recent demonstration that two *rpoD* mutations (Glu^{575} to Lys and Asp^{570} to Gly) prevent activation of several *E. coli* operons by wild-type PhoB (*17*). However, those *rpoD* mutations can also affect promoter recognition directly, because they enhance recognition of the wild-type *lac* promoter in the absence of CAP-cAMP (*18*). The Glu^{575} to Lys mutant also raises the activities of several mutants in the *lac* promoter (±CAP-cAMP) and P22 *ant* promoter (which has no activator) (*8*). In contrast, the Arg^{596} to His mutant has no effect on the activities of 20 P_{lac} alleles (±CAP-cAMP) and 18 P_{ant} alleles (*8*). Thus, all known properties of σ^{70}-RH596 indicate that it does not affect promoter recognition directly but affects positive control by λ cI and AraC. It is likely that further evidence for the role of σ^{70} in positive control will be forthcoming (*19*).

REFERENCES AND NOTES

1. M. Ptashne, *A Genetic Switch: Phage λ and Higher Organisms* (Cell Press and Blackwell Scientific, Cambridge MA, 1992).
2. B. J. Meyer and M. Ptashne, *J. Mol. Biol.* **139**, 195 (1980); D. K. Hawley and W. R. McClure, *ibid.* **157**, 493 (1982); J.-J. Hwang and G. N. Gussin, *ibid.* **200**, 735 (1988); R. S.-C. Fong, S. Woody, G. N. Gussin, *ibid.*, in press.
3. L. Guarente, J. S. Nye, A. Hochschild, M. Ptashne, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2236 (1982).
4. A. Hochschild, N. Irwin, M. Ptashne, *Cell* **32**, 319 (1983).
5. D. K. Hawley and W. R. McClure, *ibid.*, p. 327.
6. F. D. Bushman, C. Shang, M. Ptashne, *ibid.* **58**, 1163 (1989).
7. S. Jordan and C. O. Pabo, *Science* **242**, 893 (1988).
8. D. A. Siegele, J. C. Hu, W. A. Walter, C. A. Gross, *J. Mol. Biol.* **206**, 591 (1989).
9. T. Gardella, H. Moyle, M. M. Susskind, *ibid.*, p. 579; A. J. Dombroski, W. A. Walter, M. T. Record Jr., D. A. Siegele, C. A. Gross, *Cell* **70**, 501 (1992); J. Keener and M. Nomura, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1751, (1993).
10. F. D. Russo and T. J. Silhavy, *J. Biol. Chem.* **267**, 14515 (1992); A. Ishihama, *J. Bacteriol.* **175**, 2483 (1993).
11. The phage P22-3957 carries P_{RM}-*kan1*, a substitution replacing DNA between a Taq I site near gene *9* and a Sau 3AI site in *sieA* with a fusion of λ P_{RM} to the *kan* gene from pUC71K. From 5′ to 3′ (right to left in Fig. 3), one strand of P_{RM}-*kan1* contains a 26-nucleotide (nt) Cla I–Eco RI segment of pBR322 (nts 23 to 4359, with the Cla I site joined to the compatible Taq I site of P22); wild-type λ P_{RM} and O_R (nts 38,150 to 38,041); and *kan* DNA from the start codon to a downstream Bam HI site, with the Bam HI site joined to the compatible Sau 3AI site of P22. P22-3957 also carries Δ*MM*, an Mlu I–Mam I deletion.
12. The substitution P_{lac}-*clA* replaces DNA between a point in *sieA* and an Eco RI site near *erf* with a fusion of the *E. coli lacZ* promoter and ribosome binding site to λ *cI*. The promoter lacks part of the CAP-cAMP binding site and part of the operator. From 5′ to 3′ (left to right in Fig. 3), one strand of P_{lac}-*clA* contains *bla* of pBR322 (nts 3146 to 2); P_{lac} (nts −59 to −7 fused to nts +15 to +36); λ *cI* (nts 37,943 to 36,967); nts 27 to 369 and 652 to 2066 of pBR322; CTAGAG; and Hind III–Eco RI *att-int* segment of λ (nts 27,480 to 31,752), with the Eco RI site in λ *exo* joined to the Eco RI site near P22 *erf*. Phages P22-3997 through −4000 carry pc^+, *pc1*, *pc2*, and *pc3* versions of λ *cI*, respectively. They also carry four *cI* mutations that do not change the amino acid sequence but create restriction sites.
13. The *rpoD* plasmid pMS1297 (Tet^R, 5.0 kb) (Fig. 2) contains CTCTAGAG; Mun I(filled in)–Stu I fragment of P22 *sieA*; CTCTAGAGAATT; P_{lac}*UV5*, −59 to +36; CCCATGGG; Hpa I–Apa LI(filled in) fragment containing wild-type *E. coli rpoD*, followed by a G residue; and nts 4359 to 1429, 2297 to 3232, and 3428 to 3540 of pBR322. The *rpoA* plasmid pMS1250 (Tet^R, 4.3 kb) is identical, except it has wild-type *E. coli rpoA* (Hpa I–Apa I, made blunt-ended by T4 DNA polymerase, followed by a C residue) instead of *rpoD*. In both plasmids, the 208-bp *sieA* segment is dispensible, contains a unique Ssp I site, and is flanked by Xba I sites. For PCR mutagenesis, we linearized plasmid DNA with Ssp I and amplified by 30 cycles of standard PCR using Taq DNA polymerase and *sieA*-derived primers (Fig. 2). Products were cleaved with Xba I, producing sticky-ended plasmid DNA, which was circularized with T4 DNA ligase, precipitated with ethanol, dissolved in water, and electroporated into *Salmonella typhimurium* JR501 [S. P. Tsai *et al.*, *J. Gen. Microbiol.* **135**, 2561 (1989)]. Cells carrying pMS1297 were always propagated at ≤30°C. Electroporated cells (~40 μl) were diluted with 0.9 ml of SOC medium (Bio-Rad), incubated for 2 hours (at which time cultures contained ≥10^4 Tet^R transformants), diluted with 5 ml of LB broth + tetracycline (50 μg/ml), and incubated overnight. Plasmid DNA was extracted and electroporated into MS4020, which is JR501 carrying P22-3957 (P_{RM}-*kan1*) (*11*) and P22-3999 (P_{lac}-*clA-pc2*) (*12*). Outgrowth was repeated as above. Overnight cultures were plated on LB broth + tetracycline (20 μg/ml) + kanamycin (6 μg/ml) plates. About 0.05% of Tet^R transformants grew on this concentration of kanamycin, but most did not have a mutant plasmid. For each of eight PCR reactions, ~500 Kan^R colonies were pooled. Plasmid DNA extracted from each pool was electroporated into MS4020, and outgrowth and plating were repeated. If mutant plasmids were present, the Kan^R frequency was now at least 100-fold that in the previous step. One plasmid from each pool was analyzed.
14. The phage P22-4229 carries P_{RM}-*lac8*, a substitution replacing DNA between a Hpa I site in gene *9* and a Sau 3AI site in *sieA* with a fusion of λ P_{RM} to *E. coli lacZ*. We derived P_{RM}-*lac8* from P_{RM}-*kan1* by replacing an Xho I–Nru I *kan* fragment with *lac* sequences. From 5′ to 3′ (right to left in Fig. 3), one strand of P_{RM}-*lac8* contains GTT of the Hpa I site joined to GACTCTAGAG; wild-type λ P_{RM} and O_R (nts 38,107 to 37,941); nts 1 to 35 of *kan* coding sequence, followed by TTAA, which creates a stop codon for *kan*; wild-type *lacZ* and *lacY* (nts 1268 to 6274 of *lac*), except the Eco RI site in *lacZ* is mutated and *lacZ* codons 7 to 9 are replaced with CCC; AATT; and Nru I–Bam HI fragment carrying the 3′ end of *kan*, with the Bam HI site joined to the compatible Sau 3AI site in *sieA*. P22-4229 also carries Δ*AB*, an Acc I–Bbs I deletion.
15. A. E. Silverstone, M. Goman, J. G. Scaife, *Mol. Gen. Genet.* **118**, 223 (1972).
16. J. C. Hu and C. A. Gross, *ibid.* **199**, 7 (1985).
17. K. Makino, M. Amemura, S.-K. Kim, A. Nakata, H. Shinagawa, *Genes Dev.* **7**, 149 (1993).
18. D. A. Siegele, J. C. Hu, C. A. Gross, *J. Mol. Biol.* **203**, 29 (1988).
19. R. Ebright, unpublished results; R. Hayward, unpublished results; A. Hochschild, unpublished results.
20. D. Graña, P. Youderian, M. M. Susskind, *Genetics* **110**, 1 (1985).
21. Cells were grown at 30°C to mid-logarithmic phase in M9CAA [H. O. Smith and M. Levine, *Proc. Natl. Acad. Sci. U.S.A.* **52**, 356 (1964)] and tetracycline (20 μg/ml) and assayed as described [J. H. Miller, *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972)].
22. C. Waldburger, T. Gardella, R. Wong, M. M. Susskind, *J. Mol. Biol.* **215**, 267 (1990).

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Catalytic Activity of an RNA Domain Derived from the U6-U4 RNA Complex

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U6 RNA contains two regions that are essential for proper splicing of nuclear precursor messenger RNA (pre-mRNA). A comparison of putative secondary structures of the U6-U4 RNA complexes from different phyla revealed a conserved domain that is similar to the catalytic hammerhead RNA motif. Although no catalytic activity was detected in the mammalian U6-U4 RNA complexes, two nucleotide changes in U6 RNA and one in U4 RNA conferred cleavage activity to the complex. Furthermore, the highly conserved domain of the wild-type complex, without the accompanying flanking regions, cleaved an RNA substrate and exhibited other characteristics of the hammerhead ribozyme. The possible involvement of this structure in pre-mRNA splicing is also discussed.

The splicing of nuclear pre-mRNA is a complex process involving both proteins and small nuclear RNAs (snRNAs) in a multimolecular structure called the spliceosome. The requirement for RNAs with highly conserved sequences, higher order structures, or both, has led to speculation that pre-mRNA splicing may have originated from, or may still rely on, catalytic properties of RNA. The lariat intermediates in pre-mRNA splicing and in the self-splicing group II introns have fostered these notions because if these intermediates denote a common evolutionary origin, then pre-mRNA splicing should be based on RNA chemistry despite the requirement for protein factors (*1*, *2*).

To clarify this possible relation, atten-

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tion has focused on the five snRNAs (U1, U2, U4, U5, and U6) required for pre-mRNA splicing. Because the size and sequence of U6 RNA (~105 nucleotides) are well conserved among organisms as diverse as yeast and humans, its putative role in catalysis has been of interest (*3*). Researchers have reported that U6 RNA of yeast is similar to the catalytic center of the hairpin ribozyme found in the negative strand of the satellite RNA of tobacco ring virus (*4*). Mutational analyses suggest that U6 snRNA is base-paired to U4, U2, or both. In the case of U2, it forms a structure that has been likened to parts of group II introns (*5*) and to the guanosine-binding site of group I introns (*6*). Because base pairing between U6 and U4 RNAs is required for assembly of the U6-U4 snRNA protein complex into the spliceosome (*7*), one model suggests that in the chronology of splicing a U6-U4 complex may be replaced by a U6-U2 complex before the chemical events of splicing occur (*5*, *8*).

From a compilation of known sequences of U6 and U4 RNAs and a putative structure of the U6-U4 complex (*9*), we identified a consensus secondary structure of the complex (Fig. 1) that is remarkably similar to the hammerhead ribozyme motif (*10*). The stem formed between nucleotides 49 through 55 of U6 and nucleotides 57 through 63 of U4 snRNA corresponds to stem II of the hammerhead. Also, the single-stranded regions ($C^{42}AGAGAA^{48}$ in U6 and $G^{64}AAA^{67}$ in U4) fit the conserved nucleotides in positions 3 through 9 and 12 through 15 of the hammerhead ribozyme (*10*, *11*). This hammerhead-like domain includes sequence CAGAG, which is essential for in vitro splicing in yeast (*3*); nucleotides A^{45} and G^{46}, which are critical in pre-mRNA splicing, correspond to nucleotides essential for activity in the hammerhead ribozyme. Thus, U6-U4 could form a hammerhead-like catalytic center if stems I and III of the hammerhead were established by base pairing between the appropriate regions of U6 and U4 with a third RNA segment. Furthermore, most small introns found in yeast U6 RNA genes are located in this region (*4*). These structural similarities raise the possibility that U6-U4 may form a catalytically active hammerhead-like motif.

To test whether the hammerhead-like motif in the U6-U4 RNA complex is capable of catalytic activity, we prepared human U6 and U4 RNAs and variants thereof by T7 RNA runoff transcription (*12*). To improve similarity to the hammerhead domain, we made a U6 RNA variant with a U (referred to here as $U^{45.1}$) inserted before position 46 and replaced A^{43} with U. In U4 RNA, A^{65} was deleted (Fig. 1). A 21-nucleotide RNA substrate, UUGGGAAAAGUC ↓ UAUCGUUCC (↓ indicates the potential cleavage site), partially complementary to U6 and U4 RNAs, was prepared by chemical synthesis (*13*).

Experiments were performed by the incubation of the radioactive 5′-labeled substrate with an excess of U6 or U4 (individually or in combination) in the presence of 20 mM magnesium chloride at neutral pH. When both snRNAs were used, we first annealed equal molar quantities by heating them to 75°C and then by slowly cooling them to room temperature before the addition of substrate. The substrate was efficiently cleaved only by the complex formed from the mutant U6 (with two mutations)and the mutant U4 (one mutation) (Fig. 2A). The radioactively labeled product, a 12-nucleotide fragment, was that expected of a hammerhead-type cleavage. Also, the 3′ fragment produced in the reaction could be 5′-labeled by [γ-^{32}P]adenosine triphosphate (ATP) with T4 polynucleotide kinase, indicating that it contains a free 5′-hydroxyl and that cleavage results in the retention of the phosphate group by the

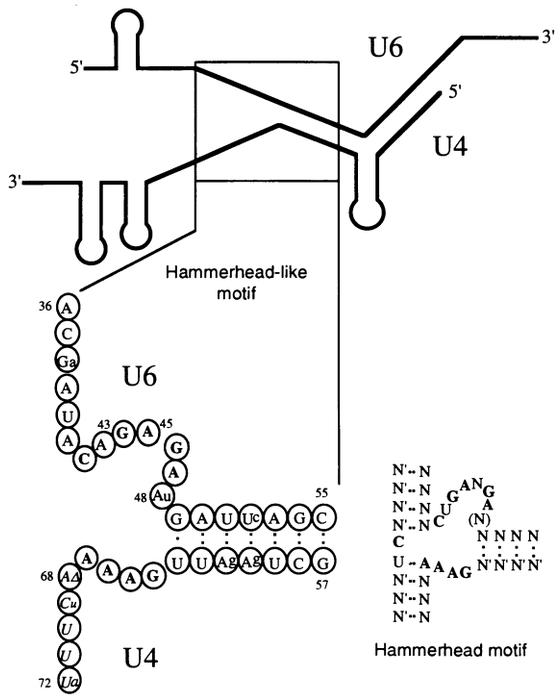

Fig. 1. Simplified secondary structure model of the human and yeast U6-U4 snRNA complex and the conserved hammerhead-like motif formed between them. The cutout shows the conserved nucleotide residues 36 through 55 of U6 and 57 through 72 of U4 represented by uppercase letters in circles. Lowercase letters show sequence variants in yeast U6-U4 RNAs. Delta (triangle) indicates a deletion of A found in yeast U4 RNA. The pattern of the hammerhead ribozyme is shown for comparison. N•N′ is the Watson-Crick base pair, and (N) is an optional nucleotide.

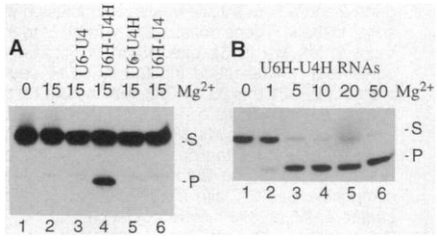

Fig. 2. Catalytic activity of U6 and U4 RNAs. We prepared wild-type genes of human U6 and U4 RNAs by polymerase chain reaction (PCR) amplification of plasmids containing genomic U6 or U4 DNA in order to remove the flanking sequences and to introduce Hind III and Eco RI restriction sites upstream and downstream of the U6 or U4 genes (*27*). We made the mutated U6 and U4 RNAs, represented as U6H and U4H, respectively, by synthesizing two overlapping oligonucleotides that were filled in with the use of *Vent* DNA polymerase (New England Biolabs) so that U6H contains an A^{43} to U mutation and a U insertion after 45; U4H has a deleted A^{68}. The PCR products, cleaved with Hind III and Eco RI, were cloned into the same sites of plasmid pGEM4 (Promega) and sequenced. U6 and U4 RNAs were produced by transcription of Eco RI–linearized plasmids with T7 RNA polymerase. Nucleotides GGAGACAAGUU and GAAUU from vector pGEM4 were added to the 5′ and 3′ terminus, respectively, to both the U6 and U4 RNA transcripts. (**A**) Reactions were run by incubation of ~1 nM of substrate UUGGGAAAAGUC ↓ UAUCGUUCC with 500 nM of U6, U4, mutant U6, or mutant U4 RNA (as indicated) for 10 hours at 37°C in 50 mM tris-HCl (pH 7.4). The millimolar concentration of magnesium chloride is indicated at the top of each lane. (**B**) The reactions were done as in (A) with varying millimolar concentrations of magnesium chloride indicated at the top of each lane. The intact substrate and the cleavage product are indicated as S and P, respectively.

upstream 5′ RNA fragment (*14*). The yield of cleavage product increased with the concentration of Mg^{2+} (Fig. 2B). The Mg^{2+} concentration that produces the half-maximum cleavage at 10 hours is approximately 5 mM, a value consistent with the K_{Mg} (dissociation constant) found for the hammerhead ribozyme (*11, 15*). The fact that a variant complex of U6-U4 with only three mutations different from the wild-type sequence has catalytic activity suggests that the wild-type complex shares at least some of the conformational properties of the hammerhead catalytic core, including stem II of the hammerhead and possibly a Mg^{2+}-binding motif (*15, 16*).

Because the long, flanking regions adjacent to the hammerhead-like motif of U6-U4 RNA could affect folding of and accessibility to the potential catalytic center, we tested a shortened version of the U6-U4 RNA complex (Fig. 3A). The core region of the synthetic hammerhead-like structure was constructed with nucleotides 36 through 53 of the wild-type U6 RNA and nucleotides 59 through 67 of the wild-type U4 RNA, nucleotides that are well conserved both in mammals and yeast. We copied the remaining nucleotides, 68 through 72, from the equivalent sequences of a family of U4 RNA pseudogenes in order to stabilize stem I (*17*). We fused the two fragments by a GUGA tetranucleotide loop (*11*) to facilitate the formation of the hammerhead structure (Fig. 3A). The substrate (UCCUG-UC ↓ UAUCGU), which can base pair to both U6 and U4 RNAs, was also made. This U6-U4 fusion product cleaves the 5′-labeled substrate in the presence of Mg^{2+} at neutral pH and 37°C (Fig. 3B, lane 1). This observation leads to the suggestion that the U6-U4 RNA complex has the capacity to form a catalytic core similar to the hammerhead although the sequence differences between this complex and the consensus hammerhead may have a negative effect on catalytic efficiency.

We made variants of the hammerhead-like domain (Fig. 3A, a through e) to determine which, if any, of the positions that differ from the hammerhead structure could restore higher activity. The replacement of A^{43} and A^{48} by U (structure b) had little effect on cleavage activity (Fig. 3B). However, insertion of U before nucleotide 46 (structure c) increased activity (Fig. 3B). Therefore, even though this position shows no nucleotide preference in the hammerhead domain (*10, 18*), the absence of this position resulted in a loss of activity. Also, the identity of the nucleotide at position 48 of U6 RNA, U in yeast but A in the human genome, did not affect cleavage significantly (Fig. 3B), as can be seen from a comparison of lanes 1 and 2 (low activity with A and U) with lanes 3 and 4 (high activity with A and U). In the case of the U4 RNA domain, the presence of an additional adenosine after A^{67}, a variant found in some human but not in yeast U4 RNA (*3, 9, 17*), affected cleavage but not in a significant way (Fig. 3B, lane 3 and 4). Therefore, sequence differences found in natural isolates of U6 or U4 RNAs affect but do not abolish the activity of the hammerhead-like catalytic domain described here.

Just as hammerhead activity is dependent on the concentration of Mg^{2+} (*10*), so are cleavages both by the hammerhead-like domain and by the mutated native U6-U4 RNAs. Both reach approximately half of their cleavage rate at a Mg^{2+} concentration between 1 and 5 mM (Figs. 2B and 4), which is approximately the optimal Mg^{2+} concentration for splicing. Because the short version of the hammerhead-like domain and the mutated U6-U4 have nucleotides identical to those found at positions 5, 8, and 9 of the hammerhead ribozyme, where 2′-hydroxyls affect the Mg^{2+}-binding constant (*15*), it is possible that this hammerhead-like structure encompasses elements of a Mg^{2+}-binding cavity.

Although we have shown that the U6-U4 complex has a low amount of catalytic activity, it is not known whether this activity relates to pre-mRNA splicing. It is unlikely that this structure is involved directly in pre-mRNA catalysis because U4 leaves the spliceosome before the first catalytic step (*8*). On the other hand, divalent metal ions are essential for RNA-based catalysis, and they are believed to be involved in the chemical step of catalysis (*19*). It is possible that the hammerhead-like domain of the U6-U4 RNA complex plays a role in pre-mRNA splicing by serving as a Mg^{2+}-binding site. Similarity of the secondary structures of the U6-U4 and U6-U2 RNA complexes (Fig. 5A) (*5*) suggests that this putative Mg^{2+}-binding motif in U6-U4 RNA could be present in the U6-U2 complex as well (*5*). A similar motif can be observed not only in the hammerhead ribozyme, U6-U4 RNA, and U6-U2 RNA, but also in the hairpin ribozyme (*20*) and in domain V and VI of group II introns (*21*) (Fig. 5A). Further-

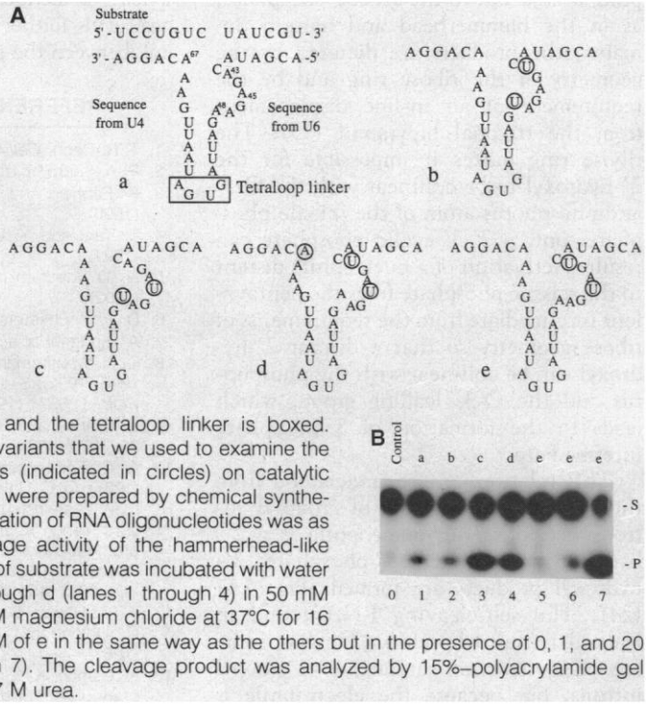

Fig. 3. Cleavage activity of the hammerhead-like domain. (**A**) Sequences and structures of the putative catalytic motifs used here. Structure a is the proposed hammerhead-like motif consisting of parts of U6 and U4 (GenBank access codes HUMU6RNA and HUMUG4PSC, respectively) fused to a tetraloop. The substrate that can base pair with this motif to form a hammerhead structure is shown above the domain and the tetraloop linker is boxed. Structures b through e are variants that we used to examine the effect of nucleotide variants (indicated in circles) on catalytic activity. All oligonucleotides were prepared by chemical synthesis. Deprotection and purification of RNA oligonucleotides was as described (*11*). (**B**) Cleavage activity of the hammerhead-like motifs. Approximately 1 nM of substrate was incubated with water (lane 0) or 500 nM of a through d (lanes 1 through 4) in 50 mM tris-HCl (pH 7.4) and 20 mM magnesium chloride at 37°C for 16 hours. We examined 500 nM of e in the same way as the others but in the presence of 0, 1, and 20 mM Mg^{2+} (lanes 5 through 7). The cleavage product was analyzed by 15%–polyacrylamide gel electrophoresis containing 7 M urea.

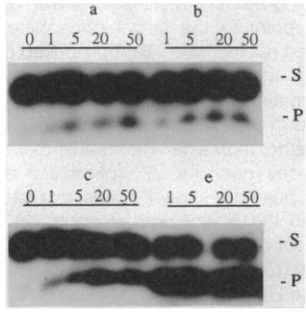

Fig. 4. Mg^{2+} dependence of cleavage. Cleavage was performed under conditions as described. Hammerhead-like domains a, b, c, and e were examined with Mg^{2+} concentrations of 0, 1, 5, 20, 50 mM as indicated at the top of each lane.

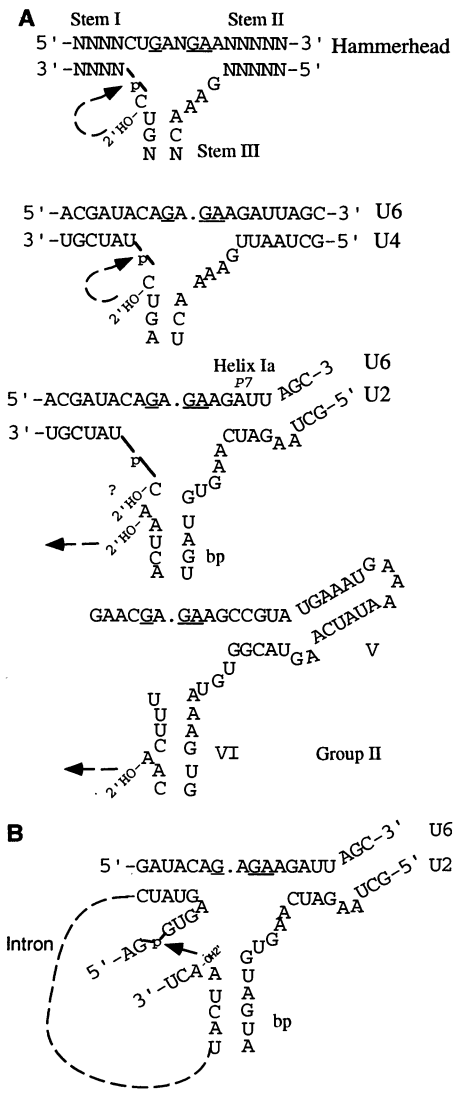

Fig. 5. (**A**) A conserved motif in the hammerhead ribozyme, the U6-U4 RNAs, the U6-U2 RNAs, and domain V-VI of the group II intron. Sequences of the U6, U4, and U2 RNAs are adapted from parts of human sequences. The base pairing model between human U6 and U2 is adapted from the model of yeast (*5*). The domain V-VI model is adapted from the chloroplast *rpS12* gene (*21*). The hairpin ribozyme containing a similar motif is not shown (*20*). The arrows point to the target phosphate of the activated nucleophile. (**B**) A proposed catalytic motif for the first step of pre-mRNA splicing. The domain of U6-U2 RNAs is essentially the same as in (A) except that a pre-mRNA intron is given rather than a hammerhead substrate. The sequences near the 5′ splice site and the branch point are shown. A possible interaction between the 5′ splicing site and U6 RNA is adapted from pre-mRNA splicing in yeast and humans (*8, 22*). A base pairing interaction between U2 and the branch point is represented by bp; the dashed line indicates the intron sequence. The conserved nucleotides in all structures are underlined. The arrow points to the phosphate at the 5′ splice site in the structures of U6-U2 and group II intron. The question mark indicates a possible adjacent nucleophile.

more, after the loss of U4 RNA from the U6-U4 complex (*8*), the hammerhead-like catalytic core could be restored by the addition of U2 RNA. Stem II in the U6-U4 hammerhead-like motif is virtually identical to that established by the proposed base pairing interaction between U6-U2 RNAs (helix Ia; *5*). Also, the important nucleotides of the U6 moiety of hammerhead-like motif CAGAGA remain in the U6-U2 complex. Our model would necessarily allow for some modification of the Mg^{2+}-binding motif in the U6-U2 complex such that the 2′-hydroxyl of the branch point nucleotide could approach this catalytic core. It remains unclear how U6 RNA and the branch nucleotide would interact with the 5′ splice site in this catalytic core, although cross-linking data suggest that this interaction does occur (*8, 22*).

For a more thorough examination of the catalytic role, the question of phosphate substitution in the products and intermediates must be considered. It has been difficult to correlate the activity of the hammerhead or the hairpin ribozymes with splicing (*4*) because the former produces 2′,3′-cyclic phosphate, and the latter produces 5′-phosphates. However, all RNA catalytic processes, with the exception of ribonuclease P hydrolysis, are based on a common phosphoryl transesterification mechanism. The link between the two types of mechanisms can be made if the domain shown here is considered to be simply a Mg^{2+}-binding motif that acts to increase the nucleophilicity of a hydroxyl group that then attacks an appropriately placed electrophile. When the nucleophile is adjacent to the scissile phosphate, as in the hammerhead and hairpin domains, the products are dictated by the geometry of the ribose ring and by the requirement of an in-line displacement from the trigonal bipyramid (*23*). The ribose ring makes it impossible for the 2′-hydroxyl to be collinear with the O-3′ and phosphorus atom of the scissile phosphate; only a 2′,3′-cyclic phosphate can result. Activation of a nucleophile distant to the scissile phosphate frees the pentavalent intermediate from the requirements of ribose geometry so that a distant 2′-hydroxyl can be collinear with the phosphorus and the O-3′ leading group, which leads to the formation of 5′-phosphate intermediates.

This relation can be understood from the splicing intermediate of group II introns in which the nucleophile is a 2′-hydroxyl even though 5′-phosphate, 3′-hydroxyl products are formed (Fig. 5A) (*24*). The self-cleaving RNA found in Varkud-1c mitochondria (*25*) has some secondary structure similarities to group I introns, but because the electrophile is adjacent, cleavage products containing a 2′,3′-phosphate and a 5′-hydroxyl are produced. Moreover, by introduction of a nonbridge sulfur to yield Rp or Sp phosphorothioate diastereomer, the stereochemistry of the transesterification reactions at both the 5′ and 3′ splice sites in pre-mRNA splicing has been found to be identical to that in the hammerhead ribozyme (*19, 26*), which may reflect an essential interaction between Mg^{2+} and the nonbridging oxygens in the scissile phosphate. Therefore, we hypothesize that the conserved motif in U6-U2 RNAs could activate a distant nucleophile, the 2′-hydroxyl at the branch point of pre-mRNAs, as illustrated (Fig. 5B).

The relation of the catalytic activity of the hammerhead-like domain to splicing is speculative because of the large number of proteins in the spliceosome complex. Because of the potential effect of these proteins on the conformation of the RNAs, one would not expect catalytic RNA motifs found in ribonucleoprotein complexes to be exactly the same as those found in cases where proteins play little or no role in catalysis. In fact, proteins could stabilize unusual base pairs or even nonpairs in such a way that it would be impossible to infer an RNA catalytic domain based solely on the sequence. The finding of a hammerhead-like domain with catalytic activity in the U6-U4 RNA and the fact that this functional structure has been evolutionarily conserved suggest that it is relevant to pre-mRNA splicing although U6-U4 itself is unlikely to be directly involved in splicing. The finding that the stereochemistry of the pathway of pre-mRNA splicing is identical to that of the hammerhead adds further evidence to our proposed link between the two processes (*26*).

REFERENCES AND NOTES

1. T. R. Cech, *Cell* **44**, 207 (1986).
2. P. A. Sharp, *ibid.* **42**, 397 (1985).
3. P. Fabrizio and J. Abelson, *Science* **250**, 404 (1990).
4. T. Tani and Y. Ohshima, *Genes Dev.* **5**, 1022 (1991).
5. H. D. Madhani and C. Guthrie, *Cell* **71**, 803 (1992).
6. D. S. McPheeters and J. Abelson, *ibid.*, p. 819.
7. A. Bindereif *et al.*, *EMBO J.* **9**, 251 (1990).
8. D. A. Wassarman and J. A. Steitz, *Science* **257**, 1918 (1992); S.-L. Yean and R.-L. Lin, *Mol. Cell. Biol.* **11**, 5571 (1991).
9. C. Guthrie and B. Patterson, *Annu. Rev. Genet.* **22**, 397 (1988).
10. A. C. Forster and R. H. Symons, *Cell* **49**, 211 (1987); O. C. Uhlenbeck, *Nature* **328**, 596 (1987); L. M. Epstein and J. G. Gall, *Cell* **48**, 535 (1987).
11. J.-H. Yang, N. Usman, P. Chartrand, R. Cedergren, *Biochemistry* **31**, 5005 (1992); J.-H. Yang, P. Perreault, N. Usman, D. Labuda, R. Cedergren, *ibid.* **29**, 11156 (1990).
12. J. F. Milligan, D. R. Groebe, G. W. Witherell, O. C. Uhlenback, *Nucleic Acids Res.* **15**, 8783 (1987).
13. N. Usman, K. K. Ogilvie, M.-Y. Jiang, R. Cedergren, *J. Am. Chem. Soc.* **109**, 7845 (1987).

14. The cleavage of the 21-nucleotide oligomer by the hammerhead ribozyme produces a 12-nucleotide fragment with a 2′,3′–cyclic phosphate and a 9-nucleotide fragment with a free 5′-hydroxyl that can be labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. The experiment was done as follows: After the 5′-labeled substrate was cleaved by the U6-U4 RNA complex as described, it was desalted, lyophilized, and relabeled with T4 kinase (*11*). The cleavage products were analyzed on a 20%-polyacrylamide denatured gel.
15. J.-P. Perreault, D. Labuda, N. Usman, J.-H. Yang, R. Cedergren, *Biochemistry* **30**, 4020 (1991).
16. S. S. Reid and J. A. Cowan, *ibid.* **29**, 6025 (1990); T. Marciniec *et al.*, *Acta. Biochim. Pol.* **39**, 183 (1989).
17. C. Bark, K. Hammartrom, G. Westin, U. Pettersson, *Mol. Cell. Biol.* **5**, 943 (1985). Also, with regard to the U4 RNA domain, there are three and four A's after position 64 for the best known yeast and human sequences (*3, 9*). Mutation analyses show that insertion of two A's in this region of the hammerhead ribozyme has little effect on the cleavage activity [C. C. Sheldon and R. H. Symons, *Nucleic Acids Res.* **17**, 5679 (1989)].
18. D. E. Ruffner, G. D. Stormo, O. C. Uhlenbeck, *Biochemistry* **29**, 10695 (1990).
19. D. E. Ruffner and O. C. Uhlenbeck, *Nucleic Acids Res.* **18**, 6025 (1990); D. W. Celander and T. C. Cech, *Science* **251**, 401 (1991); D. Smith *et al.*, *J. Biol. Chem.* **267**, 2429 (1992); T. A. Steitz and J. A. Steitz, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6498 (1993); T. Uchimaru *et al.*, *FASEB J.* **7**, 137 (1993).
20. A. Hampel, R. Tritz, M. Hicks, P. Cruz, *Nucleic Acids Res.* **18**, 299 (1990).
21. F. Michel *et al.*, *Gene* **82**, 5 (1989).
22. H. Sawa and Y. Shirmura, *Genes Dev.* **6**, 244 (1992); H. Sawa and J. Abelson, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11269 (1992).
23. R. Cedergren, F. Lang, D. Gravel, *FEBS Lett.* **226**, 63 (1987).
24. K. A. Jarrell, R. C. Dietrich, P. S. Perlman, *Mol. Cell. Biol.* **8**, 2361 (1988).
25. B. J. Wassarman and R. C. Collins, *Cell* **61**, 685 (1990).
26. M. J. Moore and P. A. Sharp, *Nature* **365**, 364 (1993).
27. M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds., *PCR Protocols: A Guide to Methods and Applications* (Academic Press, San Diego, CA, 1990), pp. 3–12.
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Mitotic Repression of RNA Polymerase III Transcription in Vitro Mediated by Phosphorylation of a TFIIIB Component

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Interphase cytosol extracts prepared from *Xenopus laevis* eggs are active in RNA polymerase III (Pol III) transcription. Addition of recombinant B1 cyclin to these extracts activates mitotic protein kinases that repress transcription. Affinity-purified p34^{cdc2}–cyclin B kinase (mitosis-promoting factor) is sufficient to effect this repression in a simplified Pol III transcription system. This mitotic repression involves the direct phosphorylation of a component of the Pol III transcription initiation factor TFIIIB, which consists of the TATA box–binding protein (TBP) and associated Pol III–specific factors. The transcriptional activity of the TFIIIB-TBP fraction can be modulated in vitro by phosphorylation with mitotic kinases and by dephosphorylation with immobilized alkaline phosphatase.

Nuclear RNA transcription becomes repressed when eukaryotic cells enter mitosis (*1*). Studies have observed both general mitotic repression of transcription and mitotic repression of specific genes transcribed by RNA polymerase II or III (*2, 3*). Mitotic repression of Pol III transcription can be reproduced in vitro (*4*) with the use of *Xenopus* egg extracts that can easily be shifted from interphase to mitosis (*5–7*). Interphase egg extracts are active in the transcription of Pol III–transcribed genes (*4, 8*), including genes encoding 5S RNA and tRNAs. In contrast, mitotic extracts, generated by the conversion of interphase cytosol to mitosis by means of purified recombinant B1 cyclin (*4, 6, 7, 9, 10*), are repressed in transcription (*4*). Mitotic repression in vitro does not require mitotic chromosome condensation, nucleosome assembly, or the binding of a general repressor protein (*4*). Instead, mitotic repression occurs even in a simplified Pl III transcription system when a mitotic kinase fraction of an egg extract is added (*4*). Action of one or more mitotic kinases is essential for inhibition because the kinase inhibitor 6-dimethylaminopurine (DMAP) blocks inhibition (*4*). Thus, mitotic repression of transcription in vitro involves the direct phosphorylation of the transcriptional machinery.

To elucidate the kinase or kinases that mediate mitotic repression in the *Xenopus* Pol III system, we used partially purified Pol III transcription factors, Pol III (*11*), and *Xenopus* mitotic kinases isolated by p13-agarose affinity chromatography (*4, 12*). The yeast p13*suc1* gene product binds the mitotic *cdc2*–cyclin B kinase (mitosis-promoting factor, MPF) and related kinases (*13*). When a mixture of transcription factors (TFIIIA, TFIIIB, TFIIIC, and Pol III) was incubated with mitotic kinases bound to p13-agarose beads and the beads were subsequently removed by centrifugation, the transcription of 5S DNA was repressed (Fig. 1A, lane 1). Similar repression of *Xenopus* tRNA^{Met1} and tRNA^{Tyr} transcription was observed (*14*). This inhibition could be prevented by including the kinase inhibitor DMAP in the reaction (lane 4) or by substituting adenosine triphosphate (ATP) with the nonhydrolyzable analog adenylyl-imidodiphosphate (AMP-PNP) (lane 3). In contrast, p13-agarose bound with interphase egg extract proteins had no effect on transcription (lane 2). Thus, immobilized *cdc2*-cyclin kinase and related mitotic kinases directly repress transcription.

Purified *cdc2*–cyclin B kinase alone caused mitotic repression in the reconstituted transcription system. The *cdc2* kinase was purified from a cyclin-activated mitotic extract by glutathione-Sepharose chromatography with the glutathione-S-transferase tag present on the recombinant cyclin B1 protein (*4, 6, 9, 10, 12*); the immobilized *cdc2*–cyclin B kinase inhibited 5S gene transcription (Fig. 2B, lane M).

To identify the target of the mitotic kinase, we performed a rescue experiment. A mixture of the transcription factors was treated with either interphase or mitotic p13-agarose in the presence of ATP, and the beads were removed after incubation. Factors treated with mitotic beads did not support transcription of the 5S RNA gene (Fig. 1B, lane 5). Each of the chromatographic fractions needed for Pol III transcription (and not exposed to the p13-bound kinase) was added back to separate reactions. DMAP was included to ensure that any secondary kinase activity present in the original factor mixture would not phosphorylate the added untreated factor or factors. Addition of the phosphocellulose fraction PC-B, which contains TFIIIB and Pol III, fully restored transcription of the 5S RNA gene (Fig. 1B, lane 7). Neither TFIIIA (lane 6) nor TFIIIC (lane 8) gave significant rescue of transcription (*15*). The same effect was observed with the tRNA^{TyrD} gene template (*14*). These results suggest that the target of the mitotic kinase is a component of the PC-B fraction and that the relevant factor or factors support transcription in the nonphosphorylated forms and are inactive in the phosphorylated forms.

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