Autocatalytic Activities of Intron 5 of the *cob* Gene of Yeast Mitochondria

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The terminal intron of the mitochondrial *cob* gene of *Saccharomyces cerevisiae* can undergo autocatalytic splicing in vitro. Efficient splicing of this intron required a high concentration of monovalent ion (1 M). We found that at a high salt concentration this intron was very active and performed many of the reactions described for other group I introns. The rate of the splicing reaction was dependent on the choice of the monovalent ion; the reaction intermediate, the intron-3' exon molecule, accumulated in NH_4Cl but not in KCl . In addition, the intron was more reactive in KCl , accumulating in two different circular forms: one cyclized at the 5' intron boundary and the other at 236 nucleotides from the 5' end. These circular forms were able to undergo the opening and recyclization reactions previously described for the *Tetrahymena* rRNA intron. Cleavage of the 5' exon-intron boundary by the addition of GTP did not require the 3' terminus of the intron and the downstream exon. An anomalous guanosine addition at the 3' exon and at the middle of the intron was also detected. Hence, this intron, which requires a functional protein to splice in vivo, demonstrated a full spectrum of characteristic reactions in the absence of proteins.

Group I introns have characteristic form and function. The form is defined by a series of conserved sequence elements. These sequence elements were first demonstrated by *cis*-dominant splicing mutations in the *cob* gene in yeast mitochondria, which encodes apocytochrome *b* (2, 13, 36). Base pairing between the conserved elements is thought to establish the secondary structure of this group of introns (7, 9, 12, 27). Among the important sequence elements is an internal guide sequence which is thought to bring the 5' and 3' exons into proximity by base pairing. The activity of group I introns involves a set of transesterification reactions initiated by the addition of a guanosine residue to the 5' end of the intron. The ensuing reactions result in the ligation of the flanking exons and, often, in the cyclization of the intron. These processes, which can occur in the absence of proteins, were first described by Cech and co-workers for the group I intron in the *Tetrahymena* large rRNA (8, 21, 37). Autocatalytic splicing of group I mitochondrial introns in *Neurospora crassa* and *Saccharomyces cerevisiae* has also been reported (15, 16, 33).

While splicing of mitochondrial introns can occur autocatalytically in vitro, proteins are required for the reaction to occur in mitochondria. Some of the evidence for the involvement of proteins comes from the analysis of *trans*-recessive mitochondrial mutations in *S. cerevisiae* (17, 25, 30). Such mutations, which map to open reading frames in the *cob* and *oxi3* (*cox1*) genes of yeast mitochondria, indicate the importance of intron-encoded proteins called maturases in the splicing of specific introns. The maturase encoded in intron 4 of the *cob* gene can even be supplied in *trans* from the nucleus, if the maturase gene is expressed there (3). Nuclear genes required for excision of mitochondrial introns have also been identified in both *S. cerevisiae* and *N. crassa* (6, 10, 14, 26, 29). The best characterized of these nuclear genes is the *Neurospora* *cyt18* gene, which appears to encode a

tyrosyl-tRNA synthetase (1). Similarly, *NAM2* (a nuclear gene of yeast) is involved in the splicing of intron 4 of the *cob* and *oxi3* genes (22, 23).

The splicing of the terminal intron of the *cob* gene of *S. cerevisiae* may require a nuclear factor encoded in the *CBP2* gene (18, 26). Nevertheless, this intron can be spliced in vitro in a protein-free reaction (15). (The terminal intron of *cob* is intron 2 in strains harboring a short form of the gene but is intron 5 [I5] in strains like ours which have the long form of *cob*.) To understand the roles of the *CBP2* product and other proteins in the splicing reactions, it is necessary to have a precise description of the autocatalytic reaction. In this paper, we report that *cob* I5 could perform many of the reactions described for other group I introns in vitro, such as cyclization of the linear introns and reopening of the circular molecules. Partial reactions, such as cleavage of the 5' exon-intron junction, could also occur with a transcript in which the 3' terminus of the intron was deleted.

MATERIALS AND METHODS

Materials. Transcription vector pT7/T3-19 was purchased from Bethesda Research Laboratories, Inc. Ribonucleotides were purchased from Pharmacia, radiolabels were purchased from Amersham Corp., and reverse transcriptase was purchased from Boehringer Mannheim Biochemicals. T7 RNA polymerase was a generous gift from Norman Pace (Indiana University). Synthetic primers were synthesized on an Applied Biosystems DNA synthesizer.

Construction of plasmid and probes. A DNA fragment containing *S. cerevisiae* *cob* I5 and its adjacent exons was derived from pBLT727 (5). pT7-bI5 was constructed by inserting a *Hind*III-*Bgl*II fragment into the *Hind*III and *Bam*HI sites in pT7/T3-19 (Fig. 1). The exon 5 probe (probe 1) was an *Ssp*I fragment from positions 1960 to 2237 in the map of Nobrega and Tzagoloff (28). The probe for the 5' end of I5 (probe 2) was an *Ssp*I fragment from positions 2237 to 2461, and the probe for the middle of I5 (probe 3) was an *Ssp*I fragment from positions 2461 to 2635. The exon 6 probe

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FIG. 1. Map of pT7-bI5 and hybridization probes. pT7-bI5 was constructed by inserting a *Hind*III-*Bgl*II fragment from pBLT727 (5) into the *Hind*III-*Bam*HI sites of pT7/T3-19. This fragment contains 296 base pairs (bp) of the 3' end of intron 4, 50 bp of exon 5 (E5), 733 bp of I5, and 43 bp of exon 6 (E6). Transcripts were made from templates linearized either at the *Sma*I site (in the polylinker region of the vector), which includes I5 and its adjacent exons, or at the *Hinc*II site, which lacks the last 15 bp of I5 as well as exon 6. Three *Ssp*I fragments were used for probing exon 5 (probe 1), the 5' end of I5 (probe 2), and the middle of I5 (probe 3). A synthetic 18-mer (probe 4) was used for probing exon 6 and for sequencing the ligated exons. A synthetic 20-mer was used for sequencing the junction of the circular introns.

(probe 4) was a synthetic 18-mer (5'TAAAATAGCATA GAATGG3').

In vitro transcription. pT7-bI5 was linearized either at the *Sma*I site (in the polylinker region of the vector) or at the *Hinc*II site which lies 15 nucleotides upstream from the 3' end of the intron. Transcription was conducted in the buffer system described by Davanloo et al. (11). Unlabeled transcripts were synthesized from 1 μ g of template in 20 mM sodium phosphate (pH 7.7)–10 mM dithiothreitol–8 mM $MgCl_2$ –4 mM spermidine–4 mM each ATP and UTP–1 mM each CTP and GTP–50 U of T7 RNA polymerase at 37°C for 2 h. Radiolabeled transcripts were synthesized in the presence of 1 mM unlabeled ATP and 10 μ Ci (410 Ci/mmol) of [α - 32 P]ATP and incubated for 30 min. The transcripts were then purified by G-50 Sephadex chromatography, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol.

In vitro splicing conditions. Transcripts were incubated at 37°C for 60 min in KCl splicing buffer (1 M KCl, 50 mM $MgCl_2$, 50 mM Tris [pH 7.6]) plus 2 mM GTP or in NH_4 Cl splicing buffer (1 M NH_4 Cl, 50 mM $MgCl_2$, 50 mM Tris [pH 7.6]) plus 2 mM GTP in a total volume of 10 μ l. The reactions were terminated by the addition of an equal volume of formamide-dye-EDTA (90% formamide, 25 mM EDTA [pH 8.0], 0.1% bromophenol blue, and 0.1% xylene cyanol) and 1 μ l of 0.2 M EDTA (pH 8.0). The samples were heated at 90°C for 1 min and fractionated on a 4% polyacrylamide (20:1 acrylamide-bis)–8 M urea gel in 89 mM Tris borate (pH 8.3)–2 mM EDTA. In the GTP labeling experiment, 1 μ g of the unlabeled transcript was incubated in the splicing buffers in the presence of 1 μ Ci of [α - 32 P]GTP. The gel was fixed, dried, and exposed to X-ray film. To study the reactivity of I5 fragments, labeled transcript from template linearized with *Sma*I was subjected to splicing conditions in KCl splicing buffer. Following electrophoresis, RNA fragments corresponding to the linear and circular forms of the intron were eluted by a method described by Waring et al. (35), filtered through Sephadex G-50 columns, extracted with phenol-chloroform-isoamyl alcohol, and precipitated with ethanol. These RNA fragments were then incubated in the splicing conditions in KCl buffer and fractionated on the same gel system.

Autoradiogram scans. The autoradiograms from the time course experiments were traced by using a Zeineh scanning densitometer and were analyzed by using Zeineh Videophoresis programs (Biomed Instruments, Inc.).

RNA transfer and hybridization. Following autocatalysis and gel electrophoresis, the RNA was electrophoretically transferred onto nylon membranes (Zetabind) in 40 mM Tris

(pH 8.3)–20 mM $Na_2H_2O_2$ –1 mM EDTA at 1 A at 4°C for 2.5 h. The membranes were then baked at 80°C for 2 h and prehybridized at 40°C for 2 h in 50% formamide–0.02% Ficoll (Pharmacia Fine Chemicals)–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin–5 $\times$ SSPE (0.9 M NaCl, 50 mM sodium phosphate [pH 7.7], 5 mM EDTA)–0.1 mg of salmon sperm DNA per ml–0.2% sodium dodecyl sulfate. The labeled probes (400,000 cpm [Cerenkov] each) were added, and hybridization was carried out at 40°C for 2 h. The membranes were then washed three times at 40°C in 15% formamide–2 $\times$ SSPE–0.1% sodium dodecyl sulfate, dried briefly, and exposed to X-ray films at –70°C. Hybridization with an oligomer was carried out in 6 $\times$ SSPE–0.04% Ficoll–0.04% polyvinylpyrrolidone–0.04% bovine serum albumin at 42°C, and washing was done in 6 $\times$ SSPE–0.1% sodium dodecyl sulfate at room temperature.

RNA sequencing. The ligated exons and the small circular intron were sequenced by the dideoxynucleotide method as described by Lane et al. (24), except that end-labeled primers were employed. Probe 4 was used as a primer for sequencing the junction of the ligated exons, and a synthetic 20-mer (5'GAGTTCACAAAGCGGAAAC3') was used as a primer for sequencing the junction of the two forms of circular intron.

RESULTS

In vitro splicing of *cob* I5. RNA synthesis from pT7-bI5 linearized with *Sma*I (Fig. 1) produced a 1,173-nucleotide transcript of I5 and its flanking exons. Similar to some other group I introns, these RNA molecules could undergo autocatalytic splicing when incubated with monovalent cation, magnesium, and GTP (Fig. 2A). The efficiency of the reaction depended on the concentration and the choice of monovalent ion. In contrast to the splicing of other group I introns, however, autocatalysis of *cob* I5 was optimal at a high concentration of the monovalent salt (Fig. 3). In addition, the reaction was more rapid in KCl than in NH_4 Cl (see Fig. 9). This reaction could also occur at low concentrations (<0.1 M) of $(NH_4)_2SO_4$, but only less than 10% of the transcript was processed in this salt. We have chosen to follow the reactions in NH_4 Cl, as well as in KCl, since the intermediate of the splicing reaction appeared to accumulate in NH_4 Cl.

The splicing products included those which we established to be the fused exons and the excised linear intron (Fig. 2A). In addition, we observed bands which corresponded to the free upstream exon and the free downstream exon (only seen in longer gels). In the NH_4 Cl reaction (Fig. 2A, lane 2), we

FIG. 2. Splicing of *cob* I5 transcript in vitro. (A) Transcripts synthesized from pT7-bI5 linearized with *Sma*I were incubated at 37°C for 60 min in 50 mM Tris hydrochloride (pH 7.6) (lane 1), NH_4Cl splicing buffer plus 2 mM GTP (lane 2), and KCl splicing buffer plus 2 mM GTP (lane 3). The samples were separated on a 4% polyacrylamide-8 M urea gel and subjected to autoradiography. (B) Transcripts synthesized from pT7-bI5 linearized at the *Hinc*II site were subjected to the same conditions described for panel A. The marker was pBR322 digested with *Hinf*I; the numbers on the left indicate nucleotide sizes (in bases). The symbols to the right of the autoradiograms indicate the identities of the splicing products, as deduced from hybridization and sequencing data.

detected a band which we later identified as the intron plus the downstream exon. This molecule did not accumulate in the KCl reaction. The 590-nucleotide fragment consisted of the 5' exon and the 5' end of the intron, and the 500- and 235-nucleotide fragments consisted of the intron sequences which were byproducts of the cyclization events (see below).

When the products of autocatalysis were separated on an acrylamide gel containing half-strength buffer, the relative positions of the bands were unchanged, with the exception of two RNA bands migrating more slowly than the primary transcript (data not shown). These two bands were further retarded in the half-strength buffer than in the full-strength buffer. Such behavior has been reported for circular RNA molecules (19). Subsequent sequence analysis confirmed that they were indeed circular molecules.

Transcription of the plasmid linearized with *Hinc*II produced a 1,072-nucleotide transcript lacking exon 6 and the terminal 15 residues of the intron (Fig. 2B). This truncated intron could also undergo autocatalytic processing when incubated under the same conditions of high salt concentra-

FIG. 3. Titration of monovalent salts required for splicing of *cob* I5 transcript in vitro. Labeled transcript synthesized from pT7-bI5 plasmid linearized with *Sma*I was incubated at 37°C for 60 min in 50 mM Tris hydrochloride (pH 7.6)-50 mM MgCl_2 with various concentrations of KCl and NH_4Cl . The splicing products were separated on 4% polyacrylamide-8 M urea gels. The bands corresponding to the primary transcripts on the autoradiograms were scanned by using a densitometer. Percent transcript processed indicates the fraction of the primary transcript converted to products in each incubation.

FIG. 4. $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ labeling of *cob* I5. Unlabeled transcripts synthesized from template linearized with *Sma*I were incubated at 37°C in NH_4Cl splicing buffer plus 1 μCi of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (lane 1) or in KCl splicing buffer plus 1 μCi of $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (lane 2). Unlabeled transcripts synthesized from template linearized with *Hinc*II were subjected to the same conditions described for lanes 1 and 2 (lanes 3 and 4, respectively). The marker (M) was pBR322 digested with *Hinf*I. Nucleotide sizes (in bases) are indicated.

tion. This processing resulted in the formation of the linear intron and the upstream exon, a 590-nucleotide fragment consisting of the 5' exon and the 5' end of the intron, and a 500-nucleotide truncated form of the intron (see below). No circular RNA molecules were detected.

The cleavage of the intron was accompanied by the addition of GTP to its 5' terminus (Fig. 4), as expected of group I introns. A 730-nucleotide band corresponding in size to the full-length intron was labeled with [α - 32 P]GTP (Fig. 4, lanes 1 and 2). In addition, an RNA of the size expected of the intron plus the downstream exon (820 nucleotides) was observed, particularly in the NH_4Cl reaction. An RNA product of 235 nucleotides, which was the 5' end of the intron released during the formation of the small circular intron, appeared in the KCl reaction. In addition, on longer gels, a band corresponding to the size of free 3' exon was also observed to be labeled with [α - 32 P]GTP (data not shown). When the transcript lacking the 3' end of the intron and the 3' exon was incubated in the presence of [α - 32 P]GTP (Fig. 4, lanes 3 and 4), the major labeled product corresponded in length to the truncated intron. A 500-nucleotide band, which was formed from guanosine addition at an internal site of the intron, was also labeled in the KCl buffer.

Identification of splicing products by hybridization. (i) Products of splicing of full-length transcript. DNA fragments corresponding to the 5' exon (probe 1), the 5' end of the

intron (probe 2), the middle of the intron (probe 3), and the 3' exon (probe 4) were employed for hybridization (Fig. 5). All probes hybridized with the 1,173-nucleotide primary transcript. Probe 1 (Fig. 5A, lanes 1 and 2) hybridized with bands corresponding to the fused exons (440 nucleotides) and the 5' exon alone (355 nucleotides). In addition, probe 1 also hybridized with a band just below the primary transcript of approximately 1,090 nucleotides and with a band of approximately 590 nucleotides.

Both intron probes (Fig. 5C and D, lanes 1 and 2) hybridized with the 730-nucleotide band expected for the linear intron, as well as with an 820-nucleotide molecule. This 820-nucleotide molecule also annealed with the 3' exon probe but not with the 5' exon probe. Consequently, we believe that this molecule is an intermediate molecule resulting from the initial removal of the 5' exon. Probe 3 hybridized to both circular forms, while probe 2 hybridized only with the larger circular molecule. In addition, probe 3 hybridized to a 500-nucleotide molecule which was the linear form of the small circular intron, while probe 2 hybridized with a 235-nucleotide fragment which was the 5' end of the intron released during the formation of the small circle. Furthermore, probe 2 (and probe 1) hybridized to the 590-nucleotide band, indicating that this molecule consisted of the 5' exon and the 5' end of the intron. Finally, the 1,090-nucleotide product migrating just below the primary

FIG. 5. Hybridization analysis of splicing products. Unlabeled transcripts were subjected to the splicing conditions in either the NH_4Cl or KCl splicing buffer, and the splicing products were separated on a 4% polyacrylamide-8 M urea gel. The RNA fragments were then transferred to nylon filters, and the filters were hybridized with probe 1 (A), probe 2 (B), probe 3 (C), and probe 4 (D). (A to C) Lanes 1 and 2 contain the splicing products of transcripts synthesized from template linearized with *Sma*I and incubated with 2 mM GTP in the NH_4Cl and KCl splicing buffers, respectively. Lanes 3 and 4 contain the splicing products from template linearized with *Hinc*II and incubated with 2 mM GTP in the NH_4Cl and KCl splicing buffers, respectively. (D) Lanes 1 and 2 contain the splicing products of the *Sma*I transcript only. Nucleotide sizes (in bases) are indicated.

transcript annealed with both intron probes (and with the 5' exon probe) but not with the 3' exon probe, suggesting that it resulted from the removal of the 3' exon from the primary transcript.

The 3' exon probe (Fig. 5D) annealed to both the 440-nucleotide band corresponding to the fused exons and the 820-nucleotide band composed of the intron plus the downstream exon.

(ii) Products of splicing of truncated transcript. Similar hybridization experiments were performed with the products of the shortened transcript. Our results (Fig. 5A to C, lanes 3 and 4) lead us to the following conclusions. The primary guanosine-labeled band with an apparent size of 715 nucleotides was the linear intron up to the *HincII* cleavage site. The 355-nucleotide band produced in the reaction of the *HincII* transcript was the upstream exon. A 500-nucleotide fragment, which was also labeled with [α - 32 P]GTP, consisted of truncated intron sequences. This fragment apparently resulted from the addition of guanosine at a site about 200 nucleotides from the 5' end of the intron. We also observed the 590-nucleotide fragment containing the upstream exon and the 5' portion of the intron which may be released from the truncated transcript after the guanosine attack. Such anomalous addition of guanosine has been reported by other investigators (19, 34). A 590-nucleotide band was also observed in the reaction of the full-length transcript, but it was derived from a different pathway (see Discussion). Finally, we observed a 235-nucleotide product derived from the 5' end of the intron. Unlike the 235-nucleotide band derived from the reaction of the full-length transcript, however, this small fragment was not labeled with [α - 32 P]GTP during autocatalysis.

Reactivity of various forms of *cob* I5. To test the reactivity of the various forms of I5 resulting from the splicing reaction, these RNA fragments were eluted from the gel and incubated in the KCl splicing buffer in the presence or absence of GTP (Fig. 6). For markers, the end lanes (Fig. 6, lanes 1 and 14) show the splicing products of the primary transcript. The large circular intron appeared to be linearized to a molecule of the size of the full-length intron (730 nucleotides) independently of KCl and $MgCl_2$ or GTP (Fig. 6, lanes 2 to 4). For the incubation at high salt concentration, upon longer exposure of the autoradiogram, we also observed a band corresponding to the small circular intron, which presumably was derived from the cyclization of the full-length intron at the internal site. A similar result was obtained from the incubation of the small circular intron (Fig. 6, lanes 5 to 7). In this case, a molecule of 500-nucleotides was produced upon incubation, independently of GTP or conditions of high salt concentration.

The linear forms of the intron were capable of undergoing cyclization when incubated at high salt concentration with magnesium (Fig. 6, lanes 8 to 13). GTP was not required for this process. Under these conditions, the full-length linear intron formed the two circular molecules, along with two minor bands which also migrated above the full-length transcript (Fig. 6, lanes 8 to 10). We believe that these bands resulted from the cyclization of the intron at additional sites. In addition, the 5' end of the intron released during the formation of the smaller circular intron was detected. When the shorter linear intron (500 nucleotides) was incubated under similar conditions, it reformed the small circular intron along with two minor bands (Fig. 6, lanes 11 to 13). These data and the RNA sequences presented below are compatible with our hypothesis that the intron cyclized at predominantly two places: at the 5' exon-intron boundary

FIG. 6. Reactivity of *cob* I5. Labeled transcript synthesized from the *Sma*I template was subjected to the splicing conditions in the KCl buffer. The splicing products were separated on a 4% polyacrylamide-8 M urea gel, and the RNA fragments corresponding to the large circular intron (fragment A), the small circular intron (fragment B), the full-length intron (fragment C), and the shorter linear intron (fragment D) were eluted. Fragment A was subsequently incubated at 37°C for 60 min in 50 mM Tris hydrochloride (pH 7.6) (lane 2), in the KCl splicing buffer (lane 3), or in the KCl splicing buffer with 2 mM GTP (lane 4). Fragments B (lanes 5 to 7), C (lanes 8 to 10), and D (lanes 11 to 13) were subjected to the same conditions described for fragment A. The samples were separated on a 4% polyacrylamide-8 M urea gel. Lanes 1 and 14 contain the splicing products of the primary transcript incubated in the KCl splicing buffer with GTP.

and at a point approximately 235 nucleotides into the intron from that junction. The formation of the minor bands suggested that there exist several other potential cyclization sites within the intron.

Reactivity of intermediate molecules. The full-length transcript was susceptible to guanosine addition at both intron-exon boundaries. Guanosine additions at these sites resulted in the formation of two intermediate molecules: the intron-3' exon molecule and the 5' exon-intron molecule. Both intermediates appeared to be reactive. Incubation of the 5' exon-intron molecule in KCl splicing buffer with magnesium (Fig. 7, lane 2) resulted in the formation of the two circular molecules, as well as the byproducts of the cyclization events. These byproducts included the linear forms of the circular molecules and the fragment consisting of the 5' exon and the 5' end of the intron (Fig. 7, arrow), which resulted from the cyclization at the internal site. We believe that the

FIG. 7. Reactivity of intermediate molecules. The RNA fragment corresponding to the 5' exon-intron molecule was isolated and incubated at 37°C for 60 min in 50 mM Tris hydrochloride (pH 7.6) (lane 1), in the KCl splicing buffer (lane 2), or in the KCl splicing buffer with 2 mM GTP (lane 3). The samples were separated on a 4% polyacrylamide-8 M urea gel. Lane 4 contains the splicing products of the primary transcript incubated in the KCl splicing buffer with GTP. The symbols indicate the identities of the bands. The arrow indicates the fragment consisting of the 5' exon and the 5' end of the intron (lanes 2 and 3).

circular molecules were formed directly from this intermediate molecule. It is unlikely that the circles were formed from the linear intron after it was released from the 5' exon, since this initial step requires guanosine. When GTP was included in the reaction (Fig. 7, lane 3), there was an increase in linear intron formed. This increase may have resulted from guanosine attack at the 5' exon-intron junction or from the reopening of the large circular intron.

The intron-3' exon molecule, which accumulated in the NH_4Cl reaction, was also reactive. When this molecule was isolated and incubated in the KCl splicing buffer, it yielded the free linear intron and the 3' exon in a reaction that was dependent on GTP (data not shown).

Sequence analysis of fused exons and two forms of circular intron. Primer extension with dideoxynucleotides was used to determine the junction sequences of the fused exons and the intron circles. The data indicated that 5'GCAUCUAU/UGUACCUGAA3' was the sequence flanking the fusion site of the exons (Fig. 8A). This result is in agreement with that for the fusion site reported by Gampel and Tzagoloff (15).

The primer used for the two circular forms of the intron was a 20-mer which ended 244 nucleotides downstream from the 5' exon-intron boundary (Fig. 1). The sequence we obtained indicated that the small circle was formed by a phosphodiester linkage between the guanosine residue at the

3' terminus of the intron and an adenosine residue 237 nucleotides downstream from the intron-exon border (Fig. 8B). As in the cyclization of the *Tetrahymena* rRNA intron (4, 31, 38), the phosphodiester which was cleaved was between a uridine residue and an adenine residue. This reaction created a circular RNA of 496 nucleotides. Hydrolysis at the cyclization site appeared to account for the linear derivative of this circle, since we detected a strong stop at this point in our primer extension reactions using the linear derivative (Fig. 8C). Similar findings with *Tetrahymena* rRNA circular intron have been reported by Zaug et al. (38). Reverse transcription of the linear derivative of the small circle proceeded beyond the stop. This fact may be attributable to the recyclization of the linear intron during hybridization with the primer in the sequencing procedure. Sequence analysis of the large circular intron indicated that the cyclization point was at or near the upstream exon-intron border (data not shown). As with the case of the small circular intron, the cyclization site of the large circle appeared to be susceptible to hydrolysis.

Kinetics of splicing in NH_4Cl and KCl buffers. The splicing reaction of *cob* 15 occurred more rapidly in KCl than in NH_4Cl (Fig. 9A and B). In KCl, the formation of the fused exons and the free linear intron reached a maximal level in 10 min of incubation, while the formation of the circular introns and the free 5' exon continued to increase for 30 min. The intron-3' exon molecule accumulated in the first 3 min of the reaction but then diminished upon further incubation. In addition, the small circular intron appeared earlier than the large circular intron. In NH_4Cl , the same reaction products were formed, but it took 60 min to obtain the same distribution of products, compared with 5 min for the KCl reaction. In addition, the intron-3' exon molecule continued to accu-

FIG. 8. Sequence analysis of junctions of ligated exons and small circular intron. The ligated exons (A), the small circular intron (B), and the linear derivative of the small circular intron (C) were sequenced by the dideoxynucleotide method described in Materials and Methods. The cDNA sequences flanking the junctions (indicated by arrows) are shown.

FIG. 9. Kinetics of splicing of *cob 15* in KCl and NH_4Cl buffers. Labeled transcript from the *Sma*I template was incubated in the KCl splicing buffer plus GTP (A) or in the NH_4Cl splicing buffer plus GTP (B). At different intervals, portions were removed, run on a 4% polyacrylamide-8 M urea gel, and subjected to autoradiography. The identities of the fragments are indicated by the line drawings. The relative amounts of the intron products (indicated by arrows in panel B) were determined by densitometry of the autoradiograms. (C) KCl reaction; (D) NH_4Cl reaction. a, Full-length intron; b, 3' exon-intron molecule; c, small circular intron; d, large circular intron. Densitometer scannings were made of several exposures of each film to ensure that the film had not been saturated.

multate over the course of the reaction in NH_4Cl . The kinetics of formation of the intron products are illustrated more clearly by the graphs derived from densitometer tracings (Fig. 9C and D).

Although intron excision occurred quite rapidly in the KCl buffer, the efficiency of the process, as judged by the formation of the ligated exons, appeared to be low. At the end of 30 min of incubation, most of the primary transcript

was consumed, but only about 15% of the exon fragments were ligated.

DISCUSSION

In these experiments, we demonstrated that the terminal intron of *cob* could be precisely spliced in a protein-free reaction. The efficiency of the autocatalytic reaction was dependent on the choice of the monovalent ion. The splicing

In the autocatalytic conditions we employed, there was an uncoupling of the cleavage and ligation reactions, which together constitute splicing. However, cleavage at the intron-exon boundaries was more efficient than formation of the fused exons. Indeed, breakage at the 5' intron boundary yielded a free upstream exon and an intron-3' exon molecule which appeared to accumulate prior to the formation of spliced exons or free intron (Fig. 9A). These intermediates and the circular forms were not detected in the previous study on autocatalytic splicing of *cob* I5 (15), probably because of the analytical method employed (nondenaturing agarose gels) and because the reaction was conducted in $(\text{NH}_4)_2\text{SO}_4$, which we found to be inhibitory at a high concentration.

Circular intron derivatives and the splicing intermediates we have described do not accumulate as a consequence of the splicing of this intron *in vivo* (26). Nevertheless, they may provide a unimolecular means for the study of the mechanism of the splicing reaction. Cyclization is a reversible and GTP-independent reaction catalyzed by the intron. Cech and co-workers have used cyclization as a model for the selection of the splice sites and the ligation of exons in the splicing pathway (4, 31). Under the conditions we employed for autocatalysis, the ligation of exons was inefficient; approximately 15% of the exons present in the primary transcript could be detected as fused exons in the steady state. Whether this inefficiency was attributable to nonproductive side reactions or to the reversibility of the reaction remains to be demonstrated. In contrast, in wild-type yeast cells, most of I5 has been spliced. McGraw and Tzagoloff (26) have established the need for a protein encoded in the nuclear *CBP2* gene for the splicing of this intron in yeast cells. Presumably, this protein, perhaps working in concert with others as yet undefined, permits the splicing reaction to proceed under physiologic conditions.

No precise role for proteins in the splicing of group I introns has been determined. One current hypothesis for the function of proteins in splicing is that they help maintain the appropriate structure of the intron (16). The high salt requirement of the splicing of I5 *in vitro* may reflect the need to minimize charge repulsion when proteins are absent. The secondary structure of *cob* I5 is stabilized primarily through A-U base pairs. Consequently, *cob* I5 secondary structure may be less stable than those of other group I introns. It will be of interest to determine whether the addition of mitochondrial proteins to the RNA will reduce the high salt requirement of the splicing reaction.

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