Mutagenesis of a light-regulated psbA intron reveals the importance of efficient splicing for photosynthetic growth

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ABSTRACT

The chloroplast-encoded psbA gene encodes the D1 polypeptide of the photosystem II reaction center, which is synthesized at high rates in the light. In Chlamydomonas reinhardtii, the psbA gene contains four self-splicing group I introns whose rates of splicing in vivo are increased at least 6–10-fold by light. However, because psbA is an abundant mRNA, and some chloroplast mRNAs appear to be in great excess of what is needed to sustain translation rates, the developmental significance of light-promoted splicing has not been clear. To address this and other questions, potentially destabilizing substitutions were made in several predicted helices of the fourth psbA intron, Cr.psbA4, and their effects on in vitro and in vivo splicing assessed. Two-nucleotide substitutions in P4 and P7 were necessary to substantially reduce splicing of this intron in vivo, although most mutations reduced self-splicing in vitro. The P7-4,5 mutant, whose splicing was completely blocked, showed no photoautotrophic growth and synthesis of a truncated D1 (exons 1–4) polypeptide from the unspliced mRNA. Most informative was the P4-3,4 mutant, which exhibited a 45% reduction in spliced psbA mRNA, a 28% reduction in synthesis of full-length D1, and an 18% reduction in photoautotrophic growth. These results indicate that psbA mRNA is not in great excess, and that highly efficient splicing of psbA introns, which is afforded by light conditions, is necessary for optimal photosynthetic growth.

INTRODUCTION

The psbA gene encodes a critical and highly conserved component of the photosystem II reaction center, polypeptide D1. This ~36 kDa polypeptide is believed to span the thylakoid membrane five times, and to bind quinone b, photosynthetic pigments and possibly metals (reviewed in 1). D1 is synthesized on thylakoid-bound ribosomes at high rates in the light (2–4), and its synthesis increases at higher light fluxes (5). The accelerated production of D1 during the daytime is part of the daily production of thylakoid components (6), and references therein], and also serves to replace damaged D1. At very high, photoinhibitory light fluxes, the rate of D1 damage exceeds the rate of removal of damaged D1, with concomitant loss of photosynthetic capacity (7).

Numerous studies have revealed the dynamic regulation of psbA gene expression that occurs in response to light (early work was reviewed in 8). Light stimulation of psbA gene expression occurs at the transcriptional level [e.g. (9–11)], and at the levels of RNA splicing (9) and translation [e.g. (4,13–15)]. Hence, in addition to its key role in photosynthesis, psbA has been an important model for understanding gene regulation in response to light.

The unicellular chlorophyte, Chlamydomonas reinhardtii, is an excellent organism for studying organelle biogenesis and regulation, since genetic, molecular and cellular approaches can be employed (16). Moreover, since photosynthesis is dispensable, and chloroplast transformation occurs exclusively by homologous recombination, chloroplast-encoded genes for photosynthesis can be manipulated, and phenotypic effects observed without interference from wild-type gene copies (17).

The psbA gene in C.reinhardtii contains four large group I introns (Fig. 1), Cr.psbA1–Cr.psbA4, that can self-splice in vitro but probably require trans-acting factors for efficient splicing in vivo (9,18,19). Interestingly, in cells growing photoautotrophically under light–dark cycles, the in vivo splicing rate of all four of the introns is very slow in the dark period, but increases 6–10-fold within 30 min of light administration via a process that requires photosynthetic electron transport (9). It was suggested that the role of light stimulation of splicing, which does not happen for the chloroplast 23S rRNA intron (Cr.LSU), is to ensure an adequate supply of spliced psbA mRNA for the high rates of translation in the light (9). However, there has been no direct evidence to support this hypothesis. The question of the developmental significance of light-promoted psbA splicing is underscored by the fact that psbA mRNA is highly abundant (3,20), and by evidence suggesting that some chloroplast mRNAs, including psbA, may be in considerable (up to 10-fold) excess of what is needed for translation (21).

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In order to address the question of whether highly efficient splicing of psbA introns is important for photoautotrophic growth, we systematically substituted nucleotides in core helices of the Cr.psbaA4 intron, and analyzed the effects of these potentially destabilizing mutations on splicing in vivo and in vitro. This intron was selected because it can be replaced in vivo with a version that has most of the free-standing open reading frame deleted, yet splices similarly to the wild-type intron (22; O.W. Odom and D.L. Herrin, unpublished results). The ORF-deleted version, which is ~500 bp shorter than the wild-type intron, facilitates the identification of homoplasmic transformants.

Most mutagenesis studies of group I introns have examined the effects of mutations on self-splicing in vitro, but only a few reports have looked at corresponding in vivo effects in the normal host organism (23–25). Those reports concerned the plage T4 thymidylate synthase intron (T4.id), and the conclusion was that the effects of the mutations in vitro and in Escherichia coli were quite similar. In a recent study of the Cr.LSU intron in C.reinhardtii, most of the nucleotide substitutions could not be effectively analyzed in vivo because of the persistent heteroplasmicity of the transformants, due apparently to the lethality of the mutations to a homoplasmic cell (19). However, since psbA is not essential in C.reinhardtii, it was possible to analyze even a very severe mutant of Cr.psbaA4 in vivo as well as in vitro.

MATERIALS AND METHODS

Culture conditions

The wild-type 2137 mt+ strain of C.reinhardtii (CC-1021, Chlamydomonas Genetics Center, Duke University), and the derived transformants were grown either mixotrophically (light + acetate) in Tris-acetate–phosphate (TAP) medium (26), or autotrophically in TAP-minimal medium (the pH was adjusted to 6.8 with HCl instead of HOAc) at 23°C; liquid cultures were shaken at 125–150 r.p.m. Spectinomycin was added to the media (100 µg/ml) for selection and growth of spectinomycin-resistant transformants. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to plates of minimal medium at a final concentration of 3 µM. The light intensity was 40–60 µmol/m²/s for bright light and 1–2 µmol/m²/s for dim light. For the growth rate experiments, liquid cultures of TAP-minimal media were gently bubbled with 5% CO₂. Cultures used for molecular analyses were always in the exponential phase of growth (0.3–3 × 10⁶ cells/ml), and cell number was determined with a hemocytometer. Stock cultures were maintained on TAP-agar (2%) plates containing 100 µg/ml ampicillin.

Generation of the intron mutations and chloroplast transformation plasmids

For mutating Cr.psbaA4, the starting plasmid was pBX4.Δ, which contains a modified BstEII–XbaI fragment of the psbA gene (Fig. 1) in pBluescript SK (22). The BstEII–XbaI insert contained 50 bp of exon 4, a 1278 bp Cr.psbaA4 intron lacking 544 bp of the ORF (22), and 263 bp of exon 5. Exon 5 also contained the DCMU4 mutation (a T6G change of nucleotide 34 of the exon), which provided resistance to DCMU (27). The nucleotide substitutions were made using the Gene Editor in vitro site-directed mutagenesis system (Promega Inc.) with the following mutagenic deoxyoligonucleotides (the substitutions are underlined): 255, 5’-GCTTCGCTCTGACATTTGTGCCT-3’ (P4-2); 256, 5’-GCCATCACCCTTCTGTCCTGCTG-3’ (P6a-2); 257, 5’-CTCAAGCTCCTCTTCTGACCTT-3’ (P7-4); 258, 5’-CTATATTTTGTGTACACCTCCT-3’ (P11-2); 259, 5’-CTTAGATGGCCTGGCCTAGTGA-3’ (P13-3); 292, 5’-GGGACTCAAGCTCCTAGCTTCG-3’ (P4-2); 294, 5’-CATCCCTATAGCCTCCCTG-3’ (P6-2); and 299, 5’-TCCCTAGCTTCCCTGCCTG-3’ (P4-3). The mutations were confirmed by sequencing the plasmid DNAs with oligodeoxynucleotides 99 and 100 [22; see also below].

For transformation and replacement of the intron in a wild-type strain, the upstream 2.1-kb EcoO109I–BstEII fragment of psbA was added to the pBX4.Δ-based plasmids (Fig. 1), by digesting them with EcoRI and DraI (which is an isoschizomer of EcoO109I and cuts in the adjacent vector sequence), purifying the large fragment by gel electrophoresis, and ligating in the DraI(EcoO109I)–EcoRI fragment of pER3. Plasmid pER3 (obtained from O.W. Odom) was derived from pEC23 (2) and contains the EcoO109I–EcoRI fragment of the psbA gene (Fig. 1). This upstream sequence was added because it greatly increased the frequency of transformants that had integrated the intron. The new plasmids were named the pEX4.Δ series (Fig. 1).
In vitro synthesis of pre-RNAs and self-splicing

The pBX4.Δ-based plasmids were linearized with NotI, deproteinized, and resuspended in H2O (1 mg/ml). The DNAs (50 μg/ml) were transcribed (10 μl volume) with T7 RNA polymerase (1500 U/ml) in 1 mM NTPs (ATP, UTP, CTP, and GTP), 1 μCi/μl of [α-32P]GTP (~3000 Ci/mmol, ICN), 6 mM MgCl2, 2 mM spermidine, 10 mM DTT, 40 mM Tris–HCl pH 8 at 37°C for 1 h. The reactions were stopped with EDTA (12 mM), extracted with chloroform, and precipitated with NH4OAc–ethanol after adding 10 μg yeast tRNA as carrier. The RNA was re-precipitated twice more with ethanol–300 mM NaOAc pH 5.2, and finally dissolved in H2O.

The standard self-splicing reactions (5 μl) contained 32P-labeled RNA (6 nM), 50 mM Tris–HCl pH 7.5, 1 mM GTP, 15 mM MgCl2, 100 mM KOAc, and were performed at 45°C (unless stated otherwise). With these conditions, the pre-RNA folds during the splicing reaction. The reactions were terminated by adding an equal volume of 90% formamide, 50 mM EDTA pH 8.0, 0.03% bromophenol blue, 0.03% xylene cyanol, heating to 65°C for 3 min and quick-cooling on ice. The RNAs were analyzed by electrophoresis on 4% polyacrylamide–8 M urea gels at 45°C; equal amounts of radioactive RNA were loaded in each lane. The gels were fixed, transferred to 3MM paper, dried and exposed to X-ray film (BioMax MS, Kodak). The films were scanned and the images were quantified using NIH IMAGE (version 1.5.2). The linearity of detection was verified by electrophoresis a dilution series of unspliced RNA and quantifying the pre-RNA band.

Chloroplast transformation and PCR analysis

Chloroplast transformation was by particle bombardment of cells in soft agar (28) with a helium-driven apparatus (Bio-Rad He 1000) as described previously (22). The pEX4.Δ-based plasmids and the parental plasmid pBX4.Δ were co-introduced along with plasmid pb4C110 (29), which contains the spectinomycin resistance marker, spr-u–16–2, in the 16S rRNA gene (30). After an overnight incubation in dim light, the cell layer was scraped off and re-spread onto plates with 100 μg/ml spectinomycin and incubated in dim light at 23°C. Spectinomycin-resistant transformants appeared within 2 weeks.

Transformants for PCR analysis were grown on selective plates and total DNA prepared as described (31). Standard protocols with Taq polymerase were used with the following primers: either no. 99, a forward primer that annealed to nucleotides 182–205 of exon 4 or no. 176, a forward primer that annealed to nucleotides 114–140 of exon 3, and no. 100, a reverse primer that annealed to nucleotides 78–53 (5′-3′) of exon 5 (22). The temperature regimen was as follows: 94°C for 4 min; 24 cycles of 60°C (1 min), 70°C (5 min) and 94°C (30 s); then 60°C for 1 min; and 70°C for 8 min. The DNA products were analyzed by electrophoresis through 1% agarose gels containing ethidium bromide. When desired, the DNA products were purified from the gel by capturing them onto 3MM paper (Whatman) supported by a dialysis membrane, eluted with 10 mM Tris–HCl pH 8.0, 1 mM EDTA and subjected to automated sequencing using the PCR primers. The homoplasmic transformants obtained with each bombarded plasmid (at least two for each plasmid) showed indistinguishable growth on minimal medium, except for the P7-4.5 mutant, which had to be grown on acetate.

RNA gel-blot hybridizations

The isolation of total RNA, agarose gel electrophoresis, capillary blotting to a nylon membrane and hybridizations were performed as described previously (32). The membrane-bound RNA was stained with Methylene Blue prior to hybridization (33) to check for equal loading. The DNA hybridization probes were a 1.5-kb PCR product amplified from the Cr.psbaA intron in plasmid pBX4 (34) using primers 102 and 103 (9), and plasmid pBA153, which contains an intronless psba gene (35). The DNA probes were labeled by random priming (36) to 0.25–1 × 109 d.p.m./μg with [α-32P]dCTP (~3000 Ci/mmol, ICN). After hybridization at 65°C with the Church–Gilbert solution (37), the blots were washed in 0.1× SSPE, 0.1% SDS (1× SSPE = 0.15 M NaCl, 50 mM NaH2PO4 pH 7.4, 5 mM EDTA) at 65°C, and then exposed to X-ray film (Kodak BioMax MS) at ~70°C with an intensifying screen. For the quantification of autoradiographs, multiple exposures of the blots were made to achieve signals within the linear response range of the X-ray film. The developed films were scanned and quantified as described above for in vitro synthesized RNAs. In some experiments, the blots were quantified with a PhosphorImage and ImageQuant software (Molecular Dynamics).

Pulse-labeling and protein analysis

For in vivo pulse-labeling with [14C]acetate, 5 × 106 cells were centrifuged at 8000 g for 5 min, washed with minimal medium and resuspended in 250 μl of minimal medium. The tubes were shaken at 125 r.p.m. under dim light (~2 μmol/m2/s), and labeling was initiated by adding cycloheximide to 10 μg/ml followed immediately by 12.5 μCi of [1-14C]acetate (60 μCi/mm, ICN). After either 5 or 30 min at 23°C, the tubes were placed on ice, centrifuged at 16 000 g for 2 min (4°C), and resuspended in ice-cold 100 mM Tris–HCl pH 8.6, 100 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride. The cells were then either frozen at –70°C, or solubilized immediately for gel electrophoresis. Radioactivity in total protein was determined by hot trichloroacetic acid precipitation (38).

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the buffers described by Laemmli (39) and an acrylamide/bisacrylamide ratio of 30:0.8. The stacking gel contained 6% acrylamide and the resolving gels were linear gradients of 7.5–15% acrylamide. Samples were prepared for electrophoresis by incubation at 60°C (10 min) in 2.5% lithium dodecylsulfate, 12% sucrose, 0.01% bromophenol blue, 60 mM Tris–HCl pH 8.6, 60 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride. After electrophoresis at 4°C, the gels were fixed and stained with Coomassie Blue to check the protein loads. They were then impregnated with Fluororhance (Research Products International), dried and exposed to X-ray film (BioMax MR, Kodak) at room temperature or ~70°C. The fluorographs were quantified as described above for in vitro synthesized RNAs.
Other

Plasmid DNAs were isolated from E.coli cultures using standard techniques. Plasmids and PCR products were sequenced at the University of Texas at Austin DNA Analysis Center using cycle sequencing, fluorescent dideoxy-nucleotides and automated gel or capillary electrophoresis-laser systems (ABI).

RESULTS

Requirements for efficient self-splicing of Cr.psbA4

Cr.psbA4 was previously shown to self-splice in vitro using a set of conditions that had worked for other group I introns (25 mM Tris–HCl pH 7.5, 100 mM (NH4)2SO4, 200 μM GTP and 25 mM MgCl2 at 45°C (18,29)). However, to compare the in vitro effects of the mutations with in vivo, we wanted to use conditions that were more similar to organellar conditions (at least as far as possible, considering that group I introns usually do not self-splice with any efficiency at temperatures that are optimal for C.reinhardtii growth). Thus, it was necessary to evaluate the effects of certain key parameters on Cr.psbA4 splicing in vitro. These effects, as well as those of the mutations (see below), were assessed using RNA synthesized from linearized pBX4.Δ-based DNAs (Fig. 1). The intron in pBX4.Δ has most of the ORF deleted from the non-conserved loop of P5b (Fig. 2). Deleting the ORF did not have an appreciable effect on Cr.psbA4 splicing in vivo (see below), although it seemed to slightly improve in vitro splicing (J.Lee, O.W.Odom and D.L.Herrin, unpublished results), and was done to facilitate identification of homoplasmic transformants. This form of the intron pre-RNA will be referred to as WTΔ.

Figure 3 shows the effects of varying the concentrations of GTP and Mg2+, as well as the reaction temperature, on Cr.psbA4 self-splicing. These reactions also contained 100 mM KOAc as monovalent salt, and were pH 7.5; typically, there is little effect of pH on self-splicing in the range of 6–9 (42). Since the reactions were for a relatively long period of time, i.e. 30 min, the data reflect the extent of splicing more so than the initial rate, and are based on accumulation of the ligated-exons product (Fig. 4A and B). Figure 3A shows that the GTP concentration was saturating at 0.1 mM, and above that concentration, the splicing efficiency declined slightly (up to 5 mM, which was the highest concentration tested). This result indicates that the ribozyme has a high affinity for GTP and that nucleotide levels are unlikely to be regulatory in vivo.

The Mg2+ concentration was varied from 0 to 25 mM and the effect on self-splicing is shown in Figure 3B. The greatest splicing efficiency was obtained at 10 mM Mg2+, and above that concentration splicing declined gradually to ~38% of maximum at 25 mM Mg2+. There was no splicing observed at 5 mM Mg2+, which was also the case for the Cr.psbA2 (43) and Cr.LSU introns (29) in the presence of monovalent salt.

Figure 3C shows the effect of temperature on Cr.psbA4 self-splicing; the optimum was around 55°C, and splicing was poor at the physiological temperatures of 23 and 30°C. The dramatic increase in splicing efficiency at 45°C compared to 37°C is not readily explained by an effect on the chemical steps of splicing, but may reflect enhanced folding of the intron at 45°C. The dramatic drop in splicing at 62°C probably reflects unfolding of tertiary structural elements (44).

Finally, it should be noted that monovalent ions (K+ and NH4+) had relatively minor effects on Cr.psbA4 self-splicing, although they increased the minimum Mg2+ requirement as expected (data not shown). Based on these data, published estimates of the nucleotide concentrations in chloroplasts (~1 mM (45)), and the optimal Mg2+ concentration for translation by chloroplast polysomes (~12 mM (41)), the chosen conditions for evaluating the effects of the mutations on Cr.psbA4 self-splicing were 25 mM Tris–HCl pH 7.5, 1 mM GTP, 100 mM KOAc, 15 mM MgCl2, at 45°C. It was also reasoned that these conditions would allow most of the mutants, except for perhaps the most severely affected ones, to self-splice.

Effects of nucleotide substitutions on Cr.psbA4 self-splicing in vitro

Figure 2 shows the locations of the nucleotide substitutions in the predicted secondary structure of Cr.psbA4 (34). The conserved ribozyme core is composed of two major, stacked-helices domains, P5-P4-P6 and P9.0-P7-P3-P8 (46); the guanosine binding site resides mainly on 1674G (WT numbering) in P7 (47). Cr.psbA4 also contains several less conserved, peripheral domains, including P5a, P5b, P6a, P7.1, P9.1 and the signature (for subgroup IA1 introns) P11 pairing (46). Initially, single-nucleotide substitutions were made in the P3, P4, P6, P6a, P7 and P11 helices, and then two more mutants were created that had two-nucleotide substitutions in P4 and P7 (the P4¢-3,4 and P7-4,5 mutants, respectively). These latter mutants were constructed because of the relatively weak effects of most of the single-nucleotide substitutions.

Figure 4A shows representative time-course splicing reactions for WTΔ (i.e. pBX4.Δ) and two of the mutant introns, P7-4.5 and P6-2, respectively. The principal products of splicing of this 1.6 kb pre-RNA are the 1.3 kb linear intron (I4) and the 0.3 kb ligated-exons (E4-E5). Quantification of the ligated-exons product is shown in Figure 4B for these reactions as well as for four other mutants (P4¢-2; P4¢-3,4; P6a-2; and P7-4). The results show that splicing of the WTΔ pre-RNA is quite fast under these conditions, being essentially finished in 10 min (kobs of ~0.15/min). The rapidly splicing fraction was ~65% of the pre-RNA, a value that was not significantly improved by a denaturation–renaturation cycle. Compared to Cr.LSU (29) and Cr.psbA2 (43), Cr.psbA4 is fairly efficient at self-splicing, particularly for such a large intron.

All six of the substituted introns showed reduced splicing compared to WTΔ; the reductions ranged from moderate, in the case of the P6a-2 and P4¢-2 mutants, to a complete loss of self-splicing with the P7-4,5 mutant. The significant effect of the P6a-2 substitution was somewhat surprising, since this paired region is not really part of the conserved core. Figure 4B also shows that the kinetics of the P7-4 mutant were unique; there was a lag period of ~3–4 min before any product was formed, and the subsequent course was biphasic. This pattern was highly reproducible under these conditions, but was not quite as biphasic under other conditions (described below). The kinetics of the P7-4 mutant are consistent with it being a slow-folding mutant. It should also be noted that heterogeneous kinetics are common for self-splicing group I introns.
(29,48), and presumably reflect the presence of conformational isomers. A fraction of relatively inactive pre-RNA is also not uncommon, and are likely molecules trapped in misfolded states (49).

The splicing efficiencies of the six mutants were also assessed in a commonly used splicing buffer (25 mM Tris–HCl pH 7.5, 100 mM (NH₄)₂SO₄, 200 μM GTP and 25 mM MgCl₂) at 45°C. Only the P7-4 and P6-2 mutants showed significant differences in their splicing efficiencies relative to WTΔ in this buffer, compared to the more physiological solution; the P7-4 mutant was ~2-fold less active, whereas the P6-2 mutant was a dramatic 8-fold lower in splicing efficiency (data not shown). Self-splicing of the other two mutants created in this study (P3'-5 and P11'-2) was also assessed with these conditions: the P3'-5 mutant was indistinguishable from WTΔ, and the P11'-2 mutant was ~80% of WTΔ (data not shown). Finally, increasing the Mg²⁺ concentration (up to 100 mM) increased the self-splicing efficiency of most of the mutants, except for the P7-4,5 mutant (which was still inactive), consistent with structural destabilization.

**Effects of nucleotide substitutions on in vivo splicing of Cr.psbA4**

To assess the in vivo effects of the nucleotide substitutions on Cr.psbA4 splicing, the mutant constructs were transformed into chloroplasts of the wild-type 2317 strain using...
co-transformation with a 16S rRNA gene that confers spectinomycin resistance, but otherwise has little effect on phenotype (26). Prior to the transformations, the upstream EcoO1091–BstEII fragment of \textit{psbA} was added to the pBX4-based plasmids to create the pEX4 series (Fig. 1); the greater amount of 5′ flanking homology greatly increased the frequency of intron replacement. The transformants were selected on acetate-containing medium in dim light, in case any were completely devoid of photosynthetic growth (like P7-4,5) or were sensitive to bright light. The homoplasmicity of the transformants was verified by PCR with primers that flank the \textit{Cr.psbA4} intron (22), and only transformants that contained exclusively the ORF-deleted form of the intron were analyzed further. The PCR products were also sequenced to verify the presence of the nucleotide substitutions in the mutant transformants (data not shown).

We were able to obtain homoplasmic transformants with each of the six intron mutations analyzed in Figure 4, and all were capable of autotrophic growth except the P7-4,5 mutant (see below). Thus, RNA gel-blot hybridizations were performed on cultures growing mixotrophically (acetate + light) under moderately bright light (~40 \text{μmol/m}^2\text{s}). Figure 5 shows the results obtained with an intronless \textit{psbA} gene (Exons 1–5 probe), and an intron-specific probe. The wild-type \textit{Cr.psbA4} intron is 1.8 kb, the ORF-deleted version is 1.3 kb, and the mature \textit{psbA} mRNA is 1.1 kb. Only the P7-4,5, P4′-3,4, P7-4, and P6-2 mutants showed evidence of reduced \textit{Cr.psbA4} splicing, based on the accumulation of a 2.4 kb pre-mRNA and corresponding decreases in the 1.1 kb mature mRNA. The hybridization with the intron-specific DNA (Intron 4 probe) confirmed that the 2.4 kb RNA is unspliced precursor. A longer exposure of the blot also showed no evidence of precursor RNA in the P6a-2 and P4′-2 mutants.

Figure 3. Effect of varying GTP, Mg\(^{2+}\) and temperature on self-splicing of \textit{Cr.psbA4}. The pre-RNA transcribed from pBX4.Δ (Fig. 1) was incubated for 30 min in the standard splicing reaction mixture, except for varying the indicated parameter, and then analyzed by denaturing gel electrophoresis. Splicing efficiency was estimated from the accumulation of the 319-nucleotide ligated-exons product (Fig. 4A). The data were normalized relative to the highest value, which was set to 100%. (A) The effect of varying the GTP concentration on \textit{Cr.psbA4} self-splicing. (B) The effect of varying the Mg\(^{2+}\) concentration on \textit{Cr.psbA4} self-splicing. (C) The effect of varying the reaction temperature on \textit{Cr.psbA4} self-splicing.

Figure 4. Effects of the nucleotide substitutions on \textit{Cr.psbA4} splicing \textit{in vitro}. The splicing reactions were performed and analyzed as described in Materials and Methods. (A) Autoradiograph of the splicing reactions for the control ORF-deleted intron (WTΔ), and the P7-4,5 and P6-2 mutant pre-RNAs. The sizes of the major RNAs indicated to the left (in nucleotides) are based on the transcribed sequence. Pre, preRNA; I4, linear free intron; E4–E5, ligated exons. (B) Time courses of self-splicing reactions by the mutants and WTΔ pre-RNAs. The ligated-exons product was quantified, and the data were normalized relative to the control pre-RNA (WTΔ), which was considered to be 100%. The time-course analysis was performed with at least two different preparations of each pre-RNA, and with similar results.
The data in Figure 5 show that splicing of the P7-4,5 mutant is essentially blocked, whereas splicing of the P4-3,4 mutant is inefficient (~40% of the WT levels of mature psbA mRNA). The numbers below the lanes of the exons-only hybridization are the levels of mature psbA mRNA relative to the control strain (WT). It is fortuitous that the sums of the mature + pre-mRNAs in the different strains are within 10% of each other, indicating that the mutations did not significantly affect RNA stability. The identities of the minor bands that migrate between the 2.4- and 1.1-kb RNAs in the splicing-deficient mutants are not known with certainty. They might be degradation products of the pre-mRNA, although the somewhat diffuse band of ~2 kb might be the intron-exon 5 splicing intermediate (9). Finally, there was no evidence of inhibition of splicing of the other psbA introns in the mutants, which is consistent with the previous conclusion that they are spliced out independently and randomly (9).

**D1 synthesis in the Cr.psbA4 splicing-deficient mutants**

To investigate the effects of the mutations on D1 synthesis, the four strains with deficiencies in Cr.psbA4 splicing (and the control strain) were grown mixotrophically as for Figure 5, and pulse-labeled for 30 min with [14C]acetate in the presence of cycloheximide. Equal numbers of cells were analyzed by SDS–PAGE, and a representative fluorograph is shown in Figure 6. The most obvious effect is the disappearance of D1 in the P7-4,5 mutant, and the concomitant, albeit somewhat surprising, appearance of a polypeptide that migrated with an apparent $M_r$ of ~24 kDa. The Cr.psbA4 intron contains an in-frame termination codon in the second subjective codon position of the intron. The predicted size of a polypeptide terminating at this site is 27 kDa. However, it is well established that D1 migrates faster than its predicted size by SDS–PAGE (8). Hence, the new ~24-kDa protein is most likely a truncated D1 (tD1) containing the first four exons. This protein can also be seen in the P4-3,4 mutant, but at a lower level than the P7-4,5 mutant. The relative rates of synthesis of full-length D1, normalized using the large subunit of ribulose-1,5-bisphosphate carboxylase (LS) to correct for slight differences in acetate incorporation and gel loading, are

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**Figure 5.** Effects of the nucleotide substitutions on Cr.psbA4 splicing in cells growing mixotrophically (light + acetate). Total RNA was isolated from early log phase cultures of the indicated chloroplast transformants and subjected to RNA gel-blot analysis. The WT strain is wild-type with respect to the substitutions, but is otherwise like the other strains in being ORF-minus and carrying the spectinomycin and DCMU resistance markers (Fig. 1). The cells were grown in liquid acetate-containing medium (TAP + 100 µg/ml spectinomycin) at a light intensity of ~40 µmol/m2/s. An equal amount of RNA (5 µg) was loaded in each lane, and the blots were hybridized with an intronless psbA probe (Exons 1–5 probe), and a PCR product from the Cr.psbA4 intron (Intron 4 probe). The positions of the major psbA precursor (2.4-kb RNA), and the mature psbA mRNA (1.1-kb RNA) are indicated. C.reinhardtii rRNAs, which were visible on the Methylene Blue-stained blots, were used as size markers. The relative amounts of mature mRNA are given below the lanes of the exons-only hybridization; they were quantified and normalized relative to the control strain (WT), which was considered to be 100%. For those mutants that showed an effect on Cr.psbA4 splicing, the mean ± standard error was obtained from gel-blot analysis of 2–3 independent RNA isolations.

**Figure 6.** D1 protein synthesis in selected Cr.psbA4 mutants. Aliquots of log phase cultures of the indicated strains, grown as described in Figure 5, were removed and pulse-labeled with [14C]acetate for 30 min in the presence of cycloheximide. Equal numbers of cells were analyzed by SDS–PAGE, and a representative fluorograph is shown. The ratios of D1/LS polypeptide labeling were determined from three gels and normalized relative to the ratio in the control strain (WT), which was set to 100%. The polypeptides identified to the right are: LS, large subunit of ribulose-1,5-bisphosphate carboxylase; D1, D2, photosystem II reaction center polypeptides; tD1, truncated D1.
shown below the lanes. The data indicate that there are reduced levels of full-length D1 synthesis in the P7-4 and P6-2 mutants, as well as in the P4'3,4 mutant. Finally, it should be noted that these results also indicate that unspliced psbA pre-mRNA is translated in vivo.

**Photautotrophic growth, psbA mRNA levels and D1 synthesis in the partially-deficient splicing mutants**

The analyses in Figures 5 and 6 were performed with cells growing mixotrophically (light + acetate), because, as demonstrated in Figure 7A, the P7-4,5 mutant did not grow at all on minimal medium. Figure 7A also shows that the P7-4,5 mutant grew slower than WTΔ under mixotrophic conditions (light + acetate), as expected.

It was important to assess the effects of the partial splicing mutations on cells growing photoautotrophically. Growth curves in liquid minimal medium (gently bubbled with 5% CO2) are shown in Figure 7B. The growth rates of the P7-4 and P6-2 mutants were similar to the control strain (WTΔ), whereas the P4'3,4 mutant showed a clear reduction in growth, having only 57% of the cell number of the control strain after 4 days. Using the exponential part of the growth curves, this corresponds to a doubling time that is 18% longer than the control strain (WTΔ). There also appeared to be a small effect on growth rate in the P7-4 and P6-2 mutants.

The growth rates of the partial splicing mutants were also analyzed in cultures growing in air (with mild shaking), rather than bubbled with 5% CO2. Although the absolute growth rates of all the strains were reduced by ~1.8-fold under these conditions, growth of the P4'3,4 mutant was still significantly (15%) slower than the control strain. We also noticed that in older cultures of the P4'3,4 mutant, senescence occurred earlier and was much more pronounced than in the WTΔ strain or the P7-4 and P6-2 mutants.

**Figure 7.** The effects of certain Cr.psbA4 substitutions on growth rate, splicing efficiency and D1 (full-length) synthesis in photoautotrophically growing cells. (A) Spot test for growth of the P7-4,5 mutant on minimal medium. Equal numbers of cells (2 x 10^4) of WTΔ and the P7-4,5 mutant were spotted onto minimal and TAP agar media, and allowed to grow for 6 days in the light (~100 μmol/m2/s). (B) Phototrophic growth curves for selected Cr.psbA4 mutants; 2 x 10^4 cells of each strain were inoculated into 200 ml of minimal medium (+100 μg/ml spectinomycin) and grown as described in Materials and Methods. The cell number at each time point represents the mean ± standard error of replicate measurements; where the error bars are not visible, they were smaller than the symbols. Doubling time (h) was calculated from the exponential phase of the curves. The arrow indicates the time at which aliquots of the cultures were removed for analyses of RNA (C) and protein synthesis (D). (C) RNA gel-blot hybridizations with a prepsbA exons probe, and the Cr.psbA4 intron-specific probe are shown in Figure 7C. In addition to the unspliced precursor and mature RNAs, this exposure shows the 1.3-kb excised intron RNA. The blots were quantified with a PhosphorImager, and relative levels of the mature mRNA are given below the lanes. The results were generally similar to those obtained with cells growing mixotrophically (Fig. 5), except that the splicing efficiency was somewhat improved. For example, the P4'3,4 mutant has ~55% of the control levels of mature psbA mRNA with these autotrophic conditions, compared to ~42% in the mixotrophic cultures (Fig. 5). As with mixotrophic growth, however, the sums of the precursor + mature RNAs in the

The psbA transcripts were also analyzed during the autotrophic growth curve (above) at the mid-log phase (arrow in Fig. 7B). RNA gel-blot hybridizations with a psbA exons probe, and the Cr.psbA4 intron-specific probe shown in Figure 7C. In addition to the unspliced precursor and mature RNAs, this exposure shows the 1.3-kb excised intron RNA. The blots were quantified with a PhosphorImager, and relative levels of the mature mRNA are given below the lanes. The results were generally similar to those obtained with cells growing mixotrophically (Fig. 5), except that the splicing efficiency was somewhat improved. For example, the P4'3,4 mutant has ~55% of the control levels of mature psbA mRNA with these autotrophic conditions, compared to ~42% in the mixotrophic cultures (Fig. 5). As with mixotrophic growth, however, the sums of the precursor + mature RNAs in the
DISCUSSION

The psbA gene of *C. reinhardtii* contains four group I introns whose splicing rates are very slow in the dark (in autotrophically growing cells), but increase coordinately at least 6–10-fold with the administration of light and resulting electron transport (9). Although this is a relatively strong effect on gene expression, the biological significance of light-promoted splicing has not been clear, in part because *psbA* mRNA is very abundant (in light or dark) (3,50), and because of evidence indicating that several chloroplast mRNAs are in excess, some as much as 10-fold, over what is needed to sustain translation rates (21). The present study, however, which describes the effects of mutations in the fourth *psbA* intron, demonstrates the importance of highly efficient splicing of Cr.psbA4, and, by inference, the other *psbA* introns.

Probably the most informative splicing-deficient mutant is the two-nucleotide substituted P4'-'3,4 mutant, which exhibited a 45% reduction in mature *psbA* mRNA, a 28% reduction in synthesis of full-length D1 and an 18% reduction in growth rate under autotrophic conditions. Clearly, a 6–10-fold reduction in splicing of this intron, which could be considered the dark or basal rate, would be expected to limit autotrophic growth even further. Moreover, it should be emphasized that we have reduced the splicing efficiency of only one of the four introns in *psbA*; a coordinate and equivalent reduction in splicing of all four introns would likely have an even greater effect, since these introns are spliced at approximately the same rates (9). These results provide strong support for the suggestion that the role of light-promoted splicing of *psbA* introns is to ensure an ample supply of spliced *psbA* mRNA for the greatly increased translation in the light (9).

Previous work with *C. reinhardtii* has shown that light and the circadian clock increase transcription of the *psbA* gene 2–3-fold in the early light period (9,51), and that light greatly (>10-fold) stimulates translation of *psbA* mRNA, which is also very low in the dark (3,50). Based on the data in this report, we propose that the documented increases in transcription, splicing and translation of the *psbA* gene are all necessary to maintain optimal photosynthetic growth in the light.

These results also suggest that most of the *psbA* mRNA is translated in the light. A previous report, which looked at the distribution of *psbA* mRNA between polysomes and non-polysomal fractions in *C. reinhardtii*, found only ~35% of *psbA* mRNA in the polysomal fraction in cells growing in the light with acetate (52). This could be explained, in part, by a reduced translation of *psbA* mRNA in mixotrophic cultures, or that the recovery of *psbA* polysomes, which are thylakoid membrane-bound (2), was not complete, or both. The isolation and characterization of chloroplast membrane-bound polysomes is not trivial, and while undoubtedly valid for some applications, has not been shown to be quantitative to our knowledge.

In a more recent report (21), a 40–50% drop in *psbA* mRNA levels, obtained by treating *C. reinhardtii* with rifampicin for several hours, was accompanied by a decrease in D1 synthesis under mixotrophic (light + acetate) conditions, but, like...
several other chloroplast-encoded proteins, an increase in D1 synthesis was obtained in autotrophic conditions. Those results would suggest that the psbA mRNA level was close to limiting in mixotrophic conditions, consistent with these findings, but in the autotrophic experiment, translation increased to compensate for the drop in psbA mRNA level. We did not see evidence of increased translation of psbA mRNA in these splicing mutants under autotrophic conditions. However, whereas we specifically reduced the level of the mature psbA mRNA (by inefficient splicing), we apparently did not decrease the total translatable psbA RNA level, since the unspliced RNA was also translated. This would suggest that the trigger for increased translation of psbA mRNA in the Eberhard et al. study (21) was the drop in translatable psbA RNA. However, since rifampicin treatment also reduced the levels of many, if not most, chloroplast mRNAs, the trigger for increased translation of psbA mRNA could also have been decreased competition among the remaining mRNAs for translation factors or ribosomes (21).

A previous attempt to perform a similar mutagenic analysis of the Cr.LSU rRNA intron was limited by the apparent lethality of most of the mutations in vivo (19). However, we can compare the in vitro effects of the substitutions in the two chloroplast introns. The mutations in Cr.psbA4 that are similar to the Cr.LSU study are the single-nucleotide substitutions in the P4, P6, and P7 regions (19); these mutations reduced Cr.LSU splicing by >95%, compared to only 50–80% for Cr.psbA4. Thus, Cr.psbA4 is more stable structurally than Cr.LSU, and appears to be similar to the Tetrahymena intron in this regard (53,54). A greater stability for Cr.psbA4 is also suggested by its ~10°C higher temperature optimum for self-splicing (29). The biological significance of a greater structural stability for Cr.psbA4 than Cr.LSU is not clear. However, one possibility is that it helps to maintain the splicing efficiency of Cr.psbA4 at high temperature. Alternatively, this difference may reflect a greater need for trans-acting factors by Cr.LSU.

The in vivo effects of the substitutions in Cr.psbA4 paralleled the in vitro effects, and were consistent with the predicted secondary structure. However, in general, the mutations had less effect in vivo, suggestive of a mechanism that stabilizes the intron’s structure. It should be noted, however, that the in vitro splicing reactions required a non-physiologically high temperature (45°C) to be efficient, and this could have had a differential effect on the mutant prerRNAs compared to the control (WTΔ). However, the fact that this difference was consistent among all of the mutations tends to support its validity. The simplest explanation for a greater in vivo stability of Cr.psbA4 is that one or more proteins bind to the partially (or completed) folded intron and steady its tertiary structure, as has been shown for several fungal mitochondrial introns (reviewed in 55). A candidate for such a protein in C.reinhardtii is the css1 gene that was recently identified using suppressor genetics (19). These nuclear mutants suppressed the splicing inefficiency of the P4–3'4 mutant intron, when it was transformed into them (19).

The effects of these mutations on splicing of Cr.psbA4 in vivo seem to differ from those of the phage T4.td intron where nucleotide substitutions in the intron’s core produced similar effects in vivo and in vitro (24,25). A likely explanation for this difference between the chloroplast and E.coli is that E.coli does not seem to encode a protein that specifically binds to the T4.td intron’s core and stabilizes its structure. Presumably, it is this feature of E.coli that makes it possible to study the ability of the group I splicing factor, CVT18, from Neurospora to suppress nucleotide substitutions in the core domains of the T4.td intron (24,25).

We were somewhat surprised to see the truncated D1 polypeptide label so well with the 30 min pulse of the P7-4,5 mutant. This would suggest that this protein, which is lacking exon 5 and therefore the 5th transmembrane domain, is not as unstable as the more severely truncated D1 generated by Preiss et al. (56), which lacked transmembrane domains 3–5.

An interesting question is how light-dependent RNA splicing evolved in the Chlamydomonas chloroplast. Based on studies with fungi, it has been suggested that group I intron splicing factors evolved from pre-existing proteins that had other functions (55). It is possible then that light-activated RNA-binding proteins (13,57) were recruited as splicing factors for psbA introns, and that these proteins have retained their light-dependent properties. Of course, this hypothesis assumes that evolution of the psbA gene involved insertion of the introns into a pre-existing psbA gene that was intronless. The isolation of splicing factors for these introns, coupled with studies of psbA genes in other Chlamydomonas species, may provide support for this hypothesis.

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