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Putative Secondary Structure of Human Hepatitis B Viral X mRNA

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Abstract: A putative secondary structure of the mRNA for the human hepatitis B virus (HBV) X gene is proposed based on not only chemical and enzymatic determination of its single- and double-stranded regions but also selection by the computer program MFOLD for energy minimum conformation under the constraints that the experimentally determined nucleotides were forced or prohibited to base pair. An RNA of 536 nucleotides including the 461-nucleotide HBV X mRNA sequence was synthesized *in vitro* by the phage T7 RNA polymerase transcription. The thermally renatured transcripts were subjected to chemical modifications with dimethylsulfate and kethoxal and enzymatic hydrolysis with single strand-specific RNase T1 and double strand-specific RNase V1, separately. The sites of modification and cleavage were detected by reverse transcriptase extension of 4 different primers. Many nucleotides could be assigned with high confidence, twenty in double-stranded and thirty-seven in single-stranded regions. These nucleotides were forced and prohibited, respectively, to base pair in running the recursive RNA folding program MFOLD. The results suggest that 6 different regions (5 within X mRNA) of 14~23 nucleotides are single-stranded. This putative structure provides a good working model and suggests potential target sites for antisense and ribozyme inhibitors and hybridization probes for the HBV X mRNA.

Key words: hepatitis B virus X gene, RNA folding program MFOLD, RNA secondary structure, X mRNA.

The hepatitis B virus (HBV) is a DNA virus that is known to cause acute and chronic hepatitis, cirrhosis and hepatocarcinoma in humans. Among the 4 open reading frames designated S, C, P and X in the HBV genome, the X has been known to encode a transcriptional transactivator (Twu and Schloemer, 1987; Spandau and Lee, 1988; Zahm *et al.*, 1988; Colgrove *et al.*, 1989; Twu and Robinson, 1989; Aufiero and Schneider, 1990). Instead of directly binding to the target DNA sequence, the X protein seems to indirectly influence transcription in a manner similar to the adenovirus E1A transactivator (Colgrove *et al.*, 1989). Recently, an oncogenic role of X protein has been proposed in studies on a transgenic mouse and through transfection experiments (Wollerscheim *et al.*, 1988; Hohne *et al.*, 1990; Seto *et al.*, 1990; Wu *et al.*, 1990; Kim *et al.*, 1991). Therefore, the inactivation of the X mRNA in HBV-infected liver cells could be an efficient way to regulate the life cycle of HBV.

The inactivation of an mRNA can be achieved by binding of antisense nucleic acids or by cleavage through ribozyme RNAs (Izant and Weintraub, 1984; Castanotto *et al.*, 1992). Cleavage by ribozymes is potentially more powerful than antisense approaches, because of the irreversible inactivation of the target RNA and because of the turnover of the ribozymes. For example, the best turnover rate for hammerhead ribozymes is 9 min^{-1} (Hendry *et al.*, 1992). The hammerhead ribozymes were originally found in self-cleaving RNAs of the avocado sunblotch viroid (ASBV), the satellite tobacco ringspot virus (sTobRV), the satellite lucerne transient streak virus (sLTSV), among others (Hutchins *et al.*, 1986; Prody *et al.*, 1986; Buzayan *et al.*, 1986; Forster and Symons, 1987). Detailed analysis of these self-cleaving RNAs has led to development of catalytic antisense RNA of the hammerhead ribozyme type that can specifically cleave target mRNA *in vitro* and *in vivo* (Forster and Symons, 1987; Uhlenbeck, 1987; Haseloff and Gerlach, 1988).

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For both the antisense and ribozyme approaches, target RNA must bind to the antisense and ribozyme nucleic acids. Thus, it is critical to design such inhibitor nucleic acids to be able to bind to the single-stranded regions of the target RNA. For this purpose, the secondary structure of HBV X mRNA was probed in this study. Typically RNA secondary structures have been determined by using single- or double-strand specific chemicals and/or ribonucleases. Also the secondary structures can be predicted by energy minimization and phylogenetic comparison methods with reasonable accuracy. Since there are no counterparts of HBV X mRNA in other organisms for a phylogenetic comparison method, a combination of the direct chemical and enzymatic methods and the computational energy minimization method was used for determination of HBV X mRNA in this study.

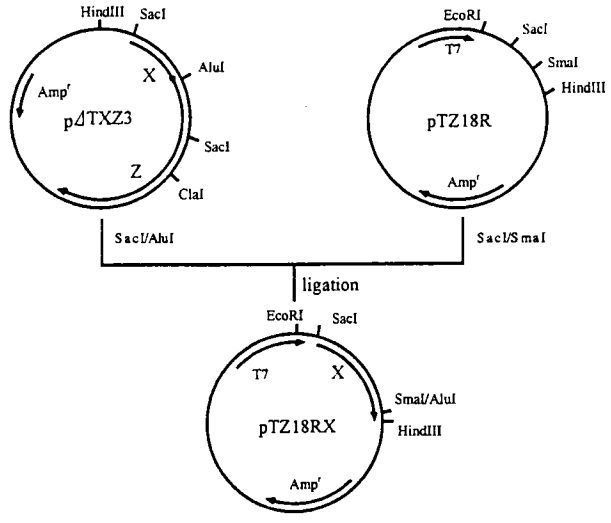

Fig. 1. The construction of pTZ18RX. The HBV X gene (X) was subcloned downstream of a T7 RNA polymerase promoter (T7) of pTX18R from the pΔTXZ3 in which it was fused to the *lacZ* gene (Z).

Materials and Methods

Materials

The enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Sac*I, phage T4 DNA ligase and AMV (avian myeloblastosis virus) reverse transcriptase were purchased from Poschem, and phage T7 RNA polymerase and RNasin from Promega. [α-^{35}S]dATP (1,000 Ci/mmol, 10 Ci/l) was purchased from Amersham. dNTP, ddNTP and rNTP were purchased from Boehringer Mannheim. Primer 1 (5′ AGGCCCAAACGGGCCCCG3′), primer 2 (5′AGGTGAAGCGAAGTGCA3′), primer 3 (5′GTATGCCTCAAGGTCGG3′) and primer 4 (5′CTCTAGAGGATCCCCCT3′) were synthesized at the Seoul National University Basic Science Research Core Facilities.

The HBV X gene of the adr subtype was cloned from the plasmid pTXZ3 (Kim and Yu, 1989), in which the X gene is fused to the *lacZ* gene, into a pBR322 derivative through various cloning measures (Lee, 19 93). The resulting plasmid of 7 kb was named pΔTXZ 3. Only the X gene was moved from pΔTXZ3 to a T7 promoter-containing vector pTZ18R (Pharmacia). The plasmid pΔTXZ3 was digested by *Sac*I, and the 2.4-kb fragment was eluted by using an NA-45 DEAE column. This fragment was then digested by *Alu*I, and the 0.5-kb fragment was eluted and inserted into the *Sac*I/*Sma*I site of the vector pTZ18R. The resulting plasmid was named pTZ18RX (Fig. 1).

In vitro synthesis of HBV X RNA

The X RNA was synthesized *in vitro* by phage T7 RNA polymerase transcription under the conditions previously described by Melton *et al.* (1984) from the template plasmid pTZ18RX that was linearized by *Hin*dIII. The 100-μl reaction mixtures contained 20 μl of 5× transcription buffer (200 mM Tris-HCl, pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine and 50 mM NaCl), 10 μl of 0.1 M dithiothreitol, 40 units of RNasin, 20 μl of 2.5 mM NTP mixture, 2~3 μg of template DNA and 30~50 units of phage T7 RNA polymerase and were incubated at 37℃ for 90 min. The samples were subsequently treated with 10 units of DNase I at 37℃ for 15 min.

Sequencing of the X RNA using reverse transcriptase

The nucleotide sequence of the *in vitro* synthesized X RNA was directly determined by the dideoxy method previously described by Sanger *et al.* (1977) using reverse transcriptase, except for its small 3′-end region whose sequence was deduced from the DNA sequence. Four 17-nucleotide long primers were designed to correspond to the regions 131~147 (primer 1), 245~262 (primer 2), 356~372 (primer 3) and 502~518 (primer 4) of the X RNA (Fig. 2). The RNA (2 pmol) was dissolved in 10 μl of the reverse transcriptase reaction buffer (34 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 50 mM NaCl and 5 mM dithiothreitol), heated at 67℃ for 3 min and slowly cooled to 42℃. After adding 7.5 units (1 μl) of reverse transcriptase, 20 μCi [α-^{35}S] dATP and 4 μl of 0.1 M dithiothreitol, a 3-μl aliquot of the mixture was added to each 3-μl ddNTP mixture. The reaction was carried out at 42℃ for 20 min and extended for another 15 min with 1 μl of the extension mixture (34 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 50 mM NaCl, 5 mM dithiothreitol and 2 mM each dNTP) added. Five μl of the stop solution (98% formamide, 10 mM EDTA, 0.3% xylene cyanole and 0.3% bromophe-

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gggcg aauuc gagcu cgaau uaauu ccccg gauca auugA UGGCU GCUCG
GGUGU GCUGC CAACU GGAUC CUGCG CGGGA CGUCC UUUGU CUACG UCCCG 100
UCGGC GCUGA AUCCC GCGGA CGACC CGUCU CGGGG CCGUU UGGGC CUCUA
CCGUC CCCUU CUUCA UCUGC CGUUC CGGCC GACCA CGGGG CGCAC CUCUC 200
UUUAC GCGGU CUCCC CGUCU GUGCC UUCUC AUCUA CCGGA CCGUG UGCAC
UUCGC UUCAC CUCUG CACGU CGCAU GGAGA CCACC GUGAA CGCCC ACCAG 300
GUCUU GCCCA AGGUC UUACA UAAGA GGACU CUUGG ACUCU CAGCA AUGUC
AACGA CCGAC CUUGA GGCAU ACUUC AAAGA CUGUU UGUUU AAAGA CUGGG 400
AGGAG UUGGG GGAGG AGAUU AGGUU AAAGG UCUUU GUACU AGGAG GCUGU
AGGCA UAAAU UGGUC UGUGC ACCAG CACCA UGCAA CUUUU UCACC UCUGC 500
aagggg ggauc cucua gaguc gaccu gcagg caugc a
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Fig. 2. The nucleotide sequence of the transcript RNA that was synthesized *in vitro* from pTZ18RX by the phage T7 RNA polymerase. The sequence for the HBV (adr subtype) X mRNA is in capital letters. The hybridization sites of the 4 primers are underlined.

nol blue) were added, and the samples were stored at −20℃.

Chemical and enzymatic probing of the X RNA secondary structure

For chemical and enzymatic determination of the secondary structure, 2 pmol of the *in vitro* synthesized X RNA were dissolved with 5 μg of carrier RNA in 200 μl of HMK solution (70 mM Hepes-KOH, pH 7.8, 10 mM $MgCl_2$, 270 mM KCl and 1 mM dithiothreitol), and the mixture was incubated at 55℃ for 10 min and then slowly cooled to room temperature. The reactions for methylation of unpaired A's and C's by dimethylsulfate were carried out at 30℃ for 5 min with 1 μl of the 50% dimethylsulfate solution (dimethylsulfate : ethanol, 1 : 1) added, and stopped by adding 50 μl of the dimethylsulfate stop solution (1 M Tris-acetate, pH 7.5, 1 M 2-mercaptoethanol, 1.5 M sodium acetate and 0.1 mM EDTA) and 750 μl of ethanol. After several washing steps, the precipitates were dissolved in distilled water to a concentration of 0.5 μM. The reactions for the kethoxal modifications of unpaired G's were carried out at 30℃ for 10 min with 5 μl of the kethoxal solution (40 g/l) added, and stopped by adding 20 μl of 3 M sodium acetate and 10 μl of 0.5 M boric acid. The ethanol precipitates, after several washing cycles, were dissolved in a solution containing 10 mM Tris-HCl, pH 7.4, 50 mM potassium borate and 0.1 mM EDTA to a concentration of 0.5 μM. In the control experiments, the dimethylsulfate and kethoxal solutions were added just before the first ethanol precipitation step to measure the extent of chemical reactions that might occur during the extraction of the modified RNA.

For the enzymatic cleavage reactions, 2 pmol of the RNA transcript were dissolved with 5 μg of carrier RNA in 40 μl of TMK solution (30 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 300 mM KCl and 1 mM dithiothreitol), incubated at 55℃ for 10 min and slowly cooled to room temperature. The reactions with either 0.01 unit of RNase T1 or 0.16 units of RNase V1 were carried out at 0℃ for 30 min. After ethanol precipitation with 150 μl of 0.3 M sodium acetate (pH 6.0) and several washing cycles, the precipitates were dissolved in distilled water to a concentration of 0.5 μM. The control experiments were carried out without addition of the ribonucleases.

Detection of probing sites by primer extension

The same 17-nucleotide long primers that were used for sequecing RNA as described above (Fig. 2) were used for detection of probing sites. The chemically modified or enzymatically cleaved RNA (2 pmol) were incubated with a primer (0.7 pmol) in the reverse transcriptase reaction buffer at 67℃ for 3 min and slowly cooled to 42℃. Then, the primer extension reaction (10 μl) was carried out with 2.5 μl of the dNTP mixture (each 250 μM in the reverse transcriptase buffer), 1 μl of 0.1 M dithiothreitol, 1 unit of reverse transcriptase, 5 μCi [α-^{35}S]dATP at 42℃ for 20 min and was extended with 1 μl of the extension mixture added for another 15 min. The reactions were stopped by adding 5 μl of the stop solution, and the mixtures were stored at −20℃. All the reaction mixtures were incubated at 70℃ for 3 min, then separated by electrophoresis on an 8% (w/v) polyacrylamide-7 M urea denaturing gel (35 cm × 42 cm × 0.04 cm) and were detected by autoradiography.

Results and Discussion

The RNA transcript, which was synthesized *in vitro* by the phage T7 RNA polymerase from the plasmid pTZ18RX linearized by *Hin*dIII consists of 536 nucleotides. It includes the 461 nucleotide-long HBV X sequence (capital letters in Fig. 2), and also 39- and 36-nucleotide plasmid-derived sequences at its 5′ and 3′ ends, respectively. The numbering starts with the first nucleotide of the RNA transcript (designated as +1). Thus, the X mRNA is from +40 to +500. In order to probe the single- and double-stranded regions of the transcript RNA, it was chemically modified or enzymatically hydrolyzed, after being thermally denatured and renatured. These modified transcripts were then subjected to primer extension analysis using 4 different primers and separated by gel electrophoresis. Control experiments of the chemical and enzymatic modifications allowed for removing the noise caused by non-specific cleavages under the experimental conditions.

Base-specific sequencing ladders were generated with ddNTPs and the same primer by reverse transcriptase. For each sample, the RNase T1, RNase V1, dimethylsulfate, kethoxal and 3 control reactions, as well as the 4 sequencing ladders were loaded in parallel. Also electrophoresis was run for both short and long periods to read all the bands of 100~130 nucleotide-long regions between the primers. Thus, 8 gels for 4 primers constituted a full set of data and each reaction was reproduced at least twice. The detection of modification and cleavage sites was limited up to about +500, because the last primer hybridized to the region from +502 to +518.

These data alone were not sufficient to reveal all the details of the secondary structure of X mRNA. However, data on certain regions were clear and reproducible enough to assign either single- or double-stranded regions. Those regions are listed in Table 1. The 20 nucleotides in 8 separate regions are clearly involved in base pairing, because RNase V1 cleaved those regions but RNase T1, dimethylsulfate or kethoxal did not react with them. Detection of RNase V1 cleavage sites indicates that the two nucleotides right next to the cleaved phosphodiester bond are involved in base pairing. Eight additional nucleotides (81, 94~98 and 114~115) in 3 regions could also be double-stranded, but the reactions were only partial.

Thirty seven nucleotides separated in 29 non-contiguous regions were found to be single-stranded (Table 1). These assignments were based on the results that RNase T1, dimethylsulfate or kethoxal reacted with the regions but RNase V1 did not cleave them. Another 11 nucleotides at 84, 138, 206, 217, 272, 323, 324, 328, 334, 335 and 394 could also be unpaired, although the results were not as evident as others. Thus, 57 nucleotides out of 461 X mRNA nucleotides were assigned for their involvement in secondary structure with high confidence (Table 1) and 19 more nucleotides could be assigned with reservation.

Although the X RNA may have a flexible structure, the transcript was not degraded significantly during the heat denaturation and thermal renaturation cycle (heating at 67℃ for 3 min and slowly cooling to room temperature). Fifteen phosphodiester bonds were especially vulnerable, as the corresponding bands were observed in all the lanes. Although the nucleotides around these bonds were more likely to be unpaired than involved in stem structures, they were not assigned either way and were not included in the computer execution as the constraints for selecting the energetically minimum structure (described below).

Artifactual bands were also observed, as reverse transcriptase prematurely stopped before reaching the ends of RNA. AMV reverse transcriptase has been reported to stop not only at chemically modified or cleaved nucleotides, but also sometimes at oligo(A) or oligo(U) tracts and at the bases of stem structures (Mierendorf and Pfeffer, 1984). Stern *et al.* (1988) have attempted to minimize these reverse transcription artifacts by changing the transcript-to-primer ratio, by adding more enzyme

Table 1. The nucleotides determined to be located in the double- and single-strand regions by probing accessibility to chemical modification and enzymatic hydrolysis

Base	Feature	Base	Feature	Base	Feature
60 C	Paired	181 G	Unpaired	322A	Unpaired
61 C	Paired	186 C	Unpaired	343 G	Unpaired
68 A	Unpaired	187 G	Unpaired	345 A	Unpaired
79 U	Paired	188 G	Unpaired	346 A	Unpaired
80 A	Paired	193 C	Unpaired	348 G	Unpaired
89 G	Unpaired	194 A	Unpaired	354 G	Unpaired
103 G	Unpaired	207 C	Unpaired	358 G	Unpaired
104 G	Unpaired	210 U	Paired	379 G	Unpaired
106 G	Unpaired	211 C	Paired	383 G	Unpaired
109 G	Unpaired	212 U	Paired	387 G	Unpaired
110 A	Unpaired	218 U	Paired	429 G	Unpaired
111 A	Unpaired	219 C	Paired	442 G	Unpaired
122 G	Unpaired	220 U	Paired	444 A	Paired
124 C	Unpaired	243 G	Unpaired	445 G	Paired
125 C	Unpaired	254 G	Unpaired	451 A	Paired
132 G	Unpaired	301 G	Unpaired	452 G	Paired
142 G	Unpaired	309 C	Paired	478 C	Paired
143 G	Unpaired	310 A	Paired	479 C	Paired
172 G	Unpaired	311 A	Paired	480 A	Paired

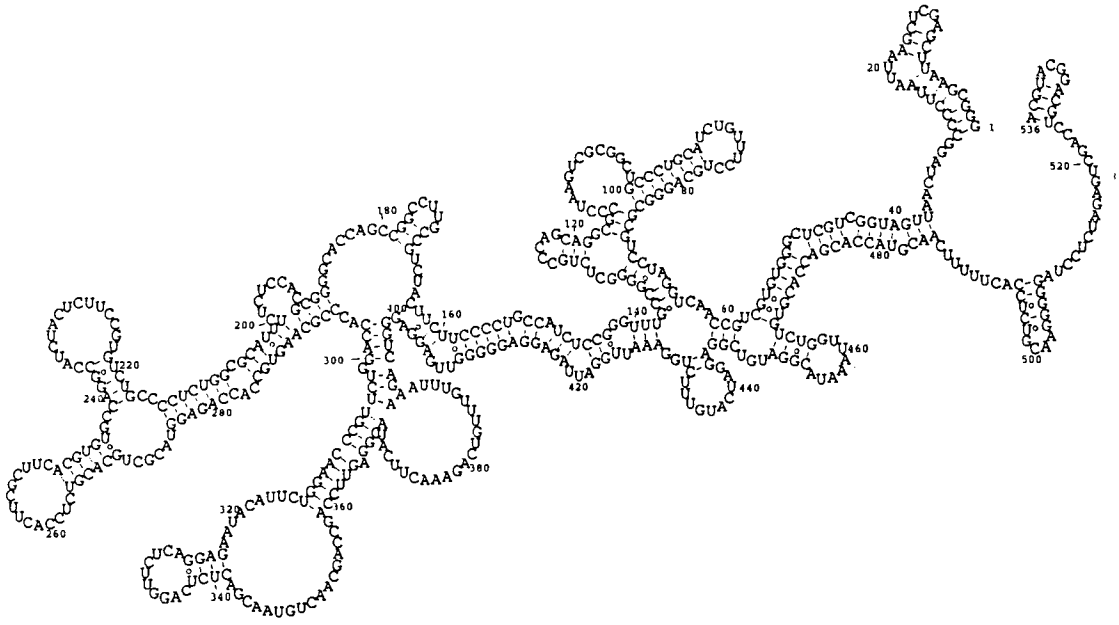

Fig. 3. The determined secondary structure of the HBV X mRNA (from +40 to +500) along with small flanking sequences. The 57 nucleotides, that were experimentally determined to be located in single- or double-stranded regions (Table 1), were forced or prohibited to form base pairs in the RNA folding program MFOLD. The output was drawn by a computer program LoopTool (developed by Gary Olsen, University of Illinois, USA). The two programs are available through Genetic Data Environment.

during the extension step, or by changing the dNTP concentrations, with little success.

Further experimental refinements are required to determine the complete secondary structure, but its laborious experimental determination has been limited to rather short RNAs such as tRNA and 5 S RNA. An alternative to the experimental approaches is to select the most energetically favorable conformation based on thermodynamic parameters for all the structural elements of RNA folding. Two types of RNA folding algorithms have been developed to find a minimum energy secondary structure. The combinatorial method (Pipas and McMahon, 1975; Gouy *et al.*, 1985) is currently limited to folding about 200 nucleotides. The recursive, or dynamic programming, algorithms (Nussinov *et al.*, 1978; Zuker, 1989) can fold up to 2,000 nucleotides. The sub-optimal RNA folding program MFOLD (Zuker, 1989; Jaeger *et al.*, 1989a; Jaeger *et al.*, 1989b) of the latter type allows for forcing or prohibiting particular base pairs.

In this study of rather long RNA, the computer program MFOLD was utilized with the constraints for the nucleotides experimentally observed above to be either single- or double-stranded. Although the MFOLD prediction alone would be only 70% accurate (90% when sub-optimal structures within 10% of the lowest free energy are included) at best (Jaeger *et al.*, 1989a), the inclusion of the experimentally determined structural elements in the running of the program would increase its accuracy tremendously. Only 20 nucleotides were forced and 37 nucleotides were prohibited to base pair with some specific other nucleotides, as supported by strong experimental evidence above (Table 1). The additional 8 nucleotides for double-stranded regions and 19 nucleotides for single-stranded regions were not included as constraints, as their experimental results could be disputed. The resulting secondary structure was drawn by the computer program LoopTool, as shown in Fig. 3.

About half (52%) the RNA nucleotides are shown to be base-paired including G:U base-pairing (Fig. 3). The longest single-stranded stretch was found to be the 23 nucleotides from +370U to +392A. Also 17-nucleotide single-stranded stretches were also found at two regions from +342A to +358G and from +508 A to +524C. Three single-stranded loops of 14~15 nucleotides were found from +101U to +114C, from +222U to +236C, and from +250C to +263C. All these substantially long single-stranded regions are within the HBV X mRNA sequence, except for the 3′ terminal region from +508A to +524C.

All the 8 nucleotides giving weak signals for base pairing turned out to be in the double-stranded regions (Fig. 3). The 19 nucleotides giving weak signals for single stranded regions are also located in the single-stranded loops, except 5 nucleotides that form terminal base pairs of stems. The 5 nucleotides showed partial unpairing, probably because the duplex ends were 'breathing'. All the 15 naturally occurring cleavage sites explained above are located either within or right

next to a loop or bulge. Thus, all the experimental data that were not included in the MFOLD program execution supported the secondary structure in Fig. 3 well.

One can argue that the presence of the sequences other than the X mRNA at the 5′ and 3′ ends of the analyzed RNA could disrupt the intact secondary structure that the X mRNA should form by itself. However, it appears to be unlikely, because the terminal sequences form hairpins by themselves, leaving the majority of the X mRNA hairpins separate from them. Exclusion of the terminal sequences in the execution of MFOLD changed only a local structure from +482 to +500.

Although the secondary structure of HBV X mRNA drawn in Fig. 3 should not be considered to be complete, it provides a good working model. The model suggests the target sites for designing the antisense and hammerhead ribozyme inhibitors and hybridization probes for the X mRNA of HBV adr subtype. For example, single stranded regions containing a GUC sequence are generally considered to be most suitable target sites for hammerhead ribozyme action (Ruffner *et al.*, 1990). Among the 14 GUC sequences in this RNA (13 within the X mRNA), the GUC from +348 to +350 appears to be the best target, because it is located in the middle of the 17-nucleotide loop from +342A to +358G.

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