Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 10400–10404, November 1992
Biochemistry

A region of group I introns that contains universally conserved residues but is not essential for self-splicing

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Communicated by Leslie E. Orgel, July 9, 1992 (received for review May 19, 1992)

ABSTRACT **The catalytic core of the self-splicing group I intron RNAs is composed of six paired regions together with their connecting sequences; these are thought to form two elongated domains, with paired regions P5, P4, and P6 aligned along one axis and P8, P3, and P7 along the other. Most of the very highly conserved residues of the group I introns lie in or near P7, but two occur in L4, the internal loop connecting P4 and P5. It is generally believed that such bases are conserved because they are essential for splicing. Mutants were created in a member of each of the two major subclasses of group I introns, in which P5, L4, and the distal portion of P4 were deleted. Splicing activity was still detected in these mutants, albeit substantially weakened; splicing was accurate and occurred by the normal group I mechanism, with addition of a guanosine molecule to the intron. Thus the deleted region, containing two universally conserved bases, is not essential but facilitates splicing. Another reaction characteristic of group I introns, hydrolysis of the 3′ splice site, was less severely affected by the deletions. The results are discussed in terms of the prevailing three-dimensional model for the core structure of the group I introns.**

Some RNA splicing is accomplished wholly or partly by autocatalytic activity of the introns themselves; self-splicing at neutral pH in the presence of magnesium ions has been demonstrated for several group I (1) and group II (2) introns in isolation from any other macromolecules. On the other hand, mRNA splicing in eukaryotic nuclei is catalyzed by the spliceosome, a large complex of multiple RNA and protein species (3). Strong mechanistic similarities between splicing by the spliceosome and by the self-splicing RNAs have led many to suspect that the catalytic functions of the spliceosome reside primarily among its RNA molecules (4, 5). Extensive study has allowed some assignment of functions to particular group I intron substructures (1). In the self-splicing RNAs, such components must be strategically arrayed within the confines of a single molecule of linear primary structure, whereas the trans-acting nature of the spliceosome allows for the partitioning of functional domains among separate molecules (5, 6).

The group I introns comprise a large homologous family; sequence alignments reveal several characteristic features of secondary structure (Fig. 1), which have been confirmed by mutational studies, and at several positions the base identity is conserved. A recent alignment identified three universally or very highly conserved bases (present in at least 85 of 87 introns) located at or near the splice sites, and eight within the intron core (7). Three-dimensional modeling (7, 10) suggests that the intron core is arranged as two elongated domains, with paired regions P5, P4, and P6 aligned along one axis and P8, P3, and P7 along the other. Two nearly universally conserved bases occur in the putative P5–P4–P6 domain of the intron core, an adenosine in each half of the internal loop L4, which connects P5 and P4 (the single exception to the conservation is an intron that apparently lacks the adenosine of the upstream half of L4); the six other very highly conserved core bases fall in or near P7. The several conserved bases and structural features of group I introns reflect the sophistication of the splicing process, in which the exogenous guanosine molecule that initiates splicing must be properly positioned, both splice sites precisely specified, and two transesterification reactions sequentially coordinated: nucleophilic attack by the 3′ hydroxyl of the guanosine molecule at the phosphorus atom of the 5′ splice site creates a 3′-hydroxyl terminus on the 5′ exon, which subsequently attacks the 3′ splice site, concertedly joining the exons and completing the excision of the intron.

The concentration of conserved bases in the vicinity of P7, including the base that has been established as part of a binding site for the guanosine molecule that initiates splicing, certainly focuses attention on the role of this region in the catalysis of splicing, but also raises the question of whether the L4 element, located in a distinct domain, is essential or auxiliary for splicing activity. Some clues to the role of L4 have emerged. In a study of the effect of single-base substitutions in the *Tetrahymena* intron on a reaction equivalent to the first step of self-splicing, substitutions of either of the two universally conserved adenosines in the L4 loop exhibited particularly deleterious effects (11). Three-dimensional modeling of the group I intron has suggested that L4 and the J8/7 sequence, which connects P8 and P7 and contains very highly conserved bases, comprise two half-sites that properly position the 5′ splice site for splicing by binding the P1 stem; recent experiments have provided evidence for the physical proximity of P1, L4, and J8/7 (12). To further examine the role of L4 in splicing, we have studied mutants of group I introns in which L4 and flanking structural elements were entirely deleted. Our data demonstrate that self-splicing activity can be retained upon deletion of universally conserved group I intron core residues.

MATERIALS AND METHODS

RNAs. RNAs were produced by transcription of linearized plasmids using T7 RNA polymerase with uniform [^{32}P]GMP labeling (13). Mutations were generated in plasmids by using the polymerase chain reaction as described (14), and verified by sequencing the DNA for the entire intron. The starting material for the *Tetrahymena* intron mutations was pTZIVSU (15); the starting material for the ΔL4 mutant of the T4 *td* intron was plasmid pΔP6-2 (9), a gift of J. Salvo and M. Belfort. The originally described (16) P5abc RNA had extraneous sequence "tails" at both the 5′ and 3′ ends; a transcription template was designed to yield a "tailless" version

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Abbreviation: nt, nucleotides.

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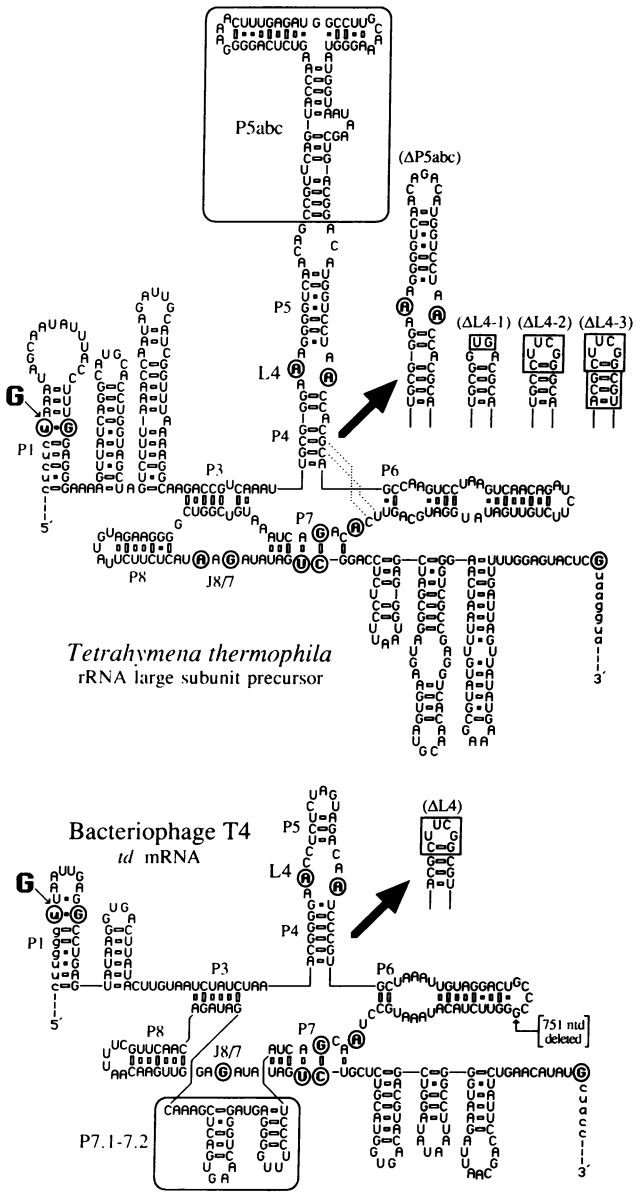

FIG. 1. RNAs used in this study. The sequences of the group I introns from *Tetrahymena* (*Upper*) and the *td* mRNA of phage T4 (*Lower*) are arranged in the standard secondary structure, with the universally or very highly conserved bases (present in at least 85 of 87 group I introns; ref. 7) in boldface and circled (note that the *td* intron lacks the very highly conserved adenosine of the J8/7 region). Intron sequences are in uppercase, and exon sequences in lowercase. The large "G" represents the guanosine molecule that attacks the 5′ splice site to initiate self-splicing. Rounded boxes indicate the structures representative of the two major subclasses of the group I intron family. Dotted lines indicate proven tertiary interactions involving the P4 stem of the *Tetrahymena* intron (8). The sequence replacing the P4–P5 arm in each deletion mutant is specified; boxes indicate altered bases. The parental version of the *td* intron used in this study had a large deletion of sequence outside the catalytic core [751 nucleotides (ntd) as indicated]; this deletion has little effect on catalytic activity (9).

of P5abc (in which the two closing base pairs were inverted from C·G to G·C). These two forms of P5abc were prepared without ^{32}P-labeling; comparative titrations showed that they were indistinguishable in their activity as cofactors for splicing by ΔP5abc (16).

Ribozyme Assay. RNA precursors were incubated at 8 nM in 10-μl reaction volumes in either hydrolysis buffer (50 mM Epps, pH 8.3/5 mM $MgCl_2$) or splicing buffer (50 mM Mops, pH 7.0/5 mM $MgCl_2$/200 μM GTP), with additional conditions specified in the figure legends. At the end of the incubation interval, the samples were analyzed by electrophoresis in denaturing polyacrylamide gels.

Assay of Splicing Accuracy. The putative ligated-exon RNA products of ribozyme assays were purified by gel electrophoresis and subjected to reverse transcription; the products were amplified by the polymerase chain reaction and sequenced with dideoxynucleotides (17).

RESULTS

The most extensively studied group I intron is that from *Tetrahymena thermophila* (Fig. 1). It contains an extension of the P5–P4–P6 domain termed P5abc, a feature representative of almost half the group I introns (7). A mutant precursor deleted of P5abc (ΔP5abc), although drastically weakened under standard self-splicing reaction conditions, still contains all the essential components for self-splicing, as observed at elevated magnesium ion concentrations and in the presence of spermidine (18). Moreover, the separately synthesized P5abc molecule can function as an activator in trans, allowing efficient splicing of ΔP5abc under mild reaction conditions (16). Gel mobility-shift assay shows that P5abc RNA binds ΔP5abc; tertiary interactions between these two parts of the intron must determine its activity, since there is no obvious opportunity for Watson–Crick base pairing. We exploited this bimolecular system to study the L4 internal loop of the group I intron core, which contains two universally conserved adenosine residues.

L4 deletion mutations of the *Tetrahymena* intron were designed based on previous studies of the flanking regions. A mutant deleted of P5, distal to L4, retained catalytic activity (19); on the other hand, disruption of the postulated base trios between the proximal portion of P4 and residues downstream from P6 (Fig. 1, dotted lines) resulted in drastic loss of activity (8). We replaced the entire portion of the wild-type *Tetrahymena* intron distal to the middle of P4, including P5abc, P5 and L4, with different types of four-nucleotide loops ("tetraloops"), retaining the proximal portion of P4 and, presumably, its capacity to form the two base trios mentioned above. Loop sequences of the formulae GNRA or UNCG (R denotes either A or G; N denotes A, C, G, or U) occur especially frequently in natural RNAs and have been found to adopt characteristic conformations that lend stability to the base-paired regions they cap (20). Replacement with the tetraloop GUGA created the mutant ΔL4-1, and replacement with UUCG created the mutant ΔL4-2; since the UUCG tetraloop structure is more stable when closed by a C·G base pair than when closed by G·C (20), the terminal base pair closing the tetraloop in the latter mutant was inverted accordingly (Fig. 1). A third mutant, ΔL4-3, was based on ΔL4-2, but the three proximal base pairs of P4 were inverted, disrupting the P4 base trios.

At slightly alkaline pH, in the absence of guanosine, the typical reaction observed for group I introns is specific hydrolysis of the phosphodiester bond at the 3′ splice site, producing 5′-phosphate and 3′-hydroxyl termini (21); the P5abc–ΔP5abc bimolecular system also performs this reaction efficiently (16). This reaction does not involve the 5′ splice site; in fact, it can occur even when all the sequence upstream of P3 is deleted (22). Due to the putative role of L4 in aligning the 5′ splice site during self-splicing, specific hydolysis appeared a more promising reaction by which to observe catalytic activity of the L4 deletion mutants. We incubated ΔP5abc and each of the three L4 deletion mutants with increasing amounts of P5abc under hydrolysis conditions (Fig. 2). All the mutants underwent specific hydrolysis, with P5abc dependence similar to that of ΔP5abc; activity reached a plateau at ≈1 μM P5abc. The unchanged P5abc

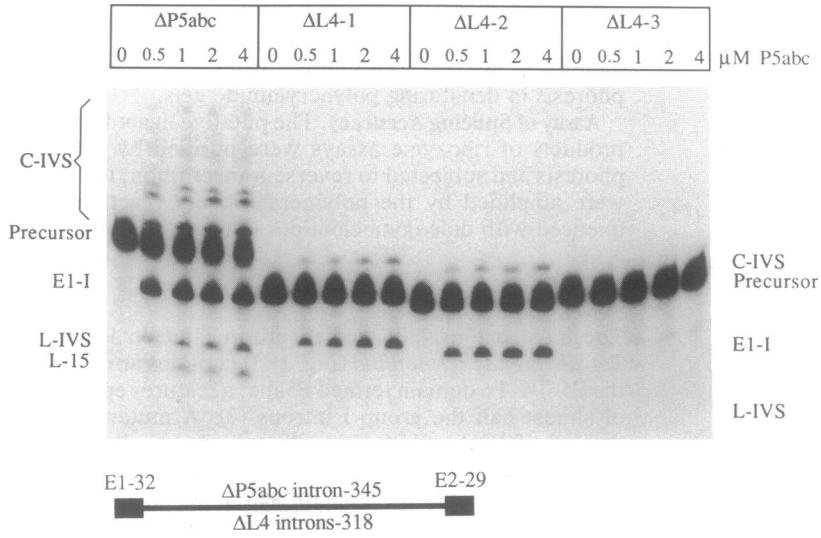

FIG. 2. Activator RNA dependence of specific hydrolysis for deletion mutants of the *Tetrahymena* intron. Precursor RNAs produced from *Hin*dIII-digested templates bearing the indicated deletion mutation of the *Tetrahymena* intron were incubated at 37°C for 60 min in hydrolysis buffer containing the indicated amounts of tailed P5abc RNA. Products of ΔP5abc, marked on the left, are 27 nucleotides longer than their counterparts from the ΔL4 mutant series, marked on the right: E1, 5′ exon; E2, 3′ exon; I, intron; L-IVS, linear form of the excised intron; C-IVS, circular form(s) of the excised intron; L-15, linear form of excised intron lacking the first 15 nucleotides. The diagram below the autoradiogram indicates the relative sizes (in nucleotides) of introns and exons.

dependence suggests that none of the deleted areas or altered bases is important for P5abc–intron binding; direct binding assays are required to test this implication. Separate kinetic analysis using excess P5abc (unpublished data) indicated that the hydrolysis rates of ΔL4-1 and ΔL4-2 were only 5.0- and 3.5-fold lower, respectively, than that of ΔP5abc; that of ΔL4-3 was ≈50-fold lower than that of ΔP5abc, presumably due to the disruption of the base trios involving the base of the P4 stem. We observed another well-known reaction of the *Tetrahymena* intron, formation of circular RNA, for ΔL4-1 and ΔL4-2; very small amounts of the linear intron were also produced, probably due to reopening of the circular form. Cyclization indicates that the mutant RNAs are capable of performing a transesterification reaction at the 5′ end of the intron.

The mutants were then assayed for self-splicing, a process requiring two sequentially coordinated transesterification reactions, by incubation in the presence of P5abc and guanosine at neutral pH (Fig. 3*A*). In addition to the guanosine-independent products of specific hydrolysis and cyclization, the guanosine-dependent products expected from normal self-splicing were observed for ΔL4-1 and ΔL4-2, but the reaction was impaired relative to ΔP5abc; in a separate kinetic experiment using excess P5abc and GTP (unpublished data), the splicing rate for ΔL4-1 was found to be 51-fold lower than that for ΔP5abc. No evidence for self-splicing was observed for ΔL4-3. To confirm splicing in the absence of L4 and to examine its accuracy, the putative self-splicing reaction product (ligated-exon RNA) of ΔL4-1 was isolated and converted to cDNA, which was amplified by the polymerase chain reaction, and sequenced (Fig. 3*B*). The only prominent bands in the sequence ladder were those expected for the correctly ligated exons, demonstrating that at least the majority of ligated-exon RNA was accurately spliced and confirming that L4 is not required for splicing by the *Tetrahymena* intron.

We examined whether our results concerning L4 extended to the other major subclass of group I introns, in which a structure equivalent to P5abc is lacking, but extra sequence exists between P7 and P3, as exemplified by the P7.1-7.2 element of the *td* intron of phage T4 (Fig. 1). Starting with the ΔP6-2 form of the *td* intron (9), which has been deleted of a large coding region that has little effect on self-splicing, we replaced the portion distal to the middle of P4, including P5 and L4, with a UNCG-type stable tetraloop closed by a C·G base pair (Fig. 1). This L4 deletion mutant was found capable of guanosine-dependent self-splicing, yet like the counterpart mutant of the *Tetrahymena* intron, its activity was drastically reduced in comparison to the parental intron (Fig. 4*A*). The ΔP6-2 *td* intron utilizes an upstream cryptic 5′ splice site (23) almost as efficiently as the correct 5′ splice site (ref. 9; Fig. 4*A*), but the L4 deletion mutant appears unable to utilize the cryptic site. The ligated-exon RNA produced by the L4 deletion mutant *td* intron was sequenced indirectly as described above for the *Tetrahymena* mutant, and again no evidence of splicing inaccuracy was found, at least for the majority of the RNA (Fig. 4*B*).

A consequence of the group I intron self-splicing pathway is that the guanosine nucleophile that initiates splicing becomes covalently attached to the 5′ end of the intron. To confirm that self-splicing in the absence of L4 follows the normal mechanism, we prepared the L4 deletion mutant *td* precursor without ^{32}P-labeling and allowed self-splicing to occur in the presence of [α-^{32}P]GTP (Fig. 5). As has been previously observed for the *td* intron (24, 25) and other group I introns (21), the primary [α-^{32}P]GTP-labeled species had the gel mobility of the linear intron, with lesser labeling of a band corresponding to the splicing intermediate lacking the 5′ exon, indicating that splicing in the absence of L4 proceeds via guanosine attack at the 5′ splice site.

DISCUSSION

The L4 internal loop contains two adenosine residues that are universally conserved among the group I introns. For both the bacteriophage T4 *td* and *T. thermophila* introns, representative of the two major group I subclasses, we have shown that L4 and its flanking sequences are auxiliary but not essential for accurate self-splicing via the normal pathway. The strategy of complete deletion used here makes the point unambiguous; not only are the bases of the deleted region nonessential, so must be any tertiary interactions involving its sugar-phosphate backbone. The particular sequence of the small loop used to replace the deleted region was not critical; two different replacement sequences (those of ΔL4-1 and ΔL4-2) yielded ribozymes with equivalent activity levels. An extensive region of the intron was deleted in the ΔL4 mutants, and in principle the observed diminution of self-splicing activity could be ascribed to any or all of the region from P5 to the middle of P4; however, we suspect that it is due primarily to the absence of the conserved L4 adenosines; a mutant deleted of P5 as well as P5abc but retaining L4 exhibits a phenotype more similar to that of ΔP5abc (18), whereas base-substitution mutants of the L4 adenosines behave more like our ΔL4 deletion mutants (ref. 11; unpublished work).

Our results can be interpreted in the context of the three-dimensional structural model of the group I intron core

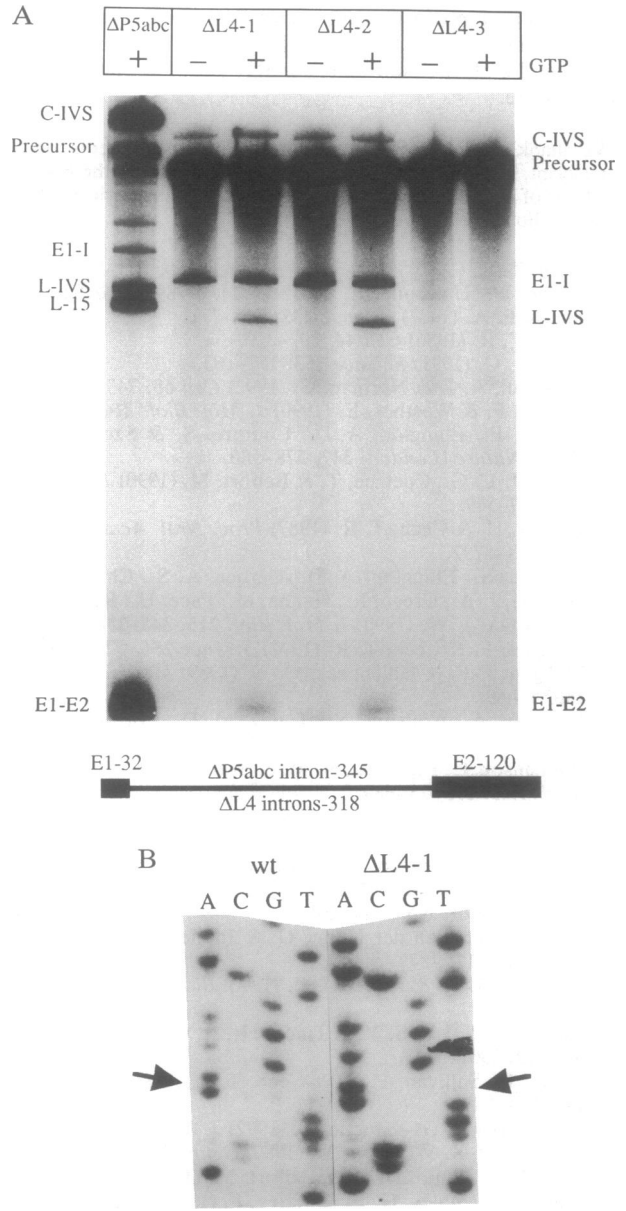

FIG. 3. Accurate self-splicing by the *Tetrahymena* intron in the absence of L4. (*A*) Precursor RNAs produced from *Pvu* II-digested templates bearing the indicated deletion mutation of the *Tetrahymena* intron were incubated at 37°C for 60 min in splicing buffer containing 2 μM tailless P5abc RNA, with GTP (+) omitted (−) as indicated. Symbols are as in Fig. 2. The diagram indicates the relative sizes (in nucleotides) of introns and exons. Minor bands migrating faster than the ligated-exon band and unrelated to the splicing reaction are not shown. (*B*) Ligated-exon RNAs were produced from wild-type (wt) and ΔL4-1 *Tetrahymena* precursors, converted into DNA, and sequenced. The dideoxynucleotide present in each sequencing reaction is indicated; arrows mark the position of the ligation junction in the sequencing ladder.

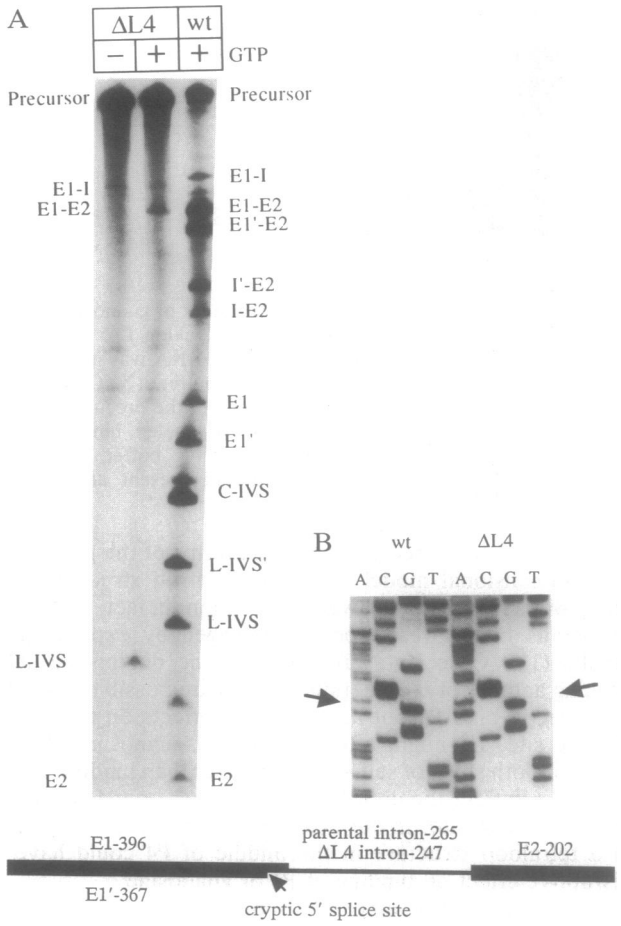

FIG. 4. Accurate self-splicing by the *td* intron in the absence of L4. (*A*) Precursor RNAs were incubated at 45°C at 8 nM in splicing buffer with GTP (+) omitted (−) as indicated, the parental *td* precursor (wild type, wt) for 10 min and the ΔL4 mutant for 90 min. RNAs containing the parental intron, marked on the left, are 18 nucleotides longer than their ΔL4 counterparts, marked on the right. Symbols are as in Fig. 2, except that the 5′ exon and intron generated by utilization of the cryptic 5′ splice site (23) are marked E1′ and I′, respectively. The diagram at the bottom indicates the relative sizes (in nucleotides) of introns and exons. (*B*) Ligated-exon RNAs were produced from the parental (wild-type, wt) and ΔL4 *td* precursors, converted into DNA (using oligonucleotides that would not allow amplification of products of the cryptic 5′ splice site), and sequenced. Symbols are as in Fig. 3*B*.

proposed by Michel and Westhof (7). In this model, the P1 paired region bearing the 5′ splice site makes tertiary interactions with highly conserved residues in two distinct regions of the core, L4 and the J8/7 sequence that joins P7 and P8. Support for the physical proximity of P1, L4, and J8/7 (as well as the guanosine-binding site in P7) comes from experiments using a guanosine nucleophile derivative that induced ribose cleavages specifically in L4 and J8/7 (12). If L4 and J8/7 are viewed as half-sites that properly position the 5′ splice site for splicing, it is not unreasonable to conjecture that with the deletion of one half-site (L4), the other (J8/7) alone could support splicing to a reduced extent. Although deletion of J8/7 may not be possible, the importance of its conserved bases for self-splicing has been demonstrated by substitution mutants (11). It is interesting that the *td* intron lacks the highly conserved adenosine residue in the J8/7 sequence (absent in only one other of the 87 group I introns listed in ref. 7); the activity of the L4 deletion mutant *td* intron demonstrates that self-splicing can occur when both L4 and one of conserved bases of J8/7 are lacking.

Self-splicing of the *Tetrahymena* intron was inhibited 10-fold more severely by the deletion of the L4 region than was specific hydrolysis at the 3′ splice site. This is consistent with the prediction from the model of Michel and Westhof (7) that the primary role of the L4 region is to bind the 5′ splice site-bearing P1 stem, since specific hydrolysis does not involve the 5′ splice site. The relatively modest inhibition of hydrolysis for the L4 deletion mutants (ΔL4-1 and ΔL4-2 relative to ΔP5abc) may still be due to a specific effect of some part of the deleted region, or the shortened P4 stem may result in a general destabilization of the intron core.

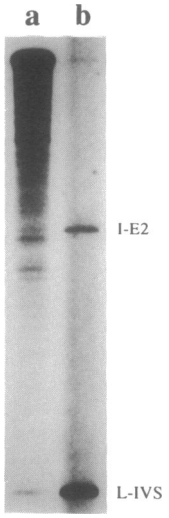

FIG. 5. Addition of guanosine to the *td* intron lacking L4. The ΔL4 *td* precursor RNA was incubated in splicing buffer at 50°C for 60 min. For lane b, the precursor RNA was not ^{32}P-labeled, but the GTP of the splicing buffer was α-^{32}P-labeled and present at 10 μM.

We have interpreted our results in terms of the prevailing three-dimensional model for group I intron structure, but many of its key features await verification; in fact, certain of its details have already been challenged by experimental results (12). Other interpretations of the diminished self-splicing activity of L4 deletion mutants are possible. L4 may not directly bind P1 but have positive effects on the actual binding site. L4 might affect binding of guanosine (during either or both steps of self-splicing) rather than binding of P1, or it might affect the coordination of either bound substrate with the active site. In our deletion mutants, the positioning of a tetraloop structure in the middle of P4 could have a disruptive effect on binding of P1 or guanosine.

The group I intron appears in some measure as a strategic assemblage of covalently linked, functionally distinct subunits. Certain large portions seem to be more important for self-splicing than for 3′ splice-site hydrolysis; this can now be said of the L4 region and has previously been shown for the sequence upstream of P3 (22). The P5abc element of the *Tetrahymena* intron, about the size of a tRNA molecule and with a similarly high degree of secondary structure, can function in trans as a general activator, both of splicing and hydrolysis, for the intron deleted of P5abc. Likewise, the correlate of the 5′ splice site in a stem–loop RNA corresponding to P1 can be utilized when the molecule is added in trans to the remainder of the intron (26, 27). One can imagine a primitive ribozyme, capable only of hydrolyzing a phosphodiester bond, integrating additional components during the course of molecular evolution that enabled recognition and coordination of two splice sites and generally improved ribozyme efficiency. It will be interesting to compare the components of the self-splicing RNAs with those of the spliceosome.

We thank E. Peter Geiduschek for critical comments on the manuscript. This work was supported by a grant from the National Institutes of Health. K.P.W. was supported by an American Cancer Society fellowship.

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