3958–3965 Nucleic Acids Research, 1994, Vol. 22, No. 19

A three-nucleotide helix I is sufficient for full activity of a hammerhead ribozyme: advantages of an asymmetric design

Martin Tabler*, Matthias Homann[1], Sergia Tzortzakaki and Georg Sczakiel[1]
Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology Hellas, PO Box 1527, GR-71110 Heraklion, Crete, Greece and [1]Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, D-69120 Heidelberg, Germany

Received June 16, 1994; Revised and Accepted August 18, 1994

ABSTRACT

***Trans*-cleaving hammerhead ribozymes with long target-specific antisense sequences flanking the catalytic domain share some features with conventional antisense RNA and are therefore termed 'catalytic antisense RNAs'. Sequences 5′ to the catalytic domain form helix I and sequences 3′ to it form helix III when complexed with the target RNA. A catalytic antisense RNA of more than 400 nucleotides, and specific for the human immunodeficiency virus type 1 (HIV-1), was systematically truncated within the arm that constituted originally a helix I of 128 base pairs. The resulting ribozymes formed helices I of 13, 8, 5, 3, 2, 1 and 0 nucleotides, respectively, and a helix III of about 280 nucleotides. When their *in vitro* cleavage activity was compared with the original catalytic antisense RNA, it was found that a helix I of as little as three nucleotides was sufficient for full endonucleolytic activity. The catalytically active constructs inhibited HIV-1 replication about four-fold more effectively than the inactive ones when tested in human cells. A conventional hammerhead ribozyme having helices of just 8 nucleotides on either side failed to cleave the target RNA *in vitro* when tested under the conditions for catalytic antisense RNA. Cleavage activity could only be detected after heat-treatment of the ribozyme substrate mixture which indicates that hammerhead ribozymes with short arms do not associate as efficiently to the target RNA as catalytic antisense RNA. The requirement of just a three-nucleotide helix I allows simple PCR-based generation strategies for asymmetric hammerhead ribozymes. Advantages of an asymmetric design will be discussed.**

INTRODUCTION

Certain satellites of some plant viruses, two viroids, and an RNA transcript of newt (rev. in 1) can assume a 'hammerhead structure' (2,3) which results in self-cleavage of the RNA at a defined position. The principle of the reaction could be exploited to generate RNA enzymes (ribozymes) that cleave a substrate RNA in *trans* (4,5). For that purpose, sequences complementary to the target RNA are attached to the actual catalytic domain of a hammerhead RNA. The flanking region found in the ribozyme RNA 5′ to the catalytic domain forms the helix I in the ribozyme/substrate complex and the 3′ terminal region forms helix III according to the conventional numbering system for hammerhead RNAs established by Hertel *et al.* (6).

A number of kinetic studies has shown that hammerhead ribozymes act as true catalysts *in vitro* and cause multiple turnover in substrate cleavage (4, 7–12). A pre-requisite for catalytic turnover is a repetitive cycle of binding, cleavage and subsequent dissociation. Hammerhead ribozymes used commonly *in vitro* for kinetic studies have flanking regions short enough to allow both, binding to the substrate RNA, as well as dissociation from it. This is achieved by helices I and III of about eight nucleotides on either side. Such ribozymes will result in catalytic turnover when cleaving an artificial short target RNA *in vitro*, but have the disadvantage that they may not compete for binding with the secondary structure of a long target RNA as found *in vivo*. For example, Cotten *et al.* (13) have reported that an U7 RNA-directed ribozyme required a 1000-fold molar excess versus the target RNA for complete inhibition of U7-dependent processing *in vivo* while a longer antisense RNA was effective already at a sixfold molar excess, suggesting that binding and not cleavage is the rate-limiting step in living cells. Similarly, Cameron and Jennings (14) described the requirements for a 1000-fold molar excess of a hammerhead ribozyme to inhibit chloramphenicol acetyltransferase in monkey cells and also L'Hullier *et al.* (15) used a 1000-fold molar excess to inhibit α-lactalbumin in mouse cells.

One way to improve binding is to increase the length of the antisense arms of the hammerhead ribozyme up to hundred or more nucleotides on either side of the catalytic domain (16–18). Since those hammerhead ribozymes can be equally considered

*To whom correspondence should be addressed

as antisense RNAs, we call them 'catalytic antisense RNAs'. They can unravel the secondary structure of the target RNA and they have proven to be still capable of cleaving the substrate RNA in *trans*. However, catalytic antisense RNAs are assumed to fail in dissociation *in vitro* and *in vivo*, so that they cleave their RNA target stoichiometrically rather than catalytically. Despite the assumed lack of multiple turnover in living cells, we could recently show that a catalytic antisense RNA performs better in suppressing the target RNA in human cells than conventional antisense RNA (17). Also other recent examples demonstrate that ribozymes with long antisense flanks are useful in gene suppression (18–20).

Since catalytic antisense RNAs had given already promising results when applied *in vivo*, we intended to study their design more systematically. Some of our mutational studies with catalytic antisense RNAs had shown that mismatches in helix I did not necessarily interfere with catalytic activity (Zoumadakis and Tabler, unpublished). This observation prompted us to test whether catalytic antisense RNAs with only one antisense flank could work similar to the symmetric onces, especially since an asymmetric design has a number of advantages as will be outlined under Discussion. As a model system we chose the HIV-1-directed catalytic antisense RNA 2as-Rz12 which had been well characterized by *in vitro* studies and inhibition assays in human cells (17).

MATERIALS AND METHODS

General techniques were carried out according to Sambrook *et al.* (21).

Construction of plasmids

In order to generate 2as-Rz12-derived asymmetric ribozymes with shortened helix I, several polymerase chain reactions (PCR) were carried out. Initially, two reactions were performed, each in a reaction volume of 100 µl containing: 20 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl, 0.05 % Tween 20, bovine serum albumin (molecular biology grade) 0.1 mg/ml, dATP, dCTP, dGTP, dTTP, each 0.1 mM, 100 pg of pBS29-Rz12 DNA (17, comp. Figure 2A), 20 pmole of the 'T7 primer' d(TAATACGACTCACTATAGGG) and 20 pmole of either the oligonucleotide Rz12/5: d(AAAAGCTTGTCCTGATGAG) or Rz12/3: d(GCAAGCTTAGTCCTGATGAG), respectively and 5 units of *Taq* DNA polymerase (Minotech, Heraklion/ Crete, Greece). The reaction was performed in a DNA Thermal Cycler (Perkin Elmer Cetus version 2.1) according to the reaction scheme: 7 minutes at 94°C, four cycles consisting of 30 seconds at 94°C, 30 seconds at 15°C, 2 minutes at 72°C. 30 cycles followed consisting of 30 seconds at 94°C, 30 seconds at 45°C, 2 minutes at 72°C, before the reaction mixtures were incubated for a final 7 minutes at 72°C. The PCR products were cleaved with *Sac*I and *Hin*dIII and ligated into the vector pT3T7*lac* (Boehringer Mannheim) at the same sites. The resulting plasmids were called pBS-Rz12/3 and pBS-Rz12/5, respectively. Relevant sequences were confirmed by sequencing. The plasmid pBS-Rz12/3 was digested with *Hin*dIII and treated with nuclease S1 to trim the protruding ends. Re-ligation generated plasmid pBS-Rz12/3H. Five additional PCR reactions were performed, using the same reaction conditions, except 100 pg of pBS-Rz12/3 instead of pBS-Rz12 and instead of Rz12/5 or Rz12/3, the DNA oligonucleotides Rz12/0: d(CCAAGCTTAAAACTGATGAGG) or Rz12/1: d(CCAAGCTTAAACCTGATGAG) or Rz12/2: d(CCAAGCTTAATCCTGATGAG) or Rz12/8: d(GGAAGCTTATTTGTCCTGATGAGG) or Rz12/13: d(GGAAGCTTAGTATTTGTCCTGATGAGG), respectively. The resulting PCR products were also cleaved with *Sac*I and *Hin*dIII and cloned into pT3T7*lac*, yielding the plasmids pBS-Rz12/0, pBS-Rz12/1, pBS-Rz12/2, pBS-Rz12/8 and pBS-Rz12/13, respectively. The relevant sequence was confirmed by sequencing. For generation of plasmid pBS-Rz12/88 the two DNA oligonucleotides SX1: d(CAAGGCTGTTTCGGCC) and SX2: d(TCGAGGCCGAAACAGCCTTGAGCT) where phosphorylated at the 5′-terminus with the aid of T4 kinase, annealed to each other and ligated into the plasmid pBS-Rz12/8 that had been cleaved with *Sac*I and *Xho*I. The correct construction was confirmed by sequencing.

The generation of plasmids pBS-UCU and pαY195 and the related plasmid pαY195inac is outlined in detail in Figure 5.

In vitro transcription of RNA

Radioactively labelled substrate RNA was obtained by transcription with T7 RNA polymerase from *Hin*dIII-linearized plasmid pBS29-CX as described earlier (17) or from *Not*I-linearized plasmid pRC-SR6 (22). For generation of ribozyme RNA, the plasmids pBS-Rz12 and pBS-Rz12/88 were linearized with *Sac*I followed by trimming with T4 DNA polymerase, all other ribozyme-encoding plasmids with *Eco*RI. All ribozymes were synthesized with T3 RNA polymerase as described (17).

Ribozyme assays

For assaying the cleavage activity, ribozyme RNAs were used in a final concentration of about 200 nM and mixed with radioactively labeled substrate RNA (final concentration about 2 nM) in a reaction volume of 5 µl, containing 20 mM $MgCl_2$ and 50 mM Tris–HCl pH 8.0. The reaction was stopped by adding EDTA to a final concentration of 25 mM, followed by addition of 1 µg tRNA and ethanol precipitation. The reaction products were separated on a 5% polyacrylamide gel containing 8M urea as described by Tsagris *et al.* (23). For separation on a native gel, the urea was omitted and samples were loaded without dissolving them in formamide and without prior boiling. After drying the gels, RNAs were visualized by autoradiography.

HIV-1 inhibition studies

For measurements of the effects of catalytic antisense RNAs on HIV-1 replication, *in vitro* synthesized RNAs at amounts of 160 ng per well and infectious proviral HIV-1 DNA pNL4-3 (24) at 40 ng, respectively, were co-transfected into SW480 cells grown semi-confluent in 48 well plates by calcium phosphate co-precipitation essentially as described recently (25). One day after transfection, 1×10^5 MT-4 cells were added and the final volume was adjusted to 1 ml with RPMI 1640 medium. Virus replication was measured 5 days after transfection with dilutions of cell-free culture supernatants by a commercial HIV-1 antigen ELISA (Organon, Holland).

RESULTS

Truncation of helix I of the catalytic antisense RNA 2as-Rz12

The parental antisense RNA 2as is directed against the 5′ leader/gag region of HIV-1 (compare Figure 1). It had been found previously to inhibit HIV-1 replication in human cells (26–28). Into this RNA, which is complementary between positions +222 and +633 of the HIV-1 genome, we had incorporated a catalytic domain of the hammerhead ribozymes such that the target RNA

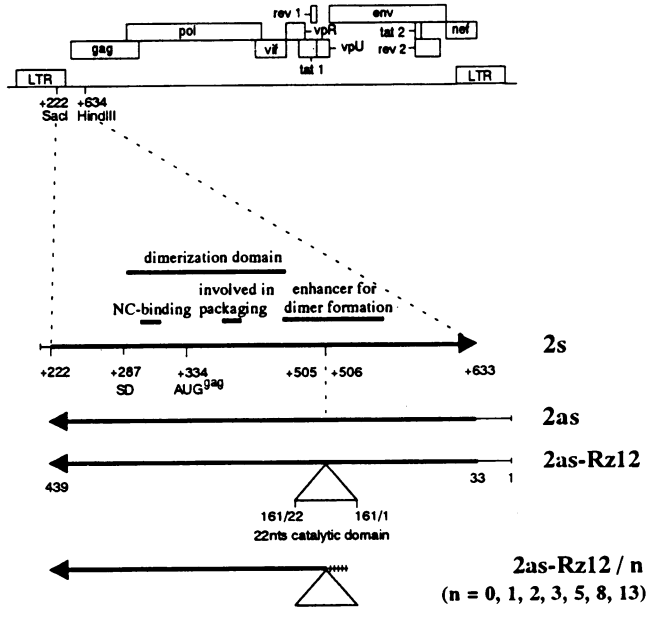

Figure 1. Schematic maps of the HIV-1 target sequence and the catalytic antisense RNAs. The top shows functional elements of the HIV-1 genome and the solid bar below details the region between nts. 222–633 which correspond to the sense RNA 2s that was used as target RNA. Functional elements within this region are also indicated. The antisense RNA 2as is complementary to RNA 2s. The catalytic antisense RNA 2as-Rz12 contains an incorporated catalytic domain of 22 nts, which causes cleavage of the target RNA between nts. 505 and 506. The bar at the bottom shows the catalytic antisense RNAs with truncated helices I.

is cleaved between nucleotides +505 and +506 (17, compare Figure 2A). When pairing to the target RNA, the resulting catalytic antisense RNA 2as-Rz12 can form a helix I of 128 base pairs and a helix III of 278 base pairs, respectively (Figure 2A). When assayed in human cells, this catalytic antisense RNA was four- to seven-fold more effective in inhibiting HIV-1 replication compared to the non-catalytic antisense RNA. A 2as-Rz12-derived ribozyme inactive in cleavage could only inhibit to levels comparable to the parental antisense RNA (17).

For the current study, a series of plasmids was constructed (pBS-Rz12/13, pBS-Rz12/8, pBS-Rz12/5, pBS-Rz12/3H, pBS-Rz12/2, pBS-Rz12/1, pBS-Rz12/0) that allow *in vitro* synthesis of catalytic antisense RNAs (2as-Rz12/13, 2as-Rz12/8, 2as-Rz12/5, 2as-Rz12/3H, 2as-Rz12/2, 2as-Rz12/1, 2asRz12/0, compare Figure 2B–H). All RNAs contain the identical catalytic domain and can form a helix III of 283 base pairs. However, the 128 base antisense sequence flanking the catalytic domain at the 5′ side — the helix I-forming region — had been truncated to 13, 8, 5, 3, 2, 1 and 0 nucleotides, respectively. The truncation of helix I does not influence the general binding properties of the catalytic antisense RNA since the remaining large helix III still prevents melting under the experimental conditions *in vitro* (and most likely *in vivo*). Consequently, neither dissociation of the ribozyme nor catalytic turnover could be expected. In order to compare the set of 5′-truncated catalytic antisense RNAs with a conventional symmetric hammerhead ribozyme with short antisense flanks, the plasmid pBS-Rz12/88 was constructed. It allowed synthesis of the ribozyme 2as-Rz12/88 that forms helices I and III of 8 nucleotides each, cleaving the substrate RNA at the same site as 2as-Rz12 (compare Figure 2I). In respect to

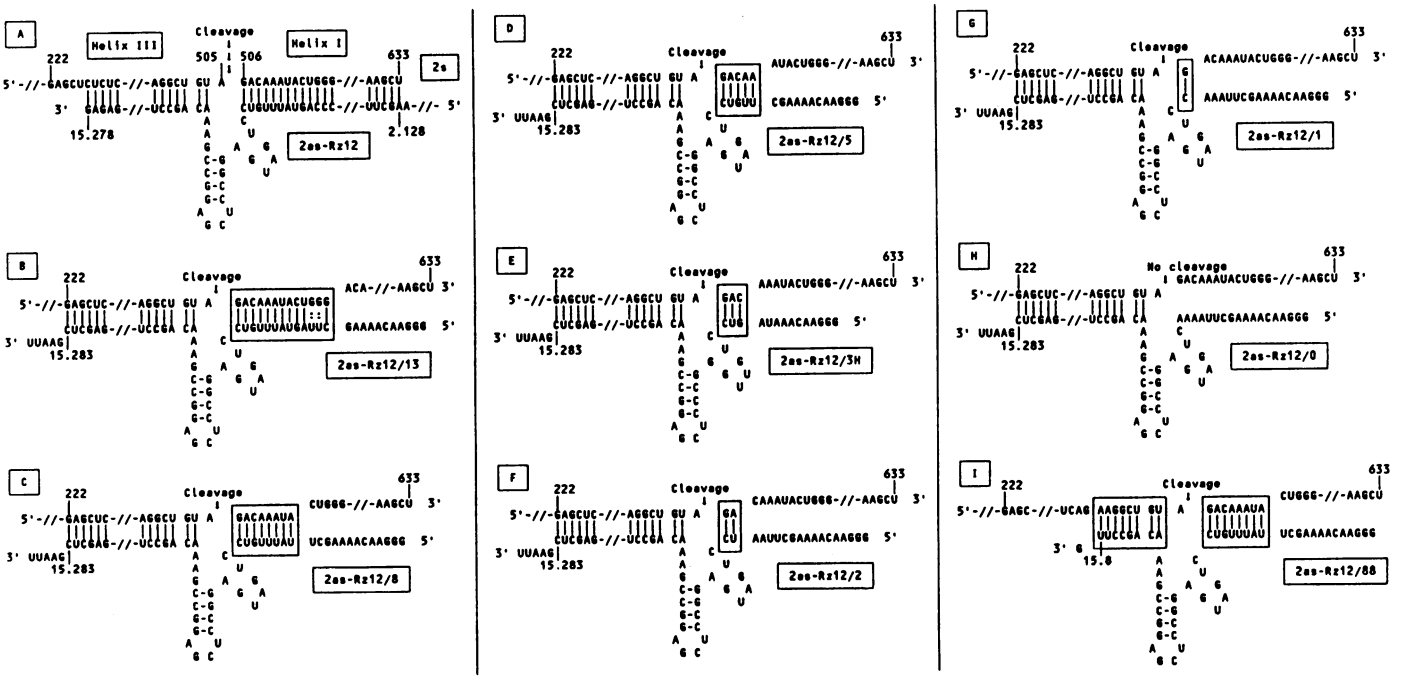

Figure 2. Depiction of the different ribozyme RNAs used. (**A**) Hammerhead complex of catalytic antisense RNA 2as-Rz12 (bottom) with the HIV-1-derived substrate RNA 2s (top); numbers given on top (222 and 633) correspond to the HIV-1 sequence, numbers given for the catalytic RNA (15.278 and 2.128) correspond to the numbering system for hammerhead RNAs according to Hertel *et al.* (6), and reflect the length of helices I and III of 128 and 278 nucleotides, respectively. (**B–I**) Hammerhead complex of the different catalytic RNAs as indicated; helix I is boxed; RNA 2as-Rz12/0 cannot form a helix I.

the length of the antisense flanks, such a design corresponds to ribozymes with catalytic turnover as introduced by Haseloff and Gerlach (5) which have been most extensively studied so far.

Cleavage rates of 5′-terminally truncated catalytic antisense RNAs

The association rate constant of all ribozyme RNAs, except transcript 2as-Rz12/88, to its target were found to be in the same range as that of 2as-Rz12 which had been determined earlier to be 1.5×10^5 $M^{-1}s^{-1}$ (17). All ribozyme assays were carried out under conditions for single turnover. The ribozyme RNAs were used in concentrations of 200 nM which was an about 100-fold molar excess versus the radioactively labelled substrate RNA. Under these reaction conditions, the substrate RNA becomes quickly associated with ribozyme RNA at similar rates for all constructs. For example, at k_{ass} 1.5×10 $M^{-1}s^{-1}$ and 200 nM of the rate limiting RNA concentration, the half life for association was in the order of half a minute. Figure 3 shows the results of the cleavage assay carried out with the set of 5′-truncated 2as-Rz12-derived RNAs. If the helix I-forming region of the catalytic RNA was three nucleotides or longer, an uniform cleavage efficiency could be detected at any reaction time. For example, the RNA 2as-Rz12/3H which can form a helix I of only three nucleotides (Figure 2E) is equally active as 2as-Rz12 that forms a helix I of 128 base pairs (compare Figure 3A,B, lanes 5 and 9). Also all other RNAs with helices I of 5−13 nucleotides in length had the same cleavage efficiency (for rate constants see Table I; compare also Figure 3A,B, lanes 6−8). The ribozyme 2as-Rz12/2, having a two base helix I-forming region (Figure 2F) delivered some cleavage products that became detectable after a reaction time of 60 minutes (Figure 3B, lane 4), the cleavage rate was, however, about 15-fold reduced (Table I). Traces of cleaved substrate RNA could even be detected on the original autoradiographs for ribozyme 2as-Rz12/1 (Figure 2G) and after longer reaction times. The ribozyme 2as-Rz12/0 (Figure 2H), which entirely lacks a matching helix I-forming region did not generate specific cleavage products.

When the ribozyme 2as-Rz12/88 (which has flanking regions of 8 nucleotides on either side, Figure 2I) was tested under the same reaction conditions as the 5′-terminally truncated 2as-Rz-derived RNAs, it did not generate any specific cleavage (Figure 3A,B, lane 10). In order to ensure that the reaction temperature was below the melting temperature for duplex RNA, the reaction was repeated at 20°C, but again neither cleavage was observed (data not shown). However, if the mixture of the substrate RNA and the ribozyme RNA 2as-Rz12/88 was heated for 5 minutes at 60°C, followed by fast chilling in ice and subsequent incubation at 37°C for 60 minutes, it became evident that this ribozyme can cleave the substrate as well as the other 2as-Rz12 derivatives (Figure 3B, lane 11). This finding indicates that without prior heat treatment, the ribozyme 2as-Rz12/88 (with an '8 plus 8 design') could not associate properly with its target RNA 2as.

When the reaction products of a ribozyme reaction were separated on a native polyacrylamide gel (Figure 4), it was observed that asymmetric catalytic RNAs released the 3′-terminal cleavage product, whereas the parental catalytic antisense RNA 2as-Rz12 remained bound to the cleaved substrate RNA. Further,

Table I. Cleavage rate constants of different catalytic antisense RNAs

RNA	Helix I	k_{cleav}[a]
	base pairs	s^{-1}
2as-Rz12/1	1	6.0×10^{-6}
2as-Rz12/2	2	1.1×10^{-5}
2as-Rz12/3	3	1.7×10^{-4}
2as-Rz12/5	5	1.7×10^{-4}
2as-Rz12	128	2.1×10^{-4}

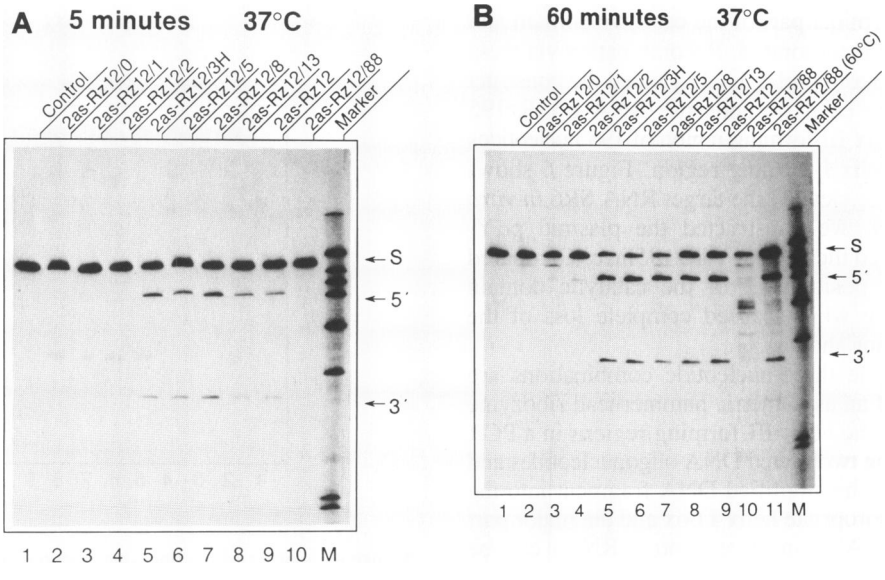

Figure 3. Cleavage analysis of a set of 2as-derived catalytic antisense RNAs. (**A**) Radiolabelled target RNA 2s was incubated for 5 minutes at 37°C alone (lane 1) or with the ribozyme RNAs indicated on top of each lane and separated on a denaturing polyacrylamide gel. Lane M contains radiolabelled *Hin*fI fragments of plasmid pBR322. The position of the substrate RNA (S) and the position of the 5′ and 3′ cleavage products are indicated. (**B**) The same reaction as in (A), except, incubated for 60 minutes. The RNAs analyzed in lane 11 were pre-heated for 5 minutes at 60°C followed by chilling in ice and normal incubation at 37°C for 60 minutes.

it was confirmed that the substrate RNA could not complex with the 2as-Rz12/88 RNA.

Generation strategy for ribozymes with a three nucleotide helix I-forming region

Generation strategies for hammerhead ribozymes may involve complete chemical synthesis of either the ribozyme RNA itself or the DNA that encodes it. Also insertion strategies using DNA cassettes that are suitable for insertion into cDNA at certain restriction sites have been described by Tabler and Tsagris (16). Since a three-nucleotide helix I is sufficient for catalytic activity, simplified approaches for ribozyme construction can be envisaged as outlined in the following.

To test this approach with another independent example for an asymmetric ribozyme in a comparable biological context, we generated a second HIV-1-directed ribozyme covering the first coding regions of the regulator genes *tat* and *rev*. This region had been targeted earlier by Homann *et al.* (22) with conventional antisense RNAs, of which a particular one (αY69) showed an enhanced inhibitory effect in human cells, so that a suitable model for quantitative studies was available. As a cleavage site we selected the GUC motif at position 301–303 of the 645 nt long target RNA (SR6) [compare (17), corresponding to positions 5595–5597 of the HIV-1 genome according to Ratner *et al.* (29)], so that the resulting asymmetric hammerhead ribozyme would contain the sequences of αY69 (compare 22) plus additional sequences to form a helix III domain of 195 nucleotides in total. The selected GUC motif is followed by an AGA sequence in the target RNA. Therefore, a ribozyme forming a three-nucleotide helix I with this target has to provide an UCU sequence 5′ to the actual catalytic domain. Figure 5 (left side) shows how the completely unrelated plasmid pBS-Rz12/0, which delivered a T3 RNA transcript that did not act as a catalytic antisense RNA (Figure 3, lane 2), was converted into the plasmid pBS-UCU in a PCR step with a matching helix I box. A second PCR reaction was performed in which the helix III-forming region of the ribozyme to be generated was amplified (Figure 5, right side). In this step also the 3′-terminal part of the catalytic domain and two restriction sites were incorporated. Recombination via these restriction sites with the plasmid pBS-UCU yielded the construct pαY-Rz195 encoding the catalytic antisense RNA αY-Rz195 (Figure 5) which has a helix III-forming region of 195 nucleotides and a three-nucleotide helix I-forming region. Figure 6 shows that the resulting ribozyme cleaved the target RNA SR6 *in vitro* as expected. In parallel, we constructed the plasmid pαY-Rz195inac which delivered the RNA αY-Rz195inac. Compared to αY-Rz195 the G at position 12 of the catalytic domain (compare 6) was missing which caused complete loss of the cleavage activity (data not shown).

Once all 64 conceivable three-nucleotide combinations are available, construction of an asymmetric hammerhead ribozyme just requires synthesis of the helix III-forming regions in a PCR amplification step applying two nested DNA oligonucleotides and subsequent subcloning of the amplified DNA fragment into the plasmid delivering the appropriate helix I box and the major part of the catalytic domain. Also inactive control RNAs can be generated with the same strategy.

Inhibitory potential of asymmetric hammerhead ribozymes

It was tested whether the successive shortening of helix I of the set of 2as-Rz12-derived constructs also influenced the extent of inhibition of HIV-1 replication. Viral replication was measured after co-transfection of infectious proviral DNA together with the asymmetric hammerhead ribozymes into human cells, essentially as described recently (25). The intention of these assays was not to obtain a large reduction in HIV-1 replication, but to compare the inhibitory potential of the different constructs. For correlating these data to that of other studies, it has to be kept in mind that greater inhibition could be achieved also in this system by choosing a higher molar excess of ribozyme or antisense RNA, respectively.

The helix I-deficient RNA 2as-Rz12/0, which had been cleavage-incompetent when tested *in vitro*, reduced HIV-1 replication to 48% (Figure 7). Due to the missing catalytic activity of this RNA, the observed inhibition must be attributed to an antisense effect. However, the degree of inhibition was clearly lower compared with the untruncated parental antisense RNA 2as. It is a well described phenomenon that the efficiency of an antisense RNA varies with its length. Depending on the termini of the antisense RNA, alternating inhibition rates have been described by Rittner *et al.* (25). For that reason, the inhibitory effect of the various truncated catalytic antisense RNAs should be compared with the inactive 2as-Rz12/0, not with the parental 2as RNA. The construct 2as-Rz12/1, which had shown some marginal cleavage activity *in vitro* (Figure 2, Table I) and which differs from 2as-Rz12/1 by a single base exchange, resulted already in a significantly increased inhibitory effect (Figure 7), which was further enhanced when asymmetric hammerhead ribozymes with helices I of 2, 3 and 5 base pairs were assayed. In no case, the inhibition rates of the parental catalytic antisense RNA 2as-Rz12 were obtained. However, as mentioned above, the catalytically inactive antisense RNA 2as is already much more effective than the likewise inactive RNA 2as-Rz12/0. Presumably, the antisense domain of the RNAs 2as and 2as-Rz12 that is missing in the helix I-truncated RNAs is involved or supports the association step in living cells.

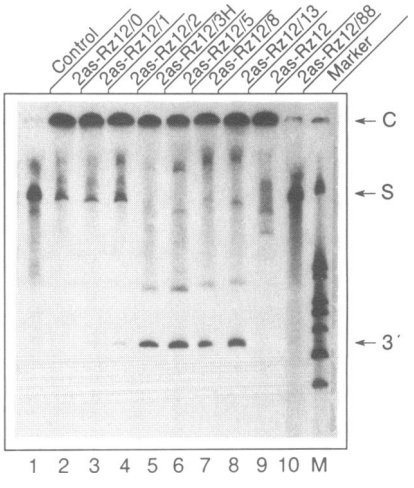

Figure 4. Analysis of the ribozyme reactions on a native polyacrylamide gel. The ribozyme reaction was carried out under condition as described in the legend to Figure 3B and the reaction products were separated on a native polyacrylamide gel. The position of the substrate RNA is indicated (S). Substrate RNA that binds ribozyme RNA forms a complex that does not migrate into the gel (C). Ribozyme RNAs analyzed in lanes 4–8 release the 3′ cleavage product (3′). Lane M contains radiolabelled *Hin*fI fragments of plasmid pBR322.

Even if the data of the truncated series cannot be compared readily with their parental counterparts, it was observed that in both groups the catalytic RNA was about four-times more effective than the inactive one (2as-Rz12 versus 2as and 2as-Rz12/5 versus 2as-Rz12/0).

In order to show that asymmetric hammerhead ribozymes indeed enhance the antisense effect, a second inhibition test was performed assaying the RNAs αY-Rz195 and the inactive control αY-Rz195inac which misses the essential nucleotide G_{12} (6) of the catalytic domain. Also in this case, the catalytically active asymmetric hammerhead ribozyme enhanced the inhibition rate from 24% residual replication to 8% (Figure 7). This indicates that asymmetric hammerhead ribozymes indeed can be used generally as tools for gene suppression.

DISCUSSION

In this work we have described hammerhead ribozymes which are characterized by different length of the two antisense arms that flank the catalytic domain. In view of the asymmetry we propose to call these RNAs 'asymmetric hammerhead ribozymes'. The asymmetry implies that binding of a hammerhead ribozyme to its target RNA is controlled by one antisense flank only. In the experiments described here, helix I was shortened and two examples were shown where a three-nucleotide helix I is sufficient for catalytic activity. It is well possible that also an inverted asymmetric design, *ie.* a short helix III and a long helix I, will behave similarly. However, preliminary data suggest that helix I tolerates more base exchanges than helix III (Zoumadakis and Tabler, unpublished). In line with this, Ellis and Rogers (30) observed a decline in activity when they truncated the helix III-forming region to four nucleotides. In this case, however, helix I was only 10 base pairs, so that the total number of base pairs might have been too small for sufficient association with target RNA.

Since a three base pair helix I is not very stable, we cannot exclude that in other ribozyme/substrate pairs temporary local secondary structures may form between the 5′ domain of the ribozyme and the substrate RNA that are not in agreement with the active hammerhead structure. In those cases the threshold length for fully efficient cleavage may be different than three. However, we have not yet encountered such a case, but three so far unpublished examples confirm the efficient cleavage rate with a three base pair helix I.

Asymmetric hammerhead ribozymes as described in this report have four advantages:

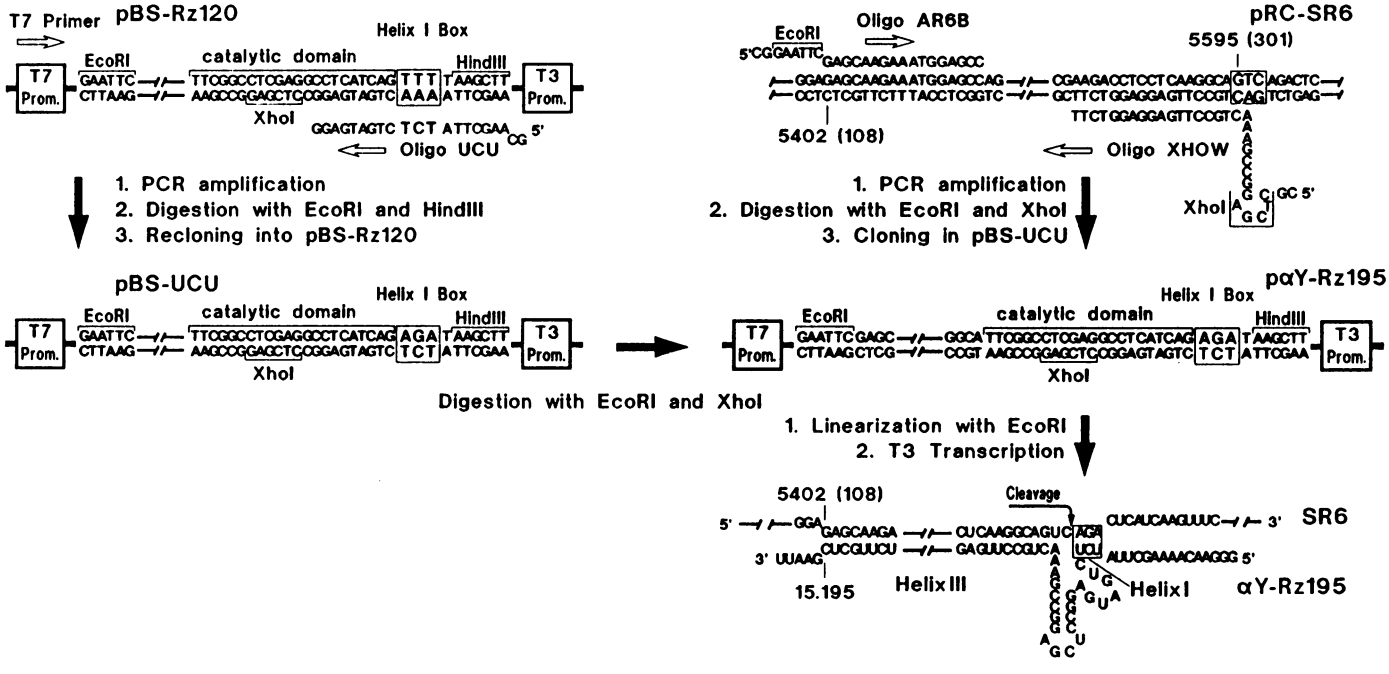

Figure 5. Strategy for simplified generation of asymmetric hammerhead ribozymes. The left panel shows the conversion of the plasmid pBS-Rz12/0 that encodes — if transcribed from the T3 promoter — the catalytic domain of a hammerhead ribozyme preceded by an AAA sequence that could form a helix I in an asymmetric hammerhead ribozyme. By using the DNA oligonucleotide UCU and the T7 sequencing primer d(TAATACGACTCACTATAGGG), a DNA fragment was PCR-amplified and re-cloned, resulting in plasmid pBS-UCU with a modified helix I box. The resulting T3 RNA transcript could pair to a target RNA having a cleavable motif that is followed by an AGA sequence. Similarly, all conceivable three-nucleotide helix I boxes can be introduced. The right panel shows the conversion of the plasmid pRC-SR6, that contains HIV-1 cDNA sequences [nucleotide numbers refer to sequence of Ratner *et al.* (29); those in parenthesis to the SR6 RNA transcript], into a ribozyme construct. The GTC sequence corresponding to the selected target site is boxed. Two nested DNA oligonucleotides AR6B and XHOW were used for PCR amplification. The latter one is given in the conformation of a hammerhead structure for clarity only. The amplified DNA was cloned via the newly introduced *Eco*RI and *Xho*I sites into the plasmid pBS-UCU that provides the matching helix I box, yielding plasmid pαY195. The related control plasmid pαY195inac was generated in a similar way, by using instead of XHOW a DNA oligo without the G residue that proceeds the AAA sequence at the 3′ terminus of the catalytic domain. After transcription with T3 RNA polymerase, the asymmetric ribozyme RNA αY195 (or αY195inac) is generated which is shown below in its hammerhead conformation together with the target RNA SR6. This hammerhead complex forms a three-nucleotide helix I and a helix III consisting of 195 nucleotides. Thus construction of asymmetric hammerhead ribozymes just requires PCR amplification, followed by subcloning into the matching vector that delivers the catalytic domain plus the target-specific helix I-forming region.

Asymmetric hammerhead ribozymes release their 3′ cleavage product

An asymmetric design ensures the release of the 3′ cleavage product (Figure 4). It remains to be seen whether the unpaired helix I-forming region can provide an entry site for binding of an uncleaved substrate RNA which might support the displacement of the bound 5′ cleavage product. Such a mechanism would also generate a catalytic turnover, despite the presence of a long region complementary to the target RNA. In view of the long helix III that we had used here, strand displacement cannot be expected under our *in vitro* conditions. However, agents that support dissociation of RNA might facilitate such a mechnism *in vitro* and it is conceivable that proteins could accelerate strand separation *in vivo*.

Asymmetric hammerhead ribozymes resemble conventional antisense RNA which ensures efficient association to the target RNA

An asymmetric ribozyme with a short helix I, in combination with a long helix III, resembles a conventional antisense RNA, just carrying a catalytic domain at its 5′ end that can function as a 'warhead'. Helix III can be chosen sufficiently long to compete with the secondary structure of the target RNA for efficient binding. Helix I just ensures the exact geometry of the catalytically active hammerhead structure at the target site, but neither influences the melting temperature, nor specific binding to the target RNA. The observation that the hammerhead ribozyme 2as-Rz12/88, with short helices I and III, became catalytically active only after heat treatment, illustrates that great care has to be taken to select the length of the flanking regions. The most plausible explanation for the lack of cleavage prior to heat treatment is the inability of the ribozyme to resolve the secondary structure of the substrate RNA. Binding cannot occur or is greatly reduced, unlike the situation when short model substrates are used as has been done in many kinetic studies on hammerhead RNAs. Reductions in cleavage efficiency was observed for other hammerhead ribozymes when short and long substrate RNAs were compared (31–33). The studies of Fedor and Uhlenbeck (7,8) indicate that changes in the overall effectiveness of hammerhead ribozymes are caused by differences in binding and not by the actual catalytic activity, but we cannot exclude that in case of catalytic antisense RNAs a slow and rate-limiting step occurs after association, but prior to actual cleavage (Homann *et al.*, submitted). In view of the inefficient binding of ribozyme with short antisense flanks to its long target RNA, it is coherent that many of them do not perform well or require an extremely high molar excess when applied *in vivo* for gene suppression (13–15). Even a high level expression of *in vitro* active hammerhead ribozymes may fail to inhibit the activity of the target gene activity at all (34). On the other hand, there are spectacular exceptions like the ribozyme-induced loss-of-function phenotype of the *fushi tarazu* gene in *Drosophila* (35). Also a hammerhead ribozyme with two flanks of twelve nucleotides could decrease α-cell glucokinase expression to 30% of the normal activity in transgenic mice (36). In general it seems to depend on the secondary structure of the target RNA whether a ribozyme with short antisense flanks can actually have access to it and function as a potent inhibitor. This is in line with the assumption made in other cases that binding and not cleavage is the rate-limiting factor of the hammerhead ribozyme (30,33). It is obvious that maximal cleavage activity *in vitro* as well as *in vivo*, is useless if the ribozyme RNA does not bind sufficiently well to its target. Moreover, L'Hullier *et al.* (15) observed that kinetic parameters determined *in vitro* for hammerhead ribozymes with 12 nucleotides in each antisense flank did not predict the *in vivo* activity. Given the limitations to predict secondary structures of RNAs as occurring *in vivo* and in view of the mixed results with ribozymes having short antisense flanks, it seems attractive to apply catalytic antisense RNAs for gene suppression, especially since their applicability has also been demonstrated in transgenic *Drosophila* by Heinrich *et al.* (18). Improvements in binding can also be achieved by introduction of 'anchor'

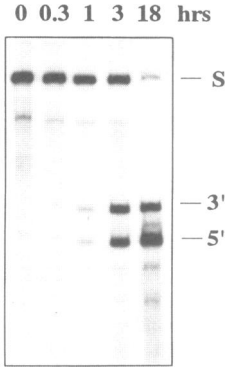

Figure 6. Cleavage of RNA SR6 by the asymmetric hammerhead ribozyme αY-Rz195. Radioactively labelled substrate RNA SR6 was incubated for different reaction times as indicated on top of each lane (in hours) with the asymmetric hammerhead ribozyme αY-Rz195 and analyzed on a polyacrylamide/urea gel and visualized by autoradiography. The position of the substrate (S) and of the 5′ and 3′ cleavage products are indicated.

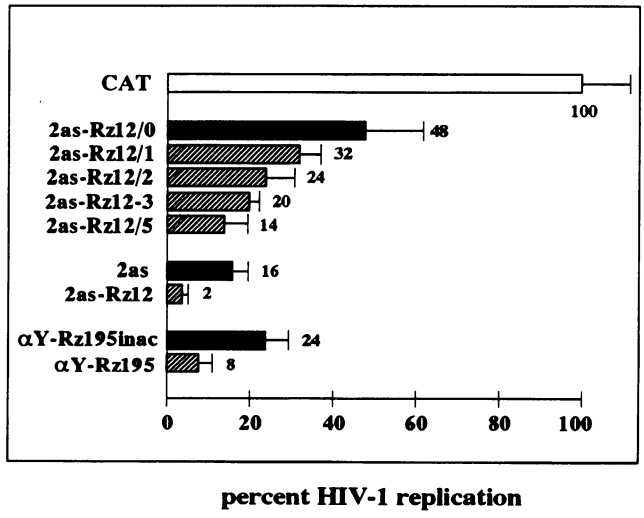

Figure 7. Replication of HIV-1 in the presence of asymmetric hammerhead ribozymes and control RNAs. All RNAs (160 ng) were tested by co-transfection in human SW480 cells with infectious proviral HIV-1 DNA (pNL4-3, 40 ng). CAT RNA served as control 100% replication. The bars represent averages of 24 measurements (16 in case of αY-Rz195 and αY-Rz195inac); the average values in % replication are given in numbers next to the bars and the standard deviations of the mean values are indicated in the diagram. Black bars represent catalytically inactive RNAs and dotted bars represent catalytically active RNAs.

sequences that help to overcome inaccessible structures of the target RNA (37). In fact, the extended helix III, that has been used in this study has the same anchor effect.

Asymmetric hammerhead ribozymes are accessible to *in vitro* selection procedures

One difficulty with longer antisense RNAs is that they themselves can assume a more or less stable secondary structure which needs to be overcome upon association with the target RNA. However, antisense RNAs are accessible to the *in vitro* selection procedure described recently by Rittner *et al.* (25). This technique, which is as well applicable to asymmetric hammerhead ribozymes, allows the identification of fast-associating and biologically effective antisense RNA species out of a pool of RNAs which have an unique 5′ terminus but differ in their 3′ end and hence in their total length. Depending on the length of the antisense RNA, differences in the binding rate constants in the order of more than two magnitudes were found. Assuming that efficient association with target RNA is equally important for catalytic RNAs, selection of a 3′ terminus which results in a fast hybridizing asymmetric hammerhead ribozyme should be beneficial for gene suppression *in vivo*.

Asymmetric hammerhead ribozymes can be generated by simple PCR procedures

A helix I of only three base pairs allows simplified PCR-based strategies for ribozyme constructions, since helix I and the major part of a catalytic domain can be provided by a pre-made vector. We are in the process to construct a set of 64 vectors containing all conceivable three-nucleotide helix I-forming regions. Construction of an asymmetric hammerhead ribozyme just requires PCR amplification of helix III and subsequent insertion into one of the pre-formed vectors. Such a strategy provides considerable flexibility to target any conceivable cleavable motif. In that way we succeeded in generating an asymmetric hammerhead ribozyme against a *Drosophila* gene for which we had no cDNA available (Heinrich and Tabler, unpublished). It was sufficient to use genomic DNA and to carry out one PCR step, followed by subcloning of the amplified DNA.

ACKNOWLEDGEMENTS

We thank Emilio Martinez who was supported by the 'COMETT' student exchange programme of the European Union (EU) for technical assistance in a part of project and H. zur Hausen for continuous support. This work was in part supported by the EU within the network activity of the 'Human Capital and Mobility' programme (ERBCHRXCT930162) (M.T. and G.S.) and by the 'BIOTECH' programme (BIO2 CT93 0400-DG12SSMA) (M.T.), as well as by the BMFT grant FKZ BGA III-007-89/FVP7 (G.S.).

REFERENCES

1. Bruening,G. (1989) *Methods Enzymol.*, 180, 546–558.
2. Forster,A.C. and Symons,R.H. (1987) *Cell*, 49, 211–220.
3. Forster,A.C. and Symons,R.H. (1987) *Cell*, 50, 9–16.
4. Uhlenbeck,O.C. (1987) *Nature*, 328, 596–600.
5. Haseloff,J. and Gerlach,W.L. (1988) *Nature*, 334, 585–591.
6. Hertel,K.J., Pardi,A., Uhlenbeck,O.C., Koizumi,M., Ohtsuka,E., Uesugi,S., Cedergren,R., Eckstein,F., Gerlach,W.L., Hodgson,R. and Symons,R.H. (1992) *Nucleic Acids Res.*, 20, 3252.
7. Fedor,M.J. and Uhlenbeck,O.C. (1990) *Proc. Natl. Acad. Sci. USA*, 87, 1668–1672.
8. Fedor,M.J. and Uhlenbeck,O.C. (1992) *Biochemistry*, 31, 12042–12054.
9. Goodchild,J. and Kohli,V. (1991) *Arch. Biochem Biophys.*, 284, 386–391.
10. Koizumi,M. and Ohtsuka,E. (1991) *Biochemistry*, 30, 5145–5150.
11. Pieken,W.A., Olsen,D.B., Benseler,F., Aurup,H. and Eckstein,F. (1991) *Science*, 253, 314–317.
12. Hertel,K.J., Herschlag,D. and Uhlenbeck,O.C. (1994) *Biochemistry*, 33, 3374–3385.
13. Cotten,M., Schaffner,G. and Birnstiel,M.L. (1989) *Mol. Cell. Biol.*, 9, 4479–4487.
14. Cameron,F.H. and Jennings,P.A. (1989) *Proc. Natl. Acad. Sci. USA*, 86, 9139–9143.
15. L'Hullier,P.J., Davis,S. and Bellamy,A.R. (1992) *EMBO J.* 11, 4411–4418.
16. Tabler,M. and Tsagris,M. (1991) *Gene*, 108, 175–183.
17. Homann,M., Tzortzakaki,S., Rittner,K., Sczakiel,G. and Tabler,M. (1993) *Nucleic Acids Res.*, 21, 2809–2814.
18. Heinrich,J.-C., Tabler,M. and Louis,C. (1993) *Dev. Genet.*, 14, 258–265.
19. Steinecke,P., Herget,T. and Schreier,P.H. (1992) *EMBO J.*, 11, 1525–1530.
20. Crisell,P., Thompson,S. and James,W. (1993) *Nucleic Acids Res.*, 21, 5251–5255.
21. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. Homann,M., Rittner,K. and Sczakiel,G. (1993) *J. Mol. Biol.*, 223, 7–15.
23. Tsagris,M., Tabler,M., Mühlbach,H.-P. and Sänger,H.L. (1987) *EMBO J.*, 8, 2173–2183.
24. Adachi,A., Gendelman,H.E., König,S., Folks,T., Wiley,R., Rabson,A. and Martin,M.A. (1986) *J.Virol.*, 59, 284–291.
25. Rittner,K., Burmester,C. and Sczakiel,G. (1993) *Nucleic Acids Res.*, 21, 1381–1387.
26. Sczakiel,G. Pawlita,M., Kleinheinz,A. (1990) *Biochem. Biophys. Res. Comm.*, 169, 643–651.
27. Sczakiel,G. and Pawlita,M. (1991) *J. Virol.*, 65, 1421–1426.
28. Rittner,K. and Sczakiel,G. (1991) *Nucleic Acids Res.*, 19, 1421–1426.
29. Ratner,L., Haseltine,W., Patarca,R., Livak,K.J., Starcich,B., Josephs,S.J., Doran,E.R., Rafalski,J.A., Whitehorn,E.A., Baumeister,K., Ivanoff,L., Petteway,S.R.Jr., Pearson,M.L., Lautenberger,J.A., Papas,T.S., Ghrayeb,J., Chang,N.T., Gallo,R.C. and Wong-Staal, F. (1985) *Nature*, 313, 277–284.
30. Ellis,J. and Rogers,J. (1993) *Nucleic Acids Res.*, 21, 5171–5178.
31. Heidenreich,O. and Eckstein,F. (1992) *J. Biol. Chem.*, 267, 1904–1909.
32. Denman,R.B. (1993) *Nucleic Acids Res.*, 21, 4119–4125.
33. Bertrand,E., Pictet,R. and Grange,T. (1994) *Nucleic Acids Res.*, 22, 293–300.
34. Mazzolini,L., Axelos,M., Lescure,N. and Yot,P. (1992) *Plant Mol. Biol.*, 20, 715–731.
35. Zhao,J.J. and Pick,L. (1993) *Nature*, 365, 448–451.
36. Efrat,S., Leiser,M., Wu,Y.-J., Fusco-DeMane,D., Emran,O.A., Surana,M., Jetton,T.L., Magnuson,M.A., Weir,G. and Fleischer,N. (1994) *Proc. Natl. Acad. Sci. USA*, 91, 2051–2055.
37. Pachuk,C.J., Yoon,K., Moelling,K. and Coney,L.R. (1994) *Nucleic Acids Res.*, 22, 301–301.