Specificity of an *Escherichia coli* RNA Polymerase-Associated NTPase

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ABSTRACT: Standard preparations of *Escherichia coli* RNA polymerase harbor a 70 kDa protein with NTPase ($\beta-\gamma$ cleavage) activity that is not a recognized polymerase subunit. The NTPase activity of this component, before and after separation from the polymerase, is strongly dependent on the presence of DNA; single-stranded polydeoxynucleotides are more effective than double-stranded. ATP and GTP are cleaved, the latter much less readily. The NTPase as it occurs with the polymerase displays cleavage preference for NTPs that are not complementary to the DNA, a fact that has led to proposals for involvement of the NTPase in transcriptional error prevention [Volloch, V. Z., Rits, L. & Tumerman, L. (1979) *Nucleic Acids Res. 6*, 1535−1546; Libby, R. T., Nelson, J. L., Calvo, J. M., & Gallant, J. A. (1989) *EMBO J. 8*, 3253−3158]. We find, however, that the lesser cleavage in the presence of complementary DNA results from competition for the NTP between the processes of incorporation by the polymerase and of cleavage by the NTPase, operating on the same substrate pool. The greater cleavage with noncomplementary DNA occurs because of the lack of incorporation by the polymerase, which then does not compete with the NTPase for the substrate pool. Thus, these findings indicate that the cleavage preference of the NTPase for noncomplementary NTPs is *not* part of a mechanism for error prevention during transcription.

Formation of RNA by the various RNA polymerase systems results in polymeric products with far greater fidelity to the parent template than can be accounted for by the necessary base-pairing interaction between template and substrate (Springgate & Loeb, 1975). How this degree of fidelity can come about has remained a perplexing question that has been considered in a number of laboratories (Loeb & Kunkel, 1982; Rosenberger & Hilton, 1983; Blank et al., 1986; Kahn & Hearst, 1989). Recent magnetic resonance studies in this laboratory with *E. coli* RNA polymerase (Eichhorn et al., 1994) led to an error-prevention mechanism involving conformational switching in the NTP binding sites on the polymerase.

In the course of our studies on the active-site properties of *E. coli* RNA polymerase (Chuknyisky et al., 1990; Beal et al., 1990; Eichhorn et al., 1994), we became aware of the presence of substantial amounts of NTPase ($\beta-\gamma$ cleavage) activity in our polymerase preparations. Under some circumstances, practical difficulties ensued, such as the cleavage of ATP to ADP at rates that limited long-term collection of NMR spectra. While the NTPase resisted removal by gel filtration, it could be efficiently resolved from core ($\alpha_2\beta\beta'$) and holoenzyme ($\alpha_2\beta\beta'\sigma$) forms (Butzow & Stankis, 1992) on the strong anion exchanger MonoQ (Hager et al., 1990). In this way, practically all of the NTPase activity in the standard RNA polymerase preparation was localized to a 70 kDa protein migrating on SDS gel electrophoresis close to but separately from the σ subunit. The NTPase had been previously observed in RNA polymerase preparations, and NTPase action on noncomplementary substrate had been proposed as a mechanism for error prevention in transcription (Ninio et al., 1975; Volloch et al., 1979; Libby et al., 1989; Libby & Gallant, 1991). Could the NTPase routinely found in polymerase preparations itself be involved in the error-constraining mechanism we were investigating?

We have now examined the DNA dependence of this NTPase activity, both in conjunction with the polymerase and separated from it, and conclude that the 70 kDa protein does not in fact support a coding-specific mechanism for error prevention. The cleavage specificity for NTP that is not complementary to template DNA can be traced to mutual competition for *complementary* NTP by polymerase and NTPase, a competition that does not occur with noncomplementary NTP.

MATERIALS AND METHODS

E. coli RNA polymerase was prepared according to the method of Burgess and Jendrisak (1975) as modified by Lowe et al. (1979). This procedure involves successive chromatography on DNA−cellulose and gel filtration. Details and characteristics of the polymerase preparation are given in Chuknyisky et al. (1990). RNA polymerase so prepared contains a 70 kDa NTPase (Butzow & Stankis, 1992); the polymerase used in this work is estimated (as described below) to contain on the order of one 70 kDa NTPase moiety per three core or holoenzyme moieties.

Although scaling up the preparation of RNA polymerase by adsorption−desorption and gel filtration methods may be expected to reduce resolution somewhat, leading to retention of minor amounts of contaminating enzymes, we note here that HPLC gel filtration of a moderate amount of crude polymerase from a large-scale DNA−cellulose step provided excellent resolution of 500 kDa RNA polymerase still containing substantial amounts of NTPase. This gel filtration analysis was performed on a 24 × 600 mm Toso-Haas G4000SW column, in 200 mM ammonium sulfate, 50 mM Tris−sulfate, 10% glycerol, and 100 μM dithiothreitol, pH 7.5. In our hands, the G4000SW column regularly resolved

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globular particles of molecular mass as close or as far apart as 1000, 600, 500, and 100 kDa.

NTPase-free RNA polymerase and separated NTPase were prepared by chromatography of up to 3 mg of standard *E. coli* RNA polymerase on a 5 × 50 mm MonoQ (Pharmacia Biotech) column (Hager et al., 1990; Butzow & Stankis, 1992), or up to 150 mg on a 20 × 160 mm HR Q-Sepharose (Pharmacia Biotech) column under similar conditions, using a Waters 650E HPLC system. The standard polymerase was introduced to the column at ~1 mg/mL protein in 250 mM NaCl, 10 mM Tris-HCl, 5% glycerol, and 100 μM dithiothreitol, pH 7.9, the column being equilibrated in this buffer at 300 mM NaCl, and a shallow gradient of 350−500 mM NaCl in this buffer was applied [as described by Hager et al. (1990)]. NTPase elutes at the beginning of the gradient, with the core and holoenzyme forms of polymerase eluting much later at ~390 and ~410 mM NaCl, respectively [see Butzow and Stankis (1992)]. The proportion of NTPase in the RNA polymerase preparation was estimated from the relative A_{280} yield of the NTPase fraction on MonoQ separation; NTPase concentrations were estimated assuming an extinction coefficient at 280 nm of 0.6 for 1 mg/mL, and a molecular mass of 70 kDa.

Polydeoxynucleotides and NTPs were obtained from Pharmacia Biotech. [α-^{32}P]NTPs were from NEN−Dupont.

RNA polymerase and NTPase activities were measured by the disappearance of [α-^{32}P]NTP and production of ^{32}P-labeled oligo- and polynucleotides or [^{32}P]NDP, respectively. These were separated by ascending thin-layer chromatography on PEI−cellulose with pH 3.7 ammonium formate: 0.5 M eluant to 2.5 cm, 1 M eluant to 5 cm, and then 4 M eluant to 20 cm. Nucleoside 5′-phosphates migrated in the order: NMP > NDP > NTP > oligo/polynucleotides (which remained at the origin). ^{32}P-labeled materials were quantified by direct scanning in a BetaGen instrument. RNA polymerase and NTPase activities were also followed directly by phosphorus NMR spectroscopy.

NMR Measurements. Phosphorus NMR data were recorded on a Varian XL-200 spectrometer operating at 81 MHz. Enzyme and poly(dA)·poly(dT) template solutions were deuterated by repeated dilution in D_2O-substituted buffer and vacuum dialysis concentration. Trace paramagnetic metal impurities were removed from all solutions by treatment with Chelex 100 (Pharmacia Biotech).

RESULTS

Our primary concern is the NTPase activity as it occurs in the standard RNA polymerase preparation. Studies of DNA dependence and its impact on the specificity of NTP cleavage were conducted, chiefly under conditions of enzyme, substrate, and divalent metal ion concentrations used in the previous studies of Volloch et al. (1979) and of Libby et al. (1989). We have compared these studies with those carried out under the conditions required for the magnetic resonance determinations used in probing the polymerase active sites (Chuknyisky et al., 1990; Beal et al., 1990; Eichhorn et al., 1994).

DNA Dependence. In the absence of polydeoxynucleotide, NTP cleavage occurs at a relatively very low rate (Figure 1). The presence of either a single- or a double-stranded polydeoxynucleotide, complementary to the NTP or not, greatly increases the rate of NDP production. The stimulation, by up to severalfold, is greater with single-stranded DNA. The NTPase activity, unlike the rho NTPase function (Seifried et al., 1992), is not stimulated by poly*ribo*nucleotide, as tested for poly(rC) with ATP (results not shown).

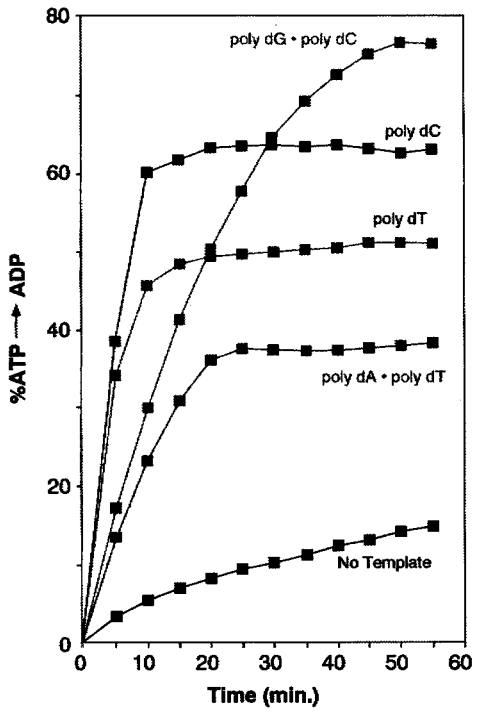

FIGURE 1: ATP cleavage to ADP by NTPase associated with RNA polymerase, in the presence of complementary or noncomplementary polydeoxynucleotides, or in their absence. Conditions: 200 nM RNA polymerase, 300 μM(res) template, 50 μM CTP, GTP, and UTP, 50 μM [α-^{32}P]ATP (400 Ci/mol), 4 mM $MgCl_2$, 14 mM 2-mercaptoethanol, 150 mM NaCl, and 40 mM Tris buffer, pH 8.0; 37 °C.

GTP as well as ATP is attacked by the NTPase, and the cleavage of GTP is likewise stimulated by polydeoxynucleotides; again, single-stranded polydeoxynucleotides produce the greater stimulation (results not shown). However, the cleavage of GTP to GDP is much slower than the cleavage of ATP to ADP: the initial rate of GTP cleavage in the presence of poly(dT) was found to be ~0.2 μM/min, as compared with 3.8 μM/min for ATP in the presence of poly(dC), under the conditions of the reactions presented in Figure 1. For this comparison, poly(dC) was used with ATP and poly(dT) with GTP to elicit a noncomplementary relationship. [Because of complementarity, as indicated in the next section, we cannot validly compare the cleavage of GTP and ATP with both stimulated by either poly(dT) or poly(dC).]

Specificity of NTPase Cleavage with Respect to DNA. Although the requirement for polydeoxynucleotide is not absolute, Figure 1 shows that the presence of bases in the polydeoxynucleotide that are not complementary to the NTP increases the rate and extent of NTP degradation. Thus, poly(dC) and poly(dG)·poly(dC) stimulate ATP cleavage much more than do poly(dT) and poly(dA)·poly(dT). This finding, without further probing, could be taken to indicate that NTPase acting together with RNA polymerase produces degradation of an NTP when that NTP is not complementary to the template, just as Volloch et al. (1979) and Libby et al. (1989) had suggested. We note, even before presenting further experiments, that specific cleavage of noncomplementary NTPs to enhance the fidelity of transcription should result in the exclusive degradation of noncomplementary

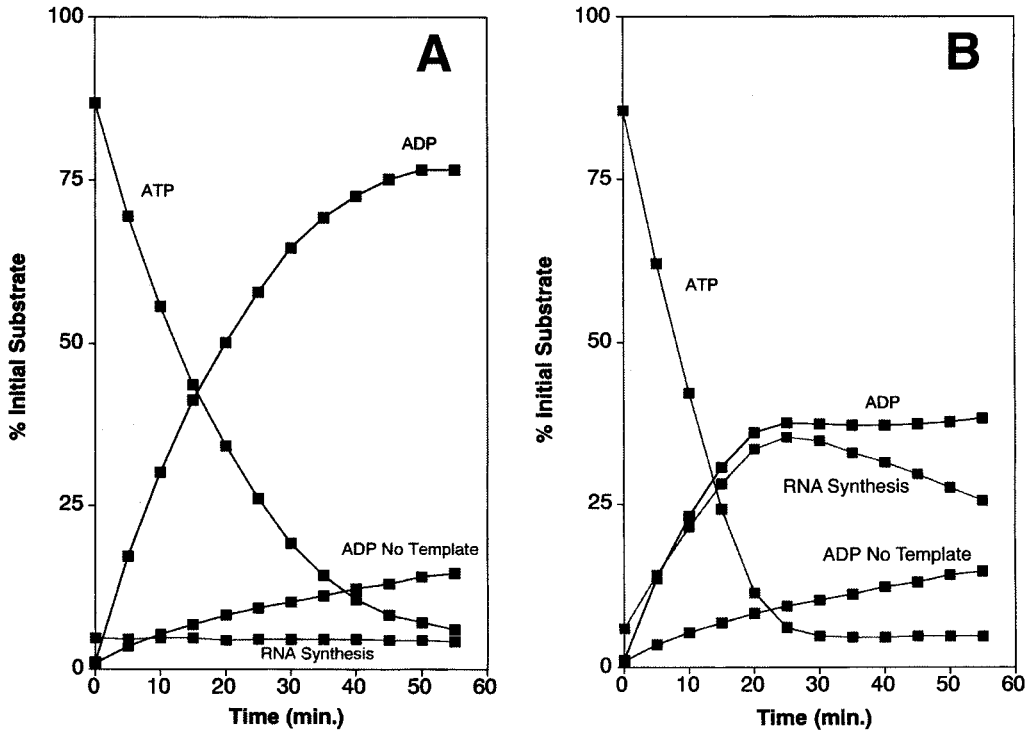

FIGURE 2: ATP cleavage to ADP by NTPase associated with RNA polymerase, and incorporation of ATP into RNA product by the RNA polymerase, in the presence and absence of (A) poly(dG)·poly(dC) or (B) poly(dA)·poly(dT). Conditions as in Figure 1.

NTPs. We have found only preferential, not exclusive degradation.

The cleavage phenomenon is examined in more detail for ATP, by following the time course of the disappearance of the nucleoside triphosphate substrate and of the evolution of products, in the presence of either noncomplementary (Figure 2A) or complementary (Figure 2B) double-stranded DNA. In these experiments, we examined not only the degradation by the NTPase but also the incorporation by the polymerase. In the presence of poly(dG)·poly(dC), in which neither strand is complementary to the substrate, ADP production consumes the initial ATP to the extent of ~75% with virtually no RNA synthesis. In the presence of poly(dA)·poly(dT), in which one of the two strands is complementary to the NTP, ADP production consumes only ~40% of the ATP while RNA synthesis consumes ~35%. Thus, the difference in the amount of cleavage between the two experiments is approximately accounted for by the occurrence of RNA synthesis in the complementary system and its absence in the noncomplementary one.

If experimental conditions are set up so that very little RNA polymerase activity occurs, even in the *complementary* situation, as was the case during our NMR studies on the polymerase active site, then little competition between polymerase and NTPase for NTPs exists. In such conditions, even a complementary substrate−template combination can be shown to lead predominantly to NTP degradation. This effect is shown in Figure 3a, in which NTPase and RNA polymerase activities are evaluated under NMR conditions, using the complementary system ATP−poly(dA)·poly(dT). Total enzyme and substrate concentrations are much higher and the divalent metal ion concentrations much lower than for the studies shown in Figures 1 and 2. In Figure 3a, ATP is consumed principally by cleavage to ADP (detected by orthophosphate production), i.e., by NTPase, and little RNA synthesis (detected by pyrophosphate production) takes place. When transcription can be enhanced by addition of a primer, ApA, Figure 3b shows that in fact transcription becomes the dominant process, as seen in this case by the pyrophosphate resonance gradually increasing while the orthophosphate resonance remains weak. The results of Figure 3b more closely resemble those of Figure 2 where NTPase action is prevented through competition by polymerase.

Cleavage Characteristics of the MonoQ-Separated NTPase. NTPase isolated by MonoQ chromatography of standard RNA polymerase preparations was examined for its activity level and the effect of various polydeoxynucleotides on ATP cleavage (Figure 4), at the same concentration (~60 nM) as in the experiments of Figures 1 and 2 where the NTPase was present with the polymerase (on the order of 1 mol of NTPase/mol of polymerase). Cleavage by the free NTPase in the absence of DNA is essentially nil. DNA stimulates the cleavage activity; however, cleavage rates are reduced by a factor of 5 or more from those produced when the NTPase was in the parent RNA polymerase preparation. Moreover, cleavage specificity with respect to complementarity with the stimulating polydeoxynucleotide is no longer observed: poly(dG)·poly(dC) and poly(dA)·poly(dT) now elicit nearly the same rate, and poly(dT) now elicits a somewhat greater rate than does poly(dC). The single-stranded polydeoxynucleotides again stimulate cleavage more than the double-stranded. There is of course no RNA synthesis since the polymerase moiety is not present.

The reduction in cleavage rate upon separation of the NTPase from the polymerase suggests either that the NTPase functions overall better in the presence of the polymerase (regardless of competition for substrate) or else that the separation leads to a conformational change in the NTPase. The remaining NTPase activity resembles what exists before separation in the continued requirement for polydeoxynucleotide. We examined the possibility that the reduction in cleavage activity could be reversed by recombining the

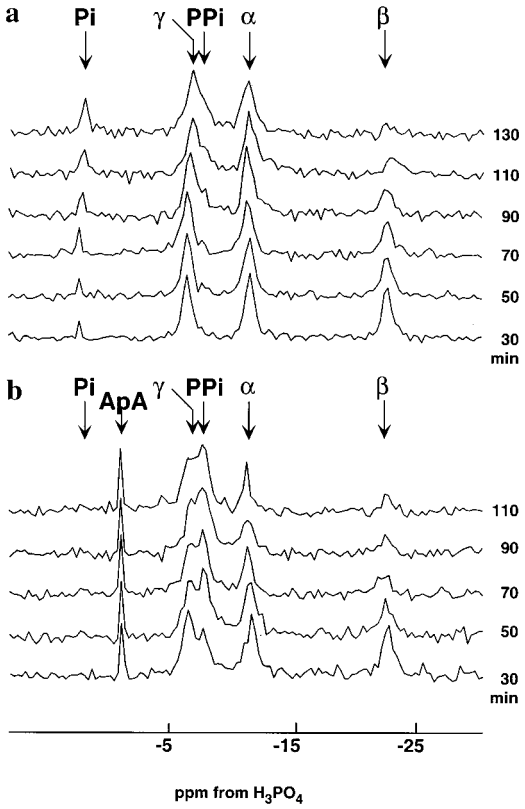

FIGURE 3: ATP cleavage to ADP by NTPase associated with RNA polymerase, and incorporation of ATP into RNA product by the RNA polymerase, in the presence of poly(dA)·poly(dT), under conditions required for NMR spectroscopy: (a) without primer and (b) with ApA. The figure shows a time sequence of phosphorus NMR spectra; positions for α, *β*, and *γ* resonances for ATP and the resonances for inorganic orthophosphate (P_i) and pyrophosphate (PP_i) are indicated. The first spectrum was collected 30 min after mixing, successive spectra at 20-min intervals thereafter. Conditions: 6 mM ATP, 2 mM ApA (when present), 40 *μ*M RNA polymerase, 2 mM poly(dA)·poly(dT), and 15 mM $MnCl_2$, in 200 mM KCl and 50 mM Tris buffer with 20% D_2O; 24 °C. This degree of D_2O substitution was required for a strong, readily obtainable lock signal in the presence of the relatively large quantity of paramagnetic metal salt.

NTPase and polymerase moieties. Little increase in cleavage activity was found either immediately on mixing or up to 20 min after incubation under reaction conditions in the presence or absence of ATP. We tentatively conclude that an irreversible conformational change occurs during separation, either to the NTPase itself or to the mode of its binding to the polymerase. (It has of course frequently been found very difficult to reconstitute multicomponent biological entities; a notable example is the reconstitution of RNA polymerases from their component subunits.)

DISCUSSION

A special NTPase activity has been invoked by Volloch et al. (1979) and by Libby et al. (1989) as a mechanism to prevent incorporation of noncomplementary NTPs, and *E. coli* RNA polymerase preparations are commonly found to harbor NTPase activity. Volloch and colleagues showed that NTPase activity disappeared when RNA polymerase was chromatographed over Cibacron Blue, and they found that the NTPase-depleted polymerase could support higher levels of misincorporation. Libby et al., investigating an *E. coli* mutant with lower transcriptional fidelity, found that RNA polymerase preparations from it also lacked NTPase activity.

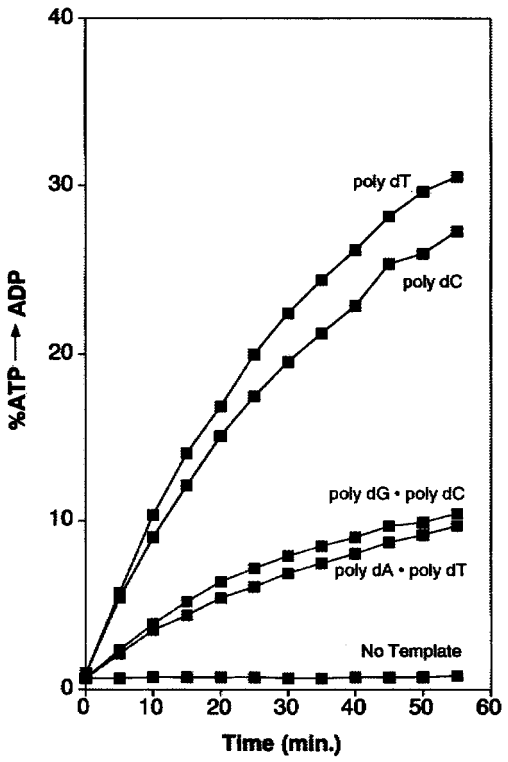

FIGURE 4: ATP cleavage to ADP by isolated NTPase. Conditions are the same as in Figure 1, with the parent RNA polymerase preparation replaced by the equivalent amount of NTPase (∼60 nM) resolved from it.

Butzow and Stankis (1992) found that practically all of the NTPase activity in the standard RNA polymerase preparation could be removed and localized to a 70 kDa protein (protomer).

We have investigated the cleavage of the NTPase. We find that NTP cleavage is very dependent on the presence of DNA both when the NTPase is present with the RNA polymerase in the parent polymerase preparation as well as in the absence of the polymerase. In the case of the NTPase associated with the parent preparation, we find NTP cleavage occurs with a much greater initial rate and extent when the DNA template is not complementary to the NTP (Figure 1). This finding is in accord with that of the earlier workers who interpreted their results as indicating a specificity for cleaving unwanted noncomplementary NTPs (thus permitting only the desired NTPs to be incorporated into the RNA product). However, our results do not indicate an all-or-none specificity, only a preference for cleaving noncomplementary over complementary NTPs (Figure 1).

Moreover, and most importantly, we find a simple explanation for this preference. With standard RNA polymerase preparations, the only enzyme activity to which noncomplementary NTP is subjected appears to be NTPase activity (Figure 2A). Complementary NTP, however, is subject not only to NTPase activity but also to RNA synthesis activity as well (Figure 2B). The amount of NTP consumed in the complementary case is approximately equal to the sum of the NMP incorporated into RNA product plus the NDP produced by NTPase degradation of NTP, and it is also approximately equal to the amount of NDP produced in the noncomplementary case by NTPase in the absence of competition by RNA polymerase. This comparison is not in line with an involvement of the NTPase in the assurance

of fidelity in RNA synthesis. Rather, it indicates that transcription and NTPase action are independent, competitive processes operating on the same substrate pool. Removal of NTP from the substrate pool by incorporation into RNA can limit cleavage to NDP, and removal by degradation to NDP can limit incorporation. Under different conditions, degradation of even complementary NTP can be enhanced, when incorporation of NMP is constrained (Figure 3a), but is then suppressed by a further change in conditions that lead to acceleration of incorporation (addition of primer, Figure 3b). Thus, the apparent preferential cleavage of noncomplementary NTP substrate is not the result of a specific RNA polymerase-linked NTPase mechanism; it appears to be an accident of competition.

If an NTPase were to play a central role in the reduction of transcriptional error, it should really be capable of readily cleaving all the common species of NTP. Volloch et al. (1979) reported that, under reaction conditions comparable to those used in our study, CTP and UTP were attacked only slightly; we find GTP to be attacked at about one-tenth the rate of ATP. Thus, it appears that ATP is the nucleotide triphosphate principally attacked by the NTPase. These studies are indeed in line with our studies on the competition between NTPase action and RNA synthesis that indicate that the NTPase does not play a part in the enforcement of transcriptional fidelity. Our own recent work on the nucleotide binding sites of *E. coli* RNA polymerase (Eichhorn et al., 1994), comparing complementary and noncomplementary NTP−DNA systems, shows that when a noncomplementary NTP occupies the incoming substrate binding site, a conformational change in the polymerase protein orients the triphosphate group away from the 2′-hydroxyl of the nascent RNA chain, disfavoring chain extension. This conformational change may be related to one detected by Erie et al. (1993) to occur upon incorporation of noncomplementary NTP, and be relieved by subsequent phosphodiester cleavage at the position of that nucleotide. We had at one time considered the possibility that cleavage of noncomplementary substrate by the NTPase could act in conjunction with the conformational change we had found in the active site of the polymerase to enhance the fidelity of transcription. For this reason, the experiments described here were conducted, leading to the conclusion that the NTPase is not involved in fidelity.

The extensive literature on *E. coli* RNA polymerase contains numerous reports of NTP-cleaving activity being present in crude polymerase preparations, including "phosphatase" activity as well as the rho transcriptional termination factor (Losick & Chamberlin, 1975), and relatively large amounts of a protein having highly aggregative properties (Paetkau & Coy, 1972; Ishihama et al., 1976a,b) that may be part of the chaperonin GroE. In addition, $\beta-\gamma$ cleavage of ATP has been proposed to be required during transcription as part of the transition from initiation to productive elongation (Fujioka et al., 1991).

The 70 kDa NTPase is not rho since, among other things, rho is stimulated by poly*ribo*nucleotides (Seifried et al., 1992), while the NTPase requires poly*deoxy*nucleotides. It is not GroE since, as far as we are aware, the ATPase activity of GroE is not stimulated by DNA; and since the molecular size of this NTPase is not the same as that of GroE or its protomers (Schon & Schumann, 1995).

Two pieces of information suggest, but do not prove, that the 70 kDa NTPase occurs in the parent RNA polymerase preparation physically bound to the polymerase: the ease of separation from the polymerase core and holoenzyme over a narrow range of salt concentration on ion-exchange as compared with gel filtration chromatography, and the reduction in NTPase activity upon separation. While gel filtration cannot by itself distinguish between a polymerase-bound NTPase and self-associated NTPase of similar hydrodynamic properties as the polymerase, it seems unlikely that facile ion-exchange separation of a free but self-associated NTPase should entail a substantial activity loss.

While we do not know the biological function of the NTPase, our evidence indicates that this function is not the enhancement of transcriptional fidelity as has been previously suggested.

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