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RESEARCH ARTICLE

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Chlamydomonas reinhardtii encodes a single sigma^{70}-like factor which likely functions in chloroplast transcription

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Abstract *Chlamydomonas reinhardtii* EST clones encoding a protein highly similar to prokaryotic sigma factors and plant sigma-like factors (SLFs) were used to isolate a BAC clone containing the full-length gene *CrRpoD*. The gene is likely to be single-copy, in contrast to small gene families encoding SLFs in plants. The *CrRpoD* mRNA comprises 3,033 nt with an open reading frame of 2,256 nt, encoding a putative protein of 752 amino acids with a molecular mass of 80.2 kDa. The sequence contains conserved regions 2–4 typically found in sigma factors, and an unusually long amino terminal extension, which by in silico analysis has properties of a chloroplast transit peptide. Expression of *CrRpoD* was confirmed by immunodetection of a 85 kDa polypeptide in a preparation enriched for chloroplast proteins. To demonstrate functionality in transcription initiation, a recombinant CrRpoD–thioredoxin fusion protein was reconstituted with *E. coli* RNA polymerase core enzyme and tested in vitro. This chimeric holoenzyme specifically bound the spinach *psbA* and *Chlamydomonas rrn16* promoters in gel mobility shift assays and exhibited specific transcription initiation from the same two promoters, providing evidence for the role of CrRpoD as a functional transcription factor.

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Introduction

Higher plant plastid genomes are transcribed by two different RNA polymerases (RNAPs): NEP, a nuclear-encoded bacteriophage-type single-subunit RNAP, and PEP, a plastid-encoded eubacterial-type multi-subunit RNAP (Hess and Boerner 1999; Liere and Maliga 2001). The transcription of photosynthesis-related chloroplast genes is primarily accomplished by PEP, whose catalytic core consists of α, β, β′ and β″ subunits. Beyond these plastid-encoded subunits, PEP is thought to require a sigma^{70}-like factor (SLF) that specifies transcription initiation downstream of promoter sequences which resemble bacterial −10/−35 promoters. SLFs are encoded by gene families in cyanobacteria (Brahamsha and Haselkorn 1992), and within the nuclear genomes of red algae (Oikawa et al. 1998) and Streptophytes (Isono et al. 1997; Tanaka et al. 1997; Tan and Troxler 1999; Fujiwara et al. 2000; Oikawa et al. 2000). Consistent with the presumed interaction of plant SLFs with PEP, import into chloroplasts has been shown in vitro for some of these factors (Isono et al. 1997; Lahiri et al. 1999; Tan and Troxler 1999; Fujiwara et al. 2000). Additionally, the conservation of the primary structure among eubacterial and plant SLFs is sufficiently high to permit the reconstitution of a functional holoenzyme with the *E. coli* core RNAP in vitro (Kestermann et al. 1998; Hakimi et al. 2000; Beardslee et al. 2002; Homann and Link 2003) and in vivo (Hakimi et al. 2000).

Plant SLFs show various types of regulation such as tissue- or organ-specific expression (Isono et al. 1997; Tozawa et al. 1998; Lahiri et al. 1999; Tan and Troxler 1999; Lahiri and Allison 2000), light-dependent expression (Liu and Troxler 1996; Isono et al. 1997; Tanaka et al. 1997; Kestermann et al. 1998; Oikawa et al. 1998; Tozawa et al. 1998; Morikawa et al. 1999), circadian

clock-controlled expression (Kanamaru et al. 1999; Morikawa et al. 1999), or plastid development-dependent expression (Lahiri and Allison 2000). These facts suggest that individual sigma factors may play a role in the control the nucleus exerts over chloroplast gene expression in response to various environmental and endogenous signals. This model has derived some support from the phenotypes of *Arabidopsis* insertional mutants lacking one sigma factor; such mutants show preferential loss of transcription from different genic subsets and, in one case, lethality due to a developmental defect (Hanaoka et al. 2003; Yao et al. 2003).

In this study we characterize a putative sigma factor from the green alga *Chlamydomonas reinhardtii*. Its 203-kb plastid chromosome has remarkable features including a preponderance of repetitive DNA, a relatively low number of coding regions, and an atypical organization of genes encoding PEP (Maul et al. 2002). Typical eubacterial-like promoters have been identified in this genome (Klein et al. 1992; Klinkert et al. 2005). It has also been suggested that *Chlamydomonas* chloroplasts, unlike those of higher plants, lack NEP, since attempts to disrupt PEP-encoding genes have failed (Goldschmidt-Clermont 1991; Rochaix 1995; Fischer et al. 1996), and because virtually all its transcription is sensitive to rifampicin, an inhibitor of PEP but not NEP (Eberhard et al. 2002). The results reported here characterize biochemically a nuclear encoded SLF that may therefore regulate all plastid transcription in *Chlamydomonas*.

Materials and methods

Chlamydomonas strains and culture conditions

Chlamydomonas reinhardtii (CC-125, mt^+) was maintained on 0.8% Tris–acetate–phosphate (TAP) agar (Harris 1989) at 25°C under constant light (70 $\mu E/m^2/s$). For protein isolation, cells were placed into 50 ml of liquid TAP medium and allowed to grow under continuous light on a rotary shaker (125 rpm) to mid-log phase (~5×10^6–1×10^7 cells/ml).

BAC screening and sequencing

A *C. reinhardtii* macroarray filter containing 15,360 independent BAC clones was obtained from Dr C. Silflow (University of Minnesota). The library was screened by hybridization with a ^{32}P-labeled probe derived from EST AV392956. BAC clones which yielded a positive signal were requested from the Clemson University Genomics Institute. A 6 kb *Sna*BI/*Ssp*I fragment from BAC 30i6 including the complete *CrRpoD* gene was subcloned into the *Eco*RV site of pBluescript SK(+) (Stratagene, La Jolla, CA) to yield the plasmid pSig1gen. To determine the gene structure, pSig1gen was sequenced along with the cDNA clone AV643470 (Kazusa DNA Research Institute) using vector and custom primers. While this manuscript was in preparation, Carter et al. (2004) reported the isolation of a *Chlamydomonas* gene encoding a sigma factor (*RpoD*; accession number AF525691), which is identical to the sequence described here (accession number AJ627180).

Chloroplast isolation and immunoblot analysis

For chloroplast isolation, CC-125 cells were grown in TAP medium under continuous light at 25°C to a density of approximately 10^7 cells/ml. Intact chloroplasts were collected from the 40/70% interface of a discontinuous Percoll gradient using a previously described method (Goldschmidt-Clermont et al. 1989). Immunoblotting was performed using a peptide antibody directed against *C. reinhardtii* RpoD, a polyclonal antibody directed against *C. reinhardtii* large subunit of ribulose-1,5-bisphosphate carboxylase (LSU) and a sea urchin monoclonal α-tubulin antibody (Sigma). The peptide designed to make the CrRpoD antibody was LADELERLLGPTTSC, where the terminal cysteine is not encoded in the *CrRpoD* gene and was added for coupling. The antibody was produced in rabbits by Quality Bioreagents (Seguin, Texas), and 5–6 injections were required to get a positive response on an immunoblot. The antibody was titered using ELISA, and pooled active fractions were affinity-purified using the immobilized peptide (Affinity Bioreagents) as a ligand. Antibody binding was detected using HRP-conjugated anti-rabbit IgG and enhanced chemiluminescence.

Overexpression and purification of recombinant CrRpoD

For expression of recombinant protein, a 2,094 nt fragment of *CrRpoD* encoding the 698 C-terminal amino acids was amplified from cDNA, and inserted into the plasmid pBAD/Thio-TOPO to yield the plasmid pBAD/Thio-$RpoD_{698AA}$. Expression was accomplished in *E. coli* TOP 10 cells (Invitrogen). Cells were grown to an OD_{600} of 0.5–0.6, and protein expression induced by addition of arabinose to a final concentration of 0.002%, followed by growth at 37°C for 3–4 h. The expression of CrRpoD in *E. coli* led to the production of insoluble inclusion bodies. Cell lysis was performed with a French Press at 20,000 psi. After centrifugation (10,000 rpm, 10 min in a SS34 rotor), the pellet was washed in PBS containing 0.2% Triton X-100, and resuspended in solubilization buffer (100 mM NaH_2PO_4; 6 M guanidine hydrochloride; 10 mM Tris, pH 8.0). After centrifugation as above, the supernatant was stored at −20°C. Refolding was performed by dilution, basically as described by Armstrong et al. (1999). The protein concentration was adjusted to 5 mg/ml with solubilization buffer, and 20 µl of this solution were added to 1 ml of refolding buffer (2 mM $MgCl_2$; 2 mM $CaCl_2$; 0.5 M glycine; 1 mM DTT; 0.5 M guanidine hydrochloride;

9.6 mM NaCl; 0.4 mM KCl). For in vitro transcription the refolded protein was used immediately.

Gel mobility shift assay

For gel shift experiments, 25 ng recombinant CrRpoD was mixed with 40 fmol ^{32}P-labeled DNA probe, 2.5 µg poly dI–dC and 100 ng *E. coli* RNAP (core enzyme, Epicentre Technologies) in 30 µl reactions containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$ and 5 mM DTT. The reactions were incubated at 25°C for 30 min and separated in 4% polyacrylamide gels. After electrophoresis, gels were dried and exposed to a phosphorimage screen and analyzed on a Molecular Imager (Bio-Rad). DNA probes of the spinach *psbA* and the *Chlamydomonas rrn16* promoter were generated by PCR from total DNA of spinach and *Chlamydomonas*, respectively, using primers psbAfw: GGGTCCGGGTTCAAGT, psbArv: GACTC CCAAGCACACAAAT, rrn16fw: CAATTATTATTT TACTGCGGAGC and rrn16rv: CAAACTCTCCAT GGATACTC.

In vitro transcription

In vitro transcription reactions (25 µl) contained 50 ng of circular DNA as template, 0–200 ng recombinant CrRpoD protein, 1 µl RNasin ribonuclease inhibitor (Promega), 1 U *E. coli* core or holoenzyme (Epicentre), 0.4 mM ATP, 0.4 mM GTP, 0.4 mM CTP and 5 µl 5×transcription buffer (5×transcription buffer = 0.2 M Tris–HCl, pH 7.5; 0.75 M KCl; 0.05 M $MgCl_2$; 0.1 mM DTT; 0.05% Triton X-100) and 8 μCi [α-^{32}P]-UTP (800 Ci/mmol, 20 mCi/ml). The DNA template pDBST7e containing the spinach *psbA* promoter and the bacteriophage T7 early terminator was described previously (Stern and Gruissem 1987). The DNA template pDBSCr16S was created from pDBST7e by replacing the *psbA* promoter with the *Chlamydomonas* chloroplast *rrn16* promoter. The *rrn16* fragment was amplified by PCR (primers rrn16-2fw: AGAATTCAATTATTAT TTTACTGCGGAGC and rrn16-2rv: ACTCGAGCA AACTCTCCATGGATACTC) and introduced into pDBST7e digested with *Xho*I and *Eco*RI. In vitro transcription products were separated in 6% TBE-polyacrylamide gels containing 42% urea. After electrophoresis, the gels were dried and analyzed on a Storm scanner.

Results

C. reinhardtii expresses a single sigma factor, RpoD

An informatic approach was used to identify putative sigma factors of *C. reinhardtii*, for which previous evidence was exclusively biochemical (Surzycki and Shellenbarger 1976). A database search revealed several ESTs encoding putative proteins with similarity to the conserved regions of classical sigma factors. An oligonucleotide specific to conserved region 2.4 was used to screen a library of 15,360 BAC clones, and six were identified. Fingerprint analyses suggested that all six clones originated from the same genomic region (data not shown). This BAC screening, as well as DNA and RNA filter hybridizations (data not shown), indicates that there is only one sigma factor encoded in the *C. reinhardtii* nuclear genome. This conclusion is in agreement with the results of Carter et al. (2004).

The *CrRpoD* sequence we obtained is identical to the one reported recently (Carter et al. 2004). The 3,033 bp cDNA encoding *CrRpoD* contains four exons comprising 2,256 nt, flanked by untranslated regions of 120 and 654 nt at the 5′ and 3′ ends, respectively. The predicted protein contains 752 amino acids with a molecular mass of 80.2 kDa. Alignments of the deduced amino acid sequences of 39 sigma factors from various species (the multiple alignment as well as results of phylogenetic analysis are available as Supplementary Material) revealed that the N-terminal region of *C. reinhardtii* RpoD exhibits very low similarity to the corresponding regions of other sigma factors, and is the longest among all reported plant sequences. Predictions using the programs TargetP (Emanuelsson et al. 2000) and PCLR (http://www.apicoplast.cis.upenn.edu/pclr/) suggested that the extreme N-terminus could direct plastid localization. The C-terminal region possesses all of the highly conserved subdomains which are known to be involved in recognition of −10 and −35 promoter elements (2.4 and 4.2), RNAP core complex binding (2.1 and 3.2), and DNA melting (2.3).

To test for expression at the protein level and confirm the supposed chloroplast localization, an antibody raised against a 14 amino acid peptide corresponding to a non-conserved region of the N-terminus of CrRpoD was used. Specificity of the antibody was confirmed by immunoblot analysis, in which the antibody reacted with a recombinant CrRpoD–thioredoxin fusion protein (see below). In immunoblots with both total protein and chloroplast isolates (Fig. 1) the CrRpoD antibody reacted with a polypeptide of approximately 85 kDa, which was clearly present in the chloroplast fraction (lane C). Minor bands of lower molecular mass showing up on the blot are probably due to non-specific reactions or protein degradation. Immunoblotting of total and chloroplast proteins with antibodies against the chloroplast protein LSU and α-tubulin confirmed the degree of chloroplast protein enrichment and ruled out possible cytosolic contamination.

Recombinant CrRpoD binds to chloroplast promoters

To test whether the identified SLF indeed had properties consistent with a role as a PEP transcription factor, gel shift mobility assays were performed with recombinant CrRpoD. To represent the protein in vivo more closely,

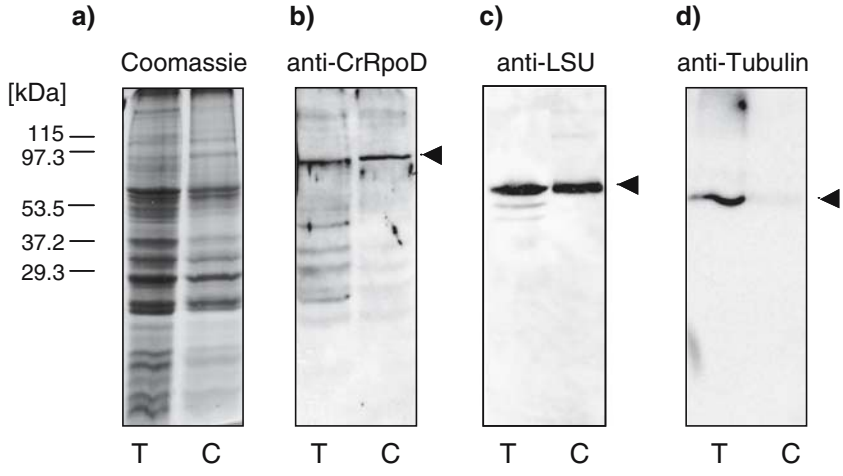

Fig. 1 CrRpoD protein expression analysis. Immunoblot analysis of *Chlamydomonas* total (*T*) and chloroplast-enriched (*C*) proteins. A Coomassie Blue-stained polyacrylamide gel is shown at the *left*. The blots were incubated with antibodies raised against a CrRpoD peptide, the chloroplast protein LSU, and sea urchin α-tubulin

an N-terminal truncated version lacking the putative transit peptide was used. These 698 C-terminal amino acids were fused to thioredoxin (pBAD/Thio-RpoD$_{698}$) and overexpressed in *E. coli*. Since expression of CrRpoD occurred predominantly in inclusion bodies, it was solubilized following isolation of inclusion bodies, which could be purified to near homogeneity under denaturing conditions (Fig. 2a). The identity of the recombinant protein was confirmed by immunodetection with an anti-peptide antibody (data not shown).

To demonstrate that recombinant CrRpoD could recognize chloroplast promoters, we used gel mobility shift assays in a heterologous system with *E. coli* core RNAP. Other recombinant chloroplast SLFs were reported to efficiently bind promoters under such conditions (Kestermann et al. 1998; Homann and Link 2003). Promoters used for the DNA binding assays exhibiting high similarity to −35/−10 bacterial promoters are displayed in Fig. 2b. As shown in Fig. 2c, a shifted band indicating a DNA–protein complex was detected when recombinant CrRpoD and *E. coli* core polymerase were incubated with the spinach chloroplast *psbA* promoter (left panel) or the *Chlamydomonas* chloroplast *rrn16* promoter (right panel). Binding was almost completely abolished in the presence of excess unlabelled promoter DNA as a competitor. None of the control reactions consisting of the probe DNA alone, or the probe DNA and the recombinant CrRpoD protein without the core polymerase, gave shifted bands. A very faint shifted band in reactions with the *E. coli* core enzyme is most probably due to traces of *E. coli* RpoD in the enzyme preparation. The gel shift experiments indicate that both promoters investigated were recognized by the reconstituted CrRpoD–*E. coli* holoenzyme.

Recombinant CrRpoD confers specific transcription initiation in vitro

Transcription assays compared the activity of *E. coli* holoenzyme with that of the *E. coli* core enzyme supplemented with increasing amounts of recombinant CrRpoD. To exclude a co-purification of transcriptionally active host proteins, the *E. coli* core enzyme was also supplemented with mock-purified proteins from cells carrying the empty expression vector. The supercoiled DNA templates used in the assays contained either the spinach chloroplast *psbA* promoter or the *Chlamydomonas* chloroplast *rrn16* promoter, followed by the bacteriophage T7 early terminator, which is recognized by the bacterial and spinach chloroplast RNAPs (Stern and Gruissem 1987). These constructs generate transcripts of predictable sizes initiating at the cloned promoter, and ending immediately downstream of the T7 terminator stem-loop.

As shown in Fig. 3, both the chimeric and homologous holoenzymes were capable of specific transcription initiation at the *psbA* promoter (Fig. 3a) as well as at the *rrn16* promoter (Fig. 3b). Transcripts of the expected sizes were obtained, and their amounts correlated with the amount of recombinant CrRpoD. In vitro transcription reactions containing recombinant CrRpoD clearly stimulated transcription in comparison to core enzyme alone, with the *E. coli* holoenzyme generating the most transcription. Based on these results, we conclude that CrRpoD conferred promoter binding and specific transcription initiation to the heterologous core. Therefore, it can be suggested that the enhanced transcription is due to the interaction of CrRpoD and the *E. coli* core polymerase and that CrRpoD exhibits biochemical properties of a sigma factor.

Discussion

A large number of plant genes encoding SLFs have been identified in recent years, and some of these proteins have been shown to be imported into plastids (Isono et al. 1997; Lahiri et al. 1999; Tan and Troxler 1999; Fujiwara et al. 2000). It is assumed that once imported, these proteins interact with the PEP to recognize the class of plastid promoters containing typical −35 and/or

−10 elements. On the other hand, prior to this and a related study (Carter et al. 2004), no SLF genes had been characterized from green algae. Our analysis of *Chlamydomonas RpoD* shows that the 752 amino acids include the universally conserved domains (2.1 and 2.3) for binding of the polymerase core complex and for DNA melting and recognition (2.3, 2.4 and 4.2). CrRpoD contains an unusually long and non-conserved N-terminal extension, which apart from chloroplast could be speculated to confer some alga-specific functions and

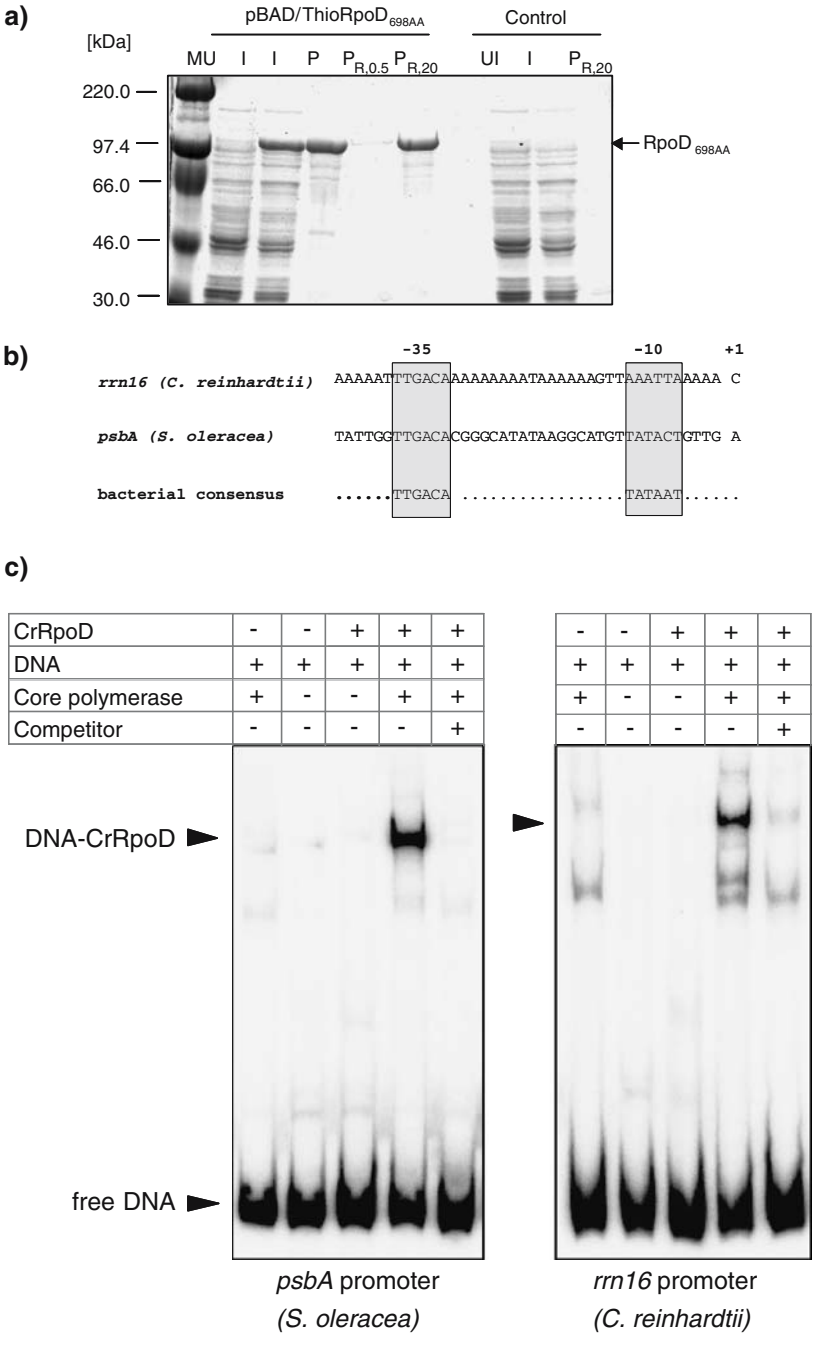

Fig. 2 Recombinant CrRpoD binds to plant and algal promoters. **a** Expression and purification of recombinant CrRpoD used for in vitro transcription. Coomassie Blue-stained SDS-polyacrylamide gel illustrating expression and purification of recombinant CrRpoD (pBAD/Thio-RpoD$_{698AA}$) and the empty expression vector (Control). *UI* uninduced bacterial cultures, *I* total protein isolated after 4 h of induction with 0.002% arabinose, *P* CrRpoD after purification under denaturing conditions, $P_{R,0.5}$ and $P_{R,20}$ 0.5 and 20 μg of recombinant CrRpoD after refolding. **b** Comparison of sequences of *Chlamydomonas rrn16* and spinach *psbA* promoters with a typical eubacterial −10/-35 promoter. **c** Gel mobility shift assays with recombinant CrRpoD. CrRpoD was incubated with ^{32}P-labeled DNA fragments containing the spinach *psbA* promoter (*left panel*) or the *Chlamydomonas rrn16* promoter (*right panel*) in the absence or presence of the *E. coli* core RNA polymerase. Competition experiments contained a 100-fold excess of unlabeled promoter fragments. Labeled DNA alone or with core RNA polymerase alone were included as controls

might be a target for other accessory factors, akin to one recently shown to interact with region 4 in *Arabidopsis* (Morikawa et al. 2002). This region might also have a more traditional function of interacting with the core, which is encoded by unusually diverged genes in *Chlamydomonas* (Maul et al. 2002).

The structural conservation of the sigma-like properties, CrRpoD enrichment in the chloroplast fraction, and the demonstration of functionality in vitro with *E. coli* core RNAP strongly suggests that CrRpoD is the principal PEP transcription factor in the *Chlamydomonas* chloroplast. The nuclear genome of *Chlamydomonas* does not appear to encode any other SLFs. Screening by Blast of the mostly completed *Chlamydomonas* nuclear genome (http://www.shake.jgi-psf.org/Chlre3/Chlre3.-home.html) did yield one additional hit over a stretch of 234 nt with moderate similarity to region 2.1–2.4 of RpoD. The lack of sequence information in the vicinity of this hit does not allow any clear conclusion whether this sequence is indeed part of a sigma factor gene.

The apparent presence of only one sigma factor in *Chlamydomonas* is in striking contrast to the abundance of up to six SLFs in land plants. The emergence of small gene families of sigma-like proteins might be linked with the transition from the unicellular stage to multicellularity. The SLFs of land plants show developmental and tissue-specific regulation (Isono et al. 1997; Tozawa et al. 1998; Lahiri et al. 1999; Tan and Troxler 1999; Lahiri and Allison 2000), a possible basis for the requirement of several sigma-like transcription factors to fine-tune plastid gene expression. *Chlamydomonas* might lack these requirements, or achieve regulatory competence by other mechanisms, e.g., via accessory factors with no homology to the sigma family. Since *Chlamydomonas* chloroplast promoters are poorly characterized, it is not known if any possess non-canonical consensus sequences.

Chlamydomonas RpoD transcription exhibits a circadian regulation (Carter et al. 2004), but it remains to be determined if the protein level varies accordingly under these or other physiological and/or environmental conditions. When detected in cells grown in rich medium under continuous light, its 85 kDa molecular mass was slightly higher than the 80.2 kDa predicted from the cDNA sequence. The calculated molecular mass of heterologously expressed Trx–RpoD protein (87.6 kDa) also differed from the 97 kDa observed following SDS-polyacrylamide gel electrophoresis. Similar discrepancies have also been reported for some bacterial sigma factors (Burton et al. 1981; Gitt et al. 1985; Brahamsha and Haselkorn 1991), and have been attributed to a large number of both acidic and basic residues and a negative net charge of the proteins. These are also features of CrRpoD.

Although CrRpoD includes all domains conserved between sigma factors, evidence of its functionality had to be provided experimentally. Two essential functions are promoter recognition and interaction with the bacterial-like core RNAP. Since plant SLFs and the *E. coli* core polymerase have been successfully reconstituted (Kestermann et al. 1998; Hakimi et al. 2000; Beardslee et al. 2002; Homann and Link 2003), we chose this approach, and were able to show that recombinant CrRpoD is capable of conferring promoter recognition and specific transcription initiation in vitro (Figs. 2 and 3). These functions were demonstrated with a plant promoter (*psbA* from spinach) and an algal promoter

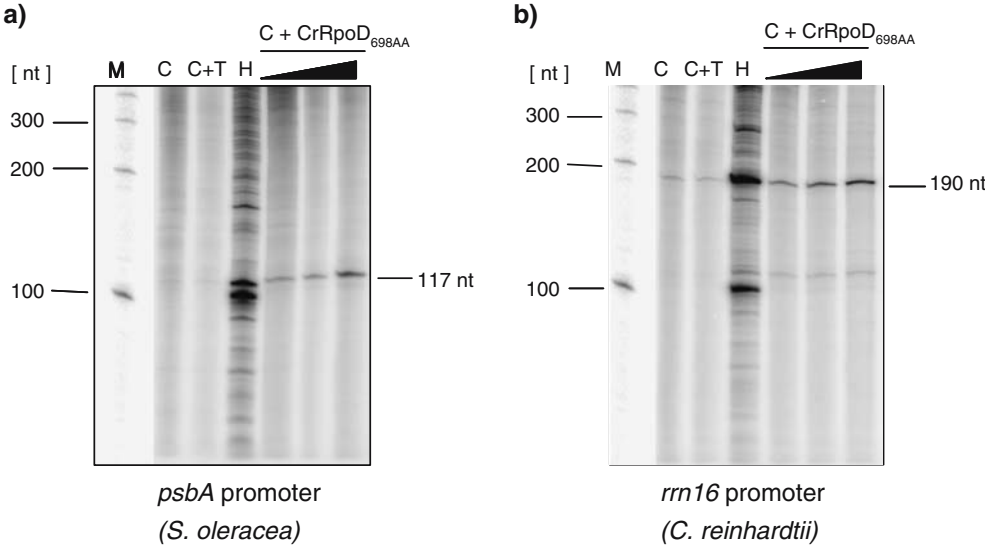

Fig. 3 Recombinant CrRpoD enhances transcription initiation in vitro. In vitro transcription assays were performed utilizing the spinach *psb*A promoter (**a**) or the *Chlamydomonas rrn16* promoter (**b**) as templates. Reactions were carried out with *E. coli* core polymerase (*C*); core plus the purified protein extracted from the pBAD/Thio-TOPO vector (*C* + *T*); the *E. coli* holoenzyme (*H*) or the *E. coli* core polymerase plus increasing amounts (25, 100, 200 ng) of recombinant CrRpoD (*C* + $RpoD_{698AA}$). The expected 117 and 190 nt products from the *psbA* and *rrn16* promoters, respectively, are marked at the *right*. RNA size markers are shown at the *left*

(*rrn16* from *Chlamydomonas*), both of which are strongly transcribed plastid promoters with a typical −10/−35 architecture. Although transcription activity was much lower than with the *E. coli* holoenzyme, this most likely reflects the very high purity of the commercial holoenzyme, and perhaps a negative effect of the thioredoxin moiety on the recombinant sigma factor, incomplete refolding, and/or a degree of incompatibility between the bacterial and eukaryotic proteins. Specific transcription in the heterologous system was, however, proportional to the amount of recombinant sigma factor added. Taken together, our results strongly support the idea that CrRpoD functions as the sole PEP specificity factor in the chloroplast.

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