cDNA cloning and gene characterization of the mitochondrial large subunit (LSU) rRNA from the liver fluke *Fasciola hepatica*. Evidence of heterogeneity in the fluke mitochondrial genome

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ABSTRACT

A cDNA clone that encodes the large subunit of mitochondrial ribosomal RNA (LSU rRNA) from the liver fluke *F. hepatica* was isolated and characterized. This RNA molecule is polyadenylated at the 3' end and represents 10% of the poly A⁺RNA in adult *F. hepatica*. Fluke LSU rRNA has significant sequence homology to mosquito mitochondria LSU rRNA and is more closely related to the mitochondrial rRNA of hermaphroditic than dioecious trematodes. Mitochondrial DNA constitutes approximately 10% of the total cellular DNA of adult flukes. This percentage is lower in non-embryonated eggs as are the levels of LSU rRNA indicating eggs have lower metabolic activity. Analysis of transcription and the number of mitochondrial genomes in *S. mansoni* shows that the LSU rRNA is more abundant in females than in males. Restriction endonuclease analysis of the fluke mitochondrial LSU rRNA genes suggests the presence of heterogeneous repeated copies in the mitochondrial genome or heterogeneity among individual genomes of mitochondria.

INTRODUCTION

Molecular dissection of parasite genomes has revealed unusual strategies for gene organization and expression (1,2,3). The information from such analysis may suggest new means to combat parasitic diseases. Trematodes are among the most wide spread and medically important parasites. Digenetic trematodes undergo numerous biochemical and morphological changes during their complex life cycle (4). They differ from other groups of parasitic worms in that one of the larval stages develops in a mollusc, and with few exceptions, the adult infects vertebrate hosts (5). Some of the most prevalent trematodes are from the genus *Fasciola* (a hermaphroditic organism) and the dioecious genus *Schistosoma*. *Fasciola hepatica* is of veterinary and medical importance and hundreds of millions of people are infected with worms of the genus *Schistosoma* (6,7). A comparative analysis of these two trematodes at the molecular level could be useful in understanding the evolution and the biochemical adaptations of these organisms during their life cycles. We have been interested in isolating and comparing the genes of trematodes, using *F. hepatica* and *Schistosoma mansoni* as model parasites. Our initial approach included the isolation of cDNA clones representing abundant RNAs in the adult form of the liver fluke *F. hepatica*.

We report that one of these cDNAs represents a copy of the mitochondrial large subunit rRNA (LSU rRNA). This RNA is polyadenylated at the 3' end and comprises approximately 10% of the total poly A⁺ RNA in adult *F. hepatica*. In addition to determining its sequence, we have analyzed the genomic organization of the LSU rRNA genes and compared it with that of other trematodes.

MATERIALS AND METHODS

Materials.

F. hepatica were obtained from bile ducts of infected cattle at a local slaughterhouse. The worms were transported and maintained in the laboratory in saline-glucose medium with penicillin and streptomycin, without the addition of serum, as described (8). Adult *S. mansoni* were collected by perfusion from bisexually infected (6 to 8 weeks after infection) anesthetized with nembutal containing heparin. Males and females were manually separated and washed before use. *Echinostoma paraense* were supplied by Dr. Paul Basch (9). *F. hepatica* eggs were collected by centrifugation from the saline solution in which the flukes were incubated overnight. Other materials were obtained from the following sources: Klenow fragment of DNA polymerase I and T4 DNA ligase from New England Biolabs or Boehringer Mannheim; restriction enzymes, New England Biolabs; radionucleotides, Amersham; nitrocellulose filters from Schleicher and Schuell.

Nucleic acids.

Total cellular RNA was extracted from adult worms by vigorous homogenization using a polytron microprobe in 6M guanidine hydrochloride dissolved in 0.1M sodium acetate, pH 5.2/10mM EDTA. The RNA was isolated by centrifugation through a CsCl cushion (10). Total RNA from *F. hepatica* eggs was isolated as reported in (11). High molecular weight DNA was purified according to the procedure of Grosschedl *et al.* (12). Mitochondrial DNA was purified using the method reported by Bogenhagen and Clayton (13). The DNA and RNA were prepared from pools of flukes.

Library screening.

To screen the *F. hepatica* cDNA library (11), duplicate nitrocellulose filters were prehybridized at 42° C in 45% formamide (deionized)/45 mM NaCl/ 45mM sodium citrate/5X Denhardt's solution (1X = 0.02% polyvinylpyrrolidone/0.02% ficoll/0.02% bovine serum albumin)/1% SDS and 100 µg of salmon sperm DNA per ml. Hybridization was performed in a similar solution at 42° C using 8X10⁶ cpm of cDNA probe per filter. Filters were washed with 30 mM NaCl/3 mM sodium citrate/0.1% SDS at 50° C.

RNA and DNA blot analysis.

For RNA gel blots, RNA was separated by electrophoresis through formaldehyde agarose gels as described (14) and transferred to nitrocellulose filters. The blot was

baked for 2 hr at 80° C in vacuum. The hybridization was performed at 42° C in 50% formamide/45 mM NaCl/75 mM NaH₂PO₄/3mM EDTA, pH 7.4/1X Denhardt's solution and 100 µg of salmon sperm DNA per ml. The filters were washed in 30 mM NaCl/3 mM sodium citrate/0.1% SDS at 50° C. To reuse the blot, bound probe was stripped by washing at 85° C for 5 minutes in 15 mM NaCl/1.5mM sodium citrate/0.1% SDS. For Southern blots, total cellular DNA from *F. hepatica*, *E. paraensi* and *S. mansoni* was digested with several restriction enzymes and separated on 0.8% agarose gels. After the transfer to nitrocellulose (15), filters were hybridized under conditions similar to those of the RNA gel blots. In the case of the [³²P] end-labelled oligonucleotide probe, the hybridization was performed in 240mM NaCl/500mM NaH₂PO₄/3mM EDTA, pH 7.4/5X Denhardt's solution/0.5% Nonidet 40/10% dextran sulfate at 42° C. The *E. paraensi* and *S. mansoni* hybridizations were performed in 40% formamide/45 mM NaCl/75 mM NaH₂ PO₄/3 mM EDTA, pH 7.4/1X Dendardt's solution and 100 µg salmon sperm DNA per ml at 42° and washed with 45mM NaCl/50 mM sodium citrate/ 0.1% SDS at 42° C. Probes were prepared by using random primers after the method of Feinberg and Vogelstein (16).

DNA manipulations and sequence.

Standard DNA manipulations were performed according to the protocols of Maniatis et al. (17). The nucleotide sequence was performed by subcloning the fragment of interest in M13mp18 or M13mp19 and then using the dideoxy chain-termination method of Sanger et al. (18). Sequence data were analyzed by the BIONETtm National Computer Resource for Molecular Biology.

Figure 1. Characterization of the RNA of the F10 clone.

A RNA gel blot containing 10 µg of total RNA per lane from whole adult *F. hepatica* (w), ventral sucker (vs), female genital complex (f), and the anterior part of the fluke (h), hybridized with random primer probe of the F10 cDNA (exposure of 5 minutes). The arrows indicate the position of the nuclear ribosomal bands.

RESULTS

Isolation and nucleotide sequence of a cDNA encoding the mitochondrial large subunit rRNA from *F. hepatica*.

The construction of a λ gt10 cDNA library using poly A⁺ RNA from the female genital complex of the liver fluke *F. hepatica* has been previously described (11). A cDNA clone was identified by plaque hybridization using labelled cDNA to the mRNA from the female genital complex as a probe. The positive plaques represented one in ten recombinants in this library. This cDNA clone, designated F10, contained an insert of 710 bp. In order to determine the size of the RNA corresponding to this clone, as well as its tissue distribution, total RNA from different tissues of the liver fluke was analyzed (Fig. 1). The regions of the fluke selected were: ventral sucker, an organ known to be rich in muscle cells; the head, which is the tip (~3mm) of the anterior part of the parasite containing muscle and ganglion nerve cells; and the female genital complex. A prominent band of approximately 1.4 kb was detected in all tissues though some differences in relative abundance were apparent. The ease with which this RNA was detected after very short exposures confirms that the F10 RNA was very abundant in total RNA preparations. No hybridization to nuclear ribosomal RNA was detected.

To further investigate the nature of the RNA encoded by the F10 clone, the complete nucleotide sequence of the cDNA was determined (Fig. 2a). The 3' end of the cDNA clone contained a tract of 18 adenines, which together with the fact that the *F. hepatica* RNA used to construct the cDNA library was isolated on Poly U-Sephadex and the cDNA primed with oligo dT, suggests that the RNA is polyadenylated. However, no large open reading frame was detected. This raised the possibility that the F10 RNA represents a structural RNA that is polyadenylated in the 3' end. A computer analysis using the IFIND program of BIONETtm revealed that the F10 RNA shares a great deal of sequence homology with the large subunit mitochondrial rRNA from the mosquito *Aedes abopictus* (Fig. 2b). The LSU rRNA from mosquito mitochondria is polyadenylated at the 3' end (19), suggesting that the F10 RNA is also the LSU rRNA of *F. hepatica* mitochondria. The presence of a polyadenylated 3' end in the F10 RNA was confirmed by primer extension experiments. A specific primer from the 5' end of the F10 cDNA clone was used to extend on the transcript the additional 700 bp which demonstrated that the poly A⁺ tract is located near or at the 3' end of the F10 transcript. In addition, the sequence of a genomic clone overlapping the 3' region of the cDNA does not contain a poly T tract, indicating that the polyadenylation occurs in a post-transcriptional fashion (data not shown). A complete sequence analysis remains to be performed with the entire LSU rRNA gene; however, the present cDNA sequence shows some relevant characteristics that are also present in the mosquito LSU RNA, e.g. 3' end polyadenylation and a low G+C content. These characteristics indicate that the

Figure 2. Homology between the F10 cDNA and the LSU rRNA from mosquito mitochondria.

- A) Partial restriction endonuclease map and sequencing strategy of the F10 cDNA; only relevant restriction enzyme cleave sites are shown. The direction and extent of sequence determinations are shown by the horizontal arrows below the clone.
- B) Sequence of the F10 clone (f) and homology with the LSU rRNA from mosquito, *A. abopictus*, mitochondria (m) is shown. The numbers of the mosquito sequence are based on reference (20). The poly A tract sequenced in the F10 clone is shown by an open box. Dots between the two sequences represent the homologous bases. Nucleotides are numbered in 5'-3' direction.

Figure 3. Genomic characterization, gene number of the LSU rRNA gene and homology with other trematodes.

- A) Hybridization of the F10 probe to total cellular DNA. Lanes a and b, *F. hepatica* DNA digested with *EcoRI* and *HindIII*, respectively; lanes c and d, *S. mansoni* DNA digested with *EcoRI* and *HindIII*, respectively; lanes e and f, *E. paraensi* DNA digested with *EcoRI* and *HindIII*, respectively; lanes g and h, rabbit DNA digested with *EcoRI* and *HindIII*, respectively. The hybridization was performed using moderate stringency (see Materials and Methods).
- B) Ethidium bromide stained agarose gel of total cellular DNA from *F. hepatica* digested with *EcoRI* (lane a) and *HindIII* (lane b). The arrows indicate the bands that hybridize with the F10 probe.
- C) Southern blot copy number analysis of the LSU rRNA gene. Lanes a, b and c are 10 μ g of *F. hepatica* DNA digested with *EcoRI*, *HindIII* and *HaeIII*, respectively. Lanes d, e and f contain different concentrations of the LSU rRNA cDNA, representing 10, 100 and 1000 copies per genome (0.05, 0.5 and 5 ng).

LSU rRNA from mosquito mitochondria is not an evolutionary extreme (19,20), as it is apparently also present in *F. hepatica*.

Characterization of the LSU mitochondrial rRNA gene.

Little has been reported about the organization of the mitochondrial genome of trematodes. We have initiated studies to characterize the LSU rRNA gene of trematodes, including *F. hepatica*, *E. paraensi* and *S. mansoni* (Fig. 3). Hybridization of the F10 cDNA to restriction endonuclease digests of total DNA from the parasites yielded detectable signals under mildly stringent conditions (see Materials and Methods);

Figure 4. Transcriptional analysis of the LSU rRNA in other trematodes and in *F. hepatica* eggs.

- A) Northern blot containing 5 µg of total RNA per lane from adult *F. hepatica* (f), *F. hepatica* egg (g), adult *E. paraensi* (e), females *S. mansoni* (sf), and males *S. mansoni* (sm), hybridized with the F10 probe.
 B) The same northern blot hybridized with nuclear ribosomal RNA as a control.

whereas, no hybridization was detected with mammalian DNA. In the case of *F. hepatica* DNA, two fragments of 10 and 9 kb were detected with both *EcoRI* and *HindIII* digestions, respectively. Larger bands with lower hybridization signals were also detected; control experiments and the presence of these bands in digests from several different DNA preparations confirmed that these fragments are not the result of partial digestions (data not shown). In the *S. mansoni* and *E. paraensi* DNA hybridizations, a band of around 20 kb was identified in both *EcoRI* and *HindIII* digestions. This experiment also showed by the signal intensity, that the LSU rRNA gene from *F. hepatica* contains more homology with the *E. paraensi* gene than with the gene from *S. mansoni*. Interestingly, ethidium bromide-stained gels of *F. hepatica* DNA digestions showed the presence of very prevalent bands (Fig. 3b), some of which comigrate with the bands detected by hybridization with the F10 clone. This suggested that the copy number of mitochondrial genomes, or at least the LSU rRNA gene, is very high in the adult form of *F. hepatica*. A reconstitution blot analysis was performed to estimate the number of copies of mitochondrial genomes represented by the LSU rRNA gene. This experiment assumes equal efficiency of DNA transfer, equal efficiency of hybridization and is based on an approximation of the size of the fluke genome as 3×10^8 bp. The result of this experiment (Fig. 3c) showed that the mitochondrial genome is present in

more than a thousand copies per nuclear genome which represents about 10% of the total cellular DNA.

Transcriptional analysis of the LSU rRNA.

To further characterize the size and abundance of the LSU rRNA of trematodes, total RNA from *F. hepatica* (adult and egg preparations), *E. paraensi* and *S. mansoni* (males and females) was hybridized with the F10 clone (Fig. 4). In all cases, a band of 1.4 kb was detected; however, the amount of hybridization differed between the adult and egg stages of *F. hepatica* and between male and female *S. mansoni* (Fig. 4). In *F. hepatica* the LSU rRNA was 15-20 times more abundant in the adult than in the eggs. In *S. mansoni*, the LSU rRNA was about 5 times more abundant in the female preparations than in the male. These differences could represent variability in the number of mitochondria genomes present in the adult and the eggs (in the case of *F. hepatica*) and in the male and female (in the case of *S. mansoni*); or alternatively, the LSU rRNA genes could have different transcript abundance. To distinguish between these two possibilities, total DNA from male and female *S. mansoni* and total DNA from adult and egg *F. hepatica* were hybridized in a Southern blot probed with the F10 cDNA and then with a chromosomal DNA probe as an internal control. The result of these experiments is shown in Fig. 5. In the case of *S. mansoni*, the copy number of the LSU rRNA gene was approximately the same in male and female suggesting that the

Figure 5. LSU rRNA gene copy number comparison in males and females *S. mansoni* and adult and egg *F. hepatica*.

A) Hybridization of the F10 probe to total cellular DNA. Lanes a and b, adult *F. hepatica* DNA digested with *EcoRI* and *HindIII*; lanes c and d, egg *F. hepatica* DNA digested with *EcoRI* and *HindIII*.

The same filter was hybridized with the F3 clone (11) as a control since it encodes a nuclear gene.

B) Hybridization of the F10 probe to *S. mansoni* total cellular DNA from female (lanes a and b) and male (lanes c and d) digested with *EcoRI*. The same filter was hybridized with clone Smf1, which encodes a putative egg shell protein, a nuclear gene (unpublished results).

differences between the number of transcripts in the two sexes is due either to more active transcription or increased stability of the LSU rRNA in the female mitochondria relative to the male. In *F. hepatica*, it was very clear that the number of copies of the LSU rRNA gene is higher in the adult than in the egg and parallels the difference in abundance of transcripts between these two stages.

Heterogeneous repeated sequences of the *F. hepatica* mitochondrial LSU rRNA gene.

Hybridization of the F10 probe with *F. hepatica* DNA digested with HaeIII or Sau3A showed 7 and 4 bands, respectively (Figs. 3 and 6). This was an unexpected result since the probe used in this experiment only contains one HaeIII site and no Sau3A recognition sequences. The same pattern was observed in different experiments with different DNA preparations. There are at least three explanations for this result. One is that the nuclear DNA contains sequences with homology to the mitochondrial LSU rRNA gene. The second possibility is that the LSU rRNA genes exist as heterogeneous, repeated sequences in the fluke mitochondria and the third is the presence of introns in

Figure 6. Sequence heterogeneity of the LSU rRNA gene.

- A) Total cellular DNA (tc DNA, lanes a and b) and mitochondrial DNA (mt DNA, lanes c and d) from *F. hepatica* was digested with EcoRI and HaeIII, respectively, Southern blotted and hybridized with clone F10 probe. A longer exposure shows the presence of other bands in both samples (data not shown).
- B) Hybridization of *F. hepatica* total cellular DNA with an oligonucleotide from F10. Lane a) HaeIII digestion and lane b) Sau3A digestion. The bottom of the figure shows the sequence of the 19-mer used as a probe and its position in the LSU cDNA. The hybridization was performed under high stringency conditions (Materials and Methods).

the gene. Mitochondrial DNA was purified in order to test whether the same DNA bands that hybridize with the F10 probe are present only in the mitochondrial DNA. The result showed that all the restriction bands that hybridized with the LSU rRNA probe are localized in mitochondrial DNA (Fig. 6). These data support the possibility that heterogeneous, repeated sequences of the LSU rRNA gene reside in the mitochondrial DNA. To confirm this idea and examine whether introns are present in these genes, HaeIII and Sau3A digestions of F. hepatica DNA were hybridized with an oligonucleotide of 19 bases (Fig. 6b) under high stringency conditions (see Materials and Methods). This experiment predicts that if more than one copy of the sequence is present and if the sequences around the gene are different, then more than one band will be detected. Fig. 6b shows that two bands were detected with the 19-mer in digestions with both enzymes. This result does not completely discard the possibility of an intervening sequence in the LSU rRNA gene. However, it is evident that the sequence which hybridizes with the oligonucleotide is repeated and confirms the presence of heterogeneous sequences of the LSU rRNA gene in the fluke mitochondria.

DISCUSSION

A cDNA clone that encodes the mitochondrial LSU rRNA from the liver fluke F. hepatica was analyzed. This RNA is polyadenylated at the 3' end and is very homologous with its counterpart in the mosquito mitochondria. The hybridization experiments presented here show that the LSU rRNA of F. hepatica mitochondria is evolutionarily closer to the LSU rRNA from hermaphroditic trematodes like E. paraensi than to S. mansoni which is a dioecious organism. The cloning and characterization of the LSU rRNA from S. mansoni could be very useful for an evolutionary analysis of the mitochondrial genomes of trematodes.

The LSU rRNA is very abundant in total RNA preparations from adult tissues of F. hepatica and other trematodes. The number of mitochondrial genomes, reflected by the number of LSU rRNA genes, is very large in the adult form of the fluke which explains the abundance of this RNA in fluke RNA preparations. In fluke eggs, however, the mitochondrial genomes and consequently the abundance of the LSU rRNA is less. The high levels of the LSU rRNA and the large number of mitochondrial genomes in the adult stage is puzzling. These parasites are predominantly anaerobes in the adult stage (21) and their mitochondria produce ATP by dismutation of malate (22). Even under aerobic conditions the adult liver fluke uses this pathway to produce ATP rather than oxidative phosphorylation (23). The efficiency of ATP production by malate dismutation is low in comparison to the oxidative phosphorylation pathway. F. hepatica is metabolically very active and produces large number of eggs daily. It is possible that an increase in the number of these cell organelles compensates for the low energy

efficiency of the metabolism in the mitochondria of adult *F. hepatica*. The eggs, which remain dormant prior to miracidial development, presumably do not need an efficient ATP generating system and therefore require fewer mitochondria (4,22).

Particularly interesting is the observation that the number of transcripts of LSU rRNA in females of *S. mansoni* is higher than in males, even though the apparent gene copy number is the same in both sexes. Perhaps the increased transcription of mitochondrial genes or greater stability of transcripts in females is related to excessive demands for ATP associated with rapid egg production (200-300 eggs per day per female worm). It will be important to analyse transcription of the mitochondrial genes in males and females of *S. mansoni* and compare this with the situation in the hermaphroditic trematodes.

The presence of heterogeneity at the DNA sequence level in different copies of the LSU rRNA is of interest. Whether multiple copies are present on the same mitochondrial chromosome or whether such polymorphisms reflect the presence of different mitochondrial genomes stably maintained in the same organism has yet to be determined. The presence of duplications and heterogeneity at sequence level has previously been described in non-coding regions of *Drosophila mauritania* mitochondrial DNA (24). The liver fluke appears to have incurred duplications of the LSU rRNA gene in mitochondrial DNA during evolution. Additional nucleotide sequence of the *F. hepatica* mitochondrial DNA must be obtained in order to test this prediction.

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