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Affinity Purification of Functional Receptors for *Escherichia coli* Heat-Stable Enterotoxin from Rat Intestine†

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ABSTRACT: Active receptors for *Escherichia coli* heat-stable enterotoxin (ST) were partially purified by ligand-affinity chromatography. The affinity column was prepared by coupling ST to biotin derivatized with an extended N-hydroxysuccinylated spacer arm prior to binding to monomeric avidin immobilized on agarose. Detergent extracts of rat intestinal mucosa membranes were quantitatively depleted of ST binding activity when chromatographed on this affinity matrix. Biotinylated ST-receptor complexes were eluted from affinity columns with 2 mM biotin and these complexes quantitatively dissociated with bile salts. Using this technique, functional ST receptors were purified maximally about 2000-fold, with about 3% of the total activity in crude extracts recovered in these purified preparations. Analysis of affinity-purified preparations by polyacrylamide gel electrophoresis and silver staining demonstrated a major protein subunit of 74 kDa. Affinity cross-linking of these preparations to ^{125}I-ST demonstrated specific labeling predominantly of the 74-kDa subunit. In addition, lower amounts of labeled ST were incorporated into subunits of 164 and 45 kDa, confirming the heterogeneous nature of ST receptors. Purified receptors bound ST in a concentration-dependent fashion, with an IC_{50} of 10^{-9} M. These studies demonstrate that ligand-affinity chromatography can be employed to purify ST receptors. The availability of purified receptors will facilitate further studies of mechanisms underlying ST-induced intestinal secretion.

Escherichia coli heat-stable enterotoxin (ST)[1] is a low molecular weight peptide which induces secretory diarrhea in humans and animals (Giannella, 1981; Levine et al., 1986; Moon, 1978). It is a major etiologic agent causing travelers diarrhea and endemic diarrhea in developing countries, the leading cause of pediatric morbidity and mortality worldwide (Giannella, 1981; Levine et al., 1986; Moon, 1978). The mechanisms by which ST alters intestinal fluid and electrolyte transport remain unclear. Previous studies demonstrated that the initial step mediating ST activity involves binding of toxin to specific protein receptors located on brush border membranes of intestinal mucosal cells (Giannella et al., 1983; Frantz et al., 1984; Dreyfus & Robertson, 1984; Kuno et al., 1986; Cohen et al., 1987). ST-receptor interaction activates particulate guanylate cyclase, resulting in intracellular cGMP accumulation (Field et al., 1978; Dreyfus & Robertson, 1984; Kuno et al., 1986; Waldman et al., 1986; Waldman & Murad, 1987; Huott et al., 1988; Hugues & Waldman, 1991). This cascade has been suggested to mediate the alterations in fluid and electrolyte transport resulting in diarrhea (Field et al., 1978; Hughes et al., 1978; Dreyfus & Robertson, 1984; Kuno et al., 1986; Waldman et al., 1986; Waldman & Murad, 1987; Huott et al., 1988). Data suggest that ST receptors are structurally and functionally heterogeneous and the mechanisms by which they induce diarrhea complex. Covalent cross-linking of radiolabeled ST to intestinal mucosal membranes results in the specific incorporation of label into protein subunits of different molecular weights (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1989; Thompson & Giannella, 1990). Also, ST-receptor populations with a high

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[1] Abbreviations: BCA, bicinchoninic acid; DSS, disuccinimidyl suberate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; cGMP, guanosine cyclic 3',5'-phosphate; NHS-LC-biotin, *N*-hydroxysuccinimide-long-chain-biotin; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ST, *Escherichia coli* heat-stable enterotoxin.

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affinity and low capacity or low affinity and high capacity for the toxin have been described (Hugues et al., 1991). Furthermore, binding to ST to receptors may induce phosphatidylinositol metabolism and protein kinase C activation (Banik & Ganguly, 1988, 1989; Weikel et al., 1990; Crane et al., 1990). Further characterization of this structural and functional heterogeneity and those mechanisms would be facilitated by obtaining purified preparations of active ST receptors. However, progress in this area has been hampered by the difficulties of purifying ST receptors by classical techniques and the inability to recover active receptors using ligand-affinity chromatography (Thompson, 1987; Hugues & Waldman, 1991). In this report, purification of functional ST receptors from rat intestine by ligand-affinity chromatography is described. These preparations provide additional evidence supporting the existence of receptor subunit heterogeneity, in close agreement with previous reports (Ivens et al., 1989; Thompson & Giannella, 1990).

Materials and Methods

Native ST was purified from *E. coli* strain 431 as described previously (Alderete & Robertson, 1978; Dreyfus et al., 1983). NHS-LC-biotin, disuccinimidyl suberate (DSS), BCA reagent for protein determinations, and monomeric avidin immobilized on 4% agarose were obtained from Pierce (Rockford, IL). Protein was visualized in polyacrylamide gels utilizing a silver staining kit, and high molecular weight protein standards were obtained from Bio-Rad (Richmond, CA). Centricon 30 concentrating devices were obtained from Amicon (Beverly, MA). All other reagents were of analytical grade and were obtained as described previously (Waldman et al., 1986; Ivens et al., 1989).

Biotinylated ST was prepared as described (Pierce Instruction Booklet, 1989). Biotin derivatized with a spacer arm was utilized since ST coupled poorly to native biotin. Briefly, native ST, containing ^{125}I-ST as a tracer, was mixed overnight at room temperature with a 20-fold molar excess of NHS-LC-biotin in 50 mM carbonate buffer, pH 9.0. Biotinylation of ST was monitored and unreacted reagents were separated by reverse-phase HPLC. Reaction mixtures were loaded onto a μBondapak C_{18} column (300 × 3.9 mm; Millipore), and the column was developed with a gradient of 10–90% acetonitrile in 50 mM ammonium acetate, pH 5.8, over 70 min. Unreacted NHS-LC-biotin presumably flowed through this column without binding, unreacted ST eluted at 52 min, and NHS-LC-biotin–ST eluted at 64 min. Immobilized monomeric avidin was utilized as the affinity support for the biotinylated toxin in these studies. Native avidin is a tetramer with an affinity for biotin of about 10^{-15} M, which is typically eluted with denaturing agents such as acidic pH and guanidinium chloride (Pierce Instruction Booklet, 1990). In contrast, monomeric avidin demonstrates a reduced affinity for biotin (about 10^{-8} M) and can be eluted under native conditions using 2 mM biotin. Biotinylated ST was bound to immobilized monomeric avidin which was prepared according to the manufacturer's instructions (Pierce Instruction Booklet, 1990).

Membranes from rat intestine were prepared by a modification of a procedure described previously (Waldman et al., 1986). All procedures were conducted at 4 °C. Briefly, rats were sacrificed; small intestine was removed and washed with 0.9% NaCl and then with buffer containing 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride (PMSF; TED buffer) and 250 mM sucrose. Mucosa was collected, homogenized in TED buffer containing sucrose, and centrifuged at 100000*g* for 60 min. Pellets were washed sequentially in (1) TED containing 500 mM KCl, (2) TED containing 0.125 M sucrose, and (3) TED. Resulting washed pellets were resuspended in TED and stored at −70 °C until use. Membranes were solubilized by rotation for 60 min in 10 mM phosphate buffer, pH 7.2, containing 1% glycerol, 0.1 mM PMSF, and 0.2% Lubrol PX (lubrol), followed by centrifugation at 100000*g* for 60 min.

Crude lubrol extracts were diluted 1:3 (v/v) with buffer containing 10 mM phosphate, pH 7.2, 150 mM NaCl, 0.1 mM PMSF, and 0.02% lubrol (buffer A) and loaded at about 1 mL/min onto the affinity resin prepared as described above. The affinity resin with receptors bound was washed with 10 column volumes of buffer A, and active receptors were specifically eluted with buffer A containing 2 mM biotin. Fractions eluted from immobilized monomeric avidin and containing biotinylated ST–receptor complexes were pooled and made 3% in chenodeoxycholate. Samples were incubated for 5 min at 37 °C, diluted 10-fold in buffer A, and concentrated by Amicon (Cambridge, MA), using a YM 30 membrane. Samples were subsequently diluted 10000–100000-fold with buffer A without lubrol and concentrated by Amicon.

Iodinated ST (1000 Ci/mmol) was prepared as described (Thompson et al., 1985). ST–receptor binding was performed by a modification of a procedure described previously (Kuno et al., 1986). Briefly, samples were incubated in buffer containing 50 mM Tris-HCl, pH 7.6, 0.67 mM cystamine, 0.1% (w/v) bacitracin, 150 mM NaCl, and 1 mM EDTA (final volume 300 μL). Assays were performed using iodinated ST (10^{-9}–10^{-8} M) with specific activities of 100 (crude extracts) or 1000 (purified receptor) Ci/mmol. Assays were processed and binding was quantified as described previously (Kuno et al., 19865). Labeled ST was cross-linked to receptors by a modification of an established protocol (Ivens et al., 1989). Briefly, receptors were incubated in buffer A containing (1–4) × 10^{-8} M labeled ST (1000 Ci/mmol). Some incubations contained a 1000-fold molar excess of unlabeled ST to determine the specificity of cross-linking. DSS dissolved in DMSO was added to a final concentration of 1 mM; reactions were incubated for 15 min at room temperature and terminated by the addition of Tris-HCl, pH 7.6, to a final concentration of 50 mM. Samples were concentrated in a Centricon 30 apparatus to a final volume of 150 μL and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) on 7–12% linear gradient slab gels by the method of Laemmli as described previously (Kuno et al., 1986). Proteins in gels were visualized using a sensitive silver stain (Bio-Rad Silver Stain Kit; Merril et al., 1981). Direct autoradiography of dried gels was performed using Kodak XARS film. Silver-stained proteins in SDS gels were analyzed by densitometry using an LKB Ultrascan XL laser densitometer and GelScan XL software (Pharmacia, Piscataway, NJ). Protein concentrations were determined using the BCA reagent unless otherwise indicated. All determinations were performed in triplicate and results are representative of at least three experiments.

Results and Discussion

ST receptors in crude detergent extracts were specifically and quantitatively retained by NHS-LC-biotin–ST bound to immobilized monomeric avidin. Indeed, greater than 80% of the total binding activity was lost from extracts chromatographed on this affinity matrix. Little ST-binding activity associated with control columns made with NHS-LC-biotin alone coupled to monomeric avidin. Extensive washing of the column with buffer A resulted in no loss of bound ST or receptor-binding activity. However, incorporation of 2 mM biotin into this buffer resulted in the specific elution of 20–30% of the total NHS-LC-biotin–ST bound to these columns.

Table I: Purification of ST Receptors from Rat Intestinal Mucosal Membranes

step	total protein[a]	total binding[b]	specific binding[c]	% recovery[d]	x-fold purification[e]
detergent extract	9100	4.8	0.53	100	1
ST–agarose	0.12	0.13	1100	2.7	2100

[a] Total protein (μg). Protein in detergent extracts was determined using the BCA reagent (Pierce). Protein concentration in affinity-purified samples was below the limit of detection of assay and was estimated by comparing the density of silve staining in these samples to that staining of different concentrations of bovine serum albumin in polyacrylamide gels. Protein staining was quantified by densitometry. [b] Total binding (pmol of ^{125}I-ST bound). [c] Specific binding (pmol of ^{125}I-ST bound/mg of protein). [d] % recovery = (total binding in fraction)/(total binding in extract). [e] x-Fold purification = (specific binding in fraction)/(specific binding in extract).

ST receptors formed a complex with the NHS-LC-biotin–ST specifically eluted from monomeric avidin. In order to detect functional receptors, these complexes required dissociation and separation of the receptor from the derivatized toxin. Preliminary studies in this laboratory demonstrated that bile salts completely dissociated ST–receptor complexes. All bile salts tested effectively dissociated ST–receptor complexes in a concentration-dependent fashion. The concentration of bile salts producing half-maximum dissociation correlated closely with their critical micellar concentrations. These data suggest that dissociation of ST–receptor interaction by bile salts reflects a nonspecific detergent effect of these agents, rather than their ability to specifically compete for receptor binding. Dissociation of ST–receptor interaction by bile salts is reversible, and 30–40% of the initial binding activity can be recovered by removing bile salts by dilution and concentration.

The yield of ST–receptor activity was about 3% after elution from monomeric avidin with 2 mM biotin and dissociation of the toxin–receptor complex with chenodeoxycholate (Table I). Maximum purification of ST-binding activity of about 2000-fold was obtained using this technique. These preparations exhibited a major silver-staining protein subunit with a molecular weight of 74 kDa by SDS–PAGE analysis (Figure 1A). This is in close agreement to the mass of a major ST–receptor subunit identified in crude membrane preparations by affinity cross-linking to labeled toxin (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1989; Thompson & Giannella, 1990). Cross-linking of these preparations with ^{125}I-ST resulted in specific incorporation of labeled toxin into the 74-kDa protein subunit (Figure 1B). In addition, lower amounts of labeled toxin were incorporated into subunits with molecular masses of 164 and 45 kDa (Figure 1B). These affinity-labeled subunits are similar in size to those reported previously in crude intestinal membranes (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1989; Thompson & Giannella, 1990). Differences between the intensity of staining and affinity labeling of proteins in purified preparations may reflect differential affinity of receptor subunits for silver or ST (Hugues et al., 1991). Unlabeled ST competed with labeled toxin for binding to purified receptors in a concentration-dependent fashion with an $IC_{50} = 1 \times 10^{-9}$ M (Figure 2). This affinity is similar to that reported for low-affinity, high-capacity receptors described previously (Hugues et al., 1991).

This is the first report utilizing ligand-affinity chromatography to purify active ST receptors. Previous efforts to purify these proteins have been hampered by the inability to recover active receptors from ST-affinity matrices (Thompson, 1987;

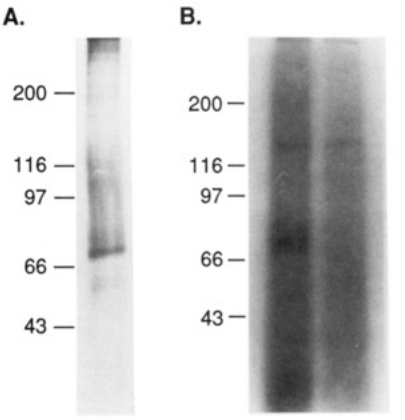

FIGURE 1: SDS–PAGE analysis of ST receptors purified by affinity chromatography. ST receptors were purified from detergent extracts of intestinal membranes (30 mg of protein) and subjected to SDS–PAGE as described under Materials and Methods. (A) Silver stain of an affinity-purified ST receptor preparation. (B) Autoradiogram of a purified preparation after affinity cross-linking to ^{125}I-ST. One milliliter of the purified preparation was cross-linked to ^{125}I-ST in the presence (right lane) or absence (left lane) of a 1000-fold excess of unlabeled ST, concentrated, and subjected to SDS–PAGE as described under Materials and Methods.

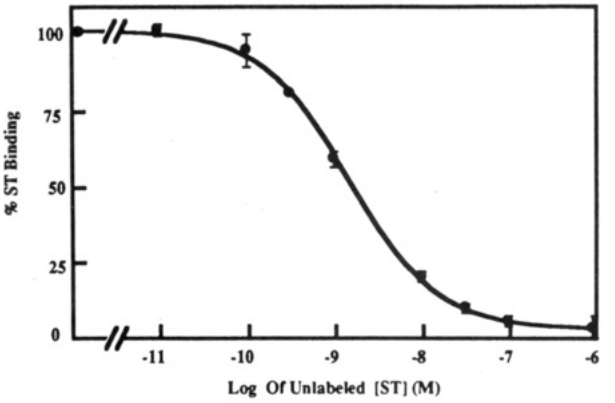

FIGURE 2: Competitive binding between labeled ST (2×10^{-9} M) and increasing concentrations of unlabeled ST to purified receptors. Experiments were performed as described under Materials and Methods and previously (Hugues et al., 1991). Results are the average of three determinations and error bars represent the SEM.

Hugues & Waldman, 1991). In addition, these receptors have been resistant to purification utilizing classical techniques In this report, active receptors were purified using NHS-LC-biotin–ST coupled to immobilized monomeric avidin as an affinity matrix. Using this novel technique, functional ST receptors from intestinal cells were purified as much as 2000-fold. The subunit composition of these preparations, defined by protein staining and specific cross-linking to labeled ligand, was similar to that previously reported for ST receptors in crude intestinal membranes (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1989; Thompson & Giannella, 1990). Proteins eluted from the affinity column were identified as ST receptors on the basis of the observations that (i) ST-binding activity was specifically and quantitatively retained by the affinity matrix, (ii) purified preparations had a subunit structure, determined by protein staining after SDS–PAGE, consistent with that defined by affinity cross-linking of ^{125}I-ST to crude intestinal membranes previously, (iii) purified preparations demonstrated specific ST-binding activity, and (iv) ST receptors in purified preparations possessed a subunit structure, measured by affinity cross-linking to ^{125}I-ST, which compared closely with that defined previously in crude mem-

brane preparations (Kuno et al., 1986; Gariepy & Schoolnik, 1986; Ivens et al., 1989; Thompson & Giannella, 1990).

The reasons for low recovery of total ST-binding activity by affinity chromatography remain unclear. Previously, functional ST receptors could not be recovered from affinity matrices which contain ST covalently cross-linked to a solid support (Thompson, 1987; Hugues & Waldman, 1991). One possibility is that ST–receptor interaction is an irreversible process. Indeed, about 40–50% of binding of ST to receptors was reported to be irreversible (Dreyfus et al., 1983; Dreyfus & Robertson, 1984; Cohen et al., 1987; Ivens et al., 1989; Hugues et al., 1991). However, in the present experiments bile salts completely dissociated ST–receptor interaction, and about 40% of that activity could be recovered. Also, these data mitigate against the possibility that ST receptors can only bind ligand one time, after which they become nonfunctional. In addition, data presented herein demonstrate that exposing crude detergent extracts containing receptors to bile salts and Amicon membranes results in the loss of about 65% of the total ligand-binding activity in the sample. This loss may be greater in preparations of pure receptors. Finally, elution of these columns with 2 mM biotin results in the recovery of only about 30% of the total NHS-LC-biotin–ST bound to the column. Thus, only 30% of the receptors specifically bound to the column will be available in purified preparations. Monomeric avidin–agarose is composed of a mixture of monomeric and tetrameric avidin (Pierce, 1990). Biotin bound to tetrameric avidin will not elute with 2 mM biotin and so is essentially irreversibly bound to the column. Consequently, receptors bound to NHS-LC-biotin–ST associated with tetrameric avidin will not elute with biotin.

ST receptors were purified as much as 2000-fold from crude detergent extracts of intestinal membranes using the described protocol. This is lower than the theoretical maximum purification of these receptors from crude detergent extracts which is about 6000 on the basis of an observed B_{max} of 1 pmol of ST/mg of protein, the assumption of a binding stoichiometry of 1, and a holoreceptor molecular weight of about 150 kDa (Kuno et al., 1986; Thompson, 1987; Ivens et al., 1989; Thompson & Giannella, 1990, Hugues & Waldman, 1991). As discussed above, significant receptor activity is lost during chromatography using immobilized monomeric avidin–biotinylated ST, elution with biotin, and dissociation of the toxin–receptor complex with chenodeoxycholate followed by dilution and Amicon filtration. Thus, the yield of receptor protein may be higher than that based on the recovery of binding activity. This suggestion is supported by the lack of other major proteins detected by silver stain and comigration of the major silver-staining and affinity-labeled proteins upon SDS–PAGE.

Recently, a protein possessing guanylate cyclase and ST-binding activity was cloned and expressed from rat and human intestinal cells (Schulz et al., 1990; de Sauvage et al., 1991). This protein appears to be a member of the particulate guanylate cyclase–peptide receptor family which includes receptors for atrial and other natriuretic peptides and egg factors produced by sperm in sea urchin (Chinkers & Garbers, 1991). These proteins share a similar general structure, possessing an extracellular domain containing peptide ligand-binding activity, a single transmembrane domain, and a cytoplasmic domain containing guanylate cyclase catalytic activity. In light of these studies, it is important to determine whether purified preparations of ST receptors possess guanylate cyclase activity, to confirm that these activities reside on a single transmembrane protein. However, there are technical constraints imposed by the present protocol which make these determinations difficult. First, ST affinity column chromatography is conducted at room temperature over several hours. Indeed, at lower temperatures or shorter times, ligand–receptor interaction is too slow for efficient binding and purification. However, intestinal guanylate cyclase is exquisitely heat-labile, and the enzyme becomes inactive after incubation for 5 min at 37 °C (Crane et al., 1990; unpublished observations). Similarly, the enzyme is labile after hours of incubation at room temperature, in contrast to ST receptors, which appear to be stable under these conditions (unpublished observations). Also, affinity purification is conducted utilizing phosphate buffer, which has been demonstrated previously to inhibit guanylate cyclase activity (Brandwein et al., 1982; Hakki & Sitaramayya, 1990). Further, purified preparations are incubated at 37 °C in bile acids to permit dissociation of ST and receptor. As pointed out above, guanylate cyclase is inactivated after 5 min at 37 °C, and bile acids have been demonstrated to be potent inhibitors of this enzyme (Waldman et al., 1986; Crane et al., 1989; unpublished observations). Bile salt inhibition of intestinal particulate guanylate cyclase was not reversible previously. These technical constraints imposed by the current purification protocol render it difficult to measure guanylate cyclase activity in preparations as they are presently processed. Currently, approaches to modify this protocol are being examined to permit measurement of enzyme activity after receptor purification.

In summary, functional ST receptors have been purified by ligand-affinity chromatography utilizing derivatized biotin and a novel monomeric avidin stationary phase. These preparations demonstrate a heterogeneous subunit composition by protein staining and covalent affinity cross-linking to labeled ligand. Highly purified receptors which retain function will be useful to further elucidate the mechanisms by which ST induces intestinal secretion. Similarly, the ability to purify active ST receptors will facilitate studies of the structural and functional relationships of different receptor populations to each other and particulate guanylate cyclase.

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Cleavage of Oligoribonucleotides by a Ribozyme Derived from the Hepatitis δ Virus RNA Sequence†

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ABSTRACT: A self-cleaving RNA sequence from hepatitis δ virus was modified to produce a ribozyme capable of catalyzing the cleavage of RNA in an intermolecular (trans) reaction. The δ-derived ribozyme cleaved substrate RNA at a specific site, and the sequence specificity could be altered with mutations in the region of the ribozyme proposed to base pair with the substrate. A substrate target size of approximately 8 nucleotides in length was identified. Octanucleotides containing a single ribonucleotide immediately 5′ to the cleavage site were substrates for cleavage, and cleavage activity was significantly reduced only with a guanine base at that position. A deoxyribose 5′ to the cleavage site blocked the reaction. These data are consistent with a proposed secondary structure for the self-cleaving form of the hepatitis δ virus ribozyme in which a duplex forms with sequences 3′ to the cleavage site, and they support a proposed mechanism in which cleavage involves attack on the phosphorus at the cleavage site by the adjacent 2′-hydroxyl group.

Hepatitis δ virus (HDV)[1] is a small single-stranded RNA virus that has been found in certain patients who are also infected with hepatitis B (Taylor, 1990). A self-cleaving sequence present in both the genomic RNA and the complementary antigenomic RNA may act to process the RNA during rolling circle replication of the viral RNAs (Kuo et al., 1988; Sharmeen et al., 1988; Wu et al., 1989). The HDV RNA, therefore, may be the first clear example of an autocatalytic RNA (ribozyme) that in its natural form functions in human cells. With the identification of self-cleaving sequences in the HDV RNAs (Kuo et al., 1988; Sharmeen et al., 1988; Wu et al., 1989), it was suggested that the HDV self-cleaving structure would have to represent a structural motif distinct from other self-cleaving RNAs (Hutchins et al., 1986; Forster & Symons, 1987; Hampel et al., 1990; Feldstein et al., 1990). Evidence in support of a potential secondary structure that is common to both the genomic and antigenomic self-cleaving sequences has been presented (Perrotta & Been, 1991; Rosenstein & Been, 1991). As with other self-cleaving RNAs, self-cleavage activity of the HDV RNA requires a divalent cation, and cleavage generates products containing a 5′-hydroxyl group and a 2′,3′-cyclic phosphate (Kuo et al., 1988; Wu et al., 1989).

Studies on the structure, substrate specificity, and kinetic mechanism of other autocatalytic RNAs have been expedited by converting the intramolecular reaction to intermolecular forms (Zaug & Cech, 1986; Zaug et al., 1986; McSwiggen

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[1] Abbreviations: HDV, hepatitis δ virus; nt, nucleotide or nucleotides; PEI, poly(ethylenimine); TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid.

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