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Evolutionary Origin of the U6 Small Nuclear RNA Intron

CLAUDIA REICH AND JO ANN WISE*

Department of Biochemistry, University of Illinois, 1209 West California Street, Urbana, Illinois 61801

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U6 is the most conserved of the five small nuclear RNAs known to participate in pre-mRNA splicing. In the fission yeast *Schizosaccharomyces pombe*, the single-copy gene encoding this RNA is itself interrupted by an intron (T. Tani and Y. Ohshima, Nature (London) 337:87–90, 1989). Here we report analysis of the U6 genes from all four *Schizosaccharomyces* species, revealing that each is interrupted at an identical position by a homologous intron; in other groups, including ascomycete and basidiomycete fungi, as well as more distantly related organisms, the U6 gene is colinear with the RNA. The most parsimonious interpretation of our data is that the ancestral U6 gene did not contain an intron, but rather, it was acquired via a single relatively recent insertional event.

The removal of introns from mRNA precursors (pre-mRNAs) in eucaryotes takes place within the spliceosome, a multimeric structure consisting of the pre-mRNA, four small nuclear ribonucleoproteins (snRNPs), and an undefined number of auxiliary proteins (31). Each snRNP consists of one (U1, U2, or U5) or two (U4 and U6) small nuclear RNAs (snRNAs) and a number of common and unique proteins (26, 42). The processing of pre-mRNA introns shares many fundamental properties with the autocatalytic splicing of group II introns (33, 47); thus, it has been postulated that the chemistry underlying the former process is also RNA-based, with the protein components serving in crucial but ancillary roles, such as facilitating spliceosome assembly (8). U1 RNA interacts with the splicing substrate via direct base pairing between its 5′ end and the conserved intron sequences at the 5′ splice site (29, 39, 40, 50). U2 RNA base pairs with the conserved branch site (32, 48, 51). U5 RNA is bound indirectly, via protein contacts, to the 3′ splice site (17, 45). U4 and U6 RNAs coexist within the same snRNP, in which they are extensively base paired (3, 4, 19); although their precise roles are not known, the interaction between them is disrupted just prior to the formation of detectable splicing intermediates (1, 10, 24, 34).

U6 is the least variable of the snRNAs (4), and its high degree of conservation points to a central role in the splicing process. U6 is unique in several other respects: it is the only snRNA functional in splicing that does not possess an Sm (core snRNP protein) binding site (36); its gene is transcribed by RNA polymerase III (23, 37); and, rather than the trimethylguanosine cap characteristic of other U-class snRNAs, its 5′ γ phosphate is esterified to a methyl group (41). Surprisingly, the single-copy gene encoding U6 RNA in the fission yeast *Schizosaccharomyces pombe* is interrupted by a 50-base-pair stretch not found in the mature RNA (44). This segment contains perfect matches to the consensus sequences identified at the splice sites and at the site of branch formation in yeast nuclear pre-mRNA introns, suggesting that the very apparatus of which U6 is an essential component is responsible for maturation of the RNA; this prediction is borne out by the finding that pre-mRNA splicing mutants in *S. pombe* accumulate unspliced U6 RNA (35). To assess whether the intron was present in the ancestral U6 gene or represents a later evolutionary acquisition, we undertook a study of this RNA and its gene in the other extant *Schizosaccharomyces* species and in an array of lower eucaryotes.

The presence of homologous introns in the U6 genes was assayed by comparing the products resulting from specific amplification of relevant genomic sequences and reverse-transcribed total cellular RNA from all identified species of the *Schizosaccharomyces* genus (namely, *S. pombe*, *S. malidevorans*, *S. octosporus*, and *S. japonicus*); the latter three species were obtained from the American Type Culture Collection, and their nucleic acids were extracted as previously described for *S. pombe* (2). Two oligonucleotides were synthesized, one complementary to the 21 3′ terminal residues of mature *S. pombe* U6 RNA (reverse primer) and another one colinear with the first 21 residues at the 5′ end of the mature molecule (forward primer). A 1- to 2-μg sample of total RNA was reverse transcribed (25) from the reverse primer (5′ AAAATGGGTTTTCTCTCAATG 3′) at a concentration of 0.5 μM in a volume of 20 μl and in the presence of the RNase inhibitor RNasin (12 U). Following incubation at 37°C for 45 min, the resulting cDNAs were subjected to polymerase chain reaction (PCR) amplification. PCR reactions (50 μl) were carried out in 50 mM KCl–1.5 mM $MgCl_2$; reverse and forward primers (5′ GATCTTCGGATCACTT TGGTC 3′) were added to 2.5 μM. Upon addition of 4 U of *Taq* DNA polymerase (Beckman Instruments, Inc., Fullerton, Calif.), the samples were subjected to a first cycle of amplification (denaturation at 92°C for 5 min, annealing at 50°C for 3 min, and extension at 65°C for 2 min), followed by 29 cycles in which the denaturation and annealing steps were shortened to 1 min. A 1- to 2- μg sample of genomic DNA was amplified as described for reverse-transcribed RNA samples.

PCR amplification of reverse-transcribed RNAs resulted in a single nucleic acid species whose length is identical to that of the mature U6 snRNA. In contrast, the single product obtained with genomic DNA from each species was larger, consistent with the amplification of an intron-containing gene (Fig. 1). These results indicate that the intron is present in the U6 gene from all fission yeasts; moreover, they suggest that there are no intronless versions of the U6 snRNA gene and that no appreciable pool of unprocessed U6 snRNA exists in the cells.

For all four *Schizosaccharomyces* species, the products obtained after PCR amplification of genomic DNA were

* Corresponding author.

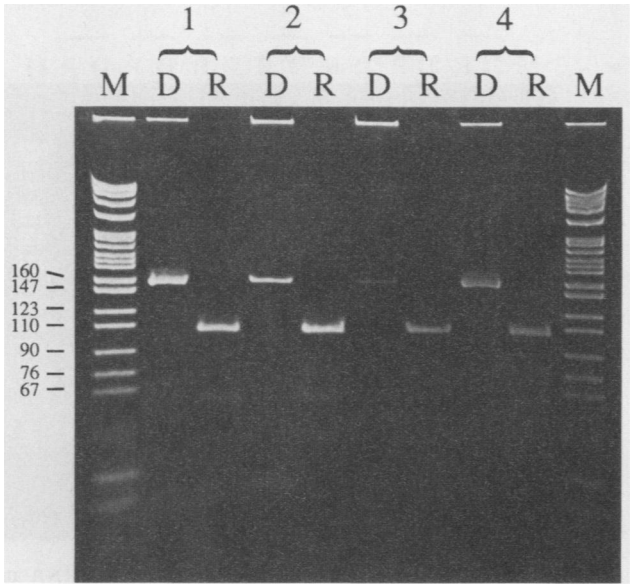

FIG. 1. PCR amplification of reverse-transcribed total RNA and genomic DNA from *Schizosaccharomyces* species. Aliquots (one-fifth) of the reactions were digested for 30 min at 37°C with a mixture of RNases A and T2; the products were resolved by electrophoresis in a native 7% polyacrylamide gel and visualized by ethidium bromide staining. Lanes: M, *Hpa*II-digested pBR322; 1, *S. pombe*; 2, *S. octosporus*; 3, *S. japonicus*; 4, *S. malidevorans*; R, total RNA; D, genomic DNA.

purified, cloned into appropriate vectors, and sequenced. Preparations of genomic DNA were amplified as described above, except that the primers used were phosphorylated. Upon completion of 30 rounds of amplification, all four deoxynucleotides were added to a concentration of 0.1 mM and the samples were incubated for 1 h at room temperature in the presence of 6 U of the Klenow fragment of DNA polymerase I. After extraction with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), the nucleic acids were recovered by ethanol precipitation and ligated to 50 pmol of phosphorylated *Bam*HI linkers. The mixture was exhaustively digested with *Bam*HI and electrophoresed in a 3% NuSieve-1% SeaKem agarose gel. The fragments were visualized by ethidium bromide staining, cut out, recovered with GelX (Genex, Gaithersburg, Md.), and cloned into the plasmid pTZ18U (27). Double-stranded plasmid DNA was prepared, and the inserts were sequenced by using the Sequenase version 2.0 enzyme and protocol (U.S. Biochemicals).

The sequences are displayed in Fig. 2. The mature domains of the RNA molecules between the amplification primers show absolute conservation (the information contained at the ends of the molecules is lost, since those regions of the cloned DNA correspond to the primers). The intron appears at the same position in all cases, and although its sequence shows considerable variation, there is sufficient relatedness to support a common ancestry. Within the intron itself, the strict conservation of those sequences shown to be important for accurate splicing is noteworthy; the only deviation is the change of the first T residue to a C in the branch site consensus region of the *S. japonicus* sequence. However, this change does not preclude its identification as a branch site since, at least in *S. pombe*, the splicing signals are not as stringently conserved as in the budding yeast *Saccharomyces cerevisiae* (28). The strict adherence of these elements to their respective consensus motifs suggests that efficient removal of the intron is of selective advantage. Outside these regions, the sequences are under no obvious selective pressure and therefore are free to diverge. Hence, their degree of dissimilarity can be construed as a measure of the evolutionary distance separating the different species. *S. pombe* and *S. malidevorans* appear to be very closely related, with the intron sequences differing at only one position; the sequences of *S. octosporus* and *S. japonicus* show more variance from *S. pombe*, with *S. japonicus* being the most divergent. The sequences of these introns have recently been reported elsewhere (16). Strikingly, there are seven differences between the *S. malidevorans* sequence and that reported here; five of them are in the otherwise conserved region 5′ of the TACTAAC box. Although it is possible that different isolates of *S. malidevorans* are this divergent, our data are most consistent with phylogenies based on other criteria. Specifically, morphological and biochemical data establish that the *Schizosaccharomyces* genus comprises three separate large clusters: one including *S. pombe* and *S. malidevorans* (sufficiently close to be proposed as varieties of the same species), one represented

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S.pombe   gatcttcggatcactttggtcaaattgaaacgatacagagaagattGTAAG  S.pombe
S.malid                     aaattgaaacgatacagagaagattGTAAG  S.malid
S.octos                     aaattgaaacgatacagagaagattGTAAG  S.octos
S.japon                     aaattgaaacgatacagagaagattGTAAG  S.japon

S.pombe   TAA--CAATATTTACCAAGGTTCGAGTCATACTAACTCGTTGTTTAGagca  S.pombe
S.malid   TAA--CAATATTTACCAAGGTTCGACTCATACTAACTCGTTGTTTAGagca  S.malid
S.octos   TACACCCCAGACGACCAAGGTTCGAGTCATACTAACTCGTTGTTTAGagca  S.octos
S.japon   TACCCCCCTAAGGTCGTGAGTTCGAGTTCCACTAACAC-CCGTCTAGagca  S.japon

S.pombe   tggcccctgcacaaggatgacactgcgacattgagagaaaacccatttt
S.malid   tggcccctgcacaaggatgacactgcga
S.octos   tggcccctgcacaaggatgacactgcga
S.japon   tggcccctgcacaaggatgacactgcga
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FIG. 2. Sequence comparison of *Schizosaccharomyces* U6 snRNA genes. Exon sequences appear in lower case; introns are in upper case. The conserved splicing signals are underlined; regions of sequence divergence are in boldface type. Abbreviations: *S. malid*, *S. malidevorans*; *S. octos*, *S. octosporus*; and *S. japon*, *S. japonicus*.

by *S. octosporus*, and a third represented by *S. japonicus*, which is sufficiently dissimilar to the other two to be proposed as a different genus (49). Our U6 snRNA intron sequence comparison supports this view; furthermore, it provides a basis for rooting the *Schizosaccharomyces* line of descent, placing *S. japonicus* as the most ancient divergence.

A comparison of known U6 snRNA sequences reveals a remarkably constant internal domain, with most of the observed variation being restricted to regions at the ends of the molecule. Since the intron lies within this domain, we designed PCR primers corresponding to universally conserved regions bracketing the site of the interruption in the *S. pombe* gene, which allowed probing of diverse organisms to ascertain whether they also possess homologous U6 introns. Since *Schizosaccharomyces* species have traditionally been classified as *Ascomycetes*, we investigated a representative organism from each of the main lines of descent within this class of fungi (9), namely *S. cerevisiae*, *Neurospora crassa*, and *Aspergillus niger*. The gene coding for the *S. cerevisiae* U6 snRNA (like those of several vertebrates [14, 22, 23, 37], an insect [13], a plant [43], and *Trypanosoma brucei*, sp., a very ancient divergence within the eucaryotes [46]) is known not to contain an intron (4), and therefore provides an experimental negative control. We also included the basidiomycete *Schizophyllum commune*; according to molecular phylogenies based on 5S rRNA (20), *S. pombe* appears to be at least as closely related to *Schizophyllum commune* as to the *Ascomycetes*. In order to provide a broader phylogenetic perspective, we analyzed the more distantly related lower eucaryotes *Physarum polycephalum* and *Tetrahymena pyriformis*.

Preparations of total RNA and of genomic DNA from these organisms were amplified as described above; the primers used were universal reverse primer 5′ TKTCAT CCTTGTGCAGGG 3′ (in which K is G or T), complementary to positions 55 to 72 in the *S. pombe* sequence, and universal forward primer 5′ TCAAWTTGAAACGATACAG 3′ (in which W is A or T), colinear with positions 20 to 38 in the *S. pombe* sequence. In all species except *S. pombe*, the major amplification products obtained from reverse-transcribed RNA or genomic DNA are identical in size, suggesting that none of the cognate genes are interrupted by an intervening sequence within the region bracketed by the universal PCR primers (Fig. 3). PCR amplification of genomic DNA sequences from *T. pyriformis* showed a second discrete product of over 600 base pairs. Upon reamplification, the purified lower-molecular-weight DNA species yielded a single product of the original size, while the purified larger species gave rise to both the long and short forms (data not shown). These results are consistent with the notion that *T. pyriformis* has two copies of the U6 snRNA gene oriented as a direct repeat and separated by approximately 500 base pairs. The appearance of a higher-molecular-weight product after amplification of reverse-transcribed RNA from *Schizophyllum commune* is presumably an artifact, since it was not reproducible (data not shown).

Thus, the occurrence of an intron in the U6 snRNA gene appears to be restricted to fission yeast, to the exclusion of other eucaryotes investigated. This, coupled with the clear relatedness of their sequences, suggests that an intron was not present in the progenitor gene but was acquired later in evolution, via a single insertional event occurring early in the divergence of the *Schizosaccharomyces* line of descent.

Two theories concerning the origin of introns have been advanced. According to one, ancestral genomes were riddled with introns; in those organisms whose genomes were streamlined towards short generation times (namely, procaryotes), they were mostly lost during evolution, while in organisms that favored specialization at the expense of longer generation times (namely, eucaryotes), they became stable and evolved to serve, for instance, such refined control mechanisms as differential expression (12). According to the second theory, introns arose after the eucaryotic line of descent was established, via insertional events (7); recent analysis of two widely distributed gene families led to the concept of a protosplice site, a consensus sequence defining positions of hypothetical intron gain (15).

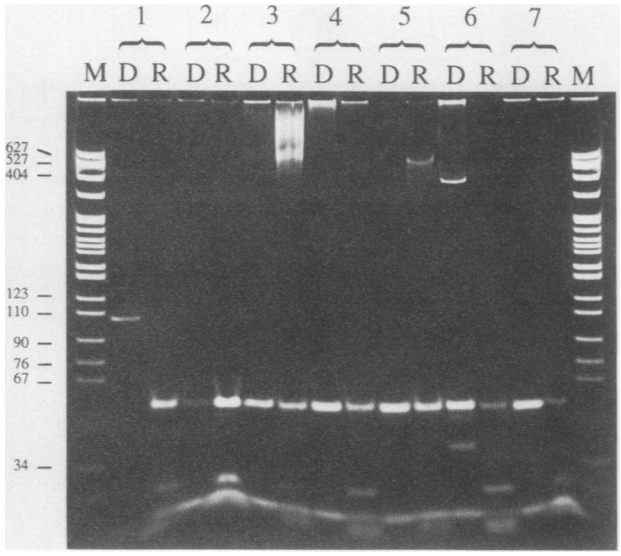

FIG. 3. PCR amplification of reverse-transcribed total RNA and genomic DNA from diverse species. Total RNA and genomic DNA from the species identified below were amplified and reaction products were resolved as described in the legend to Fig. 1. Lanes: M, *Hpa*II-digested pBR322; 1, *S. pombe*; 2, *S. cerevisiae*; 3, *A. niger*; 4, *N. crassa*; 5, *Schizophyllum commune*; 6, *T. pyriformis*; 7, *P. polycephalum*; R, total RNA; D, genomic DNA. The very faint RNA-sized band in the *S. pombe* DNA lane most likely is due to contamination from the adjacent well, since it is not present in other gel analyses of the same and other similar reactions (e.g., Fig. 1).

Our data provide a convincing example of the insertion of an intron after the divergence of the eucaryotic line of descent; in fact, the insertion appears to have occurred much later, shortly after the separation of the *Schizosaccharomyces* genus from the rest of the fungi. A tantalizing hypothesis, advanced by Brow and Guthrie (5), is that the U6 intron is the result of a mishap during pre-mRNA splicing: an intron undergoing excision inserted into the U6 snRNA molecule present in the spliceosome. This model would place U6, and in particular the site of insertion, close to the catalytic core, where the chemical events of splicing take place. Although the data presented here are consistent with such a scenario, clearly other mechanisms of intron gain can and have operated, as in the yeast nucleolar U3 snRNAs, which do not function in splicing (30).

Whatever the mechanism of its insertion, it seems most likely that the U6 intervening sequence originated and has been maintained as an intron, faithfully and efficiently removed via the pre-mRNA pathway. Other snRNAs, notably from budding yeasts (18, 38), contain extra sequence blocks which have been viewed as expansion segments (21), given the precedent set by phylogenetic comparisons of rRNAs

(11). We speculate that "extra" sequences can be maintained as expansion segments or be excised without subsequent ligation (6) when they lie within the context of a functionally malleable sequence; on the other hand, sequences which interrupt essential, highly conserved regions of the molecule would need to be spliced, thus preserving their identity as introns. By providing evidence that the ancestral U6 gene lacked an intron and that in spite of extensive divergence, the inserted sequence is efficiently spliced out in all species, our data strengthen the notion that this conserved region of U6 plays a crucial role in splicing, perhaps functioning directly in catalysis.

Nucleotide sequence accession numbers. GenBank accession numbers are as follows: *S. malidevorans*, M34104; *S. octosporus*, M34105; and *S. japonicus*, M34106.

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