Leishmania donovani is a protozoan parasite that exists as a free-living promastigote in the sandfly insect vector and as an amastigote inside the mammalian host macrophage phagolysosome compartment. The L. donovani A2 genes have been described previously as developmentally expressed in amastigotes but can be induced experimentally in promastigotes by a combination of pH and temperature shifts, conditions that mimic the phagolysosomal compartment of the macrophage cell. Considering the importance of the amastigote stage in human infections, we have examined the molecular basis for amastigote stage-specific gene expression. Our results provide evidence that A2 developmental expression during the promastigote-to-amastigote cytodifferentiation is mediated through differential RNA stability and involves the A2 mRNA 3'-untranslated region. The site of processing in the 3'-untranslated region was a major factor for the accumulation of A2 mRNAs in cells incubated under phagolysosomal conditions. The stability of reporter gene transcripts bearing the A2 3'-untranslated region was increased in cells incubated at low pH, further confirming the importance of pH shift as an inducer for A2 expression. These observations contributed to defining the mechanism of amastigote-specific gene regulation in L. donovani. We also demonstrated the feasibility of using the A2 locus to express heterologous genes differentially in the amastigote form of the L. donovani parasite.

Leishmania is a dimorphic protozoan that is responsible for a large spectrum of diseases in humans, ranging from self-curing skin ulcers to the severe pathologies associated with visceral leishmaniasis. This protozoan parasite exists as a flagellated promastigote in the sandfly vector, then as an intracellular amastigote in the mammalian host. The cytodifferentiation from the promastigote to the amastigote form occurs in the phagolysosomal compartment of the macrophage cell, and this transformation is a prerequisite for parasite survival. Amastigotes multiply within the mammalian host macrophages, and this stage of the life cycle is therefore responsible for the pathologies associated with leishmaniasis (for review, see Molyneux and Killlick-Kendrick (1987)). Leishmaniasis is considered by the World Health Organization to be one of the six major tropical diseases of developing countries (World Health Organization, 1993).

The Leishmania donovani A2 genes have been characterized previously as amastigote-specific (Charest and Matlashewski, 1994). The corresponding A2 protein is comprised predominantly of a highly conserved repetitive element and shares some characteristics with major antigens expressed developmentally by pathological forms of several other unrelated human parasites. Of particular relevance for this study, we have shown that the expression of the A2 transcripts could be induced experimentally in cultured promastigotes by a combination of temperature and pH shifts, conditions that mimic the passage from the insect vector to the phagolysosomal compartment of the macrophage cell (Charest and Matlashewski, 1994). The ability to induce A2 expression in vitro, together with the ability to transform and express exogenous DNA in Leishmania cells (Cruz and Beverley, 1990; Laban et al., 1990; Coburn et al., 1991; Curotto de Lafaille and Wirth, 1992; Beverley and Clayton, 1993) thus provides a suitable experimental framework to examine the mechanism of life cycle stage-specific gene expression in this protozoan.

To define the molecular mechanisms involved in the developmental expression of A2 transcripts in Leishmania during the promastigote-to-amastigote cytodifferentiation, the arrangement of the A2 genes within the L. donovani genome was determined. DNA sequences flanking the A2 protein coding region were then tested for their ability to modulate a developmental expression of reporter genes in transfected L. donovani cells. Transfection assays demonstrated that the 3'-untranslated region (3'-UTR\(^1\)) of the A2 mRNA could mediate a differential accumulation of reporter gene transcripts in L. donovani following induction of A2 expression by a combination of temperature and pH shifts. Moreover, reporter genes integrated into the A2 chromosomal locus by homologous recombination showed the same pattern of developmental expression as the A2 gene. Since stable DNA transfection has only been reported thus far with the promastigote stage in Leishmania, controlling gene expression using A2 untranslated sequences provides a unique system to allow amastigote stage-specific expression of transfected heterologous genes.

**MATERIALS AND METHODS**

Leishmania Strains and Culture Media—L. donovani donovani Sudanese 1S2D and L. donovani infantum Ethiopian LV9 promastigotes

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\(^1\) The abbreviations used are: UTR, untranslated region; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s); ORF, open reading frame.
were cultured at 26 °C in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% defined heat-inactivated fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and 20 μg M HEPES, pH 7.3. LV9 amastigotes were purified from infected Gold Syrian hamsters and passaged as described previously (Charest and Matlashewski, 1994).

Nucleic Acid Preparations and Analyses—Total RNA was extracted from promastigotes or the phagolysosomal products2 (Beverley and Clayton, 1993). Briefly, late log phase promastigotes of the L. donovani 152D strain were harvested by centrifugation (1,500 × g, 10 min) and resuspended in 1 M NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 4 mM ATP, 2 mM CTP, 2 mM GTP, 10 μM UTP, 10 μM phosphocreatine, 20 units/ml creatine kinase, 40 units/ml RNasin (Promega), 2 μg/ml diethiothreitol and then heated to 50 °C for 20 min. After cooling, an equal volume of nuclear run-on buffer (8 mM MgCl2, 80 mM Tris/HCl, pH 7.5, 0.1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, and 0.5% Triton X-100) and kept on ice for 10 min. The crude nuclei were then pelleted at 6,000 rpm for 10 min in a Beckman J-2-MC centrifuge (1 × 17 rotor) at 4 °C. The nuclei were washed once in lysis buffer without detergent and resuspended in an equal volume of 2 × nuclei storage buffer (100 mM Tris/HCl, pH 8.3, 80% glycerol, 10 mM MgCl2, and 0.2 mM EDTA). A 100–μl sample of nuclei (10%) containing buffer was mixed with an equal volume of nuclear run-on buffer (80 mM MgCl2, 80 mM Tris/HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 4 mM ATP, 2 mM CTP, 2 mM GTP, 10 μM UTP, 10 μM phosphocreatine, 20 units/ml creatine kinase, 40 units/ml RNasin (Promega), 2 μg/ml diethiothreitol and then denatured at 70 °C for 3 min and at 95 °C for 30 s, respectively. Primers used for the RT and PCR were: SPR, gacagcacaagacaacc (antisense, for the A2 gene); 28 MFX, cccgtgaacacagac (antisense, for the A2r gene); SL, caagctataagcatgctggacct (sense, splice leader sequence). To amplify the 5' end of the plasmid-derived transcripts, the following primers were used: 5' GATCTCT and 3' XhoI (antisense, 3' splice acceptor element (pSPYneo and pSPYPT, respectively). 3' ends of the plasmid-derived transcripts, the following primers were used: 5' GATCTCT and 3' XhoI (antisense, 3' splice acceptor element (pSPYneo and pSPYPT, respectively).

Assays were performed as described previously (Charest and Matlashewski, 1994). Cells were washed in cold phosphate-buffered saline and suspended at 4°C in lysis buffer (10 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 4 mM ATP, 2 mM CTP, 2 mM GTP, 10 μM UTP, 10 μM phosphocreatine, 20 units/ml creatine kinase, 40 units/ml RNasin (Promega), 2 μg/ml diethiothreitol and then denatured at 70 °C for 3 min and at 95 °C for 30 s, respectively. Primers used for the RT and PCR were: SPR, gacagcacaagacaacc (antisense, for the A2 gene); 28 MFX, cccgtgaacacagac (antisense, for the A2r gene); SL, caagctataagcatgctggacct (sense, splice leader sequence). To amplify the 5' end of the plasmid-derived transcripts, the following primers were used: 5' GATCTCT and 3' XhoI (antisense, 3' splice acceptor element (pSPYneo and pSPYPT, respectively). 3' ends of the plasmid-derived transcripts, the following primers were used: 5' GATCTCT and 3' XhoI (antisense, 3' splice acceptor element (pSPYneo and pSPYPT, respectively).

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shown to carry parts of two A2 gene copies: the 3' end of one copy, where the A2 cDNA mapped, followed by the 5' end of a second copy, in which the A2 protein coding region (A2/ORF II) was identified. To characterize the expression of the A2 gene, it was necessary to define further the genomic organization of the A2 genes by Southern and Northern blot analyses using fragments of the Geco 90 clone as probes (Fig. 1).

**Fig. 1. Genomic organization of A2 gene copies in two L. donovani strains.** Panel A, restriction map of the L. donovani Ethiopian LV9-derived Geco 90 EcoRI genomic clone and corresponding cDNAs. The Geco 90 insert contains parts of two A2 gene copies arranged head to tail. The A8 cDNA sequences overlap the EcoRI restriction site; the 5' end of A8 mapped on the 3' end of the genomic clone Geco 90 (Charest and Matlashewski, 1994). The open box represents the repeated section of the A2/ORF II protein coding region. Probe A (0.38-kb PstI fragment) was purified from the A2 cDNA plasmid (cDNA-inserted EcoRI/XhoI in pBluescript SK+; Charest and Matlashewski (1994)); probes B (0.5 kb), C (0.5 kb), and D (0.5 kb) were derived from Geco90 subclones; probe E consisted of a 1.1-kb BamHI fragment from the recombinant plasmid pET16b/ORFII (Charest and Matlashewski, 1994). E, EcoRI; M, SmaI; O, Xhol; P, PstI; S, SalI; X, XbaI; sl, spr, and 28mfx refer to synthetic oligonucleotides used as primers for RT-PCR RNA mapping (5' RACE); sl corresponds to the Leishmania spliced-leader; spr maps to the signal peptide coding sequence of the A2 protein product (Charest and Matlashewski, 1994); 28mfx was designed based on partial sequencing data from Geco 90 and was specific for A2rel. Further details concerning the RNA mapping are provided under "Materials and Methods." Panel B, Southern blot analyses. Genomic DNA restriction fragments were subjected to electrophoresis on a 0.7% agarose gel, transferred onto nylon membranes, and hybridized sequentially with probes A and E. L lanes are for L. donovani infantum LV9, and D lanes are for L. donovani donovani 1S2D. Panel C, karyotype analyses. Chromosomes of both L. donovani strains (LV9 and 1S2D) were hybridized with probe A. Pulsed field gel electrophoresis separated chromosomes stained with ethidium bromide in agarose gel prior to Southern blot are shown in the panel on the right. Y, Saccharomyces cerevisiae chromosomes used as molecular weight markers. Panel D, Northern blot analyses: effects of pH and temperature shifts on the expression of A2 and A2rel transcripts in L. donovani promastigotes. Total RNA was extracted from L. donovani donovani 1S2D promastigotes at several time points following their transfer from medium at pH 7.3 and 27°C into medium representing phagolysosomal conditions at pH 4.5 and 37°C (0, 3, 6, and 10 h). Ten-μg RNA samples were hybridized with probes prepared with fragment A (A2) or B (A2rel). Equal loading was verified by staining the denatured RNA with ethidium bromide in the agarose gel prior to Northern blot (shown on the bottom panel).
Fig. 1B; refer to Fig. 1A for probes) clearly showed that the repeated region within the A2 gene was responsible for most of the restriction fragment length polymorphism observed with other restriction digests. Analysis of the XbaI/XhoI double digest with probe E, which delineated the A2 coding regions, revealed a ladder of hybridization bands ranging from 1.6 to 3.0 kb. Similarly, other restriction fragments that contained this portion also produced a ladder of several hybridization bands. However, hybridization with fragment A as a probe revealed only a single band at around 3.4 kb, suggesting that A2 copies were indeed arranged similarly within the genome. Thus, the polymorphism was confined to the A2 coding regions. Restriction patterns and karyotypes were compared for two L. donovani strains used in this study: the infective L. donovani infantum Ethiopian LV9 strain, from which the A2 cDNAs and genomic clones were derived, and L. donovani donovani Suda- nese 1S2D, the noninfective strain used for subsequent transfection assays. The A2 gene loci showed a high degree of conservation between L. donovani LV9 and 1S2D strains. All of the A2 copies in both strains were contained on a 850-kb chromosome (Fig. 1C).

Northern blot analyses using the fragment B as a probe revealed another RNA encoding region (termed A2rel, 2.3 kb) lying between A2 sequences on genomic clone Geco 90. We compared the expression pattern of A2 and A2rel transcripts in promastigotes induced for an A2 developmental expression. We showed previously that a combination of temperature and pH shifts (26°C, pH 7.3, to 37°C, pH 4.5), conditions mimicking the transfer from the insect vector to the phagolysosomal compart- ment of the mammalian host macrophage cell, induces in cultured promastigotes full expression of A2 mRNAs within 10 h (Charest and Matlashewski, 1994). As shown in Fig. 1D, A2-specific mRNAs accumulated slowly in cells following trans- fer to the phagolysosomal conditions of 37°C and pH 4.5. In contrast, expression of the A2rel transcripts remained constant throughout the 10-h period. The two higher molecular weight transcripts recognized by the A2rel probe could represent mRNAs derived from copies bordering A2/A2rel clusters or from alternate trans-splicing and polyadenylation. Results of Southern blot analyses with XhoI fragments (Fig. 1B), which contained sequences of both A2 and A2rel, strongly suggest that copies of the two genes are always associated together on the genome, copies of A2 alternating head to tail with copies of A2rel. For the purpose of this study, the A2rel mRNA (2.3 kb) represented an excellent control for constitutive gene expres- sion in L. donovani.

Mapping of the A2 Transcripts—To define the sequences involved in A2 developmental expression, it was first necessary to map precisely the 5' and 3' ends of the A2 transcripts on the genomic sequences. The mapping of the 5' end was achieved using RT-PCR and DNA sequencing, and the results are in- cluded in Figs. 1A and 2A. The A2-specific antisense oligonucleotide (termed SPR) was used for cDNA synthesis and was subsequently combined for PCR with an oligonucleotide representing the spliced-leader sequence of L. donovani (SL), de- signed according to Wilson et al. (1991). The spliced-leader sequence is added by trans-splicing at the 5' end of all protein-encoding mRNAs in trypanosomatid cells (for review, see Clay- ton, 1992; Pays, 1993). Southern blot analyses of the PCR samples with an internal probe (probe D, Fig. 1A) revealed a single PCR product that was cloned and characterized. Eight clones containing the PCR product were isolated, and all displayed identical restriction enzyme patterns. The spliced- leader acceptor site was then located precisely by DNA se- quencing of the cloned PCR product and the corresponding sequences on the genomic clone Geco 90. In this manner, the A2

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Fig. 2. Panel A, schematic outlines of the A2/A2rel genomic organization and probes used for subsequent Northern blot analyses. The open box represents the repeated section of the A2/ORF II protein coding region. Arrows delineate the A2 and A2rel mature mRNA encoding regions, as determined by 5' and 3' mapping. The arrow pointing in the genomic DNA sequence shows the precise trans-splicing acceptor site for the wild-type A2 transcripts as determined by sequencing of the RT-PCR product as described under "Materials and Methods." The Pro and Tail sequences were used in subsequent plasmid constructs as shown in panel B. E, EcoRI; M, Smal; O, XhoI; P, PstI; S, SalI; X, XbaI. Panel B, schematic representation of the various DNA inserts present in the NEO plasmid series. The inserts represented the upstream (Pro) and/or downstream (Tail) untranslated sequences from the A2 gene. Refer to panel A for sizes and locations of the Pro and Tail elements. PYT refers to a synthetic trans-splicing acceptor site (92 bp). The outlined sequences were inserted into a plasmid vector in the orientation shown (5' to 3') as described under "Materials and Methods." See Fig. 3A for transcripts derived from these plasmid constructs.

trans-splicing acceptor site was located 214 nucleotides up- stream from the beginning of the A2 open reading frame as shown in Fig. 2A. The 3' end of the A2 transcript has been described previously (Charest and Matlashewski, 1994) and is also represented in Fig. 2A.

The 5' and 3' ends of the A2rel transcripts were also mapped by RT-PCR, cDNA cloning, restriction endonuclease, and partial sequencing.3 The locations of these ends on Geco 90 are also shown in Figs. 1A and 2A.

A2 Developmental Expression Involves the 3'-UTR—The genomic organization of A2 genes in L. donovani is outlined in Fig. 2A. As demonstrated in previous sections, the polymor- phism observed among A2 gene copies is restricted to the A2 protein coding regions, whereas untranslated regions were well conserved among gene copies. Based on RNA mapping results, the A2 open reading frame (ORF II) on the A2 mRNA is pre- ceded by a 214-bp 5'-UTR and is followed by a 2.0-kb 3'-UTR. With this information, it was possible to examine the involve- ment of the 5' and 3' regions flanking the A2 coding region (A2/ORF II) for their individual roles in controlling A2 gene expression in cells incubated under phagolysosomal conditions (37°C, pH 4.5).

A series of plasmids in which the neo gene was combined with 5'- and/or 3'-flanking regions of the A2/ORF II were con-

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3 H. Charest, W.-W. Zhang, and G. Matlashewski, manuscript in preparation.
structured; maps of plasmid inserts are schematized in Fig. 2B. The A2rel-A2 intergenic region (and the A2rel 3' region) were included as the A2 5'-flanking element (Pro element; 1.6 kb). Only 70 bp separated the XhoI restriction site from the translational initiating codon of the Geco 90/A2/ORF II. The 1.7-kb Tail element was constructed with sequences from the genomic clone Geco 90 and from the A9 cDNA. About 300 bp separated the translational termination codon of the A2/ORF II downstream from the XbaI restriction site. In these assays, neo was used as both the selectable marker and as a reporter gene. It has been demonstrated recently that the pSPneo plasmid that was used as the backbone for our constructs forms large oligomers that replicate autonomously in transfected Leishmania cells (Papadopoulou et al., 1994).

For each plasmid construct, polyclonal populations of stably transfected cells were established and analyzed for the differential expression of neo/A2 chimeric transcripts by Northern blot analyses. Polyclonal populations were used, rather than cloned cell lines, to average out variations that may have been displayed by selected clones. The relative abundance of neo mRNAs was compared in recombinant promastigotes cultured at 26°C, pH 7.4, or transferred into phagolysosomal conditions at 37°C and pH 4.5 for 10 h to induce full expression of A2 in cells. Results from the Northern blot analysis with the neo gene as a probe are presented in Fig. 3A. Duplicate membranes hybridized with A2 gene-specific probes confirmed that the reporter neo transcripts carried relevant sequences from the A2 5' end and/or the 3'-UTR, when these were included in the plasmid constructs (Fig. 3B).

Initially, these experiments were carried out with the plasmid constructs that lacked the pyt element at the end of the 3'-UTR (Fig. 2B, plasmids NEO T, NEO P, NEO P(-), NEO PT(-)). As shown in Fig. 3A, none of these plasmids could mediate a developmental expression of chimeric neo/A2 transcripts in the transfected cells. The sizes of these transcripts were consistent with polyadenylation occurring within the plasmid backbone sequence approximately 400 bp upstream from the pyt element (in the case of plasmid NEO T) or the Pro sequence (in the case of plasmids NEO P, NEO P(-), NEO PT(-)). Because of these initial negative results, the pyt element was added onto the 3'-UTR of plasmids NEO T and NEO PT(-), resulting in plasmids NEO T' and NEO PT'. The rationale was based on the previous observation (Lebowitz et al., 1993) that trans-splicing was linked to polyadenylation, and therefore the pyt element was included to allow trans-splicing to take place within the A2 3'UTR of transcripts derived from plasmids NEO T' and NEO PT'. As shown in Fig. 3A, plasmids NEO T' and NEO PT' did mediate developmental expression of chimeric neo/A2 transcripts under phagolysosomal conditions, and the sizes of these transcripts (around 2.3 kb) were consistent with 3' RNA processing involving the artificial trans-splicing acceptor site (pyt), which was added to the Tail element in these plasmid constructs.

Densitometric analyses using the A2rel hybridization for normalization estimated the differential expression, for both NEO T' and NEO PT', to be about five times (NIH Image, version 1.57). Results with other plasmid constructs varied between 0.75 and 1.3. These results demonstrate that the 3'UTR together with the pyt element present in plasmids NEO T' and NEO PT' is required for differential gene expression induced under phagolysosomal conditions.

The expression patterns of the A2 genes arising from chromosomal copies in transfected cells were masked by the high abundance of episome-derived neo/A2 chimeric transcripts (Fig. 3B). This clearly demonstrated that plasmid-derived A2 chimeric transcripts were much more abundant than were chromosome-derived A2 transcripts. To determine whether the
high levels of plasmid-derived transcripts carrying A2 sequences could influence the developmental expression of A2 chromosomal genes. A Northern blot analysis was performed with an A2/ORF II probe to reveal only chromosome-derived A2 transcripts. As shown in Fig. 3C, A2 gene developmental expression was still observed in induced NEO T' and NEO PT'-transfected promastigotes, suggesting that cellular factors involved in the developmental expression were relatively abundant in Leishmania cells and were not titrated out by chimeric neoA2 reporter gene transcripts.

Mapping the 5' and 3' Ends of Plasmid-derived Transcripts—The size of the neo transcripts derived from plasmids NEO T' and NEO PT' was consistent with RNA processing taking place at the artificial trans-splicing acceptor site (pyt) located at the 3' end of the Tail element. In this case, the artificial pyt element would have been removed from the mature transcript during the coupled trans-splicing/polyadenylation step (Lebowitz et al., 1993). To examine this possibility directly, the 3' end-containing region of the transcript arising from NEO T' and NEO PT' was cloned by RT-PCR, and the product was sequenced. The RT-PCR products and the sequence data for the NEO PT'-derived transcript are shown in Fig. 4. As expected, the pyt element was removed from these transcripts, and polyadenylation occurred about 400 bp upstream from the pyt element. This was also the site of polyadenylation in the NEO T'-derived neo/A2 chimeric transcript. This site of polyadenylation has never been detected in A2 cDNAs derived from wild type A2 mRNA (out of seven A2 cDNAs analyzed; Charest and Matlashewski (1994)). These results are consistent with the trans-splicing/polyadenylation model involving a cryptic polyadenylation site, as defined previously (Lebowitz et al., 1993). These data confirmed that the pyt element at the 3' end of the Tail sequence in NEO T' and NEO PT' was used for processing the 3' end of the transcripts arising from these plasmids.

We have also mapped the 5' end of the transcripts derived from the plasmids containing the Pro sequence as shown in Fig. 2, A and B, to determine which trans-splicing acceptor site was used. Mapping the 5' ends of these chimeric transcripts was performed by RT-PCR using an antisense oligonucleotide specific for the neo gene or the A2 Pro sequence and a sense oligonucleotide corresponding to the splice leader. The RT-PCR products were cloned and sequenced. The products of the RT-PCR and the sequence information are also shown in Fig. 4. These results demonstrate that the A2 trans-splicing acceptor site corresponding to nucleotide −214 (as shown in Fig. 2A) was used for processing the neo-containing transcripts derived from plasmids NEO P and NEO PT', which contained the A2 Pro sequence. The pyt element inserted between the Pro and the neo gene in the NEO P plasmid was not used for processing at the 5' end.

The preceding data demonstrate that A2 3'-UTR sequences and not the 5' upstream sequences were essential for the differential expression of the chimeric neo transcripts observed in promastigotes containing plasmids NEO T' and NEO PT' when subjected to phagolysosomal conditions. However, the 3'-UTR sequence was also included in NEO T' and NEO PT'(-) derived neo transcripts, but these were not expressed differentially when compared with the control A2rel transcript at 2.3 kb (Fig. 3A). The only difference between the NEO T'/NEO PT'(-) and NEO T'/NEO PT' constructs was the presence of the pyt element at the end of the A2 3'-UTR in plasmids NEO T'/NEO PT'. As shown in Fig. 4, this pyt element was involved in the processing of the 3' end of transcripts derived from plasmids NEO T' and NEO PT'. These data demonstrate that processing in the 3'-UTR was essential for the neo/A2 chimeric mRNA accumulation under phagolysosomal conditions. It is possible that a particular motif in the 3'-UTR could not form in the A2 3'-UTR when linked to plasmid backbone-derived sequences, as in the case of transcripts derived from NEO T' and NEO PT'(-). In conclusion, these experiments demonstrate the following. First, the A2 3'-UTR is involved in the developmental expression of A2 transcripts under phagolysosomal conditions. Second, the 3'-UTR must be processed by trans-splicing for the differential accumulation of A2 transcripts to occur in cells incubated under phagolysosomal conditions.

Mechanism of Regulation of A2 Transcripts in Phagolysosomal Conditions—We have begun to examine the molecular mechanism involved in A2 developmental expression under phagolysosomal conditions. Using nuclear run-on analysis, we assessed the RNA synthesis rates on the A2 and A2rel genomic loci. For this experiment, A2rel served as a control for constitutive gene expression. Nuclei were isolated from promastigotes cultured at 26 °C, pH 7.4, and from promastigotes incubated at 37 °C, pH 4.5, and incubated in the presence of [32P]UTP to allow elongation of transcripts. The radiolabeled nascent RNAs were extracted and used to probe the A2 and
A2rel gene sequence. As shown in Fig. 5, there was no change in A2 or A2rel RNA synthesis in promastigotes incubated at 37°C, pH 4.5, compared with promastigotes incubated at 26°C, pH 7.4. These results demonstrate that neither the A2 nor the A2rel gene is under transcriptional control.

We next tested the possibility that the A2 3′-UTR mediates an increased stability of transcripts in cells incubated under phagolysosomal conditions. It has been established previously that promastigotes maintained at pH 4.5 and 37°C will induce maximum A2 transcript accumulation, whereas promastigotes maintained at pH 7.3 and 37°C will not accumulate A2 transcripts (Charest and Matlashewski, 1994). Therefore, under these conditions, it is the reduced pH that mediates the accumulation of A2 transcripts, and these are the conditions under which we examined RNA stability. It is also noteworthy that because plasmid-derived transcripts are much more abundant that chromosome-derived transcripts, it is possible to detect readily the plasmid-derived neo/A2 chimeric sequences in promastigotes incubated at 26°C and neutral pH; this therefore represented the base line for these RNA stability experiments.

The stability of the neo/A2 mRNAs was compared in recombinant promastigotes incubated under promastigote conditions (neutral pH) or under phagolysosomal conditions (acidic pH). In these assays, promastigotes carrying plasmids NEO T or NEO PT cultured at 26°C and pH 7.3 were placed in actinomycin D (10 μg/ml) containing medium at 37°C, and pH 4.5 (lanes A) or pH 7.3 (lanes N). Total RNA was extracted from cells after 0, 1, 3, and 5 h following the addition of actinomycin, and 10-μg samples were hybridized with a neo-specific probe. Equal loading was verified by staining the RNA samples on agarose gels before transfer onto nylon membranes. Densitometric analyses were carried out using NIH Image version 1.57 software. For each pH condition, values are expressed as percentages of the amount at time 0. These results have been reproducible in two separate experiments.

Targeting the neo Gene into the A2 Gene Coding Locus—In the previous sections it was demonstrated that the developmental expression of neo/A2 chimeric mRNAs relied on processing of the A2 3′-UTR. With our series of plasmid constructs, this processing of the 3′-UTR was mediated by an artificial trans-splice acceptor site (pyt) inserted downstream of the A2 3′-UTR. To address directly the question of whether the A2-A2rel intergenic region would mediate developmental expression of a heterologous gene, we analyzed the pattern of expression of the neo reporter gene when targeted into the A2 locus. The strategy used to insert the neo gene into the A2 locus by homologous recombination is outlined in Fig. 7A. Linear Sall/Stul fragments containing the neo gene flanked by 1.0 kb of the Pro and 0.8 kb of the Tail sequences were excised from NEO PT and NEO PT(−) plasmids, and 20 μg of agarose gel-purified DNA was electroporated into promastigotes. Compared with NEO PT(−) (Fig. 2), the NEO PT plasmid contained a pyt element between the Pro sequences and the neo gene. Recombinant cells were selected with minimal doses of G418 (8 μg/ml), and monoclonal populations (R1 and R2 cell lines) were established by
limiting dilutions. Insertions of neo into the A2 locus were confirmed by Southern blot and karyotypic analyses (Fig. 7, B and C). The PstI site within the neo sequence created new PstI restriction fragments recognized by the A2 5'-specific probes (Fig. 7B). These PstI fragments differed in lengths by the pyt element (92 bp) present only in the NEO PT construct. In both R1 and R2, the neo3 probe revealed a 1.7-kb PstI fragment, as expected of a chimera of neo and A2 3' sequences. As expected, karyotype analysis shows that the neo gene was present on the 850-kb chromosome, which is where the A2 locus is located (Fig. 7C). These results are consistent with gene replacement events.

Northern blot analyses were performed to determine whether the integrated neo gene could be expressed developmentally. As shown in Fig. 7D, the neo/A2 chimeric transcripts were expressed developmentally following induction by pH and temperature shifts for both the R1 and R2 cell lines. The sizes of transcripts corresponded to a processing similar to transcripts from the A2 locus. The neo transcripts in R2 cells were slightly larger than in R1 cells, a result consistent with the presence of the pyt element in the mRNA. As for plasmid-derived transcripts, this artificial trans-splicing acceptor site added between the Pro fragment and the neo gene was not used for RNA processing. These data demonstrate that the A2-A2rel
intergenic region could mediate the developmental expression of the neo gene when targeted into the A2 locus.

**DISCUSSION**

We have previously isolated and characterized the A2 gene by virtue of its developmental expression in the amastigote stage of the *L. donovani* parasite, the causative agent of visceral leishmaniasis. A2 transcripts are expressed in infected macrophages, and expression can be induced experimentally in promastigotes by pH and temperature shifts, conditions mimicking the passage from the insect vector to the phagolysosome environment (Charest and Matlashewski, 1994). Genes such as A2 which are developmentally expressed in the amastigote stage may encode products necessary for the establishment and progression of the infection in the mammalian host. Defining the mechanism in which amastigote-specific genes are regulated will contribute to understanding the molecular basis for infection with this important human parasite.

The A2 gene system represents an excellent model to study regulation of amastigote-stage-specific gene expression in *Leishmania* cells. The key advantage for this system is that the A2 gene can be induced experimentally in promastigotes by culturing them under phagolysosomal conditions. Several major observations have been made in this study. Of particular significance, it was demonstrated that both the A2 mRNA 3′-UTR and its processing by the trans-splicing machinery were required for developmental expression under phagolysosomal conditions. It was also demonstrated that the developmental accumulation of A2 transcripts was regulated at the post-transcription level and that the 3′-UTR could mediate an increase in transcript stability in phagolysosomal conditions. These observations define mechanisms involved in amastigote-specific gene expression in *Leishmania*. Finally, the feasibility of targeting genes into the A2 locus to obtain a differential expression of heterologous genes in the amastigote stage was also demonstrated. The A2 locus would therefore be particularly suitable to control the expression of negative selection genes and may therefore establish the basis of a live attenuated vaccine.

During this study, we have examined wild type A2 expression as well as expression of neo/A2 chimeric transcripts derived from plasmid constructs and from gene replacement constructs. It was of interest to compare the level of induction of the wild type A2 transcripts with the neo/A2 chimeric transcripts under phagolysosomal conditions. The level of endogenous wild type A2 transcript induction in phagolysosomal conditions was variable between experiments but was at least 10-fold. In comparison, the neo/A2 chimeric transcript derived from the gene-targeted construct had a 10-fold induction, and from the plasmid constructs NEO T’ and NEO PT’, there was a 5-10-fold induction depending on the experiment. Although there is clearly variability between experiments, the plasmid-derived neo/A2 chimeric transcript did not appear to be as tightly controlled as the wild type A2 transcript or the gene replacement-derived neo/A2 chimeric transcript. We believe this is because of the higher background level of transcript derived from the plasmid episome compared with the chromosome-derived transcript. Alternatively, this may be due to the use of the cryptic polyadenylation site used in the NEO T’- and NEO PT’-derived neo/A2 chimeric transcripts. Nevertheless, the plasmid constructs did define the 3′-UTR as being essential for developmental expression. Future studies will define the relevant regulatory sequence within the 3′-UTR and the method in which this sequence mediates an increase in RNA stability in phagolysosomal conditions.

This is the first study carried out in *Leishmania* which directly compared the involvement of noncoding 5′- and 3′-flanking sequences for their contribution in regulating the accumulation of transcripts in amastigotes. The results provide unique insight into the involvement of the 3′-UTR in determining the relative levels of transcripts in amastigotes. A previous study examining the up-regulation of hsp 83 transcripts in *Leishmania* mexicana amastigotes likewise revealed a post-transcriptional regulation, but the individual contributions of the 5′- and 3′-flanking noncoding sequences were not defined (Argaman et al., 1994). More recently however, data have been presented showing that mRNAs encoding hsp 70 and hsp 83 in *Leishmania* major and *L. donovani* do not increase significantly following heat shock (Brandau et al., 1995). In a separate study (Flinn and Smith, 1992), a gene family that was up-regulated in metacyclic *L.* major promastigotes was shown to have conserved 3′-sequence elements, suggesting that these elements may be involved in the regulation of this gene family. Consistent with the data presented in this study, it has been demonstrated that the intergenic regions play a major role in the processing of polycistronic transcripts into polyadenylated mature mRNAs in *Leishmania* cells (Lebowitz et al., 1993). The data presented in this study now demonstrate that the 3′-intergenic region also plays an essential role in the increased accumulation of transcripts in the amastigote stage.

Gene expression in trypanosomatid cells involves the maturation of polycistronic precursor RNAs into monomeric units by trans-splicing, a molecular process restricted to lower eukaryotes (for review, see Bonen, 1993; Muhich and Boothroyd, 1988; Walton, 1987). Previous work on trypanosomes (Trypanosoma brucei) reported that transcript accumulation in the bloodstream form can be regulated at both the transcriptional and the post-transcriptional levels (for review, see Clayton, 1992; Pays, 1993). It has been demonstrated that the nature of the 3′-UTR was crucial for the accumulation of reporter transcripts in the bloodstream form (Jeffery et al., 1991; Hug et al., 1993). More recently, it has been reported that the 3′-UTR was involved in the regulation of amastigote-specific transcripts in *Trypanosoma cruzi* (Teixeira et al., 1995). Thus, processing of mRNAs within the 3′-UTRs may be an important factor in modulating the relative amounts of transcripts in trypanosomatids.

We have also characterized the A2 locus and shown that the A2 genes were arranged in tandem repeats, alternating with copies of the A2rel gene. Although A2 transcripts could be induced by temperature and pH shifts, the A2rel transcript level remained constant. Since genes repeated in tandem are often part of the same transcriptional unit in *Leishmania* (Bock and Langer, 1993; Flinn and Smith, 1992; Huang et al., 1984; Landfear et al., 1983; Lee et al., 1988; Ramamoorthy et al., 1992; Shapira and Pinelli, 1989), then the relative stability of the transcripts could be responsible for regulating the relative levels of A2 and A2rel transcripts in promastigotes and amastigotes. The data presented in this study showing the increased stability of reporter transcripts containing the A2 3′-UTR in cell cultures at 37°C and pH 4.5 are consistent with this model.

It is noteworthy that in the RNA stability experiments, the increased stability of the neo/A2 chimeric transcripts at pH 4.5 was transient. One hour after the addition of actinomycin D, the neo/A2 chimeric transcripts were degraded at similar rates in cells cultured at pH 4.5 or 7.3. This argues that the factors involved in the increased stability interacted rapidly in response to the pH shift but were relatively short lived. A rapid turnover of these factors when transcription was blocked with actinomycin D may have been responsible for this effect. Future experiments must be carried out to define the mechanism in which the 3′-UTR mediates an increase in transcript stabili-
ity and the sequences within the 3'-UTR involved in the stabilization.

It was also demonstrated that in two strains of L. donovani examined the A2 genes were located on a single chromosome of 850 kb. Polymorphism within the A2 cluster was related to the protein coding sequence, which is composed of repetitive units. We have now confirmed that there is a family of A2-related proteins ranging from 45 to 100 Kd which are only present in the amastigote stage (Zhang et al., 1996). Methods involving genetargetingwill be important in future studies to define the biological function of the A2 protein in the infection process and to determine the role it plays in the virulence of L. donovani. The present work describing the structure and regulation of the A2 genes will help in developing these future studies.

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