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Organization, function and expression of the chloroplast DNA of *Chlamydomonas reinhardii*

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Summary. Although the biosynthesis of chloroplasts depends to a large degree on the activity of the nucleocytoplasmic compartment, these organelles possess their own protein synthesizing system which includes DNA, RNA, DNA- and RNA polymerases, ribosomes and several additional factors. Among green organisms, the unicellular alga *Chlamydomonas reinhardii* is particularly suited for the study of chloroplast DNA since it can be examined by both biochemical and genetic means. This DNA is polyploid and consists of 190 kb circular molecules. The genes of ribosomal RNA, tRNA and of several chloroplast polypeptides have been localized on the chloroplast genome. Several aspects of chloroplast DNA transcription have been studied. Unique features of the chloroplast ribosomal unit of *C. reinhardii* include two genes coding for the small 3S and 7S ribosomal RNAs and the presence of an 870 bp intron within the 23S ribosomal RNA gene. Several structural properties of this intron are discussed and compared with those of other organellar and eukaryotic ribosomal RNA genes. It appears that chloroplast DNA shares common features with both prokaryotic and eukaryotic DNAs.

Why study chloroplast DNA?

It is well established that chloroplasts contain their own protein synthesizing apparatus which comprises DNA, RNA, ribosomes and several other factors (for details see Gillham, 1978). What is most interesting is that this organellar protein synthesizing system cooperates closely with the nucleocytoplasm in the elaboration of organellar proteins and cellular structures such as, for example, the photosynthetic apparatus of the chloroplast. This close cooperation between the two cellular compartments manifests itself in the structure of several organellar proteins which consist of subunits some of which are made within the organelle while others are synthesized in the nucleocytoplasm.

This point is illustrated in figure 1. Let us consider in general terms the biosynthesis of a given chloroplast protein i consisting of n subunits of chloroplast origin (C^i_1, C^i_2, ..., C^i_n) and of m subunits of nucleocytoplasmic origin (N^i_1, N^i_2, ..., N^i_m) where n and m are comprised between 0 and 5. Most chloroplast DNA molecules are circular, 120-190 kb in size (Bedbrook and Kolodner, 1979), and contain a number of genes coding for chloroplast polypeptides. These genes (C^i_1,

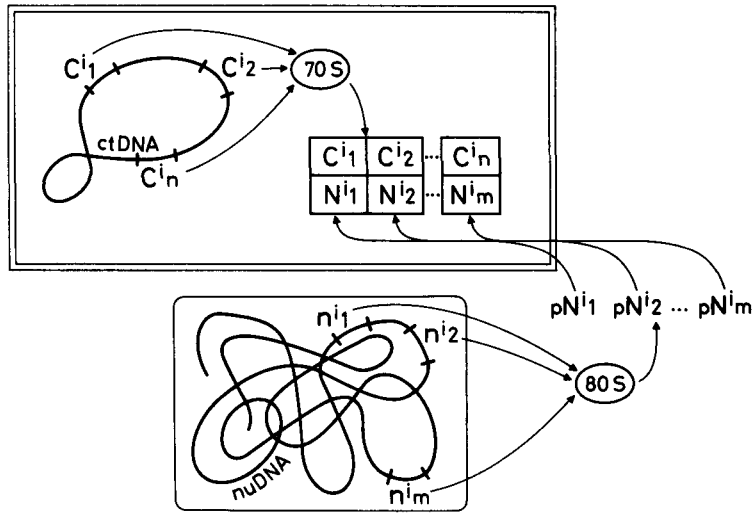

Fig. 1. Photosynthetic cell with its chloroplast (upper left, surrounded by a double membrane) and nucleocytoplasmic protein synthesizing systems. ct DNA, chloroplast DNA; nu DNA, nuclear DNA; 70S and 80S refer to the chloroplast and cytoplasmic ribosomes, respectively; for further details, see text.

C^i_2, ..., C^i_n for protein i) are transcribed by a chloroplast DNA dependent RNA polymerase and the mRNAs are translated on chloroplast 70S ribosomes which are distinctly smaller than the cytoplasmic 80S ribosomes. The genes of the cytoplasmic partner polypeptides (n^i_n, n^i_2, ..., n^i_m) are located in the nucleus. They are transcribed by a nuclear RNA polymerase and the corresponding mRNAs are translated in the cytoplasm on 80S ribosomes. Before the cytoplasmic subunits can associate with their chloroplast partner subunits, these polypeptides have to traverse the chloroplast envelope. They are synthesized as precursors which contain a specific sequence of about 40 amino acids at their N-terminus, called transit sequence, which is thought to be important for the selective transport across the chloroplast envelope and which is cleaved during or shortly after this transport (see review of Chua and Schmidt, 1979).

It is therefore apparent that the biosynthesis of chloroplast proteins requires a close cooperation between 2 distinct cellular compartments. The control of synthesis is likely to be rather complex and it could occur at the levels of transcription, translation and/or it could be post-translational. Before specific questions on the molecular mechanisms of this interplay between chloroplast and nucleocytoplasm can be answered, it is important to characterize first the chloroplast DNA in some detail.

Photosynthetic apparatus of Chlamydomonas reinhardii

C. reinhardii is a unicellular green alga containing a unique chloroplast. A common feature of chloroplasts is the presence of an inner membrane system where the primary reactions of photosynthesis occur. As in higher plants, the photosynthetic apparatus of *C. reinhardii* consists of 2 photosystems PSI and PSII (fig. 2). Light energy is captured by the antennae of these photosystems, which consist mostly of chlorophyll and carotenoids, and the energy is transferred to the reaction centers of the 2 photosystems where it is converted into chemical energy. The result of these complex reactions is the splitting of water into molecular oxygen, protons and electrons. The latter are transferred along the electron transport chain and this electron flow is coupled with ATP formation. The electron transport chain consists of electron carriers such as plastoquinone, various cytochromes, plastocyanin, ferredoxin and other compounds, some of which are still not yet well defined. At the end of this chain, the electrons are transferred to $NADP^+$ to produce NADPH. The newly formed ATP and NADPH are used to drive the Calvin cycle, a series of reactions which occur in the soluble phase of the chloroplast whose end result is the fixation of CO_2 into ribulose bisphosphate to produce phosphoglycerate. The enzyme which catalyzes this step is the ribulose bisphosphate carboxylase, thought to be the most abundant enzyme on earth (Kung, 1976). It consists of 8 identical large and 8 identical small subunits coded for by the chloroplast (Coen et al., 1977) and nuclear genome (Kawashima and Wildman, 1972), respectively. This enzyme represents the simplest example of an organellar protein made of subunits which originate from distinct cellular compartments. A more complex protein is the ATPase complex anchored in the chloroplast inner membranes which couples ATP formation to electron transport. This multimeric protein consists of 5 subunits synthesized, in the chloroplast (Mendiola-Morgenthaler et al., 1976) and of 3 subunits made in the nucleocytoplasm. The chloroplast membranes are highly complex and include at least 50 polypeptides (Chua and Bennoun, 1975).

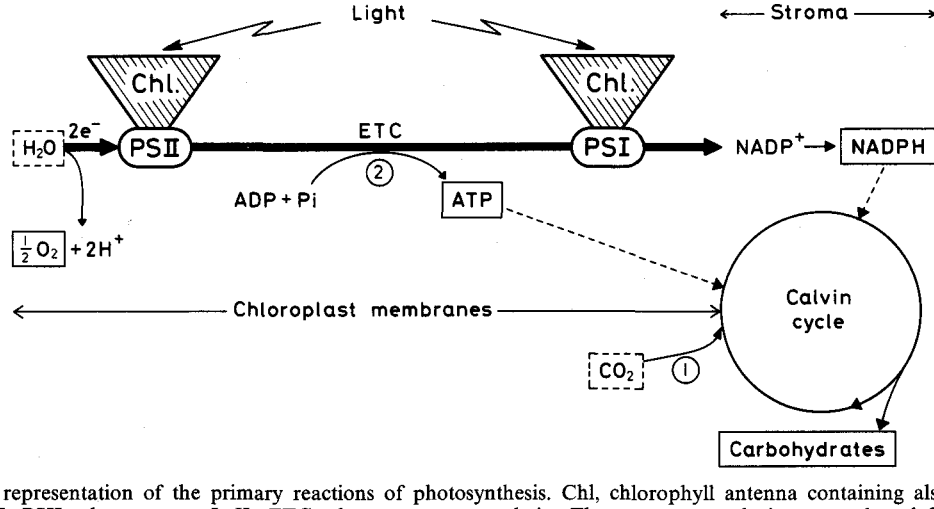

Fig. 2. Schematic representation of the primary reactions of photosynthesis. Chl, chlorophyll antenna containing also other pigments (carotenoids); PSI, PSII, photosystems I, II; ETC, electron transport chain. The enzymes catalyzing steps 1 and 2 are the ribulose bisphosphate carboxylase and the coupling factor, respectively. See text for further explanations.

Chloroplast genetics

The first report on chloroplast inheritance was already made in 1909 by Correns and Baur who discovered that differences in the photosynthetic systems of some flowering plants were inherited in a non-Mendelian fashion. It took however more than half a century until these genetic traits could be correlated with some physical organellar parameters.

C. reinhardii has played an important role in the development of chloroplast genetics. This is due in part to its life cycle (fig. 3). Cells of the 2 mating types (mt^+ and mt^-) can be grown vegetatively whereby the cells divide by mitosis. If vegetative cells are transferred into a medium which lacks a reduced nitrogen source, they differentiate into gametes. Gametes of opposite mating type fuse with each other to form a zygote and shortly after cell fusion, the 2 chloroplasts also fuse. Under appropriate light and dark regimes, the zygotes undergo meiosis and after germination 4 or 8 zoospores are produced which can start a new cycle.

In 1954 Sager discovered the phenomenon of uniparental inheritance in *C. reinhardii*. She found that certain genes, which she presumed correctly to be chloroplast genes, differed in two important ways from Mendelian genes.

1) The transmission of chloroplast genes is governed by the mt allele. Uniparental genes from the mt^+ parent are preferentially transmitted while those from the mt^- parent are not. This unilateral gene transmission cannot be explained simply by the preferential transmission of the mt^+ chloroplast since the chloroplasts of the 2 parents fuse during zygote formation.

2) In some rare cases the genes of the mt^- parent are also transmitted to the offspring giving rise to biparental zygotes. During the post-meiotic divisions the uniparental genes segregate. These biparental zygotes have been very important for studying the recombination of chloroplast genes. Two major classes of uniparental mutants have been isolated:

- Mutants resistant to a spectrum of antibiotics including streptomycin, erythromycin, spectinomycin, etc.
- Mutants which cannot grow in the absence of a reduced carbon source. These mutants are unable to fix CO_2 because of a lesion in the photosynthetic apparatus or in the chloroplast protein synthesizing system.

Many of these uniparental mutants have been mapped, mostly by Gillham, Boynton and co-workers, and they fall into a single linkage group (Gillham, 1978). Recently Spreitzer and Mets (1980) isolated a uniparental mutant which appears to be specifically affected in the large ribulose bisphosphate carboxylase subunit (LS). This mutation has been mapped relative to other uniparental mutations (Mets, personal communication). Since the gene coding for LS has been localized on the chloroplast genome of *C. reinhardii* (see below) this finding proves effectively that uniparental mutants are indeed chloroplast mutants and it also provides a first correlation site between the chloroplast genetic and physical maps. Such a correlation will undoubtedly provide more insights into the function of this DNA. If chloroplast DNA is indeed the carrier of uniparental genes, one would expect that only the mt^+ chloroplast genome survives the meiotic cycle, and is transmitted to the offspring. This expectation has been recently confirmed by Grant et al. (1980) who have shown that chloroplast DNA deletions are transmitted uniparentally.

The molecular basis of uniparental inheritance in *C. reinhardii* is still unknown. The preferential methylation of the mt^+ chloroplast DNA in gametes and in the young zygote (Burton, Grabowy and Sager, 1979; Royer and Sager, 1979) has been included in a model

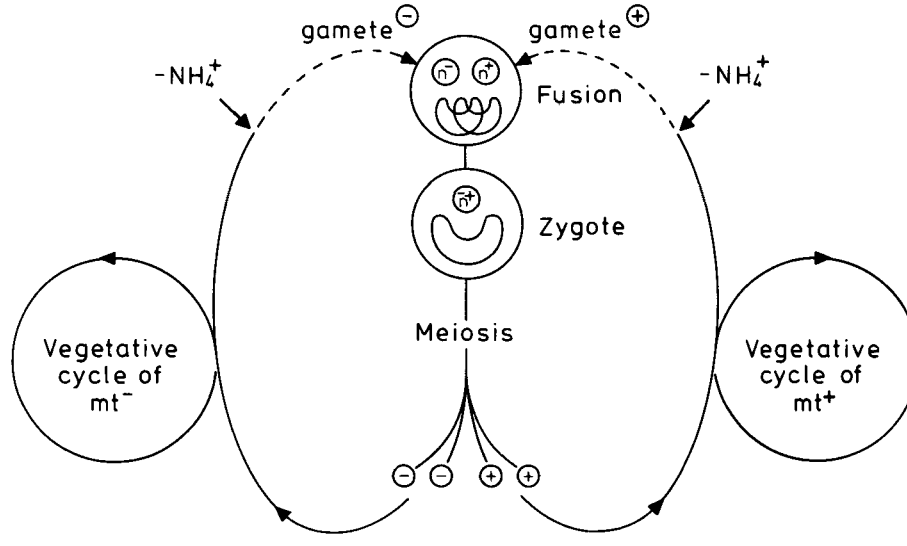

Fig. 3. Life cycle of *Chlamydomonas reinhardii*. mt, mating type; n, nucleus. See text for explanations.

Parameters of the nucleocytoplasmic, chloroplast and mitochondrial protein synthesizing systems of *C. reinhardii*

	Complexity (kb)	Genetic information %	Mass (%)	Genetics	
Nuclear DNA	$7\text{-}9\times 10^4$ (1, 2, 3)	99.7	85	Unique	Mendelian
Chloroplast DNA	190 (4, 5)	0.3	14	Repeated 50–80×	Uniparental
Mitochondrial DNA	15 (6)	0.02	1	Repeated 50×	Biparental ≠ Mendelian (13)

	S-value	Large subunit Polypeptides	rRNA	Small subunit Polypeptides	rRNA
Cytoplasmic ribosomes	80S	39 (7)	25S, 5.8S, 5S (8, 9)	26 (7)	18S (8, 9)
Chloroplast ribosomes	70S	26 (7)	23S, 7S, 5S, 3S (10)	23 (7)	16S (10)
Mitochondrial ribosomes	?	?	?	?	?

Cytoplasmic mRNA	Polyadenylated	(11)
Chloroplast mRNA	Not polyadenylated	(12)

1 Sueoka et al., 1967. 2 Wells and Sager, 1971. 3 Howell and Walker, 1976. 4 Rochaix, 1976. 5 Howell, 1976. 6 Grant and Chiang, 1980. 7 Hanson et al., 1974. 8 Hoober and Blobel, 1978. 9 Marco and Rochaix, 1980. 10 Rochaix and Malnoë, 1978. 11 Dobberstein et al., 1977. 12 Howell et al., 1977. 13 Gillham, 1978.

based on the selective degradation of the mt⁻ chloroplast DNA by a hypothetical restriction enzyme Sager, 1977). Another model assumes that the chloroplast membranes contain a limited number of attachment sites for DNA replication and that the mt⁺ chloroplast DNA occupies these sites preferentially and replicates (Gillham, 1978). The mt⁻ chloroplast DNA is competed out from these membrane attachment sites and is degraded.

In summary, there are 3 distinct protein synthesizing systems in *C. reinhardii* (and also in plants), localized in the nucleus, the chloroplast and the mitochondria. The principal parameters of these protein synthesizing systems are summarized in the table.

An attractive feature of *C. reinhardii* is the fact that mutations can be attributed to the nuclear, chloroplast or mitochondrial genome based on their unique transmission patterns (Gillham, 1978).

Gene localization on the chloroplast genome of C. reinhardii

In 1963, it was shown that chloroplast DNA preparations of *C. reinhardii* are enriched in a satellite peak

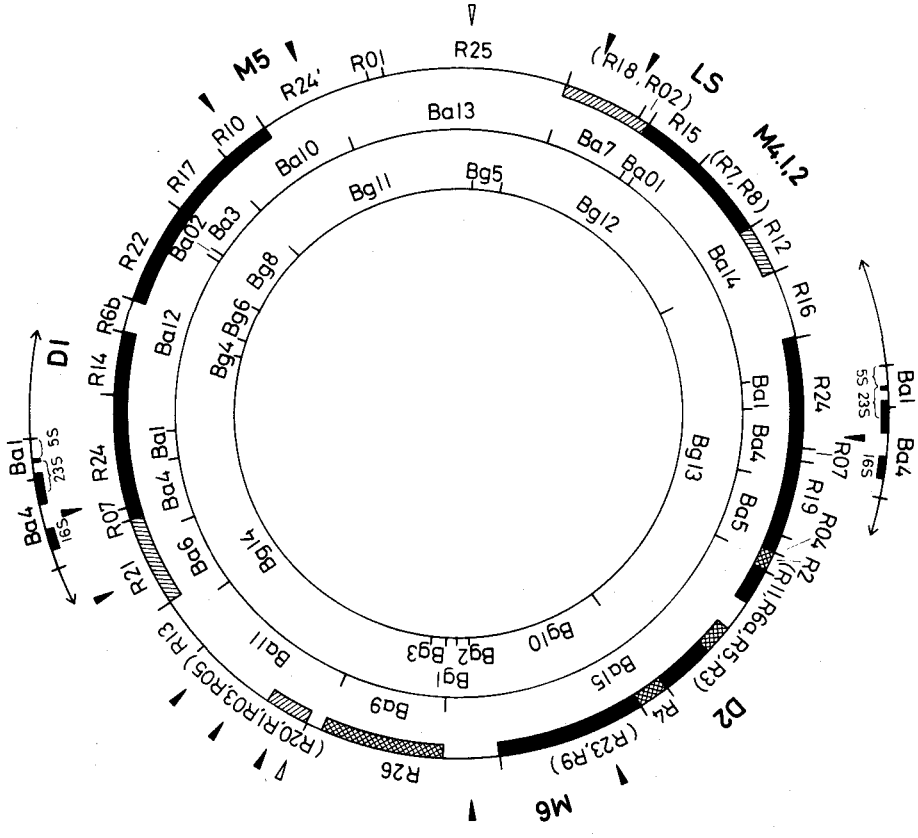

Fig. 4. Restriction map of the chloroplast DNA of *Chlamydomonas reinhardii*. The 2 inverted repeats containing the rRNA genes are indicated on the outside. The 3 circles from the inside to the outside represent the BglII, BamHI and EcoRI restriction maps, respectively. The arrows indicate the position of the 4S RNA genes (Malnoë and Rochaix, 1978). The positions of several chloroplast polypeptide genes are indicated. LS, large subunit of ribulose bisphosphate carboxylase; D1 and D2, 2 chloroplast membrane polypeptides. The assignments of M4.1, 4.2, M5 and M6 are tentative. The chloroplast membrane polypeptide nomenclature of Chua and Gillham (1977) has been used. A steady state transcription map is superimposed on the outside circle. The chloroplast DNA regions whose transcripts are present in large (■), medium (▨), and low (⊠) amounts are indicated.

with a buoyant density of 1.696 g/cm^3 in CsCl which is markedly distinct from the nuclear DNA ($\varphi = 1.724$ g/cm^3) (Sager and Ishida, 1963). This DNA was examined by reassociation kinetics and found to have a kinetic complexity close to 200×10^6 d (Bastia et al., 1971; Wells and Sager, 1971). Behn and Herrmann (1977) were able to isolate intact circular molecules with a length of 62 µm. Restriction enzyme analysis of this DNA provided a similar size estimate, 130×10^6d (Rochaix, 1976, 1978; Howell, 1976).

We recently constructed a physical map of this DNA (Rochaix, 1978). As shown in figure 4 the map is circular in agreement with the electron microscope measurements. Striking features of the map are the 2 inverted repeats 19 kb in size which contain the chloroplast ribosomal genes. A similar arrangement is also found in the chloroplast DNA, of maize (Bedbrook et al., 1977), spinach (Whitfeld et al., 1980) and tobacco (Jurgenson and Bourque, 1980) but it is by no means universal since in *Euglena* the chloroplast ribosomal units are arranged in tandem repeats (Gray and Hallick, 1978; Jenni and Stutz, 1978; Rawson et al., 1978) and in *Vicia faba* there is a single ribosomal unit per chloroplast genome (Koller and Delius, 1980). Figure 4 also shows that the chloroplast 4S RNA genes (marked by arrows in fig. 4) are interspersed throughout the genome (Malnoë and Rochaix, 1978).

Whereas the localization of the ribosomal and tRNA genes can be achieved easily by radioactively labelling these RNAs in vitro and by hybridizing them to chloroplast restriction fragments, the localization of chloroplast protein genes is more elaborate. The method we have used successfully consists of cloning chloroplast restriction fragments and then using the hybrid plasmids as templates in an in vitro coupled transcription-translation system (Zubay et al., 1970). The in vitro synthesized products can then be identified by immunoprecipitation. An illustration of this method is shown in figure 5 with the plasmid carrying the gene coding for LS (Malnoë et al., 1979). Slot (a) represents an autoradiograph of ^{35}S-methionine labelled in vitro synthesized products fractionated by electrophoresis in an SDS polyacrylamide gel. Slot (b) shows the autoradiograph obtained after immunoprecipitation of the polypeptides shown in (a) with an antibody against the purified large carboxylase subunit. Slot (c) shows that no polypeptide is immunoprecipitated when an unrelated anti-serum is used. It can be seen that the largest immunoprecipitated band co-migrates with the authentic large subunit. The identity of the in vitro and in vivo products can be examined further by comparative fingerprint analysis and/or hybrid arrested translation (Malnoë et al., 1979). By using methods of this sort we have identified several protein genes on the chloroplast genome of *C. reinhardii* (fig. 4). The assignments of some of these genes are still tentative because they are based only on immunoprecipitation of products synthesized in vitro in the coupled transcription-translation system (Malnoë and Rochaix, unpublished results).

Transcription of the chloroplast DNA

Once several chloroplast genes have been cloned and identified on the chloroplast genome, it is possible to examine their transcription.

A first approach consists of isolating cellular RNA which is separated into polyadenylated and unpolyadenylated RNA. Only the latter is of interest since the chloroplast mRNA of *C. reinhardii* is not polyadenylated. This RNA is fractionated by electrophoresis on a fully denaturing agarose gel. The RNA is then transferred and coupled to diazotized paper (Alwine et al., 1977) and hybridized to specific hybridization probes, usually gene sequences which have been labelled. This method allows one to measure the size and the steady state level of chloroplast

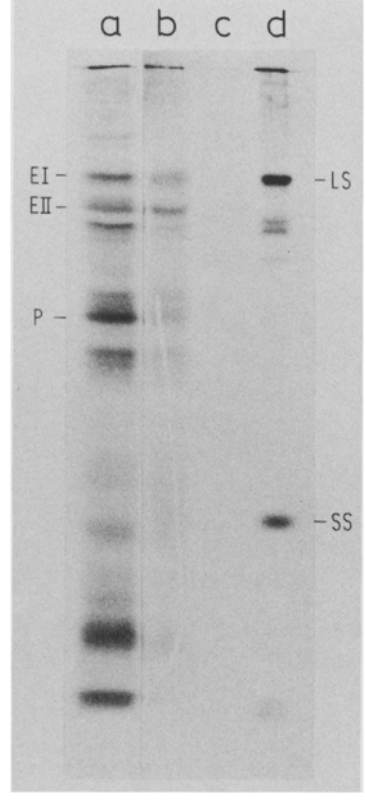

Fig. 5. In vitro synthesis of chloroplast polypeptides in a coupled transcription-translation system. (a) Fluorograph of the ^{35}S methionine-labelled products of the coupled transcription-translation of a hybrid plasmid containing the EcoRI chloroplast fragment R15 electrophoresed on a 12.5% SDS/polyacrylamide gel. (b) Immunoprecipitation of the in vitro synthesized polypeptides (a) with an antiserum against the large subunit (LS) of the ribulose bisphosphate carboxylase. (c) Immunoprecipitation of the in vitro synthesized polypeptides (a) with an unrelated serum (control). (d) Fluorograph of purified ^{35}S labelled ribulose bisphosphate carboxylase of *C. reinhardii*. SS, small subunit. (From Malnoë et al., 1979, with permission.)

mRNAs. A summary of our transcription analysis is shown in figure 6. The restriction fragments used as hybridization probes are arranged according to their order on the map. The transcripts have been ordered into 3 classes depending on whether the hybridization signal is strong, medium or weak. It can be seen that there are 6 major non ribosomal transcripts some of which correspond to genes we have identified. Some of the weaker large molecular weight transcripts could represent precursors of the strong transcripts although kinetic data are required for solving this problem. The sum of the detectable transcripts covers approximately half of the chloroplast genome if one assumes asymmetric transcription.

Another approach is to examine the transcription program of the chloroplast genome during a synchronous growth cycle. The cells can be synchronized by alternate light and dark phases and they are pulse-labelled with ^{32}P-phosphate at various periods of the cycle. The RNA is extracted and hybridized with chloroplast restriction fragments. Experiments of this sort (Matsuda and Surzycki, 1980; Schneider, unpublished results) have revealed that chloroplast transcription reaches a maximum during the first half of the synchronous cycle and declines steadily thereafter. The genes which are mostly transcribed are the ribosomal genes and the genes coding for LS and the chloroplast membrne polypeptide D1 (fig. 4).

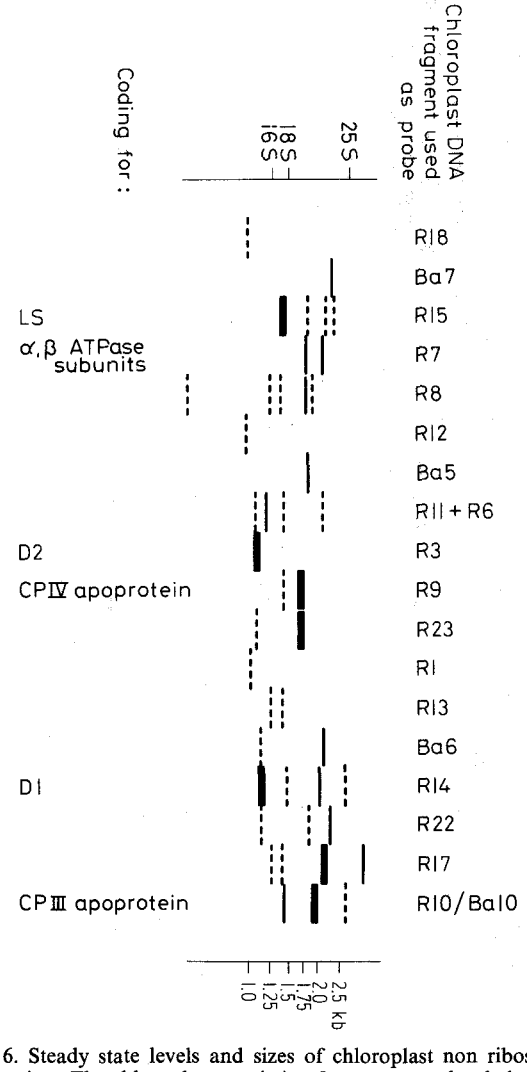

Fig. 6. Steady state levels and sizes of chloroplast non ribosomal transcripts. The chloroplast restriction fragments used as hybridization probes are indicated at the top. Transcripts which are present at high (——), medium (——) and low (– – – –) steady state levels are shown. The sizes of the transcripts increase from the bottom to the upper part of the figure. The tentative coding functions of some of the transcripts are also indicated (see fig. 4).

Chloroplast ribosomal unit of C. reinhardii

A map of the chloroplast ribosomal unit of *C. reinhardii* is shown in figure 7 (Rochaix and Malnoë, 1978). Transcription proceeds from the right to the left in the order of the genes coding for 16S, 7S, 3S, 23S and 5S rRNA. 2 unusual features of this chloroplast ribosomal unit are noticeable. 1st the gene of the 23S rRNA contains an intervening sequence of 870 bp. 2nd the presence of the genes coding for 3S and 7S rRNA is unique to *C. reinhardii*. In the ribosomal units of other green organisms these genes are absent; instead, they contain a gene coding for a 4.5S rRNA species located between the 23S and 5S rRNA genes and which is absent in *C. reinhardii* (Whitfeld et al., 1978; Bowman and Dyer, 1979).

The presence of a chloroplast ribosomal intervening sequence raises several questions:

1) Is the chloroplast ribosomal intron transcribed?

A fine restriction map of the ribosomal intron is shown in figure 7. A fragment comprised between the right HphI site and the XhoI site both of which lie entirely within the ribosomal intron was inserted into a vector plasmid. This new hybrid plasmid was used as a specific hybridization probe for detecting intron transcripts. It was radioactively labelled and hybridized with cellular non-polyadenylated RNA which had been fractionated on a fully denaturing agarose gel, transferred and bound to diazotized paper (fig. 8). It can be seen that the major hybridization occurs with an RNA of equal size as the ribosomal intron. No hybridization with larger RNAs is detectable indicating that the splicing of the 23S rRNA precursor occurs very rapidly after, or even before, transcription of the 23S rRNA gene is completed.

2) How do the chloroplast ribosomal intron flanking sequences compare with other ribosomal sequences?

The sequences of the 23S rRNA gene which flank the ribosomal intron are shown in figure 9 (Allet and Rochaix, 1979). The complete *E. coli* 23S rRNA gene sequence has been recently determined by Brosius et al. (1980). It is interesting to note that there is a remarkable sequence identity between the chloroplast flanking regions and a portion of the *E. coli* 23S

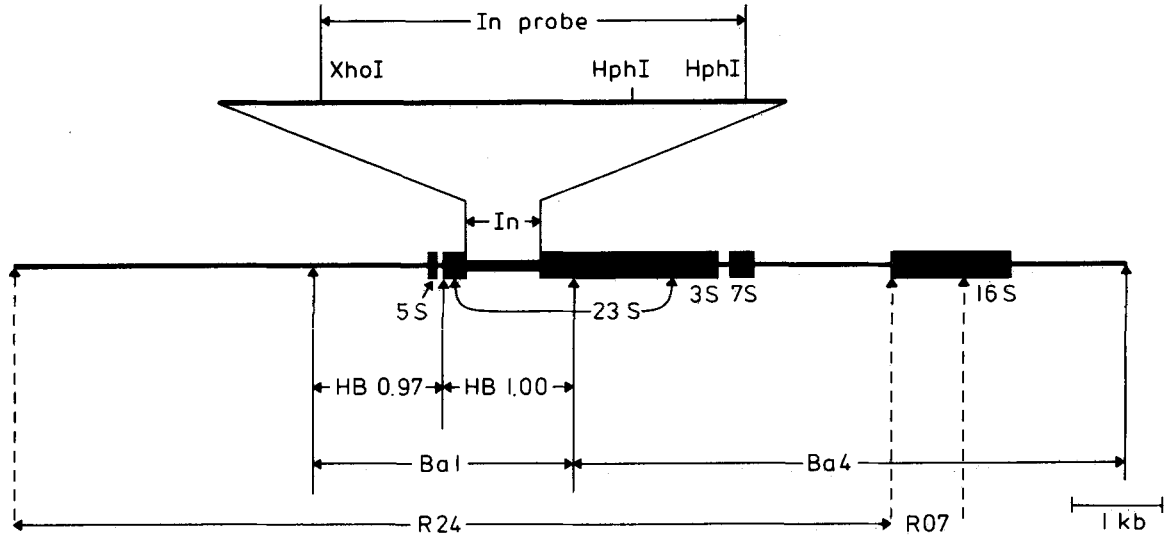

Fig. 7. Chloroplast ribosomal DNA map of *C. reinhardii*. The genes coding for the ribosomal 16S, 7S, 3S, 23S and 5S RNAs are indicated. Transcription proceeds from the right to the left. In, ribosomal intron; In probe refers to the XhoI-HphI fragment of the ribosomal intron which was subcloned and used as hybridization probe (see text).

rRNA gene (fig. 9). It is formally possible to place the chloroplast ribosomal intron between positions 2593 and 2594 of the *E. coli* sequence. Dujon (1980) recently sequenced a portion of the yeast mitochondrial 21S rRNA gene including the flanking regions of the mitochondrial ribosomal intron. A strong sequence homology is apparent between the flanking regions of the mitochondrial ribosomal intron and a region of the *E. coli* 23S rRNA gene. Here again it is possible to formally place the mitochondrial intron between position 2447 and 2449 (fig. 9). In addition, a considerable sequence homology exists between a portion of the mitochondrial 21S rRNA sequence and the chloroplast intron flanking regions (fig. 9). The ribosomal region in which the chloroplast and mitochondrial introns are located appears to be strongly conserved.
Similarly, the sequence of the ribosomal intron flanking regions of the 25S rRNA of *Tetrahymena* (Wild and Sommer, 1980) is related to a portion of the *E. coli* 23S rRNA gene sequence so that the *Tetrahymena* intron can be formally placed between positions 1925 and 1926 of the *E. coli* sequence. The same region is also conserved in the *Xenopus* 28S rRNA gene although no introns have been detected in these genes (Gourse and Gerbi, 1980). Surprisingly, the chloroplast ribosomal intron flanking regions have their counterparts in the *Xenopus* 28S rRNA gene (fig. 9) (Gourse, Clark and Gerbi, in preparation). It therefore appears that ribosomal intron flanking regions are also highly conserved between prokaryote, organelle and eukaryote systems.

3) Is the ribosomal intron sequence present elsewhere in the chloroplast or nuclear genome of C. reinhardii?
This question can be answered by hybridizing chloroplast restriction fragments with a cloned labelled restriction fragment (HB1.00) containing the entire

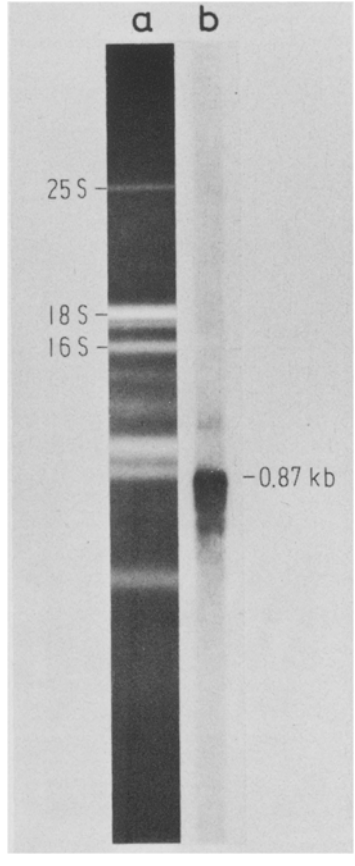

Fig. 8. (a) Fractionation of non polyadenylated RNA on a denaturing agarose gel. (b) Autoradiograph of the hybridization of the RNAs shown in (a) with the ^{32}P labelled ribosomal intron probe (see text for explanations).

ribosomal intron (fig. 7). The results presented in figure 10d show clearly that the hybridization occurs uniquely with a single BamHI (Ba1) fragment which contains the labelled restriction fragment (fig. 7). It

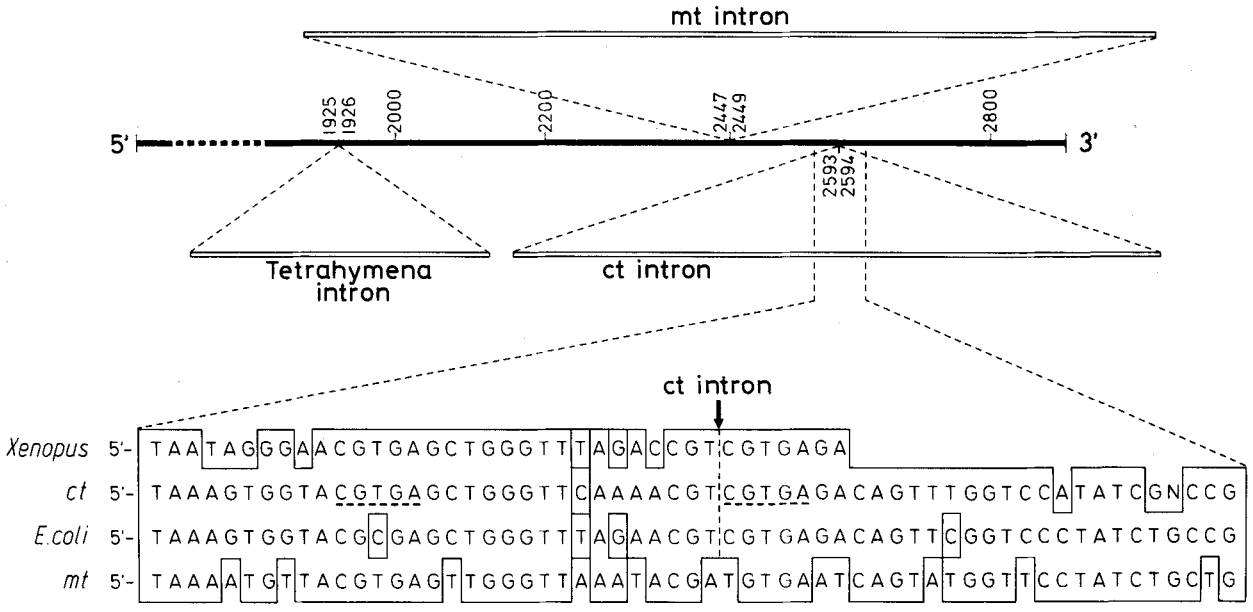

Fig. 9. Sequence homologies between the ribosomal intron flanking regions of the chloroplast of *C. reinhardii,* of the yeast mitochondria and of *Tetrahymena* with portions of the *E. coli* 23S rRNA gene. The lower part of the figure compares the chloroplast intron flanking sequences with the corresponding sequences of the genes of *E. coli* 23S rRNA, yeast mitochondrial 21S rRNA and Xenopus laevis 28S rRNA (see text for details). The *Xenopus* sequence was determined by Gourse, Clark and Gerbi (in preparation).

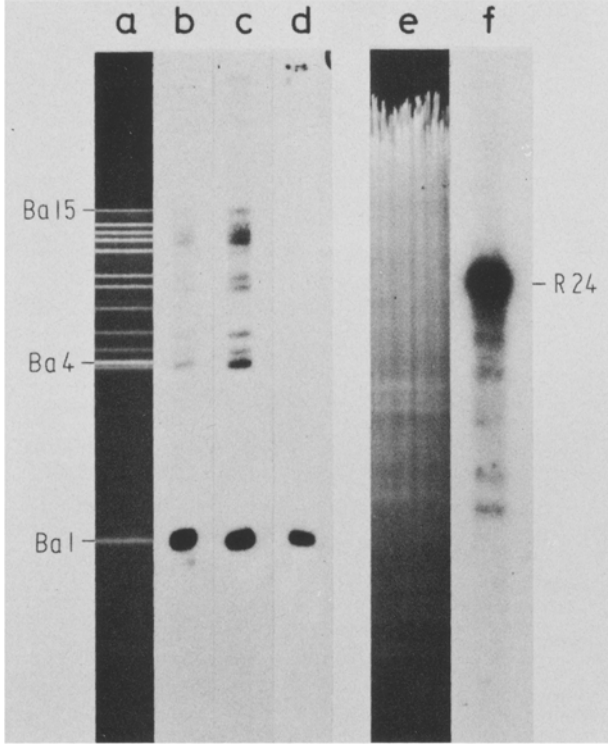

Fig. 10. Hybridizations of chloroplast intron sequences with chloroplast and nuclear DNA of *C. reinhardii* and evidence for chloroplast repetitive sequences. (a) BamHI digest of chloroplast DNA fractionated by agarose gel electrophoresis. (b) Autoradiograph of the hybridization of the fragments shown in (a) with P^{32} labelled Ba 1 fragment (cf. fig. 7). (c) Autoradiograph of the hybridization of the fragments shown in (a) with P^{32} labelled HB 0.97 fragment (cf. fig. 7). (d) Autoradiograph of the hybridization of the fragments shown in (a) with P^{32} labelled HB 1.00 fragment (cf. Fig. 7). (e) EcoRI digest of nuclear DNA fractionated by agarose gel electrophoresis. (f) Autoradiograph of the hybridization of the fragments shown in (e) with P^{32} labelled In probe (cf. fig. 7 and text for explanations).

can be concluded that the chloroplast ribosomal intron is not present elsewhere in the chloroplast genome.

Similarly when nuclear DNA is hybridized with the intron-specific hybridization probe described earlier (fig. 10f), major hybridization occurs with a chloroplast EcoRI fragment (R24) which contaminates the nuclear DNA preparation. In addition some faint hybridization is detectable with several nuclear restriction fragments. It will be possible to assess the significance of this hybridization only when these fragments have been cloned and characterized.

The fact that no sequences homologous to the chloroplast ribosomal intron can be found on the chloroplast genome outside the ribosomal region does not, of course, mean that there are no other interrupted genes in the chloroplast DNA of *C. reinhardii,* even though none has been detected until now. Introns have been found in several genes in other organellar systems. In *Zea mays* the $tRNA^{ala}$ and $tRNA^{ile}$ genes located in the chloroplast ribosomal spacer region contain large introns of 949 and 850 bp, respectively (Kössel et al., 1980). In some yeast strains, in addition to the large mitochondrial rRNA gene, the mitochondrial genes coding for cytochrome b and for subunit I of cytochrome oxidase contain also several introns (Lazowska et al., 1980).

Repetitive sequences on the chloroplast genome of C. reinhardii

When the chloroplast DNA of *C. reinhardii* is randomly sheared, treated with exonuclease III so as to digest

partially the 3′ ends of the DNA fragments, and allowed to reanneal, 10–20% of the fragments form Thomas circles (Thomas et al., 1970). This suggests that the chloroplast DNA fragments contain repetitive sequences at their ends (Rochaix, 1972).

The presence of repetitive sequences in the chloroplast genome of *C. reinhardii* can also be inferred from the observation that several cloned chloroplast restriction fragments hybridize to most of the chloroplast genome (Rochaix, 1978). Figure 10c shows that a 1.5-kb chloroplast restriction fragment (denoted HB 0.97 in fig. 7) hybridizes significantly with most chloroplast BamHI restriction fragments under standard hybridization conditions. Gelvin and Howell (1979) have detected numerous small inverted repeats 0.1–0.3 kb in size which are interspersed throughout the genome and which constitute 4–7% of the chloroplast DNA.

The function of these repetitive sequences is still unknown. They could play perhaps a role in chloroplast DNA recombination.

Conclusion

The chloroplast genome of *C. reinhardii* is a polyploid genome whose unit is a 190-kb circular DNA molecule. We have localized several genes on this genome (fig. 4), those coding for 4S RNA, ribosomal RNA and several genes of chloroplast polypeptides. Some of the protein gene assignments need to be confirmed by other means. A detailed examination of chloroplast genes is important for several reasons:

- Once a chloroplast gene is cloned and identified, it can be used as hybridization probe to study its transcriptional control. Knowledge of the transcriptional control of chloroplast genes is obviously important if we want to understand the molecular basis of chloroplast nucleo-cytoplasmic cooperation.

- The localization of several chloroplast polypeptides genes on the physical map of the chloroplast genome is required in order to achieve a correlation between the physical and genetic map. Such a correlation will allow us to use the extensive chloroplast genetic data for gaining more insights into the function of this DNA.

- Organellar systems provide unique opportunities for studying basic mechanisms, such as splicing, not only at the biochemical, but also at the genetic level. A promising system in *C. reinhardii* is the ribosomal intron. A search for uniparental mutants affected in the splicing of the chloroplast 23S RNA seems warranted. A particularly illustrative example is provided by the yeast mitochondrial system where the numerous mutants in the cytochrome b region have allowed Slonimski and his collaborators to establish attractive models on splicing (Lazowska et al., 1980).

- It is increasingly apparent that organellar protein synthesizing systems have unique features and that they have evolved according to particular selection pressures. From this point of view it is interesting to note that the chloroplast DNAs of several green organisms share common features with both procaryotic and eucaryotic DNAs. Procaryotic features of this chloroplast DNA are the absence of association with histones (Tewari and Wildman, 1969), a close resemblance to bacterial DNA in thin-section studies (Ris and Plaut, 1962), the rifampicin sensitivity of the chloroplast RNA polymerase (Surzycki, 1969), the capability of forming rifampicin-resistant preinitiation complexes with chloroplast DNA and *E. coli* RNA polymerase (Surzycki et al., 1976), the close sequence homology between portions of chloroplast and prokaryotic 23S, 16S and 5S rRNA (Allet and Rochaix, 1979; Schwarz and Kössel, 1980; Dyer and Bowman, 1979) and the presence of a Shine-Dalgarno sequence (Shine and Dalgarno, 1974) at the 3′ terminus of maize chloroplast 16S rRNA (Schwarz and Kössel, 1979; McIntosh et al., 1980). At the translational level the similarity between procaryotes and organelles is demonstrated by the sensitivity of organelle ribosomes to antibacterial drugs such as chloramphenicol, spectinomycin, erythromycin, etc. whereas inhibitors of cytoplasmic 80S ribosomes such as cycloheximide do not have an inhibitory effect (Gillham, 1978).

On the other hand, the presence of repetitive DNA segments and of intervening sequences in the chloroplast DNA relates it to eukaryotic DNAs.

It is noteworthy that Mereschkowsky proposed already in 1905 that chloroplasts may have arisen from free living organisms, similar to present day blue-green algae, which invaded primitive eucaryotic cells. This endosymbiont theory of the origin of chloroplasts has since been proposed and developed by others (Sagan, 1967). It is well documented that blue-green algae occur frequently in symbiotic association. In several cases they are incorporated into the cells of other organisms as endosymbionts (Fogg et al., 1973). In the protozoan *Cyanophora paradoxa,* intracellular structures called cyanelles are present which bear a strong resemblance to blue-green algae and chloroplasts (Hall and Claus, 1963). They can be cultured separately after extraction from their host (Provasoli and Pinter, 1953). These organelles differ however from blue-green algae in the structure of their cell wall and in other cellular components (Hall and Claus, 1963). It is possible that procaryotic intruders of this sort, forerunners of chloroplasts, gradually lost their autonomy and became more and more dependent on the nuclear gene activity of their host.

Another hypothesis takes the opposite view and holds that in the uncompartmentalized ancestor cell, the

genes coding for defined cellular functions (e.g. energy production) became associated into one or more clusters and surrounded by a membrane (Sonneborn, 1967; Bogorad, 1975). This compartmentalization of genes whithin the same cell allowed them to evolve separately even though transfer and/or substitution of genes between the nuclear and chloroplast genomes must have occurred.

Current knowledge on chloroplast protein synthesizing systems does not allow one to settle unambiguously for one theory or the other. Whichever view is correct, the study of chloroplast genes has yielded interesting insights into the evolution of protein synthesizing systems and will probably continue to do so.

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