Evidence that Two Types of 18S rDNA Coexist in the Genome of Dugesia (Schmidtea) mediterranea (Platyhelminthes, Turbellaria, Tricladida)

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Sequences of 18S ribosomal DNA (rDNA) are increasingly being used to infer phylogenetic relationships among living taxa. Although the 18S rDNA belongs to a multigene family, all its copies are kept homogeneous by concerted evolution (Dover 1982; Hillis and Dixon 1991). To date, there is only one well-characterized exception to this rule, the protozoan Plasmodium (Gunderson et al. 1987; Waters, Syin, and McCutchan 1989; Qari et al. 1994). Here we report the 1st case of 18S rDNA polymorphism within a metazoan species. Two types (I and II) of 18S rDNA have been found and sequenced in the platyhelminth Dugesia (Schmidtea) mediterranea (Turbellaria, Seriata, Tricladida). Southern blot analysis suggested that both types of rDNA are present in the genome of this flatworm. This was confirmed through sequence comparisons and phylogenetic analysis using the neighbor-joining method and bootstrap test. Although secondary structure analysis suggests that both types are functional, only type I seems to be transcribed to RNA, as demonstrated by Northern blot analysis. The finding of different types of 18S rDNAs in a single genome stresses the need for analyzing a large number of clones whenever 18S sequences obtained by PCR amplification and cloning are being used in phylogenetic reconstruction.

Introduction

Ribosomal DNA (rDNA) serves a pivotal role in the protein synthesis machinery of all prokaryotic and eukaryotic cells. The eukaryotic rDNA array typically consists of several hundred tandemly repeated copies of the transcription unit separated by nontranscribed spacers (NTS). The transcription unit codes for the 18S, 5.8S, and 28S genes and for external and internal transcribed spacers (ETS and ITS) (for a review see Long and Dawid 1980). The multiple copies of this cluster appear to be nearly identical within a given organism (Hillis and Dixon 1991; Fox, Pechman, and Woese 1977; Lane et al. 1985). This conservation of sequence presumably reflects functional constraints on the molecules that are required for optimal translational efficiency. The process postulated to keep the different copies of the repeat so similar is called concerted evolution (Dover 1982; Hillis and Dixon 1991), and two molecular mechanisms are assumed to account for it, gene conversion (Nagylaki 1984a, 1984b; Lassner and Dvorak 1986; Hillis et al. 1991) and unequal crossing over (Petes 1980; Szostak and Wu 1980; Arnheim 1983; Coen and Dover 1983).

Among the genes within each transcription unit, the 18S ribosomal DNA (18S rDNA) is increasingly being used to infer phylogenetic relationships for reasons that have been repeatedly reviewed (Woese 1987; Sogin 1991; Adoutte and Philippe 1993). The main ones are that it is sufficiently long to provide a statistically valid amount of information, it is among the slowest evolving sequences found throughout living organisms, and different regions of the molecule evolve at different rates which allows the inference of phylogenetic history across a very broad taxonomic range. Moreover, the presence of many copies of 18S rDNA per genome and its homogenization through concerted evolution greatly reduces intraspecific variation compared to what one can expect based on data of interspecific variation. The low (or negligible) levels of intraspecific variation of 18S rDNA genes avoids the extensive sampling required for most single-copy genes. Therefore, small sample sizes, even from single individuals, have been used in most phylogenetic studies of rDNA (Hillis and Davis 1988; Baverstock and Moritz 1990).

However, it is important to point out that although gene homogenization appears to be the rule, intragenomic variation is known. The most important cases seem to be growth-stage-specific rRNAs. The first cases were described in the amphibian Xenopus laevis (Wegnez, Monier, and Denis 1972; Peterson, Doering, and Brown 1980) and in the mammal Mixtusurus fossidis (Mashkova et al. 1981) where two classes of 5S rRNA transcripts that are specific to either somatic or oocyte ribosomes have been reported. More recently, it has been shown in some species of the protist Plasmodium that two different types of 18S rDNA exist, whose expression is linked to different stages of the parasitic life cycle of these organisms (Gunderson et al. 1987; Waters, Syin, and McCutchan 1989; Qari et al. 1994). Although such cases can be taken as exceptions to a general rule, small sampling sizes may hide a greater variability in 18SrDNA genes than expected. If this turns out to be the case, it may have implications for the extent and mechanisms of concerted evolution and will introduce some degree of caution in using rDNA for phylogenetic analysis.

Here we report the first case of 18S rDNA polymorphism within a metazoan species, Dugesia (Schmidtea) mediterranea, a free-living platyhelminth. D. (S.) mediterranea has sexually and asexually reproducing populations or races (Benazzi et al. 1975). Sexual populations are diploids (2n = 8) or parthenogenetic triploids (3n = 12), whereas asexual populations, also diploid (2n = 8) or triploid (3n = 12), bear a chromosomal translocation from the 1st to the 3rd pair of chromo-
asexual populations (Ribas 1990) and reproduce exclusively by fission. There is some evidence for multiple origins of these asexual lineages from sexual forms (unpublished data). The results shown here refer to diploid sexual and asexual populations.

Materials and Methods

Organisms

Specimens of the asexual populations of *D. (S.) mediterranea* were collected from a pond in the city of Barcelona (Spain). Individuals from sexual populations came from Rio di Montes in Sardinia (Italy). Prior to DNA extraction, organisms were fasted for a week.

Obtention of the Sequences

High-molecular-weight DNA was purified according to a modification (García-Fernandez, Baguñà, and Saló 1993) of the guanidine isothiocyanate method initially described for RNA (Chirgwin et al. 1979). The entire length of the 18S rDNA was amplified by the polymerase chain reaction (Saiki et al. 1985) using the primers TACCTGGTTG ATCCTGCCAG TAG and GATCCTTCCG CAGGTTCACC TAC. The reaction conditions were: 1.6 ng/μl template DNA; first denaturing step 5’ 94°C; 30” 94°C; 30” 50°C; 45” 72°C 35 cycles. The amplified DNA was electrophoresed on an agarose gel containing ethidium bromide to check the product band size, and then it was purified by DEAE-cellulose transference. DNA samples were redissolved in 400 μl of elution buffer (T.E. pH 7.5 and NaCl 1.5 M) for 2 h at 65°C. The DEAE-cellulose paper was discarded and the solution containing the DNA was washed once with 1 volume of phenol-chloroform-isooamilalcohol (25:24:1) and precipitated with 1 volume of isopropanol. The pellet was dissolved in 15 μl of sterile distilled water. The purified PCR products were ligated into pUC 18 Smal I dephosphorilated vector using the Sure Clone ligation kit (Pharmacia P-L Biochemicals). The constructs were transformed into *E. coli* JM105 competent cells. The alkaline lysis method from the small-scale preparation of plasmidic DNA protocol (Sambrook, Frisch, and Maniatis 1989) was used. In addition to the 2 primers described above, 12 internal primers, CAGGCCGCA CGGCCAGCC GCCGTTAACCC, 5F GCCGAAGCAT TTGCAAAGAA, 6F AAACCCG AAAG, 7F GCAATAACAG CTTCTGTGATG CCC, and 8F GTACCACGCCC GCGT and its antisequence analogues, called 3R, 4R, 5R, 6R, 7R, and 8R, were used to completely sequence both chains using the dideoxy chain termination method (Sanger, Nicklen, and Coulson 1977) (sequencing kit [Pharmacia]). Direct sequencing of PCR products was also performed. Single strand DNA was produced by asymmetric PCR and precipitated (McCabe 1990); the sequences were obtained by the same methods described above for cloned DNA.

Southern Blot Analyses

Total genomic DNAs of asexual (6 μg) and of sexual (7 μg) *D. (S.) mediterranea* were independently single-digested with either *EcoRI* or *HindIII* and double-digested with *EcoRI/HindIII*. DNAs were separated in 0.9% agarose gel and transferred to Hybond-N membranes (Amersham) by capillary blotting. Two oligonucleotides specifically complementary to type I or type II 18S rDNA (see fig. 2) were used as probes. The hybridization conditions were optimized in order to avoid cross hybridization between the two types. The resulting conditions were as follows: filter hybridization was carried out at 62°C (type I probe) and 45°C (type II probe) for 18 h in: 6X SSC, 5X Denhardt’s, 0.05% Na-pyrophosphate, 1% SDS, 100 μg/ml yeast RNA and 15X 1000 cpm of 32P-labeled probe. Filters were washed twice in 6X SSC, 0.1% Na-pyrophosphate: 2 × 10’ at room temperature for type I probe; and 10’ at room temperature, 10 min 50°C for type II probe.

Northern Blot Analyses

Total cellular RNA was isolated from sexual and asexual populations by the guanidine thiocyanate method (Chirgwin et al. 1979) and separated in 1.2% agarose/formaldehyde gels. After electrophoresis, the RNA was transferred to a Hybond-N membrane (Amersham) and hybridized with the same probes and in similar conditions as for Southern blot experiments. As control, rat RNA was electrophoresed and probed.

Sequence Data Comparison and Phylogenetic Trees

18S rDNA sequences from *D. (S.) mediterranea* were compared to sequences of other platyhelminthes and to those of related and distant groups, and a phylogenetic tree was obtained. Briefly, sequences of *D. (S.) mediterranea* were aligned and compared to sequences from 11 species (5 platyhelminthes and 6 belonging to other phyla). The platyhelminths were four freshwater triclads belonging to the three families of the group (Dugesidae: *D. (G.) tigrina* and *D. (D.) iberica*; Planariidae: *Crenobia alpina*; and Dendrocoelidae: *Dendrocoelium lacteum*) and one prosotrate, *Monocetes lineata*. Representatives from other phyla were: an annelid (*Lancice conchilega*, X79873), an arthropod (*Euryipelma californica*, X13457), an echinoderm (*Asterias amurenensis*, D14358), a cnidarian (*Anemonea sulcata*, X53498), a ciliate (*Colpidium campyllum*, X56532), and a sporozoan (*Theilarius parva*, L02366). Triclads were almost complete (1,630 nucleotides) sequences obtained using direct sequencing from rRNA by reverse transcription (Riutort et al. 1992). Sequence from the prosotrate (U45961) is a new complete sequence obtained by PCR. The sequences were aligned by hand using a computer editor with the help of the secondary structure. Only conserved regions that could unambiguously be aligned were used (797 positions, of which 553 were informative). Kimura’s two-parameter distances were calculated (Kimura 1980); the sequences were compared to those of related and distant groups, and a tree was obtained by the neighbor-joining method (Saitou and Nei 1987) and a bootstrap test was performed (1,000 replications) using the MEGA program (Kumar, Tamura, and Nei 1993).

Results

High-molecular-weight DNA was independently isolated from a pool of intact adult individuals of each
of the two populations of *D. (S.) mediterranea* (see Materials and Methods), 18S rDNA was independently PCR-amplified from total DNA using specific primers. Amplified bands were cloned. Full length (1,795 nucleotides) sequencing of the clones revealed the existence of two different types of 18S rDNA in both populations (fig. 1). One type was identical in sequence to the 18S rRNA partial sequences already published (Riutort et al. 1992) (which we will refer to as type I; GenBank accession number U31084). The other (type II; GenBank accession number U31085) had an overall 8% difference (130 sites) compared to type I. Positions that vary between the two sequences are not randomly dispersed throughout the length of the molecule. Six regions (nucleotides 69–81, 220–241, 645–743, 1046–1072, 1355–1375, 1465–1567; shaded background in fig. 1) showed considerable variation, and secondary structure analysis shows that most changes are conservative (when a change occurs in a stem a compensatory change in the complementary sequence restores the pairing of bases and the secondary structure is conserved) or situated in loops. The two types of 18S rDNA were also found when DNA was isolated, amplified, and cloned from a single organism in both populations. This shows that the two 18S types coexist within an individual genome.

Previous hints that different (at least two) types of 18S rDNAs coexisted within *D. (S.) mediterranea* genome came from double bands seen in particular positions after direct sequencing from PCR (fig. 2). These bands were at first thought to be artifactual; however, as they appear in constant positions giving a uniform pattern (see double bands in fig. 2 matching the four variable positions within the fragment 1713–1732 in fig. 1), a more likely explanation, it appears, is the presence of different 18S rDNA types. Further evidence came from Southern blots of genomic DNA from both races, single- and double-digested with *EcoRI* and *HindIII* and probed using two oligonucleotides matching specifically type I or II sequences (shaded boxes within the variable region 645–743, fig. 1). As expected, blots showed both types of rDNAs to be present in the genome of both races (fig. 3). The band obtained for type II when DNA was cut with *HindIII/EcoRI* (lanes 3) was around 1.3 kb, which matches the expected size for this fragment as deduced from restriction enzyme sites for type II 18S rDNA sequence (fig. 1, nucleotides 339–343 *HindIII*, 1576–1581 *EcoRI*). On the other hand, double-digested DNA probed with type I gave a band of the same size as the *EcoRI* fragment, which means that *HindIII* does not cut inside this cluster; this could also be deduced from the large band obtained (more than 21 kb, lane 2, type I) when DNA was only cut with *HindIII*. For type II, *HindIII* cuts in at least two places, one inside the 18S sequence (nucleotides 339–343, fig. 1) and another that should be located in the ITS-1 region or within the 5.8S gene (at approximately 400 bp from the 3' end of the 18S sequence). This will explain the 1.8-kb band obtained (type II, lane 2 in fig 3). Restriction patterns between both types were thus substantially different, whereas conservation of restriction sites within each type (only one band per enzyme is observed; fig. 3) indicates a lack of internal polymorphism. This strongly suggests that each type of rDNA is organized in a different array of repeat units which are independently kept homogeneous by concerted evolution.

To further prove the internal homogeneity of type I and type II genes, we sequenced, from the pool of clones obtained from a sexual single organism, 5 clones of type I and 13 clones of type II, using the 4F primer. The sequence length analyzed was 209 bp for type I clones and 190 bp for type II clones. The mean percentage of differences between type I clones was 0.188%, and that between type II clones was 0.45% (results not shown). In other words, if all the 18S molecule were as variable as it is in the zone analyzed, we would expect a mean of 3 changes between two 18S type I molecules and a mean of 8 changes between two 18S type II molecules randomly chosen from their respective rDNA clusters. Given that the 4F primer sequence comprises the most variable zone of the molecule, we can assume that the level of variability between copies within each cluster is very low.

To test whether type II sequence is of planarian origin and not from any contaminant or potential planarian parasite (e.g., protozoans), both type I and type II sequences were compared to 18S sequences from other platyhelminthes, two protostomians, a deuterostomian, an, a cnidarian and two protozoans, and a neighbor-joining tree was obtained (fig. 4). Type I sequence forms a group with the other two species belonging to the Dugesiidae family, while type II branches off after families Planaridae and Dendrocoelidae diverged from the main stem but before family Dugesiidae did. The bootstrap test clearly shows that the new sequence (type II) belongs to Tricladida (100%) and that is more related to Dugesiidae than to the other two families (80%). This result clearly indicates that type II sequence originated within the planarian genome.

From our primary and secondary structure analyses we infer that neither type I nor type II genes are pseudogenes. Indeed, differences between them are nonrandom and restricted to regions that are not evolutionary conserved, whereas a perfect match exists between those regions required for optimal functionality. Moreover, restriction patterns suggest that type I and type II genes are in different clusters and kept internally homogeneous. This led us to think of both genes as functional genes. To test this hypothesis, we compared the expression of both genes by Northern blots using the same probes as used for Southern blots. RNA was prepared from intact adult individuals of both sexual and asexual populations of *D. (S.) mediterranea*, with rat RNA as a negative control. Unexpectedly, the results show that although both types of rDNA are present in the genome of each population of *D. (S.) mediterranea* only type I is transcribed in adult intact animals (fig. 5).

**Discussion**

One of the main consequences of concerted evolution is that sequence identity between multiple copies of repeated DNA within a species is higher than that
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FIG. 1.—Sequences of Dugesia (S.) mediterranea type I and type II 18s rDNA genes. Synthetic oligonucleotides complementary to evolutionary conserved regions of the 18s rDNA were used as primers in dideoxynucleotide chain termination sequencing reactions. The type I gene sequence (GenBank U31084) is presented, and nucleotide positions that are different in the type II gene (GenBank U31085) are indicated. The six highly variable regions (nucleotides 69–82, 170–190, 280–300, 470–500, 670–690, 760–790) are indicated in shaded gray. Boxes within the variable region 645–743 show the location of the synthetic oligonucleotides which were used in Southern and Northern blot analyses. Open boxes indicate restriction sites for HindII (nucleotides 339–343) and EcoRI (nucleotides 1576–1581). Note that HindIII only cuts type II. N: uncertain nucleotide.
Fig. 2.— Autoradiography showing the results obtained when direct sequencing from PCR was achieved. Sequences where obtained using the 9R primer (nucleotides 1795–1772, fig. 1). The 20 nucleotides shown correspond to positions 1713–1732 in figure 1. It can clearly be seen that double bands correspond to the variable sites between the two types of 18S rDNA.

between members from two different species (Dover 1986). Therefore, intraspecific variations within the 18S rDNA genes, as well as within the 5.8S and 28S genes, are expected to be kept to a minimum. However, there is a growing list of exceptions to this rule (see Introduction). The finding of two different types of 18S rDNA within the genome of two different races, and even within single organisms of the metazoan D. (S.) mediterranea, a free-living platyhelminth, is a new entry to the list. To our knowledge, this is the first report of 18S sequence heterogeneity in a multicellular animal.

Sequence Comparison

Sequences of 18S rRNAs from D. (S.) mediterranea obtained using the reverse transcriptase method did not show any sequence ambiguity, indicating that they were coded from a homogeneous set of 18S rDNAs (Riutort et al. 1992). The finding here of two different types (type I and type II) of 18S rDNA sequences within the genome of D. (S.) mediterranea, with nucleotide differences nonrandomly distributed within six main regions (fig. 1), is at variance with the former data and needs to be explained. Heterogeneity of 18S rDNA may also result from undisclosed heterogeneities within the populations of the asexual and sexual Dugesia (Schmid-
**Echinodermata**

- **Arthropoda**
  - **Annelida**

- **D(S)mediterranea Type I**
  - **D(D)iberica**
  - **D(G)itigina**

- **Planariidae**

- **Dendrocoelidae**
  - **Proseriata**

**Cnidaria**

**Sporozoa**

**Ciliophora**

**Fig. 4.**—Phylogenetic tree showing the position of the type II sequence in the 18S rDNA phylogeny. Sequences from 11 other organisms were used (see Materials and Methods). Kimura's two-parameter distance was used to calculate the corrected distances and the tree was built by the neighbor-joining method. A bootstrap test was performed (1,000 replicas). Numbers at the forks represent bootstrap percentages. This is an unrooted tree.

*tear) mediterranea* (e.g., presence of chromosomal races, sibling species). That this is not the case stems from the homogeneity of their chromosomal patterns (DeVries, Baguñà, and Ball 1984; Ribas 1990), and from the lack or very low level of enzyme polymorphism (Riort 1991). A simpler and more powerful argument, however, is the finding of both 18S rDNA types in the DNA from single individuals in both populations. Another source of heterogeneity we could think of, even in single individuals, is the presence of symbionts, either parasites or commensals. Although some turbellarians are stable hosts to some endosymbionts (Saffo 1992), freshwater planarians (suborder Tricladida) are only occasionally parasitised by some protozoans, namely holotrichous ciliates (Sluys 1989) and sporozoans (Ball and Reynolds 1981), or bear some peritrich ciliates as ectocommensals (Lascombe 1973). The individuals used here came from cultures free of any protozoans as established in squashed planarians. Even if any protozoans were inadvertently included in the sample and their DNA was extracted, there are two arguments against type II being of protozoan origin. First, a much larger value than the 8% of divergence between type I and type II must be expected between 18S rDNAs from platyhelmintes and protozoans (a 35% mean distance is found between a protozoan and any of the Platyleminthes studied in Riort et al. 1993, table 3). Indeed, the phylogenetic tree obtained from sequence comparison (fig. 4) sees both type I and type II clustering within the Tricladida (100% bootstrap value), whereas protozoan sequences (from potential parasites such as ciliates and sporozoans) cluster together and far apart of platyhel-

**Fig. 5.**—Northern blot analysis of total RNA, 5 μg per lane, from intact adult *Dugesia (S.) mediterranea* asexual (*a*) and sexual (*b*) races, with rat RNA (*c*) as a negative control. The blot was hybridized with the same probes for type I and type II 18S rDNA genes as in fig. 3. Arrows mark the expected 18S size.
minthes. Second, a protozoan type II gene would be functional and, unless the number of parasites was extremely low, detectable in Northern blots, which is not the case (fig. 5). On the other hand, a low number of parasites undetectable in Northern blots cannot explain the strong bands of hybridization for type II seen in Southern blots (fig. 3).

The overall difference between type I and type II genes is around 8%. This is higher than values found in interspecific comparison between 18S rDNA sequences of different species and genera of freshwater planarians (2.5%-3.9%; table 5 in Riutort et al. 1992) and close to the values found for interfamilial comparisons of freshwater planarians (6.6%-8.9%; table 5 in Riutort et al. 1992) and to those separating classes of Vertebrata (5.0%-7.0% in table 5, Riutort et al. 1992). This means that the divergence between types I and II should be ancient. Indeed, the phylogenetic tree obtained (fig. 4) shows that the type II gene branches off before the divergence of the species of the Duisgeiidae family, but after families Planariidae and Dencrocoeliidae branched off from the main stem. This indicates that divergence between type I and type II occurred between these two events and anticipates that species of the family Dugesiidae should have both types of sequences whereas those from families Planariidae and Dendrocoeliidae should not (unpublished data).

Heterogeneity in 18S rDNA coding sequences has only been reported for the A and C genes of several species of the protozoan Plasmodium. These genes code for 18S rRNAs that differ in 4.5% of their sequences (Gunderson et al. 1987). In metazoans, most variation reported results from length heterogeneity. In the parasitic planthelminth Schistosoma mansoni, length heterogeneity revealed by extrabands of hybridization are thought to result from insertions of DNA sequences in more than one place within the 18S rDNA sequence (Simpson et al. 1984). The 28S rRNA of Drosophila melanogaster exhibit length heterogeneity due to the presence, in some copies, of a large intron (Barnett and Rae 1979). However, single bands of hybridization in Southern blots (fig. 3) for type I and type II in D. (S.) mediterranea and the extensive differences in sequence between them clearly show that nucleotide changes, and not length heterogeneity, are at the base of such polymorphism. Thus, type I and type II genes of D. (S.) mediterranea bear a striking parallelism to A and C genes of Plasmodium species.

The only planthelminth studied so far, Schistosoma mansoni, bears 100 copies per haploid genome of a standard repeat unit 10 kb long (Simpson et al. 1984; Van Keulen et al. 1985). In situ hybridization studies show its rDNA to be located in the secondary constriction region of chromosome 2 (Tanaka et al. 1995). In contrast, the rDNA organization of the protozoan Plasmodium is unusual and is thought to be required for the maintenance of the two types of ribosomal RNA transcripts encoded by the A and C genes (Gunderson et al. 1987). Thus, the number of gene copies of each type is two and they are unlinked in the genome as opposed to being tandemly repeated. In the case of D. (S.) mediter
Phylogenetic Implications

The findings described here may have important methodological implications for phylogenetic reconstruction using 18S rDNA sequences. A basic advantage of this molecule is that it occurs in multiple copies that are kept very similar through concerted evolution. Hence, small sample sizes, often single individuals, are currently used in most phylogenetic studies. The finding of divergent 18S rDNA variants in the genome of *D. (S.) mediterranea* (extensive to other platyhelminth species of different orders; unpublished data) introduces a degree of caution when using this molecule in phylogenetic studies. If 18S rDNA is polymorphic, and if different types of 18S rDNA coexist and evolve independently within a species or group, we take a risk in comparing nonhomologous sequences. This is especially true when DNA samples come from a single individual or fragments of it. The existence of these polymorphisms, however, is not a drawback for the use of 18S rDNA as a phylogenetic tool. It only stresses the need for analysis of a large number of clones when PCR amplification and cloning are used to obtain 18S rDNA sequences. Moreover, direct sequencing from PCR could be used as a first strategy (see fig. 2) to detect the existence of hidden polymorphisms.

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