Where in the cell is the minor spliceosome?

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The nucleus, a hallmark of eukaryotes, segregates the genome and the cellular machineries that transcribe and process mRNAs from the translation apparatus in the cytoplasm. Compartmentalization prevents primary gene transcripts (pre-mRNAs) from engaging in translation before they are fully matured. Thus, major mRNA processing events—pre-mRNA splicing and 3′ polyadenylation—take place in the nucleus. A recent paper by König *et al.* (1) challenged this view, claiming that the splicing of hundreds of genes occurs in the cytoplasm, after export of unspliced pre-mRNAs from the nucleus. This paper sparked intense controversy. Because the central issue of whether mRNA formation and mRNA translation occur in separate compartments is so fundamental to eukaryotic cell biology, we examine here the experimental bases for this claim and contrast them with the findings of Pessa *et al.* (2), in this issue of PNAS, which provide contradictory evidence.

The vast majority of introns are spliced by the major spliceosome: a complex RNA–protein machine containing the U1, U2, U4/U6, and U5 small nuclear ribonucleoproteins (snRNPs). Splicing can occur cotranscriptionally and is generally thought to be completed in the nucleus before mRNA export. Although the biogenesis of four of the major spliceosomal snRNPs (U1, U2, U4, and U5) involves a cytoplasmic phase, the mature snRNPs and associated protein splicing factors ultimately concentrate in the nucleus.

A minor class of introns is removed by a closely related machinery termed the "minor spliceosome." The U11 and U12 snRNPs were described in 1988 as low-abundance particles similar to the well characterized snRNPs of the major spliceosome (3). In 1993, these particles were localized, like their U1 and U2 counterparts, by *in situ* hybridization to the nucleoplasm of mammalian cells (4). In 1996, they were assigned functions in the splicing of minor-class introns (5, 6), and the U4atac and U6atac snRNAs of the minor spliceosome were identified (7). Thus, although four snRNPs are unique to the minor spliceosome, the same U5 snRNP is shared by the minor and major spliceosomes. The minor spliceosome has subsequently been studied with respect to its protein composition and mechanism (8, 9), as well as its phylogenetic distribution and evolutionary origins (10). The minor spliceosome excises ≈1 in 300 introns from human pre-mRNAs (11)—which encode proteins with a wide range of functions (12)—consistent with the lower abundance (≈1%) of its snRNPs relative to those of the major spliceosome. At least seven proteins are unique to the minor spliceosome (13). GFP-tagged versions of two—the U11/U12-associated proteins 35K and 31K (MADP-1)—were reported to concentrate in the nucleus of plant and HeLa cells, respectively (14, 15).

König *et al.* (1) called this previous work into question by localizing two minor spliceosomal snRNAs in the cytoplasm of vertebrate cells and suggesting that the excision of minor introns takes place outside the nucleus, with functional and evolutionary implications. In response, Pessa *et al.* (2) present new *in situ* hybridization and cell fractionation studies on the full set of minor snRNPs, extending prior evidence for the nuclear location of the minor spliceosomal snRNA and protein components. Although the two papers examine different sets of cells and tissues, it is highly unlikely that both conclusions can be correct.

The minor spliceosome excises ≈1 in 300 introns from human pre-mRNAs.

Contrasting Data

König *et al.* (1) concluded that splicing of the minor class of introns is cytoplasmic on the basis of two main experimental approaches. First, they conducted *in situ* hybridization employing antisense locked nucleic acid (LNA) probes for U12 and U6atac snRNAs on embryonic and adult zebrafish tissues and on mouse 3T3 cells. In all cases, the two probes showed predominantly cytoplasmic signals. No Northern analyses were included to confirm the specificity of the LNA probes under the specific hybridization conditions used for cell staining. Furthermore, control anti-U2 and anti-U5 probes did not show selective nucleoplasmic staining with nucleolar exclusion, as is well documented for components of the major spliceosome (4, 16, 17).

Pessa *et al.* (2) performed *in situ* hybridization on embryonic and adult mouse tissue sections and on human cells, using full-length antisense cRNAs verified for specificity in Northern blots under identical conditions. They found that both digoxigenin-labeled and radioactively labeled probes for U11, U12, U4atac, and U6atac snRNAs all strongly label the nucleoplasm. Importantly, under the same conditions, probes specific for the U1, U2, and U6 snRNAs of the major spliceosome exhibit strong nuclear staining with nucleolar exclusion, as expected. Comparable results were obtained by fluorescence *in situ* hybridization on HeLa cells, showing nearly complete overlap of the labeling for U2 and U12, and for U11 and U4, snRNAs.

In their second approach, König *et al.* (1) used cellular fractionation, followed by RT-PCR analysis, to reveal the presence of unspliced minor introns in the cytoplasm. The extent of cross-contamination of the respective nuclear and cytoplasmic fractions was only partially addressed. Data for three genes with minor introns clearly showed spliced mRNA in the nuclear fraction. Although the authors attributed this to "the likely presence of material derived from outer nuclear structures in the nuclear fractions," these results actually support the idea that minor-intron splicing occurs in the nucleus, although perhaps not exclusively there. Indeed, when U6atac was inactivated with an antisense oligonucleotide, the minor-intron-spliced mRNAs in the nuclear fraction disappeared; it seems unlikely that all the spliced mRNA in the nuclear frac-

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See companion article on page 8655.

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tion in untreated cells respresented cytoplasmic contamination.

Pessa *et al.* (2) also conducted nuclear/cytoplasmic fractionation of HeLa cells and quantitated the relative abundance of snRNA and protein components of the minor spliceosome. Although U6 and U6atac are approximately equally nuclear and cytoplasmic, presumably as a result of leakage of RNA polymerase III transcripts during fractionation (18), the vast majorities of the U11, U12, and U4atac snRNAs are nuclear and mirror the distributions of U1, U2, and U4, respectively. Moreover, three additional proteins that are unique to the minor spliceosome—U11-59K, -35K, and -25K—are likewise predominantly in the nuclear fraction, as revealed by Western blotting with polyclonal antibody probes (13). Additional RNA and protein controls exhibit their established nuclear or cytoplasmic locations.

Previous Findings

The results of Pessa *et al.* (2) strongly support the large body of prior evidence arguing for the nuclear location of splicing by both the major and minor spliceosomes. The biogenesis of snRNPs involves snRNA transcription in the nucleus, followed by (with the exception of U6 and U6atac) export to the cytoplasm, where snRNAs bind the common Sm proteins with the aid of the SMN complex (19). Subsequently, assembled snRNPs are reimported into the nucleus. The minor snRNAs contain all of the signals required for nuclear import, including the m_3G cap and an Sm site bound by an apparently identical set of Sm proteins. Like the major snRNPs, the Sm core of the U11 snRNP is assembled by the SMN complex (20), and the minor U4atac and U6atac snRNPs possess protein compositions identical to the major U4 and U6 snRNPs (21). Thus, for the conclusions of König *et al.* (1) to be correct, an as yet uncharacterized mechanism would be required to selectively slow or prevent the reimport of newly assembled minor snRNPs.

Contrary to the conclusions of König *et al.* (1), much functional evidence indicates that splicing of minor-class introns—like that of major-class introns—occurs predominantly, if not exclusively, in the nucleus (8, 9). Minor-intron splicing can be reproduced *in vitro* in HeLa nuclear extracts under conditions nearly identical to those used for major-intron splicing (5) but does not occur in cytoplasmic extracts except upon addition of one or more nuclear components, as is also the case for major-intron splicing (22). Moreover, exon definition involves coordinate recognition of the major and minor types of introns (23), consistent with the fact that almost all minor-intron-containing pre-mRNAs also possess major-class introns. A mutant version of the U4 snRNA can substitute *in vivo* for U4atac in the minor spliceosome (24), which would not be possible if the two splicing events occurred exclusively in separate cellular compartments. Similarly, mutation of a minor 5′ splice site in a construct expressed *in vivo* efficiently activates an internal cryptic major-class intron by weakening interactions with U11 snRNA (25); if minor-intron splicing were cytoplasmic, why wouldn't this intron always be excised by the major spliceosome before the minor spliceosome encountered it? Moreover, cytoplasmic mRNAs containing unspliced minor introns would be susceptible to nonsense-mediated mRNA decay (26), unless some selective mechanism existed to prevent their translation.

In conclusion, although science occasionally leaps forward when established principles are questioned, in this case we believe that the paradigm of nuclear pre-mRNA splicing has survived the recent challenge. The experiments of Pessa *et al.* (2) provide strong evidence that casts doubt on the claim that the minor spliceosome is localized in a different cellular compartment from the one in which its highly studied counterpart—the major spliceosome—functions. At present, the notion that pre-mRNA splicing events may occasionally occur, or be completed, in the cytoplasm, although possible, remains unproven.

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