

Alterations in cytochrome-*c* oxidase expression between praziquantel-resistant and susceptible strains of *Schistosoma mansoni*

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SUMMARY

The genetic differences between praziquantel-resistant (R) and susceptible (S) strains of *Schistosoma mansoni* (Fallon & Doenhoff, 1994) were explored using RAPD and by cloning differentially expressed mRNAs by subtractive PCR. No differences between the 2 strains were detectable by RAPD using 41 different primers indicating that no major genomic rearrangements were present. Subtractive PCR generated a number of fragments, 1 of which was shown to correspond to an over-expressed mRNA in the R strain and to encode a fragment of the subunit 1 of cytochrome-*c* oxidase (SCOX1). In the absence of a complete sequence for this gene, we used EST sequences to compile a consensus sequence for the 904 bp at the 3' end that enabled us to choose primers for semi-quantitative RT-PCR. This technique showed that SCOX1 was indeed over-expressed about 5 to 10-fold in the R strain whereas the genes encoding the 28 kDa glutathione *S*-transferase, glutathione peroxidase, NADH dehydrogenase subunit 5 and the ATP-binding cassette family protein SMDR2 were not. In contrast, cytochrome-*c* oxidase enzyme activity was 4-fold lower in the R strain than in the S strain.

Key words: *Schistosoma mansoni*, praziquantel, resistance, EST, cytochrome-*c* oxidase.

INTRODUCTION

Concern that the current reliance on 1 main drug, praziquantel (pzq), for the treatment and control of schistosomiasis may lead to the development of resistant parasite strains has received some support from recent reports of such resistance in the field (Stelma *et al.* 1995; Ismail *et al.* 1996) and the laboratory (Fallon & Doenhoff, 1994; Ismail *et al.* 1994). The first report (Stelma *et al.* 1995) of the relative inefficacy of pzq treatment came from the epidemic focus in Richard Toll (Senegal), and has been supported by the isolation of pzq-tolerant parasites from multiply-treated patients in Egypt (Ismail *et al.* 1996). Furthermore, laboratory tests of a Senegal isolate (Fallon *et al.* 1995, 1997) confirm its relative tolerance to pzq *in vivo*, even when maintained by passage in mice in the absence of drug pressure.

Initial attempts to generate pzq-resistant strains in the laboratory (Dias & Olivier, 1986; Ye, Yu & Xu, 1990) were unsuccessful but, by subjecting successive murine infections, while pre-patent to in-

creasing doses of pzq and using the eggs produced by surviving parasites for life-cycle passage, a resistant strain was selected (Fallon & Doenhoff, 1994). The parasites were not resistant to oxamniquine, showing that the resistance mechanisms toward these drugs are different. It has been suggested (Brown, 1994; Fallon *et al.* 1996) that pzq treatment of pre-patent infections may lead to the selection of late-maturing parasites since immature schistosomes are relatively insensitive to the drug (Sabah *et al.* 1986). However, when a new *S. mansoni* isolate from Kenya was compared with that from Senegal they were both found to mature at approximately the same rate, but the former was more susceptible to pzq (Fallon *et al.* 1997), thus suggesting that slower maturation is not the only reason for insusceptibility to this drug in the latter. We decided to explore genetic differences between the laboratory strain selected for pzq resistance and the parent, susceptible strain in order to determine whether the resistant worms exhibited gross DNA rearrangements, or more subtle changes in the expression levels of certain genes.

MATERIALS AND METHODS

Parasite strains and maintenance

Pzq-resistant (R) and susceptible (S) strains of *S. mansoni* obtained as previously described (Fallon & Doenhoff, 1994) were maintained in *Biomphalaria*

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glabrata snails and outbred TO strain mice. The R strain was maintained under drug pressure by treatment of mice with 300 mg/kg of pzq as an oral gavage in an aqueous suspension with 2.5% Cremophor EL (Sigma, Dorset, UK) on days 28, 35 and 37 after infection with 200 cercariae as previously described (Smithers & Terry 1965). However, the worms used in this study were perfused from untreated mice. The Puerto-Rican strain of *S. mansoni* maintained in Lille in *B. glabrata* snails and golden hamsters (*Mesocricetus auratus*) was also used in some experiments (specified in the text). Adult worms were obtained by hepato-portal perfusion (Smithers & Terry, 1965) and cercariae were released from infected snails and harvested on ice.

Preparation of nucleic acids

Total RNA was recovered by the guanidine thiocyanate-caesium chloride method (Chirgwin *et al.* 1978; Grauz *et al.* 1983) and poly A⁺RNA was purified on oligo-dT cellulose (Aviv & Leder, 1972). Genomic DNA was isolated using classical procedures after proteinase K digestion, phenol extraction and ethanol precipitation (Gros-Bellard *et al.* 1973).

Random Amplification of Polymorphic DNA (RAPD)

RAPD was performed essentially as previously described (Barral *et al.* 1993). Briefly, 20 ng of DNA from each strain was amplified in the presence of 1 of a series of single oligonucleotides (10 mers, series GEN1-70, GEN2-70 and GEN4-70, Genosys, Cambridge, UK, and oligonucleotides B5, B6, B10, B17, C2, C19, A2, A7, A8, A9 and A10 from Barral *et al.* 1993). Forty-one oligonucleotides in all were tested. PCR was carried out using 2.5 U of Taq polymerase (Promega, Charbonnières, France) in a final volume of 50 μ l using the manufacturer's reaction buffer with 3 mM MgCl₂, 200 μ M dNTPs and 0.2 μ M oligonucleotide primer. Forty cycles of amplification (94 °C, 1 min; 37 °C, 1 min; 72 °C, 2 min) preceded by a 5 min denaturation step at 95 °C and followed by a 10 min elongation step at 72 °C were carried out. PCR products were analysed on 1.4% agarose gels in Tris-borate buffer, pH 8.3.

Cloning differentially expressed mRNAs by subtractive PCR

Double-stranded cDNA was synthesized from 1 μ g of poly A⁺RNA from adult worms of the R and S strains. A subtractive Polymerase Chain Reaction (PCR) strategy was used to amplify cDNA fragments corresponding to mRNA species over-expressed in the R strain and was carried out using the PCR-Select kit (Clontech, Palo Alto, CA, USA) exactly

according to the manufacturer's instructions. In this method the strategy of hybridizing a 'tester' population of cDNA to excess of a 'driver' population is similar to that described by Lisitsyn *et al.* (1993), but with important differences. In our analysis we decided to identify fragments over-expressed in the R strain and this was therefore defined as the 'tester' as opposed to the S strain used as the 'driver'. Both tester and driver cDNAs were digested with *Rsa*I to generate shorter, blunt-ended molecules. The tester cDNA was then divided into 2 portions and each was ligated to a different cDNA adaptor. The adaptors are non-phosphorylated and only 1 strand attaches to the 5' ends of the cDNA. Two successive hybridization steps were then carried out. In the first a 20-fold excess of driver cDNA was added to each sample of tester, the samples were heat denatured and allowed to reanneal for 8 h at 68 °C. The 2 samples were then mixed, a further excess of heat-denatured driver cDNA added and the mixture reannealed overnight at 68 °C. In this way only cDNAs over-expressed in the tester population can form hybrids with different adaptors at each end. Two nested PCR amplifications using adaptor primers were then carried out, based on the suppression PCR principle (Siebert *et al.* 1995). Only annealed cDNAs with different adaptors at each end are amplified exponentially. The PCR products appeared on an ethidium bromide-stained agarose gel as a smear with several stronger bands (not shown). Rapid amplification of 3'-cDNA ends (3'RACE) was carried out using the Marathon kit (Clontech). Amplified DNA fragments were cloned into the pCR II T-vector (Invitrogen, Leek, The Netherlands) and sequenced in 1 direction (universal primer) using an automated sequencer (A.L.F., Pharmacia, Saclay, France). Sequences were analysed and sequence identity searchers carried out using the Lasergene programmes (DNASStar, Madison, WI, USA). Supplementary searches were carried out using the BLAST facility at the National Center for Biotechnology Information web site (<http://www.ncbi.nih.nlm.gov>).

Northern blotting

Ten μ g of total RNA from Puerto-Rican strain adult worms were resolved on a 1% formaldehyde-agarose gel (Lehrach *et al.* 1977) and blotted to a positively charged nylon membrane (Hybond N⁺, Amersham, UK). Northern dot blots were carried out by spotting different quantities of total RNA onto a positively charged nylon membrane using a dot blot manifold (Pharmacia) attached to a vacuum pump. In each case, the membrane was dried and RNA immobilized by exposure to 312 nm UV light for 5 min. Double-stranded DNA probes were labelled with [³²P] α dCTP (3000 Ci/mmol, Amersham, UK) by random priming (Megaprime kit, Amersham).

Table 1. Oligonucleotide primers and probes used for semi-quantitative RT-PCR

Gene	Primer	Sequence 5' → 3'	Gene accession number	Reference
<i>SCOX1</i>	5'	AGATCCCATAGTATGGTGATT	See Fig. 2 legend	This work
	3'	ACTACCCATAACTTAGAAGCA		
<i>Sm28GST</i>	Probe	AGCATTATCACTATTCTT	S71584	Pierce <i>et al.</i> (1994)
	5'	GTCCCAACAGCCAGCTTTCTGT		
<i>GPx</i>	3'	TGGCCAAAATCAAACCAACTATT	M86510	Williams <i>et al.</i> (1991)
	Probe	CCTCGTTTTACCCATC		
<i>SMDR2</i>	5'	TCACTTCTTCCTCCAAAAG	L26287	Bosch <i>et al.</i> (1994)
	3'	TGTCGCTTGCAAGGAGGCGCA		
<i>NADHDe5</i>	Probe	AAGATATTCTCCTACAACCTGCC	R95646	Unpublished
	5'	AGTCCGAGTTACCATAGCATC		
	3'	GCTCTTGGTTCGAAGGACTGCA		
	Probe	CATACAATCCGACTATTATCAC		
	5'	AGTCCGAGTTACCATAGCATC		
	3'	AGCTACTAAGAGGGCTCTATTTC		
	Probe	GAGCACAAGCCATACTAAATC		

Hybridization was carried out overnight at 65 °C in 5 × SSPE buffer containing 5 × Denhardt's solution, 0.5 % SDS and 10 µg/ml ultrasonicated herring sperm DNA (Sambrook, Fritsch & Maniatis, 1989). Blots were washed twice for 10 min at room temperature in 2 × SSPE, 0.1 % SDS and once each at 65 °C for 15 min in 1 × SSPE, 0.1 % SDS and for 10 min in 0.1 × SSPE, 0.1 % SDS. Membranes were air-dried and exposed to Kodak X-Omat AR film.

Semi-quantitative Reverse Transcriptase PCR (RT-PCR)

Semi-quantitative RT-PCR was carried out as described by Dallman & Porter (1991). Single-stranded cDNA was synthesized from 1 µg of total RNA using the Superscript cDNA synthesis kit (Gibco-BRL, Paisley, Scotland). Synthesis was checked by the incorporation of [³²P]αdCTP (1 µCi) as a tracer and the size distribution of the products also verified by subjecting an aliquot (2 µl) to agarose gel electrophoresis and autoradiography. Equal amounts of the product from the R and S strains (usually 4 µl of the reaction mixture) were amplified using *Taq* polymerase (2.5 IU) in a 100 µl reaction mixture containing the manufacturer's reaction buffer, 1 mM MgCl₂, 200 µM dNTPs, and 20 pmol of each primer (Table 1). Thirty-five cycles of amplification (94 °C 1 min, 60 °C 1 min, 72 °C 1 min) were preceded by a 5 min denaturation (95 °C) and followed by a 10 min elongation step (72 °C). In parallel, PCR reactions containing 4 µl of a blank RT reaction were also carried out. The essence of the approach toward quantification in the technique we employed is to sample product from the PCR at multiple points throughout the amplification process, ensuring analysis of product before a plateau is reached. We therefore removed 15 µl samples every 5 cycles at the end of the elongation phase, starting

from 15 cycles. PCR products were analysed both on agarose gels and dot blots. In order to verify the size of PCR products and determine that there were no extraneous bands, 5 µl of each aliquot were separated on a 1 % agarose gel in Tris-acetate buffer, pH 8.3. The gel was blotted onto a positively charged nylon membrane. After UV cross-linking, the blots were treated as described for the Northern blot, except that hybridization and high stringency washing were carried out at the melting temperature of the specific oligonucleotide probe used. The rest of the PCR reaction was used for dot blotting. Aliquots (0.1, 1 and 5 µl) of the reactions sampled at 5 cycle intervals were spotted onto a positively charged nylon membrane as for Northern dot blotting and hybridized to the corresponding specific probe as above.

Cytochrome-c oxidase enzyme assay

The cytochrome-c oxidase activity in parasite extracts was assayed as described by Capaldi, Marusich & Taanman (1995). Briefly, a 10 mM solution of horse heart cytochrome-c (Sigma) in reaction buffer (50 mM potassium phosphate buffer, pH 6.8) was reduced by 10 mM sodium dithionite for 5 min in the dark on ice. The reductant was separated from ferrocytochrome-c by passage over a Sephadex G-25M column (PD10, Pharmacia) equilibrated in the same buffer. The first part of the dark red band was collected, the ratio A₅₅₀/A₅₆₅ ratio checked to be > 6 and the concentration calculated using an extinction coefficient at 550 nm of 19.6 mM/cm. Aliquots of ferrocytochrome-c were stored at -80 °C. Adult worms or cercariae were homogenized in reaction buffer containing 1.5 % dodecyl-maltoside (Sigma) by ultrasonication (twice for 15 sec on ice, Braun Labsonic) and centrifuged for 30 min at 20000 g at 4 °C (Eppendorf 5417R centrifuge). Protein concentrations were measured using

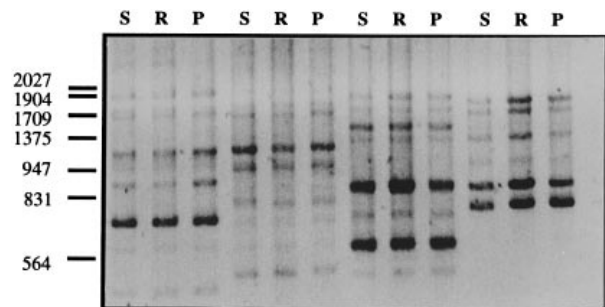


Fig. 1. RAPD analysis of DNA from Sensitive (S), Resistant (R) and Puerto-Rican (P) strains. DNA (20 ng) was amplified using 4 different oligonucleotides (from left to right C2, C19, A2 and A7—from Barral *et al.* (1993); see Materials and Methods section) and analysed on a 1.4% agarose gel.

the BCA protein assay (Pierce, Rockford, IL, USA). The cytochrome-*c* oxidase assay was performed in reaction buffer containing 0.1% dodecylmaltoside at room temperature. The sample (10 μ l) was added and pre-incubated for 1 min and the reaction started by the addition of ferrocytochrome-*c* to a concentration of 50 μ M. The initial rate of oxidation was followed by the decrease in absorbance at 550 nm and results expressed as nanomoles of ferrocytochrome-*c* oxidized/min/mg of protein.

RESULTS

Analysis of genetic differences between resistant (R) and susceptible (S) strains using RAPD

In order to detect changes in genetic organization that may have resulted from a selection of pzq-resistant parasites from the initial gene pool, we chose to carry out RAPD analysis with random primers. In all, 30 commercially available primers (see Materials and Methods section) and 11 primers shown to give efficient amplification of schistosome DNA (Barral *et al.* 1993) were tested under identical amplification conditions with genomic DNA isolated from the R and S strains, as well as from the Puerto-Rican strain maintained in Lille. Fig. 1 shows a typical result of amplification of 3 DNA sources with 4 different primers. No significant differences were detected with any of the primers used. Sometimes unique bands did appear for one or other of the strains, but these always appeared with the other DNAs when the PCR was repeated with a larger quantity of material. We were therefore unable to detect any major genetic rearrangements in the R strain.

Detection of differences in levels of expression of mRNAs using subtractive PCR

Total and poly A⁺RNA was obtained from the R and S strains and subtractive PCR performed using a

commercially available kit (see Materials and Methods section). The method generates short cDNA fragments that (in principle) derive from mRNA species over-represented in the 'test' population (in our case the R strain mRNA). Visualization of the products on an agarose gel (not shown) revealed a population of fragments with a size range of 100–500 bp. The products were subcloned into a T-vector and sequenced. Ten subclones containing different inserts were subsequently analysed on a Northern dot blot to check whether or not they appeared in fact to be over-represented in the R strain RNA. Of the 10 sequences analysed, 4 possessed significant sequence identity to cloned schistosome genes, the other 6 showing no such identity, either to schistosome genes, or to sequences present in Genbank release 102, dbEST release 102 or, in the case of derived peptide sequences to Swissprot/NBRF/Genbank translation (releases 35, 52 and 102 respectively). The 4 identified sequences corresponded to fragments of elongation factor α (SMEST0058), the 18S ribosomal RNA gene (SS18SRIBR), α tubulin (M80214) and cytochrome-*c* oxidase subunit 1 (Expressed Sequence Tags (EST) R95650 and T14506). None of these molecules would necessarily be thought a likely candidate for altered expression in the R strain, but Northern dot-blotting indicated that the mRNA detected by the cytochrome-*c* oxidase subunit 1 fragment was indeed increased by about 3 to 5-fold (not shown). In order to analyse further the significance of this finding, and in view of the limited quantity of biological material available, we used semi-quantitative RT-PCR to compare the expression levels of this mRNA and several other genes selected as controls in the R and S strains.

Analysis of the expression levels of SCOX1 and control mRNAs in the R and S strains

The fragment of the cytochrome-*c* oxidase subunit 1 gene obtained by subtractive PCR (clone SMRS39) corresponds to the 3' end of the coding sequence according to a comparison of the open reading frame with that of the *Fasciola hepatica* gene sequence (Garey & Wolstenholme, 1989). The schistosome gene (termed *SCOX1* by Skelly, Stein & Shoemaker, 1993) has not yet been fully sequenced, but PCR products corresponding to a part of the sequence (Skelly *et al.* 1993; Bowles, Blair & McManus, 1995) and several ESTs corresponding to the 3' end of the gene have been characterized. A consensus sequence was derived from all these elements (Fig. 2) that covered 904 bp and which represents about half the coding sequence, again by comparison to the *F. hepatica* gene. Translation of the consensus sequence using the invertebrate mitochondrial genetic code confirmed both its extensive identity to the predicted *F. hepatica* protein, and the

A

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TTTCAACATTTATTTTGGTTTTTTGGTCATCCAGAGGTTTATGTTTTGATCCTTCCGGGT 60
TTTGGTATAGTTAGGCATATCTGTATGAGTCTAAGGAATAAAGATTTCGTCGTTTGGTTAT 120
TATGGATTGATTTGCGCTATGGCTTCCATAGTATGCTTAGGTAGAGTAGTATGGGGTCAT 180
CATATGTTTATGGTTGGCTTTGATTCGTTAACTGGAGTGTTTTTTAGTTCCTATTACTATG 240
ATAATAGGTGTTCCCTACTGGTATTAAGGTGTTTTTCGTGACTTTATATGTTGAATAGTTGT 300
GGTATGCGAGTTTTTAGATCCCATAGTATGGTGATTAGTTGGTTTTATATTTTTTATTACG 360
GTTGGTGGTGTACAGGGGTGGCTTTATCTGCATCTGCTTTAGATATATTTATTTTCATGAT 420
ACTTGGTTTTGTTGTTGCTCATTTTCATTATGTTCTTCTTTAGGTTCTTATAGAAGAATA 480
GTGATAATGCTTAATAGGTGAAGGCCGTTTGTAGTTGGTTATAGTTTAAATAAGTATCTG 540
TTACAAGGTCACCTGGTTATTATCTATGGTTGGTTTTAATTTATGTTTTTTTCCCTATGCAT 600
TATTTAGGTATACATGGTCTTCCCTCGACGTGTAAGTTGTTATGATCAAGAATATTATTGG 660
GTTAATGTTATAAGAAGAATTGGTGGAGTATTATCGGTTGTTAGAGGTTTTGGATTATTT 720
TTTATTTTATGAGAGTCTTTGGTTGTTGGTAATCGTATAATAGGATTATGAGGTTTCAGGT 780
GGGTGCATTACGAAAGTGATGACTGTTCCCTGTTCCAGTCATGTTGATTATTTAAATGCT 840
TCTAAGTTATGGGTAGTTAAGTAATGCTAAATAATCGTAGTAGATAGGTGGCTATACAAG 900
GGTpolyA 904

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B

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FQHLFWFFGHPEVYVLLPGFGMVSHICMSLSNKDSSFGYYGLICAMASMVCLGSVWVG SCOX1
*****
FQHLFWFFGHPEVYVLLPGFGVISHICVTLTNNDSLFGYYGLILAMAAMVCLGSVWVA FHCX1
HHMFMVGFDSLTVGVFFSSITMMMGVPTGIKVF SWLYMLNS-CGMRVLDPMVWVWLVGFMF SCOX1
*****
HHMFMVGLDVHTAVVFFSSVTMVIPIPTGIKVF SWLMLLGGSSVRMWDPVVWVWIMGFIV FHCX1
LFTVGGVTVGVALSASALDMLFHDTWVVAHFHYVLSLGSYSSVMMLMWWSPFVVGYSL SCOX1
***
LFTIGGVTVGIMLSASLLDTHLHDTWVVAHFHYVLSLGSYSSVVISFIWWWPVVTVGYSL FHCX1
NKYLLQGHWLLSMVGFNLCFFPMHYLGMHGLPRRVSCYDQEYYWVNVMSIGGVLSVVS SCOX1
*
NLFMLQGHWVSMVGFNMCFPPMHYLGMAGLPRRVCVYDPDFYWLSVSVSSLGALVLAGS FHCX1
GFGLFFILWESLVVGNRMMLGWGSGGCITKVTVPVPSHVDYLNASKLWVVK SCOX1
*
AFLFVFFILWESFVVGNCVVSWSGSSSLVNLNITLPGPQHNSYMNGVGRWVF FHCX1

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Fig. 2. Consensus sequence of the 3' half of *SCOX1*. (A) A consensus sequence was obtained by assembling the sequences from ESTs designated by dbEST accession numbers R95567, R95597, R95581, T24148, R95531, R95650, R95598, H85666, H63019, N25392, T14506 and R95652, as well as PCR fragments designated by Genbank accession numbers L10109 and U22162, using the Seqman II programme (DNASTar). Sequences used to construct oligonucleotide primers and a probe are boxed and the 5' to 3' direction indicated by an arrow. A putative polyadenylation signal sequence is underlined. The polyadenylation site was located by 3' RACE from primer 1. (B) Alignment of the predicted amino acid translation of the *SCOX1* consensus DNA sequence (invertebrate mitochondrial genetic code; 267 amino acids) with the *Fasciola hepatica* COX1 peptide sequence from amino acids 224 to 509 (Garey & Wolstenholme, 1989). Identical residues are indicated by an asterisk.

mitochondrial origin of the sequence (Skelly *et al.* 1993). This sequence was used as the basis for the selection of specific primers (Fig. 2). Amplification of genomic DNA derived from Puerto-Rican strain adult worms using primers 1 and 2 produced a single amplification product of the expected size (541 bp)

and this was subcloned into a T-vector and sequenced, confirming exactly the consensus sequence derived from the ESTs. Moreover, a Northern blot of total RNA from the Puerto-Rican strain was probed with the PCR product and a single product of 1.9 kb (not shown) was detected which is slightly

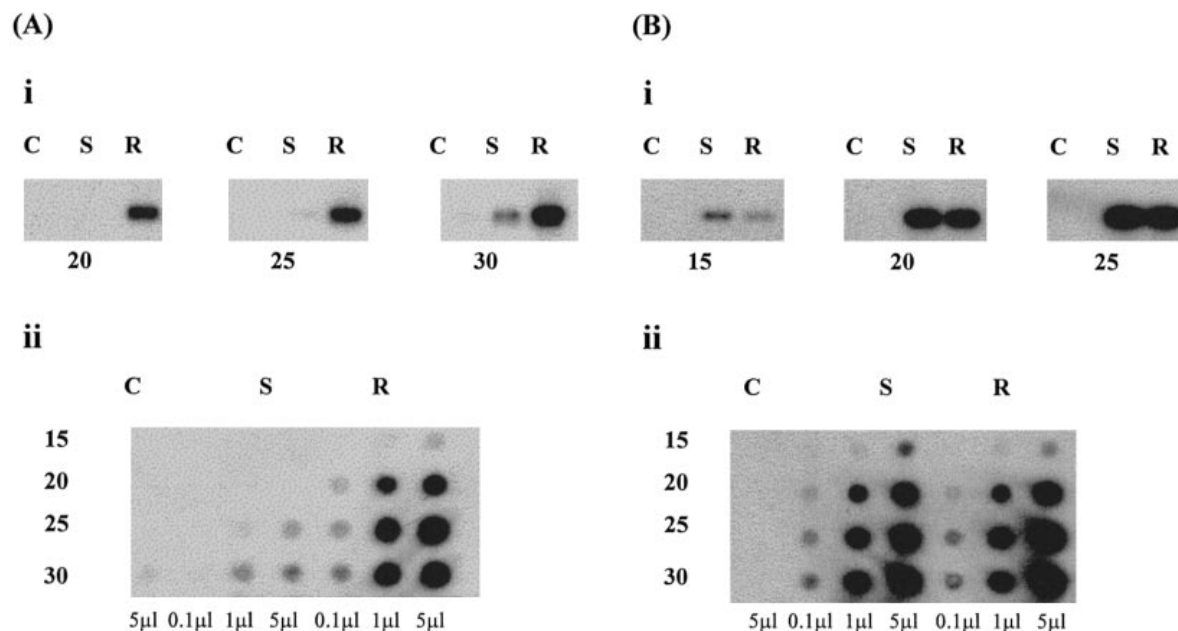


Fig. 3. Semi-quantitative RT-PCR of *SCOX1* (A) and *Sm28GST* (B) expression in the R and S strains. (i) Southern blot of 5 μ l of PCR products after 20, 25 and 30 cycles of amplification separated on a 1.4% agarose gel developed using the corresponding specific oligonucleotide probe (Table 1). C designates the control PCR reaction carried out using 4 μ l of a blank reverse transcription. (ii) Dot blot of 5 μ l of control (C) and 0.1, 1 and 5 μ l (from left to right) of RT-PCR products after 15, 20, 25 and 30 cycles from the R and S strains.

smaller than that found by Skelly *et al.* (1993) and we failed to detect the 3.4 kb transcript found in female worms by these authors.

The primers were used to assess expression levels of *SCOX1* in the R and S strains using semi-quantitative RT-PCR (see Materials and Methods Section). Single-stranded cDNA (sscDNA) was synthesized from equal amounts of R or S strain total RNA and the use of [32 P] α -dCTP as an internal tracer allowed us to assess the efficacy of the cDNA synthesis and to calibrate the amounts used for RT-PCR. In order to control this technique we also amplified 2 *S. mansoni* nuclear-encoded gene products, glutathione peroxidase (*GPx*) and the 28 kDa glutathione *S*-transferase (*Sm28GST*). Control amplifications were always done on the same amounts and the same batch of sscDNA as for the test gene product. Fig. 3 shows the results for the amplification of *SCOX1* mRNA compared to that of *Sm28GST*. The comparison of the PCR products after different numbers of cycles, separated on an agarose gel, Southern blotted and hybridized to the specific internal primer, shows that the *SCOX1* product appears much earlier (after only 15 amplification cycles) when R strain RNA is used than with the S strain (after 25 cycles). The dot blot prepared with different volumes of PCR product, again after 15, 20, 25 or 30 cycles, allows a comparison of the intensity of the signals obtained with the specific probe and indicates an approximate 10-fold increase in abundance of the product in the R strain over the S strain. In contrast, the results of the control amplification of

Sm28GST mRNA carried out at the same time show (Fig. 3) that the PCR product for this gene appears after 15 amplification cycles in both the R and S strains. In addition, the signal intensities of the dot blot indicate that the product is marginally more abundant in the S strain. The results shown are typical of 3 separate experiments and the amplification of *GPx* mRNA as a further control showed no difference in abundance between the 2 strains (not shown). These results clearly indicate that there is a significant increase in the abundance of the *SCOX1* mRNA in the R strain, confirming the results obtained by Northern dot blot.

SMDR2 and *NADH dehydrogenase subunit 5* gene expression is not altered in the R strain

In order to control further the results of RT-PCR for the *SCOX1* product, 2 other genes were selected for amplification. The first was *SMDR2* (Bosch *et al.* 1994) a schistosome homologue of the ATP-binding cassette proteins responsible for the multi-drug resistance phenotype in mammalian tumours and a possible candidate for implication in the mechanism of resistance to pzq. However, RT-PCR performed under the same conditions as for *SCOX1*, using *Sm28GST* as the control, showed that there was no difference in expression between the 2 strains (not shown). The second gene selected was the mitochondrial genome encoded NADH dehydrogenase subunit 5 (*NADHDe5*) and result of RT-PCR carried out using *Sm28GST* as the control are shown

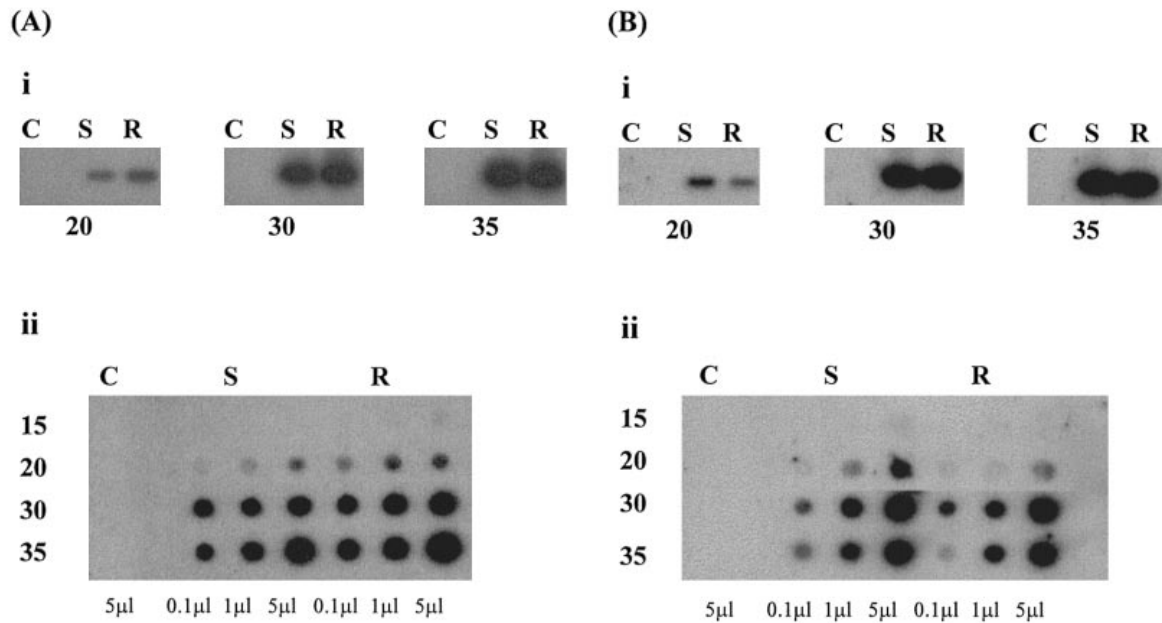


Fig. 4. Semi-quantitative RT-PCR of *NADHDe5* (A) and *Sm28GST* (B) expression in the R and S strains. (i) Southern blot of 5 μ l of PCR products after 20, 30 and 35 cycles of amplification separated on a 1.4% agarose gel developed using the corresponding specific oligonucleotide probe (Table 1). C designates the control PCR reaction carried out using 4 μ l of a blank reverse transcription. (ii) Dot blot of 5 μ l of control (C) and 0.1, 1 and 5 μ l (from left to right) of RT-PCR products after 15, 20, 30 and 35 cycles from the R and S strains.

Table 2. Specific activity of cytochrome oxidase in the R and S strains

Stage	Strain	Cytochrome-c oxidase activity (nmol/min/mg protein) \pm s.d.
Adult worm	R	27 \pm 11
	S	98 \pm 11
Cercariae	R	23 \pm 2
	S	62 \pm 13

in Fig. 4. Expression of *NADHDe5* mRNA was very similar in the R and S strains, a PCR product being detectable after 20 amplification cycles in both, although the intensity of the band, as well as the dot blot indicates a slightly higher expression in the R strain. This contrasts with the results for *Sm28GST* which again showed a slightly higher expression in the S strain. These differences are minimal, however, and there is thus no evidence for a general up-regulation of genes encoded by the mitochondrial genome in the R strain.

Cytochrome-c oxidase enzyme activity in the R and S strains

In order to determine whether the increase in *SCOX1* mRNA was reflected by a corresponding increase in overall cytochrome-c oxidase enzyme activity, we attempted to assay the activity in extracts of adult worms and cercariae from the R and S

strains. We used horse heart ferrocytochrome-c as the substrate and the results are shown in Table 2. Each value represents the mean and standard deviation for 6 separate assays in the case of adult worms and 3 in the case of cercariae. The activity values obtained for the S strain are similar to those of whole human fibroblast extracts (Capaldi *et al.* 1995). However, horse heart cytochrome-c is known not to be a good substrate for human cytochrome-c oxidase, and the situation may be similar for the schistosome enzyme. The fact that activity was detected in cercarial extracts shows that the assay detected the schistosome enzyme (and not a contaminating mouse enzyme in the adult worm extracts). It is clear that the overall cytochrome-c oxidase activity is markedly reduced in the R strain, both in adults and cercariae and this contrasts with the increase in mRNA levels for subunit 1. The adult worms used for this study came from mice that had not been treated with pzq and thus the reduction in activity measured was not due to a direct effect of the drug. Moreover, the R strain retains its resistance to pzq in the absence of drug pressure since the progeny of untreated worms are resistant (not shown).

DISCUSSION

Investigations into mechanisms of pzq resistance are hampered by the absence of a clear understanding of the mode of action of this drug on schistosomes (see Redman *et al.* 1996 for review). Indeed it has been suggested that the existence of pzq resistant schisto-

somes could help elucidate the molecular mechanism of action of the drug (Coles, 1989). Pzq is known to cause a Ca^{2+} influx across the tegument, resulting in an immediate muscle contraction (Mehlhorn *et al.* 1981). Blebbing of the tegument leading to antigen exposure and the subsequent implication of the host immune system is instrumental in causing worm death *in vivo* (Fallon *et al.* 1996). Although pzq does kill worms *in vitro* in the absence of specific antibody or immune effector cells, the importance of the immune response may be to potentiate the effects of the drug, killing parasites not lethally damaged by the drug itself (Cioli, Pica-Mattocchia & Archer, 1995).

In this study we attempted to determine the feasibility of developing genetic markers of pzq resistance. We chose to use the laboratory resistant strain (Fallon & Doenhoff, 1994) to do this, particularly due to the availability of the parental susceptible strain which, although neither strain is clonal, would reduce variation due to strain differences. This lack of variation was evident in the attempt to detect DNA rearrangements using RAPD. This technique has been shown to detect differences between different geographical isolates of *S. mansoni* (Dias Neto *et al.* 1993) and the absence of major polymorphisms between the R and S strains argues for the absence of any substantial genomic rearrangements leading to the resistance phenotype.

We therefore pursued the search for markers by looking for changes in the levels of gene expression. The PCR-based subtraction technique we employed allows the cloning of fragments of cDNA derived from mRNA species overexpressed in the 'tester' population compared to a 'driver' population. We chose to try to characterize mRNAs over-expressed in the R strain compared to the S strain, although it is evident that the basis of resistance might be the relative under-expression, or the absence of expression of a particular gene, as is the case in schistosome resistance to oxamniquine (Cioli *et al.* 1993). In fact, among the clones characterized, only 1 seemed to have a significantly higher level of expression in the R strain.

The gene that was over-expressed encoded subunit 1 of cytochrome-*c* oxidase. The sequence of the initial fragment obtained was identical to previously identified schistosome ESTs, and possessed extensive identity to the fully sequenced *F. hepatica* gene. Since the schistosome gene had not been fully sequenced, we compiled a consensus sequence from fragments in the database which include 12 ESTs and 2 fragments obtained using PCR (Skelly *et al.* 1993; Bowles *et al.* 1995). The overall sequence, which we confirmed by sequencing PCR products, encodes about half the complete molecule and is clearly derived from the mitochondrial genome as is shown by the (invertebrate mitochondrial) genetic code necessary for its translation. We have used the

name *SCOX1* for the gene as previously suggested (Skelly *et al.* 1993). Semi-quantitative RT-PCR confirmed that the level of mRNA encoding *SCOX1* is 5 to 10-fold increased in the R strain compared to the S strain. The technique we employed easily detects differences in expression of this magnitude, although a precise quantification is not possible (Dallman & Porter, 1991).

The expression of several other genes was also quantified for the 2 strains, both to control the results for *SCOX1* and to determine whether they could also be markers of resistance. Glutathione transferases are well known to bind reversibly to potential toxic agents and the *Schistosoma japonicum* 26 kDa GST has been shown to bind to pzq (McTigue, Williams & Tainer, 1995), as does Sm28GST (F. Trottein, personal communication). Increased GST activity has been associated with drug resistance in *Haemonchus contortus* (Kawalek, Rew & Heavner, 1984), and the production of Sm28GST *in vivo* was shown to increase after treatment of infected mice with xenobiotics (Van de Waa *et al.* 1993). However, more recently, Serra, Zemzoumi & Dissous (1997) have shown that the *Sm28GST* promoter is not inducible by xenobiotics or oxidants and our results show no difference in expression of the gene between the R and S strains.

SMDR2 (Bosch *et al.* 1994) is a schistosome homologue of ATP-binding cassette family proteins which are integral membrane proteins involved in metabolite disposal and which were found to be responsible for the multi-drug resistance phenotype in mammalian tumours. It is thus a candidate to be involved in the removal of pzq from the parasite. However, no difference in gene expression was noted between the R and S strains. Bosch *et al.* (1994) also showed that a schistosome strain resistant to oxamniquine and hycanthon showed no increase in expression of this gene.

SCOX1 and *NADHDe5* are both encoded by the mitochondrial genome and the presence of the latter gene indicates that schistosome mitochondrial genome organization is probably closer to that of mammals than to that of yeast, from which this gene is absent (Grivell, 1989). In mammals, the transcription of the closed circular mtDNA is polycistronic and occurs from two major promoters. Both *COX1* and *NADHDe5* transcription starts from the heavy strand promoter (Shadel & Clayton, 1993) and it would be expected that increased expression of one gene would be mirrored by increased expression of all the others initiated from the same promoter, as is the case with mouse epididymal mitochondrial gene expression under the influence of androgens (Cornwall, Orgebin-Crist & Hann, 1992). However, in our experiments this was clearly not the case and an explanation may reside in the post-transcriptional control of *SCOX1* gene expression. Both mRNA stability and trans-acting

proteins that mediate translation of the mitochondrial COX subunits may be involved (Gilham, Boynton & Hauser, 1994) and lead to differences in the levels of individual mRNAs.

Cytochrome-*c* oxidase is located in the inner mitochondrial membrane and is the terminal enzyme in the respiratory chain, catalysing the 4-electron reduction of molecular oxygen and coupling this reaction to the generation of a proton gradient across the membrane. The mammalian enzyme is composed of 13 subunits (Capaldi *et al.* 1995), the 3 largest of which (including COX1) are encoded by the mitochondrial genome. The remaining subunits are encoded in the nucleus, synthesized in the cytosol and imported into the mitochondrion. An increase in the level of *SCOX1* mRNA would not therefore necessarily reflect a concomitant increase in cytochrome-*c* oxidase enzyme activity. However, our finding that this activity was about 4-fold lower in the R strain was surprising. The reduced levels of enzyme activity measured were not due to a direct effect of the drug on the parasite, since the adult worms were obtained from mice not previously treated with pzq and this is therefore an inherited character in the R strain. The levels of specific activity measured, although broadly similar to those of human fibroblasts measured using the same substrate, should be treated with caution since horse heart ferrocycytochrome-*c* is known to be a poor substrate for the human enzyme (Capaldi *et al.* 1995) and may well not be optimal for schistosome cytochrome-*c* oxidase. Bueding & Charms (1952) used rat cytochrome-*c* as a substrate and found that the measured enzyme activity could account for only about 10% of the observed respiration rate, but here again, substrate specificity may have been a factor. We would argue that the relative activity levels between the 2 strains, measured at the same time and with the same batch of substrate, should reflect real differences, even if the absolute activity levels are under-estimates. The view that adult schistosomes are essentially homolactate fermenters, deriving no energy from oxidative metabolism, put forward by Bueding (1950) and reiterated by Bueding & Fisher (1982), has been contradicted by other studies, notably by Coles (1972) and Van Oordt *et al.* (1985) showing that up to a third of available ATP is generated by aerobic processes. Moreover, significant amounts of *SCOX1* mRNA were found in adult schistosomes (Skelly *et al.* 1993) as well as mRNAs encoding enzymes involved in oxidative glucose metabolism, supporting the view that the worms produce energy through aerobic metabolism.

The question of how a reduced level of cytochrome-*c* oxidase activity relates to the resistance phenotype of the worms remains to be answered. However, we have demonstrated a major difference at the molecular level between the laboratory R and S strains and further work will be aimed at

determining whether this is equally the case for resistant field isolates and at pursuing the search for other differentially expressed genes.

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