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Chloroplast ribosomal DNA organization in the chromophytic alga *Olisthodiscus luteus**

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Summary. There are almost no data describing chloroplast genome organization in chromophytic (chlorophyll a/c) plants. In this study chloroplast ribosomal operon placement and gene organization has been determined for the golden-brown alga *Olisthodiscus luteus*. Ribosomal RNA genes are located on the chloroplast DNA inverted repeat structure. Nucleotide sequence analysis, demonstrated that in contrast to the larger spacer regions in land plants, the 16S-23S rDNA spacer of *O. luteus* is only 265 bp in length. This spacer contains $tRNA^{Ile}$ and $tRNA^{Ala}$ genes which lack introns and are separated by only 3 bp. The sequences of the tRNA genes and 16S and 23S rDNA termini flanking the spacer were examined to determine homology between *O. luteus*, chlorophytic plant chloroplast DNA, and prokaryotes.

Key words: Chloroplast DNA – Chlorophyll a/c alga – Evolution – Ribosomal operon

Introduction

The acquisition of photosynthetic capability represents a major transition point in eukaryotic cell evolution. It is hypothesized that this event occurred approximately 570 million years ago within the Cambrian or possibly late Precambrian era. Although the paleobiological record is incomplete, early photosynthetic eukaryotes are presumed to have been algal cells. The emergence of vascular plants then followed approximately 170 million years later (Banks 1975).

Extant plants contain photosynthetic organelles that display a wide range of morphological, biochemical and genetic variation. For example, chloroplasts of chlorophytic (chlorophyll a/b), chromophytic (chl a/c) and rhodophytic plants (chl a/phycobilins [PB]) each display a distinct combination of thylakoid and outer membrane arrangement, pigment array, nucleoid structure and genome profile (Cattolico 1986; Coleman 1985; Gibbs 1981; Palmer 1985a; Whatley and Whatley 1981). Although chloroplast identity has been used to assign most plants to the three major superphyla listed above, unusual organisms with disputed phylogeny exist. These enigmatic organisms frequently display morphological and biochemical aspects of at least two phyla. For example, the unicellular alga *Cryptomonas* spp. (Gillott and Gibbs 1980; Ludwig and Gibbs 1985; Morrall and Greenwood 1982) has chl a/c and phycobilin pigments as well as a vestigial nucleus that is located between inner and outer chloroplast membrane pairs. In the Pyrrophyta, the binucleate dinoflagellate *Peridinium balticum* is composed of an aplastidic host that contains an obligately symbiotic cell. The symbiont possesses its own nucleus and cytoplasmic components, including a large array of chloroplasts (J. M. Chesnick, pers comm; Tomas and Cox 1973).

The ancestral events that could have initiated the present day spectrum of chloroplast types are the subject of much debate. Although the cluster-clone hypothesis (Bogorad et al. 1973) of chloroplast evolution has not been entirely abandoned it is more widely accepted that a symbiotic interaction between a colorless host and a photosynthetic prokaryote initiated photosynthetic eukaryote construction (Cavalier-Smith 1982; Gibbs 1981; Gray and Doolittle 1982; Raven 1970; Taylor 1979; Whatley and Whatley 1981). Proponents of the monophyletic scheme of chloroplast acquisition maintain that a single symbiotic event followed by a long and productive evolution accounts

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for chloroplast diversity. In contrast, a polyphyletic chloroplast origin proposes that plastid diversity is due to differences in the photosynthetic cell type engulfed.

To facilitate the understanding of chloroplast evolution, plastid genomes from different taxa should be analyzed using criteria applicable to all chloroplasts and their ancestors. Ribosomal RNA (rRNA) offers both a conserved nucleotide array and a highly distinct secondary structure and thus serves as an ideal choice for constructing evolutionary comparisons among diverse phyla (Fox et al. 1980; Gray et al. 1984). Although the organization of rRNA genes within an operon is highly conserved (5'-16S-spacer-23S-5S-3') in both cyanobacterial and chloroplast DNA (reviewed in Whitfeld and Bottomley 1983), it has been suggested (Cattolico 1986) that the global arrangement of this operon on cpDNA may have significant phylogenetic implication. For example, in all land plants studied to date, the ribosomal operons are localized in the inverted repeat of the chloroplast genome (Whitfeld and Bottomley 1983). There is a single exception to this observation. It has been hypothesized that the presence of only one rRNA operon in selected tribes of the legume subfamily Papilonoideae represents a mutational loss of these genes (Palmer and Thompson 1982; Palmer 1985b). Thus the presence of a single ribosomal operon in these plants serves as an excellent phylogenetic clustering device. The well studied *Euglena gracilis* system provides additional examples of how operon arrangement might serve as a taxonomic indicator. The fact that the chloroplast rDNA of *Euglena* is arranged in tandem rather than inverted array segregates this alga to a distinct evolutionary line probably well removed from other green algal representatives which are ancestral to land plants. On a more refined level, polymorphisms in the lengths of the interoperon spacers distinguish *Euglena gracilis* var. *bacillaris* from other strains of this species (Helling et al. 1979; Wurtz and Buetow 1981).

To date, there are few data that describe chloroplast rDNA operon organization of chl a/PB or chl a/c plants. Information on these algae is critical to the understanding of chloroplast evolution, and potentiates a more critical phylogenetic assessment in plants that are frequently morphologically and biochemically plastic. In this study, chloroplast rDNA gene organization of the chl a/c alga *Olisthodiscus luteus* is analyzed and the nucleotide sequence of the spacer region that separates 16S and 23S rDNA is presented.

Materials and methods

Materials. All chemicals used were reagent grade. The sources of specialized reagents and enzymes are given in the text.

Cell culture and plastid DNA preparation. Axenic cultures of *O. luteus* were grown in artificial seawater medium (McIntosh and Cattolico 1978). Approximately 2.4 l of cell culture were harvested during late log phase growth (1×10^5 cells/ml; Cattolico 1978) by centrifugation at $1{,}590 \times g$ for 5 min. The cell pellets were resuspended in extraction buffer (50 mM Tris, 50 mM EDTA, pH 8.0) at 5°C to a final colume of 9 mls and gently disrupted using a Dounce homogenizer. A mixture of 1.0 ml 20% SDS and 0.25 ml of Hoechst 33258 dye was then added (Sigma, 10 mg/ml in H_2O; Aldrich and Cattolico 1981). The detergent/dye/cell mixture was maintained at 5°C for 60 to 90 s to allow complete cellular lysis before one gram of solid CsCl (Gallard Schlesinger) per ml of lysate was added and its density adjusted to 1.66 g/cc (RI = 1.3960). The lysate was then centrifuged at $180{,}000 \times g$ in a fixed angle rotor for 18 h and DNA visualized using an UV light. The three DNA species which were separated by this procedure included nuclear (highest density), mitochondrial (middle density) and chloroplast DNA (lowest density). Chloroplast and mitochondrial DNA collected from four gradients was then pooled into a single five ml tube and recentrifuged as described above except that a vertical tube rotor was used. Chloroplast DNA was collected and centrifuged a third time using a vertical tube rotor after adding 200 µg/ml ethidium bromide (Sigma) and adjusting the solution density to 1.55 g/cc (RI = 1.3860). Following collection of the DNA from this gradient, Hoechst and ethidium bromide dye was removed by four extractions using isopropanol saturated with NaCl saturated TE (10 mM Tris, 1 mM EDTA, pH 8.0). The sample was dialyzed at 5°C against TE buffer after which 50 µl of 3.0 M sodium acetate (pH 6.0) was added. The volume of the dialyzate was then reduced to 0.5 mls by repeated extractions with 1-butanol. DNA was then precipitated at −20°C in a microfuge tube with 1.0 ml of 95% ethanol. After centrifugation, the pelleted DNA was resuspended in 50 µl TE and stored at −20°C.

Restriction endonuclease digestions. Restriction enzymes were purchased from Bethesda Research Laboratories (BRL), and were used according to the suppliers recommendations. When necessary, double enzyme digestions were carried out sequentially, by adjusting the primary enzyme reaction mix to accommodate the salt and pH requirements of the second enzyme.

Agarose gel electrophoresis and Southern transfer. Gel electrophoresis was done using gels composed of 0.6% agarose. The gel and running buffer each contained 89 mM Tris, 89 mM boric acid, 1 mM EDTA, pH 8.2 (TBE buffer). DNA samples were run adjacent to size standards of Lambda DNA *Hin*dIII digestion products and/or a commercially available one kilobase ladder marker (BRL). Following ethidium bromide staining (0.5 µg/ml), gels were placed on an ultraviolet transilluminator and photographed using a Kodak Wratten 22a orange filter and Polaroid Type 55 film. The DNA was then transferred to Gene Screen Plus using the method of Southern (1975) which has been modified by the transfer membrane supplier (New England Nuclear Inc.).

In those experiments where DNA strands were separated from one another, a gel slice containing the chosen DNA fragment was excised from an agarose gel, and the DNA denatured in situ and electrophoresed in a second agarose gel (Hayward 1972). After the bromophenol blue tracking dye had migrated halfway, the gels were photographed and Southern blotted as described above.

Preparation of DNA probes. Cloned *Euglena gracilis* chloroplast rDNA was supplied by Dr. J. Rawson (Plant Breeding Institute, Standard Oil, Ohio) as the clone pECRB4. This clone was digested with *Eco*RI and *Bam*HI, producing 2.5 and 2.7 kbp fragments which contain the entire rDNA operon. These fragments were recovered from gel slices by electroelution into dialysis membrane-bags (Maniatis et al. 1982), and impurities were removed using Elutip-R

minicolumns according to the manufacturers guidelines (Schleicher & Schuell). The DNA was then radiolabelled using nick translation (Rigby et al. 1977). Unincorporated radionucleotide was removed by Sephadex G-25 (Pharmacia) gel-filtration in 1.0 cc syringes using the spun column technique (Maniatis et al. 1982).

Alternatively, radioactive probes were generated by digesting plasmid DNA with the appropriate restriction enzymes, nick translating the resultant fragments prior to electrophoresis, and excising specific fragments from an agarose gel. These gel slices were then melted in 2.0 ml of hybridization buffer at 95°C for 10 min prior to use as probes.

DNA hybridization and autoradiography. Southern blots of gel fractionated DNA were prehybridized in 1 M NaCl, 1% SDS (1 ml per 10 cm^2 blot) for 6 to 10 h at 65°C. Probe DNA which had been denatured at 95°C for 10 min was then added to the prehybridization mixture, and incubated overnight at 65°C. Blots were then washed twice for five min in 2×SSC (1×SSC is 0.15 M NaCl, 0.015 M NaCitrate; pH 7.5) at 20°C, followed by two 30 min washes in 2×SSC, 1% SDS at 65°C. Autoradiography was accomplished by exposing the blot to Kodak X-Omat AR film for 1 to 12 h. The film was then processed according to the manufacturers recommendations.

Molecular cloning and restriction site mapping. To characterize *O. luteus* chloroplast rDNA, the two regions of the plastid DNA which contain these genes were cloned. Three µg of cpDNA and 0.1 µg of the plasmid pUC19 (Vieira and Messing 1982) were digested to completion with *Xba*I, ligated overnight at 15°C, and transformed into competent *Escherichia coli* strain HB101 as described by Maniatis et al. (1982). Transformants were plated on LB (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl) agar containing ampicillin (50 µg/ml) and X-Gal (40 µg/ml; 5-bromo-4-chloro-3-indolyl-β-D-galactoside, BRL) to yield 300 to 400 colonies per plate after 15 h of growth at 37°C. Ten plates were analyzed using the colony hybridization method of Grunstein and Hogness (1975). The radioactive DNA probe used for this screening was a 0.8 kbp *Kpn*I fragment of *O. luteus* 23S rDNA that had previously been cloned into pUC19. In earlier experiments, this fragment was found to hybridize with *E. gracilis* 23S rDNA in the clone pECRB4. Colonies which gave a positive response were grown overnight in 5.0 ml of LB broth containing ampicillin (50 µg/ml). Plasmid DNA was extracted from 1.5 ml of this culture using the alkaline lysis method of Birnboim and Doly (1979). To verify the identity of cloned DNA, restriction enzyme and blot hybridization analyses were used. The cloned rDNA was fine-structure mapped using restriction enzymes that have fewer than five recognition sites within the cloned fragment.

Preparation of RNA probes. RNA was extracted from *O. luteus* (McIntosh and Cattolico 1978). The 23S and 16S rRNA species were fractionated by electrophoresis in 0.6% agarose gels made in TBE buffer. 5S rRNA was recovered using gels composed of 25% glycerol, 8% polyacrylamide (19:1 acrylamide: bisacrylamide; BRL Ultrapure), polymerized with 0.03% w/v ammonium persulfate (BRL), 0.06% v/v N,N,N′,N′-tetramethylenediamine (BioRad) and made in TBE buffer. The 23S, 16S and 5S rRNA of *E. coli* (Boehringer Mannheim), yeast tRNA (Sigma), and ØX174 DNA *Hae*III digestion products (BRL) were used as molecular weight markers. Electrophoresis was at 5°C in TBE buffer. Gels were stained with ethidium bromide (0.5 µg/ml) and gel slices containing the desired rRNA species were excised. Electroelution at 5°C in 0.5×TBE buffer was used to transfer nucleic acid from gel slices into dialysis bags. The identity of a recovered rRNA species was verified by electrophoretic comparison with purified rRNA obtained from *O. luteus* chloroplast ribosomes. The 23S and 16S rRNAs were hydrolyzed at ph 9.5 for three min at 90°C. All rRNAs were end-labelled using T4 polynucleotide kinase (BRL) and γ-^{32}P-labelled ATP (>3,000 Ci/mM) as described by Bogorad et al. (1983).

Hybridization of rRNA probes to blotted DNA. Blots containing DNA were prehybridized in 50% formamide and 5×SSC at 43°C for 6 to 10 h. After this treatment, ^{32}P-labelled rRNA was added and hybridization continued overnight at 43°C. Blots were then washed and autoradiographed as described above.

DNA sequencing. The dideoxy technique of Sanger et al. (1977) for DNA sequence determination was used. An *Eco*RI 3.35 kbp fragment which spans the rDNA spacer region was chosen for initial sequence analysis. To subclone this fragment into the sequencing vectors M13mp18 and M13mp19 (Messing and Vieira 1982), this fragment was first cloned into pUC19, and the resultant recombinant plasmid DNA digested with *Eco*RI, *Sst*I and *Sma*I. This triple digest cleaves the cloned *Eco*RI fragment into four pieces (an *Eco*RI-*Sst*I, *Sst*I-*Sma*I, *Sma*I, and a *Sma*I-*Eco*RI fragment) which were then cloned into appropriately digested M13mp18 and M13mp19. A portion of each ligation was transfected into JM109 and the single stranded phage DNA subjected to sequence analysis. To obtain the entire sequence of the 0.9 kbp *Sma*I fragment, a systematic cloning strategy was used, which allowed the generation of a series of deletion mutations than span the region of interest (Dale et al. 1985). Materials for this procedure were obtained from International Biotechnologies (Cyclone Biosystem kit).

DNA sequence similarity analysis was accomplished using the Genepro software package (Riverside Scientific Enterprises; Seattle, WA) for MS-DOS computers. The EMBL and Genbank databases were accessed for sequences listed in Fig. 6 (Brosius et al. 1978; Douglas and Doolittle 1984; Dron et al. 1982; Graf et al. 1980; Janssen et al. 1987; Loughney et al. 1982; Markowics et al. 1988; Orozco et al. 1980; Rochaix and Darlix 1982; Schneider and Rochaix 1986; Takaiwa and Sugiura 1982a, b; Tohdoh and Sugiura 1982; Tomioko and Sugiura 1983; Williamson and Doolittle 1983; Young et al. 1979). Sequence comparison calculations treated gaps and substitutions equally.

Results

Position of rDNA on O. luteus plastid DNA

The chloroplast DNA (cpDNA) of *O. luteus* has been physically mapped (Reith and Cattolico 1986) using the restriction endonucleases *Bam*HI, *Bgl*II, *Pst*I, and *Sal*I. This algal genome is organized into large (73 kbp) and small (37 kbp) single-copy regions separated by two repeated DNA segments (each approx. 22 kbp) which are present in an inverted orientation.

Cloned *Euglena gracilis* cpDNA was used to determine the location of *O. luteus* chloroplast rDNA. The *E. gracilis* probe which contains an entire rDNA operon, hybridized to *O. luteus* cpDNA *Bam*HI 15.1 and 22; *Bgl*II 32.5 and 28.5; *Pst*I 13.5 and 21; as well as the *Sal*I 33 and 14.3 kpb generated fragments (Fig. 1). These fragments originate from the inverted repeat (IR) region of this algal cpDNA.

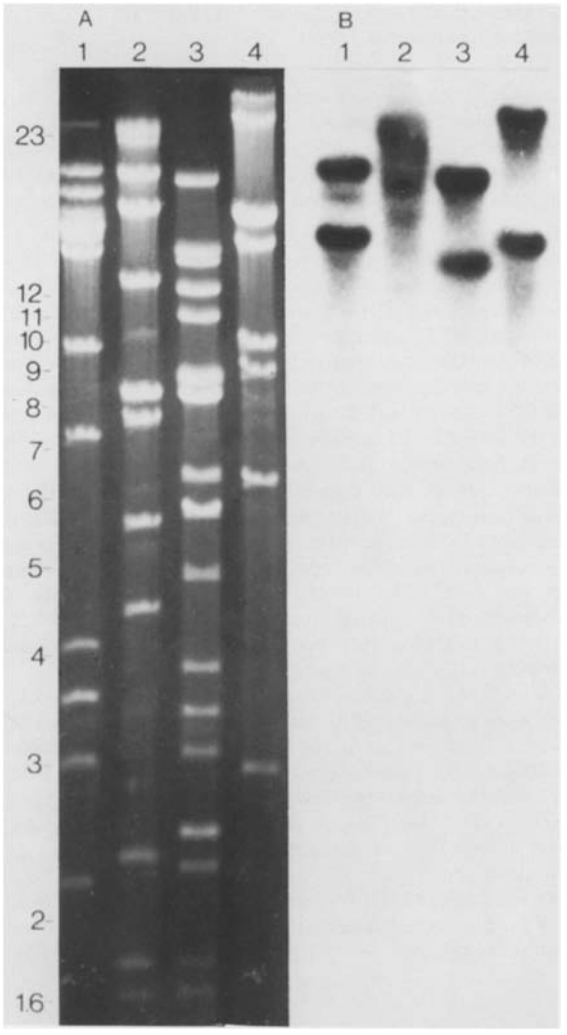

Fig. 1A–B. Hybridization of a heterologous rDNA probe to *O. luteus* plastid DNA. **A** Ethidium bromide stained agarose gel of restriction endonuclease digests of *O. luteus* cpDNA. **B** Corresponding autoradiogram of the Southern blot following hybridization to *E. gracilis* rDNA from clone pECRB4. *Lanes 1–4* represent single digests of *O. luteus* cpDNA using the enzymes *Bam*HI, *Bgl*II, *Pst*I, and *Sal*I, respectively, Size standards (kbp) are presented on the vertical axis

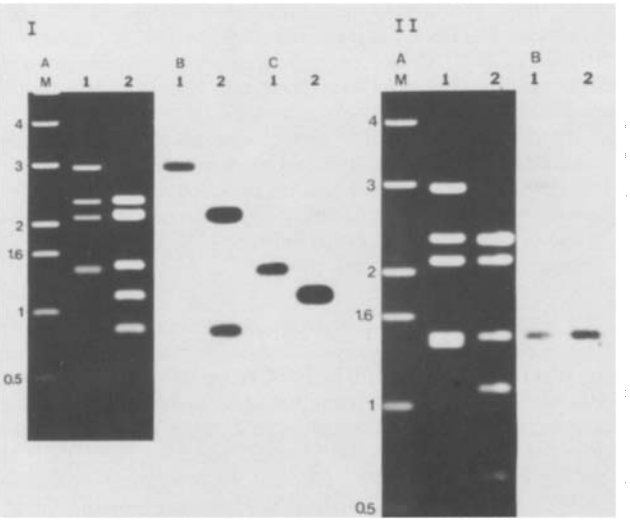

Fig. 3I–II. Hybridization of *O. luteus* chloroplast 23S, 16S and 5S ribosomal RNA to cloned *O. luteus* cpDNA. **I** (*A*) Ethidium bromide stained agarose gel; *lane M* 1 kb ladder marker with fragment sizes indicated at left (kbp); *lane 1* clone pOCX8.1 digested with *Xba*I + *Pvu*II + *Sst*I; *lane 2* clone pOCX8.1 digested with *Xba*I + *Pvu*II + SstI + *Nco*I. (*B* and *C*) Southern blot autoradiograms of replicate agarose gels when probed with 23S (*B*) and 16S (*C*) rRNA. **II** (*A*) Ethidium bromide stained agarose gel; *lane M* 1 kb ladder marker with fragment sizes indicated at left (kbp); *lane 1* clone pOCX8.1 digested with *Xba*I + *Pvu*II + *Sst*I; *lane 2* clone pOCX8.1 digested with *Xba*I + *Pvu*II + *Sst*I + *Sma*I. *B* Southern blot autoradiogram of agarose gel when probed with 5S rRNA

Fine structure mapping of the rDNA

Gel fractionation of *O. luteus* chloroplast rRNA revealed only three species. These have mobilities comparable to *E. coli* 23S, 16S and 5S rRNA. To determine the organization of these genes within the ribosomal operon of *O. luteus*, cpDNA was digested with *Xba*I and probed with a 0.8 kbp *Kpn*I fragment of *O. luteus* 23S rDNA. Two restriction fragments (8.1 and 9.3 kbp) hybridized, which originate from the left and

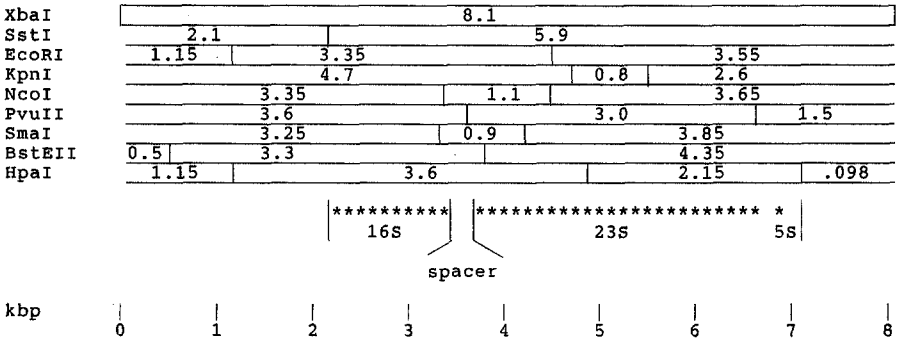

Fig. 2. Physical map of the *rrn*A operon of *Olisthodiscus luteus* plastid DNA. An *Xba*I generated fragment that spans the junction between the large single-copy and inverted repeat regions of the genome is presented. The left margin lists restriction endonucleases; cleavage sites and sizes of resultant fragments are indicated on the map (kbp). Below the map is shown the location of the 16S, 23S, and 5S rDNA

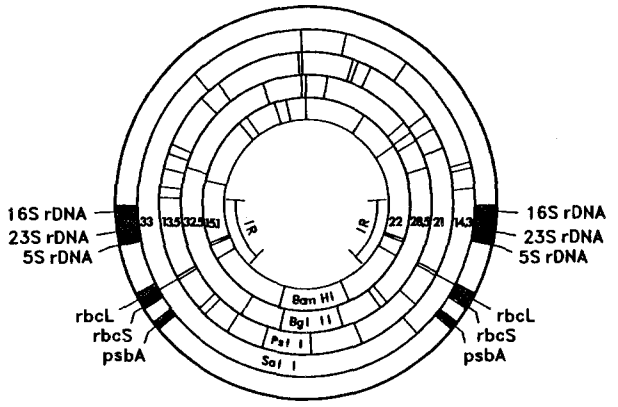

Fig. 4. Physical map of the plastid genome of *Olisthodiscus luteus*. The cleavage sites for the restriction enzymes listed are shown as radial lines. Those restriction fragments that hybridize to heterologous rDNA probes are labelled with their size in kbp (see Results). The inverted repeat (*IR*) and positions of gene loci are labelled (Reith and Cattolico 1986, and this communication)

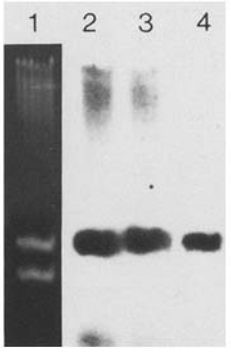

Fig. 5. Ribosomal RNA hybridization to separated strands of cloned *Olisthodiscus luteus* chloroplast rDNA. *Lane 1* Ethidium bromide stained agarose gel of denatured and fractionated rDNA (a 5.9 kbp *Sst*I + *Xba*I fragment of pOCX8.1). *Lanes 2, 3, 4* represent corresponding autoradiograms from replicates of the gel in lane 1 when probed with 23S, 16S and 5S rRNA respectively

right copy, respectively of the IR as drawn in Fig. 4. These fragments were cloned and a representative *Xba* 8.1 clone, designated pOCX8.1, was chosen for further study. Figure 2 illustrates the physical map of this region, showing the positions of the recognition sites for the enzymes: *Xba*I, *Sst*I, *Eco*RI, *Kpn*I, *Nco*I, *Pvu*II, *Sma*I, *Bst*EII, and *Hpa*I. To place the 16S, 23S, and 5S rRNA genes on this fine-structure map, Southern blots of pOCX8.1 DNA digested with the enzymes listed above were hybridized to radioactive *O. luteus* chloroplast ribosomal RNA. Results shown in Fig. 3 and summarized in Fig. 2, demonstrate that the 23S, 16S and 5S rRNA hybridize to the *Pvu*II 3.0, *Pvu*II-*Sst*I 1.45, and *Pvu*II-*Xba*I 1.5 kbp fragments respectively. The right border of 5S rDNA as drawn in Fig. 2 was positioned by the lack of hybridization of 5S rRNA to a *Hpa*I-*Xba*I 0.98 kbp fragment. Through the alignment of shared restriction sites between (a) pOCX8.1, (b) a *Kpn*I-*Sal*I 8.3 kbp clone which overlaps the *Kpn*I 2.6 kbp fragment of pOCX8.1 (data not shown), and (c) the *Bam*HI, *Bgl*II, *Pst*I, and *Sal*I map of the plastid genome generated by Reith and Cattolico (1986), it was found that the ribosomal operon is positioned in the inverted repeat such that the 16S gene lies nearest the large single-copy region, and the 23S rDNA nearer the small single-copy region of the chloroplast genome (Fig. 4).

Strand selection during rDNA transcription

It was of interest to distinguish whether *O. luteus* rRNA species are transcribed from the same or from different DNA strands. While the former organization is most common, chloroplast rDNA of *Chlorella ellipsoidea* is unusual, having 16S and 23S rDNA transcribed convergently from opposite strands (Yamada and Shimaji 1986). When clone pOCX8.1 was digested with *Sst*I and *Xba*I, three fragments were generated: a 2.7 kbp fragment which contained the vector (pUC19), a 5.9 kbp region containing the rDNA operon and a 2.1 kbp fragment representing the remainder of the cloned cpDNA. The 5.9 kbp fragment was isolated, alkali denatured and rerun on an agarose gel. This method allows clear separation of the complementary DNA strands (Fig. 5, lane 1). Southern blots of the separated strands were probed with either 23S, 16S, or 5S rRNA. As shown in Fig. 5, each of the three rRNA species recognized the same DNA strand indicating a common direction of transcription.

Molecular profile of the 16S-23S rDNA spacer region

The mapping studies presented in Fig. 3 demonstrate that 16S and 23S rDNA are separated by a short spacer of less than 400 bp. To fully understand the organization of this compact region and to allow comparison to ribosomal spacer regions which are found in other algal and land plant taxa, this DNA was sequenced. An *Eco*RI 3.35 kbp fragment of pOCX8.1 was chosen for initial sequence analysis because hybridization studies had shown that *O. luteus* 16S and 23S rRNA each hybridized to this fragment (data not shown), indicating that the spacer is contained entirely within this region. To more precisely locate the spacer region, the *Sst*I/*Sma*I digestion products of the *Eco*RI 3.35 kbp fragment were subcloned into the sequencing vectors M13mp18 and M13mp19 (see Methods). This allowed sequence determination to be made at several sites within this fragment. Subclones containing both orientations of the *Sma*I 0.9 kbp fragment, were found to

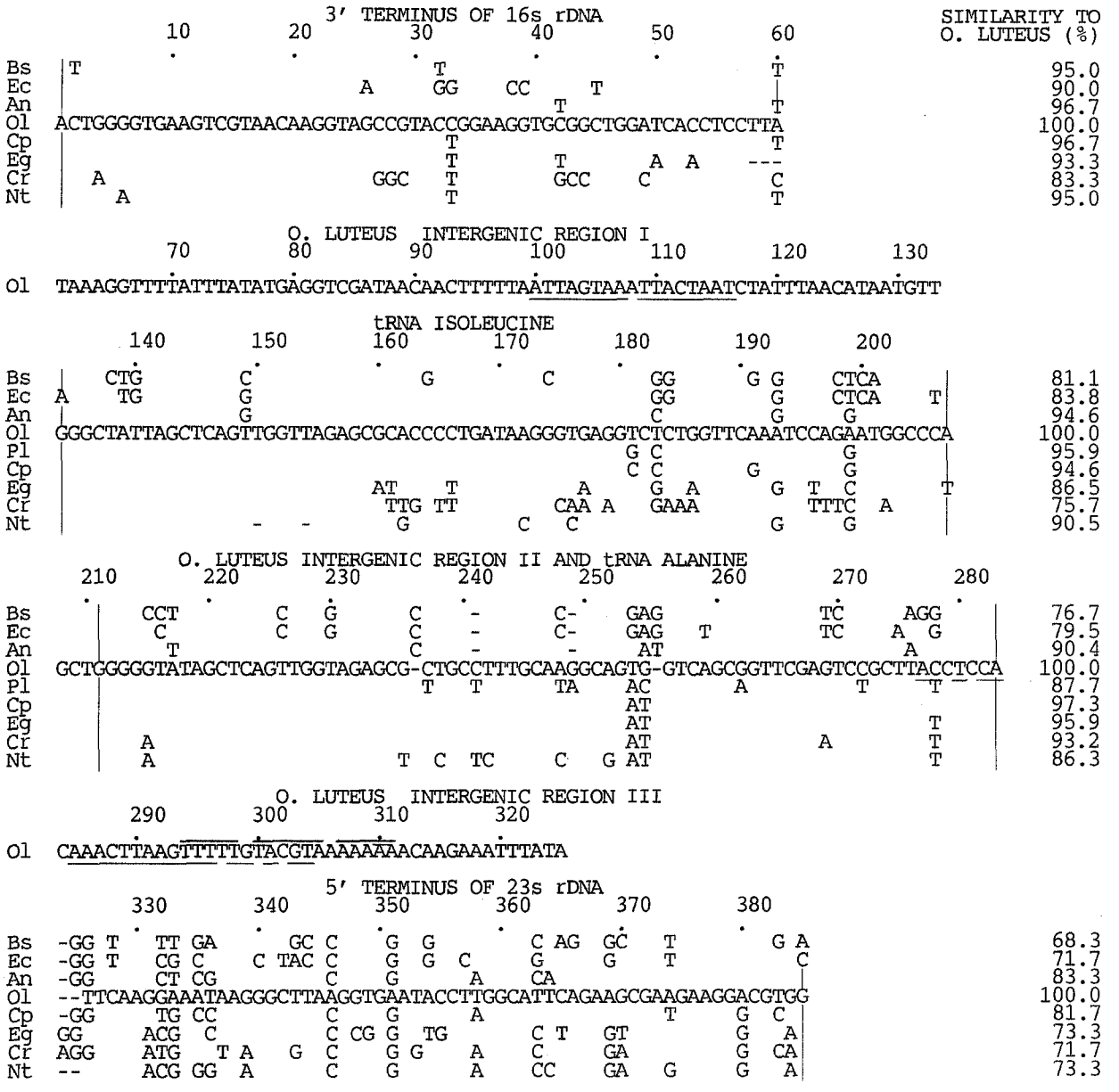

Fig. 6. Sequence of the *Olisthodiscus luteus* chloroplast rDNA spacer and flanking regions. DNA sequence similarity of *O. luteus* (01) spacer DNA to *Bacillus subtilis* (Bs), *Escherichia coli* (Ec), *Anacystis nidulans* (An), *Cyanophora paradoxa* (Cp), *Euglena gracilis* (Eg), *Chlamydomonas reinhardii* (Cr) and *Nicotiana tabacum* (Nt) 16S rDNA, $tRNA^{Ile}$, $tRNA^{Ala}$ and 23S rDNA genes. Only the tRNA genes of *Pylaiella littoralis* (Pl) are shown. The *O. luteus* sequence is listed in full, including 60 bp of the flanking 16S and 23S rDNA. For clarity, only those nucleotides that differ from the *O. luteus* sequence are typed. At the left and right limits of each reference sequence, a *vertical tick mark* indicates identity with the *O. luteus* sequence while divergent nucleotides are listed. Palindromes are indicated by *under-* and *over-lining* at positions 100-116, 277-304, and 294-311. Only those nucleotides capable of base-pairing within a stem-loop structure are *lined*

have sequence homology to either the 3′ end of 16S rDNA or the 5′ end of 23S rDNA, showing that this fragment contains the entire rDNA spacer. These clones were used in the generation of overlapping deletion mutants, which were then sequenced. The entire spacer region and portions of the flanking 16S and 23S rDNA shown in Fig. 6 were sequenced from both strands to ensure the accuracy of our results.

In summary, a total of 700 contiguous nucleotides containing the rDNA spacer and its flanking regions were sequenced, including 160 bp of the 16S and 275 bp of the 23S rDNA. Comparison of the derived sequence with sequences in the EMBL and Genbank databases have shown: (a) $tRNA^{Ile}$ and $tRNA^{Ala}$ are present in the 265 bp spacer region and that these genes are separated by only three nucleotides and do not have introns; (b)

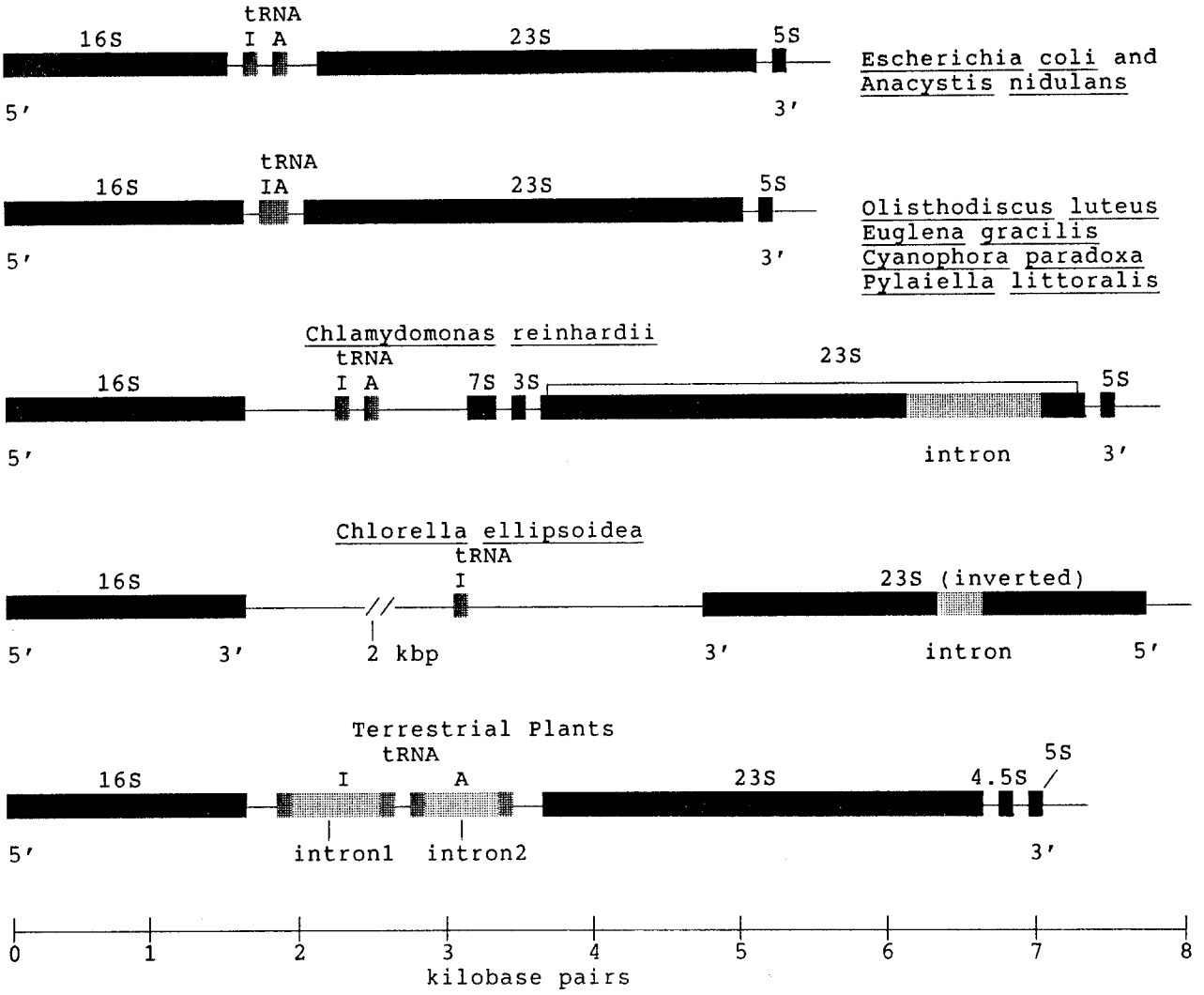

Fig. 7. Comparison of prokaryotic and plastid ribosomal operons. Polarity of coding sequences are 5′ to 3′ except *C. ellipsoidea*, where the 23S rDNA is in opposite orientation. Introns are indicated within the *C. reinhardii, C. ellipsoidea* and land plant operons. Transfer RNA genes specifying isoleucine and alanine are depicted by *I* and *A* respectively (reviewed in Bohnert et al. 1982; Palmer 1985b; Whitfeld and Bottomley 1983; Yamada and Shimaji 1986; Yamada and Shimaji 1987

the spacer region of the *O. luteus* ribosomal operon comprises 73 nucleotides of intergenic DNA 5′ to $tRNA^{Ile}$ and 42 bp of intergenic DNA 3′ to $tRNA^{Ala}$, and (c) the termini of the 16S and 23S rDNA have extensive similarity to published sequences for these genes from other sources (Fig. 6).

Discussion

O. luteus chloroplast DNA contains two ribosomal DNA operons that are localized in the inverted repeat portion of this organelle genome. Whether this arrangement will be as ubiquitous in chromophytic or rhodophytic representatives as it is observed to be in chlorophytic plants is open to speculation. The fact that *Dictyota dichotoma*, a dichotomously branched brown alga, has an inverted repeat (Kuhsel and Kowallik 1987), while the red alga *Griffithsia pacifica* lacks an inverted repeat (Li and Cattolico 1987) suggests that more variability in ribosomal operon placement might occur in these superphyla than that which is observed in chlorophytes.

The *O. luteus* spacer region between 16S and 23S rRNA genes codes for the two transfer RNAs, $tRNA^{Ala}$ (UGC) and $tRNA^{Ile}$ (GAU). These genes have been found within chloroplast spacers of every plant analyzed to date except *Chlorella ellipsoidea* (Yamada and Shimaji 1986) where only $tRNA^{Ile}$ is present. The *O. luteus* spacer encoded tRNAs are identical to those of all chloroplasts and cyanobacteria examined in their anticodon specificity (Crouse et al. 1985) and in their ability to form a characteristic cloverleaf secondary

structure (Holley et al. 1965). Sequence analysis demonstrates that the *O. luteus* tRNA genes do not include the highly conserved 3′ terminal-CCA which is found in mature tRNA. This observation suggests that these three nucleotides are added post-transcriptionally, as in chlorophytic plants (reviewed in Whitfeld and Bottomley 1983). It is interesting to note that the -CCA trinucleotide set is included in the coding sequence of the tRNA genes in most eubacteria, and that an intermediate condition is found in the cyanobacterium *Anacystis nidulans* where only $tRNA^{Ala}$ but not $tRNA^{Ile}$ codes for the -CCA terminus (Williamson and Doolittle 1983).

DNA sequence comparisons between the two *O. luteus* transfer RNAs to those found within ribosomal spacers of chloroplasts and prokaryotes were presented in Fig. 6. The *O. luteus* genes show greatest similarity to those from *C. paradoxa* and *A. nidulans*. These genes are of insufficient size to allow firm conclusions to be made regarding the phylogeny of these organelles.

Large introns in the encoded tRNAs cause chloroplast ribosomal spacer regions of land plants to be extremely large (>1.8 kbp). By contrast, nucleotide sequence data show that introns are absent in the spacer-encoded tRNAs of the few algae that have been examined. DNA sequence data showing compact rDNA spacers containing uninterrupted tRNA genes are now documented from *O. luteus, Pylaiella littoralis* (Markowics et al. 1988), *C. paradoxa* (Janssen et al. 1987) and *E. gracilis* (Graf et al. 1980; Orozco et al. 1980). Although sequence data does not yet exist describing rDNA spacers from other chromophytic and rhodophytic algae, measurements demonstrating compact ribosomal operons support the proposal that these taxa also lack intron-containing tRNAs in their chloroplast rDNA spacers. For example, the rhodophytes *Griffithsia pacifica* and *Porphyra yezoensis* (Li and Cattolico 1987; M. Shivji, personal communication) both contain rDNA within short (5 kbp) regions of cpDNA, and the brown alga *D. dichotoma* encodes the rDNA operon on a 4.7 kbp inverted repeat (Kuhsel and Kowallik 1987).

The fact that introns may be found in the spacer tRNA's of terrestrial plants but not algae and prokaryotes suggests that these structures were lost in the course of genome evolution. Conversely, the presence of introns could equally represent an acquired characteristic of chloroplast DNA architecture. This second hypothesis seems more parsimonious. The acquisition of introns within land plant cpDNA would invoke a single evolutionary event, whereas intron loss would require that precise excision of these variable sequences occurred independently throughout many diverse lines of plastid genome evolution. It should be noted that the green algal taxa having the same mode of cell division (phragmoplastic) as land plants (Pickett-Heaps 1975), have yet to be examined for intron-containing tRNAs. The identification of such introns in these green algae may identify participants in the alga-land plant transition or the absence of such introns in some land plants may demarcate a primitive lineage closely related to a green algal ancestor.

The 16S rDNA 3′ terminus adjacent to the ribosomal spacer, encodes a stemloop secondary structure in the rRNA. This sequence from *O. luteus* is most similar to analogous regions from *A. nidulans* and *C. paradoxa* (97%), and slightly less to *E. gracilis* (93%). The high conservation typically observed for this 16S rRNA region is probably due to the potential role that the sequence plays in translation initiation (reviewed in Noller 1984). The *O. luteus* 16S rDNA also contains the conserved element CCTCC (position 53-57 Fig. 6). The presence of this nucleotide array suggests that mRNA recognition by *O. luteus* ribosomes may be similar to that found in chlorophytic plant chloroplasts and in prokaryotes (for review see Bohnert et al. 1982). The 3′ end of the spacer is bordered by the 5′ terminus of 23S rDNA. This region (60 bp) of *O. luteus* is most similar (Fig. 6) to the *A. nidulans* and *C. paradoxa* counterparts (>81%), and shows much less similarity to the same region in other chloroplasts and prokaryotes (>74%). Sequence comparison data combined from the two ribosomal and two transfer RNA gene regions (267 bp), demonstrate that *O. luteus* genes have highest similarity to *C. paradoxa* (92.9%), followed by *A. nidulans* (91.4%), *E. gracilis* (87.5%), *N. tabacum* (86.5%), *Chlamydomonas reinhardii* (81.3%), *E. coli* (81.3%) and *Bacillus subtilis* (80.1%). These data suggest closer phylogenetic affinity between plastids of *O. luteus* and *C. paradoxa*, and the cyanobacterium *A. nidulans* than to green algal and land plant chloroplasts. A more definitive conclusion must await additional nucleotide sequence data from these and other non-chlorophytic algae.

The *O. luteus* rDNA spacer contains three regions of intergenic DNA, that separate 16S rDNA, $tRNA^{Ile}$, $tRNA^{Ala}$, and 23S rDNA (Fig. 6). These regions show little similarity to analogous regions of any organism examined to date. Intergenic region I (IG-I), proximal to 16S rDNA is 73 bp, while Ig-III next to 23S rDNA is 42 bp long. IG-II, which separates the two tRNA genes is comprised of just three nucleotides. This close arrangement must require a precise processing mechanism during tRNA maturation. The three intergenic regions are quite A+T rich (80% A+T) when contrasted to the tRNA coding regions (47% and 41% A+T for $tRNA^{Ile}$ and $tRNA^{Ala}$ respectively). Close clustering of tRNA genes as well as rDNA spacers having A+T rich intergenic regions are also found in plastid

DNA of the algae *C. paradoxa*, *E. gracilis* and *P. littoralis* (Graf et al. 1980; Hallick et al. 1984; Janssen et al. 1987; Markowics et al. 1988; Orozco et al. 1980).

Several regions containing palindromes are apparent when the *O. luteus* spacer region (Fig. 6) is analyzed. These structures range in size from 17 to 28 bp, and flank the tRNA gene pair. Palindromes have been found in all chloroplast rDNA spacers, including the very compact *E. gracilis* spacer. These regions may form stem-loop secondary structures in the RNA transcript, although this has yet to be conclusively demonstrated. Secondary structure elements have been shown to function in prokaryotic rRNA maturation (Young and Steitz 1978), and may prove to be important in tRNA maturation as well.

Localization to an inverted repeat structure, and linear gene order make the chloroplast ribosomal operons of *O. luteus* similar to those of most chlorophytic land plants and algae. However, as summarized in Fig. 7, ribosomal operons do differ significantly when algae and land plants are compared. The presence or absence of introns, tRNA complement, additional small rRNA species and fragmented as well as rearranged rDNA sequences are observed to vary. Additional rDNA sequence data from rhodophytes and chromophytes will be critical to the assessment of evolutionary divergence among extant plants.

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