A self-splicing group II intron in the mitochondrial large subunit rRNA (LSUrRNA) gene of the eukaryotic alga *Scenedesmus obliquus*

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ABSTRACT

The DNA sequence has been determined of the 3'terminus from the mitochondrial large subunit ribosomal RNA (LSUrRNA) gene of the eukaryotic green alga *Scenedesmus obliquus* (strain KS3/2). The gene contains two intervening sequences with characteristic sequence motifs of group II and group I introns respectively. The exon/intron boundaries of the introns have been revealed by sequence determination of the mature rRNA. During RNA processing of the precursor RNA, several abundant RNA molecules are stably maintained in addition to the mature rRNA *in vivo*. In *vitro* transcripts of the LSUrRNA gene containing the group II intron (608 bp) display a strong 'self-splicing' activity under high salt conditions. The 608 bp intron is the first group II intron reported to be integrated into a LSUrRNA gene and represents the smallest self-splicing group II intron from eukaryotic organelles so far described.

INTRODUCTION

Despite the numerous reports about mitochondrial gene organization and expression from eukaryotic organisms, algal chondrioms have been investigated in only few cases. The best studied example is the chondriome from the green alga *Chlamydomonas reinhardtii*, where about 90% of the total sequence from the 15 kb linear mtDNA has been determined (1, 2, 3, 4, 5, 6). Despite the scrambled gene organization for the large and small rRNAs (LSUrRNA, SSUrRNA), no intron sequences could be identified (5). To our knowledge, this paper reports the first example of a mosaic mitochondrial gene from an eukaryotic alga.

Recently, the isolation and molecular characterization of extranuclear DNA from eukaryotic alga KS3/2 was described (7). This alga has been identified as *Scenedesmus obliquus* (E. Hegewald, pers. communication). Using the mitochondrial 'mobile intron' from the filamentous fungus *Podospora anserina* as a probe (for review see 8), homologous DNA sequences were

detected in both mitochondrial and plastid DNA (ptDNA). Subsequent molecular analysis revealed that the plastid petD gene is interrupted by a 3.5 kb group II intron, which contains an open reading frame for a reverse transcriptase-like enzyme.

In this communication, the isolation and molecular analysis of the mitochondrial sequence homologous to the fungal intron is described. The gene for the large subunit of ribosomal RNA (LSUrRNA) contains two intronic sequences in the 3' terminal region. The upstream intergenic sequence is a group II intron, similar to those found in other organelle genes, while the downstream sequence resembles a group I intron. The group II intron is shown to possess autocatalytic RNA processing activity *in vitro*. This is the first description of a self-splicing intron in the organelle DNA from a photoautotrophic eukaryote.

MATERIALS AND METHODS

Strains and plasmids. Scenedesmus obliquus (strain KS3/2) is a wild isolate from Bochum, Germany (7) and its identification was performed by E. Hegewald (Jülich, Germany). Recombinant plasmid pAME6 contains a 3.9 kb EcoRI mtDNA restriction fragment integrated into vector pUC8. This plasmid contains the 3' region of the mitochondrial LSUrRNA gene including two intervening sequences as shown in Fig. 1. Plasmid pIG490-8 was derived from pAME6 and contains a 1.8 kb SalI-HindIII restriction fragment inserted into vector pT7-7 (9). It contains 553 bp of the 5' exon, the entire group II intron (608 bp), the 48 bp 3' flanking exon, and part of the group I intron (240 bp) (compare Fig. 2).

Isolation of nucleic acids, gel electrophoresis, hybridization conditions, oligonucleotide synthesis and standard in vitro recombinant techniques were carried out as already described (7).

Oligonucleotides used in this communication. The position of oligonucleotides is according to the DNA sequence, which was reported to the EMBL data library (accession number X17375).

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No.	Sequence	Position	Probe
102	5'- CTC GTG GAA GGA TTA TT	2598–2615	group I intron
123	5'- GCA GCA AGA GGA CAC GAT	3535–3552	3'exon
133	5'- ACC AGC CCG TCG ATT GGA	2230–2247	48 bp exon
132	5'- CGC ATG TGA TGG TAG GCT CA	1667–1686	group II intron

RNA sequencing. Total RNA from alga KS3/2 was isolated as described (7) and primed with oligonucleotide no. 123. For RNA sequencing with reverse transcriptase, the modified conditions from Tabak and coworkers (10) were used as described (11).

In vitro transcription and in vitro splicing. Transcripts uniformly labelled with ^{35}S-UTP were generated by in vitro transcription of pIG490-8, after restriction with HindIII. Transcription reactions were carried out in a finale volume of 20 μl containing 2–3 μg of template DNA, 40 mM Tris-HCl (pH 8.0), 8 mM MgCl$_2$, 5 mM DTT, 2 mM spermidine, 25 mM NaCl, 25 U RNase-Inhibitor (Amersham), 500 μM ATP, CTP, GTP, UTP, 20 μCi ^{35}S-UTP (Amersham, 400 Ci/mmol) and 40 U T7 RNA Polymerase (Boehringer) for 2 h at 37°C. After separation on 6% polyacrylamide-8 M urea gels and autoradiography, individual RNA bands were extracted and purified according to the method of Frendewey and Keller (12).

In vitro splicing of purified precursor RNA was performed under high salt conditions using NH$_4$Cl-buffer (40 mM Tris-HCl, pH 7.5, 60 mM MgCl$_2$, 2 mM Spermidine, 1.25 M NH$_4$Cl), and (NH$_4$)$_2$SO$_4$-buffer (40 mM Tris-SO$_4$ pH 7.5, 60 mM MgSO$_4$, 2 mM spermidine, 500 mM (NH$_4$)$_2$SO$_4$) respectively (13, 14). Reactions were incubated at 45°C and stopped by addition of an equal volume of gel loading buffer. Products were analysed on 6% polyacrylamide-8 M urea gels.

RESULTS

Isolation of a mitochondrial DNA fragment with homologies to a fungal 'mobile intron'

mtDNA from the eukaryotic unicellular alga KS3/2 has a size of 45 kb as calculated from restriction analysis (7). Hybridization with the 'mobile intron' from the ascomycetous fungus *Podospora anserina* (15) allowed the identification of a mitochondrial 3.9 kb EcoRI and a 3.6 kb HindIII restriction fragment with significant homology. After construction of a mtDNA library using bacterial vector pUC8, the 3.9 kb restriction fragment was isolated for further molecular analysis. The designation of the corresponding recombinant plasmid is pAME6 (Fig.1).

The 3.9 kb EcoRI restriction fragment carries the 3' region of the mosaic mitochondrial LSUrRNA gene

Sequencing of plasmid pAME6 resulted in the determination of a continuous DNA stretch of 3,888 bp (EMBL accession no. X17375). With the aid of a computer search program we revealed significant homology with mitochondrial LSUrRNA genes from plants, fungi, and animals. In comparing the algal sequence with the homologous genes from other sources, most conservative domains identified were found typically in the 3' region of various LSUrRNAs. This allows to draw a diagram which shows the secondary structure of the 3'half of the mitochondrial LSUrRNA (Fig. 2).

The secondary structural model exhibits most features which are conserved among prokaryotic and eukaryotic LSUrRNA (16, 17). At position 2233, a region of unknown structure (98 nt) is
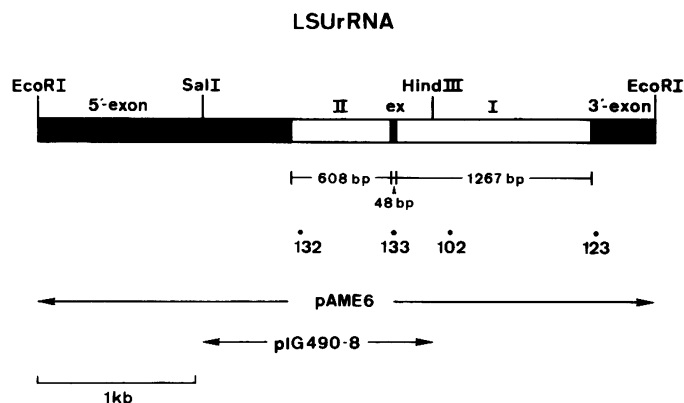
LSUrRNA

Fig.1. Physical map of the 3'region of the LSUrRNA gene containing a group II and a group I intron. Fragments are indicated which were subcloned into recombinant plasmids. The positions of oligonucleotides are given which were used in hybridization experiments.

included as a detached linear array (designated A). Except in fungal mitochondria, all other sequences contain at that position an unstructured loop of about 20 to 30 nt. The mitochondrial ribosomal RNA sequence from the yeast *S. cerevisiae* contains a 120 nt sequence at that position. Common with mitochondrial LSUrRNAs from maize and *Oenothera* is the presence of variable regions V19 and V21 in the *Scenedesmus* sequence. These domains are drastically shortened in the *Chlamydomonas reinhardtii* LSUrRNA (5).

Surprisingly, when we compared the algal sequences with those from plants (e.g. *Oenothera*, maize) two extra sequences of 608 bp and 1266 bp were observed. The 608 bp sequence contains characteristic consensus sequences of group II introns and is inserted at position 2466 in Fig. 2. This site corresponds to position 2455 of the *E. coli* LSUrRNA. The most conserved motifs were compared with corresponding sequences from various group II introns and show significant similarity (Fig. 3). These are presumably responsible for the successful hybridization between the fungal and algal intronic sequences. The 1266 bp sequence is inserted at position 2514 of the mitochondrial LSUrRNA and probably represents a group I intron, because of its homology with the group I intron found in the homologous gene from yeast (Kück, unpublished). The two intron sequences are separated by a 48 bp exon region.

During RNA processing, intermediates derived from the LSUrRNA gene are stably maintained *in vivo*

Total algal RNA was fractionated on denaturing agarose gels and subjected to Northern hybridization analysis. Using exon and intron specific probes, several RNA species of varying abundances were identified which contain exclusively exon, exon/intron, or intron sequences. From the sum of all hybridizations (Fig. 4) the following conclusion may be drawn:

1. The exon probes hybridize with an abundant transcript of 2.0 kb, representing most likely the mature rRNA.

2. Oligonucleotide no. 132, complementary to the group II intron shows homology with RNA molecule 1.8 and 1.5 kb in size. As discussed later, these RNA species probably represent RNA intermediates generated during *in vivo* RNA splicing.

3. The group I specific intron probe hybridizes with a 1.8 and a 0.5 kb RNA molecule. Comparing them with mature rRNA, the two RNA species are present at moderate abundance. This
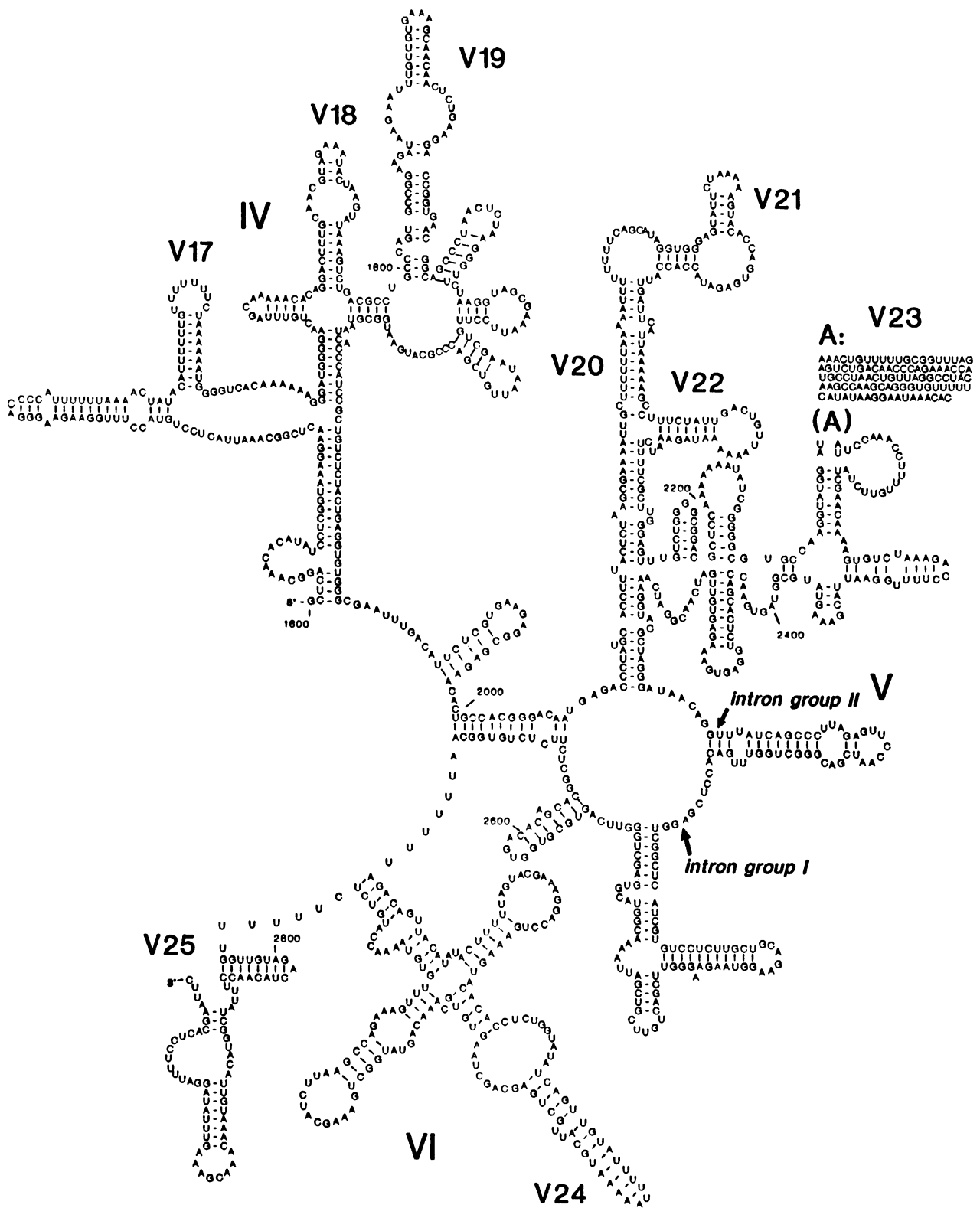
Fig.2. Proposed secondary structure of the mitochondrial LSUrRNA, 3'half, as determined from the 3'exon DNA sequence of the algal gene. The format coincides with that from *E.coli* (16). Position 1600 corresponds to position 1647 and 2352 of the *E.coli* and *Zea mays* mitochondrial 23SrRNA molecule respectively (16). Arabic numbers and Roman numerals denote variable regions and major structural domains of LSUrRNAs, as defined by Lang et al. (31) and Noller (32).

implicates that intron derived RNA molecules are stably maintained during RNA processing.

Compared with corresponding rRNAs from other organisms, the size of 2.0 kb for the *Scenedesmus* LSUrRNA is rather small. But size calibration of the RNA was performed in comparison with the homologous LSUrRNAs from fungal mitochondria

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                    exon | intron
So mt  LSUrRNA   5'-GUGCG -  527 nt - CUU GAGCCGUGUGCGGUGAAAGUCGCAUGCACGGUUC
So pt  petD-1    5'-GUGCG -3435 nt - GAG GAGCCGUAUGAGGUGAAAGUCUCACGUACGGUUC
Pa mt  COI-1     5'-GUGCG -2438 nt - GGA GAGCCGUAUGCGGUGAAAGUCGCACGUACGGUUC
Sc mt  COI-1     5'-GUGCG -2361 nt - GGA AAGCCGUAUGAUGGGAAACUAUCACGUACGGUUU
Sc mt  COI-2     5'-GUGCG -2416 nt - GGA GAGCCGUAUGAUAUGAAAGUAUCACGUACGGUUC
Zm mt  COII      5'-GUGCG -  727 nt - GGA GAGCUUUUUGCGGGGAAACUUGCAAGUCAACUUU
Zm pt  trnA      5'-UUGGG -  686 nt - GGA GAGCACAGUACGAUGAAAGUUGUAAGCUGUGUUC
Cr pt  ps1A1-1   5'-GUGCG -   50 kb - CUU GAGCCGUAUGCGAAAAAACUCGCAUGUACGGUUC
Cr pt  ps1A1-2   5'-GUGCG -   90 kb - UUU GAGCCGUGUGCAGUGAAAAUUGCAUGCACGGCUC
Eg pt  psbA-4    5'-GUGCG -  535 nt - UUU GAGCCUUGUGAUAUUAAAAAUUUCAUGCAAGGUUC
                                      ◄-----      domain V        -----►

consensus seq.  5'-GUGCG           ... PAGCY.UPUP..P..AAA.U..YAYGYA.PGUUY
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                                                         intron | exon
So mt  LSUrRNA   UAC UGGGGGGAAAGCCUG - 2- AGGGCCUACCU[A]UCCA ACU -3'
So pt  petD-1    GGC AGGGGUAAUAUGGUA -19- UAUCUUUCCCCU[A]GCC UCU -3'
Pa mt  COI-1     GGA GGAGAGUUUUUGGUU -22- GACUGGCUGCUU[A]UCC UAC -3'
Sc mt  COI-1     GGG AAAGGCUCUUUAACA -12- GGUUAAUUUGCU[A]UUU CAU -3'
Sc mt  COI-2     GGA GAGGGCUCUUUUAUA -18- GAUAGGUUUGCU[A]CUC UAC -3'
Zm mt  COII      GGG GGGAGGCGG---------------GCGUC[G]ACCC AAC -3'
Zm pt  trnA      GGG GGGGAGUUAUUGCCU -41- AGGCGGUGGUUU[A]CCC UGU -3'
Cr pt  ps1A1-1   UUU AGGAGGAUUUAAAAU -13- AAAACAAAUCCU[A]CCU GAC -3'
Cr pt  ps1A1-2   UUA AGGUUUAAAUAAAUU - 8- AGAAAAUUUA[A]CUCCU AAC -3'
Eg pt  psbA-4    UGU GACGAUAUUUUU    - 6- GAAAAUUUU[A]GUUU AAC -3'
                     ◄------       domain VI        -----►
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Fig.3. Comparison of the conserved sequence motif of domain V from various group II introns. Boxed A residues are thought to form the stem of the branch during lariat formation (18). Horizontal arrows indicate the exon/intron boundaries. Abbreviations: So: Scenedesmus obliquus (strain KS3/2); Cr: Chlamydomonas reinhardtii; Eg: Euglena gracilis; Pa: Podospora anserina; Sc: Saccharomyces cerevisiae; Zm: Zea mays; pt: plastid; mt: mitochondrial; LSUrRNA: large subunit rRNA; COI, II: subunit I, II of the cytochrome oxidase; petD: subunit IV of the cytochrome b_6/f complex; ps1A1: P700 chlorophyll a-apoprotein A1 from PSI; psbA: 32 kd protein form PSII; trnA: tRNA^{Ala}. The sequence of the petD intron is from ref. 7, all other sequences were taken from ref. 33.

(Podospora anserina). The small size seems to be reminiscent of the situation found in *Chlamydomonas reinhardtii* (5). In this alga, abundant low molecular weight RNA species ranging in size from 0.1 kb to 0.7 kb were found to be homologous with rRNA probes. A complete sequence of the *Scenedesmus obliquus* LSUrRNA gene may provide the final evidence for a discontinuous gene organization with specific rRNA modules.

Determination of the exon/intron boundaries by sequencing of the mature rRNA

From Northern hybridization experiments it becomes obvious that several RNA species may be derived from the intron sequences, which are stably maintained during RNA processing. For further molecular analysis the knowledge of the precise exon/intron boundaries is a prerequisite for detailed investigations of precursor RNA processing.

Total algal RNA was probed with oligonucleotide no. 123, which covers position 3535 to 3552 of the LSUrRNA gene sequence (Fig. 1). Generation of the cDNA by reverse transcription of mature LSUrRNA allows determination of the two intronic splice donor sites. As an example, the corresponding sequence analysis for the group II intron is shown in Fig. 5. In Fig. 2, the integration sites of intronic sequences in the mature rRNA are designated by arrows.

The 608 bp group II intron is capable of self-splicing *in vitro*

Precursor RNAs of about 1540 nt containing the entire group II intron (608 nt), a 645 nt 5' exon and a 275 nt 3' exon were obtained by *in vitro* transcription of pIG490-8 truncated at the HindIII site (Fig. 1). Following *in vitro* transcription we obtained not only the expected precursor but also several RNAs, identified as intermediates and products of a self-splicing reaction (not shown). We purified the 1540 nt RNA from a 6% denaturing
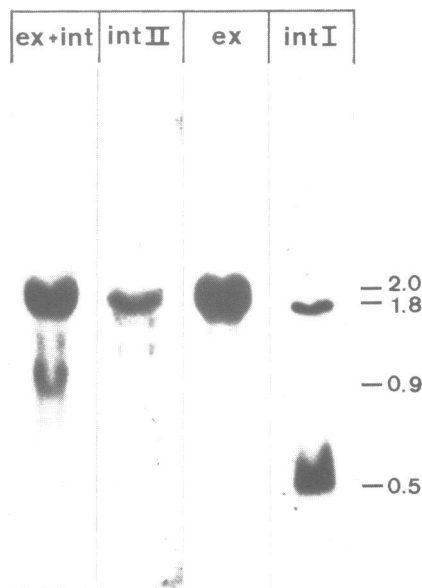
Fig.4. Northern hybridization of algal RNA with plasmid pAME6 (ex + int), exon (oligonucleotides no. 133), intron group I (olig. no. 102), and intron group II (olig. no. 132) specific probes.

polyacrylamide gel. Self-splicing activity of run-off transcripts was tested under different high salt conditions (Fig. 6a). Incubation in the presence of 1.25 M NH$_4$Cl yield several major RNAs, corresponding in length to specific splicing products and intermediates, including ligated exons (920 nt), free 5' and 3' exons (645 nt and 275 nt, respectively), the linear form of the intron (608 nt) and the linear intron-3' exon intermediate (883
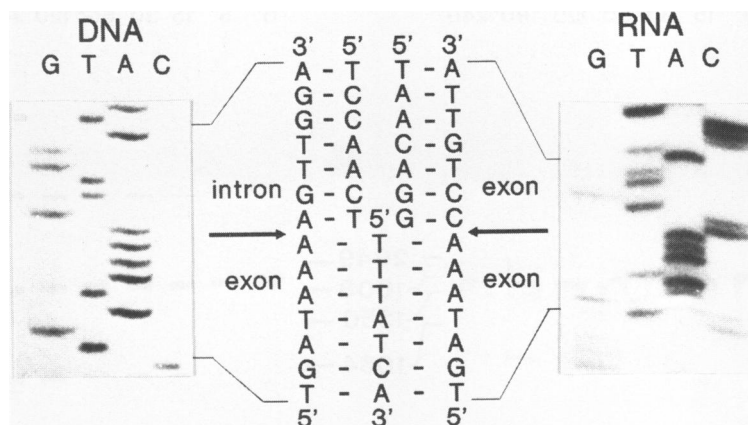
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        DNA                    3'  5'   5'  3'              RNA
      G  T  A  C             ┌─ A - T   T - A ─┐          G  T  A  C
                                A - T   A - T
                                G - C   A - T
                                G - C   A - T
                                T - A   C - G
                                T - A   A - T
                      intron    G - C   G - C  exon
                                A - T 5'G - C
                             ┌─ A -  T  -  A ─┐
                                A -  T  -  A
                      exon      A -  T  -  A    exon
                                T -  A  -  T
                                A -  T  -  A
                                G -  C  -  G
                             └─ T -  A  -  T ─┘
                                5'  3'   5'
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Fig.5. Determination of exon-intron splicing sites by plasmid DNA and rRNA sequencing. Oligonucleotide no. 123 was hybridised with recombinant clone pAME6 for DNA sequencing and with total RNA from alga KS3/2 for RNA sequencing.

nt). In addition, two RNAs can be seen, migrating more slowly than the precursor RNA, as it is characteristic of circular and lariat-shaped molecules in polyacrylamide gels. To test whether or not intron I specific self-splicing reactions are involved, we have tried to label RNA products by the addition of ^{32}P-GTP, present during the self-splicing reaction. In this case no labeled RNA products could be defected.

In yeast and other fungal mitochondria it has been shown, that group II introns are excised as lariats (18, 19, 20, 21). Furthermore for the self-splicing mitochondrial introns of *S.cerevisiae* (aI5j and bI1) a minor splicing product with lower electrophoretic mobility than the intron lariat was identified as an intermediate of the splicing reaction: the intron-3' exon lariat (22, 23).

We conclude that the most prominent slow migrating band of the splicing reaction of the LSUrRNA intron probably is the group II intron lariat, whereas the faint band could correspond to the intron-3' exon lariat intermediate.

Identical slow migrating RNAs appear when 500 mM $(NH_4)_2SO_4$ is employed for incubation. In contrast to the NH_4Cl reaction the linear intron molecule and the 5' exon are found only in minor amounts, whereas the 3' exon and linear intron-3' exon intermedi ate are not visible even after 240 min.

The identification of a slow migrating intron lariat is further confirmed when splicing products from the $(NH_4)_2SO_4$ incubation were resolved on denaturing gels with different polyacrylamide concentrations (fig. 6b). The migration of the linear precursor RNA and the ligated exons is almost identical relative to each other in 5%, 6%, and 8% polyacrylamide-denaturing gels. In contrast, the presumed lariat molecule is highly retarded in the 6% or 8% gel, but migrates close to the precursor RNA in the 5% gel. On 3.5% polyacrylamide gels, the two RNA species are not separable because of their identical electrophoretic migration properties (not shown).

DISCUSSION

Group II introns have been reported to exist in a wide range of organelles in eukaryotic organisms. This includes fungal and plant mitochondria as well as plastids from plants and algae (33). No report is available about mosaic genes from algal mitochondria, and to our knowledge, we present the first description of a

discontinuous mitochondrial gene from an eukaryotic alga. One of two intervening sequences displays typical features of group II introns with a highly conserved sequence motif (domain V) at the 3'end (Fig. 4).

The occurrence of a group II intron in the gene for the large subunit ribosomal RNA is unique, although introns of this group have been found in many structural genes from mitochondria and plastids (for review see 24). The unusual location of the group II intron supports the view of many investigators that introns represent remnants of transposable elements, which are able to move between distantly related organisms (25, 26). Consistent with the assumption that introns represent mobile elements are the autocatalytic properties of some intron RNAs (27).

Several groups of investigators have already demonstrated that yeast mitochondrial group II introns are capable of autocatalytic excision from precursor RNA *in vitro* (18, 19, 20).

Self-splicing activity of these introns has been shown to occur under low salt conditions (e.g. 10 mM Tris-HCl, 10 mM $MgCl_2$, 2 mM spermidine) in the absence of proteins via a two-step transesterification pathway. The first step leads to cleavage at the 5' splice site and formation of the intron-3' exon lariat, the second is cleavage at the 3' splice site resulting in exon-exon ligation and release of the intron lariat (for review see 28).

The products of transesterification are also predominant in splicing reactions with yeast introns aI5j (29) and bI1 (14) under high salt conditions when 500 mM $(NH_4)_2SO_4$ is provided. In contrast to low salt conditions the rate of self-splicing is increased. Using similar reaction conditions with run-off transcripts from the mitochondrial LSUrRNA group II intron of *Scenedesmus obliquus*, we obtained an almost identical product pattern, although the conversion of the algal precursor RNA seems to be not so efficient when compared with data from splicing of yeast mitochondrial introns (29, 14).

The product pattern of the splicing reaction is strikingly different, when NH_4Cl instead of $(NH_4)_2SO_4$ is used. The presence of 1.25 M NH_4Cl increases the amounts of excised linear intron and free 5' exon and moreover free 3' exon and the linear intron $-3'$ exon intermediate appears as new products. From these data we suppose that the group II intron of the LSUrRNA of *Scenedesmus obliquus* follows the same reaction pathway as the self-splicing introns of *S.cerevisiae*. As evidenced in yeast, branch point dependent 5' cleavage and lariat formation can be partially replaced by cleavage without transesterification
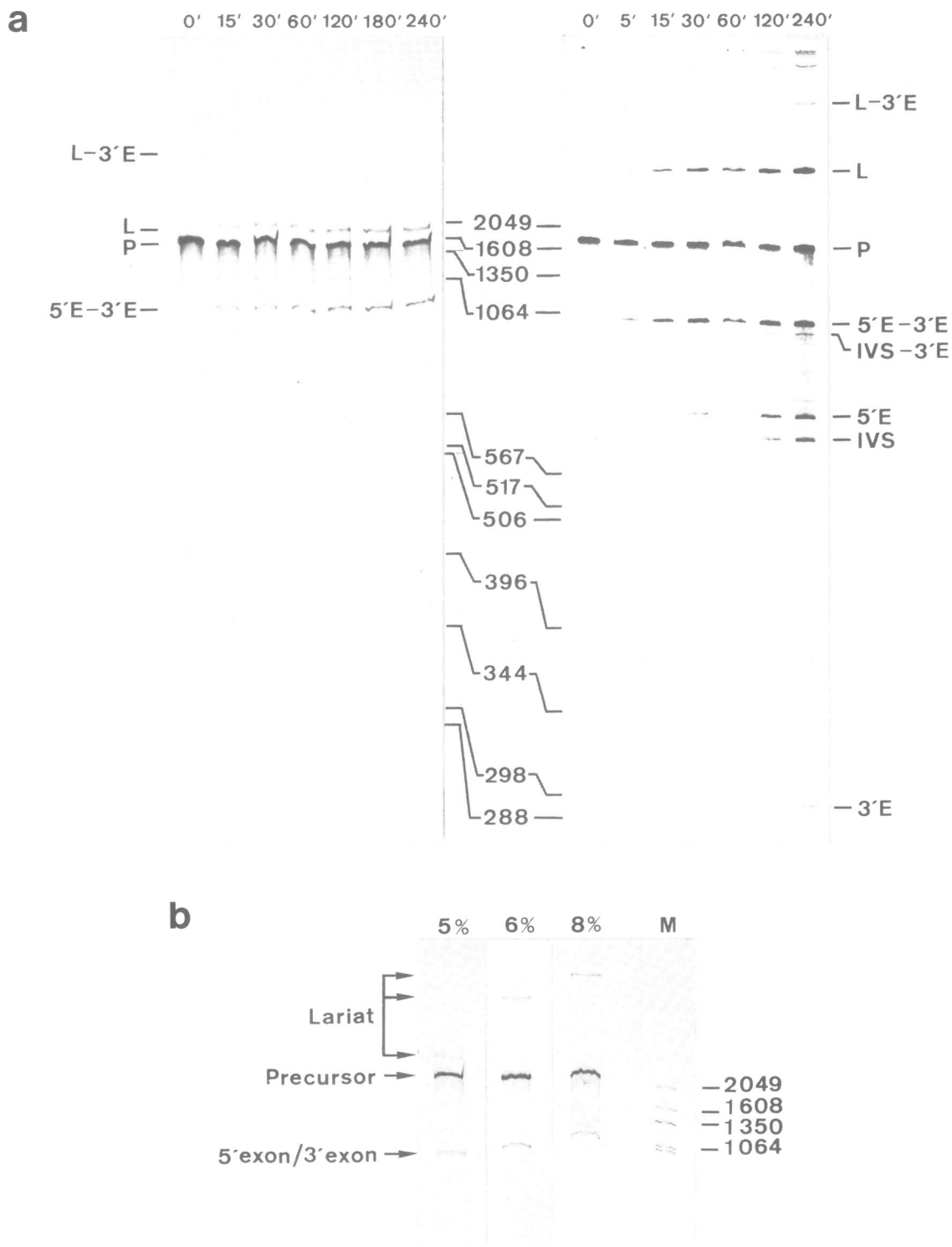
Fig.6. Autoradiogram of denaturing polyacrylamide gels on which products from self-splicing reactions were separated. *In vitro* splicing of pIG490-8/HindIII run-off RNAs. (a) Transcripts la belled with ^{35}S-UTP were incubated at 45°C for various times (0, 5, 15, 30, 60, 120, 180, 240 min) in reaction buffer containing 40 mM Tris-H$_2$SO$_4$, pH 7.5, 60 mM MgCl$_2$, 2 mM spermidine, 500 mM (NH$_4$)$_2$SO$_4$ (left), or 40 mM Tris-HCl pH 7.5, 60 mM MgSO$_4$, 2 mM spermidine, 1.25 M NH$_4$Cl (right) and separated on a 5% ((NH$_4$)$_2$SO$_4$) or 6% (NH$_4$Cl) denaturing polyacrylamide gel. (b) Separation of pIG490-8 HindIII transcripts after incubation for 1h at 45°C in (NH$_4$)$_2$SO$_4$ splicing buffer using a 5%, 6% and 8% polyacrylamide-8 M urea gel (ratio of cross-linking was 30:1). As a size marker denatured 3' labelled fragments of pMC1403 (34) digested with HinfI were used. Abbreviations: L-3'E: intron lariat with 3'exon, L: intron lariat, P: precursor, 5'E-3'E: ligated exons, IVS-3'E: linear intron with 3'exon, 5'E: free 5'exon, IVS: linear intron, 3'E: free 3'exon.

using high concentrations of KCl or NH$_4$Cl. In this case the 5′ splice junction is cleaved by hydrolysis after nucleophilic attack by water or OH⁻ (30). 5′ Hydrolysis and cleavage of the 3′ splice site by transesterification results in excision of linear intron and ligated exons. The exon ligation product can be reopened by hydrolysis in an intron-dependent trans-reaction releasing free 5′ and 3′ exon (29).

In summary, the 608 bp group II intron is the smallest self-splicing group II intron isolated from organelles described so far and it probably contains optimal sequences necessary for autocatalytic activity irrespective from the incubation conditions. Deletion mutagenesis of the intron sequence will further identify the minimal intron sequence required for self-splicing activity.

ACKNOWLEDGEMENT

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