THE INCORPORATION OF GMP-RESIDUES FROM GTP INTO THE POLYNUCLEOTIDE FRACTION DURING POLY-U DIRECTED POLY-A SYNTHESIS CATALYZED BY *E. COLI* RNA POLYMERASE

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1. Introduction

Non-complementary nucleoside triphosphates are known to inhibit the RNA polymerase reaction [1–4]. Here we observe a strong difference between the mechanism of the inhibitory action of the purine (GTP) and pyrimidine (CTP) nucleoside triphosphates in poly-U directed poly-A synthesis catalyzed by *E. coli* RNA polymerase.

Particularly extensive incorporation of GMP residues into the polymer was observed and the residues were arranged as long clusters.

2. Materials and methods

RNA polymerase was isolated from *E. coli* MRE 600 according to the procedure of Babinet [5] with some modifications [6]. Bacterial cells were harvested in the middle of the logarithmic phase. The enzyme was further purified by gel filtration on Sephadex G-75 (Phamacia, Sweden). All the experiments described below were carried out with a preparation of specific activity about 400 units per mg of protein (one unit of RNA polymerase activity was defined as the amount of enzyme which catalyzed the incorporation into acid-insoluble material of 1 nmol of [^{14}C] AMP in 20 min reaction under standard conditions [7] with chicken erythrocyte DNA (Reanal, Hungary) as template).

Poly U as well as CTP, GTP, [^{14}C] ATP and [^{14}C] GTP were prepared using the appropriate enzymatic procedures which will be published later. Poly U had a mol. wt about $1-2 \times 10^6$ as shown by gel filtration on Sepharose 4B (Pharmacia).

ATP was purchased from Reanal, DEAE-cellulose from Serva (BRD), T_1 RNase from Worthington (USA).

The reaction mixture for poly U transcription studies was as follows: 0.05 mM poly U as mononucleotides, 1.5 mM ATP (labelled or not), GTP and CTP, if present, (concentration in legends), 5 mM of each Mg^{2+} and Mn^{2+}, 40 units per ml of RNA polymerase, 0.04 M Tris–HCl buffer, pH 7.9, 0.01 M β-mercaptoethanol. The reaction was carried out at 37°C and its time course of the reaction was followed as described previously [8].

The product of the transcription was isolated by trichloroacetic acid precipitation on Whatman 3 MM paper discs and washed with trichloroacetic acid, ethanol and ether. The enzymatic hydrolysis of the product by T_1 RNase was carried out in 0.05 M Tris–HCl, pH 7.6, in the presence of 0.01 M EDTA directly with the discs after cutting them and extracting with 0.5 ml buffer containing 2 μg T_1 RNase for 24 hr at 37°C. After hydrolysis the solution was transferred into another test tube, the papers washed with water (0.5 ml) and the washes combined with the main solution. The combined solution was applied to a microcolumn (50 μl) of DEAE-cellulose (special superfine fraction obtained from commercial preparation) and eluted with a linear gradient of NaCl concentration (0–0.3 M) in 0.01 M Tris–HCl buffer, pH 7.5, containing 7 M urea. After the gradient, the column was eluted with 1 M NaCl in the same urea-buffer solution. The elution was monitored using the recording microspectrophotometer designed in the

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Novosibirsk Institute of Organic Chemistry [9].

The size of the transcription products was estimated by gel chromatography on Sephadex G-50, G-75 and G-150 (Pharmacia). Crystalline urea was added up to 7 M concentration to 0.5 ml aliquots of RNA polymerase reaction mixture after the reaction. The resulting solution was incubated for 30 min at 37°C and applied to the column (10 ml) containing the appropriate Sephadex previously equilibrated with 7 M urea, which was used as the eluent. The elution was monitored by recording the absorbance at 260 nm and the distribution of the radioactivity in the eluted material was studied with a Nuclear Chicago Mark II Liquid Scintillation Spectrometer.

3. Results and discussion

We reported earlier [6] that the time course of poly A synthesis on a poly U template depends on the enzyme-to-template ratio. It was suggested that when this ratio is relatively low (1 enzyme molecule per approx. 1000 nucleotide residues) the process is similar to real transcription. At the ratio of 1 enzyme molecule per 50 nucleotides of template the reaction seems to proceed by a reiterative mechanism because of tight packing of RNA polymerase molecules on the template. In all the present experiments we used a low enzyme-to-template ratio.

Fig.1 shows the kinetics of poly A synthesis by RNA polymerase on poly U as a template in the absence or presence of GTP (a) and CTP (b). It is seen

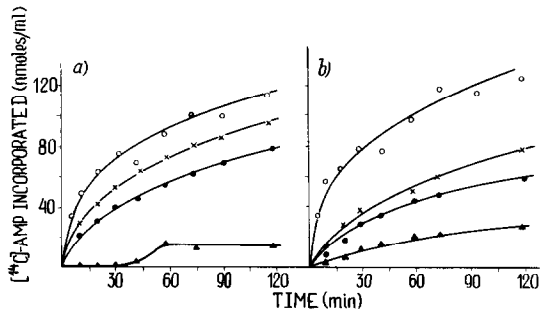

Fig.1. Kinetics of poly A synthesis on poly U in the presence of GTP (a) and CTP (b) at the concentrations of competing NTP's as follows: 0 (o–o); 0.75 mM (×–×); 1.5 mM (•–•); 5.0 mM (▲–▲).

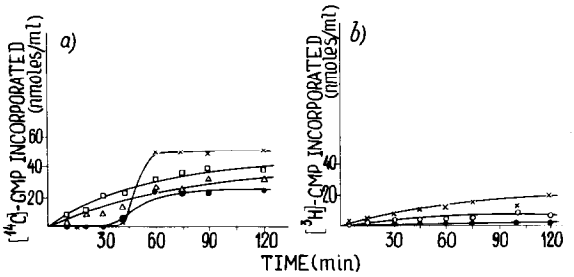

Fig.2. Kinetics of the incorporation of the non-complementary nucleotides during the poly A synthesis. The labelled NTPs and their concentrations were: (a) [^{14}C]GTP (0.2 mCi/mmol): 0.75 mM (•–•), 5 mM (×–×), 5 mM without ATP (■–■) and 5 mM without poly U (▲–▲). (b) [^{3}H]CTP (0.2 mCi/mmol): 1.5 mM (•–•), 5 mM (×–×) and 5 mM without ATP (o–o).

that both these triphosphates inhibit the reaction, but at high concentration of GTP the kinetic curve of poly A formation looks very unusual exhibiting a 30 min lag period. Moreover, when we studied similar reaction mixtures with GTP or CTP as the labelled components, considerable incorporation of GMP residues into the reaction product after a similar lag period was observed (fig.2a). Some incorporation of CMP also took place (fig.2b), but its time course was linear; it is believed to be due to some contaminations of the RNA polymerase (we failed to obtain the enzyme preparation essentially free of such an activity despite a rather good SDS-polyacrylamide gel electrophoretic pattern, not shown here). It should be pointed out that GMP incorporation with a linear time course was observed in the absence of ATP and poly U (fig.2a), but this reaction stopped immediately after adding all the components of poly A synthesis to the reaction mixture. Thus this reaction may be regarded as a non-specific function of RNA polymerase which does not work in conditions of genuine template synthesis.

The incorporation of GMP is strongly dependent upon the time of adding GTP to the reaction mixture (fig.3). Two parallel experiments with either labelled ATP or GTP were carried out. If GTP and ATP are added simultaneously, both the incorporation curves are sigmoidal and the yield of GMP incorporation is even higher than that of AMP. When GTP was introduced into the reaction mixture 3 min after ATP, the incorporation of GMP was lower but still consid-

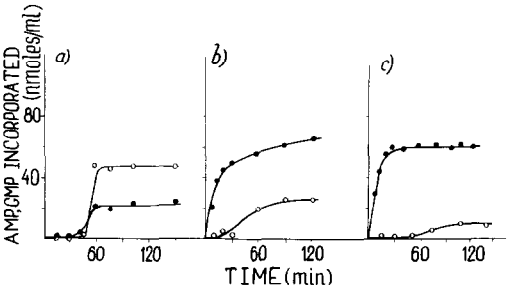

Fig.3. Incorporation of AMP (•–•) and GMP (○–○) when both ATP (1.5 mM) and GTP (5 mM) were added simultaneously (a), GTP was added 3 min (b) and 8 min (c) after ATP.

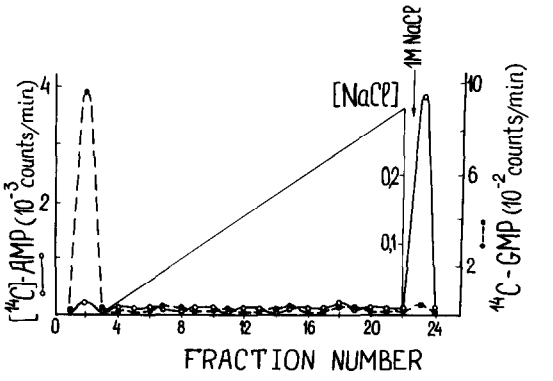

Fig.4. DEAE-cellulose chromatography of T_1 RNase digests of the RNA polymerase products obtained with ATP or GTP as labelled precursors.

erable. Lastly, the extent of GMP incorporation is very low when GTP is added 8 min after the start of the reaction.

Wu and Goldthwait [10,11] have found that there are two sites on the enzyme molecule which bind nucleoside triphosphates: one binds purine NTPs only, which are used as initiators, and the other binds all four NTPs. In this connection our data could be interpreted as follows: GTP can interfere with ATP at both initiation and elongation steps of the reaction while CTP can take its place at the elongation step only. The competition between GTP and ATP for the initiation site of the enzyme seems to be the most likely reason for the lag period in AMP incorporation at highest GTP-to-ATP ratios in the reaction mixture. As for the incorporation of GMP into the polymeric product it should be pointed out that in no case was this incorporation observed before some poly (or oligo) A synthesis. This may mean that RNA polymerase cannot use GTP as the substrate at the initiation step but does use it at the elongation one after a run of AMP residues has been formed. In this connection it was of interest to study the distribution of GMP-residues in the polynucleotide chain of the polymeric product consisting of AMP and GMP.

To simplify the experiments the polymers were obtained under the conditions shown in fig.3b after long enough runs of AMP residues had been formed before addition the GTP – for better separation of poly A runs from the products of splitting of the following sequence. The [^{14}C]AMP and [^{14}C]GMP containing polynucleotides were obtained separately but in the identical reaction mixtures and then treated by T_1 RNase as described in Materials and methods. The digests were separated by ion exchange chromatography in 7 M urea. The results (fig.4) show that the A-labelled product is practically not attacked by the nuclease. On the contrary, the G-labelled product is converted by the T_1 RNase into mononucleotides, only a small peak emerges in the 1 M NaCl fraction. These data mean that the polynucleotide product is built up of large clusters of AMP and GMP like pA–A–A– . . . –A–G–G–G– . . . –G . . . . The possibility that two polymers are formed (poly A and poly G) could not be ruled out absolutely, but is less likely as the incorporation of GMP depends on the presence of ATP. The incorporation of GMP residues between the AMP ones is very low, if any, because of the absence of discrete peaks of A-labelled material of oligonucleotide nature.

The size of the polynucleotide product was more than 100 nucleotides as shown by gel filtration experiments not shown here: the polymers were excluded by Sephadexes G-50, G-75 and G-150.

The incorporation of GMP residues may be explained from the wobbling properties of the G–U base pair.

The results of the experiments described show the high efficiency of non-complementary NTPs as inhibitors in the RNA polymerase reaction on a poly U template. This is in some discrepancy with the data of Chamberlin and Berg [1]: these authors found

practically no inhibition at GTP and CTP concentrations close to the substrate ones in the poly d(AT) directed system. There are many works showing that polyribonucleotides are poorer templates for RNA polymerase than polydeoxy ones. One may suggest that the ability of the enzyme to recognize the appropriate substrate is also lower with the polyribonucleotide templates.

Acknowledgments

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