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23. There are no good estimates of the size of recombination fragments in natural populations of *E. coli*, but if the major means of transfer is transduction-mediated by a phage similar in size to P1, the average size of transferred fragments would be approximately 1 min or roughly 45 kb of DNA (*26*). This assumption agrees with the finding of Whittam and co-workers (*27*), who observed no correlation between map distance of loci in *E. coli* and linkage disequilibrium for electrophoretic markers. This suggests that most of the fragments are not much more than 1 or 2 min of the chromosome. Milkman and Bridges (*4*, *28*) have suggested that the length of recombination fragments could be much shorter. But, even if a length as short as 1 kb is used in the calculation, the overall picture does not change because the rate of recombination remains greater than the rate of mutation. The average pairwise difference was calculated from all nongroup A strains to the nonrecombinant group A cluster for the *sppA* and *zwf* loci. The average pairwise nucleotide difference between group A and nongroup A strains was 1.53% and 1.12% for *sppA* and *zwf*, respectively (mean = 1.32%). This results in ~594 base pairs changed during every lateral transfer event. The rate of change per nucleotide per generation will then be $(3.78 \times 10^{-5}$ transfers per genome per generation) $\times$ (594 base pairs changes per transfer) divided by $(4.5 \times 10^{6}$ nucleotides per genome), which is equal to 5.0×10^{-9} changes per nucleotide per generation.
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29. We thank L. Chao, W. Eanes, D. Futuyma, A. Bely, M. McCartney, I.-N. Wang, and two anonymous reviewers for their reading and suggestions to improve the manuscript, and J. Kim and T. Morton for discussions. All nucleotide sequences are available through GenBank–European Molecular Biology Laboratory. Accession numbers for *sppA* are U13772 through U13780, U13782, U13833, and U13834. Accession numbers for *zwf* are U13783 through U13794. Supported in part by PHS grants GM30201 and AI32454 from the NIH. This is contribution number 899 from the Department of Ecology and Evolution at the State University of New York at Stony Brook.

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Catalytic Site Components Common to Both Splicing Steps of a Group II Intron

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The splicing of group II introns occurs in two steps involving substrates with different chemical configurations. The question of whether these two steps are catalyzed by a single or two separate active sites is a matter of debate. Here, certain bases and phosphate oxygen atoms at conserved positions in domain V of a group II self-splicing intron are shown to be required for catalysis of both splicing steps. These results show that the active sites catalyzing the two steps must, at least, share common components, ruling out the existence of two completely distinct active sites in group II introns.

Group II introns are found in the genomes of organelles of lower eukaryotes and plants and in bacteria (*1*). Some group II introns are able to catalyze their own excision in vitro in the presence of magnesium (*2*). Their excision follows a pathway similar to that of nuclear precursor mRNA (pre-mRNA) splicing, the intron being released in a branched lariat form (*2*). In this pathway, the substrates for the two steps are different: The first transesterification results from the attack at the 5′ splice site by a 2′ hydroxyl, and the second transesterification is initiated by a 3′ hydroxyl. The sequences at the 5′ and 3′ junctions are also different (*1*). Because in nuclear pre-mRNA introns the same phosphorothioate diastereomer (Rp) inhibits both steps when introduced at the junctions (*3*), the two transesterifications cannot be considered as the forward and reverse of the same reaction, as in group I introns (*4*). Whether distinct active sites catalyze the two chemical steps (*3*) or whether a structural rearrangement between the two steps accommodates the different substrates in a single active site (*5*) is a matter of conjecture. To address this question, we have searched for active site components involved in each step of group II self-splicing.

Group II introns are folded into six structural domains (*1*). Among these, domain V is the most conserved element in primary sequence, and deletion experiments have shown that domain V is essential for splicing (*6*, *7*). Moreover, it is able to catalyze 5′ junction hydrolysis when added in trans (*8*). For these reasons, domain V is a good candidate for carrying active site elements. We have adapted the modification interference technique (*9*) to investigate the role of structural elements of domain V in the catalysis of each splicing step (Fig. 1). To investigate the effects of modifications on each of the two splicing steps, we used two assays (Fig. 1A). (i) In a cis-splicing assay the CX fragment is annealed to a subunit composed of exon 1 and domains I to III (E1-XC fragment); under these conditions lariat formation is rate-limiting, allowing the study of the effects of modifications on

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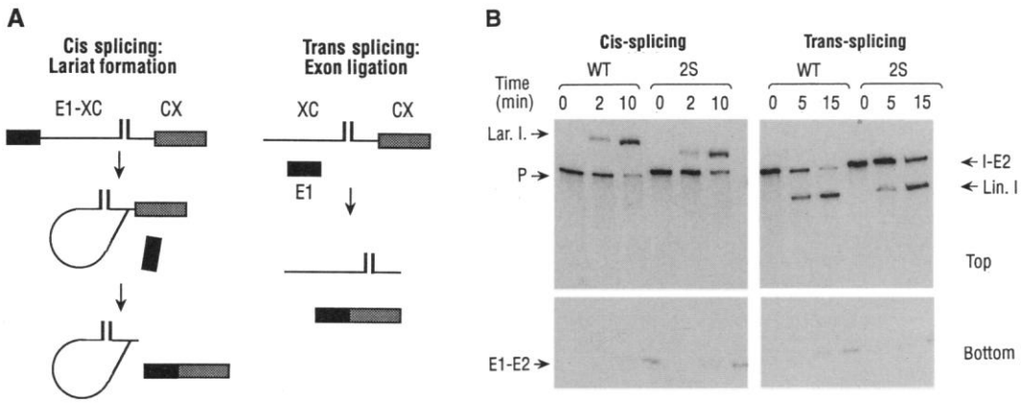


Fig. 1. Splicing of the wild-type (WT) and the two-subunit (2S) precursors. The last intron of the *Saccharomyces cerevisiae* cytochrome oxidase subunit I mitochondrial gene (intron *Sc.cox* 1/5c or al5g) was divided into two subunits that can be annealed with long GC-rich complementary sequences (GC clamps) that replace nonessential peripheral structures of domain IV. With this system, a single subunit carrying domains V and VI and exon 2 (CX subunit) can be independently labeled at the 5′ end and modified. (**A**) Scheme of the two splicing assays. E1, exon 1, represented by a black box; exon 2, a gray box; the intron, a solid line. The vertical bars intervening in the intron sequence represent complementary GC clamps. E1-XC, XC, and CX are the RNA subunits used for reconstitution of active molecules (*20*). (**B**) Time course of cis and trans splicing (*20*). P, precursor; I-E2, linear intermediate; Lar. I. and Lin. I., lariat and the linear intron, respectively; E1-E2, ligated exons. Only the top and the bottom of the gel are shown. No other products were detectable in the portion of the gel that is not shown.

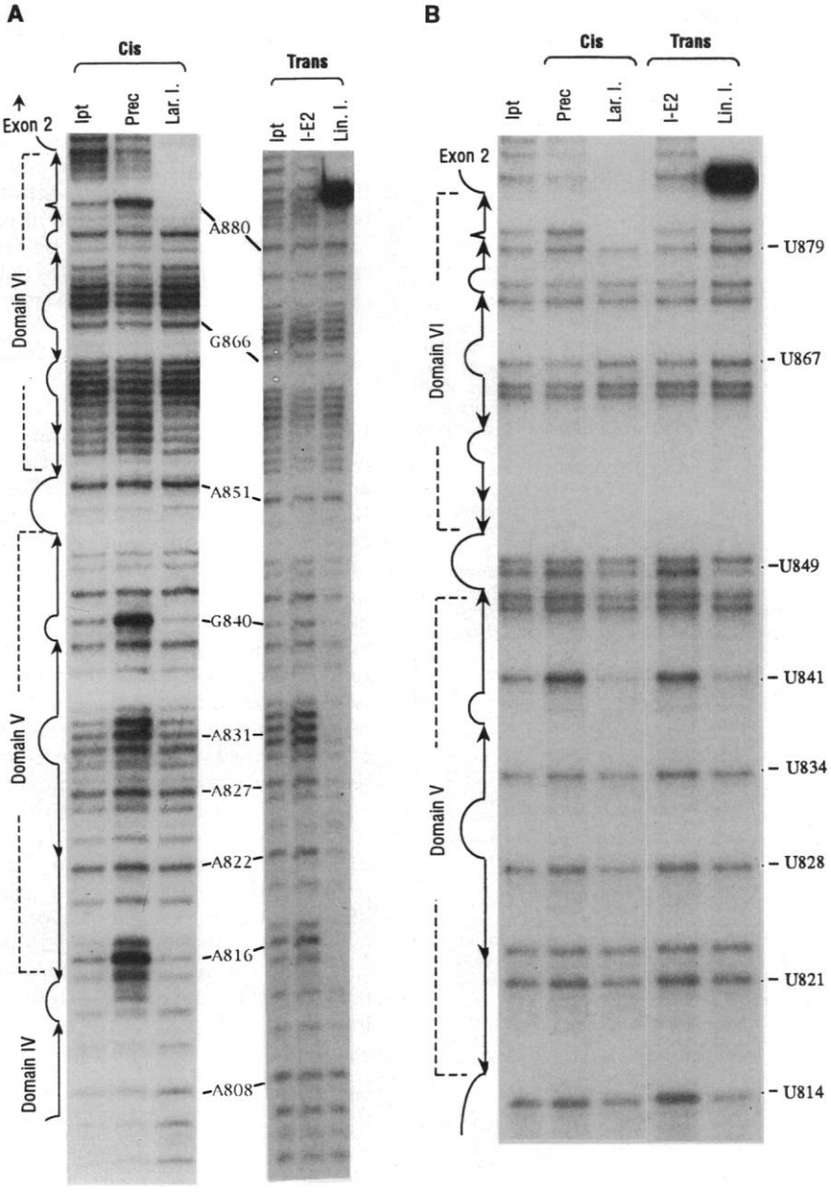


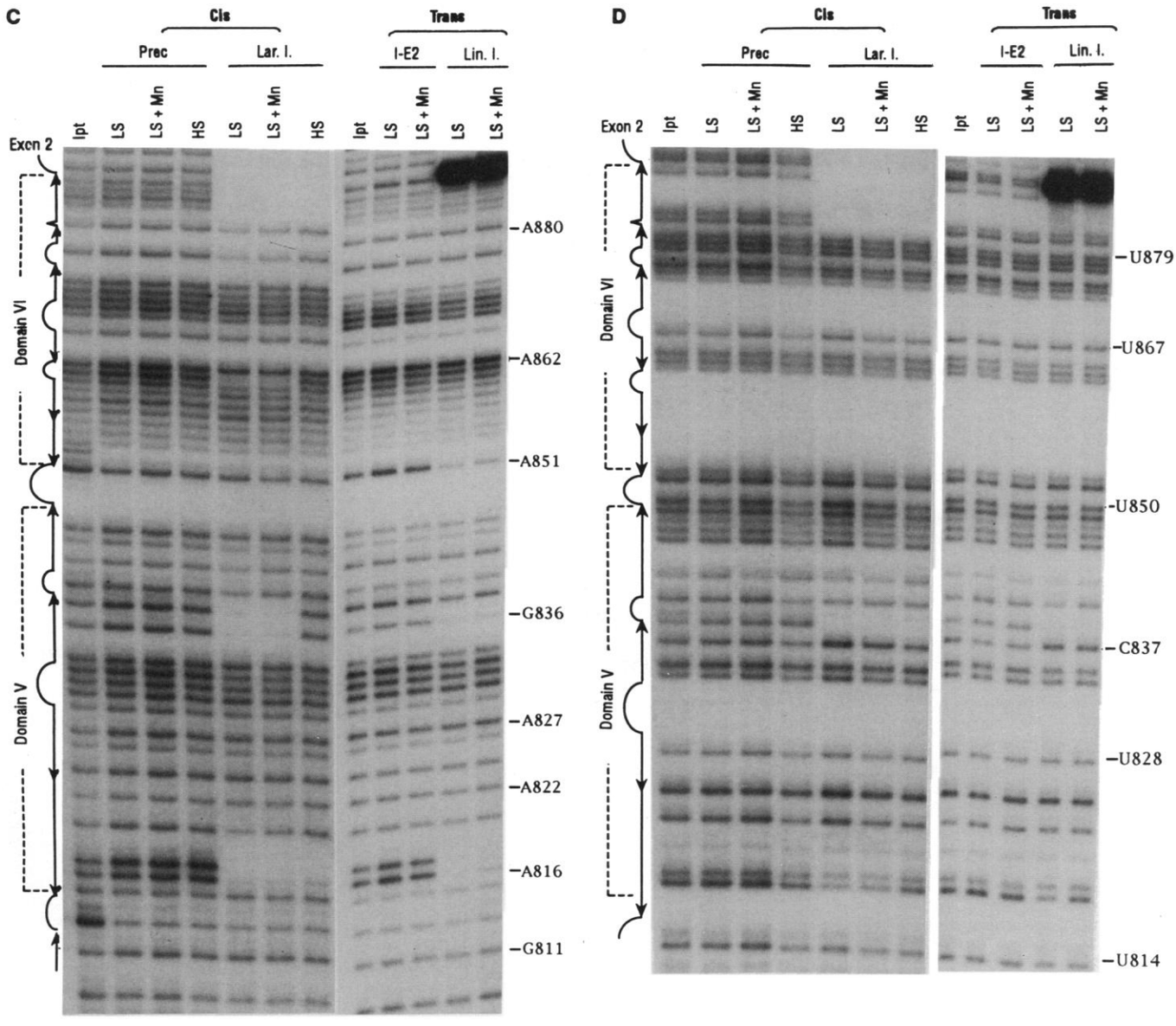
Fig. 2. Modification interference. Abbreviations are the same as in Fig. 1B except for the following: Prec, precursor, and Ipt, modified RNA without splicing. (**A**) DEPC modification. CX RNA labeled at the 5′ end was partially modified by DEPC, annealed with cold complementary subunit, and incubated at 45°C in HS buffer. The remaining precursor and products were subjected to aniline cleavage and analyzed by electrophoresis (*21*). Note that in the cis-splicing assay, positions downstream of the branchpoint were not mapped because of lariat formation that causes a strong shift in migration. (**B**) Hydrazine modification interference. Conditions are as in (A), except that hydrazine was used to remove uracil (*9*). Hydrazine-modified cytidines cannot be mapped from a 5′ end label (*9*). (**C**) (Facing page) Interference of *Rp* phosphorothioate at purines. The CX fragment was transcribed in the presence of low amounts of purine phosphorothioate nucleotides, labeled at the 5′ end, annealed with complementary subunit, and incubated at 45°C in LS buffer without (lanes LS) or with 1 mM $MnCl_2$ (lanes LS + Mn), or in HS buffer. The remaining precursor and products were subjected to iodine cleavage and analyzed by electrophoresis (*21*). (**D**) (Facing page) Interference of *Rp* phosphorothioate at pyrimidines. As in (C), except that pyrimidines phosphorothioate nucleotides were used.

the first step (*10*). (ii) In a trans-splicing assay the CX fragment is annealed to a subunit carrying domains I to III but lacking exon 1 (XC fragment); the resulting molecule corresponds to a linear intermediate that can directly undergo the second splicing step (exon ligation) on addition of exon 1 molecules, thus bypassing the first step. With this latter assay, modified positions, including those blocking the first step, can be tested for the second step. The reaction time courses of the two-subunit intron were similar to those of the wild-type intron in both assays (Fig. 1B).

The modification interference patterns in domains V and VI obtained for lariat formation (cis splicing) and exon ligation (trans splicing) are shown in Fig. 2. Diethyl pyrocarbonate (DEPC) (Fig. 2A) or hydrazine (Fig. 2B) modifications were used to analyze the involvement of bases in splicing. PhosphorImager quantification of the modification effects are summarized in Fig. 3. As expected from sequence conservation (Fig. 3A), the strongest inhibitory effects of base modification are found within domain V. The patterns of modification interference within that domain are very similar in both assays. Mild effects are observed in domain VI, but they are specific to lariat formation. This finding is not surprising because domain VI carries the branch site, one of the substrates of the first splicing step.

In addition, we looked for nonbridging phosphate oxygen atoms involved in splicing by phosphorothioate incorporation (Fig. 2, C and D). Effects of *Rp* phosphorothioate substitutions were analyzed in different buffers: a low salt (LS) buffer, a low salt plus 1 mM $MnCl_2$ (LS + Mn) buffer to look for manganese rescue indicative of divalent cation association with specific nonbridging phosphate oxygens (*11*), and a high salt (HS) buffer which is more permissive to alteration of the intron (*7*).

Sulfur substitution on phosphates 5′ to positions G817 and C839 totally blocked both steps, whatever the conditions used (Fig. 2, C and D) (*12*). Step-specific inhibitions were observed for phosphorothioates 5′ to A838 and A861 (first step) and A851 (second step). However, these defects were weak and could be totally eliminated under high salt conditions. These observations indicate that these oxygens are not directly involved in catalysis. Other inhibitory effects were also observed for both steps (phosphates 5′ to A816, C818, and G836) and could also be eliminated



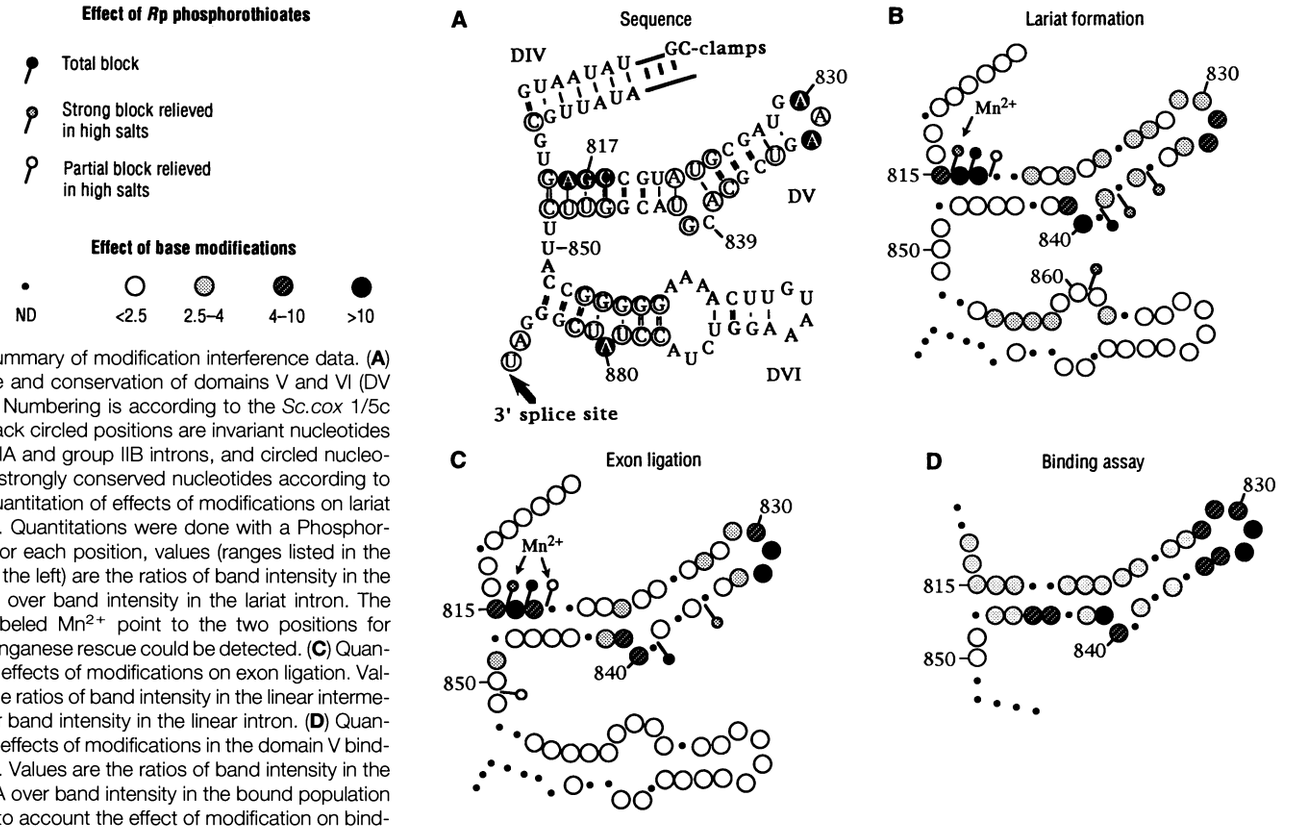


Fig. 3. Summary of modification interference data. (**A**) Sequence and conservation of domains V and VI (DV and DVI). Numbering is according to the *Sc.cox* 1/5c intron. Black circled positions are invariant nucleotides in group IIA and group IIB introns, and circled nucleotides are strongly conserved nucleotides according to (*1*). (**B**) Quantitation of effects of modifications on lariat formation. Quantitations were done with a PhosphorImager. For each position, values (ranges listed in the legend to the left) are the ratios of band intensity in the precursor over band intensity in the lariat intron. The arrows labeled Mn^{2+} point to the two positions for which manganese rescue could be detected. (**C**) Quantitation of effects of modifications on exon ligation. Values are the ratios of band intensity in the linear intermediate over band intensity in the linear intron. (**D**) Quantitation of effects of modifications in the domain V binding assay. Values are the ratios of band intensity in the input RNA over band intensity in the bound population to take into account the effect of modification on binding and on domain V folding.

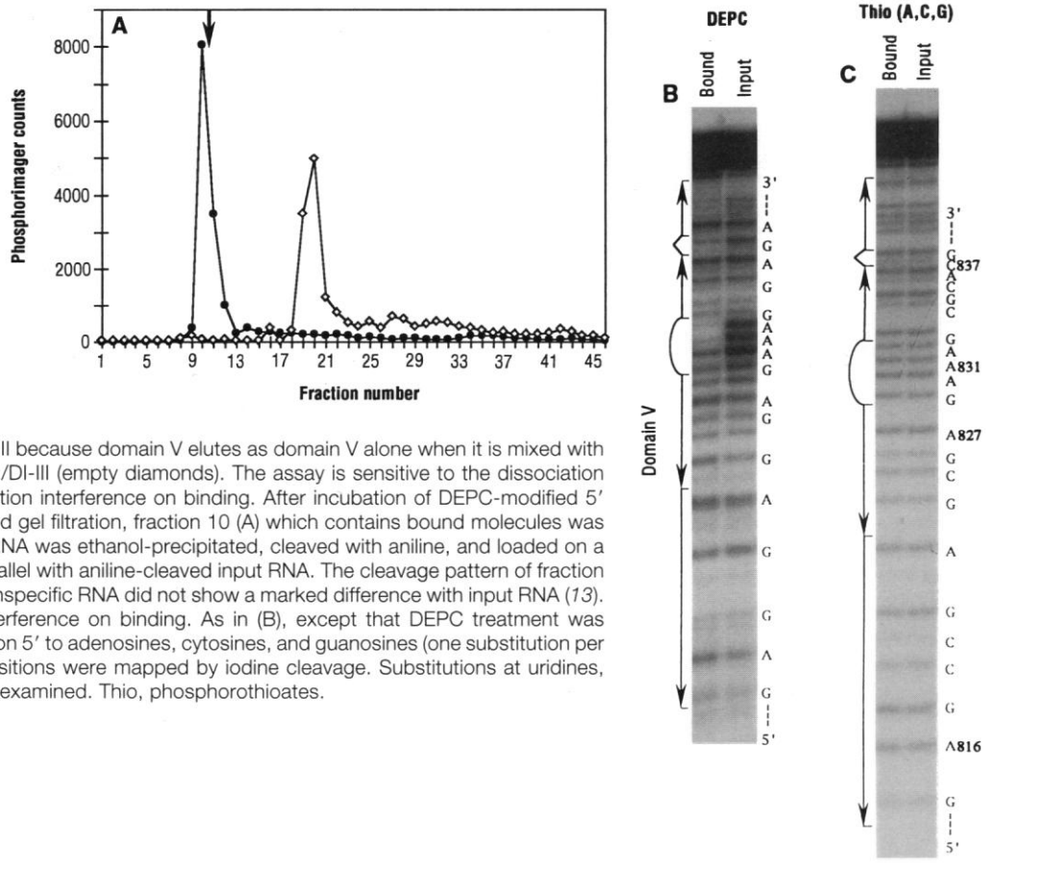


Fig. 4. Modification interference in the binding assay. (**A**) Domain V binding assay. The binding of 5′ ^{32}P-labeled domain V (0.2 mM) to unlabeled E1/DI-III (3 mM) was analyzed by a gel filtration column as described (*14*), with minor modifications (*22*). The curves represent the radioactivity (PhosphorImager counts) measured in each fraction when the ^{32}P-labeled domain V was mixed with unlabeled E1/DI-III (black dots). The peak of radioactivity coincides with the peak of elution of unlabeled E1/DI-III monitored by ultraviolet absorption at 254 nm (arrow at the top of the figure). This corresponds to the specific binding of domain V to E1/DI-III because domain V elutes as domain V alone when it is mixed with a control RNA of the same size as E1/DI-III (empty diamonds). The assay is sensitive to the dissociation constant (K_d) (*14*). (**B**) DEPC modification interference on binding. After incubation of DEPC-modified 5′ ^{32}P-labeled domain V with E1/DI-III and gel filtration, fraction 10 (A) which contains bound molecules was dialyzed against 0.1% SDS, and the RNA was ethanol-precipitated, cleaved with aniline, and loaded on a 20% acrylamide sequencing gel in parallel with aniline-cleaved input RNA. The cleavage pattern of fraction 20 (A) obtained after incubation with unspecific RNA did not show a marked difference with input RNA (*13*). (**C**) Phosphorothioate substitution interference on binding. As in (B), except that DEPC treatment was replaced by thiophosphate incorporation 5′ to adenosines, cytosines, and guanosines (one substitution per molecule on average). Substituted positions were mapped by iodine cleavage. Substitutions at uridines, which do not affect splicing, were not examined. Thio, phosphorothioates.

by high salt conditions [Fig. 2, C and D, cis-splicing assay; the suppression effect was also observed in the trans-splicing assay (*13*)]. Among these inhibitory effects, the effect of sulfur substitution 5′ to A816 and C818 could be partially compensated for (about twofold) by addition of Mn^{2+} (Fig. 2, C and D). Thus, these two positions are likely to be involved in divalent metal ion binding (*11*). The manganese rescue at position C818 was reproducible only in the trans-splicing assay, but this result does not exclude its involvement in metal ion binding during the first step as well.

In summary, only two prochiral-*Rp* phosphate oxygens (5′ to G817 and C839) are strictly required in all conditions, and this is true for both steps. This result may reflect their direct involvement in the active site, but the possibility that these phosphate oxygens, as well as the most important bases, are simply required to maintain the overall structure of domain V or its interactions with the rest of the intron (or both) could not be excluded. To address this possibility, we used modification interference in an assay developed by Pyle and Green that measures the binding of an isolated domain V to an RNA fragment carrying exon 1 and domains I, II, and III (E1/DI-III) (Fig. 4) (*14*). Some base modifications, especially at positions A831 and A832 in the GAAA tetraloop and G840 and U841 (*13*) in or near the bulge (Fig. 3D), showed a strong inhibitory effect on binding. In contrast, no *Rp* phosphorothioate significantly inhibited binding (Fig. 4C). The binding assay was performed under high salt conditions (0.5 M KCl) in which phosphorothioate inhibition was observed only for phosphates 5′ to G817 and C839 (*12*). Thus, the effects of sulfur incorporation at these two positions cannot be explained by binding or structural defects. This result does not exclude the possibility that phosphates showing a strong effect in low salt are involved in binding. With a more sensitive assay, some phosphate oxygens of domain V have been found to be involved in binding (*15*). Two of these (5′ to G836 and A838) correspond to positions showing an effect on activity in low salt conditions only.

A comparison of the results obtained with the different assays (Fig. 3) reveals that some elements clearly appear to be involved in activity for both steps but are not rate-limiting for binding or structure. These are bases A816 and G817 and prochiral-*Rp* oxygens 5′ to nucleotides G817 and C839. In agreement with our data, mutational analysis of the conserved AGC sequence at the bottom of domain V showed that this sequence is required for activity but not for binding (*16*). The essential nucleotides A816, G817, and C839 are likely to be close in space because they are on opposite sides of the helix but separated by half of a helical turn. Thus, all components found essential for activity but not for binding may be clustered in the tertiary structure and are equally required for both catalytic steps. Although it remains to be determined which elements are directly involved in catalysis, these observations suggest that the two steps are catalyzed by active sites that share at least some of their structures. This conclusion is not compatible with the existence of two completely separate active sites and is consistent with the single active site hypothesis (*5*).

In nuclear pre-mRNA splicing, helix I of the U2-U6 small nuclear RNA (snRNA) duplex contains highly conserved nucleotides and a two-nucleotide bulge that splits it into helices Ia and Ib. This helix lies just upstream of the helix carrying the branchpoint (intermolecular pre-mRNA–U2 helix, equivalent to group II domain VI). For these reasons, helix I has been proposed to be the spliceosomal counterpart of domain V in group II introns (*17*). In *Ascaris* U6 snRNA, a thiophosphate 5′ to G49 (G60 in yeast) located in the middle of the universal AGC of helix Ib, totally blocked the first step (*18*). The similarity with inhibition of group II splicing found for thiophosphate 5′ to G817 of the universal AGC sequence at the bottom of domain V suggests that these conserved elements could be functionally related. In this hypothesis, the spliceosomal counterpart of the bottom of group II domain V would be helix Ib, and not helix Ia as previously proposed (*17*).

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21. For base modifications, 15 pmol of CX RNA labeled at the 5′ end with ^{32}P was treated with DEPC or hydrazine as described (*9*) to get less than one modification per molecule and annealed with 30 pmol of complementary subunit (*20*). The precursor was incubated for 1 hour at 45°C in HS buffer. The linear intermediate was incubated 30 min at 45°C in HS buffer with 5 μM exon 1. Gel-purified unreacted precursor and products were cleaved with aniline (*9*), heated 5 min at 95°C in 98% formamide, and loaded on 6% sequencing gels. For phosphorothioate incorporations, transcriptions were performed in the presence of *Sp* phosphorothioate nucleotides (NEN) to allow an average of a single substitution per molecule (*11*). Splicing was in LS buffer [40 mM tris-Cl (pH 7.5), 5 mM $MgCl_2$, and 2 mM spermidine] with or without 1 mM $MnCl_2$, or in HS buffer. Incubation times were adapted to obtain 20% of the reacted precursor in all conditions. Gel-purified fragments were cleaved with iodine (*11*) before electrophoresis.
22. A Sephadex G75 column was used for gel filtration with a Smart System (Pharmacia). The column had a volume of 3.5 ml and a flow rate of 0.1 ml min^{-1}, and 50-μl fractions were collected (the first fraction was collected 7 min after injection).

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