Characterisation of a developmentally regulated amino acid transporter gene from *Leishmania amazonensis*

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Abstract

The metabolism of protozoan parasites of the *Leishmania* genus is strongly based on amino acid consumption, but little is known about amino acid uptake in these organisms. In the present work, we identified a *Leishmania amazonensis* gene (La-PAT1) encoding a putative amino acid transporter that belongs to the amino acid/auxin permease family, a group of H^+/amino acid symporters. This single copy gene is upregulated in amastigotes, the life cycle stage found in the mammalian host. La-PAT1 putative orthologous sequences were identified in *Leishmania infantum*, *Leishmania donovani*, *Leishmania major* and *Trypanosoma*.
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1. Introduction

Protozoan parasites of the genus *Leishmania* are the aetiological agents of leishmaniasis, a group of diseases of great morbidity and vast geographical distribution [1]. Along the life cycle, these parasites are exposed to different environments: in the gut of the insect vector, procyclic promastigotes multiply as extracellular organisms and eventually differentiate into nondividing, highly motile and infective metacyclic promastigotes; in the mammalian host, amastigotes are intracellular parasites, residing within macrophage phagolysosomes [2]. The availability of nutrients in these two environ-ments is markedly different, determining the need for metabolic adaptations. Energy production in amastig-otes has been shown to be based mainly on the con-sumption of carbohydrates and fatty acids [3], while the metabolism of promastigotes is based on the catabolism of amino acids, mainly L-proline, which is particularly abundant in the gut of the insect vector [4].

Transporters are the first cell proteins that come into contact with solutes in the surrounding media and, in several cases, they function not only as permeases carry-ing the solutes into the cytoplasm, but also as environ-mental sensors [5]. The transport of a given metabolite may be regarded as the first step of a metabolic pathway and, in some cases, transporters may work as regulators of metabolic processes, as occurs with the glucose transporter of *Trypanosoma brucei*, a regulator of the glucolytic flux [6].

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The metabolism of amino acids in trypanosomatids is particularly relevant and the characterisation of amino acid permeases, as well as their regulation along the life cycle is a major research need. The transport of L-arginine, L-proline, L-methionine and neutral amino acids in *Leishmania* has been characterised biochemically [7–13] but little is known about the molecular features involved. In fact, the only genes encoding amino acid transporters in kinetoplastids identified so far are putative members of the AAAP (Amino Acid/Auxin Permease; TC 2.A.18) family. These genes have been recently identified in *Trypanosoma cruzi*, but their function has not been biochemically demonstrated [14]. Putative orthologous sequences from *Leishmania donovani*, *Leishmania major* and *Trypanosoma brucei* were also identified in the databases [14]. The AAAP is one of the major amino acid transporter families and belongs to the "Electrochemical Potential Driven Transporters" class, including hundreds of proteins from plants, animals, yeast and other fungi. Some of these permeases transport single amino acids while others have a very broad specificity for all 20 amino acids, including D-isomers [15].

In the present work, we identified and characterized for the first time a developmentally regulated *Leishmania amazonensis* gene encoding a putative member of the AAAP family. We also demonstrated that this gene is conserved in other *Leishmania* species.

2. Materials and methods

2.1. Parasites

Leishmania promastigotes were grown in liquid culture at 25 °C in Medium 199 (Gibco BRL) supplemented with 10% fetal calf serum (FCS; Gibco BRL). Promastigote forms were collected along the exponential and stationary phase of growth. The following strains were used: *L. amazonensis* MHOM/BR/1973/M2269, *L. major* MHOM/IL/1981/Friedlin, *Leishmania chagasi* MHOM/BR/1974/M2682, *Leishmania braziliensis* MHOM/BR/1975/M2903 and *Leishmania tarentolae* TCC 017. *L. amazonensis* amastigotes were obtained from footpad lesions of experimentally infected BALB/c mice as described [16]. *T. cruzi* (Y strain) epimastigotes and *Crithidia fasciculata* (TCC 039) promastigotes were grown in LIT medium [17].

2.2. Isolation and analysis of parasite DNA

Parasite genomic DNA was prepared as described [18]. Restriction digests, alkaline transfer of DNA to nylon membranes (Hybond-XL™), preparation of radioactive probes by random oligonucleotide labelling and DNA hybridisation (using 50% formamide at 42 °C) were performed as described [19].

2.3. RNA purification and analysis

Total RNA was prepared by Trizol (Invitrogen) extraction according to the manufacturer's instructions, denatured using glyoxal and DMSO, separated in 1% phosphate buffered agarose gels, blotted and hybridised as described [19]. To control for equal RNA loading, blots were stained with methylene blue. Hybridisation washes were done at 55 °C in 1× SSC, 0.1% SDS.

2.4. Amplification of a fragment corresponding to a putative AAAP gene

The k5 fragment was amplified from *L. amazonensis* genomic DNA using two degenerated oligonucleotides (5′-GTCGTTCTTCACNAGGATGTT-3′ and 5′-RAN-GGNCTNAARTGNGATCA-3′). The PCR was performed using 100 ng of genomic DNA, 25 pmol of each primer and 2.5 mM MgCl$_2$ for 35 cycles, with each cycle consisting of 94 °C for 1 min, 50 °C for 2 min and 72 °C for 1 min.

2.5. Screening of the cosmid library

A *L. amazonensis* genomic cosmid library prepared in the vector CL-Hyg from partially digested *Sau 3A* fragments of approximately 30 kb [16] was screened with probe k5.

2.6. Sequence analysis

Assembly and analysis of the DNA sequence data, including prediction of open reading frames (ORFs), were carried out using the software package LaserGene™ and Vector NTI v. 6.0 (Informax, Inc.). The obtained sequence was deposited in the GenBank under the Accession No. AY635803. The search for conserved motifs was done using "Blocks" (www.blocks.fhcrc.org/). Global sequence alignment analysis was done using ClustalW [20]. Prediction of membrane-spanning regions was performed using "TMAP" (www.mbb.ki.se/tmap/).

3. Results

3.1. Identification of a L. amazonensis putative amino acid transporter gene

A set of degenerated oligonucleotides was used in low stringency amplification of *L. amazonensis* genomic DNA. The nucleotide sequence of one cloned product (clone k5) encoded a partial ORF that displayed significant similarity with amino acid transporters from several organisms. A *L. amazonensis* cosmid genomic library was screened with fragment k5 and several positive clones were identified. These were submitted to a

second round of screening and to restriction mapping allowing the identification of 2 strongly hybridising cosmids that possessed overlapping inserts (data not shown).

Fragments of these cosmid clones were sequenced and assembled into a 1.9-kb contig encompassing the complete coding sequence and 5′ untranslated region of the gene identified by probe k5. Analysis of this

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LaPAT1    MPGNCVPLSRTLDKDEVELKGRRFCDSNEAPAAAVEDMDPNWVDVVDDFSGVGGRKFLYECQELPMLNGVDRDTEGQPPS   80
LiPAT1    MPGNRVPLSHTLGKDEVELKGHCRSDCHEAPAAALGDMDPKWVDVDDEFSCGGGRKLLYECPDLPMQNGVDRDTEGQPLP   80
TzPAT6    -----------------------MTHAPDANEPTEQPTPMQTAGSRLPAAASAPALFTGEEV-------RVPMSGEVV     48
TbAATP11  -----------------------MTFEAAAQAPNKCPTNEATAEPLRTPNVLADNLLA-----------RSNLSEGES     44
LdAAP3    ---------------MSKPGKSIPAQAEGEVLSDSLIDGNPGNAVDKHPSGEQGN---------HLHKNGSLT          49
LmPAT     ---------------MSKPGKSIPAQAEGEVLSDSLMGGNSGNAVDKHPSGEQGS---------HLHKSGSLT          49

LaPAT1    SSQTPDEVRDAKSRRRRNVFSRISNAIIPHGGLLSGAVNLACVTLGAGIMSIPSAFNTSGIIMAVFYLVIITSLTVFSIT  160
LiPAT1    SSQAPDEVRGAKRRARRNIFSRIFNAIIPHGGLLSGAVNLACVTLGAGIMSIPSAFNTSGIIMAVFYLVIITSLTVLSIT  160
TzPAT6    PTEMESGQKNGREQPAKNCLMRLFNFIVPRGGALSGILNLASVTLGAGIISIPSAFNTSGIVMAVVYLVGVTALTVFSIK  128
TbAATP11  PEKSGEAQKP-EEQ--GNALMRCFHFILPRGGALSGIFNLASVTLGAGIMSIPSAFNTSGMIMAIIYLLLVTIVFTVFSIF  121
LdAAP3    ASSSHNENGADAAKPGRNIIFRFTGWLIPYGGVISNCFSLGSVTLGGGIISMPSSFAMSGIIMSVIYLVVITAATVYTMT  129
LmPAT     DSSSHNGNGADAAKPEHNIILRFTGWLIPYGGVISNCFSLGSVTLGGGIISMPSSFAMSGIIMSVIYLVVITAATVYTMT  129

LaPAT1    LLSKAMEKTGIYSFEGLARALFGHCGDIVAALLMWILCFGASVGFVIAIGDILKPIFAH-PRVPPFLQEKNGRRCVMGGV  239
LiPAT1    LLSNAMEKTGIYSFEGLARALFGRCGDIVAAVLMWILCFGASVGFVIAIGDILKPIFAH-PKVPPFLQEKSGRRCIMSGV  239
TzPAT6    LLVSASERSGYRSFESLARGLLGRGADIVVAILMWLLCFGDAVGYMVAVGDVLRPILEH-DGVPAYLQKDSGRRVLMSCI  207
TbAATP11  LIVSAAEKTGYRSFESMARNLLGPRADIAVGFLLWLLCFGGASGYVVAIGDVIQGLLSH-EKVPAYLQSKGGRRLLTSAI  200
LdAAP3    LLGYAMKATGCKTFEELSLVLFGRGWDYFVGFVLWLSCFGTAVAYISAVSSLLITPILEKSPGTPAYLLTTSGNRLITSLI  209
LmPAT     LLGYAMKATGCKTFEELSLVLFGRGWDYFVGFVLWLSCFGTAVAYISAVSSLITPILEKSPGTPAYLLTTSGNRVITSLI  209

LaPAT1    WLLFMLPLVLPKRINSLRYMSAVGLFFIVLFVICAIYHSIAYGLKDG-IRKDLVFVRPGNEAVSGLSIFCFSYLCCVNVG  318
LiPAT1    WLLFMLPLVLPKKINSLRYMSAVGVFFIVFFVICAIYHSIVYGLKDG-IRKDLVMVRPGNEAVSGLSIFCFSYLCCVNVG  318
TzPAT6    WLLFMFPLVLPKQVNSLRYASAVGVSFIILLFVVCVVVHSGQKMVGDGGIRSDLVLFRPGNSAVSGLSLFIFAYLCCVNCF  287
TbAATP11  WFVFIFPLTLPKRVNSLRYASAIGVSFIILPFAICVVEHSAEKMVTDGGIKQELVMFRSGNDAVAGLSLFIFAYLCHVNSF  280
LdAAP3    WLVFMVPVVIPKRVNSIRYVSAIGVFMVLYFAVTIVVHSSMNGLKEG-MRGDMKYFTSGNEAVYGLSIFIFSFLCCAVTG  288
LmPAT     WLVFMVPVVIPKRVNSIRYVSAIGVFMVLYFAVTIVVHSSMNGMKEG-MRGDMKYFTSGNEAVYGLSIFIFSFLCCAVTG  288

LaPAT1    RIIVENTKR-TTRMITLQAILSCSICATLYFLTGFFGYADFGPSLNGNILGRYDPYQSPVFFVVFPGIIVKLCASFSLDM  397
LiPAT1    RIIVENTER-TTRMITLQAILSCSICATLYFLTGFFGYADFGPSLKGNILERYSPYQSPIFFVVFPGIIVKLCASFSLDM  397
TzPAT6    KIFYEMKHR-SVSRMTRDAAVSCGTCCLIYFLVGFFGYAEFGPEVTGSIMRYINPYTAPVFFFCFIGIIVKLCAGFSLNM  366
TbAATP11  SIFFEMKKR-SVTRMTRDAAVSCSICCCVYLLTGFFGYAEFGPTVEGSVLKLYDPYANPVFFVCFIGIIVKLCAGFSLNM  359
LdAAP3    SVYFEQRPRPSVRQLTIASAISMTVCMVLYIFTGVFGYFDFADDTQDSILYNFDPVHQPYMMLAYIGMLIKICAAFAMNM  368
LmPAT     SVYFEQRPRPSVSQLTIASVISMTVCMVLYIFTGIFGYFDFADDTQDSILYNFDPVHQPYMMLAYIGMMIKICAAFAMNM  368

LaPAT1    LACRTALFQVMRWDVETMPYWKHTLVSVPIAIGALILGLFVPDINIVFGLGGAFSGGFIGFVFPPLFIMYAGSWTLKKVG  477
LiPAT1    LACRTALFQVMHWDVETMPYWKHTLVSVPMAIGALILGLFVPDINIVFGLAGALSGGFIGFVFPALFVMYAGSWNLKKVG  477
TzPAT6    LACRTALFQVMHWDVDTMPYWKHSLFSVPFAIGALVIGLFLPDINIVFGLVGAFCGGFIAFIFPALFIMYAGNWSYRSVG  446
TbAATP11  LACRTALFQVLRWDLDTMSYVRHSIVSVSFAVGSLVLGLFVPDINVIFGLVGAFCGGFIGFIFPALFIMYAGGWTRQSVG  439
LdAAP3    LPCRNFAMHCLNWDLETVPYWKHTIAILTMAVATLVCGLFIPSINTAFGLVGSLCGGFIGFIFPAYFWMYSGNWSLSSVG  448
LmPAT     LPCRNFVYHCLNWDLETVPYWKHTIVILTMAVATLVCGLFIPSINTAFGLVGSLCGGFIGFIFPAYFWMYSGNWSLSSVG  448

LaPAT1    IWYYLGTYFLLICGVISIVFGTISIYYYSFFV-- : 509
LiPAT1    IWYYLATYFLLICGVVSIVFGTVSTVYFSFFV-- : 509
TzPAT6    CVEYFLTYLLLVGVVAIVFGTGSTIFSTIQRFA  : 480
TbAATP11  WVQYILTYVLLILGVVAIVFGTSASVYYTIKRYS : 473
LdAAP3    IWHWLATYFLVVAGVIAVVFGTISTIYYSFFV-- : 480
LmPAT     IWHWLATYFLVVAGVVAVVFGTVSTIYYSFFV-- : 480
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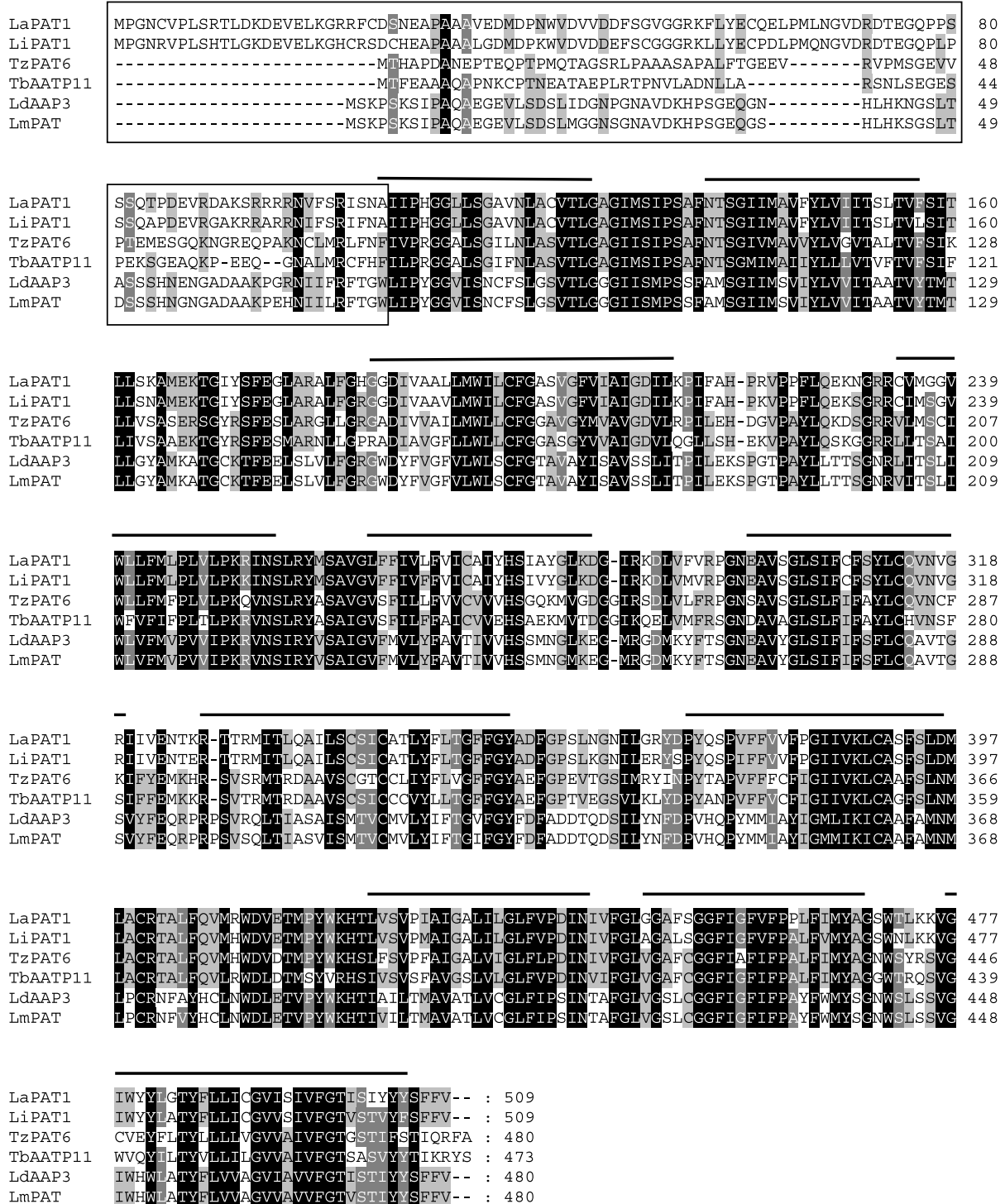
Fig. 1. Amino acid transporter sequence. CLUSTAL W alignment of the deduced amino acid sequences of putative amino acid transporters from *L. amazonensis* (La-PAT1, AY635803), *L. infantum* (LiPAT1, Contig 10204, www.genedb.org [06-08-2004]), *T. cruzi* (TcPAT6, AAS47054), *T. brucei* (TbAATP11, CAC86552), *L. donovani* (LdAAP3, AAO88094) and *L. major* (LmPAT, CAC37212). The putative extracellular region is shown in the box; lines above the sequence identify the transmembrane regions. Residue identity or conservation in all 6 sequences is marked by black boxes, in 5 sequences by white typing in grey boxes and in 4 sequences by black typing and grey boxes.

sequence identified a 1530-bp ORF encoding a hypothetical 56-kDa protein. Hydrophylicity plots and in silico prediction of protein topology suggested the presence of an intracellular N-terminal domain of approximately 100 amino acids followed by 11 *trans*-membrane spanning domains. The analysis of the putative peptide also revealed the presence of the conserved Pfam domain Aa_trans (Transmembrane amino acid transporter protein, PF01490) extending from residues 109 to 507. Aa_trans is defined as a region found in many amino acid transporters including L-proline and other amino acid permeases.

The translated coding sequence was searched against the translated GenBank protein database. The highest local amino acid identities were obtained with a protein encoded by a sequence representative of a multigenic group in *T. cruzi* named PAT6 (GenBank Accession No. AAS47054, 56% identity over 432 amino acids), the *T. brucei* putative amino acid transporter AATP11 (CAC86552, 52% identity over 429 amino acids), the *L. donovani* amino acid permease AAP3LD (AAO88094, 42% identity over 436 amino acids), and a *L. major* possible amino acid transporter (CAC37212, 40% identity over 453 amino acids). The *L. amazonensis* putative amino acid transporter nucleotide sequence (henceforth named La-PAT1) was also blasted against databases containing unfinished *Leishmania* spp. genomes at the Sanger Centre (GeneDB, www.genedb.org). A *L. infantum* contig was identified containing a complete ORF corresponding to an orthologous sequence (88.4% amino acid identity). The amino acid global sequence alignment of La-PAT1 with these trypanosomatid putative orthologues revealed a highly conserved protein with a divergent N-terminal region (Fig. 1). The predicted intracellular domain comprises 105 amino acids in La-PAT1, 66 amino acids in the *T. cruzi* PAT6 and the first 73 amino acids in the case of *T. brucei* AATP11 (Fig. 1).

3.2. Expression analysis and genomic organization of the La-PAT1 gene

The pattern of La-PAT1 transcript accumulation in the three different stages of *L. amazonensis* life cycle was evaluated by probing Northern blots containing total RNA purified from log and stationary phase promastigotes and amastigotes with fragment k5. This probe detected an approximately 5-kb transcript upregulated in amastigotes (Fig. 2), showing that La-PAT1 is a differentially expressed gene. Southern blot experiments were performed in order to establish the copy number of this gene. The hybridisation pattern observed suggests that this gene is present in a single copy per haploid genome in *L. amazonensis* (Fig. 3).
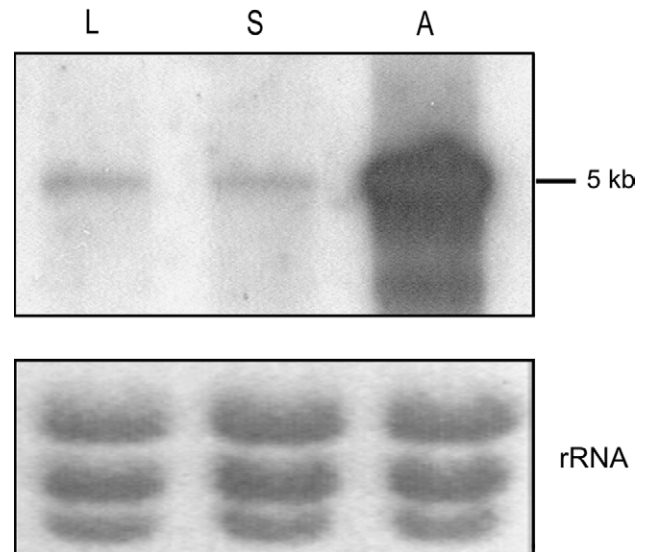

Fig. 2. Expression pattern of La-PAT1. Northern blots containing 5 μg of *L. amazonensis* total RNA from logarithmic (L) and stationary (S) phase promastigotes and amastigotes (A) were hybridised to probe k5. The approximate size of the transcript (in kb) is indicated next to the blot. rRNA:methylene-blue-stained ribosomal RNA bands.
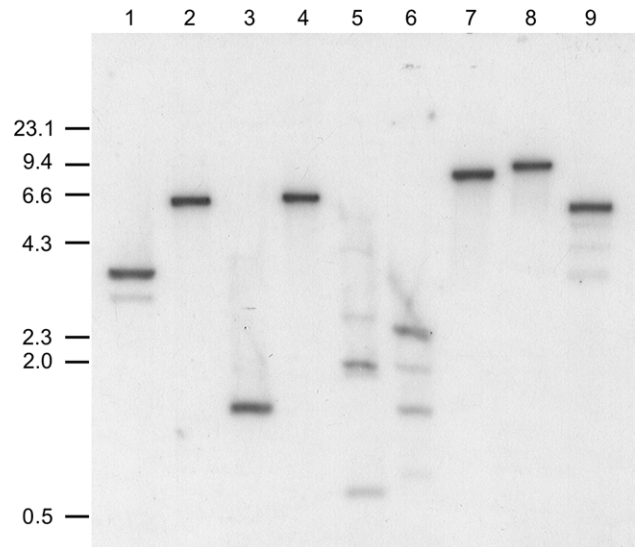

Fig. 3. Genomic organisation of La-PAT1. *L. amazonensis* genomic DNA restriction digested with *Ava*I (1), *Bam*HI (2), *Hin*cII (3), *Hin*dIII (4), *Pst*I (5), *Pvu*II (6), *Sac*I (7), *Xba*I (8), *Xho*I (9) and hybridised with probe k5.

3.3. La-PAT1 orthologous sequences in other trypanosomatids

In order to evaluate the presence of orthologous genes in other *Leishmania* species and other trypanosomatids, Southern blot analyses were performed using genomic DNA purified from *L. amazonensis*, *L. chagasi*,
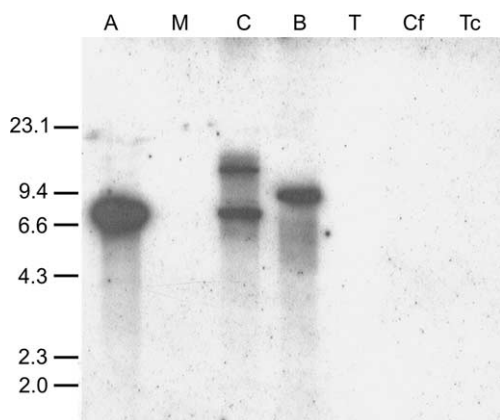
Fig. 4. Related sequences to La-PAT1 in *Leishmania* and trypanosomatids. Genomic DNA purified from *L. amazonensis* (A), *L. major* (M), *L. chagasi* (C), *L. braziliensis* (B), *L. tarentolae* (T), *C. fasciculata* (Cf) and *T. cruzi* (Tc) digested with *Hin* d III and hybridised with probe k5. Hybridisation washes were done at 65 °C in 0.1× SSC, 0.1% SDS.

L. major, *L. tarentolae*, *L. braziliensis*, *Crithidia fasciculata* and *T. cruzi*. Interestingly, positive hybridisation with probe k5 at high stringency was observed only with *L. chagasi* and *L. braziliensis* (Fig. 4). When the Southern blot assay was performed at low stringency, hybridisation bands were also evident in *L. major*, *L. tarentolae*, *C. fasciculata* and *T. cruzi* (data not shown) agreeing with similarity data obtained from sequence alignment.

4. Discussion

Amino acids are essential substrates in the metabolism of kinetoplastids. At least some amino acids have been defined for organisms of the genera *Leishmania* and *Trypanosoma* as major metabolites, as a source for energy production and as triggers to the differentiation process [21–23]. The relevance of the L-proline transport has been particularly well studied in *T. cruzi* [24,25] where it seems to work, together with other amino acids (mainly glutamate and aspartate) and glucose as a promoter of metacyclogenesis [26,27]. During this differentiation process, L-proline probably operates as a main fuel to energetically support cell remodelling [21]. Recently, L-proline and its transport systems have also been shown to be involved in *T. cruzi* intracellular differentiation [25]. Other amino acids are involved in major metabolic pathways in *T. cruzi* and *T. brucei*: an arginine kinase activity, for instance, reversibly converts arginine and ATP in phosphoarginine participating in the cell energy management and contributing to the survival of the parasite [28–30]. Whether amino acid transport systems have a similar role in *Leishmania* has not been established.

In the present work, we described for the first time a gene encoding a putative amino acid transporter in *L. amazonensis* (La-PAT1). The gene is conserved in other *Leishmania* species and trypanosomatids. Strong sequence conservation was demonstrated for the predicted sequence encompassing the *trans*-membrane domains while a highly variable region constitutes the predicted intracellular N-terminal domain.

Southern blot experiments suggest that La-PAT1 is present as a single copy per haploid genome. Interestingly, when the conceptual translation of La-PAT1 is used for searching the recently completed *L. major* genome database (GeneDB, www.genedb.org) 24 putative amino acid transporters produce high-scoring segment pairs (using a cut-off E value of 10^{-10}) suggesting that a great part of the *Leishmania* amino acid transporters belong to the same AAAP family. Accordingly, low stringency screening of the *L. amazonensis* cosmid library with probe k5 identified several positive cosmids potentially containing other members of the *L. amazonensis* transporter repertoire (data now shown).

Experimental results showed that the La-PAT1 gene is upregulated in amastigotes. Amino acids (particularly L-proline, very abundant in the vector's gut) have been shown to be a main source of energy generation for *Leishmania* promastigotes [31]. However, amastigotes also accumulate L-proline, although at a rate and extent up to 10-fold lower than promastigotes [32]. Different kinetic properties and pH sensitivity of the transport in promastigotes and amastigotes have suggested the presence of two distinct transporters with developmentally regulated expression. On the other hand, the active transport of L-proline in amastigotes, as well as in promastigotes, has been shown to be driven by proton motive force [8,32] and this activity might be particularly important to keep the intravacuolar environment acidic. So, the finding of a *L. amazonensis* putative transporter, upregulated in amastigotes and sharing characteristics with members of the AAAP family, which groups H^+/amino acid symporters [15], is supported by previous information in the literature. Further studies are in progress in order to characterise the activity of La-PAT1 and its relevance in the in vitro and in vivo infection by *L. amazonensis*.

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