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Intracellular promiscuity in *Schistosoma mansoni*: Nuclear transcribed DNA sequences are part of a mitochondrial minisatellite region

(mtDNA/variable number of tandem repeats/"promiscuous DNA")

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ABSTRACT **It has been shown that the mtDNA of the parasitic trematode *Schistosoma mansoni* is hypervariable in size. We report here that this length variation is due to a large polymorphic minisatellite composed of two types of repeated sequences of 558 bp and 62 bp. Each minisatellite repeat is made up of a large 558-bp component and a variable tandem array of the small 62-bp unit. Of more fundamental interest was the finding that both the 558-bp and 62-bp components have significant homology with a gene, SM750, previously identified in the nuclear genome of *S. mansoni*. The small 62-bp unit is identical to the nuclear polymorphic repeat element, which is apparently spread throughout the nuclear genome and is abundant among transcripts, in addition to being present in five tandem copies in SM750. The presence, in the *S. mansoni* mtDNA, of fragments of genes that are present in and transcribed from the nuclear genome raises the question of the origin of these sequences. The arrangement and the variability that the mtDNA minisatellite embodies were explored as an identity test for *S. mansoni* based on the use of PCR for tallying the relative abundance of the several repeat numbers of the tandem arrays of the 62-bp unit within the minisatellite structure.**

The digenetic trematode worm *Schistosoma mansoni* is responsible for schistosomiasis, a parasitic endemic disease that is estimated to affect at least 200 million people around the world, especially in tropical and subtropical areas (1). In Brazil alone there are at least 10 million people infected or at risk of infection, which occurs by skin contact with contaminated water. The clinical course of schistosomiasis ranges from symptomless to severe liver fibrosis with portal hypertension. The causes of this clinical pleiotropism are not known, but it is believed that genetic variation of both the human host and the parasites plays a part. Thus, the study of the genomic structure and variation in *S. mansoni* is an urgent issue for a better understanding of the factors influencing host–parasite interactions and of the molecular epidemiology of the disease.

Little is known about the genome of *S. mansoni*. The parasite is diploid, with eight chromosome pairs and sexual chromosome dimorphism (38). Less than 100 sequences of different expressed genes of *S. mansoni* have been deposited in the GenBank data base as of Release 77.0 (June 1993). The few transcriptional units that have been sequenced show scarce and small introns (2). As much as 35% of the *S. mansoni* genome may be composed of repetitive DNA (3). Spotila *et al.* (4) have ascertained from the study of a cDNA clone called SM750 a peculiar repetitive sequence family consisting of 62-bp units found in small tandem arrays in many mRNA transcripts of diverse sizes and sequences. An oligonucleotide probe directed against this 62-bp repeat, called polymorphic repeated element (PRE), recognized multiple fragments in genomic digests, suggesting that it is widely distributed in the genome.

Studies with isoenzymes (5), ribosomal RNA gene structure (6, 7), and randomly amplified polymorphic DNAs (8) have demonstrated limited genetic variability of different strains of *S. mansoni*. In contrast, Despres *et al.* (9, 10) showed extensive length polymorphism of mtDNA among six strains of *S. mansoni*, with sizes ranging from 16,500 bp to 24,900 bp. The length polymorphism was assigned to a single region, most probably the control region containing the origin of replication, which was resistant to digestion with all restriction enzymes tested and varied in size from 1500 bp to nearly 10,000 bp (10).

We report here that in the LE strain of *S. mansoni* the variable region of the mtDNA has a complex minisatellite structure involving two types of repeated sequences with 558 bp and 62 bp. Each minisatellite repeat is composed of a large 558-bp component and a variable tandem array of the small 62-bp unit. The large repeat has partial homology with the cDNA clone SM750 (4), whereas the small 62-bp unit is identical to the nuclear PRE characterized by Spotila *et al.* (4). The complex structure of the minisatellite§ can be exploited for the development of a PCR-based identity test for schistosomes.

MATERIALS AND METHODS

Parasites and mtDNA Isolation. Almost all experiments were performed with the LE strain of *S. mansoni*. This strain was isolated from a patient from Minas Gerais, Brazil, and has been maintained for more than 20 years by passaging in hamsters. The strain is known to be heterogeneous and thus should be considered an oligoclonal population. Individual clones have been obtained by infection of the intermediate host (*Biomphalaria glabrata*) with a single miracidium. Adult worms were obtained from isotonic saline perfusion of the mesenteric system of hamsters that had been experimentally infected 7 weeks previously. After perfusion, the parasites were washed in isotonic saline, and total DNA was prepared as described (8). Alternatively, the mitochondria of freshly obtained worms were prepared by differential centrifugation, and mtDNA was isolated as described by Despres *et al.* (9). DNA from clones of the LE strain was purified from cercariae obtained from clonally infected snails (8).

Restriction Enzyme Digestions. Purified total or mtDNA was digested to completion with restriction enzymes as rec-

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Abbreviations: PRE, polymorphic repeat element; ORF, open reading frame; VNTR, variable number of tandem repeats.

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§The DNA sequence reported in this paper has been deposited in the GenBank data base (accession no. L27240).

ommended by the manufacturers (BRL or Pharmacia). After digestion, mtDNA fragments were extracted with phenol/chloroform, precipitated in ethanol, electrophoresed in 4% polyacrylamide gels, and stained with silver according to Santos *et al.* (11).

Cloning of the *Dde* I 600-bp Restriction Fragment. Purified mtDNA from the LE strain was digested to completion with *Dde* I and precipitated with ethanol, and a fragment of about 620 bp was isolated by electrophoresis in low-melting-point agarose and purified with the PCR miniprep DNA system (Promega). The 620-bp fragment was repaired with the Klenow fragment of *Escherichia coli* DNA polymerase I (Sigma) and cloned in the *Sma* I site of pUC18 (Pharmacia), which was used to transform *E. coli* JM109. The clone of interest (named F21) was screened by electrophoresis of plasmid DNA isolated by the Magic miniprep DNA purification system (Promega).

Southern Blot Analysis. Ten nanograms of digested mtDNA was electrophoresed through 0.8% agarose, blotted onto Biodyne B nylon membranes (BRL), and probed with biotin-labeled clone F21 as described elsewhere (12). F21 was biotin-labeled by PCR amplification in a 100-μl reaction mixture containing 6 nmol of bio-11-dUTP (no. B6780; Sigma), 25 nmol of each dNTP, 100 ng of purified recombinant vector, and 20 pmol of M13 universal forward and reverse primers (Sigma). PCR was carried out in a MJ Research (Cambridge, MA) model PTC-100 thermal cycler using 35 cycles of 92°C for 2 min, 50°C for 2 min, and 72°C for 5 min, followed by 5 min at 72°C. After amplification, the probe was purified in Sephadex G-50. Hybridization was in 45% (vol/vol) formamide at 42°C, and the stringency of washing was 0.1× standard saline citrate/0.1% SDS for 30 min at 65°C.

Sequencing. All sequencing was performed with inserts cloned in the pUC18 cloning vector. The clones were sequenced from both ends with commercial kits based on T7 DNA polymerase and the dideoxy chain-termination method (AutoRead kit; Pharmacia) and a Pharmacia A.L.F. automatic DNA sequencer.

Computer Analysis. Consensus sequences were assembled from individual sequences using the program DNASIS (Pharmacia). After obtaining the sequences of recombinant clones, we searched for homology with nucleotide sequences deposited in GenBank using the BLASTN program through the electronic mail server of the National Center for Biotechnology Information (Bethesda).

PCR Primers and Reactions. Based on the F21 sequence, we designed two primers to amplify across adjacent minisatellite units. They were F21U (5′-GATGTAAAAATAGGATTTAGGG-3′) and F21L (5′-TAGTCGACAGACTACAAACATCTTATCTGC-3′). The latter primer had eight extra bases with a restriction site for *Sal* I to help in cloning the PCR products. The location of PCR primers within the F21 sequence is shown in Fig. 3. For PCR, 10 ng of *S. mansoni* mtDNA was amplified in a 25-μl reaction mixture containing 1.5 mM $MgCl_2$, 50.0 mM KCl, 0.1% Triton X-100, 10 mM Tris·HCl (pH 9.0), 200 μM dNTPs, 25.0 pmol of each primer, and 1.0 unit of *Taq* DNA polymerase (Promega). PCR was carried out in a MJ Research PTC-100 thermal cycler using 30 cycles of 92°C for 3 min, 55°C for 1 min, and 72°C for 2 min, followed by 5 min at 72°C. PCR products were analyzed by 4% PAGE and silver staining.

Cloning and Sequencing of PCR Products. The fragments resulting from amplification of mtDNA of the LE strain with primers F21U and F21L were electrophoretically resolved on agarose gels. The lower four bands of the 62-bp ladder were each purified by band-stab PCR (13). The pure PCR products were precipitated overnight and digested with *Hin*fI, and ends were repaired with the Klenow fragment of *E. coli* DNA polymerase I. Afterwards, they were digested with *Kpn* I and cloned in pUC18 that had previously been digested with *Sal* I, and the ends were repaired with the Klenow fragment of *E. coli* DNA polymerase I followed by digestion with *Kpn* I. The inserts were then sequenced as described above.

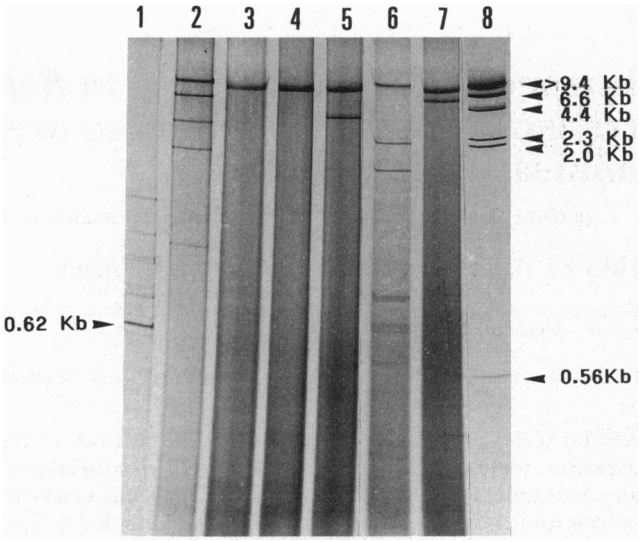

FIG. 1. Digestion of purified mtDNA of *S. mansoni* by restriction endonucleases. The products of digestion were analyzed in a 4% polyacrylamide gel and visualized by silver staining. The enzymes used with their respective lanes are lane 1, *Dde* I; lane 2, *Bsp*RI; lane 3, *Hae* II; lane 4, *Ava* I; lane 5, *Eco*RV; lane 6, *Alu* I; lane 7, *Cla* I. Molecular weight markers, which are the *Hin*dIII fragments of λ phage, are shown in lane 8. Note that the gel does not permit resolution of fragments above 9.4 kb. The arrowhead on the left indicates the major 620-bp fragment generated by *Dde* I digestion.

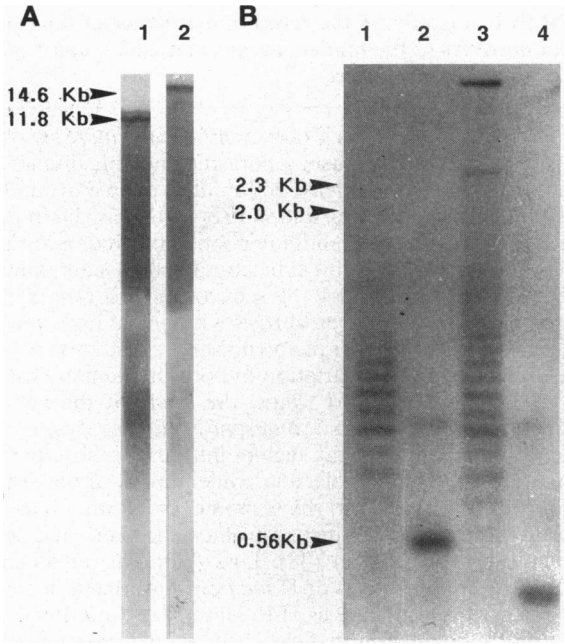

FIG. 2. Southern blots on nylon membranes hybridized at high stringency with biotinylated F21 used as probe. (*A*) Whole DNA from two different strains [LE (lane 1) and SJ (lane 2)] of *S. mansoni* was digested with *Bsp*RI, run on a 0.6% agarose gel, blotted, and hybridized with F21. The calculated molecular size of the major bands visualized is indicated. (*B*) Purified mitochondrial DNA of *S. mansoni* was digested with restriction enzymes, run on a 1% agarose gel, blotted, and hybridized. The enzymes were as follows: lane 1, *Hin*fI; lane 2, *Hin*fI plus *Dde* I; lane 3, *Kpn* I; lane 4, *Kpn* I plus *Dde* I. The migration of the three smaller *Hin*dIII digestion products of bacteriophage λ (2.3, 2.0, and 0.56 kb) is indicated at left.

RESULTS

A Minisatellite in *S. mansoni* mtDNA. mtDNA from the LE strain of *S. mansoni* was purified from mitochondria isolated by differential centrifugation. The mtDNA had a size of ≈23 kb in agarose gels. Digestion with a variety of restriction enzymes followed by PAGE showed a large (>9.4 kb) undigested fragment with most endonucleases used: *Bsp*RI, *Hae* II, *Ava* I, *Eco*RV, *Alu* I, and *Cla* I (Fig. 1) and *Xho* I, *Bgl* II, and *Eco*RI (data not shown). With *Dde* I digestion, however, the large fragment vanished and a prominent fragment appeared with a size of ≈620 bp, with several other minor bands in addition (Fig. 1). This fragment was cloned in pUC18, and the insert was named F21.

The F21 insert was labeled with biotin by PCR and used to probe fragments produced by *Bsp*RI (an isoschizomer of *Hae* III) digestion of total DNA from clones of the LE strain of *S. mansoni*. The main band recognized by this probe was a polymorphic fragment varying in size from about 11.8 to 14.6 kb (Fig. 2*A*). Identical results were obtained in Southern blots of purified mtDNA. Together with the data from Despres *et al.* (9), this was persuasive evidence that the large variable region of *S. mansoni* was a polymorphic minisatellite with a repeat unit of about 620 bp. Interestingly, the major band in the blots was followed by a large smear (Fig. 2*A*), suggesting that many other sequences, most of them probably of nuclear origin, also hybridized with F21.

Sequence Analysis of the F21 Clone. The F21 insert was sequenced in both directions using a fluorescent automatic sequencer. The sequence was exactly 620 bp long, with a 70% A+T content (Fig. 3*A*), and was deposited in GenBank (accession no. L27240). This was compared with other sequences in GenBank using the BLASTN program of the National Center for Biotechnology Information, applying a filter because of the high A+T content. The results showed a highly significant homology with the known SM750 cDNA (4), extending from nucleotide 1 to 66 and 518 to 620 (underlined and shaded in Fig. 3*A*). The homologous segment included a region from nucleotides 1–50 and 609–620 (double underlined in Fig. 3*A*) that showed 100% homology with the PRE that had been described by Spotila *et al.* (4) as an abundant transcribed nuclear repeat and that was present in five tandem copies within SM750 (Fig. 3*B*). This suggested that the *Dde* I site used to obtain the F21 insert was located within the PRE sequence (Fig. 3*B*). In consequence, if two sequences of F21 are placed in tandem, the region homologous to PRE and SM750 be-

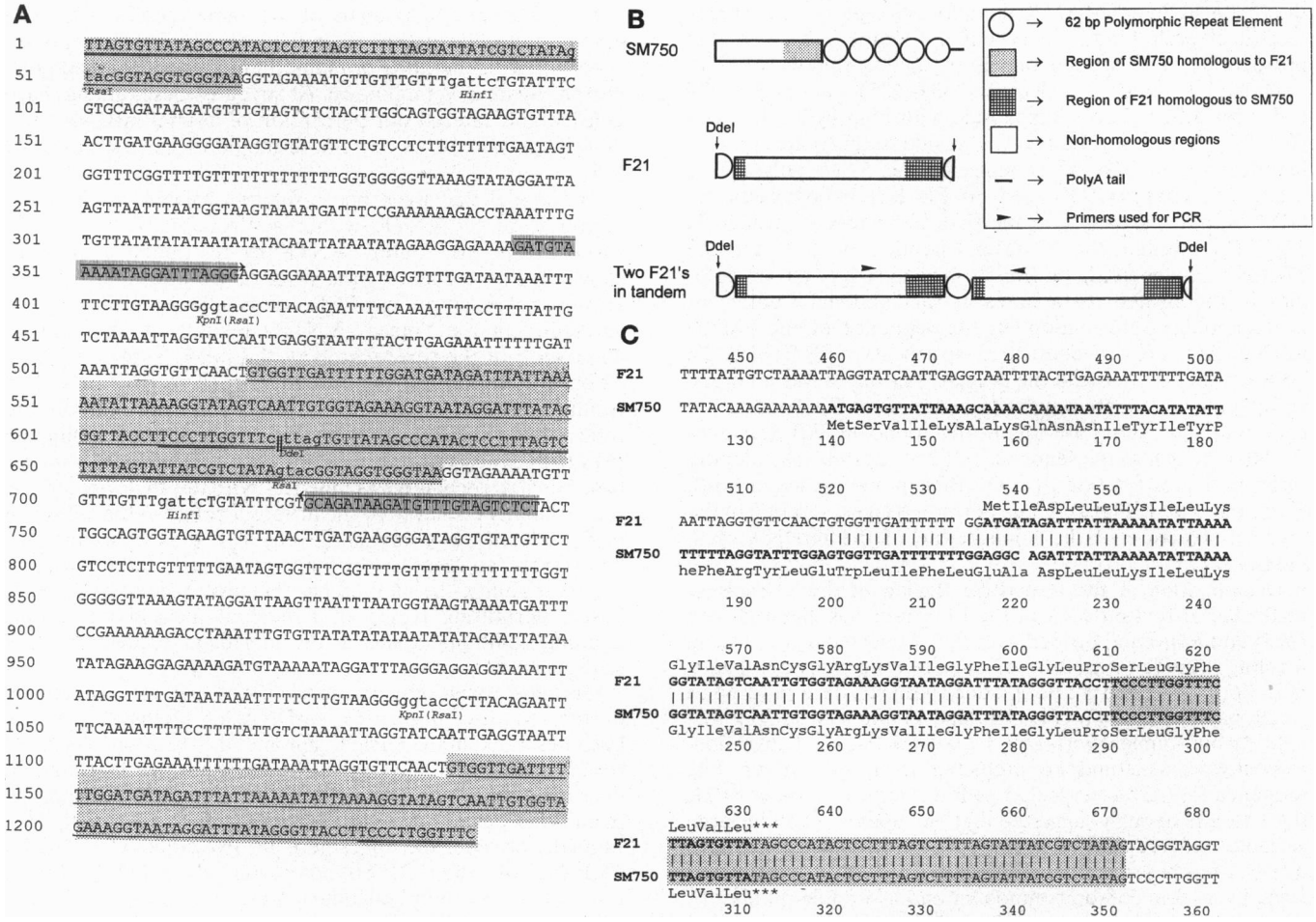

FIG. 3. (*A*) Nucleotide sequence of the 620-bp *Dde* I fragment of the LE strain of the *S. mansoni* mtDNA molecule. Two sequences are placed in tandem duplication (see scheme in *B*) to demonstrate the continuity of the region homologous to SM750 (underlined and shaded), which includes the segment identical to the 62-bp nuclear polymorphic repeat element (double underlined). The boundary between the two F21 sequences is indicated with two short vertical lines. Numbering begins at the first nucleotide of the cut site of *Dde* I. Restriction sites are displayed in lowercase letters, with the name of the endonuclease underneath. The sequences of the primers F21U (residues 103–124) and F21L (345–366) are highlighted with an overhead arrow and shading. (*B*) Diagrams showing the basic structure of SM750 and F21. The homologous regions are shaded. The drawing of two F21s in tandem depicts the arrangement of the sequence in *A*. (*C*) The homologous part of SM750 and duplicated F21 are aligned. Two gaps (blank spaces) were introduced to maximize homology. The amino acid sequences of the predicted translation products of the two ORFs are shown together with the nucleotide sequences. The 62-bp PRE region is shaded. Please note that this shading does not correspond to the shading in *A* or *B*.

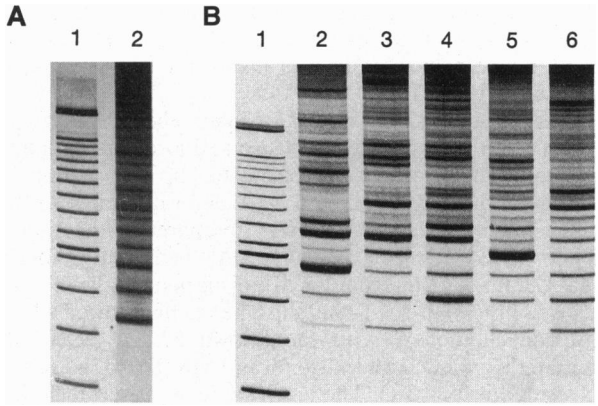

FIG. 4. Results of the PCR amplification of total *S. mansoni* DNA with primers F21U and F21L. The amplification products were run on a 4% polyacrylamide gel and stained with silver. (*A*) Strain LE of *S. mansoni*. (*B*) DNA from cercariae of five clones obtained from strain LE of *S. mansoni*. Lane 1 of both *A* and *B* shows a 100-bp ladder, starting with the 300-bp marker as the lowest rung.

comes continuous (Fig. 3 *A* and *B*). Two short open reading frames (ORFs) beginning with an ATG can be found in F21: the first is in the non-SM750 homologous regions and extends from nucleotide 156 to 243, potentially coding for a 28-residue polypeptide. The second one is in the continuous SM750-homologous region (nucleotides 534–635) and potentially codes for a 32-residue polypeptide with high hydrophobicity (Fig. 3*C*). Except for the two first residues, this polypeptide is identical to the last 30 residues of the 57-aa polypeptide predicted from the ORF of SM750 (Fig. 3*C*). In both cases, the ORF extends partially into the first 62-bp repeat (shaded in Fig. 3*C*). Neither the 32-aa polypeptide of F21 nor the 57-residue polypeptide of SM750 showed significant homologies in the protein data bases at the National Center for Biotechnology Information. In the sequence of the SM750 mRNA, there are five identical 62-bp repeats of PRE (Fig. 3*B*); between the last one and the poly(A) tail, there was a unique 16-bp sequence (containing at its beginning an *Rsa* I restriction site) that also could be identically found in F21 (residues 51–66; Fig. 3*A*). In the sequence of F21, we could also identify restriction sites for *Dde* I (in the PRE-homologous portion), *Hin*fI, and *Kpn* I (Fig. 3). These permitted an exploration of the internal structure of the minisatellite of the mtDNA of *S. mansoni*.

Organization of the Repetitive Region of the *S. mansoni* mtDNA. Purified mtDNA of the LE strain was digested with *Hin*fI and *Kpn* I and probed with F21. Many bands were seen, forming a ladder with an ≈62-bp difference between bands (Fig. 2*B*, lanes 1 and 3). Both ladder patterns were reduced to single bands of 534 bp (Fig. 2*B*, lane 2) and 414 bp (Fig. 2*B*, lane 4) by double *Hin*fI–*Dde* I and *Kpn* I–*Dde* I digestion, respectively, as could be predicted from the known F21 sequence (Fig. 3). Interpreted with the sequence data of F21, these results strongly suggested that the repeat unit of the large variable number of tandem repeats (VNTR) of *S. mansoni* mtDNA was heterogeneous, being constructed, in most part at least, by a large 558-bp component and a variable number of the small 62-bp units arranged in tandem.

This model was tested by PCR using primers F21U and F21L, designed to amplify a region between residues 345 and 747 (Fig. 3 *A* and *B*), with a minimum product size of 408 bp between adjoining large repeat units. Again, a ladder with ≈62-bp steps was seen (Fig. 4*A*). The ladder pattern could again be reduced to a single band by *Dde* I digestion (data not shown). We obtained absolute confirmation for the model, at least for its most simple configurations, by cloning and sequencing the first four smaller steps of this ladder, which, respectively, showed one to four 62-bp PRE units in tandem arrangement (data not shown). However, the minisatellite may contain considerably more complex higher order structures, as suggested by the breakdown of the regular ladder pattern at larger molecular sizes (Figs. 2*B* and 4*A*).

Repeats in Different Clones of the LE Strain of mtDNA. We had shown that different clones of the LE strain could vary in the total size of the large VNTR (Fig. 2*A*). We then tested if the relative amounts of the several heterogeneous units with different numbers of repeats of the 62-bp unit varied in different clones. To do that, we subjected total DNA of each clone to PCR with primers F21U and F21L. A ladder of regular 62-bp steps resulted, which was nevertheless highly polymorphic in the relative staining intensity of each step band, thus indicating variability between clones (Fig. 4*B*).

DISCUSSION

The polymorphic minisatellite that we have characterized in the mtDNA of *S. mansoni* is not by itself very unusual. Substantial length variation, which has been attributed to variable copy number of tandem repeats, has been found in or near the control region or D-loop of the mtDNA of a number of metazoan species (14–25). This variability occurs among species, between individuals of the same species, and even among molecules within an individual. The mechanisms responsible for generating or maintaining this widespread polymorphism are as yet unknown. At any rate, these findings have considerably altered our perception of animal mtDNA, previously considered an extreme example of genetic economy (26).

The model that we propose for the mtDNA minisatellite region is of "a VNTR within a VNTR." We can score variability in the "outer" VNTR by restriction analysis as shown in Fig. 2*A* and by the work of Despres *et al.* (9, 10). However, we also can use PCR across adjacent units to study variability in the "inner" VNTRs by tallying the relative abundance of the several sizes of the tandem repeats of the 62-bp unit within the minisatellite structure (Fig. 4*B*). This results in an internal scoring of the size of the minisatellite repeat unit, similar to the minisatellite variant repeat mapping (MVR-PCR) described by Jeffreys *et al.* (27), which is however based on heterogeneity of sequence. With this PCR procedure, the variability displayed by the different clones of the LE strain of *S. mansoni* was outstanding (Fig. 4*B*). This technique may be useful as an identity test for *S. mansoni*. In addition to its hypervariability, the method has the advantage of being PCR based, permitting typing of individual miracidia and thus opening additional vistas in the molecular epidemiology of schistosomiasis.

Our most remarkable finding was that sequences that made up the repetitive unit of the mtDNA VNTR had previously been described in the nuclear genome of *S. mansoni* as part of the SM750 cDNA (4). This cDNA has a peculiar structure. It does not contain any single ORFs; the longest ORF beginning in an ATG was 171 bp long, corresponding to 57 aa (Fig. 3*C*). Immediately after the ORF there are five copies of the 62-bp PRE (actually the ORF extends partially into the first PRE), followed by a 16-bp oligonucleotide and the poly(A) tail (diagram in Fig. 3*B*). Sequences hybridizing with an oligonucleotide probe complementary to PRE are apparently spread throughout the genome and are abundant among transcripts (4). In our laboratory, we have been conducting a systematic program of sequencing of expressed sequence tags (EST) from a directional size-selected cDNA library of *S. mansoni*. Thus far, a total of 607 ESTs have been obtained from one or both cDNA ends, encompassing 429 distinct clones (28). Twelve (2.8%) of these cDNA clones contained only several copies of the PRE in their EST, and in every case there was the presence

of the same 16-bp oligonucleotide between the last repeat and the poly(A) tail.

The presence in *S. mansoni* mtDNA of fragments of genes that are present and transcribed in the nuclear genome raises the question of the origin of these sequences. Numerous reports have been made of transfer of DNA sequences from mitochondria to the nucleus of eukaryotic cells (29–33). This is in agreement with the widely accepted endosymbiont theory of the origin of mitochondria (34), which implies that most, if not all, nuclear genes encoding mitochondrial proteins have been transferred from mitochondria to the nucleus over evolutionary time. The name "promiscuous DNA" has been given to those DNA sequences that occur in more than one of the membrane-bound organellar genetic systems of eukaryotic cells (30, 35). Other types of intracellular promiscuity have also been demonstrated with, for instance, mitochondria receiving DNA fragments from chloroplasts (36). On the other hand, transfer of DNA sequences from the nucleus to mitochondria has never, to our knowledge, been reported. Thorsness and Fox (33) estimated that such transfer, if it occurs at all, happens with a frequency less than 100,000 times smaller than from mitochondria to nucleus. This consideration is important, because in the case of *S. mansoni* the precise composition of the sequences suggests that the traffic may have occurred in the direction nucleus → mitochondria. Specifically, the mitochondrial repeat unit appears to contain only a fragment of the SM750 cDNA, with only part of the ORF of the latter (Fig. 3*C*). Moreover, the nuclear sequences SM750 and PRE are transcribed while the mitochondrial VNTR region, as is the case with most minisatellites (37), appears unlikely to be expressed. However, we cannot rule out the possibility that the sequences were transferred from mitochondria to nucleus, and yet a third prospect exists—namely, that the sequence was acquired simultaneously by nucleus and mitochondria from an extracellular "virus-like" source. We hope that intense study of the nuclear genomic organization and expression of PRE may help us solve this fascinating problem.

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