Characterization of products derived from self-splicing of intron $\text{al5}\alpha$ which is located in the mitochondrial COX I gene of *Saccharomyces cerevisiae*

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ABSTRACT

We have characterized the in vitro self-splicing of intron $\text{al5}\alpha$ containing precursor RNA from the yeast mitochondrial gene coding for cytochrome oxidase subunit I. This intron follows the rules for group I self-splicing introns and all the characteristic products have been identified. In addition we have detected abnormal RNA products with features that indicate that the self-splicing behaviour of this intron is more complex. Two intron circles are formed by use of a major and minor intron-internal site for circle closure. A cryptic 5'-splice site located in the 3' exon results in guanosine nucleotide mediated opening at a position 30 nt downstream of the normal 3' splice site. The reactions can all be explained on the basis of the "splice guide" model proposed by Davies et al (1982 Nature 300 719-724). Although the sequence motifs at cyclization and splice sites occur more often in this intron, only some of them are allowed to interact with the internal guide sequence, suggesting that both primary structure and spatial folding of the RNA are involved in formation of productive reaction sites.

INTRODUCTION

Saccharomyces cerevisiae mt DNA contains three genes interrupted by intervening sequences (introns). All these introns can be assigned to two groups on the basis of proposed secondary structures (1,2,3). An increasing number of fungal mitochondrial introns have been shown to be self-splicing in vitro: in *Neurospora crassa* the first intron of the cytochrome b precursor (4) and in *S. cerevisiae* the intron of the precursor to the large rRNA (5,6,7), introns al3 and $\text{al5}\alpha$ from the precursor of the COX I gene (6,8) and intron bl5 of the cytochrome b precursor (9) are self-splicing group I introns. Introns $\text{al5}\gamma$ and bl1 from the precursors of the COX I and cyt. b gene respectively, are the only two examples of self-splicing group II introns reported thus far (10,11,12).

The mechanism by which group I introns are excised from precursor RNA was first elucidated by Cech and coworkers, in a study of the intron in the large ribosomal RNA of *Tetrahymena* (13,14), but the mechanism applies to all self-splicing group I introns studied thus far (15). It involves two transesterification reactions. In the first one a guanosine nucleotide attacks the 5' exon-intron junction, resulting in a free 5' exon and intron+3' exon product. The intron+3' exon product carries the extra added guanosine residue at its 5' end. In the second transesterification, the 3' end of the 5' exon attacks the intron+3' exon junction, which leads to exon ligation and linear intron excision. The linear intron can then undergo cyclization by attack of its 3' terminal guanosine residue at the cyclization site in the 5' part of the intron. In this transesterification reaction a short stretch of nucleotides including the extra guanosine is split off from the 5' part of the intron.

In this report we present the properties of products of COX I $\alpha 5\alpha$ intron self-splicing in more detail. We demonstrate that in addition to the expected intermediates and products, a number of extra products are formed. Their formation can be explained along the lines of the splice-guide model in which sequences complementary to the Internal Guide Sequence (IGS) are positioned close to or in the reactive centre (2). Although there are multiple sequences which can be aligned with the IGS, only some of them are used in the side reactions we describe. These findings indicate that not only primary sequence complementarity is important but also the structure of the RNA in which these sequence motifs are embedded.

MATERIALS AND METHODS

Materials. Restriction enzymes were from New England Biolabs or Boehringer Mannheim. T4 polynucleotide kinase and *E. coli* DNA polymerase according to Klenow were also from Boehringer Mannheim. [α 32 P]-GTP (400 Ci/mmol), [α 32 P]-UTP (3000 Ci/mmol), [γ 32 P]-ATP (3000 Ci/mmol) and SP6 RNA polymerase were from Amersham. AMV reverse transcriptase was from Life Science. T7 RNA polymerase was home made, using an overproducing *E. coli* strain kindly provided by F.W. Studier, according to the protocol from Davanloo et.al. (16).

Plasmid constructions. The plasmid constructions were made using mtDNA from petite mutant LH30-5A from *S. cerevisiae* strain KL 14-4A (17). pSP $\alpha 5\alpha/\beta$ was made by ligating a 2093 bp Bgl II-Msp I fragment

from LH30-5A mtDNA in a Bam HI -Acc I cut pSP 65 vector (24,fig. 1). pT7 $\alpha 5\alpha/I$ was made by treating Hind III digested pSP $\alpha 5\alpha/\beta$ with nuclease BAL 31 and cloning the Eco RI-Bal 31 fragment behind the T7 promoter in Eco RI-Sma I cut pT713 DNA (GIBCO BRL catalog, 1987 p. 62). pSP $\alpha 5\alpha/II$ was made by cutting pSP $\alpha 5\alpha/\beta$ with Nco I and Hind III, filling the recessed 3' ends using Klenow polymerase and ligating the blunt ends. The newly made insert-vector junctions in pT7 $\alpha 5\alpha/I$ and pSP $\alpha 5\alpha/II$ were checked by sequencing. pT7 $\alpha 5\alpha/I$ still contains 32 nt of $\alpha 5\beta$ intron sequences.

In vitro transcription. pSP $\alpha 5\alpha/\beta$ and pSP $\alpha 5\alpha/II$ were linearized with Pvu II, pT7 $\alpha 5\alpha/I$ was linearized with Hind III. After phenol extraction and precipitation with ethanol, the template was dissolved in 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA. In vitro transcription was carried out in a 20 μ l reaction with 1 μ g template in 40 mM Tris-HCl pH 7.5; 6 mM $MgCl_2$; 20 mM NaCl; 10 mM DTT; 4 mM spermidine; 0.5 mM rNTP's and 10 μ Ci [$\alpha^{32}P$]-UTP. Transcription was started by adding 5 Units SP6 RNA polymerase or T7 RNA polymerase. Incubation was at 37 $^{\circ}C$ for 30 minutes. To stop the reaction, SDS was added to a final concentration of 1 % in a total volume of 100 μ l. The transcription mixture was phenol extracted, passed through a sephadex G-50 column and nucleic acids were precipitated with ethanol. Precursor RNA was isolated from low melting point agarose when appropriate.

In vitro self-splicing. In vitro self-splicing was carried out at 40 $^{\circ}C$ in 20 μ l in a mixture containing 0.2 mM GTP; 100 mM NH_4SO_4 ; 50 mM $MgCl_2$ and 50 mM Tris-HCl pH 7.5. GTP end-labeled molecules were formed by incubating unlabeled precursor RNA with 30-40 μ Ci [$\alpha^{32}P$]-GTP under the same conditions. The reaction was stopped by adding EDTA to a final concentration of 75 mM in a total volume of 100 μ l. The products of self-splicing were recovered by passing this mixture through a sephadex G-50 column and precipitating the RNA with ethanol.

RNAse T₁ digestion and primer extension analysis. Partial T₁ digestion was carried out according to Van der Horst and Tabak (6). Primer extension analysis was carried out according to Tabak et. al. (5).

RESULTS

The yeast mitochondrial gene coding for cytochrome oxidase subunit I in strain KL14-4A is interrupted by various group I and group II introns.

Earlier experiments indicated that some of the group I introns can be excised *in vitro* by self-splicing. This was based on the observation that precursor RNA present in RNA extracted from mitochondria could self-splice *in vitro*, resulting in formation of excised introns 5' end-labeled with [α^{32} P]-GTP derived from the incubation mixture. One of these labeled introns was al5 α .

Initial experiments to study the self-splicing properties of intron al5 α were carried out using pSP al5 α / β , linearized with Pvu II. This recombinant DNA consists of intron al5 α + flanking exon sequences plus a large part of intron al5 β (18, fig. 1). After transcription and purification by electrophoresis on agarose, precursor RNA was incubated under self-splicing conditions. Figure 2 compares the results of experiments carried out with internally labeled RNA (lanes 1-4) with unlabeled RNA incubated in the presence of [α^{32} P]-GTP (lane 5). Apart from the predicted self-splicing products and intermediates (intron+3' exon; linear intron; circular

Figure 1. Plasmid constructions.

Schematic representation of three plasmid constructions containing intron al5 α . Black boxes represent exons, open boxes intronic open reading frames. Thick lines are yeast sequences, thin lines are vector sequences. The promoter is indicated with a flag. A is the intron specific primer (5'-TGATAATTTCTGATC-3'), B is the exon specific primer (5'-GGCAACACCAGTTAAACC-3'). The sizes of the 5' exon, intron and 3' exon of the artificial precursor are indicated. E=Eco RI; H=Hind III; N=Nco I; P=Pvu II.

Figure 2. In vitro self-splicing of pSP a15 α / β precursor RNA.

Internally labeled ($[\alpha^{32}\text{P}]\text{-UTP}$) precursor RNA was incubated under self-splicing conditions for 30, 60, 120 and 180 minutes respectively (lanes 1-4). Lane 5: unlabeled precursor RNA incubated under self-splicing conditions in the presence of $[\alpha^{32}\text{P}]\text{-GTP}$ (3 hours). The structure of the products is indicated with cartoons. The indicated lengths are approximate.

Figure 3. In vitro self-splicing of pT7 al5α/I and pSP al5α/II precursor RNA.

A. Self-splicing products of pT7 al5α/I precursor RNA separated on a 4% PAA-urea gel. Internally labeled ($[\alpha^{32}\text{P}]\text{-UTP}$) precursor RNA was incubated under self-splicing conditions for 0; 30; 60 and 180 minutes respectively (lanes 1-4). Lane 5: unlabeled precursor RNA incubated under self-splicing conditions in the presence of $[\alpha^{32}\text{P}]\text{-GTP}$. The structures of the products are indicated with cartoons.

B. Lane 1: internally ($[\alpha^{32}\text{P}]\text{-UTP}$) labeled precursor RNA from pT7 al5α/I was incubated under self-splicing conditions for 2 hours and separated on a 8 % PAA-urea gel. Lane 2: unlabeled precursor RNA, incubated under self-splicing conditions in the presence of $[\alpha^{32}\text{P}]\text{-GTP}$ and separated on the same gel.

C. Transcription mixtures of pT7 al5α/I and pSP al5α/II (panel A and B

respectively) were incubated under self-splicing conditions and separated on a 4% PAA-urea gel. Lane 1: 0 min. and lane 2: 30 min. of incubation. The structure and lengths of the products are indicated. The arrowhead points to a transcript formed by SP6 RNA polymerase due to a premature stop.

intron and ligated exons), a number of RNA species are visible that cannot easily be explained (those indicated with * in fig. 2 will be discussed later). Two RNA species with decreased mobility are found. One of these products is the circular intron (as shown below); but the structure of the second product, migrating somewhat faster is unknown. Furthermore a GTP labeled product with a length of 820 nt, approximately 30 nt shorter than the expected 3' exon, is found and finally a faint band of approximately 740 nt was detected. The product of exon ligation is labeled by GTP (lane 5), but its occurrence can be explained by an intron mediated exchange reaction, in which the 5' terminal G of the precursor is exchanged with [$\alpha^{32}\text{P}$]-GTP from the reaction mixture (19).

To rule out the possibility that some of these products were caused by activity of the $\text{al5}\beta$ part of the transcript (which contains all the conserved group I elements of intron $\text{al5}\beta$), the $\text{al5}\alpha$ intron was subcloned in the vectors pT713 and pSP 65 to give the plasmid constructions pT7 $\text{al5}\alpha/\text{I}$ and pSP $\text{al5}\alpha/\text{II}$ (fig. 1). pT7 $\text{al5}\alpha/\text{I}$ contains the entire exon $\text{A5}\beta$, pSP $\text{al5}\alpha/\text{II}$ contains only the first 14 nt of $\text{A5}\beta$. In reactions carried out with transcripts lacking the $\text{al5}\beta$ intron part, the 740 nt RNA is not observed any more among the products of self-splicing (fig. 3A). Since the length of this product corresponds in size with the distance from the 5'-splice site to the Pvu II site used for terminating the precursor, the 740 nt RNA could very well be the result of GTP attack at the $\text{A5}\beta$ - $\text{al5}\beta$ exon-intron junction, but this was not further investigated.

To detect also the possible smaller products of self-splicing, products derived from pT7 $\text{al5}\alpha/\text{I}$ precursor RNA were separated on an 8% denaturing polyacrylamide gel in a short run (fig. 3B). This reveals three RNA products that were not observed before: a product corresponding in length with the predicted 5' free exon (fig. 3B lane 1) and two GTP end-labeled products of ± 35 and ± 40 nt (fig. 3B lane 2). The GTP labeled products were isolated from the gel and subjected to partial RNase T_1 analysis to determine the positions of the guanosine residues in these products (fig. 4). This experiment shows that the short molecules are 35 and 41 nucleotides long, and produce the same partial T_1 products as the

Figure 4. Partial RNase T₁ analysis on self-splicing products. Isolated, 5'- labeled RNA species were subjected to partial digestion with RNase T₁. The products were separated on a 16 % PAA-urea gel. Lane 1: linear intron; lane 2: 3' exon; lane 3: cryptic 3' exon; lane 4: 41 nt product; lane 5: 35 nt product.

5' end-labeled intron. The nature of these molecules will be explained below. The 199 and 169 nt products also observed in figure 3 show T₁ products, different from the intron. The T₁ pattern of the 199 nt product

Figure 5. Reincubations of isolated self-splicing products.

Products from a self-splicing reaction, using precursor RNA from a Hind III linearized pT7 $\alpha 5\alpha/1$ template were isolated and reincubated under self-splicing conditions for 2 hours. The resulting products were separated on a 3.3 % PAA-urea gel. The structure of the products is indicated. Panel a: slowly migrating products (circle); panel b: precursor RNA; panel c: linear intron; panel d: truncated linear intron. Lanes 1: input RNA, lanes 2: incubated RNA. Tr. lin. intron = truncated linear intron.

corresponds to the guanosine residues in the 3' exon after GTP addition at the 5' end of the 3' exon. The 199 nt product is therefore produced by GTP attack at the intron+3' exon border, a reaction that was also observed for other introns (4,6,19). The 169 nt product shows a T₁ pattern that cannot be directly assigned to any expected product of self-splicing. It arises from a cryptic 5' splice-site in the 3' exon, as will be shown below. To see if the preference for opening at this cryptic splice-site could be changed

to the normally found 3' splice-site, experiments were also carried out with pSP a15 α /II. The cryptic splice-site is not present in this molecule. The self-splicing reaction indeed shows only opening at the normal 3' splice-site (fig. 3C).

A number of RNA products derived from self-splicing were isolated from the gel for further characterization. First, products were rerun to determine more accurately the molecular weight after release of topological constraints. Second, products were incubated under self-splicing conditions and rerun to study precursor-product relationships. A rerun of the isolated circle is shown in figure 5 (lane a1). About half of the molecules were broken in the isolation procedure, resulting in linear molecules. Whether this linear product was produced by site-specific or random cleavage was not further investigated. Reincubation of this circular RNA preparation under self-splicing conditions does not change the ratio between these two molecules significantly, so the circle itself is not very reactive under the conditions used. The broken circle molecules comigrate with the product migrating just below the linear intron (lane b2). This product therefore corresponds with the broken circle. Reincubation of isolated linear intron RNA confirms this interpretation. The excised linear intron (lane c1) converts to the circular form (lane c2). The circular molecule can then hydrolyse to give rise to its linear counterpart (a 5'-truncated form of the excised linear intron) also observed in lane c2. This truncated intron gives no other products when incubated under self-splicing conditions (lane d2). The size difference between the linear intron and the truncated linear intron is approximately 40 nt, so the 5' part of the linear intron that is split off in the circularization reaction must also be approximately 40 nt. The 35 and/or 41 nt products that were observed in the GTP end-labeling experiments described before (fig. 3B, lane 2) are the most likely candidates for this oligomer. Determination of the sequence at the position of circle closure using primer A (see fig. 1) shows that circles 35 and 41 nt shorter than the linear intron are indeed formed. Panel A of figure 6 shows a sequence starting with the 5' part of the intron which 35 nt before the end of the intron is reached, changes into the 3' part of the intron. Faintly present among the major dideoxynucleotide terminated products, minor products are visible derived from reverse transcription on a smaller circle with a circularization point 41 nt removed from the 5'- end of the intron. These findings are in agreement with 2 positions at which circle closure can

occur: a major one between U₃₄ and A₃₅ and a minor one between U₄₀ and A₄₁ (fig. 8). Circularization at these sites gives two circles of 1331 and 1325 nt respectively (probably comigrating in the gels shown (fig. 2 and 3)) and two RNA molecules of 35 and 41 nucleotides including the 5' terminal G.

The other products and intermediates of self-splicing were also subjected to sequence analysis. The isolated linear intron gave a sequence identical to the 5' part of the intron (fig. 6 panel B). The sequence ladder stops at a length 1 nucleotide longer than the 5' end of the intron. This is explained by the extra G nucleotide added to the intron in the first step of the self-splicing reaction: opening of the 5' splice-site. The same sequence was found for the intron+3' exon intermediate (not shown). When the sequence of the 169 nt product was determined using primer B, a sequence identical to that of the 3'-exon was found, but ending 30 nucleotides short of the 3'-splice site (fig. 6 panel C). When the sequence within the 3' exon at this point is examined, a sequence of 5 nucleotides identical to the 5' splice-site is found. This site, which can be opened by GTP attack (fig. 3C lane 2) is at the same position in the pentanucleotide sequence as in the corresponding pentanucleotide sequence of the 5' splice-site. We therefore propose that this product is formed in a side reaction using a cryptic 5' splice-site located in the 3' exon. The last product that was sequenced using primer B was the ligated exon product. Inspection of this sequence shows the expected sequence for a correctly ligated exon product (fig. 6 panel C).

DISCUSSION

We have studied the self-splicing properties of the group I intron *al5 α* , occurring in the cytochrome oxidase subunit I gene of *S. cerevisiae* mtDNA. It shows the classical repertoire with some abnormal products due to side reactions described previously and some due to reactions typical for this group I intron.

Formation of all the observed RNA products can be explained using the Internal Guide Sequence (IGS) model, originally proposed by Davies et al. (1). The IGS is a short sequence element in the intron which is thought to function as a binding site for the splice- and cyclization sites (20). The IGS aligns these sites in the reaction centre of the RNA molecule. Due to the sequence specificity of the IGS, sequence specific opening and ligation reactions are possible. The proposed structures for alignment of splice-

sites (21) and cyclization sites as proposed here are drawn in figure 7. The guanosine nucleotide attack takes place at the indicated site next to the UG basepair. This basepair is conserved in the IGS of all self-splicing group I introns. Figure 7a shows the proposed structure for the splice-site alignment. After opening of the 5' splice-site the 5' exon is ligated to the 3' exon. The sequence of the ligated exon product as determined with reverse transcriptase is in perfect agreement with this structure.

The two circularization reactions that were observed can also be explained using the IGS model. Two identical alignment structures for the two sites of circle closure can be drawn (fig. 7b). In both cases an AAUAA sequence binds to the IGS. The 3' terminal G attacks the phosphodiester bond between the U and A residues, thus producing a circular intron and a short 5' GTP labeled RNA molecule. In the proposed secondary structure for intron *al5 α* (21, fig.8) these 2 sequences are located in loop L1. When the intron is excised, the AAUAA sequence could bind to the IGS, facilitating the circularization reactions. Apart from the two indicated AAUAA sequences (arrows), another AAUAA motif with identical sequence surroundings as the previous ones and a GAUAA motif that can also basepair with the IGS are found in the same loop (arrowheads). There is however no evidence that these are used in circularization reactions. Therefore, the primary structure alone cannot be responsible for the selection of the two used sites. Most probably, the RNA folding restricts the intron to use only two sites, with unequal preference.

If attack of the GTP does not take place at the 5' splice-site but at the 3' splice-site (fig. 7c), a GTP labeled 3' exon is generated. This product which was indeed found, has also been observed in other self-splicing introns (19). Since there is no clear purpose of this reaction *in vivo*, it is considered to be a side reaction of the *in vitro* system.

Internal opening of the 3' exon, 30 nt downstream from the 3' splice-site can be explained with an alignment structure analogous to the 5' exon

Figure 6. Primer extension analysis of self-splicing products.

A. cDNA sequence of the circle products (1) and precursor (2). **B.** cDNA sequence of the precursor (1) and linear intron (2). **C.** cDNA sequence of the ligated exon product (1) and the cryptic 3' exon product (2). Lower case letters indicate exon sequences, upper case letters intron sequences. The C residues indicated with an arrow in panel A, are complementary to the 3' terminal G of the intron.

Figure 7. Alignment structures.

The alignment structures are drawn according to Davies et al (1). Boxed sequences represent the Internal Guide Sequence (IGS). Lower case letters indicate exon sequences, upper case letters intron sequences. The putative binding of the 3' exon to the IGS is indicated with dashed lines.

alignment structure (compare fig. 7d with 7a). Since the GAUUA sequence is occurring several times within the intron (with a maximum homology to the 5' splice-site of 10 nt), and the GAUUA sequence in the 3' exon is the only one used beside the 5' splice-site, again this reaction cannot be explained in terms of sequence homology alone. There must be structural restrictions that prevent the other sites from being opened. Price et al (22) also found a cryptic 5' splice-site in the 3' exon of a mutant derivative of the Tetrahymena rRNA intron. They showed that opening of this site is followed by ligation of the 3' terminal nucleotide to the normal 3' splice-site, thus resulting in formation of a small circular RNA. We have not yet investigated this possibility and cannot say whether this

The structure is derived from Waring and Davies (21), and redrawn according to the new conventions described by Burke et al (23). Splice sites and conserved sequence elements are indicated. The binding of the 3' exon to the IGS is not drawn in this picture. For explanation of arrow(head)s see text.

Although we have proven the existence of two circular intron products it is unlikely that the two slowly migrating products represent these molecules (fig. 2 and 3). The size difference is too small to explain the rather large difference in migration between the two products. Since both circles can be broken to a linear molecule, migrating to the same

position after electrophoresis (fig. 5), it is unlikely that they can be resolved when migrating as circles. The intensities of the two 5' GTP labeled products that are formed in the circularization reactions (fig. 3B lane 2) also do not correspond to the ratio in which the two slowly migrating products are found. The second slowly migrating product must therefore have another structure. We have not been able to determine its structure yet. We are currently investigating whether this product is a lariat or a circular form of the complete intron. Lariats are abnormal products in group I mediated reactions and were found as side products in the self-splicing rRNA intron from *S. cerevisiae* (7). When the products of a self-splicing reaction of intron $\alpha 5$ are examined under the electron microscope we do find lariat molecules in substantial amounts (A.C. Arnberg, results not shown).

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