AUA-Cleaving Hammerhead Ribozymes: Attempted Selection for Improved Cleavage†

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ABSTRACT: In order to find a ribozyme which can cleave the AUA triplet efficiently, the specificities and rates of intermolecular cleavage by the ribozyme of the satellite RNA of the barley yellow dwarf virus have been determined. Although it cleaves the AUA triplet in the plus strand of the viroid RNA, cleavage of AUC and AUU is more efficient, and AUG is essentially not cleaved. Attempts were made to increase cleavage efficiency by *in vitro* selection with randomization at positions 7, 10.1, and 11.1 in the core region. Fifteen clones were analyzed, two of which showed increased AUA cleavage efficiency. They have a $G^{10.1}$·$C^{11.1}$ base pair and a pyrimidine at position 7. This corresponds to the sequence of the consensus hammerhead ribozyme. Attempts to further increase cleavage efficiency by *in vitro* selection of the consensus hammerhead ribozyme with randomization of the 10 nucleotides in the core region or of the sBYDV ribozyme with 12 core nucleotides randomized were not successful.

Catalytic RNA molecules (ribozymes) act as ribonucleases to cleave RNA substrate in a sequence-specific fashion. One of the smallest catalytic RNAs is the hammerhead ribozyme (Figure 1), which has been characterized in much detail (Symons, 1992). Ribozymes have generated much interest due to their potential utilization, particularly in therapeutic applications (Rossi, 1992; Rossi et al., 1992). While the sequences of helices I and III seem to serve only to direct the ribozyme to a specific target and are thus relatively noncritical, one limitation to the use of ribozymes therapeutically is the small number of specific cleavage sequences. For example, the hammerhead ribozyme is very active against its native GUC cleavage site and can also cleave other sites, i.e., GUA, GUU, CUC, and UUC, but it is relatively inactive against most other sites, with AUC cleavage being controversial (Haseloff & Gerlach; 1988; Ruffner et al., 1990; Perriman et al., 1992). It would be of interest, therefore, to develop active ribozymes acting on other sequences that might be of greater interest therapeutically and might also yield more information on the structure–function requirements. Of RNA sequences that would have widespread applicability from a therapeutic perspective AUG, as the translation initiation codon, of course comes immediately to mind. However, another sequence that caught our attention is AUA, which is ubiquitously found in the AAUAAA motif of the recognition sequence for the polyadenylation of eukaryotic mRNA (Jackson & Standart, 1990). The sequences around this motif differ for individual genes. Such highly conserved short regions of RNA are particularly important for targeting viral agents such as HIV which have a high frequency of mutation in most regions of the genome.

To date, all naturally occurring hammerhead ribozymes have been found to cleave at a GUC site, with two exceptions, GUA in the lucerne transient streak virus (sLTSV) (Foster & Symons, 1987) and the satellite RNA of the barley yellow dwarf virus (sBYDV), which cleaves at an AUA (Miller et al., 1991). The sBYDV structure differs from the consensus hammerhead ribozyme by the presence of two additional bases in the central core preceding helix II and by the very complex structure associated with stem–loop II (Figure 2). Miller and Silver (1991) studied the nature of the complex structure, finding that an interesting pseudoknot structure is apparently formed which may be involved in control over cleavage. When stem–loop II is shortened to eliminate the formation of a pseudoknot or when the structure is mutated to prevent its formation, the rate of AUA cleavage is considerably increased. The cleavage was studied only intramolecularly, and the rate of cleavage was estimated to be much lower compared to the consensus hammerhead ribozyme cleavage on GUC.

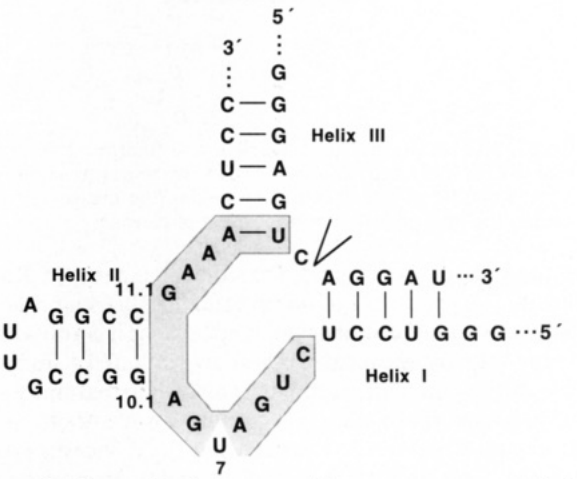

FIGURE 1: Hammerhead ribozyme with consensus sequence. Shaded area, central core; arrow, position of cleavage. Numbering is according to Hertel et al. (1991).

In vitro selection by the use of molecular evolution techniques is proving to be a powerful technique and has been very successful in elucidating structural requirements and altering specificity and other properties of RNA, particularly in the hairpin ribozyme [see, for example, Berzal-Herranz et al. (1993)] and the *Tetrahymena* group I ribozyme [see, for example, Lehman and Joyce (1993)]. To date, no *in vitro*

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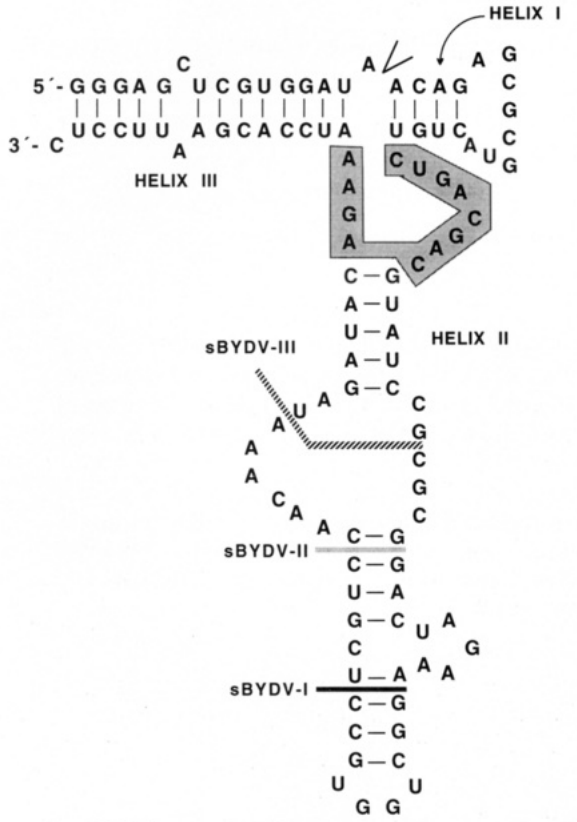


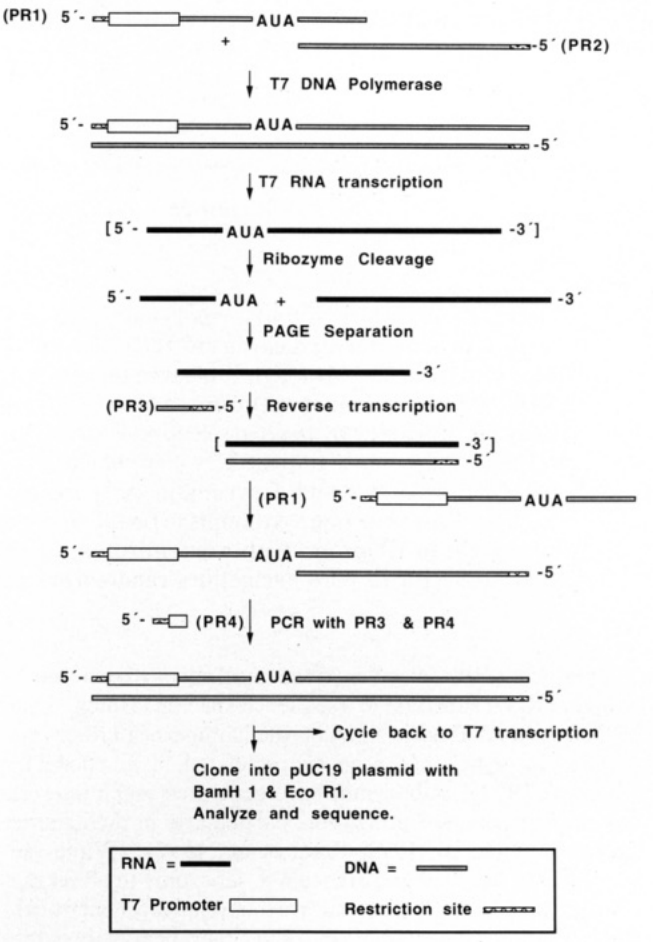
FIGURE 2: sBYDV hammerhead-like ribozyme. Shortened constructs labeled sBYDV-I, -II, and -III are generated by removal of the portions noted and closure of the structure by linking the nucleotides just preceding the lines shown; arrow, position of clevage.

selections have been reported on the hammerhead RNA structure. A great deal of information has been elucidated about the hammerhead ribozyme, and because it is also within the range of easy chemical synthesis, we initiated a study on AUA-cleaving hammerhead ribozymes. The naturally occurring hammerhead ribozyme cleaving an AUA site has a central core structure that varies from the consensus GUC ribozyme. This suggests that other structural changes might further improve the catalytic ability toward hydrolysis of an AUA site. In order to achieve increased efficiency, we investigated the selection of ribozymes from pools with either total or partial randomized sequences in the core region. We report here our findings on *in vitro* selection toward an AUA-cleaving ribozyme and the comparative activities of hammerhead ribozymes on the cleavage of this triplet.

MATERIALS AND METHODS

Nucleoside triphosphates and 2′-deoxynucleoside triphosphates (special quality for molecular biology) were purchased from Boehringer Mannheim. [α-^{32}P]ATP, [α-^{32}P]GTP, and [α-^{32}P]dCTP (specific activity, ca. 3000 Ci/mmol); [γ-^{32}P]-ATP (specific activity, ca. 5000 Ci/mmol); and [α-^{35}S]dATP (specific activity, ca. 1000 Ci/mmol) were obtained from Amersham Buchler. X-ray film (X-OMAT XAR-5) was purchased from Kodak. Radioanalytical scanning was perfomed with a Fuji BAS2000 Bio-Imaging analyzer. Taq DNA polymerase was purchased from Amersham and MoBiTec (Göttingen). Taq DNA 10× buffer was purchased from Amersham. T7 DNA polymerase was from Promega. M-MLV reverse transcriptase, human placental ribonuclease inhibitor, Sequenase and the accompanying sequencing kit, and T4 DNA polynucleotide kinase were purchased from United States Biochemical (USB). *Eco*RI, *Bam*HI, and *Cla*I

Scheme 1



restriction endonucleases were purchased from NEB. Plasmid DNA purification columns and PCR QiaQuick spin columns were obtained from Diagen (Düsseldorf, Germany).

T7 RNA polymerase was isolated from an overproducer, kindly provided by W. Studier, Brookhaven National Laboratories, Upton, NY, and was purified as described previously (King et al., 1986).

T4 DNA ligase was isolated from the overproducer *Escherichia coli* NM989, kindly provided by Dr. F. Winkler, essentially as described (Knopf, 1977).

Synthesis of Oligonucleotides. Oligodeoxyribonucletotides were prepared on an Applied Biosystems 380B DNA synthesizer and normally purified by reverse-phase HPLC with the dimethoxytrityl group on. For positions that were randomized, an equimolar mixture of the four deoxynucleoside phosphoramidites was introduced at the desired location in the sequence. Oligoribonucleotides were prepared and purified as described by Tuschl and Eckstein (1993).

Selection Protocols. The procedure for selection is diagrammed in Scheme 1.

T7 DNA Polymerization. The two oligonucleotide primer–templates for producing the randomized in *cis* cleaving ribozyme pool, PR1 [5′-CCT**GGATCC**T TAATACGACT CACTATAGGG AGCTCGTGGA TAACAGAGCG CG-TACTGT-3′] (which contains a *Bam*HI site for cloning, shown in boldface; the T7 promoter, underlined; and the cleavage site, in outline) and PR2 [5′-GAGGAATTCG TG-GATTTCXG **ATCGAT**TGAT CXTCXTCAGA CAG-TACGCGC TCTGT-3′] (which contains an *Eco*RI site for cloning, underlined; the randomized bases, in outline; and an internal *Cla*I restriction site, in boldface, for checking

selection), were annealed by heating to 70 °C for 5 min and allowed to cool to room temperature. The solution was then made 65 mM in Tris·HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT, 0.38 mM in each dNTP, and 2 μM oligos; 10 units of T7 DNA polymerase was added, and the reaction mixture was incubated at 37 °C for 60 min. The reaction mixture was purified by ethanol precipitation or passed through a QiaQuick spin PCR[1] purification column.

T7 RNA Transcription and Intramolecular Ribozyme Cleavage. Because the conditions under which the transcription was conducted also promote ribozyme cleavage, these steps could not be separated. The T7 RNA transcription was conducted essentially as described by Milligan and Uhlenbeck (1989) using [α-^{32}P]ATP or [α-^{32}P]GTP to label the transcript and 16–25 mM Mg^{2+} to promote the intramolecular cleavage of the transcribed RNA. Incubation times varied from 30 min to 3 h depending upon the degree of cleavage desired.

The reaction mixtures were applied to an 11% polyacrylamide–8 M urea gel. To identify the position of the RNA cleavage product, a control T7 transcript of an intermolecularly cleaving ribozyme of known size was also applied. The RNA cleavage band was excised from the gel, and the RNA was isolated essentially as described by Rubin (1975).

Reverse Transcription–PCR. The PCR–RT oligonucleotide PR3 [5′-GAGGAATTCG TGGAT-3′] was designed to anneal with the RNA ribozyme cleavage product and produce the DNA complement by primer extension with reverse transcriptase. The full-length ds-DNA was subsequently produced by adding a small amount of the oligonucleotide PR1 to restore the cloning restriction site, the T7 promoter site, and the sequence lost during ribozyme cleavage. The second PCR oligomer was then added, PR4 [5′-CCTG-GATCCT TAATACG-3′], and PCR was conducted to amplify the product.

To 10 μL of PCR–RT oligomer PR3 (2.5 μM) was added the RNA cleavage product, heated to 90 °C for 2 min and quickly cooled to 42 °C. Immediately, a solution of dNTP, 10× PCR buffer (Amersham), ribonuclease inhibitor, and M-MLV reverse transcriptase was added and mixed with the annealed solution. Final concentrations for the reaction mixture were 100 mM KCl, 20 mM Tris·HCl, pH 8.3, 3 mM $MgCl_2$, 0.67 mM each dNTP, 30 units/μL ribonuclease inhibitor, and 120 units of M-MLV reverse transcriptase in a 30-μL reaction volume. The reverse-transcription reaction was allowed to proceed at 42 °C for 1 h; the mixture was then heated to 75 °C for 10 min to destroy the reverse transcriptase and cooled on ice for PCR. To this reaction mixture was added 1 pmol of PR1 primer; the other PCR primer, PR4; PCR reaction buffer; and Taq DNA polymerase. PCR was conducted in a Biometra Trio Thermobloc. Final concentrations for the PCR reaction were 0.25 μM PCR oligomers, 50 mM KCl, 10 mM Tris·HCl (pH 8.3 at room temperature), 1.5 mM $MgCl_2$, 200 μM each dNTP, and 2.5 units of Taq DNA polymerase in a 100-μL reaction volume. The PCR reactions were conducted in MoBiTec PCR reaction tubes that required no oil or wax overlay.

The DNA from this step was then purified by passing it through a QiaQuick spin column. In addition to observation of the length of the final product by agarose gel electrophoresis, the product produced by RT–PCR was treated with *Cla*I to check for cleavage. The DNA could subsequently be cycled back to the T7 RNA transcription step for further selection for as many generations as desired.

[1] Abbreviations: PCR, polymerase chain reaction; RT, reverse transcription; ds-DNA, double-stranded DNA.

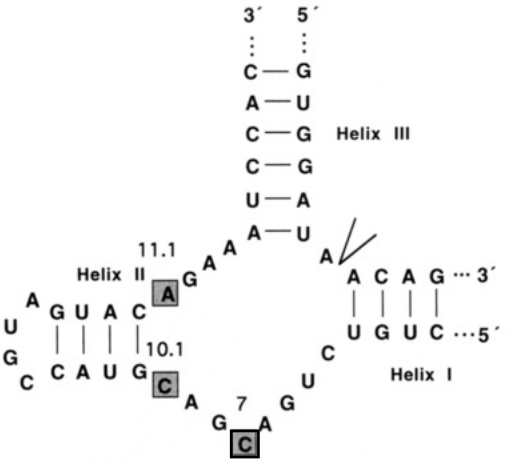


FIGURE 3: SsBYDV hammerhead ribozyme for *trans* cleavage. Boxed nucleotides are different from the consensus hammerhead ribozyme sequence. Even though positions 10.1 and 11.1 are not part of helix II in this structure, this numbering facilitates comparison with the consensus hammerhead ribozyme.

Cloning, Characterization, and Sequencing. After the desired amount of selection, the ds-DNA from RT–PCR was inserted into pUC19 plasmid by standard double restriction cleavage protocols (Sambrook et al., 1989) using the terminal *Bam*HI and *Eco*RI cleavage sites. Both plasmid and insert DNA were purified by low-melting agarose gel electrophoresis (Ogden & Adams, 1987) before ligation by T4 DNA ligase and transformed into SMH50 *E. coli* competent cells. Colorless colonies were selected, and plasmid DNA was isolated from 4-mL minipreps (Sambrook et al., 1989) by Qiagen P20 column purification. The plasmid DNA was analyzed for the presence of insert by differential restriction endonuclease analysis, again using the presence of the *Cla*I site as a positive marker. Active ribozyme-producing sequences were established by T7 RNA run-off transcriptions using *Eco*RI to linearize the cloned plasmid DNA using the protocol described by Aurup et al. (1992).

Sequencing was performed using pUC19 reverse sequence primer and the USB Sequenase sequencing kit. Heat denaturation of the cloned plasmid DNA by the method of Andersen et al. (1992) was used to initiate the sequencing.

Kinetics of Intermolecular Ribozyme Cleavage. Kinetic studies were limited to the determination of k_{obs} since stems I and III were not varied and K_m's should be relatively constant. The procedures described by Tuschl and Eckstein (1993) were used to determine turnover rates, k_{obs}, at substrate saturation. Substrate concentration was generally 500 nM, and ribozyme substrate concentration ranged from 5 to 50 nM depending on the activity of the ribozyme.

RESULTS

An obvious starting point to search for efficient AUA-cleaving ribozymes is the sBYDV ribozyme, as this triplet is its natural target. Considering that shorter constructs of this ribozyme might be more amenable to selection, we turned to the shorter variants of this ribozyme, as had been constructed by Miller and Silver (1991) (Figure 2, sBYDV-III). The ribozymes were produced by T7 RNA transcripts and confirmed many of their findings. Significantly, we were able to show that the short construct of the sBYDV ribozyme, SsBYDV, could cleave an RNA substrate intermolecularly (Figure 3; Table 1). In fact, several modifications made to helix and loop II of SsBYDV were found qualitatively to have little effect on the ability to cleave the AUA substrate. Of

Table 1: Comparison of Kinetics of Intermolecular Cleavage of AUN Triplets by AUA Ribozymes[a]

	k_{obs} (min^{-1})			
ribozyme	AUA	AUC	AUU	AUG
SsBYDV[b]	0.011	0.29	0.26	0.0001
	[280]	[11]	[12]	[31 000]
consensus central core	0.042	0.86	0.47	0.0006
	[74]	[3.6]	[6.6]	[5000]

[a] Rates of cleavage were determined at 500 nM substrate as described in Materials and Methods. Bracketed numbers = k_{obs}(GUC consensus)/k_{obs}(AUN); k_{obs}(GUC consensus), 3.1 min^{-1}, taken from Tuschl and Eckstein (1993). [b] SsBYDV structure as shown in Figure 3.

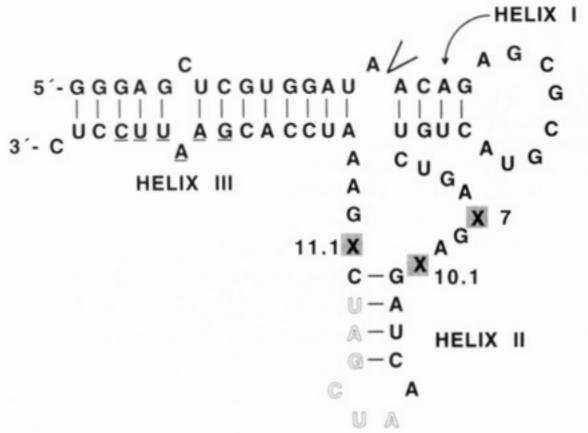


FIGURE 4: Sequence of SsBYDV transcript for selection of AUA-cleaving efficiency. X, randomized positions; underlined region, *Eco*RI cleavage site for cloning PCR DNA; open letters, *Cla*I site for selection identification.

some interest in the intramolecularly cleaving sBYDV RNA is the observation that the longer constructs sBYDV, sBYDV-I, and sBYDV-II (Figure 2) all cleaved at the AUA site in the absence of Mg^{2+}, albeit slowly.

The efficiency of the SsBYDV hammerhead ribozyme (Figure 3) on the cleavage of AUN triplets was also determined and compared to that of the consensus hammerhead ribozyme modified to target the AUN site. Substrate saturation kinetics were conducted with these ribozymes on RNA substrates which contained AUA, AUG, AUC, and AUU as cleavage targets (Table 1). Surprisingly, results show that the consensus hammerhead sequence cleaves all triplets better than the SsBYDV ribozyme. The cleavage rates are all less than that of the consensus hammerhead acting on GUC, but significant activity is observed for AUC, AUU, and AUA, whereas AUG is cleaved 100 times more slowly than AUA.

With this characterization of the sBYDV ribozyme and its shorter constructs in hand we turned to its improvement for AUA cleavage by *in vitro* selection. For this purpose, SsBYDV (Figure 3) was redesigned for *cis* cleavage (Figure 4). The strategy for the selection is outlined in Scheme 1. The randomization was limited to the three bases at positions 7, 10.1, and 11.1 of the central core (marked X in Figure 4), which are different from the central core of the consensus GUC-cleaving hammerhead ribozyme. Although with 64 possible variants this was expected to concomitantly limit the possibility of selecting a more active ribozyme, this strategy was chosen because the higher yield of expected cleavage product should facilitate isolation for further amplification. This assumption has actually been borne out, as discussed below for the selection experiments with the consensus hammerhead ribozyme. We conducted the *in vitro* selection process for three generations, by which time an active pool had been selected. One generation is the RNA product produced by T7 transcription. For analysis and sequencing of active ribozymes, we chose two different groups: pool I was obtained from the cleavage product isolated during the third-generation transcription, and pool II was obtained from the RNA cleavage product of full-length RNA of the second-generation transcription treated for cleavage by heat shock and the addition of Mg^{2+}. Both RNAs were reverse transcribed, amplified by PCR, and then cloned into pUC19. Bacterial cells were transfected, and colonies were picked and used for isolation of DNA for transcription and sequencing. Figure 5 shows the T7 run-off transcripts of 23 *Eco*RI-cleaved pUC 19 plasmid clones, 15 from pool I and 8 from pool II. Fifteen of the clones showed some degree of activity, and these were selected to sequence. The results of the sequences found and their cleaving efficiencies are presented in Table 2. As can be seen, the sBYDV-type sequence is frequently selected. Apparently $A^{10.1}$ and $C^{11.1}$ of the wild type can be interconverted with no apparent loss of activity. In fact positions 10.1 and 11.1 can apparently have nearly any combination of A's and C's with approximately the same activity of 53–39% cleavage on transcription as long as a pyrimidine is at position 7 (I-9, I-13, I-1, I-4). Activity is intermediate when a purine is at this position (I-5, I-6, I-7, II-5, I-11). Other combinations at the three randomized positions yield ribozymes with rather low activity (II-3, II-4). A ribozyme with a U at position 10.1 is the least active (I-14).

As this limited selection only produced ribozymes with moderate AUA cleavage efficiency, we attempted to improve on it by *in vitro* selection of the consensus hammerhead ribozyme (Figure 1), which is most active on the natural GUC target site (Ruffner et al., 1990). Since we desired to find a very active AUA-cleaving ribozyme and since the main influence on activity comes from the central core of this hammerhead, the selection system was designed with an AUA at the cleavage site and randomization at all 10 bases of the central core. The *in vitro* selection was attempted along with a control, nonrandomized system. Even with a variety of changes in conditions, protocols, and sequences we were only able to select out a short transcript product accidentally of the same length as the expected RNA cleavage product. Attempts were also made to improve the SsBYDV hammerhead-like ribozyme structure (Figure 4) for cleavage of its natural target by randomization of all 12 nucleotides in the central core. Again the same type of selection product was produced, rather than an active AUA-cleaving sequence, regardless of the many changes made to the protocols.

DISCUSSION

Ribozymes are of interest not only for the as yet unresolved structure–function relationship and the details of the reaction mechanism but also for their potential application for the interference with gene expression. However, at present the sequences which can be cleaved by ribozymes are limited (Haseloff & Gerlach, 1988; Ruffner et al., 1990; Perriman et al., 1992). The selection of ribozymes with different cleavage specificities from a pool of randomized sequences is therefore desirable. We have chosen the hammerhead-type ribozyme for such a selection with the aim of identifying ribozymes with the ability to cleave the AUA sequence efficiently. This sequence was considered of particular interest, as it is contained in the polyadenylation signal in eukaryotic mRNAs (Jackson & Standart, 1990).

The hammerhead-like structure contained in the plus strand of the sBYDV satellite virus is known to cleave at AUA in

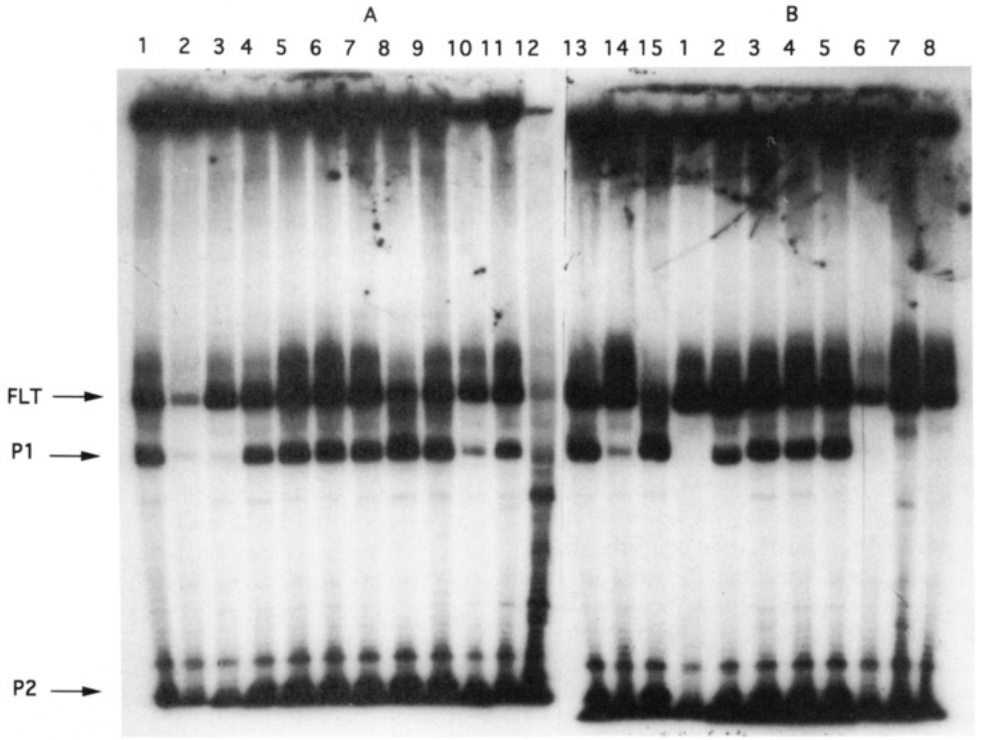


FIGURE 5: Gel analysis of transcription of DNA of selected clones. Clones on the left, lanes 1–15, are from the third generation, and those on the right, lanes 1–8, are from the second generation. FLT, full-length transcript; P1 and P2, cleavage products.

an intramolecular reaction and was considered a good basis from which to begin a search for a ribozyme with high efficiency AUA cleavage. This ribozyme was first studied by Miller and Silver (1991). We confirm that the large structure can be reduced by shortening the complex extended extra stem to produce sBYDV-I and -II. We extended their studies and found that the shortened sBYDV-II form can also cleave RNA substrates at AUA intermolecularly, i.e. in *trans*. This was a gratifying result, as it indicated that this structure could be the basis for the selection of a more efficient AUA-cleaving ribozyme which could also be useful for the modulation of gene expression. The general behavior of this ribozyme parallels that of the consensus ribozyme. Variations in the helix–loop II structure tend to have little effect on the activity of this ribozyme. For example, the following sequences for helix–loop II were all shown to be active:

5′-GUAUCCGUAGAUAC-3′ (i)

5′-GUACCGUAGUAC-3′ (ii)

5′-GGCCGUUAGGCC-3′ (iii)

These in *trans* cleaving ribozymes have respectively 5, 4, and 4 bp in helix II, and the last sequence (iii) is identical to that found in the consensus ribozyme (Figure 1).

Our studies on the intramolecular cleavage of the sBYDV constructs showed that they cleave succesively faster as the RNA is shortened: sBYDV-III > sBYDV-II > sBYDV-I > sBYDV. The question arises as to why the longest hammerhead sequence was selected by the barley yellow dwarf virus for plus strand cleavage. It is likely that the complex structure has some functional value such as control of the cleavage, as suggested previously (Miller & Silver, 1991), or perhaps as a binding site for a control agent that is necessary for the viability of the virus.

To help complete a picture of the intermolecular AUA cleavage as well as the ability of the same ribozymes to cleave other AUN sequences, kinetics were conducted to compare

Table 2: Efficiencies of Selected AUA-Cleaving Ribozymes

Clone[a]	Sequence[b] 3′-AAGX[11]--Helix II--X[10]AGX[7]AGUC-5′	% Cleavage[c]
I--15	------C----------G-------C----	69
I----8	------C----------G-------U----	68
I----9	------C----------A-------U----	53
I--13	------A----------C-------U----	46
I----1	------C----------A-------C----	44
I----4	------A----------C-------U----	39
I----5	------C----------C-------C----	37
I----6	------C----------A-------A----	35
I----7	------A----------C-------A----	34
II---5	------A----------C-----(G/C)----	30
I--11	------C----------A-------A----	29
II---3	----(A/C)----------C-------G----	29
II---4	------A----------A-------G----	28
II---2	------C----------G-------A----	15
I--14	------C----------U-------U----	approx. 9

[a] Ribozymes: I, 3rd generation; II, 2nd generation. [b] Parentheses indicate positions where two bands appeared in the sequencing gel. [c] Approximate cleavages during a 90-min transcription at 37 °C based on radioactive density in gel phosphorimaging.

the consensus hammerhead ribozyme modified to recognize AUN and the short sBYDV hammerhead ribozyme on AUN substrates (Table 1). The hammerhead ribozyme with the consensus core is quite active against AUC and AUU cleavage sites, being only 4–7 times slower compared to GUC cleavage, and about 75 times less efficient in cleaving AUA. Cleavage of AUG is extremely poor by either ribozyme. Both ribozymes show the same order of cleavage: AUC > AUU > AUA > AUG. In both of these ribozyme constructs a purine at the point of cleavage is not favorable, with a G being particularly poor. The bulkiness of the purine base may account for some of this loss of activity, and the 2-amino group of G may disrupt the structure the most, as evidenced by the fact that inosine at the GUX cleavage site cleaves well (Koizumi & Ohtsuka, 1991) but 2-aminopurine cleaves very poorly (T. Tuschl and F. Eckstein, unpublished results). Our results are at least in partial agreement with those for the GUC-cleaving ribozyme, where GUG is also cleaved very poorly, but differ for an A

at the cleavage site where GUA is cleaved nearly as well as GUC (Ruffner et al., 1990). The AUN cleavage by the AUA-cleaving ribozyme is thus not entirely analogous to GUN cleavage by the GUC-cleaving ribozyme, showing that some interactions are perturbed by the difference in structure.

With this background information in hand we started the *in vitro* selection process with the redesigned SsBYDV ribozyme for *cis* cleavage (Figure 4). As previously noted, the selection of hammerhead ribozymes poses a particular problem in that the cleavage product is isolated with all other RNA molecules produced of the same length, including short transcripts and transcripts produced from shorter PCR artifacts. Most of the other *in vitro* selection processes for ribozymes have much better selection methodologies. For example, *in vitro* selections conducted on the hairpin ribozyme (Berzal-Herranz et al., 1993) and the *Tetrahymena* group I intron (Lehman & Joyce, 1993) are able to use a ligation reaction to introduce a new RNA (or DNA) sequence which can be uniquely recognized for purification and subsequent amplification. We thus anticipated that because of this complication, the selection of a hammerhead ribozyme would work best when applied to a system where a relatively large number of potentially active sequences can be expected in the randomized pool. Rather than randomizing the entire core region of the SsBYDV, we decided to first limit selection to the three positions where the sequence differs from the consensus hammerhead structure. Indeed, randomization at positions 7, 10.1, and 11.1 of the SsBYDV ribozyme (Figure 4) selected out a complete range of activities within 2–3 generations. Comparing the cleavage efficiencies of the members of pool I versus those of pool II clearly indicates that selection is occurring (Table 2; Figure 5). Thus, 11 out of 15 clones in pool I are active, versus 4 out of 8 in pool II. Of the active ribozyme sequences only one from the group I pool has activity lower than the activities of the entire group II pool. The approximate relationship between the efficiency of intramolecular cleavage and the kinetics of intermolecular cleavage can be inferred from Table 1; i.e., the consensus ribozyme with a k_{obs} of about 0.04 min^{-1} for AUA cleavage *in trans* has an intramolecular efficiency of around 70% during a 90-min transcription at 37°C. The 90-min time includes transcription and cleavage and was chosen as ample time for transcription to be completed with additional time for ribozyme cleavage.

Examination of the selected sequences shows that the consensus GUC-cleaving hammerhead ribozyme central core was also the most active when selected for AUA cleavage with either U or C located at position 7. One clone (II-2) was isolated with the consensus core sequence and an A^7 which was much less active, by about a factor of 5, but fell within the range of attenuated activity observed for changes at this position in the GUC hammerhead (Ruffner et al., 1990). In this consensus sequence, $G^{10.1}$ and $C^{11.1}$ become base-paired as part of helix II to form five base pairs. It was previously reported that reversal of this base pair in the GUC-cleaving ribozyme severely retarded catalytic ability (Tuschl & Eckstein, 1993), and this is borne out for AUA cleavage as well, since no $G^{11.1}$-containing sequence was discovered in this group of clones. Interestingly, our active pool did not yield a G or U at position 11.1, and the single $U^{10.1}$-containing sequence was poorly cleaved and contained a C at positon 11.1. These data indicate that the $G^{10.1}$·$C^{11.1}$ base pair cannot be replaced by A·U or U·A either, at least for an AUA cleavage site. This is actually a difference from cleavage of GUC by the consensus hammerhead where $A^{10.1}$·$U^{11.1}$ supports cleavage at a reasonable rate (Ruffner et al., 1990). Unfortunately, an interpretation of this difference is not possible at present.

The next most active group of AUA-cleaving ribozymes selected were basically related to the sBYDV sequence. The wild-type sequence contains C^7, $C^{10.1}$, and $A^{11.1}$, which is unfortunately not actually found in our pool of selected clones. Very similar combinations are found however for $C^{10.1}$, $A^{11.1}$, and U^7 (I-13 and I-4) and $A^{10.1}$ and $C^{11.1}$ with C^7 (I-1) or U^7 (I-9). Nearly all other combinations of A and C are found to possess at least some activity. To summarize the results of the selection study, the consensus GUC-cleaving central core sequence is also the most active AUA-cleaving sequence with a pyrimidine at position 7.

While a limited selection provided some improvement for AUA cleavage over that of the naturally occurring sequence, we would have preferred to have found a more efficient ribozyme. We thus turned to the selection of new sequences by randomizing all 12 nucleotides in the central core of the SsBYDV ribozyme as well as randomizing the 10 nucleotides in the core of the consensus ribozyme. In both systems we encountered difficulties in that we consistently found that the selection process yielded transcripts of a size similar to that expected of the cleavage reaction during transcription. These problems appear to center around the selection process. After the T7 RNA transcription–ribozyme cleavage stage, the selection of the active pool is based solely on the isolation of RNA of the correct length for the expected cleavage product. The latter, resulting from a small number of active sequences in our randomized pool, is apparently isolated in too small a quantity to selectively amplify in the RT–PCR step. The selection scheme works very well on a control system over any number of generations, using a nonrandom sequence known to cleave. Thus the difficulties apparently occur in the earliest rounds of selection when the amount of ribozyme cleavage product is smallest relative to some of the other products, and it cannot be selectively and sufficiently amplified subsequently.

Unfortunately, the *in vitro* selection did not select a ribozyme sequence that was more active than the GUC-cleaving consensus hammerhead, although it yielded variants of the sBYDV ribozyme with better cleavage of AUA than the wild type. The results do point out some important information of a general nature, however. The limited selection process is much more rapid at detecting critical bases and positions than the systematic method of synthesizing or transcribing and analyzing each possible variant individually. The results confirm the subtle complexity contained in the relatively small structure of the hammerhead ribozyme. Although our pool of variants was small, the change of the cleavage site from GUC to AUA did not select a different central core sequence to compensate for this change. We have, of course, restricted our search to the size of the central core. It could well be that by increasing the number of nucleotides in this region a sequence with better AUA cleavage potential might be found. Combining this information with the large amount of data available suggests that the evolution of the hammerhead ribozyme is close to optimal, and with the limited size of this small catalytic RNA, a more active sequence than the consensus central core seems unlikely. This is also indicated by the k_{cat}/K_m value of approximately 38 $\mu M^{-1}min^{-1}$, which is close to the theoretically expected value (Pörschke et al., 1973). On the other hand, some structural changes in the hammerhead still permit good if not optimal activity, and in fact a wide range of activity is observed. Each change made in the hammerhead sequence can apparently alter the properties in subtle and as yet poorly understood ways.

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