

POINT OF VIEW



## Early history of circular RNAs, children of splicing

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### ABSTRACT

In this commentary we briefly summarize early work on circular RNAs derived from spliceosome mediated circularization. We highlight how this early work inspired work on the basic mechanisms of nuclear RNA splicing, the possible function of circular RNAs and the potential uses of circular RNAs as tools in biomedicine. Recent developments in the study of circular RNAs, summarized in this volume, have brought these questions back to the foreground.

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The 1977 discovery of pre-mRNA splicing (Berget et al., 1977; Chow et al., 1977) immediately led to the realization that there was an exon (or intron) definition problem: If an organism fails to define exons stringently, RNA processing could result in (1) exon skipping by ligation of a 5' splice site to a far-downstream 3' splice site and other forms of alternative splicing, (2) trans-splicing by ligation of a 5' splice site to a 3' splice site in another pre-mRNA, or (3) RNA circularization by ligation of a 5' splice site to an upstream 3' splice site. Indeed, all of these are now known to occur and to play critically important roles in providing plasticity to the coding capacity of genomes. Although much attention was given to alternative pre-mRNA splicing and some to trans-splicing, exon circularization was not appreciated immediately and indeed remained woefully understudied for decades. Acknowledging that several different mechanisms are utilized to form circular RNAs, here we review the early history of circular RNAs formed from exon circularization or 'back splicing': circular RNAs that are the children of splicing.

About a decade following the discovery of self-splicing introns (Cech et al., 1981), Puttaraju and Been (1992) showed that a self-splicing group I intron, permuted by placing the 5' splice site downstream of the 3' splice site, yielded circular RNAs *in vitro*. Indeed, this work confirmed observations by Price et al. (1987), who detected an RNA circle believed to be formed by the splicing of a cryptic 5' splice site downstream from a canonical 3' splice site in a group I splicing substrate. Ford and Ares (1994) showed that circular RNAs obtained by group I splicing can be expressed *in vivo* in *E. coli* and yeast. Circular RNAs were, and still are, believed to be more stable *in vivo* than linear RNAs due to their resistance to end-dependent degradation (Puttaraju et al., 1993). Importantly, by 1996 Puttaraju and Been, using the permuted group I intron method, showed that trans-acting RNA enzymes (the hepatitis delta virus ribozyme and *B. subtilis* RNase P RNA) can be expressed *in vivo* as circular RNAs that retain catalytic activity.

The experiments involving RNA circularization by permuted group I introns prompted the prediction of similar mechanisms for group II and pre-mRNA exons. Consistent with these predictions, splicing-mediated exon circularization was observed with permuted group II introns (Jarrell, 1993). The existence of spliceosome mediated RNA circularization, however, went against the general view that splice sites were used in the order in which they appeared in nascent transcripts – the 'first come, first served' model (Aebi et al., 1986; Beyer and Osheim, 1988). Nonetheless, RNAs with 'scrambled exons', consistent with either exon circularization or trans-splicing, were observed (Nigro et al., 1991; Cocquerelle et al., 1992), although these events were rare. Importantly, Capel et al. (1993) showed that transcripts of the testis-determining gene *Sry* were efficiently circularized in mouse adult testis and that this circularization prevented their translation. Capel et al., also showed that unique inverted repeats in the *Sry* transcript approximated the splice sites in a long exon and posited that RNA circularization was mediated by the spliceosome.

Spliceosome-mediated RNA circularization was demonstrated with single exon substrates in yeast extracts (Schindewolf et al., 1996) and mammalian nuclear extracts (Pasman et al., 1996). One difference between the yeast and mammalian reactions was that the yeast system seemed to produce only the spliced product, whereas the mammalian reactions produced, in addition to the spliced circular exon, low levels of many other non-linear RNAs. The significance of these products remains unclear, but these could very well reflect the looser definition of splice sites in mammalian pre-mRNAs and be the products of cryptic reactions as proposed for group I introns by Price et al. (1987).

The discovery of spliceosome mediated exon circularization raised questions about the mechanism of splice site approximation and about how the splicing machinery commits to a specific pairs of splice sites in alternative splicing.

Similar implications were suggested by earlier work of Konarska et al. (1985), Solnick (1985), and Chiara and Reed (1995), who showed that 5' and 3' splice sites on separate conventional pre-mRNAs can be spliced, producing the expected linear product. These splicing reactions, termed trans-splicing, were consistent with the idea that splicing does not require continuous intron sequences between the splice sites (although reaction kinetics were not yet addressed). These experiments suggested a 3-dimensional diffusion model of splice site approximation, which postulates that the splice sites are brought into proximity via interaction of complexes assembled at the splice sites sequences irrespective of the intron sequences separating them. A scanning model, in contrast, postulates that, once assembled, complexes at the splice sites are brought together in a process that involves one-dimensional scanning of intron sequences. The scanning model is reminiscent of the eukaryotic translation process involving 5' RNA cap recognition followed by one-dimensional scanning to the translation start codon. These two models were tested directly by Pasmán and García-Blanco (1996). Using several lines of evidence, including splicing precursors in which portions of the intron were replaced with a flexible non-nucleic acid polymer, the results supported the 3 dimensional diffusion model. It should be noted that while the splicing machinery does not bring the splice sites together by scanning, subcomponents of this machinery piggyback on the DNA dependent RNA polymerase II (RNAP II) that scans splicing precursors as it synthesizes them (reviewed in Goldstrohm et al., 2001). This may explain how splice sites that are large distances away can be brought into close spatial proximity, resulting in accurate and rapid splice site pairing via a 3-dimensional diffusion mechanism.

It is clear that early studies of circular RNAs contributed to the broad understanding of basic nuclear RNA splicing mechanisms. Indeed, some of the questions raised by these early experiments remain unanswered, particularly regarding the commitment to different splice site pairs during alternative splicing. Early studies also led to questions regarding the function of many circular RNAs – questions that awaited the development of novel technologies. New insights into the function of circular RNAs, which are eloquently presented in this volume, echo the very special function of Sry circularization in gene expression, but now in trans on the function of other RNAs. Finally, it should be pointed out that synthetic circular RNAs were proposed to have special uses for biomedical applications (Bohjanen et al., 1996) and this continues to be a promise today.

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No potential conflicts of interest were disclosed.

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### References

- Aebi M, Hornig H, Padgett RA, Reiser J, Weissmann C. Sequence requirements for splicing of higher eukaryotic nuclear pre-mRNA. *Cell* 1986; 47(4):555-65; PMID:3779836; [https://doi.org/10.1016/0092-8674\(86\)90620-3](https://doi.org/10.1016/0092-8674(86)90620-3)
- Berget SM, Moore C, Sharp PA. Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc Natl Acad Sci U S A* 1977; 74(8):3171-5; PMID:269380; <https://doi.org/10.1073/pnas.74.8.3171>
- Beyer AL, Osheim YN. Splice site selection, rate of splicing, and alternative splicing on nascent transcripts. *Genes Dev* 1988; 2(6):754-65; PMID:3138163; <https://doi.org/10.1101/gad.2.6.754>
- Bohjanen PR, Colvin RA, Puttaraju M, Been MD, Garcia-Blanco MA. A small circular TAR RNA decoy specifically inhibits Tat-activated HIV-1 transcription. *Nucleic Acids Res* 1996; 24(19):3733-8; PMID:8871552; <https://doi.org/10.1093/nar/24.19.3733>
- Capel B, Swain A, Nicolis S, Hacker A, Walter M, Koopman P, Goodfellow P, Lovell-Badge R. Circular transcripts of the testis-determining gene Sry in adult mouse testis. *Cell* 1993; 73(5):1019-30; PMID:7684656; [https://doi.org/10.1016/0092-8674\(93\)90279-Y](https://doi.org/10.1016/0092-8674(93)90279-Y)
- Cech TR, Zaug AJ, Grabowski PJ. In vitro splicing of the ribosomal RNA precursor of Tetrahymena: involvement of a guanosine nucleotide in the excision of the intervening sequence. *Cell* 1981; 27(3 Pt 2):487-96; PMID:6101203; [https://doi.org/10.1016/0092-8674\(81\)90390-1](https://doi.org/10.1016/0092-8674(81)90390-1)
- Chow LT, Gelinis RE, Broker TR, Roberts RJ. An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell* 1977; 12(1):1-8; PMID:902310; [https://doi.org/10.1016/0092-8674\(77\)90180-5](https://doi.org/10.1016/0092-8674(77)90180-5)
- Chiara MD, Reed R. A two-step mechanism for 5' and 3' splice-site pairing. *Nature* 1995; 375(6531):510-3; PMID:7777062; <https://doi.org/10.1038/375510a0>
- Cocquerelle C, Daubersies P, Majérus MA, Kerckaert JP, Bailleul B. Splicing with inverted order of exons occurs proximal to large introns. *EMBO J* 1992; 11(3):1095-8; PMID:1339341; PMC556550
- Feldstein PA, Bruening G. Catalytically active geometry in the reversible circularization of "mini-monomer" RNAs derived from the complementary strand of tobacco ringspot virus satellite RNA. *Nucleic Acids Res* 1993; 21(8):1991-8; PMID:7684131; <https://doi.org/10.1093/nar/21.8.1991>
- Ford E, Ares M. Synthesis of circular RNA in bacteria and yeast using RNA cyclase ribozymes derived from a group I intron of phage T4. *Proc Natl Acad Sci U S A* 1994; 91(8):3117-21; PMID:7512723; <https://doi.org/10.1073/pnas.91.8.3117>
- Goldstrohm AC, Greenleaf AL, Garcia-Blanco MA. Co-transcriptional splicing of pre-messenger RNAs: considerations for the mechanism of alternative splicing. *Gene* 2001; 277(1-2):31-47; PMID:11602343; [https://doi.org/10.1016/S0378-1119\(01\)00695-3](https://doi.org/10.1016/S0378-1119(01)00695-3)
- Jarrell KA. Inverse splicing of a group II intron. *Proc Natl Acad Sci U S A* 1993; 90(18):8624-7; PMID:8378340; <https://doi.org/10.1073/pnas.90.18.8624>
- Konarska MM, Padgett RA, Sharp PA. Trans splicing of mRNA precursors in vitro. *Cell* 1993; 42(1):165-71; PMID:3848348; [https://doi.org/10.1016/S0092-8674\(85\)80112-4](https://doi.org/10.1016/S0092-8674(85)80112-4)
- Nigro JM, Cho KR, Fearon ER, Kern SE, Ruppert JM, Oliner JD, Kinzler KW, Vogelstein B. Scrambled exons. *Cell* 1991; 64(3):607-13; PMID:1991322; [https://doi.org/10.1016/0092-8674\(91\)90244-S](https://doi.org/10.1016/0092-8674(91)90244-S)
- Puttaraju M, Been MD. Circular Ribozymes Generated in *Escherichia coli* Using Group I Self-splicing Permuted Intron-Exon Sequences. *J Biol Chem* 1996; 271:26081-7; PMID:8824250; <https://doi.org/10.1074/jbc.271.42.26081>
- Pasmán Z, García-Blanco MA. The 5' and 3' splice sites come together via a three dimensional diffusion mechanism. *Nucleic Acids Res* 1996; 24(9):1638-45; PMID:8649980; <https://doi.org/10.1093/nar/24.9.1638>
- Pasmán Z, Been MD, García-Blanco MA. Exon circularization in mammalian nuclear extracts. *RNA* 1996; 2(6):603-10; PMID:8718689; PMC1369399

19. Price JV, Engberg J, Cech TR. 5' exon requirement for self-splicing of the *Tetrahymena thermophila* pre-ribosomal RNA and identification of a cryptic 5' splice site in the 3' exon. *J Mol Biol* 1987; 196(1):49-60; PMID:2443717; [https://doi.org/10.1016/0022-2836\(87\)90510-9](https://doi.org/10.1016/0022-2836(87)90510-9)
20. Puttaraju M, Been MD. Group I permuted intron-exon (PIE) sequences self-splice to produce circular exons. *Nucleic Acids Res* 1992; 20(20):5357-64; PMID:1279519; <https://doi.org/10.1093/nar/20.20.5357>
21. Puttaraju M, Perrotta AT, Been MD. A circular trans-acting hepatitis delta virus ribozyme. *Nucleic Acids Res* 1993; 21(18):4253-8; PMID:7692400; <https://doi.org/10.1093/nar/21.18.4253>
22. Schindewolf C, Braun S, Domdey H. In vitro generation of a circular exon from a linear pre-mRNA transcript. *Nucleic Acids Res* 1996; 24(7):1260-6; PMID:8614628; <https://doi.org/10.1093/nar/24.7.1260>
23. Solnick D. Trans splicing of mRNA precursors. *Cell* 1985; 82(1):157-64; PMID:3848347; [https://doi.org/10.1016/S0092-8674\(85\)80111-2](https://doi.org/10.1016/S0092-8674(85)80111-2)