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Characterization of two chloroplast RNA polymerase sigma factors from *Zea mays*: Photoregulation and differential expression

(maize/plastid/Sig1/Sig2/transcription)

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ABSTRACT **Two distinct cDNAs encoding putative σ factors of plastid RNA polymerase were isolated from *Zea mays*, a C4 plant. The deduced amino acid sequences of both cDNAs possess all four highly conserved domains proposed for recognition of −10 and −35 promoter elements, core complex binding, DNA binding, and melting. These two cDNAs are designated *sig1* and *sig2*. Phylogenetic analysis of available plastid σ factors indicated that they were probably the descendants of cyanobacterial principal σ factors. Southern blots probed with *sig1* and *sig2* revealed that both genes exist in the maize nuclear genome as single-copy genes, but low-stringency hybridization suggested the presence of a multigene family of maize plastid σ factors. Transcription of *sig1* and *sig2* is light inducible and tissue specific. Transcripts of *sig1* and *sig2* were abundant in greening leaf tissues; *sig2* (but not *sig1*) was barely detectable in etiolated leaves and neither was detectable in roots. Immunological studies using a peptide antibody against an epitope in subdomain 2.4 of Sig1 revealed 50-kDa and 60-kDa immunoreactive proteins in maize chloroplasts. Reduced levels of the 60-kDa immunoreactive protein were detected in etioplasts, and no immunoreactive proteins were observed in roots. Collectively, the data suggest that the nuclear genes, *sig1* and *sig2*, may play a role in differential expression of plastid genes during chloroplast biogenesis.**

Plastids of algae and higher plants are semiautonomous organelles that possess their own genetic information. Plastids contain circular, double-stranded DNA genomes that encode up to 120 genes for transcription, translation, and photosynthesis (1). Transcription of plastid genes depends on plastid-specific, DNA-dependent RNA polymerases (RNAPs). Two independent plastid RNAPs have been characterized, the bacterial-type multisubunit RNAP (2) and the bacteriophage-type single subunit RNAP (3–5). The holoenzyme of bacterial RNAP consists of a core complex ($\alpha_2\beta\beta'$) and a σ factor. The σ subunit confers the holoenzyme with the ability to recognize −35 and −10 promoter motifs, leading to accurate initiation of transcription (6).

The functional enzymes of bacterial-type RNAP have been isolated from plants, and genes encoding the core subunits have been identified in plastid genomes (2). However, complete sequences of the plastid genomes of several species did not reveal any ORF homologous to σ factors. Because −35 and −10 elements exist widely in plastid gene promoters, and immunological evidence indicates that chloroplast RNAPs contain polypeptides that crossreact with antibodies against a principal cyanobacterial σ factor, it seems likely that RNAP σ factors are encoded in the nuclear genomes of plants (7). Recently, nuclear genes encoding σ factors of plastid RNAP have been isolated from red algae (8, 9) and higher plants (10–12).

Induction of plastid gene transcription by a light signal is a complex process, probably involving numerous proteins in a cascade of reactions that are not well understood (13). Plastid σ factors may play a key role in the sequence of events at an early stage of chloroplast development. Characterization of σ factors from higher plants provides a tool to examine the mechanism of spatial and temporal control of plastid gene expression. In this study, we have chosen the species *Zea mays* (maize) for isolation and characterization of genes for plastid σ factors, as a step to understand the control of plastid gene expression mediated by σ factors. Of special interest in C4 plants such as maize is to examine the differential effects of σ factors in the development and functions of the two distinct photosynthetic tissues: mesophyll cells (MCs) and bundle sheath cells (BSCs). In this paper, we present the characterization of two cDNAs encoding maize plastid σ factors and their involvement in chloroplast development.

MATERIALS AND METHODS

Plant Materials. Maize seedlings (*Z. mays*, FR9 cms × FR37; Illinois Foundation Seeds, Champaign, IL) were germinated in vermiculite and grown in the dark for 7 days. Leaves and roots of etiolated seedlings were harvested under a green safe light, frozen immediately in liquid nitrogen, and stored at −80°C. For greening tissues, etiolated seedlings were illuminated for 20 hr before leaves and roots were harvested as above. For developmental experiments, etiolated maize seedlings were illuminated for different time periods before harvesting of leaves for RNA isolation. Etioplasts and chloroplasts were isolated as described (7).

Isolation of Nucleic Acids and Gel Blot Analyses. For isolation of total RNA from both etiolated and illuminated maize, leaf or root tissues (1 g) were first ground in liquid nitrogen and quickly resuspended in 5 ml of 4 M guanidinium isothiocyanate, 0.75 M sodium citrate (pH 7.0), and 0.1 M β-mercaptoethanol. The mixture then was homogenized in three 30-sec bursts by using a Polytron (Brinkmann). The homogenate was centrifuged at 8,000 × *g* for 10 min, and the supernatant (≈3.5 ml) was removed. After addition of 175 μl of 10% Sarkosyl and 350 μl of 3 M sodium acetate (pH 5.2), the mixture was extracted three times with 3.5 ml of water-

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Abbreviations: RACE, rapid amplification of cDNA ends; MC, mesophyll cell; BSC, bundle sheath cell; RNAP, RNA polymerase; RT, reverse transcriptase.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF058708 for *sig1* and AF058709 for *sig2*).

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saturated phenol and 0.7 ml of chloroform/isoamyl alcohol (49:1). The RNA in the final aqueous phase was precipitated with an equal volume of isopropanol, and the pellet was washed in 75% ethanol. The final RNA pellet was dissolved in 200 μl of 0.1% SDS and stored at −80°C. Total RNA from MCs and BSCs was prepared by the method of Sheen and Bogorad (14). Maize genomic DNA was isolated as described (43).

Southern blot analyses were performed basically as in Sambrook *et al.* (15). The probe for *sig1* was a DNA fragment of 427 bp (nucleotides 506–932 of *sig1*), and the probe for *sig2* was a DNA fragment of 381 bp (nucleotides 339–719 of *sig2*). Probes were labeled with a Prime-a-Gene Labeling System (Promega) and [α-^{32}P]dCTP (10 μCi/μl, 3,000 Ci/mmol; DuPont/NEN). Low-stringency washing was in 1× SSC, 0.1% SDS three times at 48°C, 15 min each time. After exposure to BIOMAX MS film (Kodak) at −80°C, the same membrane was washed under high stringency conditions (0.1× SSC, 0.1% SDS at 68°C for 30 min) and exposed again.

Aliquots (10 μg) of RNA were denatured at 65°C for 10 min and fractionated on a 1% agarose gel containing 2% formaldehyde (15). The RNA was transferred onto a Hybond-N$^+$ membrane and hybridized with the *sig1* and *sig2* probes as described above. Final wash conditions were 0.1× SSC, 0.1% SDS at 60°C for 3 × 20 min.

Library Construction, Screening, and cDNA Sequencing. A maize cDNA library was constructed in the λ ZAP II vector by using poly(A)$^+$ RNA from greening leaves primed with both random primers and oligo(dT) for the first-strand cDNA synthesis according to manufacturer's protocols (Stratagene). After the second-strand synthesis, cDNAs were ligated to *Eco*RI adaptors and cloned bidirectionally into the *Eco*RI site of λ ZAP II. The maize cDNA library was screened for potential sigma factors of chloroplast RNA polymerase as described by Sambrook *et al.* (15) with a heterologous probe (GenBank T88387) from the *Arabidopsis thaliana* expressed sequence tag database (16), which was kindly provided by the Arabidopsis Biological Resource Center (The Ohio State University, Columbus). The pBluescript plasmids were excised from the λ ZAP II of the putative clones by using the helper phage R408 (Stratagene). The cDNA inserts of two of the longest clones (representing *sig1* and *sig2*) were sequenced completely on both strands by using an automated sequencer (Applied Biosystems, model 373A, version 1.2.1). DNA sequence analysis was performed by using the Sequence Analysis Software Package, version 9.0, UNIX, of the University of Wisconsin Genetics Computer Group (GCG), Madison.

Rapid Amplification of cDNA Ends (RACE). RACE was performed by using the Marathon cDNA Amplification Kit (CLONTECH). First-strand cDNA was synthesized by priming total RNA with a poly(dT) primer using Moloney murine leukemia virus reverse transcriptase (RT). The primers used for *sig1* 3′-RACE were: first primer 5′-TCACTGGATCAACAGGCGTTCCCCT-3′ and nested primer 5′-ACGGCCTACCTGGGGATACACTCCA-3′. The primers used for *sig2* 3′-RACE were: first primer 5′-CGAGAGGTTGTTAGCAATTCGTGGTGC-3′ and nested primer 5′-GCAGAGTCGCTCAATATATCGGTA-3′. PCR products were cloned into pCR 2.1 vector with the TA Cloning Kit (Invitrogen) and sequenced as described above.

RT-PCR. Total RNA (2 μg) from 7-day-old etiolated leaves and roots, from leaves and roots after illumination for 20 hr, and from MCs and BSCs of illuminated leaves was reverse-transcribed by using Moloney murine leukemia virus RT following manufacturer's protocols (GIBCO/BRL). The gene-specific antisense primer used for *sig1* was 5′-AGCAGCCCTATCAGGCCACCCTGAA-3′ and for *sig2* was 5′-TACCGATATATTGAGCGACTCTGC-3′. As a positive control, the maize 18S rRNA gene was included (GenBank U42796), and the primer used was 5′-CCCGGCCCAAGGTCCAACTACGAGC-3′. The cDNAs then were amplified by PCR for 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The sense primer for PCR of *sig1* was 5′-TTCGAGTTGGAGTTCGAGTCCTCG-3′, and the antisense primer was 5′-GAGGTTGTCGTACTTCTGGGCAATG-3′ (PCR product 529 bp); the sense primer for *sig2* was 5′-TGATAACCTCGGTGTCGACTTGGC-3′, and the antisense primer was as above (PCR product 289 bp). The 18S rRNA sense primer was 5′-ACTGTGAAACTGCGAATGGCTCAT-3′, and antisense primer was as above (PCR product 574 bp).

Expression and Purification of Sig1 Protein. The region encoding the C-terminal 397 residues of *sig1* was amplified by PCR using primers with cloning adapters. The amplified DNA fragment was cloned into the *Eco*RI and *Hin*dIII sites of pET23b(+) expression vector (Novagen), which introduced a His tag at the C-terminal end of the recombinant protein. After sequence confirmation, the vector was used to transform an expression host BL21(DE3) pLysS, and the expression of Sig1 was induced by isopropyl β-D-thiogalactopyranoside for 3 hr. The expressed Sig1 recombinant protein was purified from the insoluble portion of the host cell extracts by column chromatography of His-Bind resin following manufacturer's protocol (Novagen).

Antiserum Preparation and Western Blot. A polypeptide with the sequence RQGVSRALADNSRSFRLPT corresponding to subdomain 2.4 of Sig1 was synthesized, and a polyclonal antiserum was prepared in New Zealand white rabbits (Research Genetics, Huntsville, AL). Rabbit polyclonal antibodies against the principal σ factor (SigA) of *Synechococcus* sp. PCC 7002 was kindly provided by D. A. Bryant (The Pennsylvania State University, University Park). Western blots of maize proteins from etioplasts, chloroplasts, and root tissues were probed with these antisera by using routine methods.

Phylogenetic Tree. For the construction of the phylogenetic tree, all the sequences first were aligned with the PILEUP program of the GCG software package as described above. After inspection of the alignment, a stretch of 110 amino acid residues, which is highly conserved and includes subdomains 2.1–2.4 (see Fig. 4), was chosen for calculation of the evolutionary distance with the PROTDIST program of the PHYLIP software package using the PAM matrix method (17). The distance data were used to construct the phylogenetic tree with the NEIGHBOR program in the same package by using the neighbor-joining method (18). Bootstrap analysis was performed to determine the supporting percentage of the branching pattern with SEQBOOT and CONSENSE programs in PHYLIP.

RESULTS

Cloning of *sig1* and *sig2* cDNAs. A search of GenBank with the rpoD box sequence, TYAMWWIRQA, of *Cyanidium caldarium rpoD1* (8) identified three *A. thaliana* expressed sequence tag (EST) entries (GenBank N65838, N97044, and T88387) with similarity to putative σ factors. One of the EST clones (T88387) was used as a DNA probe to screen the maize cDNA library as described above. From 300,000 plaque-forming units screened, 19 positive clones were obtained, and partial sequence analysis indicated that 16 clones contained portions of two different genes encoding potential σ factors. The longest clone for each potential σ factor was fully sequenced, and these were named *sig1* and *sig2*. Both cDNA clones encode partial sequences of the *sig1* and *sig2,* and sequences of the C-terminal region of both σ factors were determined by 3′ RACE.

Relatedness of Maize Sig1 and Sig2 with Other σ Factors. The deduced amino acid sequences of conserved regions in Sig1 and Sig2 of maize could be aligned with six other σ factors from rice, sorghum, mustard, and *A. thaliana* (Fig. 1). The

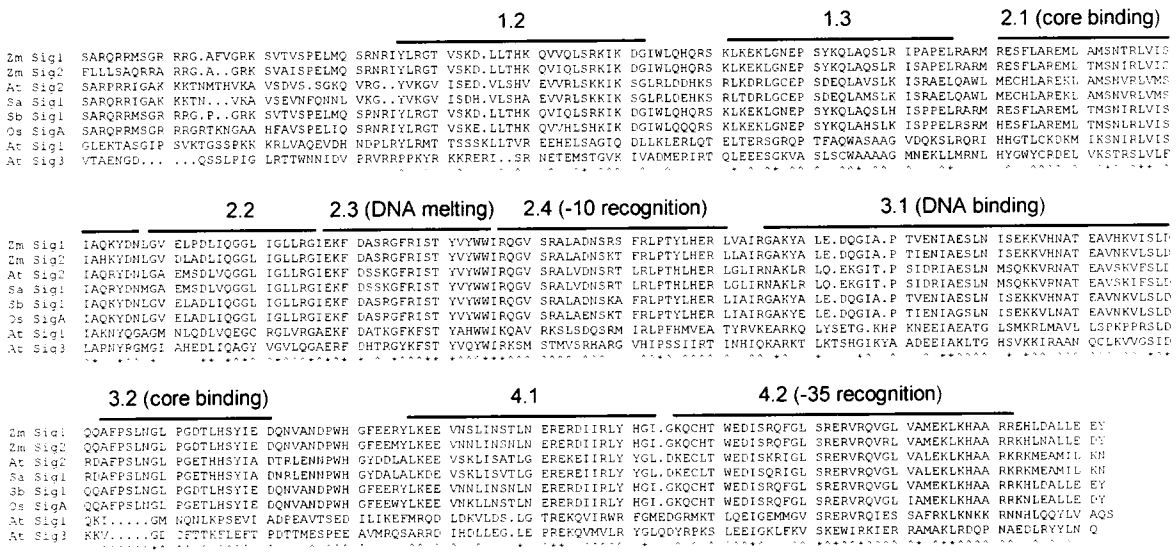

FIG. 1. Alignment of the deduced amino acid sequences of the genes encoding σ factors from different organisms. Only the conserved C-terminal two-thirds of all sequences are shown in this alignment. The conserved subdomains are marked above the sequences and their postulated functions are indicated. Residues marked with a star indicate that identical residues occur at the same position in all eight aligned sequences, and the symbol ^ indicates that six or seven of the eight aligned sequences have the same residue at the position. The dots within the sequences indicate gaps introduced for the best alignment. Zm Sig1 and Zm Sig2, gene products of *sig1* and *sig2* of *Zea mays* (AF058708; AF058709); Sa Sig1, gene product of *sig1* of *Sinapis alba* (Y15899); Sb Sig1, gene product of *sig1* of *Sorghum bicolor* (Y14276); Os SigA, gene product of *sigA* of *Oryza sativa* (AB005290) (12); At Sig1, At Sig2 and At Sig3, gene products of *sig1*, *sig2*, and *sig3* of *A. thaliana* (AB004820, AB004821, AB004822, respectively) (10, 11).

maize σ factors contained all highly conserved subdomains thought to be involved in recognition of -10 and -35 promoter elements (domain 2.4 and 4.2), in core complex binding (2.1 and 3.2) and in DNA binding and melting (3.1 and 2.3), as defined for eubacterial and cyanobacterial σ factors (19, 20). An additional conserved stretch between subdomains 1.2 and 2.1 was found in this alignment, which was marked subregion 1.3. This alignment strongly supports the proposal that *sig1* and *sig2* genes of maize encode σ factors of chloroplast RNAP.

Inspection of the sequence alignment also reveals that Sig1 and Sig3 of *A. thaliana* (bottom two σ factors) are not aligned as well as other six σ factors (Fig. 1). Analysis by the GAP program of GCG also indicates that the four σ factors of monocotyledonous plants (maize, rice, and sorghum) share a sequence similarity above 93%. To further examine the evolutionary relationships among various σ factors, a phylogenetic tree was constructed with eight σ factors from higher plants, three from red algae, and five from cyanobacteria (Fig. 2). As predicted from sequence alignment in Fig. 1, the four σ factors from monocotyledonous plants, *A. thaliana* Sig2 and *Sinapis alba* Sig1 are clustered together, whereas the other five plastid σ factors are variously branched. It is noteworthy that all of the plastid σ factors are clustered with the principal (group 1; ref. 21) σ factors in cyanobacteria (An SigA and 7002 SigA), and this branching pattern is strongly supported by bootstrap analysis data (99%). By contrast, the other three group 2 σ factors (21) in cyanobacteria are isolated from all of the plastid σ factors in the phylogenetic tree.

Analysis of Southern Blots. Southern blots of maize genomic DNA were probed with maize *sig1* and *sig2* cDNAs under high stringency conditions to determine the gene copy number in the maize nuclear genome (Fig. 3, *Left*). The same blots also were probed under low stringency conditions to examine the possible existence of a gene family for σ factors (Fig. 3, *Right*). Hybridization under high stringency revealed only one strong band for both *sig1* and *sig2*, indicating that they exist in the maize nuclear genome as single-copy genes. The double bands in lane 4 of the *sig1* blot are caused by an *Xho*I site in the *sig1* coding sequence, and the double bands in lane 2 of *sig2* probably are caused by a *Hin*dIII site in an intron of the *sig2* gene (data not shown). Similar patterns of multiple bands were identified for both *sig1* and *sig2* after hybridization

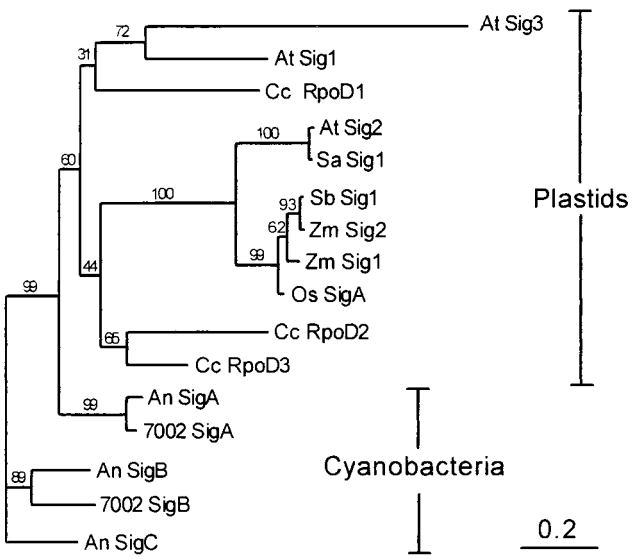

FIG. 2. Phylogenetic analysis of five σ factors from cyanobacteria, three from red algae, four from monocotyledonous plants, and four from dicotyledonous plants. All 16 sequences were aligned by using the PILEUP program of GCG package. Only the highly conserved 110 amino acid residues (containing subdomains 2.1–2.4 as shown in Fig. 1) were chosen for the calculation of the evolutionary distances by the PROTDIST program of the PHYLIP package by using the PAM matrix method. The unrooted tree was constructed from the distance data by the NEIGHBOR program in the same package by using the neighbor-joining method. Bootstrap analysis was performed by resampling the data set 100 times, and the number at each node of the tree indicates the supporting percentage of the branching pattern. The scale bar represents 20% substitution, which is proportional to horizontal distance of the tree branches. The higher plant sequences are the same as in Fig 1. Cc RpoD1, Cc RpoD2 and Cc RpoD3, σ factors of the red alga *C. caldarium* (L42639; AF050634; D83179). An SigA, An SigB, and An SigC, σ factors of *Anabaena* sp. PCC 7120 (M60046; M95760; M95759) (25, 41). 7002 SigA and 7002 SigB, σ factors of *Synechococcus* sp. PCC 7002 (U15574; U82435) (21, 42).

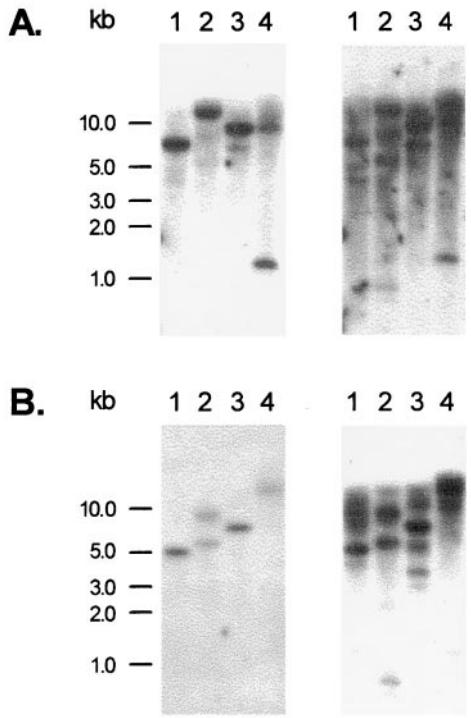

FIG. 3. Southern blot analysis of maize genomic DNA digested with various restriction enzymes and hybridized to maize *sig1* (*A*) and *sig2* (*B*) gene probes under both high stringency (*Left*) and low-stringency (*Right*) conditions. The sizes of DNA markers are shown at the left of the blots, and the restriction enzymes used are as follows: lane 1, *Eco*RI; lane 2, *Hin*dIII; lane 3, *Xba*I; and lane 4, *Xho*I.

under low-stringency conditions, suggesting the existence of a multigene family of σ factors in the maize nuclear genome.

Differential Expression of *sig1* and *sig2* Genes. To study the involvement of Sig1 and Sig2 σ factors in the biogenesis of chloroplasts, transcript levels in total RNA from leaves and roots of etiolated and greening seedlings were examined by Northern blotting (Fig. 4*A*). Transcripts of *sig1* and *sig2* (each approximately 2.0 kb) were abundant in greening leaf tissues, whereas *sig2* (but not *sig1*) was also detectable, but in greatly reduced amounts, in etiolated leaves. No *sig1* and *sig2* transcripts were detectable in roots from either etiolated or illuminated seedlings. Similar results were obtained by RT-PCR, a more sensitive method for detecting transcripts in total RNA (Fig. 4*B*).

As a C4 plant, maize has two unique functional tissues involved in photosynthesis: MCs and BSCs (23, 24). To examine the possible involvement of Sig1 and Sig2 σ factors in the regulation of transcription in MC and BSC chloroplasts, Northern blots and RT-PCR were performed with total RNA from these tissues isolated from 9-day-old etiolated seedlings that had been illuminated for 20 hr (Fig. 4). Both *sig1* and *sig2* showed a similar pattern of transcript accumulation in MCs and BSCs although it is possible that Sig1 and Sig2 are involved in transcription of distinct sets of photosynthetic genes. Further experiments are necessary to provide direct evidence for the differential promoter recognition of σ factors in the regulation of plastid gene transcription.

Another Northern blotting experiment was carried out to examine transcript levels of *sig1* and *sig2* in leaves of etiolated seedlings after exposure to light (Fig. 5). Transcripts of both *sig1* and *sig2* were not detectable in etiolated leaves, were visible after illumination for 3 hr, and reached maximal levels after illumination for 6 hr and declined thereafter. In Figs. 4 and 5, *sig1* transcripts appeared as a double band, possibly because of posttranscriptional processing of mRNA. A similar

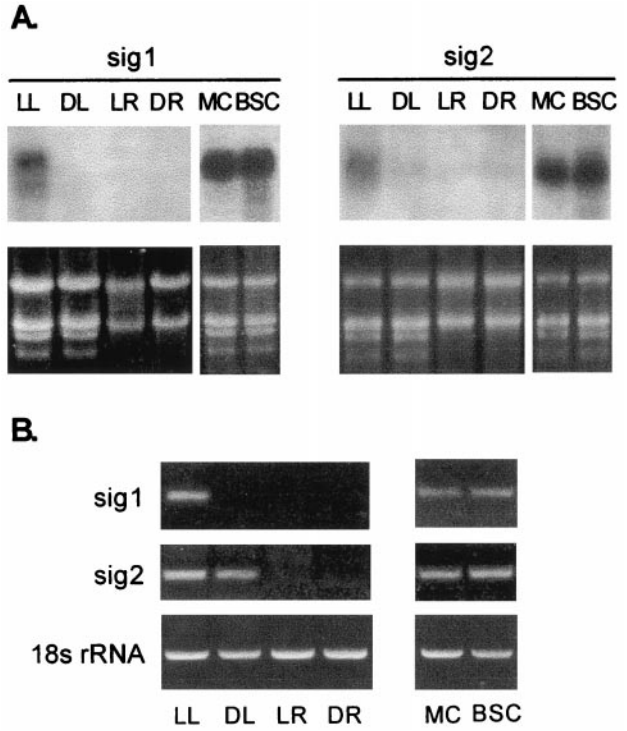

FIG. 4. (*A*) Northern blot analysis of *sig1* and *sig2* transcript levels in total RNA (10 μg/lane) isolated from different tissues of etiolated and illuminated maize seedlings. RNA samples were from leaves (LL) and roots (LR) of illuminated seedlings (20 hr under light), leaves (DL), and roots (DR) of etiolated seedlings, and MCs and BSCs of illuminated seedlings (20 hr under light). Essentially equal loading of RNA was shown by ethidium bromide staining of RNA gels. (*B*) Further assessment of *sig1* and *sig2* transcripts in total RNAs from *A* by RT-PCR. RNA was reverse-transcribed by using Moloney murine leukemia virus RT with *sig1* and *sig2* gene-specific primers, and PCR was performed to amplify the cDNA. RT-PCR also was performed for the 18S rRNA gene as a positive control. The definitions of the six different tissues are the same as in *A*.

banding pattern also has been observed during Northern analyses of *sigC* from *Anabaena* sp. PCC 7120 (25).

Identification of σ Factors in Plastids. To investigate differential expression of σ factor proteins in maize tissues and

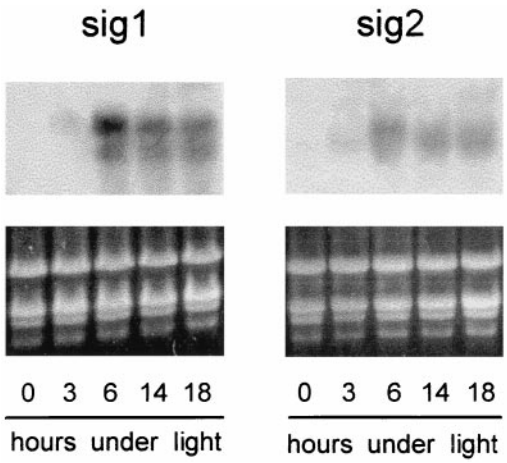

FIG. 5. Northern blot analysis of light induction of *sig1* and *sig2* genes in maize. Etiolated maize seedlings were transferred to light, and leaves were harvested at various time points after the transfer. Total RNA was isolated from all of the leaf samples, and Northern blot analysis was performed with *sig1* and *sig2* gene probes. The ethidium bromide-stained rRNAs show essentially equal loading of RNA samples (10 μg) in each lane.

plastids, a polyclonal antiserum against conserved subdomain 2.4 (−10 promoter recognition motif) of maize Sig1 was prepared by using a synthetic peptide. In addition, Sig1 was overexpressed in *Escherichia coli* to determine the specificity of the peptide antibody. The expression studies are summarized in Fig. 6*A* and show that recombinant Sig1 was induced by isopropyl β-D-thiogalactopyranoside (lane 1 vs. 2), was not in the soluble phase (lane 3) but occurred predominantly in inclusion bodies (lane 4), and was affinity purified to near homogeneity on His-Bind resin (lane 5). SDS/PAGE showed that recombinant Sig1 had an apparent molecular mass of 50 kDa, and the identity of recombinant Sig1 was confirmed by N-terminal sequencing (data not shown).

A Western blot of total protein from roots of etiolated and illuminated seedlings probed with maize anti-Sig1 antibodies did not reveal immunoreactive proteins (Fig. 6*B*, lanes 1 and 2). In contrast, a Western blot of total protein from isolated etioplasts and chloroplasts contained a 60-kDa anti-Sig1 immunoreactive protein (Fig. 6*B*, lanes 3 and 4). Chloroplasts also contained a 50-kDa immunoreactive protein not seen in etioplasts. Maize recombinant Sig1 crossreacted strongly with both maize anti-Sig1 peptide antibodies (Fig. 6*B*, lane 5) and cyanobacterial anti-SigA antibodies (Fig. 6*B*, lane 6). In addition, the cyanobacterial anti-SigA antibodies also detected the 50-kDa and 60-kDa proteins in chloroplasts (data not shown). These results strongly suggest that the 60-kDa protein in etioplasts, and the 50-and 60-kDa proteins in chloroplasts, are indeed σ factors, and the absence of these proteins in roots strongly points to their role in regulation of chloroplast gene expression.

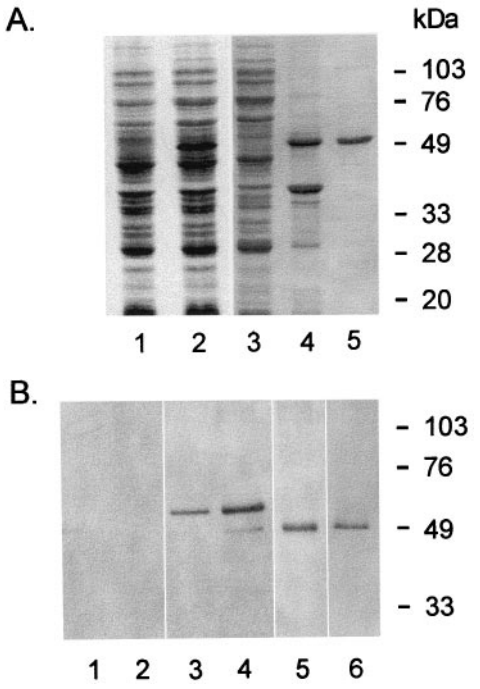

FIG. 6. (*A*) Expression and purification of recombinant Sig1 protein. Total protein was extracted from uninduced cells (lane 1) and the induced cells by isopropyl β-D-thiogalactopyranoside (lane 2). In cell lysates, recombinant Sig1 was barely detectable in the soluble fraction (lane 3) and enriched in inclusion bodies in the cell pellet (lane 4). Inclusion body proteins were solubilized in 6 M urea, and recombinant Sig1 was affinity-purified on a His-Bind column (lane 5). Samples were subjected to SDS/PAGE on 10% polyacrylamide gels, and gels were stained with Coomassie brilliant blue. (*B*) Western blot of total protein in root tissue, plastid extracts, and recombinant maize Sig1 probed with antibodies against maize Sig1 (lanes 1–5) or against the principal σ factor from *Synechococcus* sp. PCC 7002 (lane 6). Protein extract of roots from etiolated (lane 1) and illuminated (lane 2) seedlings (10 μg protein/lane); protein extract of etioplasts (lane 3) and chloroplasts (lane 4) (5 μg protein/lane); recombinant maize Sig1 probed with maize anti-Sig1 antibodies (lane 5) and cyanobacterial anti-SigA antibodies (lane 6) (0.2 μg/lane).

DISCUSSION

By screening a maize cDNA library with a heterologous σ factor cDNA probe, we have isolated two distinct cDNAs encoding putative σ factors of maize chloroplast RNAP. Evidence was first presented that σ factors were components of chloroplast RNAP (7), and this report describes nuclear genes encoding σ factor-like proteins from a C4 plant. Sequence alignment with σ factors from other species shows that the maize σ factors contain all highly conserved regions proposed for the various functions in eubacterial σ factors (Fig. 1). We therefore propose that these two cDNAs, *sig1* and *sig2,* encode σ factors of maize chloroplast RNAP.

It has long been hypothesized that modern chloroplasts arose by an endosymbiotic association of a eukaryotic organism and a photosynthetic prokaryote, presumably a cyanobacterium (26–28). Five genes encoding cyanobacterial σ factors have been identified from *Synechococcus* sp. PCC 7002 (21) and *Synechocystis* sp. PCC 6803 (29). It also has been speculated that σ factors of plastid RNAP were encoded by a multigene family (8). There are two possibilities for the origin of plastid σ factors based on the endosymbiotic theory. The cyanobacterial σ factor genes all could have migrated to the nuclear genome of a eukaryotic host and independently diversified into modern day σ factors. Alternatively, only one of the cyanobacterial (perhaps the principal) σ factor genes could have been transferred to nuclear genome followed by gene duplication and diversification of this gene while the σ factor genes remaining in the organellar genome were lost (30). To shed light on the evolutionary relationships of σ factors from plastids and cyanobacteria, a phylogenetic tree was constructed with 16 σ factors from higher plants, red algae, and cyanobacteria (Fig. 2). The results support the latter possibility. Only the two principal σ factors of cyanobacteria are tightly clustered (with a bootstrap value of 99%) with all of the plastid σ factors, no matter how diversified they are among themselves. This evolutionary relationship between plastid and cyanobacterial σ factors was initially identified from phylogenetic analysis although further clarification awaits characterization of more σ factors from photosynthetic eukaryotes.

Regulation of chloroplast gene expression is a complex process in which transcription of distinct sets of genes is under precise control of environmental, spatial, and temporal cues (13, 31–34). To cope with such a complicated situation, plastid σ factors must be functionally quite diversified to be involved in initiation of chloroplast gene transcription. One mechanism could be to covalently modify (e.g., phosphorylation/dephosphorylation) existing σ factor proteins (22, 35), and another could be to use a family of σ factor genes that are expressed at different developmental stages and in different tissues (such as MCs and BSCs of maize). Evidence presented in this study (Fig. 3) suggests that a multigene family for σ factors may exist in the maize nuclear genome, consistent with the latter possibility.

Transcripts of chloroplast genes have been shown to increase significantly in a variety of plant species after reception of light signals (36–39). Light induction of maize *sig1*, and partially *sig2*, transcripts in etiolated leaves suggests the possible involvement of σ factors in light-dependent increases of plastid gene transcription. Furthermore, this study shows that transcripts of maize σ factor genes reach maximal abundance relatively early in chloroplast development before induction of plastid genes for photosynthesis (37, 40). This finding is consistent with the assumption that in response to a light signal, nuclear encoded σ factor proteins are expressed and transported into chloroplasts, leading to initiation of transcrip-

tion of photosynthetic genes. In this regard, differential expression of *sig1* and *sig2* in maize occurred in a tissue-specific manner (Fig. 4 *A* and *B*). Sig1 and Sig2 transcripts were abundant in greening leaves and undetectable in roots, consistent with a major function of σ factors in chloroplast development. However, the presence of *sig2* transcripts in etiolated leaves suggests that at least one of the σ factors may be involved in transcription of housekeeping genes in etioplasts. This finding would appear to contradict a previous proposal (13) that only the bacteriophage-type RNAP functions in etioplasts before initiation of chloroplast development in light. Alternatively, *sig2* expression may occur in a cell-specific manner and be involved in either MC or BSC plastid development.

Immunological identification of anti-Sig1 immunoreactive proteins correlates with the pattern of transcript accumulation observed on Northern blots. Two immunoreactive proteins, 50 kDa and 60 kDa, were detected in maize chloroplasts but were not found in root tissues. The 50-kDa protein occurred only in chloroplasts, suggesting that it may be involved in expression of plastid genes for photosynthesis. The 60-kDa protein was observed in both etioplasts and chloroplasts and likely represents Sig2 protein because *sig1* transcripts were undetectable in the dark. Silver-stained gels of highly purified maize chloroplast RNAP contain several bands in the 60- to 65-kDa region (44). One of these bands could be the 60-kDa protein that crossreacted with our maize anti-Sig1 peptide antibodies and with the *Synechococcus* sp. PCC 7002 anti-SigA antibodies. Further investigation is needed to definitively correlate the *sig1* and *sig2* gene products with anti-Sig1 immunoreactive proteins in chloroplasts. It is also possible that the 60-kDa "band" detected with this antibody contains more than one protein. Cloning of maize *sig1* and *sig2* genes has provided opportunities for investigating regulation of chloroplast gene expression and the role of σ factors in control of chloroplast development.

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