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STIMULATION OF POLY(dT) TRANSCRIPTION BY *Bacillus subtilis* RNA POLYMERASE IN THE PRESENCE OF ADENOSINE MONOPHOSPHATE

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SUMMARY: The rate of synthesis of poly(A) on a poly(dT) template by *Bacillus subtilis* RNA polymerase is a function of ATP concentration and is expressed as a sigmoidal curve. The addition of millimolar concentration of AMP to low concentrations of ATP stimulates synthesis of poly(A) twenty fold and raises the rate of synthesis to the levels obtained at high ATP concentrations. The reaction is completely dependent upon the presence of poly(dT) and requires the complementary mononucleotide. Stimulation of poly(A) synthesis by AMP is more evident with the holoenzyme. Analysis of poly(A) products by acrylamide gels showed that the poly(A) synthesized in the presence of AMP has an higher molecular weight than poly(A) synthesized in the absence of AMP.

INTRODUCTION: initiation of RNA chains *in vivo* is restricted to specific promoter sites on the DNA template. To achieve the same specificity of transcription *in vitro* the enzyme RNA polymerase should contain the functional sigma subunit (1,2). Sigma subunit has also a pronounced effect on the initial rate of RNA synthesis. This is true also when synthetic copolymers or homopolymers are used as templates (3). The bimolecular nature of initiation of RNA chains which involves binding of two substrates at two different binding sites, suggests that initiation is the rate limiting step of the polymerization reaction (7,8). The transcription reaction directed by single-stranded homopolynucleotide templates shows a sigmoidal dependence on nucleoside triphosphate concentration (4-6); the sigmoidal pattern can be partially modified to a hyperbolic one by addition of complementary oligonucleotides, by substitution of the single-stranded template with the corresponding double-stranded template or by mantaining a constant ATP value to divalent ion ratio (4-6).

Experiments on transcription of homopolymers by *Micrococcus luteus* RNA polymerase showed that the initial rate of RNA synthesis can be greatly increased when a complementary nucleoside monophosphate is added to the reaction mixture containing the nucleoside triphosphate substrate (Ref. 9 and L.Grossman, personal communication).

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Here we investigate the mechanism of mononucleotide activation with single-stranded synthetic polydeoxynucleotide template. In addition, since three forms of RNA polymerase can be separated by DNA-cellulose chromatography from Bacillus subtilis vegetative cells (10), poly(dT) directed poly(A) synthesis was investigated to evidentiate peculiar features of the three enzymatic forms of RNA polymerase and to elucidate the mechanism of the mononucleotide activation.

MATERIALS AND METHODS: Chemicals: unlabelled ribonucleoside mono-di and triphosphates were purchased either from Sigma Chemical Co. or from Serva Feinbiochemica. [^{3}H]-ATP(25 Ci/mmol) was from Radiochemical Centre Amersham. Poly(dT) (1000 nucleotides long) was a gift of Dr.L.M.S.Chang of the Dept. of Biochemistry, U.S.U.H.S., Bethesda, U.S.A. Electrophoresis grade acrylamide, N,N'methylenbisacrylamide, TEMED (N,N,N',N' tetramethylethylendiamine) and ammonium persulphate were purchased from Eastman Kodak Co. All other chemicals were analytical grade.
Poly(dT) transcription assay: the standard reaction mixture contained in 0.115 ml: 50 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 50μg/ml poly(dT), 0.1 mM [^{3}H]-ATP(10 cpm/pmol), 1 mM (except otherwise stated) of 5'-AMP and 2μg of Bacillus subtilis RNA polymerase. Samples were incubated 10 min at 37°C. Reactions were terminated by pipetting aliquots on GF/C glass fiber disks and processing for acid insolubility as described by Bollum (11).
Gel electrophoresis of poly(A) products: acrylamide gel electrophoresis was performed as described by Maniatis et al. (12). Samples were processed as following: 0.5 ml of a standard reaction mixture were boiled 2 min and, after addition of 0.5 A_{260} of microsomal RNA from rat hepatocytes and NaCl to 0.15 M, the RNA was precipitated at -20°C by addition of ethanol. The precipitated was resuspended in 0.025 ml of 99% formamide and electrophorized at 100 volts. After electrophoresis the gel was cut in 0.3 cm strips and each strip incubated at room temperature in 0.4 ml of soluene. After 24 hours, 5 ml of toluene containing 1.25% PPO were added and radioactivity determined in a scintillation counter.

RESULTS

Three different forms of Bacillus subtilis RNA polymerase with the following subunit composition are separated by DNA-cellulose chromatography: enzyme A-$\alpha_2,\beta\beta',p^{28},p^{21}$ (1:1:1:2); enzyme B-$\alpha_2,\beta\beta'$ (1:1); enzyme C-$\alpha_2,\beta\beta',\sigma$ (1.1:1:0.9) (10).
Poly(A) synthesis by Bacillus subtilis RNA polymerase (forms A, B and C) directed by a poly(dT) template was analyzed as a function of ATP concentration in the presence or absence of AMP (Figure 1). A pronounced activation of homopolymer synthesis at low ATP concentrations is observed when holoenzyme is used (Fig. 1C). Figures 1A and 1B show the effect of AMP on the reaction catalyzed by enzyme A and B. The mononucleotide activation is also observed, but at a lower extent, suggesting that σ subunit is essential to increase the initial rate of polymerization at low ATP concentrations. The higher rate of poly(A) synthesis observed when enzyme A is used is of difficult interpretation. Recent results (13,14) suggest the P^{21} polypeptide present in enzyme A is identical to the delta protein

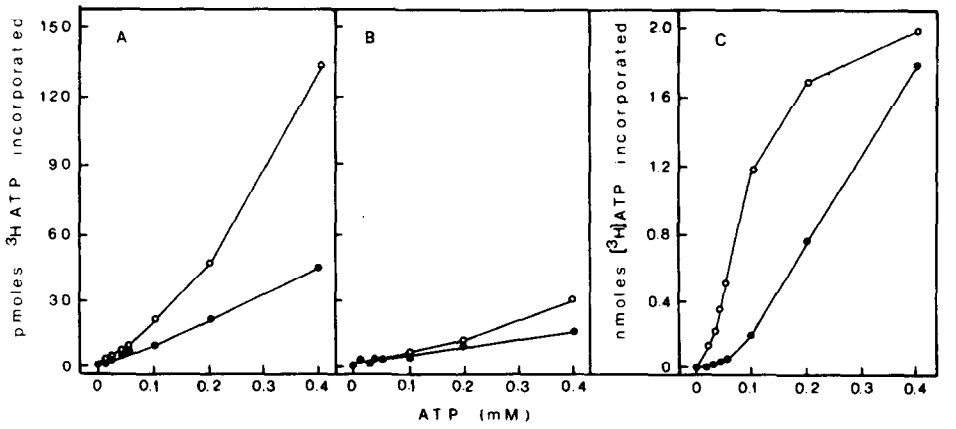

Figure 1 : poly(dT) directed poly(A) synthesis by Bacillus subtilis RNA polymerase forms A, B and C at different ATP concentrations. The standard reaction mixture (see Materials and Methods) contained 1.5μg of enzyme A (Panel A), 1.7μg of enzyme B (Panel B), 1.2μg of enzyme C (Panel C). Poly(A) synthesis was measured in the absence (●—●) or in the presence (○—○) of 2.5 mM 5'-AMP.

of Pero et al. (15). It is very likely that the function of this protein is to facilitate the binding of the core enzyme to the DNA template.

To determine optimal conditions of stimulation of homopolymer synthesis, poly(dT) transcription kinetics catalized by RNA polymerase holoenzyme were analyzed using a fixed amount of ATP and variable concentrations of AMP. The results shown in Figure 2 indicate that the activation of poly(A) synthesis by AMP depends upon the mononucleotide concentrations. At a ratio of AMP to ATP of ten, AMP enhances 20 fold the rate of poly(A) synthesis.

The specificity of the mononucleotide activation by AMP was also tested. A non complementary mononucleotide such as GMP , or the deoxyribonucleoside monophosphate dAMP, do not exhibit any activation effect on poly(dT) transcription catalyzed by Bacillus subtilis RNA polymerase holoenzyme . On the contrary, ADP can also act as an activator of the poly(dT) transcription reaction. A 15 fold stimulation is obtained when ADP and ATP are present at a concentration of 1 mM and 0.1 mM respectively (data not shown).

Stimulation of poly(dT) directed poly(A) synthesis by ADP raised the possibility that in fact the stimulation by AMP was due to the conversion of AMP into ADP catalyzed by adenylate kinase and subsequent polymerization of ADP catalyzed by polynucleotide phosphorilase. In order to verify this hypotesis we tested for ADP formation during poly(A) synthesis in the presence or absence of AMP . A sample of the reaction was analyzed on paper chromatography and nucleoside mono-di and triphosphates were separated. The concentration of the ADP formed during the reaction was 1000 times lower than the ADP concentration neces-

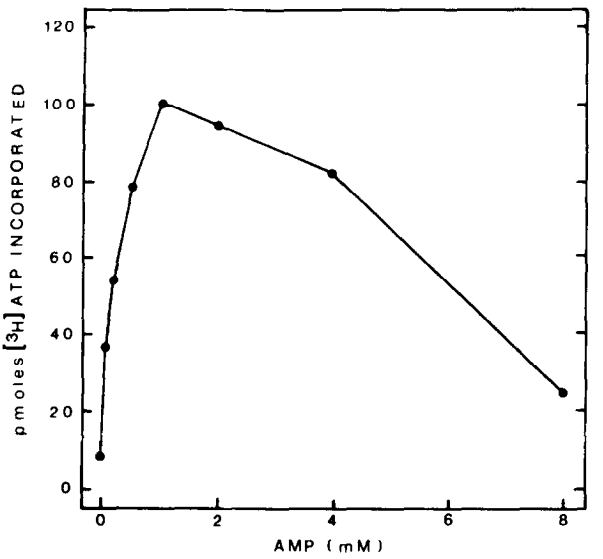

Figure 2 : stimulation of poly(dT) directed poly(A) synthesis at variable AMP concentration. The standard reaction mixture (see Materials and Methods) contained 1.2μg of enzyme C (RNA polymerase holoenzyme) and 0.1 mM [^{3}H]-ATP (10 cpm/pmol).

sary to obtain maximum stimulation of poly(A) synthesis. This result rules out the possibility that AMP stimulation might be due to a massive conversion of AMP into ADP. Moreover, stimulation of homopolymer synthesis by AMP is completely dependent on a DNA-like polymer template. Poly(A) synthesis is at zero level in the absence of poly(dT) for all the concentrations of enzyme tested (from 1 to 10μg per assay). That the RNA synthesis observed in the absence or presence of AMP is really transcription of poly(dT) is further supported by the demonstration that poly(A) synthesis is completely suppressed by rifampicin, a specific inhibitor of bacterial RNA polymerase (data not shown).

To verify if the presence of the nucleoside monophosphate only affects the total amount of poly(A) synthesized, or also biases synthesis of poly(A) molecules of longer chain length, the products of poly(dT) transcription by Bacillus subtilis RNA polymerase holoenzyme in the presence or absence of AMP were analyzed by acrylamide gel electrophoresis under denaturing conditions and the results are shown in Figure 3. When the mononucleotide activator is present, a pronounced increase of high molecular weights products is observed and the appearance of such forms of products proportionally increases with the incubation times. The appearance of larger poly(A) products is also observed when saturating concentrations of ATP were used in the poly(dT) transcription reaction. In the presence of high concen-

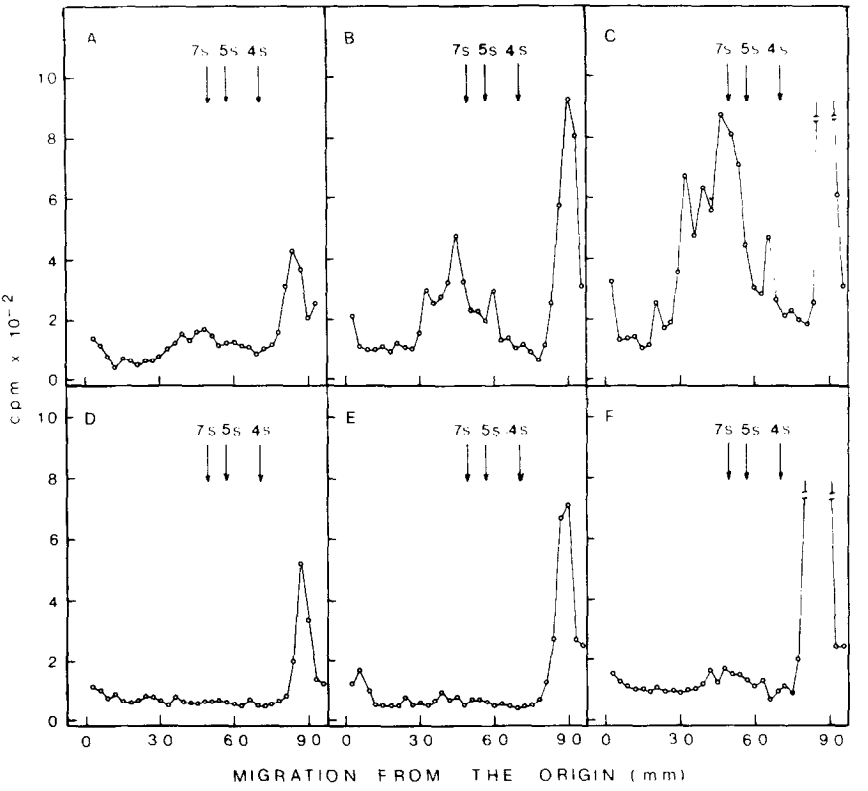

Figure 3: gel electrophoresis analysis of poly(A) products. Standard 0.5 ml reaction mixtures containing 6 μg of RNA polymerase holoenzyme were incubated in the presence of 1 mM AMP for 10 min (Panel A), 20 min (Panel B), 40 min (Panel C). Alternatively, incubation was allowed to proceed for 10 min (Panel D), 20 min (Panel E) and 40 min (Panel F) in the absence of AMP. Samples to be electrophorized were processed as described in Materials and Methods. The arrows indicate the migration of standard RNA moleculars.

trations of ATP, the larger poly(A) products were synthesized both in the presence or in the absence of AMP (data not shown).

DISCUSSION

The results described indicate that 5'-adenosine monophosphate (AMP) greatly enhances the rate of poly(dT) directed poly(A) synthesis catalyzed by Bacillus subtilis RNA polymerase. The reaction order is affected not only by ATP concentrations, but also by AMP concentrations. AMP substitutes for ATP when ATP concentration is low, and the reaction changes to a first order behaviour approaching at 0.4 M ATP zero order. This pattern, that is observed with holoenzyme (enzyme C), is not similarly detected with core enzyme (enzyme B). Core enzyme, which has very low capacity of transcribing poly(dT) is also

poorly sensitive to AMP stimulation. On the contrary, enzyme A which contains a p^{28} and a p^{21} polypeptides associated with core enzyme, is capable of limited transcription and, what is more, is sensitive to AMP stimulation. As we have previously shown (10), the presence of small polypeptides increases the specificity of phage SPP1 DNA transcription by core enzyme. Moreover, it has been demostrated that delta factor can displace sigma factor from the holoenzyme molecule and the delta-core enzyme binds more tightly to the DNA (14). The response to AMP observed with enzyme A is probably the result of an increased affinity of this enzyme form to the poly(dT) template.

As regards the products of poly(dT) transcription by holoenzyme, the net increase in poly(A) synthesized in the presence of AMP in respect to the amount synthesized in the absence of AMP could be due either to an increase in the rate of growth of poly(A) chains or to an increased rate of initiation or both.

It has been demonstrated that AMP can play the role of the initiating purine nucleotide (3); more recently, the role of AMP in transcription has been confirmed in the abortive initiation reaction either on natural or alternating d(A-T) copolymer (16). Moreover, adenosine or nucleotide coenzymes NAD and FAD are also capable to initiate to some extent RNA synthesis (17). From these results it would be reasonable to predict that the stimulation of transcription by AMP may be essentially the result of a better initiation. However, when poly(A) products synthesized in the reaction containing low ATP is compared with the product of the corresponding reaction containing 1 mM AMP, a large difference is observed in the pattern of molecular lenght. In the presence of AMP essentially two fractions of poly(A) are detected: short oligonucleotides and polymers with a sedimentation constant greater than 4 S. The fraction of large polymers constitutes at longer time the major fraction, about 70%, of the total poly(A) synthesized. On the contrary, in the absence of AMP and at low ATP concentration the major portion of poly(A) (more than 90%) is made by short molecules. In the presence of AMP, synthesis of large molecules is specifically promoted and account for the difference in total RNA synthesized observed when AMP is absent. Poly(A) chains longer than 4 S are detected, in the absence of AMP, only when saturating amounts of ATP are present in the reaction mixture.

The analysis of the products synthesized at low ATP concentrations suggest that not only initiation but also the efficient elongation of poly(A) chains is affected, since the majority of the products synthesized are abortive RNA molecules smaller than 4 S. Because larger

poly(A) chains are the predominant products synthesized in the presence of AMP, the mononucleotide activator will affect the rate of initiation as well as the efficiency of the elongation reaction.

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