Bacteriophage T4 *Alc* Gene Product: General Inhibitor of Transcription from Cytosine-Containing DNA

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The *alc* gene of bacteriophage T4 was originally defined on the basis of mutations which allow late protein synthesis directed by T4 DNA containing cytosine rather than hydroxymethylcytosine. The question remained whether the normal *alc* gene product (*gpalc*) also blocks the transcription of early genes from cytosine-containing DNA. Complementation experiments were performed between hydroxymethylcytosine-containing phage which direct *gpalc* synthesis but carry mutations in a given gene(s) and cytosine-containing phage carrying that gene(s). The required protein would then have to be directed by the cytosine-containing DNA: it is looked for directly on polyacrylamide gels or through its physiological effects or both. For all early proteins examined in this way, no synthesis was observed when 95 to 100% of the hydroxymethylcytosine was substituted by cytosine in the infecting DNA, whereas there was significant synthesis with 75% substitution or less. The results indicate that *gpalc* is carried in with the infecting DNA or is made and acts very early to block transcription of all cytosine-containing DNA.

The DNA of bacteriophage T4 normally contains 5-hydroxymethylcytosine (HMC) rather than cytosine (C). When the phage-directed deoxycytidinetriphosphatase (dCTPase) is inactivated by mutation, C can be incorporated into T4 DNA (9, 25). However, such DNA fails to direct the synthesis of T4 late proteins, apparently because late transcription is blocked (2, 8, 25). Late gene expression is inhibited even when the two C-specific endonucleases normally induced by T4, endo II (17) and endo IV (18), have been eliminated by mutation so that C-containing DNAs are not degraded (8). Snyder et al. (22) described recessive mutations that circumvent this block in late protein synthesis and thus permit the synthesis of viable T4 phage with C rather than HMC in their DNA; such mutants are termed *alc*, for allows lates on cytosine-DNA. These mutations all map in a single gene lying between the genes for RNA ligase and 5'-polynucleotide kinase-3'-phosphatase in the T4 genetic map. The mode of action of the normal *alc* gene product (*gpalc*) is still not understood.

Possible explanations for the observed transcriptional block by *alc* can be divided into two classes.

(i) The *alc* gene might effect, or interact with, one or more of the T4-directed alterations in RNA polymerase necessary for the transcription of T4 late genes, such as those effected by genes 33, 55, or 45 (5, 15, 23), which could coincidentally block the polymerase from reading C-containing DNA.

(ii) The *alc* protein might block the transcription of C-containing DNA as part of a T4 mechanism for shutting off host transcription or excluding other phage or both, either by altering the RNA polymerase or by modifying the DNA directly.

In the second case only, the normal *alc* protein should also inhibit the reading of T4 early genes from C-containing DNA, since these genes apparently have promoters that can be recognized by the unmodified host RNA polymerase (3, 28). A direct analysis of whether C in T4 DNA would affect early RNA synthesis in the presence of the normal *alc* protein is complicated by the fact that synthesis of early T4 proteins is directed primarily or exclusively by the infecting phage DNA, so that the infection must be carried out by largely C-containing phage. However, the only way to make the needed C-containing phage is by using *alc* mutants of T4. Furthermore, no amber mutants or other suitable con-

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ditional-lethal mutants of *alc* have been isolated. Therefore, to look for the synthesis of specific gene products directed by infecting C-containing DNA in the presence of the functional *alc* protein, we have used complementation experiments of the form shown in Fig. 1.

<i>am56</i> ⁻	<i>denB-ac</i>	<i>denA</i>	<i>alc</i>	X ⁺	C-DNA
			X		
56 ⁺	<i>rII-denB</i>	<i>denA</i>	<i>alc</i> ⁺	<i>amX</i>	HMC-DNA

FIG. 1. Complementation experiment protocol to detect synthesis of specific gene products directed by infecting C-containing DNA in the presence of functional *alc* protein.

Here, *denA* and *denB* are the genes for endo II and endo IV, respectively. *denB* mutations are always deletions extending into *rII* or *ac* (acri-flavin resistance). Gene 56 specifies dCTPase, and *amX* is a mutation in some early gene(s) X, whose protein product can be monitored. The ability of C-containing DNA to direct the synthesis of the X protein can be measured directly by quantitating the appropriate band after polyacrylamide gel electrophoresis when the protein is resolvable in this way. Alternatively, the synthesis can be measured indirectly by monitoring the function(s) for which X is needed, such as DNA synthesis or late protein synthesis. Since most early gene products are catalytic, the latter type of measurement can sometimes provide a

more sensitive test of gene expression than does the direct measurement of the protein product.

MATERIALS AND METHODS

Bacterial and phage strains. *Escherichia coli* K803 (*supE* *r_K⁻m_K⁻*) (30) was used for growing most phage stocks and for plating. *E. coli* B834 (*met sup⁺ r_B⁻m_B⁻*) (3) was used for producing C-containing phage and for complementation tests. Note that both K803 and B834 lack the restriction modification system that otherwise degrades C-containing DNA. *E. coli* 5K(R1), which carries a highly amplified *EcoRI* plasmid (29), was obtained from G. Wilson.

Table 1 indicates the sources of the various mutations that were used to construct appropriate phage strains for complementation experiments. The desired amber or deletion mutation was crossed into appropriate *denA-denB* mutants to prevent degradation of C-containing phage DNA in complementation tests. The selective plating methods used are described by Morton et al. (11). Since the *denB* mutation displays no readily recognized phenotype of its own, strain construction and genetic analysis were simplified by using extended deletions covering *denB* and either the neighboring *rII* region or the *ac* gene—both readily identifiable in plate assays. In the complementation experiments, the use of these two types of *denB* deletions allowed us to ascertain that both parents were present in productive infections and also to look for synthesis of the *rII* proteins directed by C-containing DNA.

Multiple mutants are routinely designated in this paper by listing gene names and numbers or the af-

TABLE 1. Mutations used in preparing endo II⁻ and endo IV⁻ multiple mutants for complementation experiments

Gene	Function	Mutation	Source
32	Helix destabilizing	<i>amHL618</i>	J. Wiberg
42	dCMP HMase	<i>amN55</i> × 5 ^a	J. Wiberg
43	DNA polymerase	<i>amB22</i> × 5 ^a	J. Wiberg
45	Required for DNA synthesis	<i>amE10</i>	J. Wiberg
52	Required for normal initiation of DNA synthesis; topoisomerase	<i>amH17</i>	J. Wiberg
55	Required for late transcription	<i>amBL292</i>	E. T. Young
56	dCTPase	<i>amE51</i> × 5 ^a	J. Wiberg
<i>rII-denB</i>	Endonuclease IV	<i>ΔrIIH23</i> ^b	N. Cozzarelli
<i>rII-denB</i>	Endonuclease IV	<i>ΔrIIDD2</i> ^b	L. Snyder
<i>ac-denB</i>	Endonuclease IV	<i>saΔ9</i> ^b	N. Cozzarelli
<i>denA</i>	Endonuclease II	<i>nd28</i>	H. Warner
<i>alc</i> ^c	Required for late-protein synthesis on T4 C-DNA	<i>alc1</i>	L. Snyder
		<i>alc2</i>	L. Snyder
		<i>alc10</i>	L. Snyder
		<i>alc14</i>	L. Snyder
		<i>alcE2</i>	Isolated in this laboratory
		<i>Δ(39-56)</i> ^d	J. Weil
Various "nonessential" functions			

^a The designation × 5 means that the strain was genetically purified by backcrossing five times against the wild type.

^b The extents of these deletions are indicated in reference 7.

^c *alc1* and *alc2* were originally isolated by L. Snyder from a 56 *ΔrIIDD2* mutant and crossed into [56 *denA denB-ac*] in this laboratory. *alc10* and *alc14* were also isolated by L. Snyder from [56 *ΔrIIH23 denA*]. *alcE2* was isolated in this laboratory from [56 42 *ΔrIIH23 denA*].

^d See reference 4.

infected proteins, if known, inside square brackets. We use the designation CT4 for any T4 strain with the basic genotype [*alc denB* 56 (dCTPase)] when such strains are grown under restrictive conditions (as in B834); the HMC in their DNA is substantially or entirely replaced by C. Any CT4 strain grown under permissive conditions (as in K803) and therefore having ordinary HMC-DNA is designated CT4^H. Specific CT4 strains may be designated by listing their distinguishing mutations in square brackets. (It should be noted that most CT4 strains are hybrids of T4B and T4D.)

Media. All media used were described by Morton et al. (11). Bacteria were grown and infected at 37°C in M9 medium supplemented with 0.1% Casamino Acids, except in those experiments involving incorporation of radioactive amino acids.

Monitoring of extra mutations in CT4 stocks. Under restrictive conditions, CT4 strains carrying a *denA* mutation normally make DNA with C substituted for about 95% of the HMC (22). However, such strains tend to pick up extra mutations that reduce the fraction of C to 50 to 80% (Fig. 2). These uncharacterized mutations, termed *hi*, occur at at least two different loci (Kutter, unpublished data). Since this variation in the C/HMC ratio would confuse the interpretation of complementation experiments, we routinely monitored the extent of C substitution by plating each stock on *E. coli* 5K(R1). On this strain, fully C-containing CT4 plates with an efficiency of about 10⁻⁴, whereas *denA*⁺ CT4 stocks, with about 70% C in their DNA, plate with an efficiency near 10⁻², and T4D⁺ plates with an efficiency of 1. For some experiments, we crossed a gene 42 (deoxycytidylate hydroxymethylase [HMase]) amber mutation into the CT4 strains to ensure that their infecting DNA will have 100% substitution of C for HMC (Fig. 2), since the

only pathway for making HMC requires HMase. Note that such a gene 42 mutation will not complicate our complementation experiments, since it will be complemented by its wild-type allele on the HMC-containing phage (11).

RESULTS AND DISCUSSION

Substituting C for HMC blocks T4 early enzyme synthesis. In the presence of the normal *alc* protein, substituting C for HMC in the infecting DNA has a drastic effect on the ability of the DNA to direct the synthesis of every T4 early enzyme examined to date, as determined by the type of complementation experiment outlined above. The experiments summarized in Table 2 examined this complementation by monitoring DNA replication and phage production. The latter is partly a reflection of the ability of T4 to synthesize late, or structural, proteins. Note that in all of the experiments in this set, except the one testing the production of dCMP HMase, the CT4 also carried a gene 42 mutation to ensure that C replaced 100% of the HMC (see Fig. 2, which analyzes the pyrimidine content of several T4 strains). This mutation is responsible for the low DNA and phage production observed in the CT4 control infection in these cases, particularly at the early time (Table 2) a phenomenon discussed by Morton et al. (11). In the mixed infections (Table 2), the HMase mutation was complemented by the wild-type allele on the HMC-containing phage, so DNA and phage production should be similar to those seen for T4D⁺ unless the C in the CT4 DNA interfered with its transcription. Such interference was, in fact, observed for all genes tested.

Genes 32, 43, and 45 direct the synthesis of proteins in the DNA replication complex (1). In all three cases, DNA replication was reduced to less than 2% of the wild-type control level when these genes were transcribed from C-containing DNA in the presence of the normal *alc* protein (Table 2). The small amount of residual [³H]-deoxyribosylthymine incorporation probably reflects synthesis in a few cells infected by the CT4 alone. This also appears to be the explanation for the few phage eventually synthesized (0.1 to 0.3 phage per cell at 60 min after infection).

Mutations in gene 52 led to a delay in both DNA synthesis and phage production. There was a similar delay and inhibition of both DNA and phage synthesis in the complementation experiments (Table 2 and unpublished data). DNA synthesis was actually somewhat lower than after infection with the mutant by itself. Gene 55 controls one of the RNA polymerase-binding proteins needed for late protein synthesis, and it should have no effect on DNA replication. As expected, there was extensive repli-

FIG. 2. Determination of the pyrimidine compositions of various T4 strains. The method of labeling with [³H]uridine, DNA hydrolysis, and ascending paper chromatography is described by Morton et al. (11). Bars at the top of the figure show the approximate positions of pyrimidine standards run along with the DNA hydrolysates. Symbols: ●, T4D⁺; ■, CT4 [*alcE2* 56 *denA* *rIIH23-denB* 42]; ○, CT4 [*alc1* 56 *denA* *denB* *hiA*]. The *hiA* mutation (for HMC increased) appeared spontaneously in a CT4 [*alc1* 56 *denA* *denB*] stock in whose DNA C replaced 95% of the HMC (data not shown). The mutation reduces the level of C replacement to approximately 55%.

TABLE 2. DNA and phage production in complementation tests and controls^a

Gene	DNA (cpm per 2.5×10^7 cells)				Phage produced per cell			
	Mutant alone ^b	Complementation	T4C alone ^c	T4D ⁺ control	Mutant alone ^b	Complementation	T4C alone ^c	T4D ⁺ control
32	15	45	830	2,800	<0.01	0.3	5.6	105
42	205	75	1,940		<0.01	<0.01	33	
43	60	110	470	5,360	<0.1	0.1	1.0	450
45	74	90	470	5,360	<0.02	0.2	1.0	450
52	1,400	500	150		6.1	5.5	0.6	
55	3,900	1,900	470	5,360	<0.05	1.1	1.0	450

^a B834^{mut} was grown at 37°C in M9 medium plus 20 μ g of L-methionine per ml to a concentration of 5×10^8 cells per ml and chilled. It was supplemented with 40 μ g of L-tryptophan per ml to enhance phage adsorption, prewarmed for 3 min at 37°C, and added to a flask containing a single phage stock at multiplicity of infection of 12 or a uniform mixture of the two phage stocks at a multiplicity of infection of 6 each. Samples were taken periodically to measure phage production and DNA synthesis through incorporation of [³H]thymidine, as previously described (8). Data shown are for 60 min after infection. Note that four separate experiments are compiled here. (Genes 32, 42, and 52 were each analyzed in separate experiments, and genes 43, 45, and 55 were run at the same time.)

^b [*denA denB amX*] containing HMC-DNA, where X is the gene being tested.

^c [*alc denA denB* 56 42] or, in the case of the gene 42 complementation, [*alc denA denB* 56], in either case with C-DNA.

cation in the mixed infection between this mutant and CT4, although, again, DNA production was substantially lower than that seen with the gene 55 mutant by itself. The somewhat reduced DNA replication here and in gene 52 (above) may indicate direct inhibition of replication by fully C-containing DNA. About one phage per cell was produced, of both parental types, indicating that replication of the C-containing DNA was not specifically inhibited. The fact that so few phage were produced appears to argue for the widespread belief that early T4 messages must be transcribed primarily from the infecting DNA. We reached this conclusion because the progeny DNA must contain primarily HMC, since the parent with HMC-DNA is 56⁺. If the progeny DNA could direct functional early transcription, one would expect to see extensive RNA polymerase modification by gp55 directed by this DNA, leading to extensive late protein synthesis and phage production. Pulitzer (13), using temperature-sensitive gene 55 mutants, has shown that providing functional gene 55 protein as late as 15 min after infection (after extensive DNA synthesis has occurred) can still largely rescue phage production. Thus, it appears unlikely that our observed effect can be explained simply in terms of timing problems.

There was also essentially no complementation observed for the dCMP HMase, as measured by either DNA or phage production (Table 2), although the CT4 DNA in this case had C substituted for only 95% of its HMC. (Note that HMase is essential for DNA replication as long as the highly active T4 dCTPase is present to exclude dCTP as a substrate.)

Direct gel electrophoresis analyses confirm the above conclusions from the functional studies. For example, no gp45 or gp42 (HMase) can be detected under conditions where their synthesis would have to be directed by C-containing DNA in the presence of a functional *alc* protein, even though in this particular experiment the CT4 DNA contained C substituted for only 95% of the HMC (Fig. 3). Under similar conditions, where the phage with HMC-DNA was 43⁻ or *rIIB*⁻, densitometry (Fig. 4) indicated the synthesis of very small amounts of gp43 (DNA polymerase) and the *rIIB* protein. Similar results have been observed in several experiments for the *rIIA* protein, but that is not apparent in Fig. 4 because the amber fragment made by this particular gene-43 mutant is about the same size as the *rIIA* protein and masks it on gels. Quantitation of these gels indicated that less than 1% as much gp43 was made here as was made after infection with T4D⁺ or CT4 alone. We find that even this low level of protein synthesis is largely eliminated when the CT4 DNA has C substituted for 100% of its HMC.

Control experiments established that the lack of complementation seen when the CT4 DNA contained C was a direct consequence of the C content and not some secondary consequence of an unsuspected mutation carried by the mutant phage. Each of the CT4 strains used for these studies was also grown in *E. coli* K803, which suppressed the amber mutations in genes 42 and 56 so phage were produced containing little or no cytosine in their DNA. These HMC-containing phage (CT4^H) are thus isogenic with the CT4 phage used in the experiments described above.

FIG. 3. Inability of C-containing DNA to direct the synthesis of H_Mase and of gp45. Tracks 1 to 6 are samples labeled 3 to 6 min after infection, and tracks 7 to 12 are samples labeled 6 to 9 min after infection. Tracks 1 and 7, [denA denB 42] (HMC-DNA); tracks 2 and 8, complementation of [denA denB 42] (HMC-DNA) and CT4 [alc2 denA denB 56] (95% C); tracks 3 and 9, [denA denB 45], tracks 4 and 10, complementation of [denA denB 45] (HMC-DNA) and CT4 [alc2 denA denB 56] (95% C); tracks 5 and 11, CT4 [alc2 denA denB 56] (95% C); tracks 6 and 12, T4D⁺. For each infection, B834 at a concentration of 5×10^6 cells per ml was supplemented with tryptophan at 40 μ g/ml, prewarmed for 3 min at 37°C, and added to a flask containing a single phage stock at a multiplicity of infection of 12 or a uniform mixture of two phage stocks at a multiplicity of infection of 6 each. The labeling and electrophoresis techniques have been described previously (8, 11). Shown here is a section of a long (27-cm) gel containing a 9 to 17% gradient of acrylamide and a 9 to 15% sucrose gradient as described previously (11).

In a number of analogous complementation experiments between these phage stocks and the various amber-mutant strains, the amount of complementing protein synthesized ranged between 35 and 65% of the normal level; 50% would be expected if the two types of infecting DNA were transcribed at the same rate. DNA and phage syntheses were virtually normal, confirming that the gene 42 mutation could be complemented in mixed infections (see above).

The exclusion is a function of the *alc* protein and not some other aspect of competition between the HMC- and C-containing T4 DNA. In control experiments (data not shown) in which the HMC-containing parent also contained an *alc*-gene mutation, good complementation was observed for mutants in both genes 43 and 55.

Time of action of the *alc* protein. The results discussed above show that the *alc* protein

strongly restricts the transcription of various early T4 genes from C-containing DNA. A more detailed analysis of this effect should indicate the functional kinetics of the *alc* protein and might provide greater insight into early T4 transcriptional control mechanisms.

Early T4 genes can be divided into at least three classes on the basis of transcriptional timing and control mechanisms: immediate early, delayed early, and middle or quasi-late. There has been much confusion in the use of these terms; their meaning and suggested employment have been reviewed by Rabussay and Geiduschek (14). In brief, immediate early proteins appear within the first few minutes after infection; their transcripts are produced even when chloramphenicol has been added to the cells before infection. Delayed early proteins apparently are transcribed on distal parts of the same messenger RNAs that carry immediate early

FIG. 4. Inability of C-containing DNA to direct the synthesis of gp43 and rII_B proteins. The methods used were identical to those for Fig. 3, except that in this case a 10% acrylamide gel was used, and the bands were quantitated with a Schoeffel scanning densitometer and a Hewlett-Packard integrator by plotting on a logarithmic scale for increased sensitivity and integrating the areas under the peaks on a linear scale. The labeling period was 6 to 9 min after infection. Top curve: complementation of [denA rII_B denB 43] (HMC-DNA) with [CT4 alc10 denA denB-alc] 56 (95% C). Bottom curve: T4D⁺.

gene transcripts, possibly with some sort of regulatory signals in between which make the expression of delayed early genes susceptible to chloramphenicol. Both of these classes of early proteins are produced *in vitro* in coupled transcriptional-translational systems with the *E. coli* RNA polymerase from uninfected and infected cells. In contrast, the middle, or quasi-late, class of genes is not transcribed *in vitro*. Recognition of a new set of so-called middle promoters is apparently required, along with the product of the T4 *mot* gene (10). Some genes, such as *rIIB*, can be transcribed from either early or middle promoters.

The protein products we have examined include representatives of all three classes. The products of genes 42 and 45 show immediate early kinetics. The *rIIA* protein shows typical delayed-early behavior, whereas *rIIB*, 43, and 45 are mainly transcribed from middle promoters, although they are also transcribed to a limited extent in a delayed early mode. Gene 32 appears to be transcribed only from a middle promoter. Synthesis of all of these early enzymes appears to be inhibited almost totally by C in the infecting DNA, implying a very early and global action of the *alc* protein.

The immediate early genes can actually be subdivided into two groups. Most early enzymes show extended synthesis past their usual shutoff times when DNA synthesis is blocked. However, there is one group, among the first to appear, whose synthesis stops by about 5 min after infection, whether DNA synthesis proceeds or not. All of these are so-called nonessential genes whose absence normally has little or no effect on phage physiology, and most of the proteins can only be detected in crude lysates by enzymatic function, not on gels. However, one such protein, with a molecular weight of about 14,000, is coded by a gene lying in the region between genes 39 and 56 which is missing in the deletion $\Delta(39-56)_{12}$ (4). Synthesis of this protein is greatly reduced when it must be directed by C-containing T4 DNA in the presence of the *alc* protein (Fig. 5). It appears that a very small amount of synthesis may still be occurring, but a neighboring minor band complicates the analysis. Under the same conditions of infection, there is no synthesis of the other three proteins whose bands are identifiable on gels and whose genes also lie in this same 10-kilobase deletion (Fig. 5, arrows). This agrees with our results on other early enzymes.

The earliest labeling time shown here is 3 to 6 min after infection. The high residual bacterial protein synthesis during the first 2 to 3 min after infection complicates the interpretation of similar complementation experiments at earlier

FIG. 5. Inability of C-containing DNA to direct the synthesis of certain nonessential proteins. Tracks 1 to 4 are samples labeled from 3 to 6 min after infection and tracks 5 to 8 are samples labeled 6 to 9 min after infection. Tracks 1 and 5, [*denA denB-ac* $\Delta(39-56)_{12}$]; tracks 2 and 6, complementation between [*denA denB-ac* $\Delta(39-56)_{12}$] (HMC-DNA) and CT4 [*alc14 denA rII-denB 56*] (95% C); tracks 3 and 7, CT4 [*alc14 denA rII-denB 56*]; tracks 4 and 8, T4D⁺. Experimental conditions and gel types were the same as those for Fig. 3.

times (data not shown). The UV irradiation of cells that is required for analysis at this time itself reduces protein synthesis and might complicate the observations. However, if significant transcription of these genes had occurred during that period, we would expect to detect the translation of that RNA during the 3- to 6-min pulse of protein labeling. Analysis with two-dimensional protein gels may be necessary to finally resolve this question.

The observed effects on immediate early protein synthesis imply that the *alc* protein acts very early in infection, since it must modify most or all of the RNA polymerase (or DNA) molecules before they become extensively involved in T4 transcription. *alc* could be one of the first proteins made, and it could act very rapidly. The protein could also be carried in with the infecting DNA as is gp2, which protects the linear T4 DNA from *E. coli* exonuclease V (19), and gpalt, which ADP-ribosylates the host RNA polymerase (16).

Our observations do not fit the reported kinetics of appearance of the 15k polymerase-bind-

ing protein (24), which Sirotkin et al. (20) suggested might be the *alc* protein. (Since this protein cannot be resolved on one-dimensional gels, it has been measured only through antibody coprecipitation of the RNA polymerase.) However, more recent experiments by Goff and others suggest that *gpalc* is not this 15k protein (7).

The apparent kinetics of appearance of the *alc* function are also more rapid than those reported for the kinetics of activity of the *unf* protein, responsible for unfolding the host genome (21), although there is strong evidence (20, 26) that the *unf* function is actually a second function of the *alc* protein. This might be another case analogous to that reported by Tomich et al. (27) for dCMP HMase where there is a discrepancy between the kinetics of synthesis of a protein and the kinetics of its function *in vivo*. In that case, it was suggested that the *in vivo* activation involves interaction with other proteins.

Recently, Parson and Snyder (12) reported that the *alc* protein leads to rapid inhibition of lambda late transcription after superinfection with T4, the inactivation being at least 95% complete within 5 min. This is in general agreement with the time course we observe, particularly considering that the lambda transcription is already in progress. However, they observe little *alc*-specific inhibition of lambda early proteins. It is not yet clear whether the difference is due to the high temperature of that portion of their experiment or to inherent differences between T4 and lambda early transcription.

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