Development and comparison of procedures for the selection of *delta* ribozyme cleavage sites within the hepatitis B virus

Lucien Junior Bergeron and Jean-Pierre Perreault*

RNA Group/Groupe ARN, Département de Biochimie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec J1H 5N4, Canada

ABSTRACT

***Delta* ribozyme possesses several unique features related to the fact that it is the only catalytic RNA known to be naturally active in human cells. This makes it attractive as a therapeutic tool for the inactivation of clinically relevant RNAs. However, several hurdles must be overcome prior to the development of useful gene-inactivation systems based on *delta* ribozyme. We have developed three procedures for the selection of potential *delta* ribozyme target sites within the hepatitis B virus (HBV) pregenome: (i) the use of bioinformatic tools coupled to biochemical assays; (ii) RNase H hydrolysis with a pool of oligonucleotides; and (iii) cleavage assays with a pool of ribozymes. The results obtained with *delta* ribozyme show that these procedures are governed by several rules, some of which are different from those both for other catalytic RNAs and antisense oligonucleotides. Together, these procedures identified 12 sites in the HBV pregenome that can be cleaved by *delta* ribozymes, although with different efficiencies. Clearly, both target site accessibility and the ability to form an active ribozyme–substrate complex constitute interdependent factors that can best be addressed using a combinatorial library of either oligonucleotides or ribozymes.**

INTRODUCTION

The goal of gene therapy is to modulate the expression of a specific gene. The ability of ribozymes (i.e. RNA enzymes) to specifically recognize an RNA substrate, and subsequently catalyze its cleavage, makes them attractive as therapeutic tools for the inactivation of both viral RNAs and the mRNAs associated with various diseases (1–3). Several successful applicable ribozyme models have been tested both *in vitro* and in a cellular environment (1–3).

Delta ribozyme possesses several unique features that are all related to the fact that it is the only naturally occurring catalytic RNA that has been discovered in humans (4,5). In

contrast to other catalytic RNAs, several hurdles remain to be surpassed before we generate a useful gene-inactivation system based on *delta* ribozyme. The initial step in the development of a ribozyme capable of cleaving a natural RNA molecule is the selection of the cleavage site with the greatest potential for targeting. The specificity of recognition is derived from Watson–Crick base pairs formed between the substrate and the ribozyme. For example, we have engineered a 57 nt *delta* ribozyme derived from the antigenomic strand of the hepatitis *delta* virus (HDV) (Fig. 1A) (6,7). According to its double-pseudoknot secondary structure, which is well supported by experimental data, the substrate specificity depends on the formation of the P1 stem (i.e. the sequence binding domain of the ribozyme), which includes one GU wobble base pair followed by six non-specific Watson–Crick base pairs (Fig. 1A) (4). Secondary and tertiary structures of a target that are characterized by significant intramolecular base pairing are important features, which can influence the cleavage activity of ribozymes (8–10). Thus, even though the appropriate target site is present, effective cleavage at that site depends on its accessibility to the ribozyme. Target sequences located in single-stranded regions of an mRNA have a higher potential because they should be more accessible to ribozyme binding than those in double-stranded regions. Within the double-stranded regions the ribozyme might compete unfavorably with intramolecular base pairing when trying to bind its substrate (8–10). The development of several procedures identifying potential target sites in RNA molecules has been reported for various nucleic acid drugs, including other ribozymes (11–22). Based on these reports, we present three procedures developed in order to identify the sites with the greatest potential for cleavage by *delta* ribozyme in a model RNA. These procedures include the use of: (i) bioinformatic tools coupled to biochemical assays (13,15,16,22); (ii) ribonuclease H (RNase H) mapping in the presence of a library of DNA oligonucleotides (11,20); and (iii) cleavage assays using a library of ribozymes (12,14).

The pregenome RNA from the hepatitis B virus (HBV) was chosen as model target because it offers several advantages. We selected an RNA virus, instead of an mRNA, as our target because the use of an mRNA would have limited our search for potential cleavage sites to those near the 5′-end in order to ensure that cleavage resulted in the production of an inactive protein. HBV has an extremely compact organization as all

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*To whom correspondence should be addressed. Tel: +1 819 564 5310; Fax: +1 819 564 5340; Email: jperre01@courrier.usherb.ca
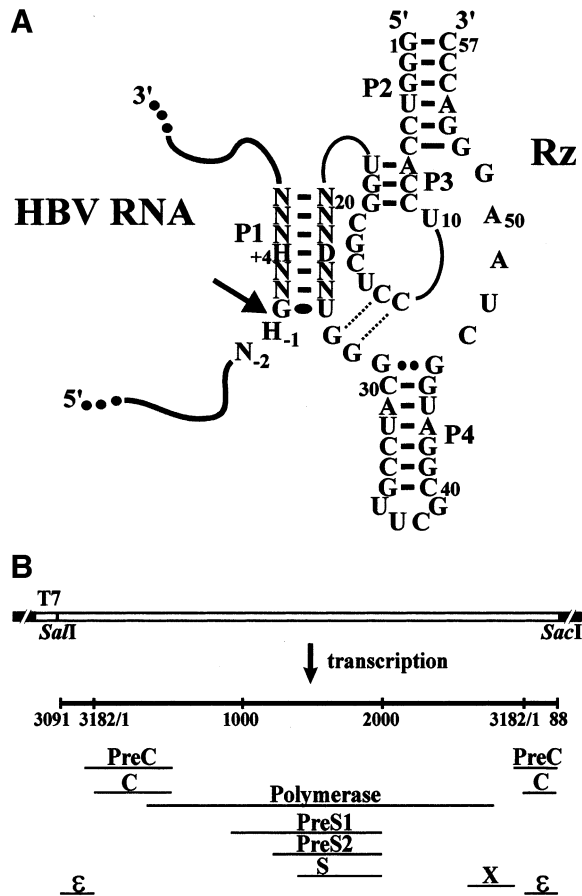
A

B

Figure 1. Illustration of *delta* ribozyme and HBV RNA pregenome. (**A**) Secondary structure of the engineered *trans*-acting *delta* ribozyme bound to the HBV target. The pseudoknot P1.1 is illustrated by the dotted lines. The homopurine base pair at the top of the P4 stem is represented by two large dots, while the wobble base pair is represented by a single large dot. Adjacent and in the P1 stem only the identity of the nucleotides essential for cleavage to occur are shown: H indicates A, C or U; D indicates A, G or U; and N indicates A, C, G, U. The cleavage site is indicated by an arrow. (**B**) Organization of the pHBVT7 vector. The four overlapping open reading frames are shown (C, P, S and X).

nucleotides have coding function (Fig. 1B) (23). The 3.2 kb DNA genome replicates via reverse transcription involving an RNA pregenome of ~3.4 kb whose secondary structure is unknown. Moreover, HBV is a model target that has clinical relevance as 350 million people worldwide are chronic carriers (24,25).

MATERIALS AND METHODS

Bioinformatic analysis of HBV RNA

The sequence of the HBV variant used corresponded to the insert of the plasmid pCHT-9/3091, which was kindly provided by Dr M. Nassal (26). The most stable secondary structures, in terms of energy, of this HBV variant were predicted using the program RNA Structure 3.5 (http://rna.chem.rochester.edu) (27). The resulting file was then analyzed for the probability of binding to complementary RNA oligonucleotides 7 nt long (which corresponds to the size of

the P1 stem formed by a substrate and a *delta* ribozyme) using the software OligoWalk (13).

Eleven HBV sequences from various genotypes [DDBJ/EMBL/GenBank accession numbers: A1 (X70185, X80924, X97848, X97849, X97850, X97851 and Z35717), B1 (D00329 and D00330), C1 (X75665) and C2 (L08805)] were retrieved from the database, aligned with the HBV sequence present in plasmid pCHT-9/3091 (26) using the ClustalW package (28), and minor adjustments introduced manually (http://penelope.med.usherb.ca/labojp/pdf/hbv.pdf). This sequence alignment was used to identify potential target sites conserved in most of the genotypes. Other criteria, including substrate specificity for *delta* ribozyme cleavage, were also used in order to identify the sites with the greatest potential (see Results).

HBV and ribozyme DNA constructs

The plasmid pCHT-9/3091 contains a full-length copy of the HBV pregenome (26). The HBV pregenome insert was subcloned downstream of the T7 RNA promoter within the vector pBlueScript SK (+/–) (Stratagene) using the *Sal*I and *Sac*I restriction sites (Fig. 1B). The resulting plasmid was named pHBVT7, and its identity was confirmed by DNA sequencing.

The construction of all ribozymes was performed as described previously (10). Briefly, pairs of complementary and overlapping DNA oligonucleotides, which corresponded to the P1 stem region, were synthesized (Invitrogen), annealed and ligated to *Pst*I and *Sph*I co-digested pδRzP1.1, yielding a plasmid harboring a *delta* ribozyme referred to as pδRz-HBVX, where X corresponds to the position of its potential cleavage site within HBV. The sequences of all ribozyme minigenes were confirmed by DNA sequencing.

RNA synthesis

Ribozymes and HBV RNA were transcribed *in vitro* using *Sma*I-linearized pδRz-HBVX and pHBVT7, respectively, as templates. Run-off transcriptions were performed in the presence of purified T7 RNA polymerase (10 μg), RNAguard (24 U, Amersham Biosciences), pyrophosphatase (0.01 U, Roche Diagnostics) and linearized plasmid DNA (5 μg) in a buffer containing 80 mM HEPES–KOH pH 7.5, 24 mM MgCl$_2$, 2 mM spermidine, 40 mM DTT, 5 mM of each NTP and with or without 50 μCi [α-^{32}P]GTP (New England Nuclear) in a final volume of 100 μl at 37°C for 4 h. Upon completion, the reaction mixtures were treated with DNase RQ1 (Amersham Biosciences) at 37°C for 20 min, purified by phenol:chloroform extraction and the nucleic acid precipitated with ethanol. The HBV RNA products and ribozymes were respectively fractionated by denaturing 5 and 7.5% polyacrylamide gel electrophoresis (PAGE; 19:1 ratio of acrylamide to bisacrylamide) in buffer containing 45 mM Tris–borate pH 7.5, 7 M urea and 1 mM EDTA. The reaction products were visualized either by UV shadowing or autoradiography. The bands corresponding to the correct sizes for the ribozymes and HBV RNAs were cut out and the transcripts eluted overnight either at room temperature (ribozymes) or at 4°C (HBV RNA) in a solution containing 0.5 M ammonium acetate and 0.1% SDS. The transcripts were desalted on Sephadex G-25 (Amersham Biosciences) spun-columns, and were then precipitated by the addition of 0.1 vol

3 M sodium acetate pH 5.2 and 2.5 vol ethanol, washed, dried, resuspended in ultrapure water and the quantity determined by either absorbance at 260 nm or ^{32}P counting.

Synthesis of a combinatorial library of ribozymes

The library of *delta* ribozymes with a random P1 stem was constructed using a PCR-based strategy (see inset in Fig. 4). Three DNA oligonucleotides were used: (i) the antisense oligonucleotides (Rz-pool; 5′-GGGTCCCTTAGCCATC-CGCGAACGGATGCCCANNNNNNNACCGCGAGGAGGT-GGACCC-3′, where N = A, C, G or T) served as the primary templates for the library of ribozymes; (ii) the sense primer (T7-5′Rz; 5′-TTAATACGACTCACTATAGGGTCCACCT-CCTCGCGGT-3′) permitted the incorporation of the T7 RNA promoter upstream of the minigene; and (iii) the antisense primer (3′Rz; 5′-GGGTCCCTTAGCCATCCGC-GAACGG-3′) amplified the template. The PCR mixtures contained 20 mM Tris–HCl pH 8.8, 6 mM MgCl$_2$, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 0.1% Triton X-100, 0.2 mM of each dNTP, 2 µM of each primer and 1 U Vent DNA polymerase (New England Biolabs) in a final volume of 100 µl. After an annealing step of 1 min at 94°C, 20 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 76°C were performed. The PCR products were purified by phenol:chloroform extraction, the nucleic acid precipitated with ethanol, resuspended in water, and then *in vitro* transcriptions and purifications of the ribozymes were performed as described above.

RNase H hydrolysis

RNase H reactions with ^{32}P randomly labeled HBV RNA were performed as a biochemical assay coupled with the data obtained from the bioinformatic tools. HBV RNA (~0.1 µM, ~20 000 c.p.m.) and DNA oligonucleotides (7 nt, 5 µM) were preincubated for 10 min at 25°C in a final volume of 8 µl containing 20 mM Tris–HCl pH 7.5, 20 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA and 0.1 mM DTT. RNase H (0.5 U; United States Biochemicals) was then added (2 µl) and the samples incubated at 37°C for 30 min. The reactions were quenched by adding 3 µl loading buffer (97% formamide, 0.025% xylene cyanol and 0.025% bromophenol blue) and the mixtures fractionated through denaturing 5% PAGE gels (19:1), which were analyzed with a PhosphorImager (Molecular Dynamics).

RNase H reactions were also performed with randomized oligonucleotides (8 nt, 5 µM) and non-radioactive HBV RNA (0.5 µM) under the conditions described above. After the RNase H reaction, water (90 µl) was added and the mixture phenol:chloroform extracted, the nucleic acids precipitated with ethanol, washed, dried and conserved for further analysis by primer extension.

Ribozyme cleavage assays

Cleavage reactions were carried out under single turnover conditions ([Rz] > [S]). Internally ^{32}P-labeled HBV RNA (50 nM) was mixed with ribozyme (1 µM) in a 10 µl mixture containing 50 mM Tris–HCl pH 7.5 and 10 mM MgCl$_2$, and then incubated at 37°C for 3 h. The reactions were stopped by the addition of loading buffer, electrophoresed on denaturing 5% PAGE gels and analyzed with a radioanalytic scanner. The specificity of cleavage of several ribozymes was also verified by primer extension.

The cleavage of full-length HBV RNA (50 nM) was also performed in the presence of the pool of *delta* ribozymes (10 µM) in a final volume of 10 µl, under the conditions described above. After the reaction, the volume was made up to 50 µl by adding water and the RNA precipitated with ethanol, washed, dried and conserved for further analysis by primer extension.

Primer extension analysis

A collection of DNA oligonucleotides complementary to the HBV RNA was purchased from Invitrogen. The nomenclature adopted referred to their respective complementary sequences on the full-length HBV: HBV199–184 (5′-TCATTAGTT-CCCCCC-3′), HBV343–329 (5′-CTGTTTCTCTTCCAA-3′), HBV488–474 (5′-GGCGAGGGAGTTCTT-3′), HBV646–632 (5′-AGGAAAAGATGGTGT-3′), HBV875–861 (5′-ATA-TACCCGCCTTCC-3′), HBV1058–1044 (5′-GTTGGGATT-GAAGTC-3′), HBV1245–1231 (5′-GTGGAGACAGCG-GGG-3′), HBV1495–1481 (5′-AAAAACCCCGCCTGT-3′), HBV1698–1684 (5′-GCAGGATGAAGAGGA-3′), HBV2040–2026 (5′-CCCAATACCACATCA-3′), HBV2499–2485 (5′-ACCAAGCCCCAGCCA-3′), HBV2784–2730 (5′-AGA-CGGAGAAGGGGA-3′), HBV3028–3014 (5′-CCCCCA-ACTCCTCCC-3′). The oligonucleotides (10 pmol) were 5′-end labeled in a mixture containing 10 µCi [γ-^{32}P]ATP (3000 mCi/mmol; New England Nuclear), 50 mM Tris–HCl pH 7.6, 10 mM MgCl$_2$, 10 mM 2-mercaptoethanol and 12 U of T4 polynucleotide kinase (United States Biochemicals) at 37°C for 30 min. The end-labeled oligonucleotides were purified on denaturing 20% PAGE gels, the relevant bands excised from the gel and eluted overnight at room temperature, passed through G-25 spun-column, ethanol precipitated, washed, dried and resuspended in water (60 µl). 5′-^{32}P-labeled primer (6 µl) and 10× reverse transcription buffer (0.6 µl of 500 mM Tris–HCl pH 8.3, 800 mM KCl and 100 mM MgCl$_2$) were used to dissolve the pellets resulting from either an RNase H hydrolysis or a ribozyme cleavage assay performed with a pool of either oligonucleotides or ribozymes, respectively. The primer annealing step was performed by successively incubating the mixtures at 65°C for 2 min followed by 2 min on ice. The reactions were initiated by adding 0.8 mM of each dNTP, 3.3 mM DTT and Superscript™ II Reverse Transcriptase (100 U; Invitrogen) in a final volume of 12 µl. The samples were incubated at 45°C for 30 min, then ethanol precipitated, washed and analyzed through 5% sequencing PAGE gels. DNA sequencing reactions using the same primer were migrated on the same gels in order to allow for identification of both the primer extension stops and the cleavage sites of the ribozymes. The results were visualized with a PhosphorImager.

RESULTS

Bioinformatic tools coupled to biochemical assays

Initially, an experimental procedure composed of three steps, including the use of bioinformatic tools and biochemical assays, was developed (Fig. 2A). The first step consists of the prediction of the RNA secondary structure and the subsequent identification of the sequences most likely to be in single-stranded regions. Using the RNA folding algorithm in RNA
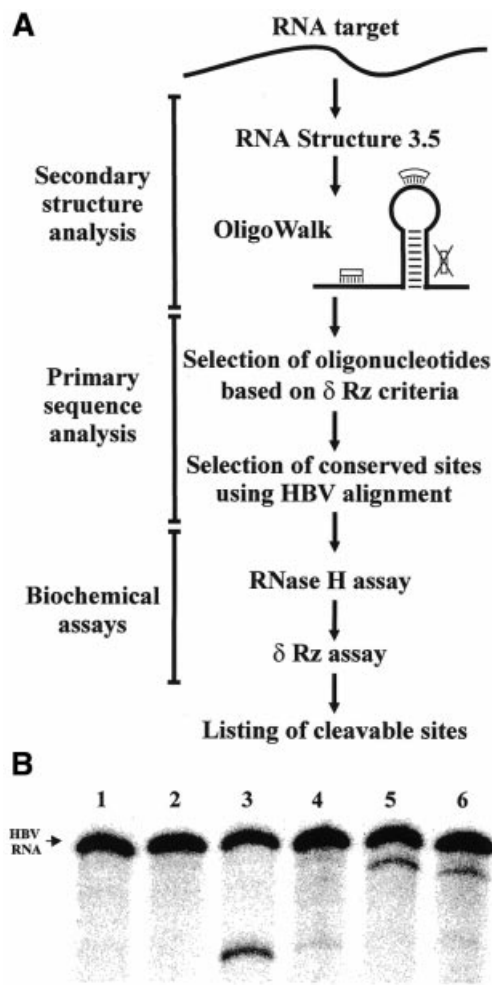
Figure 2. Selection of the sites with the greatest potential for *delta* ribozyme cleavage based on the use of bioinformatic tools coupled to biochemical assays. (**A**) Scheme of the steps that compose this procedure. Adjacent to the OligoWalk is an illustration of an example RNA structure that includes two oligonucleotides binding sites located in single-stranded regions and one (indicated by the large X) that is unavailable because it is located in a double-stranded region. (**B**) Typical results from biochemical assays for two different sites. Lanes 1 and 2 are the negative controls for the RNase H and ribozyme assays, respectively. In each case the HBV RNA was incubated under the reaction conditions, but either the oligonucleotide or the ribozyme were lacking. Lanes 3 and 5 are the RNase H assays in the presence of the oligonucleotide directing the hydrolyses at positions 1154 and 1543, respectively. Lanes 4 and 6 are the ribozyme assays for cleavage at positions 1154 and 1543, respectively.

Structure 3.5 (27), eight structures were derived for the HBV RNA pregenome (data not shown). With the exception of minor local differences, the structures were similar and their stabilities, based on the values of relative free Gibbs energy [ΔG determined by free energy minimization (27)], varied by <5%. Regardless of the HBV natural sequence variant in question, the overall organizations of the structures obtained were similar, suggesting that no important differences can be attributed to a given genotype. The eight resulting structures were then analyzed using the OligoWalk program (13). This algorithm predicts the equilibrium affinity of an RNA oligonucleotide for a given sequence, taking into consideration the predicted stability of the oligonucleotide-target helix

and the competition with the predicted secondary structures of both the target and the oligonucleotide. In our case the oligonucleotides correspond to the 7 nt composing the P1 binding domain of the *delta* ribozyme. The HBV sequence is 3358 nt in size and there are N − L + 1 complementary oligonucleotides of length L (where N and L are the lengths of the RNA target and oligonucleotide, respectively, that is 3358 − 7 + 1). Thus, the output consisted of a file of 3352 possible oligonucleotides, for each of which a ΔG value (corresponding to an overall free Gibbs energy value estimated for the interaction between an oligonucleotide and a given target) was calculated. Using an arbitrary cut-off of $\Delta G < -6.5$ kcal/mol, 343 sequences were retained as potential target sites.

The second step consists of screening the sites at the nucleotide sequence level based on two criteria. The first of these involved searching for specific sequence features known to be critical for efficient cleavage by a *trans*-acting antigenomic *delta* ribozyme. These features include: (i) the first base pair of the P1 stem must be a GU wobble base pair (29,30); (ii) there cannot be a guanosine at the cleavage site (position −1) as this results in an uncleavable substrate (6,7,30); (iii) the presence of two consecutive pyrimidines at position −1 and −2 dramatically reduces the cleavage efficiency of a substrate (30); (iv) the presence of a cytosine at position +4 (i.e. in the middle of the ribozyme strand of the P1 stem) significantly reduces the level of cleavage (31). Searching the 343 sequences for those that possess these four features reduces the list to 15 potential target sites. The second criteria we used was to analyze a sequence alignment of the natural HBV variants in order to identify which of the potential target sites are conserved. The identification of the highly conserved sequences in an RNA species should lead to the development of nucleic acid drugs that have the ability to specifically bind to all, or most, variants of this species. Twelve HBV variants from representative genotypes were aligned (Materials and Methods). This alignment revealed that nine of the 15 potential target sites are relatively highly conserved (i.e. conserved in at least 9 out of the 12 variants). These sequences are listed in Table 1.

In order to give a biochemical dimension to the procedure, the potential sites were then investigated using two assays in solution. First, RNase H hydrolysis using 7 nt long DNA oligonucleotides corresponding to the recognition domain of the ribozyme (i.e. N_6C) were performed. RNase H specifically cleaves the RNA of an RNA–DNA duplex and can be used to verify whether or not the binding of an oligonucleotide occurred and is specific to a target sequence (10). Randomly labeled HBV RNA was pre-incubated with unlabeled DNA oligonucleotides, RNase H digested and the mixtures analyzed on denaturing PAGE gels. A typical gel is shown in Figure 2B. The presence of the oligonucleotides corresponding to positions 1154–1160 and 1543–1549 allowed the RNase H to cleave the HBV RNA, although at different levels (lanes 3 and 5). These results are summarized in Table 1 (upper part). Three types of results were obtained: (i) the oligonucleotide corresponding to position 1147 did not result in any detectable cleavage under the conditions used; (ii) those for positions 409 and 2808 gave several non-specific products, suggesting that it bound to the HBV RNA at more than one position, a result that may be explained by the fact that RNase H requires only four

Table 1. Compilation of the potential sites identified by the computer method

Cleavage position	HBV P1 stem	ORF targeted[a]	ΔG (kcal/mol)	RNase H assay[c]	Rz assay[c]
409	GCCCCUA	C and P genes	−6.6	n.s.	n.d.
523	AGAUCUC	P gene	−8.0	++	−
524	GAUCUCA	P gene	−8.0	+	−
946	GCAUGGG	PreS1 gene	−8.5	+	−
1147	GCCCUCA	PreS1 gene	−9.7	−	n.d.
1154	GGCUCAG	PreS1 gene	−8.5	+++	+
1543	GGACUUC	S genes	−7.1	+++	++
2250	GGCCUAU	P gene	−8.3	+	−
2808	GCACCUC	X gene	−8.1	n.s.	n.d.
212[b]	GGGUGGG	C genes	−0.3	+++	+
486[b]	GCCUCGC	P gene	−0.9	+++	−
513[b]	GCGUCGC	P gene	−3.0	+++	+++

n.d., not determined; n.s., not specific.
[a]See Figure 1B.
[b]These sites were suggested not accessible.
[c]Relative level of cleavage.

consecutive base pairs to be active (32); and (iii) the six others that resulted in specific cleavage, albeit at different levels.

Secondly, the cleavage activity of the ribozymes was tested. *Delta* ribozymes with appropriate recognition sequences were synthesized by *in vitro* transcription from a minigene under the control of the T7 RNA promoter cloned into pUC19 for each of the six potential sites that gave specific RNase H hydrolysis. The ability of each ribozyme to cleave the labeled HBV RNA was tested under single-turnover conditions ([Rz] > [S]). After incubation at 37°C, the reactions were quenched and analyzed on PAGE gels. Typical results are shown in Figure 2B. The Rz-1154 cleaved the HBV RNA poorly, while the Rz-1543 appeared to be more efficient. Only two out of the six ribozymes tested exhibited cleavage of the HBV RNA (i.e. Rz-1154 and Rz-1543; see Table 1).

Thus, from the nine potential sites identified in the bioinformatic approach, only two were positive for *in vitro delta* ribozyme cleavage. Clearly, the biochemical assays are essential in order to validate the predictions derived from the bioinformatic analyses. The robustness of the initial computer predictions were further tested by analyzing seven additional sites with $\Delta G > -6.5$ kcal/mol (data not shown). The sequences of these sites respected the nucleotide requirements considered previously. Among these sites, three appear to be accessible based on the RNase H assay (Table 1, lower part). The ribozymes corresponding to these three sites were synthesized and their cleavage activities accessed. Rz-486 did not detectably cleave the HBV RNA, but both Rz-212 and Rz-513 did, although at different levels. Clearly, this approach appears to have important limitations, at least in the case of the *delta* ribozyme.

Biochemical approaches based on the use of libraries

Target site accessibility and the ability to form an active ribozyme–substrate complex are two interdependent factors whose relationship is complex. Such a relationship can be better addressed by using a combinatorial method. Consequently, we developed two procedures with a *delta* ribozyme based on previous reports for both the hammerhead and hairpin ribozymes (12).

A library of oligonucleotides. Figure 3A is a schematic representation of the procedure using a library of oligonucleotides. In theory, all accessible sites within an RNA molecule (i.e. those in single-stranded regions) would be specifically bound by an oligonucleotide and the resulting RNA–DNA heteroduplex subsequently hydrolyzed by RNase H (examples in 11,20). The cleavage sites can then be identified by electrophoresis of primer extension reactions using 5′-end-labeled primers and the RNase H reaction products. From the sequence of the substrate (HBV RNA), the sequence at the cleavage site may be determined and, thus, that of the complementary oligonucleotide (i.e. that acting as the P1 domain in the ribozyme). Finally, the corresponding ribozymes are synthesized and the cleavage activity tested.

The library used was composed of oligonucleotides 8 nt in length corresponding to one residue before the cleavage site, which has to be single-stranded (i.e. position −1), and the seven residues of the recognition domain of the ribozyme (i.e. the P1 stem). The libraries were designed taking into account the sequence specificities described earlier for *delta* ribozyme cleavage. More specifically, the nucleotide at position −1 relative to the cleavage site cannot be a guanosine (30), therefore the 3′-end nucleotide of the oligonucleotide cannot be a C. The first base pair of the P1 stem has to be a GU wobble pair (29,30), therefore the seventh nucleotide of all oligonucleotides was a cytosine (i.e. allowing the formation of a GC base pair). The larger library of oligonucleotides corresponds to the sequence 5′-N_6CD-3′ (i.e. 12 288 different sequences), while the smaller one corresponds to the sequence 5′-SSNDMSCD-3′ (i.e. 576 different sequences) (where N indicates A, C, G or U; D indicates A, G or U; S indicates C or G; and, M indicates A or C). The latter library was designed to include more of the sequence preferences of *trans*-acting *delta* ribozymes, and to favor stronger binding of the substrate to the ribozyme due to the presence of a higher percentage of GC residues.

A typical example of the identification of a potential site is illustrated in Figure 3B. In this case the primer extension was performed using an oligonucleotide complementary to positions 632–646 of the HBV RNA. In the absence of any
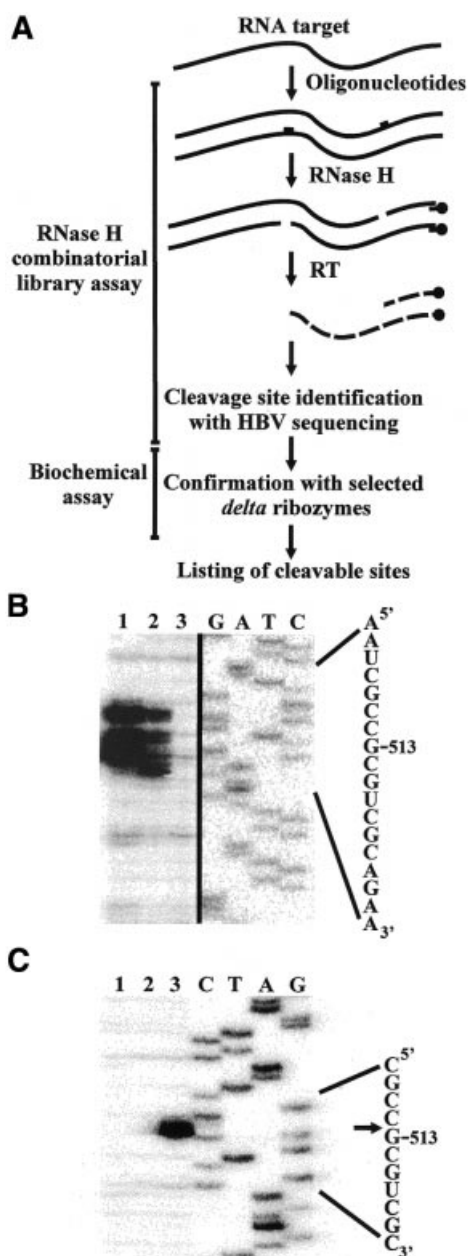
Figure 3. Selection of the sites with the greatest potential for *delta* ribozyme cleavage based on the use of an RNase H assay in the presence of a library of oligonucleotides. (**A**) Scheme illustrating the procedure. HBV RNA and cDNA are represented by the full and dashed lines, respectively. RT is reverse transcriptase. (**B**) Autoradiogram of a sequencing gel revealing the single-stranded region that includes position 513. Lanes 1 and 2 correspond to the reactions performed in the presence of the 5′-SSNDMSCD-3′ and 5′-N$_6$CD-3′ libraries, respectively (where N = A, C, G or U; D = A, G or U; M = A or C; and S = C or G). Lane 3 is the reaction performed in the absence of any oligonucleotide. Adjacent to the gel is a DNA sequencing reaction, and the corresponding sequence is shown on the right side. (**C**) Autoradiogram of a sequencing gel of the cleavage assay for Rz-513. Lanes 1 and 2 are reactions performed in the absence of either ribozyme or MgCl$_2$, respectively. Lane 3 is the complete reaction. After the cleavage reaction, primer extension was performed and migrated beside a sequencing reaction. The sequence is shown adjacent to the gel. The arrow indicates the cleavage site.

oligonucleotide no band was observed in the specific region as compared with lanes 1 and 2 (lane 3). In the presence of the oligonucleotides from the 5′-N$_6$CD-3′ library, several bands were detected (lane 2). This indicates the presence of a single-stranded region accessible to several oligonucleotides binding on different copies of the RNA target, and results in cleavage at various contiguous positions. In the presence of oligonucleotides from the 5′-SSNDMSCD-3′ library (lane 1), fewer bands were observed. This reflects the smaller expansion of the sequences composing the library. However, since each sequence is present in a higher concentration (i.e. for the same absolute amount of oligonucleotides) the concentration of cleaved RNA, and consequently cDNA, is larger. This assay suggested that a ribozyme may be engineered for cleavage at position 513 (i.e. Rz-513). Rz-513 specifically and efficiently cleaved the HBV RNA as shown by the primer extension assay in Figure 3C.

Since each primer produced a readable sequence of 200–300 bases, several primers were required in order to map the full HBV RNA. An average of two potential sites were detected for each primer extension reaction (i.e. per ~200–300 bases) based on the detection of either one, or stretches of, band(s). Most of the time, either one or two consecutive bands of weak intensity were detected. Preliminary experiments have shown that such bands do not indicate productive sites. When we considered the detection of three or more consecutive bands in primer extension, we retrieved only six potential sites (Table 2). In each case, the region was analyzed to determine the most appropriate sequence in terms of cleavage by a *delta* ribozyme (i.e. position –1 to the cleavage site must be A, C or U). With the exception of Rz-887, five of the six ribozymes exhibited a relatively efficient cleavage of the HBV RNA under the conditions used (Table 2). Of these five active ribozymes, four were conserved (only Rz-574 was not) with respect to the sequence alignment of the HBV variants (i.e. present in at least 9 out of the 12 variants).

A combinatorial library of ribozymes. Finally, we tried an approach based on a library of *delta* ribozymes (Fig. 4A). The most important advantage of this approach should be that the detection of a cDNA band is directly related to the cleavage of a ribozyme, whereas the bands produced by the previous RNase H-based assay were an indirect indication. Consequently, this method is theoretically better because the selection is based not only on a mimic of the binding site, but rather on both binding and cleavage by the ribozyme.

A pool of ribozymes was synthesized using a minigene under the control of a T7 RNA polymerase promoter (see inset, Fig. 4A). The minigene was made from a pool of antisense oligonucleotides in which the positions corresponding to the P1 stem were randomized, and the second strand synthesized using a sense primer that formed the T7 RNA promoter. The library was based on the randomization of the six positions of the P1 stem (i.e. 5′-N$_6$U-3′, giving 4096 ribozymes). This library took into account the requirement for a U for the formation of the GU wobble base pair adjacent to the cleavage site (29,30). The ribozymes (i.e. 2.4 nM of each individual ribozyme) were incubated with HBV RNA (50 nM), and the cleavage sites identified by electrophoresis of primer extension reactions as was done for the RNase H approach

Table 2. Compilation of the potential sites identified by the RNase H method

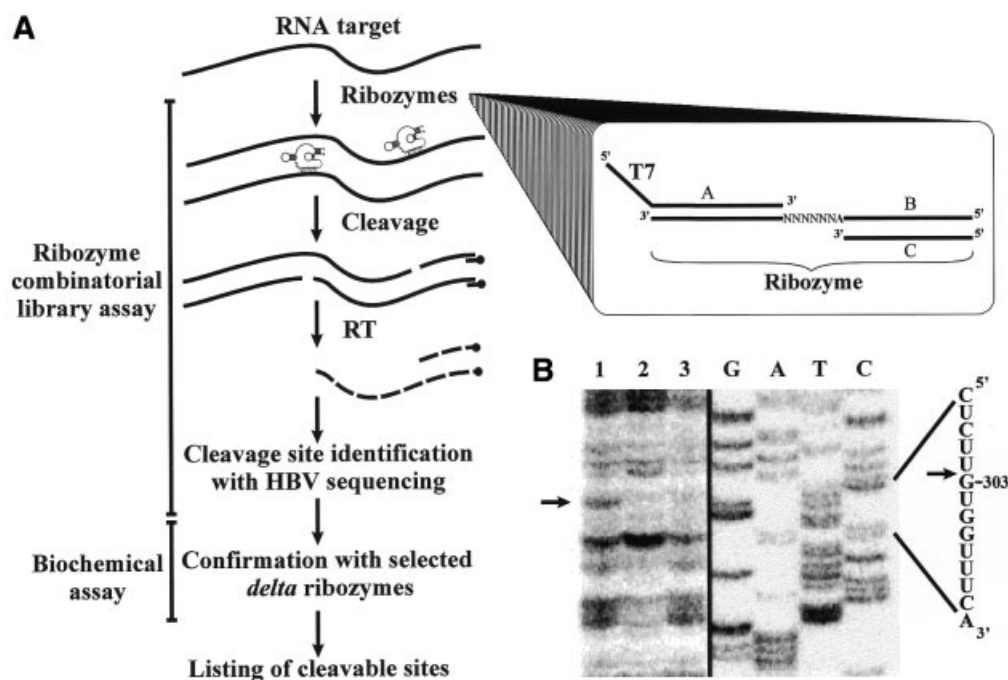| Cleavage position	HBV P1 stem	ORF targeted[a]	RNase H assay (N)[b]	Rz assay[c]
167	GGCAAGC	C genes	+ (3)	+++
279	GGGCCUA	C genes	+ (4)	+++
461	GGUCCCC	P gene	+ (4)	++
513	GCGUCGC	P gene	+ (9)	+++
574	GGGGAAC	P gene	+ (3)	+
887	GAAACAA	P gene	+ (7)	−

[a]See Figure 1B.
[b]N indicates number of cleavage bands.
[c]Relative level of cleavage.

Figure 4. Selection of the sites with the greatest potential for *delta* ribozyme cleavage sites based on a ribozyme cleavage assay performed in the presence of a ribozyme library. (**A**) Scheme illustrating the procedure. HBV RNA and cDNA are represented by the full and dashed lines, respectively. RT is reverse transcriptase. The PCR strategy used to synthesize the library is shown in the inset in which oligonucleotides A and C served as sense and antisense primers, respectively, to amplify the randomized sequences of the templates (oligonucleotide B). (**B**) Autoradiogram of a sequencing gel revealing the cleavage site at position 303. Lane 1 is the reaction performed in the presence of ribozymes and MgCl₂, while MgCl₂ and the ribozyme were omitted in the reactions in lanes 2 and 3, respectively. Adjacent to the gel is a DNA sequencing reaction, and the corresponding sequence is shown on the right side. The arrow indicates the cleavage site.

described above. A typical example of the identification of a site is illustrated in Figure 4B. In this case the primer extension was performed using an oligonucleotide complementary to positions 329–343 of the HBV RNA. The presence of the pool of ribozymes resulted in the detection of an additional band corresponding to cleavage at position 303 (lane 1) as compared with the results obtained in the absence of either MgCl₂ (lane 2) or the ribozyme (lane 3). Scanning the entire length of the HBV RNA revealed six potential sites (Table 3). Subsequently, specific *delta* ribozymes were made from the minigenes and their cleavage activities assessed. Five out of the six cleaved the HBV RNA, with the Rz-303, Rz-1543, Rz-2833 and Rz-2861 recognizing conserved sequences of the HBV variants (i.e. present in at least in 9 out of 12 variants).

DISCUSSION

Procedures for the selection of sites

This work presents the development of three procedures for the identification of *delta* ribozyme cleavage sites within an RNA target, a step essential to the development of a gene therapy. The first approach consists of an experimental scheme composed of three steps, including the use of bioinformatic tools coupled to biochemical assays (Fig. 2). At its heart, this procedure is based on computer prediction of the RNA secondary structure, a procedure that is the most popular method of determining the structures of all kinds of RNA (13,16) and elucidating the regions that are most likely to be single-stranded. Of the nine potential sites obtained by the computer-assisted analysis, six were both specific and

Table 3. Compilation of the potential sites identified by the library of *delta* ribozymes

Cleavage position	HBV P1 stem	ORF targeted[a]	Rz pool assay[b]	Rz assay[b]
303	GUGGUUU	C genes	++	+++
1365	GUAAACC	Pre S2	+	−
1543	GGACUUC	S genes	++	++
2624	GUUGUCC	P gene	+	++
2833	GUCUGUG	X gene	+	+++
2861	GUGCACU	X gene	+	+++

[a]See Figure 1B.
[b]Relative level of cleavage.

effective for the RNase H hydrolysis (Table 1). However, only two members of this subset were cleaved by the appropriate ribozymes. Although the two first steps were performed rapidly, as the coupling of the biochemical approach is essential to providing relevant results, this procedure did not result in any significant gains in terms of time. We concluded that this bioinformatic approach has severe limitations, at least in the case of *delta* ribozyme. Many reasons can be put forth to explain the discrepancy between the computer predictions and sites effectively cleaved by the ribozymes, including the fact that the tertiary structures of both the ribozyme and the RNA target were not taken into account by the software. However, the most important reason is the fact that, even today, the prediction of RNA structure remains a complicated task for long RNA molecules (8–10).

Based on our results, a combinatorial method is the best means of overcoming the complexed interdependent nature of target site accessibility and the ability to form an active ribozyme–substrate complex. In the RNase H procedure, the presence of combinatorial libraries of oligonucleotides mimics the binding of the ribozyme. Taking into account some of the substrate specificity features of the *delta* ribozyme cleavage allowed us to both limit the expansion of the library and to 'personalize' it to this catalytic RNA. Preliminary results showed that only the presence of a stretch of bands, corresponding to consecutive stops of the primer extension, was indicative of a productive site. The binding domain of the substrate is restricted to seven bases, but a larger single-stranded domain is required in order to both satisfy the tertiary structure of the ribozyme and permit its molecular mechanism (which involves a conformational transition of the ribozyme–substrate complex) (7). The procedure based on a combinatorial library of *delta* ribozymes randomized for their recognition sequences offers the advantage of selecting sites that are bound by a ribozyme and subsequently cleaved. In that case only single bands were seen. Both combinatorial methods allowed us to identify six potential sites, five of which were cleaved *in vitro* by the appropriate ribozymes in each case. However, none of the selected sites were identified by both the oligonucleotide and ribozyme libraries. This observation indicates that none of the methods permits the identification of all potential sites, which is clearly a limiting factor.

Substrate specificity for *delta* ribozyme cleavage

Using small model substrates, the specificity for *delta* ribozyme cleavage has been determined *in vitro* (29–31,33). Briefly, the P1 stem is composed of the 7 nt that base pair with the ribozyme; hence, these nucleotides can be defined as internal determinants of substrate specificity. Moreover, it has been demonstrated that the nucleotide composition at positions −1 to −4 from the scissile bond influences the efficiency of cleavage of small substrates (31,33). For example, the presence of two, single-stranded, consecutive pyrimidines at positions −1 and −2 decreases the levels of cleavage to varying degrees depending on the identity of the residues in question. The nucleotides in positions −1 to −4 do not directly contribute to substrate recognition (e.g. substrate binding), but the identity of these residues is essential for efficient cleavage (10,30). Therefore, they contribute to substrate specificity, likely as external determinants. In this study, the nucleotides at positions −1 to −4 had various compositions. Most of the cleavage sites retained do not possess consecutive pyrimidines at positions −1 and −2, or if they did they were inefficiently cleaved, supporting the idea that the rules established with small substrates are consistently respected in a long RNA target. However, we also observed exceptions to these rules: for example, the Rz-513 exhibited an efficient cleavage even though two cytosines are located at positions −1 and −2. This result shows that the situation of a small substrate is slightly different to that of a long molecule such as HBV RNA. In fact, there seems to be a complex situation in which several factors, including the secondary structure motifs in close proximity to a potential site, as well as long interactions, modulate the efficiency of the cleavage.

Any contribution to the substrate specificity for *delta* ribozyme cleavage is important for further development *in vivo*. As any given 7mer that corresponds to the length of the P1 stem (the binding domain) might be found at a frequency of one in 4^7 nt (i.e. 16 384), duplicate targets might be present in the population of cellular mRNA, and cleavage of these RNAs could prove toxic to the cell. Therefore, the modulation of cleavage efficiency caused by the sequences in positions −1 to −4 of the cleavage site might be of primary importance in minimizing this problem. The net effect of this is to extend from 7 to 11 contiguous nucleotides the domain of the substrate that contributes to determining the ability of an RNA molecule to be cleaved by *delta* ribozyme. Furthermore, it is important to remember that the complexity of an RNA structure drastically reduces the number of potential sequences that can be considered as potential sites to target. In this study, the use of a combinatorial library including thousands of different ribozymes did not allow us to retrieve too many active ribozymes even though the sequence complementary to the recognition sequence was present within the HBV RNA. Clearly, the secondary and tertiary structures of the RNA molecules, as well as their complexation with other cellular

components (e.g. with proteins forming RNPs) also make important contributions to minimizing the problem of lack of specificity.

Targeting HBV

Together, the three procedures used in this work yield a collection of 12 *delta* ribozymes with the potential to target throughout the HBV RNA pregenome. Comparing the sites identified here, within the HBV RNA, with those of other reports, based on the use of other catalytic RNAs like the hammerhead and the hairpin ribozyme (14,24,25,34–37), shows no similarities. Moreover, when we consider the sites targeted in other HBV works it appears impossible to engineer *delta* ribozyme cleaving at these positions, primarily because the *delta* ribozyme has different characteristics than other catalytic RNAs. On the other hand, the extension of this work would be to show whether or not some, or all, of the *delta* ribozymes of the collection would be effective in controlling the propagation of HBV *in vivo*. However, previous reports have demonstrated the existence of relatively good correlations between *in vitro* and *in vivo* for both oligonucleotide and ribozyme effects (19,20,22,38).

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