Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 10898–10900, December 1993

Commentary

RNA enzyme-directed gene therapy

Sidney Altman

Department of Biology, Yale University, New Haven, CT 06520

Inhibition of the replication of human immunodeficiency virus (HIV) through the inactivation of viral RNA with RNA enzymes (ribozymes) has been achieved in tissue culture by several groups (1–3). Three papers that highlight the continuing promise of such RNA enzyme-directed gene inactivation methods have appeared recently in this journal (4–6). Yu *et al.* (4), using strong polymerase III promoters to control expression of an RNA enzyme, have shown that the production of an important marker for the replication of HIV in tissue culture cells can be decreased by >90% in comparison with control transfected cells that have no added RNA enzyme. These authors have also shown that several strains of HIV can be inhibited during transient infections in tissue culture with one RNA enzyme construct that attacks a regulatory sequence common to all the viral strains, not an insignificant result in the struggle with a virus that has a high mutation rate. Cantor *et al.* (5) have produced similar results for inhibition of products encoded by bovine leukemia virus (BLV), a virus that resembles human T-cell leukemia viruses (HTLV-I and -II), in infected bat lung cells. In this issue, Ohkawa *et al.* (6) demonstrate that several ribozymes, each directed against a different target in the HIV genome and acting independently in a "shotgun" manner, markedly increase the efficiency of HIV RNA cleavage *in vitro*, a result that holds promise for similarly efficient ribozyme function *in vivo*.

The strategies used in these papers for destruction of specific RNAs, and those for most methods that employ RNA enzymes, are very similar. They are based on the observation that the structural domain that is responsible for self-cleavage in certain RNA enzymes can be divided into two entities represented in two separate oligonucleotides: one oligonucleotide can be regarded as the catalytic surface; another oligonucleotide contains the site of cleavage and is, therefore, regarded as the substrate (7–10). These two oligonucleotides have primary sequences that enable them to hydrogen bond with each other to form the structural domain (which may contain other, non-hydrogen-bonded interactions as well) that allows the cleavage event to take place (Fig. 1). Accordingly, a particular sequence in the target RNA to be cleaved in cells in tissue culture is designated as the substrate for the enzyme, which is custom designed to form the appropriate complex with the substrate. The sequence encoding the enzyme is delivered to the cells on a plasmid and is bounded by other sequences required for the initiation and termination of transcription. Thus, a synthetic gene is created to deliver and express the enzyme inside cells.

The catalytic domains of two RNA enzymes derived from pathogenic agents found in plants have been used in the experiments reported recently. Yu *et al.* (4) used the hairpin motif found in tobacco ringspot virus satellite RNA (Fig. 1*A*; ref. 9) to attack the 5′ "leader" regulatory sequence encoded by the HIV genome, whereas Cantor *et al.* (5) used the hammerhead motif found in many viroid RNAs (Fig. 1*B*; refs. 7 and 8) to cleave the mRNA coding for the *rex* and *tax* regulatory gene products of BLV. Similarly, Ohkawa *et al.* (6) used multiple hammerheads with different flanking sequences to attack simultaneously several targets in HIV RNA. In all cases, cleavage of the target RNA was demonstrated first *in vitro* with a custom-designed RNA enzyme. Subsequently, the production of various viral markers, HIV and BLV coat protein antigen (p24) and RNA, HIV *tat* gene product and BLV reverse transcriptase activity and *tax* gene product, were measured as an indication of productive viral infection in tissue culture. Inhibition of HIV p24 was as high as 95%, depending on the HIV strain used or the specific RNA sequence that was targeted (ref. 4; K. Taira, personal communication). Inhibition of BLV p24 was about 65% and of reverse transcriptase activity and *tax* gene product, >90% (5). Inhibition of HIV *tat* gene product was about 80% (4). In all cases, reduction of the intact viral RNA target was at least 70% as measured by RNA PCR methods. These data represent improvements in the target range and levels of inhibition of viral product production compared with previous reports (1–3). Success was undoubtedly due, in part, to the choice of targets—regulatory rather than structural genes. "Control" RNA enzyme sequences that have been engineered to lack catalytic function show no such dramatic effects. It should be noted that control of transcript size and amount is no trivial task in the design of the expression vectors: strong polymerase III promoters such as those associated with tRNA genes appear to be the most uniformly effective in this regard, but promoters more easily subject to regulatory control should prove very useful in some instances.

Three questions arise concerning these experiments. (*i*) Are the observed effects due to "conventional" antisense mechanisms? (*ii*) What advantages do these methods have over antisense or "triplex" methods (12, 13)? (*iii*) Will these methods work *in vivo*, as antisense methods seem to do in some cases (14–17)?

All the authors carried out a number of control experiments *in vitro* that demonstrate that cleavage of the target RNA was due to the RNA enzyme. These experiments involved the use of RNA enzymes designed to hybridize to sequences other than the target sequence or RNA enzymes mutated in regions known to be essential for catalytic activity. A complete characterization of the inactivation phenomenon in tissue culture cells should have included similar experiments. Yu *et al.* (4) do state that an inactivated RNA enzyme sequence did not have the inhibitory effects they observed with intact RNA enzyme, while Cantor *et al.* (5) utilized a control RNA enzyme with the catalytic domain intact but with "nonsense" flanking sequences to prevent hybridization with substrate. In the latter case, one can argue that any inhibitory effect of the intact RNA enzyme could have been an antisense effect but the authors, through the utilization of RNA PCR, show that intact target RNA, which contains sequences on either side of the presumed site of cleavage, represents only about 25% of that measured for RNA that contained only the sequence upstream from the site of presumed cleavage by the RNA enzyme. A more direct approach, of course, is an identification of the intact cleavage products of the target RNA and kinetic measurements of their intracellular concentration. Unfortunately, fragments of RNA are degraded extremely rapidly in mammalian cells, so only indirect measurements of the survival of the sequence

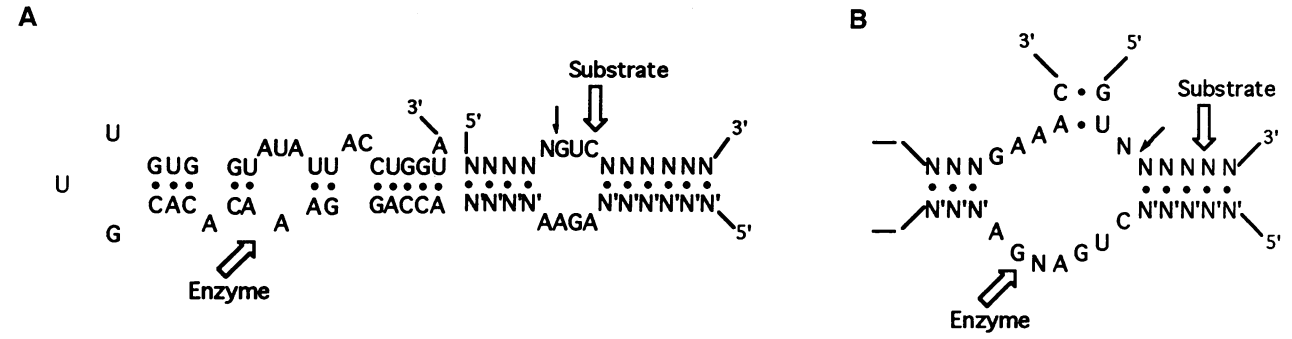

FIG. 1. Schematic diagrams of the catalytic domains of two self-cleaving RNA enzymes showing how these domains can be separated into enzyme and substrate segments. The large open arrows indicate the catalytic RNA and the target substrate. The small solid arrows indicate the sites of cleavage in the substrate sequences. Nucleotides that do not have a unique identity in order for cleavage activity to be preserved are indicated as N and their Watson–Crick base-pair partners are shown as N'. (*A*) Hairpin derived from tobacco ringspot virus satellite RNA. Redrawn from ref. 9. (*B*) Hammerhead derived from plant viroid RNAs. Redrawn from ref. 11.

containing the site of cleavage, or fragments that contain the upstream or downstream sequences, are possible. Similar measurements had been made in earlier experiments in which less efficient gene inactivation had been reported with the hammerhead RNA enzyme (1) and an external guide sequence in conjunction with RNase P (18).

Why is so much effort being devoted to developing these RNA enzyme-directed techniques when it has already been shown that antisense and triplex methods have at least a reasonable hope of success for use in gene therapy? What advantages do RNA enzyme-directed methods of gene inactivation have over "conventional" antisense and triplex methods? Certainly, in every case mentioned here, there is the possibility of a conventional antisense effect, since one RNA sequence must form a double-stranded structure with another at some point in the formation of an enzyme–substrate complex. Thus, RNA enzyme cleavage events are an addition to whatever level of inhibition due to an antisense effect already exists. Furthermore, the RNA enzyme surface, in principle, is capable of releasing from the complex after cleavage has occurred and recycling (turnover). Finally, it is likely that the RNA enzyme (or guide sequence in the case of RNase P) is more stable inside cells than is a single-stranded antisense RNA, since the RNA enzyme rapidly becomes part of a structure with complex higher-order structure that is relatively resistant to intracellular nucleases.

The chief disadvantage to the use of RNA enzyme-directed therapies lies in the generally larger sizes of the RNAs that are needed compared with antisense RNAs if the former RNAs are delivered to cells exogenously. Of course, if various delivery vehicles such as liposomes are used, this difficulty is marginalized. Because of the requirements for specific higher-order structures or the necessity for specific chemical groups (e.g., 2'-hydroxyl, unmodified phosphodiester bonds) to appear at certain positions in a sequence that has catalytic potential, it may not be possible to substitute RNA enzymes so freely with various chemical groups (2'-*O*-methyl, phosphorothioates, methylphosphonates, etc.) to provide chemical stability to exogenously administered RNA as is the case with antisense RNAs (11). However, it is equally likely that many potential applications of RNA-targeted gene therapy will involve delivery of the RNA via one or a few doses of an expression vector rather than by topical or intravenous delivery methods that necessitate many doses of high concentrations of RNA.

The armory of RNA enzymes contains several weapons: the hairpin (9) and the hammerhead (7, 8), derived from plant pathogens, and the axehead, derived from hepatitis δ RNA (19), which has a natural tropism for hepatocytes, the group I intron (9), and RNase P (18), an essential component of all cells. The efficacy of all these elements has been proven *in vitro*. Their usefulness for experiments with prokaryotic mRNAs in prokaryotes is in doubt (Ying Li, Cecilia Guerrier-Takada and S.A., unpublished experiments; K. Taira, personal communication), but their promise for use in eukaryotes seems brighter, possibly because translation is more clearly uncoupled from transcription in eukaryotes. In at least one case, it has been reported that an RNA enzyme has been modified by directed evolution *in vitro* to cleave efficiently a bacteriophage DNA (20). Indeed, such experiments have also led to the synthesis of very efficient guide sequences for RNase P from HeLa cells (Yan Yuan and S.A., unpublished experiments), but these new constructs have not yet been tested in tissue culture. Nevertheless, directed evolution offers a means of selecting highly efficient RNA enzymes or guide sequences that may enable this methodology to become the method of choice for RNA-targeted gene therapy.

The results reported in the three recent papers in the Proceedings are encouraging, but much has yet to be learned about the intracellular events that underly the phenomenon of RNA enzyme-directed inhibition of mammalian virus production. More information, for example, is needed about the intracellular location, stability, and amount of transcript from the synthetic genes, as well as the turnover of these products during catalysis, if there is to be a rational basis for pharmacology. Finally, the efficacy of these agents must be evaluated in animals.

1. Sarver, N., Cantin, E. M., Chang, P. S., Zaia, J. A., Ladne, P. A., Stephens, D. A. & Rossi, J. J. (1990) *Science* **247**, 1222–1225.
2. Dropulic, B., Lin, N. H., Martin, M. A. & Jeang, K.-T. (1992) *J. Virol.* **66**, 1432–1441.
3. Weerasinghe, M., Liem, S. E., Asad, S., Read, S. E. & Joshi, S. (1991) *J. Virol.* **65**, 5531–5534.
4. Yu, M., Ojwang, J., Yamada, O., Hampel, A., Rapapport, J., Looney, D. & Wong-Staal, F. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 6340–6344.
5. Cantor, G. H., McElwain, T. F., Birkebak, T. A. & Palmer, G. H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10932–10936.
6. Ohkawa, J., Yuyama, N., Takebe, Y., Nishikawa, S. & Taira, K. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11302–11306.
7. Forster, A. C. & Symons, R. H. (1987) *Cell* **50**, 9–16.
8. Uhlenbeck, O. (1987) *Nature (London)* **328**, 596–600.
9. Hampel, A., Tritz, R., Hicks, M. & Cruz, P. (1990) *Nucleic Acids Res.* **18**, 299–304.
10. Zaug, A., Grosshans, C. & Cech, T. (1988) *Biochemistry* **27**, 8924–8931.
11. Symons, R. H. (1990) *Semin. Virol.* **1**, 117–126.
12. Wickstrom, E., ed. (1991) *Prospects for Antisense Nucleic Acid Therapy of Cancer and AIDS* (Wiley, New York).
13. Hélène, C. & Toulmé, J.-J. (1990) *Biochim. Biophys. Acta* **1049**, 99–125.

14. Han, L., Yun, J. S. & Wagner, T. E. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 4313–4317.
15. Ratajczak, M., Kant, J. A., Luger, S. M., Hijiya, N., Zhang, J., Zon, G. & Gewirtz, A. M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 11823–11827.
16. Kitajima, I., Shinohara, T., Bilakovics, J., Brown, D. A., Xu, X. & Nerenberg, M. (1992) *Science* **258,** 1792–1795.
17. Trojan, J., Johnson, T. R., Rudin, S. D., Ilan, J., Tykocinski, M. L. & Ilan, J. (1993) *Science* **259,** 94–97.
18. Yuan, Y., Hwang, E.-S. & Altman, S. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8006–8010.
19. Branch, A. & Robertson, H. D. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 10163–10167.
20. Beaudry, A. & Joyce, G. (1992) *Science* **257,** 635–641.