Detection of multiple, unspliced precursor mRNA transcripts for the Mr 32,000 thylakoid membrane protein from *Euglena gracilis* chloroplasts

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**ABSTRACT**

The psbA gene is the coding locus for a polypeptide of 32 kilodaltons that is involved in electron transport through photosystem II. The 4.9 kilobasepair (kbp) EcoRI restriction endonuclease fragment EcoI from the 145 kbp *Euglena gracilis* chloroplast DNA was shown to encode psbA. Five transcripts of size 3.1, 2.8, 2.3, 1.8, and 1.2 kilobases were detected by hybridization of psbA probes to nitrocellulose filter blots of electrophoretically separated RNAs. This same pattern was observed when the hybridization probe consisted of only exon sequences from this split gene. A synthetic, intron specific probe hybridized to all RNA precursors except the 1.2 kb mature RNA. These results and psbA DNA sequence data lead to the conclusion that the four higher molecular weight transcripts are unprocessed precursors of the 1.2 kilobase RNA, some of which contain unspliced intervening sequences. There is an increase in psbA transcripts during light induced maturation of the chloroplasts.

**INTRODUCTION**

The "32 kilodalton" polypeptide of chloroplast thylakoid membranes is a rapidly metabolized polypeptide thought to be a "proteinacious shield" associated with photosystem II.1,2,3 It appears to be involved in the transport of electrons from photosystem II, and may confer sensitivity to the herbicides diuron and atrazine in PS II.2,4 The gene coding locus (psbA) for the 32 kilodalton (kd) polypeptide is found in the chloroplast genome, and has been localized and identified in maize,5 spinach,6 Nicotiana debneyi7 and *Euglena*8,9 chloroplasts.

The psbA gene polypeptide product has been studied in many organisms. Comparisons have been made between the photogene protein from maize,10 peak D from peas,11 protein D-1 from *Chlamydomonas*,12 and the 32 kd polypeptide from *Spirodela*.2 They were found to be virtually identical. Significant homologies have been found in the structural properties of the 32 kd protein of all angiosperms studied to date.13

The complete DNA sequence for the psbA gene is known in spinach, *N.*
debneyi,\textsuperscript{7} and Amaranthus hybridus.\textsuperscript{14} The amino acid sequence for the 32 kd protein derived from the gene sequence of spinach and N. debneyi is 100% conserved. There is one amino acid substitution in A. hybridus, and one additional change in the atrazine resistant biotype.\textsuperscript{14} In Spirodela, the 32 kd protein is synthesized in amounts comparable to the large subunit of ribulose-bisphosphate carboxylase. The protein has an extremely rapid turnover rate, 50 to 60 times more rapid than that of the large subunit.\textsuperscript{15} It appears that there is an abundance of the psbA mRNA, but comparatively low levels of the protein itself. The 32 kd polypeptide levels appear to be induced and controlled by light.\textsuperscript{2,16} This makes the psbA gene an especially good candidate for studies on gene regulation during light induced chloroplast development.

*Euglena* is a particularly suitable organism for the study of chloroplast encoded mRNAs. It may be grown in the absence of light, subsisting solely upon organic carbon sources. When grown in the dark, the chloroplasts revert to a proplastid state. Upon exposure to light, these proplastids develop into functional chloroplasts over a period of 72 hours.\textsuperscript{17,18} Changes in *Euglena* thylakoid polypeptides during chloroplast development have been described.\textsuperscript{19,20} *Euglena* RNA transcripts may be isolated at varying periods during this greening process. It has been shown that some transcripts decrease in abundance during development while others increase.\textsuperscript{21,22}

In this paper, we will report on our studies on the 4.9 kbp EcoRI restriction endonuclease fragment of *EcoI* of the 145 kbp *Euglena gracilis* chloroplast DNA. We have identified three gene loci within *EcoI*. One of these loci, the psbA gene, was chosen for extensive study. Through experiments involving the hybridization of radiolabeled psbA probes to cellulose nitrate filter blots of *Euglena* RNAs, we have detected at least five psbA transcripts. Studies were undertaken to determine the effect of light induced chloroplast development on these psbA gene transcripts.

**MATERIALS AND METHODS**

*Isolation and Identification of Nucleic Acids*

Chloroplast DNA was isolated in the intact superhelical form from *Euglena gracilis* Klebs, Strain 2 Pringsheim as previously described.\textsuperscript{23,24} Spinach (*S. oleracea*) recombinant chloroplast PstI clones and total chloroplast DNA were generously provided by Drs. W. F. Thompson and J. D. Palmer.

*Euglena* chloroplast DNA restriction fragments were ligated into plasmid vector restriction sites by standard procedures.\textsuperscript{25} pEZC514 consists of the
EcoI fragment from *Euglena* chloroplast DNA ligated into pBR322. pEZC25 consists of the HindIII 10 fragment, which is a 3.6 kilobasepair (kbp) internal fragment of EcoI, ligated into pBR325. Individual fragments from this plasmid were purified from polyacrylamide gel slices by a modification of a crush and soak procedure.26

Whole cell *Euglena* RNA was purified, as previously described,27 with aurintricarboxylic acid employed as a nuclease inhibitor. Prior to RNA extraction, aliquots of cells from the different developmental stages were stored at -70°C.

**Restriction Endonuclease Mapping and Localization of psbA**

Localization of psbA within *EcoI* was accomplished through a heterologous membrane filter hybridization approach. Restriction nuclease fragments were transferred from agarose gels to nitrocellulose filters.28 The solution for heterologous hybridization reactions contained 0.75 M NaCl, 0.075 M sodium citrate, 15% formamide, 0.075M NaPO₄ (pH 7.0), 200 μg/ml degraded Herring Sperm DNA, 1 X Denhardt's solution,29 and one or more psbA specific radio-labeled DNA probes. Hybridizations were carried out in a minimal volume in a Seal-a-Meal bag at 41°C for 24 hours. The filters were washed in the bag with three 50 ml washes of 0.75 M NaCl, 0.075 M sodium citrate, 0.1% sodium dodecyl sulfate, for 20 minutes each at room temperature.

**RNA Electrophoresis, Transfer to Nitrocellulose, and Hybridization Conditions**

RNA electrophoresis was carried out in 0.8% or 1% agarose, 2.2 M formaldehyde gels essentially as described by Rave et al.30 Before transfer to nitrocellulose, one lane containing purified *Euglena* ct RNA was trimmed away from the rest of the gel, ethidium bromide stained, and photographed. The remainder of the gel was left unstained and transferred to nitrocellulose as described by Thomas.31 Hybridization was carried out in either 50% (for homologous reactions) or 15% (for heterologous reactions) formamide, 0.75 M NaCl, 0.075 M sodium citrate, 50 mM NaPO₄ (pH 7.0), 1 X Denhardt's and 250 μg/ml degraded Herring Sperm DNA. Homologous reactions were carried out at 42°C. Heterologous reactions were at 37°C. Hybridization reactions involving 32p-labeled synthetic oligonucleotides were for 3 days at 22°C in 0.6 M NaCl, 0.06 M Na citrate, 1 X Denhardt's, and 200 μg/ml yeast RNA (Sigma, Type XI).

**Preparation and Purification of 32p-Labeled DNA Probes**

Hybridization probes were labeled by the nick translation activity of DNA polymerase I32 or with polynucleotide kinase using [γ-32p]-ATP as substrate.26 Purification was accomplished by first running a sample containing the fragment of interest on a 3.5% polyacrylamide gel. The fragment was visualized
and cut out with as little acrylamide as possible. This piece was then placed directly into a well of another 3.5% polyacrylamide gel. After electrophoresis and visualization, the fragment was once again cut out. The DNA was purified from the acrylamide by the crush and soak procedure, as referenced above. Nick translated fragments may be obtained in greater than 95% purity using this method.

RESULTS

Localization of the Gene for the 32 kd Thylakoid Membrane Protein in *Euglena* Chloroplast DNA

One approach we have taken for the localization of *Euglena* chloroplast protein genes involves heterologous hybridization. Previously characterized higher plant\(^8,3\) or bacterial\(^3\) protein gene segments are radiolabeled and hybridized to membrane filter blots of *Euglena* chloroplast DNA fragments. The methods and strategy for these studies have been described for locating the intron-containing gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (rbCL),\(^3\) and elongation factor EF-Tu (tufA).\(^3\) The *Euglena* chloroplast psbA locus was determined by using specific psbA DNA probes derived from the spinach psbA gene. The spinach chloroplast psbA gene, which is located in the large single copy DNA region adjacent to one of the inverted repeat DNAs,\(^6\) has been completely sequenced.\(^7\)

An 8.4 kbp Pst I restriction fragment of spinach chloroplast DNA which contains the 3'-terminal 805 base pair (bp) of the 1062 bp psbA coding sequence was radioactively labeled via nick translation and used as a hybridization probe for the initial screening of *Euglena* chloroplast DNA. A subclone of the 8.4 kbp fragment containing the psbA gene was also constructed. The spinach subclone contains a 1300 bp Sal I-Pst I fragment which has, in order, the 805 bp 3'-terminal psbA coding sequence, a 141 bp intergenic spacer, tRNA\(_{His}\) gene, and approximately 200 bp of spinach inverted repeat DNA\(^7\) (Gerard Zurawski, personal communication).

A gel photo of an *EcoRI* digest of total *Euglena* chloroplast DNA is shown in Figure 1, Lane A. The result of hybridization with the \(^{32}P\)-labeled spinach 1300 bp psbA probe is shown in the corresponding autoradiogram. The 4.9 kbp *EcoRI* fragment *EcoI* hybridizes specifically to the 1300 bp probe. Also shown in the gel photo (Lane B) is a *HindIII* digest of total *Euglena* chloroplast DNA. In the corresponding autoradiogram, the *HindIII* fragment *HindIII* 10 is shown to be complementary to the spinach psbA probe. *HindIII* 10 is located entirely within *EcoI*. Therefore, it was concluded that the *Euglena* psbA gene
Fig. 1. Location of the psbA gene on the Euglena chloroplast genome. The gel photos are as follows: A. Euglena chloroplast DNA digested with EcoRI. B. Euglena chloroplast DNA digested with HindIII. C. Plasmid pEZC514 triply digested with the restriction endonucleases PvuII, HindIII, and EcoRI. The corresponding autoradiograms are of hybridization with the $^{32}$P-radiolabeled spinach psbA probe. Agarose gel fragment size markers to the left of the figure are in kilobasepairs. Actual fragment sizes from DNA sequence analysis may vary from estimates based on agarose gel mobility. Fragments are named to the right of the figure.

is within EcoI. This result has been presented as a preliminary report, and was confirmed by Keller et al.

In order to study the psbA locus and its transcripts in more detail, EcoI DNA was purified and cloned in pBR322. This recombinant plasmid is designated pEZC514. Further hybridizations were done to localize the gene within EcoI. In lane C of Figure 1 the resulting psbA probe hybridization to EcoRI-PvuII-HindIII digestion products of pEZC14 DNA is shown. Shown in the autoradiogram is the psbA probe hybridization to a PvuII-HindIII fragment and the adjacent 1.1 kbp HindIII fragment which are both internal to EcoI. A detailed restric-
Fig. 2. Restriction endonuclease map of the 4.9 kbp EcoRI fragment EcoI of Euglena gracilis chloroplast DNA. (a) Cleavage site maps for the enzymes AccI, HhaI, HindIII, PvuII, and SacI. The largest internal HindIII fragment is the 3.5 kbp HindIII-10. (b) The maximum boundaries of hybridization with the probe derived from the 3'-end of the spinach psbA gene are defined by the 2.0 kbp PvuII-HindIII and 1.1 kbp HindIII fragments internal to EcoI. (c) Cleavage site map for HindI. Individual HindI fragments were used in the hybridization experiments shown in Figure 5. (d) The location and organization for the psbA gene and adjacent tRNAleu gene as determined from DNA sequence analysis. The psbA gene is composed of 5 exons (filled boxes), interrupted by 4 introns (open boxes). (G. D. Karabin, M. Farley, J. O. Narita, and R. B. Hallick, manuscript in preparation.)

tion map of the EcoI fragment and the maximum boundaries of the region hybridizing to the psbA probe are shown in Figure 2. A circular map of Euglena chloroplast DNA showing the location of EcoI and the psbA gene in relation to previously mapped genes is in Figure 3.

In order to confirm the identification of the psbA locus in EcoI, the complete DNA sequence of EcoI has been determined. This work will be described elsewhere (G. D. Karabin, M. Farley, J. O. Narita, and R. B. Hallick, manuscript in preparation). The results most germane to the gene expression studies to be described below are as follows: (1) The Euglena chloroplast psbA amino acid sequence derived from the DNA sequence has 87% homology to that of spinach and N. debneyi.7 (2) The Euglena psbA locus is interrupted by four introns ranging in size from 434-617 bp. The organization of exons and introns, and the gene polarity are illustrated in Figure 2d. (3) The minimum length of the gene from the AUG-start codon to the UAA-termination codon is 2967 bp. (4) Exons 4 and 5, which are encoded on the 1.1 kbp HindIII fragment internal to EcoI, are small (33 and 13 codons, respectively),
Fig. 3. Circular restriction endonuclease map of *Euglena gracilis* chloroplast DNA. The 4.9 kbp *EcoRI* fragment *EcoI* is located at approximately one o’clock in the outermost circular map. Also noted on this map are the locations of other known protein coding loci, known tRNA genes, and the three tandemly repeated rRNA operons.

and gave only weak hybridization signals with the spinach *psbA* probe compared to the *HindIII* 10.

**Multiple RNA Transcripts of the *Euglena* Chloroplast *psbA* Locus**

In order to study RNA transcripts from the *psbA* locus during light-induced chloroplast development, RNA was isolated from dark-adapted *Euglena gracilis* cells (0-hour), and from cells following 12, 24, 36, 48, 60, and 72 hours of light induced chloroplast development. The 0-hour cells contain immature proplastids, and the 72-hour cells contain mature chloroplasts.17 Five micrograms of each RNA were electrophoresed in adjacent lanes in a formaldehyde/agarose gel. Electrophoretically separated RNAs were transferred to cellulose nitrate filters by blotting.31 Putative transcripts of the *psbA* locus were initially detected by hybridization with pEZC514 DNA, 32P-labeled by nick translation. Results are shown in Figure 4. The expected size of the *psbA* polypeptide mRNA is 1.2 kilobases (kb).7,9,36 There is a major transcript found upon hybridization with *EcoI* of 1.2 kbp. This RNA shows distinct developmental regulation, with a marked increase in hybridization signal during chloroplast maturation. In addition to the major 1.2 kb RNA transcript, at
Fig. 4. Hybridization of a radiolabeled psbA probe to a cellulose nitrate filter blot containing electrophoretically separated RNAs from different stages of Euglena chloroplast development. The probe was nick translated pEZC514, which consists of Euglena chloroplast DNA fragment EcoI ligated into the vector pBR322. The lanes in the blot contain, from left to right, 5 micrograms each of whole cell RNAs from the 0, 12, 24, 36, 48, 60, and 72 hour stage of Euglena chloroplast development. The sizes of the RNAs detected are given to the left in kilobases.

At least 5 additional, distinct RNAs are evident, with sizes 3.1, 2.8, 2.3, 1.8, and 1.5 kb. The sum of these RNA lengths, 12.7 kb, is larger than the double strand complexity of the 4.9 kbp EcoI DNA.

Several explanations for these multiple RNAs were considered. First, some or all of the hybridization could be due to a false positive signal due to the plasmid vector DNA present in the 32P-pEZC514 DNA probe. To test this, EcoI DNA and the pBR322 vector DNA were individually purified, radiolabeled, and hybridized to identical RNA blots. The hybridization pattern with the EcoI DNA probe was identical to that found with pEZC514 DNA. None of these RNAs were detected when 32P-pBR322 DNA was used as the probe (data not shown). Second, some of the RNAs detected with the EcoI probe might be transcripts of other regions of the chloroplast genome that fortuitously hybridized to EcoI DNA, despite the high stringency hybridization conditions. This could not be rigorously excluded for every region of chloroplast DNA. However,
Fig. 5. Correlation of EcoI hybridization patterns to the internal HinfI fragments of EcoI. Shown above is the HinfI restriction endonuclease map of the EcoI restriction fragment from Euglena gracilis chloroplast DNA. Below are single lanes from the hybridization of each HinfI fragment to a cellulose nitrate filter blot of electrophoretically separated RNAs from seven stages of Euglena chloroplast development. The individual lanes are from either the 60 or 72 hour developmental stage. Sizes of the transcripts are given to the right, in kilobases. Sizes of the restriction fragments are given in base pairs.

A number of other cloned Euglena chloroplast DNA fragments containing known genes gave hybridization patterns clearly distinct from that of EcoI. These included subclones of EcoA (rbcL), EcoD (atpB), EcoN (tufA), EcoG (an unidentified open reading frame, urf 91), EcoP (16S rRNA), and tRNA coding loci of EcoV, EcoH, and EcoG (M. J. Hollingsworth, unpublished observations). We therefore conclude that all of the RNAs are wholly or in part encoded in EcoI. Third, it is possible that some of the six RNAs are products of a different gene or genes besides psbA, which are located in EcoI. Finally, some of the RNAs in the 1.5-3.1 kbp size range might be unprocessed precursor mRNA transcripts of psbA or of other loci in EcoI. With the characterization of psbA as an intron-containing gene, the interesting possibility that unspliced
psbA mRNA precursors are being detected is evident. As shown below, the multiple RNAs complementary to EcoI are due both to multiple genes within EcoI and unprocessed psbA precursor mRNAs.

Correlation of psbA Precursor mRNA Transcripts with Internal Restriction Fragments of EcoI DNA

To obtain additional information on the identity and interrelationship of the multiple EcoI RNA transcripts, the seven individual HinfI DNA subfragments of EcoI were purified, mapped (Figures 2c, 5), and 32P-radiolabeled by nick translation. Each probe was hybridized to identically prepared membrane filter blots containing electrophoretically separated RNA from the seven stages of chloroplast development described above. The results for either the 60 hour or 72 hour RNA sample with each of the 32P-DNAs are shown in Figure 5. The HinfI fragments of 1100-1500-700-140-900 bp, which are adjacent on the EcoI physical map (Figures 2c, 5), all hybridize to the same five RNAs of size 3.1, 2.8, 2.3, 1.8, and 1.2 kb. Based on physical mapping and the DNA sequence data described above and illustrated in Figure 2, these HinfI fragments are known to include the psbA coding locus and psbA intervening sequence(s). These RNAs must all therefore be derived from the psbA transcription unit. The primary transcript is at least 3.1 kb in length. The 2.8, 2.3, 1.8, and perhaps the 1.5 kb RNAs must be partially processed precursors of the mature 1.2 kb psbA mRNA. At least some of these precursor transcripts, including the 3.1 kb pre-mRNA, contain unspliced introns.

A second EcoI gene transcript was detected by hybridization to the adjacent 260-340-1100 bp HinfI fragments (Figure 5). This gene is separated from the psbA locus by a tRNA coding region containing a tRNALeu gene in the 1100 bp HinfI fragment (G. D. Karabin and R. B. Hallick, submitted for publication). The major transcript detected by membrane filter hybridization is 1.5 kbp (Figure 5). This RNA was also detected with pEZCS14 DNA as probe. In addition, a weak 1.2 kbp RNA hybridization signal is obtained with the 260 and 340 bp 32P-labeled HinfI DNA probes. This may be a consequence of minor psbA sequence contamination in the 260 and 340 bp DNA preparations, breakdown of the 1.5 kbp RNA transcript, or a third gene within EcoI.

Hybridization of a psbA Exon-specific DNA Probe to Unspliced psbA Precursor and Mature mRNAs

To obtain further evidence that the transcripts of EcoI are at least in part unprocessed psbA mRNA precursors, a hybridization probe of defined exon sequence was prepared. With reference to the restriction map of EcoI (Figure 2), the 270 bp HinfI-RhaI fragment contains exon sequence homologous to codons

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140-231 of the spinach psbA locus, but lacks intron sequences (G. D. Karabin and R. B. Hallick, unpublished observations). These restriction nuclease sites are also present in spinach chloroplast psbA. This fragment was radio-labeled in two different strand specific reactions. The 1500 bp HinfI fragment was first 5'-32P end labeled with polynucleotide kinase, and then treated with HhaI to yield 5'-end label on the DNA template strand. The 5'-end labeled 270 bp HinfI-HhaI DNA fragment was subsequently purified via polyacrylamide gel electrophoresis. Alternatively, the 5'-end labeling was initially at the HhaI site, followed by HinfI digestion and fragment purification, to yield, as a control, a probe with the "mRNA-like" sequence radio-labeled.

The two 32P-DNAs were then hybridized to membrane filter blots of RNAs from various chloroplast development stages as described above. The DNA probe 5'-end labeled on the template strand, and thus complementary to psbA mRNA, hybridized to RNAs of 3.1, 2.8, 2.3, 1.8, and 1.2 kb as expected for the psbA transcripts. The gel region containing the 1.5 kb RNAs was not clear in this experiment. Furthermore, the changes in hybridization signal during development parallel those described above (Figure 6). The control probe, 5'-end labeled on the RNA-like strand, gave a very weak signal at 1.2 kb. It took six times longer for this very faint signal to appear than it did for the very strong DNA template signal. We conclude that the weak signal is due to slight contamination of the RNA-like probe by DNA template specific psbA sequences. This confirms the conclusion that the 3.1, 2.8, 2.3, and 1.8 kb EcoI transcripts are all psbA mRNA precursors, some of which must contain unspliced intervening sequences.

Hybridization of a psbA Intron-specific DNA Probe to Unspliced psbA Precursor mRNAs

As a final confirmation that the 3.1, 2.8, 2.3, 1.8, and 1.5 kb RNA transcripts of EcoI are unspliced psbA pre-mRNAs, a defined intron-specific sequence was employed. The synthetic oligonucleotide 5'-GATGTTATTCTTTC was constructed to complement transcripts of intron 2 (G. D. Karabin, M. Farley, J. Narita, and R. B. Hallick, in preparation). The tetradecamer was radio-labeled at the 5'-end with polynucleotide kinase, and hybridized to a blot of electrophoretically separated RNAs. In order to determine the position of pre-mRNAs, this blot had previously been hybridized to a psbA probe containing DNA from the 5'-leader region through exon 3. The results of this experiment are shown in Fig. 7. The probe that contains sequences from exon 1-3, introns 1-2, and some 5'-leader sequence hybridizes to RNAs of 3.1, 2.8, 2.3,
Fig. 6. Hybridization of an exon specific probe for psbA to a cellulose nitrate blot of electrophoretically separated RNA from seven stages of *Euglena gracilis* chloroplast development. The probe was a 270 base pair HhaI-HinfI restriction endonuclease fragment that has been shown by DNA sequence analysis (G. D. Karabin, unpublished observations) to contain only sequences within the coding region of psbA. The autoradiogram to the left results when only the HinfI restriction endonuclease cleavage site is radiolabeled, resulting in only the DNA template strand of the fragment having radioactivity. The lanes are marked above with the developmental stage from which the RNA was isolated. Sizes of the transcripts are given to the left in kilobases. The autoradiogram to the right is the result of hybridization of the HhaI-HinfI fragment when it has been radiolabeled only at the HhaI restriction endonuclease site, resulting in only the "RNA-like" strand being radioactive. The cellulose nitrate filter blot for this probe contained electrophoretically separated *Euglena gracilis* chloroplast RNA. Although the specific activity of the two probes was the same, the "RNA-like" probe gave only this faint signal, even after an exposure to x-ray film that was six times longer than that of the DNA-template probed autoradiogram. We suggest that the signal from the "RNA-like" strand of the psbA exon probe was due to minor contamination from a DNA template probe.

1.8, 1.5 (doublet), and 1.2 kb. The major signal at 1.2 kb is the mature psbA mRNA. The intron 2 specific oligonucleotide did not hybridize to the 1.2 kb mature mRNA, but did hybridize to the same precursors of 3.1, 2.8, 2.3 and 1.5 kb. In addition, a faint signal at 0.4 kb is also evident. This may be the excised intervening sequence from intron 2. In a control experiment (data not
Fig. 7. Hybridization of a radiolabeled psbA probe and a chemically synthesized, intron 2 specific probe to a filter blot of RNAs from different stages of *Euglena* chloroplast development separated on 1% formaldehyde/agarose gels. Left, the probe was a nick translated 1337 bp *ThaI*-*HhaI* fragment, which contains DNA sequences from the trnL-psbA spacer (*ThaI* site) to an internal site in exon 3 (*HhaI* site). Right, the same blot was treated at 95°C in 0.1 X SSC (1 min) and then washed in 20 X SSC at 22°C to remove the probe, re-exposed to X-ray film to determine that all radioactivity had been removed, and then re-hybridized with 5'-32P-GATGTTATTCTTTC.

shown) an intron 1 specific synthetic oligonucleotide, complementary to the opposite strand of DNA as the intron 2 probe, gave no detectable hybridization to any chloroplast RNA.

DISCUSSION

Gene Mapping on *Euglena* Chloroplast DNA Fragment *EcoI*

It has previously been reported that the 4.9 kbp *EcoRi* fragment *EcoI* of *Euglena* chloroplast DNA contains genes for one or more tRNAs,37,38,39 including a tRNA*Leu*40 *EcoI* has also been reported to contain genes for the 32 kd polypeptide associated with photosystem II, designated psbA,8,9,33 and a
second polypeptide of molecular weight 46,000 daltons. As is illustrated in Figure 2, the locus of these genes can now be described. The tRNA coding locus has been previously mapped via Southern hybridization experiments to the 1.8 kbp EcoRI–PvuII fragment of EcoI, more recently to the 500 bp SacI–PvuII region, and has now been sequenced (G. D. Karabin and R. B. Hallick, submitted for publication). The 3.0 kbp region to the side of the tRNA gene(s) that includes the unique HhaI restriction site is the locus of the psbA gene. On the opposite side of the tRNA locus is a portion of a gene with a 1.5 kb RNA transcript, which most likely encodes the 46,000 kd polypeptide identified by Keller et al.

RNA Transcripts of the psbA Locus

The most unexpected result of this study was the identification of at least six distinct RNA transcripts, ranging in size from 1.2–3.1 kb. This hybridization pattern for psbA transcripts is to date unique for Euglena chloroplast DNA. In a study of psbA transcripts from 19 angiosperms, Palmer et al. found that the major RNA in each case was 1.2 kb. A larger transcript was also present in only one species, sweet pea, but the size was only 1.4 kb. Dix and Rawson have also described EcoI transcripts based on Northern hybridization data, but did not associate these RNAs with specific genes. The RNAs were of 1.4, 2.0, 2.5, and 3.2 kb. These RNAs most likely correspond to the 1.2, 1.8, 2.3, and 3.1 kb RNAs described in the present study. Keller et al. also described major EcoI transcripts of 14S and 17S which we believe correspond to our 1.2 and 1.5 kb RNAs. They also noted the appearance of faint bands larger than 17S, but did not further characterize these RNAs. It is apparent from a comparison of the hybridization data of Keller et al. to the present results that our RNA is significantly less degraded. We believe that this is due to our use of aurintricarboxylic acid as an RNase inhibitor during RNA isolation.

We conclude that the larger transcripts of psbA in Euglena chloroplasts represent unprocessed precursors of the mature mRNA for the 32 kd photosystem II polypeptide. The mature mRNA size, which is the major transcript in Euglena, is 1.2 kb, the same as that found in higher plants. At least five processing events are required to produce the mature mRNA if a sequential processing pathway is assumed. Processing would proceed 3.1 kb → 2.8 kb → 2.3 kb → 1.8 kb → 1.5 kb → 1.2 kb.

The processing of the primary psbA transcript could involve three types of events. These are cleavage of a 5'-nontranslated leader sequence, removal of a 3'-nontranslated trailer sequence, and splicing of intervening sequences.
in the mRNA. From partial sequence analysis of the gene, there is evidence that psbA contains four intervening sequences. Therefore four of the psbA mRNA processing events are presumed to involve splicing reactions. We predict that most of the precursor transcripts detected are partially spliced mRNAs. A more detailed analysis of psbA mRNA precursor processing reactions is in progress. We hope to more precisely define mRNA splicing sites and the processing pathway. The detection of multiple, unspliced pre-mRNAs with an intron 2 probe is an initial indication that processing may not simply involve sequential removal of intervening sequences in the primary RNA transcript. It has already been possible to demonstrate that the intron 2 probe will serve as a primer for reverse transcription of intron 2 cDNA using total chloroplast RNA as template (U. Johanningmeier and R. B. Hallick, unpublished observations).

This is the second example of analysis of RNA transcripts of a split chloroplast protein gene. The gene for the large subunit of ribulose-1,5-bisphosphate carboxylase (rbcL) of Euglena has at least nine introns.35,43 Unlike the psbA RNA, unspliced rbcL transcripts are not readily detected.

Developmental Regulation of psbA mRNA

There is a significant increase in the level of psbA mRNA transcription during the course of light induced chloroplast development. The increase has not been rigorously quantitated. Based on densitometry of X-ray films from various Northern hybridization experiments, we estimate approximately a fivefold increase in RNA during the 72 hour period of chloroplast development. The largest increase in mRNA occurs during the zero hour to twelve hour time interval. There is no major shift during chloroplast development in the proportion of transcripts of the various precursor size classes.

Rawson et al.22 have used a solution hybridization approach to study developmental changes in EcoI transcripts in Euglena. They find that the abundance of EcoI RNAs, which would include both psbA and the 1.5 kb mRNA, increases from 50 to 350 copies of transcript per cell during 48 hours of greening. This sevenfold increase is in good agreement with the present findings. We can therefore conclude that there is a temporal change in the cellular content of psbA mRNA levels in Euglena gracilis during light induced chloroplast development.

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