Searching for the ideal partner

Fritz Eckstein

Sequence-specific inhibition of gene expression by the antisense methodology is a fast-growing area, with the first molecules currently undergoing clinical trials. This is not to say that all problems of this new technology are solved. One of them—the identification of the most suitable site on the mRNA for binding of the antisense construct—has been the subject of a recent commentary[1] and is now addressed in two papers[2,3] in this issue, each using a different type of antisense construct and a different approach to provide clues as to good targets.

The basic principle of the antisense method (and of ribozymes) relies on the prevention of translation by binding a complementary sequence to the target mRNA, which can be freely chosen. On this basis, numerous sites on the target mRNA could be amenable to binding by these molecules. However, experience shows that the efficiency of interference with gene expression can differ widely between sites. For example, of 100 oligodeoxynucleotides tested for the inhibition of herpes simplex virus growth, only six showed acceptable activity[4]. Similarly, out of 34 oligonucleotides directed against C-*raf* kinase, only one showed reduction of mRNA levels to more than 90%[5]. This is attributed to the secondary structures adopted by the mRNA, which leaves only a few sequence stretches accessible for annealing. The question that then arises is how to best predict or identify such sequences on the target RNA?

The Sczakiel laboratory has already shown that artificial antisense RNAs that anneal faster in vitro are more efficient inhibitors of the replication of HIV-1 in human cells than those annealing slowly. In their present paper, they develop a computer program for the design of antisense RNA constructs[2]. It is based on the selection of parameters from effective hepatitis B virus-directed antisense sequences, which include, inter alia, terminal unpaired nucleotides as potential nucleation sites. A hypothesis derived from these data was used to guide the design of antisense RNA molecules of approximately 100 nucleotides in length directed against HIV-1 replication in human cells. Indeed, the prediction was validated in that both the rate of annealing could be estimated and the fast annealing molecules were most effective. It should be borne in mind that for short antisense oligodeoxynucleotides, efficacy is dependent not only on annealing rates, but also thermodynamic stability. Thus, this program is at present not directly applicable to antisense oligodeoxynucleotides commonly in use, which are between 15 and 20 bases in length.

In the second paper, Ho et al.[3] further develop a concept, described by them earlier, using a set of four chimeric oligodeoxynucleotides composed of three randomized 2-deoxynucleotides in the center, and a fourth position fixed with one of the four normal nucleotides, flanked by 8 randomized 2-O-methylnucleotides at either site. These were annealed to a transcript of the angiotensin type-1 receptor to activate cleavage of the RNA by the addition of RNase H. Using primer extension, the site of cleavage can be identified. As the semirandomized oligodeoxynucleotide has no sequence preference, the whole transcript was probed for accessibility. The authors show that phosphorothioate oligodeoxynucleotides directed against sites identified by this approach do indeed give efficient inhibition of expression of the receptor not only in cell culture, but also in an animal model.

Other methods for the identification of oligonucleotide-accessible sites are also available. One is very similar to that described by Ho et al. in that it uses a completely randomized library of oligodeoxynucleotides in combination with RNase H[6]. A very intriguing approach has been described by the Southern laboratory[7], where an array of approximately 2000 oligodeoxynucleotides was prepared on a glass plate (see ref. 1 for commentary). The sequences were complementary to the first 122 nucleotides of rabbit β-globin mRNA. Essentially, only one of these oligonucleotides annealed efficiently to the target and showed good inhibition in a translation experiment. Elegant as this approach is, it is probably not practicable for most laboratories, unless such arrays become more widely available.

There is, of course, always the concern that site identification on transcripts will not reflect the situation in the cell, where other secondary structures might form or proteins might obscure such sites. The methods described in the two papers are encouraging in that the sites selected in vitro are also shown to be accessible in vivo. The database is too small at present to generalize this observation, but the fact that these methods study transcripts that have not been passed through denaturing gels—and are therefore in a relatively native state—may mean that their folding resembles that in the cell, although this argument would diminish a role for associated proteins in folding in vivo.

Ribozymes also have to anneal to the target RNA and thus face the same problem concerning target site selection. Several approaches have been adopted to solve the problem here. Lieber and Strauss randomized the arms of a GUC-cleaving hammerhead ribozyme, giving rise to 10^9 sequences, to screen sites for cleavage of the human growth hormone RNA in cytoplasmic extracts[8]. They identified five ribozyme cleavage sites that were all situated in large loops or single-stranded regions predicted by the MFold program.

Such agreement is, however, not general. Successful site selection for a group I intron ribozyme has been achieved through the use of a GN_5 (where N is any nucleotide) library[9]. In addition, our laboratory has used a very similar approach to that of Ho et al., but with a completely randomized 10-mer oligodeoxynucleotide, in order to find suitable sites on a human acetylcholinesterase transcript for cleavage by hammerhead ribozymes[10]. We identified five such sites that had not been detected by any other method.

Understanding of the annealing process between short oligonucleotides and structured RNAs is still in its infancy and it would be highly beneficial if we could attain predictive insight into this process, as the approach of Patzel et al.[2] seems to provide for the annealing of longer antisense RNAs. Until then, either trial and error or the use of randomized oligomers remains the answer.

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