Fooling Mother Nature

Correcting defective genes at the mRNA level shows promise for the treatment of cystic fibrosis.

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When gene therapy was evolving as a therapeutic concept in the mid- to late 1980s, the initial vision was to correct hereditary disorders by replacing the abnormal gene, or at least its mutated segment, with the normal sequence. But despite major technological gains over the past 15 years, homologous recombination at the DNA level has not been achieved at a sufficiently high frequency for treating human genetic disorders. An alternative approach being pursued by several groups is to attack the problem at the RNA level. One strategy, known as "*trans*-splicing", involves transferring a DNA sequence coding for an RNA decoy that fools the cell into combining the decoy RNA sequence into the relevant messenger RNA (mRNA) as the cell processes the pre-mRNA into the mature message (Fig. 1). In this issue, Liu *et al.*[1] use *trans*-splicing to correct *in vitro* and *in vivo* models of the cAMP-dependent Cl^- channel abnormality of the airway epithelium that characterizes cystic fibrosis.

Cystic fibrosis is an autosomal recessive hereditary disorder characterized by lung and pancreatic disease and by infertility in males[2]. The disease manifests itself when mutations in the two parental cystic fibrosis transmembrane conductance regulator (CFTR) genes are sufficient to reduce CFTR levels and/or function in exocrine epithelia to <10% of normal[3]. Liu *et al.*[1] hypothesized that the consequences of the common ΔF508 mutation in exon 9 of the *CFTR* gene could be corrected in airway epithelia by transferring to cells a mini-gene coding for a decoy RNA molecule that mimics the 3′-part of intron 9, followed by the normal coding sequences for exons 9 through the remaining 3′-CFTR coding sequence. Using a recombinant adenovirus vector to transfer the DNA sequence of this decoy to human cystic fibrosis ΔF508 homozygote polarized bronchial epithelia *in vitro*, they restored the cAMP short-circuit current (a parameter that measures the Cl^- conductance of the CFTR molecule) to 16% of that exhibited by normal bronchial epithelia. Molecular analyses confirmed that the decoy functioned as predicted. This strategy also partially restored CFTR function in human cystic fibrosis bronchial xenografts in nude mice, an *in vivo* model that mimics the dysregulation of CFTR using airway epithelial cells derived from the airways of individuals with cystic fibrosis.

To design an effective decoy, it is necessary to understand the mechanisms of normal *cis*-splicing of RNA within the nucleus as the primary transcript of the gene is processed into mature mRNA (ref. 4). For most mammalian genes, one or more introns are spliced out of the primary transcript[5] (Fig. 1). Pre-mRNA splicing is mediated by the spliceosome, a large protein–RNA complex localized in the nucleolus and the nuclear membrane in structures called "speckles"[6]. Introns are removed in a two-step process. First, cleavage occurs at the 5′-splice site (splice donor), allowing the 5′-phosphate to immediately ligate to a 2′-OH at a branch-point located close to the 3′-splice site (splice acceptor) within the intron. Second, there is cleavage of the splice acceptor site with the simultaneous ligation of the 3′-OH of the upstream exon to the 5′-phosphate of the downstream exon, and the intron is released as a lariat.

The specificity of *cis*-RNA splicing—and the key to designing strategies for therapeutic *trans*-RNA splicing—is dictated by sequences in the pre-mRNA at the intron/exon junctions[7,8]. Each of these sites has consensus sequences that are recognized by spliceosomal components that mediate the splicing process. The crux of therapeutic *trans*-splicing for a hereditary disorder is to trick the various biological players within the spliceosome into recognizing the *trans*-splicing sequences coding for the normal sequence rather than the mutant sequence in the pre-mRNA transcript (Fig. 1).

In addition to correcting the consequences of a mutated gene, *trans*-RNA splicing has the added bonus that it corrects at the mRNA level, thereby taking advantage of the cell's elaborate mechanisms for regulating mRNA levels. In contrast, gene therapy as usually practiced uses constitutive promoters. Thus, *trans*-splicing has the possibility of correcting a hereditary defect in a fashion that mimics the normal variations of mRNA levels, a feature that may have advantages over conventional gene therapy.

The strategy of correcting mutations at the mRNA level is not new, nor is the specific strategy of *trans*-splicing. Ribozymes have been used for this purpose, as have antisense approaches[9]. *Trans*-splicing has been observed as a normal, albeit rare process in mammals, trypanosomes, nematodes, and plants[10,11]. Therapeutic *trans*-splicing, in which mRNAs are "reprogrammed" to achieve a therapeutic result such as correction of a mutation, has been demonstrated in principle in several models including reporter genes and CFTR or collagen minigenes[9,12–14].

While therapeutic *trans*-RNA splicing sounds terrific in theory, and clearly works to some extent in model systems, three significant hurdles will need to be overcome if it is to be successful in treating hereditary disorders: efficiency, fidelity, and persistence.

First, the *trans*-splice sequences must be designed to fool the spliceosome into preferentially recognizing the *trans*-splice sequences over the endogenous 3′-sequences that would normally be linked to the 5′-donor site. Although many hereditary disorders, including cystic fibrosis, only require 5–10% of normal mRNA transcripts to correct the abnormal phenotype[3], this is still a significant challenge for most gene transfer vectors. At present, the design of *trans*-splice sequences is probably not optimal. Efforts to improve their efficiency are partly based on knowledge of consensus splicing sequences and of sequences favoring RNA–RNA hybridization, but in reality, trial-and-error assessment of candidate constructs dominates current *trans*-splicing experimentation. The inefficiency of *trans*-splicing can be seen in the Liu *et al.* paper[1], with the data suggesting 10-fold less *trans*-splicing–corrected mRNA compared with endogenous mRNA.

Second, if *trans*-splicing is to reach the clinic, it is critical that the process be not only efficient, but foolproof—that is, without errors that could compound the consequences of the primary mutation or direct the synthesis of harmful proteins. The spliceosome consists of >50 proteins as well as small nuclear RNAs, and the splicing process involves RNA–protein, protein–protein, and RNA–RNA interactions. Splicing is normally carried out with great fidelity, and it is unknown to what extent the addition of therapeutic *trans*-splicing decoys will affect this finely tuned mechanism. For example, promiscuity of pre-mRNA *trans*-splicing has been observed in a model *in vitro* system designed to repair a proto-oncogene sequence[15]. Achieving high fidelity is critical for any strategy that is focused on an eventual clinical application, and proving that there are no mistakes will be a challenge.

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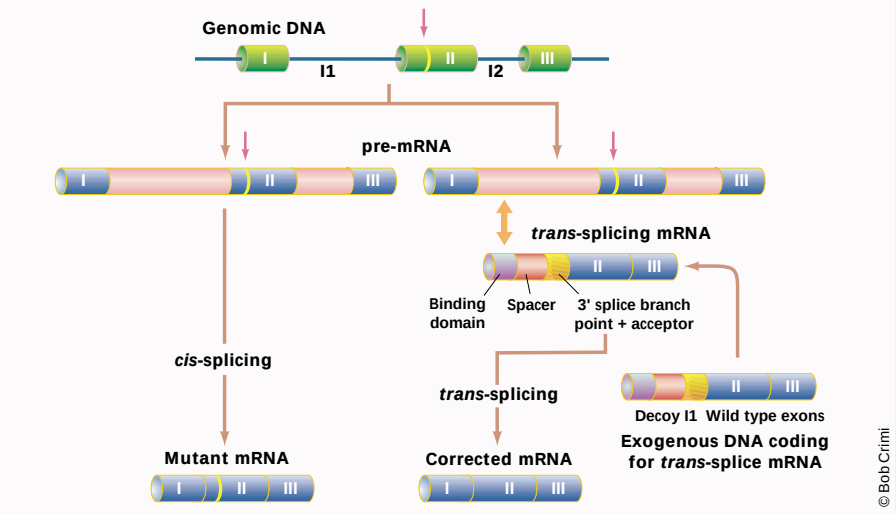

Figure 1. The use of *trans*-splicing to correct a hereditary disorder at the RNA level. Shown at the top is a 3-exon (I–III), 2-intron (I1, I2) gene with a mutation (arrow) in exon II. (Left) *Cis*-splicing. The primary transcript pre-mRNA normally undergoes *cis*-splicing to remove the two introns. The result is a 3-exon mature mRNA containing the mutant sequence. (Right) *Trans*-splicing. The *trans*-splice sequences are designed to include (5′ to 3′) a binding domain that hybridizes to the intron 5′ to the mutation site, a spacer, and a 3′-splice branchpoint and acceptor 5′ to exon II. The acceptor is spliced to the donor site 3′ to exon I, thus joining exon I of the endogenous mRNA to exons II and III of the *trans*-splice mRNA, which is transcribed following transfer to the cell of an exogenous DNA. The result is a mature mRNA in which the mutation has been corrected.

Third, the DNA that codes for the *trans*-splicing sequences must get to the nucleus and be expressed on a persistent basis. In the Liu *et al.* paper[1], this was accomplished using an adenovirus gene transfer vector. While adenovirus vectors are the gold standard for efficient gene transfer with subsequent high-level expression in most cells, host responses to the adenovirus vectors limit gene expression to only one to two weeks at these levels[16,17]. Furthermore, adenovirus vectors transfer their therapeutic gene cargo (e.g., the *trans*-splicing sequences) to the nucleus in an epi-chromosomal position, so the genetic correction will be diluted as the cells divide and only one daughter cell retains the epi-chromosomal DNA coding for the corrective *trans*-splicing sequence. Thus, for *trans*-splicing to be useful for human hereditary disorders, the chosen gene-transfer vectors will need to mediate incorporation of the *trans*-splicing sequences into the genome of the target cells. Despite more than a decade of work, this remains a huge hurdle for the gene therapist, although there is encouraging data regarding persistent expression following gene transfer with adeno-associated virus and lentivirus vectors[18,19].

Will *trans*-RNA splicing be useful to the gene therapist or will it languish like so many other clever molecular strategies as a laboratory model that is intellectually compelling but doomed to fail as a clinical strategy? Fooling Mother Nature is not easy, particularly when it comes to the central dogma of a gene begetting the protein it encodes—something cells probably defend with a vengeance. On the other hand, if the efficiency, fidelity, and persistence of *trans*-RNA splicing can be sufficiently improved, then the work by Liu *et al.* [1] is a step in the right direction toward making this interesting technology a reality.

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