MAJOR ARTICLE

Novel Mechanism of Drug Resistance in Kala Azar Field Isolates

Neeloo Singh,[1] Ramsing Th. Singh,[1] and Shyam Sundar[2]

[1]Division of Biochemistry, Central Drug Research Institute, Lucknow, and [2]Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Clinical resistance to pentavalent antimonial drugs in the form of sodium antimony gluconate (SAG) has become a major problem in the treatment of kala azar (visceral leishmaniasis) in India. The mechanism of resistance is unclear in these clinical isolates, although work has been conducted with *Leishmania* species mutants selected in vitro by stepwise increase of drug concentration, using antimony-related metal arsenic and, more recently, SAG. In the present study, we investigated the molecular aspect of drug resistance in clinically confirmed SAG-resistant field isolates. Our results show that the mechanisms of resistance postulated for laboratory mutants of *Leishmania* species are not operating in field isolates of *Leishmania donovani*. Instead, we identified a novel gene amplified in these drug-resistant parasites whose locus is on chromosome 9. The significant finding was that this isolated fragment confers antimony resistance to wild-type *Leishmania* species after transfection. We speculate that protein phosphorylation may play a role in signal transduction pathway in the parasite after exposure to drug-conferring resistance.

Leishmania species are protozoan parasites that are found worldwide. Approximately 1.5–2.0 million persons annually suffer from this disease, and the death toll is estimated to be 57,000 persons/year (World Health Organization; Centers for Disease Control and Prevention [1]). Along with Brazil, Sudan, and Bangladesh, India contributes to 90% of the global burden of visceral leishmaniasis (VL). Conventional therapy for VL consists of pentavalent antimony (sodium antimony stibogluconate; sodium antimony gluconate [SAG]; Albert David), despite its requirement of long courses of parental administration and increasing levels of resistance [2]. Alternative drugs also are not free from harmful side effects. With the advent of miltefosine, which provides cure rates of 98%, phase 4 operational trials in several districts of Bihar (India) will initiate the use of miltefosine as the first-line drug for a program of kala azar elimination [1].

Drug resistance has emerged as a major problem in treating VL. In India, there has been an epidemic of primary resistance. In Muzaffarpur, the epicenter of the outbreak in 2000, >60% of previously untreated patients were unresponsive to antimonial drugs [2]. To date, work on drug resistance in *Leishmania* species has been on laboratory mutants, mostly *L. tarentolae*, in which resistance was introduced in vitro by use of the antimony-related metal arsenic [3]. Thus, it is usually possible to get an idea of the gene involved in resistance. By this method, several mechanisms that contribute toward conferring resistance in the parasite have been described elsewhere [4–6].

The drug in clinical use against *Leishmania* species infection consists of pentavalent antimony, the active form of the metal being the trivalent form [7]. Reduction of the metal may take place in either the parasites or the macrophages, or in both. Some studies have suggested that reduction takes place in the host cells [8], whereas others have suggested that it occurs in the parasite [9]; thus, a loss in reductase activity could lead to drug resistance. In vitro resistance to pentavalent

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Reprints or correspondence: Dr. Neeloo Singh, Div. of Biochemistry, Central Drug Research Institute, Chattar Manzil Palace, PO Box 173, Lucknow 226001, India (neeloo888@yahoo.com).

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antimonial drugs in *Leishmania* species depends on transformation of the pentavalent compound to a trivalent compound, formation of a conjugate with an elevated level of intracellular thiol, and extrusion by elevated levels of ATP-binding cassette (ABC) transporters [10,11]. What is the situation in field isolates of *L. donovani* that remains to be determined? By understanding the resistance mechanisms to SAG in *L. donovani,* we can find strategies to avoid or overcome the problem of drug resistance.

Determining the drug sensitivity of *L. donovani* isolates obtained from patients is always open to the criticism that removal of microorganisms from the host and adaptation to culture medium immediately selects for a subpopulation of pathogens best suited for growth in that medium. Therefore, the drug sensitivity of parasites should be tested immediately after isolation from the patient. However, a major drawback to this approach is that promastigotes are not sensitive to pentavalent antimonial drugs [7]. The amastigote-macrophage assay offers the only model that is able to correlate clinical response to the sensitivity of the isolate; thus, we have previously demonstrated [12] the clinical resistance in *L. donovani* field isolates. Using these isolates, we tested the resistance mechanisms prevailing in field strains isolated from patients who were unresponsive to pentavalent antimony treatment.

PATIENTS, MATERIALS, AND METHODS

Patients. The patients were selected from the Kala Azar Medical Research Center of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, and also from its affiliated hospital at Muzaffarpur, Bihar. The criteria for VL diagnosis was the presence of Leishman Donovan (LD) bodies in splenic aspirations performed, which was graded according to standard criteria, as described elsewhere [13]. After diagnosis, the patients were administered intravenously a course of SAG (20 mg/kg of body weight once daily for 30 days). Response to treatment was evaluated by repeating splenic aspiration at day 30 of treatment. The designation of responsive patients was based on the absence of fever, clinical improvement with reduction in spleen size, and the absence of parasites in the splenic aspirate. Patients who showed presence of parasites were considered to be unresponsive. These patients were subsequently treated successfully with amphotericin B.

Culture conditions. The splenic aspirates of the responsive and unresponsive patients with VL were inoculated into NNN medium and passaged every seventh day into a tube containing fresh NNN medium. Positive cultures then were progressively adapted to RPMI 1640 medium supplemented with 10% fetal calf serum for mass culture. *Leishmania amazonensis* (LV78) promastigotes of virulent clone 12-1 were cultured at 25°C in medium 199, 10% heat-inactivated fetal bovine serum (HIFBS), and 25 mmol/L HEPES (pH 7.4). Macrophages of the J774A1 line were cultured at 35°C in HEPES-buffered RPMI 1640 medium (pH 7.4) with 20% HIFBS.

Nucleic acid isolation and blotting. Nuclear DNA was isolated from parasites by use of DNAZOL (Boehringer). Southern blotting was done by use of the DIG DNA labeling and detection kit (Boehringer), as described elsewhere [14]. Cloned probes for genes amplified in drug-resistant *Leishmania* species were obtained as gifts.

Isolation of the amplified DNA sequence, cloning, and sequencing. DNA (4.0 μg) was digested to completion with restriction endonuclease *Hin*dIII. After electrophoresis, the band with amplified DNA was cut, and DNA was eluted by use of USB Bioclean MP kit (catalog no. 74200). This *Hin*dIII-digested amplified *Leishmania* DNA then was ligated to *Hin*dIII-digested dephosphorylated PUC plasmid vector. After transformation, plasmids with *Leishmania* DNA inserts were isolated by use of the High Pure plasmid isolation kit (Boehringer 1754777). DNA sequencing was performed on an AB 373 DNA automated sequencer (Bangalore Genei).

Polymerase chain reaction (PCR). Primers designed from the amplified DNA sequence were 5′-TGGGCGATCTTTGCTTGCGAATAC-3′ (forward) and 5′-CGCTTCGTTTCGCTCTGCC-3′ (reverse). By use of these primers, ~900 bp of amplified DNA sequence from clinical isolates was amplified in a 100-μL reaction volume performed by use of the GeneAmp PCR System 2400 (Perkin Elmer).

Plasmid constructs. The *Leishmania*-specific vector P6.5MCS [15] was used for cloning the amplicon in 2 different orientations with reference to that of *nagt.* The plasmids were expanded in *Escherichia coli* and isolated by use of the Qiaquick kit (Qiagen).

Transfection of* Leishmania *species. Promastigotes of *L. amazonensis* grown to late log or stationary phase were transfected with ~20 μg of the following plasmids: vector alone (P6.5) and P6.5 with the amplicon in the correct orientation (P6.5+C) or in the reverse orientation (P6.5+R). Cells were transfected by electroporation with a Gene Pulser (Bio-Rad) under conditions described elsewhere [15]. Transfectants were allowed to recover in drug-free medium for 24 h and then were selected for resistance to tunicamycin at 5, 10, and 20 μg/mL.

In vitro assay for drug sensitivity. Promastigotes of the transfectants were subjected to axenic amastigote formation by growing them for 4–5 days at 35°C in Grace's medium plus 20% HIFBS (pH 5.3). The transfectants were used to infect J774 macrophages at a host-to-parasite ratio of 1:5. Specifically, 4×10^6 macrophages were mixed with 2×10^7 axenic amastigotes in 4 mL of medium in a 25-cm^2 flask to begin the infection at 35°C. On the third day, the level of infection was quantitated,

and 0.5 mL of the cells (10^6) were plated in duplicate in 24-well tissue culture–treated polystyrene plates. Different concentrations of drug (SAG) obtained from the same commercial source, as used for patient treatment, were added to final concentrations of 0, 12.5, 25, and 50 μg/mL. The cultures were incubated for an additional 24 h. The level of infection was quantitated by microscopic counting of macrophages and parasites. The total number of intracellular parasites per well was estimated for each drug concentration used in duplicate as follows: total number of macrophages $\times$ percentage of infected cells $\times$ average number of intracellular parasites/macrophage. The percentage of infected cells and the average number of intracellular parasites per cell were tallied microscopically by continuous examination of consecutive areas in a given drug concentration until the infected cells examined reached 50. In a separate experiment, these infected macrophages were inoculated into M199 with 20% HIFBS with different drug concentrations, as used in an earlier experiment, with or without tunicamycin to enable the amastigotes to differentiate into promastigotes. Microscopic counting of parasites was done when the culture of promastigotes with no drug reached turbidity.

RESULTS

DNA amplification. Digestion of equivalent amount of DNAs from the clinical isolates (table 1) revealed DNA amplification in the unresponsive isolates R-5 and R-6 on ethidium bromide–stained gel by the appearance of a >1.0-kb fragment that was completely absent in the responsive/sensitive isolate (figure 1). Therefore, gene amplification was determined in our SbV-resistant clinical isolates, although interpreting data from a group consisting of only 1 member, as in this case with 1 sensitive isolate, is debatable. Using cloned probes for genes amplified in drug-resistant laboratory mutants of *Leishmania* (which were obtained as gifts), we carried out Southern blotting to determine whether this amplified gene hybridized to *mdr*

Table 1. Clinical isolates used in this study.

Clinical isolate	LD bodies (at the time of splenic aspirate collection)
R-1	3+
R-2	2+
R-3	2+
R-4	2+
R-5	2+
R-6	2+
R-7	3+
R-8	2+
Responsive	Cured

NOTE. LD, Leishman Donovan; R-1 to R-8, unresponsive clinical isolates.

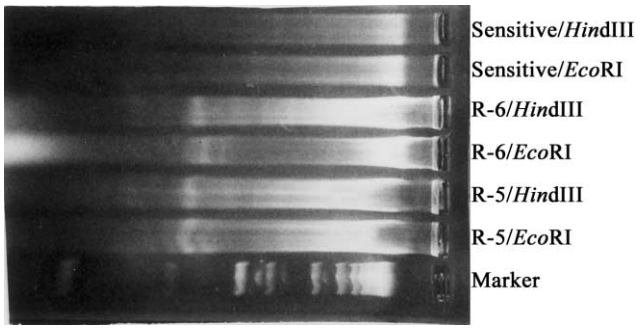

Figure 1. Ethidium bromide–stained agarose gel showing gene amplification in sodium antimony gluconate (SAG)–unresponsive clinical isolates R5 and R6. The DNAs of drug unresponsive and responsive isolates were digested with *Hin*dIII or *Eco*RI and migrated in an agarose gel. Size was determined with the λ DNA-digested *Hin*dIII marker, as shown.

(multidrug-resistance gene), *gsh1* (γ-glutamylcysteine synthetase), and *pgpA* (an ABC transporter gene) probes. The amplified gene identified in drug-unresponsive clinical isolates did not hybridize to the *mdr* or *pgpA* probe, which indicates that this gene was not linked to the ABC transporters involved in conferring resistance in laboratory mutants. A faint signal with the *gsh1* probe was obtained (data not shown).

The amplified band from *Hin*dIII-digested nuclear DNA of unresponsive isolate R-5 was cloned in *Hin*dIII site of the PUC 18 plasmid vector. Recombinants were selected on the basis of ampicillin resistance and were confirmed by restriction enzyme digestion. Nucleic acid sequencing of this cloned DNA sequence was carried out and found to be 1.254 kb (figure 2). A notable feature of this amplified sequence was the polypyrimidine–rich region 226–251 and 622–636. The finding of >1 pyrimidine-rich tract indicates that there is >1 open-reading frame (ORF) in the sequenced fragment. This was also seen by use of the ORF finder at http://www.ncbi.nlm.nih.gov (table 2). However, on the basis of the codon usage table for *Leishmania* at http://www.kazusa.or.jp/codon/, as well as the prediction tools used, an ORF of 305 nt from 760 to 1065 was identified in this amplified sequence of 1.254 kb. The other ORFs were relatively random, because ORFs occur frequently in the sequenced fragment, but whether they are real or not is why we used gene prediction software. Gene prediction was done by use of the FramePlot program, as implemented at http://www.nih.go.jp/jun/cgi-bin/frameplot.pl. The gene prediction tools did not identify any other ORFs. Overlapping ORFs were not considered to be meaningful, because only the ORF of 305 nt from 760 to 1065 showed the confirmed coding sequence (CDS)—that is, from the ATG start codon to the stop codon. This ORF also has the better Kozak consensus for initiation of translation. The prediction of the correct initiation codon (ATG) is done by choosing an initiation codon that is preceded by a stretch of pyrimidines (as shown in figure 2) or part of a Kozak se-

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CCGCGGCGGAAGAAGTTGGTGGTGTAAGTGGATGATGTGCCCCGCGCCGGGCACTTGGTTGTTGCTT

GGGGGCTGTACGATGCATTCCTGCGGACGATGCTGTGGGCGATCTTTGCTTGCGAATACTTGCGCCGT

GCGGTGCATCGCGGTGCCCGTGCGGGCGCCGTGCGTTGTGAGACAAAGCCGTCGCCGTACAAAACTT

CTATGCATTCCCTTTTTTTGCCGCCTCCCCTCCCCCTTCCCCTCCCTCCGCTCCTCCTTGTCCGGCACTT

CGTGGGATGCGCGGCAGAGCTGCGGGGGGGGGGGGGCGCTGCGCTTTAAAGAGGGAGACAACTGAA

ATGAGAGGGCAAGTGGGGCATCGGACAGGAAACGTTGCTCCCATGACCGGCTATCGACGGCTCCAG

GTGCTGCGTACTGCGTGGGCACCGAATACGAACGCCTTGTGGCGGCATCCATGACAAGTTCCCCTTTC

TGCTTGCGCCATACATTGGCAGAGGGGTTGAAACAGGGGGAGATCGTGGGGGCGACAGCGCTACCC

AAAGCATTCAGAGCGGGCTCCCGTAGGCGGAGTGCGTGTTCTACCGTTTGCTCCCTACACTTCCACTC

CTCCCCCCTCGCTGACCTTCTTTTTTTTTTGGTTTTCTGTCGATTCTCCATTTTGAGGGGTGCCCTCGTG

GGACATTTGGCCTCACCTCGTTCTTGGTGCTGCTCACTGGGGATGCCTTTGTAGGAGGGGAAGGACAC

GTATC***AG***CTGCCCAAATGTCAAATCGCTTTTTTCAAAAGTTCTACCTGCGGTGCGGTAACTGCACTGC

AATTCAGCGCAGTGCTCAGGGCTACAAACCCATTGCGAACCCCATTCTTTTCAAGTCAGACGAGCACT

GTCGCAACTACCATGACGAGCAGCGCCGTGCGGCGGGCTACGCAGGCATGATGGTGACTACTCGCTG

TGATAAGTGCAACCGCGTACACTCCAACTGGAAGGTGCTGGATGCTCAGGAGTTCCTCGATGTGAAG

CTGTCGTTGACGCCGGCAGAGCGAAACGAAGCGTTTGTGGGCCAGCTCTAAGTGAGGAAGAAGAGA

AGATGAAGGGTGGAGAGGCGATGAGGCCGTAGCTGCGCGTCTGCTTTATGGAGACGCTTCATCATGT

GTGCCCGCCGCCTCTTGATGGACTGGTGCTGTAGTATAGGAGCACGCAGAGAGAGGCAGATGTTTGC

GCTTCTTCTCTTTGGTTTACACACTTGCGAAGGAAAGCTT

1254

Figure 2. DNA sequence of amplified gene isolated from a drug-unresponsive field isolate. GenBank accession no. AF 273843. Polypyrimidine-rich regions are boxed (226–251, 622–636, and 1068–1083 [complement]). Splice acceptor site AG is underlined and in italic type. CDS 760–1065 is underlined.

quence. If this cannot be identified, the most upstream initiation codon was chosen. No specific promoters were required for gene expression in *Leishmania*. The only requirements were intergenic sequences that serve in RNA maturation by trans-splicing and polyadenylation. Therefore, the maturation of this gene was assessed by the stretch of pyrimidine tract and splice acceptor site AG that is present upstream of the ORF. The transcription starts from A of ATG with the start codon ATG in the direction of transcription, extends to the stop codon TAA, and could encode a protein of 101 aa with a molecular weight of 11.7 kDa.

Characteristics of the encoded protein. Computer evaluation of the protein predicted an isoelectric point of 8.86. The entire sequence consisted of mostly predicted hydrophobic amino acids (A, I, L, F, W, and V). Sequence analysis did not convincingly support the presence of putative transmembrane domains that are essential features of transport proteins. Transmembrane domains were predicted, as implemented at the TMHMM server http://www.cbs.dtu.dk/services/TMHMM-1.0/. The sequence was not a signal peptide. Prediction of the subcellular location indicated that the sequence contains a mitochondrial targeting peptide. No obvious BLAST hits with any

Table 2. Open-reading frames (ORFs) within the amplified sequence from drug-unresponsive isolate of *Leishmania donovani*.

ORF	Range, bp	Length, bp
−1	1–657	657
+1	760–1065	306
+2	32–337	306
+2	455–730	276
−2	318–545	228
+1	97–321	225
−2	1–197	197
−3	1–181	181
+3	279–458	180
−3	203–382	180
+1	1144–1253	111
−3	1142–1252	111

protein implicated in drug resistance emerged with this sequence. The only similarity (84%) was with the genomic survey sequence (GSS), (AQ848517), *L. major* FV1 random genomic library. This corroborated the fact that we had not obtain any signals on hybridization of this sequence with probes *(pgpA* and *mdr)* implicated in drug resistance in laboratory mutants. Therefore, this gene appeared to be conferring resistance by a novel mechanism. One structural feature that may suggest how this sequence confers resistance was the prediction of phosphorylation sites Ser 0, Thr 3, and Tyr 1 (http://www.cbs.dtu.dk/services).

PCR. From the 1.254-kb DNA sequence, primers designed to amplify ~900 bp were used in PCR with nuclear DNA isolated from the unresponsive and responsive isolates (table 1) and served as the template. Results (figure 3) showed strong amplification in the unresponsive isolates. All unresponsive isolates showed amplification, although they are not all shown in the figure. The responsive isolate showed a very faint PCR product that could be attributed to the fact that amplification increases the copy number of the sequence in drug-unresponsive isolates, although the same is present in a wild-type or responsive isolate at low frequency.

Recovery of resistant phenotype. To establish a correlation between this DNA amplification and resistance, transfection experiments were performed. This amplicon was transfected within a leishmanial transfection vector (figure 4) into sensitive parasites. The choice of using *L. amazonensis* instead of *L. donovani* in the transfection experiments was based on its ease of manipulation. The level of antimony resistance of transfectants was determined, which was done in 2 ways: (1) by comparison of transfectants obtained for SAG resistance by their growth as intracellular amastigotes in J774 macrophages directly and (2) by the differentiation of macrophage-derived amastigotes into promastigotes. The results of these experiments showed that the amplicon appears to render *L. amazonensis* 2–3 times more resistant to SAG (figures 5 and 6). Results from 6 independent experiments with these transfectants showed the same trend. Two sets of representative results are presented.

Identification of a gene locus. An *L. major* cosmid library in transfectable vector cLHYG was screened with this gene sequence probe to isolate the cLHYG shuttle cosmid that contains the locus. Sixteen cosmids that were positive on colony hybridization with this amplicon as probe were obtained, although the signals were quite faint, which suggest that this locus is quite diverged in *L. major.* However, there were 6 clones that were all part of the same contig on chromosome 9. Therefore, the preliminary results indicate that the locus is on chromosome 9. Unfortunately, one of the chromosome-sequencing projects is only beginning in Brazil.

DISCUSSION

Although some of the mechanisms of metal resistance (induced in vitro in the laboratory by use of arsenite, an oxyanion related to antimony) [16] are beginning to be understood in *Leishmania* species, it remains to be determined whether similar mechanisms operate in field isolates that are exposed to antimony. *Leishmania* mutants selected for resistance to antimonial drugs in the laboratory show amplification of the ABC transporter gene *pgpA* [5, 16]. To determine whether this also was the case with clinical isolates, we ran digested DNAs of unresponsive and responsive isolates in parallel in an agarose gel that was then probed with cloned genes of ABC transporters. Amplification of these genes was not observed in the field isolates. Instead, a novel amplicon was characterized in drug-

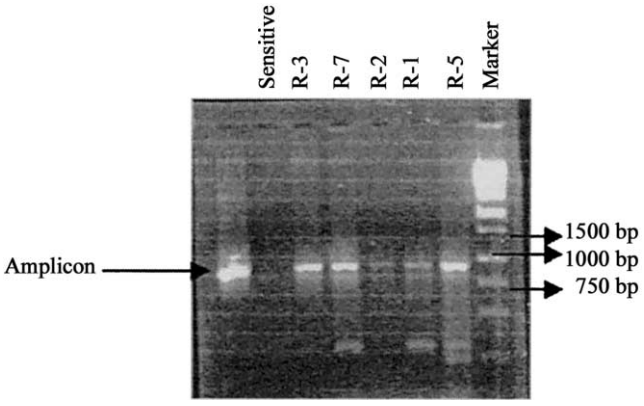

Figure 3. Polymerase chain reaction analysis showing the presence of amplified gene in resistant isolates. *Lane 1,* Amplification from positive control template (PUC plasmid with gene sequence); *lane 2,* nuclear DNA from responsive isolate showing very faint amplification of gene sequence; *lanes 3–7,* nuclear DNA from unresponsive isolates R-1 to R-5, showing strong amplification; *lane 8,* marker.

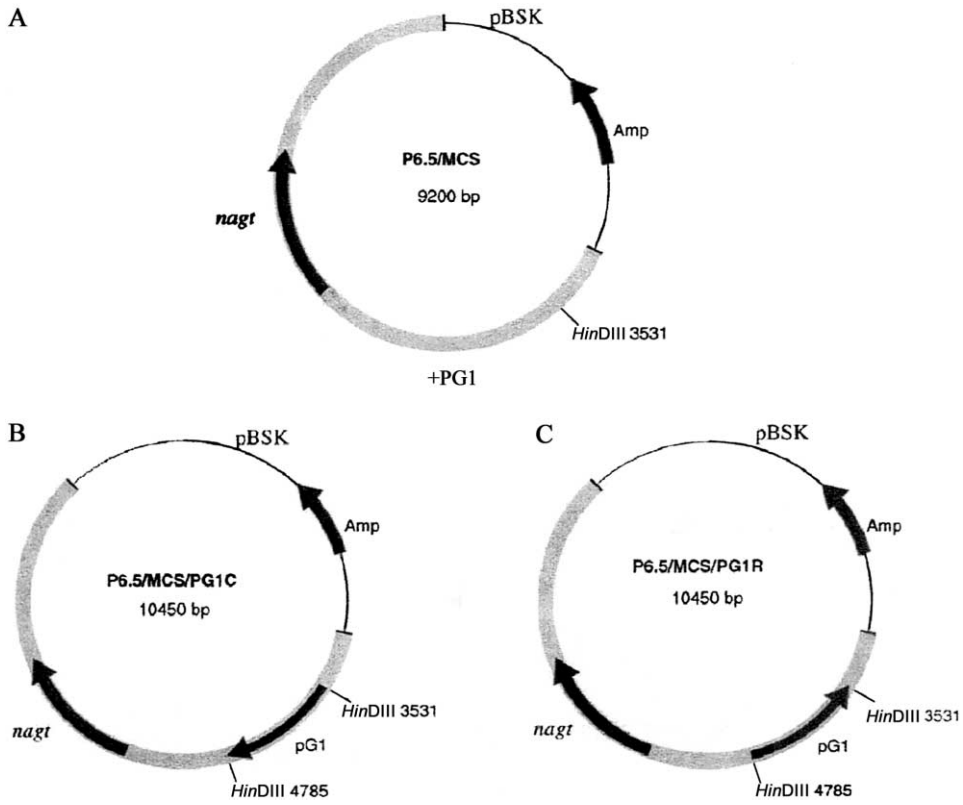

Figure 4. Plasmid constructs. *Leishmania*-specific vector P6.5MCS was used for cloning the amplicon in 2 different orientations with reference to that of *nagt* (amplicon in correct orientation with respect to *nagt* of *Leishmania* expression vector [C] and amplicon in reverse orientation with respect to *nagt* of *Leishmania* expression vector P6.5 [P6.5 R]).

unresponsive isolates after digestion with enzymes recognizing AT-rich sequences (thus rarely cutting *Leishmania* DNA with its 65% guanine-cytosine content), such as *Hin*dIII and *Eco*RI.

This finding was consistent with a previous study [16] that determined that *pgpA* is not involved in resistance in SbIII resistant laboratory mutants, although putative P glycoprotein expression in arsenite-resistant *L. donovani* was observed [17]. Also, it was observed [18] that an *L. tropica* line selected for resistance to sodium stibogluconate had a DNA amplification that was not related to P glycoproteins. Similarly, molecular analysis of total DNA isolated from patients unresponsive to antimony therapy in South America [19] suggested that *pgpA* amplification is not involved in antimony resistance to field isolates. Drug resistance in human cases of *Trypanosoma cruzi* also has shown that it is not associated with amplification or expression of P glycoprotein genes [20]. However, in both types of laboratory mutants SbIII and arsenite, trypanothione (TSH) levels are increased and are essential for resistance. It has been shown that, in arsenite-resistant mutants, the increase in TSH is mediated by *gsh1* amplification [21] and ornithine decarboxylase (ODC) overexpression [22]. Thus, our finding of weak hybridization of amplified sequence with the *gsh1* probe in field isolates is consistent with the fact that, even in SbIII-resistant laboratory mutants, the increase in TSH levels is mediated, in part, by *gsh1* amplification and by a novel mechanism that remains to be determined [23]. Therefore, because this amplified DNA in the genome of drug-unresponsive isolates appeared to not be related with the known drug-resistant genes, as documented in laboratory mutants, more information was obtained by DNA sequencing of this amplified DNA. Although the amplified DNA sequence seemed to be ORF rich, only 3, however, appeared to be in +1 frame: ORF-1 (760–1065), ORF-2 (97–321), and ORF-3 (1144–1253). The other ORFs overlapped in reading frames. ORF-2 was contained within the reading frame, as was ORF-3, but these 2 ORFs did not contain a predicted initiator methionine (MET). Only ORF-1 contained a predicted initiator MET that was located at the usual distance from the trans-splicing site in *Leishmania* genes [24], which suggests that this could be a potential coding sequence. Also, to determine which of these ORFs was most likely to code for the gene product, they were compared by codon usage, using a table of *Leishmania* codon use [25]. Codon usage in ORF-1 correlated very well with *Leishmania*-coding sequences, in which the third position was usually a G or C. With other

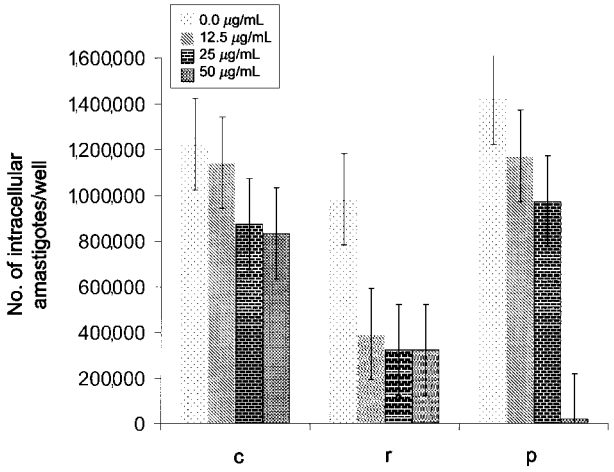

Figure 5. Sodium antimony gluconate (SAG) resistance of transfectants as amastigotes in J774 macrophages ED50 of P6.5+C (>50 μg/mL; when gene in correct orientation with respect to *nagt* of *Leishmania* expression vector P6.5), ED50 of P6.5+R (10 μg/mL; when gene in reverse orientation with respect to *nagt* of *Leishmania* expression vector P6.5); and ED50 of P6.5 (28 μg/mL; when *Leishmania* expression vector P6.5 alone was used for transfection)

ORFs, codon usage appeared to be random and not consistent with other *Leishmania*-coding sequences. Furthermore, the GC content of ORF-1 was 63.4%, which is consistent with *Leishmania*-coding sequences [26], whereas, for ORF-2 and ORF-3, the GC content was only 54.25%. The potential splice acceptor signal was preceded by a tract of pyrimidines previously implicated in trans-splicing [27]. On the basis of these criteria, this amplified DNA was predicted to contain 306 bp encoding a predicted 101-aa polypeptide.

The predicted amino acid sequence was not similar to any sequence related with drug resistance in the current databases, thus constituting a novel protein. This also was consistent with the finding that no hybridization signals in Southern blots of this amplicon with probes for drug-resistance genes were observed. Computer analysis did not identify any signal peptide. Efflux seems unlikely, because no clear transmembrane domains are present. The sequence contains a mitochondrial targeting peptide. One structural feature that may suggest how this sequence confers resistance was the prediction of phosphorylation sites Ser 0, Thr 3, and Tyr 1. It has been reported elsewhere [27] that P glycoproteins and homologous multidrug transporters contain a phosphorylated link sequence that is proposed to control drug efflux. Another study [28] also reported that phosphorylation is enhanced in resistant strains, compared with wild-type strains. Heat stress–induced modulation of protein phosphorylation in virulent promastigotes of *L. donovani* also has been examined [29]. The predicted phosphorylation sites in our sequence awaits experimental demonstration. It may be that protein phosphorylation is playing a role in signal transduction pathway in the parasite after exposure to drug and thus confer resistance.

PCR with primers designed from this amplified sequence was able to show clear-cut bands in drug-unresponsive cases, which, again, strongly indicates the association between this gene sequence and drug resistance.

The resistant phenotype was recovered in wild-type cells by transfection experiments. Because susceptibility to SbV is stage specific, promastigotes, are relatively resistant, whereas, in contrast, amastigotes are highly susceptible [30]; intracellular amastigotes were used to evaluate the direct effects of SbV on the transfectants. When these macrophage-derived amastigotes were allowed to differentiate into promastigotes in drug-containing medium, the resistant phenotype of the gene was maintained. In the present study, by use of drug-resistant field isolates, the in vivo resistance that leads to 2–3 times in vitro resistance is what would be expected, as opposed to the massive resistance generated by in vitro selection techniques.

In conclusion, we did not see any amplification of genes in the field isolates used in the present study that were reported to be present in laboratory mutants selected for arsenite resistance. However, we observed a DNA sequence that was amplified within several unresponsive isolates whose locus is on chromosome 9, and a transfection experiment established a link between drug resistance and this sequence. This isolated fragment, when amplified, conferred antimony resistance by a novel mechanism that remains to be confirmed. The stress-related stimuli (in this case, drug antimony) might be an activating

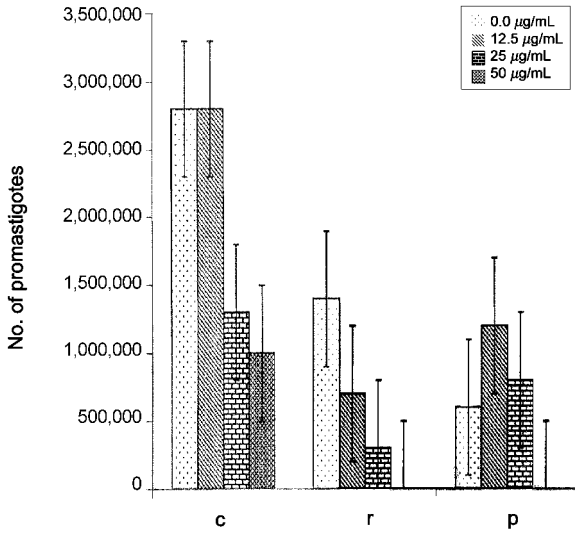

Figure 6. Sodium antimony gluconate (SAG) resistance of transfectants assessed by their differentiation from macrophage-derived amastigotes into promastigotes ED50 of P6.5+C (25 μg/mL; when gene in correct orientation with respect to *nagt* of *Leishmania* expression vector P6.5) and ED50 of P6.5+R (12.5 μg/mL; when gene in reverse orientation with respect to *nagt* of *Leishmania* expression vector P6.5).

protein kinase cascade that amplifies signals generated at the cell surface into complex biological responses, such as gene amplification associated with resistance.

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