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# Requirement for the $\beta,\gamma$-Pyrophosphate Bond of ATP in a Stage between Transcription Initiation and Elongation by *Escherichia coli* RNA Polymerase†

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ABSTRACT: A linear fragment of DNA was fixed to acrylamide or agarose beads by its ends. When a fragment containing the λ $P_R$ promoter is immobilized and transcribed, the RNA products are unchanged from those obtained on the unfixed DNA. Transcription from the immobilized fragment can be interrupted by diluting the reaction mixture into a large volume of the same buffer. Brief centrifugation allows isolation of the transcription complex with the immobilized DNA. If interruption occurs during elongation, the elongation can be resumed upon a second addition of substrates. If ATP is replaced by a $\beta,\gamma$-unhydrolyzable analogue in the second addition, the elongated products are similar to those obtained when the substrate contain ATP. When ATP is replaced by the analogue at the initiation step, however, the yield of elongated products is decreased to less than one-sixth and that of short abortive products is increased. Thus the ATP analogues are good substrates once elongation has been established in the presence of ATP, but not good enough to get past a stage just after initiation in the absence of ATP. We conclude that the $\beta,\gamma$-pyrophosphate bond of ATP is important for preparation of efficient elongation.

Transcription of genes is mediated by a large protein·DNA complex, and the DNA-dependent RNA polymerase is the major functional component. To investigate the relationship between the structure of the transcription complex and the RNA product, one has to freeze the structure and stop elongation simultaneously. Conventional methods using chelating reagents, substrate analogues, or truncated templates are not always satisfactory. The addition of EDTA stops not only RNA synthesis but also every reaction requiring magnesium ion. When 3′-deoxy substrates or truncated DNA templates are used, the rapid and simultaneous cessation of RNA chain elongation is impossible, and it thus complicates the kinetic interpretation of the results.

To overcome this difficulty, we have devised a novel kinetic method, in which transcription is directed from a linear template DNA fixed to resin beads by its ends, and the transcription complex can be isolated by rapid dilution and centrifugation. Elongation of the RNA chain can thus be interrupted without changing ionic conditions and can be resumed with different substrates. A DNA fragment is easily labeled with biotin (Langer et al., 1981), and thus labeled fragment can be strongly bound to avidin linked to a gel matrix. Such an immobilized DNA fragment was used to isolate preinitiation complex in HeLa nuclear extract where the requirements for ATP in preinitiation and initiation were studied (Arias & Dynan, 1989).

A nascent RNA chain is elongated by the addition of a nucleoside monophosphate to its 3′-end, and $\alpha,\beta$-pyrophosphate bonds in substrates are absolutely required. On the other hand,

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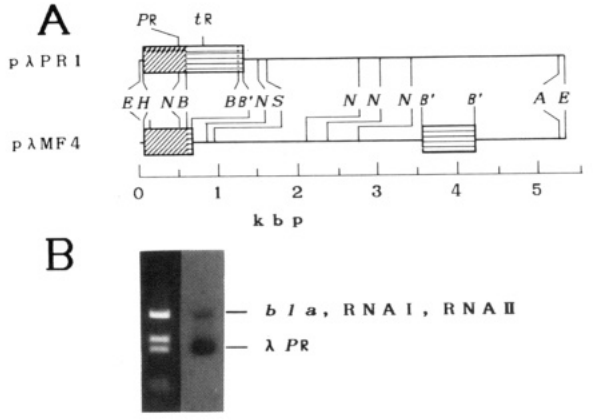


FIGURE 1: Panel A: Plasmid DNAs constructed and used in this study. The λ DNA fragments containing $P_R$ promoter (518 bp, hatched box) and a region downstream (713 bp, horizontally striped box) were inserted into pBR322 to make 5245 bp plasmids. The location of $t_{R1}$ is also shown ($t_R$). The cleavage sites are indicated for *Eco*RI (*E*), *Hin*dIII (*H*), *Bgl*II (*B*), *Aat*II (*A*), *Sal*I (*S*), and *Nsp*(7524)I (*N*). The symbol *B'* denotes the destroyed *Bgl*II sites. Panel B: Southern blotting of *Nsp*(7524)I fragments of pλPR1 probed with total 20-s transcripts which were prepared as described in panel B of Figure 3. The left lane shows the separated fragments visualized with ethidium bromide. Their sizes are 2352, 1254, 980, 365, and 294 bp. The right lane shows the autoradiogram of the blotting. The vector sequence encodes β-lactamase (*bla*) and replication-related RNAs, RNA I and RNA II. Fragments expected to hybridize with these transcripts and RNAs are also shown at the right.

the requirement for β,γ-pyrophosphate bonds has been questioned for *Escherichia coli* RNA polymerase (Nierman & Chamberlin, 1980). We have applied the rapid kinetic procedure to determine the requirement for the β,γ-pyrophosphate bond of ATP in transcription initiation and elongation.

## Materials and Methods

*Enzymes and Substrates.* *E. coli* RNA polymerase was prepared as described by Shimamoto et al. (1986). Restriction endonucleases were purchased from Toyobo (Tokyo). Nucleoside triphosphates were obtained from Yamasa Shoyu (Tokyo). ADP and ATP analogues were obtained from Sigma, except dATP (Boehringer Mannheim). β,γ-Imido analogues of UTP, CTP, and GTP (PL Biochemicals) were gifts of Dr. Katsuya Shigesada.

*Preparation of Nucleic Acids.* Plasmid DNAs were extracted by the alkaline–SDS method (Davis et al., 1986) followed by digestion with RNase A, two phenol extractions, and gel permeation chromatography using an HPLC column (TSK4000SW, Toyo Soda). *E. coli* tRNA was obtained from Boehringer Mannheim. The concentrations of RNA and plasmid DNA were determined spectrophotometrically by using extinction coefficients of 7500 and 6000 $M^{-1}$ $cm^{-1}$, respectively.

*Plasmid Constructions and Cell Transformations.* A *Hin*dIII–*Bgl*II fragment of bacteriophage λ DNA, from 37 584 to 38 814 in λ coordinates, was inserted between *Hin*dIII and *Bam*HI of pBR322 to make pλPR1. The *Bgl*II fragment of pλ PR1 was then moved to another location within the same plasmid, namely, the *Sau*3AI site at pBR322 coordinate 3226, to make pλMF4 (Figure 1). The lysogen DH1 (λ $ind^-$) was used for primary cloning and maintenance of plasmids carrying the $P_R$ promoter.

*Preparation of Avidin-Immobilized Resins.* One milligram of avidin (Vector Laboratories DN, Sigma, or Taiyokagaku) was dissolved in 1 mL of 1 M potassium phosphate buffer (pH 7.5) containing 0.1% sodium azide. The solution was added to 0.2 g of oxirane–acrylic beads, Eupergit C (Röhm Pharma, Weiterstadt), and left at room temperature overnight. The resin was washed three times with 0.1 M potassium phosphate buffer (pH 7.5), and residual oxirane was blocked by overnight incubation with the washing buffer containing 5% 2-mercaptoethanol. Avidin–agarose was purchased from Sigma. Both kinds of resin were ground with a 2-mL Teflon homogenizer to make the beads finer. The ground beads were suspended in 10 mM Tris-HCl (pH 7.9) containing 0.5 M NaCl and 1 mM EDTA (0.5TE buffer).

*Labeling with Biotin and Immobilization of DNA.* Plasmid DNA (25 μg in 25 μL) was linearized with *Eco*RI or *Sal*I, which generates 5′-protruding ends, followed by inactivation of the enzyme at 70 °C. Tris base and salts were added to make the solution 67 mM Tris-HCl (pH 8.8), 7 mM $MgCl_2$, 17 mM ammonium sulfate, and 10 mM 2-mercaptoethanol. The protruding ends were filled by use of 0.4 unit of T4 DNA polymerase at 37 °C for 0.5 h in the presence of 20 μM biotin-11-dUTP (Bethesda Research Laboratories) and 0.1 mM each of the other three deoxyribonucleoside triphosphates (Langer et al., 1981). An alternative method to internally label *Eco*RI ends was accomplished by adding T4 DNA polymerase as an exonuclease in the presence of 0.1 mM dCTP for 15 s, and then providing biotin–dUTP and the other two nucleotides. The reaction was stopped by adding EDTA and *E. coli* tRNA to final concentrations of 40 mM and 0.2 mg/mL, respectively. The mixture was extracted with phenol, and unincorporated biotin–dUTP was removed by three ethanol precipitations in the presence of 2 M ammonium acetate. The labeled DNA was incubated with avidin beads in 0.5TE buffer and gently shaken for 1 h.

*Analysis of Transcription Complex and Product RNA.* The *Nsp*(7524)I fragments of pλPR1 were separated on 0.7% agarose gel, denatured, and transferred to a Nitroplus 2000 filter (Micron Separation) using Milli Blot (Millipore). The filter was hybridized to the radiolabeled transcripts described below for 12 h in 5 × SSPE containing 50% formamide [1 × SSPE was 0.18 M NaCl, 10 mM sodium phosphate (pH 7.7), and 1 mM EDTA] at 42 °C and then washed with 0.1 × SSPE containing 0.1% SDS at 55 °C.

Rapid kinetics of transcription was performed as described by Shimamoto et al. (1986) with the following modifications. Purified holo RNA polymerase (12–50 ng) was preincubated for 4 min at 37 °C with a stoichiometric amount (in terms of promoter) of immobilized template DNA in 5 μL of 50 mM Tris-HCl (pH 7.9), 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 mM dithiothreitol (T buffer). Substrates (see below) in 5 μL T buffer were added and mixed with a Pipetman tip preequilibrated at 37 °C. If the reaction was stopped by EDTA, 10 μL, of 50 mM EDTA was added. The total transcripts were obtained by a standard ethanol precipitation with tRNA carrier. To get transcripts liberated from the transcription complex and those present in the complex, the EDTA-containing mixture was diluted with 0.18 mL of 25 μg/mL tRNA and centrifuged for 2 min at 12000*g* with a Tomy MC15 (Tokyo). The liberated and unliberated transcripts were obtained by ethanol precipitation of the supernatant and the precipitate suspended in 10 μL water, respectively.

To stop the reaction by rapid dilution, the reaction mixture was transferred at given times with the same tip into 10 mL of T buffer. The transcription complex was concentrated and recovered by centrifugation for 10 min at room temperature at 2000*g* with a Tomy LC06. The pellet was suspended in 0.5 mL of T buffer, washed with 0.3 mL of T buffer, and

transferred into an Eppendorf tube. The transcription complex was finally concentrated to 5–10 μL by centrifugation for 2 min at 12000*g*. If necessary, the second step reaction was started by adding a 5-μL solution of unlabeled nucleotides.

The labeled transcripts were suspended in 5–25 μL of deionized formamide. The resultant suspension was incubated at 60 °C for 5 min before electrophoresis. Length markers were synthesized with T7 RNA polymerase (Promega) and Bluescript pSKM13 plasmids (Promega) linearized with several restriction enzymes.

## Results

*Immobilization of DNA.* The DNAs with their maps shown in Figure 1 were labeled with biotin–dUTP and incubated with avidin acrylamide beads (see Materials and Methods). After centrifugation of the mixture, the fraction of unbound DNA was measured by electrophoresis of DNA in the supernatant to determine the efficiency of immobilization. Immobilization was independent of the commercial sources of avidin or the kinds of resin, agarose or acrylamide, but dependent on the sites of DNA linearization and the method of biotin labeling. By the straightforward fill-in labeling method, for example, 50% of pλPR1 DNA linearized with *Sal*I was bound to avidin–agarose, but less than 10% of *Eco*RI ends were bound. A longer incubation did not yield further immobilization. The simple fill-in labeling of *Eco*RI ends appears to be inefficient because T4 DNA polymerase acts as both polymerase and exonuclease to add and remove biotin–dUMP at the 3′-end of *Eco*RI sites at the low concentration of biotin–dUTP present. But the site for biotin–dUMP at a *Sal*I end is not the 3′-end, and the enzyme acts as only polymerase at high concentrations of downstream substrates, dCTP, dGTP, and dATP. When an internal T was replaced by biotin–dU (see Materials and Methods), more than 95% of DNA linearized with *Eco*RI was bound (panel A of Figure 2). Thus it appears that DNA molecules that incorporated biotin–dUMP were quantitatively immobilized. This is consistent with a previous observation that only a few biotin moieties are sufficient to bind modified DNA to avidin (Langer et al., 1981). The difference in efficiency of immobilization, therefore, may be due to a difference in the efficiency of biotin incorporation.

The plasmid pλMF4 (Figure 1) was linearized with *Eco*RI and internally labeled at the ends, bound to avidin–acrylamide, and then digested by various restriction enzymes that cut near either end (panel B of Figure 2). Most of the DNA was released as a large fragment by cutting at a unique *Bgl*II restriction site 537 bp from the left end; but little was released as a large fragment by cutting at an *Aat*II site 78 bp from the right end. Double digestion with *Bgl*II and *Aat*II released the same amount of DNA as the single digestion with *Bgl*II. Almost all of the DNA was released by the *Bgl*II digestions, because a similar amount of DNA was released by phenol extraction without digestion, and because very little residual DNA was released by phenol extraction after *Bgl*II digestion (panel B of Figure 2). Note that if most fragments were fixed at both their ends, neither enzyme alone could release much DNA. Thus we concluded that the left end of pλMF4 was preferentially fixed to the beads. Such asymmetry was not found in fixing the pλPR1 in the same method although these plasmids have the common sequence of more than 500 bp at *Eco*RI ends (panel A of Figure 1). The reason for this asymmetry is unclear at present.

Notably, almost all pλMF4 DNA was released by *Ban*III, *Hin*dIII, or *Bgl*II digestion. *Ban*III and *Hin*dIII have unique targets at positions 24 and 29 from the left end or 18 and 23 bases from the biotinylated base, respectively (panels A and C of Figure 2). These results indicate that although the fragments were fixed to the beads at their left ends, sites very near the ends were accessible to restriction enzymes. The immobilization, therefore, is likely to cause minimal steric hindrance of protein binding. This property of the immobilized DNA is important for its use as a transcription template.

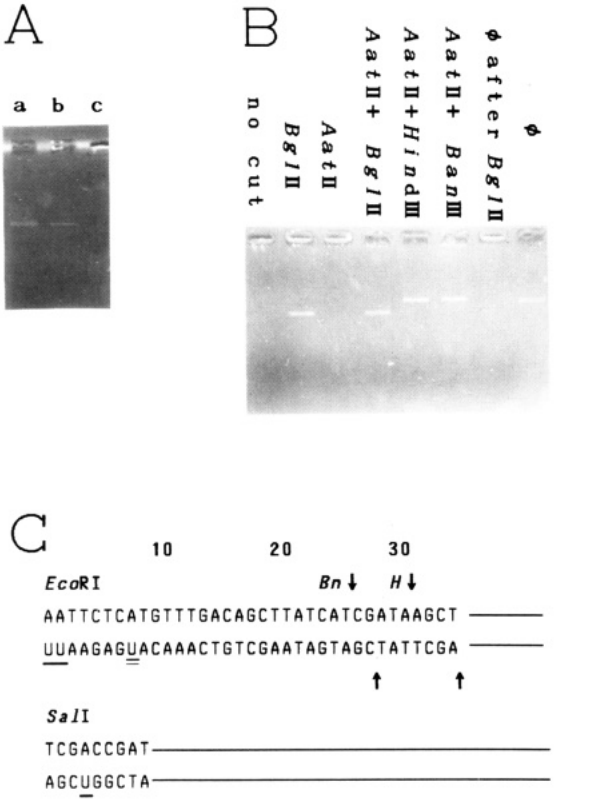


FIGURE 2: Immobilization of biotin-labeled DNAs. Panel A: Biotin–dUTP was incorporated near the *Eco*RI ends of pλMF4 by internal labeling. The DNA (0.2 mg) was dissolved in 0.5 mL of 0.5TE buffer, and an aliquot (solution) was analyzed by electrophoresis in 0.7% agarose (lane a). The rest was incubated with avidin–acrylamide suspended in 0.5TE and centrifuged. Certain volumes of the supernatant were also analyzed to give the same amount of DNA in lane a if 5% and 100% of DNA remained unbound (lanes b and c, respectively). The gel was stained with ethidium bromide. Panel B: Immobilized pλMF4 was digested with restriction enzymes indicated or extracted with phenol (φ). Phenol after *Bgl*II denotes DNA extracted from beads which had been incubated with the enzyme and washed. Panel C: Primary structures of labeled pλMF4 linearized with *Eco*RI (coordinate 1) or *Sal*I (coordinate 885). Sites for biotin incorporation are denoted as U. Sites labeled by simple fill-in labeling are underlined with single lines and a site for internal labeling by using the exonuclease activity of T4 DNA polymerase with a double line. Cleavage sites for *Ban*III (*Bn*) and *Hin*dIII (*H*) are indicated by arrows.

*Rapid Kinetics of Transcription on Immobilized Template.* To check that the λ $P_R$ promoter directs most of the transcription on pλPR1, we blotted its *Nsp*(7524)I fragments and hybridized them to its own total transcripts used in this study. The 980 bp fragment which contains the λ $P_R$ promoter hybridized most of the transcripts (panel B of Figure 1). The 2352 bp fragment which contained the other major promoters (Morita & Oka, 1979; Itoh & Tomizawa, 1980; Stuber & Bujard, 1981; Harley et al., 1988) hybridized only one-tenth to one-seventh of the total, and no radioactivity was detected at any other fragment. Thus, in this system most transcription started from the λ $P_R$ promoter.

The products of transcription on immobilized DNA templates were compared with those obtained by conventional methods (panel A of Figure 3). At first, transcription in vitro

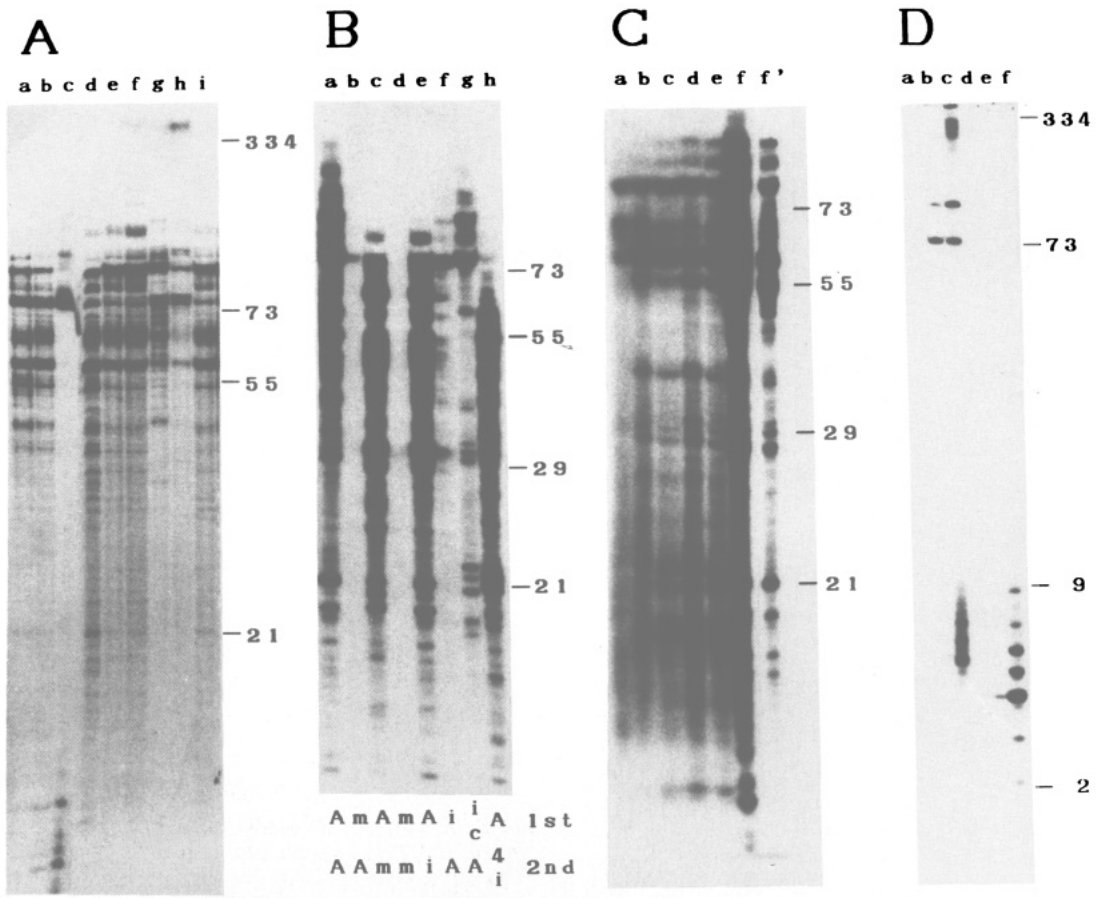


FIGURE 3: Rapid dilution and substrate substitution using immobilized template. Numbers denote the length of marker RNA. Panel A: Rapid dilution as a new method for quenching RNA synthesis on an immobilized DNA template. Transcription was synchronously started by adding 5 $\mu$L of T buffer containing 5 $\mu$Ci of [$\alpha$-$^{32}$P]UTP, 20 $\mu$M UTP, and 0.2 mM each of three other substrates to 5 $\mu$L of a preincubated mixture containing 13 ng of holoenzyme and 25 fmol (in terms of promoter) of immobilized p$\lambda$PR1. The template DNA was free (lane a) or immobilized with acrylamide beads (lanes b–h). Reactions were stopped after 30 s by adding 50 mM EDTA (a–d). The total transcripts (a and b), the liberated transcripts (c), and the transcripts coprecipitated with immobilized DNA (d) are shown. Twice as many radioactive counts were loaded on lanes c and d. The transcription was quenched by rapid dilution in T buffer at 30 s (e and i, duplicate experiments) and at 35 s (f). Transcription complexes were collected by centrifugation, incubated in a second reaction with 0.1 mM each of $\beta,\gamma$-imido analogues of four triphosphates (g), or the four natural triphosphates (h), for 30 s, and then 50 mM EDTA was added. All samples were analyzed in a 17% polyacrylamide containing 7 M urea in Tris–borate buffer. Panel B: Substrate substitution experiment. The key components in the first and the second reactions were listed below the autoradiogram with the following abbreviations: A, ATP; m, AMPPCP; i, AMPPNP; c, CpA; and 4i, the four $\beta,\gamma$-imido analogues of natural triphosphates. The nucleotides were added at 0.1 mM each, unless noted otherwise. Transcription was started in the first step reaction with 5 $\mu$Ci of [$\alpha$-$^{32}$P]UTP (5 $\mu$M), CTP, GTP, and either ATP (lanes a and c) or AMPPCP (lanes b and d). Reactions were stopped by rapid dilution at 20 s. The collected transcription complex was incubated in the second step reaction for 5 s with UTP, CTP, and GTP plus ATP (lane a and b) or plus AMPPCP (lane c and d). Instead of AMPPCP, AMPPNP was used in the first step reaction (lane f) or in the second step reaction (lane e). In the similar experiment shown in lane f, CpA (200 $\mu$M) was further added with AMPPNP in the first step reaction (lane g). Elongation nucleotides, in an experiment similar to lane a, were replaced by AMPPNP, UMPPNP, CMPPNP, and GMPPNP (lane h). Panel C: Substrate substitution in the presence of CpA. The substitution experiment was carried out as in lanes a and b of panel B. The substrates in the first step reaction included 0.1 mM AMPPCP (lanes a–e) or 0.1 mM ATP (lane f). The initiator dinucleotide CpA was added to the initiating nucleotides at 25 $\mu$M (lane b), 50 $\mu$M (lane c), 100 $\mu$M (lane d), and 200 $\mu$M (lane e). The autoradiogram shown in lane f is shown again at one-fifth exposure in lane f'. Panel D: Accumulation of abortive products. Transcription was started by adding the same substrates in the first step reaction as in lanes a and b of panel B, including either 0.1 mM ATP (lanes a–c) or 0.1 mM AMPPCP (lanes d–f). The reaction mixture was incubated for 20 s (lanes a and d), 2 min (lanes b and e), or 20 min (lanes c and f). The reactions were stopped by adding 50 mM EDTA, and transcripts were precipitated by ethanol.

was carried out with free and immobilized p$\lambda$PR1 DNA as templates with 10 $\mu$M [$\alpha$-$^{32}$P]UTP and 0.1 mM other substrates. In both cases, RNA synthesis was stopped after 30 s with EDTA, and the total products precipitated with ethanol were analyzed. No significant differences were observed (compare lanes a and b). This similarity of transcripts was also observed when transcription was carried out for 20 min (data not shown). Next, transcription was started on the immobilized template in the same manner but stopped either by adding EDTA (lane d) or by rapid dilution with 1000 volumes of T buffer (lanes e and i). The transcription complexes were then collected by centrifugation, and the products in the complex were analyzed. Again the distributions of RNA species were very similar (compare lanes d and e, or d and i), but somewhat different from that of transcripts stopped at 35 s in longer RNA (lane f). In these dilution experiments, unlabeled UTP was added to the dilution buffer to give the same final concentrations for all four substrates. Thus elongation was interrupted at 0.1 $\mu$M of each substrate.

The total tarnscripts (lanes a and b) were slightly different from the transcripts kept in the complex (lanes d, e, and i). This is because some RNA species were liberated from the complex and stayed in the supernatant after the centrifugation. The liberated products (lane c) corresponded well to the difference between the whole products and the products in the complex.

The transcription complex collected by centrifugation was incubated again with four unlabeled substrates for 30 s, and

then EDTA was added. The whole product was precipitated with ethanol and analyzed by electrophoresis (lane h). The transcripts shorter than 73 nucleotide were less abundant, and ones longer than 334 appeared. Therefore, most of the transcription complexes resumed elongation. Otherwise, short transcripts should be left behind. Thus the collected transcription complex remained competent for elongation. When four imido analogue substrates were used in the second elongation, elongation still occurred with a slower rate and with a different pausing pattern (lane g). Therefore, substrate substitution during transcription is possible.

*Substrate Substitution during Transcription Elongation.* Transcription was started by adding a substrate set containing [$\alpha$-$^{32}$P]UTP (the first step reaction). The reaction was then stopped by rapid dilution at 20 s. Transcripts in the collected transcription complexes were further elongated by adding the second set of unlabeled substrates for 5 s (the second step reaction). The requirement for the $\beta,\gamma$-pyrophosphate bond of ATP was examined by several combinations of the first and the second substrate sets containing ATP or a $\beta,\gamma$-unhydrolyzable analogue, AMPPCP[1] or AMPPNP (panel B of Figure 3). When ATP was present in the first step reaction, significant amounts of long transcripts were observed irrespective of the substrates used in the second step reaction (lanes a, c, e, and h). In the second step reaction the $\beta,\gamma$-unhydrolyzable analogues were almost equivalent to ATP except that the rate of elongation was slightly smaller (compare lane a with lanes c and e). Moreover, elongation was not retarded much even when all four substrates were $\beta,\gamma$-imido analogues (lane h). In the first step reaction, however, AMPPCP and AMPPNP were poor replacements for ATP (lanes b, d, and f). Overall, UMP incorporation decreased to about one-tenth with AMPPCP or AMPPNP. In addition, the transcripts were found to be shorter (compare lane a with lane f).

During the first step reaction, both initiation and elongation, that is, the binding of initiator and the processive additions of mononucleotides, take place. The transcript from $\lambda$ $P_R$ has a sequence of AUGUACUAAG-- and starts with A. Thus either ATP or an ATP analogue has to be bound first for initiation to take place. The binding of ATP at the initiation site is much weaker than the binding at substrate sites used in elongation (Shimamoto et al., 1981). Thus the analogues might initiate more slowly than ATP due to a reduced affinity for the initiator binding site. This possibility was excluded by the experiment in which the dinucleotide CpA [cytidylyl(3′–5′)adenosine] was added as an additional initiator to the substrate set containing AMPPCP in the first step reaction (lanes a–e of panel C). The second step reaction was carried out with unlabeled normal nucleotides. The transcripts became slightly longer with increasing concentration of CpA, showing a saturation at 100 $\mu$M. At saturating concentrations of CpA, the total UMP incorporation was still only one-sixth of that observed in which ATP is used in the first step reaction (lanes f and f′) although the distribution of transcripts became very similar to that with ATP. When a saturating concentration of CpA was added with another analogue, AMPPNP, total UMP incorporation again remained to be one-sixth of that in which ATP is used in the first step reaction (compare lanes g and a of panel B). As a control, the addition of CpA with ATP in the first step reaction affected neither the UMP incorporation nor the length distribution (data not shown). Thus, saturating the initiator binding site caused only minor changes in the results of the substrate substitution experiments. Therefore, the absence of the $\beta,\gamma$-pyrophosphate bond of ATP clearly reduces the amount of elongation complex which is going to produce long transcripts mainly through some mechanism independent of the affinity to the initiator binding site.

Which steps in elongation are inhibited by the absence of the $\beta,\gamma$-pyrophosphate bond of ATP? A prolonged incubation with the first substrate set containing ATP produced long transcripts of about 300 nucleotides and even longer readthrough transcripts (lanes a–c of Figure 3, panel D). Detectable amounts of short abortive products were also seen. Notably, the first substrate set containing AMPPCP caused many rounds of abortive initiation, with the main products being 2–9 nucleotides in length (lanes d–f), and a slight amount of full-length transcripts as minor products which were observed in the overexposed lane f. The amounts of abortive products were not related to the position of A in the transcript (1st, 5th, 8th, 9th, and so on). Therefore, the absence of the $\beta,\gamma$-pyrophosphate bond of ATP does not inhibit early additions of mononucleotides but relatively enhances abortive product release. These results taken together clearly show that the $\beta,\gamma$-pyrophosphate bond of ATP is required for preparation of efficient elongation.

## Discussion

We have developed a method for immobilizing a transcription unit on avidin-bound resin beads and studied transcription from the template under physiological conditions. Most of template DNA molecules can be immobilized with a minimum steric hindrance for the interaction with proteins. The immobilization methods have several advantages in studying transcription mechanism. First, transcription complex can be concentrated by a brief centrifugation and, thus, separated from free products or free proteins. Second, new kinetic techniques like rapid dilution and substrate substitution can be used. These techniques are suitable to determine requirements for factors with minimum perturbation in reaction components. They are also suitable for analyzing the association/dissociation kinetics of a component of the transcription complex. They also provide powerful tools for work with crude extract. In addition, template DNA with both ends fixed to a bead could be useful for understanding events under topological constraints. For example, topoisomerases may remove positive and negative superhelices resulting from translocation of transcription machinery along the topologically constrained DNA of the chromsome, and immobilized templates should provide a good model system. Moreover, this technique should also be applicable to study translation on immobilized mRNA, replication, and recombination. Thus it will provide a unique tool for analyzing transient interactions between protein and nucleic acid.

Early elongation of transcription was significantly affected when ATP was replaced with a $\beta,\gamma$-unhydrolyzable analogue (panels B and C of Figure 3). Prolonged incubation with AMPPCP produced trace amounts of long transcripts, suggesting that productive initiation/elongation might take place, although inefficiently, in the absence of any normal ATP. Alternatively, trace amounts of ATP might be contaminating the analogues used in this study. In substitution experiments with AMPPCP in the first step reaction, only one-tenth to one-sixth of transcript molecules were kept in the transcription complex. Many transcription complexes seem to have produced abortive products 2–9 bases in length (panel D of Figure 3). Thus the transcription complex seems to take two con-

[1] Abbreviations: AMPPNP, adenosine 5′-($\beta,\gamma$-imidotriphosphate); AMPPCP, adenosine 5′-($\beta,\gamma$-methylenetriphosphate); CpA, cytidylyl-(3′–5′)adenosine.

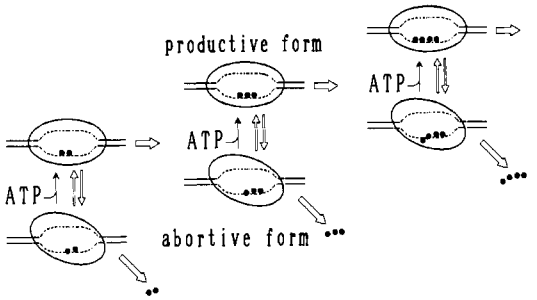


FIGURE 4: Model for the requirement for $\beta,\gamma$-pyrophosphate bond of ATP in a transcription. The transcription complex can adopt a productive or an abortive conformation at each step for elongation. Substrate binding and phosphodiester bond formation are omitted for simplicity. The two conformations coexist only in the early stages of elongation, and the $\beta,\gamma$-pyrophosphate bond of ATP favors the productive one. Short oligo-RNAs (closed circles) are released only from the abortive forms, which are symbolically illustrated as distorted configurations between RNA polymerase (big ovals) and DNA.

formations in the early stages of elongation, a productive and an abortive form (Figure 4); the productive form retains transcripts tightly and produces long RNA molecules, but the abortive form releases truncated products during elongation. The $\beta,\gamma$-pyrophosphate bond of ATP favors the productive conformation, and these two forms are likely to coexist until the elongation mode is established. Thus a small number of abortive products were detectable even in the prescence of ATP, and on the other hand, trace amounts of fully elongated products existed in the presence of the ATP analogues. The $\beta,\gamma$-unhydrolyzable analogues are good substrates for late elongation but less active substrates for early elongation.

Two conformations of the initiation complex or the early elongation complex have been considered since abortive initiation was first analyzed (Johnston & McClure, 1976). Pre- and posttranslocation complexes were suggested by the finding that a downstream substrate (a substrate of the next or the second next phosphodiester bond formations) and its diphosphate analogue enhance productive initiation/elongation with inhibited abortive initiation (Shimamoto et al., 1981). Thus the binding of a downstream substrate may stabilize the productive conformation. The two conformations are likely to coexist until the RNA product is elongated to a certain length, which may be dependent on the promoter (Carpousis & Gralla, 1980; Kinsella et al., 1982).

To examine the ATP requirement in initiation and elongation, the substitution assay is more crucial than conventional assays for the following reasons. Conventional assays require long reaction times, and during these reactions radioactive incorporation may be increased by trace amounts of contaminating nucleoside triphosphates or by the reincorporation of abortive oligonucleotides. The substitution assay requires short incubation, and such artifacts are minimized. Furthermore, large dilution prevents reincorporation. These factors may partly explain why the obtained results contradict those previously obtained (Nierman & Chamberlin, 1980), in which AMPPNP was supposed to replace ATP perfectly. In the previous work, RNA synthesis was initiated either with natural substrates or with four $\beta,\gamma$-imido analogues at low concentrations, and excess amounts of natural substrates were added for further elongation. Then total CMP incorporations during the second elongation were compared. All the active RNA polymerase was supposed to be converted into the elongation complex during 1-h incubation in the first reaction at low concentrations of the initiator (ATP or AMPPNP), where the initiator binding would be far from saturated. With this method, the accumulation of elongation complex may be measured, but such kinetic effects as we observed cannot be detected.

A remaining question is whether the $\beta,\gamma$-pyrophosphate is the target of a protein kinase or an ATPase, or whether it is just an allosteric effector. Phosphorylation of *E. coli* RNA polymerase was first observed in T7 infected cells (Ponta et al., 1974; Zillig et al., 1975). The phosphorylation of the $\beta$ and $\beta'$ subunits of RNA polymerase in uninfected *E. coli* has been reported (Enami & Ishihama, 1984), but its role in transcription is not known. On the other hand, a low level of ATPase was previously detected in highly purified RNA polymerase, but its relevance in initiation was questioned (Nierman & Chamberlin, 1980). Recently, however, NTPase activity including ATPase was found to be altered by a mutation of the $\beta$ subunit, and participation of the activity in transcriptional fidelity was suggested (Libby et al., 1989).

A requirement for the $\beta,\gamma$-pyrophosphate bond of ATP in transcription initiation has been found in eucaryotic systems [for example, see Conaway and Conaway (1988)]. It will be interesting to compare initiation mechanism of procaryotic and eucaryotic transcription by the immobilized operon procedure.

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# Effects of Antineoplastic Drugs on the Post-Strand-Passage DNA Cleavage/Religation Equilibrium of Topoisomerase II†

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ABSTRACT: The post-strand-passage DNA cleavage/religation equilibrium of *Drosophila melanogaster* topoisomerase II was examined. This was accomplished by including adenyl-5′-yl imidodiphosphate, a nonhydrolyzable ATP analogue which supports strand passage but not enzyme turnover, in assays. Levels of post-strand-passage enzyme-mediated DNA breakage were 3–5 times higher than those generated by topoisomerase II prior to the strand-passage event. This finding correlated with a decrease in the apparent first-order rate of topoisomerase II mediated DNA religation in the post-strand-passage cleavage complex. Since previous studies demonstrated that antineoplastic drugs stabilize the pre-strand-passage cleavage complex of topoisomerase II by impairing the enzyme's ability to religate cleaved DNA [Osheroff, N. (1989) *Biochemistry 28*, 6157–6160; Robinson, M. J., & Osheroff, N. (1990) *Biochemistry 29*, 2511–2515], the effects of 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA) and etoposide on the enzyme's post-strand-passage DNA cleavage complex were characterized. Both drugs stimulated the ability of topoisomerase II to break double-stranded DNA after strand passage. As determined by two independent assay systems, *m*-AMSA and etoposide stabilized the enzyme's post-strand-passage DNA cleavage complex primarily by inhibiting DNA religation. These results strongly suggest that both the pre- and post-strand-passage DNA cleavage complexes of topoisomerase II serve as physiological targets for these structurally disparate antineoplastic drugs.

Physiological processes such as DNA replication, transcription, and recombination generate a number of topological structures in nucleic acids which must be resolved in order for the cell to transmit its genetic material faithfully from one generation to the next (Vosberg, 1985; Wang, 1985). In the eukaryotic cell, topological relationships in DNA are modulated by highly conserved enzymes known as topoisomerases (Vosberg, 1985; Wang, 1985; Osheroff, 1989a). The type II topoisomerase is an essential enzyme (DiNardo et al., 1984; Goto & Wang, 1984; Uemura & Yanagida, 1984; Holm et al., 1985) which is required for the segregation of daughter chromosomes (DiNardo et al., 1984; Uemura & Yanagida, 1984, 1986; Holm et al., 1985; Uemura et al., 1987) and the maintenance of proper chromosome structure (Berrios et al., 1985; Earnshaw & Heck, 1985; Earnshaw et al., 1985; Gasser & Laemmli, 1986; Gasser et al., 1986).

The catalytic cycle of topoisomerase II can be broken into a number of discrete steps (Osheroff, 1989a). (1) In the absence of either a divalent cation or ATP, the enzyme is capable of forming a noncovalent complex with its DNA substrate. (2) If a divalent cation (magnesium is used in vivo) is present, topoisomerase II rapidly establishes a double-stranded DNA cleavage/religation equilibrium prior to strand passage. When the DNA is cleaved, topoisomerase II is covalently attached to the newly generated 5′ termini. (3) Upon ATP binding, the enzyme passes an intact DNA helix through the transient break in the nucleic acid backbone. (4) Following this strand-passage event, topoisomerase II once again establishes a DNA cleavage/religation equilibrium. (5) Hydrolysis of the bound ATP cofactor triggers (6) enzyme turnover and allows topoisomerase II to initiate a new round of catalysis.

Recent studies indicate that topoisomerase II is the primary cellular target for a number of antineoplastic drugs (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989) which are widely used for the clinical treatment of human cancers (Cassileth & Gale, 1986; van Maanen et al., 1988; Fleming et al., 1989). The chemotherapeutic efficacies of the above agents correlate with their abilities to stabilize covalent topoisomerase II–DNA cleavage complexes (Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). This drug-induced shift in the enzyme's DNA cleavage/religation equilibrium toward the cleavage event has been observed both in the absence (Chen et al., 1984; Pommier et al., 1985; Osheroff, 1989b; Robinson & Osheroff, 1990) and in the presence of ATP (Nelson et al., 1984; Tewey et al., 1984; Yang et al., 1985; Zwelling, 1985; Glisson & Ross, 1987; Liu, 1989). From studies performed in the absence of the enzyme's high-energy cofactor, it is clear that antineoplastic agents alter the DNA cleavage/religation equilibrium of topoisomerase II which occurs prior to strand passage. Recent

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