Transcriptional Mapping of the Amplified Region Encoding the Dihydrofolate Reductase-Thymidylate Synthase of *Leishmania major* Reveals a High Density of Transcripts, Including Overlapping and Antisense RNAs

GEOFFREY M. KAPLER1,2 AND STEPHEN M. BEVERLEY1*

Department of Biological Chemistry and Molecular Pharmacology1 and Department of Genetics,2

Harvard Medical School, Boston, Massachusetts 02115

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We have examined the transcriptional organization of the R region of the protozoan parasite *Leishmania major*. This region encodes the bifunctional enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) and is frequently amplified as a 30-kilobase (kb) extrachromosomal circular DNA in methotrexate-resistant lines. Northern (RNA) blot analysis shows that the R region encodes at least 10 stable cytoplasmic polysomal poly(A)+ RNAs, ranging in size from 1.7 to 13 kb and including the 3.2-kb DHFR-TS mRNA. Transcriptional mapping reveals that these RNAs are closely spaced and collectively cover more than 95% of the 30-kb amplified R region. The organization is complex, including several overlapping RNAs' 3' of DHFR-TS and two examples of antisense RNAs' 5' of DHFR-TS. The R region RNAs can be grouped into two empirical domains, with eight contiguous RNAs transcribed in the same direction as that of DHFR-TS and two contiguous RNAs transcribed in the orientation opposite to that of DHFR-TS. The two 5'-most RNAs of the DHFR-TS-containing domain overlap the RNAs transcribed from the opposite strand. These data are relevant to models of transcription, including recent studies suggesting polycistronic transcription in trypanosomatids. The abundance of R region RNAs increases uniformly 10- to 15-fold in the amplified R1000-3 line relative to the wild type, and no new RNAs were observed. This suggests that all elements required in *cis* for DHFR-TS expression are contained within the 30-kb circular DNA. Quantitative analysis reveals that the steady-state DHFR-TS mRNA and protein levels are not growth phase regulated, unlike the monofunctional mouse DHFR. DHFR-TS is developmentally regulated, however, declining about fivefold in lesion amastigotes relative to promastigotes.

Trypanosomatid protozoan parasites comprise a group of eucaryotic organisms which use a number of unconventional strategies for gene expression. These include obligatory trans-splicing of a common nucleotide sequence, the mini-exon, onto the 5' ends of all mRNAs (17, 57, 63, 70, 74; reviewed in 11), as well as editing of mitochondrial mRNAs (4, 26). An increasing body of data suggests that some protein-coding mRNAs may be synthesized as larger polycistronic precursors, up to 60 kilobases (kb) in length, which are subsequently cleaved and processed into mature mRNAs. Evidence supporting this view includes the following: (i) determination of the size of a variant surface glycoprotein gene transcriptional unit by UV inactivation (38); (ii) demonstration of comparable transcription rates for flanking, mRNA-coding, and intergenic regions (30, 46, 72); and (iii) the identification of a putative polycystronic mRNA precursor (56). Whether this process is ubiquitous and what DNA sequences are involved in transcription initiation and mRNA processing remain to be determined because of the lack of striking sequence homology with consensus eucaryotic elements and the lack of in vitro or in vivo functional assays such as DNA transfection.

We have examined the gene encoding the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) (8, 31) of *Leishmania major* (order, Kinetoplastida; family, Trypanosomatidae) in order to gain insight into the genetic organization of a housekeeping gene in these parasites. The study of this trypanosomatid gene offers several advantages: monofunctional DHFR and TS genes and proteins have been characterized in numerous prokaryotic and eucaryotic species, since these enzymes are useful targets for chemotherapy and genetic analysis. Much is known about the regulation of monofunctional DHFR and TS enzyme and mRNA levels, the structure of the mature mRNAs (which are often heterogeneous), and the *cis*-acting elements required for their expression (19, 24, 29, 36, 37, 41, 48, 50, 58). A second advantage is that the leishmanial DHFR-TS gene is contained within a 30-kb segment of DNA (the R region) which is frequently amplified in the form of an extrachromosomal circular DNA in antifolate-resistant mutants (reviewed in 9).

In R region-amplified lines, DHFR-TS protein and mRNA levels are elevated 10-fold or more, thereby facilitating analysis of these relatively scarce molecules (14, 28, 39, 75). A final advantage is that all *cis*-acting sequences necessary for DHFR-TS expression, replication, and segregation are likely to reside on the 30-kb circular amplified DNA.

Previous work on R region transcription has shown that mature DHFR-TS mRNAs are heterogeneous at their 5' ends, as is commonly observed for the monofunctional DHFR and TS mRNAs (39). DNA sequence analysis of the upstream region did not reveal striking homology to upstream sequences genetically shown to provide promoter function in DHFR and TS genes of other species nor to upstream regions of other trypanosomatid genes. This raised the possibility that the DHFR-TS promoter could be located further upstream. In this paper we have expanded our perspective of the transcriptional organization of the R

* Corresponding author.
region by using Northern (RNA) blot analysis to map and study the regulation of stable transcripts throughout the R region. We have also examined the growth phase and developmental regulation of the DHFR-TS mRNA and protein. *Leishmania* spp. are digenetic parasites, alternating between the extracellular flagellated promastigote stage borne within the digestive tract of the sand fly vector (the culture form closely resembles this stage) and the intracellular amastigote form residing within the macrophage phagolysosome of the vertebrate host. They are widespread tropical parasites which cause a spectrum of pathology ranging from mild cutaneous lesions to lethal visceral forms (76).

**MATERIALS AND METHODS**

Preparation of promastigotes and amastigotes. The wild-type LT252 line of *L. major* (10, 20) and its methotrexate (MTX)-resistant derivative R1000-3 line bearing R region amplification (7) were studied. Promastigotes were propagated in M199 medium as previously described (14). The medium was supplemented with 1 mM MTX for the R1000-3 line. Stationary-phase cells were harvested 48 h after reaching maximal cell density. Cell viability was >95% on the basis of light microscopic examination of morphology and motility.

Intracellular amastigotes of the wild-type LT252 strain were obtained from lesions initiated by intradermal inoculation of 2 × 107 stationary-phase promastigotes into the dorsal hind foot of BALB/c mice. Amastigotes were purified from the infected tissues by Percoll gradient centrifugation five to six months after infection (35).

**RNA isolation and quantitation.** Total cellular RNA was prepared by lysing parasites in 5 M guanidinium isothiocyanate; this procedure was followed by precipitation of RNA with 4 M lithium chloride (12). Cytoplasmic RNA was prepared by lysing parasites with 1% Nonidet P-40, and nuclei were pelleted through sucrose as previously described (25). Poly(A)+ RNA was prepared by chromatography on oligo(dT)-cellulose (3) and was quantitated by hybridization to [3H]poly(U) (66). Total RNA was quantitated by UV absorbance and methylene blue staining of Northern blot filters prior to hybridization (49).

**RNA filter hybridization.** RNA preparations were analyzed by Northern blotting as previously described (39), with the following modification: RNA was bound to GeneScreen Plus membranes (DuPont, NEN Research Products) by either baking for 3 h at 80°C or by cross-linking with UV light for 5 min (13). Double-stranded DNA probes were radioactively labeled with [α-32P]dCTP by using random oligonucleotides to prime synthesis (27). Strand-specific DNA probes were prepared from recombinant M13 clones by using either single-stranded M13 DNA templates and DNA sequencing primers or linearized replicative forms and reverse sequencing primers as previously described (New England BioLabs, Inc.; 52). Strand-specific RNA probes were prepared by using [α-32P]GTP, T3 or T7 RNA polymerase, and template DNA cloned into a Bluescript vector (Stratagene; 51).

**S1 nuclease mapping.** S1 nuclease mapping was performed as previously described (5, 39) with 5 μg of R1000-3 total cellular poly(A)+ RNA and DNA restriction fragments whose 5' ends were labeled with T4 polynucleotide kinase and [γ-32P]ATP or whose 3' ends were labeled with terminal deoxynucleotidyl transferase and [α-32P]dATP. Protected fragments were separated on 4% acrylamide–8 M urea gels.

**Hybrid selection.** Gel-purified DNA fragments were covalently attached to diazobenzylisoxymethyl paper as previously described (69). A 20-μg portion of poly(A)+ RNA from R1000-3 total cellular RNA preparations was annealed to DNA-containing filters at 42°C for 15 h in 50% formamide–0.1% sodium dodecyl sulfate–4× SET (1× SET is 20 mM Tris [pH 7.8]–1 mM EDTA–120 mM NaCl); filters were then washed repeatedly in the above solution at 34°C, and bound RNA was eluted in 100 mM Tris (pH 7.8)–90% formamide at 67°C for 15 min (53).

**Polysome isolation and characterization.** Log-phase promastigotes were suspended at a concentration of 107/ml in ice-cold lysis buffer (50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.4], 0.3 M KCl, 10 mM MgCl2, 10 mM diethiothreitol, 10 μg of cycloheximide per ml, 10 μg of heparin per ml, 50 U of RNAsin per ml [Sigma Chemical Co.]) and lysed by addition of 0.1 volume of 10% Triton X-100 in lysis buffer (15). Nuclei were removed by centrifugation for 5 min at 12,000 × g, and 200 μl of postnuclear supernatant was centrifuged for 1.5 h at 150,000 × g on an 11-ml 15 to 50% sucrose gradient containing 50 mM HEPES (pH 7.4), 150 mM KCl, 40 mM MgCl2, 10 mM diethiothreitol, 10 μg of cycloheximide per ml, and 5 U of RNAsin per ml. For EDTA release experiments, the postnuclear supernatant was incubated for 5 min with EDTA at a final concentration of 30 mM prior to centrifugation. Several attempts to obtain puromycin release in vitro were unsuccessful with 10 mM puromycin, which rapidly and completely inhibited promastigote growth and protein synthesis in vivo. Gradient fractions were monitored by UV absorbance and combined into four pools: (i) free RNA, (ii) monosomes, (iii) small polysomes (2- to 7-mers), and (iv) large polysomes (>7-mers), extracted sequentially with phenol-chloroform (1:1) and chloroform, and precipitated with ethanol. Resuspended pools were denatured for 15 min in 50% formamide–6% formaldehyde at 67°C and transferred to GeneScreen Plus membranes (DuPont, NEN Research Products) by using a slot blot apparatus (Schleicher & Schuell, Inc.).

**DHFR-TS protein determination.** Cell extracts were prepared as previously described (14) and were assayed for DHFR-TS by quantitative binding of high-pressure liquid chromatography-purified [3H]MTX (Amersham Corp.; purified and specific activity determined as described in reference 22). Total protein concentrations were determined after precipitation with trichloroacetic acid (65).

**RESULTS**

**Northern blot analysis.** Hybridization of cytoplasmic total and poly(A)+ RNAs from the wild-type and R1000-3 lines with a probe encompassing the complete 30-kb R region (pK300; 9) revealed at least seven polyadenylated, cytoplasmic RNAs in both wild-type and R1000-3 *L. major*, ranging in size from 1.7 to 13 kb and including the 3.2-kb DHFR-TS mRNA (Fig. 1A; 39; a larger hybridizing band evident in the R1000-3 preparation shown in this figure was not observed in most preparations). Only four of these RNAs had been detected previously by other workers (75). The hybridization patterns of wild-type and R1000-3 RNAs were identical, except for being uniformly increased 10- to 15-fold in the R1000-3 line, as shown by quantitative Northern blotting with a DHFR-TS-specific probe (Fig. 1B). This showed that the sizes and relative levels of the RNAs were not affected by the DNA rearrangement created by amplification of the R region (Fig. 1C; the hatched boxes correspond to the rearrangement sites which are joined to give the circular R region map shown in Fig. 9).
FIG. 1. Identification and quantitation of R region RNAs in wild-type and R1000-3. L. major. (A) Identification of RNAs. Cytoplasmic RNAs from R1000-3 and wild-type L7252 promastigotes were analyzed by Northern blotting with the radiolabeled probe pK300 (Table 1), which contains the entire amplified R region (6). T. Total RNA (8 μg); A−, poly(A)− RNA (8 μg); A+, poly(A)+ RNA (150 ng). Exposure times: R1000-3, 20 h; wild type, 60 h. The sizes of the RNAs visualized are shown on the left. Estimated from molecular weight markers consisting of mouse and Leishmania ribosomal RNA and HindIII-digested bacteriophage lambda DNA. (B) Quantitation of DHFR-TS RNA levels. Northern blot analysis of total cellular R1000-3 and wild-type (WT) RNA was performed using the DHFR-TS-specific probe pRBgl10 (Table 1). A 10-μg portion of wild-type RNA and a twofold dilution series starting with 10 μg of R1000-3 RNA (lanes labeled 1, 1/2, etc.) were electrophoresed. (C) Restriction map of the wild-type chromosomal R region. The heavy line indicates sequences which are amplified in the R1000-3 line. Hatched boxes correspond to the regions which are joined to form the circular amplified R region (shown in Fig. 9). The DHFR-TS protein-coding region is indicated by the stippled box immediately below the restriction map. Map coordinates (in kb) are displayed above the restriction map, with the 0 coordinate corresponding to the DHFR-TS start codon (39). E. EcoRI; Bg, BglII; H, HindIII. The R region was divided into three subregions for RNA localization: the downstream, DHFR-TS, and divergent RNA subregions. Detailed restriction maps for each subregion can be found in Fig. 2B, 3B, and 3B, respectively.

TABLE 1. Nomenclature and probe description

| Probe name | Description* | Map location | Strand specificity*
<table>
<thead>
<tr>
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<tr>
<td>pK300</td>
<td>Entire 30-kb R region</td>
<td>+8.5 to -21.5</td>
<td>—</td>
</tr>
<tr>
<td>pRBgl10</td>
<td>0.9-kb BglII-EcoRI</td>
<td>+0.65 to -0.25</td>
<td>—</td>
</tr>
<tr>
<td>2a</td>
<td>3.4-kb BglII-BglII (pBG34L)</td>
<td>+7.4 to +4.0</td>
<td>—</td>
</tr>
<tr>
<td>2b</td>
<td>3.4-kb BglII-BglII (pBG34L-I)</td>
<td>+7.4 to +4.0</td>
<td>—</td>
</tr>
<tr>
<td>2c</td>
<td>3.2-kb EcoRI-BglII** (pRBgl32)</td>
<td>+10.6 to +7.4</td>
<td>—</td>
</tr>
<tr>
<td>2d</td>
<td>0.5-kb Sphl-ApaI′</td>
<td>+8.7 to +8.2</td>
<td>—</td>
</tr>
<tr>
<td>2e</td>
<td>1.0-kb BglII-PsrI</td>
<td>+7.4 to +6.4</td>
<td>—</td>
</tr>
<tr>
<td>2f</td>
<td>1.8-kb PstI-PsrI</td>
<td>+6.1 to +4.3</td>
<td>—</td>
</tr>
<tr>
<td>2g</td>
<td>0.3-kb PstI-BglII</td>
<td>+4.3 to +4.0</td>
<td>—</td>
</tr>
<tr>
<td>2h</td>
<td>0.5-kb BglII-Sall</td>
<td>+4.0 to +3.5</td>
<td>—</td>
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<tr>
<td>2i</td>
<td>0.6-kb Sall-Sphl</td>
<td>+3.5 to +2.9</td>
<td>—</td>
</tr>
<tr>
<td>2j</td>
<td>0.3-kb Sphl-SsrI</td>
<td>+2.9 to +2.6</td>
<td>—</td>
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<tr>
<td>2l</td>
<td>1.3-kb Sphl-Sphl</td>
<td>+2.9 to +1.6</td>
<td>—</td>
</tr>
<tr>
<td>3b</td>
<td>1.9-kb Sphl-Sphl</td>
<td>+1.6 to -0.26</td>
<td>—</td>
</tr>
<tr>
<td>3c</td>
<td>0.7-kb EcoRI-Sall</td>
<td>-0.25 to -1.0</td>
<td>—</td>
</tr>
<tr>
<td>3d</td>
<td>1.0-kb Sall-BamHI</td>
<td>-1.0 to -2.0</td>
<td>—</td>
</tr>
<tr>
<td>3e</td>
<td>2.8-kb EcoRI-Palu (pRK28)</td>
<td>-2.3 to -5.1</td>
<td>—</td>
</tr>
<tr>
<td>3f</td>
<td>6.0-kb KpnI-HindIII (pKH60)</td>
<td>-5.1 to -1</td>
<td>—</td>
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<tr>
<td>3g</td>
<td>5.8-kb BglII-Palu</td>
<td>+0.7 to -5.1</td>
<td>f</td>
</tr>
<tr>
<td>4</td>
<td>2.8-kb SsrI-EcoRI</td>
<td>+2.6 to -0.25</td>
<td>—</td>
</tr>
<tr>
<td>5a</td>
<td>4.5-kb HindIII-HindIII (pH45)</td>
<td>-11.1 to -15.6</td>
<td>—</td>
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<tr>
<td>5b</td>
<td>3.2-kb Sall-Sall (pS32)</td>
<td>-15.3 to -18.5</td>
<td>—</td>
</tr>
<tr>
<td>5c</td>
<td>5.0-kb Sall-Sall** (p50)</td>
<td>-18.5 to -22.5</td>
<td>—</td>
</tr>
<tr>
<td>5d</td>
<td>1.4-kb Sphl-SsrI</td>
<td>-11.9 to -13.3</td>
<td>—</td>
</tr>
<tr>
<td>5f(l)</td>
<td>0.8-kb Sphl-Sphl</td>
<td>-13.0 to -13.8</td>
<td>f</td>
</tr>
<tr>
<td>5f(r)</td>
<td>0.8-kb Sall-Sphl</td>
<td>-13.0 to -13.8</td>
<td>f</td>
</tr>
<tr>
<td>5(t)</td>
<td>0.6-kb Sphl-Sphl (mSp5.5)</td>
<td>-13.8 to -14.4</td>
<td>f</td>
</tr>
<tr>
<td>5(f)</td>
<td>0.6-kb Sphl-Sphl (mSp5.5)</td>
<td>-13.8 to -14.4</td>
<td>f</td>
</tr>
<tr>
<td>5(t)</td>
<td>0.6-kb Sphl-Sphl (mSp5.5)</td>
<td>-13.8 to -14.4</td>
<td>f</td>
</tr>
<tr>
<td>5(g(l))</td>
<td>0.4-kb PstI-SsrI (pMT4)</td>
<td>-14.4 to -14.8</td>
<td>f</td>
</tr>
<tr>
<td>5(g(r))</td>
<td>0.4-kb PstI-SsrI (pMT4)</td>
<td>-14.4 to -14.8</td>
<td>f</td>
</tr>
<tr>
<td>5(h)</td>
<td>0.7-kb SsrI-HindIII (mTH7)</td>
<td>-14.8 to -15.5</td>
<td>f</td>
</tr>
<tr>
<td>5(i)</td>
<td>0.9-kb Sall-PsrI (mSP9)</td>
<td>-15.3 to -16.2</td>
<td>f</td>
</tr>
<tr>
<td>5(i)</td>
<td>0.9-kb Sall-PsrI (mSP9)</td>
<td>-15.3 to -16.2</td>
<td>f</td>
</tr>
<tr>
<td>5(j)</td>
<td>1.1-kb PstI-PsrI</td>
<td>-16.2 to -17.3</td>
<td>—</td>
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* Fragment size and restriction site endpoints of probes. Plasmid and M13 clones (p and m designations in parentheses) are listed on the right. All other probes are subfragments of plasmid or M13 clones.

† Direction of transcription of hybridizing mRNAs: 1 and r, leftward and rightward, respectively (see map, Fig. 1C). — Double-stranded probes.

‡ RNA probe prepared by T7 polymerase-directed transcription in a Blue-script vector.

§ Contains both amplified and nonamplified DNA sequences.

| Contains a repetitive element which recombines with the opposite end of the R region to generate the circular R1000-3 amplification.

′ DNA synthesis primed by strand-specific oligonucleotide.

To map the R region transcripts, a series of smaller hybridization probes encompassing the entire R region were used in Northern blot analysis of cytoplasmic R1000-3 poly(A)+ RNA (see Fig. 2 through 5). The sizes of all RNAs identified by these smaller probes were identical in the R1000-3 and wild-type RNA preparations (data not shown).

The physical map of the wild-type chromosomal R region is shown in Fig. 1C (7), with position 0 being the start codon of the DHFR-TS protein and increasing in the direction of DHFR-TS transcription. The segment which forms the circular amplified R region DNA is indicated by the heavy line. The names, map locations, and strand specificities of these probes are summarized in Table 1 and are diagramed in subsequent figures. For clarity in presenting RNA mapping data obtained with nearly 40 hybridization probes, the R region has been divided into three arbitrary subregions (Fig. 1C): (i) the downstream subregion 3′ of the DHFR-TS mRNA; (ii) the central DHFR-TS subregion; and (iii) the divergent RNA subregion 5′ of the DHFR-TS mRNA (so-
Transcripts of the downstream subregion. The downstream subregion spans from about position +9 to +3 on the R region map (Fig. 1C); the restriction map of this subregion and hybridization probes used for Northern blot analysis are diagramed in Fig. 2B. Probe 2a, centrally located within the downstream subregion, identified four RNAs of 3.2, 2.8, 2.5, and 2.3 kb (Fig. 2A). As shown below, the 3.2-kb RNA identified by this probe is distinct from the 3.2-kb RNA encoding DHFR-TS. All of these RNAs are transcribed in a leftward direction, since they hybridized to the strand-specific probe 2b (Fig. 2A) and not to an opposite-strand probe (data not shown). Probes 2c through 2j (Fig. 2B), proceeding progressively from the left, more precisely localized these RNAs within the downstream subregion. Probe 2c, which includes the left-end rearrangement site of the R region amplification, contains both amplified R region sequences and nonamplified flanking sequences; this probe identified the 2.3- and 2.5-kb RNAs. Probe 2d, a 0.5-kb subfragment of probe 2c, also identified the 2.3- and 2.5-kb RNAs. Southern blot hybridization and DNA sequencing have shown that probe 2d contains a repetitive element which is also present at the opposite end of the R region amplification (corresponding to the hatched region shown in Fig. 1C); the two repetitive elements are joined together to generate the circular amplified R region (J. S. Cordingley and S. M. Beverley, manuscript in preparation). Since the RNAs identified by probe 2d in wild-type and amplified lines were identical, it is likely that they terminate within the repeat element. The next probe, 2e, also identified the 2.3- and 2.5-kb RNAs, while probe 2f hybridized to all four RNAs. Probes 2g and 2h hybridized strongly to the 3.2- and 2.8-kb RNAs and less intensely to a 2.3-kb RNA. Finally, probe 2i identified the 3.2- and 2.8-kb RNAs, and probe 2j (data not shown) did not identify any RNA. Southern blot hybridization of restriction enzyme-digested R1000-3 genomic DNA with probes 2e through 2g did not reveal cross-hybridization among these fragments (data not shown).

These results are summarized in Fig. 2C, associating the RNAs with the map positions of the probes identifying them. One straightforward interpretation of these data is shown in Fig. 2D, which localizes the 3.2-, 2.8-, and 2.5-kb RNAs to contiguous regions which overlap one another to various extents. Surprisingly, a 2.3-kb RNA was identified by probes separated by at least 4.2 kb (from the Apal site at position +8.5 to the BglII site at position +4.3; probes 2d and 2h, Fig. 2B). We interpret these data to indicate that two distinct RNAs, both 2.3 kb in length, arise from the downstream subregion. Another interpretation, that a single 2.3-kb RNA is processed from a precursor which spans this subregion, seems less likely since cis-splicing has not been observed in any trypanosomatid gene thus far (11). Further characterization of the organization of downstream subregion transcripts will require direct analysis of RNAs by cDNA cloning or nuclease mapping.

Transcripts of the DHFR-TS subregion. Northern blot analysis was used to localize RNAs within the DHFR-TS subregion (Fig. 3A), which spans from about positions +3 to −11 on the R region map (Fig. 1C). The restriction map of this subregion and the diagram of hybridization probes are shown in Fig. 3B. Previous work localized the DHFR-TS mRNA between positions −0.37 and +2.6 kb (8, 39).

Probe 3a abuts the downstream subregion probe 2i, includes probe 2j, and extends into the DHFR-TS mRNA coding region (Fig. 3B); the adjacent probe, 3b, extends into...
FIG. 3. Localization of DHFR-TS subregion RNAs. (A) Northern blot hybridization of R1000-3 cytoplasmic poly(A)+ RNA to the probes indicated in panel 3B and described in Table 1. (B) Restriction map of the DHFR-TS subregion. The location of the DHFR-TS subregion within the R region is indicated by the coordinates (in kb) above the restriction map (see also Fig. 1C). B, BamH I; Bg, Bgl II; E, EcoRI; H, HindIII; K, KpnI; S, SalI; Sp, SphI; T, SstI. The locations of SalI, SphI, and SstI sites to the right of the EcoRI sites are not shown. The DHFR-TS protein-coding region is indicated immediately below the restriction map. The probes used in the Northern blot analyses shown in panel A are shown below the restriction map (see also Table 1). Probes 2i and 3j from the downstream subregion are also shown. Double-stranded probes are indicated by open boxes. Strand-specific probes are represented as solid lines, with the arrow indicating the direction of transcription of RNAs identified by the probe. (C) Interpretation of Northern blotting data. RNA species are identified on the right, with arrows indicating the direction of transcription. Parentheses indicate the furthest limit of the RNA as defined by hybridization data. Vertical arrows on the 5′ end of the DHFR-TS mRNA indicate the location of the major 5′ termini, previously determined by S1 nuclease mapping (39); the 3′ end of this RNA was inferred from analysis of a cDNA (8). Included is the 5′ terminal region of the 3.2- and 2.8-kb downstream RNAs (see also Fig. 2).

The 5′ noncoding region of the DHFR-TS mRNA (39) and contains the DHFR-TS protein-coding region. A single 3.2-kb RNA was identified by these two probes (Fig. 3A). The adjacent probe, 3c, begins at the EcoRI site in the 5′ noncoding region of the larger DHFR-TS mRNA (39) and extends 5′-ward an additional 700 base pairs. This probe hybridized to two RNAs: the 3.2-kb DHFR-TS mRNA and a 13-kb RNA. The next three probes, 3d through 3f, which successively span 9.5 kb of DNA upstream of DHFR-TS, hybridized only to the 13-kb RNA. Finally, the strand-specific probe 3g hybridized to both the 13-kb and DHFR-TS RNAs.

The results of the Northern blot analysis of the DHFR-TS subregion and previous work on cDNA sequencing and S1 nuclease analysis of 5′ ends of the DHFR-TS mRNA (8, 39) are summarized in Fig. 3C. Both the 3.2-kb DHFR-TS and 13-kb RNAs are transcribed in a leftward direction on the R region map, in the same orientation as the downstream RNAs. The RNAs are closely spaced, with the distance separating the DHFR-TS mRNA and downstream RNAs being 0.3 to 0.9 kb and that separating the 13-kb and DHFR-TS RNAs being less than 0.6 kb.

The downstream subregion encodes a 3.2-kb RNA distinct from the 3.2-kb DHFR-TS mRNA. Because a 3.2-kb RNA was identified by both downstream subregion probes and DHFR-TS subregion probes (probe 2a and others, Fig. 2; probes 3a through c, Fig. 3; also see reference 75), we investigated whether there were two distinct, similarly sized 3.2-kb RNAs or a single RNA created by processing of a precursor RNA. Previous work by others suggested that a
single 3.2-kb RNA spanned the DHFR-TS and downstream subregions (75). Hybrid selection was used to isolate RNAs which shared homology with the DHFR-TS and downstream subregions; Northern blotting was used to ascertain if the selected RNA were identified by the other nonselecting subregion of DNA. As expected, the downstream subregion probe 2a (Fig. 2B and 4A) identified four RNAs of 2.3, 2.5, 2.8, and 3.2 kb which were hybrid-selected with DNA from this subregion (Fig. 4B). However, the downstream probe did not hybridize to RNA selected with the DHFR-TS fragment which includes the entire DHFR-TS mRNA (Fig. 4B). In addition, no RNAs were identified with this probe in samples hybrid-selected with control plasmid pUC \( \pi \) DNA. Control hybridization of a DHFR-TS-specific probe to RNA hybrid-selected with the DHFR-TS subregion fragment revealed the expected hybridization to a 3.2-kb RNA (Fig. 4B). In a separate experiment, Southern blotting did not reveal cross-hybridization of any downstream subregion probes to DNA from the DHFR-TS subregion (data not shown). Collectively, these data indicated that there are two distinct 3.2-kb RNAs arising from the downstream and DHFR-TS subregions.

Transcripts of the upstream divergent RNA subregion. Probes from the divergent RNA subregion span from about positions -11 to -23 on the physical map, with the R region amplification terminating at position -21. Probes 5a through 5c successively span this subregion (Fig. 5B). The leftmost probe, 5a, identified the 13-kb RNA previously detected by the adjacent probe, 5f, from the DHFR-TS subregion, as well as a 1.7-kb RNA (data not shown; see probes 5d through 5h below). The central probe, 5b, hybridized strongly to a 6.2-kb RNA and weakly to a 1.7-kb RNA, while the rightmost probe, 5c, hybridized only to a 6.2-kb RNA (Fig. 5A). The 5-kb probe, 5c, contains a 0.6-kb repetitive element located at position -21, which is also present at position +9 in the downstream subregion, as described earlier. The repetitive element embedded within probe 5c failed to hybridize to the 2.3- and 2.5-kb RNAs (see Fig. 2A, probe 2d for comparison). No additional RNAs were detected with probe 5c in wild-type poly(A)\( ^{+} \) RNA (data not shown).

A series of hybridization probes was used to localize and determine the orientations of the 13-, 1.7-, and 6.2-kb RNAs. Strand-specific probes from the divergent RNA subregion have been given the suffix (\( l \)) and (\( r \)) to designate the leftward or rightward orientation, respectively, of the hybridizing RNAs. Probe 5d identified only the 13-kb RNA. Adjacent probe 5e(l) hybridized to both 1.7- and 13-kb RNAs, while the opposite-strand probe 5e(r) failed to hybridize to any RNA (data not shown). Adjacent probe 5f(l) hybridized to the 1.7-kb RNA and not to the 13-kb RNA, as did the opposite-strand probe 5f(r). These results suggested that there might be two 1.7-kb RNAs transcribed from opposite strands. Similarly, the next adjacent probes 5g(l) and 5g(r) identified a 1.7-kb RNA. Probe 5h(r) identified a 1.7-kb RNA, while probe 5i(r) hybridized to both a 1.7- and 6.2-kb RNA. Probe 5i(l) also hybridized to both a 1.7- and 6.2-kb RNA, suggesting that two overlapping opposite-strand 6.2-kb RNAs are also transcribed from this region. Finally, the...
FIG. 5. Localization of divergent subregion RNAs. (A) Northern blot hybridization of R1000-3 cytoplasmic poly(A)$^+$ RNA to the probes depicted in panel 5B and described in Table 1. The arrows above lanes 5e through 5i indicate the orientation of RNAs hybridizing to these strand-specific probes. (B) Restriction map of the upstream divergent RNA subregion. The location of the divergent RNA subregion within the R region is indicated by the coordinates (in kb) above the restriction map (see also Fig. 1C); sequences amplified in the R1000-3 line are depicted by the heavy line, and the hatched box corresponds to one of the rearrangement sites used to form the amplified R region. Bg, BglII; H, HindIII; P, PstI; S, Sall; Sp, SphI; T, SstI; X, XhoI. PstI and SphI sites to the right of coordinate $-18$ are not shown. Probes used in the Northern blot analyses are depicted below the restriction map (see also Table 1). Double-stranded probes are indicated by open boxes. Strand-specific probes are represented as solid lines with the arrows and suffix designations, (l) and (r), indicating the direction of transcription of hybridizing RNAs (leftward and rightward, respectively). (C) Summary of Northern blotting data. RNA species are shown on the right. The regions identifying a given RNA are indicated by the open boxes; regions not tested are indicated by broken lines. (D) Interpretation of the hybridization data. RNA species are shown on the right, with the direction of transcription indicated by arrows. Parentheses indicate the furthest limit of the RNA as defined by hybridization data. The vertical arrows above the 1.7-kb RNAs correspond to the 5′ and 3′ termini of the l-1.7-kb and r-1.7-kb RNAs, respectively, as determined by S1 nuclease mapping (Fig. 6).

double-stranded probe 5j hybridized only to the 6.2-kb RNA, indicating that the termini of the two putative 1.7-kb RNAs lie to the left of this probe. These results are summarized in Fig. 5C.

Preliminary sequence analysis of the divergent transcript subregion ruled out the existence of an inverted repetitive sequence as an alternate explanation for the 1.7-kb RNA hybridization data (K. Zhang, unpublished data), supporting the existence of opposite-strand transcripts. S1 nuclease mapping was used to verify the existence of the 1.7-kb overlapping RNAs, designated l-1.7 kb and r-1.7 kb, and to determine their rightmost termini. S1 nuclease mapping of R1000-3 poly(A)$^+$ RNA with the 2.6-kb Sall-XhoI fragment (map positions $-15.3$ to $-17.9$; Fig. 6C), labeled uniquely at the 5′ end of the Sall site, identified two protected fragments of 150 and 155 nucleotides, corresponding to the 5′ termini of the l-1.7-kb RNA (Fig. 6A). S1 mapping with the same DNA fragment, labeled uniquely at the 3′ end of the Sall site, revealed two protected fragments of 620 and 630 nucleotides, corresponding to the 3′ termini of the r-1.7-kb RNA (Fig. 6B). Consideration of the size of the two 1.7-kb RNAs and the Northern data presented in Fig. 5 suggests that the
overlapping across most of their length. As mentioned earlier for the DHFR-TS mRNA subregion, adjacent RNAs are closely spaced, with the separation between the 13-kb and r-1.7-kb RNAs being less than 0.8 kb and that between the r-1.7-kb and r-6.2-kb RNAs being less than 0.2 kb.

**Relative abundance of R region RNAs.** The relative abundances of R region transcripts were quantitated by Northern blot hybridization of R1000-3 poly(A)⁺ RNA by using several equimolar combinations of various purified restriction fragments from the plasmid pK300 (Table 1) which allowed the simultaneous examination of a number of RNAs. Densitometric scanning of these autoradiograms revealed that DHFR-TS and the 1.7-kb RNAs (the sum of r-1.7-kb and r-1.7-kb RNAs) are similarly abundant, constituting the most abundant R region RNAs (data not shown). The steady-state levels of the other RNAs relative to DHFR-TS (100%) are 13 kb, 10%; 6.2 kb (the sum of r-6.2 kb and r-6.2 kb), 40%; 3.2 kb (downstream), 25%; 2.8 kb, 25%; 2.5 kb, 50%; and 2.3 kb, 50%. Northern blot analysis indicates that the r-1.7-kb RNA is similarly abundant to the r-6.2-kb RNA [Fig. 5A, probe 5i(r)], while quantitative S1 nuclease mapping with a probe that distinguishes between the r-6.2-kb and r-6.2-kb RNAs shows that the r-6.2-kb species is approximately 10-fold more abundant than the r-6.2-kb RNA (data not shown). These data indicate that the leftwardly transcribed species constitute most of these similarly sized, opposite-strand RNAs. Comparison of R1000-3 and wild-type RNA preparations with probes specific for each RNA indicated that each RNA was approximately 10- to 15-fold more abundant in the R1000-3 line (data not shown), confirming the results shown for the total R region probe pK300 shown in Fig. 1A.

**Polysome association of R region RNAs.** Polysome preparations from the R1000-3 line were fractionated on sucrose gradients and assayed with probes specific for each R region RNA in order to determine which RNAs were associated in polysomes. UV absorbance profiles (A260 values) of these gradients revealed a typical polysomal pattern, with approximately one-third of the absorbance in the monosome and one-third in the polysome fractions (Fig. 7A). Addition of 30 mM EDTA to samples prior to gradient centrifugation shifted the bulk of the absorbance to the top of the gradient, as expected for bona fide polysomes (44). Gradient fractions were combined into four pools: (i) free RNA (top of the gradient), (ii) monosomes, (iii) small polysomes (disomes to 7-mers), and (iv) large polysomes (>7-mers), as shown in Fig. 7A. RNA was isolated from each pool, with the recovery being proportional to that expected from the UV absorbance profiles. Methylene blue staining of electrophoretically separated RNAs indicated that the ribosomal RNAs were intact, and Northern blot analysis with a DHFR-TS probe verified that the DHFR-TS mRNA was intact at that the preparations were devoid of DNA contamination (data not shown).

The RNAs recovered from the gradient pools were analyzed by slot blot hybridization by using probes specific for different R region RNAs. DHFR-TS-specific hybridization was observed in all four gradient fractions, with the monosome and polysome fractions accounting for approximately 15 and 55% of the total hybridization (pool 2 and pools 3 plus 4, respectively; Fig. 7B and C). EDTA treatment altered the distribution of DHFR-TS mRNA so that approximately 90% of the hybridization localized to the free RNA pool (Fig. 7B and C). The other R region RNAs display this basic pattern, since each RNA was detected throughout the gradient, including the large polysome region, and redistributed towards the top of the gradient upon addition of EDTA (Fig.

**FIG. 6.** S1 nuclease mapping of r-1.7-kb and r-1.7-kb RNAs. (A) S1 nuclease mapping of the 5' end of the r-1.7-kb RNA with the 2.6-kb Sall-Xhol fragment labeled uniquely on the 5' end of the Sall site (see panel C for probe). Size markers (in nucleotides) are indicated on the left, with the size of the protected fragments indicated on the right. (B) S1 nuclease mapping of the 3' end of the r-1.7-kb RNA with the 2.6-kb Sall-Xhol fragment labeled uniquely on the 3' end of the Sall site (see panel C for probe). Size markers are indicated on the left, with the size of the protected fragments indicated on the right. (C) Restriction map of the divergent RNA subregion encompassing the r-1.7-kb and r-1.7-kb RNAs. Coordinates (in kb) are given above the map (see also Fig. 1C). H, HindIII; S, Sall; T SsrI; X, Xhol. The Sall-Xhol fragments used for S1 nuclease mapping are shown. The r-1.7-kb and r-1.7-kb RNAs are shown, the heavy line on each RNA corresponding to the region protected from S1 nuclease digestion (see Fig. 6A and B) and wavy lines corresponding to the remaining portions, assuming a total size of 1.7 kb and a poly(A) tail of 200 nucleotides.
FIG. 7. Polysomal analysis of R region RNAs. (A) UV absorbance profiles of intact (solid line) and EDTA-treated (dashed line) polysomes fractionated on 15 to 50% sucrose gradients. Gradient fractions were combined into four pools as indicated: 1, free RNA region; 2, monosome region; 3, small polysome region; and 4, large polysome region. (B) Slot blot filter hybridization of intact and EDTA-treated polysome gradient pools with RNA-specific probes. Probes: DHFR-TS, pRBglO (Table 1); 13 kb, the 2.2-kb HindIII-SstI fragment within probe 5a (spanning probe 5d) from the divergent RNA region (Fig. 5B); I-1.7 kb, probe 5g(i) (Fig. 5B); and r-1.7 kb, probe 5g(c) (Fig. 5B). Numbers correspond to the pools shown in panel A. (C) Densitometric scanning of the DHFR-TS hybridization to the gradient pools shown in panel B (DHFR-TS) and the average of hybridization to all R region RNAs (AVERAGE), including the four hybridizations shown in panel B as well as for other R region probes not shown. The range of values obtained for all RNAs is indicated in the AVERAGE panel.

7B for 13-kb, l-1.7-kb, and r-1.7-kb RNAs; 6.2-kb [the sum of l-6.2-kb and r-6.2-kb] DNA and downstream RNAs; data not shown). The average and ranges for the distribution for R region RNAs in intact and EDTA-treated polysome preparations are shown in Fig. 7C. The proportional representation of these RNAs in large polysomes was somewhat less than that observed for the DHFR-TS mRNA, and small differences in the sensitivity to EDTA were observed for the different R region RNAs (Fig. 7B and C).

FIG. 8. Growth phase and developmental regulation of R region RNA. Northern blot analysis with probes specific for DHFR-TS (pRBglO; Table 1) or the 6.2-kb RNAs (probe 5c, Table 1) are shown. Where appropriate, methylene blue staining of ribosomal RNA is shown. (A) Analysis of poly(A)+ RNA from R1000-3 and wild-type promastigotes. Poly(A)+ RNA derived from total cellular RNA preparations and quantified by hybridization to [3H]poly(U) was loaded in each lane (100 ng for R1000-3, 250 ng for wild-type preparations). log, Log-phase cells; stat, Stationary-phase cells. The presence of 100 μM MTX in the culture medium is indicated by +. (B) Analysis of total cellular RNAs from R1000-3 and wild-type promastigotes. RNA input: R1000-3, 5 μg; wild type, 10 μg. (C) Analysis of total cellular RNA from wild-type promastigotes and lesion amastigotes. Promastigote, twofold dilution series from left to right beginning with 10 μg (lanes labeled 1, 1/2, etc). Amastigote, 10 μg of RNA from two independent preparations (lanes A and B).

Growth phase regulation of DHFR-TS mRNA. Northern blot analysis of R1000-3 poly(A)+ RNA [quantitated by hybridization with [3H]poly(U)] revealed that DHFR-TS mRNA levels were somewhat elevated (1.6-fold) in stationary-phase relative to log-phase promastigotes (Fig. 8A; Table 2). The addition of 100 μM MTX to the growth medium of log-phase cells revealed a slight 1.2-fold increase in DHFR-TS mRNA. Similar results were obtained in total RNA preparations, which were quantitated by methylene
blue staining of ribosomal RNAs (Fig. 8B). Quantitative Southern blotting of log- and stationary-phase R1000-3 DNAs did not detect changes in R region gene copy number, in contrast to the stationary-phase increase in copy number of certain bacterial plasmids (data not shown). Analysis of wild-type total or poly(A)+ RNA showed a 1.3-fold increase in DHFR-TS mRNA abundance in stationary phase relative to that of log phase (Fig. 8A and B; Table 2), comparable to the 1.6-fold growth phase increase observed in R1000-3 promastigotes. This suggested that amplification of the R region had not altered the normal pattern of DHFR-TS mRNA expression.

Developmental regulation of DHFR-TS mRNA levels. DHFR-TS mRNA levels were measured in wild-type amastigotes isolated from lesions formed in BALB/c mice. Comparisons of the hybridization of a DHFR-TS probe to two different preparations of total cellular amastigote RNA against a twofold dilution series of total cellular log-phase promastigote RNA showed that DHFR-TS mRNA levels were three- to fourfold lower in amastigotes relative to those in promastigotes (Fig. 8C; Table 2).

Regulation of DHFR-TS enzyme. DHFR-TS levels were assayed by quantitative binding of [3H]MTX to protein extracts. As expected, the R1000-3 line had about 10-fold more DHFR-TS protein than the wild-type line did (Table 2). Comparisons of log- and stationary-phase promastigotes revealed little difference in DHFR-TS protein levels in either the wild type or R1000-3 line. In contrast, in wild-type amastigotes DHFR-TS protein levels declined to about 16% of that observed for log-phase promastigotes. Overall, comparisons of the relative abundances of DHFR-TS mRNA and protein in all lines, stages, and phases revealed a good correspondence between RNA and protein levels (Table 2).

Regulation of other R region RNAs. We examined the regulation of several of the R region RNAs by rehybridizing the Northern blots of R1000-3 and wild-type total and poly(A)+ RNA shown in Fig. 8 with other probes. A probe which identified the 6.2-kb RNAs revealed that these RNAs also increased somewhat in stationary-phase promastigotes (2.2-fold for R1000 and 1.3-fold for wild type; Fig. 8A and B)....
mRNAs upstream of the I $\text{STAT}1.1$ VSG has been reported (2). However, at this time we regard the protein-coding capacity of the other nine RNAs encoded by the R region as an open question.

**Regulation of DHFR-TS protein and mRNA.** We find that DHFR-TS mRNA and protein levels increase somewhat in stationary-phase *L. major* promastigotes. This observation contrasts with results obtained with mouse DHFR, which show a 7- to 20-fold reduction in both protein and mRNA levels in stationary-phase cells (the monofunctional DHFR and TS genes also show significant cell cycle regulation, being maximal during S phase; 24, 29, 36, 37, 41, 48, 50). This finding may reflect basic differences in the regulation of housekeeping genes in *Leishmania* spp. relative to that in higher eucaryotes. Alternatively, perhaps the DHFR-TS complex functions in new or unusual roles during stationary phase. This latter possibility is suggested by work showing that many aspects of folate metabolism in trypanosomatids differ considerably from those of other cells (22, 40, 60, 61, 64, 68). It is notable that *Leishmania* spp. possess the capacity for growth phase regulation within the folate metabolic pathway, since the activity of the folate transporter declines more than 20-fold in stationary phase (22). Interestingly, growth phase-dependent changes in the abundance of a specific surface protein and concomitant changes in the infectivity of *L. major* promastigotes have been reported previously (67). Collectively, these data suggest that the study of growth phase regulation may offer new insights into the life cycle of the parasite.

We find that both the DHFR-TS enzyme and mRNA levels appear to be developmentally regulated, decreasing three- to sixfold in amastigotes. Previous work showed that this regulation does not involve changes in the use of the multiple 5' termini of the DHFR-TS mRNA (39). Similar decreases in DHFR and TS activity have been observed in amastigotes from *Leishmania donovani* and *L. mexicana mexicana* (68). Studies of the developmental regulation of leishmanial genes such as DHFR-TS are potentially complicated because lesion amastigotes are heterogeneous in many respects, including growth phase and nutritional status. Since we have shown that DHFR-TS protein and mRNA levels are largely unaffected by promastigote growth phase, it is unlikely that the decrease in DHFR-TS levels in the amastigote stage results from changes in the rate of cellular growth. Thus, the alternations in DHFR-TS levels represent bona fide developmental regulation. At this time, the potential contribution of growth phase regulation to developmental changes in the expression of other *Leishmania* genes is unknown.

**Effect of gene amplification upon R region transcription.** Our data show that gene amplification leads to a uniform increase in the levels of all RNAs encoded by the R region and has no effect on the structure of these transcripts. It, therefore, seems probable that all cis-acting elements necessary for transcription and processing of R region RNAs reside in this amplified domain, located within map positions +9 to −21 (Fig. 1). Another independent R region amplification exhibiting DHFR-TS overproduction has a rightmost site of DNA rearrangement mapping to position −16 (the D7B-R1000 line; 9, 33). Collectively, these data suggest that the cis-acting elements necessary for DHFR-TS expression may lie within a 25-kb interval (+9 to −16). Amplification mutants thus provide a genetic approach towards bounding the DHFR-TS transcriptional unit in trypanosomatids. organisms for which traditional genetic approaches are currently lacking.

One unanticipated finding is that while the increase in DHFR-TS gene copy number is approximately 80-fold in the R1000-3 line (7), the levels of DHFR-TS mRNA and protein are only elevated 10- to 15-fold (Table 2). A similar lack of correspondence between gene amplification and enzyme or mRNA overproduction has been reported in this and other amplified lines of *Leishmania* spp. (28, 39, 43). The basis for this finding is unknown.

**Antisense RNAs.** Northern blotting with strand-specific probes and S1 nuclease mapping have shown that the R region encodes two overlapping complementary 1.7-kb RNAs, located 13 kb upstream of DHFR-TS. The l-1.7-kb RNA is comparably abundant to DHFR-TS and overlaps the 10-fold-less-abundant r-1.7-kb RNA by about 1.1 kb. In addition, the adjacent region encodes opposite-strand 6.2-kb RNAs. We do not know whether these opposite-strand RNAs are transcribed within the same cell nor what their protein-coding or other potential roles are. Antisense RNAs are uncommon in the viral, organelle, and nuclear genomes of eucaryotes, with only a few reported examples (1, 32, 45, 47, 59, 71). In procaryotes, antisense RNAs have been shown to mediate regulation at both the transcriptional and translational levels (55, 62). Antisense RNAs introduced into eucaryotic cells have been shown to affect transcription (21), nuclear export (42), and mRNA stability (73). Stable complementary RNAs have not been previously described in kinetoplastid protozoans, and whether they play a role in regulating R region gene expression is a matter of speculation.

**Comparison of DHFR-TS 5’-flanking regions in L. major, Crithidia fasciculata, and mammalian cells.** Hughes et al. (34) have recently characterized the 5' region of the DHFR-TS gene of the nonpathogenic trypanosomatid *C. fasciculata*. These workers detected an opposite-strand RNA immediately upstream of the DHFR-TS mRNA which was variable in appearance. This situation is reminiscent of the organiza-
tion of the monofunctional DHFR gene in mammalian cells (16, 23, 54). In contrast, opposite-strand transcripts do not occur until 13 kb 5' of the DHFR-TS gene in L. major (Fig. 9), starting with a reproducible 1.7-kb RNA. Southern blot analysis with a probe specific for the opposite-strand transcript of C. fasciculata did not reveal homology to DNA sequences encoding the divergent transcripts of L. major (unpublished data). Hughes et al. also reported sequence similarity of the Crithidia 5'-flanking region with the corre-
sponding regions of various mammalian DHFR genes. How-
ever, when similarity searches are restricted to sequences shown genetically to function as mammalian DHFR or TS promoters, the similarity among the 5'-flanking regions of the two trypanosomatid DHFR-TS genes and mammalian promoters is limited and similar to that shown by Kapler et al. (39). The significance (or lack thereof) of any of these organizational or sequence similarities is currently unknown and must await functional tests (39).

Transcriptional organization of the amplified R region. Examination of the transcriptional map of the R region reveals a provocative pattern in the organization of stable transcripts, consisting of two domains of contiguous trans-
scripts arising from different strands (Fig. 9). The leftward domain extends counterclockwise around the entire amplified R region, encompassing the -6.2-kb, -1.7-kb, 13-kb, DHFR-TS, and downstream RNAs. The 5' end of the -1.7-kb RNA, at position -15.5, is located close to the right most limit of the amplified region in the D7B-R1000 mutant (position -16.9). The rightward domain begins at position -14.2 and proceeds clockwise to position -21 and includes the -1.7-kb and -6.2-kb RNAs. Within each domain the RNAs closely abut one another.

We have considered two contrasting scenarios for the transcriptional organization of the R region. In the first scenario each RNA within the two domains is transcribed and regulated independently from separate promoters. In the second scenario we postulate that adjacent RNAs in the same orientation are transcribed as part of larger polycis-
tronic precursor RNAs, which are then processed to yield the mature RNAs. These processing events would be com-
plex, since our data reveal the presence of antisense RNAs (Fig. 5 and 9) and multiple overlapping RNAs (the down-
stream subregion; Fig. 2 and 9), whose abundances can differ and show developmental changes. In this scheme, as few as two promoters could control R region transcription, one responsible for each domain and localized to the divergent RNA subregion. Models invoking polycistronic precursor RNAs have been proposed to explain transcription patterns of other protein-coding trypanosomatid genes (30, 38, 46, 56, 72).

The validity of either of these two scenarios (or combina-
tions thereof) for transcription of the R region is currently unknown, and we regard these as working hypotheses. Resolution of this question and the identification of the true functional promoter(s) for DHFR-TS and the other R region RNAs could be accomplished by a variety of approaches, such as quantitative analysis of transcriptional rates with the runoff assay or identification of putative polycistronic pre-
cursors. Perhaps the most attractive approach would be the use of DNA transfection-based methodologies for assaying promoter function in vivo. The studies of R region transcription presented in this work, in combination with the plasmid nature of the extrachromosomal circular amplified R region (9), may prove to be useful for addressing questions concerning both transcription and transfection.

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