An RNA Polymerase Mutant with Reduced Accuracy of Chain Elongation†

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ABSTRACT: A new *Escherichia coli* RNA polymerase mutant was isolated which exhibited reduced accuracy of chain elongation in vivo and in vitro. The novel isolation procedure consisted of simultaneous selection for rifampicin resistance and screening for increased leakiness of an early, strongly polar nonsense mutation of *lac*Z, one of a special class of mutations whose leakiness reflects mainly transcriptional rather than translational errors. The spontaneous mutant thus isolated displayed a 3–4-fold increase in the leakiness of two different *lac*Z mutations of this class. Transduction analysis indicated that a single mutation, mapping in or very near the *rpo*B gene for the β subunit of RNA polymerase, conferred both rifampicin resistance and increased nonsense leakiness. In an in vitro fidelity assay, homogeneous RNA polymerases from the mutant and parent strains exhibited error rates of $1/0.90 \times 10^5$ and $1/2.0 \times 10^5$, respectively, for the poly[d(A-T)]·poly[d(A-T)]-directed misincorporation of noncomplementary GMP. These error rates were verified by product analyses which further revealed that GMP was misincorporated in place of AMP in the synthesis of poly[r(A-U)]. The error rate of wild-type K12 RNA polymerase from a different source was $1/2.0 \times 10^5$, while that of a hybrid RNA polymerase, containing mutant core enzyme and wild-type σ subunit, was $1/0.64 \times 10^5$. These error rates confirmed the selection of a transcriptional accuracy mutant. The error frequencies observed are much lower than those reported in other in vitro assays. The safeguards used to avoid artifactually enhanced misincorporation, and to thereby quantitate lower error rates, are discussed.

Transcription of DNA by *Escherichia coli* RNA polymerase is a complex sequence of events including template binding, RNA chain initiation, RNA chain elongation, and chain termination. The specificity and mechanisms of template binding and of chain initiation and termination have been studied extensively [e.g., see McClure (1985), von Hippel et al. (1984), Chamberlin et al. (1982), Fisher & Yanofsky (1983), and Ryan & Chamberlin (1983)]. In contrast, less is known about the accuracy of chain elongation and its determinants. Recently, in vivo error rates of $1/4.0 \times 10^3$ to $1/1.2 \times 10^5$ have been estimated for chain elongation at several nonsense codons in the *lac*Z gene for β-galactosidase. Accuracy within this large range varied for different nonsense codons in the same position, and for the same codon at different positions (Rosenberger & Hilton, 1983). Although measurement in vitro of the accuracy of chain elongation has not been possible with DNA templates, error rates have been estimated for transcription of synthetic polydeoxynucleotide templates [reviewed by Chamberlin (1974) and Anderson & Meninger (1986)]. These error frequencies vary widely, the lowest being $1/4.2 \times 10^4$ (Springgate & Loeb, 1975).

The genetic determinants and biochemical mechanisms which govern the accuracy of chain elongation by RNA polymerase are not well-defined. The specificity of the enzyme has been investigated primarily by the use of nucleoside triphosphate substrates or polynucleotide templates containing base analogues (Chamberlin, 1974; Anderson & Meninger, 1986). Elucidation of the mechanisms controlling fidelity of chain extension would be facilitated by analysis of RNA polymerase mutants with altered accuracy. Yet we know of no RNA polymerase mutations which have been shown to cause errors in elongation both in cells and in vitro. We introduce here a screening procedure to identify RNA polymerase fidelity mutants through detection of errors in chain elongation in vivo. One such mutant was isolated, and to validate the screen, its RNA polymerase was purified and characterized. The mutant polymerase exhibited a modest increase in the frequency with which GMP was incorporated in place of AMP during in vitro transcription of poly[d(A-T)]·poly[d(A-T)]. The success of the screen forecasts its utility for isolation of additional RNA polymerase mutations which affect the fidelity of chain propagation. Study of altered RNA polymerases should help to define the genetic determinants and biochemical mechanisms which maintain the accuracy of the elongation process. Such investigation may be especially timely, since our work, together with the in vivo data of Rosenberger and Hilton (1983), indicates that *E. coli* RNA polymerase is capable of greater accuracy of chain propagation than has been previously shown.

Materials and Methods

Bacterial Strains. The accuracy mutant described here was derived from *E. coli* CP79-U118 (*thr*⁻ *leu*⁻ *his*⁻ *arg*⁻ *thi*⁻ *rel*A-2 *lac*Z-U118). Construction of the parent and other strains used is described in Gallant et al. (1982) (see also Table I). The rationale for the mutant isolation procedure is given under Results. The accuracy mutant was isolated by plating about 10^{10} cells of an exponential culture of the parent strain, without mutagenesis, on glucose minimal medium containing 1% nutrient broth, 100 μg/mL rifampicin, 5 mM cAMP, 2 mM isopropyl β-D-thiogalactoside, and 400 μg/mL final

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concentration of the indicator 5-bromo-4-chloro-3-indolyl β-D-galactoside. On this concentration of indicator, colonies which are rifampicin resistant but have normal levels of β-galactosidase synthesis remain white, with the faintest hint of blue, after 3 days of incubation; increased β-galactosidase synthesis yields colonies which are visibly bluer. Several hundred rifampicin-resistant colonies were examined, and four were picked which seemed to have the blues. Three were false positives, but one exhibited a stronger indicator reaction on restreaking and proved to have a 4-fold increase in β-galactosidase activity. This mutant is the one described herein. Cell cultivation and genetic methods were as in Gallant et al. (1982).

Materials. Poly[d(A-T)]·poly[d(A-T)] was prepared by de novo synthesis with *E. coli* DNA polymerase I (Schachman et al., 1960). After phenol extraction and ethanol precipitation, the reaction product was chromatographed on a column of Sephadex G-100 [Pharmacia, 0.7 × 45 cm, equilibrated with 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl),[1] 0.1 M KCl, and 1 mM EDTA, pH 7.5]. Material eluting in the void volume was used.

Radioactive nucleoside triphosphates were from New England Nuclear. The [8-^{3}H]GTP preparation used exclusively in this study (lot 1550-287) was concentrated 2–3-fold at 0 °C under a gentle stream of nitrogen. Its nominal specific activity (10.5 Ci/mmol) was corroborated by measurement of radioactivity and ultraviolet absorbance. Radiochemical purity of this lot of [8-^{3}H]GTP and of [α-^{32}P]UTP (nominally >99.9% and 97% upon initial preparation, respectively) was monitored by quantitative thin-layer chromatography; following completion of experiments, ≥95% of the counts recovered chromatographed as GTP and UTP, respectively. Importantly, the [8-^{3}H]GTP preparation showed no radioactive contaminants which migrated as nucleoside triphosphates upon chromatography and autoradiography of 5 μCi. The plates used were PEI–cellulose (Brinkmann), and the solvents were those in the stepwise LiCl and sodium formate procedures described in Figure 88 of Randerath (1966). Three other radioactive GTP preparations from the same supplier—two [α-^{32}P]GTP and one [8,5′-^{3}H]GTP—were examined in preliminary fidelity assays and gave ca. 5–25 times the apparent misincorporation recorded in Table II for the parent RNA polymerase. One of these three preparations was highly heterogeneous on chromatography, and the other two were not examined. These three preparations were synthesized by enzymatic phosphorylation of guanosine in the presence of kinases and an ATP-generating system. In contrast, the exceedingly pure [8-^{3}H]GTP used in this work was synthesized by catalytic dehalogenation of 8-Br-GTP with ^{3}H gas. Nonradioactive ATP, UTP, and GTP preparations were estimated to be ≥95% pure by visual inspection of chromatograms.

Purification of RNA Polymerases. Electrophoretically homogeneous RNA polymerase holoenzyme from *E. coli* K12 cells (purchased from Grain Processing, Muscatine, IA) was purified as previously described (Lowe et al., 1979; Burgess & Jendrisak, 1975). The mutant strain CP79-U118 rif^{r,b} and its parental strain CP79-U118 were grown at 37 °C in Bactotryptone–yeast extract medium to an A_{550} of 0.7–1.0 and harvested with yields of 200–400 g/150 L. Portions (100 g) were used to prepare RNA polymerase as described by Burgess and Jendrisak (1975), through ammonium sulfate precipitation of the Polymin P eluate. The dissolved and diluted sample (325 mL) was then loaded on a 35-mL single-stranded DNA–agarose column prepared as described by Lowe et al. (1979). After the column was washed with TGED + 0.25 M NaCl, core polymerase was eluted with TGED + 0.40 M NaCl, and then holoenzyme was eluted with TGED + 1.0 M NaCl, at a flow rate of 50 mL/h. The holoenzyme peak was concentrated by ammonium sulfate precipitation and chromatographed on a 500-mL Sephacryl S300 (Pharmacia) gel filtration column equilibrated with TGED + 0.50 M NaCl. The peak was dialyzed against storage buffer and the concentration determined spectrophotometrically. Both mutant and parental polymerases were free of detectable impurity bands upon SDS gel electrophoresis of 5 μg (Lowe et al., 1979). A hybrid RNA polymerase was obtained by combining equimolar quantities of σ subunit from the commercially obtained K12 cells and core polymerase from the mutant strain, both purified to electrophoretic homogeneity as described in Lowe et al. (1979).

The specific activities, at 37 °C, of homogeneous RNA polymerases from the mutant and parent strains and from the purchased K12 cells were 1.47×10^3, 1.47×10^3, and 1.53×10^3 units/mg of protein, respectively (assay procedure described below). At 42 °C, the specific activities were 1.34×10^3, 1.96×10^3, and 2.25×10^3 units/mg of protein, respectively. RNA polymerase from the rifampicin-resistant mutant thus exhibited an altered temperature dependence of the rate of transcription, relative to its rifampicin-sensitive parent.

Assay of RNA Polymerase Activity. RNA polymerase activity was assayed by measuring the poly[d(A-T)]·poly[d(A-T)]-directed incorporation of radioactivity from [α-^{32}P]-UTP into acid-insoluble material [e.g., see Burgess (1969) and Berg et al. (1971)]. Assay mixtures contained, in 100 μL, 100 μM poly[d(A-T)]·poly[d(A-T)], 25 μM ATP, 25 μM [α-^{32}P]UTP (35–55 cpm/pmol), 50 mM Tris-HCl (pH 7.6), 150 mM KCl, 10 mM $MgCl_2$, 0.4 mM potassium phosphate, 0.4 mM EDTA, 0.1 mM dithiothreitol, and 0.6–1.7 μg of RNA polymerase. Reactions were carried out for 10 min at 37 °C and were terminated by addition, with vigorous mixing, of 100 μL of ice-cold denatured calf thymus DNA (2 mg/mL) followed by 1 mL of ice-cold perchloric acid–0.1 M sodium pyrophosphate. After standing for 15 min on ice, precipitates were collected on glass fiber filters (Schleicher & Schuell, 2.4 cm) and washed 5 times with 2 mL of cold 1 M HCl–0.1 M sodium pyrophosphate and twice with 2 mL of cold 95% ethanol. Filters were dried and counted in 3.5 mL of Econofluor (New England Nuclear) in a Beckman LS7500 scintillation counter. Incorporation of ^{32}P was linear with added enzyme up to at least 1.7 μg (ca. 400 pmol of UMP incorporated). Assays were also carried out at 42 °C and were linear with added enzyme up to at least 500 pmol of UMP incorporated. One unit of activity is defined as 1 nmol of UMP incorporated per hour under the assay conditions. Each enzyme preparation was assayed at two or at three concentrations, the standard deviation of the determinations always being less than 5.4% of the mean.

Assay of RNA Polymerase Fidelity. Fidelity was determined by using a modification of the method of Springgate and Loeb (1975). The assay employed poly[d(A-T)]·poly[d(A-T)] as template-primer and measured the simultaneous incorporation of radioactivity from complementary [α-^{32}P]-UTP and from noncomplementary [8-^{3}H]GTP into a high molecular weight transcription product(s). The ratio of noncomplementary to complementary nucleotidyl residues incorporated under the assay conditions was defined as the

[1] Abbreviations: TGED, 0.01 M Tris-HCl (pH 7.9), 5% (v/v) glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; PEI, poly(ethylenimine); SDS, sodium dodecyl sulfate.

error rate. Reaction mixtures (100 μL) were the same as for RNA polymerase activity assays but included 30 μM [8-^{3}H]GTP (2.33 × 10^4 dpm/pmol) in addition to 25 μM each of ATP and [α-^{32}P]UTP (0.2–0.4 cpm/pmol) and 3 μg of enzyme. Reactions were incubated for 45 or 90 min at the indicated temperatures and were terminated by addition of a reagent (50 μL, preequilibrated at 37 °C) containing 17 mM EDTA (Na^+) (pH 7.6), 2.6 mM GTP, 15% glycerol, and Blue Dextran (A_{550} = 1.8, Pharmacia). Terminated reaction mixtures were incubated 5 min further at 37 °C and then placed in ice. (Mixtures could be frozen at this point without an effect on fidelity.)

To separate polymeric transcription products from the nucleoside triphosphate substrates, each terminated reaction mixture (150 μL) was applied to a column of Sephadex G-100 (0.7 × ca. 45 cm) and chromatographed in 0.1 M Tris-HCl, 0.15 M KCl, and 25 mM EDTA (pH 7.5). Fractions (15 drops, ca. 0.45 mL) were collected, and those four to six fractions containing visible Blue Dextran were pooled. The same number of fractions, eluted 10 fractions prior to the Blue Dextran peak and containing column buffer alone, was pooled to serve as a blank. Both blank and product-containing pools were counted for 50 min in 15 mL of Aquasol-2 (New England Nuclear).

Characterization of Transcripts. To obtain products for characterization, poly[d(A-T)]·poly[d(A-T)] was transcribed as in the fidelity assays; incubation was for 45 min at 37 °C (e.g., reactions 1a and 1b, Table II). However, to increase the amount of ^{3}H available for analysis, the concentration of [^{3}H]GTP was doubled. To isolate transcripts, terminated reactions were first chromatographed on Sephadex as for fidelity assays. The pooled Blue Dextran containing fractions were then made 2 M in ammonium acetate and 67% in ethanol, held overnight at −70 °C, and centrifuged at 0 °C for 1 h at 86000*g* or for 2 h at 38000*g*.

For digestion with RNase A, the pelleted material from 100 μL of the initial transcription reaction mixture was resuspended in 33 μL of 10 mM Tris-HCl and 1 mM $MgCl_2$ (pH 7.5). DNase I [Worthington, 2 μL of a 20 μg/mL solution in activating buffer (Rigby et al., 1977)] was first added, and the mixture was incubated for 30 min at 37 °C; this preliminary step was included to degrade the poly[d(A-T)]·poly[d(A-T)], which might have protected transcripts from RNase attack. Four microliters of a solution containing 13 μg/mL RNase A (Worthington, electrophoretically purified) was added, and the mixture was incubated for 2 h, or for 6 h, at 37 °C. Digests were subjected to anion-exchange high-pressure liquid chromatography as described in the legend to Figure 1.

For digestion to 3′-mononucleotides, the pelleted material from 100 μL of the initial transcription reaction mixture was resuspended and treated with DNase I as described above. Four microliters of a solution containing 13 μg/mL RNase A and 9 units/mL RNase T_1 (ca. 30 μg/mL, Bethesda Research Laboratories) in 0.1 M EDTA (Na^+) (pH 7.0) was added, and the mixture was incubated for 2 h at 37 °C. Finally, 11 μL of a solution containing 127 units/mL RNase T_2 (ca. 4.4 μg/mL, Bethesda Research Laboratories) in 0.23 M potassium acetate (pH 4.0) was added, and the digest was incubated for 2 h at 37 °C. Digests were subjected to reversed-phase high-pressure liquid chromatography as described in the legend to Figure 2.

Results

Isolation and Characterization of Mutant with Increased Errors in Vivo. (*a*) *Rationale.* Our screen for RNA polymerase fidelity mutants employs a parent strain with a strongly polar nonsense allele in the *lacZ* gene for β-galactosidase. Nonsense alleles which are not strongly polar are inappropriate for our screen for the following reason. Leaky enzyme synthesis by nonsense mutants comprises two linearly independent processes: errors in transcription and errors in the translation of correctly transcribed mRNA. Translational errors are overwhelmingly predominant for most nonsense mutations, as shown by the fact that the *rpsL*-A1 (formerly called *strA*1) mutation, which increases ribosome accuracy, reduces leakiness by a factor of 10 or more (Strigini & Brickman, 1973). If 90% or more of background leakiness reflects translational errors, then moderate increases in transcriptional errors made by RNA polymerase will be undetectable above this background.

In contrast, the translational component of background leakiness appears to be diminished in strongly polar nonsense mutations located near the beginning of a gene. These alleles permit very little full-length mRNA to be made, because transcription terminates shortly beyond the position of premature translation termination (Adhya & Gottesman, 1978). The consequent reduction in full-length mRNA would be expected to reduce the contribution of translational errors to background leakiness. Indeed, strongly polar nonsense alleles exhibit a background leakiness about 20 times lower than the average for all nonsense mutations (Rosenberger & Foskett, 1981; Kurland & Gallant, 1986). Strong circumstantial evidence indicates that the leakiness of this class of nonsense alleles is principally due to transcriptional errors (Rosenberger & Foskett, 1981; Rosenberger & Hilton, 1983). In agreement with this interpretation, we have found that the leakiness of one of these mutant alleles, U118 at position 18 of the *lacZ* coding sequence, is reduced only slightly by the presence of the *rpsL*-A1 allele which increases ribosome accuracy (data not shown). We conclude that the leakiness of strongly polar nonsense mutations should provide a reasonably sensitive index of the frequency of transcriptional errors and, hence, a screen for RNA polymerase fidelity mutants.

(*b*) *Observations.* Our screen employs a parent strain carrying the strongly polar U118 allele mentioned above. We selected rifampicin-resistant mutants, which are almost exclusively in the *rpoB* gene coding for the β subunit of RNA polymerase, and screened them for increased leakiness of *lacZ*-U118. Leakiness was detected visually on selective plates containing both rifampicin and a chromogenic substrate for β-galactosidase. Out of several hundred rif^r colonies, we found one which showed increased leakiness, i.e., increased blue color, on the selective plates. We then assayed β-galactosidase activity in a liquid culture of this mutant strain, designated CP79-U118 rif^{r,b}. As recorded in Table I, the mutant exhibited a 4-fold increase in the leakiness of U118, i.e., in the specific activity of β-galactosidase (entries 1 and 2), although wild-type enzyme synthesis in a *lacZ*$^+$ derivative was unaffected (entries 7 and 8).

The rif^{r,b} mutation was outcrossed by transduction with the following results: (1) Fifteen rif^{r,b} transductants selected for rifampicin resistance all showed increased leakiness of U118 (Table I, rows 3 and 4), indicating that the two phenotypes were due to the same or very closely linked mutations; as the mutation was spontaneous, rather than mutagen induced, the chance that two closely linked mutations were responsible for both phenotypes is negligible. (2) Transduction of rif^{r,b} into a strain carrying another early, strongly polar nonsense mutation in *lacZ* also increased its leakiness (Table I, rows 5 and 6). (3) To determine whether the rif^{r,b} mutation mapped in the *rpoB* gene, as expected, we transferred rifampicin resist-

Table I: Effect of the rif^{r,b} Mutation on β-Galactosidase Activity[a]

strain	sp act. (units/mg of protein × 10^3)	standard error	no. of replicates
CP79-U118	1.17	0.10	9
CP79-U118 rif^{r,b}	4.37	0.50	4
MO-U118	1.14	0.07	3
MO-U118 rif^{r,b} (15 independent transductants)	4.63 (range 3.8–6.4)	0.17	15
CP79-Z2	1.4		1
CP79-Z2 rif^{r,b}	3.9		1
CP79-U118 *lacZ*$^+$	90000	5000	4
CP79-U118 *lacZ*$^+$ rif^{r,b}	105000	7300	3

[a] β-Galactosidase specific activity was measured according to Gallant et al. (1982). Briefly, cells were grown in liquid culture, and the *lac* operon was induced by addition of isopropyl thiogalactoside and cAMP. For nonsense mutants, induced cells were centrifuged and sonicated, and crude extracts were assayed for β-galactosidase activity with *o*-nitrophenyl galactoside as a substrate. For *lacZ*$^+$ strains, induced cells were permeabilized with toluene and Triton X-100 and added to assay mixtures. The origins of the *E. coli* K12 strains are also described in Gallant et al. (1982). CP79-U118 is the parent strain used for isolation of the rif^{r,b} mutation. U118 is an extreme polar ochre (UAA) allele at the codon specifying amino acid 17 of the β-galactosidase monomer. The rif^{r,b} mutation was transferred to MO-U118 by P1 phage transduction. CP79-Z2 carries the extreme polar ochre mutation *lacZ2* at the codon specifying amino acid 23 [see Rosenberger & Foskett (1981)]. The rif^{r,b} mutation was transduced into CP79-U118 *lacZ*$^+$, an isogenic revertant derived from U118.

ance into an *arg*H$^+$ *thi*$^+$ recipient strain and tested for cotransduction of the donor *arg*H$^-$ and *thi*$^-$ alleles, which flank the *rpo*B gene. Out of 60 rifampicin^r transductants, 14 were *arg*$^+$ *thi*$^+$, 21 were *arg*$^+$ *thi*$^-$, 10 were *arg*$^-$ *thi*$^+$, and 15 were *arg*$^-$ *thi*$^-$. These cotransduction frequencies indicate that the rifampicin resistance mutation in rif^{r,b} maps in between *arg*H and *thi*, about 0.5 min from the former and 0.3 min from the latter, in close agreement with the location of *rpo*B (Bachman, 1983).

The most direct explanation for the above results is that a single mutation in the *rpo*B gene conferred both rifampicin resistance as well as a modest increase in nonsense leakiness due to errors in chain elongation. Other phenotypic properties of the mutant include an ca. 40% reduction in growth rate and enhanced sensitivity to 5-fluorouracil and 5-azacytidine, relative to the parent strain (data not shown).

Reduced Fidelity in Vitro of the Mutant RNA Polymerase. To test the interpretation of the genetic analysis that the rif^{r,b} mutation causes increased errors in chain elongation, RNA polymerases were isolated from the rifampicin-resistant mutant CP79-U118 rif^{r,b} and from its rifampicin-sensitive parent CP79-U118. Accuracy of the mutant and parent RNA polymerases, both purified to apparent homogeneity, was assessed by using a modification of the fidelity assay of Springgate and Loeb (1975). Employing poly[d(A-T)]·poly[d(A-T)] as template-primer, the assay utilizes misincorporation of noncomplementary [8-^{3}H]GTP as an index of error. As indicated in Table II, describing fidelity assays at several temperatures, RNA polymerase from the mutant strain exhibits an error rate twice that of RNA polymerase from the parent strain. The assays described in Table II were conducted in pairs (e.g., reactions 1a and 1b), the two members of each pair differing only in the source of polymerase. Reaction pairs 1 through 4 were carried out on different occasions and represent four separate comparisons of enzymes from the mutant and parent strains. The 2-fold increase in the misincorporation of [^{3}H]GTP catalyzed by the mutant polymerase is thus reproducible, the difference in error rates for the parent and mutant polymerases being highly significant ($P < 0.005$) when analyzed as either paired or independent samples by Student's *t* test. The enhancement in error frequency is commensurate with the increment in β-galactosidase activity found for the mutant strain (Table I).

The results in Table II thus support the conclusion of the genetic analysis that the rif^{r,b} mutation confers a modest increase in the inaccuracy of chain elongation. In addition, the data reveal that *E. coli* RNA polymerase can elongate chains with high fidelity. The error rate measured for the mutant RNA polymerase at 37 °C is $1/0.90 \times 10^5$, and that for the parent enzyme is $1/2.0 \times 10^5$. These error frequencies are lower than any previously reported for an RNA polymerase.

Verification of Error Rates by Characterization of Transcripts. The foregoing fidelity assays demonstrate that there is a difference in accuracy between the mutant and parent RNA polymerases and that, as anticipated, the difference is small. The error rates found for both enzymes are low and, especially for the parent RNA polymerase, approach the limit of sensitivity of the assay. Therefore, to provide confirmation of these error frequencies, a detailed characterization was

Table II: Error Rates of Mutant and Parent RNA Polymerases in Fidelity Assays[a]

reaction	polymerase	incubn condn		^{3}H incorpn			^{32}P incorpn		
		°C	min	cpm[b]	fmol total	fmol net[c]	cpm[b]	pmol[d]	error rate[g]
1a	mutant	37	45	219	25.4	19.5	129	1761	$1/0.90 \times 10^5$
2a	mutant	30	45	241	27.9	22.0[e]	195	1777	$1/0.81 \times 10^5$
3a	mutant	24	45	204	23.6	17.8[f]	163	1571	$1/0.88 \times 10^5$
4a	mutant	19	90	85	11.1	5.3	83	435	$1/0.82 \times 10^5$
1b	parent	37	45	143	16.5	8.0	118	1601	$1/2.0 \times 10^5$
2b	parent	30	45	144	16.7	8.2[e]	174	1592	$1/1.9 \times 10^5$
3b	parent	24	45	116	13.5	6.0[f]	109	1054	$1/1.8 \times 10^5$
4b	parent	19	90	77	10.1	2.6	76	395	$1/1.5 \times 10^5$
control	mutant	37	0.5	45	5.9		0		
control	mutant	19	1.0	45	5.8		0		
control	parent	37	0.5	64	8.5		0		
control	parent	19	1.0	57	7.5		0		

[a] Error rates are based on simultaneous incorporation of label from complementary [α-^{32}P]UTP and noncomplementary [8-^{3}H]GTP in assays with poly[d(A-T)]·poly[d(A-T)] as a template-primer. Mutant and parent RNA polymerases were analyzed pairwise (e.g., reactions 1a and 1b), the source of polymerase being the only variable in two transcription reactions conducted simultaneously and identically. [b] cpm are corrected for a column blank (see Materials and Methods) and ^{3}H cpm for spillover from the ^{32}P channel (4.3–4.8%). [c] Net incorporation was determined by subtracting the femtomoles observed in control incubations where no incorporation of complementary nucleoside triphosphate was detected (last four rows in table). [d] Picomoles of nucleotidyl residues incorporated from both UTP and ATP. [e] Femtomoles observed in the 0.5-min control reaction at 37 °C was subtracted. [f] Femtomoles observed in the 1.0-min control reaction at 19 °C was subtracted. [g] Error rates are not corrected for a small pool bias, i.e., a 20% molar excess of GTP over ATP and UTP.

undertaken of the transcripts synthesized by the mutant and parent RNA polymerases.

(*a*) *Transcripts Are Poly[r(A-U)] Containing Rare GMP Residues in Place of AMP.* Error rates calculated in the fidelity assay are based on the assumption that both ^3H and ^{32}P labels are incorporated into the same polynucleotide, namely, poly[r(A-U)] containing rare guanylyl residues. This assumption was examined with respect to two other possibilities. One is that guanylyl residues are sequestered in poly(rA) sequences synthesized by a contaminant poly(rA) polymerase (Edmonds & Winters, 1976) or by RNA polymerase itself. The second is that guanylyl residues are correctly incorporated in a product(s) transcribed from DNA fragments contaminating one or both of the RNA polymerase preparations. These possibilities were tested by analyzing the products obtained from digestion of transcripts with pancreatic RNase A. The radioactive oligomers expected from RNase A digestion of possible transcripts are outlined in Table III, where it is noted that the ^3H-containing oligomers generated from poly[r(A-U)] will depend upon whether [^3H]GTP is (mis)-utilized in place of ATP or in place of UTP. Inspection of Table III reveals a cardinal feature of the predicted digestion products: If, and only if, [^3H]GTP is utilized in place of ATP for synthesis of poly[r(A-U)], will both ^{32}P and ^3H be recovered exclusively in dimers. For other transcription products, ^3H will reside partially or entirely in longer fragments. With this distinguishing feature in mind, RNase A digests of transcripts were subjected to high-pressure liquid chromatography under conditions (Gait & Sheppard, 1977) modeled after those of the classic Tomlinson and Tener (1963) separations where oligonucleotides are fractionated primarily on the basis of chain length (net charge) and where nonionic interactions are diminished.

As shown in Figure 1A, an exhaustive RNase A digest of tRNA provided markers for the elution regions of mononucleotides, dinucleotides, and longer fragments. The identity of peaks was ascertained by analogy with comparable profiles in the literature [e.g., see Katz & Dudock (1969) and Holley et al. (1965)] and was corroborated by chromatography of additional markers and digests as described in the figure legend. The elution pattern of radioactivity in an RNase A digest of transcripts synthesized by the mutant polymerase is shown in Figure 1B. Both ^3H and ^{32}P eluted in two peaks, one within and one ahead of the dinucleotide region. Most importantly, no detectable ^{32}P or ^3H eluted in regions corresponding to longer oligomers. The ^{32}P peak in the dinucleotide region coincided with the ApUp peak observed for tRNA exhaustively digested either with RNase A alone (Figure 1A) or with a combination of RNases A and T_1 (not shown). The ^3H peak in the dinucleotide region coincided with the GpUp peak found in tRNA exhaustively digested with RNase A alone, but absent from tRNA digested with both RNases A and T_1. The early ^{32}P and ^3H peaks eluted in the region of net charge equal to 2−, where dinucleotides terminating in 2′:3′-cyclic phosphate are expected (Rushizky et al., 1964). The ^{32}P peak was therefore identified as the ApU>p intermediate in RNase A digestion and, similarly, the ^3H peak as GpU>p. This assignment was corroborated by the observation that the proportion of counts appearing either as the 2′:3′-cyclic phosphate intermediates or as the 3′-phosphoryl terminated end products depended upon the time of digestion with RNase A. For example, Figure 1C shows the elution profile of radioactivity in a shorter (2 vs. 6 h) digestion of transcripts synthesized by the parent RNA polymerase. In this digest, ca. 19% of the total ^{32}P counts was converted to the ApUp end

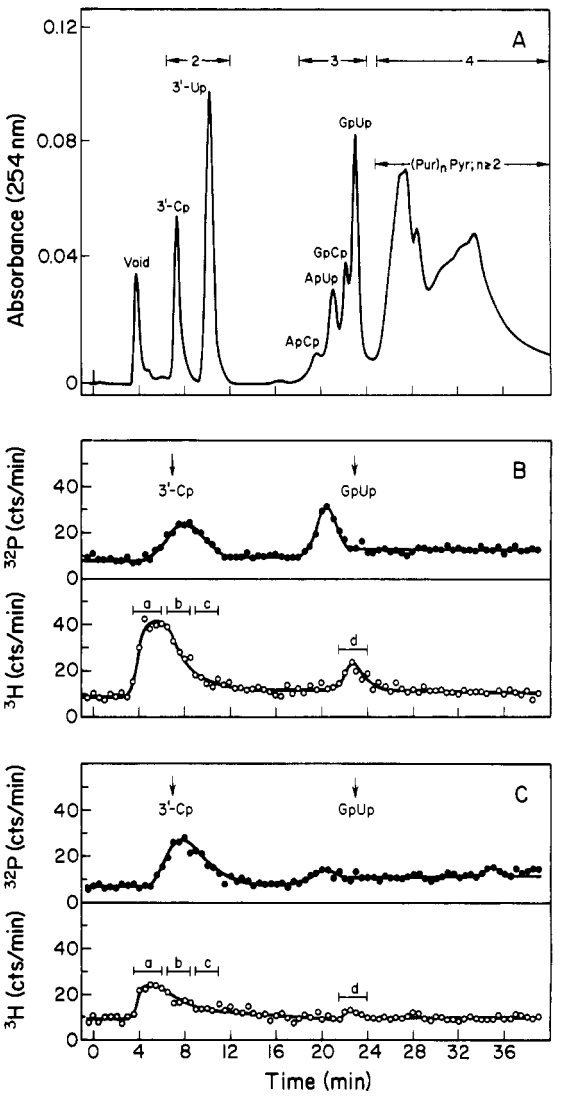

FIGURE 1: Anion-exchange chromatography of RNase A digested transcripts. (A) Absorbance standards. Column: Whatman Partisil-10 SAX (0.46 × 25 cm). Solvents: (A) 0.01 M potassium phosphate–0.01 M KCl (pH 6.8), and 20% methanol; (B) 0.15 M potassium phosphate–0.015 M KCl (pH 6.8), and 20% methanol (Gait & Sheppard, 1977). Gradient: 0–16% B, linear, 7 min; 16–100% B, linear, 11 min; 100% B, 64 min. Flow rate: 1 mL/min. Temperature: ambient. Detection: 254 nm. The first 40 min of the gradient, containing all but trailing absorbance, is shown. Standards were generated by digestion of *E. coli* tRNA (8.2 mg/mL) with RNase A (10 μg/mL) for 6 h at 37 °C in 30 mM Tris-HCl and 10 mM EDTA (Na^+) (pH 7.6). Digestion of tRNA with both RNases A and T_1 generated peaks (not shown) with retention times of 6 (3′-Cp), 9 (3′-Up), 12.5 (3′-Gp), 19 (ApCp), 20.5 (ApUp), and 23 min (ApGp), followed by partially resolved, longer oligomers. Additional markers (not shown) included guanosine, 3′–5′-adenylyluridine (ApU; Sigma Chemical Co.), and 3′–5′-guanylyluridine (GpU; Sigma); these compounds eluted in the void volume. (B and C) Digests of transcripts synthesized by the mutant (B) and parent (C) RNA polymerases. RNase A digests of material isolated from ca. 100 μL of initial transcription reactions, together with the absorbance standards shown in (A), were chromatographed as in (A). Fractions (0.5 mL) were collected and counted for 10 min in 3 mL of Aquasol-2 (New England Nuclear). Radioactivity profiles include background. To estimate recovery of ^3H, fractions were pooled as shown in (B) and (C); i.e., the indicated fractions, previously mixed with scintillation fluid and counted individually, were combined. Each pool was counted for 50 min along with a blank of the same volume and solvent composition. Counting efficiency, determined by addition of an internal toluene standard, was 0.34.

product, whereas for the 6-h digest shown in Figure 1B, containing essentially the same number of ^{32}P counts and the same quantity of transcripts, ca. 57% of the label appeared

Table III: Putative Polyribonucleotides Synthesized in Transcription Reaction Mixtures and Radioactive Products Resulting from Their Digestion with RNase A[a]

polyribonucleotide	^{32}P-labeled digestion product(s)	^{3}H-labeled digestion product(s)
poly[r(A-U)] (GMP in place of AMP)	Ap*Up	G*pUp
poly[r(A-U)] (GMP in place of UMP)	Ap*Up	ApG*pApUp
poly(rA)	none	purine polymers, e.g., ppp(Ap)$_n$G*p(Ap)$_n$A
copied from DNA	heterogeneous, e.g., Up*; (Rp*)$_n$Up	heterogeneous, e.g., (G*p)$_n$Up; (Ap)$_n$(G*p)$_n$Up

[a] Transcription reaction mixtures contain poly[d(A-T)]·poly[d(A-T)], electrophoretically homogeneous RNA polymerase, complementary ATP and [α-^{32}P]UTP, and noncomplementary [^{3}H]GTP. Were contaminating poly(A) polymerase to be present, synthesis of poly(rA) containing guanylyl residues is possible. Were contaminating DNA fragments to be present, synthesis of heterogeneous polyribonucleotides is expected. Digestion of the putative polyribonucleotides with pancreatic RNase A would yield the products shown; the ^{32}P-labeled (middle column) or ^{3}H-labeled (right column) moieties within these digestion products are indicated by asterisks. R designates a purine nucleoside residue.

as the ApUp end product. Likewise, fewer ^{3}H counts were converted to the GpUp end product in the 2-h than in the 6-h digest. When the transcripts synthesized by the parent RNA polymerase (Figure 1C) were digested for 6 h, a distribution of counts comparable to that found in Figure 1B for the mutant polymerase was observed (profile not shown). (Note that ApU>p undergoes more extensive hydrolysis to end product than does GpU>p, at least in part because it is present in digests in ca. 10^4-fold higher concentration.)

To ascertain that recovery of ^{3}H in the RNase A digests was comparable to that observed in the fidelity assays (Table II, reactions 1a and 1b), ^{3}H-containing fractions were pooled as indicated in Figure 1B,C, and the pools were counted as described in the figure legend. Twice the amounts of ^{3}H observed in the fidelity assays was anticipated, since a 2-fold greater concentration of [^{3}H]GTP was used in synthesis of transcripts. Total ^{3}H recoveries equal to 48 and 21 pmol were estimated for the transcripts synthesized by the mutant and parent polymerases, respectively. These recoveries, in slight excess of those expected, ensured that the great majority, if not all, of the ^{3}H counts associated with transcripts had been characterized.

Returning, then, to the putative transcription products under consideration (Table III), the results of RNase A digestion are consistent with incorporation of label into poly[r(A-U)] and are inconsistent with incorporation either into poly(rA) sequences or into sequences transcribed from DNA. The results also reveal that both mutant and parent RNA polymerases utilize noncomplementary GTP in place of ATP, i.e., that they catalyze a transition-type misincorporation in our assay.

(*b*) *^{3}H Resides in Covalently Incorporated GMP Residues.* Error rates derived in the fidelity assay are based on two further assumptions. One is that ^{3}H associated with transcripts derives exclusively from [^{3}H]GTP. The second is that ^{3}H resides in covalently incorporated guanylyl residues rather than in adventitiously bound GTP.

These assumptions were addressed by digestion of transcripts with a combination of RNases A, T_1, and T_2, followed by high-pressure liquid chromatography to resolve and identify the labeled digestion products. The premise of the experiment was this: If ^{3}H has been incorporated into poly[r(A-U)] from pure [8-^{3}H]GTP, label will be recovered in 3′-GMP; also, if ^{32}P has been incorporated from pure [α-^{32}P]UTP, label will be recovered in 3′-AMP, since ^{32}P from the incoming nucleoside triphosphate is transferred to the preceding nucleoside in the course of RNase digestion.

Conditions for chromatography of the RNase-digested transcripts were chosen to permit resolution of other possible products, including the following: (1) the cyclic mononucleotides [^{3}H]G>p and [^{32}P]A>p, which could remain as intermediates of RNase digestion; (2) [^{3}H]guanosine and [^{32}P]P_i, either or both of which could result from the action of a nucleotidase or phosphatase contaminating one or more of the enzyme preparations; (3) other 3′-mononucleotides which could result from incorporation of label from nucleoside triphosphates contaminating the [^{3}H]GTP and [α-^{32}P]UTP preparations.

Figure 2 illustrates chromatography of the RNase-digested transcripts and shows that ^{3}H and ^{32}P eluted almost exclusively as products expected from incorporation of pure [8-^{3}H]GTP and [α-^{32}P]UTP. Figure 2A shows the elution positions of chromatographic standards including the anticipated digestion products. Panels B and C of Figure 2 show the elution patterns of radioactivity in the RNase digests of transcripts synthesized by the mutant and parent RNA polymerases, respectively. In both digests, ^{32}P eluted almost entirely with the cochromatographed 3′-AMP standard, essentially none being detected in the void volume, nor elsewhere in the gradient, nor in the 20 mL of final methanol wash. ^{3}H eluted with the cochromatographed 3′-GMP and guanosine markers; a small unidentified peak appeared just after the void volume in both runs where [^{3}H]GTP would be expected. No detectable ^{3}H chromatographed as 5′-GMP which could have arisen from [^{3}H]GTP. Neither ^{32}P nor ^{3}H was recovered on chromatography of a digest of a control transcription reaction carried through the entire procedure but incubated without RNA polymerase, providing evidence that [^{3}H]guanosine is not derived from unincorporated [^{3}H]GTP. That guanosine arises by dephosphorylation of the 3′-GMP formed during enzymatic hydrolysis of the transcripts is shown by its absence from previous digests containing RNase A alone. These data support the assumption that ^{3}H associated with transcripts resides in covalently incorporated guanylyl residues.

(*c*) *Error Rates Based on Chromatographically Characterized Products.* To confirm the error frequencies observed in the fidelity assays (Table II), error rates were determined from the chromatographically characterized digestion products shown in Figure 2. ^{32}P eluted as 3′-AMP was used as an index of complementary nucleotidyl residues incorporated and ^{3}H eluted as 3′-GMP and as guanosine as a measure of noncomplementary nucleotidyl residues incorporated. Radioactive fractions representing each of these products were combined as shown in Figure 2B,C, and the pools were counted as described in the figure legend. Total radioactivity in each peak is recorded in Table IVA; error rates calculated for the mutant and parent polymerases are based on half the recovered ^{3}H, to account for the doubling of [^{3}H]GTP in the transcription reactions (see Materials and Methods and Table IVB). Comparing error rates found for the mutant polymerase, the fidelity assay yielded a frequency of $1/0.90 \times 10^5$, while the chromatographic analysis of digestion products yielded a frequency of $1/0.86 \times 10^5$. For the parent polymerase, the fidelity assay gave a frequency of $1/2.0 \times 10^5$ and the analysis of digestion products a frequency of $1/1.8 \times 10^5$. The agreement between the error rates obtained in the two pro-

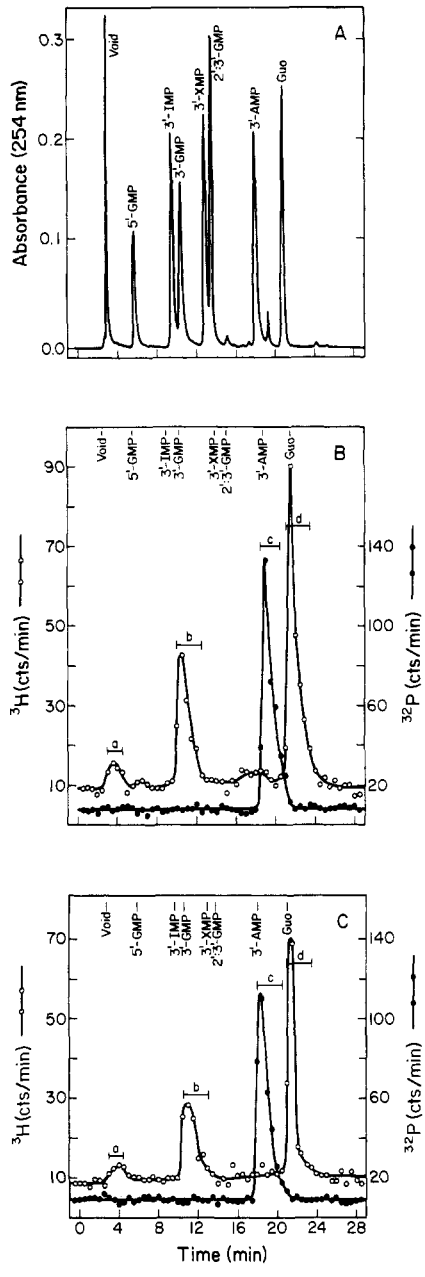

FIGURE 2: Reversed-phase high-pressure liquid chromatography of transcripts digested with RNases A, T_1, and T_2. (A) Absorbance standards. Column: Beckman Ultrasphere ODS (C_{18}, 0.46 × 25 cm). Solvents: (A) 0.1 M potassium acetate, pH 4.0; (B) 100% methanol. Gradient: 0–13% B, linear, 26 min; 13–100% B, linear, 9 min; 100% B, 20 min. Flow rate: 1 mL/min. Temperature: ambient. Detection: 254 nm. Standards were applied in 50 μL of solvent A. The first 29 min of the gradient containing all the absorbance standards is shown. 3'-XMP was prepared according to Levene and Dmochowski (1931). (B and C) Digests of transcripts synthesized by the mutant (B) and parent (C) RNA polymerases. RNase digests of material isolated from 100 μL of initial transcription reactions, together with absorbance standards, were chromatographed as in (A). Fractions (0.5 mL) were collected and counted for 10 min in 3 mL of Aquasol-2. Radioactivity profiles include background; ^{3}H cpm are corrected for spillover from the ^{32}P channel (6.1%). To quantitate radioactivity in peaks a–d, fractions were pooled as shown in (B) and (C); i.e., the indicated fractions, previously mixed with scintillation fluid and counted individually, were combined. Each pool was counted for 50 min along with a blank of the same volume and solvent composition. Blanks contained 20–21 ^{3}H cpm and 12–15 ^{32}P cpm. Net cpm, recorded in Table IVA, are corrected for these blanks. ^{3}H cpm in pools c and d are also corrected for spillover from the ^{32}P channel (6.1%). Counting efficiency for ^{3}H, determined by addition of a [^{3}H]toluene standard, was 0.30.

cedures corroborates the 2-fold greater incorporation of guanylyl residues catalyzed by the mutant polymerase and confirms the error rates found in the fidelity assays.

(d) Error Rates of Wild-Type Holoenzyme vs. a Hybrid Holoenzyme. Error rates were determined for two additional enzyme preparations by using the analysis of digestion products illustrated in Figure 2 and Table IV. The enzymes used were homogeneous *E. coli* K12 RNA polymerase from commercially obtained cells and a hybrid polymerase containing wild-type σ subunit from these cells, together with mutant core enzyme. As in the previous comparison of mutant and parent polymerases, transcription reactions were conducted simultaneously and identically, and the transcription products were digested and chromatographed under identical conditions. The error frequencies found for the wild-type holoenzyme and for the hybrid holoenzyme were $1/2.0 \times 10^5$ and $1/0.64 \times 10^5$, respectively. Two aspects of this experiment are noteworthy. First, the wild-type RNA polymerase examined here and that from CP79-U118 displayed similar fidelities in our assay. Second, the mutant core enzyme used in this experiment was not derived from the holoenzyme of the previous analyses but from an independent purification, thus establishing the reduced fidelity of a second preparation of mutant enzyme.

Discussion

The mutant we observed is the first RNA polymerase mutant to exhibit decreased accuracy of chain elongation both in vivo and in vitro. The screening procedure we developed for isolating the mutant could be used to detect a spectrum of RNA polymerase mutants with decreased fidelity of chain extension, as well as mutants with enhanced accuracy, detected by decreased leakiness. The present mutant manifests its lesion in vivo by decreased accuracy with concomitant read-through as well as by decreased growth and enhanced sensitivity to 5-fluorouracil and 5-azacytidine. The lesion is manifested in vitro by increased misincorporation of GMP (for AMP) during transcription of poly[d(A-T)]·poly[d(A-T)], by a decreased rate of transcription at 42 °C relative to wild-type enzyme, and also by a reduced half-life of RNA polymerase activity at elevated temperature (data not shown).

The assay for measurement of in vitro fidelity was chosen to reduce the possibility of artifactual enhancement of error rates (Loeb & Kunkel, 1982). Poly[d(A-T)]·poly[d(A-T)] was used as template-primer because it can be polymerized extremely accurately by de novo synthesis with *E. coli* DNA polymerase I (Agarwal et al., 1979). GTP was used as the incorrect nucleoside triphosphate, rather than CTP, to avoid complications of the facile deamination of cytosine to uracil. This particular assay discriminates effectively, but narrowly, between the mutant and parent polymerases. A larger difference in fidelity might be achieved under conditions where some other nucleoside triphosphate serves as the incorrect substrate. In fact, the enhanced sensitivity of the mutant to 5-fluorouracil and 5-azacytidine suggests that the corresponding nucleoside triphosphates might yield a greater difference in in vitro error rates. It should be noted that the G for A substitution we measured in vitro cannot cause the enhanced leakiness at an ochre (UAA) codon, which is the basis of our screen, because each such substitution yields a termination codon. However, a U to C transition, as well as transversions, would presumably cause read-through and leaky enzyme synthesis, since any amino acid misinserted at the 18th coding position is likely to yield active β-galactosidase [see Rosenberger & Hilton (1983)]. Our results thus suggest that the structural alteration in the mutant polymerase can cause more than one kind of base substitution.

Table IV

(A) Quantitation of Radioactive Products Recovered on Chromatography of RNase-Digested Transcripts (Figure 2B,C)[a]

peak	coeluted absorbance standard	mutant transcript (Figure 1B) ^{3}H cpm	^{3}H fmol	^{32}P cpm	^{32}P pmol	parent transcript (Figure 1C) ^{3}H cpm	^{3}H fmol	^{32}P cpm	^{32}P pmol
a	none	26	3.7	2	5	17	2.4	2	6
b	3′-GMP	103	14.8	2	6	66	9.4	2	4
c	3′-AMP	6	0.9	319	854	9	1.3	304	816
d	guanosine	203	29.0	34	90	69	9.9	13	35

(B) Calculation of Error Rates

	mutant RNA polymerase	parent RNA polymerase
pmol of ^{3}H (3′-GMP + Guo)/pmol of ^{32}P (3′-AMP)[b]	$43.8 \times 10^{-3}/944$	$19.3 \times 10^{-3}/851$
error rate[c,d]	$1/0.86 \times 10^5$	$1/1.8 \times 10^5$

[a] Poly[d(A-T)]·poly[d(A-T)] was transcribed by the mutant and parent RNA polymerases in the presence of complementary [^{32}P]UTP and ATP (25 μM each) and noncomplementary [^{3}H]GTP (60 μM). Transcripts were isolated, digested with RNases A, T_1, and T_2, and chromatographed as shown in Figure 2. Fractions from peaks a–d in Figure 2B (mutant polymerase) and Figure 2C (parent polymerase) were pooled and counted as described in the figure legend. [b] cpm in [3′-^{32}P]AMP include the major portion eluted in peak c and the minor, trailing portion eluted with peak d. [c] The error rate is calculated as half the total picomoles in 3′-GMP and in guanosine divided by the total picomoles of AMP and UMP incorporated. The factor of one-half is included because transcription reactions contained a 2-fold molar excess of GTP relative to that used in fidelity assays. [d] Error rates can be calculated without [^{3}H]guanosine, supposing that it appears in digests by some mechanism other than dephosphorylation of 3′-GMP. The error frequency of the mutant polymerase then becomes $1/2.6 \times 10^5$ and that of the parent polymerase $1/3.6 \times 10^5$. These error rates, while lower than those observed in the fidelity assays, also show increased misincorporation of guanylyl residues by the mutant polymerase.

The error rate observed for wild-type *E. coli* RNA polymerase ($1/2.0 \times 10^5$) is lower than previously reported. With poly[d(A-T)]·poly[d(A-T)] as template-primer and Mg^{2+} as a metal activator, the frequency of GMP misincorporation per total nucleotides polymerized has been reported to be $1/8.5 \times 10^2$ (Ozoline et al., 1980), $1/1.8 \times 10^3$ (Bick, 1975), and $1/4.2 \times 10^4$ (Springgate & Loeb, 1975). Possible sources of error in these measurements are heavily biased toward overestimation of error rates (Loeb & Kunkel, 1982). This is so because low levels of contamination of reagents—i.e., contamination too low to be detectable in routine biochemical analyses—can significantly enhance apparent misincorporation. For example, a 0.004% contamination of our [^{3}H]GTP with [^{3}H]ATP would have elevated the apparent error rate for wild-type enzyme 5-fold and would have obscured the difference between the mutant and parent polymerases. Avoidance of artifacts is thus crucial, not only for accurate determination of error rates but also for identification and characterization of altered polymerases. Appropriate analyses of in vitro transcripts will reveal artifactually enhanced misincorporation and are required to verify the results of fidelity assays which use synthetic polynucleotide templates and radioactive substrates (Loeb & Kunkel, 1982). These analyses should disclose the radiochemical identity of the labels taken as indexes of incorrect and correct incorporation and demonstrate that incorrect and correct nucleotides are covalently incorporated in sequences specified by the template-primer. That our fidelity assays have been verified by such product analyses lends credence to our measurements of the poly[d-(A-T)]·poly[d(A-T)]-directed misincorporation of GMP. Additional verification is provided by the 2-fold increase in misincorporation which accompanied transcription in the presence of a 2-fold increase in the concentration of incorrect GTP (compare Tables II and IVB). The low error rate of the wild-type enzyme is lent further reliability by the enhanced misincorporation observed for an altered RNA polymerase from a mutant which maps in the *rpo*B gene coding for a catalytic subunit of the enzyme.

The frequency in vivo of transcriptional errors at different nonsense codons in the *lac*Z gene has been estimated to vary between $1/4.0 \times 10^3$ and $1/1.2 \times 10^5$ (Rosenberger & Hilton, 1983). These estimates equate nonsense leakiness, assessed by residual β-galactosidase activity, with true read-through frequency and thus require assumptions about the association of *lac*Z monomers to form catalytically active tetramers. Our work supports the implication that RNA polymerase can achieve very high accuracy in vivo. We have shown that leaky enzyme synthesis by U118 is comparable to true read-through frequency by using immunoprecipitation and gel electrophoresis to measure production of full-length *lac*Z monomer (unpublished results). The leakiness of U118 we observed here is about $1/10^5$ (Table I) and is in fact similar to that measured by Rosenberger and Hilton (1983), and to the error rate we measured in vitro. This similarity is significant, suggesting that RNA polymerase holoenzyme—without accessory fidelity-enhancing activities (Volloch et al., 1979)—can achieve the transcriptional accuracy estimated for whole cells. Of course, in vivo and in vitro error rates must be compared with caution, because the conditions of transcription are very different.

The accuracy of chain elongation by RNA polymerase is governed by multiple determinants including the structure of the base in the nucleoside triphosphate substrate, the structure of the corresponding base in the template, and the structure and function of the polymerase [reviewed by Chamberlin (1974) and Anderson & Meninger (1986)]. The enhancement in accuracy achieved by the enzyme, beyond that ensured by Watson–Crick pairing, can be conjectured upon in view of energetics of base pairing in solution. With the use of recent data as a guide [see Aboul-ela et al. (1985) and references cited therein], the difference in free energy between correct and incorrect base pairs can be estimated as −2.7 to −4.4 kcal/mol at 37 °C, from which error frequencies of $1/0.95 \times 10^2$ to $1/1.4 \times 10^3$ can be predicted [see, e.g., Loeb & Kunkel (1982)]. An experimentally observed accuracy for RNA synthesis in solution, in the absence of polymerase, is $1/2 \times 10^2$ for the Zn^{2+}-catalyzed, poly(rC)-directed condensation of guanosine 5′-phosphorimidazolide (Bridson et al., 1980). Assuming then that base pairing provides an accuracy of $1/10^3$ and that RNA polymerase achieves an accuracy of $1/10^5$, one can suppose that the polymerase is capable of enhancing fidelity by 2 orders of magnitude. The mechanisms by which the enzyme enhances accuracy have been examined in incorporation studies employing substrates and templates containing base analogues (Chamberlin, 1974; Anderson & Meninger, 1986). This work has shown that RNA polymerase does not require a narrowly restricted chemical structure of its substrate and template bases for polymerization to occur.

Nor does the enzyme require formation of Watson–Crick pairs for polymerization (Singer & Spengler, 1981). The inference has been drawn that RNA polymerase contains a site which accommodates base pairs having the overall dimensions, though not necessarily the chemical identity, of Watson–Crick pairs; selection of the incoming base for incorporation may be determined primarily by its ability to fit exactly with the template base into this site (Chamberlin, 1974; Berg & Chamberlin, 1964). Although it is generally believed that RNA polymerase has no exonucleolytic (proofreading) activity, we are unaware of sensitive measurements to detect the generation of very low levels of noncomplementary nucleoside monophosphates. It is also possible that the interaction between the α and β subunits of the polymerase is involved in maintaining the accuracy of chain elongation, as suggested by the findings of Ishihama et al. (1980).

In conclusion, we raise a question suggested by recent results: Why might *E. coli* RNA polymerase be able to add nucleotides to a growing chain with an error frequency as low as $1/10^5$? While the frequency of errors in RNA synthesis must not exceed the frequency of errors in protein synthesis ($1/2.5 \times 10^3$ to $1/5 \times 10^3$; Ellis & Gallant, 1982; Anderson & Meninger, 1986), why might it be much lower? Since enhanced fidelity of any biological process requires energy, such enhancement would not be maintained through evolution without function. One possibility is that certain species of RNA (e.g., rRNA, which repetitively serves a general, complex function in protein synthesis) must be synthesized more accurately than mRNA. If this is so, the several RNA polymerases in eukaryotic cells might have inherently different accuracies, since each is responsible for the synthesis of different classes of RNA. Another possibility is that a crucial process(es) mediated by transcripts [e.g., chain termination involving a stem–loop structure in the nascent transcript (Ryan & Chamberlin, 1983)] require(s) very accurate synthesis of certain nucleotide sequences.

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