Synthesis of dinucleoside tetraphosphates by RNA polymerase B (II) from calf thymus

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Highly purified RNA polymerase B (II) from calf thymus catalyses the synthesis of dinucleoside tetraphosphates from ribonucleoside triphosphates in the absence of an oligonucleotide primer or additional protein factors. The reaction requires a DNA template and bivalent cations such as Mn^{2+} or Mg^{2+}. It is strongly inhibited by heparin and high concentrations of α-amanitin but not by rifampicin. On a given template various dinucleoside tetraphosphates of different sequence are formed although the yield depends on the nature of the template.

RNA polymerase B (Calf thymus) Abortive initiation Dinucleoside tetraphosphate α-Amanitin Heparin

1. INTRODUCTION

In vitro highly purified mammalian RNA polymerase B (II) initiates synthesis of specific RNAs only in the presence of additional proteins [1–3]. Without these proteins only unspecific RNAs of all sizes are synthesized. Similar to plant RNA polymerase B (II) [4] the RNA is covalently linked to the 3′-hydroxyl terminus of nicks in the DNA, which is used as primer [5]. The purified enzyme from calf thymus catalyses a process which has been designated as abortive elongation [6]. In this reaction a single 3′,5′-phosphodiester bond is formed between a primer such as an oligo- or mononucleotide and the nucleotide moiety of a ribonucleoside triphosphate in the presence of an artificial DNA template such as poly[d(A-T)] [7]. It is not known, however, if the purified mammalian enzyme initiates phosphodiester bond formation de novo on a natural DNA template with only ribonucleoside triphosphates as substrate, without addition of a primer and protein factors. To answer this question we have studied the catalysis of formation of dinucleoside tetraphosphates according to the equation

$$\text{pppN} + \text{pppN}' \xrightarrow{\text{DNA, } M^{2+}} \text{pppNpN}' + \text{pp}$$

by highly purified RNA polymerase B (II) from calf thymus. This reaction has been designated as abortive initiation [8] and is highly characteristic of the de novo start of RNA polymerase.

2. MATERIALS AND METHODS

The standard mixture (25 μl) contained 10 mM Hepes (pH 7.9), 5 μg RNA polymerase B (II) from calf thymus, purified according to Hodo and Blatti [9] through the phosphocellulose step and 0.25 μg DNA of the plasmid pSmaF [10] cleaved with the restriction endonuclease *Sma*I, or 0.1 pmol of the plasmid pβ1HR16 containing a fragment of the chicken β-globin gene (including the promoter) [11], cleaved with *Eco*RI and *Hin*dIII or 0.1 pmol of the plasmid pBR322 containing the *Pst*Ia fragment of the lysozyme gene [12], cleaved with *Pst*I. The mixture also contained 0.6 mM creatine phosphate and 7.5 μg creatine phosphokinase, to regenerate ATP (which is necessary due to the

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presence of an unspecific 5′-phosphohydrolase activity [7]), 1 mM $MnCl_2$, 60 mM NaCl, 10% glycerol, 0.25 mM dithiothreitol, 0.5 mM ATP and 25 μM [α-^{32}P]UTP (1 μCi; Amersham Buchler, Braunschweig). Incubation was carried out at 37°C for 3 h. Electrophoretic analysis was performed in 25% polyacrylamide gels as described [13]. For identification and sequence analysis, the product was digested with calf intestinal alkaline phosphatase and the dinucleoside monophosphate formed analysed by chromatography on poly(ethyleneimine)-cellulose thin-layer foils [13].

3. RESULTS

3.1. *Dinucleotide synthesis*

When RNA polymerase B (II) from calf thymus is incubated at 37°C for 3 h with ATP and [α-^{32}P]UTP in the presence of $MnCl_2$ and the template DNA pSmaF [10] (which had been cleaved with *SmaI*) a labeled dinucleoside tetraphosphate is synthesized (fig.1, lane c). It is identified as pppApU by the methods mentioned in section 2. The synthesis continues for hours. Simultaneously pppApA is also formed but it is detectable only when labeled ATP is used as substrate (not shown). The dinucleotide pppUpU is synthesized only to a small extent (fig.1, lane k). The same holds for pppUpA (not shown). These observations are in agreement with the notion that mammalian RNA polymerase preferentially initiates RNA chains with a purine nucleotide [14]. In the absence of template or ATP pppApU is not formed (fig.1, lanes h,i). When ATP is replaced by a primer such as 5′-AMP the product pApU is formed although at a slower rate than with the substrate ATP (fig.1, lanes a,c). It suggests that the enzyme catalyses abortive elongation less efficiently than abortive initiation. When Mn^{2+} is replaced by Mg^{2+} the yield of pppApU drops to 10%.

3.2. *Effect of inhibitors*

The synthesis of dinucleoside tetraphosphates is strongly reduced in the presence of 2 μg/ml heparin and disappears at higher concentrations (fig.1, lanes e,f). Rifampicin, an inhibitor of eubacterial RNA polymerase, does not interfere with the reaction (fig.1, lane g). Vaisius and Wieland [7] have shown that 1 μM α-amanitin does not significantly inhibit the formation of a phosphodiester bond during abortive elongation by the calf thymus enzyme whereas RNA synthesis is completely blocked. Here abortive initiation is as insensitive to this concentration of the drug as abortive elongation (fig.1, lanes d,b). However, at higher drug concentration the inhibition of abor-

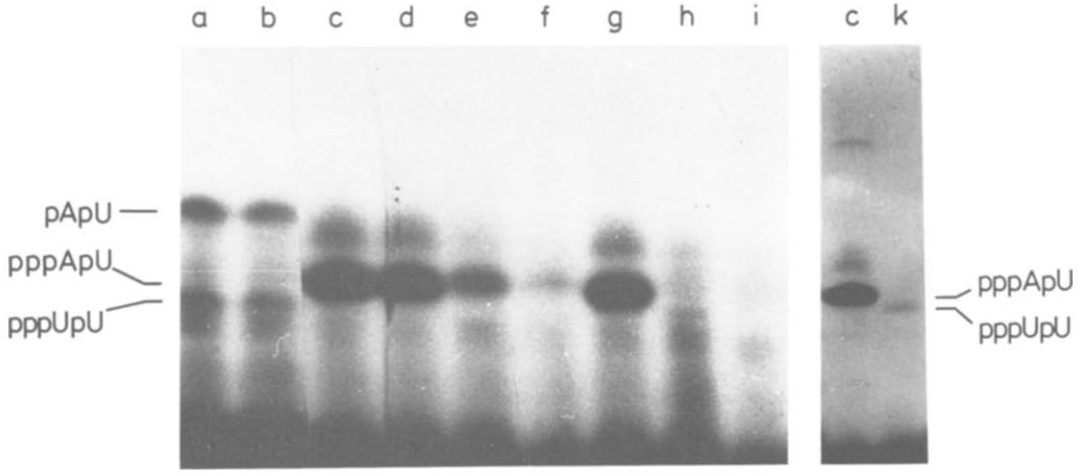

Fig.1. Synthesis of dinucleotides by RNA polymerase B (II) from calf thymus on pSmaF as template. The standard incubation was varied as follows: ATP was replaced by 5′-AMP, simultaneously creatine kinase and creatine phosphate was omitted (lanes a,b); with 1 μg/ml α-amanitin (lanes b,d); with heparin (2 μg/ml, lane e; 100 μg/ml, lane f); with 2 μg/ml rifampicin (lane g); without DNA (lane h); without ATP (lane k).

Fig.2. Synthesis of dinucleoside tetraphosphates with various labeled ribonucleoside triphosphates as substrates on different templates. The standard incubation was varied as follows: [α-^{32}P]UTP was replaced by [α-^{32}P]CTP (lanes a,d,g), by [α-^{32}P]GTP (lanes b,e,h). Templates used: pSmaF [10] (lanes a–c), pβ1HR16 [11] (lanes d–f) and pBR322 containing the lysozyme gene fragment *Pst*Ia [12] (lanes g–i).

tive initiation is more pronounced and reaches almost 90% at 27 μM α-amanitin.

3.3. *Variation of substrate and template*

The results obtained with ATP and UTP as substrates suggest the existence of several start sites on the template. To strengthen this conclusion [α-^{32}P]UTP was replaced in the incubation mixture with labeled CTP or GTP of identical specific radioactivity. Again, labeled dinucleoside tetraphosphates (pppApC, pppGpG) are synthesized (fig.2, lanes a,b). It is evident that several different dinucleotides are formed on the pSmaF template. These results indicate that initiation can occur at different sites. Moreover, the yield of the different products depends on the nature of the template used (fig.2; cf. yields of the various radioactive dinucleotides).

4. DISCUSSION

The experiments here demonstrate that purified RNA polymerase B (II) from calf thymus does not require nucleotide primers or additional protein factors to catalyse the starting reaction of RNA synthesis on a natural DNA template. In this property the mammalian enzyme differs from eubacterial RNA polymerase lacking the sigma factor. Only in the presence of the sigma factor is this enzyme able to initiate with ribonucleoside triphosphates specifically. On the other hand, the calf thymus enzyme lacks distinct selectivity in dinucleoside tetraphosphate synthesis, similar to the enzyme from plants [13]. In this respect it is interesting to remember that also in vivo, in the presence of all protein factors, the vertebrate enzyme has a relatively low specificity in selecting precise start sites [12]. The same has been observed in vitro during dinucleotide primed transcription by the mammalian enzyme [3]. The preference for the synthesis of pppApU may be caused by the property of mammalian RNA polymerase B (II) to form preferentially complexes with regions of the template rich in A and T, such as the TATA box, where it may even start RNA synthesis in vitro without addition of cell extracts [15].

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