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In vitro genetic analysis of the structural features of the pre-tRNA required for determination of the 3′ splice site in the intron excision reaction

E.Bufardeci, S.Fabbri, M.I.Baldi[1], E.Mattoccia[1] and G.P.Tocchini-Valentini[1,2]

Istituto Guido Donegani SpA, Laboratori di Biotecnologie, 00015 Monterotondo, Rome, [1]Institute of Cell Biology, CNR, Viale Marx 43, 00137 Rome, Italy and [2]Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637, USA

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During processing of intron-containing pre-tRNAs, the *Xenopus laevis* splicing endonuclease binds the precursor and cleaves it at both the 5′ and 3′ splice sites. *In vitro* selection was used to determine structural features characteristic of precursor tRNA molecules that are active in this reaction. We performed two types of selection, one for molecules that are not cut, the other for molecules that are cut at only one site. The results shed light on various aspects of the intron excision reaction, including the importance of the three-dimensional structure of the mature domain for recognition and binding of the enzyme, the active role played by the single-stranded region of the intron, and the importance of the cardinal positions which, although not necessarily occupied by the same base in all precursors, nevertheless play a fundamental role in the splicing reaction. A precursor can be cut at the 3′ site if a base in the single-stranded loop of the intron is allowed to pair (A–I pair) with the base of the 5′ exon situated at the position immediately following the anticodon stem [first cardinal position (CP1)]. The nature of the bases involved in the A–I pair is important, as is the position of the base in the single-stranded loop of the intron. We discuss the role of the cardinal positions in the reaction.

Key words: pre-tRNA/recognition/selection/tRNA splicing endoribonuclease

Introduction

In *Xenopus*, a single endonuclease can cleave several tRNA precursors and create substrates for subsequent ligation (Otsuka *et al.*, 1981). The enzyme acts (i) by specific recognition and binding of the precursors and (ii) by cleaving the two sites with consequent formation of the two halves and of the intron (Baldi *et al.*, 1986). The enzyme must therefore be dealing either directly or indirectly with features shared by all the precursors (Baldi *et al.*, 1986; Gandini Attardi *et al.*, 1989). The three-dimensional structure of any precursor has yet to be defined; however, calculation of free energy minima (Tinoco *et al.*, 1973) and the use of chemical and enzymatic structure-specific probes (Wrede *et al.*, 1979) suggest that all the precursors examined share a common tertiary structure (Swerdlow and Guthrie, 1984; Lee and Knapp, 1985). In this consensus structure, the tRNA portion of the precursor maintains the L-shaped conformation, stabilized by the interaction between the D and T ψ C loops. The 3′ splice site junction is always single-stranded. The introns occupy the same relative location, interrupting the anticodon loop one base after the anticodon. There are no conserved sequences at the splice junctions.

The exact way in which endoribonuclease recognizes the precursors has not yet been determined, but appears to require many interactions. We have previously shown that mutations in the mature domain affect precursor recognition. In particular, U8 and C56 appear to be probable contact points between protein and RNA (Mattoccia *et al.*, 1988).

These results led to the notion that the endonuclease recognizes the splice sites by binding to one or more sites in the mature domain which are common to all pre-tRNAs and measures the distance to the equivalently positioned intron–exon junctions. This hypothesis was supported by experiments that involved the engineering of changes in the distance from the mature domain to the splice sites (Mattoccia *et al.*, 1988; Reyes and Abelson, 1988). At the time, there was no evidence of a contribution of the intron to the specificity of splice site recognition. In what seemed a convincing demonstration of this point, an intron containing mostly U residues was inserted into a pre-tRNA and was spliced normally (Reyes and Abelson, 1988).

We subsequently showed that the intron is instead an active participant in the splicing reaction (Baldi *et al.*, 1992). In addition to the conserved bases, there are positions in the mature domain which, although not necessarily occupied by the same base in all pre-tRNAs, nevertheless participate in the splicing reaction. We termed these positions cardinal positions (CP). The *Xenopus* endonuclease is able to cut a precursor at the 3′ site if a base in the single-stranded loop of the intron is allowed to pair (A–I pair) with the base of the 5′ exon situated at the position immediately following the anticodon stem (position 32 in yeast pre-tRNA^{Phe}). This position is the first cardinal position (CP1). A pyrimidine, either uracil or cytosine, is normally found at CP1, but we found that a purine can, in some cases at least, accomplish the same function (Baldi *et al.*, 1992). A second cardinal position (CP2) is localized at the 5′ end of the 3′ half and a third (CP3) at the 3′ end of the 5′ half (Mattoccia *et al.*, in preparation). The function of the cardinal positions is governed by a complicated set of formal rules (Baldi *et al.*, 1992) that were deduced by creating and testing a number of individual mutants.

In this paper we study splice site selection of the yeast tRNA^{Phe} precursor carrying mutations within CP1, CP2 and three positions within the intron (positions −3, −4 and −5) that are potential partners within the A–I base pair (see Figure 2). We have generated these mutant substrates by *in vitro* genetic analysis (Tuerk and Gold, 1990; Bartel *et al.*, 1991) because the number of possible combinations of mutations at five sites is too large to construct and test each mutant individually. We have selected tRNA precursor mutants that are capable of being cleaved at least once by

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the splicing endonuclease and then asked, by sequencing, if particular nucleotides are preferred at CP1 and CP2, if a particular intron position is preferred for A−I pairing and, if so, the nature of this pairing. Our results confirm the requirement for the A−I base pair and specify its typology. Mutants that cannot form a proper A−I pair are not cleaved at the 3′ site and their reactivity at the 5′ site may be quantitatively affected as well. We also show that the A−I base pair contributes to the affinity of the precursor for the enzyme.

Results

Selection of mutants

The starting material for selection (Figure 1) was a randomized pool of RNAs obtained by *in vitro* transcription of synthetic DNA templates by T7 RNA polymerase. The initial pool of RNAs (1.2×10^{11} molecules) contained an end-matured yeast pre-$tRNA^{Phe}$ sequence which had five positions occupied by random nucleotides, one (Figure 2) was located in each of the exons (corresponding to CP1 and CP2) and three clustered in the single-stranded region of the intron (corresponding to positions −3, −4 and −5). These bases within the intron are potential partners for pairing with the base at CP1, to form the A−I base pair (Baldi *et al.*, 1992).

The RNA was circularized with T4 RNA ligase and gel purified (Pan and Uhlenbeck, 1992). After renaturation, the circular RNA was allowed to react with the *Xenopus* splicing endonuclease and incompletely cleaved molecules, either singly cut full-length linear or uncut circular, were purified in a denaturing gel. The uncut circular molecules were reconverted (Figure 1) into DNA templates by reverse transcriptase and PCR (selection 1). The linear RNAs were recircularized by the combined action of T4 polynucleotide kinase and T4 ligase and then reconverted into DNA (selection 2) (Pan and Uhlenbeck, 1992). A similar selection for mutants that are cleaved at both splice sites would be impossible, since the randomized positions would then be physically separated.

Gel purifications were carried out at three different stages of each cycle of the two selection procedures: purification of RNA from the transcription reaction, purification of circular RNA after the circularization reaction and purification of the desired species following reaction with endonuclease (Pan and Uhlenbeck, 1992).

Unexpected mutations and incorrect folding in uncut RNA molecules

After four rounds of selection 1, for uncut molecules, 50 isolates were sequenced. Variation at the initially randomized sites was observed, as expected, but more striking was the large number of unexpected mutations occurring at sites that were not initially randomized. In fact most sequences could not be folded into the cloverleaf pattern. Only 19 (38%) could still be folded into the cloverleaf; these also contained many unexpected mutations, primarily in the central core of the tRNA (Figure 3) (Pan *et al.*, 1991), at positions directly involved in tertiary base pairing (Quigley and Rich, 1976; Kim, 1978).

A direct physical assay that we developed previously (Baldi *et al.*, 1986) was used to analyse endonuclease pre-tRNA binding. The assay is based on the separation of specific RNA−protein complexes from unbound RNA by electrophoresis through low percentage, low strength polyacrylamide gels. The ability of the vast majority of the pre-$tRNA^{Phe}$ mutants derived from selection 1 to form complexes with the endonuclease is dramatically affected (data not shown).

In general, therefore, the selection for uncut molecules yields molecules that do not bind the enzyme, probably due to the fact that they do not fold properly. Folding of mature $tRNA^{Phe}$ can be monitored by measuring its specific cleavage by lead (Behlen *et al.*, 1990). This reaction is very sensitive to the folded structure and quite useful, therefore, for examining the folding of $tRNA^{Phe}$ and of its variants. Pre-$tRNA^{Phe}$ is also cleaved by lead, but the vast majority of the mutants derived from selection 1 are not (data not shown). The molecules selected on the basis of insensitivity to endonuclease generally do not fold properly.

Linear RNA molecules are not cut at the 3′ site

Selection 2 yields variants that tend to be cut only once. Figure 4 shows that even prior to selection, the mutant pool is largely defective in cleavage at the 3′ splice site. After three rounds of selection, practically no 3′ half is produced. We tested individual isolates and found that the selected RNA molecules are cut only at the 5′ site; the products are 5′ halves and 2/3 molecules consisting of an intron plus a 3′ half. Examples are shown in Figure 5 (lanes 8, 9, 10 and 11). The specificity of the cleavage was verified by sequencing (data not shown). Figure 6 shows that in the majority of the mutants the rate of production of the 5′ half was also affected.

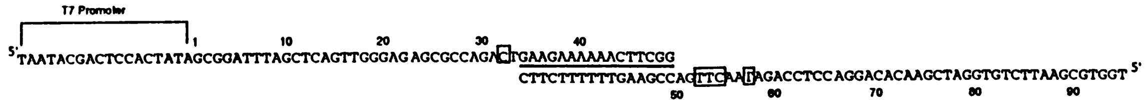
A striking feature of the mutants derived from selection 2 is that contrary to those derived from selection 1, the majority do not present any unexpected mutations; the changes observed generally occur only in the five randomized positions. Among the 110 isolates that did not contain unexpected mutations, there were 75 unique sequences (Table I). The total number of possible combinations is 1024 (4^5), but on the basis of our sample, it is already possible to draw some important conclusions. At the second cardinal position (CP2) we found an A in 44 cases and a C in only four cases; this correlates with our previous findings on the base specificity of CP2 function (Baldi *et al.*, 1992) and emphasizes its importance when the bases of the A−I pair are varied.

Typology of the anticodon loop−intron (A−I) base pair

We have already presented results indicating that the endonuclease recognizes a structural feature of the intron induced by the properties of the A−I base pair (Baldi *et al.*, 1992). Table II lists all possible pairings of the base at CP1 with one of the bases opposite, i.e. those at positions −5, −4 and −3 of the intron (see Figure 2). In >50% of the mutants that are uncut at the 3′ site (see Table I) the A−I base pair cannot form because the canonical requirements for pairings do not exist.

In all cases in which an A−I base pairing was possible among the mutants listed in Table I, only the types that are uncircled in Table II were found. Plainly, these A−I base pairs do not result in an active 3′ splice site. What instead can be said about those A−I base pairs that are missing from our collection? Given that not enough is known about the

tRNA^{Phe} Gene Construction



[] randomized nucleotides

Extension (MuLV Rev. Transcrip.)

SELEX

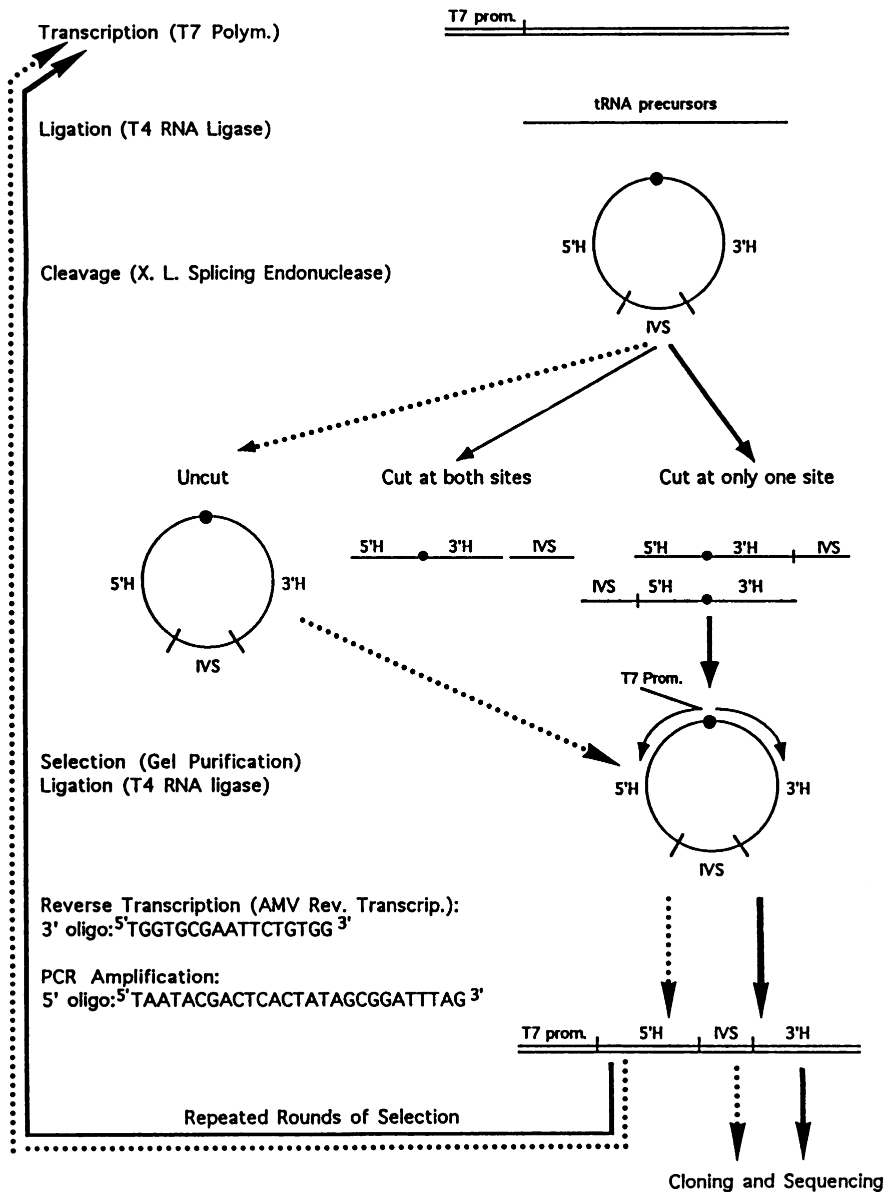


Fig. 1. Experimental design of the *in vitro* selection of RNAs.

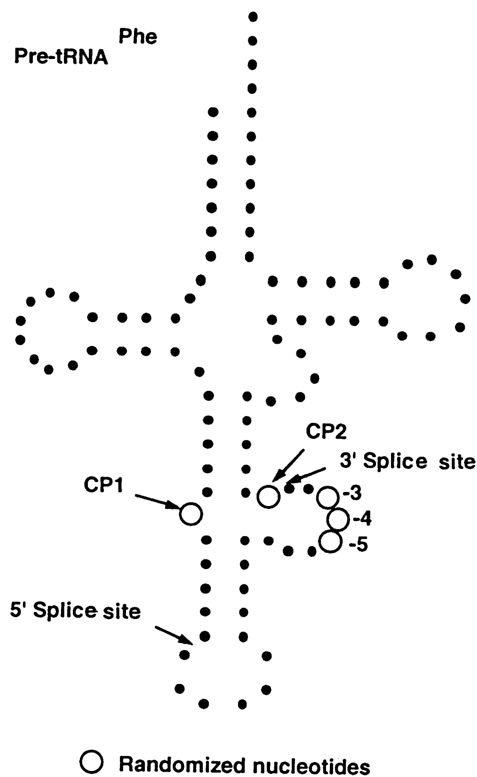


Fig. 2. Starting pool of five randomized nucleotides shown as circles in the background sequence of yeast pre-tRNA^{Phe}.

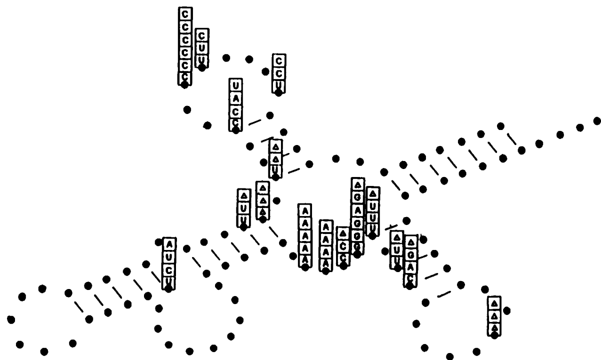


Fig. 3. Sites at which unexpected mutations occurred superimposed on the secondary structure of pre-tRNA^{Phe}. The data are based on 19 clones; sites at which only one mutation was found are not reported. Δ indicates deletion of one base. Nucleotide sequences of individual clones are available on request.

mechanisms at work in the selection procedure, it is difficult to speculate on this point. What is undisputable, however, is that those A−I base pairs that are essential for the 3′ splice site to be active must be included in the 'missing' set, i.e. the set involving the intron bases that appear circled in Table II. In the table, the circled intron bases are clustered on the right, corresponding to positions −3 and, to a much lesser extent, −4. None are to be found in correspondence to position −5. It is not only the nature of the bases (A−U, G−C) involved in the pairing that is significant (at position −4 only G−C and C−G base pairs are in the missing set), therefore, but also the position of the base on the single-stranded loop of the intron. This is precisely what might have been expected of A−I pairs responsible for an active 3′

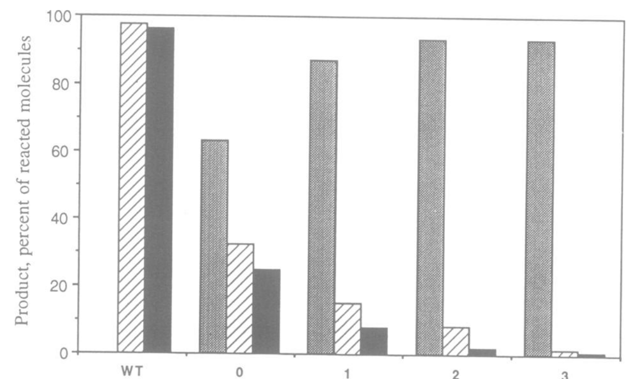


Fig. 4. Changes in the distribution of reaction products throughout the course of selection 2. Uniformly [^{32}P]UMP-labeled precursor RNAs of the wild-type and of mutant pools, prior to selection or after 1, 2 or 3 rounds of selection for singly cut molecules, were assayed for reactivity with *Xenopus* splicing endonuclease (conditions in Materials and methods). The reaction products observed were: 2/3 molecule consisting of intron +3′ half (■), intron (■), 3′ half (▨) and 5′ half. The relative radioactivity in the corresponding gel bands was measured in a phosphorimager and corrected for U content of products. One deduces that essentially all the reacted precursor molecules were cleaved at the 5′ splice site. The yields of the other products were therefore normalized to that of the 5′ half. Reaction extents for the precursors were: 81, 38, 53, 56 and 61% for wild-type and round 0, 1, 2 and 3 pools, respectively.

splice site, since the base at intron position −3 is located just opposite that at CP1 and is therefore more suitably situated to interact with it than a base at position −4, and even more so than one at position −5.

The A−I base pair contributes to enzyme binding

Two variants isolated from selection 2, which cannot form the A−I base pair and are cleaved only at the 5′ splice site, were tested for their ability to compete with wild-type precursor for enzyme binding. Figure 7 shows that the ability of the mutants to compete is severely reduced. This finding correlates with direct measurements of K_m; values for the wild-type and the two variants are 6, 117 and 155 × 10^{-10} M respectively. The K_{cat} values for the variants are increased, by 3.5 and 4.7 times, relative to the wild-type, indicating that cleavage at a single site occurs more rapidly than cleavage at two sites.

Discussion

We used *in vitro* genetic analysis to determine structural features of pre-tRNA that are important for cleavage at the 3′ splice site. Although the splicing endonuclease was purified from *Xenopus*, we used the yeast precursor pre-tRNA^{Phe}, for two reasons. First, the structure of the mature form of this RNA has been determined and second, from what is so far known, the yeast and *Xenopus* endonucleases behave quite similarly. We chose a small number of positions for randomization. We wanted to minimize perturbation of the general structure of the pre-tRNA while maximizing variation at the limited number of bases of interest (Baldi *et al.*, 1992). Despite this plan, the variants obtained in selection 1 had many unexpected mutations outside the randomized region. Their analysis is difficult because they are so far removed from tRNA^{Phe} sequence space. The mutations that characterize those that can be represented in the cloverleaf layout are localized in the central core of the

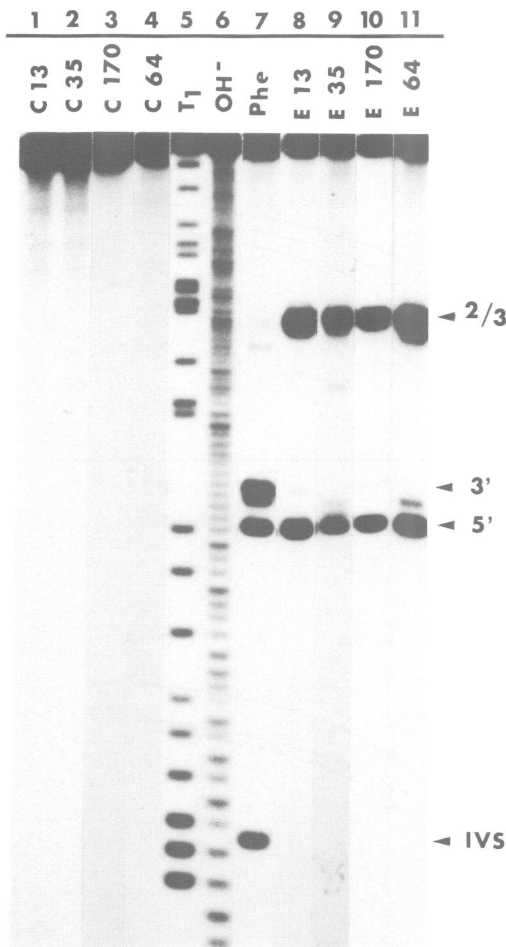


Fig. 5. Digestion of some selected mutant pre-tRNAs derived from selection 2, by purified *Xenopus* splicing endonuclease. Lanes 1, 2, 3, 4 (C) show the electrophoretic separation of the precursor incubated with buffer only. Lanes 8, 9, 10, 11 (E) show the electrophoretic separation of the products of the endonuclease reaction. Symbols: T1, partial endonuclease T1 digestion of pre-tRNA^{Phe}. OH$^-$, partial alkaline hydrolysate of pre-tRNA^{Phe}. Phe, splicing endonuclease digest of pre-tRNA^{Phe}.

molecule in the region that has previously been shown to be important for folding. These mutants cannot fold properly and it is therefore to be expected that they are defective in binding, since the multiple sites of the pre-tRNA that must interact with the enzyme are presumably not properly aligned.

Selection 2, for singly cut molecules, could have yielded molecules cut at the 5′ splice site alone, but we obtained only molecules that were cleaved at the 3′ splice site alone. This is probably not a consequence of a sequential cleavage pathway; Miao and Abelson (1993) have shown that the yeast enzyme cleaves the two sites in a random order. We therefore conclude that the 3′ cleavage-defective phenotype is a direct consequence of the base changes at the randomized positions. *In vitro* selection allowed us to test several mutation combinations at once and confirmed the rules that we formerly deduced utilizing site-directed mutagenesis (Baldi *et al.*, 1992). The endonuclease recognizes a structural feature of the intron mediated by the A−I base pair. It is not only the nature of the bases (A−U, G−C) involved in the pairing that is significant, but also the position of the base on the single-stranded loop of the intron. Position −3 of the intron is located just opposite CP1; the base at −3 is therefore more suitably situated for participation in the A−I base pair than the bases at −4 or −5.

Table I. Sequences at the randomized positions of isolates from selection 2

CP1	−5	−4	−3	CP2	CP1	−5	−4	−3	CP2
A	A	A	A	A	U	G	U	C	U
A	U	A	A	A					
A	U	U	A	A	U	U	U	U	G
A	G	U	A	A	U	A	U	G	G
A	C	U	A	A	U	U	U	G	G
A	G	G	A	A	U	U	G	U	G
A	A	C	A	A	U	U	C	U	G
A	U	C	A	A					
A	U	C	G	A	U	C	U	U	C
A	G	A	C	A	U	U	C	U	C
A	U	U	C	A	U	U	U	C	C
A	G	U	C	A					
A	C	U	C	A	G	U	U	A	A
A	A	G	C	A	G	U	A	G	A
A	A	C	C	A	G	A	G	G	A
A	U	C	C	A					
A	G	C	C	A	G	G	U	A	G
A	C	C	C	A	G	C	U	A	G
					G	G	G	U	G
A	C	A	C	U					
A	A	U	C	U	C	C	U	A	A
A	A	G	C	U	C	A	C	A	A
					C	U	C	A	A
A	A	U	A	G	C	G	C	A	A
A	G	G	A	G	C	C	U	U	A
A	A	C	A	G	C	C	C	U	A
A	U	C	A	G	C	A	A	C	A
A	C	C	A	G	C	G	U	C	A
A	A	U	C	G	C	U	C	C	A
A	A	C	C	G					
					C	U	C	U	U
U	G	A	U	A	C	U	U	C	U
U	A	G	U	A					
U	A	C	U	A	C	A	U	A	G
U	U	C	U	A	C	C	U	A	G
U	G	C	U	A	C	G	C	A	G
U	C	C	U	A	C	U	C	U	G
U	U	U	G	A	C	C	U	C	G
U	G	U	G	A	C	C	C	C	G
U	C	U	C	A					
U	A	G	C	A	C	A	A	C	C
U	G	G	C	A					
U	A	C	C	A					
U	G	C	C	A					
U	C	C	C	A					

In these isolates sequences outside the randomized positions were identical to wild-type pre-tRNA^{Phe}.

Table II. Summary of A−I base pairs

CP1	−5	−4	−3
A	U	U	**U**
U	A	A	**A**
G	C	**C**	**C**
C	G	**G**	**G**

Matrix of possible A−I base pairs involving the base at CP1 and the intron bases at positions −3, −4 and −5. Bold type marks the missing set, i.e. the A−I pairs that were not observed among the isolates from selection 2.

Fig. 6. Time course of the digestion of some selected mutant pre-tRNAs derived from selection 2, by purified *Xenopus* splicing endonuclease; insets show the nucleotides at the randomized positions (CP1, −5, −4, −3, CP2, see Figure 2). Symbols: ● wild-type 5′ half; ○ wild-type 3′ half; ▲ mutant 5′ half.

Our results show that pairing is necessary for activity, but we cannot conclude that it is therefore also sufficient. The A−I base pair might simultaneously pair with a third base to form a triple; or it might be the detailed structure of the A−I base pair, which presumably depends on the sequences, which is important for activity. We have shown here that the A−I base contributes directly to the affinity of the precursor for the enzyme.

What role might the cardinal positions play in the intron excision reaction? We will describe elsewhere additional results supporting a model of the splicing endonuclease. According to the model the enzyme is characterized by two catalytic sites: one active on the 3′ site, the other on the 5′ site. The binding of the endonuclease to pre-tRNA requires many interactions; probable contact points are a set of invariant bases in the mature domain, the A−I base pair and structural features related directly or indirectly to the bases occurring at CP2 and CP3. The CP2 site is adjacent to the catalytic site active on the 3′ site; the A−I site accepts base pairs involving the base at CP1 and a base of the single-

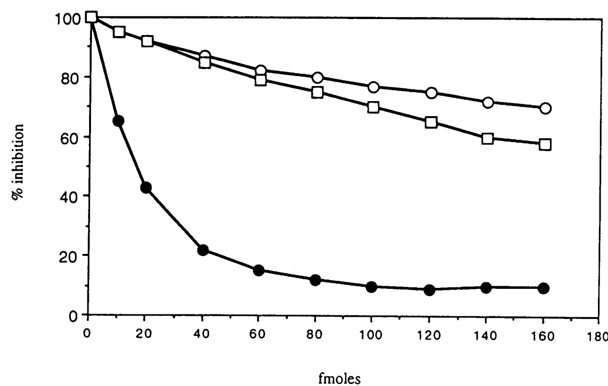


Fig. 7. Reduced affinity for the enzyme of mutants unable to form the A−I base pair. ^{32}P-labelled wild-type precursor (5 fmol) was mixed with increasing amounts of wild type (●), variant 1 (○) or variant 2 (□) precursor RNAs and incubated with splicing endonuclease according to Materials and methods. The nucleotides at the randomized positions (CP1, −5, −4, −3, CP2, see Figure 2) are CAACA and CUCAA for variant 1 and variant 2 respectively.

stranded loop of the intron, and it is located three bases downstream of the catalytic site active on the 3′ junction. The CP3 site is adjacent to the catalytic site active on the 5′ junction.

To understand the intron excision reaction we must understand the detailed three-dimensional structures of the pre-tRNA, its dynamics and the specific contributions of the molecule's individual components. What is the physical interpretation of the formal rules that have been deduced by way of genetic analysis? The requirement of an A–I base pair and the role of the base at CP2 in the determination of the 3′ splice site both point to the existence of specific local structural features which are required for cleavage. Artificial phylogeny of the type described in this paper may, through the analysis of a larger number of mutants, help to elucidate the details of the structure of the 3′ site, but such an approach has its limitations. For example, it is difficult to use phylogeny to predict accurately noncanonical base pairs since the rules governing the formation of such pairs are not well-defined. Inevitably, direct structural information obtained from NMR spectrometry and/or X-ray crystallography will be required.

Materials and methods

Preparation of site-specifically randomized pre-tRNA precursors

The starting population of pre-tRNA precursor used for the selection was generated by *in vitro* T7 RNA polymerase transcription of double-stranded DNA templates made by annealing and extending the two randomized synthetic oligodeoxynucleotides shown in Figure 1 (Pan and Uhlenbeck, 1992). The complementary sequences are underlined and N represents the five randomized bases.

50 pmol of each oligonucleotide were annealed for 5 min at 80°C and extended with 500 U of M-MLV reverse transcriptase (Gibco BRL) in 50 μl containing 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$ and 500 μM each dNTP at 37°C for 60 min. The reaction was phenol extracted and ethanol precipitated. 2.5 pmol of double-stranded template DNA were transcribed. Transcription by T7 RNA polymerase was carried out in 40 μl containing 40 mM Tris–HCl, pH 8.0, 6 mM $MgCl_2$, 10 mM DTT, 2 mM spermidine, 500 μM each rNTP, 25 mM 5′-GMP, 0.1 μM [α-^{32}P]UTP (800 Ci/mmol), [α-^{32}P]UTP, 180 U T7 RNA polymerase (Pharmacia) and 40 U RNasin (Pharmacia) at 37°C for 90 min. The 5-fold excess of 5′-GMP over GTP was included in the reaction to generate RNA with 5′-monophosphate. The reaction product after phenol extraction and ethanol precipitation was purified on a 10% denaturing polyacrylamide gel, eluted and ethanol precipitated (Pan and Uhlenbeck, 1992).

Circularization of linear RNA molecules was carried out in 50 mM Tris–HCl pH 7.5, 10 mM DTT, 15% DMSO, 200 μM ATP and 1 U/μl of T4 RNA ligase (Pharmacia) at 37°C for 120 min. The RNA concentration was 1 μM. The reaction was phenol extracted and ethanol precipitated, and circular RNA was purified from unreacted linear RNA on a 10% denaturing polyacrylamide gel. Circular RNA molecules were eluted and ethanol precipitated.

Selection procedure

In each cycle of selection 200 fmol of purified circular pre-tRNA were incubated in 900 μl containing 10 mM HEPES, pH 7.5, 7 mM $MgCl_2$, 70 mM NH_4Cl, 0.1 mM EDTA, 2.5 mM DTT and 10% glycerol at 22°C for 60 min in the presence of sufficient *Xenopus* splicing endonuclease to process to >90% the wild-type circular precursor (Gandini Attardi *et al.*, 1989). After phenol extraction and ethanol precipitation the products of the reaction were separated on a 10% denaturing polyacrylamide gel. Linear and circular RNA molecules were eluted from the gel and ethanol precipitated.

Circular RNA was directly reverse transcribed using as primer the oligodeoxynucleotide shown in Figure 1. After 10 pmol of primer were annealed by heating for 2.5 min at 93°C followed by 3 min in ice, the reverse transcription was performed in 20 μl containing 20 mM Tris–HCl, pH 8.3, 40 mM KCl, 10 mM DTT, 200 μM each dNTP and 5 U of AMV reverse transcriptase (Gibco, BRL) at 37°C for 30 min.

Linear RNA molecules that had been cut only once by the *Xenopus* splicing endonuclease were circularized according to Pan *et al.* (1991) as follows: linear RNA was incubated in a 20 μl reaction mixture containing 30 mM Tris–HCl, pH 8.0, 15 mM $MgCl_2$ and 1.5 U/μl of T4 polynucleotide kinase at 37°C for 45 min to remove 2′, 3′, cyclic phosphate. Upon addition of 30 mM Tris–HCl, pH 7.5, 8 mM DTT and 40 μM ATP, the reaction mixture in 25 μl was further incubated at 37°C for 15 min to introduce a 5′ phosphate. The same reaction mixture was then adjusted to RNA ligase conditions described above in a volume of 40 μl. The reaction mixture was phenol extracted, ethanol precipitated and reverse transcribed as above. After reverse transcription RNA was degraded by NaOH hydrolysis followed by HCl neutralization and cDNA was phenol extracted and ethanol precipitated.

Amplification of the two cDNAs by PCR was performed using as primers the oligodeoxynucleotides shown in Figure 1 in a 40 μl reaction containing 60 pmol of each oligo, 250 μM dNTP, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 2.5 U of Taq DNA polymerase (Perkin Elmer). PCR conditions were the following: 95°C for 30 s, 55°C for 10 s, 72°C for 120 s, 18 cycles.

Seventy-five percent of the DNA obtained after phenol extraction and ethanol precipitation was transcribed and the RNA precursor was used for the next round of selection or subcloning.

Cloning and sequencing

After four cycles (selection 1) and three cycles (selection 2) the respective PCR amplified DNAs were purified on a 12% nondenaturing gel. The eluted DNAs were then cloned into the *Sma*I site of pUC13. Plasmid DNA from single colonies was obtained according to standard alkaline lysis procedure (Sambrook *et al.*, 1989) and sequenced in the Automatic Sequencer (Applied Biosystem Model 373A) using Taq DyeDeoxy terminator protocol provided by the manufacturer.

Characterization of variant pre-tRNA molecules

The plasmid DNA of the variants to be characterized was amplified by PCR using the same conditions as for the amplification of cDNA. DNA, or the PCR product of a selection round, was transcribed with T7 RNA polymerase in the presence of [α-^{32}P]UTP. The transcription product was gel purified, eluted and ethanol precipitated. For the *Xenopus* endonuclease assay 5–10 fmol of labelled precursors (5×10^6 c.p.m./pmol of RNA precursor) were incubated in 30 μl reaction mixture containing 10 mM HEPES, pH 7.5, 7 mM $MgCl_2$, 70 mM NH_4Cl, 0.1 mM EDTA, 2.5 mM DTT, 10% glycerol and 10 μl of endonuclease at 22°C for 60 min, unless indicated otherwise (Gandini Attardi *et al.*, 1989).

For the Pb^{2+} cleavage assay, alkaline phosphatase treated RNA precursor was 5′-^{32}P-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. A 10 μl reaction containing 15 mM MOPS, pH 7.0, 1.5 mM spermine, 1 μg carrier tRNA, 15 mM $MgCl_2$ and 0.4 mM Pb $(OAc)_2$ was incubated at 25°C for 6 min (Behlen *et al.*, 1990). The products of the reactions were analyzed on 10% denaturing polyacrylamide gels.

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