An Ultraviolet-Inducible Adenosine–Adenosine Cross-Link Reflects the Catalytic Structure of the *Tetrahymena* Ribozyme†

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ABSTRACT: When a shortened enzymatic version of the *Tetrahymena* self-splicing intervening sequence (IVS) RNA is placed under catalytic conditions and irradiated at 254 nm, a covalent cross-link forms with high efficiency. The position of the cross-link was mapped by using three independent methods: RNase H digestion, primer extension with reverse transcriptase, and partial hydrolysis of end-labeled RNA. The cross-link is chemically unusual in that it joins two adenosines, A57 and A95. Formation of this cross-link depends upon the identity and concentration of divalent cations present and upon heat–cool renaturation of the IVS in a manner that parallels conditions required for optimal catalytic activity. Furthermore, cross-linking requires the presence of sequences within the core structure, which is conserved among group I intervening sequences and necessary for catalytic activity. Together these correlations suggest that a common folded structure permits cross-linking and catalytic activity. The core can form this structure independent of the presence of P1 and elements at the 3′ end of the IVS. The cross-linked RNA loses catalytic activity under destabilizing conditions, presumably due to disruption of the folded structure by the cross-link. One of the nucleotides participating in this cross-link is highly conserved (86%) within the secondary structure of group I intervening sequences. We conclude that A57 and A95 are precisely aligned in a catalytically active conformation of the RNA. A model is presented for the tertiary arrangement in the vicinity of the cross-link.

The self-splicing intervening sequence (IVS) of *Tetrahymena thermophila* is well-known for its catalytic properties. In vitro, the *Tetrahymena* IVS can splice itself from the large rRNA precursor without the aid of an auxiliary protein. All that it requires is magnesium ion and a guanosine nucleotide (Kruger et al., 1982). This IVS is one member of a large family of intervening sequences identified as group I, which share conserved sequences, similar secondary structures, and the same mechanism for splicing themselves from their precursor RNAs (Michel & Dujon, 1983; Waring et al., 1983; Cech & Bass, 1986).

Shortened versions of the IVS can catalyze additional reactions such as site-specific endoribonucleolytic cleavage (Zaug et al., 1986), nucleotidyl transfer (Kay & Inoue, 1987; Been & Cech, 1988), transfer of phosphate monoesters (Zaug & Cech, 1986), and RNA ligation (Doudna & Szostak, 1989). These molecules therefore represent true RNA enzymes. While the activities of the *Tetrahymena* IVS and its variants have been well characterized, its folded structure is only beginning to be revealed. The ultraviolet-inducible cross-link described here provides information about the three-dimensional structure of one region of the IVS.

UV cross-linking in any RNA depends upon a proper orientation of the participating bases. In situations of specific, highly efficient cross-linking, such alignment is provided by the stably folded conformation of the RNA. These cross-links are then reproducible and can be used to ascertain the folded structure within the RNAs that gave rise to them. In the 16S rRNA of *Escherichia coli*, such cross-links have provided a means for modeling the three-dimensional structure of this RNA within the 30S ribosomal subunit (Atmadje & Brimacombe, 1985). In tRNA, UV cross-linking has been used to identify tertiary interactions (Delaney et al., 1974) and to detect retention or alteration of folding caused by base substitutions (J. A. Sampson and O. C. Uhlenbeck, personal communication). Furthermore, such cross-links can identify recurrent structural motifs in highly structured RNAs (Branch et al., 1985).

In the following studies, a UV-induced cross-link has been mapped within a shortened version of the *Tetrahymena* IVS, the L-21 *Sca*I IVS (Zaug et al., 1988). This molecule differs from the full-length *Tetrahymena* IVS in that it lacks the first 21 nucleotides from the 5′ end and 5 nucleotides from the 3′ end. The internal guide sequence, part of the active site, makes up the new 5′ end, and the core structure that characterizes group I intervening sequences is still present. The L-21 *Sca*I IVS retains catalytic activity including that of site-specific cleavage of single-stranded RNA in a reaction that mimicks the first step of splicing. The cross-link that can be induced in this molecule forms efficiently, reproducibly, and between nucleotides separated in sequence. This cross-link therefore appears to be the consequence of a stably folded structure. Furthermore, the conditions and sequences required for cross-linking suggest that the stably folded structure is a catalytically active conformation of the molecule.

Materials and Methods

Preparation of L-21 ScaI IVS. The procedure used is described by Zaug et al. (1988) with the following exceptions. The 10× transcription buffer was composed of 200 mM Tris-HCl (pH 7.5), 75 mM $MgCl_2$, 25 mM DTT, and 10 mM spermidine. A 1-mL transcription reaction contained 100 μL of 10× transcription buffer, 100 μL of 10× nucleotide mix, 5 μg of linearized template (pT7L-21), and 1000–2000 units of T7 RNA polymerase. The L-21 *Sca*I IVS was precipitated in 0.25 M NaCl plus 3 volumes of absolute ethanol and then

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gel-purified. The RNA was eluted from the gel slice by rocking in 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.25 M NaCl. The RNA in the eluate was then precipitated with 2.5–3 volumes of absolute ethanol.

Cross-Linking of RNA. The RNA was first denatured by heating in deionized water, 3 min at 96 °C. A tenth volume of buffer was injected into the IVS solution to introduce the desired concentration of cations [e.g., 10 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris-HCl (pH 7.5)]. Immediately thereafter, the solution was placed on ice (experiments of Figure 7), or it was allowed to cool over the course of 2–30 min to 0 °C (experiments of Figures 1 and 6B). (Heating the RNA to any temperature above 50 °C and cooling either quickly or gradually gave the same extent of cross-linking.) The heat-cooled IVS samples were irradiated at a concentration of 0.2 mg/mL or less as droplets on plastic wrap over ice water or, for the experiment of Figure 4, placed in a capped NSG quartz cuvette with a path length of 2 mm, partially submerged in a temperature-controlled water bath. The 254-nm radiation source was a UVG-11 mineral lamp (UVP Inc.). The UV-irradiated IVS was ethanol-precipitated and subjected to electrophoresis on a denaturing gel (6% or greater polyacrylamide/7 M urea). The cross-linked and un-cross-linked products were excised as separate bands, frozen, and crushed, and then soaked overnight at 4 °C in 0.25 M NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA to elute the RNA. The RNA was then precipitated with 2.5–3 volumes of absolute ethanol.

End Labeling of RNA. For 5′ end labeling, 100 pmol of substrate RNA was incubated for 30 min at 37 °C in 50 μL of 50 mM Tris-HCl (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, and 5–10 units of calf intestinal phosphatase (New England Nuclear). This reaction mixture was then subjected to phenol extraction, extraction with 24:1 chloroform/isoamyl alcohol (v/v), and two ether extractions. The ether was then blown off with a stream of filtered air, NaCl was added to a concentration of 0.25 M, and the RNA was ethanol-precipitated. The RNA was resuspended in 50 μL of 50 mM Tris-HCl (7.5), 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA with 20 units of T4 polynucleotide kinase (from USB) and 7000 Ci/mmol crude [γ-^{32}P]ATP (from ICN) and incubated for 30 min at 37 °C.

For 3′ end labeling of IVS RNA, [^{32}P]pCp (10 mCi/mL from New England Nuclear) was ligated to the 3′ end of the RNA according to the method described by England and Uhlenbeck (1978).

After labeling, the RNA was ethanol-precipitated with NaCl and 3 volumes of absolute ethanol. The pellet was dried, resuspended in loading buffer, and subjected to electrophoresis on a 7 M urea denaturing gel for gel purification. The desired band was visualized by autoradiography, cut from the gel, frozen, and crushed; then the labeled RNA was recovered by ethanol precipitation of an overnight soak of the fragments in 0.25 M NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA.

Preparation of the Cross-Linked Fragment for Enzymatic Sequencing. For mapping the 3′ partner of the cross-link (Figure 3), fragments were made and 3′ end labeled as follows. UV-irradiated but un-cross-linked L-21 *Sca*I IVS (4.4 nmol) and the cross-linked form (3.7 nmol) were each subjected to an RNase H digest. Each RNA was combined with 29 nmol of a synthetic deoxyoligonucleotide complementary to nucleotides 101–125 of the IVS in 100 μL of 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM DTT, and 0.01% BSA. This solution was heated to 96 °C for 2 min and put on ice for 1 min; then 4 units of *E. coli* RNase H (Pharmacia) was added, and the solution was incubated for 1 h at 37 °C. The solution was heated, cooled, and incubated with 4 units of RNase H two more times to ensure that the RNA was digested to completion. The entire digests were ethanol-precipitated and loaded on a 6% polyacrylamide/7 M urea sequencing gel. Individual bands were visualized by UV shadowing, excised, frozen and crushed, and then soaked overnight at 4 °C in 0.25 M NaCl, 10 mM Tris-HCl (pH 7.5), and 5 mM EDTA while rocking. The RNA was ethanol-precipitated and used for 3′ end labeling.

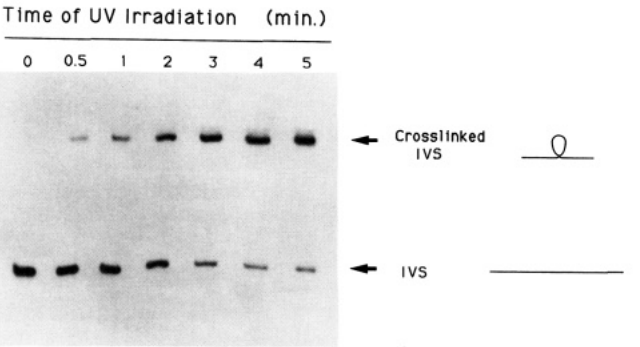

FIGURE 1: L-21 *Sca*I IVS cross-linked by ultraviolet light. 5′-^{32}P-Labeled L-21 *Sca*I IVS was heat-renatured and then irradiated at 254 nm for the times specified as 10-μL droplets at 0 °C (see Materials and Methods). The 254-nm irradiation converts the IVS into a cross-linked species that migrates more slowly on a denaturing gel (10% polyacrylamide/0.5× TBE/7 M urea).

RNA Sequencing. Enzymatic sequencing, including partial alkaline hydrolysis, was performed on 5′ or 3′ end-labeled RNA according to the methods of Donis-Keller et al. (1977) and Donis-Keller (1980). Some variations were made in the procedure: 0.01 unit of RNase T1 or U2 or 1 unit of Phy-M was included in a 5-μL reaction mixture containing 0.2 mg/mL tRNA that was incubated 15 min at 50 °C. The T1 and U2 RNases were purchased from Sankyo, and the RNase PhyM was from Bethesda Research Laboratories. Sequencing by primer extension was performed according to the procedure given in Zaug et al. (1984).

RESULTS

UV Cross-Linking of the IVS. L-21 *Sca*I IVS RNA was heated (50–100 °C) and then cooled (0 °C) in the presence of 10 mM $MgCl_2$ and 100 mM NaCl, the same salt concentrations that permit catalytic activity. When irradiated at 254 nm, the RNA was converted to a single cross-linked species as evidenced by its retarded electrophoretic mobility on a denaturing 10% polyacrylamide gel (Figure 1). This procedure of heating and cooling prior to irradiation proved necessary for optimal cross-linking efficiency (maximum of 77%). Presumably this procedure permits the IVS to assume a stably folded structure that is competent for cross-linking. Without the benefit of heating and cooling, the same period of irradiation typically yielded only 5% cross-linked product.

The rate of cross-linking did not change appreciably over a 30000-fold variation in IVS concentration [(9×10^{-5})–2.4 μM], suggesting that the cross-linking reaction is an intramolecular event (data not shown). Therefore, the cross-link most likely forms between two nonadjacent nucleotides, such that under denaturing conditions a loop is created from the segment between them. Presumably it is this looped structure that retards the migration of the cross-linked IVS in denaturing gels. A single retarded band is also generated by UV irradiation of longer transcripts including the full-length *Tetrahymena* IVS with flanking exons (data not shown).

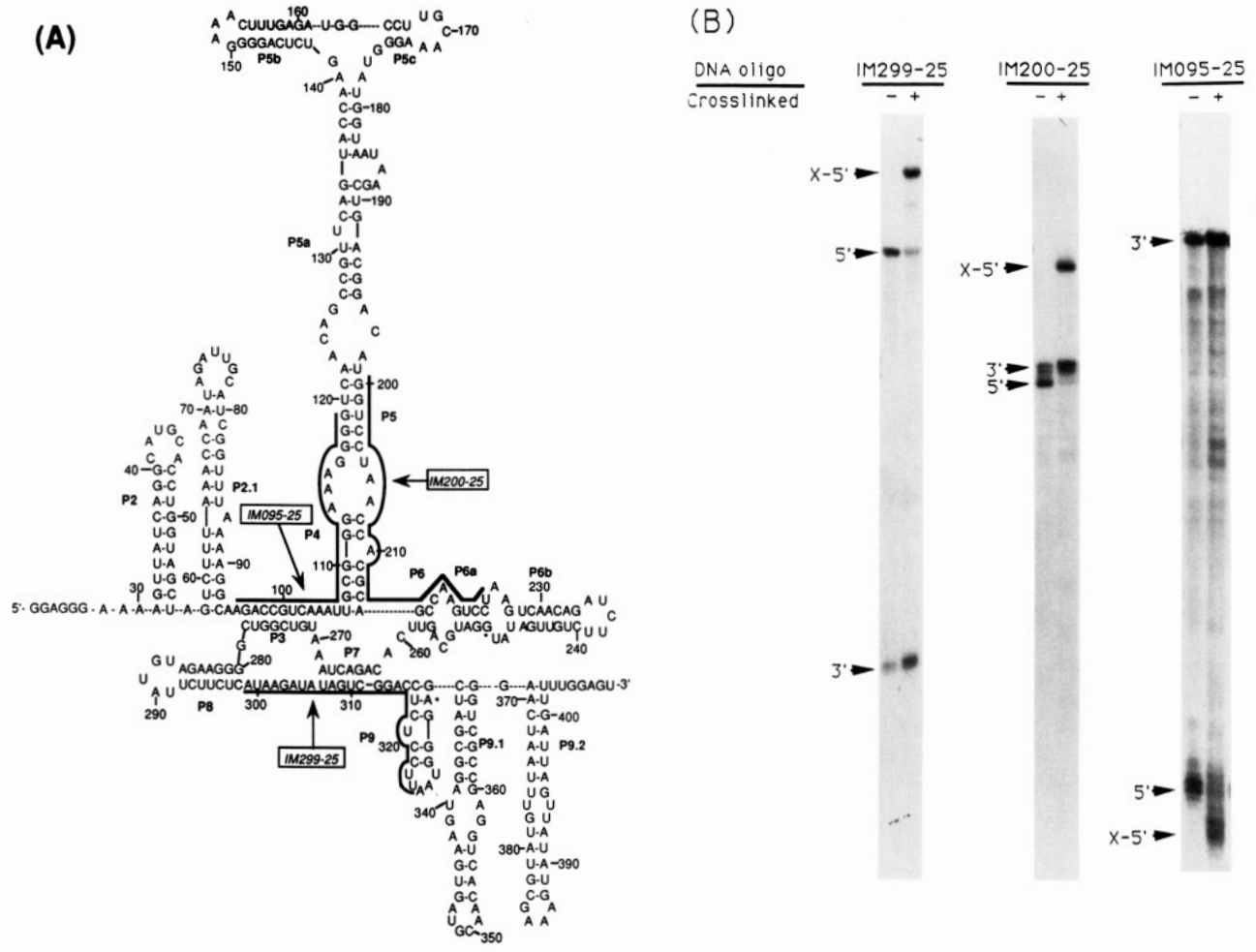

FIGURE 2: Locating the cross-link from RNase H digests. (A) Deoxyoligonucleotides used to direct RNase H cleavage. The three synthetic deoxyoligonucleotides are depicted as heavy lines where they anneal to the L-21 *Sca*I IVS RNA. (B) Products of RNase H digestion of un-cross-linked and cross-linked L-21 *Sca*I IVS. Parallel digestions were performed on un-cross-linked and cross-linked uniformly labeled L-21 *Sca*I IVS that had been gel-purified from a single irradiated sample. For each digest, approximately 1 pmol of IVS was combined with 200 pmol of one of the deoxyoligonucleotides depicted in (A) in 10 μL containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, and 0.1% BSA. This mixture was heated to 96 °C and then cooled over 20 min to 37 °C. *E. coli* RNase H (0.25 unit) was added, and the reaction was incubated for 90 min at 37 °C. In each case, multiple cleavages were made in the IVS in the region of hybridization, and two families of labeled RNA fragments were created from the segments 5′ and 3′ of those cuts. These products were separated on a 10% polyacrylamide/7 M urea gel. Arrows indicate un-cross-linked fragments containing the 5′ end or 3′ end of the RNA, and cross-linked 5′ fragments (X-5′).

Following UV-induced cross-linking to saturation (77% conversion), the un-cross-linked fraction of IVS was isolated and again heated and cooled on ice, and then taken through another round of cross-linking to saturation. In this second round of UV irradiation, 23% was converted to cross-linked product. Our interpretation is that the heat–cool protocol permits the IVS to assume an equilibrium distribution of conformations that do not readily interconvert, where not all conformations are competent for cross-linking. The second round of heating and cooling permits some of the misfolded molecules to reequilibrate their conformation which then allows them to be cross-linked. However, over the course of UV irradiation, the RNA accumulates photolesions apart from the observed cross-link; these photolesions disrupt the cross-linkable conformation.

Mapping the Cross-Link. Three methods, RNase H digestion, enzymatic sequencing, and primer extension, were consistent in identifying a single cross-link. Each method was applied to both the cross-linked and the un-cross-linked species resulting from UV irradiation. Both species should possess the same assortment of photolesions with the exception of the cross-link responsible for the slower migration on denaturing gels.

To determine what portion of the IVS contains the cross-linked structure, the RNA was cleaved into specific subfragments which were then examined on a denaturing gel. Cleavage was accomplished by annealing deoxyoligonucleotides to three specific regions of the IVS (Figure 2A) and treating with RNase H to selectively degrade the RNA within the hybrid duplex. Since the enzyme can degrade RNA at any point within the hybrid duplex and the complex will likely fall apart before digestion is complete, a family of similarly sized fragments rather than a single product is actually generated. In all cases tested, the fragments 3′ of the cleavage site were released and migrated identically in digests of both the un-cross-linked and cross-linked molecules (Figure 2B), suggesting that this portion of the IVS does not contain the cross-link. Only the fragments 5′ of each cut in the cross-linked molecule displayed an aberrant gel mobility. When cleavage was directed by the deoxyoligonucleotides IM200-25 and IM299-25, the 5′ fragments from the cross-linked IVS displayed a slower migration than the analogous fragment from the un-cross-linked molecule. Meanwhile cleavage directed by IM095-25 released fragments that migrated faster in the case of the cross-linked IVS than those cleaved from the un-cross-linked IVS; there is precedence for circular RNAs migrating faster

than the corresponding linear molecules if their molecular weight is sufficiently low (Kaufman et al., 1974). The most 5′ cut made utilized IM095-25, which is complementary to nucleotides 95–119 of the IVS (Figure 2A). Therefore, the cross-link must occur 5′ of nucleotide 119.

The nucleotides composing the cross-link were identified by the method of enzymatic sequencing. Accordingly, cross-linked and un-cross-linked RNA molecules were each end-labeled and then partially hydrolyzed with base-specific nucleases and alkali. These partial digests were run in parallel lanes on a denaturing gel, and the sequence was read. A gap in each sequence ladder was expected to occur at the site of cross-linking. The assumption was made that any fragment generated by partial digestion that was long enough to include a cross-linked nucleotide should carry with it all or a portion of the associated loop structure. Therefore, these larger sequencing fragments were expected to migrate exceptionally slowly on a sequencing gel, introducing a gap above the shorter fragments that comprise a normal sequence ladder.

5′ end labeled L-21 *Sca*I IVS was sequenced to identify the 5′-most partner in the cross-link. A gap unique to the cross-linked IVS was found as predicted (Figure 3A). Since alkaline hydrolysis is not restricted by base specificity or steric constraints as enzymes are, cleavage was expected to yield a readable ladder up to the cross-linking nucleotides in the alkali lanes. The location of the phosphodiester bond immediately preceding the cross-link was judged to be between A56 and A57 from the alkaline hydrolysis ladder, thereby identifying A57 as one of the cross-linking nucleotides.

The other nucleotide of the cross-link was identified by performing enzymatic sequencing on 3′ end labeled fragments of the L-21 *Sca*I IVS. These fragments were generated by subjecting cross-linked and un-cross-linked IVS molecules to an RNase H digest. A 5′ fragment with a 3′ terminus at U106 was recovered from the multiple products of each digest and was labeled at its 3′ end with [^{32}P]pCp. Enzymatic sequencing of these fragments again revealed a gap unique to the cross-linked molecule (Figure 3A). Examination of the alkaline hydrolysis ladder identified the 3′ cross-linking partner as A95.

Primer extension was also performed over most of the cross-linked IVS. Reverse transcriptase has been observed to stop elongation at modified bases within the template (Hagenbuchle et al., 1978; Youvan & Hearst, 1979; Barta et al., 1984), including cross-linked nucleotides (Denman et al., 1988). Therefore, primer extension was not expected to traverse the branched structure at the site of the L-21 *Sca*I cross-link. As expected, strong stops were observed only when the cross-linked IVS was used as template and only at two locations (data not shown). In both cases, the stop occurred one nucleotide 3′ of the cross-linked nucleotide identified by enzymatic sequencing and the partial alkaline hydrolysis ladder (data not shown). Likewise, others have observed stops one nucleotide 3′ of modified bases (Denman et al., 1988; Youvan & Hearst, 1981). Between enzymatic sequencing and primer extension, 93% of the cross-linked L-21 *Sca*I IVS was examined and revealed this single cross-link. The only nucleotides not examined by these methods lie between nucleotides 67 and 95 as illustrated in Figure 3B.

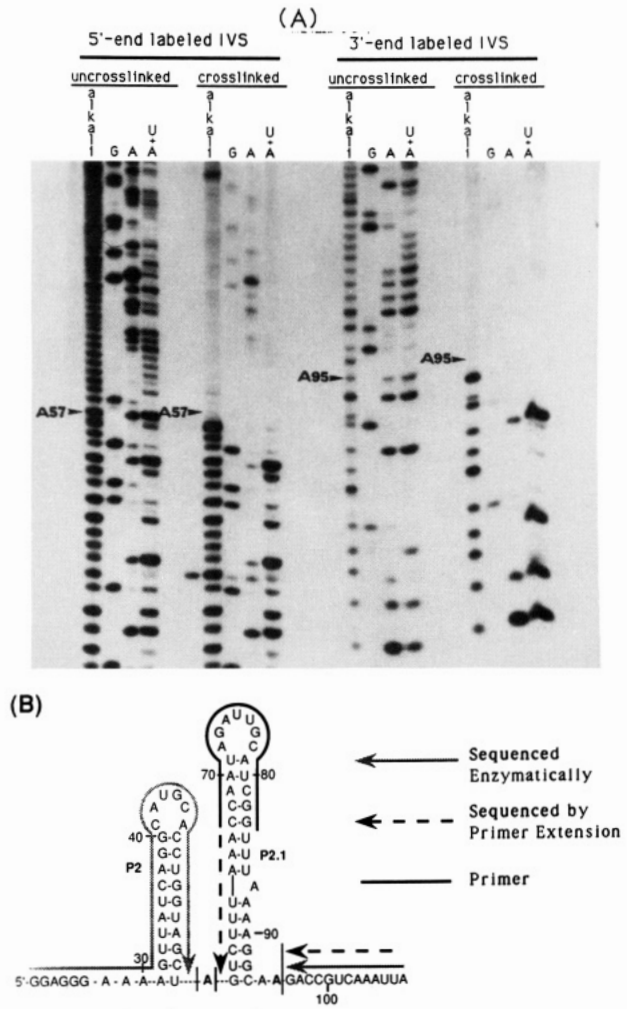

FIGURE 3: Identification of the two nucleotides participating in the cross-link. (A) 5′ end labeled L-21 *Sca*I IVS was gel-purified as un-cross-linked and cross-linked molecules following UV irradiation. 3′ end labeled un-cross-linked and cross-linked IVS fragments were prepared as described under Materials and Methods and include only nucleotides 21–106. The four end-labeled RNAs were subjected to partial alkaline hydrolysis according to the protocol of enzymatic sequencing (Donis-Keller et al., 1977). Alkali was used to generate a complete sequence ladder representing cleavage 3′ to every nucleotide. Base-specific cleavage was achieved by partial digests with RNase T1 to cleave 3′ to guanosine, RNase U2 to cleave 3′ to adenosine, and RNase Phy-M to cleave 3′ to uridine and adenosine. The markers A57 and A95 point to the expected positions of products of cleavages releasing these nucleotides (and the sequence connecting them to the end label) from the remainder of the native IVS. Sequencing of cross-linked molecules does not generate products of these sizes because A57 and A95 are cross-linked and therefore remain tethered to the remainder of the IVS even after alkaline hydrolysis of phosphodiester linkages. Sequencing was performed on a 0.5-mm 20% polyacrylamide/7 M urea denaturing gel. (B) Diagram illustrating the only portions of the cross-linked L-21 *Sca*I IVS where readthrough was not possible by enzymatic sequencing and primer extension. Arrows indicate the direction of sequencing. A vertical line indicates the last phosphodiester bond cleavage that can be identified from the alkali hydrolysis ladder in the case of enzymatic sequencing. Alternatively, the vertical line marks the point of a strong stop in primer extension. Adenosines identified as cross-linked from this analysis are shown in boldface.

Divalent Cation Requirement for Cross-Linking. To help determine whether the same structure is required for cross-linking and catalysis, the effects of magnesium ion concentration on both activities were tested and compared. Catalytic activity was assayed by the endoribonuclease reaction, in which the L-21 *Sca*I IVS carries out site-specific cleavage of a single-stranded substrate RNA following the sequence CUCU (Zaug et al., 1986). This reaction recapitulates the first step of splicing, cutting at the 5′ splice site. For both cross-linking and the endoribonuclease reaction, the same buffered magnesium solutions and temperature (49 °C) were used. The cross-linking reactions differed only in that they lacked 0.5 mM guanosine and GGCUCUCUA$_5$ RNA oligonucleotide, because these substrates absorb strongly at 254 nm. In earlier

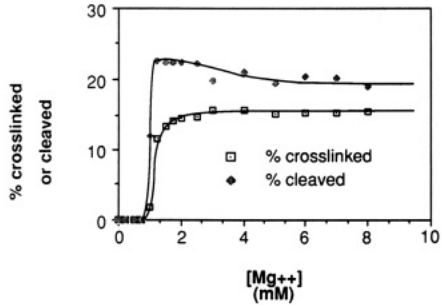

FIGURE 4: Effect of $[Mg^{2+}]$ on cross-linking and catalysis. To assay cross-linking, <0.4 pmol of 5′-^{32}P-labeled L-21 *Sca*I IVS was dissolved in 60 μL of 50 mM Tris-HCl (pH 7.5) and 0–8 mM $MgCl_2$. Each IVS solution was preincubated for at least 1 min in a 49 °C water bath and then irradiated 1 min at 254 nm. To assay catalytic activity, cleavage of [5′-^{32}P]GGCUCUCUA$_5$ substrate (2 μM) by L-21 *Sca*I IVS (0.025 μM) was measured. L-21 *Sca*I IVS, [5′-^{32}P]GGCUCUCUA$_5$, and 0.5 mM guanosine were equilibrated in 16 μL of deionized water to 49 °C. To initiate the reaction, 4 μL of a 5× buffer was added to introduce 50 mM Tris-HCl (pH 7.5) and 0–8 mM $MgCl_2$. After 1-h incubation at 49 °C, 20 μL of 10 M urea/50 mM EDTA was added to stop the reaction. For both cross-linking and endoribonucleolytic cleavage, the percent reacted was determined by performing PAGE on all samples and quantitating counts on the dried gel with the Ambis radioanalytic system.

Table I

divalent cations present	cross-linking capability	endonuclease activity[a]
1.0 mM Mg^{2+}	–	–
1.0 mM Mn^{2+}	–	–
1.0 mM Ca^{2+}	–	–
1.5 mM Mg^{2+}	+	+
1.5 mM Mn^{2+}	+	+
1.5 mM Ca^{2+}	+	–
1.0 mM Ca^{2+} + 0.5 mM Mg^{2+}	+	+
1.0 mM Ca^{2+} + 0.5 mM Mn^{2+}	+	+
1.5 mM Fe^{2+}	–	–
1.5 mM Co^{2+}	–	–
1.5 mM Zn^{2+}	–	–
1.5 mM Sr^{2+}	+	–
1.5 mM Ba^{2+}	+	–

[a] Data from Grosshans and Cech (1989).

experiments, these substrates had been shown not to alter the rate of cross-linking beyond the extent attributable to their absorption of UV radiation (data not shown). Short time points were used so that the percent substrate cleaved and percent cross-linking approximated initial rates for these processes. The two reactions shared a very similar magnesium ion concentration dependence (Figure 4). In both reactions, there is a sharp increase in activity between 1.0 and 1.5 mM Mg^{2+}, suggesting that multiple binding sites for Mg^{2+} cations are filled in the same concentration range.

The presence of magnesium ion alone in a heat-renatured L-21 *Sca*I IVS solution is sufficient to permit cross-linking. However, other divalent cations can substitute for Mg^{2+} to permit cross-linking. This repertoire of cations includes the group IIA metal ions Ca^{2+}, Sr^{2+}, and Ba^{2+}, as well as Mn^{2+} (Table I). The transition metal cations Fe^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} could not substitute for magnesium. The metal ions that support cross-linking are the same ions previously found to substitute for magnesium in supporting endoribonuclease activity of the L-21 *Sca*I IVS (Grosshans & Cech, 1989). In the cases of Ca^{2+}, Sr^{2+}, and Ba^{2+}, a small amount of magnesium ion or manganese ion (i.e., 0.5 mM) is still needed to permit endoribonuclease activity.

A57–A95 Cross-Link Inhibits Catalytic Activity. Shown in Figure 5 are three time courses of the endoribonuclease reaction using three preparations of L-21 *Sca*I IVS as enzyme.

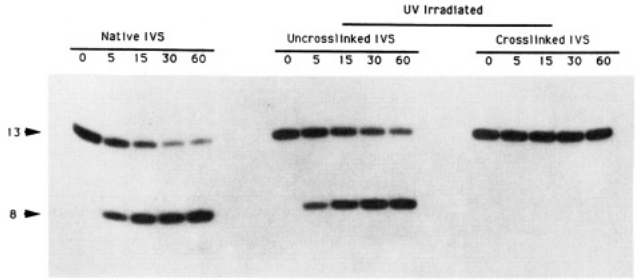

FIGURE 5: Inhibition of catalytic activity due to the cross-link. Three endoribonuclease reactions were performed in parallel using different preparations of the L-21 *Sca*I IVS as the enzyme. Native IVS was unirradiated L-21 *Sca*I IVS. Un-cross-linked and cross-linked IVS were gel-purified from IVS that had been cross-linked to an extent of 20%. The substrate for this reaction is a 13-mer, [5′-^{32}P]-GGCUCUCUA$_5$, that is cleaved by the IVS to produce an 8-mer containing the label. At each time point (0–60 min), 5 μL was taken from a 100-μL reaction including 0.15 μM IVS, 1 μM 13-mer substrate, 0.5 mM guanosine, 50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM $MgCl_2$, and 2.5 M urea incubated at 50 °C. The products were separated on a 20% polyacrylamide/7 M urea gel.

The reaction conditions chosen were optimal for the native enzyme. In the first set of lanes, the RNA substrate [5′-^{32}P]GGCUCUCUA$_5$ was incubated with native L-21 *Sca*I IVS. The next two set of lanes show the products of incubation of the same substrate with UV-irradiated IVS, the un-cross-linked and cross-linked fractions, respectively. The substrate incubated with untreated L-21 *Sca*I IVS is cleaved to yield a shorter labeled product, previously identified as pGGCUCUCU (Zaug et al., 1986). The UV-irradiated but un-cross-linked IVS retains approximately half of its endoribonucleolytic activity as judged by densitometry of the autoradiogram. In contrast, the cross-linked L-21 *Sca*I IVS does not demonstrate endoribonuclease activity over the same time period.

Both the un-cross-linked IVS and the cross-linked IVS (the second and third set of lanes) were recovered from a single UV irradiation, and, therefore, both IVS preparations should possess the same assortment of photolesions with the exception of the A57–A95 cross-link. Yet the UV-irradiated but un-cross-linked IVS shows endoribonuclease activity approaching that of the native L-21 *Sca*I IVS while the cross-linked IVS is inactive. Thus, the A57–A95 cross-link is sufficient to eliminate endoribonucleolytic activity under these reaction conditions. At lower temperature (42 °C) in the absence of urea, activity is largely restored (data not shown).

Sequences Involved in Determining Cross-Linkable Conformation. IVS transcripts with different deletions were tested for their ability to cross-link to ascertain what sequences or structural elements of the L-21 *Sca*I IVS contribute to the folded structure necessary for cross-linking. The L-21 *Sca*I IVS is generated in vitro by runoff transcription using T7 RNA polymerase; the DNA template is cut at a *Sca*I restriction site to provide the 3′ termination site. A number of IVS transcripts with different 3′ deletions were made by cutting the DNA template at restriction sites more proximal to the T7 promoter (Figure 6A). Of the runoff transcripts tested, only the L-21 *Sca*I IVS and the L-21 *Nhe*I IVS were still able to cross-link (Figure 6B). Upon comparison of time courses for cross-linking, the L-21 *Nhe*I IVS displayed almost the same rate (90%) and fully the extent of conversion observed in the L-21 *Sca*I IVS (Figure 7A). We conclude that the majority of P9.1, P9.2, and 3′ terminus of the L-21 *Sca*I IVS is not necessary nor makes a significant contribution to the cross-linking conformation. However, transcripts lacking additional sequences from the 3′ end were not competent for

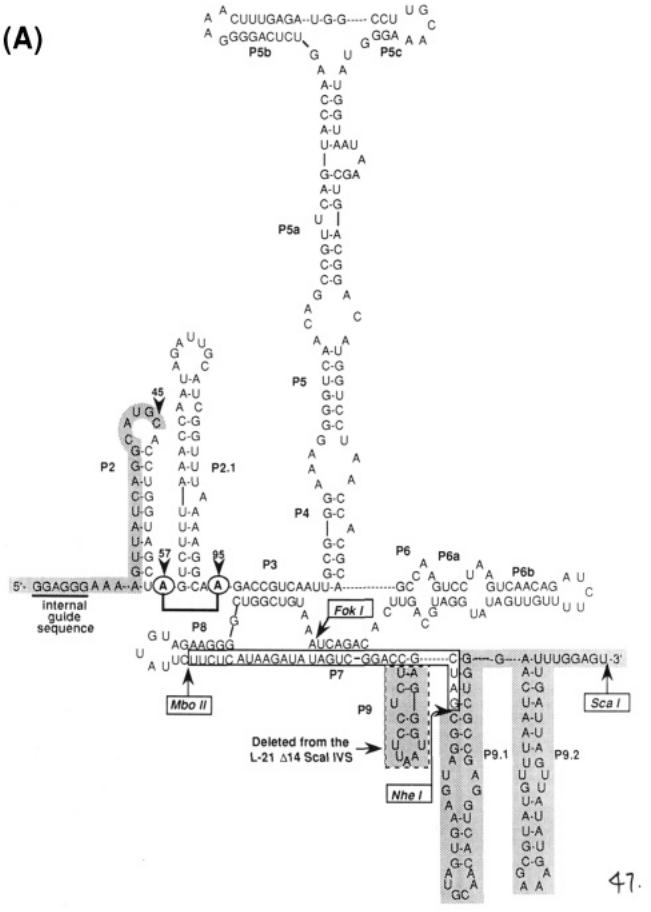

FIGURE 6: Sequences participating in the cross-linkable conformation. (A) Shaded regions indicate portions of the L-21 *Sca*I IVS not necessary for cross-linking as determined from deletion mutants. Labeled arrows indicate the 3′ termini of runoff transcripts generated by cleavage of the pT7L-21 DNA template by the restriction endonucleases shown. The open box marks one portion of the IVS determined to be necessary for cross-linking. (B) Cross-linking in transcripts of the IVS lacking 3′ sequences. Four different variants of the L-21 IVS were made by runoff transcription from the pT7L-21 template following treatment with one of four restriction enzymes. The expected 3′ termini vary as shown in (A). The products of cross-linking were separated on a 10% polyacrylamide/0.5× TBE/7 M urea gel, an autoradiograph of which is shown. Arrowheads indicate the cross-linked products.

cross-linking. This holds true for transcripts with 3′ termini at the *Mbo*II and *Fok*I sites (Figure 6A). Therefore, between the *Mbo*II and *Nhe*I sites is one region of the sequence that appears necessary to form the cross-linking structure. This segment includes the conserved S-sequence element and with it P9 and portions of P7 and P8, all elements of the core structure defining group I intervening sequences (Waring & Davies, 1984).

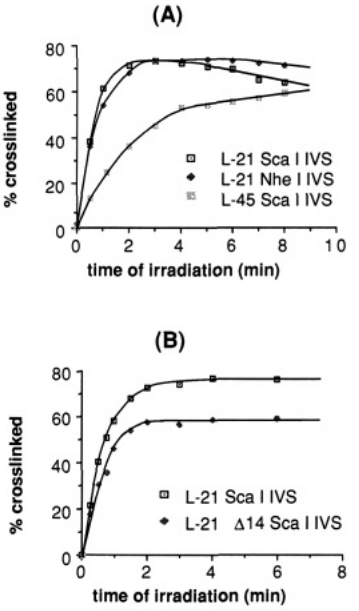

FIGURE 7: Rate of cross-linking of variants of the L-21 *Sca*I IVS. For each time course, the labeled transcript was taken through the cross-linking protocol and irradiated at 0 °C (see Materials and Methods). Droplets (5–10 μL) were removed from irradiation at various times. The products of irradiation were then separated on a 10% polyacrylamide/0.5× TBE/7 M urea gel and quantitated by using the Ambis radioanalytic system. L-21 *Sca*I IVS was cross-linked under identical conditions to provide a standard for comparison. (A) L-21 *Nhe*I IVS and L-45 *Sca*I IVS. (B) L-21 Δ14 *Sca*I IVS.

Another deletion mutant, L-21 Δ14 *Sca*I IVS, that lacks the P9 helical element (Figure 6A) is also capable of cross-linking (data not shown). Examination of the time course for cross-linking of the L-21 Δ14 *Sca*I IVS resulted in only 75% of the extent conversion observed in the L-21 *Sca*I IVS (Figure 7B). However, the initial rate of cross-linking was in agreement between the two transcripts when considering only the fraction of each population of molecules that did eventually cross-link. An explanation is that the presence of the P9 sequences results in more molecules assuming a cross-linkable conformation but once in such a conformation, the L-21 Δ14 *Sca*I IVS can cross-link with the same efficiency as seen in the L-21 *Sca*I IVS.

Some sequences at the 5′ end of the L-21 *Sca*I IVS also are not necessary for cross-linking. The L-45 runoff transcript lacks the internal guide sequence and the 5′ strand of the P2 helix (figure 6A), yet can still be cross-linked. The initial rate of cross-linking is only one-third that of the L-21 *Sca*I IVS and proceeds to only 80% the extent (Figure 7A). Thus, the 5′ region missing from the L-45 *Sca*I IVS, or some portion of it, contributes to the formation or stabilization of the cross-linking conformation, though it is not an essential component.

Enzymatic sequencing from a 5′ end label identified the same nucleotide, A57, participating in the cross-link of all three of the deletion mutants examined (L-21 *Nhe*I IVS, L-21 Δ14 *Sca*I IVS, and L-45 *Sca*I IVS; data not shown) as in the L-21 *Sca*I IVS (Figure 3A).

Discussion

Cross-Linking and Catalysis Require a Common Tertiary Structure. The UV-induced cross-link of the L-21 *Sca*I IVS, like other long-range RNA cross-links studied previously (Ninio et al., 1969; Atmadja & Brimacombe, 1985; Branch et al., 1985, 1989), forms as a consequence of the folded structure of the molecule. Tertiary interactions within the IVS

bring together the cross-linking nucleotides that are separated in sequence; at least one other region distant in sequence is required to align them for cross-linking. Once folded, the IVS cross-links very rapidly and efficiently. The rate of cross-linking has been compared to that of tRNA, another example of an RNA whose structure is determined by a large number of tertiary interactions. The YF0 transcript, a synthetic yeast $tRNA^{Phe}$, forms a C48–U59 cross-link when irradiated at 254 nm (J. A. Sampson and O. C. Uhlenbeck, personal communication). However, the L-21 *Sca*I IVS cross-links at greater than 8 times the rate of the YF0 transcript when the two are present in the same reaction (Downs and Cech, unpublished results).

The tertiary structure implied by the A57–A95 cross-link is of interest inasmuch as it reflects a catalytically active conformation of the *Tetrahymena* IVS. Three lines of evidence indicate that the conformation of the L-21 *Sca*I IVS competent for cross-linking is the same or very similar to that competent for catalysis: the effect of heat renaturation, the effect of divalent cations, and the requirement of certain sequence elements.

First, heating and cooling of the L-21 *Sca*I IVS in the presence of magnesium not only maximize the extent of cross-linking at 0 °C but also optimize catalytic activity at low temperatures. This method of heat renaturation was originally discovered as a means of making gel-purified *Tetrahymena* IVS RNA catalytically more active (S. A. Walstrum and O. C. Uhlenbeck, personal communication). The catalytic activity assayed was addition of tetrauridylate to the circular form of the IVS. In this case, the rate of reaction at 0 °C was increased up to 1000-fold when a procedure of heat renaturation identical with that used to optimize cross-linking was first applied to the RNA. At 0 °C, in the presence of 10 mM Mg^{2+}, gel-purified IVS cross-links to an extent of only 5%, but this is increased to 77% when the RNA is pretreated with the heat–cool procedure. At 50 °C, the heat–cool treatment has little effect on cross-linking (Downs and Cech, unpublished results) or on catalytic activity (Walstrum and Uhlenbeck, personal communication). Presumably the IVS must be allowed to assume a particular stable conformation for both reactions, and higher temperatures permit the IVS to shift to this conformation.

Second, the cross-linking and catalytic behaviors of the L-21 *Sca*I IVS mirror each other under different ionic conditions. Both cross-linking and endoribonucleolytic activities are undetectable at 0–1.0 mM Mg^{2+} and then increase between 1.0 and 1.5 mM Mg^{2+}, indicating a cooperative binding of magnesium ions at many sites is required for both reactions. Previous work of Grosshans and Cech (1989) showed that while Ca^{2+}, Sr^{2+}, and Ba^{2+} can partially substitute for Mg^{2+} in an endoribonuclease reaction, Mg^{2+} or Mn^{2+} is still required for this activity. One possible explanation is that Ca^{2+}, Sr^{2+} and Ba^{2+} can substitute for Mg^{2+} in folding the IVS but cannot serve a necessary chemical role in catalyzing the reaction. Ca^{2+}, Sr^{2+}, and Ba^{2+} also support the cross-linking conformation, suggesting that they do participate in a structural role and that structure is the same for both cross-linking and catalysis. Manganese alone can completely replace magnesium in both its structural and its chemical roles. Likewise, it will also support cross-linking.

Finally, cross-linking in the L-21 *Sca*I IVS depends upon at least one region of the core that includes portions of P7 and P8 and J8/7, the conserved joining sequence between them. Similarly, the splicing activity of the *Tetrahymena* IVS depends upon these same regions of the conserved core structure (Burke et al., 1986; Szostak, 1986; Williamson et al., 1987, 1989). The subset of sequences necessary for formation of the cross-linkable structure might be defined as a structural domain, while regions outside of the core, P1, P2, P9.1, and P9.2, are not necessary for the RNA to fold into this unit. When P9.1 and P9.2 are deleted, the *Tetrahymena* IVS not only is competent for cross-linking but also retains catalytic activity (Szostak, 1986; Barfod & Cech, 1988).

The cross-linking data also provide evidence that the catalytic core folds independent of the presence of P1. Reactions catalyzed by the *Tetrahymena* IVS and variants of this molecule typically rely upon base pairing of the RNA undergoing guanosine attack to the internal guide sequence, forming part or all of the P1 helix. The internal guide sequence is present but not as a helix in the L-21 *Sca*I IVS, and it is not even present in the L-45 *Sca*I IVS; yet both these molecules remain capable of cross-linking. Chemical cleavage and protection from this cleavage have suggested a pattern of folding within the IVS core (Latham & Cech, 1989). This pattern is not altered when the 5′ strand of P1 is supplied in trans, also indicating that the core of the IVS folds independently of P1 (Latham & Cech, 1989). Likewise, P2, which is not conserved among group I intervening sequences, is not necessary to the folding of the cross-linking domain. These structural studies are in agreement with functional studies showing that the core sequences and the P1/P2 segment need not be part of the same transcript but can be combined as separate molecules to reconstitute catalytic activity (Szostak, 1986; Doudna & Szostak, 1989).

Because the cross-linked IVS is catalytically inert under standard reaction conditions (2.5 M urea), it might be argued that cross-linking occurs only in molecules folded into an inactive structure. Once cross-linked, these molecules would then be trapped in this noncatalytic form. However, folding conditions that optimize catalysis also permit three-fourths of the IVS molecules to cross-link. Furthermore, activity is restored under less destabilizing conditions. We therefore think it likelier that the alteration in structure of the adenosines upon cross-linking disrupts the folded structure of the IVS. Cross-linking in tRNA can measurably alter its tertiary structure (Favre & Michelson, 1971), and psoralen adducts to nucleotides have been modeled as disrupting the DNA double helix (Kim et al., 1982).

Nature of the Cross-Link. The cross-link of the *Tetrahymena* IVS is unusual in that it involves two adenosines. (Although the cross-link is likely to involve the adenine bases, this has not been established.) Purines are generally not as photoactive as pyrimidines (Kochetkov & Budovskii, 1972), but irradiation at 254 nm has yielded photoproducts. One reported example of UV-inducible adenine–adenine cross-linking occurs within single-stranded polydeoxyadenosine (Porschke, 1973). The photoproduct recovered from that reaction has been identified as a dehydrodimer with a single bond between the C(8) of two adjacent adenines (Gasparro & Fresco, 1986). A photoproduct of d(ApA) involves a cross-link between the N(7) and C(8) of one adenone with the C(5) and C(6) of a 3′-adenine with the addition of a water molecule (Kumar et al., 1987). Purine–purine cross-linking has also been observed in rRNA (Atmadja & Brimacombe, 1985); however, to our knowledge, the cross-link of the *Tetrahymena* IVS represents the first example of an adenosine–adenosine cross-link mapped within a folded RNA.

Tertiary Structure Indicated by the Cross-Link. A95, one of the cross-linking nucleotides, is of particular interest because of its location just 5′ of P3. This proximity to an element of

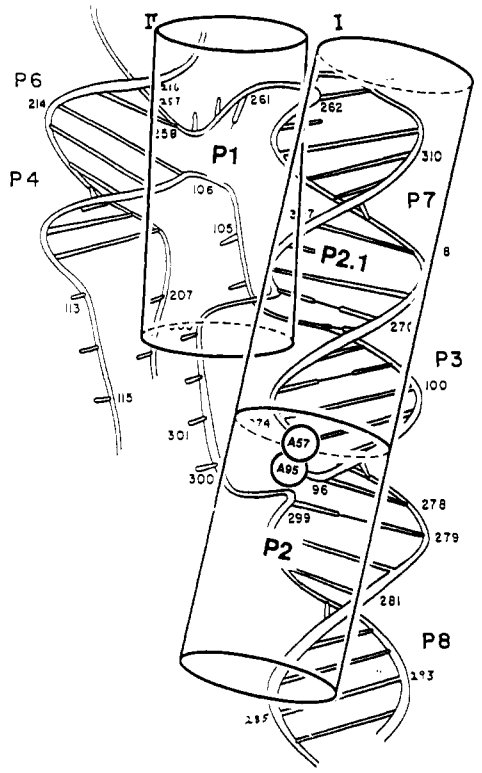

FIGURE 8: Three-dimensional model of the IVS in the region of the cross-link. Helical elements P1, P2, and P2.1, represented as columns, are superimposed on a proposed tertiary structure of the folded core in the *Tetrahymena* IVS (Kim & Cech, 1987). P2 and P2.1 are envisioned as divergent coaxial helices that pack at an angle against P3 and P7. This arrangement accommodates the cross-link between A57 and A95 and the patterns of protection from nuclease and chemical cleavage discussed in the text (Cech et al., 1983; Inoue & Cech, 1985; Latham & Cech, 1989). The placement of P1 is restricted due to its linkage to the base of P2 by three nucleotides. Furthermore, the 5′ splice site within P1 is placed proximal to the guanosine binding site at G264 (Michel et al., 1989).

the core is intriguing. In addition, 51 out of 59 (86%) group I intervening sequences examined share adenosine as the first unpaired nucleotide 5′ of P3. Therefore, this adenosine on the fringe of the core structure may play a conserved role. On the basis of current work, that role may be to form a catalytically active tertiary structure. The other partner, A57, does not appear to be conserved among group I intervening sequences.

Figure 8 shows a proposed structure that incorporates the cross-link into the provisional three-dimensional model for the folded core of the *Tetrahymena* IVS proposed by Kim and Cech (1987). A95 emerges from the base of P3. The cross-link between A95 and A57 places the ends of three helices, P2, P2.1, and P3, into close proximity. P2 and P2.1 are envisioned as being coaxial and packing against the P3/P7 coaxial helix. This arrangement not only accommodates the cross-link but also is supported by patterns of protection of P2, P2.1, P3, and P7 from chemical probes and nucleases (Cech et al., 1983; Inoue & Cech, 1985; Latham & Cech, 1989). The complementary patterns of protection on the predicted faces of the P2/P2.1 and P3/P7 helices suggest that the helices are packed at a slight angle relative to one another. Such tilting may be the consequence of electrostatic repulsion between phosphates of the backbones.

Incorporation of the cross-link extends the Kim/Cech tertiary model from the core 5′ through P2 and P2.1. Although the cross-link does not fix the P1 helix within the structure, it does limit its possible location. This is due to the fact that P1 is tethered to P2 by a three-nucleotide joiner, J1/2. According to the model, J1/2 would emerge from P2 into the heart of the folded core. This may well be expected since P1 contains the internal guide sequence, part of the active site of this ribozyme. Another major constraint on the location of P1, provided by the identification of G264 as the guanosine binding site (Michel et al., 1989), has been incorporated into Figure 8 by appropriate juxtaposition of U−1 in P1 and G264.

Further Applications. In addition to the information the cross-link has already provided, the cross-linking reaction may prove useful in the future as an assay for folded structure in the *Tetrahymena* IVS. The cross-linking reaction allows one to test for the native conformation in splicing-defective mutants of the IVS and to monitor the structure of the IVS under different solution conditions. It will also be of interest to determine if the cross-link is a common feature of group I intervening sequences, since this family of RNAs displays conservation within the core.

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Camptothecin-Stabilized Topoisomerase I–DNA Adducts Cause Premature Termination of Transcription†

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ABSTRACT: The antitumor agent camptothecin stabilizes type I topoisomerase–DNA complexes. One of the primary cellular responses to camptothecin exposure is rapid cessation of RNA synthesis. Results obtained by using an in vitro transcription system supplemented with eukaryotic topoisomerase I show that this inhibition can be attributed to physical blockage of the RNA polymerase by camptothecin-stabilized topoisomerase I–DNA complexes on the DNA template. The site of premature termination is located 10 base pairs upstream of the topoisomerase I linked nucleotide residue on the coding strand, corresponding closely to the border of the protected area obtained in a micrococcus nuclease footprint of topoisomerase I. The RNA polymerase transcribes unimpeded through complexes attached to the noncoding strand. No inhibitory effect of camptothecin on RNA transcription was observed with human topoisomerase I isolated from a camptothecin-resistant cell line. Taken together, the data suggest that part of the cytotoxicity of camptothecin is mediated through adduct formation on transcribed DNA, resulting in interference with transcriptional elongation.

DNA topoisomerases I and II are ubiquitous enzymes influencing essential processes such as replication, chromatid segregation, and transcription through regulation of DNA topology (Wang, 1985; Vosberg, 1985). Recently, DNA topoisomerases have been identified as the intranuclear target for a number of important chemotherapeutic agents (Bodley & Liu, 1988; Liu, 1989). Mechanistically, topoisomerases act by transiently breaking the phosphodiester backbone, and antitumor drugs are thought to interfere with the topoisomerization process by stabilizing a covalent enzyme–DNA reaction intermediate in the normal catalytic cycle (Hsiang et al., 1985). The stabilization of the DNA–topoisomerase complex by antitumor drugs results in inhibition of catalytic activity and DNA breakage in the presence of a protein denaturant.

Camptothecin and its derivatives are topoisomerase I targeting cytotoxic alkaloids with antineoplastic activity (Gallo et al., 1971; Tsuruo et al., 1988; Giovanella et al., 1989).

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