# An AU at the first base pair of helix 3 elevates the catalytic activity of hepatitis delta virus ribozymes

Zhi-Shun Huang[a,b], Yueh-Hsin Ping[b], Huey-Nan Wu[b,*]

[a]*Graduate Institute of Biotechnology, Chinese Culture University, Taipei, Taiwan*
[b]*Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan*

Received 1 July 1997

**Abstract A mutational analysis of the helix 3 (H3) region of hepatitis delta virus (HDV) ribozymes disclosed that an AU at the first base pair of H3 elevates the catalytic activity of various *cis*- and *trans*-acting HDV ribozymes. A GC to AU substitution at this position of H3, which is located at the junction of three of the four helices of the pseudoknot-like structure model, altered the structure of HDV ribozymes. This substitution in the H3 did not change the independence of the cleavage rate to pH nor the sensitivity to formamide treatment of the ribozymes.**



*Key words:* Ribozyme; Hepatitis delta virus (HDV) ribozyme; *cis*-Cleavage; *trans*-Cleavage

## 1. Introduction

Hepatitis delta virus (HDV) contains a 1.7-kb single-stranded circular RNA as its genome [1–3]. HDV genomic RNA and its complementary sequence, the antigenomic RNA, *cis*-cleave in vitro [4]. The site-specific cleavage process allows the generation of HDV monomer RNA's during replication [5]. The autocatalytic activity of HDV RNA requires magnesium ion or other divalent cations, and the cleavage products contain a 2′,3′-cyclic phosphate and a 5′ hydroxyl group [4,6,7].

Structural probing and mutational analysis studies illustrate that the two HDV autolytic domains have common features that are different from other catalytic RNA's [4,8–19]. The HDV autolytic domain contains four helical regions that may fold into a pseudoknot-like structure [10]. The characteristics of these helices are: H1 and H3 are restricted in size; H2 can be extended to twice original length; and H4/Lp4 can be replaced by a much shorter hairpin loop. Several regions of HDV autolytic domains, such as the cleavage site, the residues enclosed by H3, and the residues connecting helices 1 and 4 ($J_{1/4}$) and helices 4 and 2 ($J_{4/2}$), have stringent sequence requirements. These residues are proposed to be involved in tertiary interaction(s) or to participate in catalysis.

Several substitution/deletion mutants of HDV autolytic domains that *cis*-cleave efficiently have been constructed [12,13]. These variants, including ribozymes Rz 1 and Rz a1 of this study (Fig. 1), may retain the essential structure and sequence elements of HDV autolytic domains. Moreover, the *cis*-acting HDV ribozymes have been converted into various bimolecular constructs. The enzyme subdomain can catalyze the site-specific cleavage of the substrate subdomain in trans indicating that the autolytic domains of HDV ribozymes can be reconstituted through the base-pairing interactions of two subdomains [14].

The H3 region is presumed to stack with H2 and the coaxial helices directly link H1 in the pseudoknot-like structure model of the HDV autolytic domain. In this study, we analyzed the sequence and structural requirements of the first base pair of H3 that locates at the junction of three helices. We found that an AU pair at this position significantly elevates the activity of HDV *cis*-acting ribozymes and that the substitution to other sequences either decreases or abolishes ribozyme activity. The structural features associated with ribozymes containing an AU H3 were examined. The characteristics of the *cis*-cleavage reactions of AU H3-ribozymes were investigated under various conditions. Finally, the effect of an AU H3 on the *trans*-cleavage reaction of a bi-molecular construct was evaluated.

## 2. Materials and methods

### 2.1. Construction of mutants

The cDNA of each ribozyme and/or variant resided between sites *Kpn*I and *Hin*dIII. Mutants of Rz 1 and Rz a1 were constructed by PCR (polymerase chain reaction)-mediated mutagenesis with the corresponding ribozyme cDNA as the template and synthetic DNA oligos as primers. Each PCR product which contained a copy of the T7 promoter upstream of the cDNA was subcloned to pUC19. The sequence of each construct was confirmed [20].

### 2.2. RNA synthesis

Each α-32pC-labeled Rz 1, Rz a1, and their mutants were *Hin*dIII-run off transcripts of T7 RNA polymerase. In most cases, the in vitro transcription reaction was performed in the presence of 12 mM $MgCl_2$ at 37°C for 1 h and the transcripts were purified from polyacrylamide gel containing 7 M urea as described [6]. To obtain more full-length RNA for mutants that *cis*-cleaved efficiently during synthesis, [$MgCl_2$] was decreased to 4 mM, the temperature was changed to 20°C, and the incubation time was elongated. RNA37, RNA37AU, and RNA73 were synthesized by T7 RNA polymerase using synthetic DNA fragments as templates [21]. After purification, RNA's were resuspended in TE buffer (10 mM Tris-HCl and 0.1 mM EDTA). The concentration of RNA of *trans*-cleavage reaction was calculated from the radioactivity of each RNA fragment and the specific activity of [α-$^{32}$P]CTP.

### 2.3. cis-Cleavage reaction

In general, reactions were performed in 12 mM $MgCl_2$ and 40 mM Tris-HCl (pH 7.5) at 50°C for a maximum of 10 h after RNA was heat denatured and renatured. The reaction was terminated by the addition of an equal volume of 50 mM EDTA in 7 M urea. The extent of cleavage ($E$) is defined as the molar ratio of RNA that undergoes *cis*-cleavage reaction. The $t_{1/2}$ (half life) of the *cis*-cleavage reaction was determined. The $E$ values of each RNA before the *cis*-cleavage reaction was initiated ($E_0$), after the RNA was incubated with $MgCl_2$ for a certain period ($E_t$), and when the *cis*-cleavage reaction leveled-off ($E_f$) were determined. The slope of the plot of log $[1-(E_t-E_0)/(E_f-E_0)]$ versus reaction time is the cleavage rate ($k$), and $t_{1/2} = \ln 2/k$.

*Corresponding author. Fax: (886) (2) 782-6085.
E-mail: hnwu@gate.sinica.edu.tw


*PII* S0014-5793(97)00902-2

*2.4. Structural probing*

The structure of the 5′-labeled RNA was probed by RNases. The labeling of RNA fragments and all reaction conditions followed the procedures of Lee et al. [15].

## 3. Results and discussion

### *3.1. H3 mutants of Rz 1 and Rz a1*

Ribozymes Rz 1 and Rz a1 are the wild type *cis*-cleaving ribozymes of this study. Rz 1 and Rz a1 were derived from the autocatalytic domains of HDV RNA by internal substitution and deletion. Two ribozymes have common sequences in the 5′ tail, H1, $J_{1/4}$, and H4/Lp4 regions. The $J_{1/2}$, H2, H3, Lp3, and $J_{4/2}$ regions of Rz 1 and Rz a1 contain the native sequences of HDV RNA of genomic and antigenomic senses, respectively (Fig. 1A and B). Rz1 and Rz a1 were homologous in sequence, they likely have similar secondary structures, and they *cis*-cleaved at similar rates (Fig. 1A and B).

The sequence and structural requirements of the first base pair of H3 that located at the junction of H1, H2, and H3 of the pseudoknot-like structure of the HDV ribozyme were investigated. In general, the replacement of the G17C30 of Rz 1 with other sequences decreased *cis*-cleaving activity (Fig. 2): variants *A17C30*, *C17U30*, and *U17U30* *cis*-cleaved ~5-fold slower than that of Rz 1; variants *U17C30*, *A17G30*, and *G17U30* *cis*-cleaved at least 30 to 50-fold slower than that of Rz 1; the *cis*-cleaving rate of variant *U17A30* was extremely low; and variants *G17G30*, *A17A30*, and *G17A30* lost *cis*-cleaving activity (data not shown). Thus, the maintenance of the base pairing interaction of the first base pair of H3 is not the only factor that determines the *cis*-cleaving activity of HDV ribozymes. It is possible that due to the steric constraint associated with the junction of three helices, the hydrogen bonds of this location simply cannot form. Still, certain sequences are more favorable than others. Mutants with unfavorable sequence at the first base pair of H3, especially the ones with a purine-purine pair, may be trapped in certain inactive conformations that can barely or cannot at all convert to the active pseudoknot-like structure under the assay condition as described in Materials and methods.

Remarkably, variant *A17U30* *cis*-cleaved with a rate at least 10-fold faster than that of Rz 1 (Fig. 2; Table 1). Moreover, the substitution of the A884U872 of Rz a1 to a GC pair decreased the *cis*-cleaving rate ~10-fold (variant *Rz a1.A884GU872C*, Fig. 1B). The results illustrate that an AU at the first base pair of H3 (AU H3 in short) has a positive effect on the *cis*-cleavage reaction of HDV ribozymes. The GC to AU substitution at H3 may affect the folding process and/or change the structure of the catalytic core, consequently, the rate limiting step of *cis*-cleavage reaction is altered.

### *3.2. Structural probing*

The structures of the 3′-cleavage products of ribozyme Rz 1 and variant *A17U30* were probed by RNase's (Fig. 3). Since only one residue upstream of the cleaving point is required for the *cis*-cleavage reaction of HDV ribozymes, we assume that the structure of the 3′-cleavage product is very similar, if not identical, to that of the precursor RNA. With magnesium ion, the G's in $J_{1/2}$, $J_{1/4}$, $J_{4/2}$, and near the 3′ terminus of Rz 1 were susceptible to the single strand specific RNase T1. In addition, the 5′ strand of H2, the 3′ strand of H1, as well as the H4/Lp4 region were accessible to helix-specific RNase V1. In the case of variant *A17U30*, the C15, G28, G29, and G33 of the proposed helical regions of the pseudoknot-like structure became accessible to RNase A or T1 equally well with or without magnesium ion. In addition, the pyrimidines and G25 of Lp3 together with the C54 of $J_{4/2}$ were susceptible to the single-stranded specific RNase's in the presence of magnesium ion. Thus, the GC to AU substitution at H3 altered the structure of Rz 1. The structures of several Rz 1 mutants were probed. The results show that the G25 of Lp3, the G28

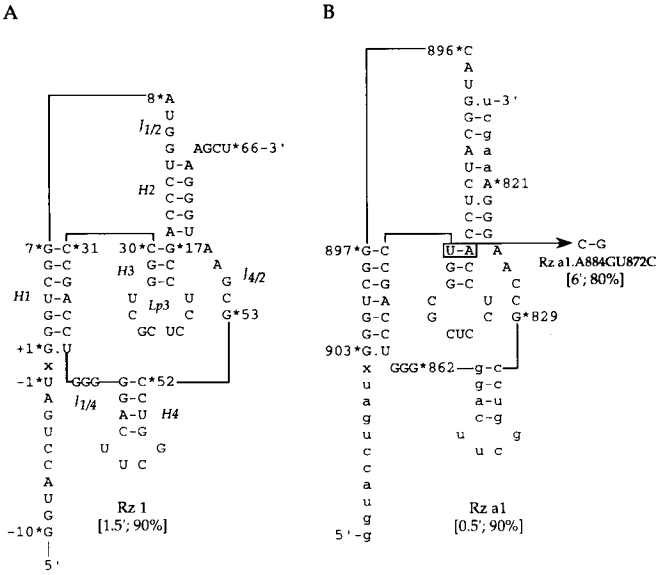


Fig. 1. (A) Sequence and proposed secondary structure of Rz 1. 'x' is the cleaving point; H1, H2, H3, and H4 are the double-stranded regions; and $J_{1/2}$, $J_{1/4}$, and $J_{4/2}$ are the single-stranded regions connecting different helices. (B) Rz a1 and its H3 mutant *Rz a1.A884GU872C*. Capital letters represent the sequence of HDV antigenomic sense RNA (the nomenclature is according to Makino et al. [3]), other residues are not HDV sequence. The $t_{1/2}$ of the *cis*-cleavage reaction of reactive RNA species and the fraction of reactive RNA molecules of each ribozyme are shown.

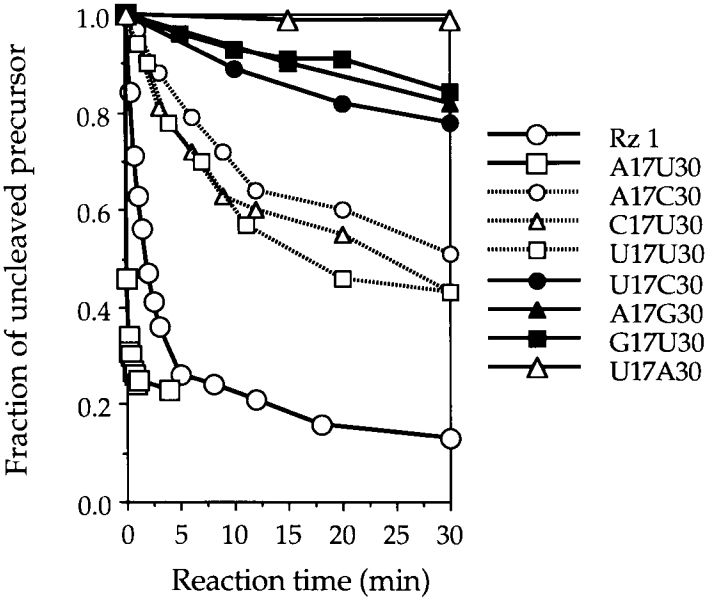


Fig. 2. The sequence of the first base pair of H3 (positions #17 and #30) affects the *cis*-cleavage reaction of Rz 1. The fraction of uncleaved precursor RNA versus reaction time is plotted.

and G29 of the 3′ strand of H3, and the G33 of the 3′ strand of H1 of variants *U58G* and *G61A* (discussed in Section 3.4) became accessible to RNase T1 when their GC H3's were converted to AU H3's (data not shown). Thus, the AU H3 ribozymes possess a less compact secondary structure than the corresponding GC H3 ribozymes. The structural alteration associated with AU H3 may facilitate the alignment of certain essential functional groups that accelerate *cis*-cleavage reaction.

### 3.3. Characteristics of GC H3 and AU H3 ribozymes

The *cis*-cleavage rates of Rz 1 and variant *A17U30* were not altered when the hydroxide ion concentration was increased 100-fold between pH 6.5 and 8.5 at 42°C (Fig. 4). The independence of the cleavage rate to pH of HDV ribozymes is similar to that of the hairpin ribozyme [22,23]. Thus, although the cleavage rates of Rz 1 and variant *A17U30* are significantly different, the chemical mechanisms of the cleavage reaction of GC H3 and AU H3 ribozymes seem to be similar and the rate limiting step does not involve a hydroxide ion. In addition, unlike the hammerhead ribozyme, the cleavage rate of HDV ribozymes may not reflect the base-catalyzed chemical step of phosphodiester hydrolysis [24]. Furthermore, at all pH values tested, the *cis*-cleavage rates of Rz 1 and variant *A17U30* increased as [$MgCl_2$] increased (Fig. 4). Magnesium ion may be essential for ribozyme folding and/or directly participate in catalysis.

An AU H3 and an elongated H2 are the only modifications of HDV ribozymes identified so far that elevate catalytic activity [15]. The *cis*-cleavage reactions of four ribozymes were performed in the presence of formamide to investigate whether an AU H3 acts as an elongated H2 that enhances the resistance to denaturant treatment [15]. In the presence of 2.5 and 6.25 M formamide, ribozymes containing an AU H3 (*A17U30* and Rz a1) *cis*-cleaved much faster than the corresponding ribozymes with a GC H3 (Rz 1 and *Rz a1.A884GU872C*) (data not shown). However, with 6.25 M formamide, the *cis*-cleavage rate of each ribozyme decreased significantly. In addition, all ribozymes barely *cis*-cleaved in the presence of 10 M formamide (data not shown). Thus, an AU at the first base pair of H3 does not elevate the resistance to formamide treatment. Although the AU H3-ribozymes possess higher catalytic activity, their catalytic cores may not be more stable than the corresponding GC-H3 ribozymes. The findings of structural probing support this speculation.

Table 1
The *cis*-cleaving activities of ribozyme Rz 1 mutants: effect of a GC H3 and an AU H3 on *cis*-cleavage reaction

| GC H3 | | | AU H3 | | |
|---|---|---|---|---|---|
| Variant | $t_{1/2}$ | Active RNA | Variant | $t_{1/2}$ | Active RNA |
| Rz 1 | 1.5′ | 90% | *A17U30* | <0.05′ | 80% |
| *U58G* | | $50\%_{(10\ h)}$ | *U58G.AU* | 30′ | 80% |
| *G61A* | | $50\%_{(10\ h)}$ | *G61A.AU* | 12′ | 75% |
| *A56U* | – | | *A56U.AU* | | $40\%_{(6\ h)}$ |
| *A56C* | – | | *A56C.AU* | 17′ | 70% |
| *A57U* | – | | *A57U.AU* | 45′ | 50% |
| *A57C* | – | | *A57C.AU* | 80′ | 40% |

'$t_{1/2}$' and 'active RNA' represent the half-life of the first order *cis*-cleavage reaction and the fraction of the reactive RNA molecules of each mutant with 40 mM Tris-HCl and 12 mM $MgCl_2$ at 50°C. '–' indicates the RNA did not *cis*-cleave. '$A\%_{(B\ h)}$' illustrates the fraction of the reactive RNA molecule (*A*%) after *B* h of incubation with $MgCl_2$.

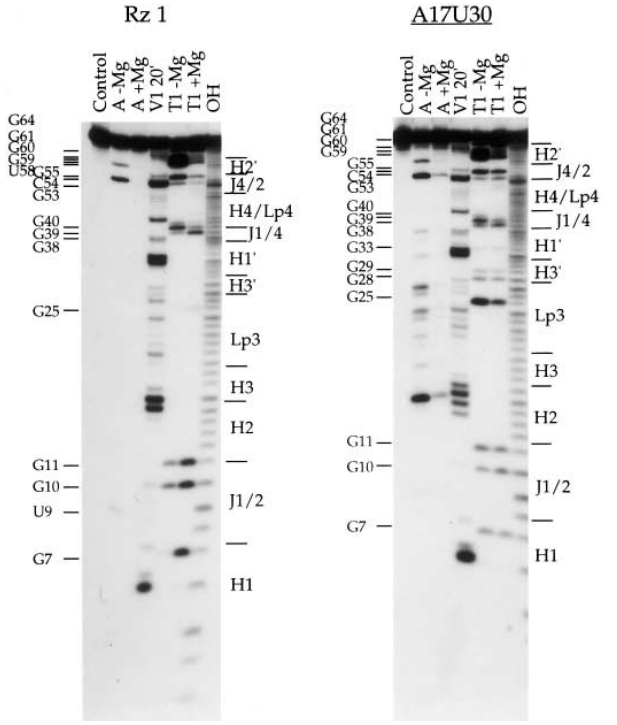


Fig. 3. Probing the secondary structures of the 5′ labeled-3′ cleavage product of Rz 1 and variant *A17U30*. 'Control' represents the RNA without any treatment; 'OH' is the alkaline hydrolysis reaction; 'A−Mg' and 'A+Mg' are the RNase A digestion reactions without and with 10 mM $MgCl_2$; 'T1−Mg' and 'T1+Mg' are the RNase T1 digestion reactions without and with 10 mM $MgCl_2$; and 'V1 10' and 'V1 20' indicate the RNA was treated by RNase V1 for 10 and 20 min, respectively. H1, H1′, H2, H2′, H3, H3′, Lp3, H4-Lp4, $J_{1/2}$, $J_{1/4}$, and $J_{4/2}$ are the different sequence domains of ribozyme molecules.

### 3.4. *An AU H3 compensates the mutations of HDV cis-cleaving ribozymes*

Whether the positive effect of an AU H3 can compensate for certain unfavorable mutations of HDV *cis*-cleaving ribozymes was examined. Variants *U58G* and *G61A* contained mutation in the H2 of Rz 1 that destabilized the essential helical region [15]. In addition, variants *A56U*, *A56C*, *A57U*, and *A57C* had substitution at $J_{4/2}$ which is one of the regions of the HDV ribozymes that has stringent sequence requirements [16,17]. Two H2 mutants *cis*-cleaved slowly and all four $J_{4/2}$ mutants were inactive (Table 1). It is notable that the GC to AU substitution at the first base pair of H3 resulted in the elevation of the *cis*-cleaving activity of each mutant: the cleavage rates of both H2 mutants were enhanced; in addition, all $J_{4/2}$ mutants became active and they *cis*-cleaved at various rates (Table 1). Thus, an AU H3 significantly elevates the *cis*-cleaving activities of various HDV ribozymes. Similar to that of an elongated H2, and AU H3 can compensate for the unfavorable mutations of HDV ribozymes.

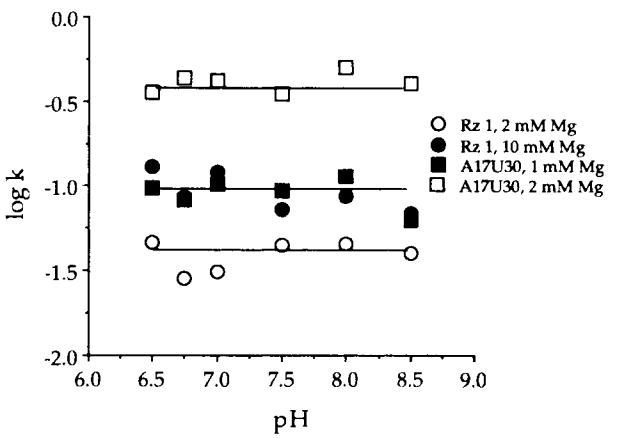


Fig. 4. Effect of pH on the *cis*-cleavage reactions of Rz 1 and variant *A17U30*. To obtain accurate *cis*-cleavage rates, the reactions were carried out at 42°C. The Tris-HCl buffer was replaced by 40 mM sulfonate buffer of different pH [Mes (pH 6.5), Mops (pH 6.75–7.5), and Tap (pH 8.0–8.5)]. The log of *cis*-cleavage rate (*k*) was plotted versus pH.

### 3.5. *An AU H3 elevates the activity of an HDV trans-acting ribozyme*

The effect of an AU H3 on a *trans*-acting HDV ribozyme was investigated. RNA73/RNA37 is a bi-molecular construct that has a *cis*-cleaving HDV ribozyme divided into an enzyme subdomain and a substrate subdomain from $J_{1/2}$ and Lp4 after the wild type 5′ and 3′ termini were ligated [14]. The pseudoknot-like structure can be reconstituted when two subdomains interact with each other through the formation of H1, H2, and H4 (Fig. 5A) [14]. Constructs RNA73/RNA37AU and RNA73/RNA37 have an equal number of intermolecular base pairs: RNA37 contains a GC H3 while RNA37AU contains an AU H3 (Fig. 5A). Both enzymes catalyzed the site-specific cleavage of RNA73 in trans. However, when the enzymes were in excess of the substrate (50 nM vs. 5 nM), twice the amount of RNA73 was cleaved by RNA37AU compared to that of RNA73 cleaved by RNA37 (Fig. 5B). Moreover, the cleavage of RNA73 catalyzed by

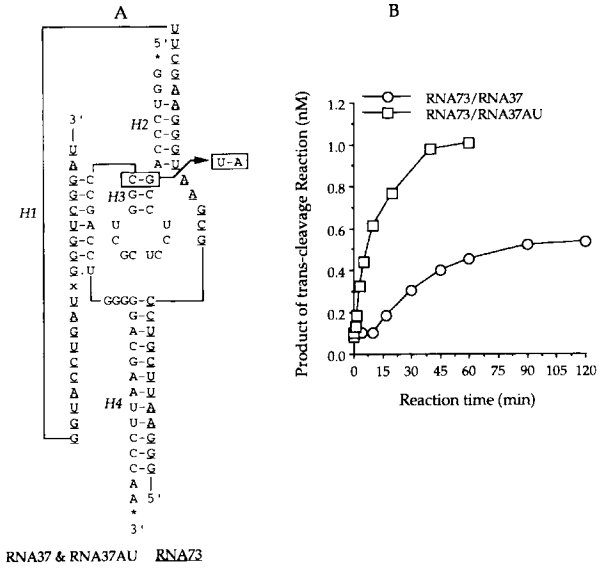


Fig. 5. (A) Sequence and proposed secondary structure of bi-molecular constructs RNA73/RNA37 and RNA73/RNA37AU. Residues of the substrate subdomain (RNA73) are underlined. RNA37AU has an AU instead of a GC at the first base pair of H3. (B) The cleavage of RNA73 catalyzed by RNA37 or RNA37AU. 5 nM of substrate and 50 nM of enzyme were individually denatured-and-renatured and incubated at 37°C for at least 5 min before the initiation of *trans*-cleavage. The reactions were carried out in 40 mM Tris-HCl and 12 mM $MgCl_2$ at 37°C. The concentration of *trans*-cleavage product was plotted versus reaction time.

RNA37AU occurred at a rate at least 5-fold higher than that of RNA37 with 12 mM $MgCl_2$ at 37°C. There was no product release process under the single turnover condition. Therefore, when incubated with RNA73, the AU H3 enzyme may promote the reconstitution of HDV's autocatalytic domain, consequently, construct RNA73/RNA37AU has a higher proclivity of cleavage. Moreover, the specific structural features associated with the AU H3 enzyme may accelerate the chemical step of cleavage reaction.

In conclusion, the mutational analysis shows that an AU at the first base pair of H3 has a positive effect on the HDV ribozymes. Compensation can be made for certain mutations, and the catalytic activities of various *cis*- and *trans*-acting ribozymes can be enhanced. Therefore, like that of the elongated H2 [15], an AU H3 can be used to improve the activity of HDV ribozymes for therapeutic application. The GC to AU substitution at H3 alters the ribozyme structure, and H3 locates at the catalytic core in the 3D structural model of the HDV ribozyme [19]. Further studies are needed to discern the molecular mechanism of the elevation of catalytic activity of AU H3 ribozymes.

*Acknowledgements:* This work was supported by Academia Sinica, ROC and a grant from National Science Council, ROC (NSC-84-2311-B-001-075). The authors would like to thank Mr. D. Platt for English editing.

## References

[1] Kos, A., Dijkema, R., Arnberg, A.C., van der Meide, P.H. and Schellekens, H. (1986) Nature (London) 323, 558–560.
[2] Wang, K.S., Choo, Q.L., Weiner, A.J., Ou, H., Najarian, R.C., Thayer, R.M., Mullenbach, G.T., Denniston, K.J., Gerin, J.L. and Houghton, M. (1986) Nature (London) 323, 508–513.
[3] Makino, S., Chang, M.F., Shieh, C.K., Kamahora, T., Vannier, D.M., Govindarajan, S. and Lai, M.M.C. (1987) Nature (London) 329, 343–346.
[4] Kuo, M.Y., Sharmeen, L., Dinter-Gottlieb, G. and Taylor, J. (1988) J. Virol. 62, 4439–4444.
[5] Macnaughton, T.B., Wang, Y.J. and Lai, M.M.C. (1993) J. Virol. 67, 2228–2234.
[6] Wu, H.N., Lin, Y.J., Lin, F.P., Makino, S., Chang, M.F. and Lai, M.M.C. (1989) Proc. Natl. Acad. Sci. USA 86, 1831–1835.
[7] Perrotta, A.T. and Been, M.D. (1992) Biochemistry 31, 16–21.
[8] Rosenstein, S.P. and Been, M.D. (1991) Nucleic Acids Res. 19, 5409–5416.
[9] Wu, H.N., Wang, Y.J., Hung, C.F., Lee, H.J. and Lai, M.M.C. (1992) J. Mol. Biol. 223, 233–245.
[10] Perrotta, A.T. and Been, M.D. (1991) Nature (London) 350, 434–436.
[11] Kumar, P.K.R., Suh, Y.A., Miyashiro, H., Nishikawa, F., Kawakami, J., Taira, K. and Nishikawa, S. (1992) Nucleic Acids Res. 20, 3919–3924.
[12] Wu, H.N. and Huang, Z.S. (1992) Nucleic Acids Res. 20, 5937–5941.
[13] Wu, H.N., Lee, J.Y., Huang, H.W., Huang, Y.S. and Hsueh, T.G. (1993) Nucleic Acids Res. 21, 4193–4199.
[14] Lai, Y.C., Lee, J.Y., Liu, H.J., Lin, J.Y. and Wu, H.N. (1996) Biochemistry 35, 124–131.
[15] Lee, C.B., Lai, Y.C., Ping, Y.H., Huang, Z.S., Lin, J.Y. and Wu, H.N. (1996) Biochemistry 35, 12303–12312.
[16] Suh, Y.A., Kumar, P.K., Taira, K. and Nishikawa, S. (1993) Nucleic Acids Res. 21, 3277–3280.
[17] Kawakami, J., Kumar, P.K., Suh, Y.A., Nishikawa, F., Kawakami, K., Taira, K., Ohtsuka, E. and Nishikawa, S. (1993) Eur. J. Biochem. 217, 29–36.
[18] Thill, G., Vasseur, M. and Tanner, N.K. (1993) Biochemistry 32, 4254–4262.
[19] Tanner, N.K., Schaff, S., Thill, G., Petit-Koskas, E., Crain-Denoyelle, A.-M. and Westhof, E. (1994) Curr. Biol. 4, 488–498.
[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 1074–1078.
[21] Milligan, J.F. and Uhlenbeck, O.C. (1989) Methods Enzymol. 180, 51–62.
[22] Hampel, A. and Tritz, R. (1989) Biochemistry 28, 4929–4933.
[23] Hegg, L.A. and Fefor, M.J. (1995) Biochemistry 34, 15813–15828.
[24] Dahm, S.S., Derrick, W.B. and Uhlenbeck, O.C. (1993) Biochemistry 32, 13040–13045.