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Chloroplast RNA polymerase from spinach: purification and DNA-binding proteins

Silva Lerbs*, Jean-Francois Briat & Regis Mache**
Laboratoire de Physiologie cellulaire végétale, CNRS ERA n° 488 Université de Grenoble I, BP 53 X, F-38041 Grenoble Cedex, France

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Abstract

Spinach DNA dependent RNA polymerase was purified from isolated chloroplasts by two different procedures. Analysis of the protein composition of the two preparations by SDS-polyacrylamide gel electrophoresis always shows six abundant polypeptides with Mr of 150, 110, 102, 80, 75 and 38 Kd and one less abundant polypeptide of 25 Kd. Some other proteins ranging from 40–70 Kd in Mr are also detected but in a minor and variable amount. The two preparations have an optimum of enzyme activity at 30 °C and at 15 mM $(NH_4)_2SO_4$ when tested with denatured calf thymus DNA.

Binding experiments with two different nick translated fragments of spinach chloroplast DNA show that the 80 and 75 Kd polypeptides possess a strong DNA binding capacity.

Introduction

RNA polymerase is a key enzyme for the understanding of gene expression. In this respect a lot of work has been done to isolate the enzyme from different organisms. But our knowledge of chloroplast RNA polymerase is still very limited. The enzyme was solubilized and partially characterized from wheat leaves (18). In maize, the enzyme has been solubilized and purified from isolated chloroplasts (1, 2, 20) as well as from cell homogenates (13). The maize chloroplast RNA polymerase is resistant to α-amanitin and to rifamycin SV. It has a temperature optimum of 46–48 °C and consists of 14–16 polypeptides with M_r values ranging from 27 Kd to 180 Kd (13).

Also transcriptionally active DNA-protein complexes have been isolated from chloroplasts of spinach and *Euglena gracilis* (5, 11, 19). Recently, we developed a new procedure to obtain a crude, but highly active RNA polymerase fraction from spinach chloroplasts. This crude RNA polymerase is resistant to α-amanitin and to rifampicin and is able to initiate transcription in the promoter region of the 16S rRNA gene (7). In the present paper we report on the further purification of the spinach chloroplast RNA polymerase and the determination of the DNA-binding proteins.

Materials and methods

Crude RNA polymerase preparation. Crude RNA polymerase was prepared from spinach chloroplasts as already described (7). Each preparation was made from 5 Kg of spinach leaves. Briefly, isolated chloroplasts were disrupted in a buffer containing 50 mM Tris–HCl, pH 7.8, 0.1 M $(NH_4)_2SO_4$, 4 mM EDTA, 1 mM dithiothreitol and 0.05 mM phenylmethylsulfonyl fluoride. The suspension was homogenized and after addition of glycerol to a final concentration of 25%, the suspension was cen-

* On leave from the Akademie der Wissenschaften der DDR, Institut für Biochemie der Pflanzen, Halle 4010 Halle (Saale), DDR.
** To whom reprint requests should be sent.

trifuged at 80 000 **g** for 15 min. The supernatant was treated with 50 μg/ml of DNase I (Sigma) in the presence of 11 mM Mg acetate for 30 min at room temperature and then loaded on a Heparin–Sepharose CL-6B (Pharmacia) column (9 × 0.9 cm). An active RNA polymerase fraction was eluted from the column and dialysed for 5 h against 2 l of buffer A [50 mM Tris–HCl, pH 7.8, 25% glycerol, 0.1% Triton X-100, 0.1 mM EDTA, 1 mM dithiothreitol containing 15 mM $(NH_4)_2SO_4$] prior to storage at –70 °C. Properties of this crude fraction are described elsewhere (7).

Polymin P (polyethyleneimine) precipitation. A 0.1% (v/v) solution of Polymin P was prepared from a 10% stock solution. The precipitation was performed according to Burgess & Jendrisak (8). Polymin P was added to the crude RNA polymerase preparation at a concentration found to be optimal for the precipitation of the enzyme (see results). After vigorous shaking the samples were cooled on ice for 15 min and centrifuged at 10 000 **g** for 30 min. Pellets were suspended in buffer A containing 300 mM $(NH_4)_2SO_4$ and adjusted to 50 mM $(NH_4)_2SO_4$ by dilution with buffer A before loading on DEAE-cellulose column.

DEAE-cellulose chromatography. A 6-ml DEAE-cellulose (Whatman DE 52) column of 0.9 cm diameter was prepared and equilibrated with buffer A containing 15 or 50 mM $(NH_4)_2SO_4$. Either the crude preparation [15 mM $(NH_4)_2SO_4$] or the resuspended Polymin P pellet [50 mM $(NH_4)_2SO_4$] was applied to the column at a flow rate of 5 ml/h. After loading, the column was washed with 12 ml of loading buffer and then eluted with a 40 ml linear gradient from 15 or 50 mM to 600 mM $(NH_4)_2SO_4$ in buffer A. Fractions of 2 ml were collected and assayed for RNA polymerase activity.

Phosphocellulose chromatography. A 3-ml column of phosphocellulose (Whatman P 11) was equilibrated with buffer A containing 15 mM $(NH_4)_2SO_4$. The column was loaded with the enzyme prepared from the crude preparation followed by further purification on DEAE-cellulose. Elution was performed with a 20-ml linear gradient from 15 to 400 mM $(NH_4)_2SO_4$ in buffer A. Fractions of 1.5 ml were collected and tested for RNA polymerase activity.

RNA polymerase assays. The enzyme activity was measured in a final volume of 100 μl containing 50 mM Tris. HCl, pH 7.8, 20 mM $MgCl_2$, 0.3 mM each of ATP, GTP and CTP (Boehringer) and 8 μCi ^{3}H-UTP (37 Ci/mM, ICN, Paris-Labo), 10 μl of each enzyme fraction to be tested and 10 μg of denatured calf thymus DNA. Incubation was performed at 30 °C for 1 h. Samples were loaded on DE 81 filter discs (Whatman), washed and counted as previously described (5).

Electrophoresis and transfer of proteins on nitrocellulose filters. Proteins were precipitated with 5 volumes of acetone for 3h at –20 °C and electrophoresed in 7.5 to 15% SDS-polyacrylamide exponential gradient gels (200 × 190 × 1 mm) according to Laemmli (14). Proteins were stained with the silver technique described by Oakley *et al.* (16). The protein content of the different fractions was determined by the method of Bradford (4).

Protein blotting to nitrocellulose sheets was achieved by electrophoresis according to Towbin *et al.* (21). The transfer of polypeptides for the DNA binding has been verified by staining with Amidoblack after autoradiography.

DNA binding. The Bgl II–Pvu II chloroplast DNA fragment containing 691 base pairs upstream the 16S rRNA gene was prepared from the clone pJFM1 as described elsewhere (7). The Pvu II–Eco RI chloroplast DNA fragment was prepared from the clone pSocB10 (7) and contains *ca.* 1100 base pairs of the 16S rRNA gene. The DNA was nick translated according to Maniatis *et al.* (15). The DNA binding to blotted proteins was done according to Bowen *et al.* (3) using *ca.* 10^5 cpm of nick translated DNA per ml of incubation medium for 1 h at room temperature. After washing with 50 mM or 500 mM NaCl, filters were dried and exposed to Kodak X AR5 films.

Results

RNA polymerase purification. Crude RNA polymerase preparations were obtained as described in Materials and methods, using Heparin–Sepharose chromatography. Starting with this preparation, RNA polymerase was purified following two procedures, A and B, as indicated in Table 1.

Table 1. RNA polymerase activity at different purification steps.

Fraction	Specific activity[a]	Purification	Yield of recovery (%)
Crude RNA polymerase	0.75	1	100[b]
Procedure A:			
DEAE-cellulose	8.8	12	70
Phosphocellulose	17.4	23	38
Procedure B:			
Polymin P pellet	8.2	11	60–70
DEAE-cellulose	25.4	34	60–70

[a] pM UTP incorporated × 10/μg proteins.
[b] Used as reference.

In procedure A, the crude preparation was applied to a DEAE-cellulose column. The enzyme elutes at 250 mM $(NH_4)_2SO_4$ as can be seen on the elution profile given in Fig. 1A. 70% of the enzyme activity is recovered and the specific activity of the pooled fractions is 12 times that of the crude fraction (Table 1). The active fractions obtained after DEAE-cellulose were further purified on phosphocellulose. The enzyme elutes at 180 mM $(NH_4)_2SO_4$ (Fig. 1B). The specific activity of the enzyme is doubled, but 46% of the activity is lost (Table 1).

In procedure B, 0.0005% to 0.01% Polymin P were added to small aliquots of the crude RNA polymerase fraction. RNA polymerase activity was assayed in order to estimate the inhibition of several concentrations of Polymin P. After centrifugation, supernatants and resuspended pellets were tested for RNA polymerase activity (Fig. 2). There is a concentration-dependent inhibition of RNA polymerase activity by Polymin P, but the inhibition is never higher than 50%.

The optimum of enzyme activity found in the pellet corresponds to a narrow range of Polymin P concentration which varies from preparation to preparation but was generally between 0.001 and 0.003% of Polymin P. At this optimum, between 60 and 70% of the enzyme activity was precipitated. When the crude RNA polymerase preparation was centrifuged without addition of Polymin P, a visible pellet was also obtained, but this pellet had no RNA polymerase activity. Therefore it can be concluded that there is a specific precipitation of the enzyme by Polymin P.

After we had determined the exact concentration of Polymin P for optimal precipitation of the enzyme, Polymin P precipitation was done with the whole crude RNA polymerase preparation. The Polymin P precipitation was solubilized and loaded on a DEAE-cellulose column. The elution profile is slightly different from that obtained when the crude RNA polymerase is directly loaded on the

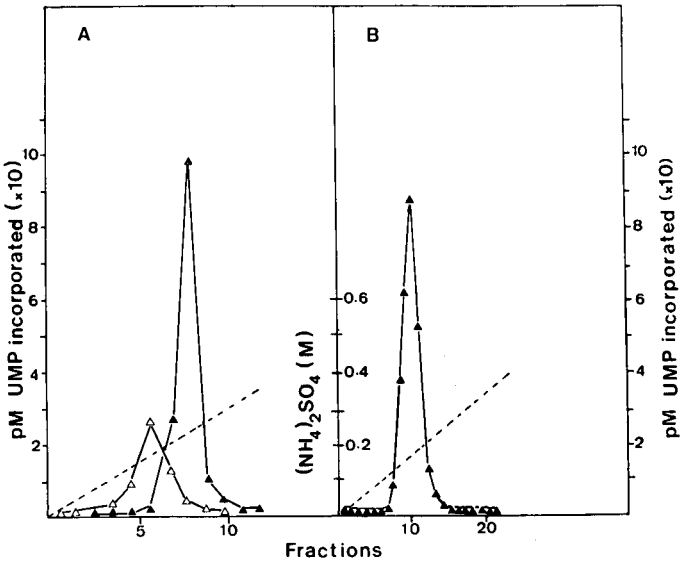

Fig. 1. Elution profiles of the RNA polymerase chromatographied on different columns. Preparations were made according to Table 1. A: DEAE-cellulose chromatography, procedure A (▲—▲) and procedure B (△—△). B: phosphocellulose-chromatography.

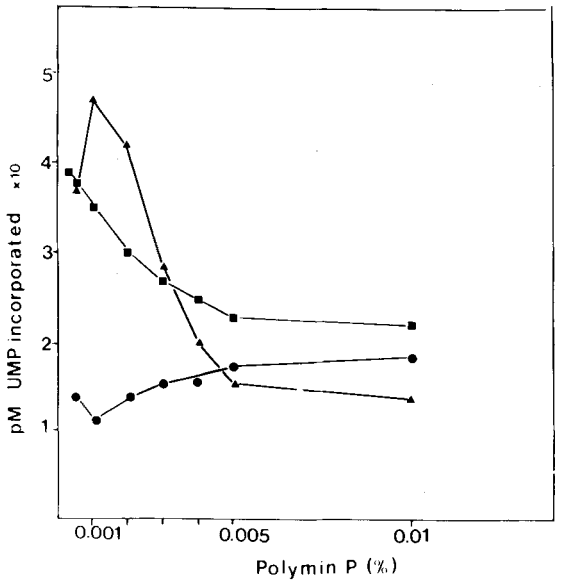

Fig. 2. Polymin P precipitation of the RNA polymerase from the crude preparation. Effect of the Polymin P concentration on the activity of the RNA polymerase before centrifugation (■—■). Activity recovered in the pellet (▲—▲) and in the supernatant (●—●) after centrifugation.

DEAE-cellulose column (Fig. 1A). The recovery of enzyme activity is 100% (compare the two last lines in Table 1) and the specific activity is 34 times higher than that of the crude RNA polymerase fraction. This procedure yields 200 μg of RNA polymerase containing proteins per Kg of leaves. The activity is stable for months if stored at −70 °C. Filtration of the RNA polymerase preparation obtained by procedure B on Ultrogel AcA 22 or Sephadex G-100 results in complete loss of activity (not shown).

RNA polymerase properties. Some properties of the two RNA polymerase preparations are shown in Fig. 3. Both preparations have an optimum of activity at 30 °C (A) and at an ionic strength of 15 mM $(NH_4)_2SO_4$ (B). The transcription reaction continues for more than 60 min (C).

Polypeptide composition. The SDS-gel electrophoretic pattern of the two RNA polymerase preparations are shown in Fig. 4D and F. Six major polypeptides with M_r of *ca.* 150, 110, 102, 80, 75, 38 and

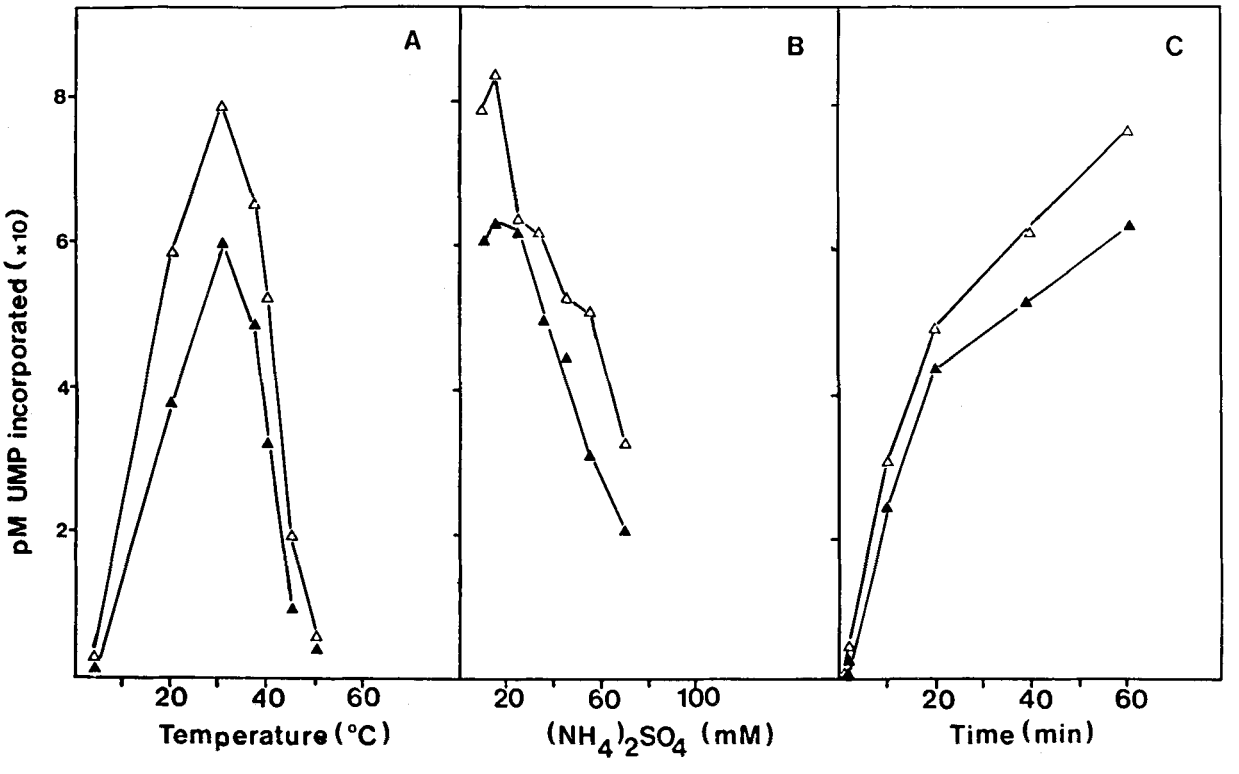

Fig. 3. RNA polymerase properties. A: influence of temperature on the activity of RNA polymerase. B: influence of ionic strength on the activity of RNA polymerase. C: time dependence of transcription reaction. Procedure A (▲—▲), procedure B (△—△).

one minor polypeptide of 25 Kd were always present in the preparations. The 25 Kd polypeptide is more abundant in procedure B than in procedure A. Some additional proteins with M_r ranging from 40 to 70 Kd are also present in the preparations but in contrast to the seven above-mentioned polypeptides their amount varies from one preparation to another. For comparison the polypeptide composition of the crude RNA polymerase preparation is given in Fig. 4B. The high *Mr* proteins which are considered to belong to RNA polymerase (150, 110, 102, 80 and 75 Kd) are not visibe yet, because they represent too small an amount relative to the total protein of the crude preparation, but considerable accumulation of the enzyme is achieved already after the first purification step in both isolation procedures (see Fig. 4C and E).

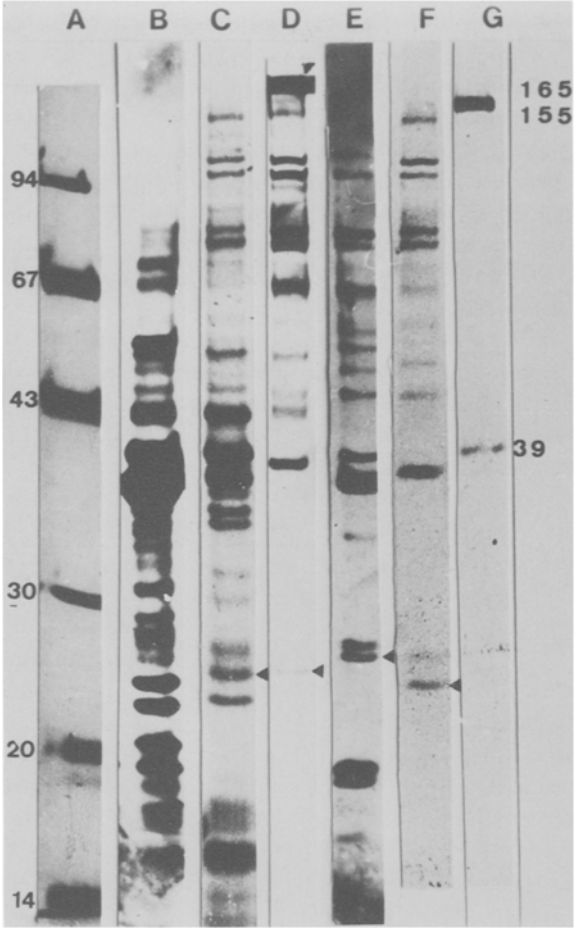

Fig. 4. SDS-polyacrylamide electrophoresis of RNA polymerase. A: markers - phosphorylase, 94 Kd; bovine serum albumin, 67 Kd; ovalbumin, 43 Kd; carbonic anhydrase, 30 Kd; trypsin inhibitor, 20 Kd. B: crude enzyme preparation after Heparin-Sepharose chromatography (25 μg of proteins; activity: $1{,}4 \times 10^5$ cpm). C: procedure A - DEAE-cellulose fraction (15 μg of proteins; activity: 10^6 cpm). D: procedure A - phosphocellulose fraction (7.5 μg of proteins; activity: 10^6 cpm). E: procedure B - Polymin P pellet of 250 μg of proteins of the crude RNA polymerase preparation (activity: 10^6 cpm). F: procedure B - DEAE-cellulose fraction (5 μg of proteins, activity: 10^6 cpm). G: *E. coli* core RNA polymerase. Black triangles indicate the position of the 25 Kd. The major bands in F have an *Mr* of 150, 110, 102, 80, 75 and 38 Kd.

DNA-binding proteins. Two fragments obtained from spinach chloroplast DNA have been used to investigate which of the RNA polymerase polypeptides are involved in the enzyme binding to the DNA. One is the 831 bp fragment containing 140 bp of the 16S rRNA gene and 691 bp upstream this gene with a promoter region of the 16S gene that has been recently cloned and sequenced (7). The other is a *ca.* 1100 bp fragment derived from the clones pSocB10 (7) by cleavage with Pvu II and Eco RI. It contains only part of the 16S rRNA gene.

The enzyme fractions obtained by procedures A and B were electrophoresed, transfered to nitrocellulose and incubated with the nick-translated DNA fragments. Figure 5 shows the result obtained with the promoter containing fragment. It clearly binds to the 80 and 75 Kd polypeptides (B and C). Washing of the filter with 500 mM NaCl reduces the radioactivity but a considerable part of the DNA remains attached to the proteins (D).

In order to know if this binding was promoter specific we repeated the experiment with the *ca.* 1100 bp DNA fragment. It binds also to the 80 and 75 Kd polypeptides with the same affinity (not shown). Therefore, the binding properties of the two polypeptides are not promoter specific, at least under the experimental conditions which were used.

Discussion

RNA polymerase was further purified from a crude enzyme preparation by two different procedures. Enzyme preparations obtained by these different procedures have the same temperature and ionic strength optima as the crude preparation (7) and the transcriptionally active DNA-protein complex (5).

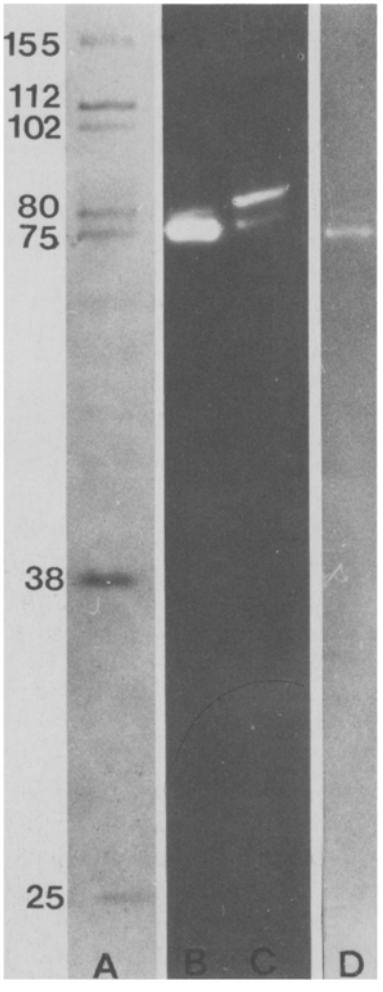

Fig. 5. Binding of the nick translated chloroplast DNA fragment containing the promoter region of the 16S rRNA gene to polypeptides of RNA polymerase. A: blot of RNA polymerase according to procedure B shown by staining with Amido-black. B: polypeptides of the RNA polymerase purified by procedure B, which have bound the nick translated chloroplast DNA fragment at 50 mM NaCl. C: the same as (B) but polypeptides of the RNA polymerase purified by procedure A. D: the same as (B) after washing with 500 mM NaCl. 125 μg of proteins of each RNA polymerase preparation were loaded on the gel before transfer to the nitrocellulose sheet.

The polypeptide composition of RNA polymerase isolated by procedure A or B is very similar. Six major polypeptides with *Mr* of *ca.* 150, 110, 102, 80, 75 and 38 Kd are always detectable in almost the same amount in the preparations. Therefore, they are considered to belong to RNA polymerase. One polypeptide with *Mr* of 25 Kd is more abundant in enzyme preparation B than in A, but is nevertheless always detectable.

Some minor bands ranging from 38 to 70 Kd are also regularly detected in the preparations A and B, but the amount of these proteins varies with each preparation. Therefore, it is suggested that they do not belong to RNA polymerase.

The polypeptide composition of the spinach chloroplast RNA polymerase described here differs from the polypeptide composition of the enzyme isolated from the transcriptionally active DNA-protein complex (6). This difference can be explained if we consider the specific activity of the enzyme fraction isolated from the complex. It is not higher than that of the crude RNA polymerase preparation described above. Therefore, RNA polymerase polypeptides cannot be detected above the background of other polypeptides.

Jolly & Bogorad found that the maize chloroplast RNA polymerase preferentially transcribes maize chloroplast DNA in the presence of a 27 Kd protein (12). In our case, preparation B is more active than preparation A (see Table 1) indicating a possible correlation of the 25 Kd protein with the transcriptional activity, but in as far as denatured calf thymus DNA was used as template we cannot compare with the results obtained with maize. Further experiments have to be performed.

If we further compare the spinach to the maize chloroplast RNA polymerase (13), we have to refer to three polypeptides with *Mr* of 38, 40 and 42 Kd which are observed in the maize enzyme. If the crude RNA polymerase preparations from spinach are purified only by DEAE chromatography or by Polymin P precipitation (first step of procedure A or B) three different polypeptides are also detected (*Mr*: 36, 38 and 40 Kd; Fig. 4C and E). But two of them (36 and 40 Kd) are completely removed by the next purification step (Fig. 4D and F). Since 100% of the enzyme activity of the Polymin P pellet were recovered after DEAE cellulose chromatography these two polypeptides should not be part of the RNA polymerase of spinach chloroplasts. In addition, the maize enzyme contains more polypeptides of high molecular weight (larger than 100 Kd) than the spinach enzyme. Besides these differences some polypeptides of similar *Mr* are found in maize and spinach chloroplast RNA polymerase: 110, *ca.* 100, *ca.* 80 and 75 Kd.

If we compare the spinach enzyme with the *E.*

coli RNA polymerase some polypeptides are similar in size. The 150 Kd spinach polypeptide has a size close to the β (155 Kd) subunit of the *E. coli* RNA polymerase. The 38 Kd protein from spinach has a size comparable to the α subunit (39 Kd) of the *E. coli* enzyme.

Two of the six main polypeptides of the spinach RNA polymerase (*Mr* of 80 and 75 Kd) possess high DNA binding capacity (see Fig. 5). However, no binding specificity to the promoter-containing DNA fragment could be shown. But this result is not surprising since it is known for *E. coli* RNA polymerase that only the holoenzyme is able to interact specifically to promoter-containing DNA in order to form an "open complex" (9, 22). The σ subunit (*Mr* of 82 000) plays an important role in the specific recognition of *E. coli* RNA polymerase to promoter sites. It would be particularly interesting to know if there are structural and functional similarities between the two DNA-binding proteins of spinach RNA polymerase and the σ subunit of the *E. coli* enzyme and, in addition, between the α subunit (39 Kd) of the *E. coli* RNA polymerase and the 38 Kd protein of the spinach enzyme.

Three different types of RNA polymerases are present in the nucleus. The polypeptide composition of the three enzymes was determined in several organisms and 9 to 14 polypeptides were found to be present with the exception of the Cauliflower RNA polymerase I, which contains only 6 subunits (17). From the low number of polypeptides found in the spinach enzyme no conclusion can be made whether this enzyme is closer to procaryotic or to eucaryotic RNA polymerase.

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