A Photo-Cross-Linkable Tertiary Structure Motif Found in Functionally Distinct RNA Molecules Is Essential for Catalytic Function of the Hairpin Ribozyme†

Samuel E. Butcher and John M. Burke*

Department of Microbiology and Molecular Genetics, Markey Center for Molecular Genetics, The University of Vermont, Burlington, Vermont 05405

Received September 14, 1993; Revised Manuscript Received November 9, 1993®

ABSTRACT: We have identified an essential UV-sensitive tertiary structure domain within the hairpin ribozyme. Irradiation at 254 nm produces two cross-linked RNA species that are resolved from the unmodified structure by denaturing gel electrophoresis. One cross-link forms at high efficiency and maps between nucleotides G_{21} and/or A_{22} and U_{41}, all essential bases located within an internal loop joining helices 3 and 4. A second cross-link forms between nucleotides A_{20} and U_{42} as a result of ribozyme dimerization at concentrations greater than 0.5 μM. Both cross-linked species retain cleavage activity and so presumably reflect catalytically proficient structures of the ribozyme. Formation of the intramolecular cross-link is independent of Mg^{2+} and substrate and is blocked by base substitutions within the reactive domain that inhibit catalysis. A 36-nt RNA fragment containing the photoreactive domain but lacking the substrate binding domain also cross-links with high efficiency and maps between G_{21} and U_{41}, as observed with the intact molecule. The sequence and cross-linking sites of the UV-sensitive internal loop are strikingly similar to those found in several other RNA molecules, including loop E of 5S rRNA. These results suggest that the loop E-like structure may be a common RNA folding domain that is utilized in a variety of functionally important RNA molecules.

The catalytic activity of both protein enzymes and RNA enzymes (ribozymes) is dependent on formation of a specific three-dimensional structure. Several tertiary contacts within self-splicing group I introns have been elucidated through intensive work in a number of laboratories by comparative sequence analysis, mutational studies, *in vitro* selection, modeling, UV cross-linking, and chemical modification [see for example Michel and Westhof (1990) and Cech *et al.* (1992)]. In contrast, very little information concerning the tertiary structure of small, *trans*-acting ribozymes such as the hammerhead and hairpin ribozymes has emerged.

The hairpin ribozyme is a 50-nt catalytic domain derived from the minus strand of the satellite RNA associated with tobacco ringspot virus [Feldstein *et al.*, 1989; Hampel & Tritz, 1989; Haseloff & Gerlach, 1989; for a review, see Burke (1994)]. The ribozyme acts as a site-specific endoribonuclease, cleaving RNA substrates using a transesterification mechanism to generate products containing 5′-hydroxyl and 2′,3′-cyclic phosphate termini. The secondary structure of the ribozyme–substrate complex has been established through mutation, *in vitro* selection, and the engineering of ribozymes that cleave different substrates (Hampel *et al.*, 1990; Chowrira & Burke, 1991; Berzal-Herranz *et al.*, 1993; Joseph *et al.*, 1993). This structure is characterized by four short helical segments separated by two internal loops (Figure 1) of unknown structure. The substrate binds to the ribozyme through two helices of six (helix 1) and four (helix 2) base pairs. Cleavage occurs to the 5′ side of a guanosine within the symmetrical internal loop separating helices 1 and 2 (loop A). A second internal loop (loop B) separates the two intramolecular helices (helix 3 and helix 4).

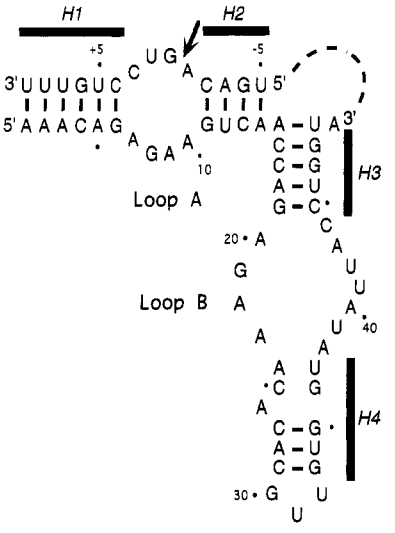

FIGURE 1: Hairpin ribozyme structure. The ribozyme and substrate used in this study were as described (Chowrira & Burke, 1991). The arrow indicates the substrate cleavage site. The dashed line indicates the pentacytidine linker used in experiments involving self-cleaving constructs (Berzal-Herranz *et al.*, 1992; Feldstein *et al.*, 1993).

Selection and mutation studies have shown that most bases within loops A and B are essential for catalytic activity (Chowrira *et al.*, 1991; Berzal-Herranz *et al.*, 1992, 1993). Within loop B, nine of eleven nucleotides (all except A_{20} and U_{39}) are essential. There are four essential bases within loop A, three in the ribozyme (G_8, A_9, and A_{10}), and one in the substrate (G_{+1}). All characterized point mutations at each of these positions reduce catalytic activity by 1–5 orders of magnitude. In contrast, only one nucleotide within the four helices (G_{11}) is important for catalysis (Joseph *et al.*, 1993; Joseph & Burke, 1993).

† This work was supported by research grants from the National Institutes of Health.

* Address correspondence to this author at the Department of Microbiology and Molecular Genetics, Stafford Hall, University of Vermont, Burlington, VT 05405. Voice: (802) 656-8503. Fax: (802) 656-8749. E-mail: jburke@moose.uvm.edu.

® Abstract published in *Advance ACS Abstracts,* January 1, 1994.

0006-2960/94/0433-992$04.50/0

Very little is known about the higher order folding of the hairpin ribozyme. Studies involving the insertion of linkers between the 3′ end of the ribozyme and the 5′ end of the substrate indicate that helices 2 and 3 are not coaxially stacked; rather, a bend or hinge occurs at or near the A_{13}–A_{14} linkage (Feldstein & Bruening, 1993). On the basis of this observation and the finding that many nucleotides in loop B are essential for catalysis, we believe that loops A and B are likely to interact.

Here, we describe photo-cross-linking experiments that provide insights into the tertiary structure of the hairpin ribozyme. We find that the ribozyme contains a highly reactive structural domain within loop B that results in the formation of both intramolecular and intermolecular cross-links across the loop. The sequence and photosensitivity of ribozyme loop B are strikingly similar to those of RNA structural domains characterized in a conserved viroid internal loop (Branch *et al.*, 1985) and in a region of eukaryotic 5S rRNA that functions in the binding of transcription factor TFIIIA and ribosomal protein L5 (loop E; Wimberly *et al.*, 1993). A tertiary structure model for 5S rRNA loop E, based on NMR data, has recently been proposed (Wimberly *et al.*, 1993). Similar structures are also conserved at two sites within large subunit (23S-like) rRNA (Gutell & Fox, 1988). On the basis of these observations, it appears that the "loop E-like" structure may represent an RNA tertiary structure element that is widely used in a variety of functionally important RNA molecules.

MATERIALS AND METHODS

RNA Transcription and End-Labeling. Ribozymes were transcribed from synthetic DNA templates using bacteriophage T7 RNA polymerase, as described (Milligan & Uhlenbeck, 1989; Chowrira & Burke, 1991), or from cloned templates obtained from in vitro selection experiments (Joseph *et al.*, 1993; Berzal-Herranz *et al.*, 1993). All RNAs were purified using denaturing polyacrylamide gel electrophoresis in Tris–borate–EDTA buffer, eluted by diffusion, and precipitated. RNA was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase following dephosphorylation with calf intestinal phosphatase (Midgley & Murray, 1985). RNA was 3′-end-labeled with [5′-^{32}P]pCp and T4 RNA ligase (England & Uhlenbeck, 1978). Heterogeneity at 3′ ends resulting from the addition of nontemplated nucleotides by T7 RNA polymerase was eliminated in the case of the full-length ribozyme by using a self-cleaving RNA construct (Berzal *et al.*, 1992; Joseph *et al.*, 1993), generating a uniform 2′,3′-cyclic phosphate, which was decyclized with 10 mM HCl at 25 °C for 120 min prior to labeling (Abrash *et al.*, 1967). The 36-nt loop B fragment of the hairpin ribozyme was purified on 22% denaturing polyacrylamide gels to generate a population of RNAs with uniform 3′ ends. Substrate RNA was internally labeled during transcription in the presence of [α-^{32}P]CTP.

UV Cross-Linking. Gel-purified RNA species were resuspended in 40 mM Tris-HCl (pH 7.6) and 12 mM $MgCl_2$ (unless otherwise noted) and renatured prior to UV irradiation by heating to 70 °C for 2 min and then rapidly cooling on ice for 15 min to ensure proper folding (Downs *et al.*, 1989; Walstrum *et al.*, 1990; Behlen *et al.*, 1992). Twenty-microliter aliquots were placed into the wells of a Falcon 96-well microtiter plate resting on ice to prevent heating of samples during UV irradiation. Samples were irradiated with a hand-held 254-nm UV light (Model UVG-54, UV Products Inc.; measured output, 2200 μW/cm^2) at a distance of 1 cm and then directly electrophoresed on denaturing polyacrylamide gels. Fifteen percent polyacrylamide gels were used to purify full-length hairpin ribozyme cross-linked RNAs, and 22% polyacrylamide gels were used to purify cross-linked RNAs from the 36-nt loop B fragment of the hairpin ribozyme. cross-linked RNA products were visualized by autoradiography, excised from the gel, eluted by diffusion in 0.5 M ammonium acetate, and precipitated with 3 vol of ethanol. Quantitation of cross-linking was done on a Betascan radioanalytic imaging instrument (Betagen).

Mapping of Cross-Linked Bases. To map the cross-links, the gel-purified, end-labeled cross-linked RNAs were subjected to partial RNase T1 digestion and limited alkaline hydrolysis. Samples were dissolved in 50 mM $(NH_4)_2CO_3$ buffer (pH 10), heated to 90 °C for 2 min, and loaded onto a 15% denaturing polyacrylamide gel (for full-length ribozyme) or a 20% denaturing polyacrylamide gel (for the loop B fragment). Non-cross-linked RNA was partially digested with base-specific ribonucleases T_1, U_2, Cl_3, and Phy_M (U.S. Biochemical) and subjected to identical limited alkaline hydrolysis to serve as markers in identifying cross-linked bases.

trans-Cleavage Assay. [5′-^{32}P]End-labeled, gel-purified ribozyme RNA (1 pmol, 2.5×10^5 dpm) was renatured and incubated with an equimolar (1 pmol) amount of gel-purified substrate RNA, internally labeled with [α-^{32}P]CTP during transcription. The reaction mixture was incubated at 37 °C for 1.5 h in a 20-L reaction volume containing 40 mM Tris-HCl (pH 7.6), 12 mM $MgCl_2$, and 2 mM spermidine. Reactions were stopped by addition of an equal volume of 90% formamide and 1 mM EDTA, and the reaction mixtures were then heated to 90 °C and loaded directly onto a 10% denaturing polyacrylamide gel.

UV Cross-Linking of Catalytically Inactive Point Mutants. Inactive point mutant RNAs were transcribed from plasmids obtained from previously described *in vitro* selection experiments (Berzal *et al.*, 1993). These constructs have substrates covalently attached to the 3′ end of the ribozymes via short linker sequences. RNAs were 5′-end-labeled, and 4 pmol of each RNA (1×10^6 dpm) was renatured in 20 μL of 40 mM Tris-HCl, pH 7.6, and 12 mM $MgCl_2$. Samples were UV-irradiated for 30 min and then electrophoresed through 15% denaturing polyacrylamide gels.

RESULTS

UV-Induced Cross-Linking of the Hairpin Ribozyme. Renatured hairpin ribozymes were UV-irradiated in standard reaction buffer and analyzed by denaturing gel electrophoresis, as described in Materials and Methods. Two cross-linked products were detected at an RNA concentration of 1 μM (Figure 2A). The major product (XL1) has slightly lower mobility than the unmodified ribozyme. The XL1 species forms with high efficiency (initial rate of 0.02 min^{-1} when irradiated as described in Materials and Methods) and accumulates to 42% of the total RNA at 40 min when RNA concentration is decreased to 50 nM (Figure 2B). The high efficiency of XL1 formation indicates that, under these conditions, the cross-linkable conformation is a major coformational isomer of the ribozyme.

A second cross-linked product (XL2) is also observed and has a much lower mobility (Figure 2). After 40 min at an RNA concentration of 1 μM, XL2 represents only 2% of the total cross-linked RNA. At 50 nM RNA concentration, however, XL2 is not observed and the rate of XL1 formation is increased (Figure 2B). Together, the apparent concentration dependence and the low electrophoretic mobility of XL2 suggest that it may result from intermolecular cross-linking. To test this possibility, cross-linking was done in the presence

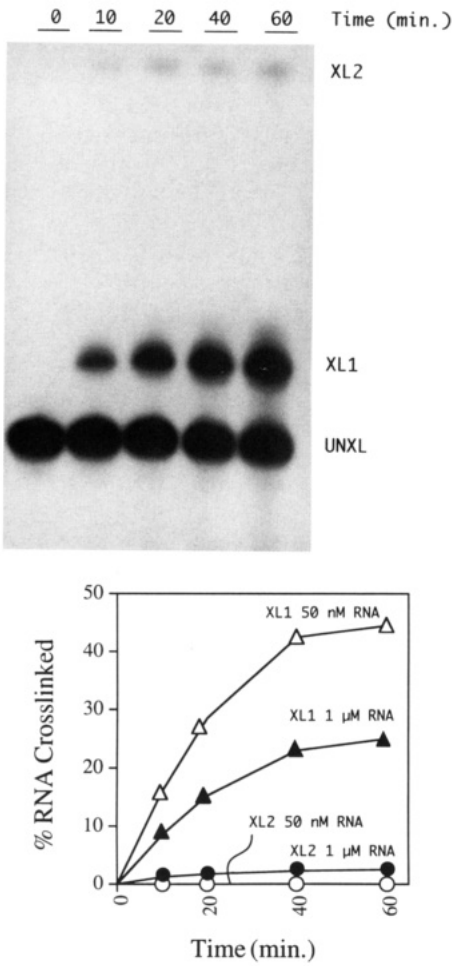

FIGURE 2: UV cross-linking of the hairpin ribozyme. (A, top) Time course of cross-linking. 5′-^{32}P-Labeled hairpin ribozyme (1 μM) was renatured and irradiated for the indicated times in a solution containing 40 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, and 2 mM spermidine. An autoradiogram of a 15% denaturing polyacrylamide–urea gel is shown. (B, bottom) Kinetics of formation of XL1 and XL2 cross-linked species at 1 μM and 50 nM concentrations: open triangles, XL1 formation at 50 nM; closed triangles, XL1 formation at 1 μM; open circles, XL2 formation at 50 nM (none is observed); closed circles, XL2 formation at 1 μM. The electrophoresis gels were dried and quantitated with a Betascan instrument.

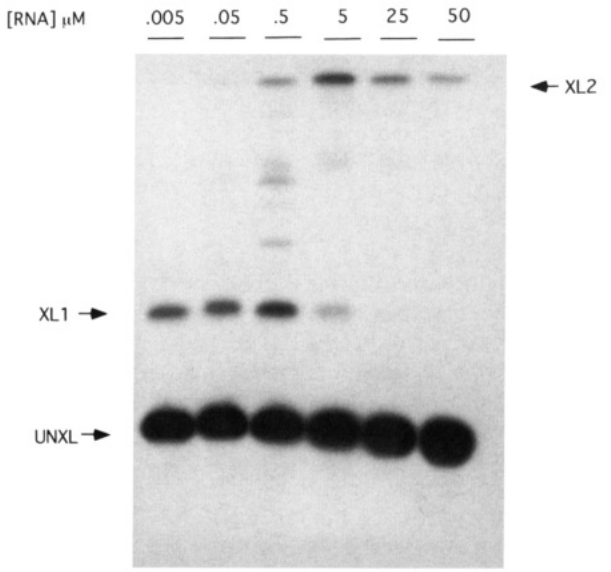

FIGURE 3: Concentration dependence of XL1 and XL2 formation. The experiment was carried out as described in the caption to Figure 2, except that 5 nM labeled ribozyme was used, and unlabeled ribozyme was added to the final concentrations indicated.

of increasing concentrations of unlabeled RNA (Figure 3). Results show that formation of XL2 is highly concentration dependent. XL2 predominates at ribozyme concentrations greater than 5 μM, while XL1 predominates at concentrations below 5 μM. These results are consistent with the formation of an intermolecular cross-link and suggest that the hairpin ribozyme is able to self-associate in solution, possibly by dimerization, at concentrations $\geq$ 0.5 μM. This result was confirmed by mapping the cross-linked bases, as described below. The absence of XL1 formation at ribozyme concentrations $\geq$ 5 μM indicates that little or no ribozyme is present as the native monomeric species under these conditions.

Mapping of XL1, An Intramolecular Cross-Link. The gel-purified cross-linked RNA species XL1 was mapped by partial RNase digestion and alkaline hydrolysis of 5′- and 3′-end-labeled material. 5′-end-labeled XL1 produces a hydrolysis ladder with a distinct gap after nucleotide G_{21} (Figure 4). However, the hydrolysis patterns of XL1 reproducibly show nucleotide G_{21} to be underrepresented in the ladder, such that nucleotide A_{20} may also represent a major stop in the ladder. This indicates that hydrolysis products to the 3′ side of nucleotides A_{20} and G_{21} result in RNA fragments with a lower electrophoretic mobility due to the cross-linking. Therefore, we conclude that nucleotides G_{21} and A_{22} are 5′ components of the cross-link XL1. 3′-End-labeled XL1 shows a single gap in the ladder above U_{42}, indicating that U_{41} is the 3′-cross-linked nucleotide in all cases; thus, XL1 results from cross-linking of G_{21}/A_{22} to U_{41}, three bases within loop B of the hairpin ribozyme. Previous studies have shown that single-base substitutions at G_{21}, A_{22}, and U_{41} each strongly inhibit catalysis (Berzal *et al.*, 1993). Because UV-inducible cross-links have not been observed between canonical and wobble base pairs, it is improbable that these nucleotides form Watson–Crick pairings. Our interpretation is that the nucleotides lie in close proximity within the tertiary structure of the ribozyme and may possibly be stacked over one another.

Mapping of XL2, An Intermolecular Cross-Link. Mapping of 5′-end-labeled XL2 reveals a specific gap in the hydrolysis ladder after U_{41}, indicating that U_{42} is the 5′-cross-linked base. The 3′-end-labeled XL2 ladder contains a gap above G_{21}, indicating A_{20} to be the 3′-cross-linked nucleotide (Figure 4). The 5′- and 3′-cross-linked nucleotides of XL2 are reversed in the order of the primary sequence of the ribozyme. Such a cross-link could only form between two RNAs in intermolecular association, consistent with observations concerning XL2 mobility and concentration dependence. It is important to note that the cross-link is specific for the nucleotides U_{42} and A_{20} and is therefore the result of specific intermolecular interactions and not simply random aggregation.

Because the ribozyme is self-complementary within helices 3 and 4, we propose that dimerization results from the formation of intermolecular versions of these helices (Figure 5). This mode of dimerization would preserve all essential secondary structure elements of the molecule. Although the loop capping helix 4 is not preserved in the proposed dimer, it is known to be dispensable for activity (Chowrira & Burke, 1992; Vinayak *et al.*, 1992; Berzal *et al.*, 1993). The cross-linked bases of both the XL2 dimer (A_{20}–U_{42}) and the XL1 monomer (G_{21}/A_{22}–U_{41}) are found within loop B. The observation that the cross-linked sites in the monomer and dimer are proximal but not identical indicates that loop B remains UV-sensitive, although there must be a conformational difference between the loop B structure of the monomer and dimer.

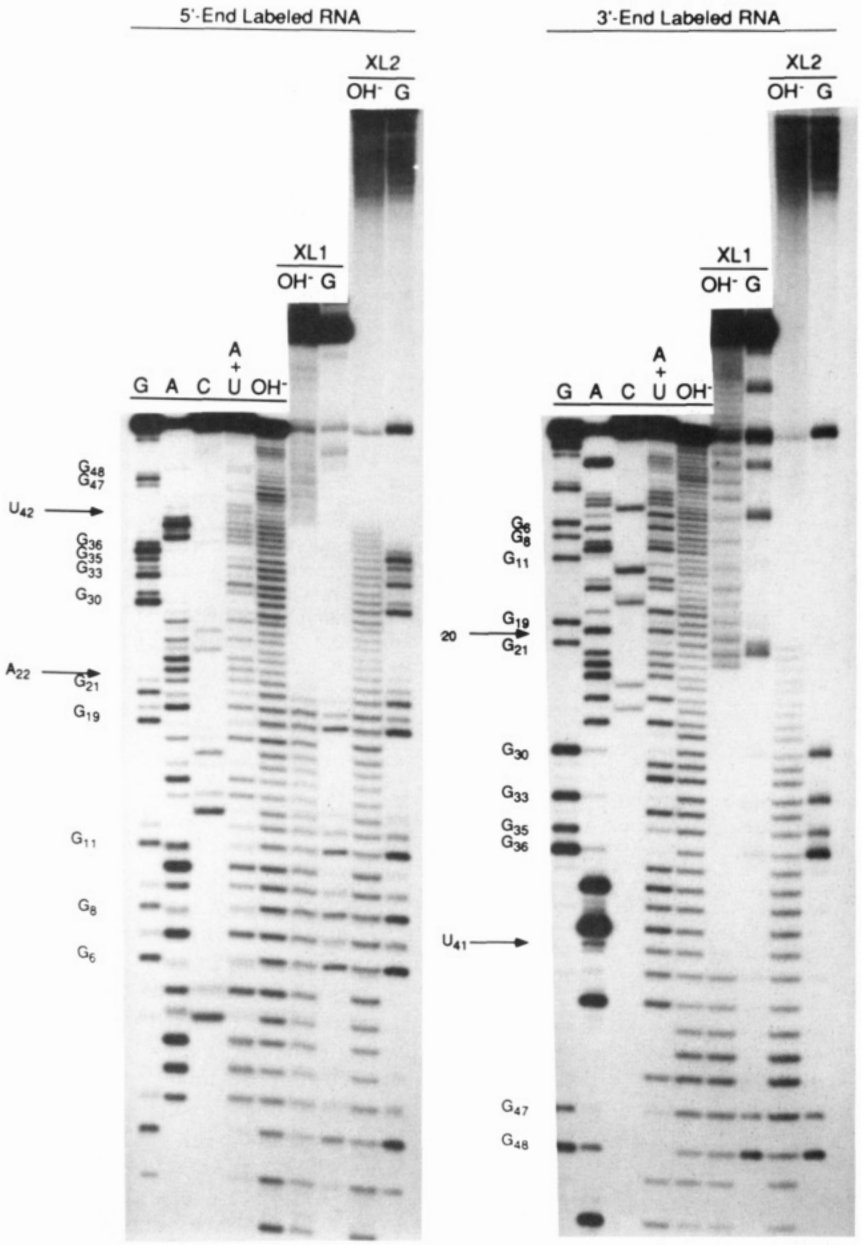

FIGURE 4: Identification of cross-linked residues in XL1 and XL2 species. The cross-linked residues were mapped by partial digestion with RNase T1 and limited hydrolysis with alkali (pH 10). End-labeled non-cross-linked RNA was partially digested with RNase T_1 (G), U_2 (A), Cl_3 (C), or PhyM (A + U) and partially hydrolyzed with alkali as a marker. G residues within the ribozyme are indicated, and the cross-linked sites are marked with arrows.

When the self-cleaving ribozyme is used for cross-linking experiments, dimerization is observed at lower concentrations than for the ribozyme alone (0.5 vs 1 μM) The additional stabilization of the dimers is likely to result from the formation of a third intermolecular helix (H2) in the dimer, utilizing the substrate sequences U_{-5} through C_{-2} that are present on the 3′ end of the self-cleaving RNA.

Cross-Linked RNAs Retain Catalytic Activity. To determine whether the cross-linked RNAs are representative of catalytically active conformations, the purified cross-linked RNA monomers and dimers were incubated with internally labeled substrate RNA under cleavage conditions and reactivity was analyzed. Both cross-linked ribozymes are able to cleave the substrate RNA (Figure 6), although in each case the amount of cleavage observed was significantly reduced relative to the nonirradiated and the irradiated but non-cross-linked ribozyme controls.

We carefully examined the XL1 preparation for the presence of non-cross-linked ribozyme that could result in the observed activity; no non-cross-linked material is detectable on over-exposed autoradiograms, and the cross-linked products remain stable throughout the purification. Together, the retention of catalytic activity by the cross-linked XL1 monomer and the observation that the cross-linked species is a major conformational isomer are evidence that the intramolecular cross-link results from proximity of the photoreactive bases within the active folded structure of the enzyme.

The activity of the cross-linked XL2 dimer is much greater than that of the XL1 monomer. This activity may be attributed to the presence of a non-cross-linked domain within the dimer. Because an unmodified catalytic domain may be retained in the dimer, we are unable to prove that the A_{20}–U_{42} cross-link reflects a catalytically active structure of the modified domain.

Cross-Linking Is Independent of Magnesium Ions and Substrate RNA. We wished to determine whether formation of the UV-sensitive tertiary structure element requires the presence of magnesium ions or substrate RNA. 5′-^{32}P-end-labeled hairpin ribozyme RNA was renatured at low con-

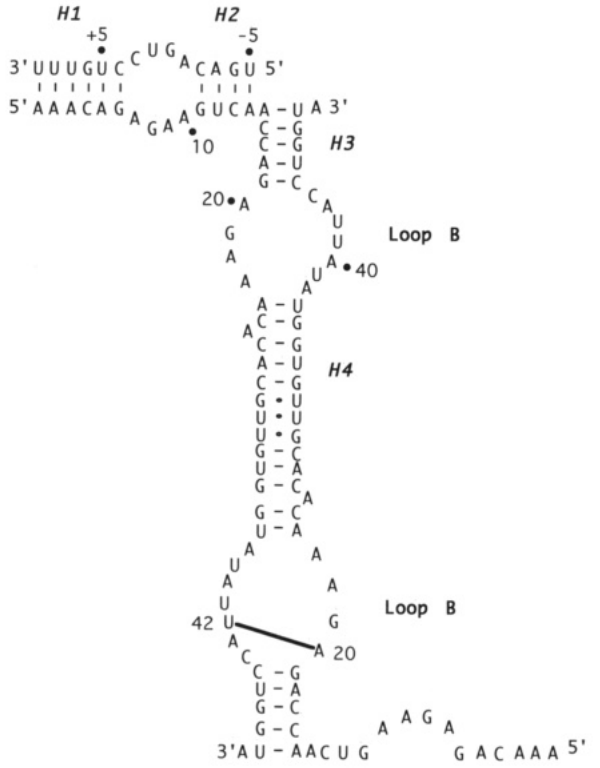

FIGURE 5: Proposed structure of a hairpin ribozyme dimer. Two hairpin ribozymes are shown in dimeric association mediated by complementary helices 3 and 4, resulting in the observed 5′-U_{42}–A_{20}-3′ intermolecular cross-link.

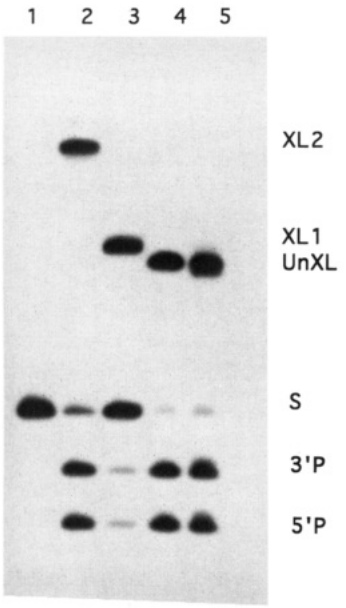

FIGURE 6: Activity of cross-linked ribozymes. 5′-^{32}P-End-labeled cross-linked ribozyme was gel purified and then incubated with an equimolar amount of internally labeled substrate RNA at 37 °C for 1.5 h in cleavage buffer: lane 1, substrate only; lane 2, XL2 (intermolecular) cross-linked RNA and substrate; lane 3, XL1 (intramolecular) cross-linked RNA and substrate; lane 4, UV-irradiated, non-cross-linked RNA and substrate; lane 5, nonirradiated ribozyme and substrate; 3′P, 3′ cleavage product; 5′P, 5′ cleavage product.

centration (50 nM) in the presence of 40 mM Tris-HCl (pH 7.6) and increasing amounts of Mg^{2+} or substrate RNA. The samples were then irradiated for 30 min. Our findings indicate that the presence or absence of Mg^{2+} and substrate RNA had no significant effect on formation of the XL1 monomer cross-link (Figure 7). Efficient cross-linking can occur when the

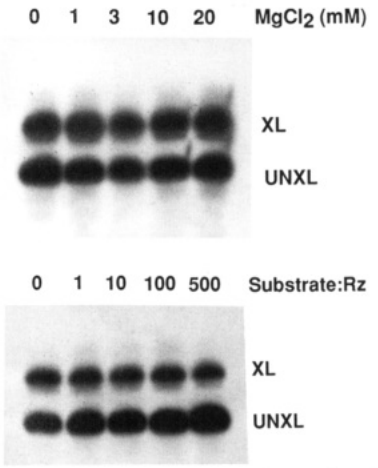

FIGURE 7: Intramolecular cross-linking is independent of magnesium ions and substrate RNA. Cross-linking of 5′-^{32}P-end-labeled hairpin ribozyme RNA renatured in the presence of 40 mM Tris-HCl (pH 7.6) and increasing concentrations of (A) $MgCl_2$ in 40 mM Tris-HCl (pH 7.6) and (B) substrate RNA in standard cleavage buffer. Samples were irradiated with UV light for 30 min. XL, intramolecular cross-link, UNXL, non-cross-linked RNA.

ribozyme is irradiated in the presence of 40 mM Tris-HCl (pH 7.6) alone (Figure 7A). These results demonstrate that the loop B region is able to fold into the specific photoreactive structure independent of the binding of magnesium ions and substrate RNA.

Point Mutations at Essential Sites in Loop B Inhibit Cross-Link Formation. To determine which nucleotides affect folding of loop B into the cross-linkable structure, we analyzed the photoreactivity of ribozymes containing single-base substitutions that eliminate catalytic activity. The constructs used derive from *in vitro* selection studies and comprise a ribozyme tethered to the 5′ end of the substrate (Berzal-Herranz *et al.*, 1992, 1993). As a positive control, a wild-type hairpin ribozyme with a tethered, noncleavable substrate ($G_{+1}A$) was cross-linked. Both XL1 and XL2 are observed (Figure 8), and mapping experiments show that the cross-linked nucleotides in this construct are identical to those described above (data not shown).

Inactivating base substitutions at essential sites within loop B ($A_{22}G$, $A_{23}U$, $A_{24}C$, and $A_{43}G$) greatly inhibit formation of the G_{21}/A_{22}–U_{41} intramolecular cross-link (Figure 8). Therefore, we conclude that these nucleotides are essential for formation of the UV-sensitive tertiary structure domain. In contrast, mutations at essential sites within internal loop A do not affect formation of the cross-link (Figure 8, $G_{+1}A$, A_9G, $A_{10}U$). The intramolecular (XL1) and intermolecular (XL2) cross-links involve different nucleotides (G_{21}/A_{22}–U_{41} vs A_{20}–U_{42}, respectively) and are sensitive to different point mutations. A point mutation at A_{22} interferes with intramolecular cross-link formation, as expected, but does not affect the intermolecular cross-link. Only nucleotide A_{43} appears to be critical for both inter- and intramolecular cross-link formation. Dimerization apparently induces a conformational change within loop B that decreases UV sensitivity and shifts the cross-linking site relative to the intramolecular loop B.

Loop B Is a Tertiary Structure Domain That Can Fold Independently. The observation that point mutations within ribozyme loop A do not inhibit cross-linking suggests that the 3′-terminal segment of the ribozyme (nt 15–50) may fold independently into the cross-linkable conformation. To test this possibility, a 36-nt RNA fragment containing helices 3 and 4 and loop B was synthesized and assayed for cross-link formation. This fragment cross-links at a rate (0.01 min^{-1}) and to an extent (30% after 30 min) similar to those of the

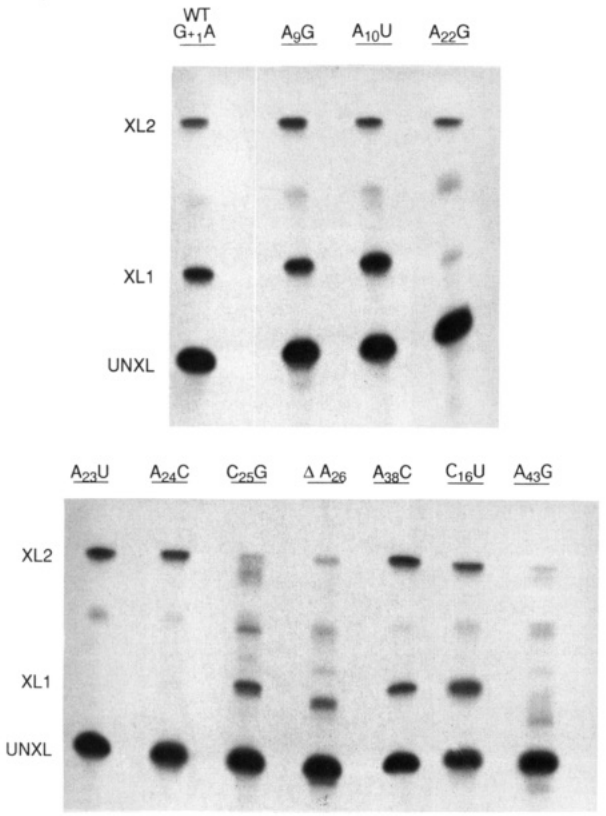

FIGURE 8: Point mutations at catalytically essential nucleotides inhibit cross-linking. Hairpin ribozyme variants containing single-base substitutions that block catalysis were 5′-^{32}P-end-labeled, renatured in 40 mM Tris-HCl (pH 7.6) and 12 mM $MgCl_2$, and exposed to UV light for 30 min. Base substitutions are shown above the individual cross-linking reactions.

entire ribozyme (Figure 9). We conclude that this loop B-containing ribozyme fragment can fold into a photoreactive tertiary structure in the absence of the substrate binding domain (nt 1–14). The location of the cross-link maps to nucleotides G_{21} and U_{41} (Figure 9), as observed for the full-length monomer. The loop B fragment, however, produces no partial cross-linking at nucleotide A_{22}. It is possible that tertiary interactions between the substrate binding domain and loop B induce a conformational change that shifts a fraction of the 5′-cross-linked bases from G_{21} to A_{22}. We have not mapped the cross-linked bases in the panel of mutants tested in the experiment of Figure 8; it is possible that these mutations might disrupt a contact within loop B and shift the cross-link back to G_{21}.

DISCUSSION

We have identified a specific photosensitive tertiary structure domain within internal loop B of the hairpin ribozyme. Cross-linking studies have been carried out in another laboratory using photosensitizing modified nucleotides (dos Santos *et al.*, 1993a,b). This work provides potentially important information concerning the proximity of specific bases in the complex. However, it is unclear whether or not all of the cross-links observed by the Favre group are representative of catalytically active structures.

The observed G_{21}/A_{22}–U_{41} cross-link forms at high efficiency (0.02 min^{-1} to an extent of 44% cross-linked after 40 min.). The observed efficiency is similar to that observed with a yeast $tRNA^{Phe}$, where cross-linking occurred at a rate of 0.06 min^{-1} to an extent of 70% cross-linked after 40 min (Behlen *et al.* 1992). In the case of $tRNA^{Phe}$, the cross-link correlates well with the crystal structure, occurring between C_{48} and U_{59}, two bases directly adjacent to one another in the tRNA tertiary structure. The fact that cross-link formation in the hairpin ribozyme is also highly efficient suggests that nucleotides G_{21}/A_{22} and U_{41} are in close proximity to each other within the major structural conformation of the hairpin ribozyme. We have been unable to cross-link more than 50% of the total RNA population. There are at least two possible reasons for this. First, UV-induced photolesions or partial denaturation during irradiation may prevent cross-linking of the ribozyme. Second, a fraction of the molecules may be folded into a conformation that cannot be cross-linked.

The maximum length for a covalent bond is typically less than 2 Å (Saenger, 1984). However, the tRNA C_{48}–U_{59} cross-link observed by Behlen and co-workers occurs between the 5–6 double bonds of the pyrimidine rings, 4.6 to 4.7 Å apart in the uncross-linked molecule. Therefore, cross-linking can itself introduce a significant structural transition. UV-induced cross-linking has been found to destabilize the *Tetrahymena* group I ribozyme, although the cross-link is thought to result from the active tertiary structure (Downs & Cech, 1990).

The residual activity in the monomeric cross-linked hairpin ribozyme, although quite low, provides good evidence that the cross-links reflect the active folded structure of the ribozyme. We have been unable to detect non-cross-linked RNAs contaminating the purified cross-linked RNAs. The cross-links are stable throughout purification and renaturation procedures and are not photoreversible upon exposure to visible light. However, we cannot rule out the possibility that an undetectable amount of non-cross-linked material remains and contributes some activity in our cleavage assay.

The intermolecular cross-link A_{20}–U_{42}, arising from the proposed dimerization between two hairpin ribozymes mediated by the existing Watson–Crick complementarity of helices 3 and 4, is interesting and significant for several reasons. First, the relatively high catalytic activity resident within the cross-linked dimer indicates that the intermolecular association of two ribozymes does not sterically disrupt one of the two catalytic centers within the molecule, although the shift in cross-linked bases is indicative of a conformational difference. Second, dimerization is essentially quantitative at concentrations $\geq 5\ \mu M$ ($\geq 1\ \mu M$ for the self-cleaving construct). This finding may be particularly important for biophysical studies of the ribozyme. Furthermore, we have found that activity can be partially reconstituted from two hairpin ribozymes containing different inactivating mutations ($A_{10}U$, $A_{38}C$) at concentrations where dimerization is favored (data not shown). We believe that most of the catalytic activity associated with the dimer can be attributed to a non-cross-linked catalytic domain.

Intramolecular cross-linking is independent of substrate RNA and magnesium ion concentration. This suggests that the photoreactive nucleotides G_{21}/A_{22} and U_{41} exist in close proximity prior to substrate and metal binding, indicating that at least part of the hairpin ribozyme is able to fold independently. Similar results have been obtained in previous NMR studies involving the hammerhead ribozyme, indicating that magnesium ions serve primarily a catalytic rather than a structural role, with the magnesium binding site already existing in the magnesium-free complex (Heus & Pardi, 1991). The hairpin ribozyme may therefore be similar to the hammerhead regarding metal ion involvement and structure formation. Previously, we have shown that the hairpin ribozyme is able to bind substrate RNA efficiently in the absence of magnesium (Chowrira & Burke, 1993). Here, we

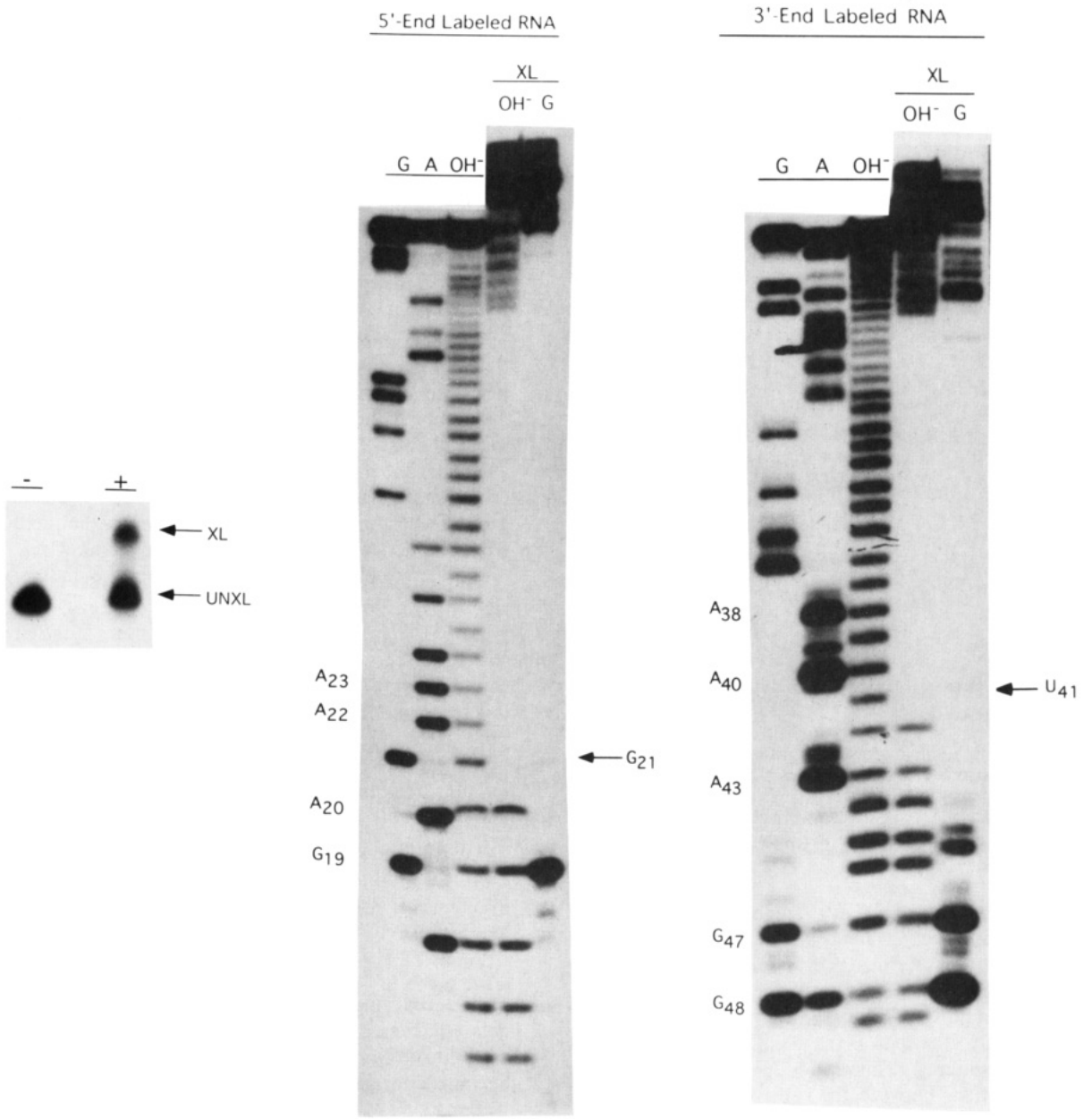

FIGURE 9: An RNA fragment that lacks the substrate binding domain retains photoreactivity. (A, left) UV cross-linking of the 36-nt RNA fragment renatured in 40 mM Tris-HCl (pH 7.6) and 12 mM $MgCl_2$ without (–) and with (+) UV irradiation for 30 min. (B, right) Identification of cross-linked nucleotides by partial digestion with alkali and RNase T1. Cross-linked nucleotides are shown with arrows.

show that folding of a specific tertiary structure domain also occurs in the absence of magnesium.

Together with results from cross-linking of mutant ribozymes, the finding that deletion of the substrate-binding domain does not eliminate cross-linking supports the conclusion that loop B represents an independently folding domain of tertiary structure. We believe that the conservation of photoreactivity accompanied by minor shifts in the cross-link positions may reflect small conformational changes in otherwise similar structures.

A striking sequence similarity exists between loop B of the hairpin ribozyme and photoreactive sequences within the conserved central domains of viroid RNAs and loop E of eukaryotic 5S rRNA (Figure 10). The cross-linkable molecules all contain a loop flanked by two helices. The sequences of the internal loop segments on the opposing strands are 5′-GAA–3′ and 5′-UANUA–3′. In all cases, the internal loops are flanked by two helices comprising canonical base pairs.

The tertiary structure of the loop E domain was recently examined using NMR methods by Wimberly and co-workers (1993), who have proposed a model in which the cross-linked nucleotides are stacked in a helix composed of non-Watson–Crick base pairs (Figure 10). In the loop E structure, strong evidence for a G_{98}–A_{77} base pair and a reverse Hoogsteen A_{99}–U_{76} pair were obtained; these base pairs extend one of the canonical helices into the loop. A_{74}–A_{100} and U_{73}–U_{100} pairs are also proposed, with chain direction at A_{74} reversed into a locally parallel duplex. An important feature of the loop E model is that G_{75} is bulged into the major groove. Although the base is not well-determined, the NMR data are consistent with formation of a base triple with the A_{99}–U_{76} pair through a contact between the 2-amino group of G_{75} and U_{76}. In this domain, photo-cross-linking is reported to occur between G_{98} and U_{76} (Branch *et al.*, 1985).

Loop B of the hairpin ribozyme shows striking homology to 5S rRNA loop E in both sequence and photoreactivity; each of the nucleotides in the loop E structure has a clear counterpart in the ribozyme (Figure 10). Furthermore, *in vitro* selection results show that each of these bases is important for catalytic function, and we have shown here that the same

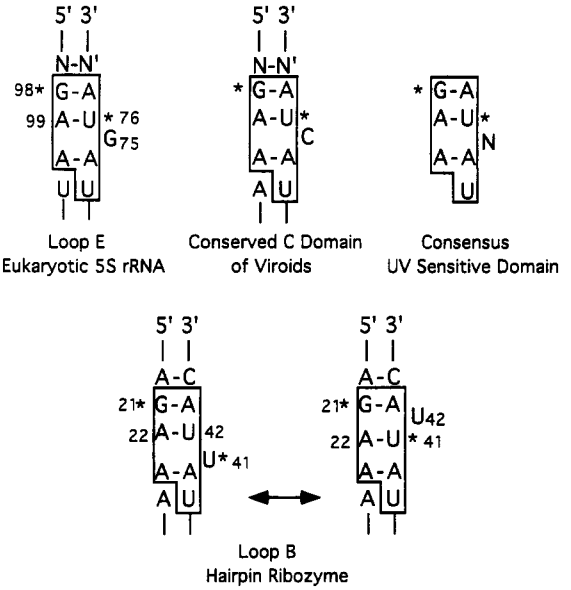

FIGURE 10: Consensus model of the UV-sensitive domain. The sequences of three UV-sensitive RNA loop domains are shown in comparison with the shared consensus sequence. The pairings for the hairpin ribozyme loop B and the viroid C domain are hypothetical and aligned according to the NMR structure of the eukaryotic 5S rRNA loop E as proposed (Wimberly *et al.*, 1993). Asterisks indicate nucleotides that are cross-linked in the hairpin ribozyme (this work), in the viroids, and in loop E of 5S rRNA (Branch *et al.*, 1985). For the hairpin ribozyme, an alternative structure is shown that may be relevant to formation of the G_{21}–U_{41} cross-link. The shifted cross-link observed with the hairpin loop B structure can be explained if the proposed extrahelical U residue can shift with an adjacent U in the A–U reverse-Hoogsteen pairing. Such a model maintains the consensus G–A pairing, A–U reverse-Hoogsteen pairing, and A–A pairings, and also accounts for the cross-links observed in the hairpin ribozyme, by placing all UV-sensitive nucleotides in close proximity to one another.

bases are essential for photoreactivity. From these results, we infer that the tertiary structure of loop B within the hairpin ribozyme may closely resemble that of loop E within 5S rRNA. Because internal loops with similar sequences and photoreactivity are found in the conserved central domain of viroids, and in other rRNA molecules, we believe that the loop E-like structure may represent a ubiquitous domain of RNA tertiary structure that is utilized in a number of functionally important RNA molecules.

The proposed extrahelical nucleotide in the hairpin ribozyme (U_{41}, corresponding to G_{75} in 5S rRNA) is adjacent to U_{42}, and either nucleotide may be bulged in the hairpin loop B while still maintaining the consensus pairings observed for the photoreactive domains. Although the bulged nucleotide may participate in a $U_{41}U_{42}$–A_{22} triple, it is a likely candidate for interaction with the substrate binding domain of the ribozyme. We believe that such an interaction may be elucidated through a combination of methods that are currently available.

REFERENCES

Abrash, H. I., Cheung, C.-C. S., & Davies, J. C. (1967) *Biochemistry 6*, 1298–1303.

Behlen, L. S., Sampson, J. R., & Uhlenbeck, O. C. (1992) *Nucleic Acids Res. 20*, 4055–4059.

Berzal-Herranz, A., Joseph, S., & Burke, J. M. (1992) *Genes Dev. 6*, 129–134.

Berzal-Herranz, A., Joseph, S., Chowrira, B. M., Butcher, S. E., & Burke, J. M. (1993) *EMBO J. 12*, 2567–2574.

Branch, A. D., Benenfeld, B. J., & Robertson, H. D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6590–6594.

Burke, J. M. (1994) *Nucleic Acids Mol. Biol.* (in press).

Cech, T. R., Herschlag, D., Piccirilli, J. A., & Pyle, A. M. (1992) *J. Biol. Chem. 267*, 17479–17482.

Chowrira, B. M., & Burke, J. M. (1991) *Biochemistry 30*, 8518–8522.

Chowrira, B. M., Berzal-Herranz, A., & Burke, J. M. (1991) *Nature 354*, 320–322.

Chowrira, B. M., Berzal-Herranz, A., & Burke, J. M. (1993) *Biochemistry 32*, 1088–1095.

dos Santos, D. V., Fourrey, J. L., & Favre, A. (1993a) *Biochem. Biophys. Res. Commun. 190*, 377–385.

dos Santos, D. V., Vianna, A. L., Fourrey, J. L., & Favre, A. (1993b) *Nucleic Acids Res. 21*, 201–207.

Downs, W. D., & Cech, T. R. (1990) *Biochemistry 29*, 5605–5613.

England, T. E., & Uhlenbeck, O. C. (1978) *Nature 275*, 560–561.

Feldstein, P. A., & Bruening, G. (1993) *Nucleic Acids Res. 21*, 1991–1998.

Feldstein, P. A., Buzayan, J. M., & Bruening, G. (1989) *Gene 82*, 53–61.

Hampel, A., & Tritz, R. (1989) *Biochemistry 28*, 4929–4933.

Hampel, A., Tritz, R., Hicks, M., & Cruz, P. (1990) *Nucleic Acids Res. 18*, 299–304.

Haseloff, J., & Gerlach, W. L. (1989) *Gene 82*, 43–52.

Heus, H. A., & Pardi, A. (1991) *J. Mol. Biol. 217*, 113–124.

Joseph, S., & Burke, J. M. (1993) *J. Biol. Chem. 268*, 24515–24518.

Joseph, S., Berzal-Herranz, A., Chowrira, B. M., Butcher, S. E., & Burke, J. M. (1993) *Genes Dev. 7*, 130–138.

Michel, F., & Westhof, E. (1990) *J. Mol. Biol. 216*, 585–610.

Midgley, C. A., & Murray, N. E. (1985) *EMBO J. 4*, 2695.

Milligan, J. F., & Uhlenbeck, O. C. (1989) *Methods Enzymol. 180*, 51–62.

Saenger, W. (1984) in *Principles of Nucleic Acid Structure*, Springer-Verlag, Inc., New York.

Vinayak, R., Anderson, P., McCollum, C., & Hampel, A. (1992) *Nucleic Acids Res. 20*, 1265–1269.

Walstrum, S. A., & Uhlenbeck, O. C. (1990) *Biochemistry 29*, 10573–10576.

Wimberly, B., Varani, G., & Tinoco, I., Jr. (1993) *Biochemistry 32*, 1078–1087.