The EMBO Journal vol.8 no.10 pp.3153-3158, 1989

Transcriptional proofreading in *Escherichia coli*

Randell T.Libby, Jeffrey L.Nelson, Joseph M.Calvo[2] and Jonathan A.Gallant

University of Washington, Department of Genetics SK-50, Seattle, Washington 98195, USA, [2]On sabbatic from the Department of Biochemistry, Cornell University, Ithaca, NY 14853, USA

Communicated by D.Richter

A novel transcriptional proofreading mechanism associated with the β-subunit of wild-type RNA polymerase from *Escherichia coli* is suggested from the following data. The purified holoenzyme contains an NTPase activity which specifically converts noncognate NTPs to their corresponding NDP in a template-dependent manner during *in vitro* transcription of synthetic single- and double-stranded templates. In contrast, purified enzyme from an *rpoB* mutant which shows increased transcriptional error lacked template-dependent NTP hydrolytic activity. The NTP hydrolytic activity of wild-type enzyme was critically dependent on the integrity of the initiation complex, and required continued transcriptional elongation. Transcription and translation of the *lacZ* gene proceeded 17% faster in the mutant than in its wild-type parent. These results are discussed in terms of a proofreading model in which the rate of transcription is limited by proofreading events that involve recognition and hydrolysis of noncognate NTPs before they can be misincorporated into RNA.

Key words: E.coli/NTPase activity/*rpoB* mutant/transcriptional proofreading

Introduction

In *Escherichia coli* the fidelity of transcription is determined by the interaction of correct and incorrect ribonucleoside triphosphates (NTPs) with a site on the RNA polymerization complex. If initial binding of NTPs were reversible, then its accuracy could achieve a maximum determined by the free energy difference between equilibrium binding of noncognate and cognate NTPs; this is of the order of 1–3 kcal, corresponding to an expected maximum error frequency of 10^{-3} to 10^{-2} (Loeb and Kunkel, 1982; Anderson and Menninger, 1986). Nevertheless, much lower error frequencies in transcription, of the order of 10^{-5}, have been observed both *in vivo* and *in vitro* (Blank *et al.*, 1986; Rosenberger and Hilton, 1983; Springate and Loeb, 1975). Such relatively high levels of accuracy suggest that some form of proofreading occurs during transcription. The possibility that a mechanism exists for the correction or prevention of transcriptional errors is, therefore, of considerable importance. Yet, convincing evidence in support of a transcriptional editing process mediated by the RNA polymerization complex has not been presented.

RNA polymerase, like DNA polymerase I, has previously been observed to convert noncognate nucleoside triphosphates (NTPs) into nucleoside diphosphates (NDPs) during *in vitro* polymerization on synthetic templates (Ninio *et al.*, 1975). Conversion of NTPs to NDPs was reported using impure (Volloch *et al.*, 1979) as well as pure (Ninio, 1975) preparations of RNA polymerase when the NTP lacked base complementarity with the template. Such template-dependent hydrolysis may be related to a transcriptional proofreading mechanism. The report here, involving analysis of a mutant with an altered RNA polymerase, strengthens the link between NTP hydrolysis and proofreading.

We previously reported the isolation of a spontaneous *rpoB* mutation which caused resistance to rifampicin and which reduced transcriptional accuracy (Blank *et al.*, 1986). The mutation, hereafter referred to as *ack-1* (for ackuracy), increased the leakiness of three different polar ochre mutations in *lacZ*, as well as an amber mutation in gene *1* of bacteriophage T7. *In vitro*, the mutant RNA polymerase exhibited an increase in the misincorporation of GTP into RNA when directed by a poly[d(A-T)·d(A-T)]template (Blank *et al.*, 1986). The reduced accuracy displayed by the mutant suggests that the β-subunit of RNA polymerase, encoded by *rpoB*, influences the accuracy of transcription.

Here, we confirm that purified wild-type RNA polymerase is capable of efficiently hydrolyzing incorrect NTPs to

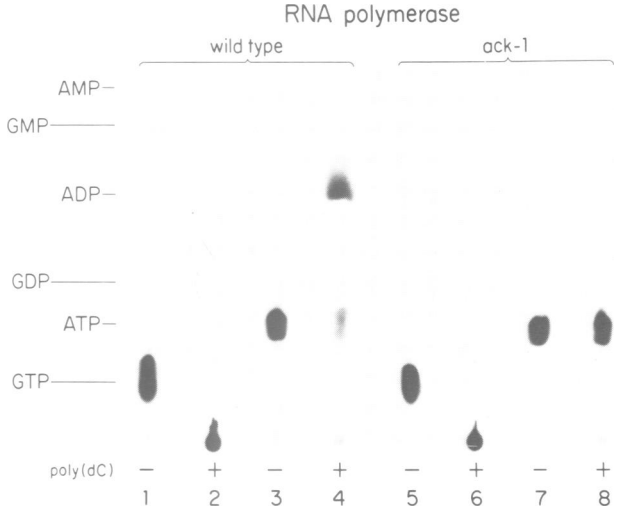

Fig. 1. Autoradiogram of *in vitro* transcription products specified by wild-type and *ack-1* RNA polymerase. Purified wild-type (**lanes 1–4**) or *ack-1* (**lanes 5–8**) RNA polymerase were used during *in vitro* transcription of poly(dC) in the presence of [α-^{32}P]GTP (**lanes 1, 2, 5** and **6**) or [α-^{32}P]ATP (**lanes 3, 4, 7** and **8**). The percentage of total counts in the NDP positions, as determined by densitometry scanning of each lane of the autoradiogram is as follows: **lane 1**, GDP <2%; **lane 2**, GDP <2%; **lane 3**, ADP <2%; **lane 4**, ADP 67%; **lane 5**, GDP <2%; **lane 6**, GDP <2%; **lane 7**, ADP <2% and **lane 8**, ADP 3.6%. The direct counts, determined by scintillation counting of NDP spots cut from the thin-layer chromatogram, reflect the same pattern of counts as the densitometry scans. Reaction products were resolved by TLC as described in Materials and methods.

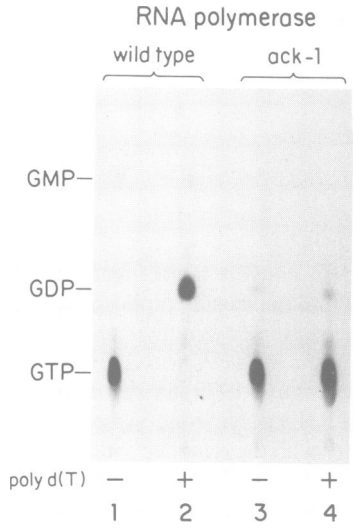

Fig. 2. *In vitro* transcription of poly(dT) in the presence of [α-^{32}P]GTP by either wild-type or mutant RNA polymerase. The percentage of total counts in the GDP positions, as determined by densitometry scanning of each lane of the autoradiogram, is as follows: **lane 1**, <2%; **lane 2**, 98%; **lane 3**, 2.6% and **lane 4**, 4.8%. Reaction conditions were as described in Materials and methods.

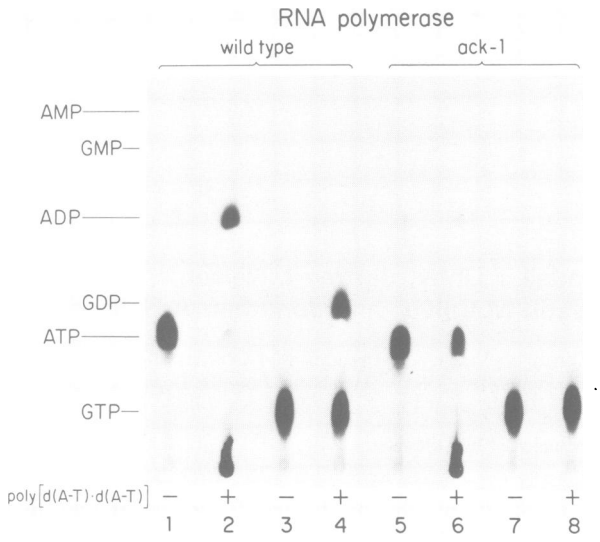

Fig. 3. Poly [(d(A-T)·d)(A-T)] directed *in vitro* transcription. Purified wild-type (**lanes 1–4**) or mutant (**Lanes 5–8**) RNA polymerase were used during *in vitro* transcription in the presence of either [α-^{32}P]ATP (lanes 1, 2, 5 and 6) or [α-^{32}P]GTP (lanes, 3, 4, 7 and 8). The percentage of total counts in the NDP positions (as determined by densitometry scanning as described in the legend to Figure 1) is as follows: **lane 1**, ADP <2%; **lane 2**, ADP 33%; **lane 3**, GDP <2%; **lane 4**, GDP 32%; **lane 5**, ADP 2.2%; **lane 6**, ADP 2.2%; **lane 7**, GDP <2% and **lane 8**, GDP <2%. Scintillation counting of spots corresponding to positions of GDP reflect the same pattern of counts as the densitometry scans. Reaction products were resolved by TLC as described in Materials and methods.

their corresponding NDPs in a template-dependent manner during *in vitro* transcription of single- and double-stranded templates. Secondly, we demonstrate that the enzyme purified from the *ack-1* mutant does not carry out template-dependent hydrolysis of noncognate NTPs. Thirdly, we provide evidence that the rate of transcription *in vivo* is slightly faster in the mutant than in the parent strain.

Results

Our initial experiments employed poly(dC) or poly(dT) as templates. *E.coli* RNA polymerase is known to use such single-stranded molecules as templates when Mn^{2+} is included in the reaction mixture (Singer and Kröger, 1979). Note that irrespective of the templates employed, all reaction mixtures contained all four NTPs, with one of the four labeled with ^{32}P in the α position.

During poly(dC) directed transcription [α-^{32}P]GTP was efficiently incorporated into product RNA (Figure 1, lanes 2 and 6). The noncognate substrate ATP was not incorporated into RNA at a detectable rate but, on the contrary, was efficiently hydrolyzed to ADP (Figure 1, lane 4). The K_m for the hydrolysis of ATP stimulated by poly(dC) was ~2 mM (data not shown); this is close to the binding constant for the initial, nonspecific binding of triphosphates by RNA polymerase (Chamberlin, 1974) and also close to the intracellular concentration of triphosphates (Gallant and Harada, 1969; Erlich *et al.*, 1975).

Enzyme purified from the *ack-1* mutant, in contrast, was deficient in the ability to hydrolyze ATP to ADP on poly(dC) (Figure 1, cf. lanes 4 and 8), although it polymerized the correct NTP into RNA as efficiently as wild-type enzyme (Figure 1, lanes 6 and 8). With poly(dT) as template, wild-type enzyme hydrolyzed the noncognate substrate GTP to GDP, and the mutant enzyme was again deficient in this activity (Figure 2). In parallel incubations, we monitored poly(dT) directed misincorporation of GTP into TCA precipitable product. When the cognate substrate ATP was present, misincorporation of GTP was not detectable. In the absence of ATP, however, significant poly(dT) dependent misincorporation was observed during a 30 min incubation: 1.25 pmol (corresponding to an error frequency of about 10^{-3}) by wild-type enzyme, and 9.2 pmol, 7.4 times higher, by the mutant enzyme. Thus, we confirm the error-prone character of transcription by the mutant enzyme.

Transcription reactions were also performed using double-stranded templates and reaction mixtures containing Mg^{2+} and no Mn^{2+}. In the presence of poly[d(A-T)·d(A-T)] wild-type polymerase catalyzed both the incorporation of ATP into product and its hydrolysis: (Figure 3, lane 2). This is not unexpected, for ATP is cognate to the T-residues in the template, but noncognate to the A residues. In contrast, when the labeled NTP was GTP, a considerable fraction was hydrolyzed to GDP, and very little was incorporated into the polymerized product (Figure 3, lane 4). Purified polymerase from the mutant strain, on the other hand, did not hydrolyze either ATP or GTP during transcription directed by poly[d(A-T)·d(A-T)] (Figure 3, lanes 6 and 8).

To investigate hydrolysis and misincorporation of NTPs with more natural templates, we prepared a G-less cassette (a sequence whose transcript contains no G residues) containing the *tac* promoter. A 740 bp *Eco*RI–*Sma*I G-less cassette, derived from plasmid pRL1100 (Figure 4), was used as the template during *in vitro* transcription in the presence of labeled ATP or GTP. When the labeled NTP was ATP, it was again both polymerized into product RNA and hydrolyzed to its corresponding NDP (Figure 5, lane 2). Labeled GTP, on the other hand, was exclusively hydrolyzed to GDP (Figure 5, lane 4). Once again, RNA polymerase from the mutant strain was devoid of the non-cognate NTPase activity (Figure 5, lanes 6 and 8).

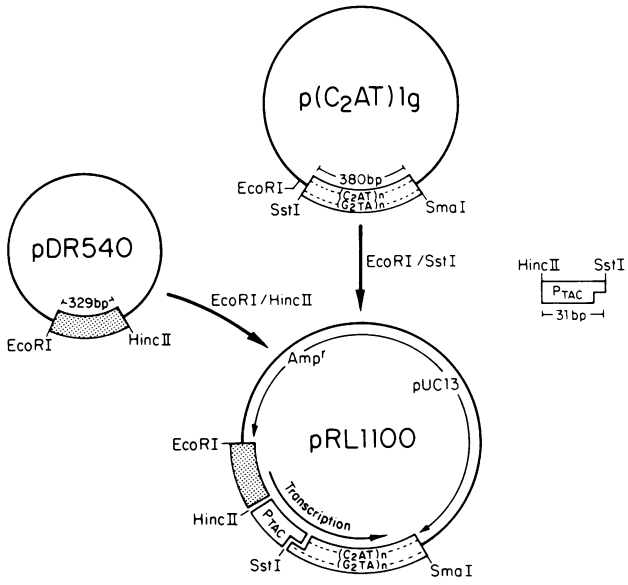

Fig. 4. Schematic construction of the G-less transcription vector. Plasmid pRL1100 (lacking G residues in the transcript of the *Hin*cII/*Sma*I cassette) was constructed from plasmid p(C_2AT)1g as represented in the figure. Abbreviations: P_{TAC}, *trp–lac* hybrid promoter; p($C_2AT)_n$, represents a 380 bp random sequence having the indicated composition.

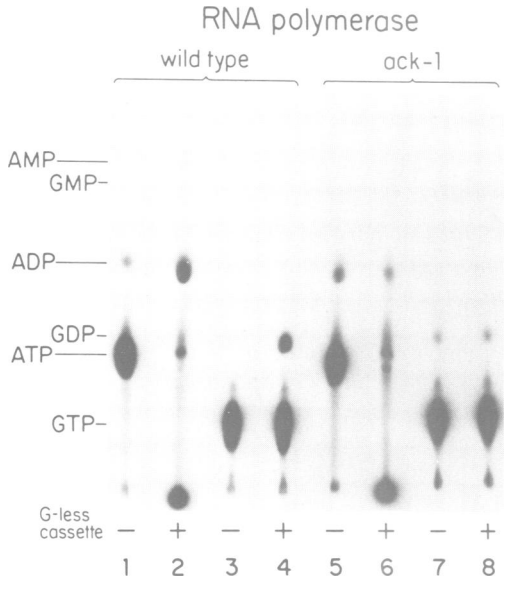

Fig. 5. *In vitro* transcription products directed by the G-less DNA cassette. The G-less cassette was purified from plasmid pRL1100, and used during *in vitro* transcription with purified wild-type (**lanes 1–4**) or mutant (**lanes 5–8**) RNA polymerase as described in the legend to Figure 3. The percentage total counts in the NDP positions (determined by densitometry scanning as described in the legend to Figure 1) is as follows: **lane 1**, ADP, 3.9%; **lane 2**, ADP 27.3%; **lane 3** GDP, <2%; **lane 4**, GDP 13.5%; **lane 5**, ADP 7.5%; **lane 6**, ADP 5.4%; **lane 7**,GDP <2% and **lane 8** GDP 3.0%. Reaction conditions and resolution of products were as described in Materials and methods.

Functional requirements of NTPase

The template dependence of NTPase activity implies that it ought to depend on the binary association of template and enzyme. A high salt concentration reversibly inhibits this association, although it does not inhibit chain elongation once the initiation complex has formed (Zillig *et al.*, 1970; Chamberlin, 1974). As Table I shows, the presence of 0.4 M NH_4Cl abolished NTPase activity on poly[d(A-T)·d(A-T)]. Qualitatively similar results were obtained when heparin was used instead of NH_4Cl to block formation of the initiation complex (data not shown).

Table I. Quantitation of NDP formation by wild-type RNA polymerase

Template	Untreated		NH_4Cl treated		Streptolydigin treated	
	ADP	GDP	ADP	ADP	ADP	GDP
poly [d(A-T)·d(A-T)	37%	65%	ND	ND	–	–
poly(dC)	25%	ND	–	–	2.3%	ND

Transcription assays were performed as described in Materials and methods. NDP spots were localized by UV absorption of thin-layer chromatograms run in the presence of 0.05 μmol of the corresponding NMP, NDP and NTP. Regions of the thin-layer chromatograms containing the NDP were cut out, placed in a scintillation vial with 3 ml of scintillation fluor and counted on a Packard 1500 tri-carb liquid scintillation analyzer. Quantitation of NDP formation is presented as a percentage of total counts following subtraction of background counts from parallel reactions lacking template.
ND, not detectable.
(–) reaction not performed on this template.

In order to determine whether noncognate NTPase activity depends on polymerase movement, we examined the effect of inhibiting transcriptional elongation. When wild-type enzyme was prevented from elongating poly(dC) directed transcripts by the addition of streptolydigin (McClure, 1980), a nearly complete inhibition of ATP hydrolysis was observed (Table I).

Kinetics of β-galactosidase induction

The ability of wild-type enzyme, but not *ack-1* mutant enzyme, to hydrolyze noncognate NTPs specifically during transcription suggests the existence of a transcriptional editing step. We therefore considered the possibility that the wild-type enzyme could transcribe a given gene at a slower rate than the mutant enzyme, and examined the time required to produce the first β-galactosidase molecules following induction of the *lac* operon. As Figure 6 shows, the first β-galactosidase molecules appeared 3 min after induction in a wild-type strain, but ~20–30 s earlier in an isogenic *ack-1* strain. Since the two strains differ only at *rpoB*, and *rpoB* mutations are not known to affect translation directly, this difference is most likely due to different rates of transcription by RNA polymerase.

In order to test this conclusion directly, we used Northern hybridization to assess the time required to complete the *lacZ* message in wild-type and *ack-1* mutant cells. Figure 7 shows that mRNA complementary to a probe at the C-terminal end of the gene begins to appear 2.0 min after induction in the mutant, but does not appear until after this time in the parental strain. (Note that accumulation of mRNA complementary to an N-terminal probe appears earlier, as expected, in both strains.) The kinetics are not sufficiently detailed to permit estimation of the time delay in wild-type relative to *ack-1* in completion of the *lacZ* message, but it is clear that there is one. The more detailed kinetics provided by the enzyme induction experiments (Figure 6) suggest a delay of 20–30 seconds out of 3.0 min, corresponding to

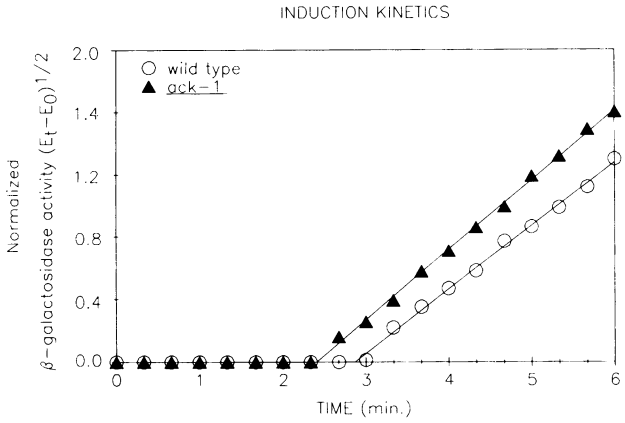

Fig. 6. Kinetics of β-galactosidase induction. *E.coli* strain CP79 bearing the wild-type (○) or *ack-1* (▲), mutations were grown in LB medium and *lacZ* transcription induced following addition of IPTG and cAMP as described in Materials and methods. The data are presented according to the square root plot of Schleif (Schleif *et al.*, 1973) in order to linearize the induction kinetics, where $E_{(0)}$ represents the basal enzyme level at time zero, and $E_{(t)}$ represents the amount of enzyme present at time t. Data are representative of four separate experiments.

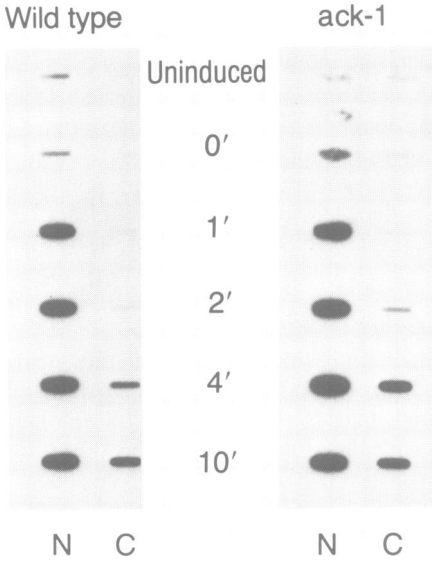

Fig. 7. *LacZ* mRNA induction kinetics. Total mRNA extracted at various times following induction from either wild-type (left panel) or *ack-1* mutant (right panel) was hybridized with either a ^{32}P-labeled PCR amplified N-terminal (**lanes N**) or C-terminal (**lanes C**) *lacZ* probe as described in Materials and methods. The percentage of maximum hybridization to the probe at each time point was calculated after subtraction of background hybridization from the uninduced sample and setting the 10 min same point to 100% hybridization. Densitometry scanning thus reflected the following levels of hybridization at each time point: wildtype (left panel) [**lane N**: uninduced, <1%; 0′, <1%; 1′, 95%; 2′, 100%; 4′, 100%; 10′, 100% and **lane C**: uninduced, <1%; 0′, <1%; 1′, <1%; 2′, <1%; 4′, 48%, 10′, 100% and *ack-1* mutant (right panel) [lane N: uninduced, <1%; 0′, 18%; 1′, 91%; 2′, 100%; 4′, 100%, 10′ 100% and lane C: uninduced, <1%; 0′, <1%, 1′, <1%; 2′, 15.2%; 4′, 100%; 10′ 100%].

an 11−17% difference in the average RNA chain growth rate.

Mapping the ack-1 mutation

The mutation which confers rifampicin resistance was localized to a small segment of *rpoB* by the deletion mapping

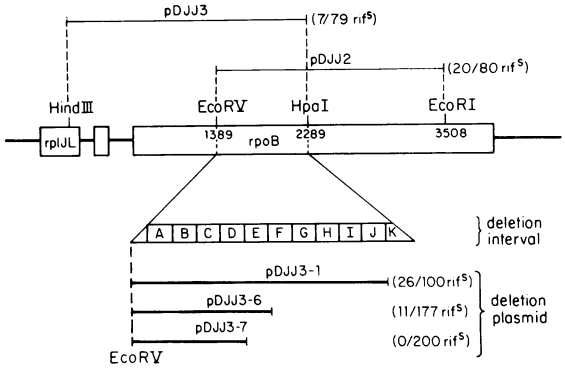

Fig. 8. Deletion plasmid mapping of the *ack-1* mutation,. The top line represents the *rplJL* operon containing the *rpoB* gene. Numbers within the *rpoB* gene indicate nucleotide positions for the *Eco*RV, *Hpa*I and *Eco*RI sites (Ovchinnikov *et al.*, 1981). Horizontal lines represent the amount of *rpoB* DNA remaining in different deletion plasmids (Jin and Gross, 1988). Strain DS410 was transformed with the *rpoB* deletion plasmids in the figure above and ampicillin resistant, rifampicin sensitive recombinants were scored following three rounds of cycloserine selection (Jin and Gross, 1988). Numbers to the right indicate the fraction of rifampicin sensitive cells observed following interaction with the indicated deletion plasmids.

method of Jin and Gross (1988). The *ack-1* strain was transformed with plasmids carrying deletions of various regions of the wild-type *rpoB* gene, and the formation of rifampicin sensitive recombinants was assessed. As Figure 8 shows, the mutation falls in the 'E' deletion interval contained on plasmid pDJJ 3-6, and missing in plasmid pDJJ 3-7, representing amino acids 565−576 of the RNA polymerase β-subunit.

Discussion

RNA polymerase purified from wild-type *E.coli* was found to hydrolyze NTPs which are not complementary to the DNA template. In contrast, purified enzyme from our previously described *ack-1* mutant was entirely devoid of the noncognate NTPase activity. Inasmuch as the mutant enzyme also exhibits an increase in transcriptional errors, the noncognate NTPase activity appears to be connected with the accuracy of transcription. A model consistent with our results is that the noncognate NTPase activity serves a proofreading function i.e. wild-type polymerase compares NTPs entering the elongation complex for base complementarity with the template, and ejects noncomplementary bases through a discard branch reaction (Ninio, 1975; Ninio *et al.*, 1975) following their hydrolysis to NDPs.

Several lines of evidence indicate that the NTPase activity is not due to a nonspecific hydrolase contaminating the polymerase preparation. First, hydrolysis of NTPs is only seen in the presence of DNA, and hydrolysis depends upon the nature of the template, which is to say it is instructed by the template. This feature is not easily reconciled with the notion of a simple contaminating hydrolytic activity. Second, we have detected the noncognate NTPase activity in three different preparations of wild-type polymerase, and one from another rifampicin resistant mutant which does not exhibit an increased error frequency (data not shown). Third, SDS−PAGE analysis of the wild-type RNA polymerase preparations revealed no bands other than the expected RNA polymerase subunits. Fourth, the NTPase is not affected by 0.5 mM pyrophosphate (data not shown), which abolishes

rho ATPase (Kent and Guterman 1982). Fifth, the NTPase activity is blocked by substances that rather specifically inhibit RNA polymerase, such as 0.4 M NH_4Cl, which reversibly inhibits template binding and streptolydigin, which inhibits chain elongation. The latter results suggest that NTP hydrolysis requires both association of RNA polymerase with a DNA template and active movement along the template, and therefore that it may be part of the transcription process itself.

The kind of proofreading mechanism we postulate involves a discrimination step in the selection of correct NTPs before their incorporation into RNA. This discrimination step must take a certain amount of time. Its absence in the mutant raised the formal possibility that transcription might proceed faster than normal in the mutant. Consistent with this possibility, transcription of the *lacZ* gene does proceed slightly faster in the mutant strain than in its wild-type parent (Figures 6 and 7). As RNA polymerase is known to spend a significant amount of time pausing during transcription (Kassavetis and Chamberlin, 12981; Greenblatt *et al.*, 1981), the present results raise the question of whether pausing by the elongation complex is in part a reflection of proofreading at noncomplementary bases.

The mutation which lowers accuracy and abolishes the putative proofreading NTPase maps to a small segment of the *rpoB* gene. Several other rifampicin resistant mutant alleles which do not affect accuracy map to different segments entirely (data not shown). Therefore, the E segment of the *rpoB* gene may be one domain which is responsible for maintaining transcriptional accuracy through the NTPase proofreading pathway.

Materials and methods

Reagents and general procedures

Alpha-^{32}P-labeled ATP, GTP and CTP were obtained from the Amersham Corporation and used without further purification., Poly (dC), (dT), and [d(A-T)·d(A-T)] were purchased from Pharmacia. Restriction enzymes and T4 DNA ligase were obtained from Boehringer-Mannheim or Bethesda Research Laboratories and used according to the manufacturer's suggestions. DNA fragments were separated following electorphoresis through low-gelling agarose (FMC Corporation), and purified on Elutip^{TM}-d columns (Schleicher and Schuell). Isopropyl-β-D-thiogalactoside (IPTG) and cyclic adenosine monophosphate (cAMP) were obtained from Sigma. Streptolydigin was a gift from the Upjohn Company (Kalamazoo, Michigan). Purified RNA polymerases (wild-type and rif^r holoenzymes) were generously provided by R.Burgess (University of Wisconsin) and were purified to homogeneity as described by Burgess and Jendrisak (1975) through ammonium sulfate precipitation of the polymin P eluate followed by chromatography through DNA−agarose and Sephacryl S300 as previously described (Blank *et al.*, 1986). PEI-cellulose was purchased from E.M.Science. Densitometry scanning of autoradiograms was performed on a Quick Scan Flur-Vis densitometer (Helena Laboratories, Beaumont, TX) according to the manufacturer's instructions. Hybridization solutions were prepared as previously decribed (Maniatis *et al.*, 1982).

Bacterial strains, plasmids and media

Escherichia coli strains CP 79, *relA2 leu thr arg his pro*λ supE44 (Gallant *et al.*, 1982), and an isogenic *ack-1* mutant (Blank *et al.*, 1986) were used for the *in vivo* assay of β-galactosidase activity. The *ack-1* mutation was transferred by P1 mediated transduction from strain CP79 to strain DS410 (F$^-$ *ara azi ton A^r minA minB str xyl mtl thi*), selection being for resistance to 50 μg rifampicin/ml on LB plates. Plasmids (pDJJ 3, pDJJ 3-1, pDJJ 3-6, pDJJ 3-7 and pDJJ 2) containing regions of the wild-type *rpoB* gene kindly provided by D.-j.Jin, University of Wisconsin, were used to map the *ack-1* mutation in strain DS 410 as described (Jin and Gross, 1988). Plasmid p(C_2AT)1g, containing a 380 bp G-less cassette (Sawadago and Roeder, 1985) was provided by M.Sawadago (Rockefeller University) while plasmid pMLB 1113.215, bearing an intact *lacZ* gene and which is closely related to plasmid pMLB1069 (Sisk *et al.*, 1986) was generously provided by M.Berman (Molecular Oncology, Rockville, MD). LB medium was prepared as previously described (Miller, 1972).

Transcription assays

In vitro transcription reactions were performed as previously described (Volloch *et al.*, 1979) with the following modifications: reactions were performed at 37°C for 30 min in 25 μl of reaction mixture containing 40 mM Tris−HCl pH 7.9, 150 mM NaCl when poly(dC), poly[d(A-T)·d(A-T)] or the G-less containing cassette were used as template or 70 mM NaCl when poly(dT) was present, 4 mM $MgCl_2$, 10 mM $MnCl_2$ (when either poly(dC) or poly(dT) were used as template), 14 mM β-mercaptoethanol,100 μg synthetic template/ml (or 10 μmol of the G-less containing cassette), 50 mM of each NTP, 0.4 Ci/mmol ^{32}P-labeled NTP and 2.5 μg of *E.coli* RNA polymerase. Noncognate hydrolysis of [α-^{32}P]ATP → [α-^{32}P]ADP, directed by poly(dC), was linear to 30 min. Error rate measurements involved incorporation of [α-^{32}P]GMP into RNA during polyd(T) transcription as previously described (Strniste *et al.*, 1973).

Reactions were terminated on ice and products resolved by ascending TLC on PEI-cellulose in 10% TCA for measurement of incorporation into RNA or in a stepwise pH 3.4 sodium formate solvent for resolution of nucleotides as follows: chromatography was initially allowed to proceed in 0.5 M Na^+HCOO^- for the first 2.5 cm followed by chromatography in 2 M Na^+HCOO^- for the next 2.5 cm and finally chromatographed to completion in 4 M Na^+HCOO^-.

Construction of the G-less transcription vector

Plasmid pRL1100, lacking G residues in the anti-sense strand, was constructed following restriction of the G-less containing vector, p(C_2AT)1g (Sawadago and Roeder, 1985), with *EcoRI and SstI*. The resulting vector fragment was ligated in a three-way reaction with a 329 bp *Eco*RI/*Hin*cII fragment from plasmid pDR540 (Russell and Bennett, 1982) which provided the 5′ half of the −35 region and a 31 bp *Hin*cII/*Sst*I ended oligonucleotide which introduces the remainder of the *trp* promoter −35 region and the *lac* UV5 promoter −10 region (P_{TAC}), as outlined in Figure 4. Transcription assays were performed with the 740 bp G-less cassette fragment following restriction and subsequent purification of plasmid pRL1100 with *Eco*RI/*Sma*I.

Kinetic measurements

V_{max} and K_m for hydrolysis of noncognate NTPs in the presence of 80 μg poly [d(A-T)·d(A-T)]/ml was determined with the wild-type (rif^S) RNA polymerase at five different ATP concentrations. Hydrolysis reactions were carried out for 5 min and terminated following addition of an equal volume of 10% TCA and reaction products resolved by TLC on PEI-cellulose in the step sodium formate solvent and counted in a liquid scintillation counting system.

In vivo β-galactosidase assays

E.coli strain CP79 containing the wild-type or *ack-1 rpoB* gene were grown to exponential phase (OD_{700}, ~3 × 10^8 cells/ml = 0.25) in LB or LB supplemented with 50 μg rifampicin/ml at 30°C. β-galactosidase was induced following addition of IPTG and cAMP (to 4 mM and 2 mM respectively) and 400 μl aliquots removed every 20 s for 5 min. Further synthesis of β-galactosidase was prevented by addition of 100 μg chloramphenicol/ml and chilling on ice. Samples were permeabilized by addition of 20 μl of lysis mixture (Putman and Koch, 1975) and β-galactosidase activity determined as previously described (Gallant *et al.*, 1982).

Construction of N- and C-terminal lacZ probes

Plasmid pMLB1113.215 was used as template to amplify two 250 bp DNA fragments corresponding to the extreme N- and C-terminal regions of *lacZ*. Each polymerase chain reaction (PCR) was primed with two 22 mer oligonucleotides chosen on the basis of *lacZ* sequence published by Kalnins *et al.* (1983). N-terminal primers 1(5′-GGCCGTCGTTTTACAACGTCGT3′) and 2(5′-GCGCATCGTAACCGTGCATCTG-3′) correspond to coordinate positions 1307−1329 and 1556−1578 respectively; C-terminal primers 3(5′-GAAAACGGTCTGCGCTGCGGGA-3′) and 4(5′-CCAACTGGTAATGGTAGCGACC-3′) correspond to coordinate positions 4086−4108 and 4316−4338 respectively. In these coordinates the *lacZ* ORF initiates at position 1282 and terminates at position 4356. Oligonucleotides were synthesized on an applied Biosystems Model 380B DNA synthesizer (Foster City, CA) according to the manufacturers specifications. The N-terminal (i.e. 1 and 2 above) and C-terminal (i.e. 3 and 4 above) oligonucleotide pairs were used in a primer-directed enzymatic amplification as previously described (Saiki *et al.*, 1988) using a GeneAmp kit (Perkin Elmer Cetus, Norwalk CT) and automated DNA-Thermal cycles (Perkin Elmer Cetus, Norwalk CT). N- and C-terminal amplified regions

were subsequently nick-translated with [α-^{32}P]dATP (NEN, Boston, MA) according to established procedures (Maniatis *et al.*, 1982).

RNA blot analysis

E.coli strain DS410 wild-type (or *ack-1* mutant) containing plasmid pMLB1113.215 was grown at 37°C, in 100 ml LB media supplemented with 50 μg ampicillin/ml, to an OD_{700} = 0.5. Cultures were split and half of the culture was induced with IPTG and cAMP as described above. Twenty milliliter aliquots were subsequently removed at 0, 1, 2, 4 and 10 min following induction and RNA synthesis was terminated by addition of 5 ml of 1 M KCN on ice. Total RNA was subsequently extracted according to the hot-phenol method of von Gabain (1983). Two micrograms of total mRNA from each time point (including an uninduced control) was denatured at 55°C for 15 min in a mixture containing 30 μl of 20 × SSC and 30 μl of 37% formaldehyde. Samples were applied in duplicate (see Figure 7, lanes N and C) to a Nitroplus 2000 nitrocellulose membrane (Micron Separations Inc., Westbore, MA) previously sealed in 20 × SSC using a multi-slot filtration manifold (Hoefer Scientific Instruments, San Fransciso, CA). Following immobilization of the RNA at 80°C for 1 h, the N- and C-terminal lanes were separated by cutting with a razor blade. Filter strips bearing the duplicate samples were prehybridized separately in heat sealed freezer bags containing 3 ml of hybridization cocktail for 1 h at 42°C. Denatured ^{32}P-labeled N- and C-terminal *lacZ* specific probes were added to each respective prehybridization mixture at 1 × 10^6 c.p.m./ml, and hybridization continued at 42°C for 18 h. Membranes were removed, washed down to 0.2 × SSC/0.1% SDS as previously described (Maniatis *et al.*, 1982) and exposed to Kodak X-Omat™ AR film, with enhancing screens for 24 h at −70°C.

Acknowledgements

We thank Richard Burgess for preparation of the purified RNA polymerase, D.-j Jin and Carol Gross for providing and instructing us on the use of RNA polymerase deletion plasmids prior to publication. We thank Ann Blank for providing us with poly [d(A-T)·d(A-T)] and for helpful discussion as well as Elizabeth (PMA) Yelverton for critical reviewing of the manuscript. Peggy Green and Rosalinda Szliuga are gratefully acknowledged for preparation of the manuscript. R.T.L. is a National Research Service Awardee (No. AG00057-10). This work was supported by grants NP-279J from the American Cancer Society and 5 ROI 6M 13626 from the NIH.

References

Anderson,R.P. and Menninger,J.R. (1986) In Kirkwood,T.B.L., Rosenberger,R.F. and Galas,D.J. (eds), *Accuracy in Molecular Processes*. Chapman and Hall, New York, pp. 159−189.

Blank,A. Gallant,J.A. Burgess,R.R. and Loeb,L.A. (1986) *Biochemistry*, **25**, 5920−5928.

Burgess,R.R. and Jendrisak,J.J. (1975) *Biochemistry*, **14**, 4634−4638.

Chamberlin,M.J. (1974) *Annu. Rev. Biochem.*, **43**, 721−775.

Denhardt,D.T. (1986) *Biochem. Biophys. Res. Commun.*, **23**, 641−646.

Erlich,H., Lazzarini,R. and Gallant,J. (1975) *J. Biol, Chem.*, **250**, 3057−3061.

Gallant,J. and Harada,B. (1969) *J. Biol. Chem.*, **244**, 3125−3132.

Gallant,J. Erlich,H., Weiss,R., Palmer,L. and Nyari,L. (1982) *Mol. Gen. Genet.*, **186**, 221−226.

Greenblatt,J., McLimont,M. and Hanley,S. (1981) *Nature*, **292**, 215−220.

Jin,D.-j. and Gross,C.A. (1988) *J. Mol. Biol.*, **202**, 45−58.

Kalnins,A., Otto,K., Ruether,U. and Mueller-Hill,B. (1983) *EMBO J.*, **2**, 593−597.

Kassavetis,G.A. and Chamberlin,M.J. (1981) *J. Biol. Chem.*, **256**, 2777−2786.

Kent,R.B. and Guterman,S.K. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3992−3996.

Loeb,A.A. and Kunkel,T.A. (1982) *Annu. Rev. Biochem.*, **52**, 429−457.

Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

McClure,W.R. (1980) *J. Biol. Chem.*, **255**, 1610−1616.

Miller,J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ninio,J. (1975) *Biochimie*, **57**, 587−595.

Ninio,J., Bernardi,F., Brun,G., Assairi,L., Lauber,M. and Chapeville,F. (1975) *FEBS Lett.*, **57**, 139−144.

Ovchinnikov,Y.A., Monastryrskaya,G.S. Gubanov,V.V., Guryev,S.O., Chertov,O.Y., Modyanov,N.N., Grinkevich,V.A., Makarova,I.A., Marchenko,T.Y., Polovnikova,I.N., Lipkin,V.N., Sverdlov,E.D. (1981) *Eur. J. Biochem.*, **116**, 621−629.

Putman,S.L. and Koch,A.L. (1975) *Anal. Biochem.*, **63**, 350−360.

Rosenberger,R.F. and Hilton,J. (1983) *Mol. Gen. Genet.*, **191**, 207−212.

Russell,D.R. and Bennett,G.N. (1982) *Gene*, **20**, 231−243.

Saiki,R.K., Gelfand,D.H., Stoffel,S,. Scharf,S.J., Higuchi,R. Horn,G.T., Mullis,K. B. and Erlich,H.A. (1988) *Science*, **239**, 487−491.

Sawadago,M. and Roeder,R. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4394−4398.

Schleif,R., Hess,W., Finkelstein,S. and Ellis,D. (1973) *J. Bacteriol.*, **115**, 9−15.

Singer,B. and Kröger,M. (1979) *Prog. Nucleic Acids Research Mol. Biol.*, **23**, 151−194.

Springate,C.F. and Loeb,L.A. (1975) *J. Mol. Biol.*, **97**, 577−591.

Sisk,W.P., Chirikjian,J.G., Lautenberger,J., Jorcyk,C., Papas,T.S., Berman,M.L., Zagursky,R. and Court,D.L. (1986) *Gene*, **48**, 183−193.

Strniste,G.F., Smith,D.A. and Hayes,F.N. (1973) *Biochemistry*, **12**, 603−608.

Volloch,V.Z., Rits,S. and Tumerman,L. (1979) *Nucleic Acids Res.*, **6**, 1535−1546.

von Gabain,A. Belasco,J.G., Schottel,J.L., Chang,A.C.Y. and Cohen,S.N. (1983) *Proc. Natl. Acad. Sci, USA*, **80**, 653−657.

Zillig,W., Zechel,K., Rabussay,D., Schachner,M., Sethi,V.S., Palm,P., Heil,A. and Seifert,W. (1970) *Cold Spring Harbor Symposia on Quantitative Biol.*, XXXV, 47−58 Cold Spring Harbor, NY.

Received on May 5, 1989