Two distinct transcriptional activities of pea (*Pisum sativum*) chloroplasts share immunochemically related functional polypeptides

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An RNA polymerase activity has been purified from pea (*Pisum sativum*) chloroplast extracts with a distinct transcriptional specificity for a chloroplast messenger gene. This activity (ms-RNA pol) differs from the pea RNA polymerase preparation reported by Sun, Shapiro, Wu & Tewari [(1986) *Plant Mol. Biol.* 6, 429–439], which specifically transcribes only the rRNA gene (rb-RNA pol). The specificity of transcription has been assessed by the synthesis *in vitro* of discrete transcripts of predicted sizes using cloned promoter regions of the chloroplast *psbA* and 16 S rRNA genes. The ms-RNA pol preparation, with polypeptides ranging in apparent molecular mass from 22 to 180 kDa, correctly initiates transcription from recombinant plasmids containing the *psbA* promoter and does not support 16 S rRNA promoter-directed transcription. The two activities differ also in their response to Mn^{2+} ions. To investigate whether the two transcriptional activities share common functional polypeptides, monoclonal antibodies were developed against the rb-RNA pol preparation. Three clones were selected on the basis of their ability to inhibit transcription *in vitro* of the 16 S rRNA gene by rb-RNA pol. The antibodies from these clones independently recognized three polypeptides with molecular masses of 27, 90 and 95 kDa on immunoblots. Antibodies cross-reacting with the 90 kDa polypeptide completely eliminated the specific retardation of an end-labelled 16 S rRNA promoter fragment in a mobility-shift assay, whereas the antibodies against the 95 kDa polypeptide resulted in the formation of a ternary complex (enzyme–DNA–antibody). The antibodies cross-reacting with the 27 kDa polypeptide, however, did not alter the mobility of the retarded DNA–enzyme complex on the gel. These antibodies also inhibited transcription *in vitro* of the *psbA* gene by ms-RNA pol and recognized polypeptides of identical molecular masses in the ms-RNA pol. These results show that the three polypeptides are functional components of the chloroplast transcriptional complex and appear to be involved in the transcription of both rRNA and mRNA genes. Transcriptional specificity is probably conferred by ancillary transcription factor(s) which remain to be identified.

INTRODUCTION

The chloroplast DNA of pea (*Pisum sativum*) is approx. 120 kbp in size [1] and contains all necessary rRNA and tRNA genes, and about 40–60 open reading frames, many of which are known to code for chloroplast proteins [2,3]. DNA sequences important for accurate expression of various chloroplast genes have been assessed by deletion analysis of the 5' regions and through the use of both homologous and heterologous 'in vitro' transcription systems. Homologous systems have employed the endogenous DNA-dependent RNA polymerase that was first reported in tobacco *Nicotiana glauca* chloroplasts [4]. However, the structure and organization of the plastid polymerase still remains obscure. Early experiments suggested that chloroplasts contain two RNA polymerases [5–8]. One RNA polymerase was found to be associated with the transcriptionally active chromosome fraction (TAC polymerase), was tightly bound to DNA, primarily transcribed rRNA genes, and did not accept exogenous DNA as template [5,9,10]. This enzyme was subsequently solubilized [11] and shown to initiate and synthesize selectively only the 16 S rRNA from supercoiled recombinant DNA, but failed to initiate transcription of tRNA genes which were carried on the same recombinant plasmids [12]. The other RNA polymerase which could be solubilized transcribed chloroplast tRNA genes [5,13] and mRNA genes [14–18]. On the basis of general transcriptional activity assayed by nucleotide in-

corporation into acid-insoluble material, attempts at purification of RNA polymerase from chloroplasts of pea, spinach (*Spinacia oleracea*), maize (*Zea mays*) and tobacco have resulted in preparations containing 7–14 polypeptides with molecular masses ranging from 22 to 180 kDa [19–22]. Fairly recently, Hu & Bogorad [23] determined the N-terminal sequence of the 180, 120, and 38 kDa polypeptides purified from a SDS/polyacrylamide gel of a maize chloroplast RNA polymerase preparation and showed these to correspond to the amino acid sequence deduced from the maize *rpoC2*, *rpoB* and *rpoA* genes. Rajasekhar *et al.* [24] purified an RNA polymerase from pea chloroplasts using ion-exchange and chloroplast 16 S rRNA promoter-affinity column chromatography. This enzyme polymerized specific transcripts of predicted size with the 16 rRNA gene, which were revealed by 'run-off' assays, but failed to do so with mRNA genes. The transcription of a mRNA gene could only be shown by promoter-protection assays. Promoter-protection assays do not permit the detection of transcripts synthesized at random from the template DNA, and therefore cannot be used to demonstrate the specificity of transcription. We now report the isolation of a RNA polymerase activity which accurately initiates transcription at the putative start site of the chloroplast messenger gene (*psbA* gene, which codes for a 32 kDa Photosystem II polypeptide). However, it is not known whether the two distinct transcriptional activities of the chloroplast RNA polymerase can be attributed to the existence of more than one

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; mAbs, monoclonal antibodies; PBS (phosphate-buffered saline; 50 mM-phosphate/150 mM-NaCl, pH 7.5); EtBr, ethidium bromide.

Fig. 1. Schematics of recombinant plasmids 5A/1T and 5A/30T

Plasmid 5A/1T contains the *psbA* promoter and the putative 3' terminator region of the *psbA* gene, and p5A/30T contains the 16 S rRNA promoter and the putative *psbA* gene 3' terminator-region gene. The transcription 'start' sites are indicated by arrows.

functional form of the enzyme or is conferred by the interaction of different specificity factors with a general RNA polymerase. In order to identify some of the functional polypeptides of the chloroplast transcription complex, and to address the question of common polypeptides in the two transcriptional activities, we adopted an immunological approach. The present paper also describes the isolation and characterization of three stable hybridoma clones that produce antibodies which inhibit chloroplast 16 S rRNA and *psbA* transcription *in vitro*, and the use of these antibodies in identifying some of the functionally significant polypeptides of the chloroplast transcription complex.

MATERIALS AND METHODS

Isolation of chloroplast RNA polymerase activity specific for mRNA gene transcription

Chloroplasts were isolated from buds (300 g) excised from 7–10-day-old pea (*Pisum sativum* L. var. Arkel) seedlings as described by Sun *et al.* [12]. The chloroplasts were lysed with 2.5% Triton X-100 in the presence of 0.5 M-sucrose in 50 mM-Tris/HCl (pH 8.0)/15 mM-MgCl₂/25 mM-2-mercaptoethanol/0.2 mM-PMSF (phenylmethanesulphonyl fluoride)/pepstatin (5 mg/l)/leupeptin (5 mg/l), and pelleted at 6000 *g*. The supernatant was loaded on a DE-52 column (2 cm × 12.5 cm) equilibrated with Buffer A [50 mM-Tris/HCl (pH 8.0), 25% (v/v) glycerol/70 mM-2-mercaptoethanol/0.2 mM-PMSF] containing 0.5 M-(NH₄)₂SO₄ to remove the endogenous chloroplast DNA. The RNA polymerase activity appeared in the flow-through fraction; DNA remained bound to the column. The proteins were concentrated by (NH₄)₂SO₄ precipitation (70% satn.) and exhaustively dialysed against Buffer A containing 0.05 M-(NH₄)₂SO₄ (Buffer B). The dialysed fraction was then loaded on to a Sephacryl S-200 (Pharmacia) column (1.8 cm × 30 cm) equilibrated with Buffer B. RNA polymerase activity was eluted in the void volume. The fractions were pooled and loaded on to a phosphocellulose column (1.5 cm × 3 cm) equilibrated with Buffer B. The column was extensively washed with Buffer A containing 0.08 M-(NH₄)₂SO₄, and the RNA polymerase was step-eluted with 0.2 M-(NH₄)₂SO₄, flash-frozen

and stored at –80 °C. This preparation specifically transcribed a chloroplast mRNA gene and is referred to as 'ms-RNA pol'.

Isolation of chloroplast RNA polymerase activity specific for rRNA gene transcription

Pea chloroplast RNA polymerase with a transcriptional specificity for the chloroplast 16 S rRNA gene was isolated as described by Rajasekhar *et al.* [24] by sequential chromatographic fractionation on ion-exchange columns. The Triton X-100 lysate of chloroplasts was loaded on a DE-52 column (2 cm × 12.5 cm) equilibrated with Buffer A containing 0.1 M-(NH₄)₂SO₄. After extensive washing with the same buffer, RNA polymerase activity was step-eluted with 0.3 M-(NH₄)₂SO₄. The fractions containing the enzyme activity were pooled, dialysed against Buffer A containing 0.1 M-(NH₄)₂SO₄ and loaded on to a second DE-52 column (2 cm × 7 cm) pre-equilibrated with the loading buffer (100 ml). Enzyme activity was eluted with Buffer A containing 0.3 M-(NH₄)₂SO₄. The enzyme-containing fractions were dialysed against Buffer A containing 0.1 M-(NH₄)₂SO₄ and loaded on to a phosphocellulose column (1.5 cm × 6 cm) in the same buffer. The column was extensively washed with Buffer A containing 0.1 M-(NH₄)₂SO₄. RNA polymerase activity step-eluted with 0.2 M-(NH₄)₂SO₄. The transcriptionally active fractions were pooled, flash-frozen and stored at –80 °C. This preparation specifically transcribed the chloroplast 16 S rRNA gene and is referred to as 'rb-RNA pol'.

Template DNA

The *psbA* gene and the 16 S rRNA gene, which are among the most actively transcribed chloroplast genes [25], were selected to study messenger and ribosomal gene transcription *in vitro* respectively. The recombinant plasmid (5A/1T) consists of the *psbA* promoter (–154 to +30 bp) and the 3' downstream region (650 bp) of the pea *psbA* gene cloned into pUC 19. The 3' terminus includes a region of nucleotides in dyad symmetry, which would result in the formation of a stem-loop structure in the corresponding part of the transcript. This structure serves as a termination sequence in pea chloroplast *psbA* transcription [26] and in bacteria [27]. Recombinant plasmid (5A/30T) with the

putative *psbA* terminator sequence placed downstream of the 16 S rRNA promoter [28] was used as a supercoiled template to assay ribosomal promoter-directed transcriptional activity. The restriction maps of the recombinant plasmids are shown in Fig. 1.

Isolation of supercoiled plasmid DNA and Southern blotting

Isolation of supercoiled plasmid DNAs and Southern hybridizations were carried out as described in [26,29].

Assays of transcription *in vitro*

Radiolabelled transcripts were synthesized in a reaction mixture containing 10 mM-Tris/HCl, pH 8.0, 5 mM-MgCl₂, 5 μ M each of ultrapure ATP, GTP and CTP, 30 μ Ci of [α -³²P]UTP (sp. radioact. 3000 Ci/mmol), 1 mM-dithiothreitol, 1 μ g of BSA, 1 μ g of template DNA, and 10 μ l of the RNA polymerase extract (phosphocellulose fractions of rb-RNA pol or ms-RNA pol) in a final volume of 100 μ l. For *psbA* transcription, the nucleotide triphosphate concentration was increased to 200 μ M, and 5 mM-MnCl₂ was added to the reaction mix. The incubations were carried out for 30 min at 25 °C. The reaction was terminated with 0.2% SDS (final concn.) and the solution was adjusted to 2.5 M-ammonium acetate. Yeast tRNA (Sigma) (1 μ g) was added as carrier, and the mixture was extracted twice with a phenol/chloroform (1:1, v/v) mixture containing 0.1% 8-hydroxy-quinoline, equilibrated with 10 mM-Tris/HCl (pH 8.0)/100 mM-NaCl/10 mM-EDTA. The RNA was precipitated with 3 vol. of ethanol and washed three times with 70% (v/v) ethanol. The pellet was finally dried and suspended in 95% formamide, containing 0.05% xylene cyanol and 0.05% Bromophenol Blue, and analysed on a 0.4 mm-thick 6% (w/v) polyacrylamide/7 M-urea gel in a Bio-Rad Gel Sequencing Cell. The transcripts synthesized *in vitro* were revealed by autoradiography at -80 °C using Kodak X-Omat film. ³H-UTP (1 μ Ci, sp. act. 35 Ci/mmol) was substituted for α -³²P-UTP and a 20-fold higher concentration of NTPs was used in the trichloroacetic acid precipitation assays [11].

Isolation of monoclonal antibodies (mAbs)

Adult female BALB/c mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were injected intraperitoneally with three injections of rb-RNA pol (about 200 μ g of protein) administered at 3-week intervals. The first injection was emulsified with Freund's complete adjuvant (Difco), and the remaining injections were emulsified with Freund's incomplete adjuvant (Difco). The immune response was monitored not only by e.l.i.s.a. and Western-blot analysis, but also by the ability of the antibodies to inhibit rb-RNA pol activity. Spleen cells from the immunoresponsive mouse were fused with SP 2/0 myeloma cells (American Tissue Culture Collection), using 35% (w/v) poly(ethylene glycol) 1450, by standard procedures [30]. Hybridomas were screened for RNA-polymerase-reactive antibodies by e.l.i.s.a. and Western blots using rb-RNA pol as an antigen. The hybridomas were cloned twice by limiting dilution and once by agarose cloning. For the production of ascites fluid, hybridomas were injected into BALB/c mice that had been primed with Freund's incomplete adjuvant 24 h previously. The sub-class of each antibody was determined by using an e.l.i.s.a. isotyping kit (Calbiochem). For the control reactions, IgM and IgG, antibodies purified from mineral-oil-induced plasmocytoma ascites fluids, were purchased from ICN Biochemicals.

Purification of mAbs

The IgG₁ antibodies were purified by chromatography on a 50 ml S-Sepharose Fast Flow column (Pharmacia) equilibrated with 50 mM-Mes buffer, pH 6.0, containing 10 mM-NaCl (Buffer C). Ascites fluid (10 ml) was dialysed against Buffer C and, after

Fig. 2. Purification profile of the antibody IgG 95 on an S-Sepharose column

Ascites fluid (10 ml), dialysed against Buffer C, was applied to a 50 ml S-Sepharose column. After extensive washing with Buffer C, the antibody was eluted with a linear gradient of 10–500 mM-NaCl. The inset shows the SDS/polyacrylamide-gel profile through an e.l.i.s.a.-positive protein peak.

centrifugation, applied to the S-Sepharose column. The column was washed with Buffer C and the bound proteins were eluted with a linear gradient of 0.01 M-0.5 M-NaCl in 50 mM-Mes buffer, pH 6.0. Fractions were analysed by SDS/PAGE and e.l.i.s.a. Immunologically active (Fig. 2) fractions were pooled, concentrated and dialysed against Tris-buffered saline and stored in aliquots at -80 °C.

The IgM antibodies were precipitated from ascites fluid by the addition of solid (NH₄)₂SO₄ to 45% saturation. The precipitated material was dissolved in Tris/HCl buffer (50 mM, pH 7.5) containing 150 mM-NaCl. The undissolved material was removed by centrifugation, and the supernatant fluid was loaded on to a Bio-Gel A column (2.0 cm × 100 cm; Bio-Rad). The fractions were monitored by SDS/PAGE and e.l.i.s.a. Appropriate fractions from the void volume were pooled, and the IgM was concentrated by centrifugation in a Centricon 30 (Amicon Corp.) apparatus. Purified antibodies were dialysed against Tris-buffered saline and were stored in aliquots at -80 °C. Antibody concentrations were determined at 280 nm by using an $A_{280}^{1\%}$ value of 13.8.

ELI.S.A. procedure

The rb-RNA pol contained in 10 mM-carbonate buffer, pH 9.6, was coated on to the wells of polystyrene microtitre plates and left overnight at room temperature. The plates were blocked with 200 μ l of 1% gelatin (Bio-Rad) contained in phosphate-buffered saline (PBS) for 1 h at room temperature. After washing with PBS containing 0.1% Tween 20 (Sigma), 50 μ l of antibody solution was added and allowed to react for 1 h at room temperature. Subsequently, washing with PBS containing 0.1% Tween 20, 50 μ l of an appropriate dilution of anti-mouse IgG (Calbiochem), prepared in goats and conjugated to horseradish peroxidase, was allowed to react for 1 h at room temperature. After extensive washing, the reaction was detected by addition of enzyme substrate, 2 mM-H₂O₂, and 0.16 mM-2,2'-azinobis (3-ethylbenzthiazoline-6-sulphonic acid (Sigma) in 0.05 M-citric acid, pH 4.0. The plates were read spectrophotometrically at 414 nm using Flow Titertek Multiskan.

PAGE and immunoblotting

Proteins were fractionated by SDS/PAGE [31] on 7.5–15% (w/v) gradient gels. For immunoblots, proteins were transferred to nitrocellulose (Bio-Rad) [32]. The blots were washed for 15 min in 50 mM-phosphate buffer, pH 7.5, containing 150 mM-NaCl and 0.1% Tween-20 (PBST buffer). The blots were blocked

with 2% BSA in PBST buffer for 1 h at room temperature, and then the strips were incubated with mAbs at appropriate concentrations (1–50 $\mu\text{g}/\text{ml}$) in PBST under the same conditions. Blots were washed extensively in PBST with three or four changes, and the antibody was detected with secondary anti-mouse IgG antibody (1:5000 dilution) conjugated with peroxidase (Calbiochem) for 1 h at room temperature. After extensive washing with PBST, peroxidase activity was detected by reaction with 0.03% 4-chloro-1-naphthol (Bio-Rad) and 0.001% H_2O_2 [32].

Effect of mAbs on chloroplast transcription *in vitro*

Phosphocellulose fractions containing rb- or ms-RNA pol activity were incubated with the mAbs for 30 min at 25 °C. The enzyme-antibody complex was then added to the transcription reaction mix and assayed for transcriptional activity as described above. In the controls, the purified immunoglobulin fraction (IgG₁ and IgM) from normal mouse serum was substituted for the specific antibodies. To check for contaminating activities of RNAase and DNAase, mAbs were incubated independently with 'in vitro'-synthesized 16 S rRNA and *psbA* transcripts, or the template plasmid DNA at 25 °C for 30 min, and analysed on 6%-polyacrylamide/7 M-urea gels or on 0.8% agarose gels respectively.

DNA-mobility-shift assays

The DNA-protein complexes were detected by a mobility-shift assay as described previously [28]. In brief, the rb-RNA pol (~0.1 μg of protein) was incubated with 10 ng of 5' ^{32}P -end-labelled DNA fragment (a 66 bp synthetic oligonucleotide containing functional 16 S rRNA promoter sequence) in the presence of 10 mM-Tris/HCl, pH 8.0, 5 mM-MgCl₂, 2.5 mM-KCl, 0.5 mM-dithiothreitol, 0.01% Triton X-100 and 0.05 μg of poly(dI-dC) in a final volume of 50 μl for 30 min at 25 °C [28]. The complexes were analysed on 4%-polyacrylamide gels (acrylamide/bisacrylamide, 80:1, w/w). To evaluate the effect of antibodies, the rb-RNA pol was preincubated with appropriate concentrations (10–50 $\mu\text{g}/\text{ml}$) of the three antibodies for 15 min at 30 °C, and then incubated with the radiolabelled DNA before analysis by gel-retardation assays. The retarded bands were revealed by autoradiography at –80 °C.

RESULTS

Optimization and characterization of transcriptional specificities of chloroplast RNA polymerase preparations *in vitro*

Chloroplast RNA polymerase activities were isolated by two different procedures to optimize transcriptional specificity respectively for chloroplast 16 S rRNA and *psbA* genes. The phosphocellulose-eluted enzyme fractions (rb-RNA pol and ms-RNA pol) were analysed for their transcriptional specificities by assays of transcription *in vitro*. Placing the putative *psbA* terminator downstream of the 16 S rRNA promoter (p5A/30T) or the *psbA* promoter (p5A/1T) in pUC-based plasmids generated supercoiled templates for the synthesis of transcripts of a discrete size. Supercoiled template DNA in these assays provides an advantage over linearized plasmids as supercoiled DNA has optimal topology for transcription *in vitro* [12]. The precise site of transcription initiation for the 16 S rRNA [28] and *psbA* RNA [14,26], and for transcription termination at the stem-loop-encoding sequence of the *psbA* gene, were reported previously [28]. On the basis of this information, the plasmid 5A/30T was predicted to produce transcripts of 236 bases if the rb-RNA pol accurately recognized the initiation and termination signals. The predicted size of the *psbA* transcript for p5A/1T was close to 124 bases. The rb-RNA pol and ms-RNA pol distinctly differed in

specificity of transcription of the two templates (Fig. 3). The rb-RNA pol yielded an irreproducible set of bands with p5A/1T, none of which corresponded to the correct transcript size (lane 1), thereby suggesting an inability to transcribe the *psbA* promoter-containing plasmid with any degree of specificity. With p5A/30T, however, rb-RNA pol synthesized transcripts of 236 bases in length (lane 2). The template-preference and specificity of the ms-RNA pol are shown in lanes 3 and 4. The ms-RNA pol did not generate transcripts with p5A/30T (lane 3), pUC 19 vector DNA or other recombinants of the 16 S ribosomal gene (results not shown). However, using p5A/1T, ms-RNA pol synthesized transcripts of 124 bases (lane 4). This would be within the range of transcript size expected if the polymerase initiated transcription at the putative 'start' site and recognized the termination sequence. A difference was observed in the specific activities of the two enzyme preparations, which was about 8–10-fold higher in rb-RNA pol. The tRNA_{HIS} gene is located downstream of the *psbA* gene in p5A/1T, and would be expected to encode for RNA 73 bases in length. Transcripts of corresponding length were observed on the autoradiograms of the ms-RNA pol only on long exposure of the gel (not shown in Fig. 3). Owing to the low radioactivity of the tRNA transcripts, these could not be characterized.

One feature which distinguished ms-RNA pol from rb-RNA pol was its differential stimulation by Mn^{2+} . The transcripts synthesized *in vitro* by rb-RNA pol using p5A/30T as template are shown in Fig. 4(a). The addition of 5 mM-MnCl₂ to the ribosomal reaction mix had only a marginal effect on both the 236-base transcript synthesized under stringent transcriptional conditions and total transcriptional activity assayed by the incorporation of [^3H]UMP into the trichloroacetic acid-precipitable fraction. [The 100% of the rb-RNA pol in Fig. 4 refers to about 2% acid-precipitable radioactivity of the total [^3H]UTP (1 μCi) added to the reaction mixture]. On the other hand,

Fig. 3. Transcriptional specificities of chloroplast RNA polymerase preparations

Autoradiogram showing transcripts synthesized by rb-RNA pol (lanes 1 and 2) and ms-RNA pol (lanes 3 and 4) *in vitro* using recombinant plasmids 5A/1T (lanes 1 and 3) and 5A/30T (lanes 2 and 4) analysed on a 6%-(w/v)-polyacrylamide/7 M-urea gel. The size of the transcripts was estimated by comparison with a pBR322-HaeIII digest (lane 5).

Fig. 4. Effect of MgCl_2 on the specific transcriptional activities of chloroplast RNA polymerase

The rb-RNA pol (a) and ms-RNA pol (b) were assayed for transcriptional activities of chloroplast RNA polymerase using p5A/30T and p5A/1T respectively as templates. The autoradiograms of the specific transcripts synthesized *in vitro* during each assay using $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ are shown. The $[\text{H}]\text{UMP}$ radioactivity incorporated during trichloroacetic acid assay in the absence (–) or presence (+) of MnCl_2 is shown.

Fig. 5. Southern hybridization of the transcripts synthesized *in vitro* to template DNA

(a) EtBr-stained agarose gel showing pUC 19 vector DNA (lane 1) and p5A/30T recombinant DNA (lane 2) digested with *Bam*HI and *Eco*RI. (b) pUC 19 vector DNA (lane 1), and p5A/1T recombinant DNA (lane 2) digested with *Xba*I and *Eco*RI. Autoradiograms of the Southern blots with ^{32}P -labelled 16 S rRNA transcripts (a) and *psbA* transcripts (b) are shown with the vector DNAs (lanes 3) and recombinant plasmids (lanes 4).

addition of MnCl_2 to the messenger reaction mix significantly enhanced the synthesis of the 124-base transcript and resulted in about 3-fold enhancement of $[\text{H}]\text{UMP}$ incorporation in the trichloroacetic acid-precipitable fraction (Fig. 4b).

Southern hybridization

The authenticity of the transcripts synthesized *in vitro* by the two chloroplast RNA polymerase preparations, rb-RNA pol and ms-RNA pol, was confirmed by Southern hybridization (Fig. 5). Lane 1 in Fig. 5(a) shows the EtBr-stained *Bam*HI–*Eco*RI-digested vector pUC 19 DNA and lane 2 shows *Bam*HI–*Eco*RI-digested p5A/30T DNA, which contains a fragment of the 16 S rRNA gene. The 236-base transcripts generated during the 16 S rRNA promoter-directed transcription *in vitro* were gel-purified and hybridized under stringent conditions to *Bam*HI–*Eco*RI-digested vector and the 16 S rRNA promoter recombinant plasmid. The transcripts hybridized to the 1.5 bp fragment which contains the 16 S rDNA promoter and 124 bp of

the coding region (Fig. 5a, lane 4). No hybridization was observed with the DNA fragment of 2.68 kbp, originating from the vector pUC 19 DNA (Fig. 5a, lane 3).

The *psbA* promoter-directed transcripts synthesized *in vitro* by ms-RNA pol were characterized by hybridization with the *Xba*I–*Eco*RI-digested fragments of p5A/1T. Fig. 5(b) shows the *Xba*I–*Eco*RI digests of the EtBr-stained vector pUC 19 DNA (lane 1) and p5A/1T DNA (lane 2). Radiolabelled transcripts synthesized by ms-RNA pol were gel-purified and used as a probe for hybridization with the *Xba*I–*Eco*RI-digested vector DNA and recombinant plasmid 5A/1T under stringent hybridization conditions. The transcripts (124 bases) synthesized *in vitro* hybridized with both the 180 bp and 630 bp fragments of p5A/1T (Fig. 5b, lane 4). This was as predicted, since the 180 bp DNA fragment contained the *psbA* promoter along with 27 bp of the coding region, and the 630 bp fragment contained the 3' region of the *psbA* gene. Hybridization was not observed with the vector pUC 19 DNA (Fig. 5b, lane 3). These results suggested that the specific 16 S rRNA and *psbA* transcripts were generated by the two transcriptionally active preparations from the corresponding templates under optimal 'in vitro' transcriptional conditions. Identical results were obtained when total transcripts synthesized *in vitro* by the two preparations from their respective templates were used as probes for the Southern hybridizations.

Polypeptide composition of chloroplast RNA polymerase preparations

The rb-RNA pol and ms-RNA pol preparations were analysed on an SDS/7.5–15% polyacrylamide gradient gels. Fig. 6 shows the silver-stained polypeptide patterns of ms-RNA pol (lane 1) and rb-RNA pol (lane 2). The molecular masses of polypeptides of ms-RNA pol ranged from 22 to 150 kDa, and of rb-RNA pol from 27 to 150 kDa. The two transcriptional activities showed some polypeptides of identical molecular mass, whereas some were distinctly different. A comparison of the protein profiles of the rb- and ms-RNA pols made it apparent that such data could not be used for identifying functional components of the two transcriptional activities. Therefore, to assess whether the two RNA polymerase preparations share common functional subunits, we adopted the approach of immunologically identifying the polypeptides involved in the transcription *in vitro* of the *psbA* and 16 S rRNA genes.

Use of mAbs to study chloroplast transcription

mAbs from BALB/c mice immunized against the native rb-RNA pol were purified and characterized as described in the Materials and methods section. Since this study necessitated the identification of mAbs that recognize functional polypeptides of the chloroplast transcriptional complex in both native and denatured states, the hybridoma supernatants were screened by their ability to interfere with RNA polymerase activity as well as by e.l.i.s.a. and Western blots. The three clones selected on this basis were IgG₁ 19/19, IgG₁ 23/55 and IgM 13/13. The use of the numbers is arbitrary and only for identification of the different clones. The clones which showed positive results with e.l.i.s.a. and Western blots and recognized low-molecular-mass polypeptides, but did not interfere with transcription, were set aside for future screening for other polypeptides which could be related to transcriptional activity. For comparative analysis of the results the Western blot data for both rb- and ms-RNA pols are shown together.

Western blotting of chloroplast RNA polymerase polypeptides by mAbs

The purified antibodies ($\sim 1 \mu\text{g/ml}$) clones were tested for their ability to detect common antigenic determinants in rb- and

Fig. 6. SDS/PAGE profiles of ms-RNA pol and rb-RNA pol isolated from pea chloroplasts

Silver stained linear-gradient 7–15% polyacrylamide/SDS gels of ms-RNA pol (lane 1) and rb-RNA pol (lane 2) are shown.

Fig. 7. Immunocross-reactivity of mAbs determined by Western blots

The rb-RNA pol (lanes 1) and ms-RNA pol (lanes 2) blotted on to nitrocellulose were probed with antibodies from normal mouse serum (a), IgG₁ 19/19 (b), IgM 13/13 (c), and IgG₁ 23/55 (d).

ms-RNA pol by Western blotting. The two transcriptionally active preparations were fractionated by SDS/7.5–15% PAGE and transferred to nitrocellulose membranes. Fig. 7 shows the Western blots of rb-RNA pol (lanes 1) and ms-RNA pol (lanes 2) probed with different antibodies. Antibodies isolated from normal mouse serum (1:500 dilution) did not exhibit any immunocross-reactivity with the chloroplast RNA polymerase preparations (Fig. 7a, lanes 1 and 2). When nitrocellulose strips were probed with the antibody from IgG₁ 19/19, it revealed a single immunoreactive polypeptide with a molecular mass of 27 kDa in both preparations (Fig. 7b, lanes 1 and 2). Probing of similar strips with IgM 13/13 antibody showed a distinct immunocrossreactivity with a polypeptide of 90 kDa (Fig. 7c, lanes 1 and 2). A weak immunoreactivity was also detected in the position corresponding to 75 kDa. The third antibody, IgG₁ 23/55, cross-reacted with the 95 kDa polypeptide in both preparations (Fig. 7d, lanes 1 and 2). The antibodies were checked for immunoreactive specificity by Western blot of *Escherichia coli* and viral (T3 and T7 phage) RNA polymerases, wheat-germ nuclear RNA polymerase and bacterial DNA polymerase, and

Fig. 8. Effect of mAbs on transcription *in vitro*

Autoradiogram showing the effect of antibodies on truncated transcripts synthesized *in vitro* by rb-RNA pol from p5A/30T (a) and by ms-RNA pol from p5A/1T (b). (a) Control reaction with no addition (lane 1); pre-incubation with antibodies isolated from normal mouse serum (10 µg/ml, lane 3); post-incubation of transcripts with all three antibodies (10 µg/ml each, lane 4); IgG 27 (10 µg/ml, lane 5); IgM 90 (10 µg/ml, lane 6); and IgG 95 (10 µg/ml, lane 7). (b) Control reaction with no addition (lane 2); post-incubation of transcripts with IgG 27 (50 µg/ml, lane 3); IgM 90 (50 µg/ml, lane 4); IgG 95 (50 µg/ml, lane 5); pre-incubation of ms-RNA pol with IgG 27 (10 µg/ml, lane 6; 50 µg/ml, lane 9); IgM 90 (10 µg/ml, lane 7; 50 µg/ml, lane 10); and IgG 95 (10 µg/ml, lane 8; 50 µg/ml, lane 11). A pBR 322–*Hae*III digest used as sizing markers is shown in (a) lane 2 and (b) lane 1.

did not cross-react with any polypeptide of any of these enzymes (results not shown). It is unlikely that these polypeptides are breakdown products, as polypeptides of identical molecular masses were also observed on Western-blot analysis of the crude lysate prepared by directly homogenizing the pea leaves in SDS/PAGE sample buffer (results not shown). The immunoblot data thus show that the two distinct transcriptional activities share at least three immunocross-reactive polypeptides. Our repeated attempts, however, to immunoprecipitate the enzyme preparation with any or all these mAbs were not successful.

mAb inhibition of chloroplast transcription *in vitro*

Antibodies purified from the three clones which cross-reacted with the 27, 90 and 95 kDa polypeptides were examined for their effect on transcription of chloroplast genes *in vitro*. Fig. 8 shows the effect of the three mAbs on promoter-dependent activity of rb-RNA pol (a) and ms-RNA pol (b) with their respective templates, p5A/30T and p5A/1T. The two transcriptionally active RNA polymerase preparations were incubated independently with each of the three antibodies (10–50 µg/ml) for 30 min at 25 °C and subsequently assayed for transcriptional specificity *in vitro*. Incubation of rb-RNA pol alone (lane 1) or control mouse myeloma proteins (IgG₁ and IgM) (lane 3) did not effect the synthesis of the 16 S rRNA *in vitro*. As an additional control reaction, the transcripts synthesized *in vitro* by rb-RNA pol were incubated with all three antibodies (10 µg/ml each). Lane 4 shows that the purified antibodies were not contaminated with RNAase activity. Incubation of rb-RNA pol with the antibodies,

Fig. 9. Effect of mAbs on DNA mobility-shift assay

Autoradiogram of a mobility-shift assay performed with rb-RNA pol (0.1 μ g of protein) which was incubated with a 5' ^{32} P-labelled 66 bp oligonucleotide (10 ng) and poly(dI-dC) (lane 1); rb-RNA pol pre-incubated with antibodies isolated from normal mouse serum (lane 2), with IgM 90 (10 μ g/ml, lane 3), with IgG 95 (10 μ g/ml, lane 4), and with IgG 27 (10 μ g/ml, lane 5; 50 μ g/ml, lane 6). The probe alone is shown in lane 7.

however, resulted in inhibition of transcription (IgG 27, lane 5; IgM 90, lane 6; and IgG 95, lane 7). A pBR322-*Hae*III digest, used as sizing markers, is shown in Fig. 8(a), lane 2, and Fig. 8(b), lane 1. (b) Shows the effect of the antibodies on ms-RNA pol activity. The control reaction is shown in lane 2. Post-incubation of the transcripts synthesized by ms-RNA pol with the purified antibodies IgG 27 (lane 3), IgM 90 (lane 4) and IgG 95 (lane 5) did not reveal any RNAase contamination. The transcriptional-inhibitory effect of the antibodies on ms-RNA pol is shown in lanes 6–11. The enzyme was pre-incubated with IgG 27 (10 μ g/ml, lane 6; 50 μ g/ml, lane 9), IgM 90 (10 μ g/ml, lane 7; 50 μ g/ml, lane 10), and IgG 95 (10 μ g/ml, lane 8; 50 μ g/ml, lane 11). These results suggested that the two distinct chloroplast transcriptional activities could be inhibited by the same antibodies. The reasons for a higher amount of antibody required for inhibition of ms-RNA pol are not clear. It is possible that, owing to a lower specific activity of this preparation, more proteins are present in this reaction mixture and thus required relatively higher concentrations of the antibodies for immunocross-reactions. The possibility of DNAase contamination of the antibodies was checked by preincubating the template DNA (1 μ g/20 μ l) with the antibody ($\sim$ 10 μ g/20 μ l) for 30 min at 25 $^{\circ}$ C. No change in the mobility or degradation of DNA was observed on agarose gels (results not shown).

Effect of antibodies on DNA mobility-shift assay

The [γ - ^{32} P]ATP-labelled double-stranded DNA fragment containing the pea 16 S rRNA promoter (–54 to +12) was incubated with rb-RNA pol and analysed on a low-ionic-strength native 4%-polyacrylamide gel. This assay was utilized to determine if any of the mAbs were immunoreactive with polypeptides involved with the retardation of the 16 S rRNA promoter. Fig. 9 shows the interference of the antibodies in the mobility of the 16 S rRNA promoter fragment by rb-RNA pol. Retardation of the promoter by rb-RNA pol is shown in lane 1. As reported previously, both the primary and secondary retardations were

competable with increasing amounts of the 66-mer [28]. Incubation of the rb-RNA pol with IgG and IgM antibodies isolated from normal mouse serum did not alter the mobility of the DNA–polymerase complex (lane 2). Addition of IgM 90 (10 μ g/ml) to the DNA–protein binding reaction completely blocked the formation of both the primary and secondary complexes (lane 3). Antibody IgG 95 (10 μ g/ml) resulted in the formation of a DNA–polypeptide–antibody ternary complex and further decreased the mobility of the DNA–protein complex (lane 4). IgG 27 did not appear to alter the mobility of the promoter–polypeptide complex (10 μ g/ml; lane 5; 50 μ g/ml, lane 6). The probe alone is shown in lane 7.

DISCUSSION

We have resolved from pea chloroplasts two RNA polymerase activities which are distinct in their transcriptional specificity for chloroplast mRNA and rRNA genes. These activities, exhibiting a differential response to Mn^{2+} ions, have been characterized in terms of accurate initiation and termination of transcription. The purification and characterization of the RNA polymerase preparation, which was shown to specifically transcribe chloroplast rRNA genes but not mRNA genes by *in vitro* 'run-off' assays, was reported recently [24]. The transcription of mRNA genes, however, could only be demonstrated by promoter-protection assays [24]. The transcription of mRNA genes by chloroplast RNA polymerase preparations solubilized from other systems has also been described by DNA–RNA hybridizations [15–18]. However, unlike the *in vitro* 'run-off' assays, the promoter-protection experiments do not provide information about the specificity of transcription in terms of accurate initiation and recognition of termination sequences by the RNA polymerase. By an alternative procedure we have obtained an RNA polymerase activity capable of specific transcription of a chloroplast messenger gene. The ms-RNA pol appears to recognize the transcription initiation and termination sequences and synthesizes transcripts of 124 bases, but does not support 16 S rRNA promoter-directed transcription *in vitro*. It has not been possible to conclusively ascertain whether the ms-RNA pol can also transcribe tRNA genes *in vitro*. The template DNA employed in this study for the *in vitro* transcription assay for ms-RNA pol contains a fragment of chloroplast DNA with the tRNA_{HIS} gene. If transcribed, the predicted size of the tRNA would be about 73 nucleotides long. We have observed RNA of corresponding length to be synthesized during the transcription of the *psbA* gene, but have been unable to characterize it, owing to its low radioactivity, which could be attributed to the inhibitory effect of manganese on tRNA synthesis [5]. The rb-RNA pol, under conditions optimized for the transcription of ribosomal genes, does not transcribe the tRNA_{VAL} gene located upstream of the 16 S rRNA promoter [28].

Characterization of the RNA synthesized *in vitro* by rb- and ms-RNA pols only by Southern-blot analysis appears to be adequate, because the precise sites for transcription initiation for the pea chloroplast 16 S rRNA gene (158th nucleotide upstream of the 5' end of the coding region [28]) and *psbA* gene (92nd–93rd nucleotide upstream of the coding region [14,26]), and recognition of the stem-loop termination sequence have already been reported [28]. Capping experiments with guanylyltransferase have verified that both the 16 S rRNA and *psbA* are primary transcripts and are not processed at the 5' ends [12,28]. It could be considered that the vectors used for Southern analysis do not show whether the RNA are transcribed from the coding or the non-template strands. However, because the start sites of both genes have been mapped, and the *in vitro* transcriptional assays employed in these studies allow assessment of transcriptional

specificity in terms of the predicted size of the transcripts, the Southern-blot data seem to be sufficient in establishing that transcripts revealed on the gels correspond to the coding regions of the 16 S rRNA and *psbA* genes.

We have repeatedly attempted to isolate the two forms of the polymerase by a single protocol, but the two transcriptional specificities appear to diverge from each other during an early stage of purification when other contaminating factors prevent the accurate assay of transcriptional specificity. Greenberg *et al.* [5] separated the ribosomal (TAC) and tRNA (soluble polymerase) transcriptional activities. The purification was followed by a trichloroacetic acid-precipitation assay which does not reveal transcriptional specificity of the RNA polymerase preparations. In the present study the Sephacryl S-200 fraction is the only fraction in which both activities are observed with some degree of transcriptional specificity, but transcripts revealed are occasionally of various sizes, possibly owing to contaminating nucleases. Consequently, the transcription of both templates by the Sephacryl S-200 fraction, although with a variable specificity, may reflect the presence of all factors essential for the transcription of both classes of chloroplast genes.

Although it has been possible to separate the two transcriptional activities by conventional methods of protein purification, this approach has its limitations. Comparison of the SDS/PAGE profiles of the two transcriptional activities have provided no information regarding the precise composition of RNA polymerase. Little & Hallick [33] have used antibodies raised against fusion products of the chloroplast *rpoA*, *rpoB* and *rpoC* genes to determine the relationship between the putative chloroplast RNA polymerase subunit genes and the TAC and 'soluble' RNA polymerase activities. These antibodies strongly inhibited the transcription of tRNA genes, but did not alter TAC RNA polymerase activity. The authors concluded that the chloroplast genes homologous to the *E. coli* RNA polymerase *rpoA*, *rpoB* and *rpoC* are expressed *in organello* and that the protein products specify at least three of the components of soluble RNA polymerase. Unfortunately, the presence of the corresponding polypeptides in either of the two preparations could not be shown by Western blots.

The approach which we have adopted to unravelling the complexity of the chloroplast RNA polymerase has been to develop and select transcriptionally inhibitory mAbs which can recognize epitopes common to both, native and SDS-denatured polypeptides of the enzyme. The antibodies described in this paper have therefore necessarily been screened not only by e.l.i.s.a. and Western blots, but also by their ability to interfere with transcriptional activity, and have been shown to inhibit transcription of both ribosomal and messenger genes. The Western-blot data establishes immunocross-reactivity of the three functional polypeptides of both transcriptional activities. It is also significant to note that the effect of the mAbs has been examined on the transcriptional specificity of the two preparations *in vitro*, and not merely on total RNA polymerase activity assayed by the incorporation of radiolabelled NTPs in the acid-precipitable fraction.

Previous studies had shown the specific retardation of an end-labelled DNA fragment of the 16 S rRNA promoter (−54 to +12) by a pea chloroplast RNA polymerase [28]. The −35 and −10 elements upstream of the transcriptional start site were found to be essential for this mobility shift, as nucleotide substitutions of the TTGACA (−35) and TATAAT (−10) elements completely eliminated this retardation. In the present study we show that the antibody IgM 90 completely eliminated the specific retardation of 16 S rDNA promoter. The 90 kDa polypeptide recognized by this antibody possibly plays a role in the recognition of these elements. The antibody IgG 95 did not

eliminate the retardation, but interacted with the transcribing polymerase to form a ternary complex, which suggested that the epitope recognized by this antibody may not be essential for the DNA-binding domain of the enzyme. Since the IgG 27 did not alter the mobility of the enzyme–DNA complex, it probably does not play a role in promoter–polymerase interaction. We have been unable to optimize binding conditions for the retardation of the *psbA* promoter by either of the two preparations. Whether this is due to the lower specific activity of the ms-RNA pol or to a lower affinity for the template, is not clear. By using a photoaffinity-labelling approach, we have, however, been able to demonstrate that a 150 kDa polypeptide present in both rb- and ms-RNA polys, interacts with the 16 S rRNA and *psbA* promoters [34].

The present data thus provide evidence that three polypeptides, with apparent molecular masses of 27, 90 and 95 kDa, are common functional components of the pea chloroplast transcriptional complexes. The specificity of transcription of each type of gene is probably conferred by other polypeptides.

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