Termination of Transcription by *Escherichia coli* Ribonucleic Acid Polymerase in Vitro. Effect of Altered Reaction Conditions and Mutations in the Enzyme Protein on Termination with T7 and T3 Deoxyribonucleic Acids†

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ABSTRACT: Both bacteriophage T7 and the related bacteriophage T3 have strong termination sites for bacterial RNA polymerase located near 20% on the standard genome map. These termination sites are used with 90% efficiency in vivo, even in cells which contain a defective ρ protein. Under normal reaction conditions in vitro, *Escherichia coli* RNA polymerase terminates with 90% efficiency at the T7 terminator site but shows little or no termination at the corresponding T3 locus. Thus, the two templates form an ideal in vitro test system with which to study the parameters that govern transcriptional termination. Termination at these sites has been monitored by following the time course of RNA synthesis under conditions where only a single transcriptional cycle is carried out and by following the size distribution of RNA chains by gel electrophoresis. Termination of the T7 termination site is unaffected by a large variety of changes in reaction conditions, by quantitative cleavage of the nascent RNA during the reaction with a mixture of single- and double-stranded specific ribonucleases, or by a number of different mutations in the subunits of RNA polymerase, including σ. Similarly, a large variety of changes in reaction conditions fail to enhance termination at the T3 terminator site, including changes in temperature, $MgCl_2$ concentration, and glycerol concentration or the addition of dimethyl sulfoxide, ethanol, or spermidine to the reaction. However, in the presence of elevated salt concentrations, at low ribonucleoside triphosphate concentrations, and in the presence of formamide, efficient in vitro utilization of the T3 terminator is seen. Changes in the RNA polymerase protein can also enhance utilization of the T3 site. A class of rifampicin-resistant *rpoB* mutants has been identified which produce a rifampicin-resistant RNA polymerase which is able to utilize the T3 terminator site in vitro. Similarly, the normal *Bacillus subtilis* RNA polymerase utilizes the T3 terminator site in vitro with high efficiency.

The synthesis of an RNA chain by *Escherichia coli* RNA polymerase is a complex reaction that can be divided into a number of distinct steps (Chamberlin, 1974; Chamberlin, et al., 1976). Each of these steps in transcription is itself a complex reaction. Since the regulation of transcription may occur at any of these steps, an understanding of the reactions catalyzed by RNA polymerase is necessary for an understanding of potential mechanisms for the control of bacterial gene expression.

The termination step in RNA synthesis can be thought of as three distinct reactions: (1) cessation of RNA chain elongation; (2) release of the RNA chain; (3) release of active RNA polymerase. For the purpose of studying this step in the transcription cycle, a template with a simple promoter and terminator structure is advantageous in that the fate of the enzyme molecules and the nascent RNA chain can be easily monitored.

The termination of transcription by *E. coli* RNA polymerase at the end of the early region of T7 DNA in vitro has been well established (Millette et al., 1970; Dunn & Studier, 1973a,b). Under normal conditions RNA polymerase holoenzyme initiates transcription at one of the three major promoter sites located at 1.25 (A_1), 1.55 (A_2), and 1.84% (A_3) of the standard T7 genetic map (Hsieh & Wang, 1976; Studier et al., 1979). The enzyme molecules then transcribe the early region of the T7 genome until they reach the termination site at 18.8% (Studier et al., 1979). At this site the major fraction (70–90%) of the enzyme molecules terminate RNA synthesis; the remainder of the molecules (10–30%) read through the stop signal into adjacent regions of the DNA (Chamberlin et al., 1979). This gives rise to a terminated RNA molecule of average length 7000 nucleotides or 2.4×10^{-6} molecular weight (Millette et al., 1970; Dunn & Studier, 1973a).

Transcription of the homologous bacteriophage T3 DNA is also initiated predominately at the left end of the genetic map (Koller et al., 1974), but there is little or no termination at the end of the early region in vitro (Dunn et al., 1972; Chakrabordy et al., 1974; Neff & Chamberlin, 1978), in contrast to what is observed in vivo (Issinger & Hausmann, 1973). There is no effect of ρ mutations in the bacterial host on termination of early transcription on either T7 or T3 genomes in vivo (Kiefer et al., 1977). Hence, comparison of transcriptional patterns in vitro employing T3 and T7 DNAs as template offers an ideal way of monitoring the factors which either reduce or enhance reading of transcriptional terminator signals by a particular RNA polymerase (Neff & Chamberlin, 1978).

E. coli RNA polymerase is a complex enzyme of five subunits ($\beta\beta'\alpha_2\sigma$). Extensive genetic and reconstitution studies have demonstrated that these subunits do constitute the active enzyme in vivo and in vitro (Burgess, 1976; Scaife, 1976; Zillig et al., 1976). Mutations have been found in all the subunits, and the map positions of all the structural genes are known (Bachmann et al., 1976; Scaife, 1976; Harris et al., 1977).

Since a number of RNA polymerase mutations are available as well as a number of different bacterial enzymes (Wiggs et al., 1979), a collection of *E. coli* RNA polymerase mutant enzymes and other bacterial enzymes was tested to determine

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the effect of structural variations in the enzyme on the efficiency of termination of transcription with T3 and T7 DNAs in vitro. Two spontaneous Rif^R mutants stimulate termination with T3 DNA in vitro substantially while a number of other RNA polymerase mutants have no detectable effect.

Materials and Methods

Enzyme Purification. Wild-type *E. coli* RNA polymerase holoenzyme or rif-15 RNA polymerase holoenzyme was purified by the method of Burgess & Jendrisak (1975), and fraction D was chromatographed on phosphocellulose in the presence of 50% glycerol to isolate RNA polymerase holoenzyme (Gonzalez et al., 1977). The wild-type holoenzyme used in these experiments had specific activities from 15 000 to 25 000 units/mg, where 1 unit is the rate of incorporation of 1 nmol of labeled CMP per h in the standard assay (Gonzalez et al., 1977). The rif-15 enzyme used in these experiments had specific activities from 10 000 to 20 000 units/mg. The rif-1 RNA polymerase was prepared by the method of Berg et al. (1971), and holoenzyme was isolated by the procedure of Gonzalez et al. (1977). Protein concentrations given were determined from the extinction coefficient of *E. coli* RNA polymerase as described by Chamberlin et al. (1979).

RNA polymerase was partially purified on a small scale by the method of Gross et al. (1976). Cells were grown in 100 mL of LB broth (Bertani, 1951) plus 0.2 or 0.4% glucose to an A_{600nm} of 1.0 and were harvested by centrifugation. The DEAE-cellulose chromatography step was omitted.

The purification and properties of the other bacterial enzymes are described by Wiggs et al. (1979). RNA polymerase from *Bacillus subtilis* was provided by Barry Davison (Davison et al., 1979).

RNA Polymerase Assays.[1] DNA templates and radioactively labeled ribonucleoside triphosphates were prepared as described previously (Neff & Chamberlin, 1978). RNA polymerase assay conditions included 40 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 10 μg of T7$^+$ or T3$^+$ DNA, and 0.4 mM each of the four ribonucleoside triphosphates in a final reaction volume of 0.1 mL. Except where noted, reactions contained from 0.5 to 1 μg of RNA polymerase holoenzyme which was added to initiate the reaction ("free starting" conditions), followed by incubation at 37 °C. This amount of enzyme gives a molar ratio of 10–20 total RNA polymerase molecules/DNA molecule or about one-half that number of active RNA polymerase molecules. Specific activities were defined in 4-min reactions. Where indicated, heparin was added to the transcription reaction at 1 min after RNA synthesis had begun (0.05 or 0.1 mg/mL final concentration). For assays of rifampicin resistance, rifampicin was either mixed with the enzyme or added to the complete reaction mixture prior to addition of the enzyme to initiate RNA synthesis.

Rifampicin (Calbiochem, grade B) was prepared as a stock solution of 0.4–3.6 mg/mL in 10 mM Tris-HCl, pH 8, plus 0.1 mM EDTA, protected from light, and stored at −20 °C. When rifampicin was added to bacterial media for selection or testing of Rif^R, 0.3 mL of a stock solution of 10 mg/mL in 95% ethanol was spread on each plate just before use and allowed to dry. The extinction coefficient used for standardizing the stock rifampicin solutions was 1 mg/mL = 31.6 at 333 nm (Rhodes & Chamberlin, 1975).

Transition temperatures for the wild-type and rif-15 enzymes were measured essentially by the method of Mangel & Chamberlin (1974) except that heparin was substituted for rifampicin (0.05 mg/mL final concentration), the incubation at the specific temperatures was only 5 min, and the RNA chains were elongated at 30 °C for 2 min. This assay provides a direct measure of the fraction of RNA polymerase molecules which are able to interact with promoter sites and form heparin- or rifampicin-resistant or "open" complexes (Chamberlin, 1974; Chamberlin et al., 1976).

Assays for the Termination of Transcription. (*1*) *Time Course of RNA Synthesis.* Standard RNA polymerase conditions were employed with either rifampicin or heparin added to inhibit reinitiation of RNA chains. The reaction component or the reaction condition to be tested for its effect on termination was added or adjusted after 1 min of RNA synthesis. RNA synthesis reactions were stopped at the specific time points and analyzed for acid-insoluble material (Berg et al., 1971). The slope of each phase of RNA synthesis was determined [cf. Chamberlin et al. (1979)], and the ratio of slope 4 (5–12 min) to slope 2 (1–4 min) was determined as an estimate of the efficiency of termination (see Figure 1 below).

(*2*) *Polyacrylamide–Agarose Gel Electrophoresis.* RNA polymerase reaction conditions were employed with heparin added as described, and the component or reaction condition to be studied was added or adjusted at 1 min after RNA synthesis had begun. The reactions were allowed to continue until the time points indicated in the figure legends. Samples were then analyzed by polyacrylamide–agarose gel electrophoresis (Golomb & Chamberlin, 1974).

(*3*) *Pyrophosphate Release and Preparation of RNase A Dimers.* RNA synthesis was monitored under the conditions described above except that [γ-^{32}P]ATP was used as the radioactively labeled ribonucleoside triphosphate and RNA synthesis was measured by following the production of labeled pyrophosphate after absorption of labeled nucleotides to Norit. Reactions (0.1 mL) in capped 1.5-mL plastic microfuge tubes (West Coast Scientific) were incubated at 37 °C and were stopped by the addition of 0.4 mL of cold H_2O at appropriate times. After the addition of 0.1 mL of 0.1 N HCl, 0.2 mL of carrier solution, and 0.2 mL of Norit (Zimmerman & Kornberg, 1961), the tubes were incubated on ice for 10 min and the charcoal was removed by spinning the tubes for 2 min in a Brinkman microfuge. Aliquots (0.1 or 0.2 mL) of the supernatant fraction were spotted on glass fiber filters (Whatman GF/C), dried, and counted (Berg et al., 1971).

RNase A dimers were prepared by the method of Wang et al. (1976) from Sigma RNase A, type IIA, and were assayed with $(rI)_n \cdot (rC)_n$ as substrate (Zimmerman & Sandeen, 1965). The preparation contained a mixture of approximately 50% RNase A dimers and 50% RNase A monomers [as judged by $NaDodSO_4$–polyacrylamide gel analysis (Ames, 1974)] and was added to the standard reactions where noted at 0.1 mg/mL final concentration.

(*4*) *RNA Chain Release.* Reaction conditions described above for RNA synthesis with heparin were employed, and reactions were stopped at specific times by the addition of 1 mL of ice-cold filtering buffer (20 mM Tris-HCl, pH 8, 20

[1] Since completion of these studies in 1978, we have developed a modified assay for bacterial RNA polymerases which allows direct determination of the fraction of RNA polymerase molecules which are active together with several other enzymatic reaction parameters. The assay procedure described here is closely analogous to the procedure described by Chamberlin et al. (1979), except that the temperature was 37 °C instead of 30 °C which increases the rate of chain elongation from 17 to 25 s^{-1} and shortens the period of the single-step transcription reaction. The enzymes employed here had specific activities that would correspond to fractions containing from 40 to 50% active molecules by the quantitative assay of Chamberlin et al. (1979).

Table I: Bacterial Strains

strain no.	relevant genotype	origin or ref
AB257	*metB* HfrC	A. J. Clark
BG-1	*metB* HfrC *rpoB*	spontaneous Rif^R mutant of AB257 isolated by B. Gordon in 1969; the strain was lost
JC7623	*recB recC sbcB argE leu*	A. J. Clark
C2121	*rpoA*	R. Calendar (Sunshine & Sauer, 1975)
KY1330	*stl* permeable	T. Yura (Iwakura et al., 1973)
KY1344 and KY1345	*rpoB*	Stl^R derivatives of KY1330
2016	*purD*	T. Yura
2016 rif-267	*purD rpoB*	Rif^R derivatives of 2016
2016 rif-268	*purD rpoB*	
2016 rif-270	*purD rpoB*	
NN1500	*recB recC sbcB leu rpoB argE*	Rif^R derivative of JC7623 (rif-15)
SA1030	*his str*	A. Das
NN1003	*his str rpoB argE*	transduction of NN1500 into SA1030, selecting Rif^R and then Arg^-
P90A5c	*argG lac thi*	R. Calendar (Isaksson et al., 1977)
285c	*argG lac thi rpoD*	ts derivative of P90A5c

mM $MgCl_2$, 20 mM KCl, and 10 mM 2-mercaptoethanol). The diluted reaction solution was filtered through a nitrocellulose filter (13 mm, Schleicher and Schell, type B-6) with a very low suction (3–5 mmHg) such that the flow rate was ~0.5 mL/min. The filter was washed with 1 mL of cold filtering buffer, and the wash and the filtrate were collected in a tube containing 1 mL of cold 10% perchloric acid and 0.3 M sodium pyrophosphate. The acid-insoluble material that passed through the filter was collected and counted (Berg et al., 1971).

Bacterial Strains and Phage Stocks. The origin and relevant genotype of the bacterial strains used are listed in Table I. All bacterial strains are *E. coli* K12 derivatives except for C-2121 which is *E. coli* C. Genetic nomenclature follows that of Bachmann et al. (1976).

$T7^+$, T7, and T3 deletion mutants were obtained from F. W. Studier. $T3^+$ phage was obtained from R. Hausmann. The P1 phage used for transduction was a gift of Brian Sauer. P1 (high-frequency transducing mutant 607H; Wall & Harriman, 1974) was grown for two cycles on NN1500 and used to transduce SA1030 by using the methods described by Bowden et al. (1975), selecting for Rif^R (100 μg/mL) and Arg^-.

Transformation with Linear E. coli DNA. JC7623 was transformed with DNA extracted from BG-1 by the procedure of Cosloy & Oishi (1973), selecting for Rif^R (10 μg/mL, then 100 μg/mL) or Leu^+ as a control for transformation efficiency.

Results

Polyacrylamide–Agarose Gel Analysis and Time Course of RNA Synthesis in Vitro. (1) Analysis of Termination by E. coli RNA Polymerase on T3 and T7 DNAs in Vitro. RNA synthesis under standard conditions can be easily followed by monitoring the size of RNA chains by polyacrylamide–agarose gel electrophoresis (Neff & Chamberlin, 1978; Chamberlin et al., 1979). It takes ~4 min at 37 °C for RNA polymerase molecules elongating an RNA chain from one of the three A promoters to reach the terminator, from which a mean elongation rate of 25 nucleotides/s is calculated. Quantitative analysis of the RNA made under these conditions with T7 DNA by slicing RNA gels (Table II) shows that 70–80% of the enzyme molecules terminate an RNA chain and the remainder read through to yield higher molecular weight RNAs. Less than 5% of the label is found in lower molecular weight regions, indicating that there is little or no premature termination of T7 RNA chains under these conditions. When T3 DNA is used as the template, however, 90% of the enzyme molecules read through the termination site and only 10% terminate.

Table II: Quantitative Analysis of T7 and T3 DNA Transcription Products Isolated by Gel Electrophoresis after 15 min of RNA Synthesis[a]

DNA template	total cpm of RNA loaded on gel	total cpm recovered from gel track	% cpm in T7 A RNA	% cpm in "rt" RNA	molar ratio A/rt
T7	60 000	24 500	47	37	2.8
T3	60 000	22 300	5	74	0.1

[a] Average molecular weight of rt or read-through RNA species is 5×10^6 and the molecular weight of the T7 A RNA species is 2.3×10^6 with T7 late mRNAs as molecular weight markers (Golomb & Chamberlin, 1974). RNA gel tracks similar to Figure 3, tracks a and f, were sliced into 0.5-cm pieces, placed in 2 mL of Bray's scintillation fluid (Bray, 1960), and counted in a scintillation counter. The *E. coli* RNA polymerase fraction employed was the same as that in Figure 1.

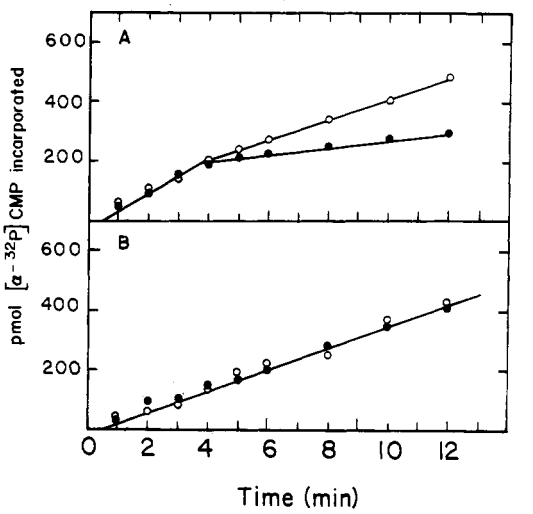

FIGURE 1: Time course of RNA synthesis by *E. coli* RNA polymerase with T7 and T3 DNAs in vitro. Part A is transcription with T7 DNA, and part B is with T3 DNA. The open circles denote transcription without any heparin present. The closed circles denote transcription with heparin added at 1 min. Standard reaction conditions were employed as described under Materials and Methods with 0.28 μg of enzyme in each reaction mixture.

This pattern of RNA synthesis can also be seen by simply following incorporation of a labeled ribonucleoside triphosphate into acid-insoluble material as a function of time (Figure 1). At ~4 min for transcription with T7 DNA, there is a distinct break in the rate of incorporation or RNA synthesis and then continued synthesis at another rate (Figure 1A). With T3 DNA, however, no break in the rate of RNA synthesis is seen

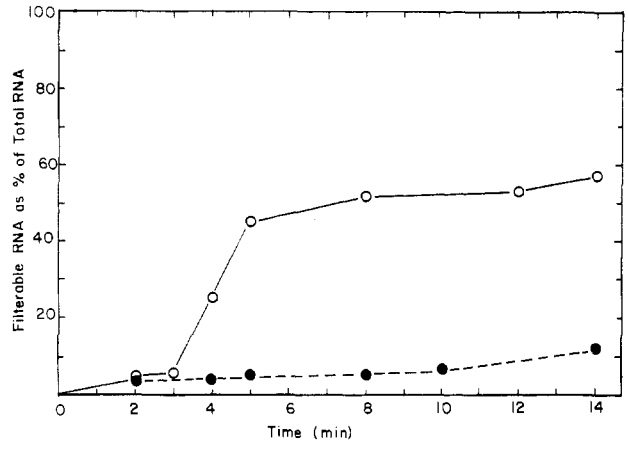

FIGURE 2: RNA chain release assay. The acid-insoluble counts passing through the nitrocellulose filter as a function of time of RNA synthesis are expressed as the percentage of the total acid-insoluble counts. Standard conditions were employed (Figure 1), and typical values for total RNA made as a function of time of RNA synthesis are shown in Figure 1. The open circles designate filterable RNA from RNA synthesis with T7 DNA, and the closed circles designate filterable RNA from RNA synthesis with T3 DNA. Heparin was added to all samples at 1 min.

(Figure 1B) [see also Neff & Chamberlin (1978) and Chamberlin et al. (1979)].

Since recycling of the enzyme is inhibited in all reactions by the addition of excess rifampicin or heparin, the incorporation and gel assays represent one round of transcription. The break in the rate of RNA synthesis, therefore, represents the termination of transcription on T7 DNA and the second rate of synthesis represente the synthesis of read-through RNA. The ratio of the slope of the second or read-through phase of RNA synthesis to the slope of the initial phase of RNA synthesis gives an estimate of the fractions of molecules which read through the termination site, assuming that all enzyme molecules are synthesizing RNA chains at a more or less constant rate. With T7 DNA this fraction ranges from 10 to 30% with different *E. coli* RNA polymerase preparations (Chamberlin et al., 1979). For any given preparation the fraction of read through is highly reproducible and the value read from the slopes is in good agreement with the value obtained from measurement of the RNA chains directly (Table II).

(*2*) *RNA Chain Release.* A population of RNA polymerase molecules, synchronously elongating an RNA chain with T7 DNA, should exist in a ternary complex (RNA–DNA–RNA polymerase) from the time of initiation until it approaches the termination site. At the termination site the complex dissociates in some manner, yielding a released RNA molecule, the reformed DNA template, and RNA polymerase. With T3 DNA as template the RNA polymerase molecules should remain in a ternary complex until they run off the end of the DNA, encounter some obstruction, or terminate randomly.

If ternary complexes are filtered through a nitrocellulose filter, the radioactively labeled RNA chain should be retained on the filter since it is tightly associated with RNA polymerase. Once the RNA chain is released from the enzyme it should pass through the filter. Assays of this sort with both T3 and T7 DNAs (Figure 2) show that the time of release of the labeled RNA chains from the T7 ternary complexes agrees well with the time of termination found in Figure 1. Retention of the labeled RNA chains on the filters with T3 ternary complexes continues until at least 14 min, which agrees well with the low efficiency of termination with T3 DNA in vitro found in Figure 1. The increase in the background level of filterable T3 RNA chains between 10 and 14 min may be due to shearing of the large RNA chains during the filtration process or to traces of RNase activity in the reaction.

Table III: Factors That Have No Substantial Effect on Termination by *E. coli* RNA Polymerase with T3 or T7 DNA in Vitro

factor	range tested	factor	range tested
dimethyl sulfoxide	0–20% (v/v)	$MgCl_2$	10–200 mM
ethanol	0–20% (v/v)	spermidine	0–20 mM
glycerol	0–20% (v/v)	temperature	20–45 °C

The amount of released RNA passing through the filter at 14 min agrees well with the quantitation of the efficiency of termination with T7 DNA shown in Table II. The percentage of the total radioactivity found in the released RNA in Table II is 37%, and the amount of RNA passing through the filter at 14 min in Figure 2 is 45% (subtracting a 12% background value from the T3 DNA value). The distinct break in the time course assay and the appearance of distinct RNA species after electrophoresis agree well with the time course of RNA chain release and the quantitation of the termination reaction. These data demonstrate a good correlation between termination as measured by RNA gels and time course assays and actual RNA chain release.

(*3*) *Factors Which Affect Termination with T3 DNA in Vitro.* Since RNA polymerase terminates quite efficiently in vitro with T7 DNA as template and very inefficiently in vitro with T3 DNA, these two DNAs were used to look for changes in the reaction conditions that would suppress termination with T7 DNA or stimulate termination with T3 DNA. The reaction component or condition to be studied was added or changed at 2 min after RNA synthesis had begun to preclude effects on the promoter binding or initiation reactions. Since heparin or rifampicin was present, no recycling of the enzyme could occur and the effect of the component on just the elongation and termination reactions could be monitored.

Many different reaction components and additions were tried. As summarized in Table III, the termination reaction is relatively insensitive to substantial changes in temperature and $MgCl_2$ concentrations and to addition of glycerol, dimethyl sulfoxide, ethanol, and spermidine. Here the qualitative presence or absence of termination was judged by assays such as that shown in Figure 1 and additionally by analytical gel electrophoresis of the resulting transcripts. There are, however, several factors that do affect the termination of transcription. Addition of increasing amounts of KCl to the transcription reaction has the effect of greatly enhancing termination with T3 DNA and gives a discrete transcript of M_r 2.5×10^6, corresponding roughly to the early region of the phage genome (Figure 3). Stimulation of termination on T3 DNA by *E. coli* RNA polymerase in the presence of elevated salt concentrations has been reported by others (Dunn et al., 1972; Adhya et al., 1979). Addition of amounts of formamide (MCB) up to a concentration of 20% to the transcription reaction has a very similar effect (Figure 4), but addition of formamide to a final concentration of greater than 20% (v/v) appears to block elongation immediately at the time of addition (data not shown). This block can be reversed by diluting the reaction mixtures (Figure 5).

Lowering the ribonucleoside triphosphate concentration in the transcription reaction also stimulates termination with T3 DNA (Figure 6). Both changes in the temperature of the reaction and in the ribonucleoside triphosphate concentration have a dramatic effect on the elongation rate of RNA polymerase (Table IV); however, only lowering the triphosphate

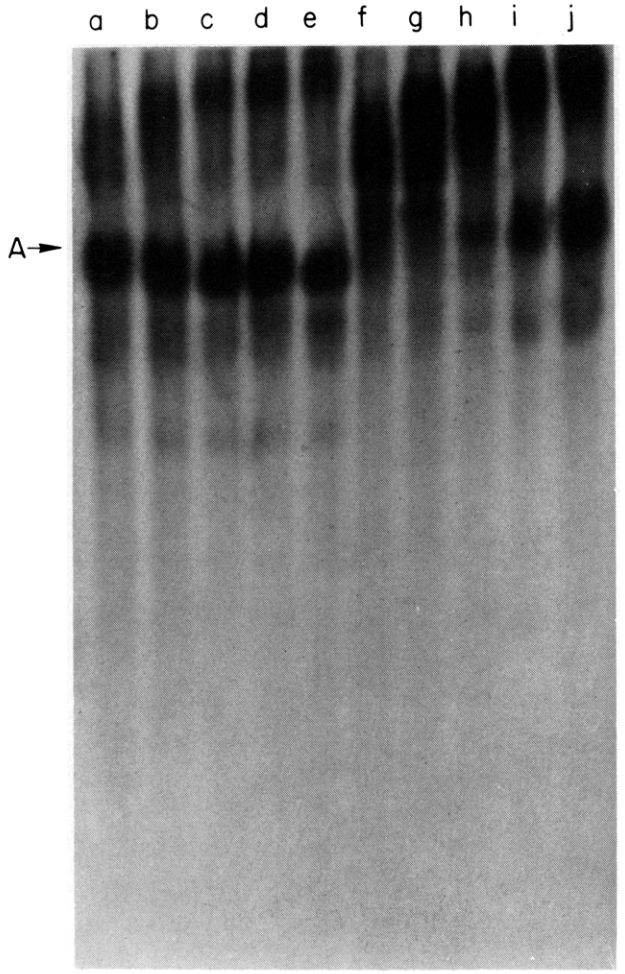

FIGURE 3: Polyacrylamide–agarose gel electrophoresis of T7 and T3 RNAs transcribed by *E. coli* RNA polymerase in the presence of increasing KCl concentrations. RNA synthesis, carried out as described under Materials and Methods, was stopped after 10 min of synthesis, and an aliqout of each reaction mixture was loaded on separate tracks of a 2.2% polyacrylamide–0.7% agarose gel. The total amount of CMP incorporated into RNA in each reaction was 0.6–0.7 nmol. Approximately 50000 cpm of labeled RNA was loaded on each track. Tracks a–e are transcription with T7 DNA, and tracks f–j are transcription with T3 DNA. The reactions contain the following: tracks a and f, no added KCl; b and g, 50 mM KCl; c and h, 100 mM KCl; d and i, 200 mM KCl; e and j, 500 mM KCl. The T7A transcript appears smaller than the salt-stimulated T3 transcript because the T7 DNA used in this experiment was not wild type but contained a 2.2% deletion of the 0.7 gene or T7 protein kinase gene.

Table IV: Elongation Rates with T7 and T3 DNAs in Vitro under Different Reaction Conditions[a]

	nucleotides		[NTP]	nucleotides	
temp (°C)	T7	T3	(μM)	T7	T3
15	2	2	5	0.6	0.3
25	10	10	10	1.5	1
30	17	17	25	3	2.5
37	25	25	50	5	4
42	28	28	400	25	24

[a] Variations of temperature experiments were performed with 400 μM NTPs, while variations of [NTP] experiments were performed at 37 °C. Mean chain elongation rates were determined by gel electrophoretic analysis of the transcripts formed under standard reaction conditions at several time points as described by Chamberlin et al. (1979).

concentration has a detectable effect on termination under the conditions tested. Hence, there is no tight relationship between the rate of RNA chain elongation and the frequency of chain

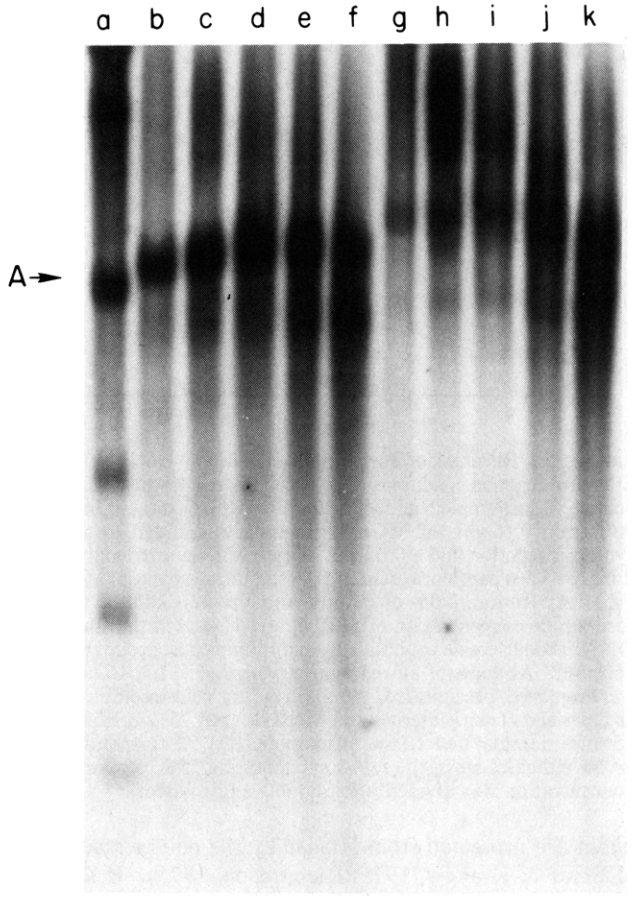

FIGURE 4: Polyacrylamide–agarose gel electrophoresis of T7 and T3 RNAs transcribed by *E. coli* RNA polymerase in the presence of increasing formamide concentrations. RNA synthesis, carried out as described under Materials and Methods, was stopped after the times specified, and an aliquot of each reaction mixture was loaded onto a 1.75% polyacrylamide–0.7% agarose gel. Approximately 50000 cpm of labeled RNA was loaded on each track. Tracks b–f are transcription with T7 DNA; tracks g–k are transcription with T3 DNA. The total amount of CMP incorporated into RNA and the times of incubation of each reaction were as follows: tracks b and g, 1 nmol, 20 min; tracks c and h, 0.8 nmol, 30 min; tracks d and i, 0.7 nmol, 60 min; tracks e and j, 0.6 nmol, 80 min; tracks f and k, 0.5 nmol, 80 min. The final concentration of formamide in each reaction was the following: tracks b and g, 0% formamide; tracks c and h, 10% formamide; tracks d and i, 15% formamide; tracks e and j, 17.5% formamide; tracks f and k, 20% formamide. The RNAs made by the T7-specific RNA polymerase from the late region of T7 DNA are included in track a as molecular weight markers (Golomb & Chamberlin, 1974). The major A transcripts in each track did not migrate together across the gel due to the variation in the formamide concentration in each gel sample.

termination for the T3 or T7 terminators.

Digestion of nascent T7 RNA chains by pancreatic RNase A or dimers of RNase A also has no effect on the efficiency of transcriptional termination at the T7 terminator. One model for the termination of transcription (Lee & Yanofsky, 1977; Stauffer et al., 1978; Rosenberg et al., 1978) postulates the formation of a highly base-paired RNA hairpin loop near the 3′ end of the terminated RNA chain. If there is a large RNA loop formed near the termination site on T7 DNA and if it is required for the termination of transcription, then preventing the formation of the loop or removing it during the transcription reaction should suppress termination. If a large amount of RNase A is present in the transcription reaction from the start, the RNA chains should be digested as soon as they are accessible to the ribonuclease, and it has been shown that no more than 12 nucleotides of the nascent RNA

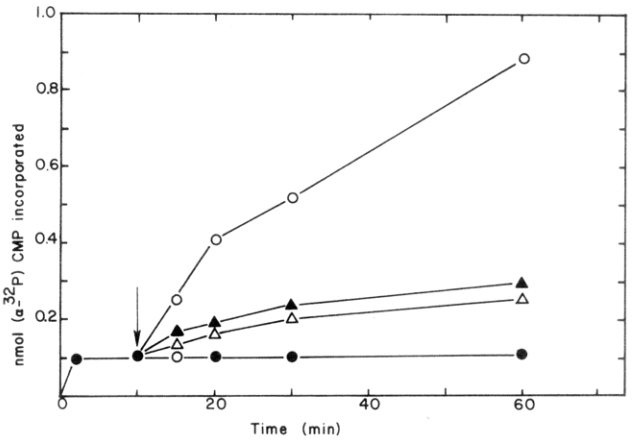

FIGURE 5: Reversal of formamide elongation block by dilution. Standard reaction conditions were scaled up to 0.45-mL final reaction volume with rifampicin added at 30 s after RNA synthesis had begun. At 2 min 0.15 mL of MCB formamide was added to the RNA synthesis reaction and a 0.05-mL aliquot was removed and added to 2 mL of 3.5% perchloric acid–0.1 M sodium pyrophosphate (PCA–PP_i). At 10 min, 0.05- or 0.1-mL aliquots were diluted to a final formamide concentration of 5, 12.5, and 15% such that the buffer, DNA, ribonucleoside triphosphate, and rifampicin concentrations were constant. Aliqouts of all reaction mixtures (5, 12.5, 15, and 25%) were removed, precipitated, and analyzed for acid-insoluble material as described under Materials and Methods. (●) 25% formamide; (△) 15% formamide; (▲) 12.5% formamide; (○) 5% formamide. The arrow indicates when the reaction containing 25% formamide final concentration was divided into aliquots and diluted.

chain are protected from RNase by the polymerase protein (Kumar & Krakow, 1975; Richardson, 1975). If RNase A monomers are cross-linked with dimethyl suberimidate, the resulting dimers can also digest double-stranded RNA (Wang et al., 1976). If dimers and monomers are present from the beginning of the RNA synthesis reaction, then all the RNA formed in the reaction except for a run of consecutive purines will be susceptible to digestion.

RNA synthesis in the presence of ribonuclease was monitored by $^{32}PP_i$ release using Norit to remove labeled nucleotides. The time course of $^{32}PP_i$ release was the same as that found by incorporation of labeled nucleotide whether RNase A monomers or RNase A monomers and dimers were present (Figure 7). This result shows that digestion of all RNA accessible to double strand or single strand specific ribonucleases has no effect on the termination of transcription with T7 DNA in vitro.

Attempts to detect possible large RNA fragments in such an RNase digestion were carried out by gel electrophoresis of the digest. With either RNase A or RNase A dimers all of the RNA digestion products migrated with a mobility consistent with a size of less than eight nucleotides (Neff, 1978).

Alterations of the Bacterial RNA Polymerase Protein. (1) Alteration of Termination by Mutations to Rifampicin Resistance. Our interest in the effects of alterations of the RNA polymerase protein on termination at T3 and T7 terminators was first stimulated by the observation that a preparation of rifampicin-resistant *E. coli* RNA polymerase available in the laboratory (rif-1 enzyme) was able to read the T3 terminator with great efficiency while the wild-type enzyme could not. The rif-1 RNA polymerase had been prepared in 1969 by B. Gordon from a spontaneous rifampicin-resistant mutant of *E. coli* B/1 (designated BG-1) as part of a laboratory course in molecular biology. While the rif-1 RNA polymerase holoenzyme was available in quantity, the bacterial strain BG-1 had not been saved. As a result we tried first to isolate the

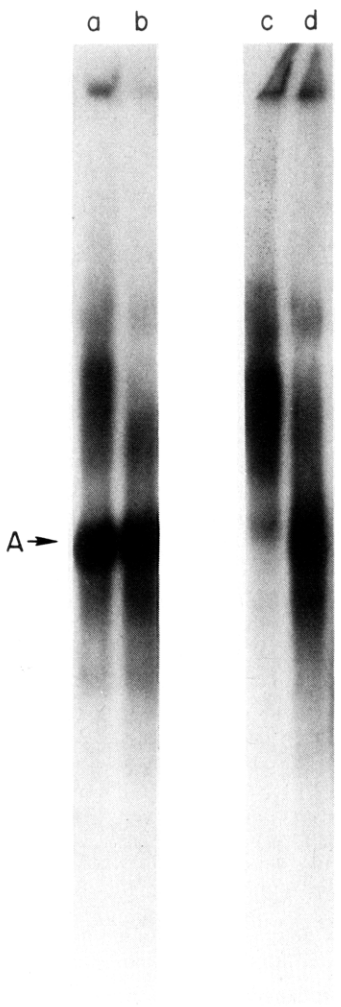

FIGURE 6: Polyacrylamide–agarose gel electrophoresis of T7 and T3 RNAs transcribed by *E. coli* RNA polymerase at 0.4 and 0.025 mM ribonucleoside triphosphate concentration. Standard RNA synthesis conditions were employed except that in tracks b and d, all four ribonucleoside triphosphates were at 0.025 mM final concentration; tracks a and c contained 0.4 mM ribonucleoside triphosphates. Tracks a and b are transcription with T7 DNA; tracks c and d are transcription with T3 DNA. The amount of CMP incorporated into RNA in each reaction and the time of incubation for each reaction were as follows: tracks a and c, 1.2 and 0.1 nmol, respectively, 20 min; tracks b and d, 0.6 and 0.06 nmol, respectively, 60 min. The position of the major T7 A transcript is indicated in the left-hand margin.

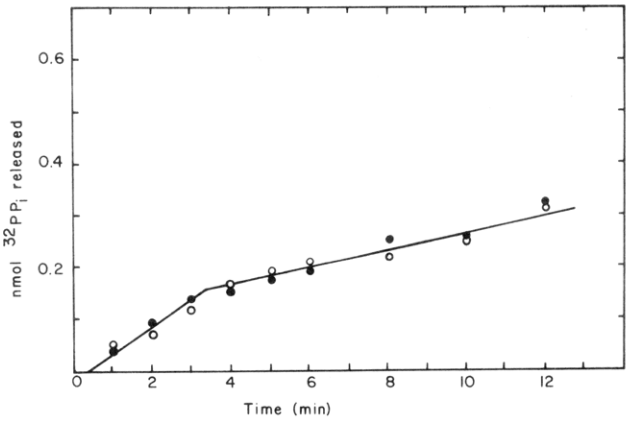

FIGURE 7: Time course of T7 RNA synthesis by *E. coli* RNA polymerase in the presence and absence of pancreatic RNase A. RNA synthesis was monitored by following the release of [^{32}P]pyrophosphate from [γ-^{32}P]ATP as described under Materials and Methods; 0.28 μg of enzyme was used. (○) Transcription without ribonuclease; (●) transcription with ribonuclease A dimers.

rif-1 allele by transformation from frozen rif-1 cells and subsequently characterized RNA polymerases from a number of separate rifampicin-resistant *E. coli* mutants for their termination behavior with T7 and T3 DNAs.

Attempts to isolate the rif-1 allele by transformation were carried out by the procedure of Cosloy & Oishi (1973) using JC7623 as recipient for DNA extracted from 5 g of frozen BG-1 cells. The spontaneous mutation frequency of JC7623 from Rif^S to Rif^R was 5×10^{-8}. DNA from BG-1 transformed JC7623 from Leu^- to Leu^+ at a frequency of 10^{-7}. DNA from BG-1 appeared to transform JC7623 from Rif^S to Rif^R at a frequency of 5×10^{-7}.

Sixteen possible transformants were picked and purified, and a small amount of RNA polymerase was purified from each. Only two showed termination with T3 DNA in vitro (as we discuss below, the properties of these strains and of the isolated RNA polymerases were somewhat different from those of the original rif-1 strain; hence, it is likely that these are simple spontaneous rifampicin-resistant mutations rather than rif-1 transformants). One of these was designated NN1500, and RNA polymerase holoenzyme (rif-15) was purified from 50 g of cells by the large-scale purification procedure of Burgess & Jendrisak (1975).

Properties of NN1500 and rif-15. The *rpoB* mutation from NN1500 was moved into SA1030 by P1 transduction. The frequency of P1 transduction expected is $\sim 10^{-5}$/infected cell (Miller, 1972). The frequency found for conversion of SA1030 from Rif^S to Rif^R was 10^{-6}. This was lower than expected; however, out of 30 possible transductants tested, 20 were also Arg^-. This is consistent with the linkage found for *rpoB* and *argE* (Bachmann et al., 1976). Eight of these 20 transductants were picked for further characterization by small-scale enzyme purification. All eight enzymes from these strains terminated with T3 DNA in vitro.

The rif-15 enzyme was tested to see if it shows altered transcriptional properties in vitro other than that of termination. The enzyme does not appear to show any altered promoter recognition with T7 DNA. At low (0.1–1) and high (10–20) enzyme/DNA ratios, the rif-15 enzyme utilizes the A promoters over the other minor promoters on T7 DNA (Stahl & Chamberlin, 1977) (data not shown). This is in contrast to the rif-1 RNA polymerase holoenzyme which utilizes T7 promoters A1 and C with equal efficiency even at molar enzyme/DNA ratios well below 1 (George Kassavetis, unpublished observation). Hence, rif-15 is not the original rif-1 mutation but almost certainly a spontaneous Rif^R mutation. The rif-15 enzyme shows the same transiton temperature with T7 DNA (~17 °C) as the wild-type enzyme (Mangel & Chamberlin, 1974; Chamberlin et al., 1976). The enzyme shows the same effect of KCl on activity (maximal activity at 200 mM KCl) as the wild-type enzyme. The termination of the rif-15 enzyme with T3 DNA is enhanced by 300 mM KCl as is that of the wild-type enzyme (Figure 8).

The rif-15 enzyme forms heparin-resistant rapid-starting complexes on T7 DNA (100% with 50 μg/mL heparin) (Mangel & Chamberlin, 1974) but appears to form them more slowly than the wild-type enzyme; $t_{1/2}$ at 30 °C is 10 s for site selection by the wild-type enzyme and 15 s for the rif-15 enzyme. The rif-15 enzyme also elongates more slowly than the wild-type enzyme as measured at 30 °C (10 nucleotides/s as compared to 17 nucleotides/s).

The rif-15 enzyme is also extremely rifampicin resistant in vitro (Figure 9). Even when 2.7 mg/mL rifampicin is present in the assay mixture, there is still 6% of the original activity remaining. Thus, the enzyme appears to have both an activity

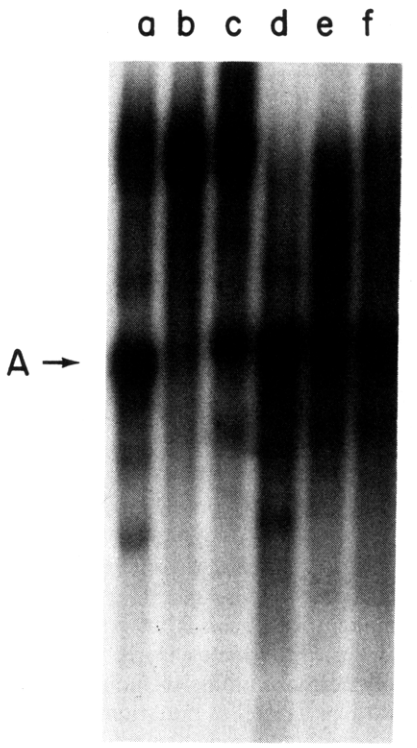

FIGURE 8: Polyacrylamide–agarose gel electrophoresis of T3 and T7 RNAs transcribed by wild-type *E. coli* RNA polymerase and rif-15 RNA polymerase in vitro. RNA synthesis was stopped after 25 min of synthesis at 30 °C, and an aliquot of each reaction mixture was laded on separate tracks of a 1.75% polyacrylamide–0.7% agarose gel. The total amount of [α-^{32}P]CMP incorporated into RNA in each reaction was from 1 to 1.5 nmol. Approximately 80 000 cpm of labeled RNA was loaded on each track. The position of the major A transcript (2.4×10^6 molecular weight) made by wild-type *E. coli* RNA polymerase with $T7^+$ DNA is indicated in the left-hand margin. Tracks a–c show RNAs made by wild-type *E. coli* RNA polymerase holoenzyme, and tracks d–f show RNAs made by rif-15 RNA polymerase holoenzyme. Tracks a and d are transcription with $T7^+$ DNA. Tracks b and e are transcription with $T3^+$ DNA. Tracks c and f are transcription with $T3^+$ DNA with KCl added to 0.3 M final concentration at 2 min after RNA synthesis had begun.

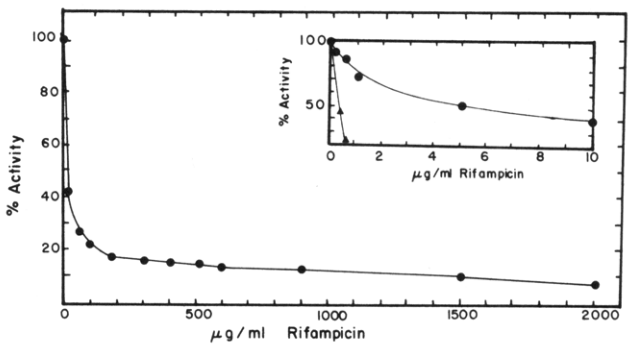

FIGURE 9: Activity of wild-type *E. coli* RNA polymerase and rif-15 RNA polymerase as a function of rifampicin concentration. Rifampicin was added to standard transcription assays to the final concentrations indicated, and RNA synthesis was measured after 5 min at 30 °C. The circles represent the activity of the rif-15 enzyme, and the triangles represent that of the wild-type enzyme. The amount of [α-^{32}P]CMP incorporated by the rif-15 enzyme and the wild-type enzyme was ~1 nmol when no rifampicin was present.

or a form that is rapidly inactivated by rifampicin and one that is hardly inactivated at all. This might be due to the existence of two enzyme conformations which are in slow equilibrium [cf. Bahr et al. (1976)] or to actual heterogeneity in the enzyme as isolated. The enzyme does not show any altered transcription pattern with T7 DNA when rifampicin is added

Table V: Summary: Termination of T7 and T3 RNAs in Vitro by Mutants of *E. coli* RNA Polymerase

strain (mutation)	terminates with T7 DNA	terminates with T3 DNA
BG-1 (rif-1)	yes	yes
NN1500 (rif-15)	yes	yes
2016 (*rpoB*$^+$)	yes	no
2016 rif-267 (*rpoB*)	yes	no
2016 rif-268 (*rpoB*)	yes	no
2016 rif-270 (*rpoB*)	yes	no
KY1330 (*rpoB*$^+$ Stl^S)	yes	no
KY1344 (*rpoB* Stl^R)	yes	no
KY1345 (*rpoB* Stl^R)	yes	no
C2121 (*rpoA*)	yes	no
P90A5c (*rpoD*$^+$)	yes (30 and 45 °C)	
285 ($rpoD_{ts}$)	yes (30 and 45 °C)	

Table VI: Bacterial RNA Polymerases Tested for Termination with T3 and T7 DNAs in Vitro

bacterial strain	enzyme source	terminates with T7 DNA	terminates with T3 DNA
E. coli (wild type DG156)	purified in this lab	yes	no
C. crescentus	gift of L. Shapiro	yes	no
A. vinelandii	gift of J. Krakow	yes	no
M. smegmatis	purified by J. Bush	yes	no
R. rubrum	purified by J. Bush	yes	no
S. typhimurium	purified by J. Bush	yes	no
B. subtilis	purified by J. Jaehning or B. Davison	yes	yes

to the enzyme before DNA and NTPs are added, or to the enzyme and the DNA, or during elongation.

(*2*) *Test of Other E. coli RNA Polymerase Mutant Enzymes.* Using the method of Gross et al. (1976), it is possible to purify small amounts of *E. coli* RNA polymerase rapidly. This procedure was used to isolate enzyme from a number of *E. coli* RNA polymerase mutants including other rifampicin-resistant mutations as well as mutations in the *rpoA* and *rpoD* genes for the ability to terminate with T3 DNA. T7 and T3 RNAs made in vitro by the purified mutant RNA polymerases were analyzed by gel electrophoresis to determine qualitatively whether they read the T7 and/or T3 terminators (Neff & Chamberlin, 1978), and the results are summarized in Table V. Termination in this case is scored visually by comparing the efficiency of termination by the wild-type *E. coli* with T7 DNA (Figure 3, track a) to the efficiency of the other enzymes with T3 DNA. A positive result listed in the table shows that the enzyme reads the termination site on T3 DNA with high efficiency (60–80%). All of the mutant RNA polymerase enzymes tested terminated quite well with T7 DNA and not with T3 DNA and hence were not detectably different from the wild-type enzyme.

It has been suggested (Schafer & Zillig, 1973) that the σ subunit of *E. coli* RNA polymerase may be involved in displacing the RNA chain from the ternary complex at the termination site. RNA polymerase was isolated from a strain carrying a temperature-sensitive σ mutation ($rpoD_{ts}$) and its parental strain (Isaksson et al., 1977; Harris, 1978) to test whether σ has any role in termination with T7 DNA. This enzyme fails to carry out selective transcription initiation at the nonpermissive temperature of 45 °C.

Termination at the permissive temperature (30 °C) and the restrictive temperature (45 °C) was assayed by two methods. (1) RNA synthesis was initiated at 30 °C, and after 1 min the chains were elongated at 45 or 30 °C. The transcription products were then analyzed by polyacrylamide–agarose gel electrophoresis. Both the mutant and the wild-type enzyme terminated at both the restrictive and the permissive temperature with T7 DNA. Termination was also monitored by the time course assay described above. A longer incubation period at the restrictive temperature was included to be sure that the σ subunit was completely inactivated. (2) RNA synthesis was initiated at 30 °C and after 2 min EDTA was added to stop the reaction. After 10 min of incubation at either 30 or 45 °C, $MgCl_2$ was added to allow elongation and termination of the RNA chains. Both the mutant and the wild-type enzyme lost ~15% of their initial elongation activity on prolonged incubation at 45 °C, but both enzymes still terminated at 30 or 45 °C (data not shown). These experiments suggest that the σ subunit does not play a major role in the termination of transcription with T7 DNA in vitro.

(*3*) *Termination of Transcription by Other Bacterial RNA Polymerases.* A large variety of purified bacterial RNA polymerases from divergent bacterial RNA species all utilize the major T7 A1 promoter site and the T7 termination site at 19.3% on T7 DNA in vitro (Wiggs et al., 1979). As a further step in studying the effect of protein alterations on the efficiency of termination, these enzymes were tested to see if they might read the termination site on T3 DNA in vitro, where the *E. coli* enzyme does not. The RNAs made from T3 DNA by the enzymes listed in Table II were analyzed by polyacrylamide–agarose gel electrophoresis. From the results summarized in Table VI, only the *B. subtilis* enzyme reads the termination site on T3 DNA with high efficiency, suggesting that either this enzyme is enhanced in its termination efficiency relative to the *E. coli* enzyme or the T3 termination signal is more closely homologous to those normally used by *B. subtilis*.

Discussion

The DNAs of bacteriophages T7 and T3 provide an ideal system for studies of the parameters that affect transcriptional termination in vitro. Under the appropriate conditions, essentially all of the transcription initiated by *E. coli* RNA polymerase begins at the strong early promoter region at the left end of the T7 or T3 genome and proceeds through the early genetic region to encounter a termination signal near 20% on the standard physical map. With T7 DNA, termination at this site is highly efficient in vitro and in vivo, while with T3 DNA termination is highly efficient in vivo but weak or nonexistent under normal synthetic conditions in vitro. Hence, by following the termination reaction in vitro with both templates, one can easily monitor, in parallel reactions, factors that enhance termination (with T3 DNA) or reduce termination (with T7 DNA).

Experimentally the overall termination process can be followed by measurements of the rate of ribonucleotide incorporation, under conditions of synchronous initiation where only one round of transcription is permitted, or by analysis of the size and amount of the in vitro transcripts by using polyacrylamide gel electrophoresis. Individual steps of the overall termination process, such as RNA chain release or enzyme release from DNA, can also be followed by using filter binding methods.

Using these procedures, we find that transcriptional termination at the strong terminator sites on T7 and T3 DNAs is a relatively difficult reaction to perturb. Termination at the T7 terminator remains highly efficient under all of the

reaction conditions we have tested and with all of the mutant *E. coli* RNA polymerases we have screened. In addition, the T7 terminator is read efficiently by a variety of other prokaryotic RNA polymerases [Wiggs et al. (1979) and this communication]. However, utilization of the T7 terminator is affected by the nature of the *E. coli* RNA polymerase preparation (Chamberlin et al., 1979), with termination efficiencies ranging from 90% with our best preparations to 60% with others.

Interaction of a ternary enzyme complex with the T7 terminator leads rapidly to release of the nascent T7 RNA chain and to release of RNA polymerase in a form that can initiate another RNA chain [Chamberlin & Ring (1972) and this communication]. This termination, release, and reinitiation process does not require either ρ factor or elevated ionic strength in vitro.

Reading of the T3 terminator site, which is normally very inefficient in vitro, is not enhanced by large changes in most reaction conditions (Table III). However, at elevated ionic strengths, in the presence of formamide, or at very low concentrations of ribonucleoside triphosphates, reading of the T3 terminator becomes quite efficient. Reading of the T3 terminator is also enhanced by replacement of CTP with BrCTP as substrate (Neff & Chamberlin, 1978; Adhya et al., 1979).

Several kinds of alterations of the RNA polymerase protein lead to enhancement of the reading of the T3 terminator. The *B. subtilis* RNA polymerase reads the T3 terminator quite efficiently, suggesting that it may have an intrinsically greater efficiency of interaction with termination signals than the *E. coli* enzyme. The enhancement of termination seen with the rif-1 and rif-15 RNA polymerases is extremely interesting and implicates the β subunit of RNA polymerase in the termination process. Such mutants appear to be fairly common among spontaneous rifampicin-resistant mutants of *E. coli*. Several other rifampicin-resistant, *rpoB* mutants have been described which alter transcriptional termination in vivo (Guarente & Beckwith, 1978; Das et al., 1978); however, in at least one instance the purified mutant enzyme does not show enhanced transcriptional termination in vitro but shows an altered interaction with ρ protein (Das et al., 1978). The potential to produce a mutant RNA polymerase which is greatly enhanced in its intrinsic termination efficiency suggests that some rifampicin resistance mutations could dramatically alter the pattern of transcription in vivo. It is interesting that the rif-15 RNA polymerase terminates efficiently at a mutant *trp* attenuator region (Stauffer et al., 1978) which is characterized by its inability to be utilized by the normal *E. coli* RNA polymerase in vitro (C. Yanofsky, personal communication). This suggests that there is a common mechanism for termination at the strong, ρ-independent T7 and T3 terminators and the *trp* attenuator.

It would be attractive to discuss the factors that alter the termination reaction between *E. coli* RNA polymerase and the T3 and T7 terminators in terms of a molecular mechanism for the process. There are probably a number of different interactions involved in the overall termination process including interactions between RNA polymerase, RNA, the DNA template, and finally the ρ factor. Two general steps seem rather likely: (1) a long pause or hesitation at the termination site and (2) a sequence-dependent recognition event leading to termination and release of the RNA chain and RNA polymerase. Rosenberg et al. (1978) have shown that RNA polymerase pauses at the ρ-dependent λ terminator P_L even in the absence of ρ, and there are single base changes in several terminators which abolish terminator function (Rosenberg et al., 1978; Stauffer et al., 1978). However, the exact molecular mechanism which controls these events is not yet understood.

Studies of the sequences involved in the terminator region and of the effect of different conditions on the efficiency of terminator have led to two general kinds of models for the specific molecular interactons in the termination process. The first stresses the involvement of GC-rich sequences just upstream from the transcriptional termination site (Gilbert, 1976). At low concentrations of nucleoside triphosphates, RNA polymerase hesitates at such sequences during normal chain elongation and many known terminator sequences have such a GC-rich region just upstream of the terminator [see Gilbert (1976) and Adhya & Gottesman (1978) for reviews]. Hybrid DNA–RNA structures involving GC pairs are far more stable than their DNA–DNA counterparts (Chamberlin, 1965), and formation of long DNA–RNA hybrids during transcription strikingly enhances transcriptional termination (Maitra et al., 1967; Chamberlin, 1970; Neff & Chamberlin, 1978). Also, transcriptional termination is reduced by incorporation of IMP into the RNA (Neff & Chamberlin, 1978; Adhya et al., 1979; Lee & Yanofsky, 1977); IC hybrid structures are far less stable than the corresponding GC DNA–DNA structures (Chamberlin, 1965).

However, Rosenberg et al. (1978) have shown that for the λ terminator t_{R1} there is no GC-rich region upstream of the stop site and their studies, taken with the work of Nayofsky and his collaborators (Lee & Yanofsky, 1977; Stauffer et al., 1978) on the *trp* operon attenuator, focus attention on the formation of a stem–loop structure in the RNA just upstream of the terminator. In particular, mutational substitutions which would block formation of such a structure relieve termination even when a GC-rich region is present and this leads to a model for termination in which it is supposed that interaction between such an RNA stem–loop structure and RNA polymerase acts as an essential element to trigger termination.

This model is similar in some respects to a model proposed originally by Stent & Brenner (1961) to account for the stringent response, in which uncharged tRNA was postulated to bring about transcriptional arrest. This kind of "product inhibition" was studied in some detail in the early 1960s (Tissieres et al., 1963; Fox et al., 1965). These studies concluded that RNA polymerase in elongating complexes is insensitive to inhibition by RNA, and later studies have extended this to show that ternary elongating complexes are not affected even by high concentrations of the polyanionic inhibitor heparin (Walter et al., 1967). These studies, taken with the rather short region of the nascent RNA protected by RNA polymerase from RNase digestion (Kumar & Krakow, 1975; Richardson, 1975), argue against the existence of a large binding site ($\sim 30 \times 40$ Å) on RNA polymerase specific for binding of a loop–stem RNA structure of over 20 nucleotides. Furthermore, nucleic acid binding by proteins that interact with DNA duplex structures is reduced and then eliminated as the concentration of counterion is increased, especially when the interaction does not involve specific nucleotide sequences (Record et al., 1976; de Haseth et al., 1977). Yet termination at the T7 terminator is not eliminated by elevated salt concentrations and termination at the T3 terminator is greatly enhanced, contrary to the result expected if the process involved an RNA duplex–protein interaction. Hence, both models for the interactions involved in RNA chain termination have serious lacunas in their current form.

The data presented here add to our knowledge of the factors that influence in vitro termination but do not discriminate between the two extreme models noted above. The stimulation

of termination with T3 DNA in the presence of very low nucleotide concentrations (Figure 6) might be interpreted as due to an increase in the hesitation time of the enzyme at the termination site on T3 DNA induced by the low nucleotide concentrations. It is possible that the GC-rich region of the T3 termination site is shorter than the sequence at the T7 terminator, which contains a 10 base pair run with 9 GC pairs (J. Dunn, personal communication). The observation that low nucleotide concentrations stimulate hesitation has been reported previously (Maizels, 1973; Gilbert, 1976; Darlix & Horaist, 1976). However, the reduction of pausing or termination at low substrate concentrations is probably not simply due to a reduction in the chain elongation rate since equivalent reduction of the elongation rate brought about by reduced temperatures does not enhance termination.

How KCl and formamide act to stimulate the termination reaction on T3 DNA is not known with certainty. Formamide reduces the relative stability of DNA duplexes as compared to hybrid RNA–DNA duplexes (Thomas et al., 1976), and the addition of 40% formamide to transcribing complexes on T7 DNA leads to the formation of long hybrid loops between DNA template and nascent RNA at the growing point (Bick et al., 1972). It is attractive to suppose that the ability of formamide to enhance hybrid stability inhibits the strand displacement reaction catalyzed by RNA polymerase in which the nascent RNA is displaced from the template DNA strand with re-formation of the original template duplex. This would lead to formation of a long DNA–RNA hybrid structure and might slow or completely block RNA chain elongation as we have suggested before (Neff & Chamberlin, 1978). The fact that RNA chains can be stopped reversibly without RNA chain release is consistent with the idea that several kinds of interactions are needed for the overall termination process. Presumably, when RNA polymerase is forced to halt at a sequence which is not a terminator sequence, the information needed for the RNA chain release reaction is not normally present. However, most studies on the effect of formamide on nucleotide base pairing have been done at higher concentrations of formamide than we have employed. Also, the effect of formamide on the structure of the enzyme itself or on RNA–RNA pairing interactions is not known. The effects of formamide and KCl on transcriptional termination are probably not the same; formamide concentrations of 20% or greater can block elongation at any point on DNA whereas KCl concentrations up to 800 mM do not.

Addition of increasing amounts of KCl to the transcription reaction has two striking effects. It not only stimulates termination and reinitiation (Bremer & Konrad, 1964; Richardson, 1970; Millette et al., 1970; Maitra et al., 1970; Schafer & Zillig, 1973) but also increases the elongation rate of the enzyme. The read-through RNA band(s) near the top of the tracks in Figure 3 is progressively larger in size as the KCl concentration in the reaction is increased. KCl obviously does not increase the hesitation time of the enzyme and may actively decrease it. Salt might act in part to stimulate the dissociation of the ternary complex at the terminator site by lowering the affinity of the enzyme for the DNA and the RNA. Increasing the ionic strength could also stabilize certain RNA–DNA or RNA–RNA interactions over DNA–DNA or other RNA–DNA interactions, although one might expect relatively little effect of modest KCl concentrations on the stability of a duplex structure in the presence of 10 mM Mg^{2+}.

The most dramatic result we present, in terms of discrimination between the two kinds of models for termination, is the negative finding that transcriptional termination at the T7 terminator is not altered by continual removal of the nascent RNA by ribonuclease during the reaction under conditions that should cleave to within 10–20 nucleotides of the 3′ end of the growing chain. In its simplest form the stem–loop RNA model would seem to predict that such cleavage would abolish transcriptional termination. The sequence of nucleotides at the T7 terminator does allow writing of a stemp–loop structure with eight GC base pairs in a stem located seven to eight bases from the 3′ end of the RNA (J. Dunn, personal communication). In addition, the common effect of the rif-15 mutation on reading of the T3 terminator and *trp* attenuator (cited above) suggests a common mechanism of termination for these different sites. However, the experiment is not decisive in that it cannot be ruled out that the stem–loop RNA moves into a site on the RNA polymerase protected from RNase, causes termination, and is then released to be subsequently cleaved. Consequently, more extensive studies of the protein–nucleic acid interactions at the terminator will be needed to resolve the current questions posed by the mechanism of transcriptional termination.

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