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# Synthesis of circular RNA in bacteria and yeast using RNA cyclase ribozymes derived from a group I intron of phage T4

(splicing/RNA processing/*Escherichia coli*/*Saccharomyces cerevisiae*/*td* gene)

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**ABSTRACT** **Studies on the function of circular RNA and RNA topology *in vivo* have been limited by the difficulty in expressing circular RNA of desired sequence. To overcome this, the group I intron from the phage T4 *td* gene was split in a peripheral loop (L6a) and rearranged so that the 3′ half intron and 3′ splice site are upstream and a 5′ splice site and 5′ half intron are downstream of a single exon. The group I splicing reactions excise the internal exon RNA as a circle (RNA cyclase ribozyme activity). We show that foreign sequences can be placed in the exon and made circular *in vitro*. Expression of such constructs (RNA cyclase ribozymes) in *Escherichia coli* and yeast results in the accumulation of circular RNA in these organisms. In yeast, RNA cyclase ribozymes can be expressed from a regulated promoter like an mRNA, containing 5′ leader and 3′ trailer regions, and a nuclear pre-mRNA intron. RNA cyclase ribozymes have broad application to questions of RNA structure and function including end requirements for RNA transport or function, RNA topology, efficacy of antisense or ribozyme gene control elements, and the biosynthesis of extremely long polypeptides.**

Most natural RNA molecules are linear and contain special structures at their ends. Other natural RNAs are circular, but the biological significance of circularity is not clear. Circular viroids (1) and archaeal introns (2) accumulate in cells, but the expectation that this is due to unusual stability *in vivo* has been difficult to demonstrate directly. Experiments to generate circular RNA and test its stability *in vivo* (3, 4) took advantage of the cyclization reaction of group I introns (5), producing circles that still contained the highly structured group I core. The stability of RNAs made this way can be demonstrated, but since they carry ribozyme elements, the contributions of secondary structure and circularity are difficult to distinguish (3, 4). To vary the kinds of sequences that could be made circular and to begin to study topological properties of RNA reactions, we have used splicing reactions to make and test circular RNA *in vitro* and *in vivo*.

Natural splicing usually takes place between upstream 5′ splice sites and downstream 3′ splice sites. Unusual splicing events that join a downstream 5′ splice site to an upstream 3′ splice site would make exons circular. Experiments in which new functional introns are made artificially by joining segments of different natural pre-mRNA introns demonstrate that nearly any pair of 5′ and 3′ splice sites are compatible (6). Although abundant opportunity for splicing of upstream 3′ splice sites to downstream 5′ splice sites exists in multiintron pre-mRNA transcripts, such events are only very rarely observed (7–9), arguing that there is a restriction preventing a downstream 5′ splice site from being spliced to an upstream 3′ splice site (10). In contrast, the efficient trans-splicing reactions carried out by derivatives of group I and group II introns (for example, see refs. 11–15) indicates that these splicing machineries have no mechanism for enforcing the requirement for proper splice site position within a transcript. Thus, group I and group II transcripts in which the 3′ splice site is artificially placed upstream of the 5′ splice site will carry out intramolecular cis-splicing to produce circles *in vitro* (16–18). Galloway-Salvo *et al.* (19) showed that separate transcripts, each containing half of a T4 *td* intron split in L6a (20, 21) carry out group I trans-splicing *in vivo*, suggesting that this intron might be useful for expressing circular RNA in cells.

We rearranged the group I intron from the T4 *td* gene so that the splicing reactions excise and release an internal exon RNA as a circle. We demonstrate the synthesis of circular RNA by such transcripts (called RNA cyclase ribozymes) after transcription *in vitro*, similar to other constructs using different self-splicing introns (17, 18). In addition, we show that RNA cyclase ribozymes containing foreign sequences can be built to produce circular foreign RNA. When placed in appropriate contexts for expression, RNA cyclase ribozyme constructs function in *Escherichia coli* and yeast. In yeast, RNA cyclase functions from within an mRNA-type transcript after removal of a nuclear pre-mRNA intron.

## MATERIALS AND METHODS

**Strains and Media.** *E. coli* DH5α and DH5α Δ*thy* carrying a *thyA*::*kanr* deletion insertion (provided by Jill Galloway-Salvo, Union College, Albany, NY) were used throughout. *Saccharomyces cerevisiae* IH1097 is *MATa1, GAL2, ura3-52, leu2-3,112, trp1, pep4-3, prb1, prc1*. All strains were grown in standard media (22). Yeast strains were grown in synthetic complete glycerol medium lacking uracil and were induced for expression of *GAL*-regulated genes by the addition of galactose to 3% (wt/vol) when the $A_{600}$ was 0.1–0.2 unit. After 6–8 h of growth, RNA was isolated.

**Plasmids.** Plasmid pEFC (Fig. 1*A*) was created by isolating the 580-bp *Sph* I–*Xba* I fragment of pTZtd18-2 containing the 3′ "half" of the intron starting in P6a′ through the second exon of *td* (19), blunting the ends with the Klenow fragment of DNA polymerase I (Klenow), and inserting it into the unique *Eco*RI site of pTZtd18-1. This site lies downstream of a tandem *lac*;T7 promoter and upstream of a fragment containing the 5′ "half" of the *td* gene from upstream of the translation start site and into the intron through P6a (19). pEFC contains a circularly permuted *td* gene (Fig. 1*A*) that complements an *E. coli thyA* deletion, indicating that it is functional (see ref. 19). pEFCΔNde (Fig. 1*B*) was created by cutting pEFC with *Nde* I and ligating the large fragment back together. This construct contains the intron sequence and 100 bp of *td* exon sequences. The unique regenerated *Nde* I site

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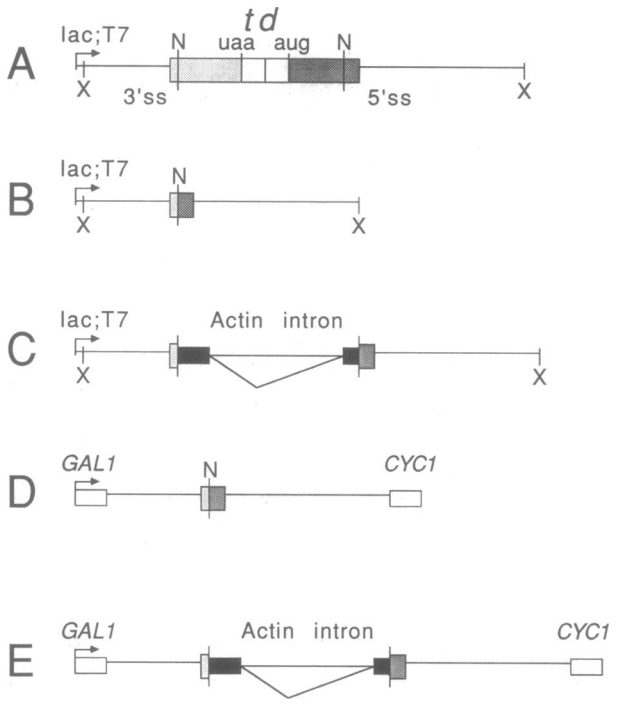


FIG. 1. Plasmid constructs used in this study. Only the relevant insert regions are shown. (*A*) pEFC, formed by introducing the 3′ half intron, 3′ splice site (3′ ss), and second exon fragment of pTZ18td-2 (19) behind the tandem *lac* and T7 promoters, and upstream of the first exon, 5′ splice site (5′ss), and 5′ half intron of pTZ18td-1 (19). Thin lines, *td* intron; light stippled box, exon 2 sequences from 3′ splice site to end of *td* coding region; dark stippled box, *td* exon 1 sequences from start of *td* coding region to 5′ splice site; open boxes, *td* flanking sequences; N, *Nde* I sites; X, *Xba* sites. (*B*) pEFC-ΔNde, formed by deleting the *Nde* I fragment of pEFC that spans most of the fused *td* exon sequences. (*C*) pΔNAct(+), formed by inserting a segment of the yeast actin gene spanning its nuclear pre-mRNA intron in the sense orientation with respect to the promoters. Solid boxes, actin exons; thin line (triangle), actin intron. (*D*) pΔN-Y(+), formed by placing the *Xba* I fragment containing all but the tandem *lac* and T7 promoters from the insert of pEFC-ΔNde into a yeast expression vector containing a *GAL1* promoter and the *CYC1* 3′ end formation signals. (*E*) pΔN-A(+)-G(+), formed by placing all but the tandem *lac* and T7 promoters from the insert of pΔNAct(+) into pYES1.2.

is used for the introduction of sequences to be included in RNA circles.

The plasmid pΔNAct(+) (Fig. 1*C*) was created by blunting the ends of a 452-bp *Sma* I–*Hin*dIII fragment of the yeast actin gene from pT7Act (23) and inserting it into the *Nde* I site of pEFCΔNde.

The rearranged *td* gene in pEFCΔNde and derivatives are carried on an *Xba* I fragment. Plasmids for expression in yeast were made by inserting *Xba* I fragments into the yeast expression vector pYES1.2 (Invitrogen, San Diego), which carries a yeast *URA3* gene, a 2-μm plasmid origin, a kanamycin-resistance gene, and an expression site consisting of a polylinker interposed between a *GAL1* promoter and a *CYC1* termination-polyadenylylation site. These plasmids were constructed by blunt-end ligation of treated *Xba* I fragments with pYES1.2 cut at the expression site with *Bst*XI, so that the *td* sequences are in the sense orientation with respect to transcription directed by the *GAL1* promoter. Plasmid pΔN-Y(+) (Fig. 1*D*) was made by inserting the 499-bp *Xba* I fragment of pEFC-ΔNde into pYES1.2 whereas plasmid pΔN-A(+)-G(+) (Fig. 1*E*) was created by inserting the 947-bp *Xba* I fragment of pΔNAct(+) into pYES1.2.

**RNA.** Total RNA was isolated from yeast by phenol extraction at 65°C as described (23). RNA is denatured under these conditions. Total RNA from *E. coli* was isolated from 50-ml cultures at mid-logarithmic phase ($A_{600}$ = 0.6) by using the same method. The DNA was removed from bacterial RNA using RNase-free DNase I (Promega).

***In Vitro* Transcription and Group I Splicing.** Transcription with T7 RNA polymerase (gift of H. Noller, University of California, Santa Cruz) was performed essentially as described by Milligan and Uhlenbeck (24), using 1–2 μg of CsCl-gradient-purified *Sal* I-cut plasmid template DNA, and three rNTPs (except UTP; each at 4 mM), and including enough $MgCl_2$ to surpass the total nucleotide concentration by 6 mM in a total volume of 10–20 μl. The UTP concentration was 0.1 mM and 10–20 μCi of [α-$^{32}$P]UTP (specific activity, 3000–1500 Ci/mmol; 1 Ci = 37 GBq; Amersham) was included. Transcripts from pEFCΔNde splice efficiently during transcription, but a fraction of the pΔNAct(+) transcripts remained unspliced. After further incubation in 40 mM Tris·HCl, pH 7.9/10 mM NaCl/6 mM $MgCl_2$/10 μM rGTP at 55°C for 30 min, additional spliced transcripts were obtained.

**Two-Dimensional Denaturing Gel Electrophoresis.** Two-dimensional denaturing gels were formed by electrophoresing samples on a low-percentage acrylamide first-dimension gel. A lane from the first gel was cut and laid between glass plates and clamped with 0.4-mm spacers, and a higher-percentage acrylamide second-dimensional gel was then cast underneath the first-dimension gel slice. As needed, sample-well formers were inserted to the sides of the first-dimension gel slice to provide lanes for one-dimensional markers in the second dimension. All polyacrylamide gels were 1× TBE (1× TBE is 89 mM Tris base, 89 mM boric acid, 2 mM EDTA) and 7.5 M urea and prepared with different percentages of acrylamide using an acrylamide/*N,N′*-methylene bisacrylamide ratio of 19:1.

**Blots.** Five micrograms of total yeast or *E. coli* RNA was separated on denaturing polyacrylamide gels, electroblotted to Nytran nylon filters according to the supplier's recommendations (Schleicher & Schuell), dried at room temperature, and irradiated with shortwave ultraviolet radiation for 20 min to crosslink RNA to the filter. Filters were preincubated at least 30 min and hybridized in 5 ml of 5× SSC/0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% bovine serum albumin/5 mM EDTA/0.5% SDS/sonicated herring sperm DNA (250 μg/ml)/50 mM Tris·HCl, pH 8.0, at 45°C. One million counts per min of *td* exon 1 oligonucleotide (5′-AAATC-CAAATAGCCATTAC-3′) labeled at its 5′ end with [$^{32}$P]phosphate using [γ-$^{32}$P]ATP (Amersham) and polynucleotide kinase (New England Biolabs) was boiled in water containing herring DNA (100 μg/ml), added, and incubated overnight. Filters were washed at room temperature three times in 5× SSC/0.5% SDS and once in 5× SSC and autoradiographed. The probe is complementary to the first exon upstream of both the correct and alternate *td* exon products (25).

## RESULTS

**Synthesis of Circular RNA *in Vitro*.** Galloway-Salvo *et al.* (19) showed that two transcripts generated by splitting the T4 *td* intron in the region of L6a (20, 21) could associate to form a group I ribozyme capable of accurate and efficient trans-splicing *in vitro* and *in vivo*. We reasoned that the two half introns should be capable of the same reactions in a unimolecular arrangement where the ends of the transcript were in L6a and the exons were internal to the transcript and fused into one (Fig. 2; see also refs. 17 and 18). The splicing reactions would join the exon at the splice sites, making it circular and freeing the two half-intron fragments. To test this, we constructed a plasmid in which the 3′-half-intron–exon 2 segment of the T4 *td* gene was placed upstream of the exon 1–5′-half-intron segment behind a tandem *lac*–T7 pro-

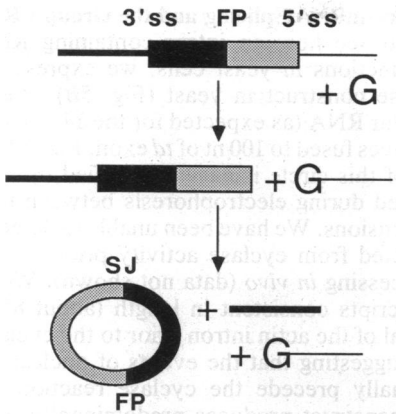


FIG. 2. Rearrangement of a group I intron so that splicing produces a circular exon. The arrows indicate the two steps of group I splicing (see also ref. 17). Stippled box, exon 1 sequences; solid box, exon 2 sequences (the two exon segments are fused, indicated by FP); thin line, 5′ half of the intron from the 5′ splice site through P6a; thick line, 3′ half of the intron from P6a′ through the 3′ splice site. The intron folds so that there is a backbone discontinuity in place of L6. G, guanosine cofactor; 3′ss, the 3′ splice site; 5′ss, the 5′ splice site; FP, fusion point of the two exons; SJ, splice junction.

moter (19). The fused exon 2–exon 1 segment carries a complete circularly permuted T4 *td* gene that requires splicing for correct expression. This plasmid (pEFC, Fig. 1*A*) is expected to produce an 1130-nt circular *td* mRNA. *In vitro* transcription using this plasmid as a template generates RNA molecules consistent with this expectation (data not shown). In addition, the plasmid complements a *thyA* deletion in *E. coli*, indicating that functional td mRNA is produced *in vivo*. To begin developing constructs generally useful for making a desired foreign RNA sequence circular, we reduced the size of the fused internal exon 2–exon 1 from 1130 residues to 100 residues by deleting an *Nde* I fragment from the exon, to produce pEFCΔNde (Fig. 1*B*).

During *in vitro* transcription of pEFCΔNde, intron-derived products of splicing accumulate (19) as do other products with anomalous migration. We employed two-dimensional denaturing gel electrophoresis to distinguish circular from linear RNA. A diagonal containing linear molecules is produced, and the circular molecules appear above the diagonal, due to the proportionally more dramatic reduction in mobility of nonlinear species in higher percentage polyacrylamide gels (Fig. 3). Circles that break between the first and second dimension run well below the diagonal, at the first-dimension coordinate of the circular species and in the second-dimension coordinate of the linear species, allowing determination of the linear length of circular molecules.

After separation of the pEFCΔNde transcription reaction on a two-dimensional denaturing gel, several products are observed migrating above the diagonal, the most abundant of which are 71 and 100 nt as estimated by the migration of their linear derivatives (Fig. 3 and data not shown). To confirm the circular nature of these products, we gel-purified and sequenced them with reverse transcriptase and the exon 1 primer (data not shown). Both give rise to multiply repeated sequences longer than template length, indicating circularity. The sequence (data not shown) indicates that the more abundant 71-residue circle is produced by splicing at a cryptic 5′ splice site in exon 1 (25) and that the larger circle is produced by splicing at the natural *td* 5′ splice site. We conclude that our rearranged T4 *td* intron splices efficiently to produce circular RNA *in vitro*, as described for other similarly rearranged group I (17) and group II (18) introns.

**RNA Circles Are Produced in *E. coli*.** To demonstrate the production of RNA circles in *E. coli*, we transformed

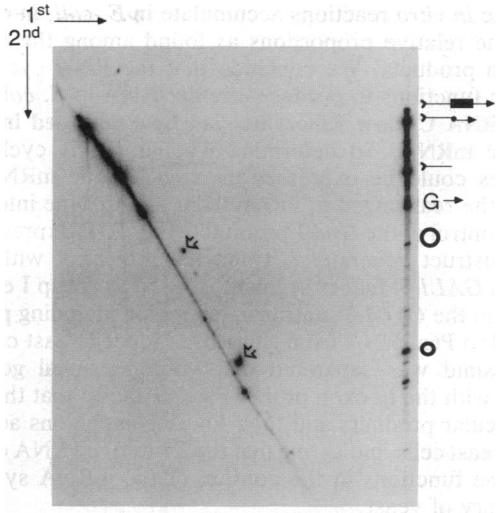


FIG. 3. Two-dimensional gel electrophoresis of uniformly labeled RNA derived by T7 transcription of *Sal* I-cut pEFC-ΔNde. The first dimension is 7.5 M urea/6% polyacrylamide and the second dimension is 7.5 M urea/8% polyacrylamide. An RNA sample from the same transcription was electrophoresed on the side of the second-dimension gel as a second-dimension marker. Open arrows indicate the 100-nt and 71-nt circular RNAs. The symbols on the right side represent products of transcription and group I splicing are the same as those used previously (19). Arrow with box, unspliced precursor; arrow with circle, linear product of intron "cyclization" reaction; arrow tail, 3′ half-intron product; G arrow, guanosine-linked 5′ half-intron product; circles, circular spliced exon products.

pEFCΔNde into DH5α, isolated RNA, separated it on two-dimensional gels, transferred it to nylon, and probed it with a labeled oligonucleotide complementary to *td* exon 1. Fig. 4*A* shows that two abundant products corresponding in mobility to the 100- and 71-nt circular RNAs characterized

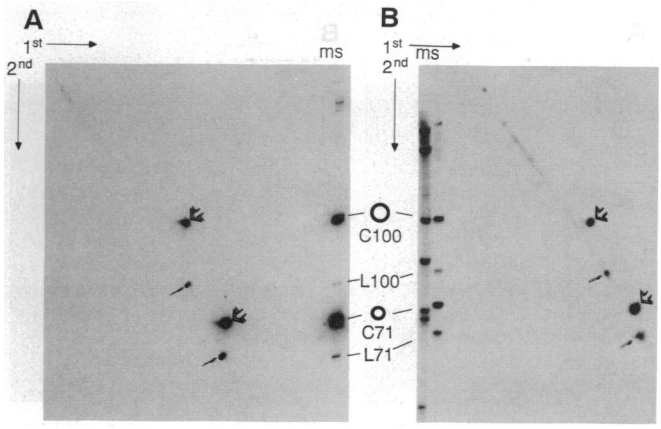


FIG. 4. Two-dimensional blots of total RNA from *E. coli* (*A*) and yeast (*B*) transformed with RNA cyclase ribozyme expression constructs. Both blots were probed with $^{32}$P-end-labeled *td* exon oligonucleotide. Second-dimension marker lanes are indicated as follows: m, end-labeled pUC13/Sau3A markers; s, the same RNA sample. Large open arrows point to the two circular products (C100 and C71) and small arrows point to their linear derivatives (L100 and L71, respectively). The migration of these products in lanes s is indicated between *A* and *B*. (*A*) Total RNA from *E. coli* DH5α transformed with pEFC-ΔNde (Fig. 1*B*). The first dimension is 5.5% polyacrylamide and the second dimension is 8% polyacrylamide. The markers do not show well in this exposure. (*B*) Total RNA from *S. cerevisiae* IH1097 transformed with pΔN-Y(+) (Fig. 1*D*). The first dimension is 6% polyacrylamide and the second dimension is 8% polyacrylamide. The largest marker DNA fragments did not transfer. Sizes of visible marker fragments from bottom are 50, 79, 82, 109, 142, 262, and 345 nt.

from the *in vitro* reactions accumulate in *E. coli*, in roughly the same relative proportions as found among the *in vitro* reaction products. We conclude that the RNA cyclase ribozyme functions to produce circular RNA in *E. coli*.

**The RNA Cyclase Ribozyme Can Be Embedded in a Eukaryotic mRNA.** To determine whether RNA cyclase ribozymes could be expressed as a eukaryotic mRNA, we placed the rearranged *td* intron from pEFCΔNde into yeast under control of the *GAL1* promoter (Fig. 1*D*). Expression of this construct generates a ribozyme transcript with extra RNA: a *GAL1* 5′ leader segment is fused to group I element P6a′, and the *CYC1* 3′ untranslated region including poly(A) is fused to P6a. RNA from galactose-induced yeast carrying the plasmid was separated on two-dimensional gels and probed with the *td* exon probe. Fig. 3*B* shows that the same two circular products and their linear companions accumulate in yeast cells, indicating that the T4-derived RNA cyclase ribozyme functions in the context of the mRNA synthesis machinery of yeast.

**Foreign Sequences Can Be Made Circular *in Vitro*.** To test whether the process of nuclear pre-mRNA splicing is compatible with the function of the RNA cyclase ribozyme, it was first necessary to demonstrate that a foreign sequence can be rendered circular by the RNA cyclase ribozyme *in vitro*. We placed a segment of the yeast actin gene including the actin intron into the *Nde* I site of pEFCΔNde to create pΔNAct(+). This site is within the region that is released as circular RNA (Figs. 1 and 2). After transcription and group I splicing, a 554-nt circular RNA containing 100 nt of *td* exon, 145 nt of actin exon, and the 309-nt actin intron was produced (Fig. 5*A*), demonstrating that an RNA cyclase ribozyme construct will convert foreign sequences into circular form *in vitro*. The introduction of actin sequence reduces the efficiency of splicing under transcription conditions, but it improves accuracy, restoring the preference for use of the correct 5′ splice site over the cryptic site (25) that was altered in the exon deletion ΔNde construct (Figs. 3 and 4).

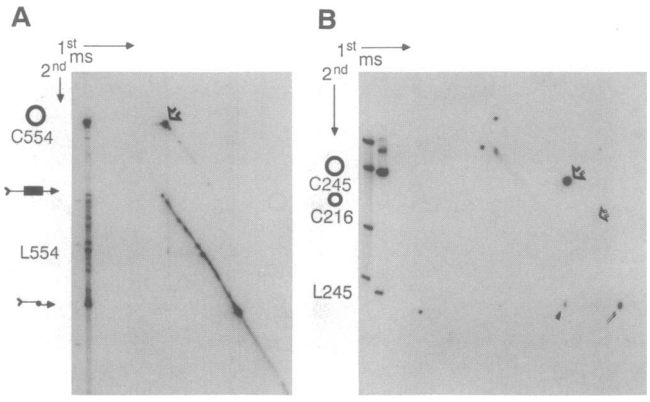


FIG. 5. Generation of circular actin RNAs *in vitro* and *in vivo*. (*A*) Two-dimensional gel electrophoresis of pΔNAct(+) (Fig. 1*C*) *in vitro* transcription and splicing products (uniformly labeled). The first dimension is 4% polyacrylamide and the second dimension is 5.5% polyacrylamide. The symbols on the left indicate transcription and splicing products, as in Fig. 3*A*. (*B*) A two-dimensional Northern blot of total RNA from *S. cerevisiae* IH1097 transformed with pΔN-A(+)-G(+) (Fig. 1*E*) probed with $^{32}$P-end-labeled *td* exon oligonucleotide. The first dimension is 4.5% polyacrylamide and the second dimension is 6% polyacrylamide. The two open arrows point to the circular exon products from use of the correct and alternative *td* 5′ splice sites (25). These transcripts lack the actin intron. The solid arrow indicates the linear derivative of the 245-residue circle. The arrowhead indicates circles that became linear between the first and second dimensions. Second dimension only lanes are indicated as in Figs. 3 and 4. Markers are end-filled pUC13/*Sau*3A fragments. Sizes are (from top) 956, 589, 345, and (*B* only) 262 nt. The smaller markers have run off the gel.

**Nuclear Pre-mRNA Splicing and the Group I RNA Cyclase Reaction.** To see how an intron-containing RNA cyclase ribozyme functions in yeast cells, we expressed the actin RNA cyclase construct in yeast (Fig. 5*B*). We observe a 245-nt circular RNA (as expected for the 145-nt spliced actin exon sequences fused to 100 nt of *td* exon, Fig. 5*B*). The linear derivative of this circle is easily identified by relating it to circles nicked during electrophoresis between the first and second dimensions. We have been unable to detect the 554-nt circle expected from cyclase activity prior to nuclear pre-mRNA processing *in vivo* (data not shown). We can detect linear transcripts consistent in length (about 850 residues) with removal of the actin intron prior to the cyclase reaction (Fig. 5*B*), suggesting that the events of nuclear pre-mRNA splicing usually precede the cyclase reaction. The actin-containing construct produces predominantly one circle *in vivo*, consistent with use of the natural *td* 5′ splice site, as it does *in vitro* (Fig. 5*A*). We conclude that the RNA cyclase ribozyme is compatible with the critical steps of mRNA synthesis and processing in yeast, including intron removal, and can be used to express foreign RNA in circular form in a eukayotic cell.

## DISCUSSION

**A Flexible Means To Produce Circular RNA *in Vitro*.** Splicing reactions catalyzed by rearranged group I (ref. 17 and this work) or group II (18) introns can be used *in vitro* to generate circular RNA molecules. We show here that constructs containing a desired foreign sequence can be made circular and free of the ribozyme structures (Fig. 5*A*). In principle, any sequence could be made circular provided it can be used to satisfy the requirements of group I exon structure. These include a small contribution to the structures P1 and P10 (20, 21) and the requirement that the exon does not interfere with folding of functional intron elements (for example, see ref. 26).

**Making Circular RNA *in Vivo*.** Upon expression *in vivo*, RNA cyclase ribozymes generate circular RNA in bacteria (Fig. 4*A*) and in yeast (Figs. 4*B* and 5*B*). Group I introns, when identified in eukaryotic nuclear genes, have been found exclusively within ribosomal DNA, which is transcribed in the nucleolus by RNA polymerase I (21). Our results indicate that cryptic termination signals for RNA polymerase II that might interfere with transcription are not found in the T4 sequences. Compared to the situation *in vitro*, function *in vivo* requires the tolerance of mRNA "leader" and "trailer" sequences (Fig. 1). With respect to the group I elements, these are added at the site of the phosphate backbone discontinuity created in L6a (19). This site is highly variable in length and sequence, even containing open reading frames in some cases (21), and we did not observe loss of function upon addition of sequence at these sites (Figs. 4*B* and 5*B*).

**Pre-mRNA Splicing Precedes Circle Formation.** We detected unreacted RNA cyclase transcripts from which the actin intron had been removed and the circular product lacking the actin intron but no circular products containing the actin intron (Fig. 5*B* and data not shown). In other experiments (data not shown), we were able to detect unspliced primary transcripts carrying both the yeast actin intron and the unreacted RNA cyclase ribozyme. Thus, these data suggest that for the majority of transcripts, the actin intron is removed prior to the cyclase (group I splicing) reaction. Because the RNA sequence of the actin intron does not block the RNA cyclase reaction *in vitro* (Fig. 5*A*), it seems unlikely that it would do so *in vivo*, at least directly. One possibility is that nuclear proteins or pre-mRNA splicing factors might associate with the intron segment of nascent RNA cyclase transcripts, precluding RNA folding events required for the group I reactions. If true, it may be that the

cyclase can only be active *in vivo* after nuclear pre-mRNA splicing is complete.

**Potential Uses of Circular RNA.** Due to the expected resistance of circular RNA to exonucleases, it seems likely that a circular RNA will be more stable than its linear counterpart (3, 4). The ability to produce a more stable RNA of conceivably any sequence may improve the efficacy or activity of RNA-based antisense or ribozyme gene regulation technologies. The cyclase reaction itself could be used to generate a new functional RNA sequence present only in the circle, serving to activate an RNA function. Circular RNA would also be useful in testing the requirements of free 5′ or 3′ ends in RNA function. Circular RNA can be a source of linear circular permutations of an RNA sequence useful for structure–function studies (27–29). Because topological information is lost upon analysis of linear products, topology must be investigated using closed RNA (e.g., circles or lariats). Reactions carried out with such substrates may then result in topologically linked products (e.g., ref. 30). In bacterial or in eukaryotic translation systems (in conjunction with an internal ribosome entry site to allow translation initiation on a circular mRNA, e.g., ref. 31), circular mRNAs with infinite open reading frames could conceivably be used to create extremely long repeating polypeptides.

RNA cyclase ribozymes may be introduced into any cell type or species for which an expression system is available. The exon sequence can be modified to carry the sequences desired as circular RNA. If RNA polymerase II transcription and nuclear pre-mRNA processing in yeast is sufficiently similar to that in other eukaryotes, our results suggest that a wide variety of prokaryotic and eukaryotic organisms could be manipulated to produce circular RNAs of many different sequences.

**Note.** A recent paper (32) presents data on the function and stability of a circular trans-cleaving form of the hepatitis delta virus ribozyme *in vitro*, made circular by the splicing reaction of a rearranged group I intron (RNA cyclase ribozyme).

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