4466–4470 Nucleic Acids Research, 1995, Vol. 23, No. 21

Two group I introns with a C•G basepair at the 5′ splice-site instead of the very highly conserved U•G basepair: is selection post-translational?

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Received June 27, 1995; Revised and Accepted September 26, 1995

ABSTRACT

In virtually all of the 200 group I introns sequenced thus far, the specificity of 5′ splice-site cleavage is determined by a basepair between a uracil base at the end of the 5′ exon and a guanine in an intron guide sequence which pairs with the nucleotides flanking the splice-site. It has been reported that two introns in the cytochrome oxidase subunit I gene of *Aspergillus nidulans* and *Podospora anserina* are exceptions to this rule and have a C•G basepair in this position. We have confirmed the initial reports and shown for one of them that RNA editing does not convert the C to a U. Both introns autocatalytically cleave the 5′ splice-site. Mutation of the C to U in one intron reduces the requirement for Mg^{2+} and leads to an increase in the rate of cleavage. As the C base encodes a highly conserved amino acid, we propose that it is selected post-translationally at the level of protein function, despite its inferior splicing activity.

INTRODUCTION

The splicing of RNA precursors containing a group I intron involves two sequential trans-esterification reactions which are catalyzed by the intron RNA itself. The first is initiated by nucleophilic attack of the 5′ splice-site (5′sS) by free guanosine (or a 5′ phosphate derivative). The resulting free 3′ hydroxyl group of the 5′ exon then attacks the 3′ splice-site to generated ligated exons and the excised intron (1). The excision of many group I introns requires the assistance of proteins factors but these are not thought to contribute directly to catalysis (reviewed in ref. 2).

Group I introns have a very similar core secondary structure but very few individual nucleotides are absolutely conserved (3). There is minimal sequence conservation at the splice-sites. At the 3′sS the terminal intron nucleotide is always a G. The 5′sS, is defined by a pairing (P1) between nucleotides flanking the 5′sS and a region within the intron known as the internal guide sequence (IGS) (Fig. 1) (3–7). The only consensus feature is a U•G basepair where the U is the last base of the 5′ exon. This basepair is so highly conserved that it is sometimes regarded as being absolutely invariant. However of ~200 group I introns sequenced to date, two have been reported as having a C•G basepair in this position. These are the first intron of the cytochrome oxidase subunit I gene in *Aspergillus nidulans* (8) and the third intron of the same gene in *Podospora anserina* (9). These exceptions have been overlooked in recent citations to the point where it has been suggested that group I introns require a U•G basepair to splice (10).

We have re-examined these two introns to determine whether they are truly exceptions to the general rule. We have recloned the intron DNA and confirmed that the published sequences are correct. The possibility that the C is converted to a U by RNA editing has also been excluded in the case of the *Aspergillus* intron. Both introns are able to self-cleave the 5′sS although this requires a high concentration of Mg^{2+} and monovalent salt. Interestingly if the C base of the *Aspergillus* intron is mutated to a U, the Mg^{2+} concentration can be reduced and the rate of cleavage is increased. We present the argument that C to U mutations are selected against *in vivo* because the C base encodes a highly conserved amino acid. That is, selective pressure at the level of protein function has prevented RNA splicing efficiency being optimized by selection for a U•G basepair.

MATERIALS AND METHODS

Strains

The strain of *Aspergillus nidulans* used to obtain mitochondrial DNA was yA2, pyroA4, cnxC3 (8). RT–PCR was performed on the strain FGSC4. Mitochondrial DNA from *Podospora anserina* strain *s* was kindly provided by Dr Leon Belcour.

Oligonucleotide PCR primers

Nox1c5: 5′-AGGAATTCTTTCTTTATGGTTATCACAGCTTTAATAG-GA-3′
Nox13: 5′-GGAAGCTTCTTATATTATTTAATCTAGGAAACC-3′
Paox15: 5′-CCCGAGCTCAATGGATGAAGATATAGTCC-3′
Paox13: 5′-CCCAAGCTTATCAGAAACTGATATTATTAAGT-3′
NoxC-1U: 5′-ATTATTTTACCATATCTGGACC-3′
Nox23: 5′-TGGAAGCTTCTATCTGTTAAAACCATAGTAATACC-3′

Cloning and mutagenesis

The AnOX1,1 intron was cloned using PCR with 1 ng of mtDNA and primers Nox1c5 and Nox13. Each cycle consisted of denaturation at 95°C for 2 min, annealing at 52°C for 2 min and polymerization at 72°C for 2 min. The reactions were hot-started

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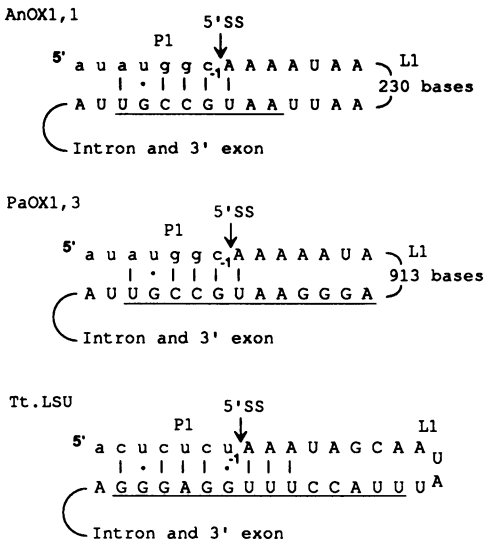

Figure 1. P1 stem–loop structure surrounding the 5′sS of AnOX1,1 and PaOX1,3. For comparison the structure of the P1 stem-loop from the *Tetrahymena* group I intron (Tt.LSU) is also shown. Exon sequence is shown in lower case. The internal guide sequence (IGS) is underlined and pairs with the 5′ exon and in many Group I introns with the start of the 3′ exon (4). Models of the complete secondary structure of AnOX1,1 and PaOX1,3 are shown in refs 8 and 9, respectively.

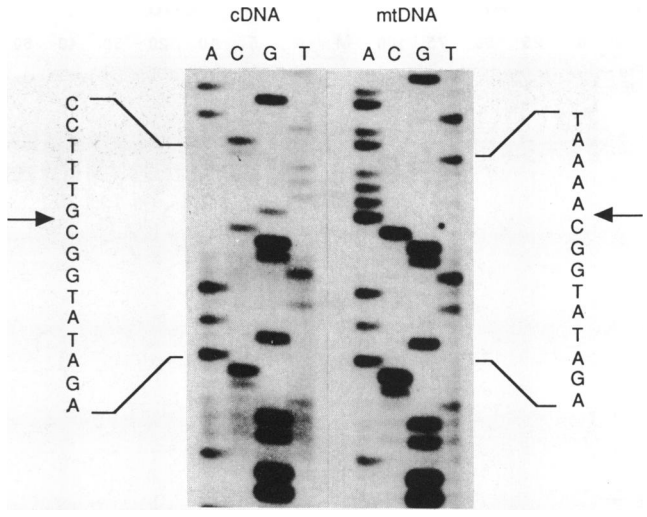

Figure 2. Sequencing data confirms that An.OX1,1 has a C at the –1 position instead of the highly conserved U. (left) cDNA from total *A.nidulans* RNA was amplified using PCR and cloned into a plasmid vector. DNA sequencing was performed by cycle sequencing. (right) *A.nidulans* mtDNA encoding An.OX1,1 and its flanking exons was amplified by PCR, cloned into a plasmid vector and sequenced. Nucleotide sequences around the 5′sS are shown at either side. Arrows indicate exon junction site and 5′sS, respectively.

at 52°C to avoid mispriming. Amplified DNA was digested with *Eco*RI and *Hin*dIII and cloned into pSP65 (Promega) which contains the promoter sequence for SP6 RNA polymerase (the *Eco*RI site is promoter proximal). The resulting plasmid was called pSPAnOX1,1. The inserted region of pSPAnOX1,1 was resequenced in its entirety.

PaOX1,3 was cloned using a similar approach with mtDNA from *P.anserina* and primers Paox15 and Paox13. A *Sac*I cloning site was used instead of *Eco*RI and the resulting plasmid called pSPPaOX1,3.

The $C_{-1}U$ mutation in AnOX1,1 was introduced as follows: oligonucleotide NoxC-1U is complementary to the sense strand and spans the 5′ exon–intron junction and has a point mutation to introduce a U instead of a C at the –1 position of the 5′ exon. A PCR reaction was performed using sense primer Nox1c5 and antisense primer NoxC-1U. pSPAnOX1,1 was used as template under standard conditions but with 20 μCi of [α-^{32}P]dCTP, 100 μM of non-radioactive dCTP and 200 μM of dATP, dGTP and dTTP. The PCR reaction generated a radiolabeled 96 base DNA fragment which contains an *Eco*RI recognition site at its exon end. After *Eco*RI digestion, the two DNA strands which differ in size by 4 bases, were separated by electrophoresis on a 4% denaturing polyacrylamide gel and visualized by autoradiography. The longer DNA strand was cut out of the gel and purified as described (7,11). A second round of PCR was performed (using the cycling conditions described above) with the purified single stranded DNA which contains a U at the –1 position as a sense primer and Nox13 as an antisense primer in the presence of template DNA (pSPAnOX1,1). The PCR amplified DNA was cloned into pSP65 and sequenced to confirm the mutation.

In vitro splicing assays

Transcription and purification of radioactively labelled precursor RNA was performed as described (7,11). Precursor RNA was incubated with splicing buffer containing 50 mM Tris-HCl, pH 7.5, $MgCl_2$, KCl and 1 mM GTP. The concentration of $MgCl_2$ was varied from 5 to 100 mM. The concentration of KCl used was 0.1–0.4 M for An.OX1,1 and 0.1 M for PaOX1,3. Reactions were started, after a 3 min preincubation, by the addition of GTP and incubated at 37°C for 1 h, unless otherwise indicated. Reactions were stopped by adding formamide dye (95% deionized formamide, 20 mM EDTA, pH 8.0 and 0.05% of bromophenol blue and xylene cyanol), heated at 65°C for 10 min and analyzed on a 5% denaturing polyacrylamide gel, which was then subjected to autoradiography. RNA molecules were quantified by a phosphorimager. In Figure 4B the rate of 5′sS cleavage was determined by measuring the amount of precursor RNA.

Reverse transcriptase PCR

Total RNA was purified from *A.nidulans* grown as described (12). Twenty micrograms RNA was treated with 1 U of DNAse for 10 min, phenolized, ethanol precipitated and then reverse transcribed using M-MLV reverse transcriptase (BRL) for 30 min at 42°C under standard conditions. To check for RNA editing of the mature mRNA, reverse transcription was primed with primer Nox23 which anneals to the exon downstream of the second intron (AnOX1,2) in the cytochrome oxidase gene. To determine the levels of precursor RNA still retaining AnOX1,1 *in vivo* (Fig. 5), reverse transcription was primed with the antisense primer Nox13. The cDNA was amplified by PCR using Nox15 as the sense strand primer in both cases.

RESULTS

Confirmation that the base upstream of introns AnOX1,1 and PaOX1,3 is a cytosine

DNA encompassing the first intron (AnOX1,1) of the *A.nidulans* cytochrome oxidase subunit 1 gene was amplified by PCR and

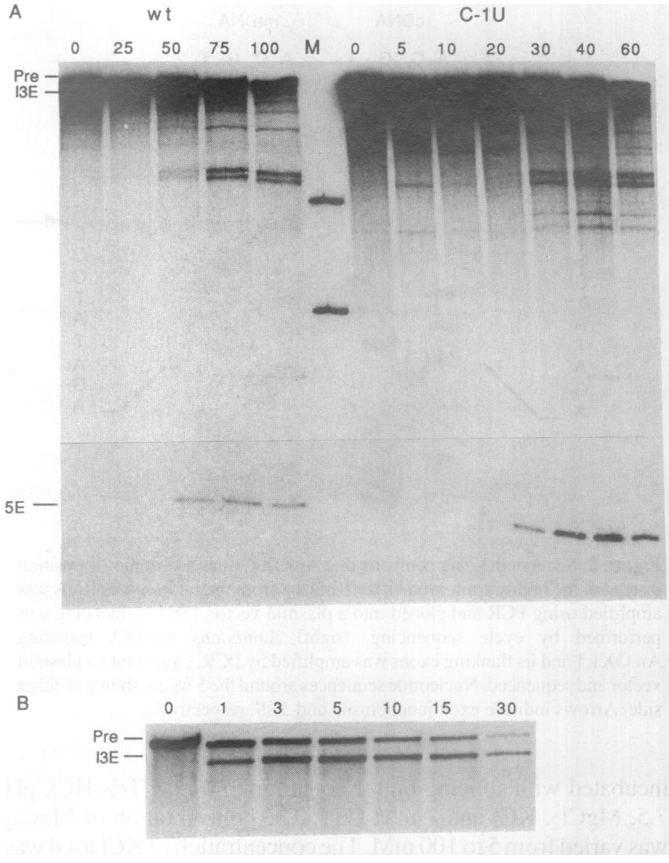

Figure 3. RNA splicing of AnOX1,1 and the $C_{-1}U$ mutant: dependence on Mg^{2+} concentration. (**A**) AnOX1,1 and its $C_{-1}U$ mutant were reacted at 37°C for 60 min in 50 mM Tris-HCl, pH 7.5, 1 mM GTP, 0.4 M KCl and $MgCl_2$ as shown. Pre, precursor; I3E, intron + 3′ exon; M, size markers. The second step of splicing is not correctly completed, other bands represent aberrant splicing products. The gel is overexposed to visualize the 5′ exon. (**B**) Time course (min) of the An.OX1,1 $C_{-1}U$ mutant as in A but in 50 mM $MgCl_2$ and 0.1 M KCl (the bands are slightly sharper with 0.1 M KCl samples; the reaction rate is very similar to that at 0.4 M).

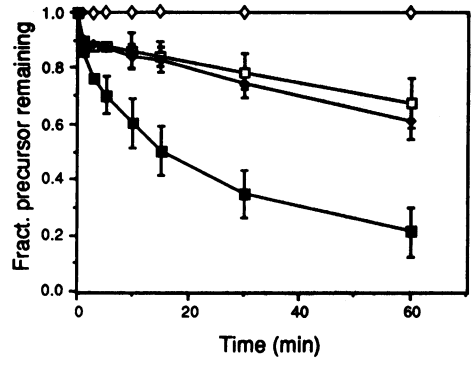

Figure 4. The $C_{-1}U$ mutant reacts faster than the wild-type. Uniformly labeled precursor RNA was incubated in 50 mM Tris-HCl pH 7.5, 0.1 M KCl, 1 mM GTP and 25 or 50 mM $MgCl_2$ at 37°C. Diamonds, wild-type; square, $C_{-1}U$ mutant. Open and closed symbols 25 and 50 mM $MgCl_2$, respectively. The mean of four replicates is shown for each reaction; the standard errors are omitted from the earlier time points for clarity.

cloned into the transcription vector pSP65 to yield the plasmid pSPAnOX1,1. This contains 864 bases of intron sequence with 81 and 24 bases of 5′ and 3′ flanking sequence, respectively. The region of the 5′sS was sequenced and the last base of the 5′ exon confirmed to be a cytosine (Fig. 2B). This sequence was also obtained from two other independent PCR reactions using different primers.

In plant and Physarum mitochondria (13,14) cytosine residues can undergo RNA editing to uracil. To determine whether the last base of the 5′ exon (C_{-1}) was edited to a U, the sequence of the cytochrome oxidase subunit 1, mature mRNA was obtained at the junction of exons 1 and 2. cDNA was synthesized from *A.nidulans* total RNA using a primer specific for exon 3 (downstream of intron 2), amplified by PCR, subcloned to yield plasmid pAnOXLE12 and sequenced. Sequencing of five independent subclones indicated that both introns 1 and 2 had been excised and that no RNA editing had occurred (Fig. 2A). The sequence data also formally confirmed the precise location of the splice-sites of this intron, previously only deduced by phylogenic comparison of derived amino acid sequences (8). This therefore also verifies that the cytosine in question is indeed the terminal base of the 5′ exon.

Using mitochondrial DNA purified by L. Belcour as a template, DNA encompassing the third intron from the cytochrome oxidase subunit 1 gene of *Podospora anserina* was amplified by PCR and cloned into pSP65 to give the plasmid pSPPaOX1,3. This contains 1260 bases of intron sequence with 90 and 32 bases of 5′ and 3′ flanking sequence, respectively. DNA sequencing confirmed that the last base of the 5′ exon is indeed a cytosine (data not shown).

Self-splicing activity of AnOX1,1 and PaOX1,3

Precursor RNAs were transcribed from either pSPAnOX1,1 or pSPPaOX1,3. Defined RNA 3′ termini were generated by first linearizing the plasmids with *Pvu*II which adds about 200 bases of vector sequence to the 3′ exon. The precursors were gel purified and subjected to self-splicing assays under various conditions. The PaOX1,3 intron readily self-spliced in 25 mM Mg^{2+} and 0.1 mM KCl. The AnOX1,1 intron completed the first step of splicing, 5′sS cleavage, in 50–100 mM Mg^{2+} and 0.1–0.4 M KCl, but did not undergo the second step of splicing, exon ligation (Fig. 3A). The first step required at least 50 mM Mg^{2+}. Further analysis of the block in the second step will be described elsewhere (Hur and Waring, in preparation).

Mutation of the C_{-1} base to the highly conserved U increases reactivity

To determine the consequences of replacing the 5′ exon terminal cytosine with a uracil, a $C_{-1}U$ mutation was constructed in the AnOX1,1 intron by PCR and assayed for self-splicing activity. The concentration of GTP used (1 mM) was close to saturating. Interestingly the $C_{-1}U$ mutant underwent 5′sS cleavage at a lower Mg^{2+} concentration than the wild type intron, activity being detected at 20 mM $MgCl_2$ (Fig. 3A). The rate of cleavage of the 5′sS was assayed by performing a time course (Fig. 3B) in 50 mM $MgCl_2$, a concentration in which both intron sequences are active. The $C_{-1}U$ mutant (U•G bp) was clearly faster than the wild-type (C•G bp) (Fig. 4). The reactions deviated somewhat from simple first order kinetics and so a quantitative analysis was not performed. The $C_{-1}U$ mutant in 25 mM $MgCl_2$ was about as active as the wild-type in 50 mM $MgCl_2$ (Fig. 4). Both these

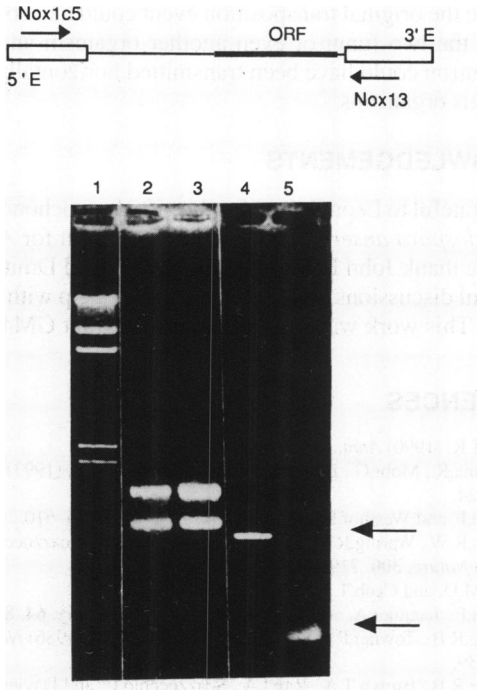

Figure 5. AnOX1,1 is efficiently spliced *in vivo*. The RNA template is shown at the top. Open boxes indicate 5′ and 3′ exons and thin line intron. Total RNA from *A.nidulans* was subjected to reverse transcription with Nox13 as a 3′ primer and then amplified by PCR with Nox1c5 as a 5′ primer. Lane 1, *Hin*dIII lambda DNA markers; Lane 2, total RNA; Lane 3, total RNA treated with DNase; Lane 4, cDNA amplified from untreated RNA; Lane 5, cDNA amplified from DNase treated RNA. Upper arrow indicates the fragment amplified from contaminating genomic DNA sequence in lane 4 and lower arrow indicates the fragment amplified from correctly ligated exons.

slower two reactions continued beyond 1 h but an end-point was not determined. Both showed an apparent small burst of splicing activity within the first minute; a possible explanation is that the rate-limiting process in the wild-type is a folding step which is accelerated by either increasing the concentration of Mg^{2+} or introducing a C to U mutation.

The C_{-1} base does not noticeably slow splicing *in vivo* since no unspliced RNA was detected by reverse transcriptase PCR (Fig. 5).

DISCUSSION

It has occasionally been stated that all Group I introns have (and even require) a U•G bp at their 5′sS. We have re-examined and confirmed reports that two of the ~200 introns sequenced to date have a natural C•G bp at the 5′sS. It has also been shown for one of these that RNA editing does not convert the C to a U post-transcriptionally.

Cleavage of the 5′sS of the *Tetrahymena* group I intron involves two distinct steps: the splice-site is first incorporated into a RNA helix (P1); this then docks with the ribozyme core structure in such a way that the scissile bond is orientated correctly in the active site (15–17). What factors determine that the correct bond is cleaved? Selection of the splice-site is determined in part by its distance from the base of the P1 stem (18) and the relationship between P1 and the next pairing P2 (15,19,20). Various basepairs distal to the 5′sS can be altered in sequence without dramatically reducing splicing (5,7) but a primary determinant of specificity appears to be the U•G bp at the splice-site. Replacement of this basepair with other base combinations has demonstrated that only a C•A basepair retains a significant fraction of the U•G activity. This has led to the proposal that the ribozyme recognizes basepairs in the wobble configuration, for example a U•G or a protonated C•A basepair (18).

Mutation of the U to a C in the *Tetrahymena* intron reduced 5′sS cleavage of an artificial RNA precursor transcript 100-fold, although the second step of splicing was barely affected (21). Analysis of a bimolecular reaction involving a 5′ truncated form of the intron and an oligoribonucleotide substrate has demonstrated that the C•G bp stabilizes formation of the P1 helix but significantly impairs subsequent docking of the helix leading to a reduction in the rate and specificity of cleavage (22,23). It has been suggested that formation of a C•G bp would withdraw the guanine N2 exocyclic amine from the P1 helix minor groove, thus eliminating a tertiary interaction between the amine and the ribozyme docking site (24).

Given these observations and the fact that the U•G bp is so highly conserved in the 200 odd group I introns sequenced to date, we examined the consequence of mutating the C_{-1} base to a U in the AnOX1,1 intron. Interestingly, introduction of a U•G bp increases the rate of 5′sS cleavage and partially alleviates the requirement for a high concentration of Mg^{2+}. These results are only indicative of the relative activities of these two basepairs because other factors may be contributing to the rate-limiting step of the reaction. At the least, this argues against the AnOX1,1 ribozyme interacting with the C•G bp in a way that is mutually exclusive to that proposed for the U•G bp in the *Tetrahymena* intron. It is consistent however with the suggestion that the ribozyme docking site promotes cleavage by realigning the C•G bp into a protonated wobble pair (22) in that any fine-tuning that may have occurred in the tertiary structure to optimized activation of the C•G bp still readily accommodates a U•G bp.

If a U•G bp is more active, why does the AnOX1,1 intron have a C•G bp?

We will consider three possibilities. First, AnOX1,1 is simply a rare example of a sub-class of intron which docks its splice-site helix and recognizes its 5′sS by a slightly different collection of tertiary interactions. This is plausible but not entirely persuasive given the results of the $C_{-1}U$ mutation. Secondly, excision of the first intron is deliberately designed to be a slow rate-limiting step in the maturation of the mRNA to regulate expression of the cytochrome oxidase subunit 1 protein or to permit expression of an ORF within the intron (8). Regulation could be conferred through the action of an ancillary splicing factor. Such factors probably assist splicing *in vivo* anyway to compensate for the slow rate of splicing (Fig. 4) and the requirement for a high concentration of Mg^{2+} *in vitro* (Fig. 3A) (see also 2,25). The absence of any detectable unspliced RNA *in vivo* (Fig. 5) does not support this hypothesis but neither does it contradict it.

The third possibility is that the C base is required after RNA splicing to encode a highly conserved amino acid. The AnOX1,1 intron and the PaOX1,3 intron are located at exactly the equivalent site and are inserted after the C of the alanine codon GCG and GCA, respectively. Mutation of the C to U would replace alanine with valine. A search of the SWISS-PROT database revealed that this alanine is conserved in 52 of 57

cytochrome oxidase subunit 1 like sequences from organisms as diverse as fungi, mammals, plants, insects, protozoa, bacteria and archaebacteria (valine is found in this position in two kinetoplastid containing organisms, *Trypanosoma brucei* and *Leishmania tarentolae*). This suggests that there is strong selection against mutation of the cytosine base and that this selection acts post-translationally rather than during RNA splicing.

How might the AnOX1,1 and PaOX1,3 introns have originated?

Endonucleases, usually intron encoded, can readily mobilize group I introns into the equivalent exon DNA sequence of intronless alleles of the same gene. This is known as homing (26). They are also thought, in very rare circumstances, to mobilize introns into novel locations because the endonucleases tolerate some variation in their lengthy recognition sequence (26,27). This is know as intron transposition. Transposed introns will initially splice less well than those which have mobilized by homing because flanking exon sequence, tolerated at the DNA level by the endonuclease may not form an optimal tertiary RNA structure for splicing (27,28).

The following scenario may describe the initial events that led to insertion of the AnOX1,1 intron. The progenitor intron originally had a U•G bp. An endonuclease mobilized the intron into its current site because the endonuclease tolerated either a C or a T at the position corresponding to the end of the 5′ exon [PaOX1,3 encodes a potential endonuclease and the P1/L1 stem-loop of AnOX1,1 encodes vestigial remains of endonuclease-like sequences (8,9)]. If, initially, the intron was poorly excised, mutations that improved splicing would have been selected for; such mutations might have mapped in DNA encoding either the ribozyme, ancillary protein factors or flanking exon sequence. By contrast, $C_{-1}U$ mutations would have been selected against post-translationally, despite improving splicing. This assumes that *in vitro* activities reflect those *in vivo* and that U is at least as active as C in the second step of splicing. However we note that a $U_{-1}C$ mutation has no effect on the second step of splicing in the *Tetrahymena* intron despite significantly inhibiting the first step by increasing the K_m for GTP 15-fold and reducing k_{cat} 7-fold (21).

AnOX1,1 and PaOX1,3, which both belong to the IB2 subgroup, share 89% sequence homology, discounting the open reading frames located in peripheral stem-loop structures (8,9). Independent transposition events may have led to insertion of AnOX1,1 and PaOX1,3. However there is circumstantial evidence for the horizontal transmission of group I introns (29). Therefore the original transposition event could have occurred in either of the two fungi or even another organism and then the group I intron could have been transmitted horizontally between the various organisms.

ACKNOWLEDGEMENTS

We are grateful to Leon Belour for the gift of mitochondrial DNA from *Podospora anserina* and John Zimmerman for *A.nidulans* RNA. We thank John Burke, Dan Herschlag and Dmitri Maslov for helpful discussions and Joel Sheffield for help with computer analysis. This work was supported by NIH grant GM41009.

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