>
</table>

We have displayed measurements made under the most restrictive conditions which yield progeny. Furthermore, for data obtained under equivalently restrictive conditions, for genes 46 and 47 we have shown the lowest recombination frequencies reported; for the other genes we have given the highest.

The data for gene 32 expression in cells infected by mutants in gene X are the average for each allele tested in Table I. These data are expressed relative to wild type gene 32 expression. Data for recombination frequencies are from the literature cited; recombination frequencies are given relative to wild type infected cells that is, $rI_{X} - X^{-}$ by $rI_{X}, X^{-}$.

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Acknowledgments—We wish to thank everyone in our lab for their generosity and ideas; we also thank Drs. Mike Yarus, Larry Soll, David Hirsh, and Glenn Herrick for valuable conversations. Tom Hill helped with some of the experiments. Henry Krisch and Dick Epstein communicated their results to us prior to publication. Dr. Bill Wood provided much needed editorial criticism.

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