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In vitro generation of a circular exon from a linear pre-mRNA transcript

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ABSTRACT

Recent findings have firmly established the existence of circular exons *in vivo*. We were interested in the possible splicing mechanism by which these unusual mRNA molecules could be created *in vitro*, though no biological relevance has been attached to their existence as yet. In this report we demonstrate that a modified synthetic linear yeast *ACT1* transcript whose sequence begins with the 3′-part of its original intron, is continued by 247 nt of exon sequence and terminates with the 5′-part of its intron will generate a circular exon when introduced to standard *in vitro* splicing reactions in whole cell splice extracts from *Saccharomyces cerevisiae*. The formation of a circular exon was found to be independent of specific circular or secondary structures of the pre-mRNA transcript. We hypothesize that circular exons which are found *in vivo* may be generated from pre-mRNAs which derive from rare events of transcription initiation within an intron.

INTRODUCTION

The fidelity of nuclear pre-mRNA splicing is influenced by many *cis*-acting elements (1–6). In a normal splicing reaction the branch point of an intron reacts with its upstream 5′ splice site and a lariat-shaped intermediate is formed via a 2′–5′ phosphodiester bond. The final products of the splicing reaction are the excised lariat-shaped intron and the ligated exons (7). However, low frequency events of exon skipping and scrambling have shown that errors in the fidelity of pre-mRNA splicing do arise, resulting in unusual splice products, such as circular exons (8–11). In the original report of 'scrambled exons' (8) it was predicted that the *in vivo* disarrangement of co-linearity between the genomic DNA of the *DCC* gene (deleted in colorectal carcinoma) and its mRNA resulted from a looping of the pre-mRNA prior to the splicing reaction. In a succeeding report the production of 'scrambled exons' by splicing was confirmed for the human proto-oncogene *ets-1* and a connection was established between an inverted order of the exons after splicing and their proximity to large introns (9). The circular nature of these 'inverted' mRNA transcripts was later demonstrated by RT–PCR reactions with mRNA derived from the human proto-oncogene *ets-1* (10) and from the *Sry* gene of the mouse (11). Circular exons have also been generated from transcripts which contained group I or group II intron sequences flanking the exon (12–14). Recently we reported the generation of a circular exon from a circular pre-mRNA *in vitro* (15). Consequently, we investigated whether a synthetic pseudocircular yeast 'pre-mRNA' transcript which started with the 3′-part of an intron, was continued by exon sequences and terminated with the 5′-part of an intron, i.e. a linear transcript with a discontinuous intron sequence, might also yield a circular exon. Surprisingly, not only a linear transcript which was able to form a pseudocircle by secondary structural interactions at its 5′- and 3′-ends was capable of producing such a circular exon in a standard *in vitro* splicing reaction, but a similar linear transcript without this pseudocircular structure also showed this inherent capability.

MATERIALS AND METHODS

Bacterial and yeast strains

The *Escherichia coli* K12 strains XL1 Blue (*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1 lac* [F′*proAB*lac*I*^{q}ZΔM15Tn*10*(*tet*)] (16) and CJ236 [*dut*1, *ung*1, *thi*1, *rel*A1:pCJ105(cm)] (BioRad) were used as hosts for the different cloning and mutagenesis experiments. The yeast strains BJ2412 (MAT**a**/α, *gal2*, *leu2*, *pep4-3*, *prb1-1122*, *prc1-407*, *trp1*, *ura3-52*) (17) and EJ101 (MATα, *trp1*, *pro1-126*, *prb1-112*, *pep4-3*, *prc1-126*) were used to prepare the whole cell extract for the *in vitro* splicing reactions (18).

Plasmid construction

The pAct1-Inv construct is derived from pM5act1 (15), a construct containing a truncated version of the *ACT1* gene of *Saccharomyces cerevisiae* (19). To invert *cis*-acting components of the intron-containing *ACT1* DNA sequence a 0.1 µg *Bam*HI–*Eco*RI *ACT1* insert of pM5act1 was blunt ended by incubation in 60 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$, 10 mM DTT, dNTPs (100 M each) with 2 U Klenow enzyme in a 10 µl volume for 30 min at room temperature. Afterwards incubation was continued in the additional presence of 100 µM ATP, 2.39 µM $[Co(NH_3)_6]Cl_3$, 30 mM KCl, 60 mM Tris–HCl, pH 7.5, 8 mM $MgCl_2$, 10 mM DTT and 4 U ligase enzyme at 4°C overnight to promote an intramolecular ligation of the blunt-ended *ACT1*

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DNA fragment. The nucleic acids were extracted with chloropane and ethanol precipitated. The circularized DNA fragments were hydrolysed with *Xho*I, which cleaves the *ACT1* intron sequence 64 nt downstream of the 5′-splice site. Afterwards the nucleic acids were again precipitated with ethanol. The *Xho*I fragment was ligated into *Xho*I-cleaved CIP-treated pBluescriptSKII(–) vector overnight at 4°C. XL1 Blue competent cells were transformed with the ligation products by electroporation. Individual clones were checked via their restriction patterns for the correct orientation of the insert. For the pAct1-ΨC construct 32 nt of the 5′-terminal sequence of the pAct1-Inv construct was mutagenized via site-directed mutagenesis with the 60mer oligonucleotide 5′-GGTAAAAGAGAAATCGAG-GTCGACGGTATCGATAAGCTTGATATCAAATTCGCCCTA-TAG-3′ to create a sequence complementary to its 3′-terminus (20). The two control constructs, pM5act1 and pM5act2, have been described previously (15). The DNA sequences of all the constructs were confirmed by analysis on a DNA sequencer from Applied Biosystems.

Synthesis and circularization of transcripts

All *in vitro* transcriptions were performed according to Melton *et al.* (21). The components of the modified *in vitro* transcription reaction [40 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 1 mM ATP, 1 mM CTP, 1 mM GTP, 493 µM UTP, 30 µCi [α-^{32}P]UTP, 16 U RNasin (Amersham), 25 U T7 RNA polymerase (Stratagene), 0.5 µg/µl BSA (Boehringer Mannheim) and either 1.7 mM m^7GpppG or 2.3 mM GMP, depending on whether or not the transcripts generated were to be ligated] were combined in a volume of 10 µl and reacted for 20 min at 37°C. The transcript was eluted in 400 µl elution buffer (500 mM NH_4Ac, 10 mM $MgCl_2$, 100 µM EDTA, 0.1% SDS) by shaking for 120 min at room temperature. The ligation reaction was performed with 1.4 pmol gel-purified linear transcript incubated in the presence of 50 mM HEPES, pH 8.3, 10 mM $MgCl_2$, 50 ng/µl BSA, 5 mM DTT, 2 mM ATP and 100 U T4 RNA ligase (Amersham) in a total volume of 10 µl at 12°C for ~14 h in a modification of the method of Uhlenbeck and Gumport (22). The phenol-extracted and ethanol-precipitated ligation reaction was subjected to gel electrophoresis on a 6% polyacrylamide (19:1)/8 M urea gel in order to separate the ligated circular product from the remaining linear transcript. The yields varied between 5 and 9% of the initial input.

Splicing reaction

In a standard splicing reaction ~130 fmol radiolabelled pre-mRNA transcript (circular or linear) was added to a total reaction volume of 10 µl according to Lin *et al.* (18). After 20 min at 25°C the splice reactions were stopped with a 1/5 volume of stop mix (1 mg/ml proteinase K, 50 mM EDTA, 1% SDS) and the mixture was incubated at 37°C for 15 min. An 88 µl aliquot of a mixture containing 50 mM sodium acetate, pH 5.0, 1 mM EDTA, 0.1% SDS and 25 µg/ml *E.coli* tRNA was then added and the proteins were removed by two extractions with equal volumes of chloropane, followed by one final extraction with an equal volume of chloroform/isoamylalcohol (24:1). The nucleic acids were precipitated with 3 volumes of ethanol, rinsed with 70% ethanol and dried. The reaction products were then analysed directly by electrophoresis on polyacrylamide urea gels and autoradiographed.

Two-dimensional gels

Splicing reactions were performed as described above and loaded onto a 5% polyacrylamide (19:1)/8 M urea gel. The experiment was then conducted according to a modification of the method described by Domdey *et al.* (7). The samples were subjected to gel electrophoresis until the bromophenol blue had migrated ~15 cm from the bottom of the gel well, whereupon the electrophoresis was stopped and the gel dismantled. Each lane was transferred to a prepared glass plate. A new 8% polyacrylamide (19:1)/8 M urea gel was then poured around the sample and allowed to set. Then electrophoresis was carried out in the second dimension to completion. Afterwards the gels were fixed in 3% glycerol, 20% ethanol, 10% acetic acid for 20 min before transfer to Whatman 3MM paper, drying at 80°C for 120 min and autoradiography.

Debranching reaction

The Y-shaped intermediate and the Y-shaped intron product were excised from the gel and the RNA eluted in 300 µl elution buffer overnight prior to precipitation with ethanol. Debranching of the purified RNAs was performed in 10 µl containing 30 mM KCl, 20 mM $MgCl_2$, 1.5 mM ATP, 3% polyethylene glycol, 20 mM creatine phosphate, 0.1 mg/ml BSA and 4 µl purified HeLa debranching enzyme for 25 min at 30°C (23). The reactions were stopped by addition of 50 µl stop mix (200 mM Tris–HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl, 2% SDS, 50 µg/ml tRNA) and 40 µl H_2O prior to incubation at 37°C for 30 min. The RNA was extracted with chloropane and ethanol precipitated. The reaction products were analysed by electrophoresis on a denaturing 8% polyacrylamide gel and autoradiography.

RESULTS

A pseudocircular pre-mRNA species was created by (i) restructuring the order of the *cis*-regulating splice sites of a synthetic *S.cerevisiae ACT1*-derived pre-mRNA transcript and (ii) creating a 32 nt complementary sequence for base pairing between its 5′- and 3′-ends (Fig. 1A). Upon transcription this synthetic Act1-ΨC pre-mRNA behaved as a circle, as demonstrated by its abnormal electrophoretic mobility on a denaturing polyacrylamide gel (data not shown).

Splicing reactions were performed with this pseudocircular pre-mRNA transcript, the RNA was extracted and separated first on a denaturing 5% polyacrylamide gel and then on a denaturing 8% polyacrylamide gel. After two-dimensional gel electrophoresis linear RNA lies on a diagonal track, because its relative electrophoretic mobility is independent of the different percentages of polyacrylamide utilized in the first and second dimension gels. As this is not true for either circular or Y-shaped RNA molecules (7,15,24), they can be recognized and identified by their abnormal electrophoretic mobility. As expected, two-dimensional electrophoretic separation of the products of a standard splicing reaction of Act1-ΨC RNA minus ATP did not display any splicing products (Fig. 1B). When the splicing reaction was carried out in the presence of ATP not only the putative excised discontinuous intron (380 nt; see Fig. 1A) appeared, but also an RNA whose migration behaviour suggested that it was a circularized exon (247 nt; Fig. 1C). Since we wished to establish the identity of the putative circular splice product, we added ~200 c.p.m. of a circularized control transcript, M5act2 (251 nt), to a standard

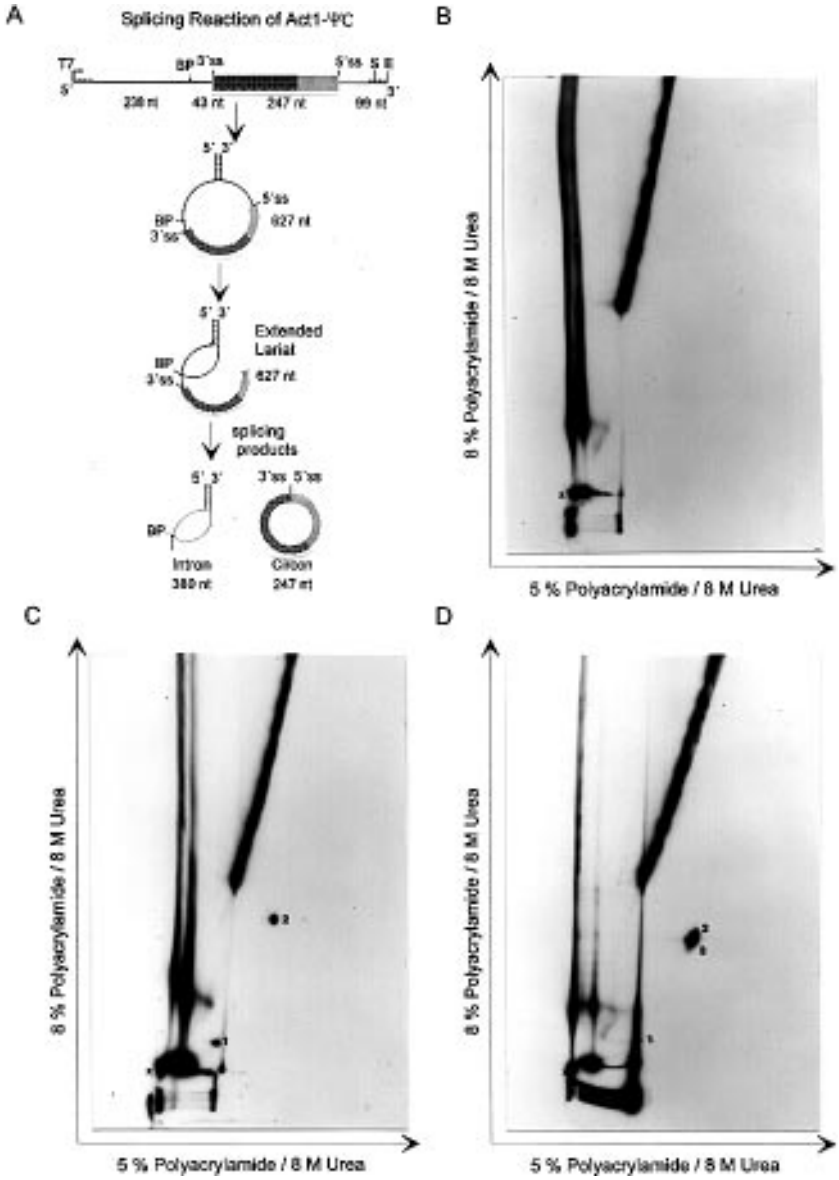

Figure 1. (**A**) Schematic representation of the splicing reaction of the transcript Act1-ΨC in a whole cell splicing extract from *S.cerevisiae*. The grey boxes denote the fused exon sequences and the line intron sequences. For the purposes of brevity and clarity we adopted the name 'circon' for the circular exon. S,*Sal*I; E, *Eco*RI. (**B**–**D**) Autoradiographs of two-dimensional gel electrophoreses of standard splicing reactions with the synthetic radiolabelled pseudocircular transcript Act1ΨC. Arrows indicate the direction of each individual electrophoresis. x designates the approximate entry positions of the RNA into the gels in the first and second dimensions. The smears seen at the bottom of the gels result primarily from successive formation and melting of possible secondary structures of the pseudocircular pre-mRNA transcript before and during electrophoresis. Spot 1, putative excised intron (380 nt). Spot 2, circon (247 nt). Spot 3, circularized transcript M5Act2 (251 nt) partially eclipsing spot 2. (**B**) Splicing reaction without added ATP. (**C**) Splicing reaction with ATP added. (**D**) Splicing reaction as in (C) with 200 c.p.m. circularized M5act2 transcript (251 nt) added as a control prior to electrophoresis.

splicing reaction of Act1-ΨC RNA prior to precipitation. The two-dimensional gel electrophoresis showed that the circular control transcript, pM5act2 (251 nt), partially eclipsed the putatively circular splice product (247 nt), which we named a 'circon' (Fig. 1D).

To confirm that this splicing reaction had taken place because of the specific secondary structure of Act1-ΨC a control experiment was performed with transcript Act1-ΨCΔ, which was derived from the same pAct1-ΨC plasmid but had been linearized with *Sal*I instead of *Eco*RI. Since the *Sal*I restriction site is 27 nt further upstream than the *Eco*RI site, the 3′-end nucleotides which had been able to base pair with the 5′-end of the Act1-ΨC transcript were no longer present in transcript Act1-ΨCΔ. Amazingly, this linear substrate was also able to generate a circular exon (spot 3 in Fig. 2A), although admittedly with low efficiency. The identities of the splice products seen (Fig. 2A, spots 2 and 3) were deduced initially from the previous experiments described in Figure 1 and from corresponding data obtained with analogous group II intron constructs reported previously (13). Also, the proposed mechanism of the putative splicing reaction of Act1-ΨCΔ (Fig. 2B) implied similarity to the *in vitro* splicing reaction previously performed with a group II

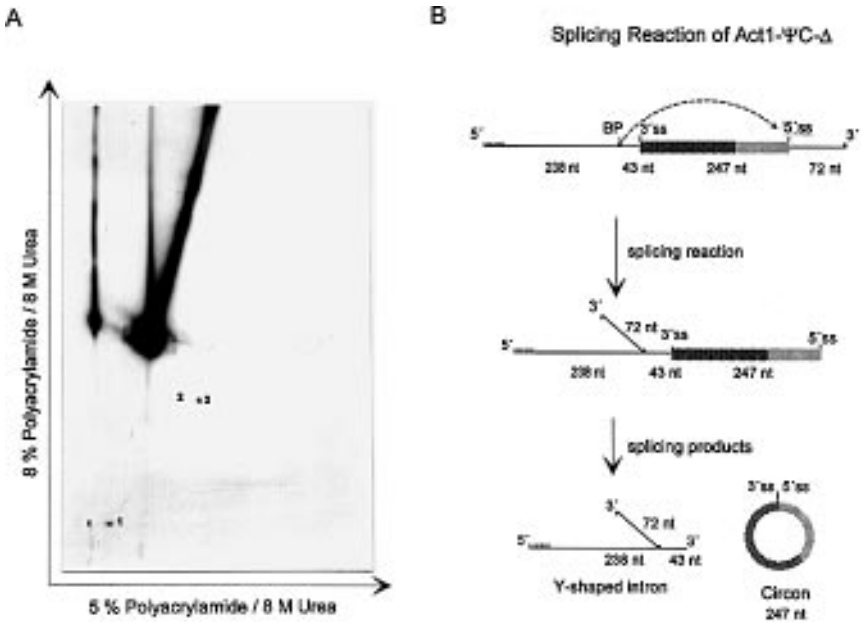

Figure 2. (**A**) Two-dimensional gel analysis of a standard splicing reaction with Act1-ΨCΔ RNA plus ATP. Spot 1, putative Y-shaped extended intermediate (600 nt). Spot 2, putative excised intron (353 nt). Spot 3, circon (247 nt). Arrows indicate the direction of each individual electrophoresis. x designates the approximate entry positions of the RNA into the gels in the first and second dimensions. (**B**) Schematic diagram of a splicing reaction with Act1-ΨCΔ RNA. Note that in contrast to the transcript Act1-ΨC, Act1-ΨCΔ RNA is 27 nt shorter at its 3′-end and thus is no longer characterized by the secondary structure present in Act1-ΨC RNA.

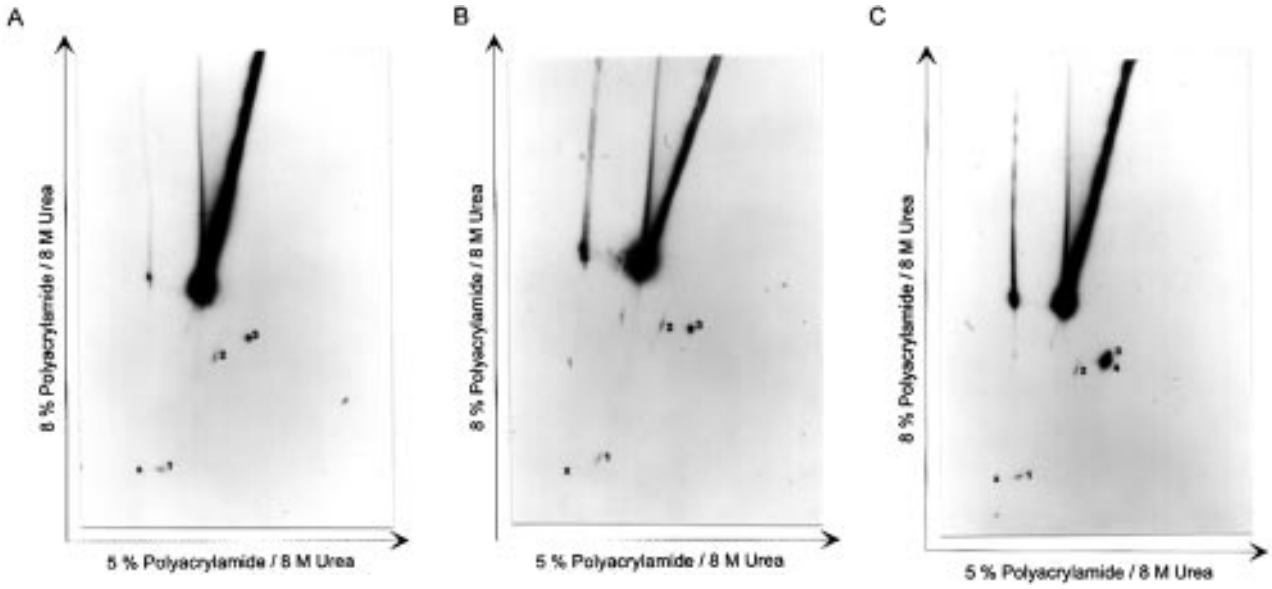

Figure 3. Two-dimensional gel electrophoresis of standard splicing reactions with transcripts Act1-Inv and Act1-InvΔ. The splicing reactions of Act1-Inv and Act1-InvΔ both have a reaction mechanism which is identical to the schematic diagram depicted in Figure 2A. Spot 1, putative Y-shaped extended intermediate. Spot 2, putative excised Y-shaped intron. Spot 3, circon. Spot 4, circularized transcript M5Act2 (251 nt) partially eclipsing spot 3. Arrows indicate the direction of each individual electrophoresis. x designates the approximate entry positions of the RNA into the gels in the first and second dimensions. (**A**) Standard splicing reaction of transcript Act1-Inv (619 nt) deriving from plasmid pAct1-Inv linearized with*Eco*RI prior to *in vitro* transcription. (**B**) Splicing reaction of transcript Act1-InvΔ (592 nt) deriving from pAct1-Inv digested with *Sal*I. Note the position of the putative excised intron relative to the circular splice product. As this intron is 27 nt smaller than that in (A) it moves correspondingly faster in the second dimension. (**C**) An equal aliquot of the splicing reaction shown in (A) was given 200 c.p.m. of circularized M5act2 transcript prior to electrophoresis to approximate the position of the predicted circular splice product.

intron construct (13). According to this model the branch point present in the 5′-proximal part of the transcript reacts with the 5′ splice site of the downstream intron, producing a Y-shaped intron and a circular exon as products (13,25).

To further substantiate our conclusions another construct, pAct1-Inv, was generated. Transcripts Act1-Inv and Act1-InvΔ, which were derived from this template after linearization with *Eco*RI and *Sal*I respectively, were similar to transcript Act1-ΨC,

but lacked the complementary sequences at either end. As the splicing intermediate and the intron of the splicing reaction of Act1-Inv were expected to be Y-shaped in form, standard splicing reactions of the Act1-Inv RNA precursor were analysed by two-dimensional gel electrophoresis in order to visualize their molecular structure (24). When Act1-Inv RNA (619 nt) was used as splicing substrate three distinct spots, representing two putative Y-shaped RNA molecules and the putative circular exon, were observed migrating beneath the diagonal of the linear RNA species (Fig. 3A). The spots designated 1 and 2 were judged to be the Y-shaped intermediate and its excised Y-shaped intron. When seen relative to the electrophoretic migrations of the products and educts of a standard splicing reaction in previous two-dimensional gel electrophoreses (15) and other data (13,24–25) it was evident that the RNA species derived from the splicing reaction of Act1-Inv, spots 1 and 2, did not have a circular, but a Y-shaped structure. Proof that spot 3 was the putative circon was provided by co-electrophoresis of the circularized control RNA M5Act2 (251 nt) with a standard splicing reaction of Act1-Inv RNA (Fig. 3C). Here the splice product (247 nt) was seen co-migrating with the circular marker, suggesting its circular character.

To once more verify the circular nature of the presumed circon (spot 3, Fig. 3A and B) the products of a splicing reaction with Act1-Inv RNA were co-electrophoresed with ~200 c.p.m. of the circularized control RNA derived from pM5act2. Here again, this control RNA (spot 4, 251 nt) migrated in a position very close to spot 3 (247 nt) (Fig. 3C).

In addition to these demonstrations of their unusual electrophoretic mobility as circular molecules, the circons deriving from Act1-ΨC and Act1-Inv transcripts were each eluted from a two-dimensional gel and reverse transcribed with a 22 nt primer starting 26 nt downstream of the 5′-end of the exon. The resulting cDNA was subjected to a polymerase chain reaction and the amplified DNA isolated after gel electrophoresis and cloned. The cloned sequence of the RT–PCR product of the eluted putative circular splice product (spot 3) confirmed our earlier conclusion that the 5′- and 3′-ends of the exon sequence had been spliced together to form a ‘circon’ (Fig. 4).

In order to demonstrate the identity of the excised Y-shaped intron, transcript Act1-InvΔ (592 nt), generated from pAct1-Inv linearized with *Sal*I, was subjected to a splicing reaction. With Act1-InvΔ RNA any splicing intermediates or products which were formed from and with the downstream intron sequence would display altered, i.e. smaller, molecular weights and migrate faster in both dimensions. In Figure 3B the putative excised Y-shaped intron (spot 2) had (i) a different position relative to the circularized exon (spot 3) and (ii) had migrated faster than shown in Figure 3A.

Lastly, a debranching experiment proved the Y-shaped structures of the splicing intermediate and the intron of Act1-Inv, confirming the existence of the 2′–5′ phosphodiester bond normally found in the intron splice product of *cis*- and *trans*-splicing reactions (Fig. 5). Debranching of these RNAs yielded the expected 273 nt RNA fragment for the Y-shaped intron and the 520 nt RNA fragment for the splicing intermediate. Since RNAs with an unprotected 5′-terminal monophosphate structure are extremely sensitive to nuclease degradation, the 99 nt RNA fragment representing the downstream intron sequence was only barely detected, especially in the debranching reaction of the splicing intermediate, where it represented <20% of the entire molecular structure of the splicing intermediate.

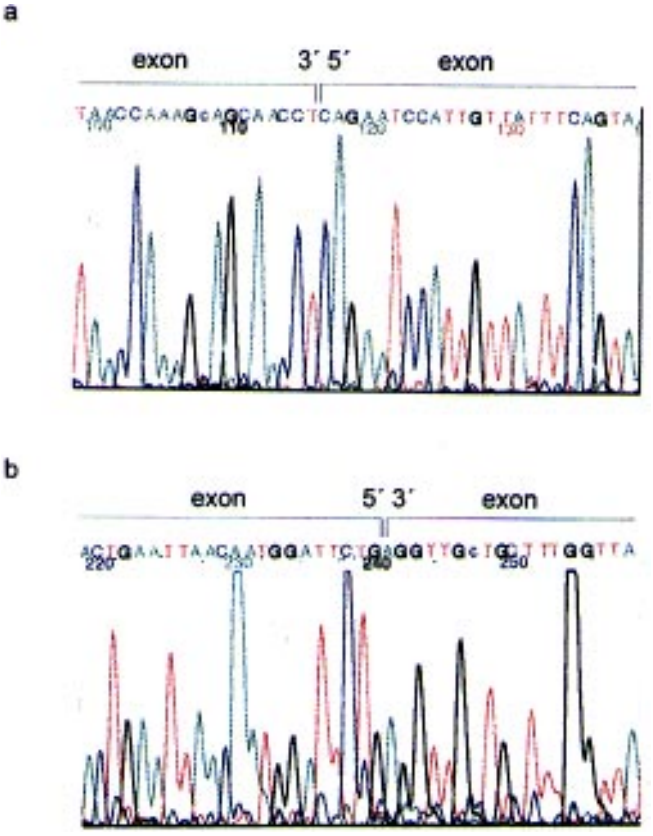

Figure 4. Sequence analyses of products of RT–PCR reactions executed such that amplification occurred across the splice junctions of the putative circular splice products of Act1-ΨC and Act1-Inv. The sequences of Act1-ΨC and Act1-Inv display opposite orientations because a blunt-end ligation was performed during cloning of the PCR products. (**a**) Sequence analysis of Act1-ΨC. The site of the splice junction lies between nt 115 and 116 (CCT/CAG). (**b**) Sequence analysis of Act1-Inv. The site of the splice junctions is located between nt 240 and 241 (CTG/AGG).

DISCUSSION

The existence of circular exons *in vivo* has been proven for several mammalian pre-mRNAs (8–11). Circular exons have also been generated artificially in several model systems both *in vitro* and *in vivo* from autocatalytic RNA intron sequences (12–14) and from circular pre-mRNA transcripts (15). While our experimental data show that the secondary structure of the pseudocircular RNA precursor Act1-ΨC seems to be relevant for an increase in the efficiency of circon formation in an *in vitro* splicing reaction, they also show that it is not an absolute necessity for the production of circons. Hence it would appear that when a single linear RNA precursor begins and ends with a discontinuous intron sequence it will form a circular exon as its product.

Our results further substantiate the conclusion that circons are created *in vivo* by the pre-mRNA splicing machinery (8–11). That Y-shaped introns can be formed during an *in vitro* splicing reaction has been either proposed or demonstrated previously (13,25–27). However, with the exception of experiments performed with the sequences of group II introns (13), all of the other splicing experiments were performed with two separate RNA precursors. The *in vitro* splicing reactions presented here have

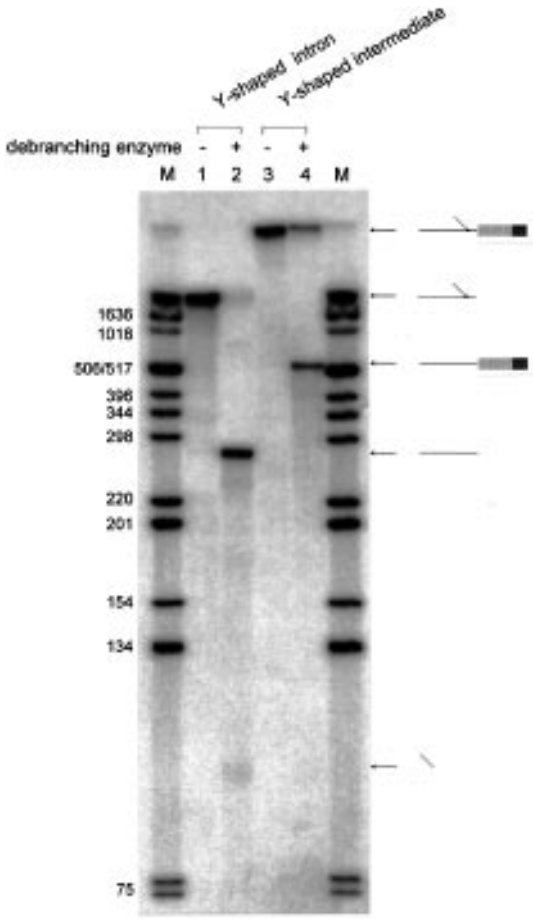

Figure 5. Debranching analysis of the Y-shaped intermediate, intron and splicing intermediate of Act1-Inv. The gel-purified Y-shaped RNA species were either treated (lanes 2 and 4) or not (lanes 1 and 3) with HeLa debranching enzyme as described in Materials and Methods. Schematic representations of the splicing intermediate and products are displayed. The 247 nt exon sequence is represented by shaded boxes and the discontinuous intron sequences of 273 nt and 99 nt are depicted by lines. The positions of the branched and de-branched RNAs are indicated. M, ^{32}P-labelled kilobase ladder marker.

been performed with a single linear transcript and our two-dimensional gels show not only Y-shaped RNA species, but also definitive circular splice products.

Thus it is apparent from our data that there are several possible sources for naturally occurring circons (8–11). Circular exons may result either from pre-mRNAs which have base pairing between neighbouring introns or pre-mRNAs which begin with the 3′-part of a discontinuous intron sequence as a result of transcription initiation within an intron. Another explanation for the generation of circons could be the 'masking' of neighbouring splice sites by either hnRNP proteins or specific proteins, such as those involved in alternative splicing (28–33). In higher eukaryotes alternative splicing has been shown to occur during developmental growth in the formation of isoforms of structural proteins (34), in the splicing of components of the immune response (35–37) and in the brain (38). Correspondingly, the original report on 'scrambled exons' showed the scrambled or circular transcripts to co-exist at relatively low levels with normal mRNA transcripts in a variety of normal and neoplastic cells of rodent and human origin, with rodent brain tissue displaying the highest level of circular transcripts (8). In *S.cerevisiae* alternative splicing in the form of exon skipping has been previously shown (39–40) and a splicing factor, *SLU7*, has been demonstrated to mediate 3′ splice site choice (41).

Despite recent evidence for *in vitro* translation of circular transcripts with internal ribosome entry sites (42), the biological relevance of these unusual mRNA species remains in question. Further studies of this unusual splicing reaction should provide information as to the nature of the mechanism by which a spliceosome produces circular exons and enlightenment as to the method by which it maintains fidelity in pairing splice sites.

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