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Editing of the mitochondrial small subunit rRNA in *Physarum polycephalum*

Ratha Mahendran, Matthew S.Spottswood, Anita Ghate, Mei-ling Ling, Karen Jeng and Dennis L.Miller[1]

Molecular and Cell Biology Program, The University of Texas at Dallas, Richardson, TX 75080, USA
[1]Corresponding author

Post-transcriptional insertion, substitution or deletion of nucleotides in RNA (RNA editing) has been observed in RNAs from a number of organisms but always in messenger RNA or transfer RNA. We report here that the 17S rRNA of the mitochondrial ribosome of *Physarum polycephalum* is edited at 40 sites with single cytidine insertions. The locations of the editing sites are fairly evenly distributed throughout the RNA and do not correspond to any obvious feature of the primary sequence or secondary structure. In addition to these cytidine editing sites are editing sites in which a nucleotide other than cytidine is inserted. At two sites a uridine is inserted and at two sites two adenosine residues are inserted. This is the first report of mixed nucleotide insertional editing. These results imply that the editing mechanism in *Physarum* may be different from those proposed for the kinetoplastid protozoa.

Key words: gene expression/mitochondria/RNA editing/ ribosomal RNA/slime mold

Introduction

RNA editing is defined as the insertion, deletion or substitution of nucleotides in an RNA such that the RNA's sequence is different from that of the DNA encoding it (Benne *et al.*, 1986). The most extensive editing in terms of the number of nucleotides affected is found in the mitochondria of the kinetoplastid protozoa (Stuart, 1991), some plants (Bonnard *et al.*, 1992) and the slime mold *Physarum polycephalum* (Mahendran *et al.*, 1991). In the kinetoplastid protozoa and *Physarum* the production of a functional mRNA is accomplished by the insertion of nucleotides, while in plant mitochondria RNA sequences are altered by the substitution of nucleotides.

RNA editing has also been observed outside of mitochondria. Hoch *et al.* (1991) have identified substitutional editing in chloroplast mRNA that is reminiscent of the editing found in plant mitochondria. In addition, a number of viral RNAs have nucleotides substituted or inserted (Cattaneo, 1991) and in at least one instance a nuclear gene (encoding apolipoprotein B) has been shown to produce two different peptides depending on whether or not a single nucleotide is substituted in its mRNA (Hodges and Scott, 1992). While all of these systems are related by the functional definition of editing, it is unlikely that the editing in these different organisms is accomplished by similar or related mechanisms.

One feature that relates most of these edited RNAs is that the fully edited RNA is a functional mRNA, where the editing has created initiation codons, eliminated frameshifts in the reading frame or changed codons so that different amino acids are encoded by the message. Certain preferences in the codons created by editing have raised questions as to whether the reading frame is involved in the editing process and if RNAs lacking reading frames can be edited (Mahendran *et al.*, 1991).

Another feature common to all insertional editing systems studied so far is that only one type of nucleotide is inserted. In the mitochondria of kinetoplastid protozoa this nucleotide is invariably uridine. Small RNAs which are antisense to edited mRNAs have been identified (Blum *et al.*, 1990). These RNAs (termed guide RNAs) are thought to specify the location of editing sites as a result of mismatches between themselves and the unedited mRNA. Because the inserted uridine can base pair with either an adenosine or guanosine in the guide RNA and because only uridines are inserted or deleted, it has been proposed that the identity of the edited nucleotide is determined not by base pairing but by the activity of a uridine terminal transferase or by the composition of a polyuridine tract at the end of the guide RNAs which inserts the uridines at sites of mismatch between the guide RNA and the mRNA (Blum *et al.*, 1991; Cech, 1991).

We report here that the mitochondrial small subunit (SSU) rRNA is extensively edited in mitochondria of *P.polycephalum*. This editing, like the editing of the RNA for the α subunit of the mitochondrial ATP synthase (αATP), is primarily accomplished by single cytidine insertions. However, several examples of non-cytidine insertion have also been observed, indicating that the mechanism of editing in *Physarum* is different at least in some respects from the models proposed for editing in the kinetoplastid protozoa.

Results

Jones *et al.* (1990) have identified a single 2.9 kb region of the mitochondrial (mt) DNA of *P.polycephalum* which hybridizes with the gene for the 18S rRNA from corn mitochondria and with the 17S rRNA of *Physarum* mitochondria. Analysis of this region revealed sequences of significant similarity to those of other mitochondrial SSU rRNAs. While the homology was significant, a number of domains were different in primary sequence or in predicted secondary structure from those previously reported for prokaryotes, eukaryotes, mitochondria or chloroplasts. This is not surprising in light of the apparent ancient divergence of *Physarum* from the plant, animal and fungal kingdoms (Hasegawa *et al.*, 1985; Johansen *et al.*, 1988). However, certain highly conserved residues in the core portion of the RNA were absent, and since RNA editing had been previously observed in the RNA for the *Physarum* αATP (Mahendran *et al.*, 1991), we decided to analyze cDNAs to the rRNA in order to determine if RNA editing occurs.

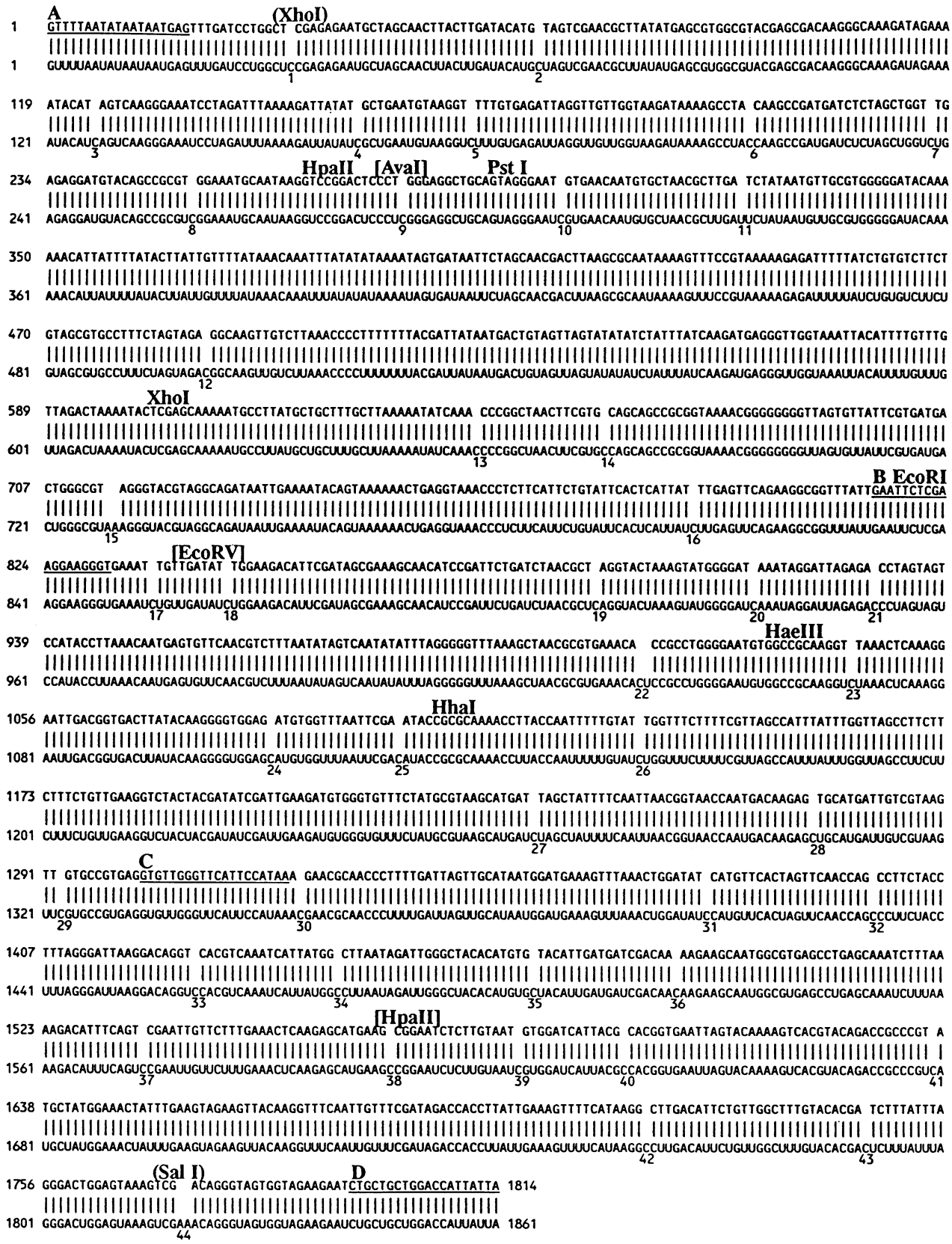

Fig. 1. Alignment of mtDNA and 17S rRNA sequences (EMBL data library accession Nos X75591 and X75592, respectively). The sequence of the mitochondrial 17S rRNA (1861 nt) as inferred from cDNA sequences is aligned with the analogous segment of mtDNA (1814 nt). Vertical bars mark nucleotide identity between the sequences. Gaps are inserted in the mtDNA sequence to maximize sequence alignment. Relative to the mtDNA 47 nt are inserted in the rRNA sequence. Cytidine residues are inserted at 41 sites, uridine residues at two sites and adenosine dinucleotides at two sites. The location of the oligonucleotide primers used in the synthesis of cDNA is indicated by the underlined sequences. Several restriction enzyme sites are shown for reference to the restriction map of Jones *et al.* (1990). Restriction sites shown in parentheses are absent in the cDNA due to nucleotide insertions. Restriction sites in brackets are absent in the mtDNA but present in the cDNA due to their creation by nucleotide insertion.

Specific cDNAs to rRNA were prepared by coupling primer extension using reverse transcriptase to the polymerase chain reaction (PCR, Saiki *et al.*, 1988). Four different primer combinations were used to produce cDNAs (Figure 1). Although these cDNA amplification products were essentially the size predicted from the mtDNA sequence, some contained restriction enzyme sites absent in the mtDNA and others lacked sites present in the mtDNA. For example, the *Xho*I site at position 31 and the *Sal*I site at position 1770 in the mtDNA are absent in cDNAs. Conversely, an *Ava*I, an *Eco*RV and a *Hpa*II site are present in cDNA, but are absent at the analogous position in the mtDNA (Figure 1).

The amplification products were cloned in plasmids and both strands of the cloned amplification products were sequenced and compared with the sequence of the mtDNA (Figure 1). The sequence of each amplification product was determined by sequencing the cloned amplification product of a plasmid from at least two independent transformants.

The cDNA sequence differed from the mtDNA sequence at 44 sites. Most of the differences were the insertion of a single cytidine in the cDNA relative to the mtDNA sequence. This is very similar to the type of editing observed in the αATP mRNA which is edited at 54 sites (Mahendran *et al.*, 1991). Also, the distribution of the editing sites was similar to that seen in the αATP RNA. Editing sites were distributed over the entire length of the RNA. The average distance between editing sites in the SSU rRNA is 40 nt with a standard deviation of ~34 nt. Both the average distance and the standard deviation are larger than the spacing of editing sites in the αATP mRNA (26 nt with a standard deviation of ~10 nt, Mahendran *et al.*, 1991). This is due in part to some clustering of the editing sites in the SSU rRNA that was not present in the αATP RNA. Six separations between editing sites are >70 nt. These separations can be used to define editing domains which have spacing similar to that in the αATP. Whether these arbitrary domains have functional significance or are just the result of the greater variation in the spacing of editing sites in the SSU rRNA is unclear. The average distance between editing sites within these arbitrary domains is 29 nt with a standard deviation of ~13 nt. These cytidine insertion sites plus the cytidine insertion sites in the αATP RNA bring the number of observed cytidine editing sites in *Physarum* to 94.

In addition to the editing sites which were a single cytidine insertion relative to the mtDNA, several other different insertions were observed. Two uridine and four adenosine nucleotides were also inserted relative to the mtDNA sequence (Figure 2). At site 11 (Figure 1) a single uridine was inserted (Figure 2A). At two other sites (sites 15 and 44, Figure 1) two adenosines were inserted either side by side or within one nucleotide of each other (Figure 2B and D). At a fourth site (site 22, Figure 1) a single uridine is inserted next to or within one nucleotide of a cytidine which is also inserted relative to the mtDNA sequence (Figure 2C). This CU insertion and the two adenosine dinucleotide insertions contain the most closely positioned nucleotide insertions observed in *Physarum* mitochondria to date. The next smallest distance between insertion sites is 9 nt and it may be that these AA and CU dinucleotides are inserted as a single editing event. All four of these sites are located at the boundary between an editing domain and an unedited region. However, their distance from the nearest cytidine insertion site is essentially the same as the average distance between cytidine insertion sites. These sites constitute the first examples of non-cytidine editing in *Physarum* and are the first example of mixed nucleotide editing in organisms with insertional editing.

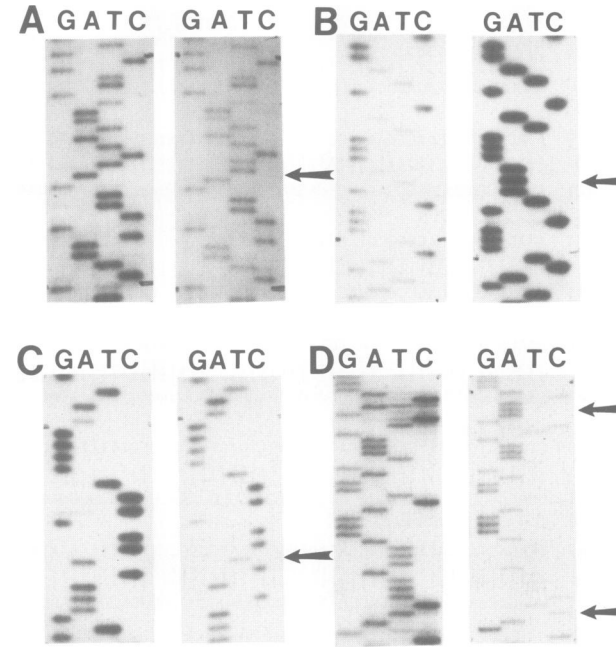

Fig. 2. Comparison of the sequence of selected regions of the SSU rRNA with the sequence of the analogous region of the mtDNA. The panels show autoradiographs produced from sequencing gels of termination products produced from mtDNA templates and from cDNAs produced from the SSU rRNA. In each panel the autoradiograph on the left is of mtDNA and that on the right is of cDNA. Arrows indicate the location of insertions in the sequence of the cDNA relative to the mtDNA. (**A**) The uridine insertion (site 11, Figure 1) and flanking sequences (positions 310–338 on the mtDNA sequence). (**B**) An adenosine dinucleotide insertion (site 15) and flanking sequences (positions 703–727). (**C**) The UC insertion (site 22) and flanking sequences (positions 1010–1031). (**D**) A cytidine insertion (site 43), an adenosine dinucleotide insertion (site 44) and flanking sequences (positions 1742–1779).

Although no other region of the mtDNA displays homology to the putative SSU rRNA gene, it is possible that DNA sequences might exist in *Physarum* which could act as classical templates for the RNA. However, no DNA of this type has been detected even with sensitive PCR-based approaches. For example, a small PCR amplification product produced from total *Physarum* DNA using primers complementary to sequences flanking the *Sal*I site at position 1770 in the mtDNA sequence, can be completely digested with *Sal*I even though the *Sal*I site is absent in cDNAs produced from the rRNA due to the insertion of two adenosines in the cDNA relative to the mtDNA (data not shown). Conversely, no digestion products can be detected after digestion of the amplification product with *Stu*I even though a *Stu*I site is present in cDNA due to the insertion of a cytidine at site 42 (data not shown). These data indicate that it is very unlikely that an alternative gene or a portion of a gene for the mitochondrial SSU rRNA exists in *Physarum* as a classical DNA template and therefore the inserted nucleotides must be added to the RNA without the use of a classical DNA template.

S1 nuclease protection and primer extension

In order to determine the location of the 3′ and 5′ ends of the SSU rRNA relative to the mtDNA sequence, S1 nuclease

protection and primer extension run-off experiments were performed. The location of the 3′ terminus was determined by measuring the sizes of protection products produced by S1 nuclease digestion of a 480 bp *SalI*−*Eco*RI fragment radiolabeled at the 3′ end of the *SalI* site which had been annealed with purified mtRNA. A reproducible pattern of protection fragments 36−42 nt in length was produced indicating that the 3′ end of the SSU rRNA is heterogeneous. The longest protection product correlates with the 3′ terminus predicted by analogy to the 16S rRNA of *Escherichia coli*.

Likewise, the location of the 5′ end of the SSU rRNA was determined by S1 nuclease digestion of a 516 bp *Hin*dIII−*Hpa*II fragment labeled at the 5′ end of the *Hpa*II site. The production of a 280 nt protection fragment gave the approximate location of the 5′ end. To determine more precisely the location of the 5′ end, primer E′ (which is complementary to a sequence near the 5′ end) was extended using reverse transcriptase, producing a 76 bp run-off product.

In order to determine the location of the 5′ end of the SSU rRNA and to confirm the insertion of a cytidine by editing, the 77 bp cDNA run-off product was isolated and sequenced directly without cloning or PCR amplification. This sequence confirmed the insertion of a cytidine at position 33 (site 1, Figure 1). The first nucleotide of the rRNA mapped at or near the guanosine at position 1 of Figure 1. This is an 8 nt extension beyond the site predicted to be the first nucleotide by analogy to the 16S rRNA of *E.coli*. A similarly sized extension has been observed in the mitochondrial SSU rRNA of plants (Chao *et al.*, 1984; Spencer *et al.*, 1984; Brennicke *et al.*, 1985).

To confirm the CU insertion at position 1017, the *Hae*III−*Hha*I fragment (positions 1033−1107) was radiolabeled at the 5′ end of the *Hha*I site and extended using reverse transcriptase. The extension product was isolated and directly sequenced. Sequence analysis confirmed the presence of the CU insertion (site 22, Figure 1).

The distance between the nucleotide on the mtDNA corresponding to the 5′ end of the RNA and the nucleotide corresponding to the 3′ end of the RNA producing the largest protection product is 1814 nt. The predicted size of the SSU rRNA after the insertion of 47 nt by editing is 1861 nt, a size consistent with that observed for the rRNA in Northern blotting experiments (Jones *et al.*, 1990).

Sequence alignment with E.coli and plant mitochondrial SSU rRNAs

The mitochondrial SSU rRNA of *Physarum* has significant similarity to SSU rRNAs of plant mitochondria (Chao *et al.*, 1984; Spencer *et al.*, 1984; Brennicke *et al.*, 1985) and *E.coli* (Brosius *et al.*, 1981). Alignment of the *Physarum* SSU rRNA sequences with those from *E.coli* (Brosius *et al.*, 1981) and *Oenothera* mitochondria (Brennicke *et al.*, 1985) defines seven regions of sequence similarity (conserved regions 1−7) interspersed with six variable regions designated V1−V6. The overall homology of the aligned regions of *Physarum* with *Oenothera* mitochondrial and *E.coli* SSU rRNAs is 71.6% (803/1121) and 69.3% (778/1123) respectively. In Figure 3, conserved regions 1 and 3−7 are shown aligned with the analogous sequences from *E.coli* and *Oenothera* mitochondria (conserved region 2 is homologous between *E.coli* and *Oenothera* only). Nucleotides inserted within conserved regions by editing are indicated. Editing sites are not confined to either the variable or conserved regions but are present in both (34 sites in conserved domains; 10 sites in variable domains). In the conserved domains the insertion of the cytidine increases the homology to other SSU rRNAs and produces highly conserved sequences. Not only is cytidine inserted in conserved regions but also uridine and the CU and AA dinucleotides are inserted in conserved regions and increase homology to other SSU rRNA sequences.

Secondary structure of the mitochondrial 17S rRNA of Physarum

RNAs with extensive secondary structure could provide their own templates for editing specificity. Editing in RNAs such as rRNA and tRNAs for which a general secondary structure has been proposed provide an excellent prototype to determine if editing is restricted to a specific feature of the secondary structure. Figure 4 shows the secondary structure predicted for the SSU rRNA of *Physarum* mitochondria based on its homology to the SSU rRNA of *E.coli* for which the secondary structure has been determined (Woese *et al.*, 1983).

The predicted secondary structure has most of the features associated with SSU rRNAs and varies from the canonical *E.coli* structure primarily by the absence of two *E.coli* features at locations designated V1 and V2 and by the elaboration of four variable domains designated V3, V4, V5, and V6 in Figure 4. The sequence insertions in the *Physarum* mitochondrial SSU rRNA relative to *E.coli* at V3 and V4 are extensive (214 and 90 nt, respectively) and are unprecedented in other SSU rRNAs although the *Saccharomyces cerevisiae* mitochondrial SSU rRNA has a 110 nt insertion at V3 (Sor and Fukuhara, 1980; Li *et al.*, 1982). The insertion at V6 (49 nt) is similar in size to the insertion characteristic of plant mitochondrial SSU rRNAs. However, plant mitochondrial SSU rRNAs lack insertions at V3 and V4 but have an extensive insertion at V5 (~325 nt; Chao *et al.*, 1984; Spencer *et al.*, 1984; Brennicke *et al.*, 1985) that is only a 20 nt insertion in *Physarum* mitochondrial SSU rRNA.

The location of editing sites is not limited to any feature of the proposed secondary structure. While most insertions (24/47) are found in RNA duplexes and could be specified by the complementary strand, other insertion sites are in the loops of stem−loop structures (3/47) or are at other locations not predicted to be base paired (17/47).

Since secondary structure could not be correlated with the location of editing sites, we looked for some aspect of the primary sequence which could be correlated. As with the αATP mRNA, we could not find any primary sequence feature which could unambiguously specify editing sites; however, as with editing in the αATP mRNA, editing sites are preferentially located downstream of purine−pyrimidine dinucleotides. Of the 40 sites with a single cytidine insertion, 13 sites are next to pre-existing cytidines so that the exact location of the inserted nucleotide is ambiguous. The remaining 27 sites were screened for common sequence elements around the insertion site. As with editing of the αATP mRNA, the composition of the nucleotides at positions −1 and −2 relative to the insertion site is not random. The −1 position is predominantly uridine (67%) and the −2 position is predominantly adenosine and guanosine (52% and 30%, respectively). This bias results in 56% of the insertion sites being downstream of purine−pyrimidine dinucleotides (Table I), more than three times the frequency one would

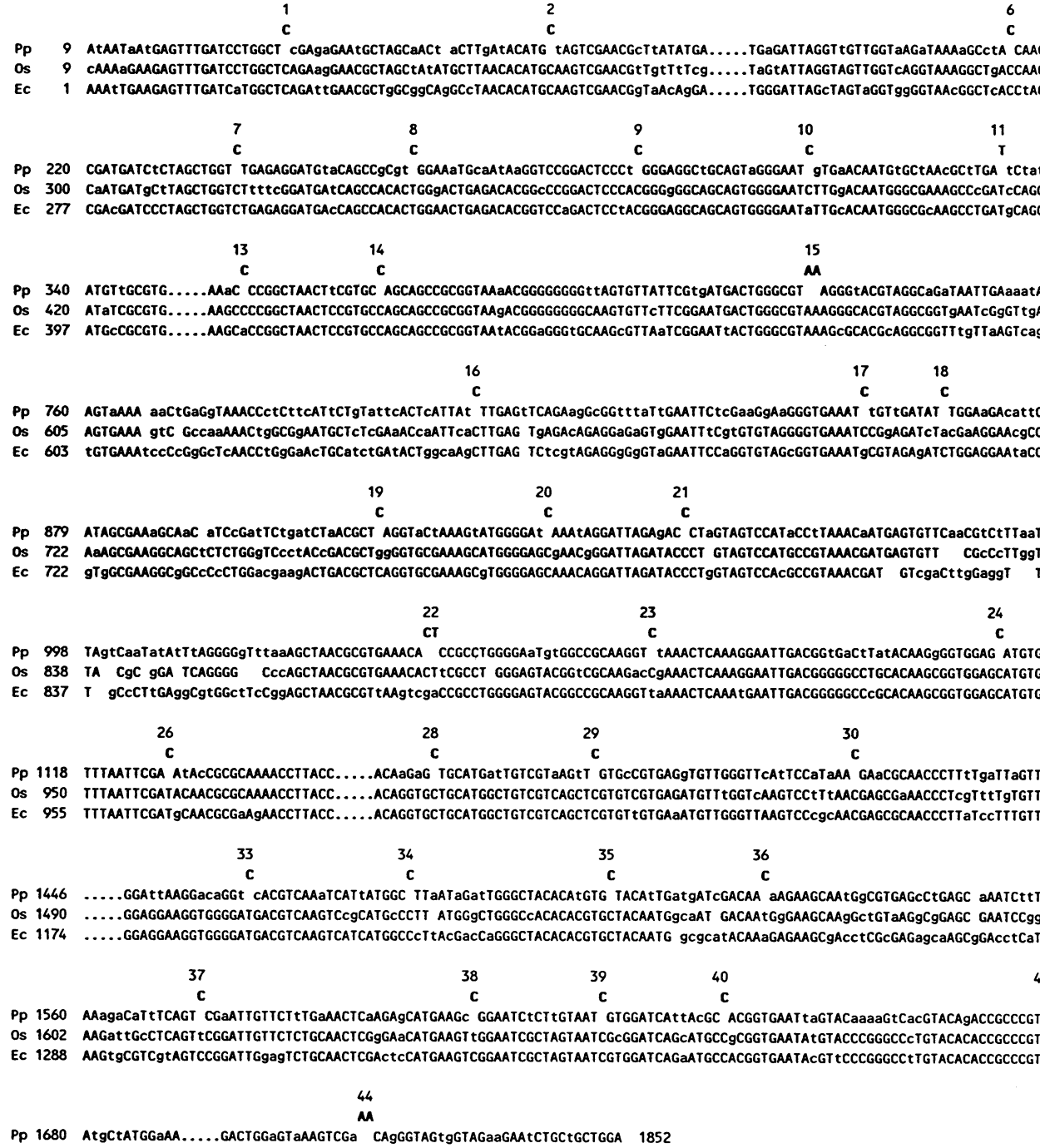

Fig. 3. Alignment of the DNA encoding the conserved regions of the SSU rRNA from *P.polycephalum* mitochondria (Pp), *Oenothera* mitochondria (Os) and *E.coli* (Ec). Nucleotides conserved in at least two of the three sequences are shown as capital letters, all other nucleotides are shown as small letters. The locations of variable regions are indicated by a series of dots in the sequences. The numbers to the left of the sequence indicate the position of the following nucleotide relative to the first nucleotide of the mature rRNA. The positions of the 34 editing sites located in conserved regions of the SSU rRNA are indicated above the line. Each editing site is marked with the number of the site as defined in Figure 1 and the identity of the nucleotide found at that position in the cDNA produced from the rRNA.

expect by random distribution based on nucleotide composition. Of the 13 insertion sites next to a cytidine, all but one site could be downstream of a purine−pyrimidine dinucleotide. This would mean that 27 of the 40 single cytidine insertion sites were directly downstream of one of the four purine−pyrimidine dinucleotides.

Discussion

We have determined that RNA editing is required for the maturation of the SSU RNA of the mitochondrial ribosome of *P.polycephalum*. This is the first report of extensive editing in an rRNA. Schuster *et al.* (1991) have reported

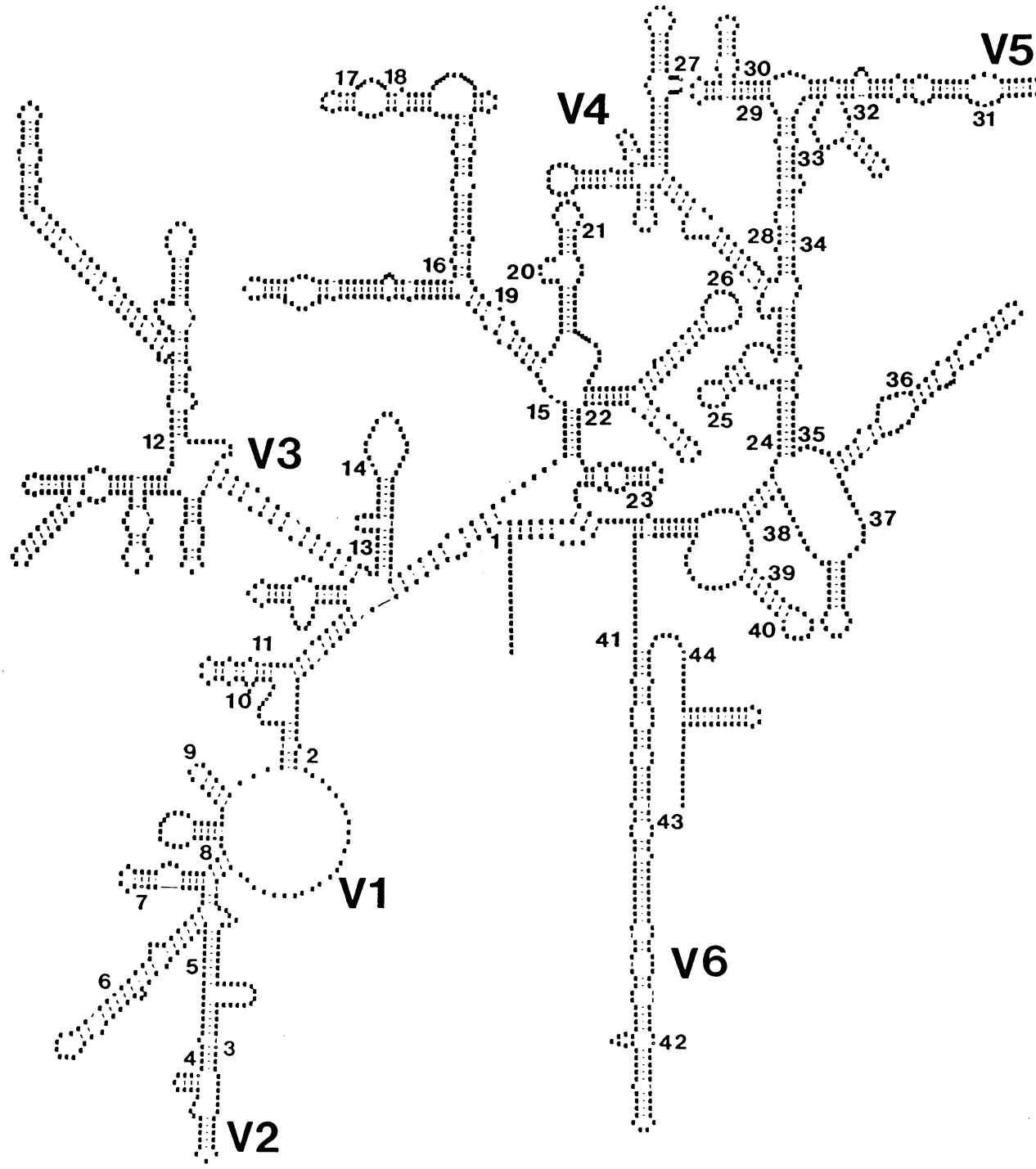

Fig. 4. Secondary structure of the mitochondrial SSU rRNA of *P.polycephalum*. The secondary structure of the conserved regions is inferred by analogy to the secondary structure of the 16S rRNA of *E.coli* as proposed by Woese *et al.* (1983). Six variable regions are indicated by V1–V6, respectively. The secondary structure shown for the variable regions is one of several possible structures. Nucleotides produced by editing are numbered and are shown as open circles. Watson–Crick base pairs are indicated by dashes; G-U base pairs are indicated by dots.

two sites on the 26S rRNA of *Oenothera* mitochondria that have U for C or C for U substitutions relative to the mtDNA sequence. These substitutions were observed in only one of five cDNAs produced from this region of the rRNA and so are probably not required for rRNA function. In contrast the SSU rRNA from *Physarum* mitochondria has nucleotides inserted at 44 sites in regions that are highly conserved among divergent organisms. The αATP mRNA from *Physarum* mitochondria has also been shown to require RNA editing (Mahendran *et al.*, 1991) and the genes for these two RNAs are located close to each other on the mtDNA. As in the αATP mRNA, the majority of editing sites in the SSU rRNA are single cytidine insertions at sites distributed fairly evenly throughout the RNA sequence. Likewise, the preferential location of editing sites downstream of purine–pyrimidine dinucleotides indicates a common mechanism and origin for the editing of these two RNAs.

A hallmark of the editing in *Physarum* mitochondria is

Table I. Distribution of dinucleotides upstream of single cytidine editing sites

Dinucleotide	Dinucleotide frequency in SSU rRNA gene (%)	Dinucleotide frequency upstream of cytidine editing sites (%)[a]
AA	10.4	7.4
AC	4.8	–
AG	6.7	7.4
AT	8.6	37.0
CA	4.5	0.0
CC	2.5	–
CG	3.5	0.0
CT	5.1	7.4
GA	6.6	11.1
GC	4.0	–
GG	5.6	0.0
GT	6.8	18.5
TA	9.0	0.0
TC	4.4	–
TG	7.2	7.4
TT	10.3	3.7

[a]Dinucleotides ending in C are not listed since the exact location of the insertion is ambiguous.

the relatively uniform distribution of editing sites within the RNA. As with the αATP mRNA, the single cytidine insertions are separated from adjacent editing sites by at least 9 nt. The single exception to this is editing site 22 where a cytidine and uridine are inserted next to each other, but this is probably the result of a generically different editing phenomenon similar to the adenosine dinucleotide insertions at sites 15 and 44. With the exception of six gaps between editing sites of >70 nt, the average spacing between editing sites in the SSU rRNA is 29 nt, similar to the 26 nt average spacing found in the αATP mRNA (Mahendran *et al.*, 1991). Whether these gaps define separate domains of editing in the SSU rRNA remains to be determined.

The SSU rRNA is especially useful for analyzing the relationship between RNA secondary structure and editing site location since the secondary structure of the *Physarum* SSU rRNA can be deduced based on its sequence similarity to the 16S rRNA of *E.coli* for which the secondary structure has been determined. Alignment of the *Physarum* SSU rRNA with the 16S rRNA of *E.coli* and the 17S rRNA of *Oenothera* mitochondria (Figure 3) reveals seven conserved domains. The location of the six variable regions that were excluded by the alignment is consistent with variable sequences observed in other SSU rRNAs. However, the size and sequence of some of these variable regions are unique to *Physarum* mitochondria. Variable regions V3 and V4 are extensively elaborated relative to *E.coli* and *Oenothera* mitochondria, whereas the large insertion at V5 characteristic of plant mitochondria is absent in *Physarum*.

The potential for secondary structure within the conserved domains of *Physarum* mitochondria is generally conserved relative to *E.coli*. There does not seem to be any correlation between editing site location and features of the secondary structure, and it seems unlikely that alternative secondary structures exist which could specify editing site location.

Thirty-four of the editing sites are located within the conserved domains (Figure 3). These insertions invariably fill gaps in the mtDNA sequence relative to the *E.coli* or *Oenothera* sequences. In addition, the identity of the inserted nucleotide is usually such that it increases the similarity of the *Physarum* SSU rRNA with other SSU rRNA sequences. The observation that editing is required to produce highly conserved sequences in the SSU rRNA argues that editing is probably required to produce functional SSU rRNAs and that it is likely to be necessary for ribosome assembly and function.

As with the αATP mRNA, cytidine insertion sites are preferentially (68%) located directly downstream of purine–pyrimidine dinucleotides. Editing sites in the αATP mRNA are also preferentially located in certain codons as well as being preferentially located at the third position of the codon (Mahendran *et al.*, 1991). In the αATP mRNA it was not clear if the preferential location of editing sites downstream of purine–pyrimidine dinucleotides was a result of the codon constraint. Since the SSU rRNA which lacks a reading frame also retains the purine–pyrimidine bias, it is likely that this bias is not a result of a codon preference, but is an intrinsic feature of *Physarum* editing. However, it should be emphasized that this preference is not sufficient to explain editing site specificity since editing sites also follow other dinucleotides and most purine–pyrimidine dinucleotides do not precede editing sites.

The failure to detect a feature of the sequence or secondary structure which could specify editing sites leads to the hypothesis that information for editing site specificity is located in a second molecule, presumably a nucleic acid, which can specify editing site location through some type of base pairing interaction. This is likely to be the case for editing in the trypanosomes where RNAs termed guide RNAs have been proposed to determine editing site location by base pairing with mRNAs (Blum *et al.*, 1990). However, our current inability to detect RNAs analogous to guide RNAs in *Physarum* mitochondria and our failure to detect DNA sequences analogous to those on minicircle or maxicircle DNA which code for the guide RNAs (Stuart, 1991), leaves open the question of how RNA editing is accomplished in *Physarum*.

The unexpected observation that nucleotides other than cytidines can be specifically inserted at some sites during editing has implications for the mechanism of editing. Since there has been no report of deletion or insertion of nucleotides other than uridine in the kinetoplastid protozoa, models have been proposed which do not predict nucleotide specificity, only specificity of location. Blum *et al.* (1990) have identified small antisense RNAs which are thought to anneal with mRNAs and direct the site of editing. Alternative models for the role of these RNAs have been proposed (Decker and Sollner-Webb, 1990; Blum *et al.*, 1990, 1991; Cech, 1991) but most models propose that only a single terminal transferase activity determines the identity of the inserted nucleotide. This is necessary since the abundant G-U base pairing predicted to occur between guide RNA and mRNA would not allow the guide RNA to specify uridines since guanosine residues could not discriminate between cytidine and uridine.

The observation that nucleotides other than cytidine can be inserted during editing in *Physarum* indicates that none of the current models for editing in kinetoplastid protozoa can account for its editing. This could mean that *Physarum* has a different RNA editing mechanism or that the current models must be modified to account for the nucleotide specificity observed in *Physarum* editing. It is possible that multiple, independent editing systems function in *Physarum*

mitochondria, one for single cytidine insertion, one for single uridine insertion and one for double adenosine insertion. Each might have a separate terminal transferase activity and would require separate signals to specify editing sites. This model would predict that the cytidine and uridine inserted at site 22 are inserted independently. Alternatively, the dinucleotide insertions may occur in a separate event from single nucleotide insertions. A third possibility is that a single editing mechanism which could specify nucleotide identity as well as editing site location may function in *Physarum*. Experiments to differentiate among these possibilities are in progress.

Materials and methods

Cell cultures

Physarum polycephalum strain M3C (originally characterized at McArdle Laboratory, Madison, Wisconsin) was generously given by R.Marsh (University of Texas at Dallas, Richardson) from the Guttes collection. Microplasmodia were grown at 27°C in the dark, with continuous agitation, in semi-defined *Physarum* medium (Daniel and Baldwin, 1964). Microplasmodia were generally harvested 84 h after inoculation.

Isolation of mitochondria

MtDNA was isolated from microplasmodia as described by Jones *et al.* (1990). Microplasmodia were harvested by centrifugation and washed with water three times before homogenization. The cells were homogenized in a Waring blender for 15 s. The homogenate was centrifuged at 650 *g* for 5 min to pellet nuclei and cell debris. Mitochondria were pelleted from the supernatant fluid by centrifugation at 8000 *g*. The mitochondria were then purified by several rounds of differential centrifugation. Purified mitochondria were then used for RNA and DNA isolations.

DNA isolation

Mitochondria were resuspended in BEST (0.1% BSA, 2 mM EDTA, 300 mM sucrose and 20 mM Tris−HCl pH 7.4) and lysed with 2% SDS (Jones *et al.*, 1990). This was followed by two phenol extractions and one chloroform extraction. Nucleic acids were precipitated with 0.1 vols of 3 M sodium acetate and 3 vols of ethanol, and then resuspended in 10 mM Tris−HCl, pH 8.0; 1 mM EDTA. The sample was then digested with RNase A at 37°C for 30 min, extracted with phenol and chloroform, and precipitated with ethanol as described above.

RNA isolation

All solutions for the isolation of RNA were made up in diethylpyrocarbonate (DEPC)-treated water. RNA was isolated from mitochondria (Locker, 1979) by lysis in an equal volume of lysis buffer (10 mM Tris−HCl, pH 8.0, 2 mM EDTA, 0.2% SDS) followed immediately by phenol extraction. The aqueous layer was separated from the phenol by centrifugation at 12 000 *g* for 10 min. The phenol extraction was repeated three times followed by a chloroform extraction. RNA was then precipitated with 0.3 vols of 2 M potassium acetate pH 4.55 and 3 vols of ethanol and stored at −20°C.

Oligonucleotide primers

The oligonucleotides shown in Table II were used for cDNA and PCR amplification. All primers were synthesized using an ABI 381A DNA synthesizer.

Primer extension

Nucleic acids isolated from purified mitochondria were treated with DNase I (RNase-free, BRL) in the presence of 50 mM sodium acetate pH 6.5, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 1 μl Inhibit-ACE (5Prime-3Prime, Inc.) at 37°C for 30 min. DNase I was removed by phenol extraction and the samples precipitated in 4.5 M sodium acetate (DEPC-treated) with ethanol. RNA and primer were resuspended in 100 mM KCl, heated at 80°C for 30 min and cooled to 50°C for 1 h or overnight. The annealed RNA and primers were resuspended in 50 mM Tris−HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.1 μg BSA, 25 mM dNTPs and 1 μl Inhibit-ACE in a final volume of 100 μl, and then extended using M-MLV reverse transcriptase (BRL) at 37°C for 1 h followed by RNase A (DNase-free) digestion and precipitation of cDNAs with ammonium acetate (2.5 M) and ethanol after phenol, phenol−chloroform, and chloroform extraction.

Table II. Oligonucleotides used for cDNA and PCR amplification

Location	Name	Sequence (5′ to 3′)
1−19	A	GTTTTAATATAATAATGAG
814−832	B	GAATTCTCGAAGGAAGGGT
814−832	B′	ACCCTTCCTTCGAGAATTC
1303−1322	C	GTGTTGGGTTCATTCCATAA
1303−1322	C′	TTATGGAATGAACCCAACAC
1795−1814	D′	TAATAATGGTCCAGCAGCAG
61−75	E′	GCGTTCGACTACATG

The oligonucleotides labeled with primes are complementary to the SSU rRNA and were used as the primer in cDNA synthesis. The oligonucleotides lacking the primes are the same sense as the rRNA

PCR and cloning

The cDNAs were amplified as described by Saiki *et al.* (1988). The cDNAs were resuspended in 98 μl of Taq polymerase buffer (50 mM KCl, 10 mM Tris−HCl pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin) to which was added 1 μl of 2.5 mM dNTPs and 1 μg of the appropriate primers. Taq polymerase (2.5 U, Perkin Elmer−Cetus) was added to each sample which was then overlaid with paraffin oil. The cycling temperatures and timing used were as follows: 94°C, 1 min; 37°C, 2 min; 72°C, 3 min. In the last cycle extension was carried out for 10 min at 72°C. Routinely, 30 cycles of amplification were performed. For cloning, the PCR products were phosphorylated using T4 polynucleotide kinase and ligated with *Hin*cII-digested pUC18, which had been previously dephosphorylated. The ligated DNA was used to transform *E.coli* strain HB101. Bacterial colonies containing plasmids with mtDNA inserts were identified by hybridization with radiolabeled mtDNA or PCR products. The positive colonies were mapped and sequenced.

S1 nuclease protection

The location on the mtDNA corresponding to the 5′ and 3′ ends of the SSU rRNA were determined using the protocol of Berk and Sharp (1978) as modified by Weaver and Weismann (1979). Appropriately end-labeled restriction fragments were mixed with mtRNA in annealing buffer (40 mM PIPES; 1 mM EDTA, pH 8.0; 400 mM NaCl, 80% formamide, pH 6.4) and heated at 80°C for 30 min. The restriction fragments were annealed to the RNA by lowering the temperature to 35°C and incubating for 2 h. Ten volumes of S1 nuclease buffer (280 mM NaCl; 50 mM sodium acetate, pH 4.6; 4.5 mM $ZnSO_4$) containing 0, 100, 250 or 425 U of S1 nuclease was added and the mixture was incubated at 37°C for 30 min. After digestion nucleic acids were precipitated with ethanol. Labeled protection fragments were resolved by electrophoresis in denaturing gels and visualized by autoradiography.

Nucleotide sequence determination

DNA sequences were determined by the dideoxy chain termination method (Sanger *et al.*, 1977) using a Sequenase kit (USB Co., Ohio, USA) and by the chemical cleavage method (Maxam and Gilbert, 1980).

Computer analysis

Sequence analysis and alignment was obtained using the MicroGenie sequence analysis package (Queen and Korn, 1984).

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