Transfer RNA Splicing in *Saccharomyces cerevisiae*

SECONDARY AND TERTIARY STRUCTURES OF THE SUBSTRATES*†

Ming-Chou Lee and Gayle Knapp‡

From the Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294

The interruption of the gene by noncoding sequences (commonly referred to as intervening sequences, IVS, or introns) occurs in genes of each of the major classes of RNA: transfer RNA, ribosomal RNA, and messenger RNA (reviewed in Refs. 1–3). These intervening sequences are removed from transcripts by the process of RNA splicing, defined as the excision of the intervening sequence from a RNA precursor and subsequent ligation of the coding fragments to yield the matured RNA. The molecular mechanism by which this process occurs is not well understood. It differs among the several classes of RNA and may also vary for the same class of RNA while the HeLa cell studies were heterologous (4, 5)). In the yeast mechanism was studied using a homologous system that this RNA undergoes autocatalytic excision and ligation. Using a variation of this methodology we have investigated the effects of both Mg"+ and spermidine on the conformation of yeast tRNA splicing.

* This research was supported by a grant from the American Cancer Society (MV-138). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom reprint requests and correspondence should be addressed.

‡ This research was supported by a grant from the American Cancer Society (MV-138). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
tRNAs (11, 24). The introns in these pre-tRNAs range in size from 14 to 60 nucleotides and, on the basis of computer or hand calculations of potential lowest free energy structures (25), may assume secondary or tertiary structures of varying complexity. We selected for this study pre-tRNAs which cover the spectrum of sizes and possible complexities of introns as well as different tRNA structure classes (26). These four pre-tRNAs are pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}}, pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{B}}, pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{C}}, and pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{D}} which contain introns of 19, 28, 32 (or 33), and 60 nucleotides, respectively. The native structures of these four uniquely end-labeled pre-tRNAs were probed using limited RNase digestion which allowed the determination of regions in the RNA molecule that were most accessible to RNase cleavage and, therefore, least involved in secondary or tertiary structure (15, 22, 27).

Aspects of the Secondary and Tertiary Structures of Yeast Pre-tRNAs—Conditions which allowed multiple cleavages of pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} by single-strand-specific RNases (see “Experimental Procedures”; data not shown) identified the single-stranded regions in this precursor. These regions can be observed in the secondary structure of pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} shown in Fig. 4A. The observed secondary structure demonstrates that pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} retains tRNA structure except in the anticodon loop area which is interrupted by the intron. In this region, two additional hairpin structures are formed. A helical stem is formed by part of the anticodon loop and a complementary sequence in the 5'-portion of the intron (we will call it the “anticodon-intron helix”). The 5' splice-site cleavage site is included in this hairpin structure. The other hairpin structure is formed by the middle portion of the intron (we call it the “extra arm”). The six nucleotides at the 3' terminus of the intron and the 3' terminal nucleotide of the anticodon loop (see Fig. 4A) form a single-stranded interior loop which includes the 3' splice-site cleavage site. Thus, both splicing cleavage sites are in single-stranded regions of the molecule.

The primary cleavage sites in pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} for different probing enzymes are shown in Fig. 1 and summarized in Fig. 4A. As these figures demonstrate, primary cleavage sites for RNase T1 (18) and duplex-specific RNase V1 (29) are located in the single-stranded loop containing the 5' cleavage-site site of the splicing endonuclease. The primary cleavage site of RNase T1 is G\text{\textsuperscript{77}} (Figs. 1 and 4A). Residue 57 of pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} was recognized by both RNase A (uridine) and RNase T1 (guanosine), confirming the sequence variation (represented by the double-headed arrows in Fig. 4A) found in two published tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} gene sequences (28, 29). This residue is in the loop of the extra arm. Since the other loop which contains the primary cleavage sites for RNase U2, RNase A, and mung bean nuclelease does not have a guanosine residue, the fact that the extra arm is cleaved under primary cleavage conditions by RNase T1 but not by RNase A, RNase U2, or mung bean nuclelease suggests that this region is exposed on the surface of the molecule but may not be the most accessible single-stranded region. Digestion of pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} with duplex-specific RNase V1 resulted in cleavage at residues G\text{\textsuperscript{22}} and G\text{\textsuperscript{23}} (Fig. 1) with the predominant cleavage at residue G\text{\textsuperscript{23}} in the stem of the extra arm. This cleavage confirms the existence of the extra arm and its relative availability on the outside of the pre-tRNA molecule.

These data indicate that the intron-anticodon region of pre-tRNA\text{\textsubscript{Phe}}\text{\textsuperscript{A}} is the most accessible portion of the precursor molecule. Furthermore, the D' and T4C loops, detected in probes of secondary structure (data not shown) but not under

\textsuperscript{2}The abbreviations used are: D, dihydrouridine; \(\Psi\), pseudouridine; T, thymidine.
Structure of Yeast Intron-containing tRNA Precursors

primary cleavage conditions (Fig. 1), appear to be involved in tertiary structure. This observation would be consistent with the tRNA moiety of the pre-tRNA, assuming a tertiary structure like that observed for x-ray crystal structure of tRNA\textsubscript{CyA} (30, 31) and inferred for tRNA solution structure in crystal-solution comparisons (32-34).

Other yeast pre-tRNAs were also studied to establish whether the structure observed for pre-tRNA\textsubscript{CyA} could be a common feature. The secondary and tertiary structures of pre-tRNA\textsubscript{PhA}, pre-tRNA\textsubscript{PhU}, and pre-tRNA\textsubscript{PhC} were probed using the same approach as for pre-tRNA\textsubscript{CyA}. Fig. 4, B to D, summarizes these data and shows the secondary structures and primary cleavage sites of different structure-probing enzymes in these pre-tRNAs.

Pre-tRNA\textsubscript{CyA} contains a class III tRNA (26) similar to pre-tRNA\textsubscript{CyU}; however, unlike the latter it has one of the shortest introns among the yeast pre-tRNAs (19 vs 32 or 33 nucleotides, respectively). The structure-probing data (not shown) demonstrate that pre-tRNA\textsubscript{CyA} (Fig. 4D) has a structure much like pre-tRNA\textsubscript{CyU} except that the extra arm of the intron is not present in this molecule. There is, however, a short single-stranded region which includes the 3' splicing cleavage site. Helix-specific RNase V1 cleaved primarily at the stem formed by the anticodon loop and complementary sequences in the intron, confirming the presence of the anticodon-intron helix.

Fig. 4C shows the secondary structure of pre-tRNA\textsubscript{PhU}. Although the large size of the intron may seem to contribute to a complex structure, the structure-probing results (data not shown) summarized here show that this pre-tRNA retains features seen in both pre-tRNA\textsubscript{CyA} and pre-tRNA\textsubscript{CyU}. The tRNA portion of the precursor has the D, TyC, acceptor, and anticodon helices. The D and TyC loops are not cleaved under primary cleavage conditions. Nucleotides of the anticodon loop are base-paired with a complementary sequence in the intron although the anticodon-intron helix thus formed contains a mismatch of the central base in the anticodon. The majority of the sequence of this large intron is found in two hairpin stem-loop structures located in place of the lower loop seen in pre-tRNA\textsubscript{CyA}. The 5' and 3' splice junctions are both single-stranded in pre-tRNA\textsubscript{PhU}.

One pre-tRNA from a population of precursors which contains the same tRNA\textsubscript{PhU} sequence was also studied. The precursor, pre-tRNA\textsubscript{PhU}, has 28 nucleotides in its intron. The intron sequence was obtained from the sequencing reactions which accompanied the structure-probing experiments (data not shown). It differs at several nucleotide positions from intron sequences previously reported. The structure-probing data (not shown) for this precursor revealed similar secondary and tertiary structure (Fig. 4D) as has been previously described for the other three pre-tRNAs. A hairpin stem-loop structure, found in the 5' portion of the intron, is very sensitive to single- and double-strand-specific nucleases. Both splice junctions are single-stranded and the 5' splice site in this precursor is the primary cleavage site of RNase T1. One potential difference was observed. Only part of the anticodon (2 out of 3 nucleotides) can form a traditional Watson-Crick helix with the complementary sequence in the 3' portion of the intron. Additionally, the anticodon portion of the precursor is very susceptible to RNase T1 cleavage including the central nucleotide of the anticodon (shown as a Watson-Crick base pair in Fig. 4D). The potential for a nontraditional G-A base pair between the third anticodon nucleotide (a guanosine) and the adenosine opposing it in the secondary structure must be considered. Deoxyguanosine-deoxyadenosine pairing has been observed and produced helices with lower stability and potentially deformed backbone structure (35). The existence of such structures in RNA and their susceptibility to RNase T1 have yet to be demonstrated but could explain our observation.

Effects of Mg\textsuperscript{2+} and Spermidine on Pre-tRNA Structure

In order to further substantiate the observation that the pre-tRNA assumes a tRNA-like tertiary structure, we looked at the nuclease sensitivity of pre-tRNAs at different concentrations of divalent cation (Mg\textsuperscript{2+}) and polyamine (spermidine). It is well known that tRNA structure is highly dependent on its ionic environment and that the tertiary structure is espe-
cally sensitive to changes in Mg$^{2+}$ and spermidine concentrations (16, 34). We postulated that if the pre-tRNA has elements of tRNA structure, stabilization of tertiary structure by Mg$^{2+}$ may be observed as has been seen in the case of tRNA (16, 17). The conditions that were established previously to give only primary cleavage of the pre-tRNA were used in these experiments. We assumed that if the pre-tRNA structure was altered we would observe the appearance of new bands, either as primary cleavages or in addition to the primary cleavage in the native structure. This is seen in Fig. 2 for pre-tRNA$^{AUA}_{FA}$. In the absence of Mg$^{2+}$ (plus 1 mM EDTA), predominant cleavages by RNase T1 were detected at guanosine residues in the D (G$^{15}$, G$^{17}$, and G$^{19}$) and TyC (G$^{39}$) loops. In addition, we detected destabilization of certain helices of the secondary structure in the absence of Mg$^{2+}$ at 37 °C. These helices include the D stem (G$^{24}$, G$^{25}$), anticodon stem (G$^{30}$), variable stem (G$^{38}$, G$^{39}$) and the stem formed by anticodon and intron sequences (G$^{50}$, G$^{51}$). Cleavages at these normally base-paired guanosines could be the result of lower stability of these secondary structures, the loss of tertiary structure, or cleavages in single-stranded regions. The latter alternatives could result in lower stability of these short helices. However, as the Mg$^{2+}$ concentration increased from 0 to 10 mM, cleavage at these guanosines seemed to disappear more rapidly than the cleavages in the D and TyC loops which supports the former hypothesis. The primary cleavage at residue 57, seen under the standard conditions, can be observed at all Mg$^{2+}$ concentrations tested.

Fig. 3 shows the effect of varying spermidine concentration on the RNase T1 cleavage pattern of pre-tRNA$^{AUA}_{FZ}$ in the presence (10 mM) or absence (plus 1 mM EDTA) of Mg$^{2+}$. Spermidine has no additional detectable effect on pre-tRNA$^{AUA}_{FZ}$ structure in the presence of 10 mM Mg$^{2+}$. The guanosine residue G$^{37}$ remains as the sole cleavage site at all spermidine concentrations. However, spermidine does substitute effectively for Mg$^{2+}$ in stabilizing secondary structure. At very low spermidine concentrations cleavage could not be detected at guanosine residues in the double helical regions previously mentioned. But, spermidine cannot substitute as efficiently in the tertiary structure. It required 3 to 5 mM spermidine but only 0.5 to 1 mM Mg$^{2+}$ to eliminate cleavage in the D and TyC loops. These data are consistent with NMR studies which suggest that Mg$^{2+}$ is more efficient than spermidine at restoring tertiary hydrogen bond resonances in tRNA$^{AUA}_{FA}$ (16). The RNase T1 primary cleavage site at G$^{37}$ was seen at all spermidine concentrations tested. These effects of Mg$^{2+}$ and spermidine on the secondary structure and the probable tertiary interactions between D and TyC loops of pre-tRNA$^{AUA}_{FZ}$ further substantiate a structure of pre-tRNA$^{AUA}_{FZ}$ which has native tRNA structure in the tRNA portion of the precursor. The cleavage of pre-tRNA$^{AUA}_{FZ}$ by RNases showed similar dependence on Mg$^{2+}$ and spermidine concentrations (data not shown).

**Consensus Secondary Structure of Yeast tRNA Precursors**

When the data gathered from structure-probing of four different yeast pre-tRNAs are compiled, it appears that although pre-tRNAs might vary in the size and sequence of their introns, several common structural features exist among these tRNA precursors. Fig. 5 shows a consensus secondary structure, which is compiled from our four structure-probing studies and the predicted secondary structures obtained from

---

**Fig. 2. Effects of Mg$^{2+}$ on pre-tRNA$^{AUA}_{FA}$ structure.** RNase T1 at 1 × 10$^{-4}$ units/µg of RNA was used to study the effect of varying MgCl$_2$ concentration of pre-tRNA$^{AUA}_{FA}$ structure. Reaction conditions were essentially the same as those in Fig. 1, except that Mg$^{2+}$ concentrations were varied as indicated on the figure. Cleavage sites in the absence of Mg$^{2+}$ (1 mM EDTA) are shown in the right panel. See legend to Fig. 1 and “Experimental Procedures” for other explanations.

---

**Fig. 3. Effects of spermidine on pre-tRNA$^{AUA}_{FA}$ structure.** RNase T1 at 1 × 10$^{-4}$ units/µg of RNA was used to study the effect of varying spermidine (Spd) concentrations on pre-tRNA$^{AUA}_{FA}$ structure either in the presence (10 mM) or absence (plus 1 mM EDTA) of Mg$^{2+}$. The reactions conducted in 10 mM MgCl$_2$ were essentially the same as those in Fig. 2 except for the presence of different spermidine concentrations as indicated on this figure. The reactions done in the absence of Mg$^{2+}$ were also the same as the RNase T1 reaction in the presence of EDTA in Fig. 2 except for the presence of different spermidine concentrations as indicated. Guanosine positions which were cleaved by RNase T1 are indicated in the right panel. These nucleotides were assigned by comparing to parallel sequencing reactions (not shown).

Spermidine has no additional detectable effect on pre-tRNA$^{AUA}_{FA}$ structure in the presence of 10 mM Mg$^{2+}$. The guanosine residue G$^{37}$ remains as the sole cleavage site at all spermidine concentrations. However, spermidine does substitute effectively for Mg$^{2+}$ in stabilizing secondary structure. At very low spermidine concentrations cleavage could not be detected at guanosine residues in the double helical regions previously mentioned. But, spermidine cannot substitute as efficiently in the tertiary structure. It required 3 to 5 mM spermidine but only 0.5 to 1 mM Mg$^{2+}$ to eliminate cleavage in the D and TyC loops. These data are consistent with NMR studies which suggest that Mg$^{2+}$ is more efficient than spermidine at restoring tertiary hydrogen bond resonances in tRNA$^{AUA}_{FA}$ (16). The RNase T1 primary cleavage site at G$^{37}$ was seen at all spermidine concentrations tested. These effects of Mg$^{2+}$ and spermidine on the secondary structure and the probable tertiary interactions between D and TyC loops of pre-tRNA$^{AUA}_{FA}$ further substantiate a structure of pre-tRNA$^{AUA}_{FA}$ which has native tRNA structure in the tRNA portion of the precursor. The cleavage of pre-tRNA$^{AUA}_{FA}$ by RNases showed similar dependence on Mg$^{2+}$ and spermidine concentrations (data not shown).
sequences of the other five pre-tRNAs (pre-tRNA^Ph^, pre-tRNA^Ch^, pre-tRNA^Y^, pre-tRNA^De^, and pre-tRNA^A^) (11, 29, 36-39). In this consensus structure, the acceptor stem, D and T/C hairpin stem-loop structures, and anticodon stem are the same as those found in the tRNA cloverleaf. The intron is always located in the same position between the sixth and seventh bases of the anticodon loop. Sequences complementary to some of the nucleotides of the anticodon loop are always found within the intron, allowing the forma-

FIG. 4. Secondary structures of the pre-tRNAs. A. The secondary structure of pre-tRNA^Leu_1^ is shown with the nucleotides numbered starting from the 5' end. Sequence variations observed in the two genes that have been sequenced (28, 29) are shown by the double-headed arrows. The three-pronged bracket indicates the anticodon triplet. P and OH are the 5'-phosphate and 3'-hydroxyl termini, respectively. Base-paired nucleotides are connected by . . Heavy arrows indicate the positions of the 5' and 3' splicing cleavage sites. The primary cleavage sites for the single-strand-specific RNases are indicated by squares and are the following residues: RNase U2 (A^6, A^4), RNase A (A^14), RNase T1 (G^5), and mung bean nuclease (A^6, A^4). The primary cleavage site for duplex-specific RNase V1 (G^5) is indicated by the diamond. The circles indicate other nucleotides which are secondary sites of cleavage under the standard-probing conditions. Triangles indicate nucleotides which become very susceptible to RNase cleavage upon removal of Mg^{2+}. The inverted triangles indicate nucleotides of lesser intensity of cleavage under the latter conditions. B. The secondary structure of pre-tRNA^Leu_2^ is shown. The symbols used in this figure are as described in A. The primary cleavage sites are as follows: RNase U2 (A^6), RNase A (U^6), RNase T1 (G^5, G^6), mung bean nuclease (U^6), and RNase V1 (G^5, U^3, A^3). C. The secondary structure of pre-tRNA^Val_1^ is presented with primary cleavage sites as follows: RNase A (U^7, C^8), mung bean nuclease (A^8, A^8), and RNase V1 (C^8). Symbols are as described in A. Cleavage

FIG. 5. Consensus secondary structure for yeast tRNA precursors. The tRNA portion of the precursor retains the conventional cloverleaf structure except in the anticodon loop where the intron is always found inserted between the sixth and seventh bases (arrows) of the loop. A sequence complementary to part of the anticodon loop can always be found within the intron forming a double helical structure. The smallest is three base pairs (found in pre-tRNA^Ch^). The 5' and 3' splice junctions (broad arrow) are located at either end of this helix and are almost always single-stranded. Nucleotide sequences which are always conserved are indicated by sequence: G, guanosine; U, uridine; A, adenine; C, cytidine; T, thymidine. Invariant purine and pyrimidine positions are indicated by R and Y, respectively. Open circles indicate conserved positions with varied sequences. X's indicate positions with varied length as well as sequence. The three-pronged bracket indicates the anticodon triplet. P and OH indicate the 5'-phosphate and 3'-hydroxyl termini, respectively. Base-pairs are indicated by . by RNase T1 (G^7, G^18, G^42, G^69) was enhanced at lower Mg^{2+} concentrations; thus, these residues were not considered “primary” sites. D. The secondary structure of pre-tRNA^Ph^ is shown and presents an intron sequence of another member of the group of precursors for tRNA^Ph_. The primary cleavage sites are as follows: RNase U2 (A^6, A^4), RNase T1 (G^6, G^9, G^16), mung bean nuclease (A^6, A^4, A^6, and U^4), and RNase V1 (U^6 and G^29). Symbols are as described in A.
tion of a double helical structure. In two of the four cases all three anticodon bases are included in traditional base pairs in this helix. In the two other cases (pre-tRNA\textsubscript{Phe} and pre-tRNA\textsubscript{Ala}), it may be possible that nontraditional purine-purine base pairs exist. The 5' and 3' splice junctions, located at either end of this helix, are single-stranded except for the 5' splice junction in the predicted secondary structure of pre-tRNA\textsubscript{Ala}. In this pre-tRNA the intron-anticodon helix may be extended by three additional base pairs involving complementary intron sequences. This places the 5' splicing cleavage site in the middle of a double helix. Differences among the introns of the various pre-tRNAs are accommo-
dated in two intron-specific regions denoted by X's in Fig. 5. These two regions can be simple single-stranded loops (e.g. pre-tRNA\textsubscript{Phe} or more complex stem and loop structures. The pre-tRNAs shown in Fig. 4 are representative of the variations that occur.

**Model for a Tertiary Structure of Yeast tRNA Precursors**

Structure probing of these four pre-tRNAs also provides insight into elements of their probable tertiary structure. The primary cleavage data and the effects on cleavage site availability by variation in Mg\textsuperscript{2+} and spermidine concentrations support the concept of an organization of the "cloverleaf" secondary structure into a tertiary structure consistent with that directly observed in the yeast tRNA\textsuperscript{Phe} crystal structure. Strikingly, the intron region is always the portion of the molecule most sensitive to cleavage by the RNases. This finding strongly indicates that the intron is an exposed region of the molecule and, therefore, is readily accessible for interaction with the splicing endonuclease. The concerted loss of cleavage of single-stranded nucleotides in the D and T\textsubscript{4}C loops as Mg\textsuperscript{2+} or spermidine concentration is increased in structure-probing reactions mimics the results obtained for tRNA\textsuperscript{Phe} (Ref. 17 and data not shown). This observation suggests that a similar tertiary structure is found in the yeast pre-tRNAs. Fig. 6 summarizes these observations in a consensus tertiary structure. In this model, the tRNA portion of the precursor retains the conventional "L"-shape conformation that is stabilized by tertiary interactions between different regions of the molecule, especially between D and T\textsubscript{4}C loops. These interactions are also stabilized by Mg\textsuperscript{2+} which specifically complexes in the crystal structure in the pocket formed by the D and T\textsubscript{4}C loops (40, 41). The stem formed between anticondon loop and intron sequences extends from the anticondon stem to form a continuously base-stacked helix on one arm of the L conformation. Additional sequences may be found at the 5' and/or 3' ends of the intron in two variable regions (hatched lines in Fig. 6).

**DISCUSSION**

RNA splicing is ubiquitous in eukaryotes, but we know little about it mechanistically in terms of how splice junctions are uniquely recognized by the enzymes which cleave and ligate. In the various systems under study, consensus sequences have been observed among only certain classes of spliced RNA precursors. However, even these consensus sequences may not have a direct role in the recognition of the associated splice junction (e.g. certain cryptic sequences unmasked by \(\beta\)-thalassemia mutations (42)). It is becoming increasingly apparent that RNA secondary and/or tertiary structure may define the selection of cleavage and ligation positions (8-10). To investigate the significance of RNA structure in tRNA splicing and to eventually understand its mechanism, we have probed the native structures of four yeast pre-tRNAs containing introns of different sizes and structural complexities.

The limited RNase cleavage technology (15, 22, 27) has been widely used for the study of RNA structure, especially those RNAs which would be difficult to study using physical methods, such as NMR or x-ray crystallography, due to limited availability of substrate. The structure of yeast tRNA\textsuperscript{Phe} obtained using this method has proved to be consistent with the structure obtained by physical methods (15, 22, 27). In using this method, it can still be argued that the specific interactions between probing enzymes and RNA may, in some cases, result in artifacts, e.g. sequence preferences or failure to detect an accessible region because of steric hinderance. To minimize this possibility, we have included in our structure-probing study a battery of enzymes with different specificities and a variety of ionic conditions in which yeast tRNA\textsuperscript{Phe} was known to exhibit dynamic changes in tertiary structure. The standard conditions were chosen because they were known to maintain the native tertiary structure of yeast tRNA\textsuperscript{Phe} (reviewed in Ref. 34) and were close to conditions used in splicing endonuclease assays.

The observation that a single enzymatic activity is responsible for the endonucleolytic cleavage of the intron from nine precursors (13) suggests that a common feature of these pre-tRNAs is being recognized. As we have shown here, the fairly diverse sequences of the pre-tRNAs still produce secondary and tertiary structures that maintain certain common features (summarized in Figs. 5 and 6). Similar observations of the
formation of base pairs between anticodon loop and intron sequences have been made for yeast precursors of tRNA\textsuperscript{3114} and tRNA\textsuperscript{3114}\textsuperscript{th} (43). This conserved double helix is flanked by single-stranded regions and by the most variable elements of pre-tRNA secondary structure. In the four structures that we studied, these two regions show diverse structural organization. Single-stranded loop, single hairpin stem-loop, and multiple hairpin stem-loop structures are found in pre-tRNAs which are all cleaved by the same partially purified splicing endonuclease. These two regions must contribute a minimum number of nucleotides to connect the conserved regions to allow for optimum structural stability of the consensus structure, i.e. particular sizes of hairpin loops and bulge loops may be thermodynamically favored (25). The single-stranded region which includes the 3' splice junction, while variable in size, never contains less than three nucleotides (e.g. Fig. 4C). Beyond these requirements the splicing endonuclease is apparently insensitive to sequence and structure of these variable regions. In several cases where multiple genes for a tRNA have been sequenced, it has been shown that introns for identical tRNAs vary in sequence (28, 29, 36, 37). These variations are always located in the variable portions of the consensus structure. In addition, in vitro mutagenesis of one cloned gene for pre-tRNA\textsuperscript{3114} has provided altered genes with insertions and deletions at the Hpa1 site (GTATAAC) located in the extra arm of the intron (44, 45). These alterations had no effect on splicing of the variant precursors except in one case. The exception was a mutant in which all of the extra arm was deleted except for one nucleotide. In this mutant the 3' end of the intron terminates with a cytosine which is not found in any of the nine yeast pre-tRNAs. This mutant also deviates from the consensus structure in that there are only two single-stranded nucleotides at the 3' cleavage site. All of the other mutants in which splicing was unaffected maintained our consensus structure.

The finding of a consensus structure, which is an extension of the conventional tRNA structure, may suggest that the splicing endonuclease recognizes some portion of the tRNA structure. Another laboratory has reported that elimination of base pairing of the D stem in a yeast pre-tRNA\textsuperscript{3114} gene resulted in loss of cleavage by the Xenopus TRNA splicing endonuclease (46). It appears that the integrity of the structure of the tRNA portion of the precursor is an essential feature. It has also been demonstrated that Mg\textsuperscript{2+} and spermidine are required for splicing (4, 13). It is tempting to speculate that this requirement may be related to the tertiary structure stabilization by these cations. Whether the tertiary structure is necessary for recognition or for the maintenance of stable helical structures more proximal to the cleavage sites remains to be demonstrated.

The 5' splicing cleavage site is shown single-stranded in our model; however, the predicted secondary structure for pre-tRNA\textsuperscript{3114} suggests that it may be double-stranded. The anticodon-intron helix and single-stranded 3' splicing cleavage site are the two invariant elements of yeast pre-tRNA structure. This strongly indicates that they have an important role for the binding site and/or active site of the yeast tRNA splicing endonuclease. Some illumination is shed by the correlation of other experimental results which utilized heterologous systems. Transfer RNA genes from some other organisms have been sequenced and shown to contain introns which cannot form the anticodon-intron helix (e.g. Refs. 47, 48). In the fusion gene, Schizosaccharomyces pombe, pre-tRNA\textsuperscript{3114} has an eight nucleotide-long intron that contributes no probable secondary structure and can be spliced in S. cerevisiae extracts although not as efficiently as S. cerevisiae pre-tRNA\textsuperscript{3114} (47). Conversely, when wild type and SUP4 pre-tRNA\textsuperscript{3114} from S. cerevisiae are compared in Xenopus oocytes the SUP4 pre-tRNA, in which the anticodon-intron helix is compromised by the suppressor mutation, appears to splice more efficiently than the wild type pre-tRNA (50). The Xenopus pre-tRNA\textsuperscript{3114} sequence cannot form the anticodon-intron helix (48). In both examples there is a correlation between the known (or predicted) structure of homologous pre-tRNAs and the relative efficiency of splicing of heterologous pre-tRNAs with dissimilar details of structure. This suggests that in the finer points of endonuclease-precursor recognition various organisms may differ.
Structure of Yeast Intron-containing tRNA Precursors
