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ARTICLE *in* PLANTA · SEPTEMBER 1984

Impact Factor: 3.26 · DOI: 10.1007/BF00410209 · Source: PubMed

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Planta (1984) 162:132–138

Planta

DNA-dependent RNA polymerase II from nuclei of suspension-cultured tobacco cells*

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Abstract. From isolated nuclei of suspension cultured cells of *Nicotiana tabacum*. DNA-dependent RNA polymerase II (E.C. 2.7.76) has been purified to homogeneity as evidenced by polyacrylamide-gel electrophoresis under non-denaturing conditions. The purified enzyme had a specific activity of more than 15 nmol $min^{-1} \cdot mg^{-1}$ with denatured calf thymus DNA as template. Sodium-dodecyl-sulfate gel electrophoresis and protein high-performance liquid chromatography revealed a subunit composition of four proteins with molecular weights of 165000, 135000, 35000 and 25000 and with a stoichiometry of 1:1:2:2. The RNA polymerase did not exhibit any detectable protein-kinase activity. The 25000 subunit binds ADP in a molar ratio of 1:1; it could not be decided whether this subunit has an ATPase activity or is merely an acceptor of ADP.

Key words: *Nicotiana* (RNA polymerase II) – RNA polymerase II.

Introduction

Whereas the properties of DNA-dependent RNA polymerases from the bacteria, especially from *Escherichia coli*, are well known, RNA polymerases from the eukaryotic kingdom are less well elucidated and this is true especially for enzymes from higher plants. The RNA polymerases from bacteria have a rather simple quaternary structure and some details of the molecular mechanisms of transcription have been elaborated. On the other hand, RNA polymerases of eukaryotic cells have a more complicated quaternary structure with a large number of subunits. The function of these subunits is largely obscure. It is known, however, that the fungal toxin α-amanitin binds to the 140000 subunit of RNA polymerase II (Brodner and Wieland 1976); this toxin obviously inhibits the elongation step after the first phosphodiester bond has been formed; for review see (Chambon 1975; Roeder 1976; Duda 1976; Guilfoyle 1980).

In our laboratory a very efficient procedure has been developed for the preparation of nuclei from suspension-cultured cells (Willmitzer and Wagner 1981). The isolated nuclei showed rather high physiological activities when different nuclear enzymes were tested. In order to elucidate the properties of RNA polymerase II from the tobacco cell, which had not been previously described, a purification procedure was elaborated starting with isolated nuclei. Using the purified enzyme the subunit structure and the function of subunits was investigated. The results give rise to the proposal of a quaternary structure which contains only six polypeptides; this is less complicated than all the former proposals for other plant RNA polymerase II enzymes (Duda 1976; Guilfoyle 1980; Jendrisak and Burgess 1977; Jendrisak and Guilfoyle 1978; Guilfoyle and Jendrisak 1978; Goto et al. 1978; Sasaki et al. 1979; Guilfoyle and Malcolm 1980; Hahn and Servos 1980; Jendrisak and Skuzeski 1983). It is shown that some of the formerly assumed smaller subunits originate from a common subunit by proteolytic digestion. It is further shown that the enzyme binds ATP and after cleavage of the γ-phosphate, ADP is very strongly bound to the 25000 subunit.

* This contribution is dedicated to Professor Fritz Cramer on the occasion of his 60th birthday

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Abbreviations: HPLC = high-performance liquid chromatography; PMSF = phenylmethylsulfonyl fluoride; SDS = sodium dodecyl sulfate

Material and methods

Chemicals and buffers. Unlabeled nucleotides were purchased from Sigma (München, FRG), calf thymus DNA from Worthington Biochem. Co. (Freehold, New Jersey, USA) and α-amanitin from Calbiochem (Giessen, FRG). [^{3}H] Uridine-5′-triphosphate (UTP), [^{3}H]ATP, α-[^{32}P]ATP and γ-[^{32}P]-ATP were obtained from Amersham-Buchler (Braunschweig, FRG). Buffer A (140 mM NaCl) and B (50 mM NaCl) contained 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) HCl (pH 7.9), 1 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 100 μM phenylmethylsulfonyl fluoride (PMSF) and 20% (v/v) glycerol.

Enzyme assays. The RNA polymerase was assayed (Rose et al. 1976) 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 ATP, guanosine 5′-triphosphate (GTP) and cytidine 5′-triphosphate CTP, respectively, 5 μM labelled UTP (45 cpm·pmol^{-1}), 10 μg denatured calf thymus DNA. Assays started by addition of the polymerase were incubated for 15 min at 30° C, 100 μl were applied onto a piece of Whatman DE 81 paper (Whatman, Springfield Mill, U.K.) and washed three times (5 min) with 5% NaH_2PO_4 solution, twice with distilled water and once with 96% ethanol. The radioactivity was counted with a Betaszint 5000 (Berthold, Wildbad, FRG). Protein kinase was assayed as described by Baydoun et al. (1982) with phosvitin as substrate; the labelled ATP concentration was 1 μM.

Gel electrophoresis and thin-layer chromatography. Sodium-dodecyl-sulfate (SDS) gel electrophoresis (8% gel) was performed according to Laemmli (1970). The gels was scanned in a LKB 2202 Ultrascan laser densitometer (LKB, Bromma, Sweden) after staining with Coomassie Blue. Gel electrophoresis under non-denaturing conditions was performed with a 7% acrylamide gel according to Sklar and Roeder (1976). Thin-layer chromatography of labeled nucleotides was performed on poly (ethylenemine)-cellulose with a 1.0 M KCl solution. The absorption at 260 nm and radioactivity was scanned using the TLC Scanner CS-920 (Shimadzu Coop., Kyoto, Japan) and Berthold Automatic TLC-Linear Analyser (Berthold, Wildbad, FRG), respectively.

High-performance liquid chromatography (HPLC)-gel filtration chromatography. The enzyme was treated with SDS as described in the legend of Fig. 2 and 50 μg were applied onto a TSK-G 3000 SW column (LKB) equilibrated and developed with 20 mM phosphate (pH 7.0) and 0.1% SDS (flow rate 0.7 ml min^{-1}). The absorbance was recorded at 280 nm. The molecular weights of the subunits were estimated through separate runs with reference proteins including fibrinogen, γ-globulin, bovine serum albumin, ovalbumin, carboanhydrase, myoglobin and aprotinin.

For the determination of binding of labelled ATP the enzyme was treated with α-[^{32}P] ATP as described in the legend of Fig. 2. The further treatment with SDS and the gel filtration (50 μg protein) conditions were the same as described above; 0.35-ml fractions were collected for counting the radioactivity.

Purification of RNA polymerase II. The origin and growth of the suspension-cultured tobacco cells have been described (Willmitzer 1979). Preparation and purification of the nuclei was performed according to Willmitzer and Wagner (1981). All operations were performed at 0–4° C. The nuclei were suspended in buffer A (1 ml per 10 g of original tissue), sonicated in a Branson (Danbury, Conn., USA) sonifier (three times 1 min at 100 W), brought up to 30% $(NH_4)_2SO_4$ concentration and, after stirring for 30 min, centrifuged at 20000 *g* for 60 min. The pellet was dissolved in buffer B, dialysed over night with the same buffer and centrifuged at 100000 *g* for 60 min. The supernatant was applied to a single-strand DNA-cellulose column and the proteins were eluted by a stepwise increase of the NaCl concentration (steps of 0.1 M) in buffer B. The main polymerase activity appeared at 0.3 M NaCl, while a small fraction was eluted at 0.4 M. The pooled active fractions were dialysed overnight with buffer B and applied onto a heparin-Sepharose column (Fig. 1A). After pooling the active fractions and dialysis overnight against buffer B a second DNA-cellulose purification step was applied (Fig. 1B). The enzyme was stored at −68° C and was stable up to one month with only a small decrease in activity.

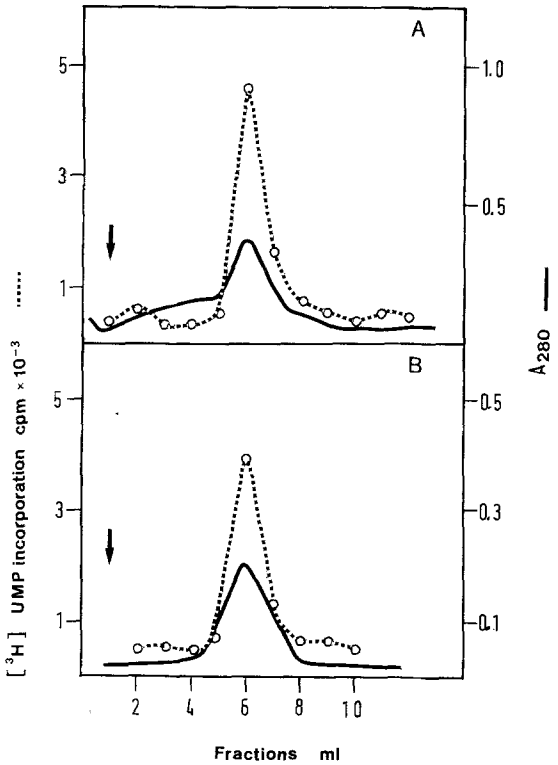

Fig. 1A, B. Purification of tobacco RNA polymerase by heparin-Sepharose (**A**) and DNA-cellulose (**B**) chromatography. Active fractions from the 0.3 M NaCl elution of the first DNA-cellulose column dialysed against buffer B were applied onto a heparin-Sepharose column (0.8 cm inner diameter, 9 cm long) which had been equilibrated with buffer B. The column was developed with a linear gradient of 0.05–1.0 M NaCl in buffer B. The pooled active fractions of the heparin column dialysed against buffer B were applied onto a single-strand DNA-cellulose column (0.8 cm inner diameter, 9 cm long). After washing with buffer B a linear gradient of NaCl (0.05–1.0 M) was applied. Fractions of 1 ml were collected and 50-μl probes were used for determination of the RNA polymerase activity. UMP = uridine 5′-monophosphate

Results

Purification of tobacco RNA polymerase II. The isolation of the RNA polymerase is based on a nuclei preparation. Buffers for extraction and puri-

Table 1. Purification of RNA polymerase II from nuclei of tobacco cell suspension cultures. The data were taken from an experiment starting with 880 g suspension-grown cells (wet weight)

Purification step	Protein (mg)	Total activity (nmol/min^{-1})	Recovery (%)	Specific activity (nmol/min^{-1} mg^{-1})	Degree of purification
Sonicated nuclei	920	7.33	25	0.008	1
$(NH_4)_2SO_4$ precipitate	270	28.8	100	0.11	3.5
DNA-cellulose	6.4	14.9	52	2.33	74
Heparin-Sepharose	1.2	14.4	50	12.0	380
DNA-cellulose	0.85	13.1	45	15.3	490

fication were supplemented with PMSF to reduce proteolytic degradation. For purification, $(NH_4)_2SO_4$ precipitation and chromatography on DNA-cellulose and heparin-Sepharose was used; the former column was applied twice (Fig. 1).

Table 1 shows the results of a typical purification experiment; 880 g (wet weight) cells from late log phase were used for preparing the nuclei (Willmitzer and Wagner 1981), resulting in 0.85 mg RNA polymerase II. There was a significant increase in the total RNA-polymerase activity after ammonium-sulfate precipitation which indicates some inhibitory substances in the crude extract. The degree of purification for this step was not, therefore, calculated from the specific activities but from the total amount of protein. This is correct under the assumption that there is no appreciable loss of activity in this step; there was no RNA-polymerase activity detectable in the supernatant of the ammonium-sulfate precipitation. The total degree of purification was about 500; this figure would be still higher if a factor for the purification of the nuclei were taken into account. A recent purificationof RNA polymerase II from whole pea seedlings (Sasaki et al. 1979) ended up with a 1500-fold purification. The present figure of 500 is therefore quite acceptable, as one would expect a factor of more than three for the purification of the nuclei.

Properties of RNA polymerase II. The RNA polymerase is of type II, as is shown by its sensitivity towards α-aminitin. The pooled active fractions of the first DNA-cellulose column exhibited an 80% reduction in activity at an inhibitor concentration of 0.5 μg ml^{-1}; the pure enzyme is completly α-aminitin sensitive. After the last chromatographic step, RNA polymerase showed a specific activity of more than 15 nmol min^{-1} mg^{-1} which is comparable to that reported for purified mammalian polymerases (Kedinger and Chambon 1972) and also for those from plant tissue (Sasaki et al. 1979; Job et al. 1982; Guilfoyle and Malcolm 1980; Goto et al. 1978; Guilfoyle and Jendrisak 1978; Hahn and Servos 1980).

The optimum concentration for Mn^{2+} ions was found to be 1.2 mM which is very nearly the same as reported for other plant RNA polymerases of type II (Duda 1976; Guilfoyle 1980). In the presence of 1.2 mM Mn^{2+} the optimum concentration of $(NH_4)_2SO_4$ is 75 mM. The purified RNA-polymerase-II fraction was also assayed for protein-kinase activity; however, under the conditions described in Material and methods no protein-kinase activity was detectable.

Subunit composition of RNA polymerase II. When 15 μg of the enzyme were applied to a 7% polyacrylamide gel under non-denaturing conditions, Coomassie-Blue staining revealed a single band with no protein impurities detectable (Fig. 2, panel 1). The SDS gel electrophoresis and Coomassie-Blue staining (panel 5) clearly indicate four strong protein bands. Applying protein references, the molecular weights of the subunits were estimated to be 165000, 135000, 35000 and 25000.

The stoichiometry of the subunits was determined by scanning the Coomassie-Blue-stained bands, the average value of three experiments gave a composition of 1:1:2:2 with a deviation of not more than 10%. This subunit structure was also confirmed by high-performance gel-filtration chromatography under denaturing conditions, i.e. in the presence of 0.1% SDS.

When a silver stain was applied, some more proteins bands were visualized (Fig. 2, panel 4). Those at 19000 and 14000 were sometimes also observed with Coomassie Blue. By separate experiments, it was shown that these bands appeared when the enzyme was, even shortly, incubated at 30° C. In contrast to the larger subunits, the molar ratio of the 25000, 19000 and 14000 subunits was found to be variable; however, the sum of these small subunits mostly turned out to be nearly two.

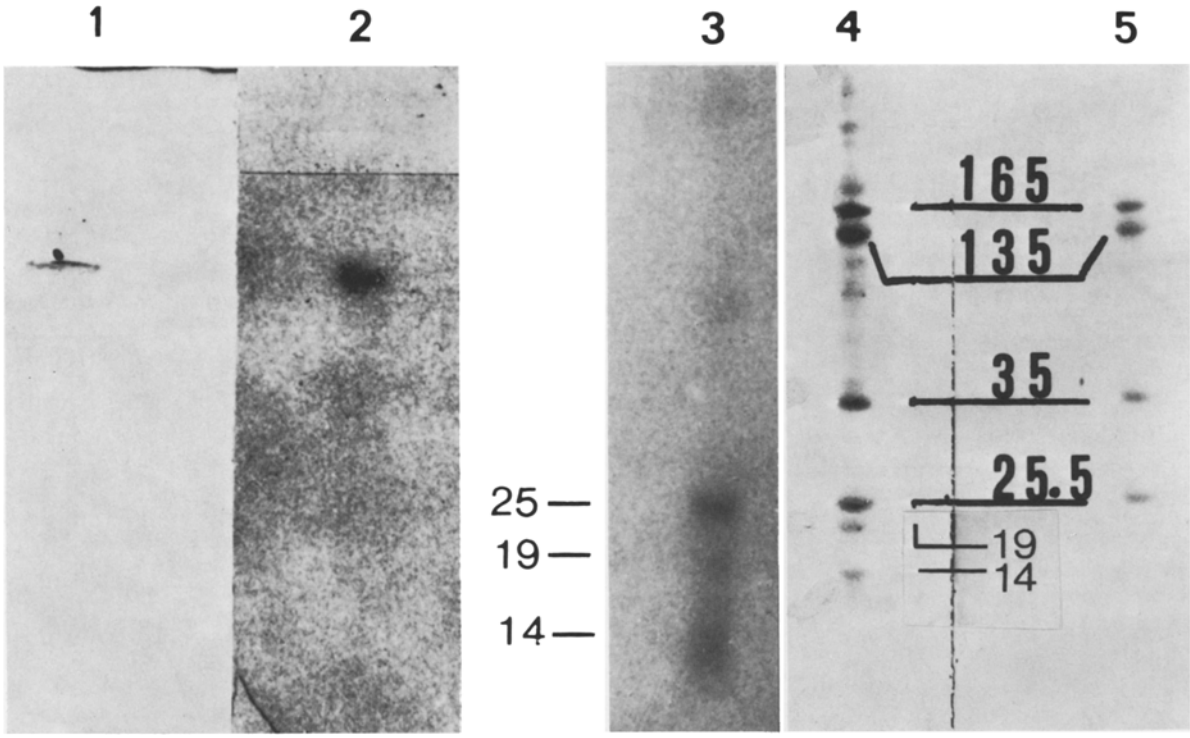

Fig. 2. Gel electrophoresis of tobacco RNA polymerase II under non-denaturing conditions (*panels 1, 2*) and in the presence of SDS (*panels 3, 4, 5*). *Panels 1* and *5* indicate Coomassie-Blue staining and *panel 4* silver staining; *panels 2* and *3* are autoradiograms after labeling with α-[^{32}P]ATP. Gel electrophoresis under non-denaturing conditions was performed according to Sklar and Roeder (1976) with a 7% acrylamide gel and 15 µg protein. For labeling (*panel 2*), the enzyme had been dialyzed over night with 50 mM Tris-HCl (pH 7.0), 2 mM DTT, 5 mM $MgCl_2$ and 10 µM PMSF and then allowed to react with 3.4 µM [^{3}H] ATP at 30° C for 30 min; thereafter 5 mM L-lysine added for quenching. For SDS polyacrylamide-gel electrophoresis the enzyme was dialysed over night against 0.1% SDS, treated at 80° C for 10 min and 10 µg protein were subjected to 8% polyacrylamide slab SDS gel electrophoresis. For reference, proteins of known molecular weights were run on parallel gel tracks (not shown); they included myosin, aldolase, β-galactosidase, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin and cytochrome c. After staining, the Coomassie-Blue tracks were scanned and the relative amount of each band was determined from the respective areas. For labeling (*panel 3*), the enzyme was treated as the probe of *panel 2* (except that the buffer had a pH of 8.0) followed by the SDS treatment

This is shown with a separate experiment (Fig. 3) when RNA polymerase was incubated at 30° C. It is clearly indicated that the appearance of the 19000 and 14000 subunits is correlated with a disappearance of the 25000 subunit. Thus, it is highly likely that the protein bands of 19000 and 14000 originate from the 25000 subunit. This is confirmed by data, discussed below, which indicate that the 19000 and 14000 proteins also bind ADP which is a specific property of the 25000 subunit (compare panel 3 of Fig. 2). The transformation of the 25000 subunit into the smaller proteins may be caused by the action of a protease in the RNA-polymerase fraction; however this protease is obviously insensitive to PMSF, pepstatin and phenandrolin, as they have no detectable effect on this transformation.

Silver staining further revealed some proteins bands with M_r values larger than 165000 (Fig. 2, panel 4). It is possible that they originate from artificial complexes of different subunit composition. Treatment of the RNA polymerase with higher concentrations of ammonium sulfate revealed, on gel filtration, protein peaks of 240000 to 370000; however, their subunit molecular weights and composition, as analysed by SDS gel electrophoresis, was rather variable. The best results were obtained when the enzyme was treated with 0.1% SDS containing 250 mM ammonium sulfate (Fig. 4). Analyses of the subunit composition on SDS gel electrophoresis showed that the 330000 peak contained subunits of 165000 and 35000 with a molar ratio of 1:3.2; the 270000 peak turned out to be an artificial complex of 135000 and 25000 subunits with the molar ratio of 1:3.4. The later findings are supported by the fact that the 270000 peak binds small amounts of ATP which is a property of the 25000 subunit.

The binding of ADP by RNA polymerase II. High-performance liquid chromatography and classical gel filtration of the polymerase in the presence of ATP revealed a slight enhancement in the absorbtion at 260 nm of the enzyme peak. Surprisingly, using γ-[^{32}P] ATP the enzyme peak exhibited only very little, if any, radioactivity. Thus, the association of a degraded form of ATP had to be considered. Gel filtration of the RNA polymerase after incubation with either α-[^{32}P]ATP or [^{3}H]ATP showed both effects, an enhancement of the absorbtion at 260 nm and also labelling of the enzyme peak. Labeling by α-[^{32}P]ATP and gel electrophoresis under non-denaturing conditions is shown in Fig. 2 (panel 2). Almost all radioactivity could be dissociated from the enzyme at pH 2.0. Based on chromatography on poly(ethyleneimine)-cellulose thin-layer plates in 1 M KCl, all the radioactivity dissociated could be assigned to ADP. It was of interest to determine which polymerase subunit is the origin of ADP binding. The HPLC gel-filtration experiments (Fig. 4) clearly demonstrated that it is the 25000 subunit which binds ADP. The bound ADP obviously does not dissociate from the 25000 subunit in the presence of SDS. These results were further confirmed by SDS gel electrophoresis and autoradiography (Fig. 2, panel 3), which revealed that besides the 25000 subunit the 19000 and 14000 bands were also labelled with ATP, but the amount of radioactivity incorporated was variable and dependent on the time of incubation. An estimate of the stoichiometry of labelling of the 25000 subunit with ATP gave a value of 1:0.9–1.2; this estimate is deduced from the HPLC experiments.

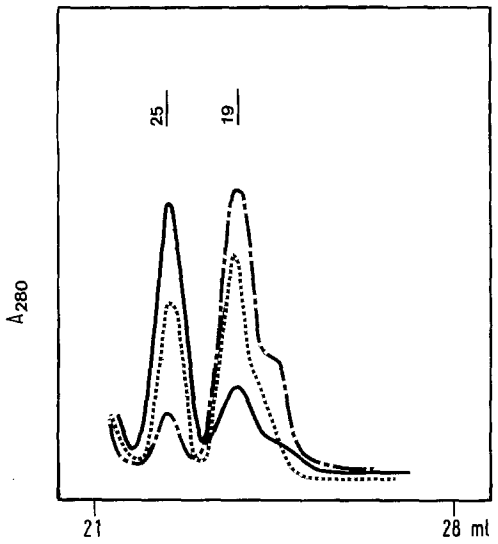

Fig. 3. Effect of incubation at 30° C on the subunit structure of tobacco RNA polymerase. The enzyme in buffer B was incubated for 3 h at 0° C (———); 40 min at 30° C (– – –); 6 h at 30° C (– – – –). Treatment with SDS and gel-filtration conditions (50 µg protein) are described in the legend of Fig. 2 and in Material and methods

Discussion

The present purification procedure for RNA polymerase II from tobacco is based on the preceeding purification of nuclei, and this may be a crucial point. The finding of a rather simple subunit composition for the tobacco enzyme, which would result in an ($\alpha\beta\gamma_2\delta_2$) quaternary structure, is in contradiction to other reports on plant and animal RNA polymerases of type II which show a more complex structure containing a larger number of subunits. This discrepancy lies mainly in the smaller subunits with M_r values less than 25000. The present proposal is based on the findings that

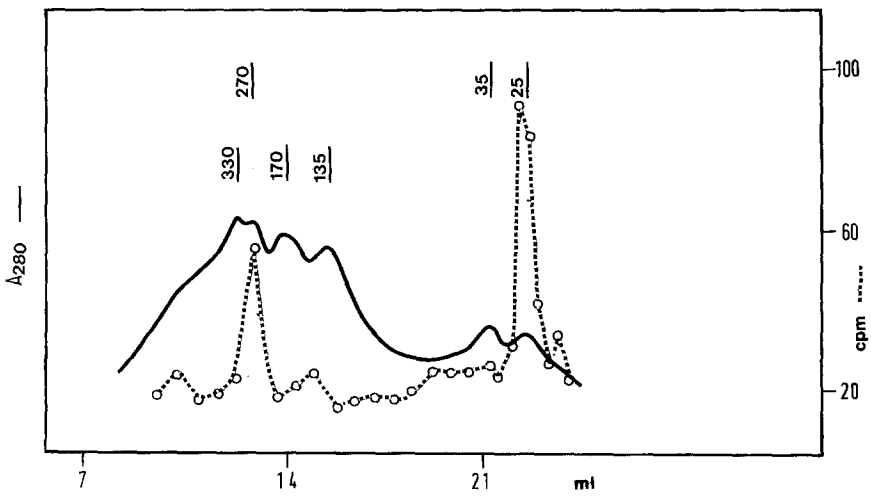

Fig. 4. Effect of elevated $(NH_4)_2SO_4$ concentrations on the subunit structure of the RNA polymerase II. The RNA polymerase was treated as described in the legend of Fig. 2 except that 0.25 M $(NH_4)_2SO_4$ was present during dialysis and gel filtration (50 µg protein). Labelling with [^{3}H] ATP was also performed as described in the legend of Fig. 2; however, ammonium sulfate was not present during the labelling procedure

the smaller subunits with molecular weights of 19000 and 14000 are generated from the 25000 subunit by proteolytic degradation. This is shown by a direct experiment (Fig. 3) but is also strengthened by the fact that these three proteins have a common property, i.e. binding of ADP, which is shown by HPLC gel filtration and autoradiography of SDS gels.

It would be very interesting to see whether the present proposal of a less complicated quaternary structure of RNA polymerase II could be further confirmed for other plant enzymes and even for mammalian polymerases of type II. On the other hand, the more complicated subunit structure with its sometimes odd stoichiometries could be consistent with the suggestion that eukaryotic RNA polymerases also use σ-like factors for the recognition of promoters and these may be lost to different degrees during the purification of these enzymes. Thus further studies are necessary to clarify these points.

Besides the subunits smaller than 25000, the M_r values of the present proposal are in accord with those from other plant RNA polymerases of type II. In his review, Guilfoyle (1980) summarizes these values as 180000 (for proliferating tissue), 140000, 40000 and 27000. The supposition that the 40000 and 27000 subunits might be present in two copies per polymerase II molecule has already been indicated in this review, which leaves open the question of the existence one or two copies (Guilfoyle 1980). On the other hand, in the case of pea polymerase II (Sasaki et al. 1979), stoichiometry values of 1.5 and 2.0 are reported for the 43000 and 26000 subunits, respectively. In the present work it is further shown that under certain conditions, e.g. at elevated ionic strength, a redistribution of polymerase subunits may occur resulting in different combinations of subunits. This may explain the occasional appearance of odd subunits in SDS gel electrophoresis especially when applying the sensitive silver stain.

A second very interesting point is the finding that the 25000 subunit strongly binds a molecule of ADP. This is the first report on nucleotide binding of an individual subunit of a eukaryotic RNA polymerase. The ADP is bound after cleavage of ATP, and it cannot be distinguished whether the ATP-cleavage activity is a function of the holoenzyme or of the 25000 subunit. At present, one can only speculate on the functional role of this nucleotide binding. In general, one assumes that RNA polymerases have two binding sites for nucleotide substrates, an initiation site for binding of the first purine triphosphate and an elongation site for the repeated binding of the next nucleotide (Wu and Goldthwait 1969).

The latter process involves the cleavage of nucleoside triphosphate into monophosphate. Enzymatic cleavage of ATP into ADP, however, could be correlated with an energy-providing process for local unwinding of the DNA double strand (Saucier and Wang 1972). On the other hand, a nucleotide triphosphatase was reported which was assumed to control the nucleotide-triphosphate pools in response to changes of the divalent-cation concentration (Grossmann et al. 1981; Grossmann and Seitz 1979). However, this conclusion stems from data obtained with whole nuclei from parsley suspension-cultured cells; thus it is unlikely that the ATPase activity found in the present work is part of the nuclear nucleotide-triphosphatase activity reported.

The present RNA-polymerase preparation does not exhibit a protein-kinase activity when assayed with an acidic protein such as phosvitin. This finding does not support recent speculations on the protein-kinase activities of subunits of RNA polymerase I from a mammalian source (Rose et al. 1981) and of RNA polymerase II from a plant source (Jankowski and Kleczkowski 1980).

Jan Szopa, on leave from the Institute of Biochemistry, Wroclaw University, Poland, acknowledges fellowship from the Alexander-von-Humboldt-Stiftung.

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Received 2 February; accepted 4 April 1984