RNA (1998), *4*:489–497. Cambridge University Press. Printed in the USA.

LETTER TO THE EDITOR

A UV-crosslinkable interaction in human U6 snRNA

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ABSTRACT

U6 snRNA is the most conserved of all the snRNAs involved in pre-mRNA splicing, and likely plays an important role in splicing catalysis. Using a U6 snRNA fragment encompassing residues 25–99, we have identified a strong, UV-sensitive tertiary intramolecular interaction. A 5′ deletion that removed sequences up to nt 37 only slightly reduced crosslinking, but further deletion of 11 bases, eliminating the nearly invariant ACAGAGA sequence, essentially abolished crosslinking, as did deletion of sequences 3′ of 82A. The crosslinked residues were mapped to 44G in the ACAGAGA sequence and to 81C, the nucleotide at the base of the U6 intramolecular helix, opposite the G of the invariant AGC trinucleotide. This interaction is striking in that it has the potential to juxtapose invariant regions of U6 believed to play critical roles in splicing catalysis.

Keywords: pre-mRNA splicing; tertiary interaction; snRNA structure

Nuclear pre-mRNA splicing requires the assembly of the pre-mRNA substrate into the spliceosome, a dynamic structure consisting of a large number of distinct proteins as well as five small nuclear RNAs (snRNAs) (U1, U2, U4, U5, and U6). These snRNAs, which are preassembled into ribonucleoprotein particles (snRNPs), form extensive interactions with each other and with pre-mRNA. These interactions play important roles not only in spliceosome assembly, but also in the two catalytic transesterification steps in pre-mRNA splicing (reviewed by Madhani & Guthrie, 1994a; Nilsen, 1994; Sharp, 1994; Ares & Weiser, 1995). Four of the snRNAs participate in base pairing interactions with the pre-mRNA, although at most only three snRNAs, U2, U6, and U5, are likely to play direct roles in catalysis (Madhani & Guthrie, 1994a; Nilsen, 1994). This conclusion is based upon two types of experiments. First, biochemical assays, mainly UV and psoralen-induced crosslinking, have demonstrated that interactions between the pre-mRNA and all three of these snRNAs are formed within the spliceosome (Sawa & Abelson, 1992; Sawa & Shimura, 1992; Wassarman & Steitz, 1992; Wyatt et al., 1992; Sontheimer & Steitz, 1993; Kim & Abelson, 1996). Second, genetic suppression studies have demonstrated that these interactions are functionally important (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989; Newman & Norman, 1991, 1992; Cortez et al., 1993; Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Sun & Manley, 1995). However, the U5-pre mRNA interaction is dispensable for the first step of splicing in vitro (O'Keefe et al., 1996), and therefore only U2 and U6 are likely to play a direct role in catalysis, at least for the first step. These RNA–RNA interactions provide similarities to contacts that exist in autocatalytic group II introns (for review see Moore et al., 1993; Madhani & Guthrie, 1994a; see also Hetzer et al., 1997), and support the view that group II self-splicing and nuclear pre-mRNA splicing involve related mechanisms.

U6 snRNA is the most highly conserved of the snRNAs. Specifically, a central region of about 40 bases is nearly invariant between yeast and mammals (Brow & Guthrie, 1988; Fortner et al., 1994). Much of this conserved central domain is base paired with U4 snRNA in the U4/U6 snRNP (reviewed by Madhani & Guthrie, 1994a). U4/U6 and U5 snRNP are recruited to the spliceosome as a tri-snRNP complex. Upon completion of spliceosome assembly, the U4/U6 duplex dissoci-

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https://doi.org/10.1017/S1355838298980141 Published online by Cambridge University Press

ates, and U4 likely leaves the spliceosome, allowing both extensive base pairing of U6 with U2 snRNA and formation of a U6 intramolecular helix. Both of these structures likely play important roles in splicing catalysis.

Genetic suppression experiments support the existence of three distinct U2-U6 helices, helix I, II, and III. Helix II was first identified by crosslinking and involves bases at the 5′ end of U2 and 3′ end of U6 (Hausner & Weiner, 1990), and the interaction was proven genetically in mammalian systems (Datta & Weiner, 1991; Wu & Manley, 1991). It may be of less significance in yeast, although a requirement for helix II was observed in a yeast strain carrying a mutation in U2-U6 helix Ib (see below), suggesting that these helices may be functionally redundant (Field & Friesen, 1996). Helix I was identified in yeast and was proposed to consist of two helices, Ia and Ib (Madhani & Guthrie, 1992, 1994b). This structure is striking in that bases involved in both U2 and U6 are adjacent to other critical bases. Helix Ia is situated just 5′ to the nearly invariant ACAGAGA in U6, in which the underlined bases are implicated in 5′ splice site pairing, and 3′ to the U2 branch recognition sequence, which pairs with the pre-mRNA branch site. Helix Ia was proven subsequently in mammalian cells by genetic suppression (Sun & Manley, 1995), although current evidence suggests that helix Ib is not essential (Datta & Weiner, 1993; Sun & Manley, 1995). Finally, a third U2-U6 helix, helix III, has been proven by genetic suppression experiments in mammals (Sun & Manley, 1995). Although the potential to form helix III is well conserved evolutionarily (Takahashi et al., 1993), and it could further stabilize a 5′ splice site-branch site interaction, it is not essential in yeast (Yan & Ares, 1996).

In addition to these intermolecular interactions, an intramolecular helix in U6 may also play an important role in splicing catalysis. Genetic experiments in both yeast (Fortner et al., 1994) and mammals (Wolff & Bindereif, 1993; Sun & Manley, 1995) have provided evidence that formation of the helix is necessary for some step(s) in splicing. Mutagenesis of human U6 revealed that the ability to participate in base pairing is compatible with function at all positions tested in this helix, and that bases not naturally paired could be incorporated into the helix without loss of function (Sun & Manley, 1997). These results suggest the role of the intramolecular helix may be largely structural and not sequence-specific.

Other studies suggest a similarity between the U6 intramolecular helix and domain 5 of group II introns. Analysis of critical phosphate oxygens in the two RNAs revealed that important phosphates were located at similar positions in secondary structure models of both RNAs (Fabrizio & Abelson, 1992; Chanfreau & Jaquier, 1994; Yu et al., 1995). Analysis of essential 2′ hydroxyls also revealed similarities between the two structures (Abramovitz et al., 1996; Kim et al., 1997), and genetic analyses have suggested that corresponding bases in U6 and domain 5 can be important for splicing (Madhani et al., 1990; Madhani & Guthrie, 1992; Boulanger et al., 1995; Peebles et al., 1995; Sun & Manley, 1995). U2-U6 helix Ib has been proposed to correspond to the bottom of domain 5 and the U6 intramolecular helix to the top, and genetic (Boulanger et al., 1995; Peebles et al., 1995) and chemical modification (Yu et al., 1995) studies are consistent with this model. Particularly important are the AGC bases at the bottom of both structures. These three bases are highly conserved in both systems and effects of mutations in both domain 5 and U6 are similar. However, a modification of this model, in which the U6 intramolecular helix is extended to replace helix Ib (Fortner et al., 1994), is more consistent with some mutational studies, at least in human U6 snRNA (Sun & Manley, 1995).

Here we describe a novel tertiary interaction, detected by UV crosslinking, in human U6 snRNA that bridges the invariant ACAGAGA sequence and the base of the U6 intramolecular helix. We show that crosslinking occurs between residue 44G, the central position in ACAGAGA, and 81C, which is at the base of the U6 intramolecular helix, opposite the G of the invariant AGC. This long-range tertiary interaction has the potential to juxtapose invariant regions of U6 that likely play critical roles in catalysis.

RESULTS

UV-induced crosslinking of human U6 snRNA

We undertook a UV crosslinking analysis of an in vitro-synthesized U6 snRNA fragment containing nucleotides 25–99 (U6 25–99). We chose this RNA because it includes the highly conserved central domain, the U6 sequences necessary for formation of U2-U6 helices I, II, and III, and the U6 intramolecular helix. In addition, bases 5′ and 3′ of this region have been shown to be unimportant for U6 function in vitro (Wolff & Bindereif, 1992). We were interested in the detection of possible interactions between and/or inside these functionally important domains, especially between ACAGAGA, helix I, and/or the U6 intramolecular helix. The U6 25–99 RNA was synthesized by in vitro transcription, dephosphorylated, and 5′ end-labeled with [γ-P^{32}]ATP. In initial experiments, U6 25–99 was resuspended in a standard low-salt buffer used previously for group II self-splicing introns, containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 2 mM spermidine (Peebles et al., 1987). The U6 RNA was first denatured at 70 °C for 2 min and then rapidly cooled on ice for 15 min. Renatured U6 snRNAs were UV irradiated and analyzed by denaturing gel electrophoresis. The heating and cooling procedure prior to irradiation was necessary for efficient crosslinking, presumably by allowing the RNA to assume a stably folded structure competent for crosslinking. Without heating and cooling, the same irradiation

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procedure yielded less than 10% the amount of crosslinked RNA obtained with the renaturation procedure (results not shown).

Using the above protocol, a strong crosslinked product was detected in an initial experiment employing an RNA concentration of 100 nM. A time-course was performed to determine the efficiency of the crosslinking (Fig. 1). The crosslinked species formed with reasonable efficiency and accumulated up to 37% of the total RNA at 90 min, as determined by PhosphorImager analysis. The efficiency of crosslinking varied from about 20% to more than 50%. The lower yield likely reflects the fact that accumulation of crosslinked product did not always increase after 30–60 min, perhaps reflecting denaturation of the crosslinkable structure by excess heating during irradiation. The efficiency of crosslink formation indicates that, under these conditions, the crosslinkable conformation was a significant form of the U6 RNA fragment employed. This efficiency is comparable to those observed with several other RNAs (e.g., Branch et al., 1985, 1989; Behlen et al., 1992; Butcher & Burke, 1994), although less than with the *Tetrahymena* group I ribozyme (Downs & Cech, 1990). To investigate whether the crosslink was intra- or intermolecular, crosslinking was performed in the presence of increasing concentrations of U6 25–99 RNA, from 0.1 nM to 10 μM. The results (not shown) indicate that the rate and extent of crosslinking did not change significantly over the 100,000-fold variation in concentration, establishing that formation of the crosslink was independent of U6 concentration, and therefore likely intramolecular.

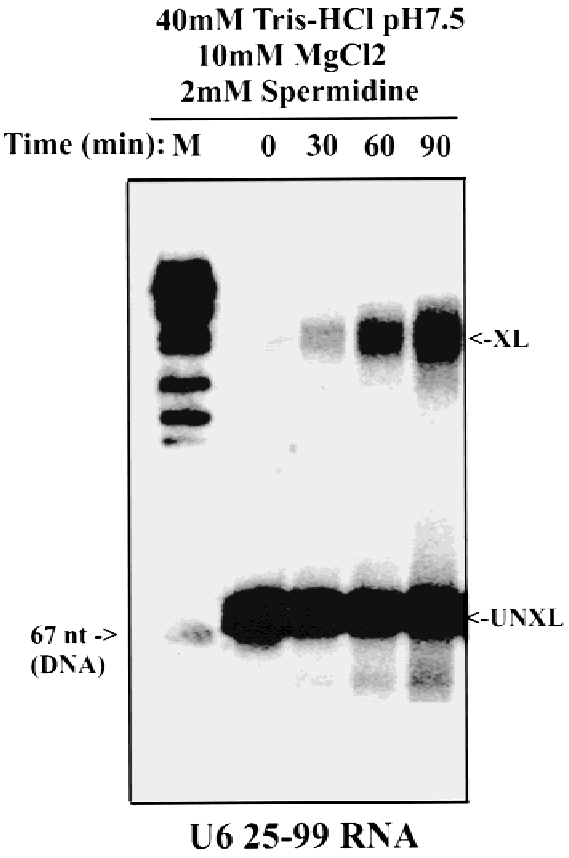

FIGURE 1. Time-course of UV-induced crosslinking of U6 25–99 RNA. UV crosslinking was performed under standard conditions (top) for the indicated times, as described in Materials and Methods. RNAs were resolved by electrophoresis through an 8 M urea–20% polyacrylamide gel. XL and UNXL indicate the positions of crosslinked and uncrosslinked U6 RNAs, respectively. M is a DNA marker from 5′-labeled pBR322-*Hpa* II digestion.

Sequence and ionic requirements for crosslinking

To investigate the regions of the U6 RNA fragment necessary for crosslinking, we examined several truncated derivatives of the U6 25–99 fragment. These include two 5′ truncations beginning at positions 37 and 48, and a 3′ truncation terminating at position 82. Note that the longer 5′ truncation retains the ACAGAGA sequence, whereas the shorter begins just 3′ to it. The 3′ truncation terminates just one nucleotide 3′ to the base of the intramolecular helix. RNAs were synthesized, dephosphorylated, 5′ end-labeled, heated and cooled, and subjected to UV irradiation and denaturing PAGE as above. Figure 2 shows the results obtained after incubation for the indicated times, in buffer containing either 10 mM $MgCl_2$ or 1 mM EDTA. As above, U6 25–99 was crosslinked efficiently and in a Mg^{2+}-dependent manner. Additional experiments (not shown) indicated that NaCl, over a concentration range of 50 mM to 1 M, could fully substitute for $MgCl_2$. These results suggest that $MgCl_2$ and NaCl function interchangeably to facilitate a structure compatible with crosslinking, likely by simple electrostatic interactions with the phosphate backbone. U6 37–99 also gave rise to a Mg^{2+}-dependent crosslinked species of similar gel mobility, although the extent of crosslinking was reproducibly a factor of 2–3 lower than observed with U6 25–99. In contrast, crosslinking was undetectable with both U6 49–99 and U6 25–82 (results with U6 24–82 not shown). These results suggest that a residue or sequence in or adjacent to the ACAGAGA and 3′ to the intramolecular helix is essential for crosslinking.

Identification of UV-crosslinked residues

To determine the identities of the crosslinked bases, both 5′ and 3′ end-labeled U6 25–99 RNAs were prepared, crosslinked under standard conditions (i.e., 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM spermidine), gel purified, and subjected to limited alkaline hydrolysis. The noncrosslinked RNAs were treated similarly, as well as subjected to partial digestion with a panel of RNases to provide a sequence ladder. Products were then resolved on 20% polyacrylamide gels, and results with the 5′ (Fig. 3A) and 3′ (Fig. 3B) labeled

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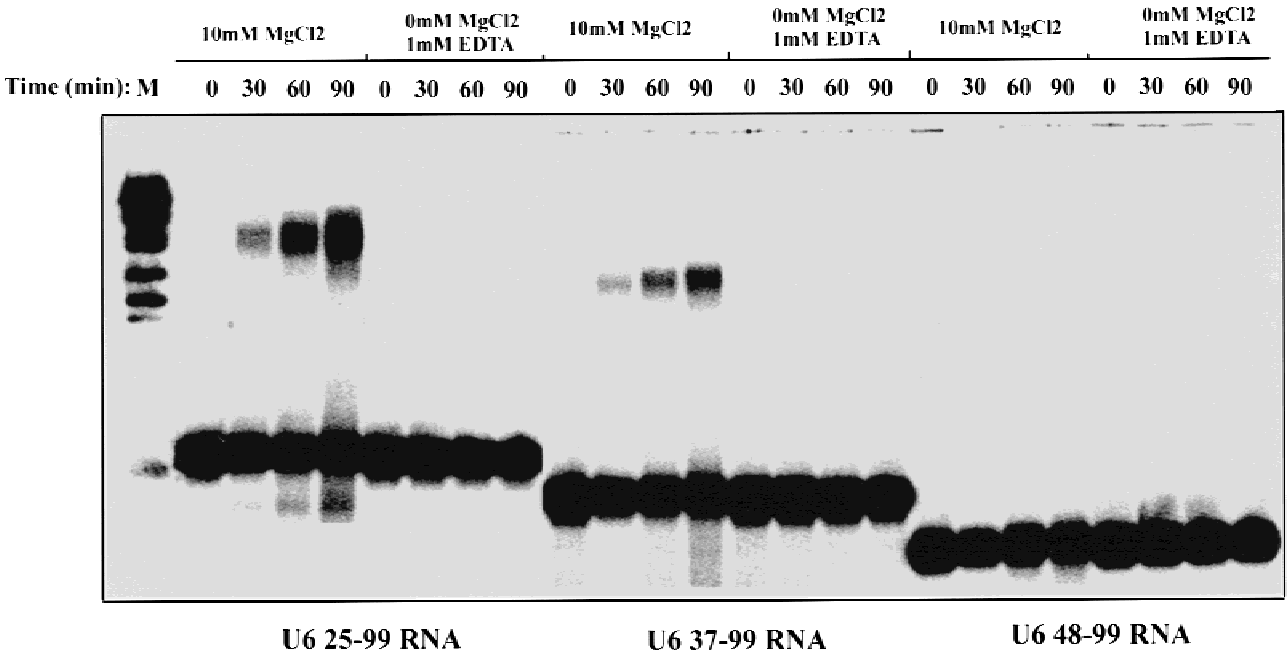

FIGURE 2. RNA sequence requirements for crosslinking. Three U6 RNA fragments, U6 25–99, 37–99, and 48–99, were UV irradiated under the conditions indicated at the top of the panel (plus 40 mM Tris HCl, pH 7.5). Crosslinking was performed for the times indicated and RNAs resolved by denaturing gel electrophoresis as above. XL and UNXL indicate the positions of crosslinked and uncrosslinked U6 RNAs, respectively.

RNAs are shown. (Note that both of the purified crosslinked RNAs contained a small amount of uncrosslinked RNA. Whether this was from contamination or from partial reversal of the crosslink is unclear, but its presence offers an explanation for a low background of hydrolysis products extending beyond the crosslinked base.) The 5′-labeled RNA revealed a distinct stop at residue A43, indicating that G44 is the major 5′ crosslinked nucleotide. Samples in lanes XLOHa and XLOHb were made with different length of time of partial alkaline hydrolysis, 4.5 min and 5.5 min, respectively, and produced essentially identical results. Crosslinked species from NaCl-containing crosslinking buffer (40 mM Tris-HCl, pH 7.5, 100 mM NaCl) resulted in an identical stop at the same position (data not shown). With the 3′ end-labeled RNA, a single stop at A82 was detected, implicating C81 as the 3′ crosslinked nucleotide. Samples in lanes U6XL1a and 2a, U6XL1b and 2b were again alkaline-treated for 4.5 min and 5.5 min, respectively. Crosslinked products in lanes U6XL1a and b were made in the presence of NaCl, whereas crosslinked products in U6XL2a and b were from standard conditions. These data indicate that G44 and C81 participate in a long-range interaction within the U6 25–99 RNA molecule. Furthermore, these assignments are consistent with the results obtained with the truncation mutants described above. It is noteworthy that C81 is the penultimate 3′ residue in the 3′ truncation. Whether the failure of this RNA to crosslink reflects the terminal position of C81 in this RNA or a specific requirement for downstream sequences is not known, but is discussed below.

DISCUSSION

Here we have described a specific UV-crosslinkable interaction between residues G44 and C81 in human U6 snRNA. Although most of our studies were performed with a truncated version of U6 snRNA, the same crosslink was detected with a full-length molecule (unpubl. data). This crosslink is similar to other tertiary RNA crosslinks described previously (e.g., Atmadja & Brimacombe, 1985; Branch et al., 1985, 1989; Downs & Cech, 1990; Butcher & Burke, 1994) in that it results from a folded structure of the U6 molecule, because it is detectable only after denaturing and annealing. The potential significance of this crosslink is enhanced because it juxtaposes G44 in the phylogenetically invariant ACAGAGA sequence and C81, which is opposite G54 in the invariant trinucleotide AGC. Thus, this interaction has the potential of bringing these two important regions of U6 snRNA into close proximity. Deletion mutations that remove either ACAGAGA or U6 sequences immediately downstream of C81 eliminated crosslinking, but single-nucleotide substitutions at either position unexpectedly had little effect (results not shown). These findings suggest that the overall structure of the region is more important for crosslinking than are the identities of the bases involved. We discuss below other

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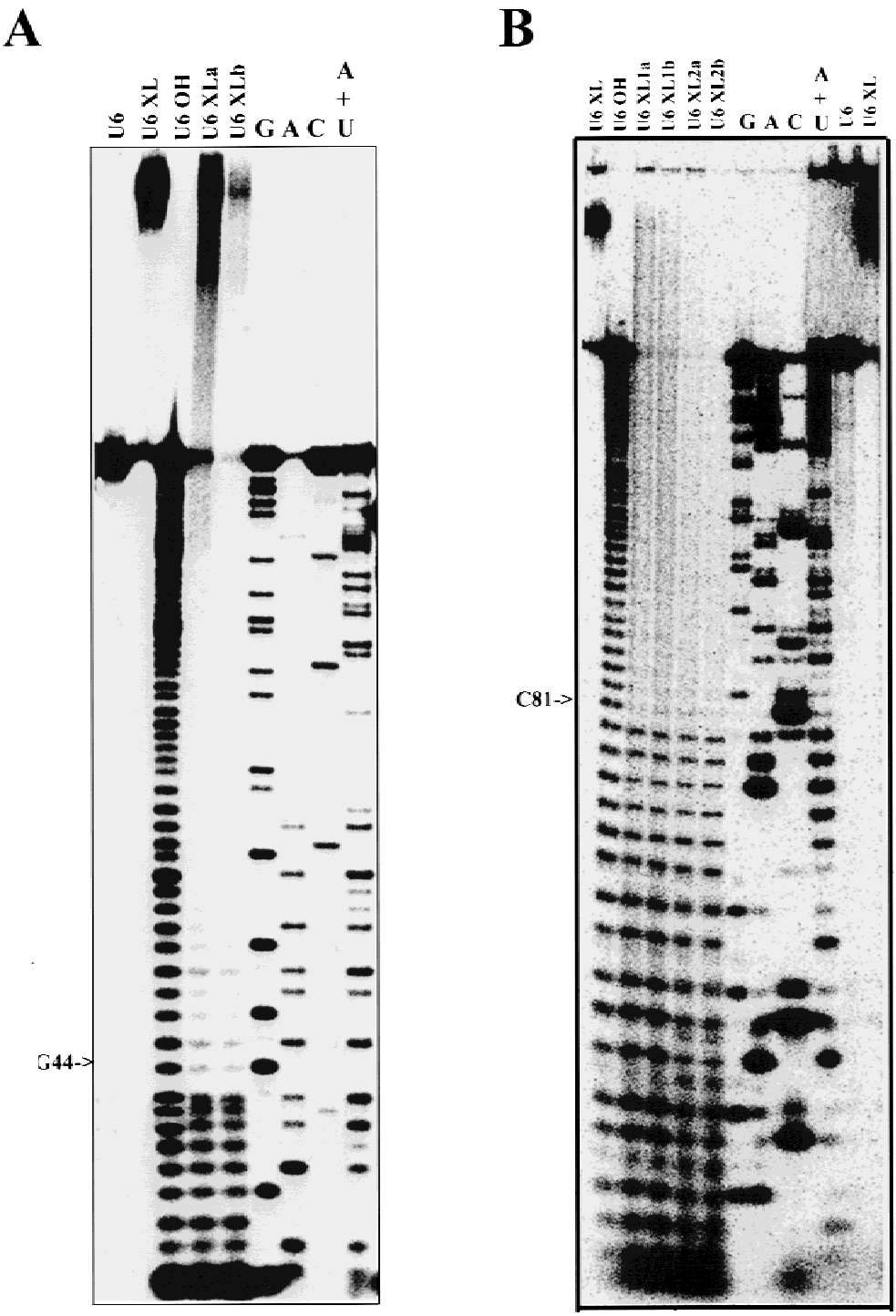

FIGURE 3. Identification of crosslinked residues in U6 25–99 RNA. The 5′ (**A**) and 3′ (**B**) crosslinked residues were mapped by limited alkaline hydrolysis (pH 12.0) of 5′ and 3′ end-labeled crosslinked and uncrosslinked products. U6 and U6XL represent purified uncrosslinked and crosslinked U6 25–99 RNA, respectively. U6 OH indicates partial alkaline hydrolysis of the uncrosslinked RNA. U6 XL a and b were subjected to alkaline hydrolysis for 4.5 and 5.5 min, respectively. In B, XL1 depicts RNA crosslinked in the presence of 100 mM NaCl, whereas XL2 is from reactions performed under standard conditions (see text). 5′- or 3′-Labeled uncrosslinked U6 RNAs were partially digested with RNases T1 (G), U2 (A), C13 (C), and PhyM (A+U) to produce a sequence ladder. RNAs were resolved by denaturing 20% PAGE. Crosslinked residues are indicated with arrows.

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potential interactions that may contribute to formation of the crosslinkable configuration.

The tertiary interaction involving G44 and C81 is interesting in that previous studies have suggested potentially important roles for these bases in splicing, especially for G44. For example, the single-base substitution G44C has been shown to abolish splicing activity in both transient expression in vivo (Datta & Weiner, 1993) and splicing complementation in vitro (Wolff et al., 1994). A G → C mutation at the corresponding position in yeast U6 snRNA led to a significant reduction in in vitro reconstitution activity (Fabrizio & Abelson, 1990), and to a *ts* growth phenotype in vivo (Madhani et al., 1990; McPheeters, 1996). Both G44U and G44A mutations can be lethal in yeast (McPheeters, 1996), although G44A had no effect on splicing in transfected human cells (Datta & Weiner, 1993). Mutation of C81 to G resulted in a decrease in splicing in a transient expression assay, although the defect could be rescued by a compensatory G → C change in the essential residue G54 (Sun & Manley, 1995). A number of previous studies have illustrated the importance of G54, not only in yeast (e.g., McPheeters, 1996) and mammalian (Datta & Weiner, 1993; Sun & Manley, 1995) splicing, but also in group II splicing, where a comparable AGC sequence is situated at the base of domain 5 (Boulanger et al., 1995; Peebles et al., 1995).

A number of additional studies have indicated the importance for splicing of bases and/or phosphate oxygens near the two positions identified in our crosslinking studies. Crosslinking experiments in yeast and humans have shown that the ACA of the ACAGAGA sequence physically contacts the pre-mRNA 5′ splice site, an interaction that has also been proven genetically in yeast and mammals (see Introduction for references). Thiophosphate incorporation at various positions in U6 RNA has identified important phosphate oxygen atoms in both yeast and nematodes (Fabrizio & Abelson, 1992; Yu et al., 1995). The phosphate oxygen between ACA and GAGA was shown to be critical for splicing activity in both organisms, which may be particularly significant in light of our crosslinking studies implicating this same position in the intramolecular crosslink. The phosphate oxygen immediately 5′ to the invariant AGC was shown to be necessary for the second step of splicing (Fabrizio & Abelson, 1992; Yu et al., 1995), whereas a thiophosphate 5′ to the G inhibited splicing in worms (Yu et al., 1995) and also in group II self splicing (Chanfreau & Jacquier, 1994). U6-81C is in close proximity to these two oxygens via base pairing with G54 (Sun & Manley, 1995), and the crosslinkable conformation we have described thus has the potential to help bring all three of these important phosphate oxygens into close proximity. An intriguing possibility is that this contributes to formation of a metal binding site essential for catalysis (e.g., Steitz & Steitz, 1993).

Several related secondary structures for free human U6 snRNA have been proposed (Epstein et al., 1980; Harada et al., 1980; Miura et al., 1983; Rinke et al., 1985). The structure illustrated in Figure 4A is similar but not identical to the earlier structures. These structures all share in common what is now known as the U6 intramolecular helix, which extends from G54 to C81. Most of the paired positions have been proven to exist at some stage during splicing by genetic suppression experiments (Wolff & Bindereif, 1993; Sun & Manley, 1995, 1997). The proposed structures also all share the lower stem, although we are not aware of direct evidence, genetic or chemical, confirming its existence or importance during splicing. The central loop and helix shown in Figure 4A have been drawn in several configurations. There is again limited evidence for or against any of these structures, although chemical modification data suggest that C42 is unpaired (Miura et al., 1983).

The secondary structure depicted in Figure 4A has a striking feature not noticed previously, which is a remarkable similarity with the currently envisioned U2-U6 secondary structure, excluding U2-U6 helix II (Fig. 4B). It shows not only structural similarity, but also nucleotide identities at several functionally important positions, such as U2-U6 helix Ia and a region overlapping the U2 branch site recognition sequence, AUGAUG (identities are lightly shaded in Fig. 4). Whether these similarities are functionally significant is unclear, but they do raise the possibility that the U6 RNA fragment we have analyzed reflects the U2-U6 structure thought to exist in the spliceosome. In addition, this structure provides a framework to consider the basis for the effects of the deletions on crosslinking. Removal of bases 5′ to 37C reduced crosslinking efficiency, and eliminated four of six bases from the bottom stem, consistent with the possibility that this helix may contribute to formation of the crosslinkable conformation. Deletion of bases 3′ to 82A, which eliminated crosslinking, would disrupt all the structure below the intramolecular helix. Future experiments will be aimed at determining in more detail the role of the proposed U6 structure in crosslinking, as well as whether the 44G-81C crosslinkable conformation exists in the spliceosome, when U6 is paired to U2.

By using an elegant in vivo bimolecular randomization-selection assay, Madhani and Guthrie (1994b) obtained genetic evidence in yeast for a tertiary interaction between the penultimate nucleotide in the ACAGAGA sequence and the 3′ base in the two-nucleotide bulge between U2-U6 helices Ia and Ib (Fig. 4C). This interaction has been suggested to contribute to formation of the catalytic center for the second step of splicing by helping to juxtapose the ACAGAGA and AGC sequences. The similarity between this interaction and the one described here is striking, and it is conceivable that both could exist simultaneously in the same folded

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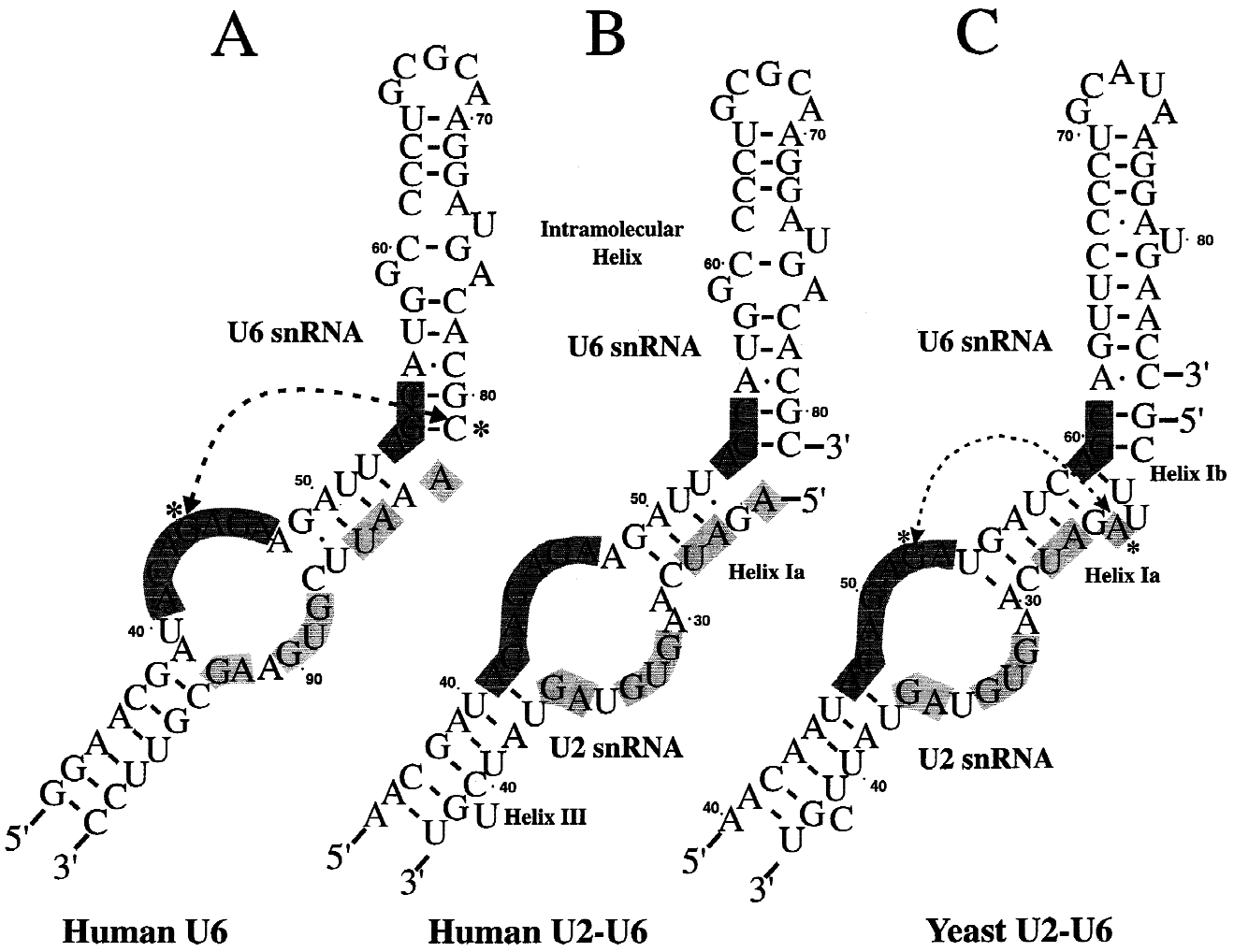

FIGURE 4. Potential U6 and U2-U6 snRNA secondary structures in human and yeast. Numbers indicate nucleotide positions from 5′ end. Dark shading highlights the invariant ACAGAGA and AGC sequences. Light shading denotes sequence identities between U6 RNA and human and yeast U2 RNA in the regions corresponding to helix Ia and the branch site recognition region (see text). **A:** U6 RNA secondary structure model, residues 33–99. Crosslinked residues are indicated by asterisks and joined by dashed arrow. **B:** Human U6-U2 structure, including helices Ia and III and the intramolecular helix. **C:** Yeast U6-U2 structure, including helices Ia and b, putative helix III, and the intramolecular helix. Residues involved in a genetically established tertiary interaction (Madhani & Guthrie 1994b) are indicated.

RNA structure. There are minor differences between the relevant regions in the proposed yeast and human secondary structures (e.g., yeast helix Ib is replaced by the extended U6 intramolecular helix in human; compare Fig. 4B and C), but these may not be functionally important, because both structures can form related secondary and, potentially, tertiary structures. However, in yeast, a U2 snRNA residue, 22C, would be predicted to play the role of U6 81C in human. Or, given the lack of importance of base identity at the crosslinkable positions, perhaps the human equivalent of the yeast interaction, which would involve U6 residues 46G and 82A, plays a key role in establishing the conformation that gives rise to the crosslink we have analyzed. In any event, it will be important in the future to determine how the tertiary interaction we have described contributes to the functions of U6 snRNA in splicing.

MATERIALS AND METHODS

RNA transcription and end-labeling

All U6 snRNA fragments were transcribed from DNA templates that were amplified by PCR on a U6-containing plasmid by a 5′-overhanging primer containing the sequence of the T7 promoter region and transcriptions were performed with bacteriophage T7 RNA polymerase. All RNAs were purified using 20% denaturing PAGE in Tris-borate-EDTA buffer, eluted by overnight diffusion, and precipitated. RNAs were 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase following dephosphorylation with calf intestinal phosphatase, or 3′ end-labeled with [5′-^{32}P]pCp and T4 RNA ligase. Heterogeneity resulting from the addition of nontemplated nucleotides by T7 RNA polymerase could be eliminated in the case of the 3′ end-labeled RNAs by long runs on 6% denaturing polyacrylamide gels (80-cm plate) at 1,600 V for 8 h.

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UV crosslinking

After end-labeling and gel purification, the appropriate U6 RNA species were resuspended in 40 mM Tris-HCl, pH 7.5, and 10 mM $MgCl_2$ unless otherwise noted. Unlabeled U6 RNAs were added to adjust the final concentration to 100 nM, unless otherwise noted. The RNAs were allowed to fold prior to UV irradiation by heating to 70 °C for 2 min and then rapidly cooling on ice for 15 min. Heating the RNA to any temperature above 60 °C and cooling either quickly or gradually gave the same extent of crosslinking (results not shown). Twenty-microliter aliquots were placed into wells of a Falcon 96-well microtiter plate resting on ice. Samples were irradiated for 10-min intervals interrupted by 10 min to prevent overheating. Irradiation was done with a hand-held 254-nm UV light (model UVG-54, UV Products Inc.; measured output, 2,200 $\mu W/cm^2$) at a distance of 1 cm. After the indicated times (0–90 min), RNAs were directly electrophoresed on 20% denaturing polyacrylamide gels. For mapping of the crosslinks, RNA products were visualized by autoradiography, excised from the gel, eluted by diffusion in 0.3 M ammonium acetate overnight, and precipitated with 2.5 volumes of ethanol. Quantitation of crosslinking was done by using a Storm PhosphorImager (Molecular Dynamics).

Mapping of crosslinked bases

To map the crosslinks, gel-purified, end-labeled crosslinked RNAs were subjected to partial alkaline hydrolysis. Samples were dissolved in 50 mM $(NH4)_3PO_4$ buffer, pH 12.0, heated to 100 °C for 4.5 or 5.5 min for hydrolysis of crosslinked RNA, and for 30 s for hydrolysis of uncrosslinked RNA. The partially hydrolyzed samples were loaded directly onto a 20% denaturing polyacrylamide gel. Noncrosslinked RNAs were partially digested with base-specific ribonucleases T1, U2, Cl3, and PhyM (U.S. Biochemical) to provide sequencing ladders to identify the positions of the crosslinked bases.

ACKNOWLEDGMENTS

We thank Yoshio Takagaki, Shou-Hua Xiao, Jin Wang, and Chi Li for their discussion and generous suggestions; Lin Ge and Xiaohong Shi for excellent technical assistance; and John Burke and Joyce Heckman for kindly providing in vitro transcription template for production of the 51-nt hairpin ribozyme, and for their experimental protocols for partial alkaline hydrolysis and partial RNase digestion. This work was supported by NIH grant GM 48259.

Received January 26, 1998; returned for revision February 16, 1998; revised manuscript received February 27, 1998

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https://doi.org/10.1017/S1355838298980141 Published online by Cambridge University Press