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A possible mechanism responsible for the correction of transcription errors

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ABSTRACT

Nucleoside triphosphate phosphohydrolase (NTPase) activity was found in a preparation of E. Coli RNA polymerase. This enzymatic activity is capable of hydrolysing all four ribonucleoside triphosphates to the nucleoside diphosphates. However, during in vitro RNA synthesis directed by poly(dC) or poly(dT), only the non-complementary nucleoside triphosphate of the same heterocyclic class was hydrolysed. No incorporation of the non-complementary precursor into RNA could be detected in these experiments. When another RNA polymerase preparation, devoid of NTPase activity, was employed, there was no hydrolysis of any nucleoside triphosphate and significant incorporation of non-complemtary precursor into RNA was observed.

These observations lead us to the conclusion that NTPase, acting in conjunction with RNA polymerase, has the function of correcting errors in transcription.

INTRODUCTION

Elongation of the RNA chain occurs from a stable ternary complex of enzyme (DNA dependent RNA polymerase) DNA and nascent RNA (1, 2).

The sequence of nucleotides added to the growing RNA chain is determined by the sequence of nucleotides in the DNA template. The relationship between template and product sequences is dictated by the Watson-Crick hydrogen bonding rules for the four naturally occuring nucleoside triphosphates. The mechanism by which selection of the correct nucleoside triphospate is effected is of considerable interest.

The specificity of the selection process is determined by the corresponding base in the DNA template, by the structure of the base in the nucleotide triphosphate substrate and also by the structure of the RNA polymerase itself.

The properties of the triphosphates of some base analogs indicate that base pairing itself is not an absolute requirement for the dictation of substrate by a template base. These studies suggest that the selection of

the incoming base for incorporation is determined primarily by its ability to fit exactly with the template base into a site on the enzyme rather than by its actual identity or by virtue of its ability to interact with the template base (3, 4, 5).

A model has been proposed (6, 7), in which selection of the correct nucleoside triphosphate proceeds through a random, weak binding of all possible substrates at a common site on the enzyme followed by a conformational change to a stronger complex when the correct triphosphate enters that site. It is conceivable, however, that a non-complementary nucleoside triphosphate may occasionally reach the elongation site of the enzyme and be bound to it.

In such a case, three possibilities can be predicted a priori:

a) such an event will terminate the elongation of RNA;
b) the non-complementary nucleoside triphosphate will be incorporated into a growing RNA chain;
c) the non-complementary NTP will be removed and replaced by a complementary one.

Cases (a) and (b) are known and have been described in a number of studies (8-14). In this communication we wish to propose a mechanism that could accomplish the function (c).

We propose that this function is carried out by an enzyme nucleoside triphosphate phosphohydrolase (NTPase), which we have detected in one of our preparation of E. Coli DNA dependent RNA polymerase. This enzyme is capable of hydrolising all four ribonucleoside triphosphates. However, during in vitro RNA synthesis only the non-complementary nucleoside triphosphates of the same heterocyclic class are hydrolysed.

MATERIALS AND METHODS

Materials. Two separate preparations of E. Coli DNA dependent RNA polymerase were used in these experiments:

Preparation A was isolated and purified by Dr. A. Goldfarb from E. Coli, strain MRE 600 as described (15).

Preparation B was isolated and purified by Dr. M. Zeevi (both from this Institute) from the same strain of E. Coli as described by Burgess and Jendrisak (16) and was further purified by affinity chromatography on Blue Dextran Sepharose column (17, 18).

Both preparation A and B of RNA polymerase were examined by polyacryla-

mide gel electrophoresis as described (16).

The highly purified preparation B has given only four bands, which correspond to the known subunits of the enzyme (16). At the contrary, the more crude preparation A has given many additional bands (data not presented).

Unlabeled nucleotides were purchased from Sigma. Tritium-labeled and ^{32}P labeled nucleotides were obtained from Amersham. Homopolymers were purchased from P and L Biochemicals, Inc. Snake venom phosphodiesterase was a product of Worthington.

Methods. RNA synthesis was performed at 30° in 50 µl of reaction mixture containing 40 mM Tris-HCl buffer, pH 7.9; 150 mM NaCl (when poly(dC) was used as template) or 70 mM NaCl (when poly(dT) was used as template); 4mM $MgCl_2$; 10 mM $MnCl_2$; 14 mM β-mercaptoethanol; 0.1 mg/ml poly(dT) or poly(dT); 0.05-0.2 mM of each NTP (^{32}P labeled NTP were taken in the same concentration as other NTP. In each experiment all four NTP were used at the same concentration. Only one ^{32}P NTP was used in separate assay) and 0.5 u/ml of E. Coli RNA polymerase.

For the error rate measurement the RNA was repeatedly extracted with 1 vol. each of phenol and 24:1 chlorophorm isoamylacohol and the incorporation of radioactive nucleotides into an acid-insoluble precipitate was measured in a liquid scintillation counter. Background counts from non-incubated assay tubes were measured after analogous treatement.

Assay for NTPase activity was carried out in the same reaction mixture as RNA synthesis, but in the absence of a template, at 30° for 2 hrs.

Thin layer chromatography was performed on PEI-cellulose plates (DC-Alufolien, Merck), using 300 mM NH_4HCO_3 as running liquid.

Chromatograms were stained by the molibdate reagent after Stahl (19). ^{32}P labeled material was made visible by autoradiography.

Hydrolysis of the reaction product by snake venom phosphodiesterase was performed at 37° in a reaction containing 100 mM Tris-HCl, pH 8.4, 5mM $MgCl_2$, 100 µg/ml snake venom phosphodiesterase, and approximately 1 µg of RNA in a total volume of 0.3 ml. Unhydrolised product was precipitated with 10% trichloracetic acid and 100 µg of yeast RNA as carrier and its radioactivity was measured in a liquid scintillation counter.

Error rate is defined as the molar ratio of non-complementary to complementary nucleotides incorporated.

RESULTS

Two preparations of DNA dependent RNA polymerase were used in this work. They were isolated from the same strain of E. Coli (MRE 600) and were purified by different methods (see Methods). NTPase activity was not found in the more highly purified preparation B, but was present in preparation A, where it acted with approximately equal efficiency on all four ribonucleoside triphosphates (Fig. 1).

We propose that this activity can carry out the function of correction during RNA synthesis, removing non-correct (non-complementary) nucleoside triphosphates from the elongation site of RNA polymerase by hydrolysing them to nucleoside diphosphates. This proposal, which assumes that NTPase will hydrolyse only non-correct nucleoside triphosphates during the prosess of RNA synthesis, was tested experimentally.

RNA synthesis was carried out in vitro with RNA polymerase preparation A, using poly(dC) as template. Four separate reaction mixtures were set up, identical in all respects except that each contained the same amount of a different $\alpha^{32}P$ labeled nucleoside triphosphate. Each mixture also contained equal concentrations of all four non-radioactive nucleoside triphospates. The reaction products were analyzed by thin layer chromatography (see "Methods")

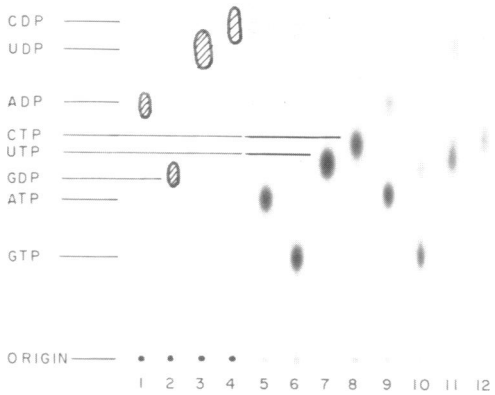

Figure 1: Assay for NTP ase activity. The reactions were performed with preparation A (9-12) or with preparation B (5-8) of RNA polymerase. 1-4 - markers (made visible by staining). $\alpha\ ^{32}P$ NTP's were used in the following order: 5,9 - ATP; 6,10 - GTP; 7,11 - UTP; 8,12 - CTP. Reaction products were analyzed by thin layer chromatography.

in order to detect products of NTPase activity, i.e., $\alpha^{32}P$ labeled nucleoside diphosphates.

The results are shown in Fig. 2. When the labeled precursor was $\alpha^{32}P$ ATP (non-complementary), labeled ADP was formed (1.3% of the corresponding GTP incorporation). No labeled GDP was produced from labeled GTP (complementary). These results fulfill the prediction of our hypothesis.

The hypothesis further predicts that radioactive CDP and UDP would be produced from their respective precursors, both non-complementary, but these products were not found. A reasonable explanation is that in these cases the template-precursor base pair is pyrimidine-pyrimidine and too dissimilar to the required purine-pyrimidine pair to be admitted to the elongation site of RNA polymerase in the first place. This implies that the most probable source of the type of error correctable by NTPase would be the substitution of one nucleoside triphosphate for the other of the same heterocyclic class.

The analogous experiment was performed with poly(dT) as template (this is

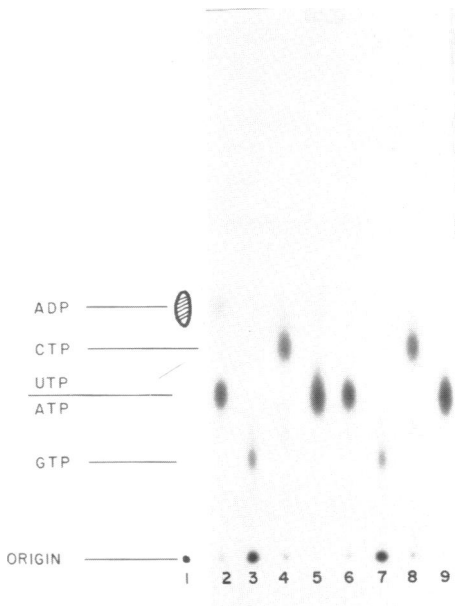

Figure 2. Thin layer chromatogrphy of the products of RNA synthesis directed by poly(dC) and catalyzed by preparation A (2-5) or by preparation B (6-9) of RNA polymerase. $\alpha^{32}P$ labeled precursors: 2,6 - ATP; 3,7 - GTP; 4,8 - CTP; 5,9 - UTP. 1 - marker (made visible by staining). Each $\alpha^{32}P$ NTP was used at the same final specific activity (0.4 Ci/mmol).

the only other homopolymer that can serve as template, since the RNA chain is initiated only by purines). In this case, labeled GDP was formed (2.2% of the corresponding α^{32}P ATP incorporation) when the precursor was α^{32}P GTP (non-complementary); while no radioactive ADP was produced from α^{32}P ATP (complementary) (Fig. 3). Again, no nucleoside diphosphates were formed from pyrimidine precursors.

When preparation B of RNA polymerase was used, no nucleoside diphosphate was produced in any case. As mentioned, this preparation was devoid of NTPase activity and therefore presumably unalbe to dephosphorylate a non-complementary precursor that had found its way to the RNA polymerase elongation site. If our assumption is true, one would predict that polymerase preparation B would exhibit a higher rate of erroneous (non-complementary) incorporation than preparation A. To test this prediction, RNA was synthesized in vitro as described above, and the newly synthesized RNA was purified (see "Methods") and analyzed for the incorporation of complementary and non-complementary nucleotides (Tables I and II).

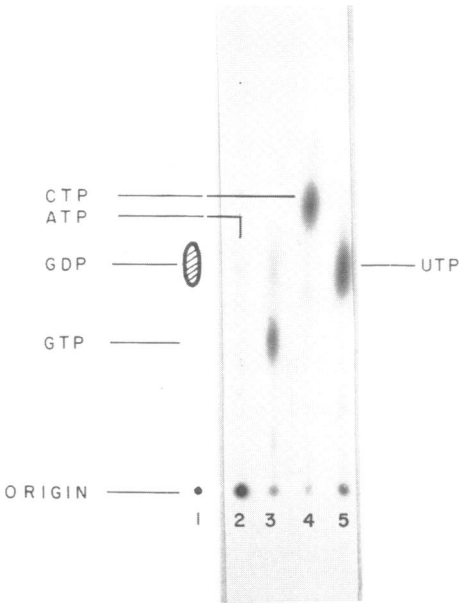

Figure 3. Thin layer chromatography of the products of RNA synthesis directed by poly(dT) and catalyzed by polymerase preparation A. α^{32}P labeled precursors: 2 - ATP; 3 - GTP; 4 - CTP; 5 - UTP. 1 - marker (made visible by staining). Each α^{32}P NTP was used at the same final specific activity (0.4 Ci/mmol).

TABLE I

Incorporation of complementary and non-complementary nucleotides by RNA polymerase copying poly(dC).

The assay conditions for measuring error rate with poly(dC) are given under Methods. Each α^{32}P NTP was used at the same final specific activity (0.4 Ci/mmol). The first five columns give the results of one experiment. The sixth column gives the observed variation in error rate for three separate experiments.

Polymerase Preparation	α^{32}P NTP	NMP Incorporated (cpm)	Background (cpm)	Error Rate (%)	Variation in Error rate (%)
A	ATP	111	97	-	-
A	GTP	311892	81	-	-
A	CTP	118	112	-	-
A	UTP	99	75	-	-
B	ATP	2762	92	0.91	0.72-0.97
B	GTP	303674	105	-	-
B	CTP	109	79	-	-
B	UTP	94	98	-	-

TABLE II

Incorporation of complementary and non-complementary nucleotides by RNA polymerase copying poly(dT).

The results are expressed as in Table I.

Polymerase Preparation	α^{32}P NTP	NMP Incorporated (cpm)	Background (cpm)	Error Rate (%)	Variation in Error rate (%)
A	ATP	316104	87	-	-
A	GTP	97	81	-	-
A	CTP	104	103	-	-
A	UTP	114	98	-	-
B	ATP	327066	112	-	-
B	GTP	4932	93	1.51	1.33-1.51
B	CTP	121	109	-	-
B	UTP	94	86	-	-

When RNA polymerase preparation A was used with either poly(dC) or poly (dT) as template, there was no detectable incorporation of non-complementary

nucleotides over background level (0.03%, non-incubated mixture). With preparation B, however, there was significant mis-incorporation. The observed error rates were 0.91% for ATP (poly(dC) as template) and 1.51% for GTP (poly(dT) as template). As expected from the earlier results, no errors were detected with UTP and CTP, using either template.

These results were confirmed in double-label experiments in which radioactive complementary and non-complementary precursors were present in the same reaction mixture (α^{32}P GTP and ^{3}H ATP with poly(dC), and α^{32}P ATP and ^{3}H GTP with poly(dT)). The results were very similar to those of the single-label experiments (data not presented).

With both enzyme preparations the incorporation of both complementary and non-complementary nucleotides was DNA dependent. In the control experiment, without DNA, no incorporation was detected. When preparation B of RNA polymerase was used, the incorporation of non-complementary nucleotides paralleled that of the complementary nucleotides with the time of incubation (Figs. 4a, 4c) or when the amount of polymerase was varied (Figs. 4b, 4d).

These results suggest strongly that non-complementary nucleotides are incorporated into RNA by the same enzyme which effects the incorporation of

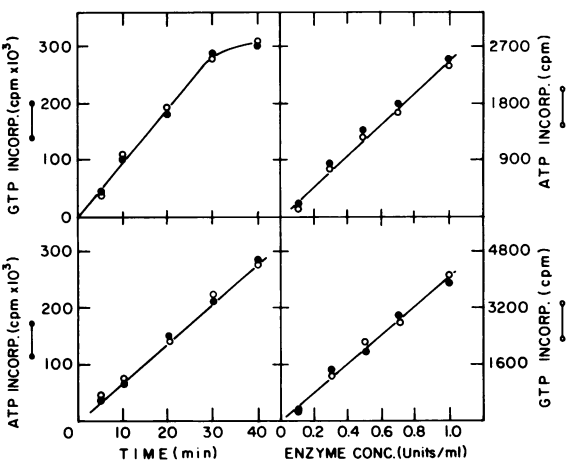

Figure 4. Effect of time incubation ((a) and (c)) and enzyme concentration ((b) and (d)) on the incorporation of complementary and non-complementary nucleotides. (a) and (b) - the reaction was directed by poly(dC). (c) and (d) - the reaction was directed by poly(dT). When the amount of enzyme was varied, incubation was for 15 min. at 30°C. The reactions were catalyzed by polymerase preparation B. The assay conditions are given under Methods. Each α^{32}P NTP was used at the same final specific activity (0.4 Ci/mmol).

complementary nuclotides.

In order to determine the distribution of the non-complementary nucleotides, the reaction products of polymerase preparation B were hydrolysed with snake venom phosphodiesterase, a processive 3' → 5' exonuclease. In these experiments phosphodiesterase was employed in saturating amount, so that hydrolysis would be initiated on all RNA molecules. Such digestion revealed that AMP and GMP were rendered acid soluble at the same rate (Fig. 5).

Thus, non-complementary nucleotides are in phosphodiester linkage and are present throughout the length of the synthesized poly-nucleotides. Together with the previous experiment these results prove also that incorporation of non-complementary nucleotides is not due to terminal addition.

DISCUSSION

Quantitation of the error rate of RNA synthesis *in vitro* depends on the purity of the nucleoside triphosphate substrates and the polynucleotide template. Routinely, the labeled NTP's used were at least 99% pure. When necessary, they were purified by chromatography (20). That the misincorporation does not result from the presence of contaminating or altered nucleotides in

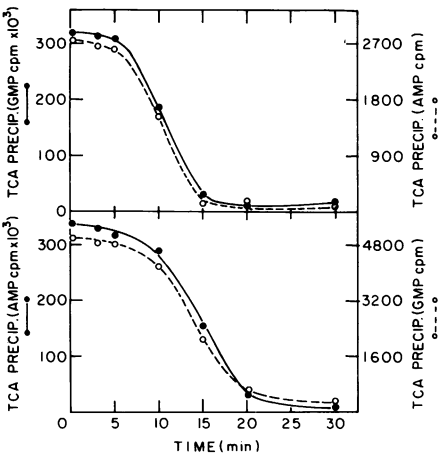

Figure 5. Time course of processive hydrolysis of the reaction product by snake venom phosphodiesterase. RNA was synthesized by preparation B of RNA polymerase using as template poly(dC) (a) or poly(dT) (b). The assay conditions are given under Methods. Each α^{32}P NTP was used at the same final specific activity (0.4 Ci/mmol).

the homo-polymer templates was indicated by the fact that the error rate can be increased by increasing the ratio of non-complementary to complementary NTP (results not presented).

But most importantly, our two preparations of RNA polymerase exhibited extremely different error rates under identical conditions.

In our study we obtained different error rates for different templates. The observation that fidelity depends on the template is consistent with published evidence that the template affects base descrimination by both RNA and DNA polymerases (21, 22, 28). The error rate can evidently differ in different conditions. When poly(dT) was used as template, a 3.3% misincorporation of GMP was observed by Streriste et al. (11) with E. Coli RNA polymerase.

The fact that NTPase activity is present in one polymerase preparation but not in another indicates that it may be a separate enzyme which works in a coordinated manner with RNA polymerase.

It is known that RNA polymerase has an absolute requirement for nucleoside triphosphates. The triphosphate group seems to be very important for the elongation site of RNA polymerase. Whereas the initiation nucleotide site is purine - specific and will bind PuTP, PuDP and PuMp (22) the elongation nucleotide site has an absolute requirement for the triphosphate group, and the NTP can be replaced only by the analogs that have this group (21, 24-28). Moreover, the importance of the triphosphate group is shown by the ability of tripolyphosphate to compete with NTP for the elongation site of RNA polymerase (8). The same is true also for the DNA polymerase triphosphate site (functionally related to the RNA polymerase elongation site) (29).

Thus, the NTP binding site active in chaim elongation seems to be primarily directed toward the triphosphate portion of the substrate, and hydrolysis of nucleoside triphospate to nucleoside diphosphate could be sufficient to remove it from this site. Our results support this assumption. We can speculate that the NTPase activity described here selects non-correct nucleotide triphosphates by the absence of complementary bonds with the template nucleotide. Correct substrates are protected against the action of NTPase, presumably by complementary interaction with the template nucleotides.

Rho factor, a protein isolated from E. Coli catalyzes hydrolysis of nucleoside triphosphates to nucleoside diphosphates (30, 31). However, the NTPase activity described above cannot be due to contamination with Rho factor, because the hydrolysis reaction of Rho factor can occur only in the presence of RNA (32). NTPase, described above, does not have this requirement.

After RNA has dissociated from the template, no correction of non-complementary incorporation is possible. It is conceivable that following transcription but before dissociation of RNA from the DNA, a repair enzyme could read down and correct errors in the RNA. However, no such enzyme has yet been reported. Thus, the mechanism described above may be responsible for the correction of transcription errors.

By combining these results with those of our previous study (20) we came to the conclusion that the biological process of RNA synthesis is catalyzed by an enzyme complex, rather than by a single enzyme (RNA polymerase). This complex contains at least three activities which belong presumably to separate enzymes working in a coordinated manner.

1. The polymerasing activity: new phosphodiester bonds are formed and prophosphates are liberated. Energy of hydrolysed "high energy" bonds is stored in newly formed phosphodiester bonds (33).

2. The pyrophosphate condensing activity: two liberated pyrophosphates molecules are condensed to one trimetaphosphate molecule and one orthophosphate molecule. Energy of "high energy" bonds of two pyrophosphates is stored in trimetaphosphate (20).

3. The "error eliminating" activity: non-complementary nucleoside triphosphates entering the elongation site of RNA polymerase are eliminated by the reaction of dephosphorylation.

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