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Biochemistry

DNA-dependent single-step addition reactions catalyzed by *Escherichia coli* RNA polymerase

(oligonucleotide synthesis/rifampicin/RNA product analysis/RNA nucleotidyltransferase)

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ABSTRACT **The addition of a single nucleotide to a short oligonucleotide, catalyzed by RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) in the presence of synthetic DNA templates, has been studied. The reactions A-U + ATP → A-U-A and U-A + UTP → U-A-U occur in the presence of poly[d(A-T)], while the reactions G-C + GTP → G-C-G and C-G + CTP → C-G-C take place in the presence of poly[d(I-C)]. These reactions proceed with a turnover of enzyme. The products U-A-U and C-G-C are formed rapidly, while A-U-A and G-C-G are formed much more slowly. Another poly[d(A-T)]-dependent reaction, which occurs with a turnover of enzyme, is U-A-U + ATP → U-A-U-A. All of these reactions are only partially inhibited by rifampicin. ATP can be replaced by 3′-deoxyadenosine 5′-triphosphate in the reactions A-U + ATP → A-U-A and U-A-U + ATP → U-A-U-A, though the rate of formation of the products becomes somewhat slower. The reactions involving 3′-deoxyadenosine 5′-triphosphate are almost completely inhibited by rifampicin, indicating that the 3′-hydroxyl group is necessary for these reactions to occur in the presence of rifampicin.**

Recently a method for amplifying the initiation step of RNA synthesis catalyzed by DNA-dependent RNA polymerase (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) of *Escherichia coli* by means of the "abortive initiation" reaction was described by Johnston and McClure (1). This reaction involves the catalytic synthesis of a 5′-triphosphodinucleotide in the presence of nucleoside triphosphates complementary to the initial two bases of the transcription sequence. In a similar manner, it would be desirable to isolate and amplify subsequent steps in RNA synthesis in a way that would make studies of the reaction mechanism possible.

In this communication we describe a DNA-dependent single-step addition reaction catalyzed by RNA polymerase in which a phosphodiester bond is formed between a short oligonucleotide and a nucleoside triphosphate. We were led to investigate this reaction by a desire to reconcile reports that the antibiotic rifampicin does not inhibit the synthesis of the dinucleotide pppA-U by RNA polymerase (1) but does inhibit the reactions U-A + UTP → U-A-U and A-U + ATP → A-U-A catalyzed by the same enzyme (2). If rifampicin truly inhibits the latter reactions and not the former, there would be interesting implications for the mechanism of action of this antibiotic.

MATERIALS AND METHODS

Materials. *E. coli* B and *E. coli* K-12 (late logarithmic phase) were purchased from Grain Processing Corp. RNA polymerase was purified from either strain by the method of Burgess and Jendrisak (3). The enzyme was >94% pure and had a σ content of approximately 60% as shown by dodecyl sulfate/polyacrylamide gel electrophoresis (4).

Ultrapure nucleoside triphosphates (purified by high-pressure liquid chromatography) were purchased from ICN Pharmaceuticals. Cordycepin 5′-triphosphate (3′-dATP) was from Miles. A-U and U-A were from P-L Biochemicals. G-C and C-G were from Sigma. ^{3}H-Labeled nucleotides were purchased from New England Nuclear or Schwarz/Mann. Poly[d(A-T)] and poly[d(I-C)] were obtained from Grand Island Biological Co. or from Miles. Ribonuclease A (R6KA) was from Worthington.

Chromatography papers used were Whatman 3 MM and Whatman DE-81 (DEAE-paper).

Chromatography Solvents. Solvent A: water/saturated ammonium sulfate, pH 8/isopropanol, 18/80/2 (vol/vol/vol). Solvent B: ethanol/1 M ammonium acetate, pH 7.5, 7/3 (vol/vol). Solvent C: 0.2 M ammonium bicarbonate/5 mM sodium tetraborate. Solvent A is identical to the WASP solvent described by Johnston and McClure (1).

Standard Abortive Elongation Reactions. The procedure used for these reactions was a modification of the method described by Johnston and McClure (1) for carrying out abortive initiation reactions. Equal volumes (usually 50 μl) of a solution I and a solution II were mixed and incubated at 37° for the indicated period. Solution I, which contained 0.08 M TrisCl at pH 7.9, 2 mM mercaptoethanol, 5 mM $MgCl_2$, DNA, and RNA polymerase as indicated, was preincubated for 5 min at 37°. Solution II contained 5 mM $MgCl_2$, a dinucleoside monophosphate at a concentration of 20 μM, and a ^{3}H-labeled nucleoside triphosphate (500–1500 cpm/pmol) at a concentration of 10 μM. Thus, the final concentrations of dinucleoside monophosphate and nucleoside triphosphate were 10 μM and 5 μM, respectively. Reactions were stopped by chilling the mixtures on ice and adding EDTA to a concentration of 50 mM.

When rifampicin was included in the reaction mixture, the drug was added to solution I before the addition of DNA, and the solution was incubated for 5 min at 37° to allow the binding of rifampicin to the enzyme. DNA was added and the usual 5 min preincubation at 37° was then carried out before the addition of solution II.

Analysis of Standard Abortive Elongation Reaction Mixtures. Paper chromatographic analysis of standard reaction mixtures was performed on Whatman 3 MM paper. Unless otherwise indicated, 10-μl aliquots of reaction mixtures were streaked at the origin of ½-inch-wide (~12 mm) strips and developed by ascending chromatography with solvent A. After being developed, chromatograms were air-dried, cut into 1-cm

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Abbreviations: Rif, rifampicin; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate or cordycepin 5′-triphosphate; 3′-dAMP, 3′-deoxyadenosine 5′-monophosphate, A-U-3′dA, adenylyluridylyl-3′-deoxyadenosine; U-A-U-3′dA, uridylyladenylyluridylyl-3′-deoxyadenosine.

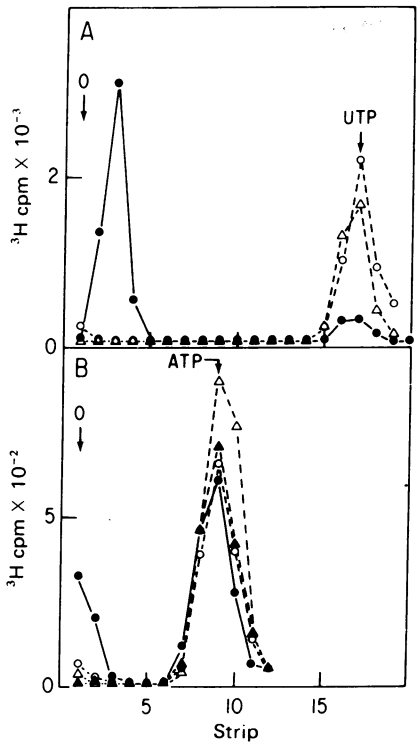

FIG. 1. Single-step addition to form U-A-U or A-U-A. Standard abortive elongation reaction mixtures were incubated for 30 min at 37° and analyzed for radioactivity. O, origin. (*A*) Reaction with U-A and [^{3}H]UTP. The mixtures of solutions I and II described under *Materials and Methods* (total volume 0.1 ml) contained 7 μg of RNA polymerase and 0.036 A_{260} unit of poly[d(A-T)]. (One *A* unit is the amount of material having an absorbance of 1.0 when dissolved in 1 ml and the light path is 1 cm.) ●—●, Complete reaction mixture containing U-A and [^{3}H]UTP; △- - -△, complete mixture minus poly[d(A-T)] (identical results were obtained if enzyme or U-A was omitted); O- - -O, reaction mixture containing A-U instead of U-A in solution II. (*B*) Reaction with A-U and [^{3}H]ATP. The mixtures of solutions I and II (total volume 0.1 ml) contained 14 μg of RNA polymerase and 0.013 A_{260} unit of poly[d(A-T)]. ●—●, Complete reaction mixture containing A-U and [^{3}H]ATP; ▲- - -▲, complete mixture minus RNA polymerase (identical results were obtained if poly[d(A-T)] was omitted); △- - -△, complete mixture minus A-U; O- - -O, reaction mixture containing U-A instead of A-U in solution II.

strips, and analyzed for radioactivity in Econofluor (New England Nuclear) in a Beckman liquid scintillation counter.

Synthesis of Radioactive A-U and U-A-U. U-A-[^{3}H]U was synthesized in a reaction mixture similar to that described in the legend to Fig. 1*A* except that the total volume of the mixture of solutions I and II was 0.6 ml. The U-A-[^{3}H]U was purified by preparative chromatography on Whatman 3 MM with solvent B. After elution from the paper with water, the trinucleoside bisphosphate was evaporated to dryness and dissolved in 0.2 ml of water.

A-[^{3}H]U was prepared by cleavage of U-A-[^{3}H]U with RNase as described in the legend to Fig. 2*A* except that the reaction mixture was increased 10-fold to a volume of 0.5 ml. An aliquot (5 μl) of this reaction mixture and an aliquot (5 μl) of a parallel reaction mixture not treated with RNase were analyzed on separate strips of DEAE-peper (Whatman DE-81) by descending chromatography. The chromatograms were developed overnight with solvent C, which readily resolved A-U and U-A-U (by 5–6 cm). The RNase-treated sample showed one main peak of radioactivity, which coincided with the position of an A-U marker. No radioactivity was detected at the positions

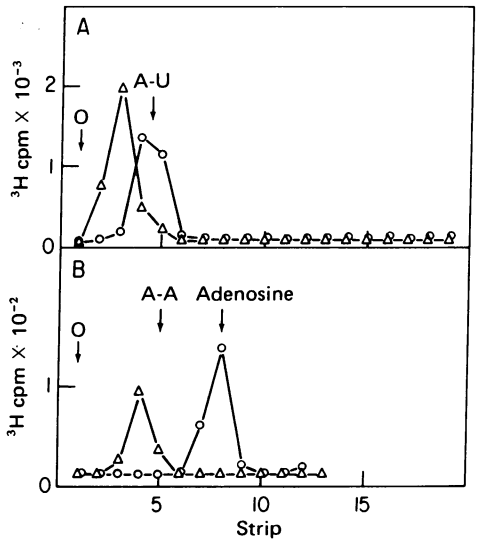

FIG. 2. (*A*) Proof of structure of U-A-U. Fifty microliters of the complete reaction mixture analyzed in Fig. 1*A* was incubated with 30 μg of RNase A for 10 min at 37°. A 10-μl aliquot of this sample and a 10-μl aliquot of the reaction mixture not treated with RNase were applied to separate DEAE-paper strips and developed with solvent C by ascending chromatography; the chromatograms were analyzed for radioactivity. △, Minus RNase; O, plus RNase. (*B*) Proof of structure of A-U-A. Forty microliters of the complete reaction mixture analyzed in Fig. 1*B* was applied to a 1-inch-wide (~2.5 cm) paper chromatogram (3 MM paper) and developed to a height of 20 cm. The chromatogram was dried, the region from 0.5 cm below the origin to 1 cm above the origin was cut out, and the radioactivity was eluted with water. The eluate (~1 ml) was divided into two equal portions, one of which was incubated with 0.3 mg of RNase A for 10 min at 37°. Aliquots (50 μl) of each sample were then applied to separate 3 MM paper chromatograms by repeated spotting. The chromatograms were developed with solvent B and analyzed for radioactivity. △, Minus RNase; O, plus RNase.

corresponding to U-A-U, indicating that the digestion had been complete. The A-[^{3}H]U was then purified by the method described above for U-A-[^{3}H]U.

RESULTS

Single-step addition reactions directed by synthetic templates

Addition of UTP to U-A. Paper chromatographic analysis of a reaction mixture in which U-A and [^{3}H]UTP were incubated with RNA polymerase and poly[d(A-T)] indicated that more than 90% of the [^{3}H]UTP was converted into material that moved as a single peak with an R_F of about 0.1 (Fig. 1*A*). The appearance of this peak was absolutely dependent on the presence of enzyme, DNA template, and U-A. When U-A was replaced by A-U (which cannot combine with UTP to give a product complementary to the DNA template), essentially all of the ^{3}H label was recovered as UTP. Thus, it seems reasonable that the radioactive peak near the origin of the chromatogram in Fig. 1*A* is due to poly[d(A-T)]-directed synthesis of U-A-U from U-A and UTP catalyzed by RNA polymerase. Because the R_F of this peak was identical to that of U-A (or A-U) in this paper chromatographic system (solvent A, data not shown), it was necessary to prove that the product was, in fact, a trinucleoside bisphosphate. To do this, aliquots of the complete reaction mixture analyzed in Fig. 1*A* were incubated with and without RNase A. These samples were then chromatographed on DEAE-paper along with unlabeled A-U as a marker (Fig. 2*A*). The mobility of the RNase-digested material was the same

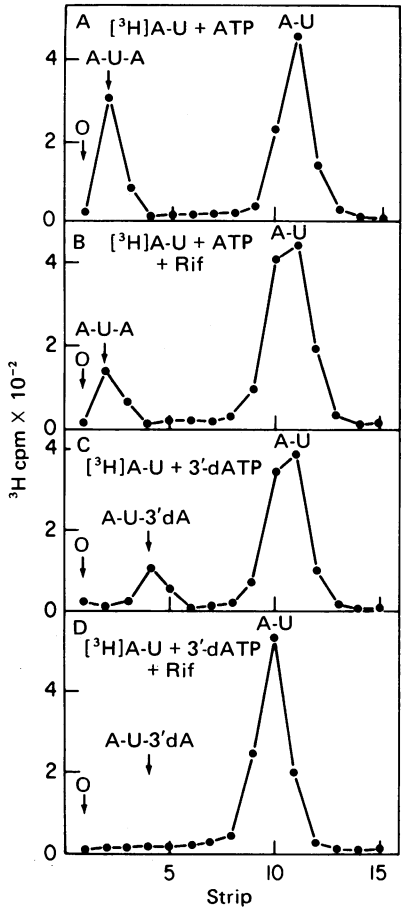

FIG. 3. Effect of rifampicin (Rif) on A-U-A or A-U-3'dA synthesis. Abortive elongation reactions were performed and analyzed as described under *Materials and Methods* except that the solution IIs contained 5 mM $MgCl_2$, 40 μM A-[^{3}H]U, and 0.4 mM ATP (*A* and *B*) or 0.4 mM 3'-dATP (*C* and *D*). Mixtures of solutions I and II (total volume 0.1 ml) were incubated 30 min at 37° and contained 14 μg of RNA polymerase and 0.03 A_{260} unit of poly[d(A-T)]. Where indicated, the reaction was performed in the presence of 12 μM rifampicin. Aliquots of the reaction mixtures were chromatographed on DEAE-paper with solvent C.

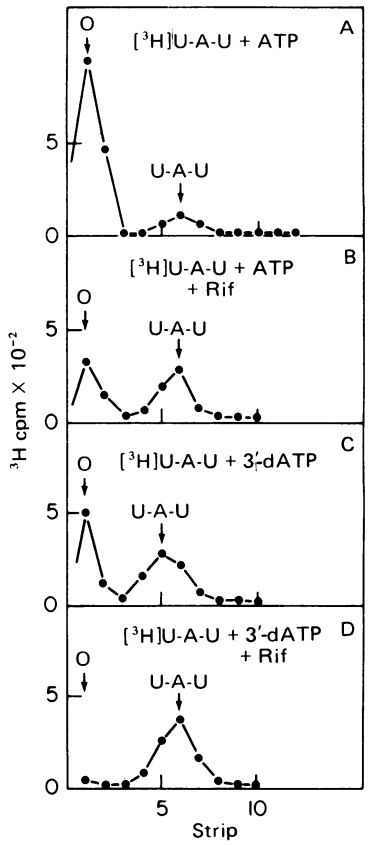

FIG. 4. Effect of rifampicin on synthesis of U-A-U-A and U-A-U-3'dA. Abortive elongation reactions were performed as described under *Materials and Methods* except that solution IIs contained 5 mM $MgCl_2$, 40 μM U-A-[^{3}H]U, and 0.4 mM ATP (*A* and *B*) or 0.4 mM 3'-dATP (*C* and *D*). The mixtures of solutions I and II (total volume 0.1 ml) were incubated 30 min at 37° and contained 14 μg of RNA polymerase and 0.04 A_{260} unit of poly[d(A-T)]. Where indicated, the incubations were done in the presence of 12 μM rifampicin. Aliquots of reaction mixtures were applied to 3 MM paper strips and developed overnight by descending chromatography.

as that of A-U, while the nondigested material moved slower than A-U. Both results would be expected if the product were U-A-[^{3}H]U. If the product were longer than U-A-U due to the addition of more than one UMP residue, RNase cleavage would have yielded A-Up, which sould migrate differently than A-U on DEAE-paper. These observations suggest that the product formed was U-A-U.

Addition of ATP to A-U. Fig. 1*B* shows the chromatographic analysis in solvent A of a reaction mixture in which A-U and [^{3}H]ATP were incubated with poly[d(A-T)] and RNA polymerase. In such reaction mixtures, about 25% of [^{3}H]ATP was converted into a form that remained near the origin of the chromatogram. The appearance of this peak depended on the simultaneous presence of enzyme, DNA, and A-U. When A-U was replaced by U-A, negligible radioactivity was detected at the origin. The radioactivity at the origin was assumed to be the A-U-[^{3}H]A, which moved slower than U-A-U on paper chromatography in solvent A.

To prove the structure of the putative A-U-A, the radioactive material near the origin of a chromatogram like the one shown in Fig. 1*B* was eluted with water. Aliquots of the eluted material were incubated in the presence and absence of RNase and then subjected to paper chromatography in solvent C (Fig. 2*B*). The radioactively labeled, RNase-treated material comigrated with adenosine as expected for the product A-U-[^{3}H]A. This result rules out the possibility that products longer than a trinucleoside bisphosphate were formed. Evidently, the product A-U-A was formed much more slowly than the product U-A-U. This can be seen best in Fig. 5 *A* and *C*, for which the two reactions were carried out in parallel under identical conditions.

Addition of CTP to C-G and GTP to G-C. Reactions analogous to those described above were also carried out with poly[d(I-C)] as template. Incubation of G-C + [^{3}H]GTP or C-G + [^{3}H]CTP with RNA polymerase and poly[d(I-C)] resulted in the formation of G-C-[^{3}H]G or C-G-[^{3}H]C, respectively (data not shown). The reaction that yielded C-G-C was nearly as efficient as the one that yielded U-A-U, while reactions yielding G-C-G were much less efficient, as was the case for reactions yielding A-U-A.

Addition of ATP to U-A-U. A single-step addition reaction with a tetranucleoside trisphosphate as the product was also observed. When the substrates U-A-[^{3}H]U and ATP were incubated with poly[d(A-T)] and RNA polymerase, a labeled product was formed which migrated more slowly than U-A-U in solvent A (Fig. 4*A*). The formation of this material was also dependent on the presence of DNA, enzyme, and ATP (data not shown). By analogy with the addition reaction of ATP to A-U, the product of this reaction was most likely U-A-U-A.

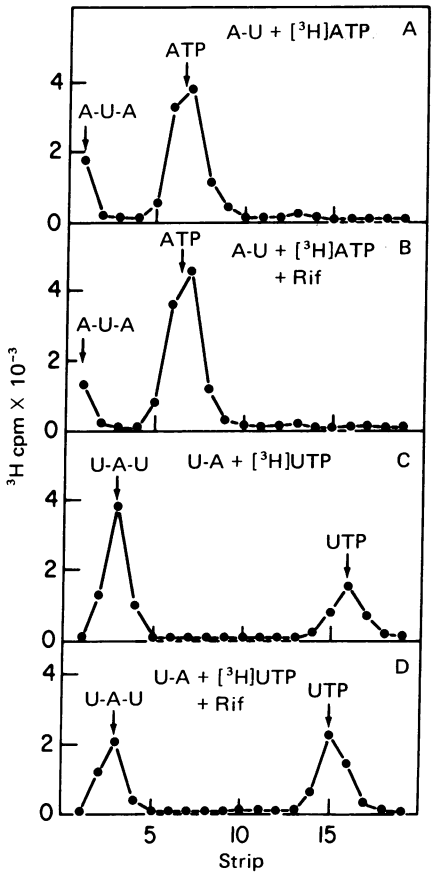

FIG. 5. Effect of rifampicin on A-U-A or U-A-U synthesis. Standard abortive elongation reactions with A-U and [^{3}H]ATP (*A* and *B*) or U-A and [^{3}H]UTP (*C* and *D*) were carried out and analyzed. In all cases the mixtures of solutions I and II (total volume 0.1 ml) were incubated 10 min at 37° and contained 14 μg of RNA polymerase and 0.03 A_{260} unit of poly[d(A-T)]. Where indicated, reactions were performed in the presence of 12 μM rifampicin.

Addition Reactions with 3′-dATP. It has been shown that 3′-dATP (cordycepin 5′-triphosphate) can be incorporated into the 3′ end of RNA chains by RNA polymerase (5). (The incorporation of this nucleotide prevents the further elongation of RNA chains due to the absence of a 3′-hydroxyl group.) Thus, single-step addition reactions of the type A-U + 3′-dATP or U-A-U + 3′-dATP should be readily observed when poly[d(A-T)] is used as the template. These reactions were observed with A-[^{3}H]U and U-A-[^{3}H]U (Figs. 3*C* and 4*C*). For comparison, parallel experiments were also performed for the reactions A-[^{3}H]U + ATP and U-A-[^{3}H]U + ATP (Figs. 3*A* and 4*A*). As can be seen, the addition reactions with 3′-dATP were less efficient than those with ATP.

Effect of rifampicin on single-step addition reactions

The effect of rifampicin on the reactions A-U + [^{3}H]ATP → A-U-[^{3}H]A and U-A + [^{3}H]UTP → U-A-[^{3}H]U is shown in Fig. 5. A shorter incubation period was used in these experiments than in those described in Fig. 1. Under these conditions, the reaction yielding U-A-U did not go to completion. The formation of U-A-U was inhibited approximately 50%, while the formation of A-U-A was inhibited to a lesser extent by the presence of 12 μM rifampicin. This partial inhibition by rifampicin is contrary to the report of So and Downey (2). They reported that 0.5 μM rifampicin completely inhibited the catalysis of either of these reactions by RNA polymerase. The reactions A-[^{3}H]U + ATP → A-[^{3}H]U-A and U-A-[^{3}H]U + ATP → U-A-[^{3}H]U-A were also partially inhibited by 12 μM rifampicin (Figs. 3*B* and 4*B*).

The effect of rifampicin on the single-step addition reactions with 3′-dATP was examined (Figs. 3*D* and 4*D*). In contrast to the reactions in which ATP is the substrate, the reactions with 3′-dATP were almost completely inhibited by 12 μM rifampicin (>95%).

DISCUSSION

We have shown that RNA polymerase catalyzes a template-directed single-step addition reaction in which a phosphodiester bond is formed between the 5′-phosphate group of a nucleoside triphosphate and the 3′-hydroxyl group of a dinucleoside monophosphate or a trinucleoside bisphosphate. The rate of turnover of the enzyme depends on the base composition of the product. In the case of poly[d(A-T)]-directed reactions, we have demonstrated a rapid turnover for the formation of U-A-U from U-A and UTP, while the formation of A-U-A from A-U and ATP is much slower. Similar observations were made for the poly[d(I-C)]-directed reactions C-G + CTP → C-G-C and G-C + GTP → G-C-G. The rate of formation of the former product was much faster than that of the latter. Thus, the reaction of a dinucleoside monophosphate with a nucleoside triphosphate does not always result in the formation of a "stable ternary complex," because products can dissociate from the enzyme–DNA complex. By analogy with the terminology "abortive initiation" (1), these reactions may be called "abortive elongation" reactions. It should be mentioned that the abortive elongation reaction consists of substrate binding, phosphodiester bond formation, and dissociation of product with or without translocation. We do not know, however, which of these processes is rate limiting.

The effect of rifampicin on the rate of product formation in these "abortive elongation" reactions was examined. Contrary to the conclusion of So and Downey (2), we have found that the poly[d(A-T)]-directed reactions U-A + UTP → U-A-U and A-U + ATP → A-U-A are not inhibited (or are only partially inhibited) by rifampicin. Their conclusion was based on the observations that incubation with A-U + ATP or U-A + UTP stabilized the poly[d(A-T)]-enzyme complex against dissociation by high salt and that rifampicin inhibited this stabilization. One possible explanation for their observations could be that the nucleoside triphosphates used were not highly purified. Because dinucleotides can stimulate RNA synthesis at low concentrations of nucleoside triphosphates (6), it is possible that their observations were due to synthesis of RNA chains longer than a trinucleotide, a process that would be sensitive to rifampicin.

We have also observed that the reaction U-A-U + ATP → U-A-U-A is only partially inhibited by rifampicin. The fact that products as long as a tetranucleotide can be formed in the presence of rifampicin makes it seem unlikely that the mechanism of action of rifampicin is simple steric blockage of the RNA product site beyond the dinucleotide binding site as has been proposed (1). The effect of rifampicin we observed here on the abortive elongation reaction is basically the same as its effect on the abortive initiation (1) or on the addition of ribonucleotides to the 3′ end of DNA catalyzed by RNA polymerase (7). The latter reaction is also insensitive to this antibiotic.

Our finding that A-U-A formation is slower than U-A-U synthesis may explain an observation by So and Downey (2). In their experiments, preincubation of RNA polymerase–poly[d(A-T)] binary complex with A-U and ATP resulted in a complex resistant to the inhibitory action of rifampicin upon subsequent addition of UTP. On the other hand, preincubation with U-A and UTP did not protect the complex from rifampicin

inhibition upon addition of ATP. Evidently the trinucleoside bisphosphate U-A-U dissociates too rapidly to afford protection, while A-U-A (or unreacted A-U + ATP) remains bound long enough to RNA polymerase to allow RNA synthesis to begin before rifampicin inhibition takes place.

The rate of the abortive elongation reaction performed with 3′-dATP and A-U was somewhat slower than the corresponding reaction carried out with ATP (Fig. 3 *A* and *C*). This is most likely not due to weak binding of 3′-dATP to the active site. With RNA polymerase from *Pseudomonas putida*, a K_i value for 3′-dATP of 2 μM was reported (8) as compared to a K_m value for ATP of 50 μM. Another 3′-substituted nucleotide analog, 3′-amino-ATP, also has a very low K_i value ($\frac{1}{20}$ that of the K_m for ATP) for *E. coli* RNA polymerase (9). Because 3′-dATP binds more tightly to the enzyme than ATP, products containing 3′-dAMP residues may be expected to bind more tightly than the corresponding products containing AMP residues. Therefore, the lower rate of formation of the products containing 3′-dAMP residues may be due to a slower release of products.

It is interesting that the abortive elongation reactions involving 3′-dATP were inhibited >95% by a concentration of rifampicin that partially inhibited (<50%) the parallel reactions using ATP. Thus it appears that the 3′-hydroxyl group of the incoming nucleoside triphosphate is essential for this reaction to occur in the presence of rifampicin (though it is not necessary for the reaction to occur in the absence of the antibiotic). It is not clear, however, whether the 3′-hydroxyl group is required for binding of substrates, formation of the phosphodiester bond, or release of products.

The analysis of single-step addition reactions described here can be used to study nucleotide analogs that cannot support synthesis of long RNA chains by RNA polymerase but can engage in the formation of one or a few phosphodiester bonds. In addition to 3′-dATP used here, such analogs include 2′-*O*-methyladenosine 5′-triphosphate (10), 8-amino-ATP (11), and perhaps other 2′- and 3′-modified nucleotides as well (9). Another method that has been employed to detect a single elongation step catalyzed by RNA polymerase is a DNA-dependent pyrophosphate exchange reaction (11) that measures the reverse reaction of the single nucleotide addition (e.g., A-U + ATP $\rightleftarrows$ A-U-A + PP_i). The technique described here for detection of products formed after addition of one or more phosphodiester bonds in conjunction with the use of the PP_i exchange reaction, various nucleotide analogs, and specific inhibitors such as rifampicin may provide means to further study the various steps involved in RNA synthesis.

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