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Three-dimensional model of the active site of the self-splicing rRNA precursor of *Tetrahymena*

(intervening sequence/intron/RNA splicing/RNA structure/"ribozyme")

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ABSTRACT **The rRNA intervening sequence of *Tetrahymena* is a catalytic RNA molecule, or "ribozyme." A tertiary-structure model of the active site of this ribozyme has been constructed based on comparative sequence analysis of related group I intervening sequences, data on the accessibility of each nucleotide to chemical and enzymatic probes, and principles of RNA folding derived from a consideration of the structure of tRNA determined by x-ray crystallography. In the model, the catalytic center has a two-helix structural framework composed of the base-paired segments of the group I conserved sequence elements. The structural framework supports and orients the conserved nucleotides that are adjacent to the base-paired sequence elements; these conserved nucleotides are proposed to form the active site and to bind the 5′ splice-site duplex and the guanine nucleotide substrate. Tests of the model are proposed.**

In self-splicing of the *Tetrahymena* pre-rRNA, the intervening sequence (IVS) mediates its own excision from the precursor with concomitant ligation of the flanking exon sequences. Self-splicing exemplifies intramolecular catalysis in that specific cleavage–ligation reactions are accelerated many orders of magnitude beyond the basal chemical rate (1–3). Shortened versions of the excised IVS RNA act as RNA enzymes ("ribozymes"), facilitating cleavage–ligation reactions on exogenous RNA substrates with multiple turnover. The ribozyme can act as a nucleotidyltransferase and a phosphotransferase, both by a transesterification mechanism (3, 4).

The mechanisms of catalysis of the *Tetrahymena* ribozyme have been partially determined. The RNA provides at least two substrate-binding sites, one for a guanine mononucleotide and one for the last few nucleotides of the 5′ exon or an alternative oligopyrimidine substrate. The guanosine-binding site has not been located within the folded RNA structure, although the functional groups of the substrate that contribute to binding have been identified (2, 5). The oligopyrimidine-binding site has been localized to nucleotides 22–27 of the IVS (6–8). Binding of the oligopyrimidine to its binding site forms the 5′ splice-site duplex. In addition to these binding sites, the IVS RNA provides a catalytic apparatus to facilitate transesterification, perhaps by transition-state stabilization and/or general acid-base catalysis (2, 3).

Not surprisingly, ribozyme activity requires a specific folded structure of the RNA. Activity is lost at high temperatures and in the presence of high concentrations of denaturants such as urea and formamide. Furthermore, small deletions and a variety of single- and double-base substitutions reduce or eliminate activity (7–12). Site-specific mutagenesis has been particularly useful in establishing structure–function relationships, because most mutations alter some aspect of the reaction (guanosine addition, splice-site activation, or specificity) while leaving the others intact.

Based on conserved nucleotide sequence elements, ≈40 IVSs have been categorized along with the *Tetrahymena* rRNA IVS as belonging to group I (13–15). In addition to nuclear rRNA genes, group I IVSs are found in fungal mitochondrial mRNA and rRNA genes, chloroplast rRNA and tRNA genes, and bacteriophage T4 mRNA genes. Self-splicing has been reported for eight other group I IVSs (cited in ref. 3); their splicing mechanism follows that established for the *Tetrahymena* rRNA IVS.

The secondary structure of the *Tetrahymena* IVS RNA was modeled by Waring *et al.* (6) and Michel and Dujon (13) based on comparative structure analysis (16, 17) with other group I IVSs. The basic features of the structure are convincingly proven by the fact that a large number of group I IVSs, nonhomologous in sequence except for four short regions, can be folded into an almost identical core secondary structure. Further, a very similar secondary structure was derived independently by computer modeling based on the results of enzymatic digestion of the native IVS RNA (18). More recently, key features of the secondary-structure model have been confirmed by analysis of splicing-defective mutations and second-site mutations that restore splicing by restoring base-pairing interactions (7, 8, 11, 12). A current version of the secondary structure model is shown in Fig. 1.

An understanding of RNA catalysis requires information at a higher level of folding, that of tertiary structure. The model presented here is an initial attempt to derive such a structure. In evaluating this model, it is important to keep in mind the limited success of such exercises. Only in the case of tRNA do we have a three-dimensional structure of an RNA molecule (reviewed in ref. 21), and in that case even the best of the many proposed models proved to be only partially successful in predicting the structure.

MATERIALS AND METHODS

Principles of Model-Building. Most of the working rules were derived from consideration of the structure of tRNA as follows. (*i*) RNA duplexes were assumed to have A-form RNA helix conformation. (*ii*) If two duplex stems were separated by fewer than three unpaired nucleotides, they were stacked colinearly. (*iii*) If one helix competed with two others for colinear stacking, the two helices separated by the least number of unpaired nucleotides were chosen to form a stacked helix. (*iv*) Non-Watson–Crick base-pairing was allowed at the junction of two helices. (*v*) Single "bulged" bases were stacked within a helix. (*vi*) If two conserved bases in a single-stranded region were in proximity, base-pairing was attempted subject to the constraints of the chemical-

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Abbreviations: IVS, intervening sequence (intron); COB, mitochondrial cytochrome *b*.

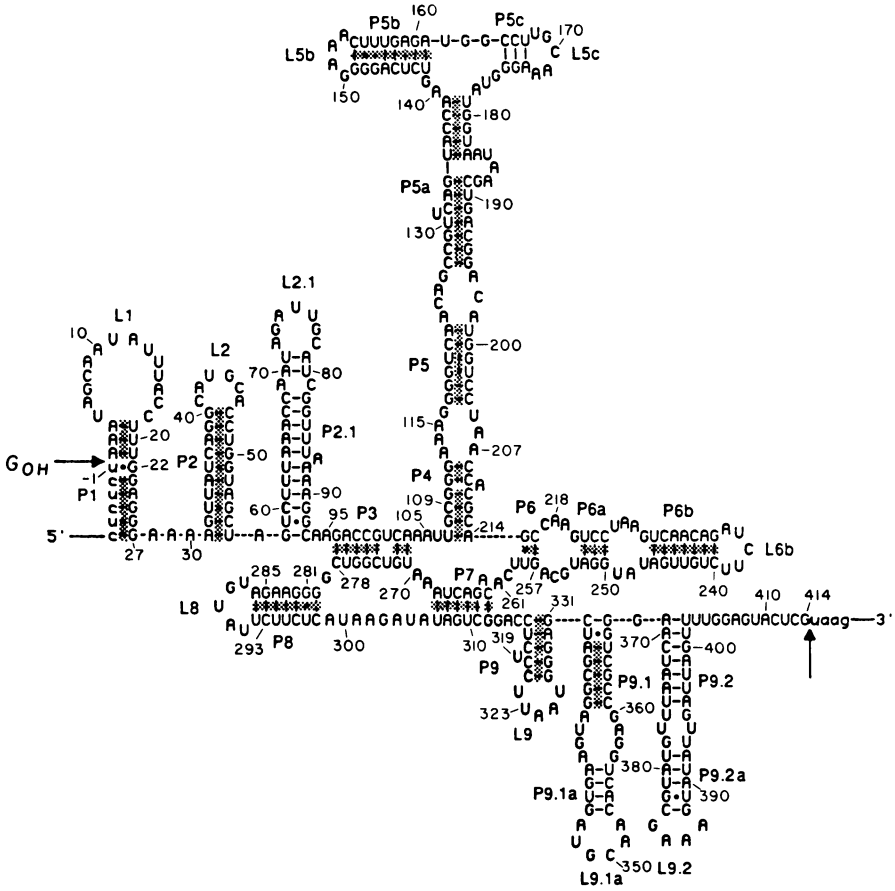

FIG. 1. Phylogenetic conservation of secondary-structure elements in the *Tetrahymena* IVS RNA. Secondary structure is drawn in the standard format for group I IVSs (19). The IVS is in uppercase letters and the exons in lowercase letters. Arrows designate splice sites. Base-paired regions proven by comparative sequence analysis (two or more base-pair changes in the paired region) are shaded. For the paired elements P*i*, the phylogenetic proof involves comparison with diverse fungal mitochondrial IVSs (6, 13–15). P5a, P5b, and P6a are not universal and are variable in length as well as sequence, so they are proven contingent upon the validity of the sequence alignments in these regions. P6b and P9.1 are proven by comparison of IVSs from *Tetrahymena thermophila* and *Tetrahymena malaccensis* (20).

modification data. (*vii*) Loop conformations were taken from those in the tRNA structure.

Molecular Models. HGS molecular models were purchased from Maruzen (Tokyo). The components were for A-form DNA with the substitution of uracil for thymine, ribose sugars, and "arms" and 19.1 spacers for the T-stem of yeast $tRNA^{Phe}$. (The arms determine the tilt of the base pair and its distance from the helix axis, while the spacers determine the rise per base pair.)

Nomenclature. Base-paired regions of the secondary structure are designated P*i* (where integer values of *i* are reserved for stems conserved among group I IVSs), and nucleotides joining two paired regions P*i* and P*j* are designated J*i*/*j*, in accordance with standard nomenclature for group I IVSs (19).

RESULTS

Structure of the Catalytic Center. The catalytic center of the IVS RNA is defined as that portion of the core structure (15) that is directly involved in binding guanosine and the 5′ splice-site duplex (P1) and in catalyzing transesterification. Because all group I IVSs undergo splicing by the same mechanism, our working hypothesis is that the catalytic center is composed of the nucleotides conserved among group I; these are found in paired regions P3, P4, P6, and P7 and the adjacent nonhelical nucleotides.

The tertiary-structure model of the catalytic center, built according to the working rules described in *Materials and Methods*, is shown in Fig. 2 *Left*. A two-dimensional version of the model is given in Fig. 2 *Right*. The catalytic center includes two major helical domains. Domain I is formed by colinear stacking of three shorter helices, P7, P3, and P8. Domain II is formed by colinear stacking of P4 and P6. P5, a 6-base-pair stem extending below residues A^{206} and A^{115} that has not been modeled, would also form part of domain II. The two domains are bridged by J3/4 (A^{104}–U^{106}) and by J6/7 (U^{259}–A^{261}), segments that are particularly rich in conserved nucleotides. The general orientation of the two helical domains is fixed by the conformation of nucleotides at the points where J3/4 and J6/7 join to the middle of duplex regions of domains I and II. These conformations are modeled after that which occurs in the tRNA structure where a single-stranded variable loop and another single strand containing residues 8 and 9 of tRNA join the continuous duplex formed by stacking of the D-stem and amino acid acceptor stem (23). The exact orientation of the two helical domains is subject to refinement; that is, the two helical axes might be nearly parallel, as shown in Fig. 2, or angled significantly with respect to each other.

Within domain I, paired regions P7 and P8 are joined by a single-stranded RNA segment (part of J8/7) composed of the nucleotides U^{300}, A^{301}, A^{302}, and G^{303}. This stretch of 4 nucleotides is able to traverse the length of the 8-base-pair helical segment P3 without difficulty, because the single-stranded region enters and exits on the same side of the P3 helix. The ability of J8/7 to span P3 appears to be general for group I IVSs. J8/7 varies in length from 3 to 5 nucleotides among 36 IVSs (ref. 15; T.R.C., unpublished work). Superposition of these same IVS sequences on the model of Fig. 2 *Right* shows that in 35 cases P3 can be drawn as a 7- or 8-base-pair helical segment. When different lengths of J8/7 and P3 are compared on the physical model, it is seen that a stretch of 3 nucleotides can span a P3 segment of either 7 or 8 base pairs very easily but would require bending or unwinding to span either shorter or longer helical segments. A stretch of 4 or 5 nucleotides can span a greater distance, with the position of closest approach occurring at 7 or 8 base pairs. Thus in 35 cases it is possible for J8/7 to span P3, so that in principle the IVSs could form an approximately coaxially stacked helix composed of P7, P3, and P8. The single exception is the fourth IVS of yeast mitochondrial

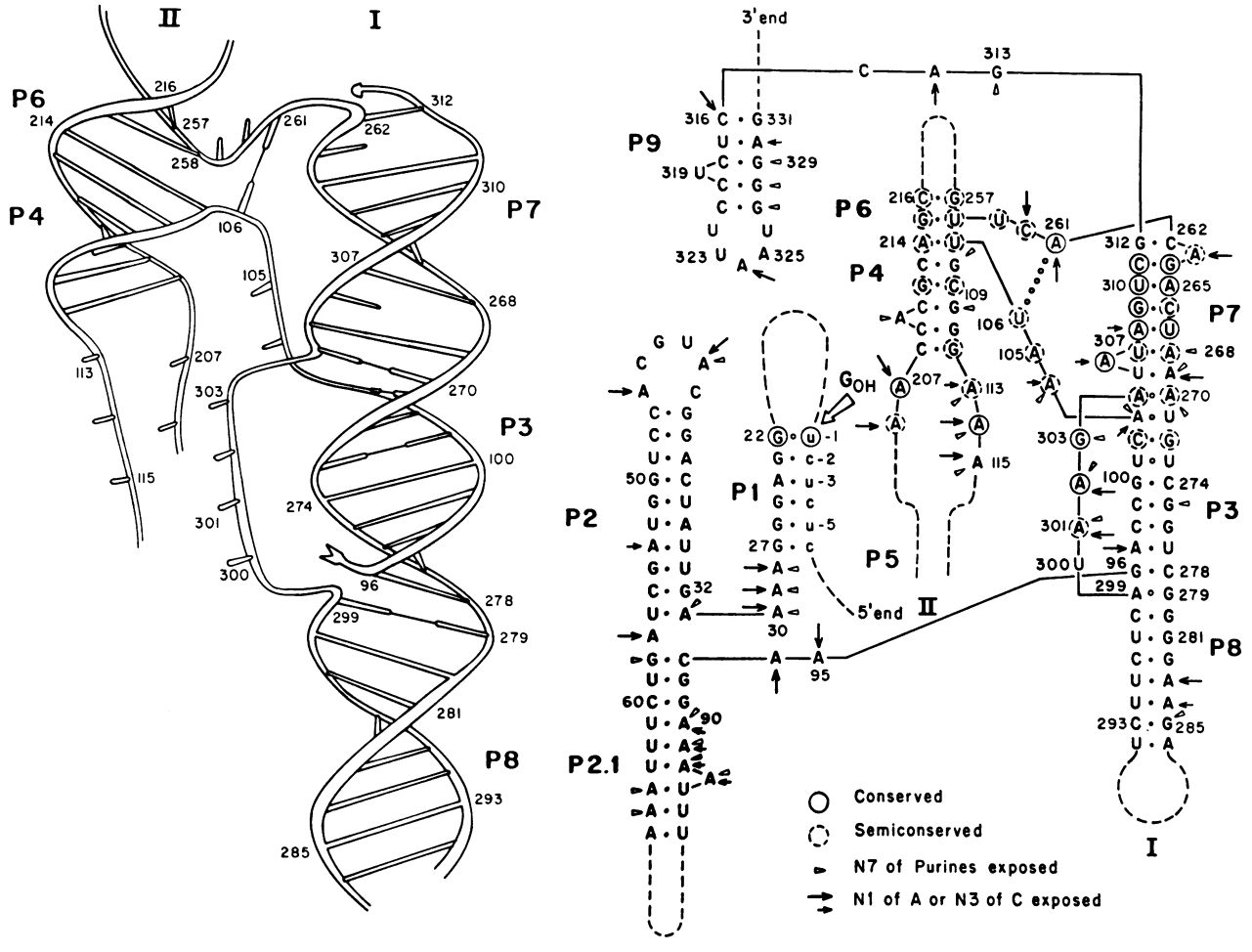

FIG. 2. (*Left*) Model of the three-dimensional structure of the catalytic center of the *Tetrahymena* IVS RNA. (*Right*) Two-dimensional version of the model, including also the paired regions P1, P2, P2.1, and P9. Nucleotides conserved among group I IVSs are indicated with full circles (conserved, >90% have the same base in a given position); broken circles show semiconserved nucleotides (semiconserved, a particular base is found in that position with >70% frequency or either of two bases is found in that position with >90% frequency). Exposure of bases to chemical modification under the standard conditions of ref. 22 is indicated as follows: triangle, modification of N-7 of guanosine by dimethyl sulfate or of N-7 of adenosine or guanosine by diethyl pyrocarbonate; long arrow, observable modification of N-3 of cytidine or strong or medium modification of N-1 of adenosine by dimethyl sulfate; short arrow, weak modification of N-1 of adenosine by dimethyl sulfate.

cytochrome *b* (COB) pre-mRNA, which has a P3 of 6 base pairs, making it less favorable to be spanned by its J8/7 of 3 nucleotides. Several possible solutions to this problem can be imagined, including partial unwinding of P8. (This IVS is unusual in that P8 contains a U/U mismatch, which might facilitate unwinding.)

Details of the Structure. Most of the base pairs in the three-dimensional model are normal Watson–Crick or G·U "wobble" base pairs occurring in helical regions, and are proven by comparative sequence analysis (refs. 13 and 15; T.R.C., unpublished work) and/or analysis of mutations and second-site suppressors (7, 8, 11, 12). In addition, possibilities for several non-Watson–Crick base pairs were directly suggested by the model building. These are admittedly speculative and need to be tested experimentally. These pairs, indicated by open dots in Fig. 2 *Right* and by thin lines in Fig. 2 *Left*, are as follows.

A^{261}·U^{106}. The model includes a reverse Hoogsteen base pair involving N^6 and N-7 of A^{261} and O^2 and N-3 of U^{106}. Such base pairing does not contradict the strong N-1 methylation of A^{261}. There is no phylogenetic basis for evaluating this interaction, because the position equivalent to 261 is occupied by an adenosine in all group I IVSs. In the position equivalent to U^{106}, most other group I IVSs have either an adenosine or a uridine; an A·A base pair similar to the proposed A·U base pair is easily accommodated by the model and provides the rationale for postulating a reverse Hoogsteen rather than a Hoogsteen or Watson–Crick base pair in the *Tetrahymena* IVS.

A^{269}·U^{305}. This interaction can be modeled as either a Watson–Crick or a Hoogsteen base pair. The strong N-1 methylation of A^{269} might indicate the Hoogsteen base pair, or the pairing might be dynamic. This type of extension of P7 is possible in only 22 of the 38 group I IVSs analyzed. In the other cases, the most common juxtapositions of bases in these positions would be U/U and C/U.

A^{270}·A^{304}. This interaction is modeled as a symmetric base pair involving N^6 and N-7 of both adenines (24). Superposition of the other group I IVSs on the model reveals that this interaction would be A·A in 27 cases, other likely base pairs (A·U, A·G, and C·A) in 10 cases, and an uncommon pair (G·G) in only one case.

U^{271}·A^{103}. This interaction can be modeled with a single hydrogen bond between O^2 of uridine and N^6 of adenosine in the case of the *Tetrahymena* IVS, but there is no phylogenetic evidence for this interaction in general.

U^{273}·U^{101}. This interaction is modeled as an asymmetric base pair (24), but nonpairing of these bases is equally tenable and need not have any major structural consequence. While P3 normally contains a Watson–Crick base pair at this position, the *Neurospora crassa* ND4L IVS also has a U/U

juxtaposition at this site (25) and there are several examples of G·U pairs.

G^{279}·A^{299}. This interaction is modeled with both bases in the *anti* orientation, as found in tRNA (21). The proposal of a G·A base pair at this position is supported by comparative sequence analysis; it is a G·U base pair in eight group I IVSs, a putative G·A pair in two others, and a standard Watson–Crick base pair in all the rest.

Interaction of the 5′ Splice-Site Duplex with the Core Structure. A major function of the catalytic center is to bind the 5′ splice-site duplex (P1) in such a way as to facilitate its attack by guanosine (2, 3, 6, 7, 26). We propose that this binding must have both a sequence-independent component and a component that specifically recognizes the G·U pair at the reaction site. Binding of the body of the helix in a largely sequence-independent manner is indicated by the ability of a variety of other base pairs to substitute for the base pairs that occur naturally in P1 (7, 8) and by the lack of phylogenetic conservation of these sequences. Specific binding of U^{-1}·G^{22} is indicated by the inactivity of other base pairs in this position (except C·G; E. T. Barfod and T.R.C., unpublished data) and the extreme phylogenetic conservation of these bases (13–15).

Although the model shown in Fig. 2 does not define a unique site for interaction of the 5′ splice-site duplex, one site of interaction seems particularly attractive. The two helical domains of the catalytic center form an open pocket or depression lined with stacked, single-stranded residues 113–115, 207–208, and 301–303; the bulged adenosine at position 306 and the nucleotides at positions 104–106 are also close enough to participate. All of these single-stranded residues are conserved or semiconserved among group I IVSs, indicative of some critical role. Most are highly accessible to chemical modification in the circular IVS RNA, which does not contain a 5′ splice-site duplex, and thus they should be available for interaction. Binding of the 5′ splice-site duplex in this pocket is shown in Fig. 3. Binding is proposed to involve mainly interactions with the phosphates and 2′-hydroxyls of the backbone of P1, thereby accounting for sequence-independent binding. It must be reiterated that this is a speculative proposal; relatively minor rearrangements of the model of the catalytic center would allow P1 to be accommodated in other sites, including a position between P5 and P7, or a position on the back side of the core structure.

The path of the RNA chain between P1 and P3 has not been modeled. The joining segment consists of five single-stranded adenosine residues as well as the ends of paired elements P2 and P2.1. This segment is long enough and, based on susceptibility to chemical modification, flexible enough to permit binding of P1 in many positions, including that indicated in Fig. 3.

P9 is a small duplex that is not conserved in sequence but is conserved in location among group I IVSs; it is almost invariably separated from P7 by 3 nucleotides. (In the *Tetrahymena* IVS, the two of these whose structure was analyzed were heavily modified and therefore presumably single-stranded.) It is possible that P9 interacts with P1 from the side opposite that of domains I and II (Fig. 3). In this manner it might help bind P1 in the active site. Structures beyond P9 are not required for catalytic activity (26) and have not been modeled.

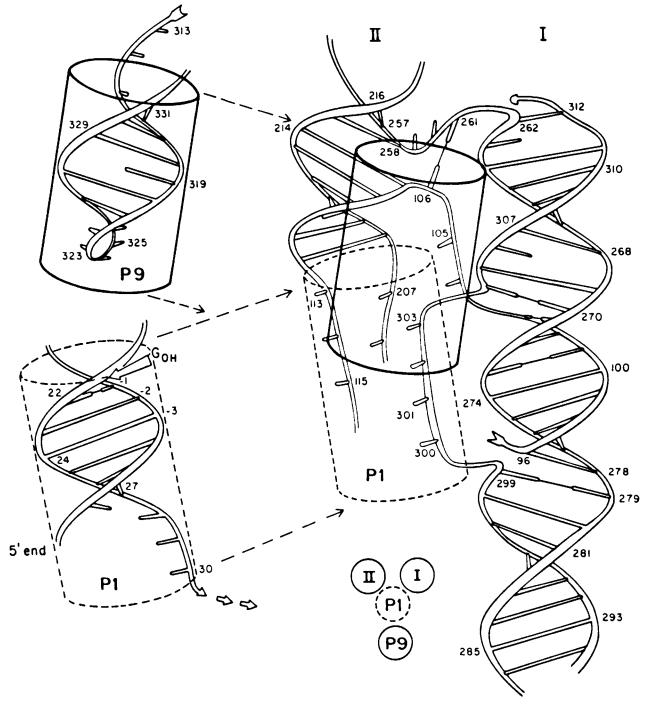

FIG. 3. Possible interaction of the 5′ splice-site duplex (P1, dashed cylinder) with the catalytic center of the *Tetrahymena* IVS RNA. Paired element P9 (solid cylinder) might interact with P1 from the side opposite that of domains I and II of the catalytic center as shown. Small diagram at bottom depicts top view of the model. See text for details.

DISCUSSION

Principles. The secondary structure of the *Tetrahymena* IVS RNA is largely established, based on extensive comparative sequence analysis (6, 13–15), characterization of splicing-defective mutants and second-site revertants (7, 8, 10–12), and determination of the accessibility of individual residues to an array of chemical and enzymatic structure probes (18, 22, 27). We have now extended the structure model to three dimensions by application of RNA-structure rules derived from a consideration of tRNA structure. Additional constraints were imposed by the results of comparative sequence analysis and chemical-modification studies; the latter proved to give information quantitatively consistent with the known structure of tRNA (28).

Comparative sequence analysis has proven to be a powerful predictor of RNA structure where functionally equivalent RNAs are being compared, as in the case of the rRNAs (16, 17, 29). Diverse group I IVSs are functionally equivalent in that they all undergo splicing by the same biochemical mechanism, in all cases directed by the folded structure of the RNA (1, 2, 15, 30). However, the reactivity of the RNA by itself is not equivalent for all the IVSs. The *Tetrahymena* IVS undergoes self-splicing with high efficiency in solutions containing 5–10 mM Mg^{2+}, whereas self-splicing of some of the other IVSs requires high Mg^{2+} concentrations (refs. 31–33; cf. ref. 34) and even then may proceed with lower efficiency (35). Protein binding is presumably necessary for these IVSs to splice with high efficiency under conditions found *in vivo*. Some group I IVSs, such as the mitochondrial rRNA IVS of *N. crassa* and COB IVS 4 of yeast, undergo no detectable self-splicing *in vitro*; in these cases, proteins are presumably essential for formation of the splicing-competent structure (30, 31, 36). Thus, we have applied comparative sequence criteria with some discretion when considering the *Tetrahymena* IVS structure. If the possibility of formation of a given base pair, such as A^{269}·U^{305}, is not phylogenetically conserved, it could indicate that protein binding compensates for the deficit in RNA structure in other IVSs.

Chemical-modification data were also applied with some discretion. Structural dynamics are presumably necessary for reactivity (2). In particular, there is evidence that P7 may be a dynamic structural element (22, 27). Thus, it would be misleading to consider all chemically modified residues as obligatorily single-stranded. It seems most reasonable to

expect chemical modification of base-paired nucleotides when they occur at the ends of helices (note A^{31}, G^{58}), in A+U-rich regions or adjacent to bulged residues within helices (note A^{64}, A^{65}, A^{87}–A^{90}, G^{110}), or adjacent to non-standard base pairs that might give some local disruption of a helical structure (perhaps A^{269}).

The Structure Model. We propose that the catalytic center of the IVS is composed of a two-helix structural framework that supports and orients the nonhelical residues conserved among group I IVSs. The nonhelical residues—which include the adenosines at positions 113–115, 206, and 207, the conserved trinucleotides at positions 301–303, 104–106, and 259–261, and the bulged adenosine at position 306 (and perhaps that at 263)—either provide functional groups that participate directly in the reaction or form tertiary-structure interactions that have not yet been established.

A striking feature of the model is the way in which it brings the conserved residues together. With the exception of the G·U pair in P1 that defines the 5′ splice site and G^{414}, which defines the 3′ splice site, all of the conserved nucleotides are either part of domain I or II or in one of the linkers between these two domains (Fig. 2 *Right*). The distances are such that many of the conserved nonhelical nucleotides could make direct contact either with the 5′ splice-site duplex or with the guanosine that must be held in place at the 5′ splice site.

An attractive feature of the model of the catalytic center of the *Tetrahymena* IVS RNA is how well it accommodates the other group I IVSs. It is expected that the helical regions be accommodated, because the conserved secondary structure is preserved in the tertiary structure. More significant is the fact that the two strands that link domains I and II are highly conserved in sequence and essentially invariant in length. The first linker (J6/7) has the sequence UCA or GYA, where Y is a pyrimidine, and invariably consists of 3 nucleotides. The second (J3/4) has been commonly shown as either a 3- or a 4-nucleotide region in secondary-structure models of various group I IVSs. However, in ND1 and ND5 IVSs of *N. crassa* (25, 37) and in ND1 IVSs 1 and 2 and the cytochrome oxidase IVS of *Podospora anserina* (38), where J3/4 appears to be 4 nucleotides long, the adjacent helix P3 begins with an unpaired cytidine (at the position equivalent to U^{271} in the *Tetrahymena* IVS). If the first adenosine of J3/4 were paired with that cytidine, P3 would be increased from 6 to 7 or from 7 to 8 base pairs, both of which are compatible with the model, and J3/4 would be decreased from 4 nucleotides to 3 nucleotides, its common length. Thus, J3/4 may be 3 nucleotides (consensus sequence AWW, where W is A or U) in almost all cases, with rare exceptions such as 2 nucleotides in *N. crassa* COB IVS 1 (39).

Testing the Model. The best test of the model would be structure determination via x-ray crystallography or NMR spectrometry. However, the IVS RNA has not yet been crystallized, and NMR seems premature until the size of the active unit can be reduced considerably. In the absence of direct structure determination, some refinement of the structure model is possible through site-specific mutagenesis, chemical modification, and active-site probes.

Additional mutagenesis has already been completed since the model was built. A triple mutant (A→C at position 301, A→C at 302, and G→C at 303) eliminates self-splicing but does not affect activation of the 3′ splice site and appears not to affect guanosine binding (12). Thus, the phenotype of this mutant is consistent with our proposal that nucleotides 301–303 might help bind the 5′ splice-site duplex.

Additional mutagenesis will serve to test the non-Watson–Crick base pairs hypothesized above. In some cases, substitution of natural base pairs will not permit a rigorous test, and substitution of individual functional groups will be required. Other aspects of the model can be tested by deletion mutagenesis. For example, we predict that deletion of 1 base pair from P3 will be tolerated, but deletion of 2 base pairs or addition of 1 base pair will inhibit self-splicing. Mutagenesis should also lead to the identification of nucleotides that form the guanosine binding site, which will provide additional constraints that can be used to refine the model.

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