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The effect of Lp3 enlargement on the folding and catalysis of hepatitis delta virus *cis*-cleaving ribozyme

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Abstract Oligonucleotides were inserted into the Lp3 region of a hepatitis delta virus (HDV) *cis*-cleaving ribozyme and the effect of Lp3 enlargement on ribozyme folding was examined by assaying the activity of each mutant. The location of insertion and the sequence of the inserted oligonucleotide had distinct effects on ribozyme activity, and the HDV ribozyme was capable of adopting the active structure for *cis*-cleavage when a pentanucleotide was inserted into the 3′ portion of Lp3. Furthermore, whether the insertion mutant *cis*-cleaved or not, the structure of Lp3 was altered by the inserted oligonucleotide. These findings disclose that the 5′ rather than the 3′ portion of Lp3 is critical for ribozyme folding and catalysis.

Key words: Hepatitis delta virus ribozyme; Ribozyme folding; Ribozyme catalysis

1. Introduction

Hepatitis delta virus (HDV) contains a single-stranded circular RNA of 1.7 kb as its genome [1–3]. The HDV RNAs of genomic and antigenomic senses *cis*-cleave in vitro [4]. The *cis*-cleavage reaction occurs in the presence of magnesium ion or other divalent cations, and the cleavage products contain a 2′,3′-cyclic phosphate and a 5′ hydroxyl group [4–6]. The *cis*-cleaving activity of HDV RNA is proposed to be involved in generating the genomic-sized RNA molecules during viral RNA replication [7].

The autolytic domains of two senses of HDV RNA are homologous in sequence and they have similar secondary structures [4,8–10]. The framework of the HDV ribozyme is composed of four stems that may fold into a pseudoknot-like structure [11]. The base pairing structure of each stem is either critical or important to *cis*-cleaving activity: H1 has to be 6 or 7 bp in length; H3 but not H2 requires specific sequences; and H4/Lp4 can be replaced by a stable hairpin loop of non-HDV sequence [10,12]. The Lp3 (the residues enclosed by H3), J1/4 (the residues linking H1 and H4), and J4/2 (the residues linking H4 and H2) regions of the HDV ribozyme contain essential nucleotides necessary for catalysis. These regions are proposed to be involved in the formation of the catalytic core [13–16]. Moreover, photo-crosslinking experiments illustrated that all these indispensable single-stranded regions are positioned in close proximity to the cleavage site [17,18].

The Lp3 of the HDV ribozyme has a surprisingly stable structure that is mainly supported by sugar hydroxyl hydrogen bonds and base-base and base-phosphate stacking interactions [19]. The first and the last residue of Lp3 form a non-Watson-Crick base pair [19], while Lp3 residues are not likely to form base pairs with other single-stranded regions of the ribozyme molecule [16]. To further investigate the importance of the Lp3 region to the structure and activity of HDV ribozyme, we inserted oligonucleotides into different locations of this loop and analyzed the *cis*-cleaving activity of each mutant. Since ribozyme activity relies on a correctly folded structure, insertion mutation analyses may elucidate the effects of Lp3 enlargement on ribozyme folding. The results indicated that the location of the insertion as well as the sequence of the inserted pentanucleotide had distinct effects on the ribozyme *cis*-cleaving activity, whereas the HDV ribozyme was capable of adopting the active structure for *cis*-cleavage when a pentanucleotide was inserted into the 3′ but not the 5′ portion of Lp3. The structural features of several Lp3 insertion mutants were probed by ribonucleases. The results showed that whether the insertion mutant *cis*-cleaved or not, the structure of the Lp3 region of the ribozyme molecule was altered to accommodate the pentanucleotide insertion. Thus, the 5′ rather than the 3′ portion of Lp3 is critical for the overall folding and catalysis of the HDV ribozyme.

2. Materials and methods

2.1. Construction of mutants

Ribozyme mutants were constructed by PCR (polymerase chain reaction)-mediated mutagenesis with the corresponding ribozyme cDNA as the template and synthetic DNA oligos as the primers. Each PCR product that contained a copy of the T7 promoter upstream of ribozyme cDNA was subcloned to pUC19. The sequence of each construct was confirmed [20].

2.2. RNA synthesis

The α-^{32}P-labeled ribozymes 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 a polyacrylamide gel containing 7 M urea [5]. For mutants that possess high autolytic activity, to obtain enough precursor RNA, the reaction condition was modified as described [21]. The precursor RNAs purified from polyacrylamide-7 M urea gels were resuspended in TE buffer (10 mM Tris-HCl and 0.1 mM EDTA).

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 4 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 cleavage products were resolved from the precursor RNA on a 7% polyacrylamide-7 M urea gel. The radioactivity of each RNA fragment was quantified using a PhosphorImager (Molecular Dynamics). The extent of cleavage (E) was defined as the molar ratio of RNA that underwent *cis*-cleavage. The half-life ($t_{1/2}$) of the *cis*-cleavage of the reactive species of each ribozyme mutant was determined as described [21].

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2.4. Structural probing

The structure of the 5′-labeled RNA was probed by RNases in the presence of 10 mM $MgCl_2$. The labeling of RNA fragments and all reaction conditions followed the procedures of Lee et al. [22].

3. Results and discussion

3.1. Evaluating the insertion mutation analysis

In this study we developed an insertion mutation analysis method to examine the effect of the enlargement of certain internal loops and the elongation of certain single-stranded regions connecting different helices on the folding of HDV ribozyme Rz 1. Rz 1 is a *cis*-cleaving ribozyme derived from the autolytic domain of HDV genomic sense RNA by internal deletion and substitution [12]. Previous studies illustrated that the J1/2 and Lp4 regions can be variable in sequence and the phosphodiester bond of these regions can individually be broken without preventing the ribozyme from adopting the catalytic structure. Moreover, these two regions protrude away from the catalytic center in the 3D structural model of the HDV ribozyme [10,16,23]. Thus, in the present study, the insertion mutants of the J1/2 and Lp4 regions were first analyzed. Rz 1 is rich in G and C residues. To prevent the potential base pairing interactions between the inserted oligonucleotide and the residues of the ribozyme molecule, the A residue rather than other sequences was used for the insertion.

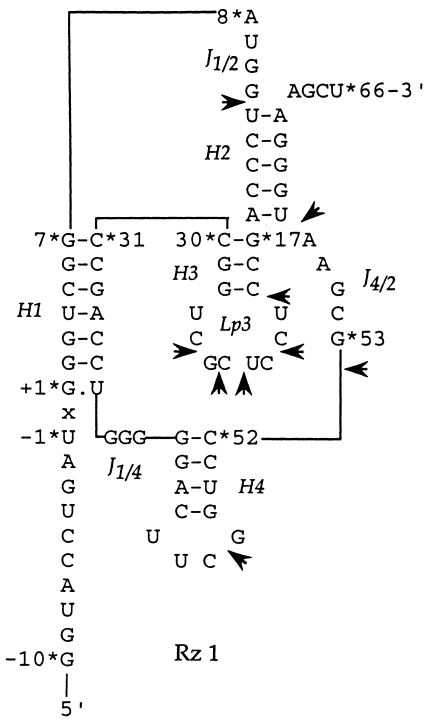
The variant with an A5 inserted into the 3′ part of J1/2 and the variants with an A3 or an A5 inserted into the Lp4 *cis*-cleaved at the same rate as that of Rz 1 (Fig. 1). The high *cis*-cleaving activity of these variants indicated that neither the elongation of J1/2 nor the enlargement of Lp4 prohibits the HDV ribozyme from adopting the active structure for *cis*-cleavage. In addition, the insertions of oligo A to J1/2 and Lp4 may not necessarily cause the formation of inactive alternative structures that decrease ribozyme activity. Therefore, the findings derived from these studies of insertion mutants agree well with those of mutational analyses and circular permutation analyses.

3.2. The Lp3 insertion mutants of ribozyme Rz 1

Lp3 insertion mutants of ribozyme Rz 1 were constructed and the *cis*-cleaving activity of each mutant was assayed. Since a correctly folded structure is required for ribozyme activity, the effect of Lp3 enlargement on ribozyme folding could then be examined.

The insertion of A5 (variant G25C26-iA5) or 5′-Ga4-3′ into the G25/C26 of Rz 1 had a moderate effect on ribozyme activity. The corresponding variants *cis*-cleaved slightly more slowly than Rz 1 (Fig. 1). Therefore, the HDV ribozyme may adopt the correct structure for *cis*-cleavage when its Lp3 is enlarged by five nucleotides. However, variants with a 5′-A4G-3′ or a 5′-UGA3-3′ inserted into the same location of Rz 1 were much less active: the former *cis*-cleaved at a significantly reduced rate while the latter barely *cis*-cleaved (Fig. 1). It is likely that these inserted sequences caused the formation of alternative structures that change the rate limiting step of ribozyme reaction.

Variants with an A5 inserted into the C21/C22 (variant C21C22-iA5) and the U23/C24 of Rz 1 (variant U23/C24-iA5) as well as variants with an A6 inserted to the C24/G25 of Rz 1 did not *cis*-cleave. Thus, the exact location of oligo A insertion rather than the enlargement of Lp3 had a more



location	inserted residues	t1/2
Rz 1	wild type	1′
G11C12	A5	1′
C47G48	A5	1′
	A3	1′
C19U20	5′-UCCUC-3′	inactive
C21C22	A5	inactive
	Py3	inactive
U23C24	A5	inactive
	5′-UCC-3′	inactive
	5′-CUG-3′	inactive
C24G25	A6	inactive
G25C26	A5	4′
	5′-A4G-3′	>120′
	5′-GA4-3′	5′
	5′-UGA3-3′	cs*

Fig. 1. Sequence, proposed secondary structure, and the *cis*-cleaving activity of ribozyme Rz 1 and its insertion mutants. 'x' is the cleaving point. H1–H4 are the helical regions, and J1/2, J1/4, and J4/2 are the single-stranded regions connecting different helices. The arrowheads represent the location of oligonucleotide insertion. Py represents the pyrimidine residues U and C. The $t_{1/2}$ of the *cis*-cleavage reaction of each variant in the presence of 12 mM $MgCl_2$ at 50°C was determined. 'cs' indicates less than 10% of the precursor RNA underwent *cis*-cleavage in 4 h, and 'inactive' indicates no cleavage product was detected.

profound effect on ribozyme folding. It has been demonstrated that the conservation of the sequences of Lp3 except C26 and U27 is essential [16]. In addition, the deletion of C26 or U27 has a much smaller effect on *cis*-cleavage reaction than the single-point deletion of other residues of Lp3 [24]. Therefore, the inactivation of these insertion mutants may be due to the disruption of the interactions between the pyrimidines of Lp3 and other regions of the ribozyme molecule [17,18] and/or the alteration of the stable structure of Lp3 [19]. We then constructed mutants that had U/C rich sequences inserted to the 5′ half of Lp3. The insertion of 5′-UCCUC-3′ to the C19/C20 of Rz 1 that duplicated the U20 to C24 of Lp3 abolished the *cis*-cleaving activity (Fig. 1). Variants with a C3, a 5′-CCU-3′, a 5′-CUC-3′, or a 5′-UUC-3′ inserted into the C21/C22 of Rz 1 as well as variants with a 5′-UCC-3′ or a 5′-CUG-3′ inserted to the U23/C24 of Rz 1 were inactive (Fig. 1). Therefore, the replacement of the inserted oligo A with U/C rich sequences of different lengths did not compensate for the deleterious effect of oligonucleotide insertion into the 5′ portion of Lp3.

3.3. The Lp3 insertion mutants of ribozyme A17U30

A previous study illustrated that an AU at the first base pair of H3 (referred to as AU H3) elevates the catalytic activity of HDV ribozymes. The corresponding mutant of Rz 1, named A17U30, *cis*-cleaved at a rate >10-fold higher than Rz 1 (Fig. 2) [21]. To confirm that the oligonucleotide insertion into the 5′ portion of Lp3 had a more profound effect on ribozyme folding, we studied the Lp3 insertion mutants of ribozyme A17U30. All of the eight variants with a 5′-(A/U)-(A/G)AA(A/G)-3′ inserted into the G25/C26 of ribozyme A17U30 *cis*-cleaved, and the $t_{1/2}$s of their *cis*-cleavage reactions were 2–7 min (Fig. 2). It is notable that unlike that of Rz 1 (Fig. 1), variants with a 5′-A4G-3′ and a 5′-UGA3-3′ insertion were as active as the remaining G25/C26 insertion mutants. Thus, an AU H3 may compensate for the negative effect of the insertion of an unfavorable sequence into the 3′ portion of Lp3. Since all of the G25/C26 insertion mutants were less active than ribozyme A17U30, the pentanucleotide insertion into the 3′ portion of Lp3 may alter the catalytic core in certain ways that decrease the *cis*-cleaving activity of the HDV ribozyme. Furthermore, similar to that of Rz 1, the insertion of A5 into the 5′ portion of Lp3 severely decreased the activity of ribozyme A17U30: the variant with an A5 inserted into C21/C22 was inactive and the variant with an A5 inserted into U23/C24 *cis*-cleaved very slowly (Fig. 2). The results confirmed that the oligonucleotide insertion into the 5′ portion of Lp3 was detrimental to the HDV ribozyme, and an AU H3 did not compensate for this deleterious effect.

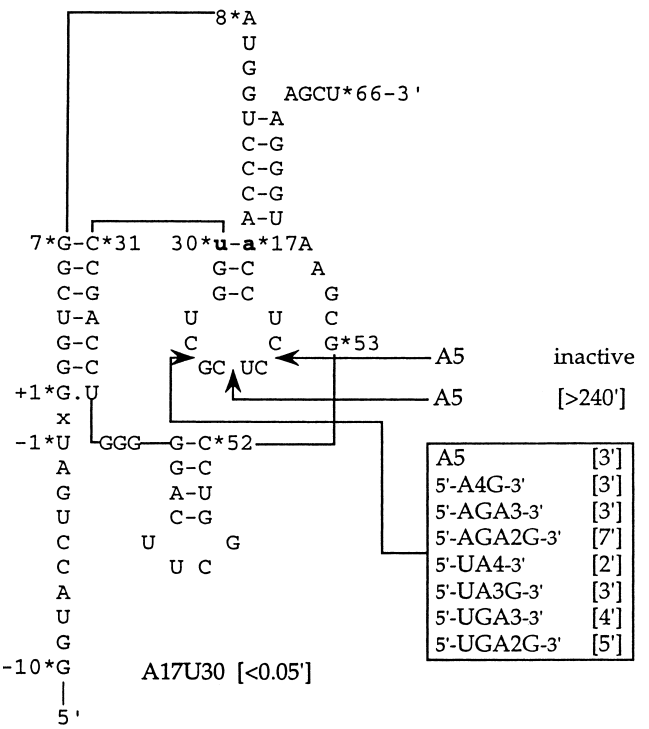


Fig. 2. Summary of the *cis*-cleaving activities of ribozyme A17U30 and its Lp3 insertion mutants. The location of the oligonucleotide insertion and the inserted sequence of each variant is shown. The data in parentheses are the $t_{1/2}$s of *cis*-cleavage reaction with 12 mM $MgCl_2$ at 50°C.

3.4. Probing the structure of Rz 1 Lp3 mutants

The structures of the 3′ cleavage products of Rz 1 and variant G25C26-iA5 and the precursor RNAs of variants C21C22-iA5 and U23C24-iA5 were probed by RNases in the presence of 10 mM $MgCl_2$. The results are summarized in Fig. 3. RNases A and T1, respectively, cut after unpaired pyrimidines and unpaired guanosine residues. RNase V1 hydrolyzes double helical RNA and nucleotide segments of the polynucleotide backbone with approximately helical conformation. Since only one residue upstream of the cleaving point is required for the *cis*-cleavage reaction of the HDV ribozyme, the structure of the 3′ cleavage product may be very similar to that of the precursor RNA. This speculation was supported by the finding that the RNase digestion pattern of the 3′ cleavage product of variant G25C26-iA5 was indistinguishable from that of the uncleaved precursor RNA (data not shown).

In the case of variant G25C26-iA5, the G residues at both strands of H3 (G17, G28, and G29) became accessible to RNase T1. Moreover, the strong RNase V1 cutting sites in the 5′ strand of H2, the 3′ strand of H1, and the H4/Lp4 region became much weaker. Furthermore, the RNase digestion pattern of the Lp3 region of this variant was different from that of Rz 1. G25, which is the residue just upstream of the inserted A5, became strongly cut by RNase T1 while the inserted A residues were not accessible to RNase V1. In addition, residues U20–C24 were weakly cut by RNase V1 whereas residues C26 and U27 were not cut by RNase A or RNase V1. However, the RNase T1 susceptible sites in the single-stranded regions other than Lp3, i.e. J1/2, J1/4, and J4/2, were the same as those of Rz 1; moreover, the relative accessibility of each of these G residues was similar to those of Rz 1. These results suggested that the pentanucleotide insertion alters the structure of Lp3 and destabilizes not only H3 but also other helical regions of the ribozyme molecule, and that variant G25C26-iA5 seems to have a less compact structure than Rz 1. Since variant G25C26-iA5 *cis*-cleaved quite well, the structural changes associated with the pentanucleotide insertion into the 3′ portion of Lp3 did not disrupt the catalytic core of the HDV ribozyme. At the same time, these structural changes may account for the lower *cis*-cleavage rate of this insertion mutant.

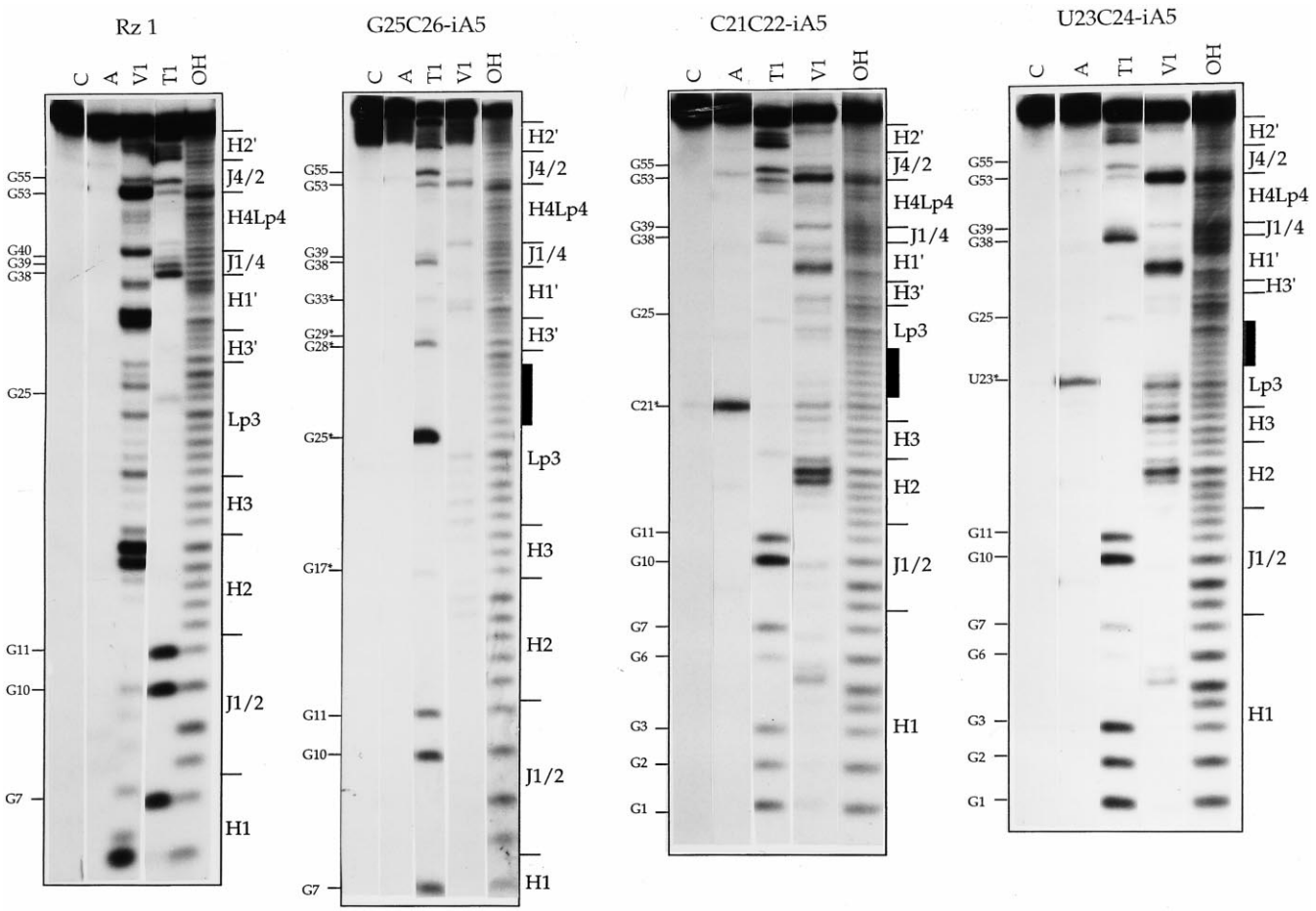


Fig. 3. Probing the secondary structure of Rz 1 and its Lp3 insertion mutants. The structures of the 5′ labeled 3′ cleavage products of Rz 1 and variant G25C26-iA5 together with the 5′ labeled precursor RNAs of variants C21C22-iA5 and U23C24-iA5 were probed. 'C' represents no treatment; 'OH' is the alkaline hydrolysis reaction; 'A', 'T1', and 'V1', respectively, are the RNases A, T1, and V1 digestion reactions in the presence of 10 mM $MgCl_2$. H1, H1′, H2, H2′, H3, H3′, Lp3, H4Lp4, J1/2, J1/4, J4/2 are different sequence domains of the HDV ribozyme, and the black box represents the inserted A5.

The most striking feature of the inactive Lp3 insertion mutants was that the pyrimidine just upstream of the inserted A5, i.e. C21 of variant C21C22-iA5 and U23 of variant U23C24-iA5, was readily accessible to RNase A. The inserted A5 of variant U23C24-iA5 was resistant to RNase V1 while some of the inserted A residues of variant C21C22-iA5 were weakly cut by RNase V1. However, similar to those of Rz 1, the remaining pyrimidines of Lp3 of each variant were accessible to RNase V1 but not RNase A, and the only purine in the Lp3 of both variants (G25) was weakly cut by RNase T1. Moreover, the RNase digestion pattern of the remaining portions of each variant was almost indistinguishable from that of Rz 1. Thus, the structural alteration associated with the pentanucleotide insertion into the 5′ portion of Lp3 appears to localize at the Lp3 region of the ribozyme molecule. The alteration seems to be sufficient to abolish the catalytic activity of the HDV ribozyme. We speculate that the orientation of certain functional groups necessary for catalysis are altered, or certain essential tertiary interactions between Lp3 and other regions of the ribozyme molecule are disrupted. The molecular mechanism, however, cannot be elucidated from this study.

The present structural probing studies illustrate that the insertion of A5 into various locations of Lp3 alters the structure of this essential single-stranded region. The insertion into the 5′ portion of Lp3 abolishes ribozyme activity, whereas the insertion to the 3′ portion of Lp3 dose not prohibit the ribozyme from adopting the active structure. Therefore, the 5′ rather than the 3′ portion of Lp3 is critical for the overall folding and the catalysis of the HDV ribozyme.

4. Conclusion

We developed an insertion mutation analysis method to investigate the effect of the extension of the J1/2, Lp3, and Lp4 regions on the folding of the HDV *cis*-cleaving ribozyme Rz 1. The elongation of J1/2 or Lp4 to twice the original size by oligo A insertion did not alter the activity of Rz 1. These findings support the speculation that the J1/2 and Lp4 regions of the HDV ribozyme do not directly participate in the formation of the catalytic core. The Lp3 region can be enlarged by five nucleotides, and the sequence of the inserted oligonucleotide together with the location of insertion have distinct effects. An A5 insertion altered the Lp3 structure: the alteration caused by the insertion into the 5′ portion of Lp3 was deleterious while the insertion to the 3′ portion was not. Therefore, we conclude that the 5′ portion of Lp3 is essential to the folding and catalysis of the HDV ribozyme. These

findings from insertion mutation analyses provide an insight into the structure and sequence requirements of the HDV ribozyme.

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References

[1] Kos, A., Dijkema, R., Arnberg, A.C., van der Meide, P.H. and Schellekens, H. (1986) Nature 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 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 329, 343–346.
[4] Kuo, M.Y., Sharmeen, L., Dinter-Gottlieb, G. and Taylor, J. (1988) J. Virol. 62, 4439–4444.
[5] 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.
[6] Perrotta, A.T. and Been, M.D. (1992) Biochemistry 31, 16–21.
[7] Macnaughton, T.B., Wang, Y.J. and Lai, M.M.C. (1993) J. Virol. 67, 2228–2234.
[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] Wu, H.N. and Huang, Z.S. (1992) Nucleic Acids Res. 20, 5937–5941.
[11] Perrotta, A.T. and Been, M.D. (1991) Nature 350, 434–436.
[12] Wu, H.N., Lee, J.Y., Huang, H.W., Huang, Y.S. and Hsueh, T.G. (1993) Nucleic Acids Res. 21, 4193–4199.
[13] 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.
[14] 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.
[15] Suh, Y.A., Kumar, P.K., Taira, K. and Nishikawa, S. (1993) Nucleic Acids Res. 21, 3277–3280.
[16] Tanner, N.K., Schaff, S., Thill, G., Petit-Koskas, E., Crain-Denoyelle, A.-M. and Westhof, E. (1994) Curr. Biol. 4, 488–498.
[17] Bravo, C., Lescure, F., Laugaa, P., Fourrey, J.L. and Favre, A. (1996) Nucleic Acids Res. 24, 1351–1359.
[18] Rosenstein, S.P. and Been, M.D. (1996) Biochemistry 35, 11403–11413.
[19] Kolk, M.H., Heus, H.A. and Hilbers, C.W. (1997) EMBO J. 16, 3685–3692.
[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 1074–1078.
[21] Huang, Z.S., Ping, Y.H. and Wu, H.N. (1997) FEBS Lett. 413, 299–303.
[22] Lee, C.B., Lai, Y.C., Ping, Y.H., Huang, Z.S., Lin, J.Y. and Wu, H.N. (1996) Biochemistry 35, 12303–12312.
[23] Lai, Y.C., Lee, J.Y., Liu, H.J., Lin, J.Y. and Wu, H.N. (1996) Biochemistry 35, 124–131.
[24] Thill, G., Vasseur, M. and Tanner, N.K. (1993) Biochemistry 32, 4254–4262.