Curr Genet (2000) 37: 234–241

ORIGINAL PAPER

Achim Schnaufer · Sandro Sbicego · Beat Blum

Antimycin A resistance in a mutant *Leishmania tarentolae* strain is correlated to a point mutation in the mitochondrial apocytochrome *b* gene

Received: 6 October / Accepted: 17 December 1999

Abstract In this paper we report the first case of antimycin A resistance in a protozoan parasite that is attributable to a mutation in the mitochondrial apocytochrome *b* (CYb) gene. We selected for, and isolated, a mutant *Leishmania tarentolae* strain that is resistant to antimycin A. This resistance was evident at the levels of the in vitro growth and enzymatic activity of the cytochrome *bc1* complex. Molecular characterisation of the mutant revealed a Ser35Ile mutation in the expected region of the CYb gene. In kinetoplastids, CYb and other structural genes of the mitochondrial genome are located on the maxicircle component of the mitochondrial DNA, which is present in 20–50 copies. Primer-extension analysis confirmed the presence of the mutation at the mRNA level. The phenotypic manifestation of the mutation implies that the CYb mRNA is edited and translated within the mitochondrion. Thus, this finding provides direct evidence that edited RNAs are translated in kinetoplastid mitochondria. Furthermore, a defined mutation conferring drug resistance to a mitochondrial gene product can be exploited for the development of mitochondrial transfection systems for trypanosomatids.

Key words *Leishmania* · Apocytochrome *b* · Drug resistance

Communicated by H. Bertrand

A. Schnaufer (✉)[1] · S. Sbicego[2] · B. Blum
Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012 Bern, Switzerland
Tel.: 1(206)284-8846488; Fax: 1(206)284-0313
e-mail: achim@sbri.org

Present addresses:
[1]Seattle Biomedical Research Institute, 4 Nickerson Street, Suite 200, Seattle, WA 98109, USA
[2]Department of Molecular, Cell and Developmental Biology, UCLA, 675 Charles Young Drive 6770, Los Angeles, CA 90095, USA

Introduction

The cytochrome bc_1 complex (cytochrome *c* reductase) of the mitochondrial respiratory chain transfers electrons from ubiquinol to cytochrome *c*. This process is thought to be coupled to the translocation of protons across the mitochondrial inner membrane, thus contributing to the electrochemical proton gradient that drives ATP synthesis. The current model for this mechanism is based on the so-called Q cycle (Mitchell 1976; Trumpower 1990). Cytochrome *b* is a highly hydrophobic subunit of the bc_1 complex. It is a transmembrane protein in which the two hemes, b_H (b_{562}) and b_L (b_{565}), form a transmembrane electron circuit between two ubiquinone (Q) redox sites responsible for proton uptake (Q_i) and proton release (Q_o). These functional predictions have contributed to models of the three-dimensional folding of the amino acid sequence of apocytochrome *b* (CYb) (Brasseur 1988; di Rago and Colson 1988) and are supported by the recent crystallisation of the several cytochrome bc_1 complexes (Xia et al. 1997; Zhang et al. 1998).

A number of inhibitors block the electron transfer from and to ubiquinol/ubiquinone, apparently by interacting with either of the oxidoreduction centres. One group, including myxothiazol and stigmatellin, is proposed to bind at, or near, the Q_o reaction centre; while a second group, including antimycin A, funiculosin, diuron and 2-*n*-heptyl-hydroxyquinoline-*N*-oxide (HQNO), is thought to bind near the Q_i site. These predictions stem from studies of numerous mutants that revealed a striking clustering of mutations conferring resistance to either of these inhibitors (Colson 1993; Gennis et al. 1993). The mutations affecting antimycin A sensitivity map to two distinct regions that are evolutionary conserved within the CYb sequence (Howell and Gilbert 1988; Brasseur et al. 1996; and Table 1). It was therefore proposed that these regions form a single functional structure in the folded protein (di Rago and Colson 1988). Other studies demonstrated that antimycin A

Table 1 Alignment of putative antimycin A binding pockets for several CYb genes and their resistance-conferring mutations. Amino acids were numbered according to the *L. tarentolae* (*L. t.*) sequence. Highly conserved residues are underlined. *S. c.*, *Saccharomyces cerevisiae; S. p.*, *Schizosaccharomyces pombe; K. l.*, *Kluyveromyces lactis; R. r.*, *Rhodospirillum rubrum*

Organism	Sequence of the putative antimycin binding pocket		Reference
	30 44	222 235	
L. t.	YGVGFSLGFFICMQI	CFCMWFYLRDMFLA	(de la Cruz et al. 1984)
S35I	I		This work
S. c.	WNMGSLLGLCLVIQI	PMHSYFIFKDLVTV	(Nobrega and Tzagaloff 1980)
N31K	K		(di Rago and Colson 1988)
G37V	V		(di Rago and Colson 1988)
K228I		I	(di Rago and Colson 1988)
M221E		E[a]	(Coppee et al. 1994)
M221Q		Q[a]	(Coppee et al. 1994)
S. p.	WNFGSLLACVLVIQI	PMNPYYTMKDLMTM	(Lang et al. 1985)
A37V	V		(Weber and Wolf 1988)
K. l.	WNLGSLLGLCLVIQI	PMHGYFIFKDLITV	(Brunner and Coria 1989)
N31K	K		(Coria et al. 1989)
K228M		M	(Coria et al. 1989)
K228I		I	(Coria et al. 1989)
L230SΔΔ		SΔΔ	(Coria et al. 1989)
Mouse	WNFGSLLGVCLMVQI	PFHPYYTIKDILGI	(Bibb et al. 1981)
G38V	V		(Howell and Gilbert 1988)
G232D		D	(Howell and Gilbert 1988)
R. r.	WNFGSLAGIAMIIMI	PFHPYYTVKDAFGL	(Majewski and Trebst 1990)
D243H		H	(Uhrig et al. 1994)
D243E		E	(Uhrig et al. 1994)

[a] Revertants from respiratory deficient M221K mutants

blocks oxidation-reduction at the Q_i site and causes changes in the optical properties of heme b_H (Dutton et al. 1972). Together, these findings led to the conclusion that the antimycin A binding pocket overlaps with the Q_i site and the suggestion that antimycin A competes with ubiquinone for binding to this site (di Rago and Colson 1988). This view is supported by recent data emerging from the crystal structure of the cytochrome bc_1 complex (Xia et al. 1997; Kim et al. 1998; Zhang et al. 1998).

CYb is probably the only subunit of the complex that is encoded by the mitochondrial DNA in trypanosomatids and other eukaryotes. The trypanosomatid and other protozoan CYb sequences exhibit only 25% identity to those of other eukaryotes (Ghelli et al. 1992). This divergence is likely to have consequences for the structure of the complex, as apparently reflected by unusual responses of protists to some of the bc_1 inhibitors. For instance, trypanosomatids are resistant to the Q_o reaction-centre inhibitors myxothiazol and stigmatellin but are highly sensitive to antimycin A (Ghelli et al. 1992). Nevertheless, no antimycin-resistant trypanosomatid strains have been described to-date.

We report here the isolation and characterisation of an antimycin A-resistant clone of the trypanosomatid *Leishmania tarentolae*. A guanosine to thymidine transversion in the CYb gene predicts a change of serine 35 to isoleucine. This position is located within the putative antimycin binding pocket, suggesting that this mutation is the basis of the resistance. We confirmed the assumed cause of resistance by performing cytochrome *c* reductase assays in vitro with isolated mitochondrial vesicles. This finding suggests a structural similarity of the Q_i site of trypanosomatids to that of higher eukaryotes despite the divergent primary sequence. The characteristics of this mutation also have implications for the significance of the RNA editing process that occurs in the mitochondria of *Leishmania*. The mutation is located downstream from editing events that create the AUG start codon for the CYb mRNA. Correct editing of the CYb mRNA is therefore required for the phenotypic manifestation of the mutation. Although postulated to occur, evidence for the actual translation of edited mRNAs has so far been controversial (Speijer et al. 1997; Stuart et al. 1997). Finally, this is the first report of a mutation in the mitochondrial genome of trypanosomatids that confers drug resistance and, hence, should contribute to the feasibility of mitochondrial transfection experiments in kinetoplastids.

Materials and methods

Cell cultivation and isolation of an antimycin A-resistant clone

L. tarentolae cells (UC strain) were cultivated in liquid BHI medium at 27 °C (Simpson and Braly 1970). An antimycin A-resistant *L. tarentolae* cell line was isolated following the procedure described by Fong et al. (1994), with the following modifications: BHI medium was used throughout the selection and antimycin A was used instead of paromomycin. In the first round of selection, cells were kept in medium containing 1, 3, 5, or 10 ng ml^{-1} of antimycin A. After 24 h, cells were spun down, re-suspended and incubated for 4–6 days in drug-free medium. The culture that had survived the highest concentration of antimycin A was chosen for the next round of selection, adjusting the range of drug concentrations whenever appropriate. After 6 months of selection, antimycin A sensitivity had dropped approximately 20-fold. The mutant *Ant-one* clone was obtained from the culture by limiting dilution in microtiter plates. For the selective growth of the mutant

strain on solid medium, we prepared agar-solidified BHI/M199 plates as described elsewhere (Sbicego et al. 1998).

Isolation of mitochondrial vesicles and cytochrome *c* reductase assay

After hypotonic cell lysis, mitochondrial vesicles were isolated on pre-formed Urografin (Schering) gradients from cells harvested at a cell density of 5–10 × 10^7 cells ml^{-1} as described (Braly et al. 1974). Vesicle pellets were re-suspended in STM buffer (0.25 M sucrose; 20 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$). Aliquots were lysed with 0.5% Triton X-100 and the protein concentration was determined with the *DC* Protein Assay (Bio-Rad). Prepared vesicles were directly used for the enzyme assay. Cytochrome *c* reductase activity was measured in a reaction containing 10 mM of succinate as substrate, 50 μM of cytochrome *c* as electron acceptor, 25 mM of sodium phosphate (pH 7.4), 1 mM of EDTA, 1 mM of KCN, mitochondrial vesicles corresponding to 50 μg of mitochondrial protein, and varying concentrations of antimycin A in a total volume of 0.5 ml. Reactions were monitored at 550 nm in a SpectraMax spectrophotometer (Molecular Devices) at room temperature.

Isolation, cloning, and sequencing of CYb DNA

Total cellular DNA was isolated as follows. Ten millilitres of a mid-log *L. tarentolae* culture, corresponding to 5 × 10^8 cells, were harvested by centrifugation at 4000 rpm in an SS-34 rotor, washed in 10 mM of Tris-HCl (pH 7.5) and resuspended in 5 ml of the same buffer, supplemented with 0.5% sodium lauroyl sarcosinate and 100 μg ml^{-1} of Proteinase K (Boehringer Mannheim). The digestion was incubated for 4 h at 50 °C and extracted with phenol/chloroform. Nucleic acids were precipitated with ethanol and dissolved in 100 μl of H_2O. In order to clone the CYb gene, 1 μl of the nucleic acid solution was used as a template in polymerase chain reactions with oligonucleotide pairs B-62/B-63 and B-70/B-119 as primers to yield two overlapping CYb fragments. The B-62/B-63 product encompasses the 5′ 220 nt of CYb DNA while the B-70/B-119 product contains the 3′ 989 nt of CYb DNA. The products of three independent reactions using the combination B-62/B-63 were gel-isolated with Gene Clean (Bio 101) and cloned separately with the TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. Seven clones were sequenced from both strands using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical) according to the manufacturer's instructions. The sequencing gels were analysed with the PhosphorImaging system (Molecular Dynamics). The products of the B-70/B-119 reactions were gel-isolated as described above and directly sequenced by Microsynth (Balgach, CH), using dye terminators.

Primer-extension analysis of CYb RNA

Primer-extension analysis was performed as described (Estevez et al. 1999) using 10 μg of kinetoplast RNA (kRNA), the oligonucleotide S3090 (see Fig. 5) and AMV reverse transcriptase.

Oligodeoxynucleotides

Oligodeoxynucleotides were purchased from Microsynth (Balgach, Switzerland). Nucleotide changes introduced for cloning purposes are underlined. Leikpmax and maxicircle entry in Genbank.

B-62 5′-TAATCGATGCTTTAATTTTTTGTGTATG (nt 5212–5239 in LEIKPMAX),
B-63 5′-GTAAAACATATATGTGTCGACGTATCAC (nt 5622–5594),
B-70 5′-GTAGGTTTTATCCTAGGTTTTTTTATTTGTATGC (nt 5459–5492),
B-119 5′-GGATCCCTTCCTATTCGCAA (nt 6561–6542).

Results

Isolation of an antimycin A-resistant strain of promastigote *L. tarentolae*

The induction and selection of antimycin resistance in *L. tarentolae* (UC strain) was performed in a stepwise fashion in liquid culture as described previously (Fong et al. 1994). A resistant clone, named *Ant-one*, was obtained after approximately 6 months of selection. Inhibitor titration curves show an IC_{50} of about 1 ng ml^{-1} for the wild-type and 10–20 ng ml^{-1} for the mutant strain (Fig. 1). IC_{50} values for cultivation in BHI/M199 medium, either in liquid culture or on plates, are approximately 50 times higher (data not shown). When plated on BHI/M199 plates containing 1 μg ml^{-1} of antimycin A, only colonies of the *Ant-one* cell line were visible after 9 days of incubation (Fig. 2). When incubated for extended periods in selective liquid medium or on selective plates (i.e., 1 week in liquid and more than 2 weeks on plates) growth of wild-type cells was observed at drug concentrations that are able to inhibit short-term growth completely (data not shown). When incubated in fresh antimycin A-containing medium, these "delayed-growth" cultures did not show any increase in drug tolerance, indicating that this phenomenon is due to decay of the drug and is not a consequence of genetic changes within the organism. Additionally, this observation points towards a reversible effect of antimycin A in *L. tarentolae*: the organism is not killed by the antibiotic but rather is inhibited in its growth as long as a critical antimycin A concentration is maintained. This is in accordance with recent findings that inhibition of the respiratory chain results in a reversible metabolic arrest

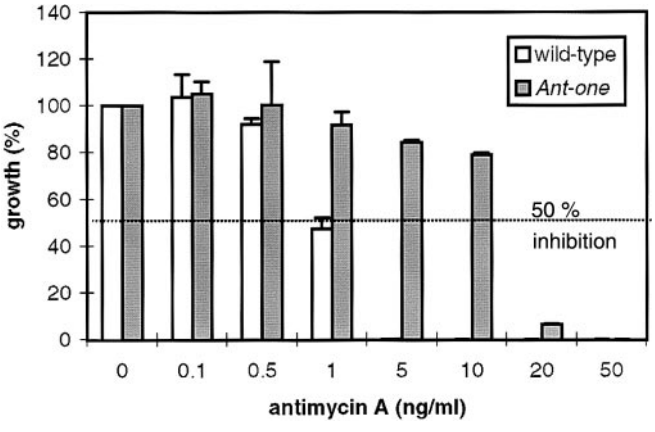

Fig. 1 Inhibitor titration experiments for wild-type *L. tarentolae* and the mutant *Ant-one* strain. The starting cell density in all samples was 2.4 × 10^6 cells ml^{-1}. Cells were incubated in BHI medium with the indicated concentrations of antimycin A at 27 °C and were counted after 66 h. Final cell densities for wild-type and *Ant-one* cells in the control cultures without antimycin A were 132.0 ± 5.7 × 10^6 ells ml^{-1} and 76.3 ± 3.9 × 10^6 ells ml^{-1}, respectively. Drug sensitivity was measured as the cell density at each antimycin A concentration compared to the one in the absence of antimycin A, which was defined as 100%

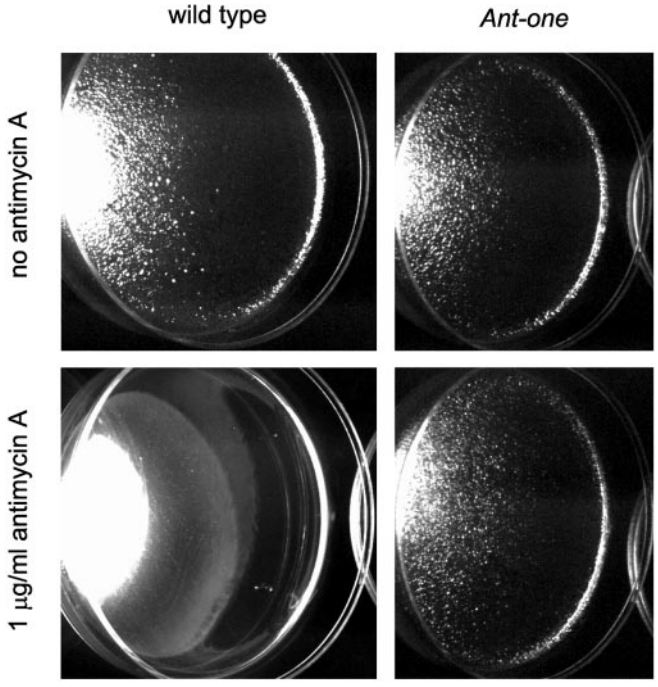

Fig. 2 Selective growth of wild-type and mutant *L. tarentolae* on plates. A total of 10^5 cells was plated on BHI/M199 plates with or without antimycin A. Plates were photographed after incubation for 9 days at 27 °C

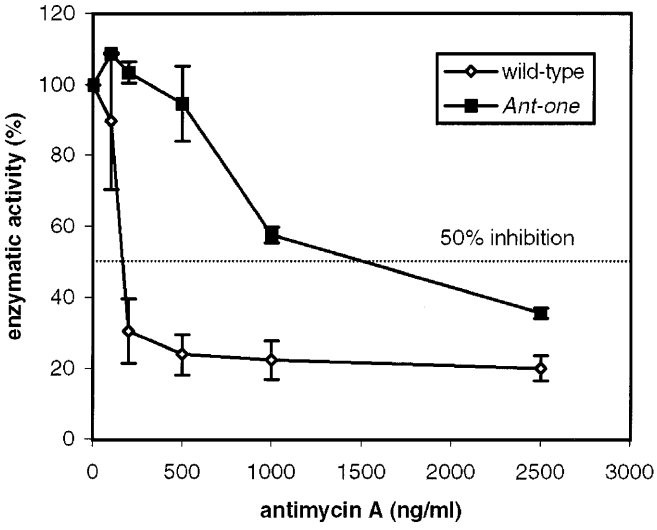

Fig. 3 Inhibitor titration curves for cytochrome *c* reductase activity in mitochondria isolated from wild-type *L. tarentolae* and the mutant *Ant-one* strain. Mitochondrial vesicles were prepared as described in Materials and methods. Cytochrome *c* reductase activity was measured using mitochondrial vesicles corresponding to 50 µg of mitochondrial protein with succinate as the substrate and cytochrome *c* as the electron acceptor as described in Materials and methods. Reactions were monitored at 550 nm. Drug sensitivity was measured as per-cent activity at each antimycin-A concentration compared with that in the absence of antimycin A, which was defined as 100%. Specific activities for cytochrome *c* reductase in control assays without antimycin A, expressed as nmol of cytochrome *c* reduced per min^{-1} g^{-1}, were 219.5 ± 3.5 for wild-type mitochondria and 232.0 ± 0.0 for mitochondria isolated from *Ant-one*. IC_{50} values given in the text were estimated from the plot

of *Leishmania* promastigotes (Van Hellemond and Tielens 1997).

Characterisation of cytochrome *c* reductase activity in isolated wild-type and mutant mitochondria

Drug resistance in prokaryotes and eukaryotes can be mediated by efflux systems located in the cell membrane (Bolhuis et al. 1997) and similar mechanisms appear to exist in kinetoplastids (Maser and Kaminsky 1998; Grondin et al. 1997; Chiquero et al. 1998). Therefore, we directly investigated cytochrome *c* reductase activity in isolated mitochondria and titrated its sensitivity towards antimycin A (Fig. 3). In this assay, mitochondria isolated from the mutant *Ant-one* strain (IC_{50} approximately 1500 ng ml^{-1}) showed a significantly higher tolerance towards the drug than those isolated from wild-type cells (IC_{50} approximately 150 ng ml^{-1}).

Cross-resistance to other inhibitors of the cytochrome bc_1 complex

We next explored the potential cross-resistance of *Ant-one* to some other Q_o- or Q_i-site inhibitors and compared the effects of myxothiazol, stigmatellin, HQNO and diuron on the growth of wild-type *L. tarentolae* and *Ant-one* in liquid cultures.

No difference between wild-type and *Ant-one* cells was found in sensitivity to the Q_o-site inhibitors myxothiazol and stigmatellin (data not shown). The same was true for the Q_i site inhibitor diuron. However, HQNO, another Q_i-site inhibitor, completely inhibited growth of wild-type *L. tarentolae* at a concentration of 5 µg ml^{-1}. By contrast, a 20-fold higher concentration of the drug was needed to completely inhibit growth of *Ant-one* cultures (data not shown). Hence, the antimycin A-resistant clone *Ant-one* exhibited cross-resistance to HQNO.

The antimycin A-resistant phenotype correlates with a point mutation in the mitochondrial CYb gene of the *Ant-one* clone

We also explored the molecular basis of the antimycin A resistance exhibited by the clone. Since antimycin A inhibition of the respiratory chain is due to interference with subunit *b* of the cytochrome bc_1 complex in all prokaryotic and eukaryotic species investigated so far, we sequenced the complete CYb region of the mitochondrial genome of the *Ant-one* clone. A single G-to-T transversion was found at position 5469 in LEIKPMAX. This mutation predicts a CYb protein with isoleucine at position 35 rather than a serine (i.e. Ser35Ile, Fig. 4). This sequence alteration was found in all seven clones obtained from three independent polymerase

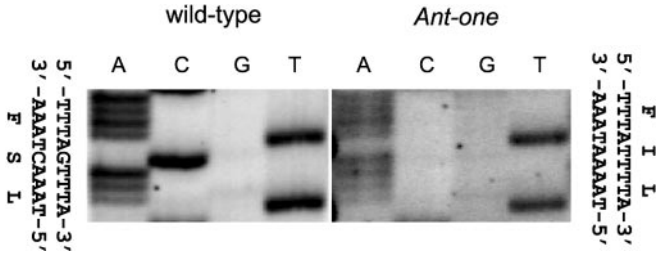

Fig. 4 Partial sequence of the CYb gene of the wild-type and mutant *Ant-one* strains. The sequence shown corresponds to nt 5465–5473 in LEIKPMAX (The *L. tarentolae* kinetoplast maxicircle DNA sequence; GenBank accession number M10126). The corresponding amino acids are indicated

chain reactions, suggesting that: (1) the mutation was not an artefact of the polymerase chain reaction, and (2) the mutation was present in the majority of the CYb alleles within each mitochondrial genome. The mutation was not found in the wild-type CYb sequence that was sequenced in parallel, nor were any other mutations detected in the CYb sequence.

No wild-type CYb RNA is detectable in kRNA isolated from *Ant-one*

In order to determine the ratio between wild-type and mutant CYb alleles more precisely, we performed a poisoned primer-extension assay on isolated *Ant-one* kRNA. As shown in Fig. 5, no wild-type extension products were detectable with this method. Hence, virtually all CYb alleles were affected by the point mutation. Furthermore, these data confirm that the mutated gene is actually transcribed.

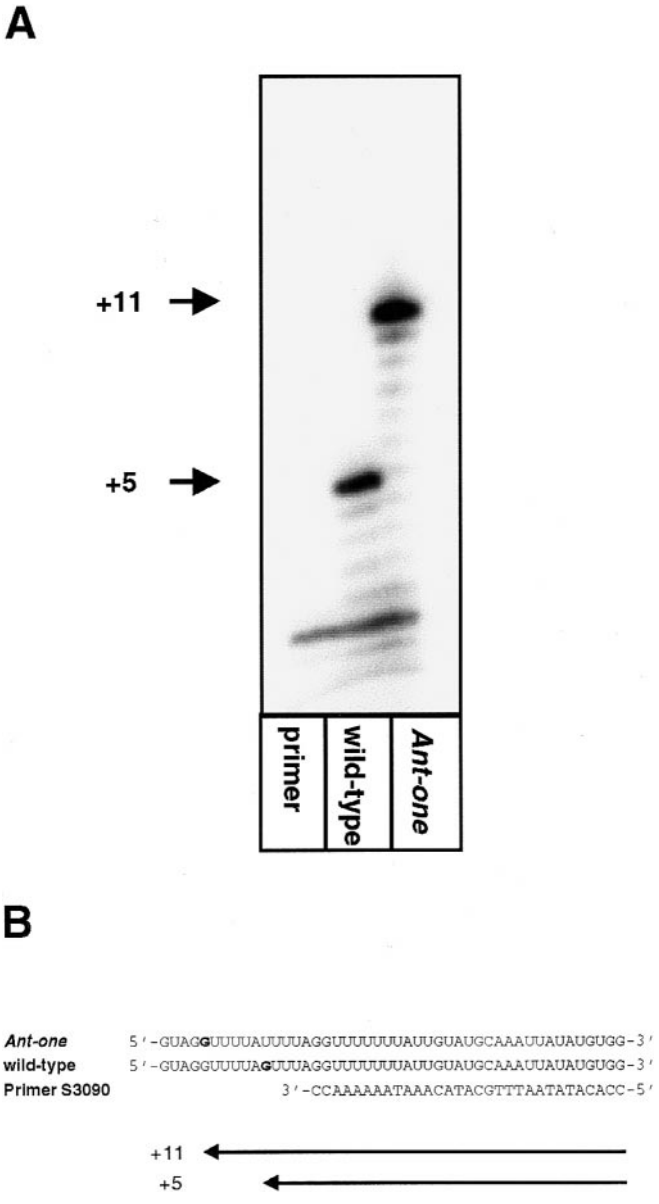

Fig. 5A, B Poisoned primer-extension analysis of CYb RNA. **A** ten micrograms of kRNA were analysed by poisoned primer extension of the oligonucleotide S3090 using ddCTP. The wild-type CYb sequence leads to an extension of five nucleotides, whereas the mutated *Ant-one* sequence leads to an extension of 11 nucleotides. No wild-type extension products above background could be detected in *Ant-one* kRNA. **B** sequence alignment of wild-type and mutated Cyb

Discussion

Our laboratory is interested in the development of a system for the stable mitochondrial transfection of *Leishmania*. One of the major obstacles in this aim has been the lack of selectable mitochondrial marker genes in this organisms. To be useful in a mitochondrial transfection system, the product of such a gene (1) has to confer a selectable phenotype and (2) its function has to be dependent on its expression within the mitochondrion. Candidate genes include, for example, mutant components of the respiratory chain that confer resistance towards inhibitors of oxidative phosphorylation. We therefore sought to isolate such mutated genes by selecting for parasites surviving in the presence of such inhibitors.

In the present study, we have used this strategy to successfully isolate and characterise a mutant *L. tarentolae* strain, *Ant-one*, exhibiting resistance towards the electron-transfer inhibitor antimycin A. Comparative growth curves between the *Ant-one* and the wild-type strain indicate an about 20-fold decrease in drug sensitivity, based on estimated IC_{50} values (Fig. 1). In the mitochondrion, antimycin A inhibits electron transfer from the cytochrome bc_1 complex to cytochrome *c*. When we compared IC_{50} values for cytochrome *c* reductase activity in isolated wild-type and mutant mitochondria, we found an approximately 10-fold decreased sensitivity for the mutant (Fig. 3). Our conclusion was that the resistance-conferring factors were located within the organelle, possibly directly involving the cytochrome bc_1 complex. This was an important finding since drug resistance is often conferred by transporter systems in

the cell membrane (Bolhuis et al. 1997) and such cases have previously been described for kinetoplastids (Grondin et al. 1997; Chiquero et al. 1998; Maser and Kaminsky 1998). Further evidence for an involvement of the cytochrome bc_1 complex in the mechanism of resistance was gained when we looked at parasite viability in the presence of other inhibitors of this complex. The two major cytochrome bc_1 inhibitors are called Q_o or Q_i inhibitors, depending on their interaction with either of the two electron transfer centres of the complex (Kim et al. 1998). We found cross-resistance of the *Ant-one* cell line towards HQNO, like antimycin A an inhibitor of the Q_i site of the cytochrome bc_1 complex (data not shown). No differences to the wild-type strain were found with respect to resistance to another inhibitor of the Q_i site, diuron, and to the Q_o-site inhibitors myxothiazol and stigmatellin. Such a cross-resistance towards some, but not necessarily all, inhibitors of the same Q site is a frequent characteristic of CYb mutants (Brasseur et al. 1996). Altogether these findings strongly pointed towards a mutation in the CYb gene. In a wide variety of organisms, mutations that confer resistance to antimycin A are clustered in only two regions of the CYb protein (Table 1; also reviewed in Brasseur et al. 1996). These regions, although far apart in the primary sequence, are believed to participate in the formation of the Q_i centre (N site) at the matrix side of the cytochrome bc_1 complex. Antimycin A is suggested to block the electron transfer from cytochrome b_H to quinone or semiquinone by binding close to this site. Recently, this view was strongly supported by crystallographic studies of several cytochrome bc_1 complexes (Xia et al. 1997; Kim et al. 1998; Zhang et al. 1998). The trypanosomatid and other protozoan CYb sequences exhibit only a low degree of homology with those of other organisms. Protein sequences of higher eukaryotes show an overall identity of about 50% when compared with each other, whereas their degree of identity with trypanosomatid sequences is about 25% (Ghelli et al. 1992). Despite this low overall homology, the presence of some highly conserved residues in the CYb sequence of *L. tarentolae* (Gly33, Gly37, Glu43, Ile44, Asp230) enabled us to roughly assign the antimycin binding pocket to amino-acid residues 30–44 and 222–235 (Table 1). As in all other eukaryotes studied so far, the *L. tarentolae* CYb gene is encoded in the mitochondrial genome. Sequencing of this gene in the mutant clone, which we called *Ant-one*, revealed a mutation of serine 35 to isoleucine (Fig. 4). This residue is located within the predicted antimycin binding pocket (Table 1).

The *L. tarentolae* CYb gene is located as a single-copy gene on the maxicircle component of the mitochondrial DNA (de la Cruz et al. 1984). These DNA molecules are the functional homologues of mitochondrial DNA in other eukaryotes and harbour the structural genes of the mitochondrial genome (Stuart and Feagin 1992). Some 20–50 copies of the maxicircle molecule, together with about 10000 so-called minicircles, are organised in the form of a huge DNA network, the kinetoplast DNA (kDNA) (Simpson 1987). Hence, the CYb gene is also present in 20 – 50 copies per cell. Although we sequenced the products of seven independent PCR reactions, all of which contained the same mutation, this number is still too limited to precisely determine the ratio of wild-type to mutated sequences within the maxicircle population. However, when we performed primer-extension analysis of kRNA isolated from the *Ant-one* strain we did not detect any wild-type CYb sequence. Altogether these data strongly suggest that the mutation had affected the vast majority, if not all, of the CYb alleles.

We can only speculate on the progression of the mutation within the maxicircle population. It seems unlikely that each of the CYb genes was altered individually by an identical mutation. Probably this transversion occurred in a single gene first and then, upon selection, was amplified either by recombination between maxicircle molecules or by an unequal distribution of replicated molecules during cell division. Such a loss of heterogeneity has been reported for *Trypanosoma brucei* maxicircles and has been explained by the stochastic segregation of these DNA molecules during mitosis (Turner et al. 1995).

We cannot absolutely rule out the possibility that the observed antimycin-resistant phenotype is the consequence of another, yet undiscovered, genetic change in the *Ant-one* clone. For instance, in yeast, multidrug resistance sometimes involves resistance to antimycin (Leppert et al. 1990). However, the fact that the mutation in *L. tarentolae* described here could be localised in a region of the CYb gene that was reliably identified as the antimycin binding region in a number of studies (see above) strongly supports a relationship between point mutation and phenotype. The formal proof of this causality has to await the availability of a mitochondrial transfection system for *Leishmania*.

To our knowledge, our results represent the first example of a correlation between a mutation in a maxicircle-encoded gene and a particular phenotype in trypanosomatids. In this particular case concerning a mutation in the CYb gene, the results have important implications on the RNA-editing phenomenon described for these organisms (for recent reviews see Arts and Benne 1996; Alfonzo et al. 1997; Kable et al. 1997; Stuart et al. 1997). Most of the maxicircle transcripts have to undergo RNA editing to form a functional mRNA. However, it has never been conclusively demonstrated that edited transcripts are indeed translated by the mitochondrial ribosomes (Speijer et al. 1997; Stuart et al. 1997). The *L. tarentolae* CYb pre-mRNA has to be edited in its 5′ region in order to restore the proper reading frame and to create a functional AUG initiation codon which is missing in the DNA sequence (Feagin et al. 1988). No other potential initiation codons are encoded in the DNA upstream of the *Ant-one* mutation. It directly follows that the CYb mRNA has to be edited in order for the mutation to have an effect. Previous experimental evidence for the translation of edited

transcripts was only circumstantial. In one report, a synthetic oligopeptide corresponding to the predicted C-terminal sequence of cytochrome oxidase subunit II was used to prepare a specific antiserum. The serum detected two polypeptides in mitochondrial lysates of *L. tarentolae*, one of which corresponded to the expected molecular weight of subunit II (Shaw et al. 1989). A similar strategy was used for the detection of mitochondrially encoded subunits 7 and 8 of NADH dehydrogenase in *T. brucei* (Beattie and Howton 1996) and for the detection of mitochondrially encoded cytochrome *c* oxidase subunit II in *Crithidia fasciculata* (Breek et al. 1997). However, these reports could not rule out the possibility that the detected proteins were imported from the cytoplasm. In fact, the subunits investigated in these studies are nuclearly encoded in some species (Fearnley et al. 1989; Nugent and Palmer 1991; Covello and Gray 1992).

The present study was fuelled by our desire to develop a mitochondrial transfection system for trypanosomatids. In these organisms, the genetic organisation of the mitochondrion shows several unusual features such as a complex DNA network composed of thousands of catenated circular DNA molecules (Shapiro and Englund 1995), the lack of encoded tRNAs (Schneider et al. 1994), and the still-enigmatic process of RNA editing (Arts and Benne 1996; Alfonzo et al. 1997; Kable et al. 1997; Stuart et al. 1997). The investigation of these phenomena should greatly benefit from the availability of a mitochondrial transfection system. Recently, we have established conditions for the transfection of *Leishmania* by particle bombardment, a technique supposed to be crucial for the targeting of DNA to organelles in vivo (Sbicego et al. 1998). The mutated CYb gene of the *Ant-one* strain described in the present report could be exploited as a selectable marker for mitochondrial transfection and thereby provide another important tool towards this goal.

Acknowledgements We are grateful to Larry Simpson and Antonio M. Estévez (Los Angeles) for their help in performing the primer-extension experiments. We thank Uli Göringer and Michael Brecht (Darmstadt), as well as Ken Stuart and Moffett Lee Kable Burgess (Seattle), for critical reading of the manuscript. We are also indebted to André Schneider (Freiburg) for calling our attention to the implications of our results on RNA editing. This work was supported by grant no. 31–37582.93 from the *Schweizerischer Nationalfonds* and a START-fellowship to B.B.

References

Alfonzo JD, Thiemann O, Simpson L (1997) The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. Nucleic Acids Res 25: 3751–3759

Arts GJ, Benne R (1996) Mechanism and evolution of RNA editing in kinetoplastida. Biochim Biophys Acta 1307: 39–54

Beattie DS, Howton MM (1996) The presence of rotenone-sensitive NADH dehydrogenase in the long slender bloodstream and the procyclic forms of *Trypanosoma brucei brucei*. Eur J Biochem 241: 888–894

Bibb MJ, van Etten RA, Wright CT, Walberg MW, Clayton, DA (1981) Sequence and gene organization of mouse mitochondrial DNA. Cell 26: 167–180

Bolhuis H, van Veen HW, Poolman B, Driessen AJ, Konings, WN (1997) Mechanisms of multidrug transporters. FEMS Microbiol Rev 21: 55–84

Braly P, Simpson L, Kretzer, F (1974) Isolation of kinetoplast-mitochondrial complexes from *Leishmania tarentolae*. J Protozool 21: 782–790

Brasseur, G, Saribas, AS, Daldal, F (1996) A compilation of mutations located in the cytochrome b subunit of the bacterial and mitochondrial *bc*1 complex. Biochim Biophys Acta 1275: 61–69

Brasseur R (1988) Calculation of the three-dimensional structure of *Saccharomyces cerevisiae* cytochrome *b* inserted in a lipid matrix. J Biol Chem 263: 12571–12575

Breek CK, Speijer D, Dekker H, Muijsers AO, Benne R (1997) Further evidence for the presence of mitochondrially encoded subunits in cytochrome c oxidase of the trypanosomatid *Crithidia fasciculata*. Biol Chem 378: 837–841

Brunner A, Coria R (1989) Cloning and sequencing of the gene for apocytochrome b of the yeast *Kluyveromyces lactis* strains WM27 (NRRL Y-17066) and WM37 (NRRL Y-1140). Yeast 5: 209–218

Chiquero MJ, Pérez-Victoria J, O'Valle F, González-Ros JM, Del Moral RG, Ferragut JA, Castanys S, Gamarro F (1998) Altered drug membrane permeability in a multidrug-resistant *Leishmania tropica* line. Biochem Pharmacol 55: 131–139

Colson AM (1993) Random mutant generation and its utility in uncovering structural and functional features of cytochrome *b* in *Saccharomyces cerevisiae*. J Bioenerg Biomembr 25: 211–220

Coppee JY, Tokutake N, Marc D, di Rago JP, Miyoshi H, Colson AM (1994) Analysis of revertants from respiratory deficient mutants within the center N of cytochrome *b* in *Saccharomyces cerevisiae*. FEBS Lett 339: 1–6

Coria R, Garcia M, Brunner A (1989) Mitochondrial cytochrome *b* genes with a six-nucleotide deletion or single-nucleotide substitutions confer resistance to antimycin A in the yeast *Kluyveromyces lactis*. Mol Microbiol 3: 1599–1604

Covello PS, Gray MW (1992) Silent mitochondrial and active nuclear genes for subunit 2 of cytochrome *c* oxidase (cox2) in soybean: evidence for RNA-mediated gene transfer. EMBO J 11: 3815–3820

Cruz VF de la , Neckelmann N, Simpson L (1984) Sequences of six genes and several open reading frames in the kinetoplast maxicircle DNA of *Leishmania tarentolae*. J Biol Chem 259: 15136–15147

Dutton PL, Erecinska M, Sato N, Mukai Y, Pring M, Wilson DF (1972) Reactions of *b*-cytochromes with ATP and antimycin A in pigeon heart mitochondria. Biochim Biophys Acta 267: 15–24

Estevez AM, Kierszenbaum F, Wirtz E, Bringaud F, Grunstein J, Simpson L (1999) Knockout of the glutamate dehydrogenase gene in bloodstream *Trypanosoma brucei* in culture has no effect on editing of mitochondrial mRNAs. Mol Biochem Parasitol 100: 5–17

Feagin JE, Shaw JM, Simpson L, Stuart K (1988) Creation of AUG initiation codons by addition of uridines within cytochrome *b* transcripts of kinetoplastids. Proc Natl Acad Sci USA 85: 539–543

Fearnley IM, Runswick MJ, Walker JE (1989) A homologue of the nuclear-coded 49-kDa subunit of the mitochondrial NADH-uniquinone reductase is coded in chloroplast DNA. EMBO J 8: 665–672

Fong D, Chan MMY, Rodriguez R, Gately LJ, Berman JD, Grogl M (1994) Paromomycin resistance in *Leishmania tropica*: lack of correlation with mutation in the small subunit ribosomal RNA gene. Am J Trop Med Hyg 51: 758–766

Gennis RB, Barquera B, Hacker B, Van Doren SR, Arnaud S, Crofts AR, Davidson E, Gray KA, Daldal F (1993) The bc_1 complexes of *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. J Bioenerg Biomembr 25: 195–209

Ghelli A, Crimi M, Orsini S, Gradoni L, Zannotti M, Lenaz G, Degli Esposti M (1992) Cytochrome *b* of protozoan mitochondria: relationships between function and structure. Comp Biochem Physiol 103B: 329–338

Grondin K, Haimeur A, Mukhopadhyay R, Rosen BP, Ouellette M (1997) Co-amplification of the gamma-glutamylcysteine synthetase gene *gsh1* and of the ABC transporter gene *pgpA* in arsenite-resistant *Leishmania tarentolae*. EMBO J 16: 3057–3065

Howell N, Gilbert K (1988) Mutational analysis of the mouse mitochondrial cytochrome *b* gene. J Mol Biol 203: 607–618

Kable ML, Heidmann S, Stuart KD (1997) RNA editing: getting U into RNA. Trends Biochem Sci 22: 162–166

Kim H, Xia D, Yu CA, Xia JZ, Kachurin AM, Zhang L, Yu L, Deisenhofer J (1998) Inhibitor-binding changes domain mobility in the iron-sulfur protein of the mitochondrial bc_1 complex from bovine heart. Proc Natl Acad Sci U S A 95: 8026–8033

Lang BF, Ahne F, Bonen L (1985) The mitochondrial genome of the fission yeast *Schizosaccharomyces pombe*. J Mol Biol 184: 353–366

Leppert G, McDevitt R, Falco SC, van Dyk TK, Ficke MB, Golin J (1990) Cloning by gene amplification of two loci conferring multiple drug resistance in *Saccharomyces*. Genetics 125: 13–20

Majewski C, Trebst A (1990) The *pet* gene of *Rhodospirillum rubrum*: cloning and sequencing of the genes of the cytochrome bc_1 complex. Mol Gen Genet 224: 373–382

Maser P, Kaminsky R (1998) Identification of three ABC transporter genes in *Trypanosoma brucei* spp. Parasitol Res 84: 106–111

Mitchell P (1976) Possible molecular mechanisms of the protonmotive function of cytochrome systems. J Theor Biol 62: 327–367

Nobrega FG, Tzagaloff A (1980) Assembly of the mitochondrial membrane system. DNA sequence and organization of the cytochrome *b* gene in *Saccharomyces cerevisiae*. J Biol Chem 255: 9828–9837

Nugent JM, Palmer JD (1991) RNA-mediated transfer of the gene *coxII* from the mitochondrion to the nucleus during flowering plant evolution. Cell 66: 473–481

Rago JP di, Colson AM, (1988) Molecular basis for resistance to antimycin and diuron, Q-cycle inhibitors acting at the Q_i site in the mitochondrial ubiquinol-cytochrome *c* reductase in *Saccharomyces cerevisiae*. J Biol Chem 263: 12564–12570

Sbicego S, Schnaufer A, Blum B (1998) Transient and stable transfection of *Leishmania* by particle bombardment. Mol Biochem Parasitol 94: 123–126

Schneider A, Martin J, Agabian N (1994) A nuclear-encoded tRNA of *Trypanosoma brucei* is imported into mitochondria. Mol Cell Biol 14: 2317–2322

Shapiro TA, Englund PT (1995) The structure and replication of kinetoplast DNA. Annu Rev Microbiol 49: 117–143

Shaw JM, Campbell D, Simpson L (1989) Internal frameshifts within the mitochondrial genes for cytochrome oxidase subunit II and the maxicircle unidentified reading frame 3 of *Leishmania tarentolae* are corrected by RNA editing: evidence for translation of the edited cytochrome oxidase subunit II mRNA. Proc Natl Acad Sci USA 86: 6220–6224

Simpson L (1987) The mitochondrial genome of kinetoplastid protozoa: genomic organization, transcription, replication, and evolution. Annu Rev Microbiol 41: 363–382

Simpson L, Braly P (1970) Synchronization of Leishmania tarentolae by hydroxyurea. J Protozool 17: 511–517

Speijer D, Breek CK, Muijsers AO, Hartog AF, Berden JA, Albracht SP, Samyn B, Van Beeumen J, Benne R (1997) Characterization of the respiratory chain from cultured *Crithidia fasciculata*. Mol Biochem Parasitol 85: 171–186

Stuart K, Feagin JE (1992) Mitochondrial DNA of kinetoplastids. Int Rev Cytol 141: 65–88

Stuart K, Allen TE, Heidmann S, Seiwert SD (1997) RNA editing in kinetoplastid protozoa. Microbiol Mol Biol Rev 61: 105–120

Trumpower BL (1990) The proton-motive Q cycle. Energy transduction by coupling of proton translocation to electron transfer by the cytochrome bc_1 complex. J Biol Chem 265: 11409–11412

Turner CM, Hide G, Buchanan N, Tait A (1995) *Trypanosoma brucei*: inheritance of kinetoplast DNA maxicircles in a genetic cross and their segregation during vegetative growth. Exp Parasitol 80: 234–241

Uhrig J, Jakobs C, Majewski C, Trebst A (1994) Characterization of antimycin-resistant mutants of *Rhodospirillum rubrum*. Biochem Soc Trans 22: 59S

Van Hellemond JJ, Tielens AGM (1997) Inhibition of the respiratory chain results in a reversible metabolic arrest in *Leishmania* promastigotes. Mol Biochem Parasitol 85: 135–138

Weber S, Wolf K (1988) Two changes of the same nucleotide confer resistance to diuron and antimycin in the mitochondrial cytochrome *b* gene of *Schizosaccharomyces pombe*. FEBS Lett 237: 31–34

Xia D, Yu CA, Kim H, Xia JZ, Kachurin AM, Zhang L, Yu L, Deisenhofer J (1997) Crystal structure of the cytochrome bc1 complex from bovine-heart mitochondria. Science 277: 60–66

Zhang Z, Huang L, Shulmeister VM, Chi YI, Kim KK, Hung LW, Crofts AR, Berry EA, Kim SH (1998) Electron transfer by domain movement in cytochrome bc_1. Nature 392: 677–684