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Promoter opening (melting) and transcription initiation by RNA polymerase I requires neither nucleotide β,γ hydrolysis nor protein phosphorylation

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ABSTRACT

With some bacterial RNA polymerases and in eukaryotic RNA polymerase II, DNA melting during initiation requires the coupling of energy derived from β,γ hydrolysis of ATP. A detailed analysis of this possible requirement for eukaryotic RNA polymerase I reveals no such requirement. However, in some cases, β,γ non-hydrolyzable derivatives (β,γ imido or methylene) of nucleotide substrates have been found to significantly inhibit transcription initiation because of their inefficient use as the first nucleotide of the transcript. In addition, the results presented here show that protein kinase activity is not required as an integral part of transcription initiation by RNA polymerase I. Prior phosphorylation of proteins participating in the process is not ruled out.

INTRODUCTION

During transcription initiation in enteric bacteria, the transition from the closed to open (melted) DNA configuration may or may not require hydrolysis of the β,γ phosphodiester bond of ATP depending upon the σ subunit associated with the core enzyme [reviewed in (1)]. Holoenzyme with the most prevalent σ subunit, σ^{70}, spontaneously catalyzes strand separation. Holoenzyme with σ^{54}, however, requires the presence of an additional protein (phospho-NTRC, nitrogen regulatory protein C or phospho-NR_I) bound to a distant enhancer site, but contacting the polymerase by forming a DNA loop (2). An analogous reaction involved in the melting of late T4 promoters has recently been shown to involve DNA tracking of the enhancing protein rather than DNA looping (3). The melting process stimulated by phospho-NTRC (4) requires the hydrolysis of the β,γ bond of ATP (5); substitution of a β,γ nonhydrolyzable ATP analog inhibits phospho-NTRC-stimulated melting.

In eukaryotic cells, the three nuclear RNA polymerases exhibit a differential ability to catalyze the melting reaction. RNA polymerase III appears to function at promoters in a manner analogous to *E.coli* holoenzyme with σ^{70}; strand separation of the DNA occurs spontaneously after assembly of the polymerase at a promoter, in the absence of added nucleotide (6). Eukaryotic RNA polymerase II, on the other hand, has a requirement for ATP hydrolysis to make the transition from the closed to open complex (7). Concomitant with this transition, a kinase or a general transcription factor (eg. TFIIH) catalyzes multiple phosphorylation of the C-terminal domain of the largest polymerase subunit, at the expense of ATP β,γ hydrolysis (8–10). Whether this reaction is involved in DNA strand separation or in aiding the release of polymerase from its association with other proteins in the vicinity of the promoter, or in other reactions, is not clear. It is known, however, that even polymerases missing the entire C-terminal domain require ATP β,γ hydrolysis for initiation (9). Therefore, ATP hydrolysis may be required both for aiding in the melting process and for other events occurring during initiation by RNA polymerase II. This phosphorylation event is required at each round of transcription initiation by this enzyme. Unphosphorylated polymerase II (IIa) enters the transcription cycle by binding to the promoter. IIa then must be phosphorylated during initiation and the transition to elongation (9,10). Polymerase II must be dephosphorylated before it can enter into another round of transcription; the phosphorylated form (II_0) cannot interact with the promoter (11).

The situation with eukaryotic RNA polymerase I is less clear. Kownin *et al.* (12) were able to show that *Acanthamoeba* RNA polymerase I binding to promoters is mediated by protein-protein contact with transcription initiation factors [TIF(s)] bound upstream of the promoter and is DNA sequence-independent. Bateman and Paule (13) showed that this complex is a closed promoter complex, requiring the addition of nucleoside triphosphates before strand separation can occur. In these experiments, however, a short RNA product was synthesized, and therefore it was not clear whether β,γ hydrolysis is required for melting and initiation. Gokal *et al.* (14) and Schnapp and Grummt (15) investigated the requirement for ATP βγ hydrolysis

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for mouse RNA polymerase I initiation. In their experiments, the formation of the first phosphodiester bond of the transcript, between ATP and CTP, rendered the resulting ternary complex insensitive to inhibition by high salt, heparin or sarcosyl. Only transcripts which had formed this first bond during a preincubation produced a runoff product in a subsequent incubation with all four nucleoside triphosphates and one of the inhibitors. Formation of resistant complex was insensitive to substitution of a nonhydrolyzable ATP analog. However, since all of the preincubation reactions, save one, also contained hydrolyzable CTP, the studies did not completely address a possible requirement for nucleotide hydrolysis. In one experiment, the preincubation of a dinucleoside phosphate, ApC, in the absence of any nucleoside triphosphate also rendered the complex insensitive to heparin during the subsequent elongation phase (14). Thus the acquisition of heparin resistance did not require β,γ hydrolysis. The acquisition of heparin resistance, however, is poorly understood in eukaryotes. In *E.coli*, attainment of heparin resistance requires sigma subunit (σ^{70}) and the same elevated temperature necessary for DNA strand separation. Resistance is reversible as temperature is cycled between 20°C and 12°C. Thus, heparin resistance seems not to be related to initiation *per se*, but rather to the presence of a small melted 'bubble' in the DNA (16,17). In mouse, ApC probably results in a region of melted DNA, as suggested by the sequence specificity of dinucleoside phosphates' effect on transcription (18,19). The attainment of heparin resistance in these experiments may only indirectly be related to the normal melting process and may not be related to normal initiation. Therefore, the experiments presented here were undertaken, and they definitively show the lack of requirement for NTP $\beta\gamma$ hydrolysis. We used the *Acanthamoeba* system and an assay in which initiation can be assayed without the addition of any $\beta\gamma$ hydrolyzable nucleoside triphosphate or RNA primer and which does not rely upon ill defined inhibitor resistance.

In addition, it has been shown that one of the transcription factors utilized by RNA polymerase I (20), and perhaps RNA polymerase I itself (21) must be in a phosphorylated state to function properly. Furthermore, experiments *in vivo* and *in vitro* have suggested that RNA polymerase I makes a transition from a transcriptionally competent form to an incompetent form at each round of transcription (22). Since Paule and coworkers (23–25) have shown previously that eukaryotic ribosomal RNA transcription is regulated by the amount of the active form of RNA polymerase I, it is important to know whether initiation by RNA polymerase I requires ATP or GTP β,γ hydrolysis, perhaps indicative of phosphorylation during each round of initiation.

MATERIALS AND METHODS

Preparation of nuclear extracts

Acanthamoeba nuclear extracts were prepared as described by Zwick *et al* (26) except that nuclei were lysed in a final ammonium sulfate concentration of 0.5 M, not 0.3 M. The extract was centrifuged at 100,000×g for 80 min at 4°C. The resulting supernatants were pooled, solid ammonium sulfate was added to a final concentration of 1.82 M, and, following a 60 min equilibration, the precipitated proteins were collected by centrifugation at 100,000×g for 35 min at 4°C. Again the supernatants were pooled and solid ammonium sulfate was added to a final concentration of 3.56 M. Following a 60 min equilibration, the precipitated proteins were collected by centrifugation as above. The 1.82 M and 3.56 M ammonium sulfate pellets were separately suspended in 1–2 ml of Buffer C (50 mM HEPES/KOH, pH 7.9; 50 mM KCl; 0.1 M EDTA; 1 mM DTT; 0.5 mM PMSF; 10% glycerol) and dialyzed against 2 liters of Buffer C for 14 hours with buffer changes at 4 and 10 hours. As previously described (26), no RNA polymerase I was detected in the suspension derived from the 1.82 M pellet. The RNA polymerase I-specific transcription factors TIF-IB and aUBF, however were in the 1.82 M ammonium sulfate fraction. In contrast, all of the RNA polymerase I activity (but no detectable TIF-IB or aUBF activity) fractionated with the 3.56 M ammonium sulfate pellet.

Fractionation of nuclear extracts

The highly purified TIF used in this study was provided by William Kubaska and was prepared as described previously (27). (TIF as described here can be further resolved on Mono Q into two components, TIF-IB and aUBF (Q.Yang and M.Paule, unpublished). For the experiments described in this study, pre-Mono Q TIF was used.) RNA polymerase I was purified from the 3.56 M ammonium sulfate fraction of *Acanthamoeba* nuclear extracts by a scheme similar to that described by Spindler *et al.* (28) except that BioRex 70 was substituted for Phosphocellulose P11 and heparin Agarose was substituted for heparin Sepharose. Briefly, the ammonium sulfate fraction (above) containing RNA polymerase I activity was sequentially fractionated over BioRex 70, DEAE Cellulose (DE-52) and heparin Agarose. Sedimentation of the pooled polymerase-containing fractions over a 17.5–35% glycerol gradient resulted in near homogeneous RNA polymerase I.

Plasmid DNAs and *in vitro* transcription reactions

Most transcription reactions presented in this study utilized 50 ng of a linearized plasmid which contained the *in vivo* *Acanthamoeba* rRNA promoter and transcription initiation site [pEBH10; (29)]. The experiment presented in Figure 4 utilized 50 ng of a linearized DNA template containing the *Acanthamoeba* rRNA promoter, but containing an A to G transition at the site of transcription initiation [pt+1 A/G;(30)]. As previously reported, this transition mutation has no appreciable affect on initiation of transcription by RNA polymerase I at the *Acanthamoeba* rRNA promoter (30). All transcription reactions were performed as previously described (25,31) except that the composition of nucleoside triphosphates was altered as indicated in individual figure legends. All nucleoside triphosphates, or their respective analogs, were used at a concentration of 500 μM and were Pharmacia Ultrapure Solution rNTPs (Pharmacia LKB Biotechnology).

RESULTS

Some but not all β,γ-nonhydrolyzable nucleotide analogs inhibit transcription

To examine whether an ATP β,γ hydrolysis-dependent reaction is required for initiation of transcription by *Acanthamoeba* RNA polymerase I, we performed *in vitro* transcription reactions in which ATP was replaced by analogs which had nonhydrolyzable β,γ phosphoanhydride bonds. Neither 5'-adenylylimidodiphosphate (AMP-PNP) nor β,γ-methyleneadenosine-5'-triphosphate (AMP-PCP) could be efficiently substituted for ATP in our reconstituted transcription system (Figure 1, lanes 3 and 4). In

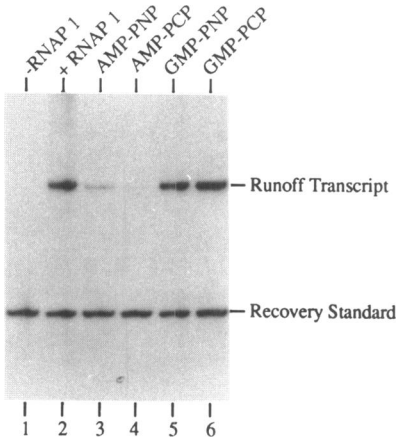

Figure 1. Inhibition of gene-specific RNA polymerase I transcription by ATP analogs containing non-hydrolyzable β-γ phosphoanhydride bonds. Lane 1, no RNA polymerase I; lanes 2–6 contain 30 mUnits RNA polymerase I. 500 μM of 5′-adenylylimidodiphosphate (AMP-PNP) (lane 3) or β-γ-methyleneadenosine-5′-triphosphate (AMP-PCP) (lane 4) was substituted for ATP. 500 μM of either 5′-guanylylimidodiphosphate (GMP-PNP) (lane 5) or β-γ-methyleneguanosine-5′-triphosphate (GMP-PCP) (lane 6) was substituted for GTP. Gene-specific *in vitro* transcription was performed as described (25,31).

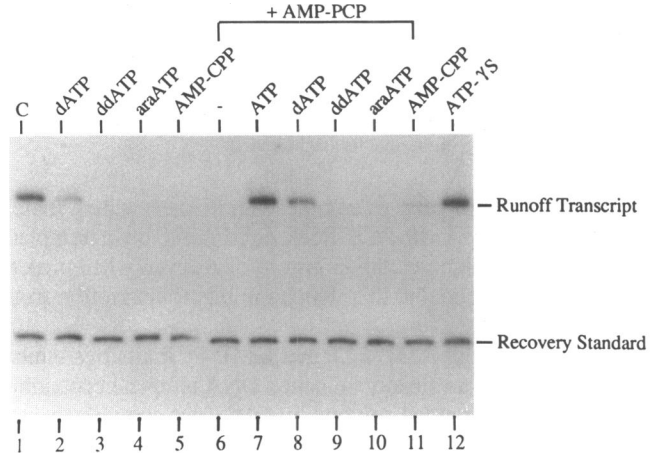

Figure 2. Inhibition by AMP-PCP can be reversed by either ATP or dATP, but not by other ATP analogs containing hydrolyzable β-γ phosphoanhydride bonds which can't be incorporated into RNA. Lanes 1–5; standard transcription reactions (25) which contained 500 μM ATP (lane 1), 2′deoxyATP (dATP) (lane 2), 2′,3′dideoxyATP (ddATP) (lane 3), adenine-9-β-D-arabinofuranoside 5′-triphosphate (ara-ATP) (lane 4) or AMP-CPP (lane 5). Lanes 6–11; standard transcription reactions which contained 500 μM AMP-PCP in place of ATP and were supplemented, following a 10 minute preincubation, with no additional nucleoside triphosphate (lane 6), or with 500 μM ATP (lane 7), dATP (lane 8), ddATP (lane 9), ara-ATP (lane 10), or α,β-methyleneadenosine-5′-triphosphate (AMP-CPP) (lane 11). Lane 12; standard transcription reaction which contained 500 μM adenosine-5′-0-(3-thiotriphosphate) [ATP-γ-S] in place of ATP.

contrast, the analogous GTP analogs, GMP-PNP and GMP-PCP, had no discernable effect on specific transcription from the *Acanthamoeba* rRNA gene promoter (Figure 1, lanes 5 and 6). Interestingly, both ATP and GTP analogs had similar effects on the ability of RNA polymerase I to catalyze RNA synthesis nonspecifically from naked calf thymus DNA. Each of the analogs reduced the nonspecific transcriptional activity of RNA polymerase I by approximately 60% (data not shown). Presumably the analogs have differential effects on specific transcription because elongation is not the rate limiting step in runoff transcription from a promoter.

As with other studies that have examined the energy requirements for initiation of transcription, the inhibition of RNA polymerase I activity we observed when substituting either AMP-PCP or AMP-PNP for ATP could be relieved when reactions were supplemented with ATP or dATP (Figure 2, lanes 7 and 8). In contrast to other studies, however, dideoxyATP, araATP or AMP-CPP, each of which contain a hydrolyzable β,γ phosphoanhydride bond but which cannot be incorporated into RNA, did not relieve the inhibitory effect of AMP-PCP (Figure 2, lanes 9–11). Therefore, we questioned whether the observed inhibitory effect of AMP-PCP on transcription initiation was due to a specific ATP-dependent energy or phosphorylation requirement.

RNA primers relieve the inhibitory effect

Since the immediate 5′-sequence of the nascent *Acanthamoeba* rRNA transcript is 5′-pppAAAGGGAC (19), we reasoned that AMP-PCP inhibition of RNA polymerase I transcription initiation might occur if the analog were a poor substrate for the initial nucleotide of the specific transcript. During this reaction, the 5′ nucleotide is occupying a site on the enzyme normally containing the 3′ OH terminus of the nascent RNA. There is extensive evidence for the uniqueness of this site in the *E.coli* polymerase [reviewed in (32)]. By analogy, a homologous site should be present in eukaryotic polymerases. The similarity of the effect of dinucleoside phosphate primers in the *E.coli* and eukaryotic polymerases (18,19) is in accord with this notion of a product terminus site. We have demonstrated, using nonspecific polymerase assays, that AMP-PCP can be utilized for elongation of RNA, albeit less efficiently than ATP (not shown). To test the idea that the analog might not be used efficiently in the product terminus site, we supplemented AMP-PCP inhibited transcription reactions with either the dinucleotide primer ApA (Figure 3, lane 4) or the trinucleotide primer ApApA (data not shown). Both RNA primers relieved the inhibitory effect. The ability of the RNA primers to restore transcription to normal levels is not a result of ATP contamination of the primer since the wrong dinucleotide primer, GpA, could not relieve the inhibitory effect of AMP-PCP (Figure 3, lane 5, and results below)and addition of the primer in place of ATP or its analog did not lead to transcription (not shown). Similarly, since no RNA synthesis was observed in the presence of the analog and a combination of the other three nucleotides, GTP, CTP and UTP (Figure 3, lane 2), we conclude that the nucleotide preparations are free of ATP.

Inhibition is due to an inefficient use of the ATP analog as the initiating nucleotide

To further investigate the idea that the ATP analogs AMP-PCP and AMP-PNP inhibit synthesis of wild-type *Acanthamoeba* rRNA by functioning as poor substrates for the initial nucleotide and not by interfering with an energy coupling or phosphorylation step, we performed *in vitro* transcription reactions on a mutant *Acanthamoeba* rDNA template (30) in which the initial nucleotide (adenosine) of the wild-type DNA was replaced by a guanosine residue in the RNA-like strand. The immediate 5′-sequence of the resulting nascent rRNA is 5′-pppGAAGGGAC. In marked contrast to transcription from the wild-type template, AMP-PCP had no inhibitory effect on initiation of transcription from this mutant template (Figure 4, lane 2). Interestingly, the analogous

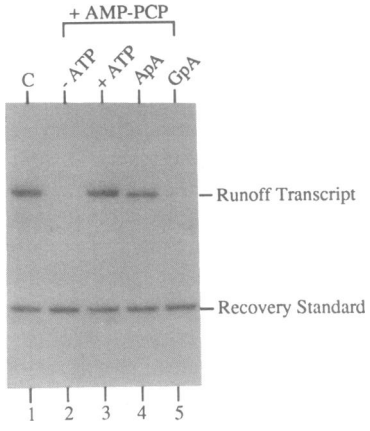

Figure 3. AMP-PCP inhibition of rRNA gene transcription can be relieved by either ATP or the dinucleotide ApA. Lane 1; standard transcription reaction containing 500 μM ATP, Lanes 2−5; standard transcription reactions which contained 500 μM AMP-PCP in place of ATP and supplemented, following a 10 minute preincubation, with no additional nucleoside triphosphate (lane 2), 500 μM ATP (lane 3), 100 μM ApA (lane 4), or with 100 μM GpA (lane 5).

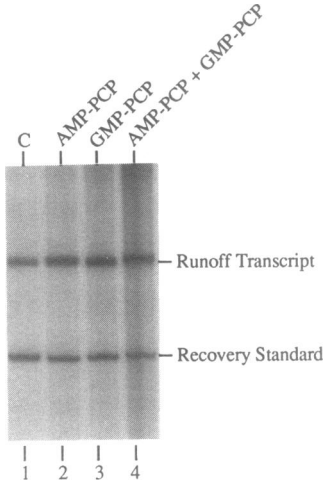

Figure 4. Neither ATP nor GTP analogs containing non-hydrolyzable β-γ phosphoanhydride bonds inhibit transcription from a mutant rRNA template containing guanosine instead of adenosine as the initial nucleotide (30). Lane 1; control. Lanes 2−4; standard transcription reactions of a +1 A/G point mutant template in which 500 μM AMP-PCP (lane 2), GMP-PCP (lane 3) or both were substituted for ATP or GTP, respectively.

GTP analog, GMP-PCP, also had no effect on transcription from the mutant template (Figure 4, lane 3). The lack of any detectable inhibition by GMP-PCP was not a result of contaminating GTP in the preparation since the level of GTP contamination, 0.2%, was not enough to allow for the observed wild-type levels of RNA synthesis when using the analog alone [data not shown and (19)]. Another ATP analog, ATP-γS, had no inhibitory effect on transcription of the wild type template by RNA polymerase I (Figure 2, lane 12) suggesting that inhibition is analog-dependent (see Discussion).

We infer that the inhibition caused by AMP-PCP on the wild type template is not due to contamination with some nonspecific inhibitor. The analog does not inhibit nonspecific transcription to the same extent as it does specific initiation at the promoter.

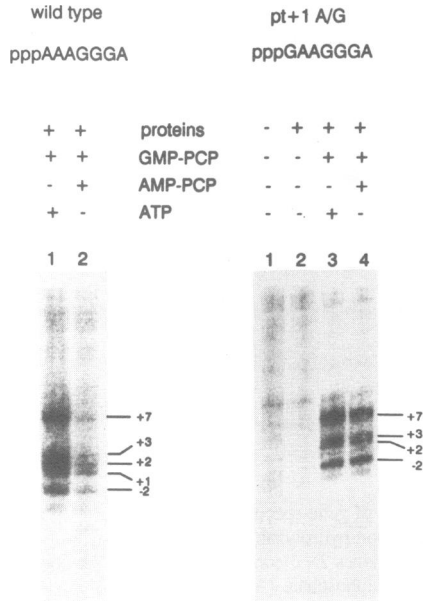

Figure 5. Complete substitution of β,γ-nonhydrolyzable nucleotide analogs has no inhibitory effect on stable DNA strand separation during initiation by polymerase I. Analysis of single-stranded DNA was carried out using diethylpyrocarbonate modification of adenosines (13) on the wild-type (left panel) or +1 A/G point mutant (right panel) templates. No pyrimidine nucleoside triphosphates are present in these assays. Purine nucleotides or analogs were added as shown. Positions modified by DEPC were determined by comparison to DNA sequencing ladders [not shown and ref (13)]. Note that in the pt+1 A/G mutant, the A at position +1 normally modified by DEPC in the wild type template is a G and is not modified. This internally confirms the assignment of the modified positions since the +1 band is missing in the mutant (right panel).

Specific inhibition is only overcome with primers which match the initiation site. AMP-PCP does not inhibit on a template encoding RNA beginning with G instead of A, even when present at a concentration equal to that which inhibits transcription from a wild type template.

Finally, while both AMP-PCP and GMP-PCP analogs inhibit elongation initiated artificially on naked DNA template containing nicks and single-stranded regions by 60% (not shown), even a combination of the two analogs has no apparent effect on specific initiation at a committed complex on the +1 A/G promoter measured by a runoff assay. This suggests that, as previously shown for mouse RNA polymerase I, the rate limiting step in a specific runoff assay is the transition from the closed initiation complex to the elongation complex, rather than the elongation rate (14,33,34).

Protein kinase activity appears unnecessary for initiation

Even a combination of both non-hydrolyzable purine nucleoside triphosphate analogs (Figure 4, lane 4) had no inhibitory effect on transcription of the mutant template further supporting the notion that no phosphorylation event by a kinase specific for purine nucleotides is required for initiation by RNA polymerase I. To further eliminate the possibility that transcription involves a reversible tyrosine phosphorylation step during initiation, either staurosporin, a potent protein kinase C and tyrosine kinase inhibitor (35), or genistein, a specific inhibitor of tyrosine protein kinases (36), was included in *in vitro* transcription reactions. Inclusion of these inhibitors at concentrations up to 10^{-5} M had no measurable effect on the amount of rRNA synthesized *in vitro* (data not shown).

Stable DNA strand separation requires addition of purine nucleotide substrates, but does not require β,γ-hydrolysis

We directly tested the effect of AMP-PCP plus GMP-PCP on melting of the wild type and +1 A/G point mutant. Bateman and Paule (13) had observed previously that melting can be detected by single-strand-specific modification of adenosine residues by diethylpyrocarbonate (DEPC). In those experiments, it was determined that the formation of a stable melted region of the template does not occur until after an RNA product of several nucleotides is synthesized. We presume, however, that rapid isomerization between closed and open states must occur to allow the first nucleotides to be aligned with the template strand. In agreement with this, we find susceptibility to DEPC (at positions −2, +2, 3, 7) of the +1 A/G mutant template is only observed after nucleotides have been added (Figure 5, right panel, compare lanes 1 and 2 with 3). As above, hydrolysis of the β,γ-phosphoanhydride bond is not required for melting because AMP-PCP plus GMP-PCP can be substituted for normal nucleotides in this reaction on the +1 A/G point mutant (Figure 5, lane 4). Note that in this assay, unlike in the transcription assays, no normal pyrimidine nucleotides are present. Thus energy coupling or phosphorylation cannot be mediated by enzymes using pyrimidine nucleoside triphosphates. This contrasts with experiments done previously in the mouse system (14,15) (see Introduction). Just as is observed for transcription, melting (at positions −2, +1, 2, 3, 7) is not as efficient on the wild type template when the analogs are substituted. A low degree of melting is detectable, however (Figure 5, left panel), in accord with the weak transcription of this template when AMP-PCP is used (Figure 1).

DISCUSSION

Results presented here demonstrate that β,γ hydrolysis of nucleotides is not required for strand separation during initiation by RNA polymerase I, in contrast to eukaryotic polymerase II. Formation of a stable melted DNA region occurs as a separate step following binding of RNA polymerases to DNA. For RNA polymerases I and II, melting also requires the addition of nucleotide precursors (7,13). The melting and initiation step of polymerase II is inhibited by AMP-PCP. However, neither AMP-PCP nor GMP-PCP, nor a combination of both of them, inhibits polymerase I because of a requirement for β,γ bond hydrolysis, even under conditions in which the two analogs are the only nucleotides in the reaction.

Our results suggest caution in interpreting inhibition by these analogs when studying transcription, however. These analogs may not serve effectively as the initiating (first) nucleotide of the transcript, a reaction in which the initiating nucleoside triphosphate binds to the 3′-OH product terminus site on the polymerase (32). This binding is inefficient as demonstrated by the requirement for a high concentration of the initial nucleotide (19,37). This product terminus site has different structural requirements that the 'elongation nucleotide site' in *E.coli*. For example, magnesium is not required, nor is a triphosphate group [reviewed in (32)]. Dinucleotide RNA primers have previously been shown effective in priming rRNA synthesis when the concentration of initiating nucleotide is insufficient (19,38), and we show in the present study that AMP-PCP-mediated inhibition of *Acanthamoeba* rRNA transcription also can be completely relieved by supplementing an AMP-PCP-inhibited reaction with the dinucleotide RNA primer, ApA (Figure 3, lane 4). Inhibition could also be relieved by ATP, dATP (to a lesser degree, see below), but not by other β,γ-hydrolyzable ATP analogs which can't be incorporated into RNA. Significantly, inhibition was completely eliminated by changing the initial nucleotide encoded in the template from A to G (Figure 4) indicating a template sequence dependence rather than a nucleotide dependence for ATP. Since the mutant template is not inhibited by GMP-PCP, perhaps inhibition by AMP-PCP on the wild type template arises because of an inability of the polymerase to properly align two ATP analogs to form the first phosphodiester bond. In contrast, alignment is not significantly anomalous when elongating a nascent RNA, dinucleoside phosphate primer or when GMP-PCP is the first nucleotide and AMP-PCP is the second. The inhibitory effect also appears to be dependent upon the specific analogs being aligned since ATP-γS can replace ATP on the wild type template (Figure 2, lane 12). The ability of dATP to partially relieve the inhibitory effects of AMP-PCP on wild type template is in accord with the finding that dATP can be incorporated as the initial nucleotide by RNA polymerase II and may also function as a poor substrate for elongation [Figure 2, lanes 2, 8 and see (39)]. Polymerase I may also follow this model. Alternatively, there may be some low level ATP contamination in our dATP preparation.

The results presented here and elsewhere (14,15) have profound implications not only for the mechanism of initiation by RNA polymerase I, but in addition, for the mechanism of regulation of rRNA expression. Preinitiation complexes form at the *Acanthamoeba* rRNA gene promoter in the absence of nucleotides (25). Results presented here indicate that neither exposure to tyrosine protein kinase inhibitors nor blocking of purine nucleoside triphosphate β,γ bond hydrolysis had detectable effects on transcription *in vitro*. Casein kinase II-related kinases characteristically require ATP or GTP as substrates. The alternative possibility that a required kinase is specific for pyrimidine nucleoside triphosphates can be ruled out because initiation occurs efficiently with both purine analogs in the absence of CTP and UTP, producing a 7 nucleotide long product, pppAAAGGGA or pppGAAGGGA (Figure 5). These observations lead us to conclude that transcription by *Acanthamoeba* RNA polymerase I requires neither factor nor polymerase phosphorylation as an integral part of each round of initiation. This is not to say that a phosphorylation mechanism may not be involved in regulation of rRNA transcription (20,21,24,25), but only that phosphorylation is not required at each round of initiation as for eukaryotic RNA polymerase II (see Introduction).

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