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Identification of the template binding polypeptide in the pea chloroplast transcriptional complex

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ABSTRACT

We have identified the template-binding polypeptide in the pea chloroplast transcriptional complex by photoaffinity labelling. This polypeptide has an apparent molecular weight of about 150 kDa and binds to both, chloroplast ribosomal (16S rRNA) and messenger (*psbA*) promoters. The 16S rRNA and *psbA* promoters were amplified from chloroplast DNA by the polymerase chain reaction and labelled with a photoactive analogue of TTP, 5-bromodeoxy UTP, as well as with α-^{32}P-dCTP. Using the filter-binding assay, the conditions for binding of the RNA polymerase complex to chloroplast promoters were optimized. The polypeptide directly interacting with the template was photo-crosslinked to it and resolved by denaturing gel electrophoresis. The photoaffinity labelling of the 150 kDa polypeptide was dependent on photoactivation by UV irradiation, and the presence of chloroplast promoters. Competition experiments showed that the protein formed a strong interaction with the plastid promoters which could not be displaced by λ-phage DNA or synthetic polynucleotides. The photo-crosslinked and nuclease-treated promoter-polypeptide complex was resistant to further digestion with DNase and RNase, but could be hydrolyzed by Proteinase K. Binding of the promoters by the 150 kDa polypeptide could not be surpressed by transcription inhibitors like rifampicin and α-amanitin. However, heparin (0.001%) inhibited the formation of the enzyme-promoter complex, and interfered with the photoaffinity labelling of the 150 kDa polypeptide. The extent of photoaffinity labelling of 150 kDa polypeptide exhibits some degree of correlation to total transcriptional activity under various salt concentrations. The results demonstrate that the 150 kDa polypeptide is a functional template binding polypeptide of the pea chloroplast transcription complex.

INTRODUCTION

The chloroplast transcription apparatus has been studied extensively and there have been several reports on highly enriched fractions of RNA polymerase from chloroplasts (1–9). These multisubunit enzymes, which have been obtained from various plant species by using different purification schemes have been shown to contain 7–14 polypeptides. The pea chloroplast RNA preparation has been reported to contain 10 prominent polypeptides of 150, 130, 115, 110, 95, 85, 75, 48, 44 and 39 kDa, and four minor other polypeptides of 90, 37, 34 and 27 kDa (5, 9). In spinach, the 150, 145, 110 102, 90, 85, 75, 38, 33 and 25 kDa polypeptides have been considered as the prominent polypeptides of the chloroplast RNA polymerase (6). In maize, two polypeptides of 180 and 140 kDa and twelve other polypeptides ranging in molecular weight from 27 to 110 kDa have been found to constitute the functional chloroplast RNA polymerase (1), and the 180, 120, 85 and 38 kDa polypeptides have been reported as prominent components (10). Since these polypeptides have not yet been functionally characterized, it is possible that many of these polypeptides are co-purifying contaminants. Thus, the precise subunit composition of the chloroplast RNA polymerase remains elusive.

The chloroplast genome of several species has been shown to contain genes (*rpo A*, *rpoB*, *rpoC*$_1$ and *C*$_2$) encoding proteins with segments about 26–50% homologous to the *Escherichia coli* RNA polymerase subunits. Recently, Hu and Bogorad have shown that 3 prominent polypeptides with apparant molecular masses of 180, 120 and 38 kDa are encoded by *rpoC*$_2$, *rpoB* and *rpoA* genes mapped on the maize chloroplast genome (10). However, the precise function of these polypeptides is far from clear.

Elucidation of the nature of interaction of the RNA polymerase with the DNA template and the nascent RNA is essential for understanding the structure of the active transcription complex. The photoaffinity labelling technique has been successfully used to functionally analyse the subunits of RNA polymerases from bacteria and higher organisms. In RNA polymerases isolated from *Drosophila*, HeLa cells and *E. coli*, the binding site for the promoter has been localized on the largest subunit of the enzyme (11, 12). The largest subunit also makes contact with the RNA during transcript elongation (13, 14). In a recent report (15), using the photoaffinity labelling approach, we have shown that the nascent transcripts contact atleast two polypeptides of about 51 and 54 kDa during the transcription of pea chloroplast DNA *in vitro* (see Discussion). We have, however, been unable to detect any radioactive RNA associated with the largest polypeptide of

the pea chloroplast transcription complex, which was photoaffinity labelled both, in the relatively purified state as well as immediately after chloroplast lysis (15). In this paper, we report the identification of the template-binding polypeptide in the pea chloroplast RNA polymerase complex by photoaffinity labelling the transcription complex with the bromo-dUMP-substituted α-^{32}P-labelled DNA fragments containing the chloroplast 16S rRNA or *psbA* promoter sequences.

MATERIALS AND METHODS

Isolation of pea chloroplast RNA polymerase complex

Chloroplasts were isolated from 250 grams of 7 – 10 day old pea leaves (*Pisum sativum* L. var Arkel) as described previously (9). The chloroplasts were lysed with 2.5% Triton X-100 in the presence of 50 mM Tris-HCl pH 8.0 containing 0.5 M sucrose, 15 mM $MgCl_2$, 25 mM 2-mercaptoethanol and 0.2 mM pmsf (phenylmethyl sulfonyl fluoride) and 5 mg/l each of pepstatin and leupeptin. The lysate was loaded on a 100 ml DEAE-cellulose column (Whatman DE-52) equilibrated in 50 mM Tris-HCl pH 8.0, 25 mM 2-mercaptoethanol and 0.2 mM pmsf, 20% glycerol (Buffer A) with 0.1 M $(NH_4)_2SO_4$. After extensive washings with the equilibration buffer the enzyme fraction was eluted with 0.3M $(NH_4)_2SO_4$ in Buffer A. Fractions were assayed for RNA polymerase activity (9), dialysed against the equilibration buffer and loaded onto a 5 ml phosphocellulose column (Whatman) equilibrated in the same buffer. The enzyme fraction was eluted with 0.2 M $(NH_4)_2SO_4$ in Buffer A and stored in 50% glycerol at −20°C or flash frozen in aliquots in liquid N_2. This preparation is referred to as the RNA polymerase complex—a complex of 14 polypeptides (27 – 150 kDa) which co-purify with RNA polymerase activity under various chromatographic conditions, including fractionation on a 16S rDNA promoter affinity column (9).

Chloroplast DNA

Chloroplasts were isolated and lysed as described above except $MgCl_2$ was not included in the chloroplast isolation buffer. The chloroplast lysate was centrifuged at 100,000×g for 16 hours and the DNA pellet was dissolved in 1 ml of 10 mM Tris pH 8.0 and 1mM EDTA (TE). The DNA solution was extracted three times with phenol chloroform (1:1) and finally precipitated with 2.5 volumes of ethanol in presence of 0.8 M $LiCl_2$. The DNA pellet was dissolved in 1 ml TE and used as a template (at appropriate dilution) for PCR amplification.

PCR Primers

Primers for the PCR amplification of segments of chloroplast DNA were synthesized on an Applied Biosystems 380B DNA synthesizer, deprotected with ammonia, extracted with phenol:chloroform (1:1), and precipitated with ethanol. The primer consisted of about 24 base sequences selected from sequencing data of clones carrying the 16S rRNA or *psbA* promoter fragments. These clones have been described earlier (16, 17) and the relevant sequence information is shown in Fig. 1.

Labelling of chloroplast promoters with bromo-dUTP during PCR amplification

PCR reactions contained 10 mM Tris pH 8.0, 50 mM KCl, 5 mM $MgCl_2$ 0.001% gelatin 50 uM each of dATP, dGTP, bromo-dUTP (or TTP) and 0.5 uM dCTP, 100 uCi of α-^{32}P-dCTP (6000 Ci/mmole), 0.5 uM of each primer, 100 ng of chloroplast genomic DNA and 2.5 units of Taq polymerase (Perkin elmer/Cetus USA) in a final reaction volume of 100 ul. Amplification consisted of 25 – 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and elongation at 72°C for 3 min. After extraction with phenol:chloroform (1:1) each reaction mixture was passed through a 1 ml spun column of Sephadex G-50 in TE. The concentration of the labelled promoter fragments were estimated by running a parallel 'cold' reaction in the absence of α-^{32}P-dCTP and comparing the band intensity with a known concentration of PBR 322 DNA—HaeIII digest on an ethidium bromide stained 6% polyacrylamide gel. A 500 bp fragment of λ-DNA was also amplified under identical conditions by using the template and the primers provided by Perkin Elmer Cetus PCR amplification kit. The sequence of the amplified fragments is shown in Fig. 1A and B. The amplified products were found to be of the predicted size (422 bp sequence of the 16S rRNA gene and a 426 bp sequence of the *psbA* gene). The fidelity of the amplified fragments was evaluated by the ability of the pea chloroplast RNA polymerase complex to use these as templates for *in vitro* 'run-off' transcription assays.

Nitrocellulose filter binding assay

The binding reactions consisted of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 uM KCl, 0.1 mg/ml BSA, 1 mM DTT, 5% glycerol and pea chloroplast RNA polymerase complex (~400 ng protein) in a final volume of 100 ul. The reaction was started by addition of α-^{32}P-bromo-dUMP—substituted probe (≥50,000 cpm; 5 – 10 ng) containing either the 16S rRNA or the *psbA* promoter sequences. After 20 minutes of incubation at 25°C, 0.5 ml of washing buffer (same as binding buffer without BSA) was added to each tube and the solution was filtered immediately through a Millipore 0.45 um nitrocellulose filter discs (HATF 25 mm diameter) presoaked in the binding buffer. The filter discs were washed 3×5 ml and were counted for

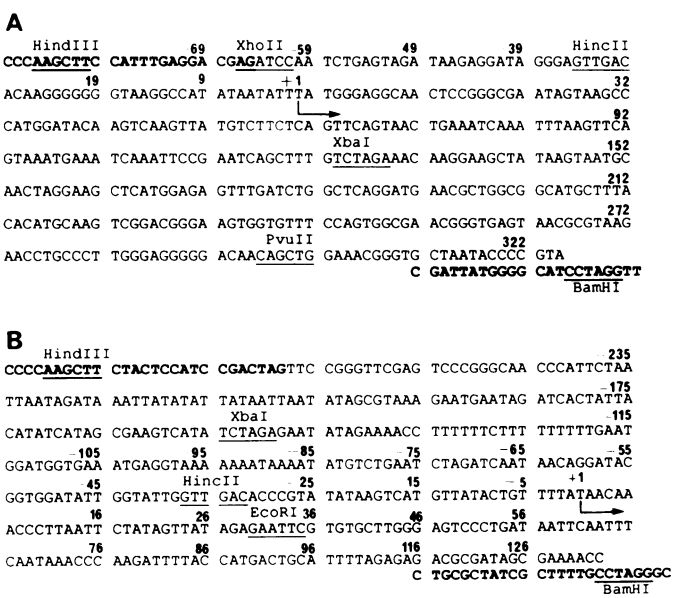

Figure 1. Sequences of the amplified fragments of chloroplast DNA. Nucleotide sequences of the amplified fragments of chloroplast DNA containing the (A) 16S rRNA promoter and (B) *psbA* promoter are shown. Primers used for the PCR amplification of these fragments are indicated by bold letters. ↳ indicates the transcriptional start sites.

retained radioactivity (Cerenkov). Blanks (no RNA polymerase complex added) were also included in each experiment to monitor the nonspecific binding of the probe to the filters (which was generally less than 3%).

Photoaffinity labelling

Reactions were set up as described above for the filter binding assay. After 20 min at 25°C, the solution was irradiated 5 cm from the ultraviolet light source for 20 min (or as specified). The UV source was an IBI transilluminator (300−315 nm) with a maximum output of 7.4 watts/sq. cm. This spectrum is specific for absorption by bromouracil. (In this wavelength range, the absorption coefficient of bromo-dUMP is 10−100 times greater than that of TMP.) Moreover there is a minimum damage to the proteins under these conditions (18).

Isolation of photo-crosslinked complexes

After UV irradiation, the samples were treated with 5 units of DNase I (Boehringer Mannheim) for 2 hours at 37°C. In some experiments, this treatment was followed by digestion with snake venom phosphodiesterase (Boehringer Mannheim), alkaline phosphatase (from calf thymus, Boehringer Mannheim), and micrococcal nuclease (Boehringer Mannheim), essentially as described in (19). The samples were analysed on a 5% SDS-polyacrylamide gel. Standard protein markers were used to ascertain the apparant Mr of the labelled proteins visualized by autoradiography.

RESULTS

Optimization of binding conditions by nitrocellulose filter binding assay

We have used a nitrocellulose filter binding assay to optimize the binding of an enriched pea chloroplast RNA polymerase complex to the 16S rRNA and *psbA* promoter—containing fragments. The assay relies on the ability of nitrocellulose to bind proteins but not double stranded DNA (20). Use of ^{32}P-labelled promoter fragments allowed quantitation of the DNA bound to the protein at various times and under different conditions. The RNA polymerase complex was mixed with the promoter fragment under optimized binding conditions. After incubation, the mixture was suction-filtered through nitrocellulose which allowed the unbound DNA to pass through while the protein-bound DNA was retained on the filter. Figure 2A shows the increase in the binding of 16S rRNA promoter fragment with increasing concentration of RNA polymerase complex when incubated for 30 minutes at 30°C. Although an increase in binding was observed with increasing concentrations of the enzyme preparation upto 400 ng protein, about 50 ng was enough for half maximal binding. This information was necessary to ensure the use of limiting quantities of the enzyme preparation for competition experiments with unlabelled promoters (see Fig. 6). Since heparin is known to interfere with most protein-DNA interactions (21−23), the chloroplast RNA polymerase was treated with heparin (0.001%) and was found to lose its ability to bind to the labelled DNA fragments (Fig.2A). However, binding to the labelled fragment of DNA was not affected by pretreatment of the chloroplast RNA polymerase complex with 0.01% each of α-amanitin and rifampicin (data not shown). A time course experiment using 400 ng of the enzyme preparation showed that maximum binding could be observed in about 45 min at 30°C, and half maximal binding was observed in about 5 minutes. The binding was slower at 4°C (Fig. 2B).

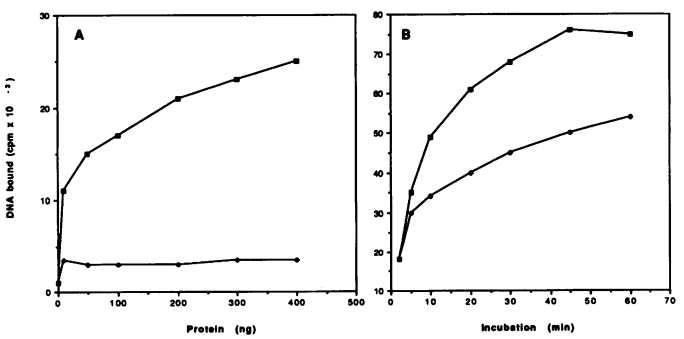

Figure 2. Nitrocellulose filter binding assay. Panel A shows the increase in binding of α-^{32}P-dCTP labelled DNA containing the 16S rRNA promoter with increasing concentrations of the chloroplast RNA polymerase complex incubated at 30°C for 10 min in the absence (◆) and presence (◆) of heparin (10 μg/ml). Panel B shows the binding of the RNA polymerase to the DNA as a function of varying incubation times at 30°C (◆) and at 4°C (◆).

Photoaffinity labelling of the 16S rRNA promoter fragment with pea chloroplast RNA polymerase complex

Under similar binding conditions as described for the filter binding assay, the RNA polymerase complex was allowed to interact with the bromo-dUMP-substituted 16S rRNA promoter fragment. Ultraviolet irradiation covalently crosslinks the proximal proteins of this complex to the bromo-UMP-substituted DNA. The irradiation displaces the bromine and produces an activated uracilyl radical. The free radical can abstract a hydrogen from the proximal protein(s) or react with the protein(s) to form a covalent crosslink (24). At 305 nm the absorption coefficient of bromo-dUMP is 10−100 times higher than that of TMP. Moreover, at this wavelength, UV damage is minimal to both the protein and the nucleic acid component of the complex. It has been reported that an extensive nuclease digestion can degrade the DNA bound to the protein while leaving intact small DNA fragments 'protected' by the enzyme, which are 15−40 base pairs

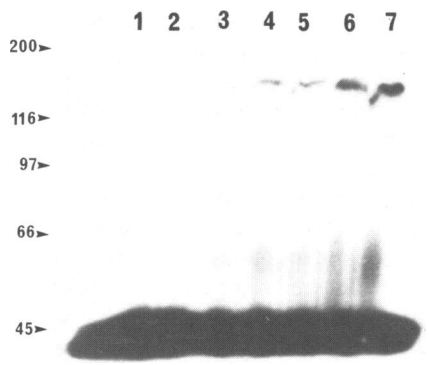

Figure 3. Autoradiographic analysis of photoaffinity labelled protein-DNA complexes on 5% SDS-polyacrylamide gels. Chloroplast RNA polymerase complex was allowed to interact with the bromo-dUTP-substituted α-^{32}P-labelled fragment containing the 16S rRNA promoter sequence at 30°C for 15 min. The complexes were irradiated for 0 min (Lane 2), 1 min (Lane 3), 3 min (Lane 4), 6 min (Lane 5), 12 min (Lane 6), 24 min (Lane 7) and 48 min (Lane 8). Lane 1 shows the probe alone. The UV-exposed complexes were nuclease-treated and analysed as described in 'Methods'.

Figure 4. Photoaffinity labelling of the chloroplast RNA polymerase complex with different promoters. Autoradiogram of a 5% SDS-polyacrylamide gel showing the photoaffinity labelling of the complex formed between the transcriptional complex and amplified DNA fragments containing the *psbA* (Lane 1) and 16S rRNA (Lane 3) promoter sequences. Lanes 2 and 4 are corresponding complexes which were not irradiated.

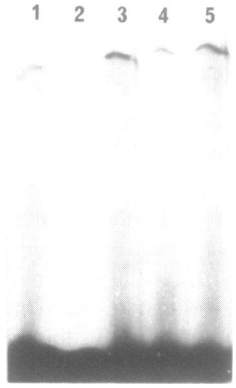

Figure 5. Effect of Proteinase K and nucleases on photoaffinity labelling of chloroplast RNA polymerase complex. The UV-exposed and nuclease-treated complexes formed by chloroplast RNA polymerase complex and 16S rRNA promoter fragment were analysed on a 5% SDS-PAG, and autoradiographed. The photoaffinity labelled complexes are shown in the absence of any further treatment (Lane 1), treatment with Proteinase K (Lane 2), DNase I (Lane 3), RNase A (Lane 4) and RNase H (Lane 5).

long (19). By treating the UV-exposed RNA polymerase-DNA complexes with the nuclease, the protected DNA fragments obtained would include those which had been photo-crosslinked to the enzyme. Furthermore, since these complexes are small, it can be expected that a protein with a small DNA fragment attached to it would have a mobility in SDS-gel electrophoresis similar to or slightly more retarded than that of the unreacted protein (19). Autoradiographic analysis of the gel would allow identification of the polypeptide photo-crosslinked to the labelled 16S rRNA promoter-containing DNA fragment. Figure 3 shows that a single polypeptide with an apparent Mr of about 150 kDa was photo-crosslinked to the DNA. The extent of crosslinking was dependent on the time of exposure to UV, and increased with time. No crosslinking was observed in the absence of the enzyme (Lane 1), reactions not exposed to UV (Lane 2), or with just TMP-substituted DNA (data not shown in figure).

In order to examine the promoter-specificity of this polypeptide, the 16S rRNA promoter fragment was substituted with another chloroplast promoter of similar length. A protein of identical molecular mass was observed when such binary complexes of the protein were formed with a radioactively labelled bromo-dUMP-substituted *psbA* promoter (Fig. 4, Lane 1). No crosslinking was observed in reactions not exposed to UV (Lane 2). Lane 3 shows the control reaction with the 16S rRNA promoter, and the same which was not exposed to UV is shown in Lane 4. These observations indicate that the radioactive DNA fragments were covalently attached to the polypeptide by UV irradiation. Under similar conditions of promoter binding, this binding could not be seen with a 500 bp λ-DNA fragment. However, a 150 kDa polypeptide was also observed to get photo-crosslinked when non-specific binary complexes were formed by binding a large excess of chloroplast RNA polymerase complex to a ^{32}P-labelled bromo-dUMP-substituted λ-DNA (500 bp fragment), or a 176 bp 3′ flanking region of the pea *psbA* gene containing the stem loop structure (data not shown).

Analysis of the photoaffinity labelled complexes by nuclease and protease digestion

The complexes were formed with 16S rRNA promoter fragments. After UV exposure and extensive DNase hydrolysis, the complexes were treated with Proteinase K (1 mg/ml) or DNase I (10 units) or RNase A (10 μg/ml) or RNase H (10 μg/ml). The samples were analysed by autoradiographic exposure of the SDS-polyacrylamide gels. Figure 5 shows that if the photoexposed, nuclease-treated binary complex was treated with Proteinase K to degrade the polypeptide, no radioactivity was detected in the complex (Lane 2). However, the complex was resistant to further hydrolysis by DNase I (Lane 3) RNase A (Lane 4) and RNase H (Lane 5). The control reaction is shown in Lane 1. These observations indicate that such photo-crosslinked complexes are composed of protein-DNA interactions, and that the mobility of the complex is determined primarily by the Mr of the protein.

Specific binding of the polypeptide to the 16S rRNA promoter

At enzyme levels which do not saturate the available promoters, the RNA polymerase holoenzyme forms specific binary complexes at promoter sites from which the initiation of a specifc RNA chain takes place (19, 25). These specific complexes are extremely stable having a half-time of dissociation longer than 20 h at 37°C (25). In contrast, the non-specific binary complexes which are formed at higher ratios of holoenzyme to DNA dissociate very rapidly (the half-time of dissociation is to the order of seconds). Taking advantage of this difference in dissociation rates, we were able to study the specific enzyme-DNA complexes. These complexes were allowed to form with limiting amounts of enzyme and labelled 16S rRNA promoter fragments in the absence and presence of 10−100 fold weight excess of unlabelled specific (16S rRNA, 422 bp; *psbA*, 426 bp promoter containing DNA) and nonspecific (500 bp λ-DNA and poly(dI-dC) DNA. The complexes were analysed by the filter binding assay. Figure 6 shows that decreasing amounts of labelled 16S rRNA promoter is retained as bound DNA in the presence of specific competitor DNA. However, similar concentrations of nonspecific could not displace the ^{32}P-labelled 16S rRNA promoter in such binary complexes. When complexes formed in the presence of 50 fold excess of specific or non-specific DNA were analysed by photoaffinity labelling, the labelling of the 150 kDa polypeptide was found to be competed with specific DNA fragments (Fig. 6 inset, 16S rRNA fragment (Lane 2), *psbA*

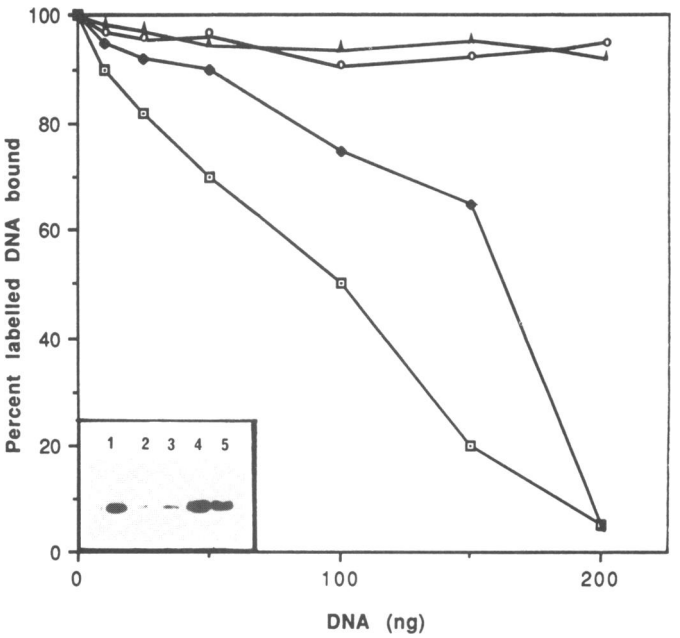

Figure 6. Specificity of binding of the chloroplast RNA polymerase complex to DNA. Filter binding assays showing competition of α-^{32}P-labelled 16S rRNA fragment bound to chloroplast RNA polymerase complex with non-radioactive DNA containing the 16S rRNA promoter (◆), *psbA* promoter (◆), λ-DNA (○) and poly (dI-dC) (▲).The radioactivity bound to the protein in the absence of any competitor DNA was treated as 100%. The inset shows an autoradiogram of the photo-crosslinked enzyme-16S rRNA promoter complex (Lane 1) competed with 50 fold excess cold 422 bp 16S rRNA promoter (Lane 2), 426 bp *psbA* promoter (Lane 3), λ-DNA (Lane 4) and poly (dI-dC) (Lane 5).

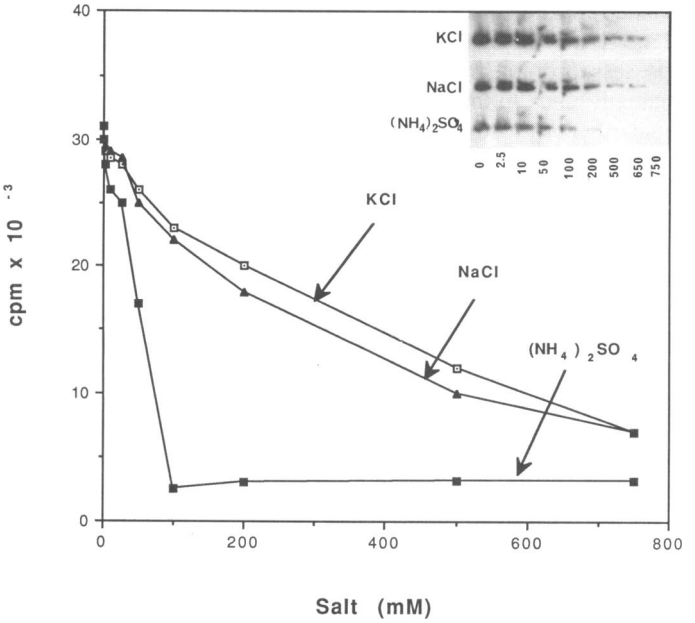

Figure 7. Effect of salts on the transcriptional activity and photoaffinity labelling of chloroplast RNA polymerase complex. Transcriptional activity of the chloroplast RNA polymerase complex using as template the DNA fragment containing the 16S rRNA promoter sequence was evaluated by measuring the TCA-precipitable radioactivity in the presence of varying salt concentrations. The inset shows an autoradiogram of the photo-crosslinked complexes formed in the presence of different salt concentrations.

promoter (Lane 3), but not with the non-specific DNA (λ-DNA (Lane 4), poly(dI-dC) (Lane 5). The control reaction, in which no cold DNA was added, is shown in Lane 1. These experiments further validate the specificity of both, the filter binding and photoaffinity labelling assays.

Effects of salts on *in vitro* transcription and photoaffinity labelling

Transcription by chloroplast RNA polymerase complex exhibits differential salt sensitivity to ammonium sulfate, sodium chloride and potassium chloride. The sensitivity was measured by analysing the template-directed TCA-precipitable incorporation of ^{32}P-UMP by the enzyme in the presence of various concentrations of the salts. Figure 7 shows the results of a typical experiment. The transcriptional activity was more sensitive to the presence of $(NH_4)_2^+$ ions than to KCl or NaCl. About 50% transcriptional activity was inhibited in the presence of 50 mM $(NH_4)_2SO_4$, 300 mM NaCl and 400 mM KCl (Fig. 7). In order to evaluate the effect of various concentrations of salts on the photoaffinity labelling of the 150 kDa polypeptide, the binary complexes were allowed to form by binding of the 16S rRNA containing fragment to chloroplast RNA polymerase complex under various salt conditions. After exposure to UV and nuclease treatment, the complexes were analysed as usual. Figure 7 inset shows the result of a typical experiment. The binding of the enzyme was relatively stable in the presence of increasing concentrations of KCl, NaCl and $(NH_4)_2SO_4$ and the polypeptide failed to get crosslinked with the DNA only at high salt concentrations. Photo-crosslinking of complexes could be observed in the presence of 100 mM $(NH_4)_2SO_4$, and 500 mM NaCl and KCl (Fig. 7 inset).

DISCUSSION

The chloroplast genes are transcribed by a multisubunit RNA polymerase, the composition of which still remains speculative. The early reports on purifying sufficient amounts of this enzyme from a variety of plants have used the criterion of closely associated polypeptides co-purifying with enzyme activity as a basis for regarding them as subunits of RNA polymerase (1–9). The purification of the pea chloroplast RNA polymerase used in the present study has been reported earlier (9). Through promoter-protection experiments, this enzyme has been shown to transcribe both ribosomal and messenger genes (9). The SDS-PAGE analysis of this enzyme has revealed the presence of 14 polypeptides ranging in molecular weights from 27–150 kDa. These polypeptides always co-purify with transcriptional activity on glycerol gradients, various ion exchange, hydrophobic and gel filtration columns, as well as on chloroplast 16S rDNA promoter affinity column (9). We have refered to this enzyme preparation as 'RNA polymerase complex'. Further attempts to purify the enzyme, including 'add-back' reconstitution experiments, have consistently resulted in inactive RNA polymerase preparations. Thus, conventional methods of purifying the chloroplast RNA polymerase have yeilded no information about the functional polypeptides that constitute the RNA polymerase complex. It seems possible that only a few of the polypeptides constitute the active enzyme and the others are co-purifying contaminants.

Photoaffinity labelling has been successfully used to identify the template, transcript and nucleotide binding subunits of

eukaryotic and prokaryotic RNA polymerases (see 12). The largest subunit of these RNA polymerases has been shown to possess promoter binding activity, while the second largest subunit binds to the nucleotides. The two largest subunits also make contact with the elongating chain of RNA (11–14).By incorporating bromo-dUMP in the promoter-containing DNA fragments during PCR amplification, the DNA can be labelled by the photoprobe at various positions. DNA molecules containing halogenated analogues of thymidine, such as bromo-dUMP, are consistently more sensisitve to UV-induced crosslinking compared to unsubstituted DNA. Irradiation of protein-DNA complexes with UV light causes covalent bonds to form between the nucleic acid and the closely associated proteins.

In this study, UV-crosslinking of the active transcription complex formed with the photoreactive DNA harbouring the chloroplast 16S rRNA or *psbA* promoters, has revealed a 150 kDa polypeptide as the template binding component of the pea chloroplast RNA polymerase complex. This is the first report on the functional characterization of the 150 kDa polypeptide of the pea chloroplast RNA polymerase complex. The specificity of the photoadduct has been determined by measuring the ability of an excess of unlabelled DNA to compete for binding sites on the proteins. The radioactive DNA fragments bound to the 150 kDa polypeptide can only be competed by chloroplast promoter-containing DNA. As expected, the binding is resistant to the presence of α-amanitin and rifampicin (Data not shown). However, the binding is eliminated by preincubation of the enzyme with low concentrations of heparin. Results from both the nitrocellulose filter binding assays and the photoaffinity labelling experiments, indicated that the enzyme shows preferential association with DNA containing chloroplast promoter sequences. Only under extremely low stringent conditions is the interaction observed with non-promoter sequences. This is expected as promoter-polymerase interactions are of higher affinity as compared to non-specific DNA-protein interactions (see 27).

The synthesis of RNA from different templates and the RNA polymerases from different bacteria shows great differences to altered ionic composition. Elevated ionic strengths alter the binding of RNA polymerase to DNA, the transition into active complex, the rates of chain initiation, elongation and termination (see 27). In an attempt to further optimize the *in vitro* pea chloroplast transcription system, the effects of commonly employed salts used in other transcription systems, were evaluated on both the template dependent transcription and on the ability of 150 kDa polypeptide to get photo-crosslinked to promoter containing DNA. Our data exhibits some degree of correlation between the two activities. Ammonium sulphate is more inhibitory to both activies as compared to sodium or potassium chloride. Moreover, since the binding is observed even at higher salt concentrations, it suggests specificity of interaction, as nonspecific binding is eliminated at higher salts.

Recently (15), using the photoaffinity labelling approach, we have also shown that two polypeptides of 51 and 54 kDa get crosslinked to the nascent transcripts during transcription of chloroplast DNA. However, on a longer (36 h) SDS-polyacrylamide (5–20%) run, in which most proteins approach zero mobility, the transcript-binding polypeptides moved to positions corresponding to 44 and 48 kDa, respectively, while the template-binding polypeptide moved to a position corresponding to 150 kDa. Therefore, until the gene sequence information for these polypeptides become available, these should only be regarded as operational values of molecular weights. Thus, the photoaffinity labelling of the active transcription complex has identified the 150 kDa polypeptide as the template binding (this report) and the 44 and 48 kDa polypeptides (15) as the transcript binding components of the pea chloroplast RNA polymerase complex.

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