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Differential Gene Expression in

C₄ plants

Research Proposal

for Period February 1, 1982 - January 31, 1983

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A. INTRODUCTION

For reasons that have been discussed previously in a letter (August 4, 1981) to Dr. Rabson, the topic of this research proposal is slightly different from that originally outlined. Specifically, instead of characterizing the genes encoding the small subunit of RuBP carboxylase and the chlorophyll a/b binding polypeptide from corn, I have been characterizing these genes from pea. The above polypeptides represent the major products of cytoplasmic protein synthesis in green leaves.

Considerable progress has been made with this project. As outlined in some detail below, cDNA clones encoding the above polypeptides have been isolated and characterized. A manuscript describing this work will appear in December, 1981 in Proc. Natl. Acad. Sci. USA. Both of these cDNA clones have now been sequenced, providing us with amino acid sequences for the carboxylase small subunit and, for the first time, for the chlorophyll a/b binding polypeptide. Pea nuclear DNA has been cloned into the lambda phage Charon 4, and cloned nuclear DNA sequences encoding the above polypeptides have been isolated. Future work will be concerned with the structural and functional characterization of these nuclear genes.

B. PROGRESS REPORT

Cloned cDNAs encoding the small subunit of RuBP carboxylase and the major chlorophyll a/b binding polypeptide We have constructed and characterized cloned cDNAs complementary to mRNAs encoding precursors to the small subunit of RuBP carboxylase and the major constituent polypeptide of the light-harvesting chlorophyll a/b protein complex. The recombinant plasmids were characterized by translation of hybridization selected mRNAs. Translation products were identified by SDS-polyacrylamide gel electrophoresis and by chloroplast uptake and processing studies.

The small subunit cDNA has been sequenced² and shown to encode 33 amino acids of the transit peptide, 123 amino acids of the mature protein and 140 nucleotides of the 3' non-coding region. An interesting finding was that the nucleotide sequence showed 21 differences in the coding region compared with the sequence reported by Bedbrook et al.³ for a pea small subunit cDNA. This may reflect an unexpected multiplicity of small subunit mRNAs. Alternatively, this discrepancy may simply reflect strain differences. In our case the mRNA was prepared from *P. sativum* (var. Progress No. 9) compared with *P. sativum* (var. Feltham First) used by Bedbrook et al.

The a/b cDNA has also been sequenced² and contains an 833 nucleotide cDNA insert encoding the chlorophyll a/b binding polypeptide. From the nucleotide sequence it has been possible to obtain the amino acid sequence of the chlorophyll a/b binding polypeptide.

Cloned nuclear DNA encoding the small subunit The small subunit cDNA was used as a hybridization probe for the selection of cloned complementary nuclear DNA sequences. Partial Eco R1 restriction digests of pea DNA were ligated to the DNA vector arms of the lambda phage Charon 4. The recombinant DNA was packaged in vitro and the phage were screened by plaque hybridization. One phage, ChPS163, which hybridized to the small subunit cDNA, was plaque purified and studied further.

DNA was prepared from ChPS163 and characterized by restriction with Eco R1,

Bam H1 and Hind 111. This analyses showed that the recombinant phage DNA contained 3 Eco R1 fragments (3.6, 4.7 and 8.0 kb) in addition to the vector arms of Charon 4 (Figure 1). Two of these fragments (3.6 and 8.0 kb), as well as two Bam H1 fragments (5.9 and 13 kb) and three Hind 111 fragments (0.5, 2.5 and 4.6 kb), were shown by Southern transfer and hybridization to contain sequences complementary to the cloned small subunit cDNA (Figure 2). The two complementary Eco R1 fragments (3.6 and 8.0 kb) and one of the complementary Bam H1 fragments (5.9 kb) were subcloned into the plasmid pBR327 to yield the recombinant plasmids pPSR4, pPSR6, and pPSB4 (Figure 3).

The complementarity of one of these subclones was tested further by translation of hybrid selected mRNA. DNA from the recombinant plasmid pPSB4, containing the 5.9 kb Bam H1 fragment, was adsorbed onto a nitrocellulose filter and this immobilized DNA was used to hybrid select complementary mRNA. Translation of this mRNA in a wheat germ cell free extract yielded the precursor for the small subunit of RuBP carboxylase (Figure 4).

Further restriction analyses of DNA from the phage ChPS163, and the subclones pPSR4, pPSR6 and pPSB4, yielded the restriction map shown in Figure 5. The two hybridizing Eco R1 fragments were shown not to be adjacent, but separated by the 4.7 kb Eco R1 fragment. This was first interpreted as possibly reflecting a large intervening sequence in the small subunit gene. However, the subclone pPSR6, containing the 3.6 kb Eco R1 fragment, was shown to hybridize to the 8.0 kb Eco R1 fragment of ChPS163 (Figure 2). The simplest interpretation of this result is that the recombinant phage ChPS163 carries two tightly linked small subunit genes. The alternative explanation is that the two Eco R1 fragments do contain a split small subunit gene and that the cross-hybridization of these fragments is due to repeated sequences flanking this gene. Heteroduplex studies or hybridization studies involving probes specific for the 3' and 5' ends of the small subunit cDNA, and ultimately, sequence studies, will distinguish these two possibilities.

Pea nuclear DNA has been restricted with Eco R1, and the fragments fractionated by electrophoresis. After Southern transfer to nitrocellulose the DNA was hybridized to ³²P-labeled small subunit cDNA. By this method the two cloned Eco R1 fragments were identified (3.6 and 8.0 kb) and three additional bands at 3.3, 5.4 and 9.8 kb were found. These observations indicate that there may be as many as five, or more, small subunit genes.

Cloned nuclear DNA encoding the chlorophyll a/b binding polypeptide By procedures essentially the same as that described above, a cloned nuclear DNA fragment encoding the chlorophyll a/b binding polypeptide has been isolated as a recombinant Charon phage (ChPA151). The phage DNA contains two Eco R1 inserts, of 3.1 and 8.0 kb, one of which (8.0 kb) hybridizes to the a/b cDNA. The phage DNA also contains two Hind 111 fragments (0.6 and 3.4 kb), and one Bam H1 fragment (25.3 kb) which hybridize to the a/b cDNA. The 8.0 kb Eco R1 fragment has been subcloned into pBR327 and translation of hybridization-selected mRNA confirmed that this fragment is complementary to mRNA encoding the precursor of the chlorophyll a/b binding polypeptide (Figure 4).

C. PROPOSAL

Characterization of the nuclear genes encoding the small subunit of RuBP carboxylase

(I) Heteroduplex and R loop analyses Pea nuclear DNA has been cloned into Charon 4 and a phage (ChPS163) has been isolated and characterized as containing two

Figure I. Restriction of DNA from phage ChPSI63. Phage DNA was restricted with Eco RI(R), Bam HI(B), and Hind III(H). The restriction fragments were separated by gel electrophoresis in 0.8% agarose gels.

Figure 2. Hybridization of restriction fragments from ChPSI63 DNA. Restriction fragments were separated by agarose gel electrophoresis and then transferred to nitrocellulose. Hybridization was with ³²P-labeled pSSI5 (small subunit cDNA) or pPSR6 (subclone of 3.6 kb Eco RI fragment from ChPSI63)

Figure 3. Subclones of Eco RI fragments from ChPSI63. The Eco RI fragments were subcloned into pBR327. The restriction digests show the 8.0 kb Eco RI fragment in pPSR4 and the 3.6 kb Eco RI fragment in pPSR6

Figure 4. Translation of hybridization-selected mRNA. Complementary mRNA was obtained by hybridization of pea poly A⁺ mRNA to DNA immobilized on nitrocellulose. Translation was in a wheat germ extract and the ³⁵S-methionine labeled polypeptides were fractionated by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. In sample 1, mRNA complementary to DNA from the phage ChPAI5I was translated (this phage is a genomic clone encoding the major chlorophyll a/b binding polypeptide). Sample 2 was translation of mRNA complementary to pPSB4 (this subclone contains the 5.9 kb Bam HI fragment from ChPSI63). Sample 3 was translation of pea poly A⁺ mRNA. p15,16 are precursors to the chlorophyll a/b binding polypeptides and pS is the precursor to the small subunit of RuBP carboxylase.

Figure 5. Restriction map of ChPSI63. The 19.8 kb and the 10.9 kb fragments represent the arms of Charon 4. The hatched areas represent restriction fragments which hybridize to pSSI5 (the cloned small subunit cDNA). They are delimited by Hind III or Eco RI restriction sites. Not all of the Hind III sites have been ordered and they are not included in this map.

ChPSI63

Bam HI ▮

RI ▲

regions of DNA which hybridize to the cloned cDNA (pSS15) encoding the small subunit of RuBP carboxylase. Eco R1 restriction fragments containing these two regions of hybridization have been subcloned into pBR327 to give the recombinant plasmids pPSR4 and pPSR6. The extent of homology within these three plasmids (pSS15, pPSR4 and pPSR6) will be examined by heteroduplex analyses.

The plasmids will be linearized with a restriction enzyme that cuts the plasmid vector once but not the insert. The restricted plasmids will be denatured and then allowed to anneal before spreading and staining according to the procedure of Davis et al⁴. The sample grids will be shadowed with Pt/Pd prior to visualizing in the electron microscope.

The heteroduplexes formed between the genomic subclones and the cDNA clone will allow us to map the regions of homology. Intervening sequences, if present, will be seen as looped regions of non-homology bounded by homologous coding sequences. Furthermore, heteroduplexes formed between pPSR4 and pPSR6 will allow us to determine whether the regions of homology known to exist between these two DNAs correspond to the regions that hybridize to the small subunit cDNA. That is, we will determine whether or not pPSR4 and pPSR6 represent distinct small subunit genes.

R loop analyses⁵ will be carried out to confirm and add to the above findings. Messenger RNA encoding the precursor to the small subunit of RuBP carboxylase will be purified by sucrose gradient centrifugation⁶. This RNA will be hybridized, under R looping conditions⁵, to DNA from the phage ChPS163 or to plasmid DNA from the subclones pPSR4 and pPSR6. Prior to R looping the DNA will be cross linked lightly as recommended by Kaback et al⁷. As with the heteroduplex analyses, these analyses will indicate intervening sequences, if present, and confirm or otherwise, whether or not pPSR4 and pPSR6 represent distinct small subunit genes. In addition, the direction of transcription may be determined, as the 3' end of mRNAs can often be seen in the electron microscope as a non-hybridizing poly A sequence. Furthermore, as the cloned cDNA is not quite full length, hybridization with the mRNA will allow us to locate the 5' CAP site and to look for the possible existence of intervening sequences at the 5' end of the gene.

(II) Restriction maps The small subunit genomic sequences have already been mapped with respect to the restriction enzymes Eco R1, Bam H1 and Hind 111 (some of the Hind 111 fragments have not yet been ordered). A more detailed restriction map will be prepared. This will be achieved, primarily, by electrophoretic fractionation of terminally labeled partial restriction digests. The Eco R1 inserts in the plasmids pPSR4 and pPSR6, and the Bam H1 insert in the plasmid pPSB4, will be excised and isolated by gel electrophoresis in low melting point agarose. The restriction fragments will be isolated from the gel by phenol extraction and then they will be labeled either at the 5' end using T4 polynucleotide kinase (after first removing the 5' phosphate with alkaline phosphatase) or at the 3' end using the Klenow fragment of E. coli DNA polymerase¹⁰. The labeled fragments will then be cleaved asymmetrically and the resulting fragments will again be separated by gel electrophoresis and isolated as above. Partial digests with, for example, Hinf 1, Sau 111A, Hpa 11 and Taq 1 will be performed. These partial digests will then be separated electrophoretically and the restriction sites ordered according to the method of Smith and Birnstiel⁹.

(III) DNA sequencing Definitive evidence concerning the structure of the cloned small subunit genes will be obtained by sequencing. It is proposed to use both

the Maxam and Gilbert⁸ and the M13¹⁰-Sanger dideoxy¹¹ procedures for DNA sequencing. We are presently using the former technique to sequence cloned cDNAs and the dideoxy procedure is routinely being used on campus in the Department of Genetics. M13 recombinant phage will be obtained from Sau 111A, Hpa 11 and Taq 1 digests of the Eco R1 inserts from pPSR4 and pPSR6. Recombinant phage will be selected by their inability to complement β -galactosidase mutants and, initially, sequence studies will be restricted to those phage which, by plaque hybridization, hybridize to either ³²P-labeled small subunit mRNA or cloned cDNA.

(IV) Sequence analyses We plan to sequence the entire coding region, the 3' and 5' non-coding region, and at least 200 nucleotides upstream from the 5' end of the small subunit genes. If the cloned genes represent expressed genes then the sequences will provide structural information concerning the N-terminus of the small subunit precursor(s). This information is not presently available as our cloned cDNA is not quite full length. In addition we hope to identify sequences which may be important in the regulation of expression of these genes.

Two regions of evolutionarily conserved sequences have been identified in eucaryotic genes as potential polymerase II transcriptional regulatory elements. The most ubiquitous of these, the TATA box, is normally located approximately 30 nucleotides 5' from the putative transcriptional start site of the structural gene^{12,13}. Because of its conserved nature, and its apparent essential role in transcription, as indicated by in vitro transcription studies, it was earlier concluded that this TATA sequence represented the major component of a structural gene promoter¹⁸. More recently however, in vivo expression assays have indicated that this sequence is dispensable and that sequences further upstream are essential for efficient levels of transcription^{14,15,16}. Computer assisted sequence comparisons have detected a pentanucleotide homology, CCAAT, that is centered roughly 80 nucleotides upstream from eucaryotic structural genes^{12,13,16}.

The sequences of the small subunit genes will be examined to see whether sequences exist analogous to those discussed above. Alternatively, homologous, potential transcriptional regulatory sequences, distinct from those characterized for animal and viral genes, may be found by comparisons within the small subunit genes and by comparisons with other plant gene sequences that will become available.

If the small subunit genes contain intervening sequences then we will compare the splicing sequences with the consensus sequences that have been derived for animal and viral genes^{17,18}. The sequences around plant splicing junctions appear, so far, to be consistent with the Breathnach-Chambon rule¹⁹ as the intervening sequences start with GT and end with AG.

Further sequences of interest will include those thought to be involved in termination and the addition of poly A²⁰, and sequences in the vicinity of the CAP site. The CAP site, at which RNA synthesis is thought to start, will be identified by using the primer extension technique²¹. Short restriction fragments will be end-labeled, strand separated, and then used to prime reverse transcripts complementary to the 5' end of the mRNA. The size of the extended primer will be accurately determined by DNA sequencing gels and this will allow the CAP site to be determined. This site is characterized in sea urchin histone mRNAs, by the short sequence homology, pyCATTCPu²², and the sequence CUUPyUG^{13,23} is common to the 5' non-coding regions of many other eucaryotic mRNAs. Sequence comparisons of the types discussed above will be aided by the necessary computer facilities and

programmes that are available on campus.

Characterization of the nuclear genes encoding the chlorophyll a/b binding polypeptides The nuclear genes encoding the chlorophyll a/b binding polypeptides will be studied, by the procedures outlined above, in parallel to the studies on the small subunit genes.

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