POLYMERASE CHAIN REACTION ASSAY BASED ON A HIGHLY REPEATED SEQUENCE OF SCHISTOSOMA HAEMATOBIUM: A POTENTIAL TOOL FOR MONITORING SCHISTOSOME-INFESTED WATER

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Abstract. We have cloned from Schistosoma haematobium genome a repeated sequence, which consists of tandemly arranged 121-bp-long units and which is highly abundant (~15% of the S. haematobium genome). By these features, the DraI repeat is similar to the Sm1–7 sequence of Schistosoma mansoni previously described by us. However, their nucleotide sequences are profoundly different. Polymerase chain reaction (PCR) primers were designed on the basis of the DraI sequence information and were used in a PCR assay by which as little as 10 fg of schistosomal DNA as well as individual cercariae were detected. The DraI repeat cross-hybridized with DNA from Schistosoma bovis, Schistosoma magna, Schistosoma mattheei, Schistosoma curassoni, and Schistosoma intercalatum, but not with DNA from S. mansoni nor from Trichobilharzia ocellata and Echinostoma sp. A potential value of this PCR assay is suggested for monitoring free-living cercariae and infected snails only in bodies free of cross-hybridizing species.

INTRODUCTION

The 3 main species of schistosomes, Schistosoma mansoni, Schistosoma haematobium, and Schistosoma japonicum, infect ~200 million people in the tropics and subtropics, and of these, S. haematobium infects close to 100 million people in 53 countries in Africa and the Middle East. Various approaches for targeting chemotherapy to human populations were not sufficient to suppress transmission, with the resulting persistence of the risk of infection. This is partly due to the overdispersed nature of snail distribution as well as the variability with regard to human infection intensity, water contact patterns, and water contamination. These variable factors, as well as seasonal fluctuations in snail population densities, infection rates, and cercarial output, call for well-focused and carefully timed intervention for reducing transmission. However, because the efficacy of focal control is also hampered by missing data on untreated people in 53 countries in Africa and the Middle East.1 Various approaches for targeting chemotherapy to human populations were not sufficient to suppress transmission, with the resulting persistence of the risk of infection. This is partly due to the overdispersed nature of snail distribution as well as the variability with regard to human infection intensity, water contact patterns, and water contamination. These variable factors, as well as seasonal fluctuations in snail population densities, infection rates, and cercarial output, call for well-focused and carefully timed intervention for reducing transmission. However, because the efficacy of focal control is also hampered by missing data on untreated patients or on transmission sites, the implementation of such a control approach requires improved means for evaluating snail-to-human transmission variables, and parasite flux from human to snail. Standard tools for monitoring of free-living cercariae and infected snails during prepregnancy have not proven sufficiently sensitive for these purposes, but the polymerase chain reaction (PCR) methods we have previously developed have the potential to fill this gap. Thus, when primers based on sequence information of the Sm1–7 repeated sequence of S. mansoni11 were employed in PCR assays, it was possible to detect as little as 10 fg of DNA. This degree of sensitivity enabled the identification of individual cercariae and of infected snails throughout prepregnancy. The feasibility of using these assays for mass screening of snails by testing pooled snail extracts was also established. Similar PCR assays that are based on corresponding repeated sequence information for detecting S. haematobium with similar sensitivity are needed. Also needed are assays for differentiating S. haematobium from a number of schistosomes with terminal-spined ova, which may inhabit the same bodies of water and the same snail hosts. Some of them may even infect humans and produce hybrids with S. haematobium in cases of coinfection.2 In the present study, we describe the cloning and characterization of a highly abundant, tandemly arranged, repeated sequence in the genome of S. haematobium and assess its potential diagnostic usefulness.

MATERIALS AND METHODS

Parasites. The Egyptian strains of S. haematobium and S. mansoni were maintained in Bulinus truncatus and Biomphalaria glabrata; golden hamsters and mice served as vertebrate hosts, respectively. Snails were exposed individually to 10 miracidia per snail, hamsters were infected by subcutaneous injection of 500 cercariae, and mice were infected by subcutaneous injection of 300 cercariae. Adult worms were collected from their respective laboratory hosts by liver perfusion.3 Schistosoma mattheei, Schistosoma magna, Schistosoma bovis, Schistosoma curassoni, and Schistosoma intercalatum adult worms were obtained from the Natural History Museum, London, United Kingdom. Trichobilharzia ocellata cercariae and Echinostoma sp. adult worms were obtained from Erlangen-Nuerenberg University, Germany.

Isolation of DNA and construction of S. haematobium genomic library. Materials for recombinant DNA technology, including cloning vectors and enzymes employed for restriction, ligation, and dephosphorylation, were from New England Biolabs (Beverly, MA) when not indicated otherwise. Conditions for these procedures were used according to the recommendations of the manufacturer. High-molecular-weight DNA was prepared from adult worms by lysis in sodium dodecyl sulfate (SDS), digestion in proteinase K, and extraction with phenol, phenol-chloroform, and chloroform in sequence as previously described. Extraction of DNA from cercariae for detection by PCR was performed by a rapid alkaline extraction method we previously described. A genomic library was prepared from S. haematobium DNA partially digested by SmaI restriction enzyme. Standard procedures were employed for the various steps of cloning.
ing as previously described. Digestion products ranging in size between 1.5 to 0.5 kb were ligated at a molar ratio of 1:1 into the BamH1 site of pBluescript plasmid DNA (Stratagene, La Jolla, CA). The plasmid was pretreated with calf intestinal alkaline phosphatase for dephosphorylation. Transformation was carried out with Escherichia coli XL1 Blue by established protocols. Transformed cells were plated out on LB agar plates containing ampicillin. Isopropyl-β-D-galactoside (IPTG) and X-Gal were included in the final plating mixture for color selection of recombinant clones.

**Selection of repetitive DNA clones from the S. haematobium genomic library.** White colonies were plated on a grid, transferred to nylon filters, and initially screened with 32P-labeled total genomic S. haematobium DNA for selecting clones exhibiting strong hybridization signals indicative of the presence of repeated sequences. Screening with S. mansoni total DNA was done similarly for excluding cross-hybridizing clones. Colony lysis, DNA denaturation, and fixation onto the filters were carried out by a single step in a microwave oven as previously described. Schistosomal DNA was radiolabeled with 32P by use of the Ready-To-Go DNA labeling kit (Pharmacia Biotech, Piscataway, NJ) according to the instruction of the manufacturer, and was used for in situ hybridization (see below) to bacterial colonies transferred onto nylon filters as described above.

**Filter hybridization.** Standard procedures were used as previously described. For colony hybridization, prehybridization was carried out for 2 hr at 42°C in a reaction buffer containing 0.8 M NaCl, 20 mM 1,4-piperazine diethylsulfonic acid (Sigma Chemical, St. Louis, MO) pH 6.5, 50% deionized formamide, 0.5% SDS, and 0.1%/vol denatured salmon sperm DNA. Hybridization was then carried out with 32P-labeled total DNA overnight at the same conditions, and the filters were washed 3 times for 30 min at 55°C, with 0.1× standard saline citrate (SSC); 0.15 M NaCl, 15 mM sodium citrate pH 7.0) containing 0.1%/vol SDS. For dot blot hybridization and for Southern blot hybridization (see below), prehybridization was carried out at 65°C for 2 hr, and hybridization was carried out with the radioactive probe at 65°C overnight. Both were done in 6× SSC and 5× Denhard solution. The first wash was carried out at room temperature with 2× SSC for 30 min, and the second and third washes were carried out with 2× SSC and 0.1% SDS for 15 min each time. The air-dried membranes were then exposed to X-ray film.

**Dot blot and Southern blot analyses.** Various dilutions of S. haematobium DNA and DNA from other schistosomes with terminal-spined ova, as well as from Trichobilharzia and Echinostoma, were dotted and probed by radiolabeled DraI in a recombinant plasmid. Comparative dot blot hybridization was performed to estimate the abundance of the repeated sequence. For this purpose, various concentrations ranging from 250 pg to 10 ng of total S. haematobium DNA and DNA of a selected recombinant plasmid were dotted and comparatively probed with labeled total DNA and with the repeated sequence DNA cloned in pSh091 (containing 3 repeat units). The DNA tested was dotted onto a nitrocellulose membrane by employing a dot blot apparatus (Bio-Rad, Hercules, CA). It then underwent denaturation and neutralization by standard procedures and was finally hybridized with a radiolabeled probe (pSh1027) as described above.

![FIGURE 1. The consensus sequence of a DraI repeat unit, obtained from sequence analysis of 50 repeat units. The primers designed from this sequence are underlined.](image)

Analysis of the dot blot hybridization signals was performed on a Macintosh computer by use of the public domain National Institutes of Health Image program (available at http://rsb.info.nih.gov/nih-image/). Southern blot analysis was performed by use of standard procedures for determining the distribution of the repeated sequence. For this purpose, 1,000 ng S. haematobium DNA was partially digested by Sau3A restriction enzyme, and after Southern blot transfer to a nitrocellulose filter, hybridization was carried out as described above by use of a recombinant plasmid (pSh1027) containing 5 copies of the repeated sequence.

**Polymerase chain reaction assay.** Polymerase chain reaction primers were designed on the basis of the sequence information of the DraI repeat unit (see Results). They were as follows: Sh1, 5′-GATCTCACCTATCAGACGAAAC-3′ and Sh2, 5′-TCACAAAGCATACGCCAAC-3′ (Figure 1). An optimal PCR buffer was selected by the Opti-Prime PCR Optimization Kit (Stratagene, La Jolla, CA). The reaction was carried out in 100 µl solution containing 10 mM Tris-HCl pH 9.2, 25 mM KCl, 3.5 mM MgCl2, 200 µM (each) dATP, dGTP, dCTP, and dTTP (Promega, Madison, WI), 0.4 µM of each of the amplification primers, 2.5 units Taq DNA polymerase (Red Hot Taq DNA polymerase, Advanced Biotechnologies, Epsom, UK), and target DNA. The thermal cycler (MiniCycler; MJ Research, Watertown, MA) was used with a thermal profile involving 5 min at 95°C, followed by 35 cycles each of 1 min at 95°C, 1 min at 55°C, 30 sec at 72°C, and a final elongation step at 72°C for 10 min.

**Nucleotide sequencing.** Plasmid Isolation Kit (Qiagen, Chatsworth, CA) was used according to the instructions of the manufacturer for obtaining DNA from recombinant plasmids selected for sequence analysis. Nucleotide sequence analysis was carried out by Dye Terminator Cycle Sequencing kit (Perkin-Elmer, Wallington, UK), according to the instructions of the manufacturer. An ABI PRISM 377 DNA sequencer and ABI Sequencing Analysis software (Perkin-Elmer, Foster City, CA) were used. The thermal profile was 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C for 25 cycles. Consensus sequence analysis was performed via the Wisconsin Package (Genetic Computer Group, Madison, WI).

### RESULTS

**Library screening and colony selection.** Approximately 4,000 colonies were initially screened by colony hybridiza-
tion with radiolabeled total DNA of *S. haematobium*, and 429 gave strong hybridization signals. These were subsequently screened by hybridization with radiolabeled total DNA from *S. mansoni* and *S. haematobium*, and 316 clones exhibiting specific hybridization signals with *S. haematobium* DNA, were identified. Approximately 20 clones, which demonstrated the highest detection sensitivity by hybridization with dilutions of total *S. haematobium* DNA, were selected for sequence analysis.

**Nucleotide sequence and arrangement of the *S. haematobium* repeated DNA.** The selected clones exhibiting specific hybridization with *S. haematobium* DNA (in comparison with *S. mansoni* DNA) were subjected to nucleotide sequence analysis. The largest of these, clone pSh0610, was found to contain a 1.3-kb DNA insert composed of 10 units of 121-bp tandemly arranged DNA sequences. A total of 50 repeat units were analyzed in order to obtain a consensus sequence of the repeat unit. Figure 1 presents the consensus sequence of one such unit of 121-bp-long repeat and depicts the corresponding PCR primers. *Dra*I repeats have a single Sau3A restriction site, as does the Sm1–7-repeated sequence of *S. mansoni*. In both cases, cloning was done with Sau3A-digested DNA. *Dra*I enzyme also cuts once within the *S. haematobium* repeated sequence units but not within Sm1–7 and was therefore selected to designate the *S. haematobium* repeated sequence. Only occasional base changes were observed in the 50 repeat units analyzed (results not shown), indicating a high intraspecies conservation of the *Dra*I repeat. Southern blot analysis of *S. haematobium* genomic DNA partially restricted by *Dra*I and probed by a *Dra*I exhibited a pattern of multiple ladder of bands with size increases of 121 bp, as shown in Figure 2. It is thus demonstrated that the *Dra*I repeat is tandemly arranged in the genome of *S. haematobium*, with proof of presence of large arrays. However, the maximal size and size variability of *Dra*I repeat arrays is as yet unknown.

**Abundance of the *Dra*I repeat.** Initially the high representation of the *Dra*I repeat in the *S. haematobium* genome was suggested by the large number of clones cross-hybridizing with the *Dra*I repeat. Thus, of the 316 clones exhibiting specific hybridization with total *S. haematobium* DNA, 274 showed hybridization signals with a *Dra*I probe (results not shown). For obtaining a quantitative estimate on the abundance of the *Dra*I repeat, we carried out a comparative dot hybridization study. The relation between DNA concentration and relative strength of the signals obtained is presented graphically in Figure 3. The DNA concentrations in the linear part of the curve (in the range of DNA quantities between 250 pg and 2.5 ng) were taken for calculating the abundance of the *Dra*I repeat. When the radiolabeled pSh091 insert, containing 3 copies of *Dra*I repeat, was used as a probe, the calculated relative signal strength for 1 ng total DNA was 2,314. This corresponded to signal strength of 3,271 with the recombinant plasmid pSh091. Thus, 1 ng of total DNA corresponded to 1.41 ng plasmid DNA. However, because the insert size (363 bp) constitutes 1.917 the size of the recombinant pBluescript plasmid, it can be calculated that the insert constitutes 15.37% of the genomic DNA. In a reciprocal experiment, dilutions of total DNA and of plasmid DNA were probed with labeled total DNA. The signal obtained with 1 ng pSh091 recombinant plasmid corresponded to the signal obtained with 0.47 ng total DNA. However, considering that the 363-bp schistosomal sequence constitutes 1.917 parts of pSh091, it was calculated that the sequence cloned in pSh091 constitutes ~23% of the schistosomal genome. This is considerably higher than the 15.37% figure obtained by employing the *Dra*I insert (from pSh091) as a probe, and 3 repeated tests gave similar results. The discrepancy could be the result of nonspecific hybridization of the total DNA probe with *Dra*I, which resulted in unduly high signals. In any event, the *Dra*I repeat constitutes at least 15% of *S. haematobium* genome. Considering that the schistosomal genome size is 270 Mb, the number of copies of the *Dra*I repeat runs in the hundreds of thousands.

**Detection sensitivity by PCR.** Amplification of 10-fold dilutions of total *S. haematobium* DNA by PCR that used *Dra*I repeat primers is demonstrated in Figure 4. The detec-
FIGURE 4. Detection sensitivity by polymerase chain reaction employing Drai primers. Lanes 1–4, amplification products of 10 pg, 1 pg, 100 fg, and 10 fg DNA, respectively; M-size markers (lambda [Greek letter uppercase] DNA/Eco471 cut).

Detection sensitivity is < 10 fg. A ladder pattern of the amplification products further demonstrates the tandem arrangement of this repeat. A direct relation between DNA concentration and number of ladder bands was demonstrated with the Sm1–7 repeat of S. mansoni. This high detection sensitivity is more than enough for the detection of individual cercariae (Figure 5).

Detection specificity by the DraI repeat. Evaluation of the specificity of detection by DraI repeat was carried out by dot hybridization (Figure 6) with several other schistosomes and with nonschistosome species. Hybridization of clone pSh1027 with homologous DNA was achieved down to a concentration of 50 pg total DNA. Cross-hybridization signals were obtained with DNA from S. mattheei, S. magrebowiei, S. bovis, S. curassoni, and S. intercalatum. No cross-hybridization signals were obtained with DNA from S. mansoni as well as with DNA from Trichobilharzia ocellata and Echinostoma sp.

DISCUSSION

We have cloned and characterized a highly abundant, tandemly arranged, repeated sequence in the genome of S. haematobium and developed a highly sensitive PCR assay for the detection of this parasite. This test is intended for monitoring free-swimming cercariae and infected snails for epidemiological purposes. In this regard, the present work is a natural extension of our previous studies on a repeated sequence (Sm1–7) of S. mansoni, which served for developing highly sensitive PCR-based detection of S. mansoni cercariae and infected snails. Although detection of infected snails was not attempted in the present study, DNA from normal snails is not amplified by DraI-based primers (results not shown). Thus, the DraI-based PCR assay should also enable, by virtue of its very high detection sensitivity, very early detection of prepatent infection in snails. As was previously demonstrated with S. mansoni.

The DraI repeated sequence, described in the present work, is similar to the Sm1–7 repeated sequence of S. mansoni by several features. Both have a single Sau3A restriction site, and the repeat units are 121 bp long (Figure 1), highly abundant, and tandemly arranged—at least partially—in large arrays (Figure 2). The possible structural or functional significance of these common features is unknown. Also unknown are the size variability of these repeat arrays, their full sequence variability, and their chromosomal localization. All these aspects warrant a special attention in schistosome genome studies, considering the high abundance of these repeated sequences. The abundance of the DraI repeated sequence, as determined by comparative dot blot hybridization, is ~ 15% of the S. haematobium genome. This is closely similar to the abundance of the analogous repeat Sm1–7 repeat of S. mansoni. The high detection sensitivity resulting from the very high abundance of the corresponding repeated sequences in the genomes of both S. mansoni and of S. hae-


FIGURE 6. Preliminary evaluation of detection specificity by the DraI repeat. The DNA from various schistosome species and from nonschistosome trematodes was probed by a DraI probe.
S. haematobium (Figure 4) enabled identification of individual cercariae of S. haematobium (Figure 5) as well as of S. mansoni. In spite of the similar features of the DraI and the SmI–I repeats, these sequences are profoundly different by base composition, exhibiting only $\approx 36\%$ of homology (data not shown). Indeed, the DraI sequence used as a probe in a dot blot hybridization study did not hybridize with total DNA of S. mansoni (or with DNA from T. ocellata and Echinostoma sp.) (Figure 6). However, cross-hybridization signals were demonstrated with DNA from S. magrebowiei, S. bovis, S. mattheei, S. intercalatum, and S. curassoni (Figure 6). Therefore, the use of the PCR assay developed in the present study for detection of S. haematobium is limited to areas where the other schistosome species tested are not present (assuming that other trematodes in these areas will not be amplified).

We expect that analysis of repeated sequences of the various cross-hybridizing schistosomes may discover species-specific repeated sequences that will enable development of PCR assays or oligonucleotide probes for a clear-cut differentiation of S. haematobium from the other species studied. Indeed, we found in preliminary studies that the S. mattheei genome contains repeated sequences similar (but not identical) to the DraI repeat, but other repeated sequences of S. mattheei do not cross-hybridize with the DraI repeat (unpublished results). It also remains to be seen whether putative species-specific variations in the DraI repeat will enable species-specific identification of schistosomes with terminal-spined ova, as was previously accomplished by means of species-specific oligonucleotide probes that were based on differences in the SmI–I repeat family for differential identification of Brugia species. The question also emerges regarding whether analysis of repeated sequences may help determine evolutionary relatedness between schistosome species and strains, as has been done by other molecular approaches. Further analyses of DraI and other repeated sequences from a variety of schistosomes with terminal-spined ova are therefore required for both diagnostic and taxonomic purposes.

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