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Stabilization and analysis of intron lariats in vivo

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Dedicated to the memory of Dr. Mary Edmonds.

Abstract

The analysis of lariats produced in vivo during pre-mRNA splicing is a powerful tool for elucidation of regulatory mechanisms and identification of natural recursive splicing events. Nevertheless, this analysis is technically challenging because lariats normally have short half-lives. With appropriate controls, RT-PCR amplification and sequencing of the region spanning the 2′–5′ phosophodiester bond at the branch junction can be a sensitive and versatile method for lariat analysis. This approach can be facilitated and enhanced by reducing the activity of debranching enzyme (DBR) in order to stabilize lariats. We have generated a set of plasmids for dsRNA-mediated knockdown of DBR under diverse conditions in transgenic *Drosophila* and in cultured cells. We describe the use of these plasmids and protocols for lariat analysis. We have generated transgenic *Drosophila* strains carrying a GAL4-regulated RNAi construct that allows selective knockdown of DBR in specific tissues or developmental stages, using the large collection of available GAL4 expression lines. These strains should prove useful for detailed developmental analyses of alternative and recursive splicing and for genetic analyses of splicing factors. Similar approaches should be readily adaptable to other organisms.

Keywords: Intron; Alternative splicing; Recursive splicing; Branchpoint; Branchsite

1. Introduction

Natural branched RNAs containing both 2′–5′ and 3′–5′ phosphodiester linkages were first described over 20 years ago in total nuclear RNA from human cells by Wallace and Edmonds [1]. Soon thereafter it was shown that RNA lariats containing these branched structures are intermediates and side-products of the pre-mRNA splicing reaction (Fig. 1) [2–5]. Lariat intermediates are generated by the 5′ splice site cleavage reaction, in which the 2′ hydroxyl group of the branchpoint adenosine (usually located 15–40 nucleotides upstream of the 3′ splice site) attacks the phosphodiester bond at the exon/intron boundary; this generates a free 3′OH group at the end of the upstream exon and a lasso-like structure that contains the intron, branch junction, and downstream exon. In the second step of splicing, the free 3′OH attacks the phosphodiester bond at the intron/exon boundary to ligate the two exons; a side product of this reaction is a free lariat, which is degraded rapidly in vivo.

Recognition of the branch site by U2 snRNP is one of the early events in spliceosome assembly. Thus, analysis of lariats can provide important information about regulatory mechanisms and the point of action of particular splicing factors. Lariats can also serve as surrogates for the corresponding mRNAs to analyze splicing. This has been exploited to obtain evidence of splicing commitment when substrates do not proceed efficiently to the exon ligation step in vitro, and also for genome-wide analysis of splicing in yeast [6]. Analysis of lariats can be advantageous when alternative splicing generates a very unstable mRNA, and it can provide evidence to distinguish between regulation of alternative splicing versus differential accumulation of alternative mRNAs in vivo [7]. In cases of recursive

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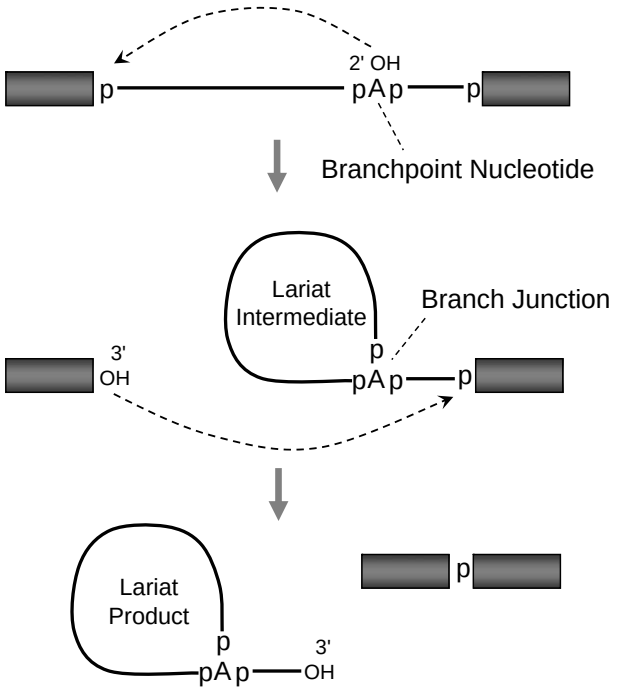

Fig. 1. Formation of the lariat intermediate and lariat product by the two catalytic steps of pre-mRNA splicing.

splicing, which leaves no trace in the final mRNA, the resulting lariats provide the only direct evidence for the splicing event [8].

The 2′–5′ phosphodiester of an intron lariat blocks reverse transcription from the 3′ side of the branch junction. This effect allows the position of the branchpoint nucleotide to be mapped by measuring the length of cDNA fragments extended from a downstream primer [9,10]. However, it is possible to extend a primer through the branched junction from the 2′ side [11]; this forms the basis of a versatile RT-PCR assay that has been used to detect the lariats and identify the branchpoint nucleotides from several spliceosomal and group II introns in vivo [7,8,11–14]. In this case, the cDNA extended through the branch junction provides a template for amplification with an antisense primer downstream of the 5′ splice site and a sense primer upstream of the branchpoint nucleotide (primers B1 and F2 in Fig. 2); these primers would be divergent on the pre-mRNA. The sequence of the resulting amplimer contains the region upstream of and including the branchpoint nucleotide (an A in the genome that is replaced by a T in the amplimer) juxtaposed to the 5′ splice site (Fig. 2). Thus, this approach allows precise identification of the branchpoint nucleotide and verification that the cDNA is derived from a bona fide lariat.

It has been difficult to analyze lariats produced in vivo because they are linearized rapidly by the 2′–5′ phosphodiesterase activity of lariat debranching enzyme (DBR) [15,16]. Genetic analysis in yeast has shown that lariats are stabilized in the absence of DBR activity and can accumulate to very high levels [16,17]. If DBR homologs are also responsible for the rate-limiting step of lariat turnover in more complex eukaryotes, it should also be possible to stabilize lariats and facilitate their analysis in such organisms by reducing or interfering with their DBR activity. Deletion

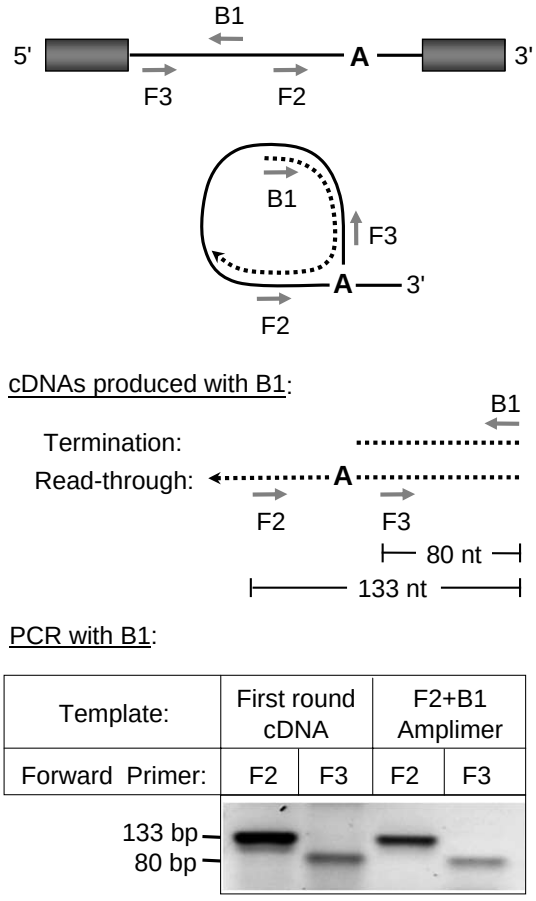

Fig. 2. Lariat analysis by RT-PCR. An example is shown to demonstrate that reverse transcription from the 2′ side extends efficiently through the branch junction. Reverse transcription was performed on a sample of RNA from a *dbr1Δ* strain of *S. cerevisiae* that accumulates high lariat levels [16]. A gene-specific antisense oligonucleotide (B1) was used to prime reverse transcription within the lariat from the *RPS14A* intron, followed by 21 cycles of PCR amplification with B1 and forward primers (F2 or F3) located on either side of the branch junction. The ratio of products obtained with the two primer pairs measures the efficiency of reverse transcription through the branch junction. As a control for inherent differences in efficiency of the primer pairs, the same sequences were also amplified from a homogeneous linear template generated by previous PCR with primers F2 + B1; this fragment spans the branch junction and contains the priming site for F3 (center of diagram; note that reverse transcription through the 2′–5′ phosphodiester incorporates an A instead of the expected T at the position of the branchpoint nucleotide). The observed ratio of PCR products with the two primer pairs was the same using the lariat template or the control linear template, indicating that reverse transcription within the lariat loop does not terminate preferentially at the branch junction. Reverse transcription through the branch junctions of *RPS14B* and *ACT* lariats is equally efficient. Note that in the experiment shown here the B1 + F3 pair can also amplify the same fragment from the unprocessed intron, but this contributes minimal signal because the stabilized lariats are far more abundant. A standard lariat assay uses primers arranged like B1 and F2, which diverge on the unprocessed intron but converge on the lariat and amplify across the branch junction. Primer sequences used: B1 GCACGTCGTCAGAAACATTC; F2 GGAAAAGCAAAGATACTATGTAAGAAT; F3 GTATGTTTA ATCACATAGTGAACATTTTT.