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

Rabah Iratni, Laurence Baeza, Alexandra Andreeva, Régis Mache, and Silva Lerbs-Mache

Laboratoire de Biologie Moléculaire Végétale, Université Joseph Fourier and Centre Nationale de la Recherche Scientifique, BP 53X, F-38041 Grenoble Cédex, France

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 rrrn 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 rrrn operon in all developmental phases under the transcriptional control of the non-E. coli-like RNA polymerase.

[Key Words: Chloroplast; rDNA; repression; transcription]

Received May 25, 1994; revised version accepted October 5, 1994.

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; Eiermann 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 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 rrrn operon [Lescure et al. 1985; Baeza et al. 1991].

The rrrn operon upstream region of the spinach plastid...
which transcription initiation at Pl and P2 is prevented. in vivo, and we used it to analyze the mechanisms by DNA-binding factor (CDF2; Baeza et al. 1991) that Therefore, this enzyme reproduces in vitro what happens of the enzyme (P2). The implications of this mechanism in vitro at 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 µl 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 indicated 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 α-like factor of RNA polymerase, as plastid α-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 (Baaza 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 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 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 μM heparin (lane 4). Both small complexes are stable at 20 μM and dissociate at 100 μM 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 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).
rDNA transcription in chloroplasts

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 μM; 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.

The presence of CDF2 in the CL fraction was further determined by DNase I footprinting, corresponds exactly to the DNA-binding site of the small complex that is 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 rrr 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. Concomitantly 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 MnlI. The two cleavage sites of MnlI are indicated by vertical arrows in Figure 6 (top). The resulting DNA construction is named Δ16S. 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 rrr 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 Δ16S 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 Mg²⁺, 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.

The entire 16S rDNA promoter was incubated with freshly prepared CL extract [lanes 1, 2] or with the Δ16S fragment [lane 4] where the CDF2-binding site is deleted by cleavage with MnlI (see Fig. 6, top), in the presence of 5 mM Mg²⁺. [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.

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
rDNA transcription in chloroplasts

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 rrr operon promoter fragment before [16S, lanes 3,5] or after [Δ16S, 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-Δ16S-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 recognition 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 rrr 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 rrr operon promoter. The three mutations, M1, M2, and M3 [top] were introduced into the rrr 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 μl 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.
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-
and we had to ask why P1 and P2 are not used in vivo. Merases: one is multimeric and used in plastids of very young spinach plants (Lerbs-Maehe, unpubl.). Therefore, we suggest that 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 concentration.

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 concentration...
Concomitant fixation of CDF2 and RNA polymerase on the DNA leads to interactions of both protein complexes in the overlapping region.

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-Sepharose chromatography, as described previously [Baeza et al. 1991]. Two-milliliter fractions were collected, dialyzed against buffer containing 50 mM Tris-Cl (pH 7.8), 1 mM EDTA, 1 mM DTT, 15 mM (NH₄)₂SO₄, 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, recloned 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 insert was cleaved with HindIII–BamHI. After purification on a 2% agarose gel the fragments were labeled by filling in with Klenow enzyme using [α-³²P]dATP and [γ-³²P]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, 0.8 mM EDTA, 1 µg of poly[d(I-C)], and 1.5 mM MgCl₂ 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 MgCl₂, 50 mM NaCl, 4 mM EDTA, 300 µM each of GTP, ATP, CTP, and 5 µM of UTP, including 20 µCi of [α-³²P]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 [³²P]dATP. After labeling, the fragment was cleaved by Hhal 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 Mg²⁺ under the same conditions as for gel retardation. For DNase I digestion the buffer was adjusted to 25 mM CaCl₂, 10 mM HEPES (pH 7.6), and 22 mM MgCl₂. 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-
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 were 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’-GAGGACTCGTGAGGTTGACGTGAGG and TCTTACTTAA-3’; oligonucleotide 2, TTACTATTCTCCGAGCACCCTAACTGCACTCCCCTT-5’; oligonucleotide 3, 5’-GATACAGTTATGGCCTTGGGGAATGAGG; oligonucleotide 4, TGGTTCAATACGGAACCTTACTTCT-5’. In transcription and gel mobility-shift experiments a tetramer of BSA. The accompanying sequence was established by chemical cleavage of phosphodiester bonds 3’ of A and G [Negri et al. 1991].

References

Baeza, L., A. Bertrand, R. Mache, and S. Lerbs-Mache. 1991. Characterization of a protein binding sequence in the promoter region of the 16S rRNA gene of the spinach chloroplast genome. Nucleic Acids Res. 19: 3577–3581.

Baumgartner, B.J., J.C. Rapp, and J.E. Mullet. 1993. Plastid genes encoding the transcription/translation apparatus are differentially transcribed in ribosome-deficient plastids. Evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J. 12: 563–571.

Hinkle, D.C. and M.J. Chamberlin. 1972. Studies of the binding of Escherichia coli RNA polymerase to DNA. J. Mol. Biol. 70: 157–185.

Hu, J. and L. Bogorad. 1990. Maize chloroplast RNA polymerase: The 78-kilodalton polypeptide is encoded by the plastid rpoC1 gene. Nucleic Acids Res. 19: 3431–3434.

Hess, W.R., A. Prombona, B. Fieder, A.R. Subramanian, and T. Börner. 1993. Chloroplast rps 15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids. Evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J. 12: 563–571.

Klein, U., J.D. De Camp, and L. Bogorad. 1992. Two types of chloroplast gene promoters in Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. 89: 3453–3457.

Lerbs, S., M. Schaffner, and G. Link. 1985. Identification and mutational analysis of the promoter for a spinach chloroplast rRNA genes does not require upstream promoter elements for transcription. Nucleic Acids Res. 14: 7541–7555.

Hess, W.R., A. Prombona, B. Fieder, A.R. Subramanian, and T. Börner. 1993. Chloroplast rps 15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids. Evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J. 12: 563–571.

Hinkle, D.C. and M.J. Chamberlin. 1972. Studies of the binding of Escherichia coli RNA polymerase to DNA. J. Mol. Biol. 70: 157–185.

Hu, J. and L. Bogorad. 1990. Maize chloroplast RNA polymerase: The 78-kilodalton polypeptide is encoded by the plastid rpoC1 gene. Nucleic Acids Res. 19: 3431–3434.

Klein, U., J.D. De Camp, and L. Bogorad. 1992. Two types of chloroplast gene promoters in Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. 89: 3453–3457.

Lerbs, S., M. Schaffner, and G. Link. 1985. Identification and mutational analysis of the promoter for a spinach chloroplast rRNA genes does not require upstream promoter elements for transcription. Nucleic Acids Res. 14: 7541–7555.

Hess, W.R., A. Prombona, B. Fieder, A.R. Subramanian, and T. Börner. 1993. Chloroplast rps 15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids. Evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J. 12: 563–571.

Hinkle, D.C. and M.J. Chamberlin. 1972. Studies of the binding of Escherichia coli RNA polymerase to DNA. J. Mol. Biol. 70: 157–185.

Hu, J. and L. Bogorad. 1990. Maize chloroplast RNA polymerase: The 78-kilodalton polypeptide is encoded by the plastid rpoC1 gene. Nucleic Acids Res. 19: 3431–3434.

Klein, U., J.D. De Camp, and L. Bogorad. 1992. Two types of chloroplast gene promoters in Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. 89: 3453–3457.

Lerbs, S., M. Schaffner, and G. Link. 1985. Identification and mutational analysis of the promoter for a spinach chloroplast rRNA genes does not require upstream promoter elements for transcription. Nucleic Acids Res. 14: 7541–7555.

Hess, W.R., A. Prombona, B. Fieder, A.R. Subramanian, and T. Börner. 1993. Chloroplast rps 15 and the rpoB/C1/C2 gene cluster are strongly transcribed in ribosome-deficient plastids. Evidence for a functioning non-chloroplast-encoded RNA polymerase. EMBO J. 12: 563–571.

Hinkle, D.C. and M.J. Chamberlin. 1972. Studies of the binding of Escherichia coli RNA polymerase to DNA. J. Mol. Biol. 70: 157–185.

Hu, J. and L. Bogorad. 1990. Maize chloroplast RNA polymerase: The 78-kilodalton polypeptide is encoded by the plastid rpoC1 gene. Nucleic Acids Res. 19: 3431–3434.

Klein, U., J.D. De Camp, and L. Bogorad. 1992. Two types of chloroplast gene promoters in Chlamydomonas reinhardtii. Proc. Natl. Acad. Sci. 89: 3453–3457.

Lerbs, S., M. Schaffner, and G. Link. 1985. Identification and mutational analysis of the promoter for a spinach chloroplast rRNA genes does not require upstream promoter elements for transcription. Nucleic Acids Res. 14: 7541–7555.
scription of a protein-coding plastid gene in a plastid in vitro system from mustard (Sinapis alba L.). EMBO J. 3: 1697–1704.

Little, M.C. and R.B. Hallick. 1988. Chloroplast rpoA, rpoB, and rpoC genes specify at least three components of a chloroplast DNA-dependent RNA polymerase active in tRNA and mRNA transcription. J. Biol. Chem. 263: 14302–14307.

McGarvey, P., R.B. Helling, J.-Y. Lee, D.R. Engelke, and M.R. El-Gewely. 1988. Initiation of rrn transcription in chloroplasts of Euglena gracilis bacillaris. Curr. Genet. 14: 493–500.

Mullet, J.E. 1993. Dynamic regulation of chloroplast transcription. Plant Physiol. 103: 309–313.

Morden, C.W., K.H. Wolfe, C.W. de Pamphilis, and J.D. Palmer. 1991. Plastid translation and transcription genes in a non-photosynthetic plant: Intact, missing and pseudo genes. EMBO J. 10: 3281–3288.

Narita, J.O., K.E. Rushlow, and R.B. Hallick. 1985. Characterization of an Euglena gracilis chloroplast RNA polymerase specific for ribosomal RNA genes. J. Biol. Chem. 260: 11194–11199.

Negri, R., G. Costanzo, and E. Di Mauro. 1991. A single-reaction method for DNA sequence determination. Anal. Biochem. 197: 389–395.

Orozco, E.M. Jr., J.E. Mullet, and N.-H. Chua. 1985. An in vitro system for accurate transcription initiation of chloroplast protein genes. Nucleic Acids Res. 13: 1283–1302.

Rajasekhar, V.K., E. Sun, R. Meeker, B.-W. Wu, and K.K. Tewari. 1991. Highly purified pea chloroplast RNA polymerase transcribes both rRNA and mRNA genes. Eur. J. Biochem. 195: 215–228.

Sexton, T.B., D.A. Christopher, and J.E. Mullet. 1990. Light-induced switch in barley psbD-psbC promoter utilization: A novel mechanism regulating chloroplast gene expression. EMBO J. 9: 4485–4494.

Sun, E., B.-W. Wu, and K.K. Tewari. 1989. In vitro analysis of the pea chloroplast 16S rRNA gene promoter. Mol. Cell. Biol. 9: 5650–5659.

Straney, D.C. and D.M. Crothers. 1985. Intermediates in transcription initiation from the E. coli lac UVS promoter. Cell 43: 449–459.

Strittmatter, G., and H. Kössel. 1984. Cotranscription and processing of 23S, 4.5S and 5S tRNA in chloroplasts from Zea mays. Nucleic Acids Res. 12: 7633–7647.

Strittmatter, G., A. Gozdzicka-Jozefiak, and H. Kössel. 1985. Identification of an rRNA operon promoter from Zea mays chloroplasts which excludes the proximal tRNAVal from the primary transcript. EMBO J. 4: 599–604.

Tiller, K., A. Eiermann, and G. Link. 1991. The chloroplast transcription apparatus from mustard (Sinapis alba L.). Eur. J. Biochem. 198: 93–99.

Tohdoh, N., K. Shinozaki, and M. Sugiura. 1981. Sequence of a putative region for the rRNA genes of tobacco chloroplast DNA. Nucleic Acids Res. 9: 5399–5406.

Zaitlin, D., J. Hu, and L. Bogorad. 1989. Binding and transcription of relaxed DNA templates by fractions of maize chloroplast extracts. Proc. Natl. Acad. Sci. 86: 876–880.
Regulation of rDNA transcription in chloroplasts: promoter exclusion by constitutive repression.

R Iratni, L Baeza, A Andreeva, et al.

*Genes Dev.* 1994, 8:
Access the most recent version at doi:10.1101/gad.8.23.2928