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Specificity of Prp24 binding to RNA: A role for Prp24 in the dynamic interaction of U4 and U6 snRNAs

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ABSTRACT

Prp24 was previously isolated as a suppressor of a cold-sensitive U4 mutation and is required for at least the first step of splicing in vitro. Our investigation of the in vitro RNA binding properties of the purified Prp24 protein shows that it binds preferentially to the U4/U6 hybrid snRNAs compared to other snRNAs. The interaction between Prp24 and the U4/U6 hybrid appears to involve two regions in the RNA: the 39–57 region of U6 and stem II of the U4/U6 hybrid. Interestingly, some U4 mutations, which destabilize stem II, increase the affinity of Prp24 for the U4/U6 RNAs compared to the wild type. This suggests that the binding of Prp24 to the U4/U6 RNAs may involve some destabilization of the RNA duplex. We also found that Prp24 can stimulate the annealing of U4 and U6, suggesting that Prp24 participates in both the formation and disassembly of the U4/U6 hybrid during splicing.

Keywords: hydroxyl-radical RNA footprinting; protein–RNA interaction; Prp24; SELEX; U4 snRNA; U6 snRNA

INTRODUCTION

The excision of intervening sequences from pre-mRNA and the ligation of exons occurs in a ribonucleoprotein complex called the spliceosome. Spliceosome assembly on the pre-mRNA substrate is a highly ordered process involving a hierarchy of interactions among five ribonucleoparticles (snRNP) U1, U2, U4, U5, and U6, as well as many non-snRNP proteins (Green, 1991; Guthrie, 1991; Ruby & Abelson, 1991; Rymond & Rosbash, 1992; Moore et al., 1993). Each snRNP is composed of a specific small nuclear RNA (snRNA U1, U2, U4, U5, or U6) and a set of proteins, some of which are common to all the snRNPs, whereas others are specific for the different RNPs.

Following the association between the 5′ splice site and the branch sequences with the U1 and U2 snRNPs, respectively, the U5 and U4/U6 snRNPs join the complex as a tri-snRNP particle. The details of the U5 snRNP interaction with the tri-snRNP particle are unknown, but genetic and biochemical evidence indicates that U4 and U6 snRNAs can base pair extensively to form two intermolecular helices termed stem I and II (Bringmann et al., 1984; Hashimoto & Steitz, 1984; Brow & Guthrie, 1988; Vankan et al., 1990; Shannon & Guthrie, 1991). The U6 snRNA also interacts with the U2 snRNA to form two helices. Helix II involves the 3′ end of U6 (Hausner et al., 1990; Datta & Weiner, 1991; Wu & Manley, 1991) and is compatible with the U4/U6 base pairing; on the other hand, formation of helix I, which involves the central region of U6 (Madhani & Guthrie, 1992), requires the dissociation of the U4/U6 snRNA hybrid. The dissociation of U4 from U6 is necessary also to form an intramolecular stem/loop in the U6 snRNA that is essential for splicing (Wolff & Bindereif, 1991; Fortner et al., 1994). Although required for spliceosome assembly, the U4 snRNA does not appear to be involved in catalytic steps and its interaction with the U6 snRNA is destabilized prior to the first step (Yean & Lin, 1991). It has recently been proposed that a possible role for the U4 snRNA may be to act as an RNA chaperone to stabilize the proper U6 snRNA conformation required for spliceosome assembly (Nilsen, 1994). In addition to the destabilization of the U4/U6 hybrid several other dynamic snRNA/snRNA and snRNA/pre-mRNA interactions occur during spliceosome assembly and catalysis (for a recent discussion see Newman, 1994; Nilsen, 1994). Prior to the first step, the association of the U1 snRNP with the spliceosome is weakened and the U1/pre-mRNA helix may be displaced to form pre-mRNA/U6 and/or pre-mRNA/U5 helices (Sawa & Abelson, 1992; Sawa & Shimura, 1992; Wassarman & Steitz, 1992; Kandels-Lewis & Seraphin,

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1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993).

The dynamic nature of the spliceosome suggests that a possible role for some of the protein factors involved in the splicing reaction is to increase the efficiency and fidelity of the RNA rearrangements. Among the first candidates for this function is a class of splicing factors sharing sequence similarity to ATP-dependent RNA helicases. RNA helicases, whose prototype is the translation initiation factor eIF-4A (Nielsen et al., 1985), can disrupt RNA duplexes in reactions dependent on ATP hydrolysis (for review see Schmid & Linder, 1992). Although unwinding activity has not been reported for any of the putative helicases involved in splicing, all of those studied show RNA-dependent ATPase activity in vitro (Schwer & Guthrie, 1991; Kim et al., 1992; O'Day & Abelson, manuscript in prep.) and are believed to catalyze conformational changes in the RNA in vivo. The genes coding for these splicing factors (*PRP2, PRP5, PRP16, PRP22, PRP28*) were isolated in *Saccharomyces cerevisiae* by screening libraries of conditional lethal mutants for cells specifically impaired in RNA processing (Vijayraghavan et al., 1989). During these screenings however many *PRP* genes were also identified that coded for proteins not homologous to helicases. Some of these factors contain putative RNA binding motifs; Prp6, Prp9, and Prp11 have zinc finger-like motifs (Chang et al., 1988; Legrain & Choulika, 1990), whereas Prp24 contains three domains similar to the consensus sequence RNA binding domain (RBD) (Shannon & Guthrie, 1991). An increasing number of RNA binding proteins capable of destabilizing and/or facilitating the formation of RNA duplexes have been described in recent years (Kumar & Wilson, 1990; Krainer et al., 1990; Pontius & Berg, 1990; Ghisolfi et al., 1992; Munroe & Dong, 1992; Lee et al., 1993; Oberosler et al., 1993; Tsuchihashi et al., 1993; Portman & Dreyfuss, 1994). Proteins with similar activity are also likely to participate in conformational rearrangements of the spliceosomal RNAs.

The PRP24 gene was also identified in a suppressor screening of the cold-sensitive U4 snRNA mutation G14C (Shannon & Guthrie, 1991). Mutations in the third RNA binding domain of Prp24 can suppress the cold-sensitive phenotype of the mutation G14C in U4 snRNA. Shannon and Guthrie (1991) showed that the U4 mutation G14C had a dramatic effect on the stability of the U4/U6 hybrid, lowering the T_m about 15 °C. Interestingly, anti-Prp24 antibodies immunoprecipitate only the U6 snRNA in wild-type extracts but immunoprecipitate both U4 and U6 in mutant extracts containing the U4-G14C mutation. These data led Shannon and Guthrie (1991) to propose that Prp24 may be stably associated with U6 snRNA and can form a transient complex with U4/U6, possibly concomitant with the annealing reaction between the two snRNAs. The short-lived complex between Prp24 and U4/U6 would become hyperstabilized in extracts containing U4 snRNA with the G14C mutation.

In the present work, we have studied the interaction of Prp24 with the U6 and U4 snRNAs in an attempt to directly test the binding specificity of Prp24 and to study its possible role in the dynamic association of the U4 and U6 snRNAs. We have found that, in vitro, the U4/U6 hybrid is the highest affinity ligand for Prp24 and that the U4/U6–Prp24 interaction depends on specific protein–RNA contacts. Our results are consistent with a model in which binding of Prp24 to the U4 and U6 snRNAs influences the association and the dissociation cycle of these two snRNAs.

RESULTS

In vitro affinity of Prp24 for the spliceosomal snRNAs

The presence of RNA binding domains in the sequence of Prp24, together with the observation that anti-Prp24 antibodies co-immunoprecipitate the U6 snRNA and that mutant Prp24 proteins can suppress a U4 mutation, suggest that Prp24 is indeed an RNA binding protein associated with spliceosomal snRNAs. We have investigated the affinity of Prp24 for the different snRNAs in vitro. The prp24 gene was overexpressed in *Escherichia coli* cells and the protein purified as described in the Materials and methods. The purified protein can complement a heat-inactivated *prp24* splicing extract (data not shown). The affinity of Prp24 for various RNAs was studied by both filter binding and gel shift techniques. The results from filter binding experiments are shown in Figure 1 and comparable results

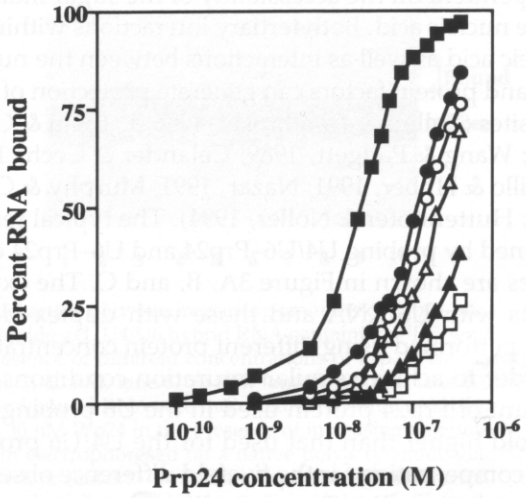


FIGURE 1. Prp24 affinity for the different snRNAS. Affinity of Prp24 for the snRNAs as determined by filter binding assay: ■, U4/U6 hybrid snRNAs; ●, U6 snRNA; ○, U1 snRNA; △, U2 snRNA; ▲, U4 snRNA; □, U5 snRNA.

were obtained by the gel shift assay (data not shown). These data show an apparently higher affinity for the U4/U6 hybrid compared to the other snRNAs tested. The affinity for the individual U6 and U4 snRNAs is 5- and 10-fold lower, respectively, compared to the affinity for the U4/U6 hybrid, suggesting that the determinants of U4/U6 higher affinity are not only in the sequence of the two RNAs but may also depend on some particular feature of the structure formed in the U4/U6 hybrid.

The specificity of the interaction of Prp24 with the U4/U6 duplex was examined in a competition experiment in which the U4/U6–Prp24 complex was allowed to form in the presence of increasing concentrations of different snRNA competitors. Figure 2A shows the result of a gel shift assay in which increasing amounts of Prp24 have been allowed to bind U4/U6 followed by native gel electrophoresis. The conditions of lane 5 have been used for competition experiments with cold U1, U2, U4, U5, U6, and U4/U6 snRNAs (Fig. 2B,C, D,E,F,G). The only efficient competitor is the U4/U6 hybrid, although the U6 snRNA in high excess also competes. We have not investigated the origin of the multiple retardation bands evident in our gel shift assays. This effect is unlikely to be due to RNA–RNA interactions because different RNA substrates can generate a similar pattern. A possible explanation may be the formation of Prp24 multimers at higher protein concentration.

Hydroxyl radical footprinting of the U4/U6–Prp24 and U6–Prp24 complexes

In a first attempt to identify the Prp24 binding site on the U4/U6 and U6 snRNAs, we used hydroxyl radical footprinting (Tullius & Dombroski, 1986; Dixon et al., 1992). The pattern of reactivity to the hydroxyl radical is dependent on the accessibility of the sugar moieties of the nucleic acid. Both tertiary interactions within the nucleic acid as well as interactions between the nucleic acid and protein factors can generate protection of specific sites (Tullius & Dombroski, 1986; Latham & Cech, 1989; Wang & Padgett, 1989; Celander & Cech, 1991; Darsillo & Huber, 1991; Nazar, 1991; Murphy & Cech, 1993; Huttenhofer & Noller, 1994). The typical results obtained by probing U4/U6–Prp24 and U6–Prp24 complexes are shown in Figure 3A, B, and C. The experiments with U6 RNA and those with duplex U4/U6 were performed using different protein concentrations in order to achieve similar saturation conditions (the amount of Prp24 protein used in the U6 probing was fivefold higher than that used for the U4/U6 probing thus compensating for the fivefold difference observed in the apparent K_d). The main difference between the protection seen with U6–Prp24 and U4/U6–Prp24 complexes is in the U6 region from base 64 to 76 (Fig. 3A, B,C). Although this region reproducibly shows moderate protection in the U4/U6–Prp24 complex, no protection is observed in the Prp24/U6 complex. The portions of the U4 and U6 sequence base paired in stem II are both protected in the U4/U6–Prp24 complex. Moreover, both the U4/U6–Prp24 and U6–Prp24 complexes show protection in the 39–56 region; however, the intensity and the pattern of protections are different. The results of these protection experiment are summarized in Figure 4.

The appearance of multiple retardation bands in the gel shift assays raises the possibility that with increasing the protein concentration, Prp24 forms multimers. The binding specificity of the multimers could be different from that of the monomers and thus a mixture of different complexes may be present in our probing experiments: for example, the protection in the 39–56 region of U6 could be due to Prp24 monomers, whereas protection in the 67–76 region could originate from RNA molecules interacting with dimers. Although we cannot rigorously rule out this possibility, we think it is unlikely because mutations in different regions of U6, which decrease Prp24 binding, do not specifically affect any individual complex band in the gel shift assay but affect all the complex bands to a similar extent.

The Prp24-dependent protection of the U4/U6 RNAs observed in the experiments shown in Figure 3 could be due to either direct contact of Prp24 with the RNA or to changes in RNA tertiary structure induced by binding of Prp24. In order to distinguish between these two possibilities, two approaches were taken. In the first, high-affinity RNA ligands for Prp24 were isolated by selective enrichment (SELEX) (Tuerk & Gold, 1990), and in the second, the affinity of Prp24 for various mutant U4 and U6 RNAs was measured.

In vitro selection of high-affinity RNA ligands for Prp24

A library of RNAs containing a sequence with 50 randomized positions flanked by defined primer binding sequences (pool 0) was generated as described in the Materials and methods. Following incubation of this random library with Prp24, protein–RNA complexes were isolated by filter binding. The RNAs retained on the filter were eluted, reverse transcribed, and amplified by PCR using a sense primer containing the sequence of the T7 RNA polymerase promoter. Following 12 cycles of selection and amplification the RNA population (pool 12) showed a K_d similar to that of the U4/U6 hybrid (Fig. 5A), and an additional cycle did not result in increased affinity for Prp24 (data not shown).

The RNA from the pool 12 was reverse transcribed, PCR amplified, and cloned. The sequences of 40 independent clones are shown in Figure 5B. The only conserved motif present in all of the clones is 5′-CCCU-3′. The binding constants for several RNAs transcribed from the individual clones were measured by filter

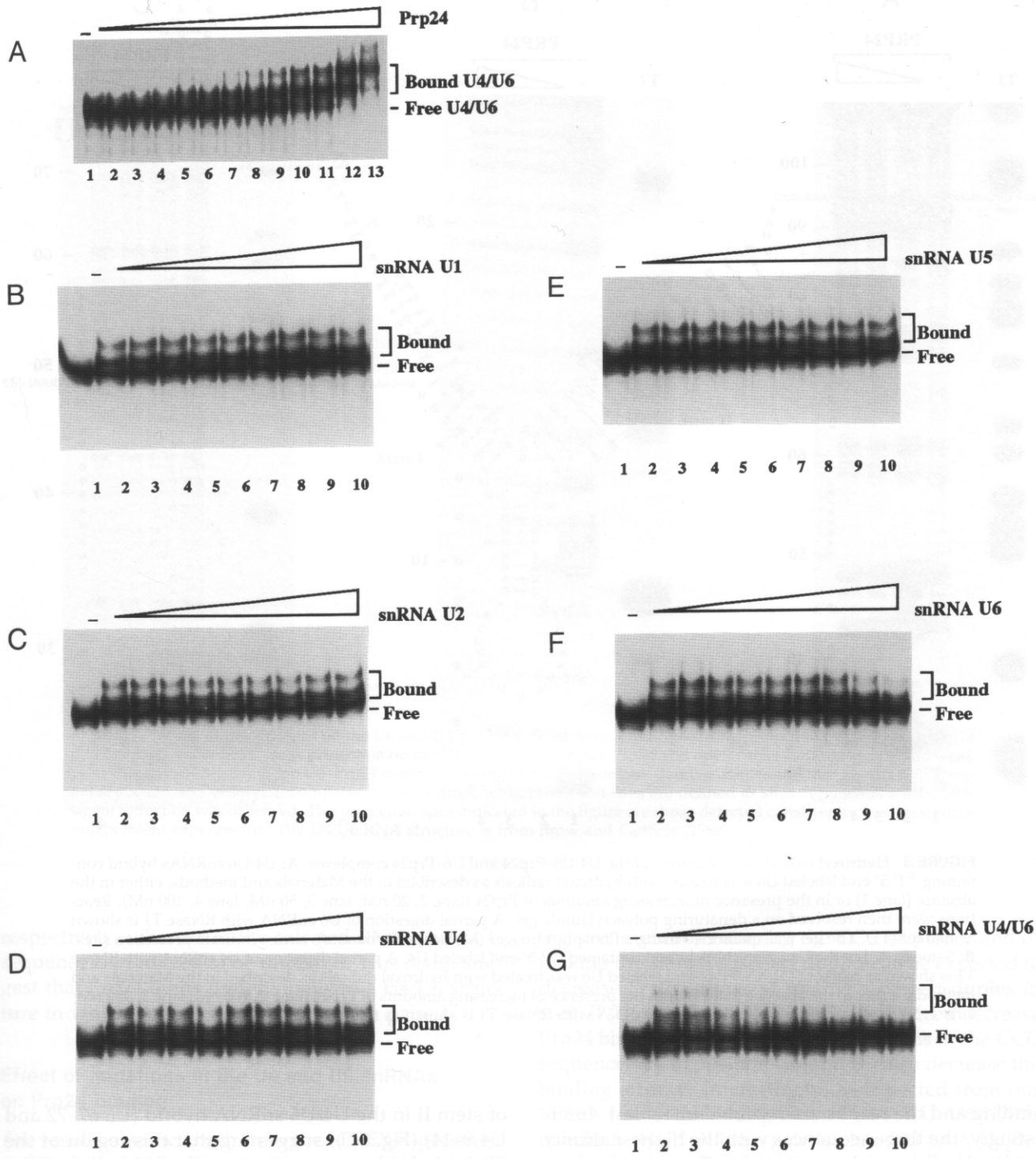


FIGURE 2. Competition among the snRNAs for Prp24 binding. **A:** Interaction between increasing amounts of Prp24 protein and the U4/U6 RNA duplex was visualized on a native polyacrylamide gel. U4/U6 hybrid RNA containing ^{32}P-5′-end labeled U6 was incubated either in the absence (lane 1), or in the presence of different concentrations of Prp24 (lane 2, 1 nM; lane 3, 2 nM; lane 4, 10 nM; lane 5, 20 nM; lane 6, 30 nM; lane 7, 40 nM; lane 8, 50 nM; lane 9, 100 nM; lane 10, 150 nM; lane 11, 200 nM; lane 12, 500 nM; lane 13, 1,000 nM). Free and bound RNAs are indicated. **B:** 35 fmol of U4/U6 snRNA hybrid containing ^{32}P-5′-end labeled U6 were incubated with 30 nM Prp24 in the presence of increasing amounts of unlabeled U1 snRNA hybrid as a competitor. Reactions were then electrophoresed on a native polyacrylamide gel. Lane 1, control, neither Prp24 or cold competitor added. All other reactions (lanes 2–10) contain Prp24 20 nM and the following amounts of U1 RNA as cold competitor: lane 2, no competitor; lane 3, 15 fmol; lane 4, 35 fmol; lane 5, 70 fmol; lane 6, 200 fmol; lane 7, 500 fmol; lane 8, 1 pmol; lane 9, 2 pmol; lane 10, 4 pmol. **C:** Same as B, but unlabeled U2 snRNA was used as competitor. **D:** Same as B, but unlabeled U4 snRNA was used as competitor. **E:** Same as B, but unlabeled U5 snRNA was used as competitor. **F:** Same as B, but unlabeled U6 snRNA was used as competitor. **G:** Same as B, but unlabeled U4/U6 snRNA was used as competitor.

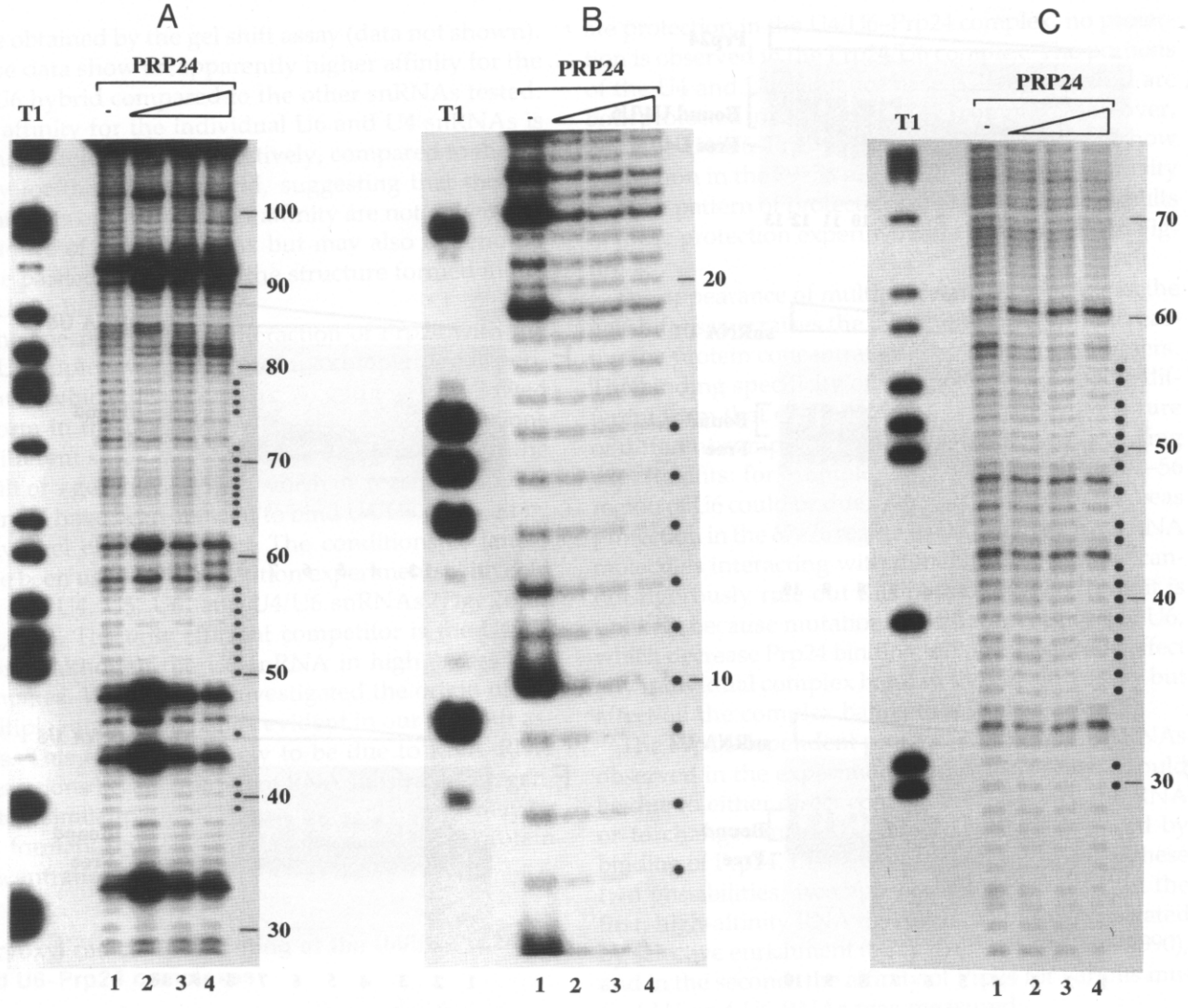


FIGURE 3. Hydroxyl radical "footprinting" of the U4/U6–Prp24 and U6–Prp24 complexes. **A:** U4/U6 snRNAs hybrid containing ^{32}P-5′-end labeled U6 was treated with hydroxyl radicals as described in the Materials and methods, either in the absence (lane 1) or in the presence of increasing amounts of Prp24 (lane 2, 20 nM; lane 3, 50 nM; lane 4, 100 nM). Reactions were then resolved on a denaturing polyacrylamide gel. A partial digestion of U6 snRNA with RNase T1 is shown as marker (T1). The gel was quantified using a Phosphor Imager (Molecular Dynamics); circles indicate protection sites. **B:** Same as A, but the U4/U6 snRNAs hybrid contained ^{32}P-5′-end labeled U4. A partial digestion of U4 snRNA with RNase T1 is shown as marker (T1). **C:** ^{32}P-5′-end labeled U6 was treated with hydroxyl radicals as described in the Materials and methods, either in the absence (lane 1) or in the presence of increasing amounts of Prp24 (lane 2, 100 nM; lane 3, 250 nM; lane 4, 500 nM). A partial digestion of U6 snRNA with RNase T1 is shown as marker (T1).

binding and the results are reported in Table 1. Interestingly, the three sequences with the highest affinity for Prp24 all have the potential to form a stem loop structure in which the CCC sequence is in the 3′ side of the loop and the U is base paired at the top of the stem (see Fig. 5B,C). These observations are consistent with the consensus binding motif shown in Figure 5C. This result suggests that binding of Prp24 may require a stem-loop structure having a CCC sequence on the 3′ side of the loop and an A-U base pair at the top of the stem. There is also a preference for G-C or C-G base pair in the second base pair from the top. Interestingly, a similar motif is present in the central region of stem II in the U4/U6 snRNA hybrid (U6 67–72 and U4 9–14) (Fig. 5C), suggesting that this region of the U4/U6 hybrid may contribute to Prp24 binding. If the selected RNAs actually correspond to a Prp24 binding site in the U4/U6 hybrid they can be expected to compete with U4/U6 for binding to the Prp24 protein. In fact, the four selected RNAs we tested can outcompete the U4/U6 hybrid for binding to Prp24 (Fig. 6A). With slightly lower efficiency the same sequences also can compete with U6 binding to Prp24 (Fig. 6B). The main difference between the structure suggested by the SELEX experiment and the U4/U6 structure is that C67 and C68 in U6 are base paired to U4 bases 14 and 13,

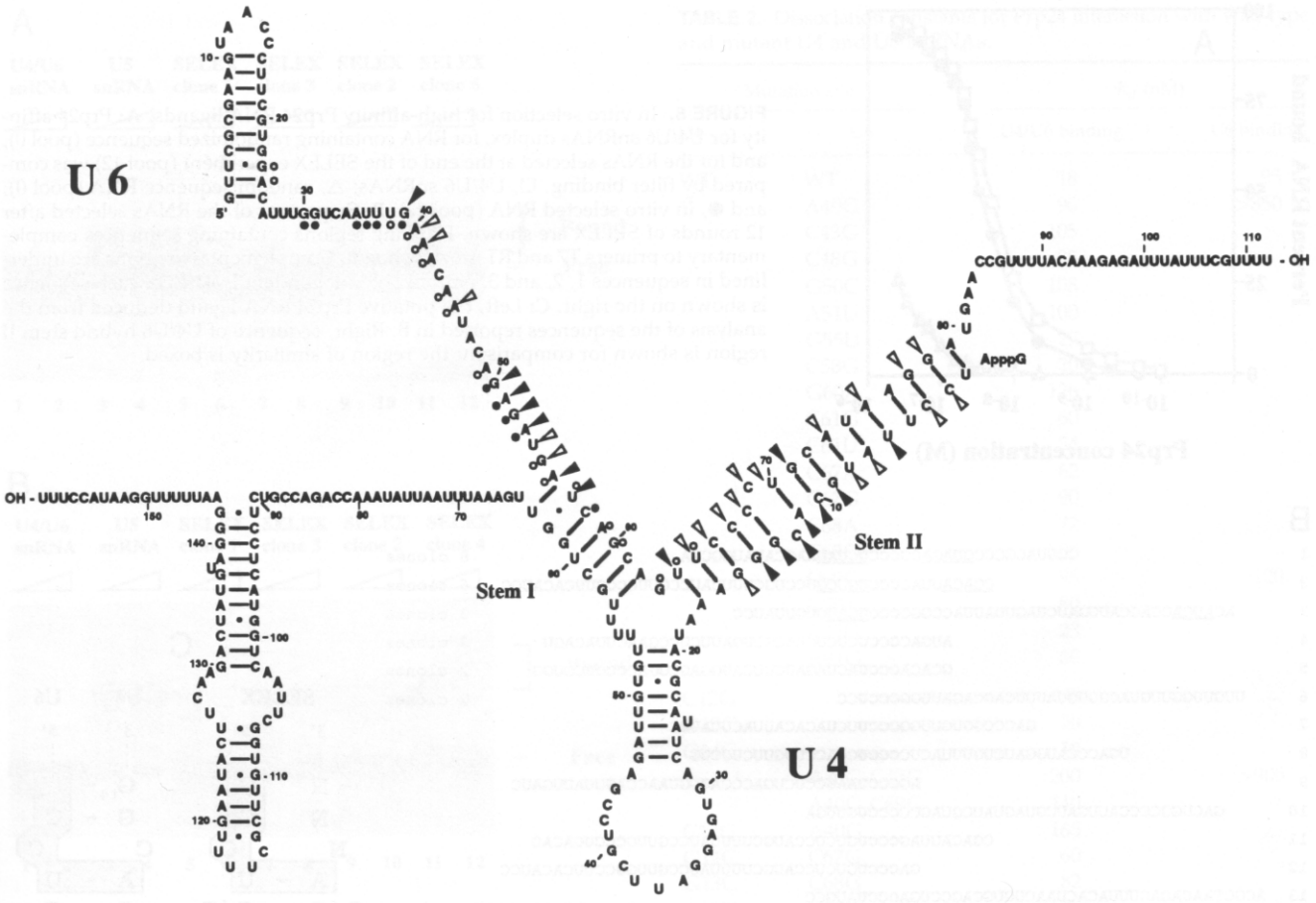


FIGURE 4. Prp24 protection sites on the U6 and U4 snRNAs. Solid arrows indicate strong protection on the U4/U6–Prp24 and open arrows indicate weak protection on the U4/U6–Prp24 complex. Solid and open circles indicate strong and weak protection, respectively, in the U6–Prp24 complex. Strong protection sites are defined as sites at which at least a 75% signal reduction was observed, compared to the control; weak protection sites are defined as sites at which at least a 50% signal reduction was observed. The protection sites indicated in the figure are those detected consistently in at least three independent experiments. The U4/U6 RNA structure is from Brow and Guthrie (1988).

respectively, whereas in the selected RNAs the CCC sequence is predicted to be unpaired. This result suggest that Prp24 binds preferentially to a U4/U6 structure in which part of stem II is not base paired.

Effect of mutations in the U4 and U6 snRNAs on Prp24 binding

The results obtained both from the SELEX experiment and hydroxyl radical footprinting suggest that stem II of the U4/U6 snRNA hybrid may be a binding site for Prp24. To test this possibility further, we measured the binding affinity of a number of U4 and U6 snRNA mutants using a gel shift assay to estimate the K_d. The results of these experiments are summarized in Table 2 (see Fig. 7 as a reference for mutation sites). One prediction of the model for Prp24 binding to U4/U6 stem II is that it requires a CCC sequence, possibly in a single-stranded conformation. If the central region of stem II in the U4/U6 hybrid is part of the Prp24 binding site, mutations altering the CCC sequence and/or making the stem II a perfect duplex can be expected to decrease Prp24 affinity. Creating a perfect duplex in stem II (U6-C69G and U4-C12G) does indeed decrease Prp24 binding to U4/U6. Other alterations in the CCC sequence of U6 (positions 67 and 68) also decrease the binding affinity. Interestingly, as expected from our SELEX results the double mutant U4-A11U, U6-U70A, in which the U-A base pair is changed to A-U, decreases binding. On the other hand, mutations in the 71–79 region of U6 have no detectable effect on the binding of Prp24.

Although the RNAs characterized in the SELEX experiment do not show any conserved motif similar to other sequences in either U6 or U4, the hydroxyl radical footprinting experiments (Figs. 3, 4) on the U4/U6–Prp24 complex indicate that the 39–57 region of U6 may also be involved in Prp24 binding. Indeed, mutations in this domain of U6 were found to decrease binding of Prp24 five- to eightfold (Table 2). Mutations in po-

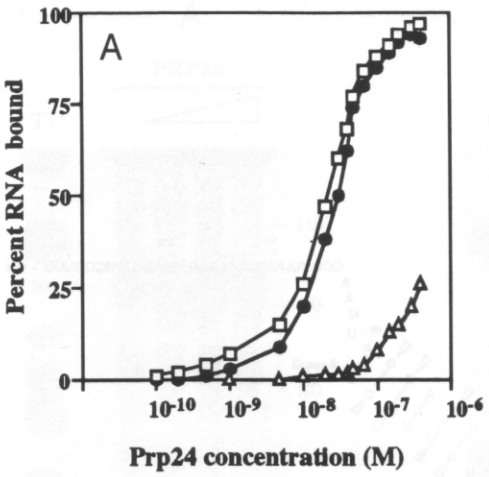


FIGURE 5. In vitro selection for high-affinity Prp24 RNA ligands. **A:** Prp24 affinity for U4/U6 snRNAs duplex, for RNA containing randomized sequence (pool 0), and for the RNAs selected at the end of the SELEX experiment (pool 12) was compared by filter binding. □, U4/U6 snRNAs; △, random sequence RNA (pool 0); and ●, in vitro selected RNA (pool 12). **B:** Sequences of the RNAs selected after 12 rounds of SELEX are shown. Flanking regions containing sequences complementary to primers T7 and RT are not shown. Complementary regions are underlined in sequences 1, 2, and 3. Number of independent isolates of each sequence is shown on the right. **C:** Left, the putative Prp24 RNA ligand deduced from the analysis of the sequences reported in B. Right, sequence of U4/U6 hybrid stem II region is shown for comparison: the region of similarity is boxed.

B

1	CCGUACGCCCGUAGACGCCCCUCUAUAAGCAUAUGGCUA	8 clones
2	CGACAUUACCCCUGUCGUCCUGCUUUUAUCCGUUCCCCUUCACAUCC	6 clones
3	ACAUCACCACCAUUGAUCUAGUUAUUACCGGCCCCUGAUGUGUUAUCC	3 clones
4	AUGACCCCUCUCUCUACGUUGAUUCUCCGAACUUACAGU	3 clones
5	GCACACCCCUACUAGAUCUUCAUGGAGCGGACCCCUCGUGG	2 clones
6	UUGUUGGUUGUACGGUGUAUUUCAGCAGAUUGGCCCCUCC	2 clones
7	GACCCGGUGUUGGCCCCUUCUACACAUUACUUAUGC	
8	UGACCCAUGGAUCUGUUUACUCCCCCUCCACCUUGUUCUCCUG	
9	AGCCCCUAAUCCUCUGACCCAGGGUAACCAGUUAUUGAUC	
10	GACUGGCCCCAUUGAUCGUAGUAUCGUAGCCCCCUGUUGA	
11	CGACAUUAGCCCCUGUCUCCAUGCUUUUAUCCGUUGCCCCUCACAC	
12	GACCCCUGUCUCCAUGCUUUUAUCCGUUGGCCUUCACAUCC	
13	ACGCTAACAGACUUUACACUAACUGUGCAGCCUGACCCCUAUGCC	
14	GACCCGGUGUGGCCCCUUCUACACAUUACUUAUGCCC	
15	AGGACCCCUGUUGUUGGGUACAGUUAUUC	
16	GUUACUACGUACCCCUAUGACCCAGGGGACCUGUCCUGU	
17	GGCCCCUGUCUCUGGACCUCUCAGCCCACUCCAUGUUCCA	
18	GACUGGCCCCAUUGAUGGUAGUAUCGUAGCCCCCU	
19	GACUACCGUACUACACUGCCCCUCAGACGUGCUCACCUCC	
20	CCCAGCUAGCCCCUCUAUCGGAUCGUACCUCUGUGAUGACCCCACAUC	
21	AGGCCCAAUAAUCCCCUGACCCAUAAUCCUCUGAGGGAGCGUAACCC	
22	UACCUUGAGAACACUACUACACUACGGUUGACCCCCU	

C

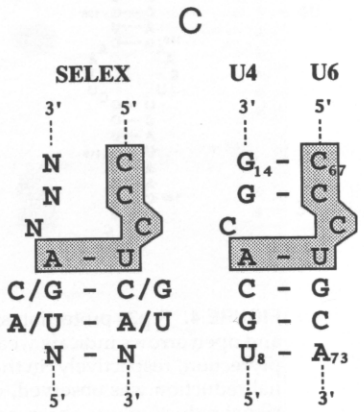


TABLE 1. Dissociation constants for the interaction between Prp24 and U4/U6 snRNA or RNAs isolated by SELEX.

snRNA	Selected RNA[a]	K_d (nM)
U4/U6		18
	1	25
	2	20
	3	45
	4	60
	5	50
	12	80
	13	70
	18	60
	19	50
	Pool 0	>500

[a] The numbers refer to the RNA sequences shown in Figure 5B.

sition 40, 43, 48, 50, and 51 in U6 all decrease Prp24 binding to U4/U6 and the double mutant A40G, C43G increased the K_d more than 10-fold compared to the wild-type RNA. Mutations in the stem I region have a negligible effect on Prp24 binding, with the exception of G60A, which increases the K_d about eightfold. The affinity of Prp24 for the U4/U6 hybrid is decreased about eightfold in the U6 double mutant A40G, C69G, and in the double mutant U4-C12G/U6-A40G. Overall, the affinities of the mutant RNAs for Prp24 indicate that, in the U4/U6 hybrid, both stem II and the 39–57 region of U6 contribute to Prp24 binding.

The affinity of Prp24 for several U6 mutants in the absence of U4 was also measured. Both U6-A40G and U6-A40G-C43G increase the K_d about 10-fold. However, the U6-C69G mutation, which has a fivefold effect

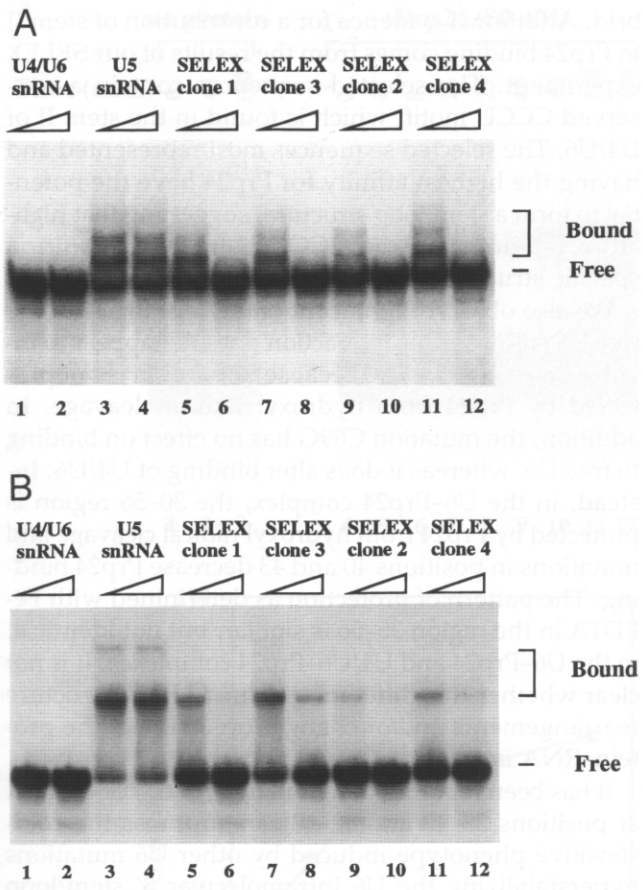


FIGURE 6. Competition of Prp24 binding to the snRNAs U4/U6 and U6 by in vitro-selected RNA ligands. **A:** A total of 15 fmol of U4/U6 snRNA hybrid containing ^{32}P-5′-end labeled U6 was incubated with 70 nM Prp24 in the presence of 10-fold (lanes 1, 3, 5, 7, 9, 11) or 100-fold (lanes 2, 4, 6, 8, 10, 12) excess of unlabeled competitor RNA. The following competitors were used: lanes 1 and 2, U4/U6 hybrid; lanes 3 and 4, U5 snRNA; lanes 5 and 6, SELEX RNA 1; lanes 7 and 8, SELEX RNA 3; lanes 9 and 10, SELEX RNA 2; lanes 11 and 12, SELEX RNA 4 (see Fig. 5B for numbering of the in vitro-selected sequences). **B:** Same as in (A), but 15 fmol of ^{32}P 5′-end labeled U6 snRNA was used.

in the U4/U6 context, does not change the affinity of free U6 for Prp24. This result is consistent with the chemical probing data and indicates that the binding determinants for Prp24 in U6 RNA are only in the 30–56 domain.

Temperature dependence of Prp24 binding to the U4/U6 hybrid

Shannon and Guthrie (1991) reported that the U4 mutation G14C confers a cold-sensitive phenotype and that in extracts from these cells anti-Prp24 antibodies can co-immunoprecipitate both the U4 and U6 snRNAs. In contrast, in extracts prepared from wild-type cells, anti-Prp24 antibodies only immunoprecipitate the U6 snRNA. Our measurements of Prp24 affinity for the U4/U6 hybrid containing the U4-G14C mutation at 23 °C indicate that the wild-type and U4-G14C mutant

TABLE 2. Dissociation constants for Prp24 interaction with wild-type and mutant U4 and U6 snRNAs.

Mutation site		K_d (nM)	
U4	U6	U4/U6 binding	U6 binding
WT	WT	18	95
	A40G	90	>850
	C43G	105	
	C48G	85	
	G50C	108	
	A51U	100	
	G55U	27	
	C58G	20	
	G60A	126	
	C61G	60	
	C61U	54	
	C67A	62	
	C67G	90	
	C68A	72	
	C68G	108	
	C69G	95	120
	U70A	80	
	C72G	23	
	A91G	60	
C12G		95	
G13C		20	
G14C		18	
	A40G C43G	200	>900
	A40G C69G	140	
C12G	A40G	168	
G14C	C67G	60	
G13C	C68G	82	
A11U	U70A	75	

RNAs bind Prp24 with identical affinity (Table 3). However, when assayed at 4 °C, a different result is obtained. At 4 °C, the K_d for the wild-type U4/U6 RNA is about fourfold higher compared to the value mea-

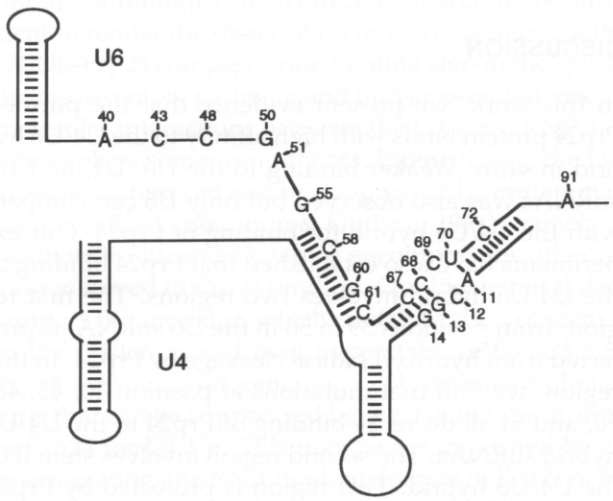


FIGURE 7. Position of the mutated bases on the U4/U6 snRNAs duplex. Mutations reported in Table 2 are shown in the context of the model of U4/U6 hybrid described by Brow and Guthrie (1988).

TABLE 3. Temperature dependence of the dissociation constant for Prp24 interaction with wild-type and mutant U4/U6 duplex.

Mutation in the U4/U6 snRNA duplex	K_d (nM)	
	4 °C	23 °C[a]
WT	76	18
U4 (G13C)	25	20
U4 (G14C)	18	16

[a] Data from Table 1.

sured at 23 °C. In contrast, the K_d for the U4/U6 hybrid containing the U4-G14C mutation does not change with temperature. We have also observed a similar effect with the U4-G13C mutation. Shannon and Guthrie (1991) also found that this mutant confers a cold-sensitive phenotype.

Prp24 can facilitate U4 and U6 snRNAs annealing

It has been suggested that one possible role for Prp24 may be to facilitate duplex formation between the U4 and U6 RNAs (Shannon & Guthrie, 1991). We have tested this possibility directly using an in vitro annealing assay. Figure 8A and B shows U4/U6 renaturation over time in the absence or presence of Prp24. In the Prp24-containing reaction, 50% of the single-stranded U4 substrate is converted to U4/U6 hybrid in 1 h. In contrast, in the absence of the protein, less than 7% of U4 was annealed to U6 over a 3-h time period. This annealing activity was detected over a wide range of protein concentrations, although it is maximal around 30 nM Prp24, which, under the conditions used, corresponds to a ratio of 60 nt of RNA/Prp24 molecule (Fig. 8C).

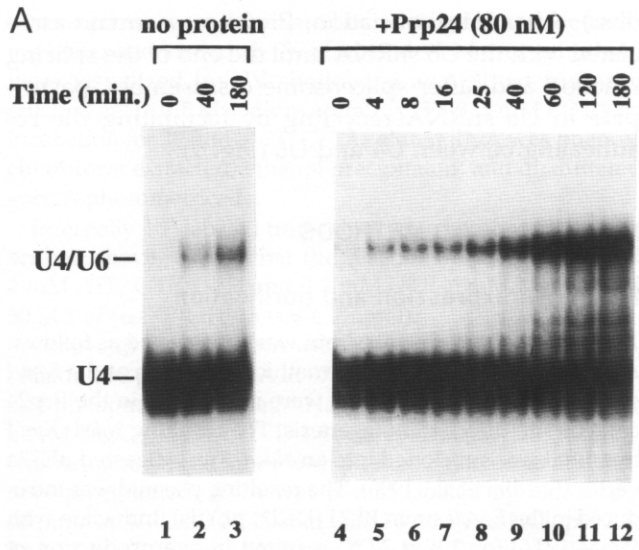
DISCUSSION

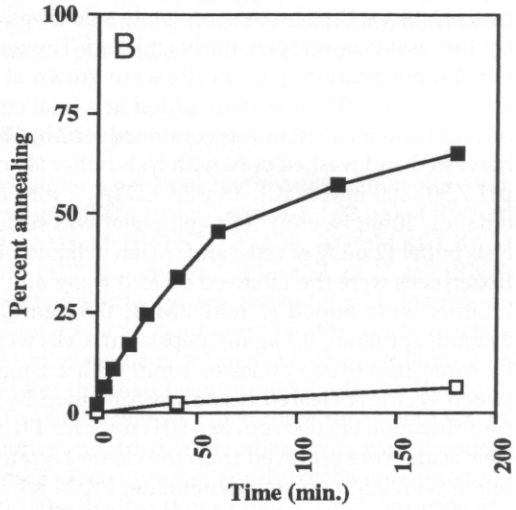
In this work, we present evidence that the purified Prp24 protein binds with high affinity to the U4/U6 hybrid in vitro. Weaker binding to the U6, U1, and U2 snRNAs was also observed but only U6 can compete with the U4/U6 hybrid for binding of Prp24. Our experiments have also established that Prp24 binding to the U4/U6 hybrid involves two regions. The first region, from positions 39 to 56 in the U6 snRNA, is protected from hydroxyl radical cleavage by Prp24. In this region, we find that mutations at position 40, 43, 48, 50, and 51 all decrease binding of Prp24 to the U4/U6 hybrid snRNAs. The second region involves stem II of the U4/U6 hybrid. This region is protected by Prp24 from hydroxyl radical cleavage and mutations in positions 67–70 in U6 as well as positions 11 and 12 in U4 decrease the affinity of the protein for the U4/U6 hybrid. Additional evidence for a contribution of stem II in Prp24 binding comes from the results of our SELEX experiment. The selected sequences contain a conserved CCCU motif, which is found in the stem II of U4/U6. The selected sequences most represented and having the highest affinity for Prp24 have the potential to form a stem-loop structure, suggesting that high-affinity binding to the CCCU motif may require a specific structural context.

We also observe significant binding of Prp24 to the free U6 snRNA. This interaction does not appear to involve bases 67–71 of U6 because this region is not protected by Prp24 from hydroxyl radical cleavage. In addition, the mutation C69G has no effect on binding to free U6, whereas it does alter binding of U4/U6. Instead, in the U6–Prp24 complex, the 30–56 region is protected by Prp24 from hydroxyl radical cleavage and mutations in positions 40 and 43 decrease Prp24 binding. The pattern of protection as determined with Fe-EDTA in the region 35–56 is similar, but not identical, in the U6–Prp24 and U4/U6–Prp24 complexes. It is not clear whether this difference is due to RNA structure rearrangements and/or changes occurring at the protein–RNA interface.

It has been reported that mutations in the U6 snRNA at positions 38–40 and 42–43 can suppress the cold-sensitive phenotype induced by other U6 mutations hyperstabilizing the U6 intramolecular 3′ stem/loop (Fortner et al., 1994). Although the positions of these suppressor mutations overlap with regions important for Prp24 binding to both the free U6 and the U4/U6 hybrid (U6 bases 39–56), it is not known whether Prp24 is involved in the suppression of the U6 mutations altering the stability of the intramolecular U6 3′ stem/loop.

Although our results show that pure Prp24 binds preferentially to the U4/U6 hybrid, this is not what is observed in yeast extracts. Shannon and Guthrie (1991) found that only U6 is precipitated by anti-Prp24 antibodies and, more recently, Jandrositz and Guthrie (1995) have observed that the 39–43 region of U6 is strongly protected (presumably by Prp24) in U6 snRNPs but not in U4/U6 snRNPs. Prp24 does bind to U4/U6 in mutants (e.g., U4-G14C) in which stem II is disrupted. There are a number of possible explanations for the discrepancy between the results obtained for the two-component system and the extract. Perhaps the most likely is that Prp24 acts to facilitate the formation of U4/U6 in the annealing reaction but leaves immediately due to the action of some other factor. Other explanations include possible modification of Prp24 that might occur in yeast but not in *E. coli*. However, the Prp24 protein synthesized in *E. coli* is active in a splicing complementation assay. A further problem in interpreting the results obtained in extracts is that we do not know what is the active form of the U4/U6 snRNP that participates in spliceosome assembly or





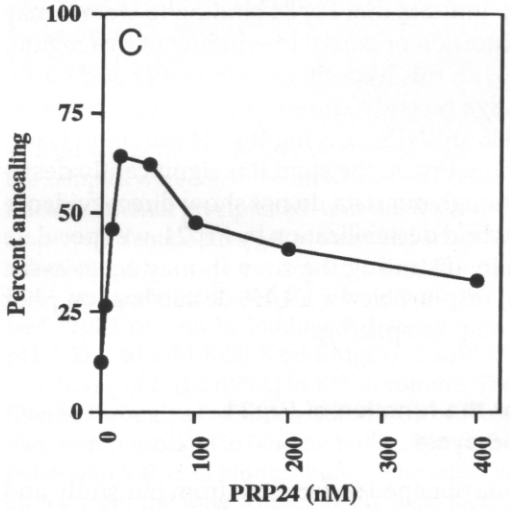


FIGURE 8. Prp24 stimulation of U4 and U6 snRNAs annealing. **A:** Time course of U4 and U6 snRNAs annealing in the absence (lanes 1–3) or in in the presence (lanes 4–12) of Prp24 protein. U4 snRNA (2 nM) was ^{32}P-5′-end labeled. Unlabeled U6 snRNA was 10 nM. **B:** Gels shown in A were quantitated by a Phosphor Imager (Molecular Dynamics) and the data plotted: open squares refer to the reactions in the absence of protein; filled squares represent the Prp24-containing reactions. **C:** U4-U6 snRNAs annealing in the presence of various concentrations of Prp24. RNA concentrations were the same as in A; reactions were incubated for 60 min.

what fraction of the U4/U6 molecules in an extract are in that form.

Interestingly, we find that binding of Prp24 to the U4/U6 hybrid at 23 °C is not affected by the G13C or G14C in U4. Both of these mutations confer a cold-sensitive phenotype in vivo, and one of them (U4-G14C) can be suppressed by mutations in the Prp24 gene. However, the affinity of Prp24 for the wild-type U4/U6 hybrid measured at 4 °C is about fourfold lower compared to that measured at 23 °C. In contrast, the U4-G14C and U4-G13C U4/U6 mutants have the same affinity at both temperatures. Thus, at 4 °C, Prp24 affinity for the mutant RNAs is about fourfold higher than for wild-type RNA. Our observation that, at low temperature, the U4-G14C or U4-G13C mutations hyperstabilize the U4/U6–Prp24 complex, are consistent with the immunoprecipitation data reported by Shannon and Guthrie (1991), which show that extracts harboring U4 with the G14C mutation accumulate U4/U6–Prp24 complexes at low temperature. This hyperstabilization of the U4/U6–Prp24 complex in the U4-G14C mutant, although a small effect in vitro could be sufficient to render the dissociation or rearrangement of the U4/U6–Prp24 complex a rate-limiting step in the spliceosome assembly pathway and impair essential macromolecular interactions between the U4/U6 snRNP and other spliceosome components. Interestingly, the U4-G14C mutation can also be suppressed by mutation in the U6 snRNA (Shannon & Guthrie, 1991), namely at position 38, 40, and 43, which encompass the other region involved in Prp24 binding. Our biochemical data support the model in which destabilization of stem II in the U4/U6 hybrid may hyperstabilize the U4/U6–Prp24 complex and generate a cold-sensitive phenotype that can be suppressed by weakening the affinity of Prp24 for U4/U6. This may be accomplished by either mutating one RNA binding domain in Prp24 or by mutating the protein binding site on the RNA.

The observation that U4 mutations having a destabilizing effect on the stem II can facilitate Prp24 bind-

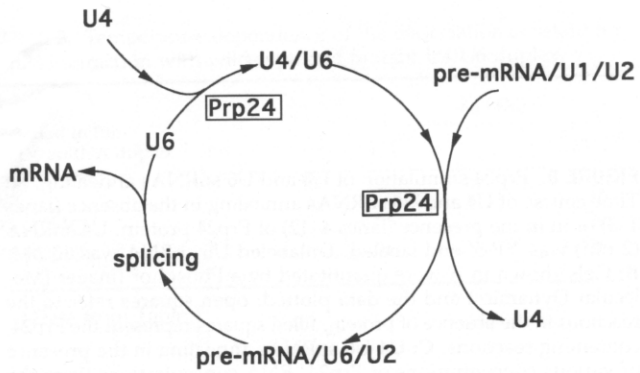


FIGURE 9. Hypothetical model of Prp24 function in splicing.

ing to U4/U6, and the apparent preference of Prp24 for unpaired CCC stretches, suggested by the SELEX experiment, indicate that Prp24 binding to stem II may require a distortion or partial unwinding of this region. Consistent with this hypothesis, Jandrositz and Guthrie (1995) have recently shown, by chemical probing, that in U4/U6 snRNPs carrying the U4 mutation G14C and containing Prp24, the stem II is significantly destabilized. Although our data do not show direct evidence for U4/U6 hybrid destabilization by Prp24, we speculate that Prp24, by distorting the stem II, may act to assist the factor(s) responsible for U4/U6 destabilization prior to the first step of splicing.

A model for the function of Prp24 in the U4/U6 cycle

All of the data obtained so far, both from our study and by Shannon and Guthrie (1991) and by Jandrositz and Guthrie (1995), support the idea that Prp24 mediates the annealing of U4 and U6. Strong support for that notion is our finding that purified Prp24 effectively mediates the annealing reaction.

A major question remains regarding the dissociation of U4 and U6 that takes place in spliceosome assembly. Though there are no published data that show an association between Prp24 and the spliceosome, our studies and those from Shannon and Guthrie (1991) and Jandrositz and Guthrie (1995) show that Prp24 binds preferentially to U4/U6 hybrids in which at least a portion of the base pairs in stem II cannot form. It therefore follows that Prp24 could also facilitate the dissolution of that stem and thereby promote the dissociation of U4 from the spliceosome. This reaction would likely require a second factor, perhaps a helicase. A prediction of this model is that, during spliceosome assembly, Prp24 should be required before and after the U4/U5/U6 tri-particle joins the spliceosome. Indeed, preliminary evidence that this is the case has recently been obtained using a temperature-sensitive *prp24* mutant extract (A. Ghetti & J. Abelson, unpubl. obs.). After U4 dissociation, Prp24 may remain associated with the U6 snRNA until the end of the splicing reaction and, after spliceosome disassembly, participate in U6 snRNA recycling by facilitating the reannealing between U4 and U6 (Fig. 9).

MATERIALS AND METHODS

Prp24 overexpression and purification

The Prp24 overproducing strain was constructed as follows. An *Nde* I site at the initiation methionine codon and a *Kpn* I site at the termination codon were introduced in the Prp24 gene by site-directed mutagenesis. The resulting *Nde* I–*Kpn* I fragment was subcloned into an *Nde* I/*Kpn* I-digested pET3a vector (Studier et al., 1990). The resulting plasmid was introduced in the *E. coli* strain BL21 (DE21, pLYSs). Induction with 1 mM IPTG for 2 h at 37 °C resulted in overproduction of Prp24 up to 20% of the total protein content of the cells. The overproduced Prp24 was completely soluble as determined by the assay developed by Nagai and Thogersen (1987). For the purification, 6 L of cells were grown at 37 °C to an $OD_{600} = 0.5$. IPTG was then added at a final concentration of 1 mM and incubation was continued for 2 h. The cells were harvested and washed once with lysis buffer (40 mM HEPES, pH 7.50, 100 mM NaCl, 10 mM EDTA, 2 mM β-mercaptoethanol, 10% glycerol). The cell pellet was resuspended in lysis buffer (2 mL/g of cells) and frozen in liquid nitrogen. The frozen cells were then thawed on iced water and protease inhibitors were added (1 mM PMSF, 0.5 μg/mL leupeptin, 2 μg/mL aprotinin, 0.7 μg/mL pepstatin). Cells were disrupted by sonication (three cycles of 1 min with a 1-min pause between each). A cleared lysate was obtained by high-speed centrifugation (45,000 rpm in a 50Ti rotor for 1 h at 4 °C). Nucleic acids were removed from the cleared lysate by precipitation with 0.5% polyethyleneimine. Prp24 remained in the supernatant and was precipitated with 45% ammonium sulfate. The ammonium sulfate pellet was resuspended at a final protein concentration of 5 mg/mL in buffer A (containing 1 mM EDTA and protease inhibitors) and dialyzed against buffer A overnight with one change of the dialysis buffer. The dialysate was then loaded onto a mono-S FPLC column and eluted with a gradient of 50–1,000 mM NaCl in buffer A. Prp24 elution peaked at 500 mM NaCl. Peak fractions were pooled, concentrated, dialyzed against buffer B (buffer A containing 50% glycerol), and stored at −20 °C. Prp24 appeared homogeneous on a Coomassie blue and silver-stained gel analysis.

RNA synthesis

U1, U2, U4, U5, and U6 snRNAs were transcribed in mixtures containing 10 μg of pT7/U1 (D. McPheeters, unpubl.), pT7/U2 (McPheeters et al., 1989), pT7/U4 (P. Fabrizio, unpubl.), pT7/U5 (D. McPheeters, unpubl.), pT7/U6 (Fabrizio et al., 1989), respectively. Reactions were incubated at 37 °C for 2–4 h in 100 μL volume and, in addition to the plasmid DNA linearized with the appropriate restriction enzyme, contained: 40 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 4 mM ATP, UTP, GTP, CTP, 1,000 units of T7 RNA polymerase, and 100 units of RNasin. Transcrip-

tion reactions were terminated by adding formamide loading buffer and loaded on 6% acrylamide–7 M urea gel. RNAs were visualized by UV shadowing and eluted from the crushed gel in 0.5 M sodium acetate, pH 5.0, 5 mM EDTA by incubation for 20 min at 23 °C. The eluted RNA was phenol–chloroform extracted, ethanol precipitated, and quantitated spectrophotometrically.

Internally ^{32}P-labeled transcripts were synthesized as described above, except that the reactions (20 μL) contained 1 mM ATP, GTP, CTP, and 0.2 mM UTP, 6 mM $MgCl_2$, and 50 μCi of [α-^{32}P]UTP (3,000 Ci/mmol).

For 5′-end labeling, RNA was first dephosphorylated by incubating 50 pmol of RNA at 37 °C for 1 h with 1 unit of alkaline phosphatase in 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 3 mM $MgCl_2$, 1 mM DTT, 1 U/μL of RNasin. The reaction was extracted three times with phenol–chloroform and ethanol precipitated. RNA was 5′-end labeled by incubating 20 pmol at 37 °C for 20–30 min with 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, and 1 unit of T4 polynucleotide kinase and 160 μCi of [γ-^{32}P]ATP (7,000 Ci/mmol). The reaction was stopped by adding formamide loading buffer and the RNA was gel purified as described above.

Filter binding assays

Protein–RNA binding reactions were carried out in 50 μL volume containing 10,000 cpm (approximately 2 pg) of RNA in binding buffer (30 mM Tris-HCl, pH 7.35, 100 mM KCl, 8 mM $MgCl_2$, 2 mM DTT, 4% glycerol, 10 ng/μL of *E. coli* tRNA, 40 μg/mL BSA) and different amounts of Prp24. Incubation was at 23 °C for 15 min. To measure binding, each reaction was applied under gentle vacuum to a 0.45-μm Millipore nitrocellulose filter that had been pre-washed with the binding buffer. The filters were washed three times with 200 μL of binding buffer and dried, and radioactivity was measured by liquid scintillation counting. For the reactions containing U4/U6 hybrid, the in vitro-transcribed RNAs were annealed by mixing the two RNAs in water, incubating 3 min at 85 °C, then adding the annealing buffer (Tris-HCl, pH 7.50, 150 mM NaCl, 2 mM EDTA), and slowly cooling down the reaction to 25 °C over 5–6 h. Glycerol (4% final) was added to the annealing reaction and the sample was loaded on a 6% polyacrylamide gel (30:1 acrylamide:bis-acrylamide). The U4/U6 hybrids were eluted from the gel in 0.3 M sodium acetate, 3 mM EDTA, and ethanol precipitated.

Gel shift assay

Binding reactions were assembled as described for the filter binding assay (see above) but using a final volume of 10 μL. Following a 15-min incubation, the reactions were loaded on a 6% polyacrylamide gel (30:1 acrylamide:bis-acrylamide) that had been pre-run for at least 2 h. Samples were electrophoresed for 4 h at 8 V/cm, in TBE buffer (89 mM Tris, 89 mM boric acid, 2.4 mM EDTA).

For the competition experiments, 35 fmol of U4/U6 snRNA hybrid containing ^{32}P 5′-end labeled U6 were incubated with 30 nM Prp24 in the same binding buffer used for the filter binding experiment in the presence of increasing amounts of unlabeled snRNA as a competitor. The reactions were electrophoresed on a native acrylamide gel as described above.

Hydroxyl radical probing

The protein was allowed to bind to the RNA by incubation for 15 min at 23 °C in a buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 4 mM $MgCl_2$, 50 ng/μL *E. coli* tRNA, 15 ng/μL BSA. Cleavage reactions were carried out essentially as described (Tullius & Dombroski, 1986) for the probing of DNA, in the presence of 100 μM iron (II), 200 μM EDTA, 0.003% hydrogen peroxide, and 1 mM ascorbate, by 2 min incubation at 23 °C. The reactions were stopped by the addition of thiourea and EDTA, followed by phenol–chloroform extraction and ethanol precipitation. Reactions were analyzed on 10% polyacrylamide–7 M urea gel.

In vitro selection

The template DNA used to transcribe the initial pool of random RNAs was generated by 5 cycles of PCR amplification of 5 μg of the oligonucleotide S1: 5′-GGCACTATTTATATCA AC$(N)_{50}$AATGTCGTTGGTGCTCC-3′ using the two oligonucleotides T7: 5′CTACGCGGATCCTAATACGACTCACTA TAGGGAGCACCAACGACATT-3′ and Rev: 5′-CCCGACA CCCGCAAGCTTAGGCACTATTTATATCAAC-3′. A total of 75 μg of the resulting double-stranded product was transcribed using the T7 RNA polymerase; after the synthesis, the samples were treated with RNase-free DNase, phenol extracted, ethanol precipitated, and the RNA was purified on an polyacrylamide-urea gel as described above. The first three selection cycles were performed using 10 nmol of random RNA and were incubated for 20 min with 100 pmol of purified Prp24 protein in binding buffer (30 mM Tris-acetate, pH 7.35, 100 mM KCl, 8 mM $MgCl_2$, 2 mM DTT, 4% glycerol, 0.5 μg of *E. coli* tRNA) in 100 μL volume. The sample was filtered through a 0.45-μm Millipore nitrocellulose filter that had been presoaked in binding buffer. The filter was washed twice with 500 μL of binding buffer. The retained RNAs were eluted from the filter as previously described (Bartel et al., 1991). Half of the eluted RNA was reverse transcribed using the Rev oligonucleotides and the RNase H- Reverse Transcriptase from BRL according to manufacturer's instructions. The cDNA was PCR amplified using both the T7 and the Rev oligonucleotides monitoring by agarose gel the amplification level to avoid over-cycling. The double-stranded DNA obtained from the PCR reaction was transcribed by T7 RNA polymerase to generate the RNA pool for the next cycle. Cycles 4–12 were performed as described, but 200 pmol of RNA and 10 pmol of Prp24 were used. The PCR products obtained from cycle 12 was digested with *Bam* HI and *Hin*d III and cloned in the pUC19 plasmid vector. After transformation, the plasmids from 50 independent colonies were isolated and sequenced using the standard dideoxy sequencing protocol.

Mutant snRNAs

Generation of the mutant U6 snRNAs C48G, G50C, A51U, G55U, C58G, G60A, C61U, and C61G has been previously described (Fabrizio & Abelson, 1990). All other mutants reported in Table 3 were constructed using the method by Deng and Nickoloff (1992) and the Transformer Site Directed Mutagenesis Kit (Clonetech Lab, Inc.). The following mutagenic oligonucleotides have been used. dU4-A11U 5′-ATGC

GTATTTCCCGAGCATAAGGATCCTATAGAG-3′; dU4-C12G 5′-ATGCGTATTTCCCCTGCATAAGGATCCTATAGAG-3′; dU4-G13C 5′-ATGCGTATTTCCGGTGCATAAGGATCCTATAGAG; dU4-G14C 5′-ATATGCGTATTTCGCGTGCATAAGGATCCTATAG-3′; dU6-A40G 5′-TCATCTCTGTATTGTTCCAAATTGACCAA-3′; dU6-C43G 5′-ATCATCTCTGTATTCTTTCAAATTGACCAA-3′; dU6-A40G-C43G 5-ATCATCTCTGTATTCTTCCAAATTGACCAAATGT-3′; dU6-C67A 5′-CATCCTTATGCAGGTGAACTGCTGATCATC; dU6-C67G 5′-CATCCTTATGCAGGCGAACTGCTGATCATC-3′; dU6-C68A 5′-CATCCTTATGCAGTGGAACTGCTGATCATC-3′; dU6-C68G 5′-CATCCTTATGCAGCGGAACTGCTGATCATC-3′; dU6-C69G 5′-CATCCTTATGCACGGGAACTGCTGATCATC-3′; dU6-U70A 5′-CATCCTTATGCTGGGGAACTGCTGATCATC-3′; dU6-C72G 5′-CATCCTTATCCAGGGGAACTGCTGATCATC-3′; dU6-A91G 5′-GAAATAAATCTCTTTGCAAAACGGTTCATC-3′.

Annealing reactions in the presence of Prp24

Before assembling the reactions, the U4 and U6 snRNAs were incubated separately in water at 85 °C for 2 min and then chilled in ice water. Reactions were carried out at 30 °C in 10-μL reactions containing 30 mM Tris-HCl, pH 7.35, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 4% glycerol, 2 nM U4 snRNA ^{32}P 5′-end labeled, 10 nM U6 snRNA unlabeled. Prp24 was added to the concentration indicated in the legend to Figure 8. After incubation, the reactions were stopped by adding 2 μL of buffer containing 0.5% SDS, 0.2 mg/mL proteinase K, tRNA 0.3 mg/mL, and incubating an additional 7 min at 30 °C. The samples were then loaded on a 6% acrylamide gel (30:1 polyacrylamide:bis-acrylamide). The gel was pre-run for at least 2 h and, after loading, was run for 4 h at 8 V/cm, in TBE buffer (89 mM Tris, 89 mM boric acid, 2.4 mM EDTA).

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