Regulation of rDNA transcription in chloroplasts: promoter exclusion by constitutive repression

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Spinach chloroplasts contain two types of RNA polymerases. One is multimeric and *Escherichia coli*-like. The other one is not *E. coli*-like and might represent a monomeric enzyme of 110 kD. The quantitative relation of the two polymerases changes during plant development. This raises the question, how are plastid genes transcribed that contain *E. coli*-like and non-*E. coli*-like promoter elements during developmental phases when both enzymes are present? Transcription of the spinach plastid *rrn* operon promoter is initiated at three sites: P1, PC, and P2. P1 and P2 are preceded by *E. coli*-like promoter elements that are recognized by *E. coli* RNA polymerase in vitro. However, in vivo, transcription starts exclusively at PC. We analyzed different promoter constructions using in vitro transcription and gel mobility-shift studies to understand why P1 and P2 are not used in vivo. Our results suggest that the sequence-specific DNA-binding factor CDF2 functions as a repressor for transcription initiation of the *E. coli*-like enzyme at P1 and P2. We propose a mechanism of constitutive repression to keep the *rrn* operon in all developmental phases under the transcriptional control of the non-*E. coli*-like RNA polymerase.

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The existence of two different types of RNA polymerases in plastids, which was suggested some years ago (Greenberg et al. 1984; Lerbs et al. 1988; Little and Hallick 1988), seems to have been confirmed recently (Hess et al. 1993; Lerbs-Mache 1993). There is evidence that one of the two enzymes is *Escherichia coli*-like (Lerbs et al. 1985, 1988) and its subunits are encoded by the plastid *rpo* genes (Little and Hallick 1988; Hu and Bogorad 1990; Hu et al. 1991). The importance of *E. coli*-like promoter elements on the plastid genome for transcription initiation has been shown by mutational analysis (Bradley and Gatenby 1985; Gruissem and Zurawski 1985a,b). Although these studies have been made using relatively crude transcriptional extracts we can assume that it is the *E. coli*-like enzyme that recognizes *E. coli*-like promoter structures on the plastid genome.

The other RNA polymerase is nuclear encoded (Morden et al. 1991; Falk et al. 1993; Hess et al. 1993). As of yet, the promoter structures that are recognized by this enzyme on the plastid genome have not been defined, but in several cases, transcription initiation at sequences lacking the *E. coli*-like consensus has been shown (Gruissem et al. 1986; Sexton et al. 1990; Klein et al. 1992). Also, unusual promoters containing prokaryotic and nonprokaryotic sequence motifs have been described

(Link 1984; Eisermann et al. 1990). Such non-*E. coli*-like sequences might be regarded as potential promoters for the nuclear-encoded enzyme. In spinach, two RNA polymerase activities can be separated by heparin-Sepharose chromatography, and the nuclear-encoded RNA polymerase might represent a monomeric enzyme of 110 kD with some phage-like properties (Lerbs-Mache 1993).

The quantitative relation of the two RNA polymerases seems to change with plant development (Lerbs-Mache 1993), and a model was proposed in which the nuclear-encoded enzyme transcribes preferentially plastid genes encoding the transcriptional/translational apparatus during early phases of plastid development to build up the transcriptional and translational machinery of the mature chloroplasts (Lerbs-Mache 1993; Baumgartner et al. 1993; Mullet 1993). During this phase the plastid-encoded *E. coli*-like RNA polymerase is made up and transcribes preferentially the genes implicated in photosynthesis. This model raises the question how plastid housekeeping genes are transcribed during the later developmental phases when both RNA polymerases are present in plastids and might compete for transcription. This question becomes of special interest when *E. coli*-like and non-*E. coli*-like promoter elements have been identified in promoter regions, as in the case of the spinach *rrn* operon (Lescure et al. 1985; Baeza et al. 1991).

The *rrn* operon upstream region of the spinach plastid

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genome contains two tandem *E. coli*-like promoter elements that are recognized in vitro by the *E. coli* RNA polymerase (Lescure et al. 1985). Transcription initiates at two different sites (P1 and P2). However, a detailed in vivo analysis of existing chloroplast ribosomal precursor RNAs from spinach revealed only 5' ends located between the "-35" and "-10" elements of the second *E. coli*-like promoter. We named this position PC (Baeza et al. 1991). The localization of PC with respect to the two *E. coli*-like promoter elements makes it unlikely that the *E. coli*-like elements are implicated in a transcription initiation in vivo. This suggests that the PC-initiated transcripts are made by the 110-kD phage-like RNA polymerase. Furthermore, it suggests that the *E. coli*-like enzyme has to be excluded from transcription initiation at P1 and P2 in leaf plastids in vivo.

Recently, we have characterized a sequence-specific DNA-binding factor (CDF2; Baeza et al. 1991) that should be implicated in the regulation of the *rrn* operon expression. Its DNA-binding site comprises the transcription start site P1, which suggests that it might function as a repressor for transcription initiation at P1. In this study we show that partially purified chloroplast *E. coli*-like RNA polymerase does not initiate transcription in vitro at the two *E. coli*-like *rrn* operon promoters. Therefore, this enzyme reproduces in vitro what happens in vivo, and we used it to analyze the mechanisms by which transcription initiation at P1 and P2 is prevented. We also show that CDF2 acts as a repressor for rDNA transcription by the *E. coli*-like chloroplast enzyme and by the *E. coli* enzyme by blocking the access to the promoter region (P1) and by complexation and inactivation of the enzyme (P2). The implications of this mechanism of transcriptional regulation during plant and chloroplast development are discussed.

Results

The heparin-Sepharose-purified E. coli-like RNA polymerase does not transcribe the rrn operon

Transcriptional extracts were prepared from 500 grams of small spinach leaves from very young plants as described previously (Lerbs-Mache 1993). In contrast to our previous experiments (Baeza et al. 1991) we analyzed each heparin-Sepharose-eluted fraction individually by gel retardation using the *rrn* operon promoter (Fig. 1; mobility shift). As shown previously (Baeza et al. 1991), two complexes of different sizes (labeled L and S) were formed: Fractions 2–4 form the large complex (L), and fractions 6–11 form the small complex (S), which we named CDF2 for chloroplast DNA-binding factor 2. The analysis of each fraction permits us to separate the two different complexes and determine their DNA-binding sites individually (see below). Aliquots of 100 μ l of fractions 2–4 and 6 and 7 were combined and used immediately for further assays or stored at -70°C until usage. We have named these combined fractions CL (2–4) and CS (6 and 7). Transcriptional activity elutes from the heparin-Sepharose column in two peaks, which are in-

Figure 1. Characterization of chloroplast transcriptional extracts after heparin-Sepharose chromatography by gel mobility-shift and transcription assays. (Top) Each heparin-Sepharose fraction was analyzed by gel retardation (F 1–14) using the 163-bp 16S rDNA promoter-containing fragment. The small and the large complex are indicated (S,L). Transcriptionally active fractions are underlined. (Bottom) In vitro transcription of the *rbcL* or 16S rRNA gene promoter region was performed using 0.5 unit of *E. coli* RNA polymerase (Boehringer Mannheim) or 8 μ l of heparin-Sepharose fractions 2, 3, 6, or 7 (cf. with gel mobility-shift assay). The sizes of the transcripts (left) correspond to transcription initiation at the *rbcL* (PLS) or the two *E. coli*-like *rrn* operon promoters P1 and P2 (indicated at right).

dicated by horizontal bars. The first peak (fractions 2–5) comprises the proteins of the large complex. When analyzed by antibody-linked polymerase assays, it was found that this peak contains the *E. coli*-like RNA polymerase polypeptides (Lerbs et al. 1985; Lerbs-Mache 1993), whereas the second peak (fractions 9–12) contains only the 110-kD non-*E. coli*-like RNA polymerase polypeptide (Lerbs-Mache 1993). We have analyzed only the first peak of transcriptional activity (i.e., the *E. coli*-like chloroplast RNA polymerase).

In vitro transcription studies were performed immediately after extract preparation with fractions 2, 3, 6, and 7 using two different DNA templates (Fig. 1; transcription). One plasmid [pTZ19-PLS-Ta; Chen and Orozco 1988] contains a plastid DNA fragment that harbors the *E. coli*-like promoter of the spinach *rbcL* gene (Orozco et al. 1985). It serves as control for transcription by the *E.*

coli-like enzyme because it represents the promoter of a photosynthetic gene. The other plasmid (pTZ19-16S-Ta) contains the 163-bp DNA fragment harboring the spinach *rrn* operon promoter. Both plastid DNA fragments are inserted upstream of the strong, *rho*-independent *E. coli* *thr* attenuator, which terminates transcription efficiently either by the *E. coli* or the chloroplast RNA polymerases (Chen and Orozco 1988; Lerbs-Mache 1993). Transcription from the *rbcL* promoter should produce a 227-nucleotide RNA; transcription from the P1 and P2 promoters (16S) should produce 197- and 137-nucleotide RNAs, respectively. RNAs corresponding to these sizes are found in control experiments performed with *E. coli* RNA polymerase. The chloroplast extract (heparin-Sepharose fractions 2 and 3) starts transcription efficiently at the *E. coli*-like *rbcL* gene promoter but not at the two *E. coli*-like promoters of the *rrn* operon. Heparin-Sepharose fractions 6 and 7 are not active transcriptionally (right).

This result shows that the chloroplast enzyme of the first peak probably reproduces in vitro what happens in vivo. Now we have to ask how the enzyme of this extract can discriminate between the *E. coli*-like promoter of the *rbcL* gene and the two *E. coli*-like promoters of the *rrn* operon. For this reason we analyzed in more detail the protein-DNA interactions (the formation of the two complexes L and S) of the chloroplast extracts with the *rrn* operon promoter region by DNase I footprinting and gel retardation.

The large complex represents an association of the E. coli-like chloroplast RNA polymerase with the DNA-binding factor CDF2

From the transcription studies presented in Figure 1 we can conclude that the small complex is not a subunit of RNA polymerase. It is also not a σ -like factor of RNA polymerase, as plastid σ -like factors do not bind directly to DNA (Tiller et al. 1991); therefore, it should represent a potential transcription regulator with specific DNA-binding properties. DNase I footprinting experiments (Fig. 2) show that the protected region of the large complex includes the -35 and -10 elements of the second *E. coli*-like promoter and the initiation site P2. It is quite similar in size and position to the region that is protected by the *E. coli* RNA polymerase and by chloroplast transcriptional extracts on the *psbA* promoter sequence (Zaitlin et al. 1989; Eisermann et al. 1990). The small complex protects the transcription start site P1 and the -35 element of the *E. coli*-like promoter P2. This protected region extends the CDF2 binding site as it was determined previously by exonuclease III digestion (Baeza et al. 1991). This might be attributable to the different methods that were used or to the fact that the large and the small complex are separated from each other. The fixation of CDF2 to the DNA should block the access of the *E. coli*-like RNA polymerase to the promoter region of P1 (see schematic representation of the two complexes, below, in Fig. 6, top). Furthermore, both complexes overlap over a region of 9 bp. This suggests that

Figure 2. Localization of the large and the small complex on the DNA by DNase I footprinting. DNA was incubated with (+) extract CL (left) or CS (right) or with BSA (–) before DNase I treatment. The DNA sequence was established by chemical cleavage of A and G (AG). (CL) Combined heparin-Sepharose fractions 2–4; (CS) combined fractions 6 and 7 (see Fig. 1).

they should exclude each other from binding to DNA or that they should interact with each other in the overlapping region.

When we characterized the strength of the protein-DNA affinity of the two complexes by raising the salt concentration and introducing heparin during the binding reaction we obtained a surprising result (Fig. 3). At 200 mM NaCl the large complex disappears and a smaller complex forms that migrates on the same gel at the same position as the small complex (Fig. 3, top, cf. lanes 2 and 6). Both complexes are stable up to 600 mM NaCl (lanes 3 and 7), start to diminish at 700 mM NaCl, and disappear completely at 1 M NaCl (lanes 4, 8 and 5, 9). The same holds true when we introduce heparin during the binding reaction (Fig. 3, bottom). The large complex transforms into the small complex at 10 μ M heparin (lane 4). Both small complexes are stable at 20 μ M and dissociate at 100 μ M of heparin (lanes 5, 11 and 2, 8).

This transformation of the large into the small complex also occurs during storage of the extracts at -20°C . Fraction CL was reanalyzed by gel retardation 3 days, 14 days, and 1 year after freezing in the presence of 1.5 (–) and 5 (+) mM Mg^{2+} (Fig. 4A). The dissociation of pro-

Figure 3. Characterization of the DNA affinity of the large (L) and the small (S) complex. Gel mobility-shift assays were performed using the labeled 163-bp 16S rDNA promoter-containing fragment. Increasing concentrations of NaCl (200 mM–1 M; top) and increasing concentrations of heparin (10–200 μ M; bottom) were introduced during incubation of the DNA fragment with fraction CL and CS (see Fig. 2 legend) for complex formation. Lanes 10 (top) and 6 (bottom) represent the DNA fragment without addition of protein extract.

teins already starts after 3 days (lanes 1,2). After 14 days, only the small complex forms at 1.5 mM Mg^{2+} . But the large complex can still be restored by increasing the Mg^{2+} concentration to 5 mM (lanes 3,4). In addition, an intermediate complex appears with the 14-day extract [lane 4, L(Δ)]. This result explains that in Figure 1, lanes 2 to 4, only the large complex is formed, although the small complex forming proteins should be present. This result tells us also that after preparation of a plastid extract we have no more than 3 days to characterize the large complex and that under this condition, it is not technically feasible to purify this complex to homogeneity. After 1 year, the extract has lost transcriptional activity completely and only the small complex forms (lanes 5,6). The DNA-binding site of this complex, as determined by DNase I footprinting, corresponds exactly to the DNA-binding site of the small complex that is formed by the CS fraction (Fig. 4B, cf. with Fig. 2, right).

The presence of CDF2 in the CL fraction was further confirmed by UV cross-linking of CS and CL fraction polypeptides to oligonucleotide hybrids corresponding to the CDF2-binding site. In both cases, two small polypeptides that migrate corresponding to 35 and 33 kD were

linked specifically to the oligonucleotide hybrids that correspond to the CDF2-binding site (Fig. 4C).

Thus far, our results show that the CL fraction contains RNA polymerase as well as CDF2. Now, we must determine whether both components compete for binding to the *rrn* promoter or whether they can bind concomitantly and interact in the overlapping region (cf. Fig. 6, top). In the case of competition we should obtain only two different types of complexes in gel retardation assays, corresponding to binding of CDF2 or RNA polymerase. Concomitant binding of both components should result in the formation of a third type of complex. The existence of three types of complexes (Fig. 4A, lane 4) indicates that L should correspond to binding of RNA polymerase plus CDF2, L (Δ) to binding of RNA polymerase, and S to binding of CDF2. If this assumption is correct, we should obtain only the L (Δ) complex, if we prevent the fixation of CDF2. Therefore, we deleted the CDF2-binding site from the DNA template by cleavage with *MnII*. The two cleavage sites of *MnII* are indicated by vertical arrows in Figure 6 (top). The resulting DNA construction is named Δ 16S. As expected, incubation of 14-day CL extract with this deletion shows only the formation of the L (Δ) complex (Fig. 5, left, cf. lanes 2 and 4). This experiment demonstrates that CDF2 is a component of the L complex. But we must also show that the L complex contains RNA polymerase in addition to CDF2. This was done by immunological cross-reaction with antibodies raised against the *E. coli* RNA polymerase (Fig. 5, right). Complexation with the antibodies causes a supershift (lane 2), thus demonstrating the presence of the *E. coli*-like chloroplast RNA polymerase in the L complex.

These results show that CDF2 complexes with the *E. coli*-like plastid RNA polymerase on the *rrn* promoter region to form the large complex. But, as the large complex is located on a DNA sequence other than the small complex (see Figs. 2 and 4), it implies that CDF2 loses its specific DNA-binding capacity when it contacts the RNA polymerase. From the transcription experiment shown in Figure 1 we know that in this form (as complex with CDF2) the RNA polymerase does not initiate transcription at P2. This suggests that it is the association of the enzyme with CDF2 that blocks transcription initiation at P2.

Complexation of CDF2 with the E. coli-like plastid RNA polymerase is necessary to prevent transcription initiation at P2

If this assumption is correct we should obtain initiation at P2 if CDF2 cannot fix to the template. Therefore, we tested the Δ 16S construction (Fig. 5, left, lanes 3,4, and Fig. 6, top) in mobility-shift and transcription assays using freshly prepared CL extract (Fig. 6). The deletion of the CDF2-binding site abolishes the fixation of CDF2 (Fig. 6, Mobility shift, lanes 1–4) and consequently, should result in the fixation of the *E. coli*-like RNA polymerase depleted of CDF2 [complex L (Δ)]. Correspondingly, incubation of the deleted DNA fragment with hep-

Figure 4. Degradation of the large complex during storage of extracts and comparison of the CL and CS fractions by UV cross-linking of proteins to the CDF2-binding site. (A) Aliquots of fraction CL were thawed after 3 days (lanes 1,2), 14 days (lanes 3,4), and 1 year (lanes 5,6) of storage at -70°C . The extracts were incubated with the 16S rDNA promoter fragment in the presence of 1.5 mM (–) or 5 mM Mg^{2+} , and complex formation was analyzed by gel shift assays. (B) The small complex that dissociates from the large and is stable for 1 year (lanes 5,6) was localized on the DNA sequence by DNase I footprinting. DNA was incubated with protein extract (+) (stored for 1 yr) or with BSA (–) before DNase I treatment. AG corresponds to the A and G cleavage of the DNA fragment. (C) Labeled oligonucleotide hybrids (5 ng, 50,000 cpm) corresponding to the CDF2-binding site (oligonucleotide 1/2) were incubated with chloroplast extract CL (lanes 1,2) (stored for 1 month) or CS (lanes 3,4) in the presence of 50 ng unlabeled oligonucleotide hybrids 1/2 as competitor (lanes 1,3) or 50 ng unlabeled hybrids 3/4 that do not include the CDF2-binding site (lanes 2,4). After cross-linking by UV treatment DNA–protein complexes were analyzed on 10% SDS–polyacrylamide gels. For an explanation of CL and CS, see the legend to Fig. 2.

arin–Sephadex fraction 3 (see Fig. 1) results in the formation of a slightly smaller complex than incubation of the nondeleted DNA fragment (lanes 5,6). In vitro transcription of the two constructions with heparin–Sephadex fraction 3 shows that the deletion of the CDF2-binding site enables the RNA polymerase to initiate transcription at P2 (Fig. 6, transcription). This result confirms our assumption that CDF2 acts as a repressor for transcription of the *rrn* operon by the *E. coli*-like RNA polymerase.

To analyze in more detail the interactions of CDF2 with the *E. coli*-like chloroplast RNA polymerase, we constructed three mutations of the *rrn* operon promoter

and separated the enzyme from the factor to perform reconstitution assays. The mutations are shown in Figure 7 (top). We changed the –10 and –35 promoter elements of P2 (M3 and M2) and two nucleotides in the most conserved part of the CDF2-binding site (M1; for sequence comparison, see Baeza et al. 1991). Mobility-shift assays show that M1 completely abolishes the fixation of CDF2 to the DNA (lanes 8–11). On the other hand, changes in the overlapping region of the two complexes (M2) do not interfere with the binding of the factor (lanes 12–15). Neither do changes in the –10 element (M3; lanes 16–19).

Transcription of the three constructs by the *E. coli* RNA polymerase gives the expected transcripts (Fig. 7, transcription). M2 and M3 largely diminish or completely abolish transcription initiation at P2; M1 abolishes initiation at P1 (lanes 1–4). The chloroplast extract does not initiate transcription on the promoter region of the wild type; however, when CDF2 cannot bind any more to the template (M1) the enzyme can initiate at P2 (lanes 5,6).

To test the two other mutations with the chloroplast enzyme and for reconstitution assays we had to separate CDF2 from the enzyme. This was achieved by stepwise ammonium sulfate precipitation (Fig. 8). The presence of CDF2 was checked in the different fractions by gel retardation assays using an oligonucleotide–tetramer of the CDF2-binding site (mobility shift). The enzyme activity was measured by transcription of the *rbcL* gene promoter (transcription, top). With 50% ammonium sulfate we precipitated only the enzyme without detectable traces of CDF2 (lanes 2,5). Between 50% and 60% the enzyme precipitates along with the factor (lanes 3,6), and at concentrations >60% we can obtain a fraction that only contains the factor (lanes 4,7). The CDF2-depleted enzyme transcribes now the wild type with the same efficiency as M1, but it does not initiate on the two constructions, M2 and M3, in which the two *E. coli*-like promoter elements have been changed (lanes 8,10–12). If

Figure 5. The large complex represents an association of CDF2 with the *E. coli*-like chloroplast RNA polymerase. (Left) Fourteen-day CL extract was incubated with the entire 16S rDNA promoter fragment (lane 2) or with the Δ16S fragment (lane 4) where the CDF2-binding site is deleted by cleavage with *MnII* (see Fig. 6, top), in the presence of 5 mM Mg^{2+} . (Lanes 1,3) The labeled DNA fragments. (Right) The entire 16S rDNA promoter fragment was incubated with freshly prepared CL extract (lanes 1–3) in the presence of antibodies raised against *E. coli* RNA polymerase (lane 2) or preimmune serum (lane 3). (Lane 4) The labeled DNA fragment.

Figure 6. CDF2 represses transcription initiation at the P2 promoter by complexation with the RNA polymerase. The binding of the small (S) and the large (L) complexes to the DNA is represented schematically at the top. Mobility-shift assays were performed with fraction CL (lanes 5,6) and CS (lanes 3,4) and the *rrn* operon promoter fragment before (16S, lanes 3,5) or after (Δ 16S, lanes 4,6) deletion of the CDF2-binding site by cleavage with *MnII*. Lanes 1 and 2 represent the two labeled DNA fragments without protein extract. In vitro transcription assays were done using plasmids pTZ19-16S-Ta (lane 1) and pTZ19- Δ 16S-Ta (lane 2) and fraction CL. For CL and CS, see Fig. 2 legend.

we reintroduce CDF2, initiation at P2 is abolished completely on the wild type (lanes 13,14), but there is still initiation on M1 (lanes 17,18). The fact that the addition of CDF2 reduces the transcription (cf. lanes 17 and 18), is probably attributable to the presence of an additional unspecified transcription inhibitor in the CDF2-containing fraction (S. Lerbs-Mache and R. Iratni, unpubl.).

CDF2 also interacts with the *E. coli* RNA polymerase

Chloroplast σ -like factors are known to interact with the RNA polymerase core enzyme of *E. coli* and to stimulate correct initiation on plastid promoters (Bülow and Link 1988; Lerbs et al. 1988; Tiller et al. 1991). This implies a similarity in one of the chloroplast RNA polymerases (which we call *E. coli*-like) with the *E. coli* enzyme. As CDF2 is not a σ -like factor but interferes with the rec-

ognition of *E. coli*-like promoter elements on the plastid genome, we wanted to know whether it could also interact with the *E. coli* RNA polymerase. Therefore, we performed transcription assays using the *E. coli* enzyme supplemented with different concentrations of CDF2 (Fig. 8, transcription). The addition of CDF2 has no effect on the transcription of the *rbcL* promoter because it does not contain the CDF2-binding site and, consequently, the factor cannot associate with the enzyme (lanes 2–4). However, transcription initiation at the *rrn* promoter is prevented by CDF2 as in the homologous system (lanes 5–7). This inhibition should be caused by CDF2 and not by other transcriptional inhibitors that could be present in the CDF2-containing fraction, as it can be overcome if the factor is “inactivated” before addition of the enzyme by binding to oligonucleotides, which correspond to the CDF2-binding site (lanes 8–11).

Figure 7. Mutational analysis of the *rrn* operon promoter. The three mutations, M1, M2, and M3 (top) were introduced into the *rrn* promoter region of the plasmid pTZ19-16S-Ta (WT). They were tested with the isolated promoter fragments by mobility-shift assays without extract (lanes 1–3) and using 4, 8, 12, and 16 μ l of chloroplast extract CS (lanes 4–7, 8–11, 12–15, and 16–19, respectively) and by in vitro transcription of the plasmids with 0.5 unit of *E. coli* RNA polymerase (lanes 1–4) and with the chloroplast CL fraction (lanes 5,6). For CL and CS, see Fig. 2 legend.

Figure 8. Separation of the *E. coli*-like chloroplast RNA polymerase from CDF2 and reconstitution assays. Ammonium sulfate fractions were tested by mobility-shift assays using an oligonucleotide-tetramer of the CDF2-binding site (lanes 1–4) and by in vitro transcription of pTZ19-LS-Ta (*rbcL* gene promoter, lanes 5–7). The RNA polymerase fraction (50% ammonium sulfate) was used for in vitro transcription of pTZ19-16S-Ta (WT) and all three *rrn* operon mutations (lanes 8, 10–12). The function of the CDF2 fraction (2 μ l of the 60–80% ammonium sulfate fraction) was tested by reconstitution with the enzyme on the wild-type plasmid (lanes 13–15) and on the M1 plasmid (lanes 16–18). For mutations, see Fig. 6. (S1) CDF2 monomer; (S2) CDF2 dimer.

If we analyze the interaction of CDF2 with the *E. coli* enzyme on the *rrn* operon promoter by mobility-shift

assays, we observe that the presence of CDF2 leads to the formation of a larger complex on the entire *rrn* promoter (mobility shift, lanes 6,7) but not on M1 where CDF2 cannot bind (lanes 4,5). This complex corresponds to the L complex of the homologous system. If the concentration of CDF2 reaches the concentration used in the transcription assay (Fig. 9, lanes 1–7), the complex that is formed by the *E. coli* enzyme disappears completely and the L complex analog is highly reduced (not shown). This result is consistent with the idea that CDF2 complexes with the *E. coli* enzyme and this association leads to the detachment of the complex from the DNA.

Discussion

Higher plant plastid rRNA operons show the typically prokaryotic gene order of 16S, 23S, and 5S rDNA. They are transcribed as large precursor RNAs that are processed subsequently into the various mature rRNA species (Strittmatter and Kössel 1984; Dormann-Przybyl et al. 1986). Very little is known of the processes engaged in transcription and processing. Transcription has been studied by expression of the genes in heterologous systems such as *E. coli* (Tohdoh et al. 1981; Lescure et al. 1985; Delp et al. 1987) and by in vitro transcription using run-on assays or highly purified chloroplast enzymes (Briat et al. 1987; Sun et al. 1989; Rajasekhar et al. 1991). For maize and pea, it was reported that transcription starts downstream of a –10 and –35 *E. coli*-like promoter sequence (Strittmatter et al. 1985; Sun et al. 1989). This sequence corresponds to the P2 promoter of the spinach *rrn* operon. We have noticed, however, that maize capping experiments were performed by hybridization of capped RNA to plastid DNA restriction fragments (i.e., the exact position of the capped nucleotide in the sequence is not determined). In pea, the capping experiment has been made after in vitro transcription with purified chloroplast RNA polymerase (i.e., the in vivo transcription start site has not been determined). To our knowledge, a clear determination of the 5' capped nucle-

Figure 9. Interactions of CDF2 with *E. coli* RNA polymerase. In vitro transcription of pTZ19-LS-Ta (lanes 2–4) and pTZ19-16S-Ta (lanes 5–7, 8–11) was performed using 0.5 unit of *E. coli* RNA polymerase (lanes 2,5, 8–11) and 2 μ l (lanes 3,6,10,11) or 4 μ l of fraction CS (lanes 4,7). In the competition experiments (lanes 8–11) transcription assays were incubated for 5 min with (lanes 9,10) or without (lanes 8,11) 50 ng of oligonucleotide-tetramer of the CDF2-binding site before addition of the *E. coli* enzyme. In mobility-shift assays the *rrn* promoter (lane 8) was incubated with 0.4 μ l (lanes 1,4,6) or 0.8 μ l (lanes 2,5,7) of fraction CS and 1 unit of *E. coli* RNA polymerase (lanes 3–7) before electrophoresis. For CS, see Fig. 2 legend.

otide of plastid precursor rRNA has only been made for *Euglena gracilis* (McGarvey et al. 1988). Here, the first nucleotide is a G, which is followed by four C residues, and the initiation site is not preceded by an *E. coli*-like promoter sequence. Furthermore, there is strong evidence that the *Euglena rrn* operon is transcribed by an RNA polymerase other than the one encoded by the plastid *E. coli*-like *rpo* genes (Narita et al. 1985; Little and Hallick 1988). This situation resembles more closely what we find in spinach plastids than what was reported for pea and maize. Therefore, at present, there are not enough data available on the *rrn* expression in other plant species to allow a generalization of this proposed model of transcriptional regulation.

In spinach, of the three transcription start sites (P1, PC, and P2; see Fig. 5), only P1 and P2 are preceded by *E. coli*-like promoter sequences and are recognized by the *E. coli* RNA polymerase and by highly purified chloroplast *E. coli*-like RNA polymerase in in vitro transcription assays (Lescure et al. 1985; Briat et al. 1987). However, in vivo transcription initiation starts exclusively at PC in plastids of all leaf types analyzed so far (Baeza et al. 1991; S. Lerbs-Mache, unpubl.). This is very surprising, and we had to ask why P1 and P2 are not used in vivo. Spinach plastids contain at least two different RNA polymerases: one is multimeric and *E. coli*-like, and the other is active as a single subunit enzyme (Lerbs et al. 1985, 1988; Lerbs-Mache 1993). Both enzymes coexist in leaf plastids of very young spinach plants (Lerbs-Mache 1993) where transcripts are initiated at PC (Baeza et al. 1991). Therefore, the *E. coli*-like enzyme has to be excluded from recognition of the P1 and P2 promoters in vivo. To elucidate the mechanisms of such transcriptional regulation we have started to characterize promoter-protein complexes formed between chloroplast extracts that contain the *E. coli*-like plastid RNA polymerase and the *rrn* operon promoter. We have demonstrated that two DNA-protein complexes of different sizes were formed. The smaller one, which was named CDF2, was further characterized by determination of its DNA-binding site and the DNA-binding proteins (Baeza et al. 1991). The DNA-binding site of CDF2 comprises the transcription start site P1. Thus, fixation of CDF2 on the DNA should prevent any initiation at the P1 promoter. However, we were never able to reproduce the results published by Briat et al. (1987). In our hands, purified chloroplast RNA polymerase, even if isolated in exactly the same way as reported by these investigators, always initiates exclusively at P2 or not at all (if σ -factors are lost).

In this paper we show that the large complex represents an association of CDF2 with the *E. coli*-like plastid RNA polymerase. It was very difficult to get a DNase I footprint of this complex because this complex is very unstable and DNA-protein interactions are very weak (Figs. 3 and 4). During storage, the two components of the complex lose their capacity to interact with each other, and the affinity of the *E. coli*-like RNA polymerase for promoter-binding ceases. Both functions can be restored partially by increasing the Mg^{2+} concentration

(Fig. 4). The affinity of the RNA polymerase to DNA is much lower than that of CDF2 (Fig. 3). At 200 mM NaCl, promoter binding of the enzyme is no longer detectable (Fig. 3, top, lane 2). This correlates with the data obtained for promoter binding of the *E. coli* enzyme, where complex formation diminishes for >90% between 50 and 200 mM of NaCl (Hinkle and Chamberlin 1972).

The DNA-binding site of the large complex comprises the two *E. coli*-like promoter elements of P2 (Figs. 2 and 5). It is quite similar in size and position (relative to the transcription initiation start site) to the *psbA* promoter sequence that has been shown to be protected by *E. coli* RNA polymerase and by chloroplast transcriptional extracts in exonuclease mapping (Eisermann et al. 1990). In the following, we discuss two models to exclude transcription from the P2 promoter. The large and the small complex overlap in a region of 9 bp (Fig. 6, top). This overlap might be sufficient to exclude the concomitant fixation of the two complexes for steric reasons. But this would not explain our finding that CDF2 is a constituent of the large complex. It is more likely to assume that CDF2 interacts with the RNA polymerase in this region. This interaction could cause conformational changes in both complexes that lead not only to the detachment of CDF2 from its binding sequence but subsequently to the detachment of the CDF2-complexed RNA polymerase from the promoter P2 (Fig. 10). This model would also explain the instability of the complex, as it is a complex that is ready to detach from the template. Our results suggest further that the interaction of CDF2 with the enzyme is dependent on the fixation of the two protein complexes to the DNA and does not occur in solution; otherwise CDF2 should also inhibit transcription of the *rbcL* gene (Figs. 1 and 8). If the protein-protein interactions between CDF2 and the *E. coli*-like RNA polymerase on the DNA template are prevented (either by deletion or mutation of the CDF2-binding site or by separation of CDF2 from the enzyme), P2 becomes accessible to initiation (Figs. 5–7).

Our findings strongly suggest a role of CDF2 as repressor for transcription initiation by the *E. coli*-like chloroplast RNA polymerase at P1 and P2. The physiological significance of this type of repression is not yet clear. However, it was shown recently that in *E. coli* the formation of active ribosomal subunits requires coupling of rRNA transcription and ribosome assembly (Lewicki et al. 1993). This coupling demands definite transcription rates for the *rrn* operon expression, and if the operon is placed under the control of a T7 instead of an *E. coli* promoter, this assembly is disturbed. Preliminary results indicate that the spinach *rrn* operon is transcribed by the 110-kD "T7-like" chloroplast enzyme, and that in this case, CDF2 acts as initiation factor or activator of transcription. The 110-kD enzyme is present in chloroplasts of all plant developmental stages analyzed thus far. The *E. coli*-like RNA polymerase is not always detected (S. Lerbs-Mache, unpubl.). Therefore, we suggest that this type of repression serves to keep the *rrn* operon under transcriptional control of the same type of RNA polymerase during all stages of plant development.

Figure 10. Schematic representation of CDF2 and RNA polymerase interactions.

The question remains open as to why the two *E. coli*-like promoters, P1 and P2, have been preserved during evolution if they are not used. It is believed that similar sequences might also have some other function than transcription initiation. Another possibility is that the two promoters are used under specific stress conditions of plant growth. A systematic screening of RNA from different plant organs of plants grown under varying physiological conditions is under way.

Materials and methods

Preparation of the spinach chloroplast extracts

Supernatant of lysed spinach chloroplasts at 48,000g was prepared and subjected to heparin–Sephacryl chromatography, as described previously (Baeza et al. 1991). Two-milliliter fractions were collected, dialyzed against buffer containing 50 mM Tris-HCl (pH 7.8), 1 mM EDTA, 1 mM DTT, 15 mM $(\text{NH}_4)_2\text{SO}_4$, 25% glycerol, and 0.1% Triton-X100, and each fraction was tested for the presence of DNA-binding proteins by mobility-shift assays.

pTZ19-16S-Ta plasmid constructions and preparation of DNA fragments

To obtain the plasmid pTZ19-16S-Ta, the 163-bp *EcoRI*–*HinPI* fragment harboring the 16S rDNA promoter (Baeza et al. 1991) was cloned into the *EcoRI*–*AccI* sites of pUC 19. The fragment was isolated by *EcoRI*–*PstI* cleavage, recombined into Bluescript KS, and resorted by *HindIII*–*PstI* cleavage. The resulting fragment was inserted into the multicloning site of pTZ19-Ta (Chen and Orozco 1988). Site-directed oligonucleotide mutagenesis was performed on this construction according to the protocol of Stratagene. For gel mobility-shift assays the inserts were cleaved with *HindIII*–*BamHI*. After purification on a 2% agarose gel the fragments were labeled by filling in with Klenow enzyme using $[\alpha\text{-}^{32}\text{P}]\text{dATP}$ and $[\alpha\text{-}^{32}\text{P}]\text{dTTP}$.

Mobility-shift assays

Gel retardation assays were performed according to Straney and Crothers (1985). One nanogram of labeled DNA was incubated in a mixture (20 μl) containing 44 mM Tris (pH 8.0), 10 mM NaCl, 0.4 mM EDTA, 0.8 mM DTT, 1 μg of poly[d(I-C)], and 1.5 mM MgCl_2 if not otherwise indicated with 4 μl of chloroplast extract at 30°C for 15 min. DNA–protein complexes were analyzed on 4% polyacrylamide gels under non-denaturing conditions. In some cases, the protein extracts were incubated for 5 min with antibodies (0.2 μl) before the labeled DNA fragment was added.

In vitro transcription

In vitro transcription was performed at 30°C in 25- μl assays containing 44 mM Tris-HCl (pH 8.0), 14 mM DTT, 14 mM MgCl_2 , 50 mM NaCl, 4 mM EDTA, 300 μM each of GTP, ATP, CTP, and 5 μM of UTP, including 20 μCi of $[\alpha\text{-}^{32}\text{P}]\text{UTP}$, 200 ng of DNA, and 8 μl of enzyme extract if not otherwise indicated. Reactions were stopped after 30 min by extraction with phenol–chloroform and precipitation with ethanol. Transcription products were analyzed on 8% acrylamide/urea gels.

DNase I protection

The 274-bp *EcoRI* fragment harboring the 16S rDNA promoter (Baeza et al. 1991) was filled in with Klenow enzyme using $[\text{P}^{32}]\text{dATP}$. After labeling, the fragment was cleaved by *HhaI* and the 163-bp promoter-containing fragment was isolated. DNA corresponding to 200,000 cpm was incubated with 14 μl of fraction CL (12 μg of protein) in the absence of Mg^{2+} under the same conditions as for gel retardation. For DNase I digestion the buffer was adjusted to 25 mM CaCl_2 , 10 mM HEPES (pH 7.6), and 22 mM MgCl_2 . DNase I treatment was done for 30 sec at 30°C using 2 units of DNase I. The reaction was stopped by adjustment of the solution to 20 mM EDTA, 250 mM NaCl, and 0.5% SDS, addition of 1 μg of tRNA, and ethanol precipitation. The control reaction was incubated for the same time in the pres-

ence of 12 µg of BSA. The accompanying sequence was established by chemical cleavage of phosphodiester bonds 3' of A and G (Negri et al. 1991).

Oligonucleotides

Labeled oligonucleotide hybrids were prepared as described previously (Baeza et al. 1991). For competition cross-linking experiments 2 µg of oligonucleotides 1 and 2 or 3 and 4 was hybridized in 30 µl of 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 mM NaCl, and 80% formamide overnight at 42°C. Hybrids were precipitated, dissolved, and used as competitors without further purification: oligonucleotide 1, 5'-GAGAATGAATAAGAGGCTCGTGGGATT; oligonucleotide 2, TTAATTATTCTCCGAGCACCTAACTC-5'; oligonucleotide 3, 5'-GATACAAGTTATGCCTTGGGAATGAA; oligonucleotide 4, TGTTCATACGGAACCTTACTT-TCT-5'. In transcription and gel mobility-shift experiments a tetramer of the CDF2-binding site was used. To construct this tetramer the following two oligonucleotides were hybridized, ligated, and cloned into the *Sma*I site of pUC 18 after blunt-end formation by Klenow treatment: 5'-GGGAAAGAATGAATAAGAGGCTCGTGGGATTGACGTGAGG and TCTTACTTATTCTCCGAGCACCTAACTGCACTCCCCCTT-5'. The tetramer was obtained by cleavage with *Eco*RI-*Pst*I and electroelution after separation on 2% agarose gels.

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