An endonuclease closely associated with DNA-dependent RNA polymerase II

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An endonuclease tightly bound to RNA polymerase II has been purified. The enzyme consists of two subunits, and is essential for the polymerase activity.

Cucurbita pepo *seedling* *DNA-dependent RNA polymerase II* *Plant endonuclease*

1. INTRODUCTION

Eukaryotic RNA polymerases are multisubunit enzymes with 10–15 distinct polypeptide chains that range in molecular mass from over 200 to less than 20 kDa [1].

Recently we have presented the purification procedure for RNA polymerase II from tobacco suspension cultures [2]. The finding of a rather simple subunit composition for the tobacco enzyme, which resulted in $\alpha\beta\gamma_2\delta_2$ quaternary structure, remains in contradiction to the other reports on plant and animal RNA polymerases of type II. This discrepancy lies mainly in the smaller subunits which are possibly generated from larger ones by proteolytic degradation.

Whereas the quaternary structure of eukaryotic RNA polymerases has been clarified, the function of individual subunits remains largely obscure. It is known, however, that α-amanitin binds to the 140 kDa subunit of RNA polymerase II [3]; this toxin obviously inhibits the elongation step after the first phosphodiester bond has been formed [4,5]. Recently it has been reported, that the 25 kDa subunit binds ADP in a molar ratio of 1 : 1. Therefore it is suggested that enzymatic cleavage of ATP into ADP could be correlated with an energy-providing process for local unwinding of the DNA double strand [2].

Recently a low molecular mass (3.2 kDa) protein, called CPPTI (*Cucurbita pepo* patissonina trypsin inhibitor) from White bush seeds (*Cucurbita pepo* var. patissonina) has been purified [6]. This protein which is widely spread within the *Cucurbita* family [7] inhibits proteases and therefore it can be used to preserve polymerase activity. We have found that CPPTI protein activates RNA polymerase II and this effect strongly depends on CPPTI concentration. We demonstrate that RNA polymerase activity is affected by CPPTI protein as a result of the activation of an endonuclease activity present in the RNA polymerase preparation. The presence of the two polypeptide chains corresponding to the endonuclease is essential for the polymerase activity.

2. MATERIALS AND METHODS

RNA polymerase II from 3–4-day-old seedlings of White bush was purified using the procedure described [2] except that chromatography on DEAE-cellulose was employed. The enzyme was assayed in a volume of 125 μl of 50 mM Tris-HCl (pH 7.8), 75 mM $(NH_4)_2SO_4$, 2.5 mM NaF, 1.2 mM $MnCl_2$, 2 mM DTT, 0.32 mM each of ATP, GTP, CTP, 0.02 mM UTP, 0.5 μCi

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[^{3}H]UTP (Amersham, Braunschweig, FRG), 10 μg denatured calf thymus DNA. A 100 μl aliquot was spotted on Whatman 3 MM filter paper discs, washed 15 min with solution containing 5% trichloroacetic acid and 5% NaH_2PO_4, dried and counted with a BETA-matic (Kontron, FRG).

One unit of RNA polymerase activity is defined as the amount of enzyme catalyzing the incorporation of 1 pmol UMP into acid precipitable product under standard assay conditions.

CPPTI protein from the seeds of White bush was purified as in [8]. SDS gel electrophoresis (10% gel) was performed according to Laemmli [9]. The gels were scanned in a ERJ 65 M densitometer (Carl Zeiss Jena, GDR) after staining with Coomassie blue. The molecular masses were determined with reference proteins including phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Agarose gel electrophoresis was performed as described [10]. *E. coli* DNA was purchased from Sigma.

3. RESULTS AND DISCUSSION

3.1. *Purification and properties of White bush RNA polymerase II*

For purification, $(NH_4)_2SO_4$ precipitation and chromatography on Heparin-Sepharose and DE-cellulose was used. The total degree of purification is about 5000. The resulting polymerase is of type II, as shown by its sensitivity towards α-amanitin. The enzyme is completely α-amanitin sensitive. Purified RNA polymerase II has a subunit composition of 195, 138, 45 and 37 kDa (to be published) and shows a specific activity of more than 260 nmol·min^{-1}·mg^{-1}, which is comparable to that reported for purified enzyme from other plant tissues [2,11–13]. The above value was obtained when the preparation was characterized within a few days after isolation. During storage at −20°C RNA polymerase activity diminished and its subunit composition was altered. Specifically, several low molecular mass polypeptides appeared presumably as a result of a protease in the RNA polymerase fraction. However the presence of protease inhibitors such as PMSF, pepstatin, phenanthroline and DFP, remained without detectable effect on this degradation.

3.2. *The effect of homologous naturally occurring protease inhibitor on RNA polymerase II activity*

Recently, a low molecular mass protein (29–32 amino acid residues), called CPPTI, from White bush seeds was purified. This protein shows three very interesting features which are advantageous from our point of view. Because of its small molecular mass (3.2 kDa), it is not precipitable with trichloroacetic acid and does not stain with Coomassie blue. These features make it very useful and allow us to characterize the polymerase components at every step of the purification procedure. The third most important feature of this protein is its ability to inhibit some proteases. We have attempted to preserve RNA polymerase activity purified from White bush seedlings employing the CPPTI protein purified from the same source. We have observed that CPPTI protein remarkably stimulates RNA polymerase activity, which was initially interpreted as an inhibition of endogenous protease in the polymerase fraction. RNA polymerase activity strongly depends on

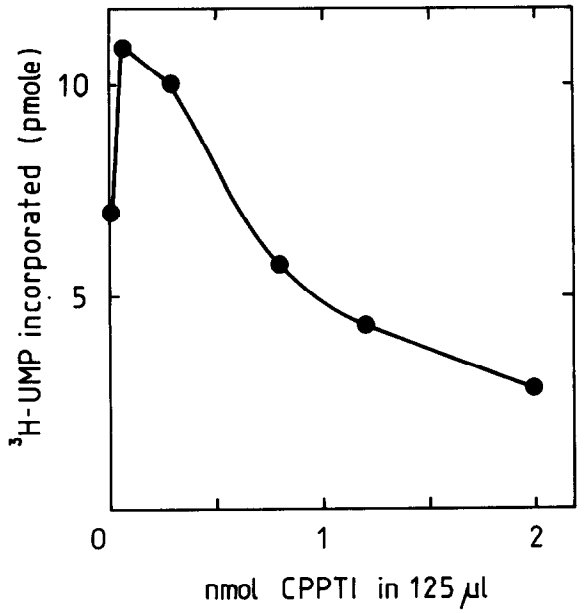

Fig.1. Effect of CPPTI concentration on RNA polymerase activity. The activity of enzyme (0.1 μg protein) was assayed as described in section 2. The transcription mixtures were incubated at 30°C for 30 min.

CPPTI concentration (fig.1). These results are very intriguing. The CPPTI protein could affect RNA polymerase activity by rearrangement of its subunit composition, which is rather difficult to demonstrate and/or by influence on DNA template. As mentioned above, our polymerase preparation does not show nuclease activity (fig.3) which was checked in a rather sensitive test with pBR322 as a substrate. Surprisingly, the polymerase preparation in the presence of CPPTI protein does show endonuclease activity. This nuclease, called paE, was isolated by chromatography on a CPPTI-Sepharose column.

3.3. *Purification and properties of polymerase associated endonuclease (paE)*

The paE nuclease was purified on CPPTI covalently attached to CNBr-activated Sepharose 4B. The amount of CPPTI bound was 20 mg/ml wet gel, as determined by trypsin inhibition. The RNA polymerase (1–2 mg) in buffer B, containing 50 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 1 mM DTT, 20% glycerol and 50 mM NaCl was applied onto a CPPTI-Sepharose column (0.5 × 8 cm). The column was washed with buffer B. Most of the proteins (92–95%) do not bind to the resin and do not show any RNA polymerase activity. The other proteins could be removed with 0.3 M NaCl in buffer B and these also do not exhibit any transcription activity, but display the same nuclease activity as found in the RNA polymerase preparation in the presence of CPPTI protein. Coomassie blue staining revealed a single band, when the nuclease was resolved on 10% polyacrylamide gel under nondenaturing conditions. The SDS gel electrophoresis and Coomassie blue staining indicate the presence of two protein bands (fig.2). Applying protein references, the molecular masses of the subunits were estimated to be 39 and 37 kDa. The stoichiometry of the subunits was determined by measuring the area of densitometric peaks and also absorption at A_{595} Coomassie blue eluted with DMSO from the protein-dye complex. The average value of 1:0.9 was calculated. RNA polymerase completely lost its activity, when the nuclease was removed. The polymerase activity could be partly restored (30–40% of original) following the addition of the nuclease preparation (but not DNase I at the same concentration, not shown).

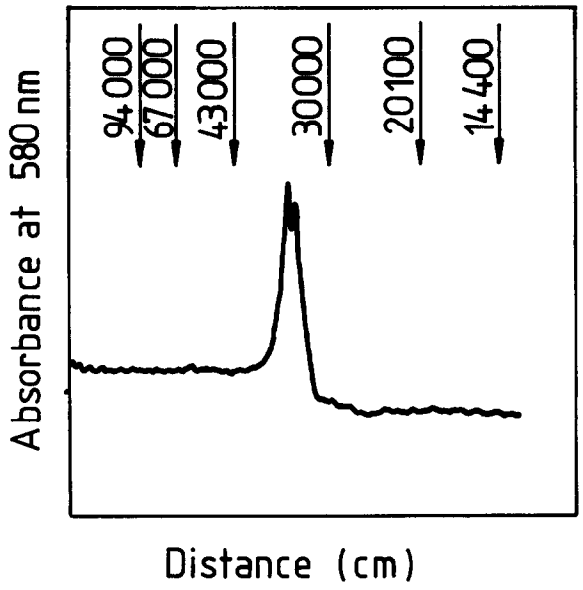

Fig.2. Densitometer tracing of paE nuclease. The enzyme was subjected to SDS-gel electrophoresis and scanned as described in section 2. The molecular masses of the paE subunits were determined with reference proteins.

SDS-PAGE of an RNA polymerase preparation treated with CPPTI-Sepharose revealed the reduction of one of the 4 major polymerase subunits ($\bar{M}_r$ 37000) by about one third. The molar ratio of 1:0.7 of polymerase to endonuclease was calculated.

3.4. *Analysis of paE nucleolytic activity*

The mode of nuclease action was investigated with the substrate *E. coli* DNA and supercoiled DNA from plasmid pBR322. Fig.3A shows the degradation of circular covalently closed DNA (cccDNA). Agarose gel electrophoresis of supercoiled DNA following 30 min treatment with nuclease indicates that the enzyme produces immediately a band, which migrates slightly faster than supercoiled DNA. This is linear DNA which is shorter than the unit-length linear molecule. The position of three forms of DNA plasmid in agarose gel was established by its sequential digestion with tobacco endonuclease and S1 nuclease [10,14] and *Eco*RI cleavage.

Some specificity in the enzyme action was observed in the digestion of *E. coli* DNA (fig.3B).

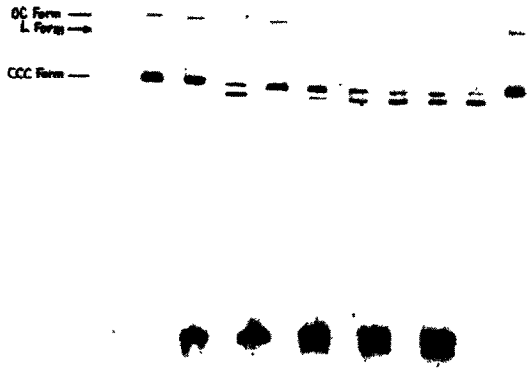

Fig.3. Agarose gel electrophoretic patterns of supercoiled pBR322 DNA and *E. coli* DNA treated with RNA polymerase II and paE endonuclease. The reaction was carried out in 50 μl mixtures containing 50 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.01 mM EDTA, 2 mM $MnCl_2$, 10% glycerol, 0.8 μg DNA and RNA polymerase or paE preparation. After 30 min incubation at 37°C, reaction was stopped by adding 10 μl of solution containing 1 mM EDTA and 0.5% SDS and the samples applied onto 0.9% agarose slab gels. Gels were run at 80 V for 4 h at about 5°C and stained with ethidium bromide. (A, top) pBR322 DNA: track 1, without enzyme, track 2, with 2.3 μg of RNA polymerase, track 3, with 2.3 μg RNA polymerase and 30 pmol CPPTI protein, track 4, with RNA polymerase fraction unadsorbed on CPPTI-Sepharose column, track 5, with 0.01 μg paE, track 6, with 0.1 μg paE, track 7, with 0.3 μg paE, track 8, with 0.5 μg paE, track 9, with 0.7 μg paE and track 10, with 300 pmol CPPTI protein. The arrow indicates the relative migration of linear DNA, run on a separate gel. (B, bottom) *E. coli* DNA: track 1, without enzyme, track 2, with 2.3 μg RNA polymerase, track 3, as track 2 with 30 pmol CPPTI, track 4, with 0.1 μg paE, track 5, with 1.0 μg paE. Numbering of tracks from left to right.

The cleavage products gave only one sharp band, a feature which distinguishes this enzyme from other plant nucleases [10,14], suggesting that nucleolytic attack is limited only to a few positions.

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