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The structure of precursor mRNAs and of excised intron RNAs in chloroplasts of *Euglena gracilis*

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Partially spliced precursor mRNAs (pre-mRNAs) in the steady-state population of RNA from chloroplasts of *Euglena gracilis* were found by electron microscopy. The structure and the frequency of the pre-mRNAs of the *psbA* gene (the gene for the 32-kd protein of photosystem II), which is split by four introns in *Euglena* chloroplasts was analysed by electron microscopy. A chloroplast DNA (cpDNA) fragment containing the *psbA* gene from *Euglena*, was cloned into a pEMBL vector. The single-stranded recombinant phage DNA of the coding strand was prepared and hybridized with cpRNA. The majority of hybrids were formed with mature mRNA, but ~8% of the hybrids were formed with pre-mRNAs. The pre-mRNAs were either unspliced or incompletely spliced. A detailed analysis of the structure and the frequency of the pre-mRNAs of the *psbA* gene showed that the four introns are neither spliced out in a strictly random way, nor in a 5'-3' or 3'-5' direction. Introns 2 and 3 are preferentially spliced out first, intron 1 intermediately and intron 4 is generally spliced out last. However, this sequence is not a strict rule. We conclude that the introns can be spliced independently, each one at a different rate. The coding strand from a fragment of the *psbA* gene was separated and annealed with low mol. wt. cpRNA, which was isolated from an agarose gel. Small circular hybrids were found at the positions of the four introns, demonstrating for the first time covalently closed circular excised intron RNAs (iRNAs) in chloroplasts.

Key words: Euglena/chloroplast/*psbA* gene/pre-mRNA/circular iRNAs

Introduction

The chloroplast genome of *Euglena gracilis* has a size of 145 kb. It contains at least 50 introns which account for ~35 kb (Koller and Delius, 1984). The introns of three genes have been characterised in detail. The nine introns of the *rbcL* gene (the gene for the large subunit of ribulose-1,5-bisphosphate carboxylase) and the four introns of the *psbA* gene (the gene for the 32-kd protein, or herbicide-binding protein of the photosystem II) have very well conserved boundary sequences, which resemble those of nuclear protein genes (Koller *et al.*, 1984; Karabin *et al.*, 1984; Keller and Stutz, 1984; Cech, 1983). Another split gene from *Euglena* chloroplasts which has been sequenced, is that for the EF-Tu protein (Montandon and Stutz, 1983).

Two genes of *Chlamydomonas* chloroplasts, the 23S rRNA gene and the *psbA* gene, are split by introns (Rochaix *et al.*, 1985; Erickson *et al.*, 1984). In higher plants introns in chloroplast genes occur much less frequently than in *Euglena*. A few tRNA genes (Koch *et al.*, 1981; Steinmetz *et al.*, 1982; Bonnard *et al.*, 1984; Deno *et al.*, 1982), and the spinach ribosomal protein gene L2 (Zurawski *et al.*, 1984) contain one intron each. The wheat chloroplast gene of the subunit I of the CFo complex of ATP synthase has recently been characterised. The gene contains one intron with border sequences, which are very similar to those found in *Euglena* chloroplast genes (Bird *et al.*, 1985).

Although a number of introns have been sequenced, very little is known about the mechanism of splicing in chloroplasts. To study the processing of RNAs in chloroplasts, *Euglena* cells are especially suitable. They grow relatively quickly compared with higher plant leaves, and the chloroplasts are constantly dividing and synthesising RNA. One of the most abundant transcripts is the *psbA* mRNA. Precursor mRNAs and excised intron sequences of the *psbA* gene were detected on Northern blots. The structure of these precursors was not analysed, and it was not clear whether the introns from a pre-mRNA are removed in a strict order, or randomly, as was suggested (Keller *et al.*, 1982; Hollingsworth *et al.*, 1984).

To study processing of pre-mRNAs in chloroplasts, RNA/DNA hybrids between the transcripts of the *psbA* gene and its genomic DNA were analysed by electron microscopy. The results presented here are the first description of the structure of partially spliced chloroplast pre-mRNAs and circular excised intron RNAs (iRNAs).

Results

Precursor mRNAs of the psbA gene

The *psbA* gene of *Euglena* chloroplasts is split by four introns. The *Eco*RI fragment I from *Euglena* cpDNA contains the whole gene and upstream of it part of an unknown gene which is transcribed from the same strand (Keller and Stutz, 1984; Hollingsworth *et al.*, 1984). The fragment was cloned into the vector pEMBL8-, and the single-stranded recombinant phage DNA of the coding strand was prepared and annealed with homologous cpRNA. Hybrids with the mature *psbA* mRNA have four intron loops, which are formed by the genomic DNA intron sequences (Figure 1a).

About 8% of the hybrids of the *psbA* gene were formed with unspliced or partially spliced pre-mRNAs (Figure 1b-f). A pre-mRNA which is completely unspliced forms a long hybrid without any loops (f). Partially spliced pre-mRNAs form hybrids with one (e), two (d) and three (b and c) loops, and additional double-stranded regions at the positions of the unspliced introns. Precursors of the unknown upstream gene have also been detected. In Figure 1g the smaller intron has not been spliced out, and in Figure 1h both introns are not spliced. The RNA tail is much longer than usual, suggesting that the gene has one or several more introns upstream of the cloned fragment which had not been spliced out.

If the introns of the *psbA* pre-mRNA were spliced out in a strictly random fashion, the partially spliced pre-mRNAs should contain the four intron sequences in equal proportions. Table I lists the different precursor structures and the number of molecules found for each of the 14 possible types. Almost all types of precursor structures have been observed, but it is appar-

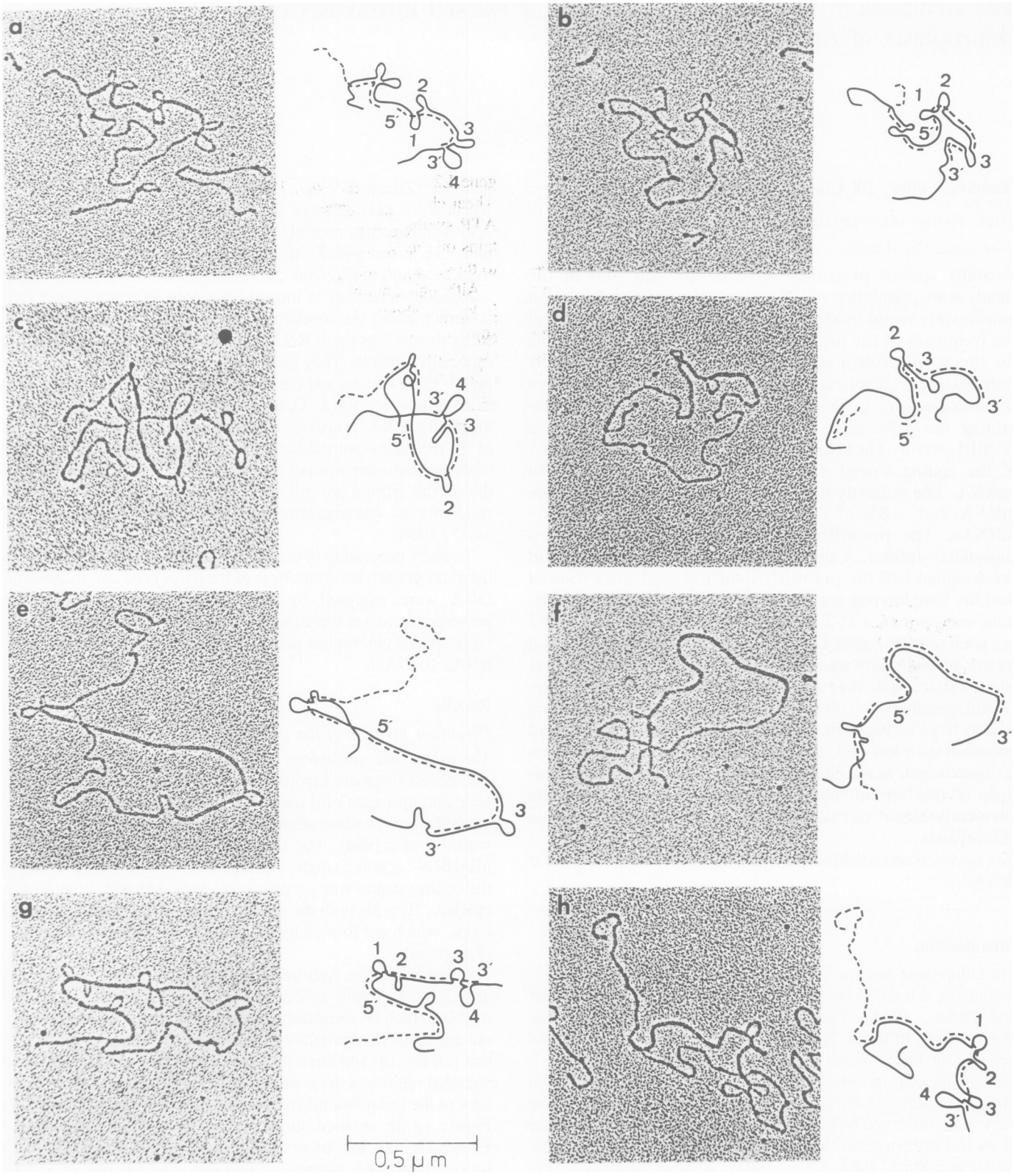

Fig. 1. Electron micrographs of hybrids between cpRNA and the *psbA* gene from *Euglena*. The cpDNA *Eco*RI fragment (see drawing in Figure 2) was inserted in the pEMBL8- vector, and single-stranded circular recombinant phage DNA was annealed with cpRNA. The cloned fragment contains part of another gene which is located upstream of the *psbA* gene in the same orientation. The 5′ end of the mRNA of this gene cannot hybridize, and the resulting single-stranded mRNA tail serves as a marker for the 5′ ends of the genes. (**a**) A hybrid with the mature mRNA of the *psbA* gene. Four intron loops are formed which are marked in the line drawing. The hybrid of the gene upstream displays two intron loops and a non-hybridized RNA tail. (**b**) – (**e**) Hybrids with partially spliced pre-mRNAs. (**b**) The fourth intron has not been removed, instead of the 4th intron loop a longer hybrid region is visible. (**c**) The 1st intron has not been spliced out. (**d**) Introns 1 and 4 have not been spliced out. (**e**) Introns 1, 2 and 4 have not been spliced out. (**f**) A hybrid with an unspliced pre-mRNA. (**g**) and (**h**) Hybrids with pre-mRNAs of the gene upstream of the *psbA* gene. In the tracings the solid lines are DNA, the dashed lines are RNA.

Table I. The occurrence of the 14 different types of partially spliced pre-mRNAs of the *psbA* gene and the frequency of unspliced introns for each intron position

Intron number	1	2	3	4	Number of molecules
One intron	+	−	−	−	5
removed	−	+	−	−	3
	−	−	+	−	2
	−	−	−	+	1
Two introns	+	+	−	−	6
removed	+	−	+	−	4
	+	−	−	+	0
	−	+	+	−	9
	−	−	+	+	6
	−	+	−	+	1
Three introns	−	+	+	+	13
removed	+	−	+	+	5
	+	+	−	+	3
	+	+	+	−	53
No. of unspliced introns	35	23	19	82	111 molecules
% unspliced	32	21	17	74	

Spliced (+) and unspliced (−) introns are indicated for each type of precursor.

ent that the unspliced intron sequences of the four introns do not occur at a random frequency. Of 111 partially spliced pre-mRNAs 53 are molecules in which introns 1, 2 and 3 have been spliced out and only intron 4 is unspliced. The ratio of spliced to unspliced introns in the partially spliced pre-mRNAs was determined for each of the four introns. The proportion of unspliced introns in the steady state pool of partially spliced pre-mRNAs varied from 17 to 21% for introns 3 and 2 to 74% for intron 4. Unspliced intron 1 occurs in 32% of the partially spliced pre-mRNA molecules. No 5′-3′ or 3′-5′ direction of the removal of introns occurs, but intron 4 is usually excised slower than introns 2 and 3, and intron 1 at a medium rate. However, some molecules are spliced at the position of the introns 1 and 4 without the complete removal of introns 2 and 3. It can be concluded that the excision of the introns 2 and 3 is not a prerequisite for the splicing of introns 1 and 4.

The data suggest that each intron is removed independently at a different rate during the maturation of the mRNA, which leads to a non-random distribution of the various types of precursors. In the group of pre-mRNAs with two spliced and two unspliced introns, six molecules were found which were spliced at introns 3 and 4, but only one molecule was found which was spliced at introns 2 and 4, although both types should occur with similar probability. This could indicate the possibility that the splicing of one intron might promote the splicing of the neighbouring intron. The *rbcL* gene (the gene for the large subunit of the ribulose-1,5-biphosphate carboxylase) in *Euglena* is split by nine introns (Koller *et al.*, 1984). In experiments similar to the ones described for the *psbA* gene, partially spliced pre-mRNAs were also found (data not shown). Again no direction for the removal of the introns was apparent, but two unspliced introns often occurred next to each other. However, it is not possible to conclude from the limited statistics that the removal of neighbouring introns is correlated.

Circular excised iRNAs

The intron loops in the hybrids with the mature mRNAs were nearly always single-stranded. Double-stranded introns were only observed in very rare cases. Even if excised introns are present as stable iRNAs in the chloroplast, they might not have been detected in normal hybridizations for two reasons. First, the concentration of iRNAs might be even lower than the concentration of pre-mRNAs. Second, their hybridization might be inhibited if they are covalently closed circles. The annealing of two circular single strands, which could be covalently closed circles, or stem/loop structures, or DNA intron loops in hybrid molecules, is strongly inhibited, because there are no free ends, to allow the winding of one strand around the other. If the iRNAs are closed circles they would not anneal very efficiently to the circular phage DNA, and even less to DNA intron loops of the mRNA/DNA hybrids. To overcome this difficulty the coding strand of the linear *Pvu*II-*Sal*I fragment, which contains most of the *psbA* gene, was prepared. Apart from the additional 16 bp downstream of the cloning site, the fragment is identical with the right hand *Pvu*II-*Eco*RI fragment, as shown in the drawing in Figure 2. To avoid the formation of hybrids with the mature mRNA, low mol. wt. cpRNA was isolated from an agarose gel and annealed with the coding strand of the linear *Pvu*II-*Sal*I fragment. Under these conditions small double-stranded circles were formed with the DNA fragment. Figure 2a–d shows the linear single-stranded DNA fragments with small circular hybrids of the 1st, 2nd, 3rd and 4th intron. The appearance of these circular loops is distinctly different from the appearance of single-stranded DNA intron loops. They have a thicker outline and usually a rigid circular structure, whereas the single-stranded DNA introns (Figure 2e) look thinner and have an irregular and elongated outline. The positions of the hybrid loops with respect to the ends of the fragment were measured in 87 molecules. They coincide with the positions of the introns and verify that the circular double strands are formed by iRNAs.

The conclusion that these iRNAs are covalently closed circles, rather than stem/loop structures is based on two arguments. First: the single-stranded DNA in cytochrome spreadings and the sequence of the *psbA* gene do not show any stable stem/loop structures, which could hold the hybrid in a circular configuration. Second: although short inverted repeats, which often have mismatches but can fold the RNA into a secondary structure, have been reported (Keller and Michel, 1985), the hybrids with pre-mRNAs show smooth double strands of the intron regions. This indicates that the base-paired regions in the RNA are unfolded during the annealing. If the excised iRNAs had a circular configuration due to their secondary structure, they would still form linear hybrids. It can therefore be concluded, that the observed circular hybrids are due to covalent links between the ends of the iRNAs.

Discussion

The unspliced intron sequences in the pre-mRNAs of the *psbA* gene in *Euglena* chloroplasts occur at non-random frequency. This seems to suggest that there is a preferential order for the removal of introns. However, the fact that this order is not a strict rule, has led us to the conclusion, that the introns can be removed independently at different rates. Similar observations were made for the splicing pattern of ovomucoid mRNA (Tsai *et al.*, 1980). The seven introns were removed from pre-mRNAs in a preferred order and the two largest introns were spliced fastest. In the case of rabbit globin mRNA and the adenovirus late leader sequences the smaller introns are usually spliced out before the larger introns (Grosveld *et al.*, 1981; Mariman *et al.*, 1983). Therefore it seems that the differences in the rates of splic-

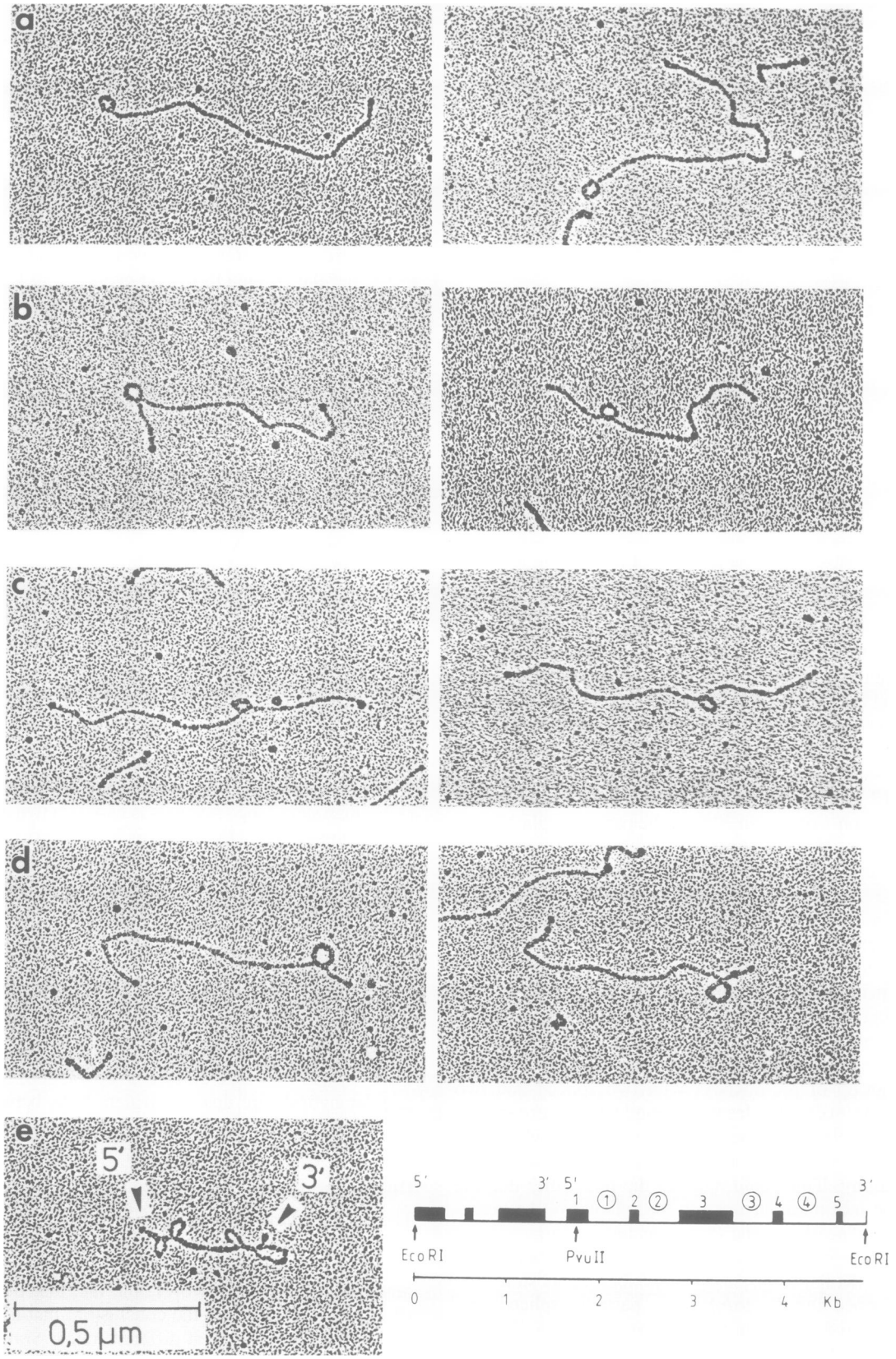

Fig. 2. Electron micrographs of hybrids between a linear single-stranded *Pvu*II-*Sal*I fragment of the *psbA* gene and excised circular iRNAs. The line drawing shows the structure of the *Eco*RI fragment which was cloned into pEMBL and used for the analysis of the partially spliced mRNAs (Figure 1). The *Pvu*II-*Sal*I fragment used here for the hybridization with iRNAs is almost identical to the right hand *Pvu*II-*Eco*RI fragment, since the *Sal*I site is 16 bp outside of the *Eco*RI fragment in the polylinker of the vector. The exons (black bars) of the *psbA* gene are numbered 1 – 5, the four introns are indicated with encircled numbers. **(a)**, **(b)**, **(c)**, **(d)** Hybrids with iRNAs of the 1st, 2nd, 3rd and 4th introns, respectively. The 1st intron is so close to the *Pvu*II site that the part of the 1st exon which is included in the fragment is not visible in the hybrid. **(e)** Hybrid of the same fragment with mature mRNA. In the hybrid with the mature mRNA a 5′ tail is visible upstream of the 1st intron. It consists of the sequences of the 1st exon and the 5′ non-coding sequence of the mRNA. The arrows indicate the 5′ and 3′ ends of the gene.

ing are not simply due to a size effect, and that large introns are not necessarily removed slower than smaller ones. In the case of the *psbA* gene, intron 1 is spliced out slower than the introns 2 and 3, although they have the same size. It is unlikely that intron 4 is spliced out slower than the others, simply because it is larger. Although the splicing system of nuclear introns may not be the same as for chloroplast introns, it was found in all cases, that the apparent order of intron removal is not obligatory.

The sequences and the potential secondary structures of the *psbA* introns have been compared. Intron 4 can be distinguished from the other three introns of this gene in several ways. The border sequence at the 3′ end of the intron 4 is TTTAAC-3′, which deviates from the consensus sequence TTTTAT-3′ in two positions. Of nine sequenced *Euglena* chloroplast introns, five introns (of the *rbcL* gene) follow the consensus sequence exactly and three (the introns 1, 2 and 3 of the *psbA* gene) only deviate in one position (Koller *et al.*, 1984; Karabin *et al.*, 1984; Keller and Stutz, 1984). Whether this correlation has any significance for the splicing reaction is not known.

Introns of pre-mRNAs which do not follow the GT...AG consensus border sequence of nuclear pre-mRNAs, have been grouped into two classes. The members of each class have common sequences and secondary structures (Davies *et al.*, 1982; Michel and Dujon, 1983). Chloroplast introns were allocated to both classes. The *Chlamydomonas* 23S rRNA gene and *psbA* gene, the *Vicia* $tRNA^{Leu}$ and tobacco $tRNA^{Val}$ genes (Erickson *et al.*, 1984; Rochaix *et al.*, 1985; Bonnard *et al.*, 1984; Deno *et al.*, 1982) have typical class I introns which show primary sequence and secondary structure similarities with the *Tetrahymena* nuclear and yeast mitochondrial rRNA introns and the intron 1 of the *Neurospora* mitochondrial apocytochrome b gene (Kruger *et al.*, 1982; Tabak *et al.*, 1984; Garriga and Lambowitz, 1984). Introns of other chloroplast genes, like the $tRNA^{Ile}$ and $tRNA^{Ala}$ from maize (Koch *et al.*, 1981) were allocated together with several yeast mitochondrial introns and the intron of the maize mitochondrial cytochrome oxidase subunit II to class II (Fox and Leaver, 1981; Keller and Michel, 1985). It was attempted to show that all four *psbA* introns are class II introns (Keller and Michel, 1985). Although the introns 1, 2 and 3 have sequence similarities with class II introns, they do not fit into the scheme very well, because their sequences could not be folded into the characteristic secondary structure. Only intron 4 could be folded into a core structure which closely resembles other class II introns. It remains to be seen whether the potential secondary structure is in any way responsible for slowing down the process of splicing, or whether other unknown factors are involved. This would be in contrast to the effect of *cis*-acting mutations in yeast mitochondrial introns, where the secondary structure is essential for the splicing (Grivell *et al.*, 1983).

Circular iRNAs derived from nuclear pre-mRNAs as well as from class I and class II pre-mRNAs have been found. The introns of *Tetrahymena* nuclear and of yeast mitochondrial rRNA genes are excised as linear RNAs and then circularised by a normal 5′-3′ phosphodiester bond (Kruger *et al.*, 1982; Zaug *et al.*, 1983; Tabak *et al.*, 1984). In contrast, the introns of nuclear pre-mRNAs are circularised during the process of splicing, by formation of a 2′-5′ phosphodiester bond which results in a lariat structure (Zeitlin and Efstratiadis, 1984; Rodriguez *et al.*, 1984; Domdey *et al.*, 1984).

Circular iRNAs of class II introns from yeast mitochondria were described (Arnberg *et al.*, 1980; Halbreich *et al.*1980; Hensgens *et al.*, 1983) and it was indicated that circularisation might not be achieved by a normal phosphodiester bond, since the enzyme reverse transcriptase does not seem to be able to proceed across the joint of the intron ends (Grivell *et al.*, 1983). This could be due to a lariat structure as in nuclear pre-mRNA introns. The *Euglena* chloroplast introns have very well conserved intron boundary sequences which resemble the consensus sequences of nuclear pre-mRNA introns (Cech, 1983). On the other hand they have sequence and structural similarities with the mitochondrial class II introns. It will be interesting to see whether the circular iRNAs in chloroplasts are formed *via* a branch structure as in nuclear mRNA introns or whether the circles are formed by a normal 5′-3′ phosphodiester bond. The observation that the excised iRNAs of introns 1, 2 and 3 are also circularised, shows that a secondary structure as was suggested for intron 4 is not necessary for circularisation. It remains to be seen whether the chloroplast splicing system is a derivation of the nuclear system or is entirely different. The fact that the intron border sequences of a wheat chloroplast protein gene are almost identical with the *Euglena* consensus boundary sequences, suggests that the splicing of *Euglena* chloroplasts may function in the same way as in higher plants (Bird *et al.*, 1985).

Materials and methods

Preparation of cpDNAs

A pBR322 clone of the *Eco*RI fragment I from cpDNA of *Euglena gracilis* strain Z was a gift from Mario Keller, Strasbourg. The insert fragment was recloned into the vector pEMBL8-. Recombinant phage DNA of the coding strand was prepared as described (Dente *et al.*, 1983).

Separated complementary single strands of the *Pvu*II-*Sal*I fragment which contains most of the *Euglena psbA* gene (see Figure 2) were prepared with the help of biotin labelling and avidin-agarose columns. Supercoiled DNA of the plasmid was linearised with *Sal*I, which cuts only in the polylinker, 16 bp downstream of the *Eco*RI cloning site. The protruding ends at the *Sal*I site were filled in with biotinylated dUTP using the Klenow enzyme. This linear DNA, which is biotin labelled at both ends, was then digested with *Pvu*II. This results in several fragments of which only two are biotin labelled at one end. One of these fragments reaches from the *Pvu*II site within the 1st exon of the *psbA* gene to the *Sal*I site 16 bp downstream of the *Eco*RI site in the polylinker (see Figure 2) and has a size of ~3 kb. The other biotinylated fragment reaches from the *Sal*I site to the next *Pvu*II site in the vector. It does not contain any insert sequences. The biotin-containing fragments were bound to an avidin-agarose column. By denaturation of the bound DNA with NaOH only the non-biotinylated strand was eluted. The biotinylated strand was subsequently eluted with guanidinium isothiocyanate/formamide. The method has been described in detail elsewhere (Delius *et al.*, 1985).

Preparation of cpRNA

Total cpRNA from *E. gracilis* was prepared as described earlier (Koller *et al.*, 1984). Low mol. wt. cpRNA was isolated from an agarose gel. 10 μg of total cpRNA were separated on a cylindrical 0.8% agarose gel in Loening buffer. Electrophoresis was for 4 h at 50 V. A 2 cm thick slice was cut out from a region ~2 mm below the 16S rRNA band to just above the 5S rRNA band. The RNA was eluted with malachite-polyacrylamide columns, as described (Koller *et al.*, 1978) and recovered in 150 μl of 10 mM Tris-Cl, 1 mM EDTA, pH 7.5.

Preparation of RNA-DNA hybrids

Recombinant phage DNA of the *Euglena psbA* gene (at a final DNA concentration 2 – 10 μg/ml) was mixed with ~1 – 2 μg of total cpRNA, or 20 μl of the low mol. wt. RNA which was eluted from the agarose gel. The hybridization mix had a final volume of 40 μl which contained 25% formamide, 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 0.2 M CsCl. The mixture was hybridized overnight at 33°C. To remove excess RNA the sample was passed over a Sepharose C1-2B column (3 × 45 mm), equilibrated in 10 mM Tris-Cl, 1 mM EDTA, pH 7.5.

Electron microscopy

Samples were spread in 50% formamide, 100 mM Tris-Cl, pH 8.5, 10 mM EDTA with cyanogen bromide-cleaved cytochrome on a hypophase containing 0.005% octyl glucopyranoside (Sigma).

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