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The A and B forms of plastid DNA-dependent RNA polymerase from mustard (*Sinapis alba* L.) transcribe the same genes in a different developmental context

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Abstract Two RNA polymerases, termed A (cp-pol A) and B (cp-pol B), are known to be present in mustard plastids. In vitro, the two enzymes have different requirements for DNA binding, but both bind to, and transcribe from, the same set of chloroplast promoters. The B enzyme is sensitive to rifampicin (Rif), whereas the A enzyme is not. When seedlings were grown in the presence of Rif, RNA pool sizes of the photosynthesis-related plastid genes *rbcL* and *psbA* were smaller than in untreated controls, whereas transcripts of the non-photosynthetic genes *rps16*, *trnG*, *rrn* and *rpoB* remained virtually unaffected by the drug. The Rif inhibition patterns of *rbcL* and *psbA* transcripts reflect the relative abundance of the A and B enzymes at different stages and light/dark conditions. These genes can thus be transcribed by either of the two enzymes in vivo, whereas the non-photosynthetic genes are transcribed mostly or exclusively by the A enzyme, or by another Rif-resistant plastid polymerase. Among several nuclear gene transcripts that were tested for Rif inhibition, only those of the *RbcS* gene family for the plastid-bound small subunit of Rubisco revealed a decrease in pool size, which may imply that mechanisms exist that serve to coordinate patterns of gene expression in the different cellular compartments.

Key words Chloroplast transcription · Etioplast · Light regulation · Multiple RNA polymerases · Nuclear-chloroplast interaction

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Introduction

Plastids contain multiple DNA copies, which code for photosynthesis-related proteins as well as for components of the plastid gene expression apparatus (Sugiura 1992). On the other hand, they are genetically semiautonomous, insofar as imported nuclear-encoded proteins form an essential part of macromolecular complexes within the organelle (Bogorad 1981). This is well established for photosynthesis-related proteins (Herrmann et al. 1994; Pakrasi 1995; Cline and Henry 1996), as well as for the translational and RNA processing machinery (Rochaix 1992). Direct evidence has recently become available showing that this is also true for the transcription apparatus (Allison et al. 1996; Liu and Troxler 1996; Tanaka et al. 1996; Hedtke et al. 1997).

Plastid DNA contains functional *rpo* genes that code for the α, β and β' homologues of prokaryotic RNA polymerase (Bogorad 1991; Igloi and Kössel 1991; Sugiura 1992). On the other hand, the parasitic plant *Epifagus* has been shown to lack plastid *rpo* sequences but nevertheless contains transcripts of other plastid genes (Morden et al. 1991). Similar results were obtained in transgenic *rpoB* knock-out strains of tobacco (Allison et al. 1996; Hajdukiewicz et al. 1997), for plastid ribosome-deficient plant systems such as heat-bleached rye seedlings (Bünger and Feierabend 1980) and the barley *albostrians* mutant (Hess et al. 1993), and for tobacco seedlings treated with inhibitors of plastid gene expression (Kapoor et al. 1997). Collectively, these data suggest that organellar transcripts observed in the absence of the *rpo* gene products must be the result of synthesis by another (nuclear-encoded) plastid RNA polymerase.

Biochemical evidence for the existence of more than one form of plastid RNA polymerase has long been available (Bogorad and Woodcock 1970; Greenberg et al. 1984; Zaitlin et al. 1989; Lerbs-Mache 1993). However, questions concerning the coding site and site of synthesis of the polymerase-associated polypeptides

have only recently been addressed experimentally (Liu and Troxler 1996; Tanaka et al. 1996). Additional clues have come from the findings that either "prokaryotic" (−35/−10-type) or "unusual" promoters (at least some of which resemble 5′ upstream control regions of eukaryotic nuclear genes), or both, are located in front of chloroplast genes (see, for example, Christopher et al. 1992; Klein et al. 1994; Vera et al. 1995, 1996).

In mustard (*Sinapis alba*) cotyledons, two different plastid RNA polymerases have been shown to exist (Pfannschmidt and Link 1994). In agreement with earlier reports on the sensitivity of chloroplast transcription to the prokaryotic inhibitor rifampicin (Surzycki 1969; Bogorad and Woodcock 1970), one of the characterized mustard polymerases (the B enzyme; cp-pol B) is sensitive to this drug, whereas the other (the A enzyme; cp-pol A) is not. Although both are multi-subunit enzymes, cp-pol A is much larger and contains more polypeptides, and in SDS gels none of the latter comigrates precisely with subunits of cp-pol B, i.e. the putative products of the plastid *rpo* genes. Together with its lack of rifampicin sensitivity, this suggests that the A enzyme is distinct from the "prokaryotic-type" B form and, in terms of its complexity, resembles the nuclear RNA polymerases.

Here we compare DNA-binding and transcriptional characteristics of mustard cp-pol A and cp-pol B in vitro. Furthermore, in an attempt to assess their functional roles in vivo, we assessed transcript levels of various plastid and nuclear genes in seedlings grown in the presence or absence of rifampicin.

Materials and methods

Cloned DNAs

The DNA fragments containing the promoters for the mustard plastid genes *psbA*, *rps16*, and *trnQ* were prepared from plasmids pSA05/H120 (Bülow and Link 1988), pSA364-ET02 (Neuhaus and Link 1990) and pSA364/H018 (Eisermann et al. 1990), respectively. Clone pSA103a/D315, which carries the intergenic region between *trnV* and the 16S rRNA gene from mustard (Przybyl et al. 1984), was constructed by inserting a 315-bp *Dde*I fragment of pSA103a into pBluescript (Stratagene). This region contains two putative −35/−10-type promoters, which flank the CDF2 binding element (Baeza et al. 1991), as well as a 'non-consensus-type' (NC-II, NEP) promoter (Vera and Sugiura 1995; Allison et al. 1996; Kapoor et al. 1997; Hajdukiewicz et al. 1997) (see Fig. 4C). The *Bam*HI insert in plasmid pSA364/B0.5 (Neuhaus and Link 1987) contains 460 bp of the mustard *trnK* intron; this fragment was used as a promoter-free control in DNA binding assays (see Fig. 3). Gene probes for detection of in vivo transcripts were as follows: pSA204-EH1.0 is an internal *rbcL* probe and pSA452a covers most of the *psbA* gene (Link and Langridge 1984). pSPTXX680 contains *rps16* sequences (Neuhaus et al. 1989), PBSE996 spans the *trnG* gene (Liere and Link 1994), pBSH895 the 5′ 16S rRNA portion of the *rrn* operon, and pBSEX630 carries an internal portion of the *rpoB* gene from mustard, all cloned into pBluescript. Plasmid pBSEH633AT contains a 633-bp *Eco*RV-*Hin*dIII fragment of a tubulin gene from *Arabidopsis* (Oppenheimer et al. 1988), pBSEX965BN the cDNA for a G-box binding protein (Waldmüller and Link 1995), and pBNPTrc2 a cDNA for an RbcS from *Brassica napus* (Fiebig et al. 1990).

RNA polymerase preparations

Plastid RNA polymerases A and B were prepared from 5-day-old mustard seedlings by heparin-Sepharose chromatography followed by glycerol gradient centrifugation as described (Pfannschmidt and Link 1994).

DNA binding assays

Gel-shift assays (Fried and Crothers 1981) were carried out as described (Tiller and Link 1993b), except that 4.5% polyacrylamide gels were used instead of 5% gels. The *psbA* promoter fragment was 3′-labelled by fill-in with [α-^{32}P]dATP (Amersham) and Klenow enzyme (BRL), and 2.5 ng of the labelled probe were used per assay. In competition experiments, unlabelled *psbA*, *rps16*, *trnQ* or *rrn* promoter fragments were also present at indicated concentrations. Some control reactions were incubated with 10 µg of proteinase K at 37° C for 10 min prior to addition of the radioactively labelled DNA fragment.

Transcription assays

In vitro transcription with glycerol gradient-purified plastid RNA polymerase A or B (2 units) was as described (Eisermann et al. 1990). One unit represents the enzyme activity that results in the production of 1000 cpm of acid-precipitable RNA under standard incubation conditions using denatured calf thymus DNA as template (Pfannschmidt and Link 1994). The DNA templates used were plasmids pSA05/H120, pSA364-H018 (Eisermann et al. 1990) and pSA103a/D315. Transcripts were purified by phenol/chloroform extraction and isopropanol/ethanol precipitation. Rifampicin stock solutions (0.2 g/ml in methanol) were freshly diluted with water before use. Controls contained the same final methanol concentration but no rifampicin.

Nuclease S1 mapping

The probes for detection of unlabelled in vitro transcripts were the insert fragments of pSA05/H120, pSA364-H018 or pSA103a/D315, each 5′ labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase. DNA/RNA hybridization was in 80% formamide at 47° C for 14 h and hybrids were then treated with 150 units of nuclease S1 (BRL) as described (Link and Langridge 1984). S1-protected products were analyzed on 6% sequencing gels alongside labelled size markers, i.e. either pBR322 restriction fragments (Fig. 4A) or chain-termination sequencing products (Sanger et al. 1977) (Fig. 4B). The latter were generated using a 20mer primer that covers the right-hand border of the pSA103a/D315 insert.

Sterile growth of seedlings

Mustard seedlings were treated with 70% ethanol for 1 min, sterilized with 5% sodium hypochlorite for 15 min, and then rinsed with sterile distilled water five times for 5 min each. Batches of 30–40 seeds were germinated in 250-ml glass jars on 50 ml of MS medium (Murashige and Skoog 1962) containing 1% Difco agar. Rifampicin stock solution (0.5 g/ml in DMSO) or DMSO alone as control were added to the autoclaved medium before it solidified. Seedlings were maintained at 25° C for the indicated periods of time either in continuous light (250 µE/m^2 per s) or in darkness.

RNA isolation

Cotyledons of seedlings were harvested and immediately frozen in liquid nitrogen. The tissue was ground in a mortar and the powder resuspended in TNS buffer (50 mM TRIS-HCl pH 7.6, 2.5 mM $MgCl_2$, 100 mM NaCl, 2% sodium triisopropylnapthalene sulphonate). RNA was prepared by phenol-chloroform extraction and ethanol precipitation as described (Hughes et al. 1987).

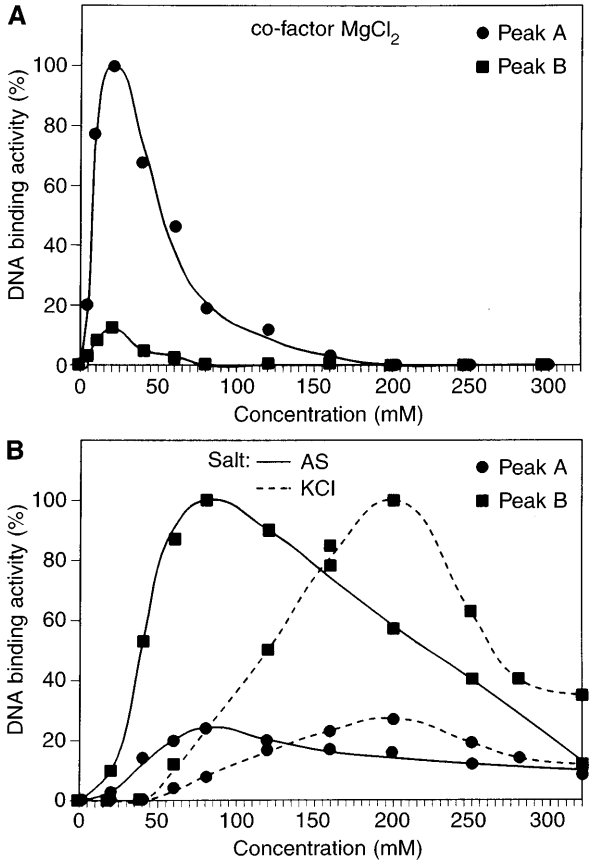


Fig. 1A, B Ionic strength and cofactor requirements for DNA binding by plastid RNA polymerases A and B. The enzymes A (from chloroplasts) and B (from etioplasts), purified by glycerol gradient centrifugation were each incubated with a labelled *psbA* promoter fragment in standard assays containing 1 μg of poly(dIdC) and increasing amounts of $MgCl_2$ (Fig. 1A), $(NH_4)_2SO_4$ (AS), or KCl (Fig. 1B). After electrophoresis, dried gels were autoradiographed and the intensity of signals from DNA/protein complexes was determined densitrometrically. The highest measured value was defined as 100%

Northern blot analysis

RNA samples (20 μg) were heat-denatured and fractionated on 1.7% agarose gels containing formaldehyde. RNA was transferred to nitrocellulose membranes (Schleicher and Schuell, BA 85S) using standard procedures (Sambrook et al. 1989). Blots were hybridized with ^{32}P-labelled RNA probes prepared by transcription of linearized plasmids with T7 or T3 RNA polymerase (Melton et al. 1984). All hybridization and washing steps were carried out as previously described (Hughes et al. 1987). After heating in 0.1% SDS and test exposure to ensure complete removal of RNA probes, filters could be re-hybridized up to five times.

Results

DNA binding of plastid RNA polymerases A and B

To determine ionic strength and cofactor requirements for DNA binding, the glycerol gradient-purified enzymes were assayed at various salt conditions (Fig. 1) with a DNA fragment that contains the *psbA* promoter (Eisermann et al. 1990). In the absence of $MgCl_2$, the

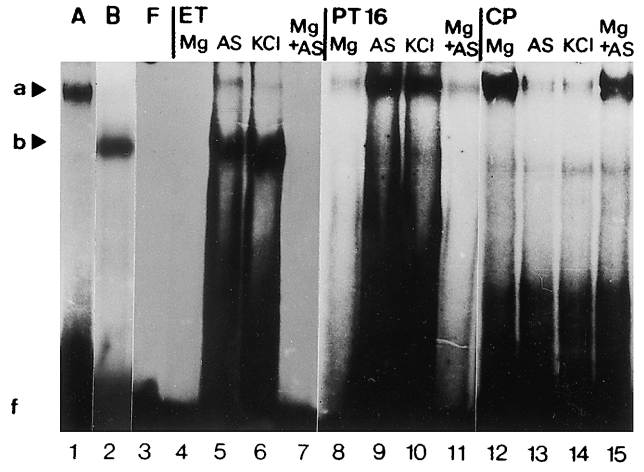
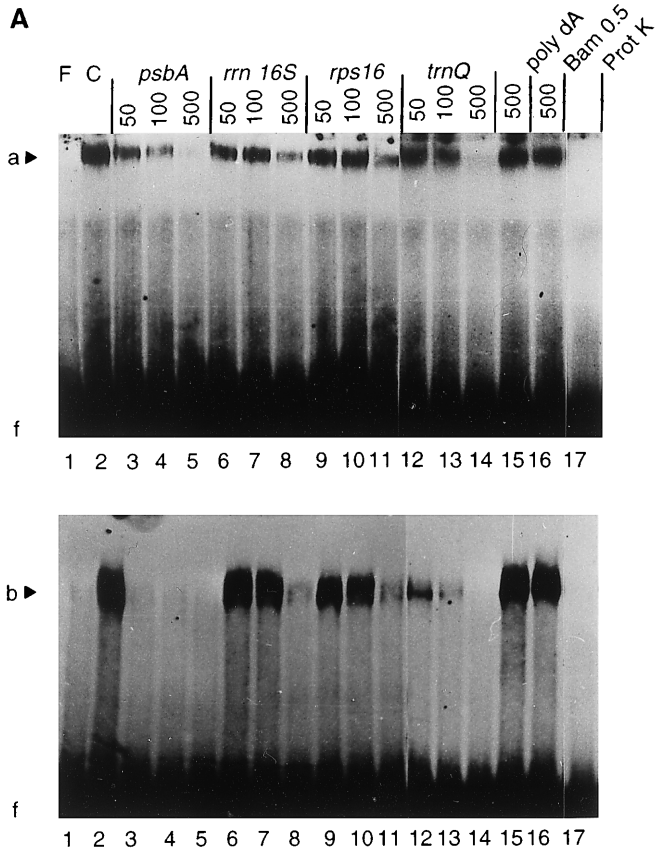


Fig. 2 DNA binding activities of enzymes A and B in partially purified preparations from different plastid types. The transcriptionally active fractions after heparin-Sepharose chromatography from etioplasts (ET), 'intermediate-type' plastids (PT16) and chloroplasts (CP) were incubated with the labelled *psbA* promoter fragment and 1 μg of poly(dIdC) in gel-shift assays. Reactions contained either 20 mM $MgCl_2$ (Mg), 80 mM $(NH_4)_2SO_4$ (AS), 200 mM KCl (KCl), or 20 mM $MgCl_2$ plus 80 mM $(NH_4)_2SO_4$ (Mg + AS). Glycerol gradient-purified enzymes A (lane A) and B (lane B) define corresponding binding signals in lanes with heparin-Sepharose fractions ('a' and 'b' in the left margin). Lane F shows the probe, carried through the procedure in the absence of protein ('f', free DNA)

binding activity of both enzymes was found to respond to ionic strength conditions, with maximal activity at 80 mM $(NH_4)_2SO_4$ or 200 mM KCl. The activity of polymerase B was, however, stimulated more efficiently than that of enzyme A, reaching at least threefold higher levels (Fig. 1B). Conversely, when the effect of $MgCl_2$ was studied in the absence of added $(NH_4)_2SO_4$ or KCl (Fig. 1A), the binding activity at optimal (20 mM) $MgCl_2$ concentrations was approx. 10-fold higher for the A enzyme than for the B enzyme.

As shown in Fig. 2, the gel-shift band formed in the presence of the A enzyme (from chloroplasts) depends on the presence of $MgCl_2$ in the binding reaction and its relative intensity is not significantly affected by the addition of KCl (lanes 12–15). In contrast, binding by the B enzyme (from etioplasts) is efficiently obtained only at elevated ionic strength and is inhibited in the presence of $MgCl_2$ (Fig. 2, lanes 4–7).

These optima were found to vary, however, depending on the type of plastid used as a source of the enzymes. As shown in Fig. 2, lanes 4–7, the A enzyme present in a partially purified polymerase preparation from etioplasts shows binding characteristics that resemble those of the B enzyme present in the same preparation more closely than those of the A enzyme from chloroplasts (Fig. 2, lanes 12–15). Conversely, the B enzyme from chloroplasts behaves more like the A enzyme in that same fraction (Fig. 2, lanes 12–15) than the etioplast B enzyme (lanes 4–7). With a plastid polymerase preparation from seedlings that were first grown in the dark and then transferred to light (PT16),



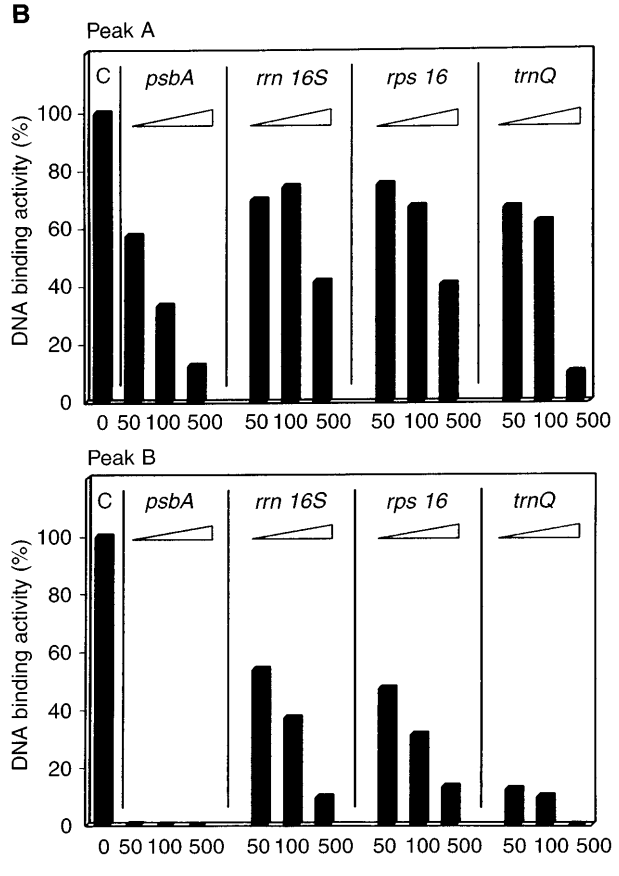


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Fig. 3A, B Competition gel-shift binding assays with the A and B polymerases. Gradient-purified A or B enzymes were incubated with *psbA* promoter fragment and 1 μg of poly(dIdC) in gel-shift assays in the absence (0) or presence of unlabelled competitors in 50-, 100-, or 500-fold molar excess. After electrophoresis, gels were dried and autoradiographed. **A** Results for enzyme A from chloroplasts (*upper panel*) and enzyme B from etioplasts (*lower panel*). Protein/DNA complexes are marked 'a' and 'b', free DNA probe as 'f'. Assays contained 20 mM $MgCl_2$ for the A enzyme and 80 mM $(NH_4)_2SO_4$ for the B enzyme. Control lanes: F, reactions without protein; C, complete reactions in the absence of competitor DNA; poly dA and Bam 0.5 indicate, respectively, poly dA and a promoterless 0.5-kb fragment from the mustard *trnK* intron (Neuhaus and Link 1987), used as competitors in 500-fold excess; 'Prot K', plastid polymerases treated with proteinase K prior to binding assays. **B** Densitometric quantitation of shifted bands in **A**

both enzymes reveal ionic strength requirements for DNA binding that resemble those of the etioplast B enzyme (Fig. 2, lanes 8–11).

To test the promoter binding specificity of purified A and B polymerases, we carried out competition gel-shift experiments using labelled *psbA* promoter probe and unlabelled competitors. As shown in Fig. 3, the homologous *psbA* promoter fragment was the most effective competitor with either the A (upper panel) or the B

▶

Fig. 4A–C In vitro transcription by plastid polymerases A and B. **A** Transcription from the mustard *psbA* (left panels) and *trnQ* promoters (right panels), followed by nuclease S1 analysis. Major S1-resistant products after transcription by gradient-purified polymerase A (lanes 1–3 and 7–9) or B (lanes 4–6 and 10–12) and hybridization with the end-labelled fragment pSA05-H120 (*psbA*) or pSA364-H018 (*trnQ*). Full-length products spanning the entire length of the probe are marked **f**; products with sizes expected for initiation at the chloroplast promoter are labelled **s**. Standard assays contained supercoiled plasmid DNA (sc), or no DNA template (−DNA). Rifampicin reactions (+Rif) contained 20 μg/ml of the drug and were preincubated for 5 min at 30° C before starting the transcription reaction by addition of the nucleotides. Positions of DNA size markers (pBR322 *Hin*fI fragments) are given in the left margin (bp). **B, C** Transcription from a mustard *rrn* promoter template. **B** S1 analysis of RNA isolated from light-grown (lane 5) and dark-grown mustard seedlings (lane 6) and of the products of in vitro transcription by cp-pol A (lanes 7–9), cp-pol B (lanes 11–13) or *E. coli* RNA polymerase (lane 10). Fragment pSA103a/D315 was used as probe. The arrowheads indicate S1-resistant fragments starting at −155/−154 and −104 (relative to the *rrn* 16S coding region) which define the P1 and P2 start sites used by *E. coli* RNA polymerase (Fig. 4C). The *filled square* and the *empty* and *filled circles* mark S1 products initiated at the putative −143 start site by plastid RNA polymerases in vivo and in vitro (PC, Fig. 4C). Larger S1-resistant products, some of which are marked by *asterisks*, are discussed in the text. The −233 position indicates the 3′ end of the *trnV* coding region; −50 a position downstream of NC-II (see Fig. 4C). G, T, A, C: dideoxy sequencing reactions. Size markers (left margin): HinfI fragments of pBR322. **C** Schematic view of the intergenic region between the 16S rRNA gene and *trnV*. The putative −35/−10 elements as well as the CDF2 and NC-II (NEP) regions are indicated by *boxes*. P1 and P2 (*arrowheads*), in vitro transcription start sites used by *E. coli* RNA polymerase; PC, plastid RNA 5′-end in vivo (*square*) and putative start sites for transcription by cp-pol A and B in vitro (*open* and *filled circles*) as indicated by the S1-resistant products in **B**. P1, P2, PC designations follow the usage of Baeza et al. (1991) as for spinach *rrn*. Note that P2 of mustard and spinach is equivalent to P1 in tobacco, where the upper −35/−10 region is not highly conserved (Vera and Sugiura 1995)

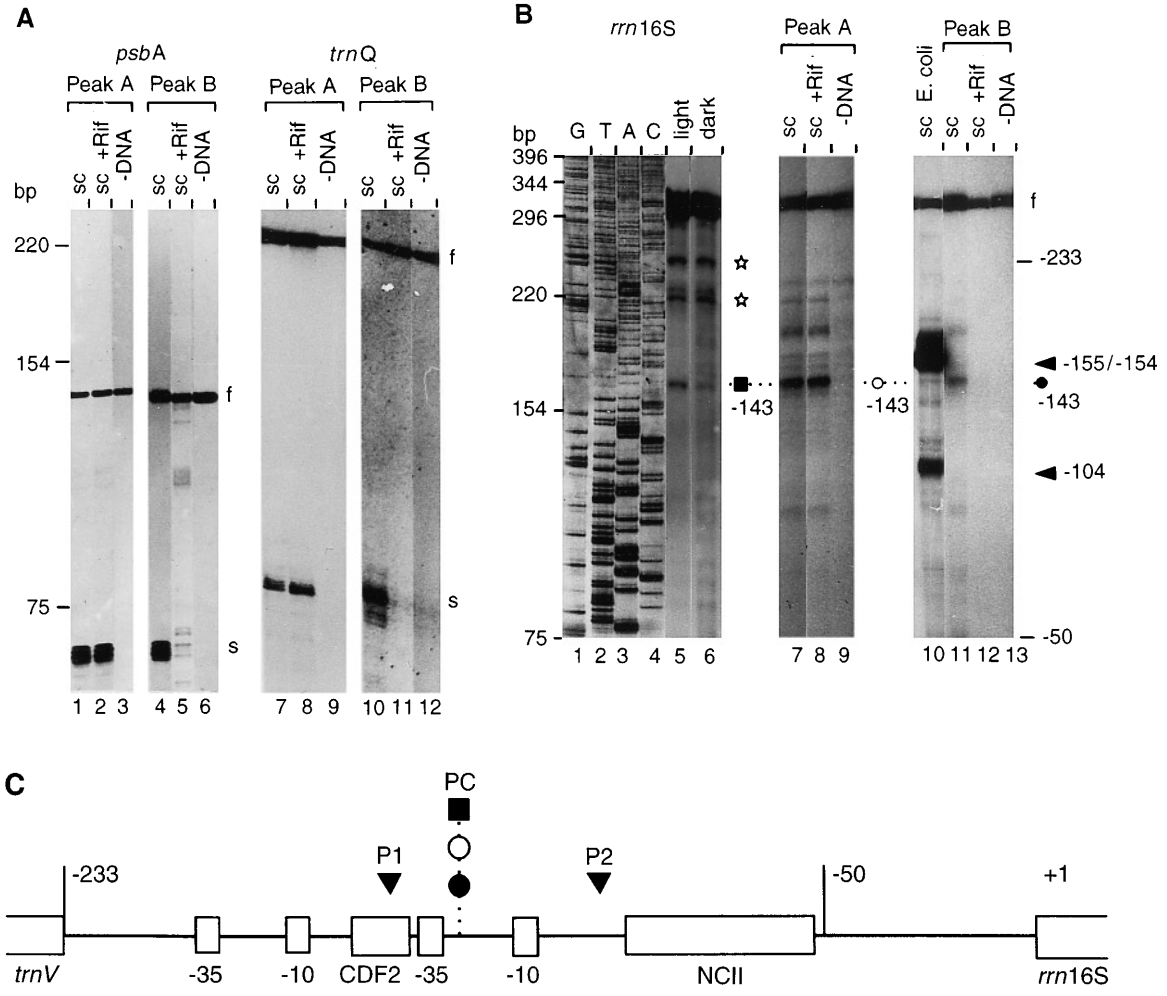
enzyme (lower panel). Of other chloroplast DNA fragments that were tested, the *trnQ* promoter fragment was a more efficient competitor than either the *rps16* and *rrn* promoter fragment. Based on competition efficiency, the binding preference of the *psbA* promoter is higher for the B enzyme (Figs. 3A and 3B, lower panel) than for the A enzyme (upper panel).

In vitro transcription by the A and B polymerases from mustard plastid promoters

In vitro transcription assays were carried out with the purified A or B enzymes and supercoiled plasmid templates that contained the *psbA*, *trnQ* or *rrn* promoter. To distinguish transcripts derived from chloroplast promoters from those driven by vector sequences, nuclease S1 analysis was chosen. As shown in Fig. 4A (left panels) for the *psbA* promoter region, both the A and B polymerases generate major transcripts of a size expected for initiation from the *psbA* promoter (Eisermann et al. 1990). The only significant difference seen is in the presence of rifampicin, which does not affect the amount of correctly-sized RNA products generated by the A enzyme (lanes 1 and 2). In the case of the B enzyme, bands at this position are greatly diminished and other minor transcripts of various sizes become visible (lane 4 and 5).

The situation for the *trnQ* promoter (Fig. 4A, right panels) is comparable to that for the *psbA* promoter. Major S1-resistant bands are generated at the same positions from transcripts synthesized by either the A (lane 7) or the B enzyme (lane 10), and the position of these bands is consistent with initiation at the +1 position downstream of the −35/−10 elements (Neuhaus and Link 1990). As in the case of the *psbA* promoter (Fig. 4A, left panels), transcription from the *trnQ* promoter by the B enzyme is sensitive to rifampicin (lanes 10 and 11) but transcription by the A enzyme is not (lanes 7 and 8).

Using the *rrn* promoter template (Figs. 4B and 4C), again no significant difference could be detected between the patterns of S1-resistant products after in vitro transcription by the A (Fig. 4B, lane 7) and B enzymes (lane 11), and only the latter products were affected by rifampicin (lane 12). The major signal in vitro at −143 (relative to the 16S RNA coding region; Fig. 4C) matches the position of an in vivo RNA end (Fig. 4B,



lanes 5 and 6). The additional, mostly larger bands may represent read-through transcripts initiated further upstream and/or processing intermediates of the upstream *trnV* gene (labelled by asterisks); those seen also in transcription reactions without template DNA (Fig. 4B, lane 9) are likely to be the result of incomplete S1 digestion at high GC or folded regions.

The *rrn* template carries a more complex set of putative promoter elements than either the *psbA* or *trnQ* template (Fig. 4C): Two −35/−10-type regions flank a CDF2 binding site (Baeza et al. 1991); further downstream is a sequence that resembles the NC-II promoter region defined for tobacco chloroplast DNA (Vera and Sugiura 1995; Kapoor et al. 1997), which includes the NEP consensus sequence (Allison et al. 1996; Hajdukiewicz et al. 1997).

The S1-protected product at −143 observed in vivo and in vitro with both the A and B polymerases (Fig. 4B, lanes 5–8, 11) corresponds to a RNA end (designated PC in Fig. 5C) that lies somewhat downstream of the more proximal −35 element. In contrast, *E. coli* RNA polymerase gave only a faint signal at this position and, instead, two major S1-protected products at −155/−154 and −104 were generated (Fig. 4B, lane 10). These bands reflect the putative transcription start sites that are designated P1 and P2 in Fig. 4C, each of which is located a few bases downstream of one of the −10-like sequence motifs. Hence, based on the patterns of S1-resistant products after transcription from the *rrn* promoter template, plastid RNA polymerases A and B are both clearly distinct from the *E. coli* enzyme. In contrast, when the bacterial enzyme was used in transcription reactions with either the *psbA* or the *trnQ* promoter template, the putative start sites were indistinguishable from those found for the plastid RNA polymerases in Fig. 4A (data not shown). With the *rrn* template (Fig. 4B), the lack of strong S1-resistant signals below position −104 suggests that none of the three enzymes can initiate efficiently from the NCII promoter (Fig. 4C).

Transcript analysis in rifampicin-treated seedlings

Based on the sensitivity of cp-pol B to rifampicin in vitro, it should be possible to inhibit this enzyme selectively in vivo by treatment of seedlings with the drug, and to investigate the resulting transcription pattern.

Initial experiments showed that mustard seedlings grown on agar remained viable for at least 120 h at rifampicin concentrations of up to 0.75 mg/ml. Although at concentrations higher than 0.1 mg/ml they showed reduced hypocotyl length and partial loss of chlorophyll, neither total RNA and protein content per fresh weight nor stained RNA and polypeptide patterns were grossly affected. Seedlings grown in the light or in the dark were similarly affected by rifampicin, suggesting that inactivation of the light-sensitive drug does not seem to play a role. This was further checked by comparing growth of seedlings on freshly prepared and pre-illuminated (for 5 days) rifampicin agar; both gave comparable results. Liquid collected from the agar was tested for its inhibitory effect on in vitro transcription by *E. coli* RNA polymerase. Again, no significant difference was detected for freshly prepared versus pre-illuminated medium (data not shown).

Based on this initial characterization, a rifampicin concentration of 0.5 mg/ml was chosen for subsequent experiments. Total RNA was isolated from cotyledons of rifampicin-treated or untreated seedlings and was then analyzed by RNA gel-blot hybridization, using probes for several chloroplast and nuclear genes.

The Northern blot analysis carried out with chloroplast probes showed reduced transcript levels in rifampicin-treated versus control seedlings for the *rbcL* and *psbA* genes (Fig. 5A, two upper horizontal panels). In dark-grown seedlings (left) these transcripts were affected by rifampicin at all developmental stages, whereas under light-grown conditions (right) inhibition was noticeable only until approx. 48–60 h after sowing.

A *rps16* probe (Fig. 5A, third horizontal panel), which detects both precursor and mature transcripts of this gene, revealed transcript levels that were not significantly lower in rifampicin-treated seedlings than in untreated control seedlings. Similarly no effects were observed on the mature *trnG* transcript or its precursor (fourth panel), on the 16S rRNA transcript (fifth panel) or the *rpoB* transcript (sixth panel).

Of the nuclear gene probes used, those representing the genes *Tubß1* for tubulin and *Gbf1a* for a G-box binding factor gave hybridization signals of similar intensity with RNA from rifampicin-treated versus control seedlings (Fig. 5B, upper and middle horizontal panels). The *RbcS* probe (representing the gene family for the small subunit of Rubisco), however, showed reduced transcript levels in rifampicin-treated seedlings (Fig. 5B, third horizontal panels). Like the chloroplast *rbcL* and *psbA* transcripts (Fig. 5A, upper panels), *RbcS* levels were reduced in dark-grown seedlings of all ages, whereas in light-grown seedlings inhibition was noticeable only until 60 h after sowing.

Discussion

We have previously reported evidence showing differential usage of the *psbA* promoter by in vitro transcription systems prepared from chloroplasts and etioplasts (Eisermann et al. 1990). This promoter element switch was found to be related to reversible phosphorylation of components of the plastid transcriptional apparatus (Tiller and Link 1993b; Baginsky et al. 1997). The subsequent separation of two forms of plastid RNA polymerase, with the A enzyme being abundant in chloroplasts and the B enzyme in etioplasts (Pfannschmidt and Link 1994), has raised the possibility that the two enzyme forms might be involved in plastid type-specific differential transcription of one and the same gene, or of sets of genes.

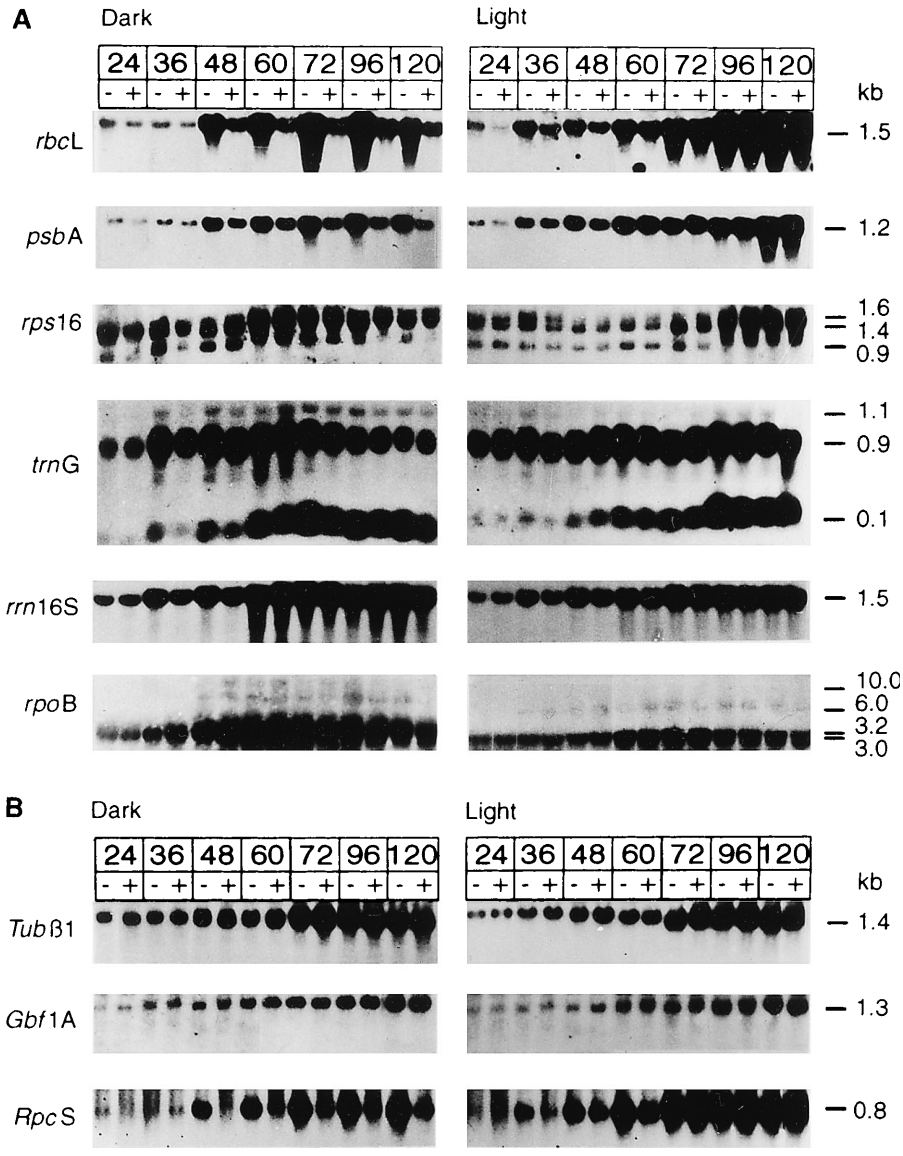


Fig. 5A, B Northern blot analysis of transcript pool sizes in seedlings grown in the presence (+) or absence (−) of rifampicin (0.5 mg/ml). Seedlings were grown on agar in the light (*right panels*) or in the dark (*left panels*) and their cotyledons were used for RNA extraction at the times indicated. Gene probes (*left margin*) and sizes of transcripts (*right margin*) are given. **A** Transcripts of chloroplast genes (from top to bottom) *psbA*, *rbcL*, *rps16*, *trnG*, *rrn*, and *rpoB*. **B** Transcripts of nuclear genes *Tubß1*, *Gbf1a*, and *RbcS*

The gel-shift experiments (Figs. 1 and 2) show that the A and B enzymes differ in their requirements for optimal DNA binding. The A enzyme (from chloroplasts) requires $MgCl_2$ for efficient binding and is relatively insensitive to changes in ionic strength, whereas the B enzyme (from etioplasts) requires elevated ionic strength but no $MgCl_2$. The latter conditions were used in previous work (Eisermann et al. 1990; Tiller and Link 1991, 1993a, b), and it could thus be argued that mainly the B enzyme was assayed in those studies.

However, as indicated by the results obtained with partially purified preparations from different plastid types, which contained both the A and B activities (Fig. 2), Mg^{2+} concentration and ionic strength do not seem to be fixed determinants of DNA binding activity. The A and B activities from etioplasts (or chloroplasts) show more similar salt characteristics than either the A or B activities from the different plastid types. Furthermore, both enzymes from (PT16) plastids of greening seedlings (Fig. 2, lanes 8–11) still show 'etioplast-type' binding requirements, yet the relative intensity of the A versus B activity is greatly increased compared to that in the etioplast fractions (lanes 4–7). This is consistent with the increase in the A/B ratio seen during chloroplast formation (Pfannschmidt and Link 1994) and could be explained by either changes in synthesis (and degradation) or by interconversion of the two enzyme forms.

Both the purified A and B enzymes are capable of binding to the promoter regions of major chloroplast genes, represented by *psbA*, *rps16*, *trnQ*, and the *rrn* operon. Based on competition efficiency (Fig. 3), the relative order of affinity for the four tested promoters was the same for both enzymes, i.e. *psbA* > *trnQ* > *rps16* and *rrn* promoters. However, the degree of preference for the *psbA* promoter (and to a lesser extent for the *trnQ* promoter) seems to be higher for the B enzyme than for the A enzyme.

The transcription experiments (Figs. 4A and 4B) show that both enzymes can initiate from *psbA, trnQ* and *rrn* promoter templates in vitro, and they both seem to use the same start sites. This correlates with the results of the promoter binding experiments (Fig. 3), indicating that indeed both the purified A and B polymerases can efficiently use these different promoters in vitro. On the other hand, the results obtained in vitro may not necessarily, and indeed do not (see Discussion below), reflect the in vivo situation. For instance, additional proteins and/or modifications, such as phosphorylation and SH-group control, could confer differential transcription efficiency even if the properties of the basal polymerases are similar.

The Northern experiments obtained with RNA from rifampicin-treated seedlings (Fig. 5) have revealed transcript patterns that can be distinguished on the basis of the selective effect of this inhibitor on plastid RNA polymerase B (Pfannschmidt and Link 1994). Transcripts of nuclear genes such as *Tubß1* and *Gbf1a* remain unaffected by rifampicin throughout seedling development. This is the expected result for these control transcripts, which indicates a lack of unspecific general effects on cellular gene expression. Among the transcripts of chloroplast genes, two groups could be distinguished in these experiments on the basis of rifampicin sensitivity. Transcript levels of the genes *rbcL* and *psbA*, both of which code for photosynthesis-related proteins, were affected by the drug, whereas those of several other chloroplast genes (*rps16*, *trnG*, 16S ribosomal RNA, *rpoB*) were virtually unaffected. Perhaps the most reasonable conclusion would thus be that the B enzyme is involved in *rbcL* and *psbA* gene expression but has a limited role in the synthesis of the second group of plastid RNAs, all of which specify products not directly related to photosynthesis. The latter genes could be transcribed by cp-pol A, or by another rifampicin-resistant plastid polymerase.

A single-subunit enzyme resembling that of phage T3/T7 or the mitochondrial enzyme is known to be present in chloroplasts (Lerbs-Mache 1993) and to be encoded by a nuclear gene (Hedtke et al. 1997). The NC-II promoter in front of the plastid 16S rRNA gene has been implicated in transcription by the nuclear-encoded (T3/T7-type) polymerase (Kapoor et al. 1997; Hajdukiewicz et al. 1997). It is interesting to note (Figs. 4B, C) that neither cp-pol A nor cp-pol B was able efficiently to transcribe from this promoter, nor was the *E. coli* enzyme.

On the other hand, the bacterial enzyme initiated at the P1 and P2 sites, whereas the purified plastid A and B enzymes each gave an S1 pattern consistent with initiation at the PC site located between P1 and P2 (Figs. 4B, C). The actual mechanism that leads to this apparent shift remains unknown but might involve binding and folding of the region at or near the start site which includes the CDF2 element (Iratni et al. 1994). No apparent differences in start site selection were detected following transcription from either the *psbA* promoter (Eisermann et al. 1990) or the *trnQ* promoter (data not shown), both of which lack the CDF2 element. Thus, although the A and B enzyme are both related to the RNA polymerase from *E. coli*, they reveal clearly distinct characteristics in the *rrn* transcription experiments (Fig. 4B and 4C). A more detailed view of the degree of relatedness will become possible as more sequence information becomes available. Our initial attempts to acquire protein sequence data for the cp-polA and cp-pol B subunits suggest that the core complexes of both enzyme forms are related (not shown), with the A enzyme recruiting additional components such as the heterotrimeric kinase factor KC (Baginsky et al. 1997).

The time-course of rifampicin inhibition of gene expression in vivo (Fig. 5) reveals that the chloroplast *rbcL* and *psbA* transcripts are strongly affected throughout seedling development in the dark. In the light, their transcript pool sizes are decreased during early but not late seedling development. This may point to an 'early' role of the B enzyme (Schrubar et al. 1991; Mullet 1993; Suck et al. 1996), which is then largely replaced by the A enzyme (Pfannschmidt and Link 1994; Link 1996). Consistent with this view is the low polymerase A/B ratio (in terms of protein and activity) in etioplasts, and the high ratio in chloroplasts (Pfannschmidt and Link 1994). With regard to transcription of photosynthesis-related genes, the B enzyme seems to play a major role in proplastids and differentiated non-green plastid types such as etioplasts. During greening it is successively down-regulated and replaced by, or converted into, the A enzyme.

A rifampicin inhibition pattern similar to those of the chloroplast *rbcL* and *psbA* transcripts is noticeable also for transcripts of the nuclear *RbcS* gene family (Fig. 5B). This is reminiscent of norflurazone and other inhibitors of plastid functions, which have been shown selectively to affect the expression of nuclear genes for products destined for the chloroplast (Taylor 1989). It is likely that inhibition of plastid RNA polymerase B leads to impairment of organellar functions and this information is then transmitted to the nucleus by still largely unknown mechanisms. It will be interesting to define the details of this intracellular signalling pathway (Mochizuki et al. 1996).

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