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A gene of bacteriophage T4 whose product prevents true late transcription on cytosine-containing T4 DNA

(transcription factors/regulation/T4 late mRNA synthesis)

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ABSTRACT **T-even coliphages have 5-hydroxymethylcytosine in their DNA instead of cytosine. In some T4 mutants, the replicated DNA contains cytosine, but then no late gene products are made. We show that the inability to make late gene products with cytosine-containing T4 DNA is due to a T4 gene product. This gene product, while probably nonessential under normal conditions, interacts with an essential part of the transcription apparatus. Mutations in this gene allow viable T4 particles to be made whose DNA has been substituted almost 100% with cytosine.**

Studies of the interaction between the T-even coliphages T2, T4, and T6 and their host have been central to the development of modern biology. One reason for their importance is that the DNA of these bacteriophages contains an unusual base, 5-hydroxymethylcytosine, instead of the usual cytosine. It was the appearance, after infection, of enzymes responsible for synthesizing this base that led to the concept of virus-induced functions (1).

The presence of hydroxymethylcytosine protects the phage DNA against the normal host restriction systems (r_K m_K or r_B m_B) but opens it to attack by other restriction systems coded by the *rgl* genes (2). In turn, the existence of virus-coded glucosyltransferases, which put glucose on the hydroxymethyl group of hydroxymethylcytosine, makes them immune to the *rgl* systems (3). Presumably because they have hydroxymethylcytosine, it is possible for the T-even coliphages to code for deoxyribonucleases that are specific for cytosine-containing DNA and therefore to degrade host DNA in the presence of bacteriophage DNA. Host DNA degradation is a nonessential source of nucleotides for bacteriophage DNA replication.

By mutating the functions required for the synthesis of hydroxymethylcytosine and the functions required to degrade cytosine-containing DNA, it should be possible to make T4 with cytosine in its DNA instead of hydroxymethylcytosine. In fact, it has been known for some time that with the right combination of mutations, T4 DNA that contains cytosine is replicated. The mutations required are apparent from the scheme shown in Fig. 1. In essence, T4 prevents cytosine from entering its DNA by producing a dCTPase (4) (gene 56) which dephosphorylates dCTP and dCDP (5, 6) and furnishes substrate (dCMP) for a hydroxymethylase (gene 42) (7). The substrate, dCMP, is also furnished by the breakdown of host DNA, which requires at least T4 endodeoxyribonuclease II (gene *denA*) (8–10) and an exonuclease activity (genes 46 and 47) (11, 12). Thus, a dCTPase⁻ mutant should have some cytosine in its replicated DNA but should have hydroxymethylcytosine as well, since dCMP substrate for hydroxymethylase is being furnished by host breakdown and, possibly, from dCDP by the reversal of the host dCMP kinase reaction. Even though it has some hydroxymethylcytosine, this DNA is degraded by the enzymes responsible for degrading host DNA because of the presence of cytosine. These enzymes are the same ones required for host DNA breakdown, except that T4 endodeoxyribonuclease IV (gene *denB*) plays a major role in the degradation of T4 DNA containing cytosine, while it only plays a minor role in host DNA degradation (13, 14). Thus, dCTPase⁻, *denB* mutants make normal amounts of T4 DNA, but this DNA has some hydroxymethylcytosine because host DNA breakdown furnishes dCMP for the hydroxymethylase enzyme. In order to decrease further the amount of hydroxymethylcytosine, it is necessary to eliminate host DNA breakdown with a *denA* (endo II⁻) mutation and/or to prevent the synthesis of hydroxymethyl dCTP with, for example, a gene 42 (hydroxymethylase⁻) mutation.

When the T4 DNA synthesized after infection contains cytosine, no phage are produced because no true late mRNA or protein synthesis occurs (12–17). True late mRNAs are defined as those that are dependent on the replication of T4 DNA and the products of genes 33, 55 (15), and 45 (18) for their appearance. Apparently, cytosine-containing T4 DNA cannot be used as a template for the synthesis of these mRNAs. We show in this paper that cytosine-containing T4 DNA cannot be used for true late transcription because of the product of a T4 gene, which we call *alc*. The *alc* gene product is probably nonessential in our laboratory conditions. We suppose that it is involved, somehow, in the shutoff of host transcription by T4.

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains. *Escherichia coli* B834 is a su^- B strain lacking the $r_B m_B$ restriction system (19). It was obtained from H. Revel. *E. coli* B40 is a B strain carrying the amber suppressor su_I^+. E. coli B^E is a su^- strain with normal restriction systems. *E. coli* K803 is an $amsu_{II}^+$ K12 strain which is $r_k^- m_k^- rgl^-$ (19).

The parent strain for the T4 dCTPase⁻, *denB*, *alc*⁻ mutants was amE51, DD2, where amE51 is in gene 56 and DD2 is a double deletion in the rII region composed of rEM 66 (*rIIA*⁻) and rPB 296 (*rIIB*⁻). rPB 296 extends into *denB*. The parent for the dCTPase⁻, *denA*, *denB*, *alc*⁻ mutant was constructed by R. Bruner and has the mutations amE51, nd 28, and r IIH23, respectively.

Crosses and Complementation Tests. Crosses were done by growing *E. coli* K803 bacteria to 4×10^8 cells per ml in M9S medium (20) at 37°. Tryptophan was added to 10 μg/ml and phage were added at a multiplicity of infection (MOI) = 5 of each. After 2 min for adsorption, they were diluted 1:100 in M9S medium at 37° and shaken for 1 hr before chloroform was added. They were diluted further and plated on *E. coli* K803. Complementation tests were performed the same way except that B834 bacteria were used for the infection. The phage to be used were always grown on *E. coli* K803 so that the parental (infecting) DNA had hydroxymethylcytosine and was glucosylated.

Table 1. Alc phenotype is recessive

Phage	Burst size	
	Amber suppressor	Nonsuppressor
Exp. I		
amE51, *denB*, *alc*	32	50
amE51, *denB*	111	0.1
[amE51, *denB*] + [amE51, *denB*, *alc*]	48	1
Exp. II		
amE51, *denB*, *alc*	28	78
amE51, *denA*, *denB*, *alc*	26	83
[amE51, *denB*, *alc*] + [amE51, *denA*, *denB*, *alc*]	42	112

Complementation tests with *alc*$^-$ mutants. Exp. I shows the phage production of a dCTPase$^-$, *denB*, *alc*$^-$ mutant by itself and with its parent dCTPase$^-$, *denB*. The number of phage produced per infected cell at 60 min is shown for both amber suppressing (B40) and nonsuppressing (B834) bacteria. Exp. II shows the complementation of dCTPase$^-$, *denA*, *denB*, *alc*$^-$ mutant with the dCTPase$^-$ *denB*, *alc*$^-$ mutant shown in Exp. I. Although there are variations in burst sizes from experiment to experiment, *alc*$^-$ mutants generally grow better on *su*$^-$ than on *su*$^+$ bacteria. We have no explanation for the rather poor phage production when the dCTPase$^-$, *denB*, *alc*$^-$ mutant coinfected with its parent on *su*$^+$ bacteria (row 3, Exp. I). As noted in *Materials and Methods*, all the phage for these experiments had been grown on *su*$^+$ bacteria.

Growing *alc* Mutant Stocks. To grow the *alc*$^-$ mutants on *su*$^-$ bacteria, B834, a small plaque was picked from a B834 plate and added to 10 ml of a 1:30 dilution of a fresh overnight culture of K803 in M9S with 10 μg/ml of tryptophan. After 6 hr of shaking at 37°, $CHCl_3$ was added and the phage were titered on K803 at 37°. The next day a much larger culture of B834 was grown to an optical density of 0.2 at 625 nm in M9S + tryptophan at 37°. Phage were added to a concentration of 6×10^7/ml. After 3 hr, the culture began to clear, $CHCl_3$ was added, and the phage was purified as described.

Thin-Layer Chromatography. DNA was prepared from virus purified by differential centrifugation and CsCl step gradients. The virus was dialyzed against 0.1 M potassium phosphate buffer (pH 7.0), and the DNA was extracted with phenol saturated with this phosphate buffer, dialyzed against 1 M NaCl and then water, and lyophilized. Two milligrams of the DNA were hydrolyzed in 88% formic acid in sealed tubes according to Bendich (21). The chromatography was run in two dimensions on 20 × 20 cm thin-layer cellulose plates from Brinkman. The buffer systems of Randerath (22) were used. The chromatograms were photographed under ultraviolet light using a UV filter on the camera. The spots were then cut out, the material was eluted with 0.2 M HCl, and the eluates were filtered before their ultraviolet spectra were determined. The spectral data of Bendich (21) were used to identify the bases and to calculate relative molar abundances. The positions of all the bases agreed with the assignments of Randerath (22). Calf thymus and wild-type T4 DNA were run as controls.

Hybridization-Competition Analysis. The DNA was prepared as it was for chromatography, but the last dialysis was against 0.01 M Tris·HCl (pH 7.9) and it was not lyophilized. Unlabeled and labeled RNA was extracted by method I of Bolle *et al.* (20). Hybridization was for 6 hr in 0.3 M NaCl–0.03 M Na citrate. The hybrids were treated with 12.5 μg/ml of RNase I (IX crystallized from Sigma) for 15 min at 37°. They were collected on presoaked Schleicher and Schuell grade B6 nitrocellulose filters, washed with 0.5 M KCl, 0.01 M Tris·HCl (pH 7.9), and 80% ethanol, and dried. Radioactivity was then determined.

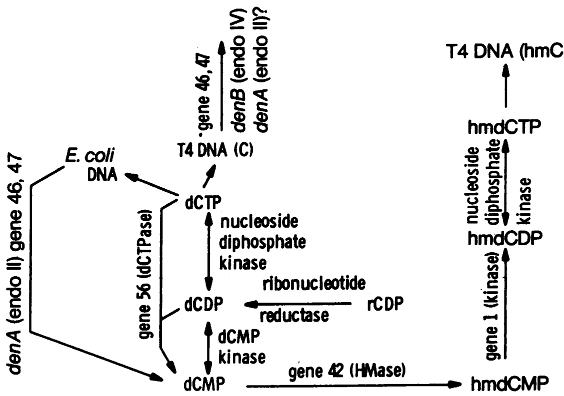

FIG. 1. Pathways to dCMP production in T4-infected cells. Genes coding for the enzymes are shown for T4-induced functions. endo II, endodeoxyribonuclease (EC 3.1.4.30); dCTPase, deoxycytidinetriphosphatase (EC 3.6.1.12); nucleosidediphosphate kinase (EC 2.7.4.6); dCMP kinase (EC 2.7.4.14); HMase, deoxycytidylate hydroxymethyltransferase (EC 2.1.2.8); ribonucleotide reductase (EC 1.17.4.2); endo IV, endodeoxyribonuclease; hm, hydroxymethyl.

Slab Gel Electrophoresis. The method has been described (23). Gels (12.5%) were used with a 5% stacking gel.

RESULTS AND DISCUSSION

As noted in the Introduction, dCTPase$^-$ mutants make T4 DNA after infection, which is rapidly broken down. This breakdown is largely dependent on the function of T4 endodeoxynuclease IV (*denB*), so that, if this gene is also inactivated, substantial amounts of concatemeric T4 DNA are made, which presumably contain largely cytosine. No virus particles are produced, however, because no late gene products are made. In hope of finding mutants that could make late mRNA on cytosine-containing T4 DNA, we looked for apparent revertants of a gene 56 amber (dCTPase), *denB* double mutant on *su*$^-$ bacteria. We chose to do this plating on nonrestricting *E. coli* B834 ($r_B{}^- m_B{}^-$) because T4 with cytosine might be subject to the r_B restriction system.

Apparent revertants do exist at a relatively high frequency (about 1 in 10^4). Most of these apparent revertants give smaller plaques than those obtained with wild-type T4. They are obtained on restricting bacteria as well, but give even smaller plaques and are less frequent.

A number of observations indicate that most of these plaques are not formed by revertants of the amber mutation in gene 56. (*i*) They are not observed when the dCTPase$^-$ single mutant is plated. (*ii*) The plaques seem to be smaller when the host restriction system is functioning, suggesting that one is dealing with phage whose DNA has substantial amounts of cytosine. (*iii*) When a stock was made from one of the plaques and it was crossed against wild type T4, about 10% of the progeny showed the amber phenotype and could not be complemented by mutants in gene 56. Thus, they will still harbor the amber mutation in gene 56. (*iv*) Their phenotype is recessive to the dCTPase$^-$ *denB* parent in mixed infections; that is, they do not grow (see Table 1). Revertants would be expected to be dominant in mixed infections.

Thus, the plaque-forming ability of these phage is not due to reversion of the amber mutation in gene 56 but to a mutation elsewhere in the genome. We call the gene in which the mutations occur *alc*, and the mutations that permit the double

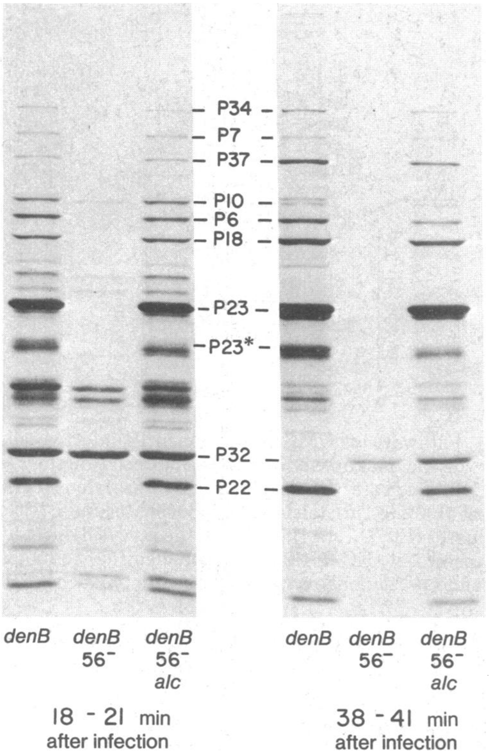

FIG. 2. An autoradiogram of a slab polyacrylamide gel electropherogram of proteins synthesized late in infection by a dCTPase$^-$ (56^-) *denB*, *alc*$^-$ mutant. The parental phage had been propagated on *E. coli* B40 so their DNA had hydroxymethylcytosine. *E. coli* B^E were grown and infected in M9 medium without amino acids at 28°. The cells were at a concentration of 5×10^8/ml, and the multiplicity of infection was 10. Labeling was with ^{14}C-labeled amino acids during the times indicated. The products of some "true late" genes are identified, as well as the product of gene 32, which is overproduced when cytosine-containing T4 DNA is made.

mutant amE51, *denB* to produce phage on *su*$^-$ bacteria, *alc*$^-$ For simplicity, we shall often refer, in the remainder of the paper, to the original triple mutant amE51, *denB*, *alc*$^-$ as "the *alc*$^-$ mutant," and the reader can assume that it is being grown on *su*$^-$ bacteria so that the dCTPase is not functioning.

As mentioned above, the Alc$^-$ phenotype requires the *denB* mutation. While we have not used *denB* point mutations but rather deletions of rII that extend into *denB*, the Alc$^-$ phenotype is not observed with point mutants in the rII gene. We have not excluded the possibility that *denB* as well as a gene close to it must be inactivated for the Alc$^-$ phenotype, but it should be possible to test this because of the existence of point mutants in *denB* (24).

The fact that *alc*$^-$ mutations are recessive makes it easy to determine if most mutations to the Alc$^-$ phenotype occur in the same cistron. If they do, then they should not complement each other. So far, we have isolated five additional independent *alc*$^-$ mutants starting from five different dCTPase$^-$, *denB* parent stocks grown from independent plaques. None of these *alc*$^-$ mutants prevented the growth of the original mutant in mixed infections. Thus, we tentatively conclude that all mutations to the Alc phenotype are in the same cistron.

Gene expression by *alc*$^-$ mutants

In contrast to the dCTPase, *denB* parent which makes almost no true late gene products, the *alc*$^-$ mutation must allow the true late gene products to be synthesized; otherwise no viruses would be produced. In fact, as shown in Fig. 2, the *alc*$^-$ mutation allows dCTPase$^-$ *denB* mutants to make almost normal amounts of true late gene products. The only important difference between the wild-type T4 pattern and the *alc*$^-$ pattern is that the *alc*$^-$ mutant overproduces the product of gene 32 on *E. coli* B^E, as does its dCTPase$^-$, *denB* parent, suggesting that cytosine-containing DNA is made and that has single-stranded "gaps" (25). We shall return to this point later. The overproduction of the gene 32 product is not observed on *E. coli* B834, so it may be due to the action of the r_B restriction system.

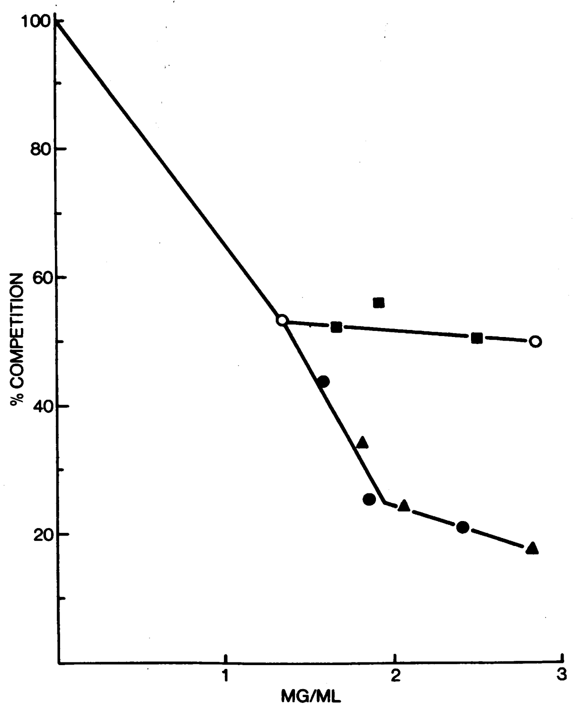

FIG. 3. Hybridization competition analysis of the RNA made late in infection by a dCTPase$^-$, *denB*, *alc*$^-$ mutant. The radioactive late RNA was labeled with [^{3}H]uridine 28–31 min after infection of K803 by wild-type T4. M9S medium at 30° and multiplicities of 10 were used throughout. The unlabeled RNAs were prepared from B^E cells chilled 20 min after infection. The open circles show the competition obtained with unlabeled RNA from cells infected for 5 min. The closed symbols show the competition obtained with 1.2 mg/ml of unlabeled RNA from cells infected for 5 min plus increasing concentrations of RNA from cells infected for 20 min with dCTPase$^-$, *denB* (■), dCTPase$^-$, *denB*, *alc*$^-$ (●), and wild-type (▲) T4. The competition by cellular RNA infected with wild-type T4 is relatively poor because the labeled RNA was from a later time than the unlabeled RNAs and the percent of true late RNA increases with time after infection. Hybridization efficiency was 10% without competitors, and 1836 cpm were hybridized without competitors; 10 μg/ml of heat-denatured DNA and 10 μg of labeled RNA were used.

We would expect the *alc*$^-$ mutation to permit the synthesis of almost normal amounts of true late mRNA (i.e., those mRNAs that code for the true late proteins). A preliminary experiment demonstrating this is shown in Fig. 3. Since some T4 early mRNAs continue to be made late in infection, it is necessary to distinguish these from the true late RNAs or the ones that are only made late in infection. They can be distinguished by a "mixed competitor" assay (20). First, the concentration of unlabeled early mRNA that is needed to compete with the early labeled sequences in a radioactive late RNA is determined. Second, this concentration of unlabeled early competitor is mixed with unlabeled late competitors to measure the latter's content of T4 true late mRNA. The effectiveness per weight for competition is a reflection of the percent of true late mRNA in the late competitor. As shown in Fig. 3, the

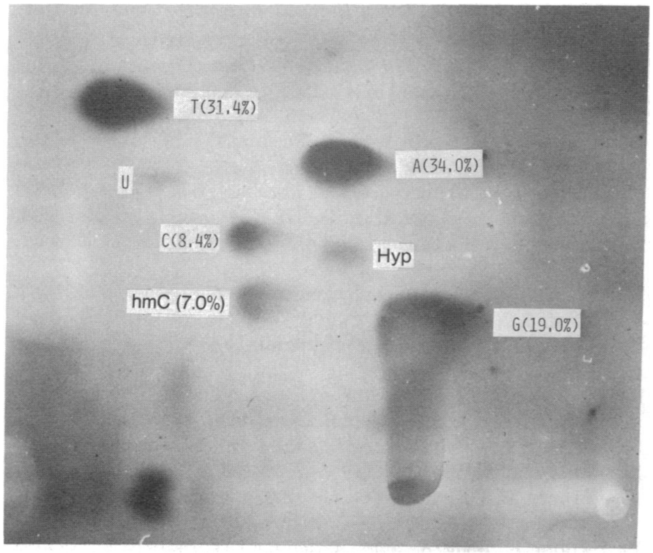

FIG. 4. Two-dimensional thin-layer cellulose chromatography of amE51 (dCTPase$^-$), *denB*, *alc*$^-$ T4 DNA. The percent and identification of each base are shown. The minor spots are probably due to uracil (U) and hypoxanthine (Hyp), which may be formed during formic acid hydrolysis by the deamination of cytosine and adenine, respectively. Guanosine (G) always gives a "tail" in our hands, but the whole spot is due to guanosine. hmC, hydroxymethylcytosine.

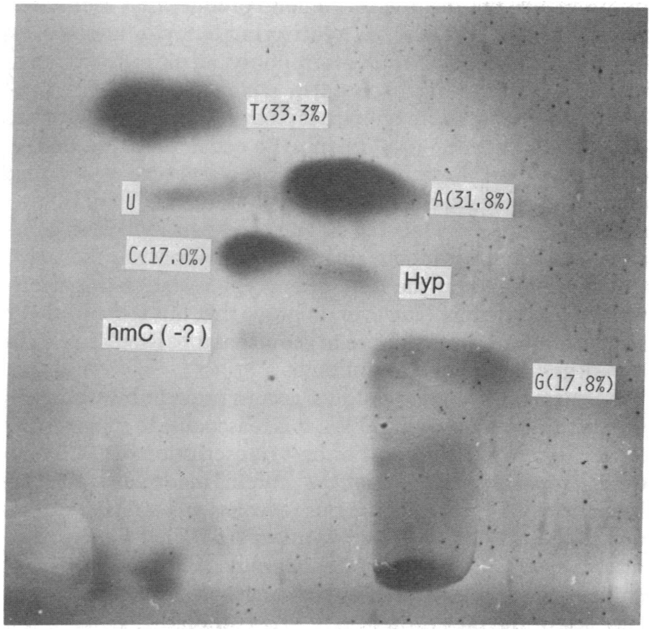

FIG. 5. Two-dimensional thin-layer cellulose chromatography of amE51 (dCTPase$^-$), *denA*, *denB*, *alc*$^-$ T4 DNA. See legend of Fig. 4.

dCTPase$^-$, *denB* parent has made, at best, little more true late RNA than exists in the early competitor while the *alc*$^-$ mutant has made about as much as wild-type T4. Therefore, *alc*$^-$ mutations affect transcription.

Measuring the cytosine content of *alc* mutants

The dCTPase$^-$, *denB* parent of the *alc*$^-$ mutant makes DNA that presumably contains substantial amounts of cytosine. The *alc*$^-$ mutation could allow phage growth either by somehow excluding cytosine from the DNA or by allowing late gene expression when cytosine-containing T4 DNA is made. A number of considerations suggest that the latter explanation is correct. No more than 20% substitution by cytosine is enough to substantially depress phage production (26). Therefore, the mutants must have less than this amount of cytosine. Also, the fact that they overproduce the gene 32 product, as does the parent (see Fig. 2), suggests that the DNA they are making has cytosine, as does the parent. Further, the *alc*$^-$ mutants are subject to the r_Bm_B restriction system to which hydroxymethylcytosine-containing phage are immune. In fact, they are restricted even if they have been grown on *E. coli* B and should have the B modification, possibly because the modification system of the host is not capable of keeping up with T4 DNA replication. The restriction of *alc*$^-$ mutants is more severe at higher temperatures, making them unable to form plaques on restricting hosts at 43°, and offering a convenient way of distinguishing *alc*$^-$ mutants from revertants of the dCTPase$^-$ mutation.

Measurements of base composition confirm that *alc*$^-$ mutants have substantial amounts of cytosine in their DNA. Fig. 4 shows the two-dimensional thin-layer chromatogram of an acid hydrolysate of *alc*$^-$ mutant DNA. The identity of each base and its percentage of the total DNA are shown. Thus, *alc*$^-$ mutants that are generated from dCTPase$^-$ *denB* mutants have about 55% of the hydroxymethylcytosine in their DNA replaced by cytosine.

To further increase the amount of cytosine in T4 DNA, *alc*$^-$ mutants were also selected starting with dCTPase$^-$ *denB*, *denA* triple mutants. As mentioned above, these mutants should have more cytosine in their DNA because the *denA* mutation retards host breakdown and therefore reduces the amount of dCMP, the substrate for the hydroxymethylase. *Alc*$^-$ mutants are obtained at about the same frequency, but they form much smaller plaques on *su*$^-$ bacteria. Complementation tests show that the mutations to the Alc$^-$ phenotype occur in the same cistron as before (see Table 1).

When the DNA from these *alc*$^-$ mutants is chromatographed (Fig. 5), the hydroxymethylcytosine spot is barely visible. Thus, the hydroxymethylcytosine in their DNA is nearly all substituted by cytosine, confirming that *alc*$^-$ mutations can permit late gene expression when DNA is made that contains almost all cytosine. We are attempting to grow T4 that have no hydroxymethylcytosine at all in their DNA.

What are *alc*$^-$ mutations?

At present, we can only speculate as to the molecular basis of the Alc$^-$ phenotype. Perhaps a clue lies in some observations concerning r-strand (late) transcription by dCTPase$^-$ mutants of T4. Even though no late proteins are made, substantial amounts of RNA are synthesized, which hybridize to the late strand of T4 DNA (17). Perhaps these RNAs are very short and cannot be translated into late proteins and *alc*$^-$ mutations prevent premature chain termination on cytosine-containing T4 DNA. Of course, other explanations are possible.

Even in *alc*$^-$ mutants, there probably is an effect of higher degrees of substitution by cytosine on T4 development. dCTPase$^-$, *denA*, *denB*, *alc*$^-$ mutants grow much less well at lower temperatures than dCTPase$^-$, *denB*, *alc*$^-$ mutants on plates of *su*$^-$ bacteria, as indicated by their much smaller plaque size. However, they multiply about as well in their first generation on *su*$^-$ bacteria (see Table 1).

The Alc$^-$ phenotype is probably due to the inactivation of a nonessential T4 gene product. There are a number of observations in support of this. (*i*) We know that *alc*$^-$ mutants are complemented by dCTPase$^-$, *denB* mutants with additional amber mutations in genes 33, 55, or 45 (data not shown), the only known essential genes required for late transcription. Thus, the *alc*$^-$ mutations do not lie in one of these genes. (*ii*) *alc*$^-$

mutations are not inactivating a gene product that is only required because T4 DNA has hydroxymethylcytosine since, as shown in Table 1, *alc*$^-$ mutants multiply, albeit rather poorly, on amber-suppressing bacteria in which the T4 DNA presumably contains hydroxymethylcytosine rather than cytosine. (*iii*) Mutations to the Alc$^-$ phenotype occur very frequently, more often than we would expect if the phenotype were being caused by a specific change in gene product. (*iv*) They behave as though they were missing a gene product function in complementation experiments, since *alc*$^-$ mutations are recessive, which is expected if the Alc$^-$ phenotype is due to the inactivation of a function. However, to prove that this is the basis for *alc*$^-$ mutations, we will have to show that a particular protein is malfunctioning in *alc*$^-$ mutants.

Because the *alc* gene product acts to prevent transcription of cytosine-containing T4 DNA, it is reasonable to suppose that it plays a role in the shutoff of host transcription by T4. However, a superficial analysis of the proteins made early after infection by *alc*$^-$ mutants has not revealed any striking effects on the shutoff of host protein synthesis (data not shown). Perhaps the *alc* gene product provides only one of many ways of shutting off the host, or is only responsible for shutting off a subclass of the host protein or RNA synthesis.

We can ask what gene product might be inactivated in *alc*$^-$ mutants. Although *alc*$^-$ mutations are probably in a nonessential gene, its product must interact with an essential part of the transcription apparatus since it is capable of blocking essentially all true late transcription. Some excellent candidates are the *mod* gene, whose product puts ADP-ribose on an arginyl residue of the α subunit of the host RNA polymerase (27), and the genes for the T4-coded polypeptides II (15,000 daltons) and III (10,000 daltons) that are bound by RNA polymerase (28). Other candidates undoubtedly exist, but these are ones that can be tested immediately. Since the only presently known phenotype for *alc*$^-$ mutations exists when they are coupled with two other mutations, they may be difficult, but not impossible, to map.

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