*DNA-DEPENDENT SYNTHESIS OF RNA BY ESCHERICHIA COLI RNA POLYMERASE: RELEASE AND REINITIATION OF RNA CHAINS FROM DNA TEMPLATES**

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Abstract.—RNA synthesis in an *in vitro* RNA polymerase system at low ionic strength soon ceases, due to inhibition by accumulated RNA. Measurement of RNA chain initiation by the incorporation of γ-^{32}P-ATP and GTP with native T2 or T4 DNA as template shows that only one RNA chain is formed per molecule of enzyme added. In contrast, when the polymerase reaction is carried out in 10 mM Mg^{++} and 0.2 *M* KCl, RNA synthesis proceeds nearly linearly for hours, resulting in a marked increase in accumulated RNA. Incorporation of γ-^{32}P-ATP also proceeds throughout the course of the reaction and the number of chains initiated per molecule of enzyme is increased severalfold. Most RNA chains formed are released from the DNA template as free RNA. Polymerase is released also in this process and acting catalytically reinitiates new chains. Rifampicin inhibits initiation of RNA synthesis and also blocks reinitiation of RNA chains without affecting growth of RNA chains already initiated.

Introduction.—The DNA-dependent RNA polymerase reaction *in vitro* can be divided into a number of discrete steps. These include (a) recognition by the polymerase of specific starting points on the template DNA at which enzyme molecules bind to DNA and (b) initiation of RNA chains. It has been shown previously[1–4] that the initiation reaction, measured by the incorporation of γ-^{32}P-labeled nucleoside triphosphates, leads to the formation of RNA chains in which the first nucleotide retains the 5′-triphosphate end. Purine nucleoside triphosphates are the only triphosphates beginning an RNA chain.

The next steps involve (c) elongation of RNA chains by the addition of ribonucleotides to the 3′-OH group of the ribonucleoside end of the initiated ribonucleotide chains in a 5′ → 3′-OH direction[1] and (d) termination of RNA chains, with the release of newly formed polyribonucleotide chains from the DNA-RNA-enzyme complex as free RNA. The enzyme molecules should be released in termination and thus be able to reinitiate new chains. Although previous reports from this and other laboratories[1–6] have presented evidence for steps (a) through (c), the termination step (d) with release and reinitiation of RNA chains has not been previously demonstrated.

Re-examination of the problem of termination and reinitiation of RNA chains was motivated by the observations of So *et al.*[7] and Fuchs *et al.*[8] They showed that at high ionic strength, nucleotide incorporation proceeds for a long period, resulting in a marked increase in the total yield of RNA synthesized.

The present communication describes experiments which show that in the presence of 10 mM Mg^{++} and 0.2 *M* KCl, incorporation of γ-^{32}P-ATP also pro-

ceeds during the entire period of RNA synthesis, resulting in several moles of RNA chains initiated per mole of enzyme added. Most of the new RNA chains formed are free of template DNA and released as free RNA. Evidence is also presented to document the release of enzyme during termination and the reinitiation of RNA chains during the course of the polymerase reaction. Preliminary reports of this work[9] and of similar findings by Millette[10] have appeared.

Materials and Methods.—γ-^{32}P-labeled nucleoside triphosphates were prepared as described previously.[2] Unlabeled and labeled ribonucleoside triphosphates were obtained from Schwarz BioResearch. RNA polymerase from *E. coli* was isolated and purified as described previously[4] through the ammonium sulfate fraction III step. Units of polymerase are defined as described before.[4] One $\mu\mu$mole of polymerase is equal to 1.1 units of enzyme, based on a molecular weight of 360,000 and specific activity of 3,000 units/mg of homogeneous protein as described previously.[4] Incubation of 10 units of polymerase with 10 mμmoles of ^{32}P-T4 DNA and 10 mμmoles of ^{3}H-f2 bacteriophage RNA at 37° for 2 hr did not alter the sedimentation constant of either radioactive polymer indicating the absence of endonucleases in the polymerase preparation. Bacteriophage T4 and T2 DNA were isolated by the procedure described previously.[4] ^{3}H-f2 bacteriophage RNA was a gift from Mr. J. Dubnoff of this laboratory and was used as a marker for 30*S* RNA in sucrose gradient analysis. The dAT copolymer was obtained from Worthington Biochemical Corporation. Rifampicin was obtained from Ciba Pharmaceuticals, New Jersey.

Measurement of the polymerase reaction: The initiation reaction was measured by the incorporation of γ-^{32}P-ATP or γ-^{32}P-GTP into an acid-insoluble RNA product while total RNA synthesis was measured by the incorporation of ^{14}C or ^{3}H-ribonucleoside triphosphates. The conditions of assay, unless otherwise specified, were as follows: reaction mixtures (0.5 ml) contained 50 m*M* Tris buffer, pH 7.8; 8 m*M* 2-mercaptoethanol; 10 mM $MgCl_2$; 14 mμmoles of DNA as deoxynucleotide residues; and 60 mμmoles each of ATP, UTP, CTP, and GTP. γ-^{32}P-ATP or γ-^{32}P-GTP (2×10^9 cpm/μmole) was used to measure initiation; ^{14}C or ^{3}H-UTP (5×10^6 cpm/μmole) was used to measure total RNA synthesis. Wherever indicated, KCl at a final concentration of 0.2 *M* was included in the reaction mixture. The order of addition of components in the polymerase reaction was as follows: DNA and enzyme were mixed, followed when indicated by KCl; the reaction was subsequently initiated by the addition of the four ribonucleoside triphosphates. After incubation as described, the reaction was terminated and the amount of radioactivity incorporated into an acid insoluble RNA product was determined by the procedures outlined previously.[1, 2]

Results.—Effect of KCl on kinetics of RNA synthesis and initiation: The effect of 0.2 *M* KCl on the initiation of RNA synthesis and on chain elongation in a T2 DNA directed reaction can be summarized as follows (Table 1): (1) nucleotide incorporation continued linearly for hours in the presence of salt, leading to a five-fold increase in the yield of RNA. (2) This salt-stimulated increase in total synthesis was quantitatively accounted for by the increase in the number of RNA chains initiated. In the absence of KCl, incorporation of γ-^{32}P-labeled ATP and GTP was virtually complete after 30 minutes, whereas in the presence of 0.2 *M* KCl this incorporation continued throughout the incubation period. (3) The average chain length of RNA synthesized was not affected by KCl. (4) The number of RNA chains synthesized per molecule of enzyme was approximately one in the absence of KCl; in its presence, however, nearly four chains were synthesized per molecule of enzyme during a 4-hour incubation period. The influence of salt in stimulating RNA synthesis and initiation was not re-

stricted to KCl. At 0.2 *M* salt concentration, 6 units of polymerase and under the conditions of the assay described in Legend to Table 1, the amounts of ^{3}H-UMP incorporated in 30 minutes, 4 hours, and 8 hours were 7.8, 37, and 58.5 mμmoles with KCl; 5.4, 20.4, and 31.3 mμmoles with NH_4Cl; 3.2, 22.4, and 40.4 mμmoles with NaCl and 2.5, 5.6, and 7.2 mμmoles with LiCl. The optimal concentration for salt stimulation was between 0.2–0.3 *M*. Higher concentrations of salt (0.4 *M*) were inhibitory.

The stimulatory effect of KCl on RNA initiation was predominantly on chains beginning with ATP; initiation with GTP was little affected (Table 2). No γ-^{32}P-UTP or γ-^{32}P-CTP was incorporated into RNA products either in the absence or presence of KCl. The stimulatory effect of salt was specific for native DNA; RNA synthesis primed with denatured DNA was not stimulated by salt but was, in fact, inhibited (Table 3). Similarly, denatured DNA-primed polyriboadenylate synthesis and initiation with γ-^{32}P-ATP were inhibited by 0.2 *M* KCl either with 10 mM Mg^{++} or 2 mM Mn^{++}. dAT primed Poly rAU synthesis was also not affected by salt.

To prove that the increase in initiation of RNA chains with native DNA as template is due to release and reinitiation, two sets of polymerase reactions were set up, one with and one without added 0.2 *M* KCl (Table 4). The polymerase reaction was started in both cases with ^{3}H-UTP (to measure RNA synthesis)

TABLE 1. *Influence of KCl on RNA synthesis.*

Time (min)	γ-^{32}P-Nucleotide Incorporated (ATP + GTP) ($\mu\mu$moles)		RNA Synthesized (mμmoles)		Average Chain Length	
	+KCl	−KCl	+KCl	−KCl	+KCl	−KCl
10	1.9	1.8	3.6	3.8	1900	2100
30	4.2	2.2	10.2	5.8	2400	2600
120	9.0	2.5	30.3	9.2	3300	3700
240	13.4	2.8	57.8	10.1	4300	3600

The conditions of the assay were as described in *Materials and Methods*. Incorporation of γ-^{32}P-ATP and γ-^{32}P-GTP were determined separately and the results added. Incubation was at 37° with 3.3 units of enzyme (equivalent to 3 $\mu\mu$moles of polymerase) and 12 mμmoles of T2 DNA. 0.2 *M* KCl was added where indicated. Average chain length was calculated as the ratio of total RNA nucleotide synthesized to total initiation obtained with ATP and GTP.

TABLE 2. *Influence of KCl on initiation with ATP vs. GTP.*

	Time (min)	γ-^{32}P Triphosphate Incorporated ($\mu\mu$moles) ATP	GTP	Ratio A/G
Expt. A				
No KCl	10	1.2	0.6	2.0
	30	1.4	0.8	1.8
	120	1.7	0.8	2.1
	240	1.9	0.9	2.1
Expt. B				
+ KCl	10	1.6	0.3	5.3
	30	3.5	0.7	5.0
	120	7.5	1.5	5.0
	240	11.2	2.2	5.1

The conditions of assay were the same as those for Table 1.

and the other three unlabeled nucleoside triphosphates. After 30 minutes of incubation at 37°, γ-^{32}P-ATP was added to each reaction mixture and the kinetics of chain initiation was followed. As shown in Table 4, in the absence of KCl, no γ-^{32}P-ATP incorporation occurred, presumably because all RNA chains were already initiated with non-radioactive triphosphates. In the presence of salt, however, reinitiation was clearly evident by the incorporation of γ-^{32}P-ATP and quantitatively accounted for the increased synthesis of RNA chains.

Further evidence for reinitiation of RNA chains in the presence of salt was obtained by the observation that the antibiotic rifampicin completely blocked the stimulating effect of salt on initiation of RNA chains. Rifampicin has been shown to combine with the enzyme and inhibit RNA synthesis by blocking initiation of RNA synthesis but not to inhibit the chain elongation reaction.[11] When rifampicin was added before the reaction was started, both RNA synthesis and chain initiation were virtually completely inhibited (Table 5). Rifampicin, added five minutes after the start of the polymerase reaction, did not inhibit RNA synthesis already initiated, whether salt was present or absent, but com-

TABLE 3. *Effect of denaturation of DNA on salt-stimulated initiation and synthesis of RNA chains.*

	Time (min)	γ-^{32}P Nucleotide Incorporated ATP ($\mu\mu$moles)	γ-^{32}P Nucleotide Incorporated GTP ($\mu\mu$moles)	RNA Synthesis ($\mu\mu$moles)
Expt. A				
No KCl	30	2.8	5.2	1974
	60	4.6	7.8	2980
	120	7.7	11.0	4278
	240	12.3	13.4	5880
Expt. B				
with 0.2M KCl	30	1.8	4.7	945
	60	3.5	5.1	1290
	120	4.5	6.1	1806
	240	6.4	8.3	2308

The conditions of the assay were the same as those for Table 1 and under *Materials and Methods* except that 12 mμmoles of heat denatured T2 DNA were used as template. The amount of RNA polymerase added to each tube was 3.3 units (equivalent to 3$\mu\mu$moles). 0.2 *M* KCl was added where indicated.

TABLE 4. *Reinitiation of RNA chains.*

Time after addition (min) of γ-^{32}P-ATP	γ-^{32}P-ATP Incorporated ($\mu\mu$moles) −KCl	γ-^{32}P-ATP Incorporated ($\mu\mu$moles) +KCl	^{3}H-UMP Incorporated (mμmoles) −KCl	^{3}H-UMP Incorporated (mμmoles) +KCl
0	...	...	1.6	3.6
30	0.2	2.4	2.4	6.1
60	0.2	4.1	2.7	8.9
120	0.3	6.2	3.1	15.1
240	0.4	9.8	3.6	22.7

One set of polymerase reaction mixture contained 0.2 *M* KCl while KCl was omitted in the other. Each tube contained 6 units of polymerase. RNA synthesis was started in both cases by the addition of 60 mμmoles each of 3 unlabeled nucleoside triphosphates and ^{3}H-UTP (to measure RNA synthesis). After 30 min of incubation at 37°, 10 mμmoles of γ-^{32}P-ATP (1×10^{10} cpm/μmole) were added to each reaction mixture yielding a final specific radioactivity of γ-^{32}P-ATP of 1.45×10^{9} cpm/μmole). Incorporation of ^{32}P and ^{3}H was followed. Other conditions of the assay were as described under *Materials and Methods*.

TABLE 5. *Influence of rifampicin on KCl-stimulated initiation and RNA synthesis.*

Additions	^{3}H-UMP Incorporated (mμmole)			γ-^{32}P-ATP Incorporated (μμmole)		
	10′	120′	240′	10′	120′	240′
None	0.90	2.50	2.80	1.0	1.2	1.7
KCl	1.20	15.60	27.30	1.8	8.5	12.1
None + Rifampicin at 0 time	0.02	0.05	0.06	0.2	0.3	0.3
None + Rifampicin at 5 min	0.90	2.40	2.90	1.0	1.1	1.2
KCl + Rifampicin at 0 time	0.02	0.06	0.08	0.2	0.3	0.3
KCl + Rifampicin at 5 min	1.10	2.10	2.90	1.8	2.1	2.3

The conditions of the assay were as described under *Materials and Methods* and as in Table 1 except that rifampicin (final concentration 0.5 μg/ml) was added at the time indicated in the table.

pletely abolished the stimulation by salt of RNA synthesis and initiation. Thus, stimulation of RNA synthesis in the presence of salt is almost entirely due to reinitiation of RNA chains.

Release of RNA chains from DNA template in the presence of KCl: The addition of KCl to the polymerase reaction mixture resulted in the accumulation of a considerable amount of free RNA (Figure 1). The amount of free RNA formed increased as the reaction progressed (time points at 30 and 60 minutes yielded results which were intermediate to those shown). After two hours of incubation, most of the RNA formed in the presence of salt were molecules of approximately 30–40*S*, free of templates (Fig. 1*B*). Some RNA was also released from the DNA template in the absence of KCl as well; this slowly accumulated during the first hour of incubation, after which there was no further increase. Quantitatively, more than seven times as much free RNA accumulated after two hours in the presence of salt. Our observation of RNA product release even under low ionic strength conditions is in contrast to the reports of Bremer and Konrad.[12] These workers found that almost all the RNA product formed in similar low ionic strength reaction mixtures cosedimented with DNA as a DNA-RNA-enzyme complex and RNA was released from such complex as free RNA only on treatment with sodium dodecyl sulfate.

Release of enzyme in salt-mediated termination-reinitiation: The question was also asked that if on termination and reinitiation of synthesis whether the enzyme is released or remains bound to the initial template. Evidence for the release of the enzyme from template was obtained by adding dAT copolymer after 30-minute incubation of a reaction mixture containing T2 DNA template and ^{3}H-GTP as the labeled substrate. If all the enzyme molecules copying T2 DNA remain irreversibly attached to this template during RNA synthesis, then the addition of the second template, dAT, should not affect the rate of ^{3}H-GMP incorporation. If, however, enzyme molecules are released from template DNA, some of the released enzyme molecules will bind to the dAT copolymer when a fresh round of RNA synthesis is resumed, and these enzyme molecules will no longer incorporate ^{3}H-GMP. In the absence of salt, there was no inhibition of ^{3}H-GMP incorporation when the dAT copolymer was added at 30 minutes, in

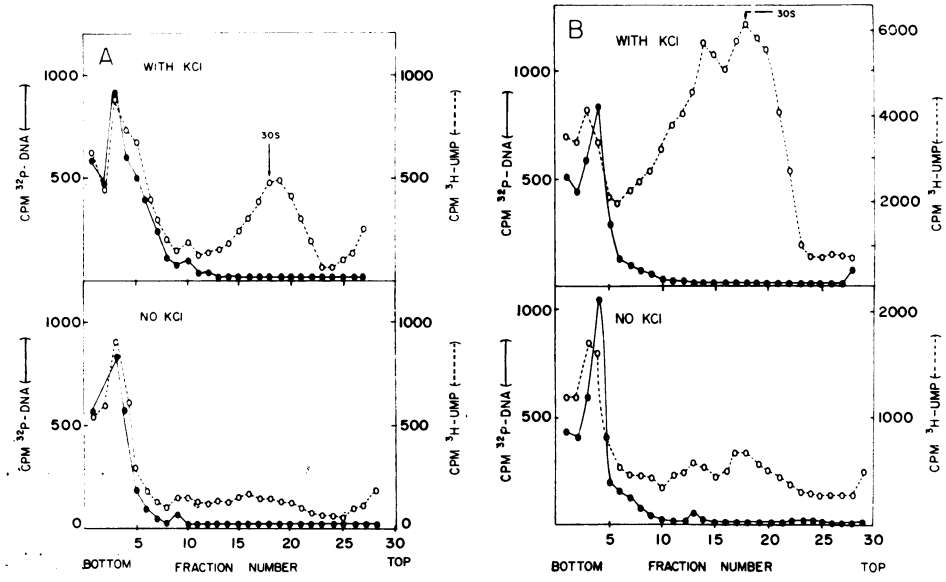

FIG. 1.—Zone sedimentation analysis of RNA polymerase reaction mixture in presence and absence of KCl. Two reaction mixtures (0.5 ml) containing 6 units of polymerase were set up as described under *Materials and Methods*. ^{3}H-UTP (2×10^7 cpm/μmole) was used as the labeled substrate and ^{32}P-T4 DNA as the template. 0.2 M KCl was added where indicated. At time intervals, 0.1-ml aliquots of chilled reaction mixture were layered onto 5–20% sucrose gradients (5.0 ml) containing 50 m*M* Tris buffer, pH 7.8, 0.1 M NaCl, and 10^{-3} M dithiothreitol. The tubes were centrifuged for 105 min at 48,000 rpm in an SW 50 rotor at 4°C. The acid insoluble radioactivity was measured in fractions (0.16 ml) collected from the bottom of the tube. The counting was carried out in a liquid scintillation counter in such a manner that no ^{3}H was detected in the ^{32}P-channel and less than 2% of ^{32}P was detected in the ^{3}H-channel. No correction was made for this low value. — ^{32}P-T4 DNA; -----^{3}H-UMP; Fig. 1*A*: 10-min. incubation; Fig. 1*B*: 120-min incubation.

contrast to the experiment in which dAT was added at zero time where there was extensive inhibition (Table 6). In the presence of salt, there was nearly 50 per cent inhibition of GMP incorporation after the addition of dAT copolymer. In this case, enzyme molecules were apparently released from T2 DNA, permitting new chains to be initiated, and accounting for the stimulation of the polymerase reaction by KCl. Quantitatively, at low ionic strength, there is a marked preference for the binding of the enzyme to dAT copolymer, presumably because the affinity of the enzyme for the copolymer is higher than for native DNA.[13] In the presence of salt, inhibition of GMP incorporation by dAT is less, presumably because the high salt concentration decreases the affinity of the polymerase for the dAT copolymer.

Discussion.—In the experiments presented above, we have subdivided the problem of RNA chain termination into three processes: (a) release of free RNA from the DNA-RNA-enzyme complex, (b) the release of free enzyme from the template which is now able to cause, (c) reinitiation of new RNA chains. In low ionic conditions with native DNA as template, there is almost a stoichiometric equivalence between RNA polymerase added and RNA chains syn-

TABLE 6. *Dissociation of RNA-polymerase-template DNA complex in presence of KCl and release of RNA polymerase.*

Template added at 0 min	Template added at 30 min	^{3}H-GMP Incorporated/120′ −KCl (mμmoles)	+KCl
T2 DNA	None	2.96	13.4
T2 DNA + dAT copolymer	None	0.55	6.4
T2 DNA	dAT copolymer	2.50	7.6

Two sets of RNA polymerase reaction mixtures, with and without added 0.2 *M* KCl, were set up. 10 mμmoles of each polynucleotide were used where indicated. RNA synthesis was initiated in both sets of reaction mixtures by addition of enzyme (5 units) and triphosphates. dAT-copolymer was added either before enzyme or at 30′ as indicated. ^{3}H-GMP incorporated into RNA product was followed with time as described under *Materials and Methods*.

thesized. With denatured DNA, though the amount of RNA formed decreases, the total number of RNA chains formed is greater than the moles of enzyme added. Thus, the absence of a duplex structure permits the polymerase to terminate and reinitiate. In the presence of native DNA, the binding of the polymerase is nearly irreversible and once RNA synthesis has begun, the enzyme is not released.[4, 5]

Results presented here show that in the presence of 0.2 *M* KCl, the rate of RNA synthesis is maintained for several hours, which is in agreement with the results first obtained by So *et al.*[7] and Fuchs *et al.*[8] In addition, we have provided conclusive evidence that the salt effect also stimulates the total yield of γ-^{32}P-labeled nucleoside triphosphate incorporation into RNA. The average RNA chain length was unaltered but the total number of RNA chains increased fivefold. In these experiments, an average of five moles of RNA chains were produced per mole of enzyme added. There is evidence (Tables 4 and 5) that this was due to reinitiation of RNA chains by the polymerase.

We have also shown that the effects of salt on the termination and reinitiation phenomena are associated with the release of both free RNA and free enzyme. The possibility is suggested that the polymerase recognizes termination sites on the DNA. If indeed the polymerase is able to recognize sites on the DNA which govern release, we might expect the 3′-hydroxy ends of the released chains to have similar nucleotide sequences. Experiments are underway to analyze the sequences in the released RNA chains.

The stimulation of the reaction by salt raises the question whether this effect is on a subunit responsible for the release of RNA or more simply due to a charge effect on the RNA which is then unable to inhibit the polymerase. Our present working hypothesis is that part of the salt effect may be due to the latter process. Even in the absence of salt, free RNA chains accumulate in the reaction mixture, and the presence of 0.2 *M* KCl serves to increase the amount of released RNA product. It is known from previous studies[4, 14] that under low ionic conditions the addition of pancreatic ribonuclease relieves end-product inhibition, suggesting that the inhibition was due at least in part to the accumulated RNA product. Similarly, we have found that addition of 0.2 *M* KCl, after the polymerase reaction in low ionic conditions has slowed down, causes resumption of RNA synthesis which continues for hours, much as in the polymerase reaction with added KCl at zero time. Reinitiation of RNA chains also occurs. All of these observa-

tions together suggest that under low ionic conditions, accumulated RNA chains combine with enzyme molecules; the effect of high salt concentration may be to prevent this association. At very high concentrations of salt (0.4 *M*), RNA synthesis is greatly inhibited, rather than stimulated, and at these concentrations, the combination of enzyme molecules with template DNA may also be prevented. The optimal concentration of 0.2 *M* KCl required to stimulate the polymerase reaction corresponds closely to the intracellular level of KCl in *E. coli*.[15] Termination and release of RNA chains may well be governed by a cellular termination-release factor which can be either part of the polymerase molecule or an additional factor not present in the polymerase preparation. Recent reports[16, 17] of the possible involvement of σ factor in RNA chain initiation are noteworthy. RNA polymerase preparation used in the studies reported here has a full complement of σ factor as assayed by the method reported by Burgess and Travers.[16, 18]

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