A group III intron is formed from domains of two individual group II introns

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A 1352-nucleotide intron within the Euglena gracilis chloroplast ycf8 gene has been characterized as a complex twintron with overlapping internal introns and alternative splicing pathways. Partially spliced pre-mRNAs were characterized by a combination of cDNA cloning and sequencing, Northern hybridization, and S1 nuclease protection analyses. In the predominant pathway, two internal group II introns (601 and 392 nucleotides) are spliced from subdomain ID of an external group II intron (359 nucleotides). In an alternative pathway, following excision of the 601-nucleotide intron, splicing of a group III intron occurs. This group III intron is recruited from sequences of the external intron and the 392-nucleotide intron. This is the first evidence that a group III intron can be derived from portions of existing group II introns. The mechanism of group III intron formation may also be relevant to the evolution of nuclear introns from putative group II intron ancestors.

[Key Words: Complex twintron; alternative splicing; group III intron evolution; nuclear intron evolution; Euglena gracilis; chloroplast]

Received April 15, 1994; revised version accepted May 18, 1994.
Because some group II introns can act as mobile genetic elements (Lambowitz and Belfort 1993), the formation of twintrons is most likely a consequence of an intron moving into a pre-existing intron. Internal introns appear to be inserted into functional domains of external introns (Copertino and Hallick 1993). Excision of twintrons follows a sequential splicing pathway. Internal introns are excised first, reconstituting the functional domain of the external intron. External introns are subsequently spliced, resulting in exon ligation. Splicing of internal group III introns of twintrons can occur from multiple 5' and 3' splice sites (Copertino et al. 1992; Drager and Hallick 1993).

The E. gracilis chloroplast ycf8 gene, encoding a 31-residue hydrophobic polypeptide of unknown function, is split by an unusual 1352-nucleotide intron (Hallick et al. 1993). When partially spliced ycf8 pre-mRNAs were characterized, intermediates from alternative splicing pathways were identified. One pathway is that of a complex twintron composed of two internal group II introns inserted into an external group II intron. In the alternative splicing pathway, a group III intron is formed from portions of two group II introns. The structural rearrangements necessary to convert domains of two group II introns into a group III intron may be a model for understanding the origin of group III introns from group II ancestors.

Results

The ycf8 intron is a complex twintron composed of two introns within a third intron

A 1352-nucleotide intron occurs in the E. gracilis chloroplast ycf8 gene in the psbB-ycf8 dicistronic operon (Figs. 1 and 2). This intron is three times larger than a typical Euglena group II intron (Copertino and Hallick 1993; Hallick et al. 1993) and contains three group II domains V and VI (described below). Because there is precedent for a group II intron within another group II intron (Copertino et al. 1991), our hypothesis was that the ycf8 intron is composed of two group II introns internal to a third group II intron.

To test this hypothesis, cDNAs from partially spliced pre-mRNAs were amplified with the polymerase chain reaction (PCR). The PCR products were cloned and sequenced. The PCR strategy and representative DNA sequence data are shown in Figure 1. The plasmid DNAs obtained are summarized in Table 1. To identify partially spliced pre-mRNAs, cDNA synthesis was primed with an oligonucleotide (C2) spanning the boundary of the 1352-nucleotide intron and the 3' exon. The cDNA was amplified with primer C2 and primer P1 in ycf8 exon 1. As shown in Figure 1, the most abundant cDNA (pEZC1062) represents a partially spliced pre-mRNA. Two internal introns, intron b (601 nucleotides) and intron c (392 nucleotides) (Fig. 2) have been removed, leaving a 359-nucleotide external intron (intron a).

To confirm subsequent splicing of the ycf8 pre-mRNA and to determine the splice boundaries of the external intron, a cDNA–PCR reaction was performed using a cDNA primer (C3) specific to ycf8 exon 2 and a PCR primer (P3) specific to psbB exon 5 (Fig. 1). Based on the sequence of pEZC1061, the 5' and 3' splice boundaries of the 359-nucleotide external intron are 5'-GUGUG and UUAUCUAAC-3' (Fig. 2).

The sizes of the three ycf8 introns are typical of Euglena group II introns (Copertino and Hallick 1993; Hallick et al. 1993). As shown in Figure 3, secondary structures for each intron were predicted based on a model proposed by Michel et al. (1989). Based on these structures, intron b is located in the loop region between D1 and D3, and intron c is located 5 nucleotides 3' of EBS1 of the external intron.

Splicing of internal introns is not ordered

Splicing of the internal introns could occur either in an ordered pathway or as two independent events. To de...
Figure 2. DNA sequence of the E. gracilis chloroplast ycf8 gene (coordinates 97051-95552 of EMBL accession no. X70810, "E. gracilis chloroplast exon 2 (C2), representing the 5'-ends of intron d and the 3'-end of intron c.

To detect intermediates lacking only intron b, cDNA synthesis was primed at a site internal to intron c (C1), and the resulting cDNA was amplified with a primer specific to ycf8 exon 1 [P1]. The cDNA in pEZC1079 represents a pre-mRNA with intron b removed and introns a and c remaining [Fig. 1]. Therefore, intron b can excise prior to intron c. When cDNA synthesis was primed at a site spanning the junction of intron a and ycf8 exon 2 [C2], and the resulting cDNA was amplified with a primer specific to intron b [P2], a cDNA [pEZC1078] representing a pre-mRNA with intron c removed and introns a and b remaining was obtained. Therefore intron c can be excised prior to intron b. A model for one RNA splicing pathway of the ycf8 complex twintron is shown in Figure 4. Intron a is split into three segments (a1, a2, and a3) by the presence of introns b and c. The excision of the two internal introns is sequential, but not ordered, resulting in two different intermediates [Fig. 4, II and III] with one intron excised. After the excision of both internal introns, the segments of the external intron are joined and the external intron is spliced to yield the mature ycf8 mRNA.

Alternative splicing of a partially spliced ycf8 pre-mRNA excises portions of two different group II introns

When cDNA synthesized from a primer (C1) within intron c was amplified using an exon 1-specific primer [P1], two additional splicing products were detected. The new cDNAs correspond to pre-mRNAs with 705 (pEZC1080) or 703 nucleotides [pEZC1081] removed [Fig. 2]. Common to each excised sequence are the 601-nucleotide intron b, and 85 nucleotides 3' of intron b. This 85-nucleotide corresponds to the entire 40-nucleotide external intron segment a2 and the 45 nucleotides of the 5' end of intron c. The 5' end of the excised sequences includes either 19 [pEZC1080] or 17 nucleotides [pEZC1081] from intron a, segment al. DNA sequence data surrounding the new splice boundaries and intron b splice boundaries are shown in Figure 5.

Because the recombinant plasmids pEZC1079, pEZC1080, and pEZC1081 were derived from the same cDNA—PCR reaction, the frequency of each cDNA is an approximate measure of the abundance of different partially spliced pre-mRNAs. Of 22 cDNAs analyzed, 15 represent splicing of intron b, 4 come from excision of 705 nucleotides [pEZC1080], and 3 from excision of 703 nucleotides [pEZC1081].

Alternatively spliced sequences are characteristic of a group II-within-group III twintron

There are at least two splicing pathways that might account for excision of 705- and 703-nucleotide RNA. One possibility is that excision occurs in a single splicing reaction. Alternatively, splicing might occur by a sequential pathway. First, intron b (601 nucleotides) is spliced, second, the remaining 104- or 102-nucleotide RNAs are spliced as group III introns. There is precedent for a group II intron internal to a group III intron in the Euglena chloroplast rps3 gene [Copertino et al 1991].

Features of the 104- and 102-nucleotide RNAs are consistent with their identification as group III introns. Models for the secondary structures of the putative group III introns are shown in Figure 6. Because multiple U residues flank either side of the excision site, the exact splice boundaries could only be determined to within ±2 nucleotides. Group III introns have characteristic 5' boundary sequences of 5' nUnnG, and a domain VI with a bulged A residue at position –8 or –7 preceding the 3' splice site [Copertino and Hallick 1993]. According to these criteria, the most likely 3' splice boundary for both group III introns is UAUUGCUUU-3' [Fig. 6]. The 5' splice boundary is 5'-UUUG for the 104-nucleotide intron and the overlapping sequence 5'-UUGUG for the 102-nucleotide intron. Multiple 5' splice boundaries are utilized in excision of internal group III introns of other twintrons [Copertino et al 1992, Drager and Hallick 1993]. Thus, our working hypothesis is that the 104- and 102-nucleotide RNAs are a single group III intron [intron d] with multiple 5' splice boundaries. Intron d is derived from part of external intron segment a1, all of segment a2, and the 5' end of intron c. A prediction from this
model is that the excised intron d should accumulate in vivo as a 102- to 104-nucleotide RNA. To test this prediction, excised putative intron d was characterized by Northern hybridization and S1 nuclease protection.

Identification of an excised group III intron derived from group II intron sequences

For Northern hybridization and S1 nuclease protection, a cDNA [pEZC1082] lacking intron b, but with contiguous intron d and flanking sequences, was used as a template for RNA probe synthesis. This cDNA clone was obtained by cloning the cDNA–PCR product amplified from the cDNA primer (C1) and PCR primer (P4) [Table 1; Fig. 7].

For Northern hybridization, Euglena chloroplast RNA [ctRNA], and ctRNA fractionated into low [soluble RNA (sRNA)] and high molecular weight [HMW] fractions were electrophoretically separated, transfected to a membrane, and hybridized with the pEZC1082 RNA probe. The results are shown in Figure 7A. The RNA probe hybridized to a RNA species migrating at 110 nucleotides, which is enriched in the sRNA fraction. This RNA is interpreted as the linear and/or lariat forms of the excised 102- and 104-nucleotide group III intron d [Copertino et al. 1992]. Precursor RNAs containing intron d sequences are present in the total and HMW ctRNAs. This RNA probe also hybridizes to excised introns a and c and their pre-mRNAs. To confirm the identification of excised intron d, an S1 nuclease protection assay was used. A RNA probe was synthesized from cDNA clone pEZC1082 and hybridized to Euglena total ctRNA. To help distinguish S1-protected RNAs derived from introns a and c, RNA probes made from pEZC1082 cDNAs modified at the 3' and 5' ends by deletions of 30 and 44 nucleotides, respectively, were also hybridized with the same RNA. The hybrids were treated with S1 nuclease and resolved by gel electrophoresis (Fig. 7B). An abundant 101-nucleotide S1 nuclease-protected fragment was detected with the full-length and Δ3 deletion RNA probes. The corresponding product was ~4 nucleotides shorter with the Δ5' DNA RNA probe. Because the Δ5' deletion includes the first 5 nucleotides complementary to the putative 5' end of intron d, and the Δ3' deletion is outside of intron d, we conclude that the 101-nucleotide products are the result of protection of each of the RNA probes by excised intron d. As judged by the relative abundance of the S1 nuclease-protected products [Fig. 7B], the accumulation of excised introns d (101-nucleotide product) and c (180-nucleotide product) are comparable. Therefore, both RNA splicing pathways contribute significantly to ycf8 pre-mRNA processing.

The alternative splicing pathway of the ycf8 complex twintron

In one splicing pathway of the ycf8 complex twintron, excision of the internal group II introns is unordered but precedes excision of the external intron (Fig. 4). In an alternative pathway, group III intron d may excise following the excision of intron b. In the formation of group III intron d, sequences from both external intron a and internal intron c (Fig. 6) are recruited. The excision of group III intron d disrupts the structure of both internal intron c and external intron a. A model for the alternative splicing pathway is shown in Figure 8. Following the excision of intron b, the structure of the intermediate may alternate between one permissive for group II splicing [Fig. 8, II] and one permissive for group III splicing [Fig. 8, II'].

What is the fate of the pre-mRNA [Fig. 8, VI] (647 or 649 nucleotides) following excision of intron d? There are at least three possibilities. First, because subdomain ID and EBS1 are removed from intron a, intron a may not be processed further, resulting in a dead end pathway, and RNA turnover. Second, even though subdomain ID and EBS1 of the external intron a have been removed, the remaining 647–649 nucleotides might still excise as a single group II intron. Finally, the remaining 647 or 649 nucleotides of the pre-mRNA might be excised by sequential splicing of individual introns. Attempts to characterize further RNA processing of intermediate VI [Fig. 8] are currently in progress.

Discussion

A new category of complex twintron with alternative core structures

A novel 1352-nucleotide intron in the chloroplast-encoded ycf8 gene from E. gracilis is composed of two
A group III intron formed from two group II introns

After the excision of internal intron b, the partially spliced pre-mRNA can assume different structures. One of these structures (Fig. 8, II) facilitates the excision of group II introns inserted into a third group II intron. The ycf8 intron is the second example of a group II intron in another group II intron, the first case of intron insertion in domain ID, and the first twintron formed from three group II introns. The properties of this intron are consistent with the emerging picture of twintron formation resulting from the insertion of one intron into a functional domain of a second intron (Copertino and Hallick 1993). The unexpected and unprecedented result of this study is the characterization of a group III intron formed from segments of two group II introns.

**Figure 3.** Secondary structure models of the three individual group II introns in the Euglena ycf8 complex twintron. (A) Intron a; (B) intron b; (C) intron c. Long-range tertiary interactions, α/α', ε/ε', and γ/γ', are represented by broken lines. The guided pair is represented by rectangles. [*] The branch site A. The insertion sites of internal introns b and c in intron a are indicated by arrows. The γ/γ' interaction of intron c is predicted to be a C-U pair. The guided pair sequence is a normal base pair only in external intron a, the EBS2-IBS2 is apparent only in introns b and c, and the α/α' pairing is only evident in intron b.

**Figure 4.** One splicing pathway of the Euglena ycf8 complex twintron. The psbB-ycf8 pre-mRNA is shown. Exons are represented by solid boxes. The shaded boxes represent intron a. Intron b is depicted as a thick line. Intron c is shown as a thin line. Excised introns are represented by broken lines.
intron c. An alternate structure (Fig. 8, II') leads to splicing of intron d. RNAs are known to switch between different conformations during splicing. During nuclear mRNA splicing, U6 small nuclear RNA (snRNA) base-pairs with U4 snRNA during spliceosome assembly (Pikielny et al. 1986; Cheng and Abelson 1987; Lamond et al. 1988; Wassarman and Steitz 1992) and subsequently unwinds from U4 and base-pairs with U2 snRNA and the 5' splice boundary (Madhani and Guthrie 1992; Kandels-Lewis and Seraphin 1993; Lesser and Guthrie 1993). Large-scale conformational changes also occur in movement of the internal guide sequence relative to the catalytic core of the *Tetrahymena* group I ribozyme (Wang et al. 1993).

Models for alternative structures of partially spliced ycf8 twintron permissive for group II versus group III splicing are shown in Fig. 9. In one intermediate (Fig. 8, II), group II intron c secondary structure and tertiary interactions form. Intron c does not have a particularly robust core structure. The proposed tertiary interactions EBS1-IBS1 and ε/ε' are not strong, and the γ/γ' and guided pairs are not normal base pairs. No α/α' interaction is apparent (Fig. 3C). Possibly as a consequence of a weak intron c structure, a group III intron can form from the same RNA intermediate (Fig. 8, II'). The group III intron is composed of a subdomain ID-like structure at its 5' end and a domain VI at its 3' end (Fig. 9). Domain VI and part of the stem of subdomain ID are contributed by intron c while the remainder of subdomain ID (including EBS1) comes from intron a.

Intron d has a reasonable EBS1 in either the 104- or 102-nucleotide form (Fig. 6). The 102-nucleotide splicing variant has the same EBS1 and a portion of the D3 stem of intron a. The 104-nucleotide variant has a different D3 stem and EBS1. One model for intron d splicing involves the use of the EBS1 loop of intron a for exon recognition. In the partially spliced intermediate lacking intron b (Fig. 4, II), EBS1 of intron a might be blocked from interaction with its normal IBS1 by the presence of intron c but be able to interact with a different IBS1. An IBS1-like sequence [IBS1'] is present at the base of intron a domain.
Origin of a group III intron

Figure 7. Northern hybridization and S1 nuclease protection of intron d. Diagrams of the cDNA–PCR product pEZC1082 and deletion clones pEZC1082(Δ3') and pEZC1082(Δ5') used for Northern hybridization and S1 protection are shown. The boxed regions in the plasmid diagrams represent intron d. (A) Northern hybridization analysis. The sizes of internal cRNA markers are indicated at left (Schnare and Gray 1990). (B) Group III intron d. (B) S1 nuclease protection of the excised group III intron d. Lanes are labeled as follows: (−) Untreated probe; (+) S1 nuclease digestion of unprotected probe; (in vivo) digestion of hybridized RNAs with 0.3, 0.5, 1.0, and 1.5 U/μl of S1 nuclease for pEZ1082, and 0.5, 1.0, and 1.5 U/μl for the A3' and AS' probe. The size of each protected band is shown at left; the identity of protected RNA species is shown at right.

The 220- and 56-nucleotide RNAs result from protection by fully unspliced pre-mRNA. This 220-nucleotide RNA is converted to smaller species by 10 and 20 nucleotides at higher concentrations of S1 nuclease. The protected fragment of ~180 nucleotides is from excised intron c.

ID. The 5' splice sites of intron d might simply be cryptic boundaries resulting from an EBS1–IBS1' interaction (Fig. 9). To complete intron d formation, domain VI would be recruited from sequences near the 5' end of intron c (Fig. 9).

Intron splicing may be regulated by trans-acting factors. In Neurospora, the CYT-18 protein binds to the highly conserved core of the group I intron and stabilizes the structure in a conformation required for splicing enhancers have been identified for the alternative splicing of nuclear pre-mRNA. These cis-acting enhancers recruit proteins to the 3' splice site of the nuclear intron to facilitate spliceosome assembly (Lavigneuer et al. 1993; Tian and Maniatis 1993). In the alternative splicing of the ycf8 complex twintron, binding of group II and group

Figure 8. The alternative splicing pathway of the Euglena ycf8 complex twintron. Solid boxes represent the psbB and ycf8 exons. Shaded boxes represent intron a. Intron b is shown as a thick line, and intron c as a thin line. Intron d is represented as a box shaded with vertical lines. The RNA intermediates [II] and [II]' have the same primary sequence but differ in structure.

Figure 9. Formation of a group III intron from sequences of two group II introns. The nucleotides from intron a and intron c are represented by thin and thick lines, respectively. The cryptic IBS1 for intron d formation is labeled IBS1'.
III intron-specific proteins may stabilize different core structures. The *Euglena* chloroplast genome contains several intron-encoded open reading frames (ORFs) (Copertino and Hallick 1993; Hallick et al. 1993) that have similarity to other intron-encoded maturases (Mohr et al. 1993). One of these *Euglena* ORFs, ycf13, resides within a group III twintron and its protein product may facilitate group III splicing (Copertino et al. 1994). Other ORFs, such as orf758 in *psbC* intron 2 and orf506 and orf281A in *psbD* intron 8, are present in group II introns. Although the mitochondrial maturases involved in group II splicing are, generally, intron specific, it is possible that *Euglena* chloroplast intron-encoded polypeptides may have a role in splicing numerous group II or group III introns (Hallick et al. 1993; Cupertino et al. 1994).

Subdomain ID in group III introns

*Euglena* group III introns are proposed to be a streamlined form of group II introns in which a group II-like 5′ splice boundary and 3′ domain VI are retained, but domains II, III, IV, V and most of domain I are absent. (Copertino and Hallick 1993). It has been suggested that many group III introns have retained the equivalent of group II subdomain ID. The evidence is twofold. First, many group III introns have EBS1-like sequences as terminal loops of a 5′ domain. Second, the putative 5′ domain is a hot spot for intron insertion among group III twintrons. Such intron insertion sites are presumptive markers for functional domains (Copertino and Hallick 1993). For group III intron d, the proposed group III subdomain ID is actually a bona fide subdomain ID recruited from group II intron a. This is additional evidence for a subdomain ID function in some group III introns. It is also noteworthy that intron b interrupts intron d in the putative subdomain ID. Intron b must be spliced prior to excision of the intron d.

Because the mechanism for group III intron splicing resembles that of group II (Copertino et al., 1994), the catalytic core for both types of introns should be similar. For group II splicing, domains I and V and the 5′ exon constitute the catalytic core for the first transesterification reaction. Domain I is the site for binding domain V, the putative ribozyme, during splicing of the yeast mitochondrial intron a5γ (Jarrell et al. 1988, Koch et al. 1992). The splicing activity of the yeast mitochondrial b1 intron is greatly reduced when subdomain ID is deleted [Bachl and Schmelzer 1990]. Some *Euglena* and *Astasia* group II introns lack subdomains IA, IB, and IC but retain ID. Domain V is absent from group III introns. The most logical explanation for the relationship between group II and group III introns is that some key group II interactions, especially that of domain V, occur in *trans* during group III splicing. The subdomain ID-like region of group III introns may be the best candidate for interaction with a *trans*-acting domain V. It is possible that the domain V from intron a or intron c might be in close enough proximity to participate in *trans* in the group III intron d splicing reaction.

On the origin of group III and nuclear pre-mRNA introns from group II introns

Based on the similarities in splicing mechanisms, it has been suggested that group II and nuclear pre-mRNA introns are evolutionarily related (Sharp 1985; Cech 1986; Guthrie 1991). The cis-encoded domains of group II introns and the trans-acting snRNAs of nuclear introns have structural and functional similarities (Zhuang and Weiner 1986; Parker et al. 1987; Jacquier 1990; Madhani and Guthrie 1992; Newman and Norman 1992; Reich et al. 1992), and may assume a similar core catalytic structure (Lesser and Guthrie 1993; Sontheimer and Steitz 1993, Wise 1993). Group II, group III, and nuclear pre-mRNA introns may all belong to the same intron superfamily. All splice via a lariat intermediate with an internal 2′ → 5′ phosphodiester bond. The U residue in the second position and G residue in the fifth position of the 5′ splice boundaries of group II (5′-GUCYG), group III (5′-NUNNG), and nuclear introns (5′-GUNNG) are conserved (Kandels-Lewis and Seraphin 1993; Lesser and Guthrie 1993; Sontheimer and Steitz 1993). The base-pairing between U2 snRNA and the branchpoint consensus sequence of nuclear introns results in a helical domain comparable to domain VI of group II and group III introns. Group II and possibly group III introns have an essential tertiary interaction between subdomain ID and the 5′ exon, comparable to the interaction of nuclear 5′ exons with U5 snRNA. Therefore, it has been suggested that the evolution of the group II and group III splicing in *Euglena* chloroplast may parallel the evolution of nuclear pre-mRNA splicing (Copertino and Hallick 1993). Similar mechanisms may have been used for transferring cis-encoded splicing elements to *trans*-acting splicing factors during evolution of both group III and nuclear pre-mRNA introns.

The discovery that domains of group II introns can be converted into a group III intron via a twintron intermediate may serve as a model for events in the evolution of nuclear pre-mRNA introns from ancestral group II introns. The group III splicing pathway, as an alternative or mis-splicing variant of a normal group II splicing reaction, would appear to require *trans*-acting RNAs as direct replacements for key tertiary interactions that occur during group II splicing. *Trans*-acting RNAs recruited from group II introns (or from within a twintron) might replace the *cis*-acting domain V, the Κ interaction of domain IC, and EBS2 during group III splicing. Domain V can act in *trans* to promote splicing of a yeast mitochondrial group II intron, albeit with very low efficiency (Dib-Hajj et al. 1993). Interactions of *trans*-acting RNAs for group III splicing might be very similar to tertiary interactions that occur during nuclear pre-mRNA splicing, requiring proteins to stabilize RNA–RNA interactions. A helix formed by base-pairing of U2 and U6 snRNAs is proposed as a *trans*-acting counterpart of group II domain V (Madhani and Guthrie 1992), and another region of U6 snRNA is the proposed counterpart of the subdomain IC Κ that interacts with the 5′ splice site. Therefore, if nuclear pre-mRNA introns evolved from a common ances-
tor with group II introns, the transition may have also occurred directly from a group II twintron-like precursor.

Once a group III, or a nuclear pre-mRNA, intron is formed, the new intron could potentially spread to new genes as a mobile genetic element. Intron mobility could involve the same events envisioned for group II mobility and twintron formation, including reverse splicing into a new RNA species, reverse transcription, and homologous recombination (Copertino and Hallick 1993).

Twintrons are not likely restricted to Euglena chloroplasts. Yeast mitochondrial intron all efficiently transposes into both a group I and a group II intron (Mueller et al. 1993). If we accept the view that functional domains at the heart of the nuclear spliceosome are derived from a common ancestor with group II introns, then the occurrence of an intron within U6 snRNA of Schizosaccharomyces pombe [Tani and Ohshima 1989] can also be viewed as a twintron-like RNA that occurs in a nuclear RNA precursor. This U6 intron is within a region that base-pairs with U2 snRNA and is proposed to be the functional equivalent of group II intron domain V (Madhani and Guthrie 1992). Domain V is also a hot spot for group II twintron formation (Copertino and Hallick 1993). In the most general view, the introduction of one catalytic RNA into a functional domain of another catalytic RNA, through a process similar to twintron formation, can result in new combinations of sequences and structural domains that might lead to new RNA catalyzed reactions significant for RNA evolution.

Materials and methods

Euglena chloroplast RNA isolation

E. gracilis ctRNA (DNA-free) was isolated from purified chloroplasts as described [Christopher 1989]. HMW RNA and sRNA were prepared by isopropanol fractionation as described [Orozco 1982].

cDNA synthesis, PCR amplification, and cDNA cloning

cDNAs were synthesized in vitro from total ctRNA as described previously [Yepiz-Plascencia et al. 1990]. Each cDNA reaction contained 10 μg of DNA-free total ctRNA and 200 ng of gene-specific oligonucleotide primer. The GeneAmp DNA amplification Kit (Perkin-Elmer Cetus) was used for PCR amplification of cDNAs using specific oligodeoxynucleotide PCR primers complementary to the cDNA and the cDNA primers. Each PCR reaction contained half the cDNA synthesis product and 200 ng of each oligodeoxynucleotide primer. Reactions were performed as previously reported [Copertino et al. 1991]. All cDNA–PCR products were ligated into the ddT-tailed pKS+ vector (Holton and Graham 1991), cloned, and sequenced. A total of three cDNA primers and four PCR primers were utilized for cDNA–PCR amplifications (Table 1).

Molecular subcloning

The 5' end of plasmid pEZC1082 was deleted by 44 nucleotides to result in plasmid pEZC1082Δ5'. The 3' end of plasmid pEZC1082 was deleted by 30 nucleotides to result in plasmid pEZC1082Δ3'. The exonuclease III deletion and cloning method has been described [Christopher 1989].

RNA probe synthesis

Probes for Northern hybridization and S1 nuclease protection were generated from plasmids pEZC1082, pEZC1082Δ3', and pEZC1082Δ5' linearized by digestion with HindIII, HindIII, and PvuII, respectively. The linearized plasmid DNAs were used as templates for 32P-labeled RNA probe synthesis, using either T3 or T7 RNA polymerase [Christopher 1989].

Northern hybridization analysis

Five micrograms each of total chloroplast RNA, HMW enriched chloroplast RNA, and the sRNA fraction were electrophoresed on a 6% polyacrylamide gel containing 8 M urea. Fractionated RNAs were transferred to GeneScreen (New England Nuclear, DuPont Co.) membranes and hybridized with RNA probes. The prehybridization, hybridization, and posthybridization washes were done as described previously [Copertino et al. 1991].

S1 nuclease protection analysis

S1 nuclease protection assays were carried out as described by Sambrook et al. (1989). 32P-uniformly labeled RNA probe (1 x 106 cpm) was hybridized to 3 μg of chloroplast RNA at 42°C for 16 hr. The hybridized samples were subsequently treated with 0.3, 0.5, 1.0, and 1.5 U/μl of S1 nuclease [U. Biochemical] at 37°C for 1.5 hr. The resulting protected fragments were size-fractionated by electrophoresis on 6% polyacrylamide/7 M urea gels and visualized by autoradiography.

Prediction of intron secondary structures

Group II intron secondary structures are predicated based on the model of group IIB introns proposed by Michel et al. (1989). Intron sequences were aligned with group II intron sequences from other species prior to folding. Conserved key features such as domains V and VI, the closure of domain I, EBS1–IBS1 pairing, e/e' and γ/γ' interactions were identified and used as the landmarks for the folding of intron secondary structures. The folding of the group III intron secondary structures was based on the primary sequence alignment with other group III introns and the conserved domain V and EBS1–IBS1 pairing.

Acknowledgments

We thank Alan Lambowitz, Carol Dieckmann, Don Copertino, Mike Thompson, Jenny Stevenson, and Kristin Jenkins for critical reading of the manuscript and stimulating discussions. We also thank Don Copertino for providing the Northern blot. This work was supported by the National Institutes of Health.

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*Genes Dev.* 1994, 8:
Access the most recent version at doi:10.1101/gad.8.13.1589

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