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Structure and Transcription of the Actin Gene of *Trypanosoma brucei*

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In *Trypanosoma brucei*, the actin gene is present in a cluster of two, three, or four tandemly linked copies, depending on the strain. Each cluster seems to exist in two allelic versions, as suggested by the polymorphism of both gene number and restriction fragment length in the DNA from cloned trypanosomes. The amplification of the gene copy number probably occurs through unequal sister chromatid exchange. The chromosomes harboring the actin genes belong to the large size class. The coding sequence was 1,128 nucleotides long and showed 60 to 70% homology to other eucaryotic actin genes. Surprisingly, this homology seemed weaker with *Trypanosoma congolense*, *Trypanosoma cruzi*, *Trypanosoma vivax*, *Trypanosoma mega*, or *Leishmania* actin-specific sequences. The mRNA was around 1.6 kilobases long and was synthesized at the same level in bloodstream and procyclic forms of the parasite. Large RNA precursors, up to 7.7 kilobases, were found in a pattern identical in strains containing either two or three gene copies. Probing of the flanking regions of the gene with either steady-state or in vitro transcripts, as well as S1 nuclease protection and primer extension experiments, allowed mapping of the 3′ splice site of the actin mRNA, 38 nucleotides upstream from the translation initiation codon. A variably sized poly(dT) tract was found about 30 base pairs ahead of the splice site. The largest detected actin mRNA precursor seemed to give rise to at least two additional stable mRNAs. The RNA polymerase transcribing the actin gene exhibited the same sensitivity to inhibition by α-amanitin as that transcribing both the spliced leader and the bulk of polyadenylated mRNAs.

Actin is an ubiquitous protein, highly conserved between eucaryotes (12). The actin gene generally belongs to a multigene family, but there are exceptions such as in *Saccharomyces cerevisiae* (8, 26) and *Tetrahymena* spp. (13). The size of the multigene family may be small, as in *Acanthamoeba* spp. (25) and *Physarum* spp. (36), which have three or four actin genes, or large, as in *Dictyostelium* spp. (34), mice (21), or humans (24, 27), which have 20 to 30 actin genes. In these latter organisms, some members of the family are probably pseudogenes. In *Dictyostelium* spp., evidence for a differential expression between family members has been provided (34).

In trypanosomes, the existence of actin has never been conclusively demonstrated. However, anti-actin antibodies seem to specifically detect cytoplasmic components present in low concentrations, suggesting that actinlike proteins do actually exist in these parasites (T. Seebeck, unpublished data).

Transcription in trypanosomes exhibits particular characteristics. It seems to proceed generally by the synthesis of large polycistronic precursors from which mature RNAs are produced by *trans*-splicing (6, 23, 40; for a review, see reference 45). So far, the promoter region responsible for the initiation of the synthesis of the precursor RNA has not been characterized. In an attempt to find such a region for the transcription of a "housekeeping" gene, we decided to clone the actin gene of *Trypanosoma brucei*, together with extensive stretches of neighboring sequences, and to characterize its structure and transcription properties.

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MATERIALS AND METHODS

The trypanosome strains and clones analyzed in this work have been characterized elsewhere (28). Trypanosomes were separated from the blood components by DEAE chromatography (19). The methods for DNA and RNA isolation have been described previously (30).

Cloning of the *T. brucei* actin gene involved a preliminary isolation, by preparative low-melting-point agarose gel electrophoresis, of 15- to 20-kilobase-pair (kb) *Eco*RI fragments of *Trypanosoma brucei gambiense* LiTat 1.6 DNA, which were found to contain sequences strongly hybridizing with a ^{32}P-labeled 2-kb *Bgl*II fragment from the *Xenopus laevis* cytoskeletal beta-actin pCY-2 clone (gift of S. Brennan). These fragments were inserted in the lambda Charon 4A bacteriophage arms. The recombinant phage DNA was packaged in vitro (14) and plated on *Escherichia coli* BHB2600 cells. The library was screened by hybridization with the *Xenopus* actin probe.

The preparation of specific probes from the cloned region was performed by subcloning of restriction fragments in derivatives of the M13 phage (47). The DNA sequence of some of these fragments has been determined on both strands by the method of Sanger et al. (35). The methods for Southern and Northern (RNA) blot hybridization have been described previously (30).

Run-on transcription assays were performed as follows. The trypanosomes were lysed by homogenization in 10 volumes of buffer A (0.5 M sucrose, 50 mM KCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT], 50 mM Tris hydrochloride [pH 7.4]). A crude nucleus preparation was obtained by a brief centrifugation at 2,000 × *g* followed by a single wash

in buffer B (same as buffer A, except for the substitution of Nonidet P-40 by 5 mM spermidine). The pellet was then suspended in 25% glycerol–5 mM $MgCl_2$–50 mM Tris hydrochloride (pH 7.4) at a final DNA concentration of 1 mg/ml. The nuclei were maintained on ice, and to 500 μl of these nuclei were added, in the following order, 200 μl of 5 mM DTT and 25 mM spermidine, 100 μl of heparin (10 mg/ml), and 100 μl of water or α-amanitin (to reach the final concentration indicated). After a 10-min incubation on ice, 100 μl of 5 mM ATP, CTP, and UTP, together with 0.5 mCi of [α-^{32}P]GTP (400 Ci/mM), was added. Elongation of preinitiated RNA was allowed to occur for 30 min at 30°C. The reaction was stopped by the addition of RNase-free DNase I (1 μg/ml) and incubation on ice for 1 h. The RNA was isolated by proteinase K digestion and phenol extraction, followed by Sephadex G50 chromatography. Hybridization with DNA blots was for 48 h at 65°C in 0.5 M NaCl–0.05 M sodium citrate–0.2% Ficoll–0.2% polyvinylpyrrolidone–0.1% sodium dodecyl sulfate–0.1% proteinase K–100 μg of yeast tRNA per ml. Subsequent washings of the filters were in 45 mM NaCl–4.5 mM sodium citrate–0.1% sodium dodecyl sulfate at 65°C for 4 30-min periods.

RESULTS

Cloning of the actin gene of *T. brucei*. A probe derived from the *X. laevis* cytoskeletal beta-actin gene hydridizes strongly with *T. brucei* DNA, even at high stringency (data not shown). In the *T. brucei gambiense* LiTat 1.6 clone, this probe hybridizes with an *Eco*RI fragment of about 17 kb, which was cloned in the phage vector Charon 4A. However, in most *T. brucei* strains, the size of the actin-specific *Eco*RI fragment was about 1.5 kb larger and turned out to be unclonable in the same vector. In these strains, the size increase of the *Eco*RI fragment is due to the presence of an additional 1.5-kb *Sal*I fragment, which also hybridizes to the *X. laevis* actin probe (data not shown). This 1.5-kb *Sal*I fragment was cloned in pBR322.

Number of gene copies in different isolates. Probes made from the cloned *T. brucei* actin sequences were hybridized to the DNA of several *T. brucei* isolates. The hybridization patterns, identical to those obtained with the *X. laevis* probe, are presented in Fig. 1. In *Bgl*II digests, the size of the main actin-specific fragments differed between isolates, being 5.4 kb in the *T. brucei gambiense* LiTat 1.3 clone (lane 1) and 6.9 kb in the *Trypanosoma brucei brucei* AnTat 1.3 clone (lane 3). In clone A of the isolate TSw 125/82 EKPI (lane 2), both the 5.4- and the 6.9-kb fragments were present, whereas in the DNA of the uncloned ENT TM KD 37 isolate (lane 4), the 6.9-kb fragment was found associated with a 8.4-kb fragment (and a faintly hybridizing 5.4-kb fragment, which was due to a minor population; data not shown). These observations reflect a variation in the number of actin gene copies, as shown in other digests. Digestion by *Sal*I generated a 1.5-kb fragment in the DNAs of TSw 125/82 EKPI, AnTat 1.3, and ENT TM KD 37 only, where it was present in 1, 2, and 3 copies, respectively (arrowhead in Fig. 1). In *Cla*I and *Cla*I-*Sal*I digests, an analogous increase in copy number (but from 2 to 5 in the four DNAs analyzed) was found for a 1.5-kb fragment and a 1.1-kb fragment, respectively. In the *Bgl*II, *Cla*I-*Sal*I, and *Cla*I digests, in addition to the main actin-specific fragments, faintly hybridizing bands were detected which were due to cross-hydridization with sequences downstream from the actin genes (see below).

These data, together with other results (data not shown), are summarized in the restriction map of Fig. 1C. The actin gene appeared to be present in two copies in LiTat 1.3 and was amplified by 1.5-kb duplications in the other isolates. We have analyzed 44 isolates for their actin gene copy numbers. The results (Table 1) show that the most common situation is three linked copies (as in lane 3 of Fig. 1A). Deviations from this pattern were particularly frequent in isolates from Ivory Coast isolates and seemed absent from East African isolates.

Evidences for allelic clusters of the actin gene. The simultaneous presence of duplicated and triplicated genes in cloned trypanosomes (as in lane 2 of Fig. 1A), indicates that at least two actin gene clusters coexist in the genome. These clusters appeared to be in the same environment over more than 75 kb, as observed by restriction site mapping with probes derived from the extremities of the cloned 17-kb *Eco*RI fragment (P. Paindavoine, results not shown). The simplest interpretation is that the two gene clusters are on homologous chromosomes.

This interpretation is strengthened by the analysis of restriction fragment length polymorphism. Upstream of the actin genes, a *Cla*I site (bracketed in Fig. 1C) was generally absent but was present in one allele only in the TSw 125/82 EKPI clone A (shown by asterisks in the digestion patterns of Fig. 1) and in the two alleles in the *Trypanosoma brucei rhodesiense* NITR/40.12 isolate (see the 2-kb fragment in lane r of Fig. 4). The faint 2-kb *Cla*I fragment in ENT TM KD 37 was due to a minor population in this uncloned isolate (data not shown). Downstream from the genes, both the *Sst*I and *Pst*I sites (bracketed in Fig. 1C) exhibited polymorphism. Both sites were absent from LiTat 1.3 DNA and present in AnTat 1.3 DNA. Again, these sites were present in only one allele of TSw 125/82 EKPI clone A, as shown for *Sst*I in Fig. 1B (arrows). The complex *Sst*I pattern shown in Fig. 1B summarizes the polymorphism of both actin gene copy number and 3′ *Sst*I site. In NITR/40.12 (Ni), the upper 12.3-kb fragment extended in the 5′ direction from the *Sst*I site marked by a dot in Fig. 1C. This fragment included three actin gene copies. The 10.8-kb fragment extended in the 3′ direction from the *Sst*I site (marked by a star). In TSw 125/82 EKPI clone A (Ts), two 5′ *Sst*I fragments were generated (12.3 and 10.8 kb), containing three and two actin genes, respectively (the *Bgl*II digests in lane 2 of Fig. 1A show that this clone contained two, probably allelic, gene clusters). In the same clone, three 3′ *Sst*I fragments, of 10.8, 9.6, and 1.2 kb, were generated because of the presence of an additional *Sst*I site (bracketed) in one allele only. In LiTat 1.3 (Li), both the 5′ and 3′ fragments were superimposed, since both alleles contained the duplicated gene cluster resulting in 10.8-kb 5′ *Sst*I fragments, and were devoid of the bracketed *Sst*I site, resulting in 3′ *Sst*I fragments which also had a length of 10.8 kb. Finally, in AnTat 1.3 (An), both alleles carried the triplicated gene cluster (12.3-kb 5′ fragment) and contained the bracketed *Sst*I site (9.6 and 1.2 kb 3′ fragments, marked by arrows). The observed stoichiometry of both the 5′ and 3′ fragments in these four clones can most easily be interpreted when assuming diploidy of the actin gene and its environment.

Actin genes in large chromosomes. The genomic location of actin genes has been examined for some *T. brucei* strains, representative of *T. brucei brucei*, *T. brucei gambiense*, and *T. brucei rhodesiense*, by pulsed-field gradient gel electrophoresis of chromosome-sized DNA molecules (37) and hybridization with probe SBg as shown in Fig. 1. Only hybridization to large chromosomes occurred (data not shown).

Sequence determination. The area between *Sph*I and *Bgl*II,

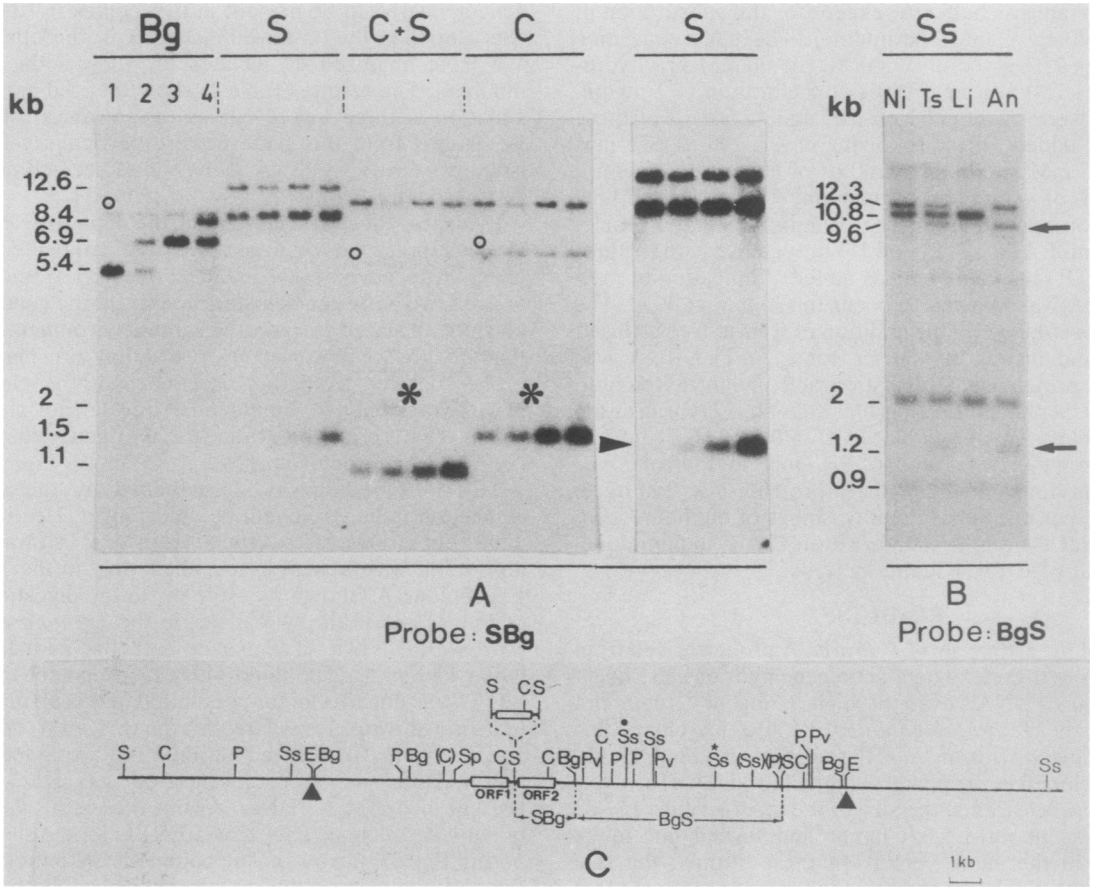

FIG. 1. Restriction mapping of the genomic environment of the *T. brucei* actin genes. (A) Variation of the number of actin gene copies. Restriction digests of the DNAs from the LiTat 1.3 clone, the AnTat 1.3 clone, the TSw 125/82 EKPI clone A, and the uncloned ENT TM KD 37 isolate (lanes 1 to 4 for each digest, respectively) have been hybridized with an actin-specific 1.9-kb *Sal*I-*Bgl*II probe as shown in the map. ○, Fragments which do not originate from the genomic region covered by the probe but from its 3′ environment (see text); *, fragments which are produced by a cleavage, in one allele only, at the bracketed *Cla*I site (see map). The increase in the amount of the 1.5-kb *Sal*I fragment (▶) is clearly shown after a longer exposure of the autoradiogram (extreme right of panel A). The increase in gene copy number has been estimated by liquid scintillation counting of the relevant nitrocellulose areas (results not shown). (B) Polymorphism of an *Sst*I site (bracketed in the map). *Sst*I digests of the DNAs from the NITR/40.12 isolate (Ni), the TSw 125/82 EKPI clone A (Ts), the LiTat 1.3 clone (Li), and the AnTat 1.3 clone (An) have been hybridized with a probe specific for the 3′ environment of the actin genes (6.7-kb *Bgl*II-*Sal*I; see Fig. 1C). The arrowed fragments are produced by a cleavage at the bracketed *Sst*I site. To interpret the restriction patterns, two *Sst*I sites are marked in the map (•, ★), as discussed in the text. (C) Restriction map showing the extent of the cloned *Eco*RI fragment (▲), as well as the origin of the probes used for the hybridizations on Southern blots. The position of the facultative 1.5-kb *Sal*I fragment is shown above the map. Depending on the presence or absence of this fragment, the DNA exhibits the *Bgl*II pattern 3 or 1, respectively (Bg in panel A). Abbreviations for restriction sites: Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; Sp, *Sph*I; Ss, *Sst*I.

which by hybridization appeared to include the two actin gene copies in LiTat 1.3 (central part of Fig. 1C), was subcloned in M13 derivatives after several rounds of restriction endonuclease digestions. The sequence of the 3.8-kb region between *Sph*I and *Bgl*II has been determined by the dideoxynucleotide chain termination method (35) and is presented in Fig. 2. Two open reading frames (ORFs) of 1,128 base pairs (bp) (ORF 1 and 2), only differing by seven nucleotides (open dots above the sequence), were separated by 387 bp. The two sequences shared homology from about 100 bp upstream from the translation initiation codon and started diverging at the stop codon. Curiously, the length of an oligo(dT) stretch, located 69 bp upstream from the initiation codon, varied between the two copies (12 and 18 bp for the first and second copies, respectively).

The arrangement of the two genes, with indication of the extent of sequence homology, is shown in a scheme in Fig. 2. The sequence of the 1.5-kb *Sal*I fragment, absent from

TABLE 1. Distribution of actin-specific DNA pattern among trypanosome isolates from different African countries

Origin of isolate	No. of isolates exhibiting actin-specific pattern[a]:			
	1	2	3	4
Ivory Coast	3	7	9	3
Liberia	1			
Congo		1	2	
Zaire			5	
Rwanda			2	
Uganda			3	
Nigeria			1	
Kenya			5	
Tanzania			2	
Total (%)	4 (9.1)	8 (18.2)	29 (65.9)	3 (6.8)

[a] Classified according to the *Bgl*II patterns shown in Fig. 1.

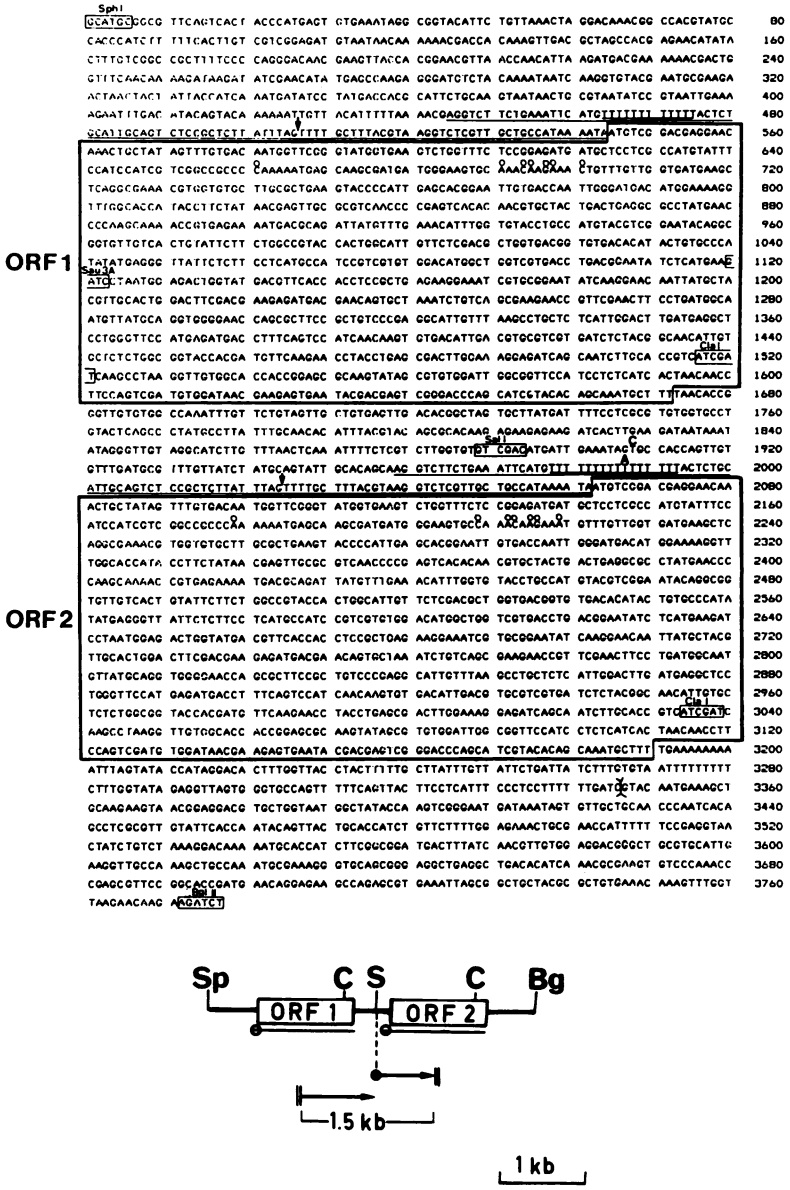

FIG. 2. Nucleotide sequence of the genomic region encompassing the two actin gene copies in LiTat 1.6 DNA. The sequence between the *Sph*I (Sp) and *Bgl*II (Bg) sites has been determined by the dideoxynucleotide chain termination method on fragments cloned in M13 derivatives. Two ORFs are boxed. O, Differences between these ORFs. A stretch (underlined) is conserved upstream of the ORFs. This stretch includes a variably sized oligo(dT) tract (doubly underlined). →, Splice site 5' to the mRNA. ⅄, Unsequenced stretch of about 20 nucleotides. A scheme summarizes the main observations. The extent of DNA duplication is indicated by the lines below the map; O, oligo(dT) tracts. The structure of the additional 1.5-kb *Sal*I (S) fragment is also shown. This fragment starts and ends at the *Sal*I site and includes a full actin gene copy identical to ORF 2. C, *Cla*I.

LiTat 1.3 but present in most DNAs that we analyzed (see Fig. 1C), has been determined by the same procedure. This sequence is entirely included in that shown in Fig. 2 but is in two pieces starting and ending at the *Sal*I site (see scheme in Fig. 2). Only two nucleotides differed between that sequence and the corresponding one in Fig. 2 (indicated above the sequence, positions 1908 and 1987).

Homology between the actin of trypanosomes and those of other organisms. Figure 3 shows the translation of the ORFs presented in Fig. 2. The two ORFs differed by four amino acids (positions 50 to 53). A comparison with actins of other organisms shows a general sequence conservation, with, however, five regions of higher divergence (positions 1 to 11, 40 to 54, 225 to 240, 262 to 280, and 307 to 330). The trypanosome actins exhibited between 61 and 73% homology with other actins, the lowest homology being with *Oxytricha* spp. (60.9%) and the highest being with yeast actin (72.9%).

Surprisingly, this homology seems weaker with actin-specific sequences of related trypanosome species, as shown in Fig. 4. Hybridization of the DNA from *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma mega*, *Trypanosoma cruzi*, or *Leishmania donovani* with the *T. brucei* actin probe was relatively poor and exhibited no restriction pattern conservation, in contrast to the hybridization of *T.*

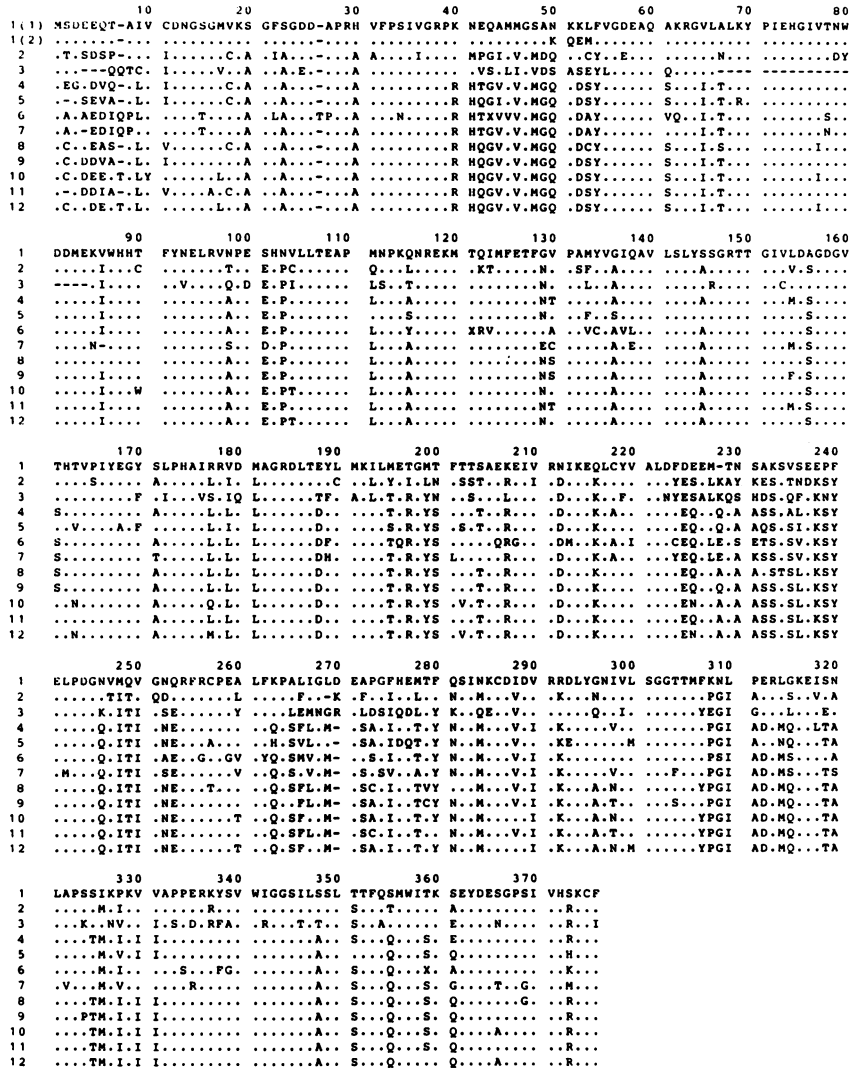

FIG. 3. Comparison of actins from *T. brucei* and other organisms. The amino acid sequences are aligned to maximize homology. Only the amino acids differing from their corresponding residues in the *T. brucei* actin are indicated. 1, *T. brucei*; 2, *Tetrahymena* spp.; 3, *Oxytricha* spp.; 4, *Physarum* spp.; 5, *S. cerevisiae*; 6, soybean; 7, maize; 8, *Drosophila* spp.; 9, sea urchin; 10, *X. laevis*; 11, cytoplasmic, human; 12, skeletal, human.

brucei or *Trypanosoma evansi* DNA with the same probe. Under the same conditions, the *T. brucei* probe strongly hybridized with *X. laevis* DNA, as well as with *Paracentrotus lividus* actin-specific DNA (data not shown).

Transcription: steady-state RNAs. To map the 5′ terminus of the mature actin mRNA, S1 protection experiments were performed, using poly(A)$^+$ RNA from either a LiTat 1.3 or a AnTat 1.6 clone, which, respectively, carry a duplicated and a triplicated actin gene cluster. A *Sau*3A fragment of the actin gene, labeled at position 578 of the coding sequence, was protected over 610 to 615 nucleotides after hybridization with either RNA (Fig. 5A). This size, measured on acrylamide gel in Fig. 5A, has been verified by electrophoresis on alkaline agarose gel (data not shown). This experiment allows the beginning of the mRNA to be mapped at about 40 bp upstream from the initiation codon.

A similar mapping by primer extension on AnTat 1.6 poly(A)$^+$ RNA gave an estimate of around 75 nucleotides upstream from the initiation codon (Fig. 5B), about 35 nucleotides more than the estimate by S1 protection. These observations suggest that the mature mRNA starts with a 39-bp "miniexon" sequence. This was verified by a sandwich hybridization experiment, in which the mRNA was hybridized first with the template strand of the actin gene cloned in M13 and then with a synthetic probe of the miniexon (Fig. 5C). The data are summarized in Fig. 5D, which shows that the 3′ splice site of the actin mRNA is most probably 38 nucleotides upstream from the initiation codon.

The steady-state transcripts have been hybridized with a battery of probes from the sequences in and around the gene copies (Fig. 6). The main mRNA was about 1.6 kb and was present in the same amount in clones differing by the number of gene copies, in bloodstream forms as well as in cultured procyclic forms. The slight differences in hybridization intensity of the 1.6-kb transcript between lanes in Fig. 6 are not significant and only reflect the relative enrichment of the RNA preparations in polyadenylated transcripts, as determined by the analysis of other RNA preparations as well as by the use of other probes on the same RNAs (data not shown). In addition to the major actin mRNA (1.6 kb), two

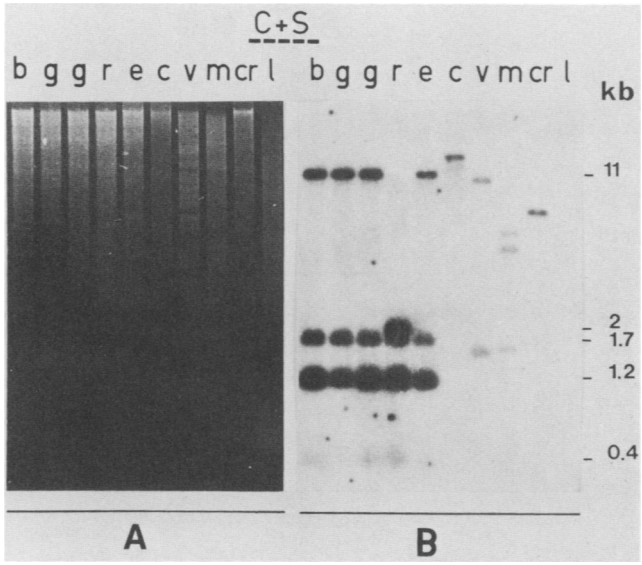

FIG. 4. Hybridization patterns of actin-specific sequences in different trypanosomatids. The DNAs from *T. brucei brucei* (b) (AnTat 1.3), *T. brucei gambiense* (g) (LiTat 1.3 and AnTat 11.6), *T. brucei rhodesiense* (r) (AnTat 12.1), *T. evansi* (e) (AnTat 3.3), *T. congolense* (c) (Ilnat 1.1), *T. vivax* (v) (Ildat 1.2), *T. mega* (m), *T. cruzi* (c), and *L. donovani* (l) have been digested by *Cla*I and *Sal*I and then electrophoresed in a 0.85% agarose gel. After ethidium bromide staining (panel A), they were transferred to nitrocellulose and hybridized with the SBg probe (panel B; see Fig. 1 for the origin of the probe).

minor bands of 3 and 3.8 kb were also detected (Fig. 6, probe 3). The 3-kb transcript was better revealed by a 5′ probe (probe 2), whereas the 3.8-kb transcript was seen with different 3′ probes, extending over 7.5 kb (probes 3 to 7). These two transcripts were most likely actin mRNA precursors and not related sequences, as they were revealed by probes recognizing only a single region in the genome (Fig. 1 and 4; data not shown). Their tentative localization is shown in Fig. 6. The detection of the 3.8-kb transcript with several 3′ probes was due to the cross-hybridization of these probes with a sequence in the actin gene region (data not shown). Detailed Southern hybridizations with these 3′ probes have shown that probes 5, 6, and 7, but not probe 4, cross-reacted with fragments spanning the actin genes (data not shown). Conversely, a probe restricted to the actin gene region faintly hybridized to fragments of the distant 3′ gene environment (Fig. 1). In the actin gene region, the cross-hybridizing sequence seems more precisely located just downstream of the last coding sequence (data not shown). Upstream of the genes, probe 1 recognized several RNAs, among them a 3-kb transcript. This transcript was not identical to one of about the same size recognized by probe 2. A close examination, as well as hybridization with a probe spanning both areas 1 and 2, has shown that the 3-kb transcript hybridizing to probe 1 was slightly larger (about 50 nucleotides) than that recognized by probe 2. Apart from this 3-kb transcript, all other RNAs recognized by probe 1 were clearly different from those hybridizing to probe 2. These results are in good agreement with those of the above S1 protection experiments, which have established that the actin mRNA starts only 38 bp upstream from the initiation codon. In a further effort to determine whether the region between the 5′ terminus of the mature mRNA and the upstream *Sph*I site is transcribed at all, we hybridized the transcripts with a 262-bp probe located just ahead of the 3′ splice site (position −500 to −238), using as a control a probe of about the same size (335 bp) but located at the beginning of the transcription unit (position −127 to +208) (Fig. 6, probes 2a and 2b). As shown in Fig. 6, probe 2a recognized the same transcripts as probe 1, in contrast to probe 2b. Thus, the pattern of transcripts changed drastically between positions −238 and −127. Finally, two large transcripts (7.7 and 5.3 kb) hybridized with both gene internal and 3′ probes (probes 3 and BgS: Fig. 6). These transcripts were also recognized by probes 2, 2b, and 2c (after longer exposure than shown in Fig. 6), indicating that they include the actin coding sequence. In contrast, they did not hybridize with 5′ probes, such as probes 1 and 2a, even after very long exposure of the autoradiograms (not shown). They seemed equally abundant in clones containing two or three gene copies (Fig. 6). On the basis of these observations, the most likely hypothesis is that the 5.3- and 7.7-kb transcripts start upstream of the last actin gene copy, as schematized in Fig. 6. However, a beginning upstream of another actin gene copy cannot be excluded, since the cross-hybridization between probes of the 3′ region does not allow precise determination of where the transcripts end.

Whatever the exact transcript processing pattern may be, it seems clear that the actin gene transcription unit extends largely downstream from these genes. In the area covered by probe 6, at least two additional stable poly(A)$^+$ RNAs could be seen (Fig. 6). These RNAs were not synthesized on sequences located elsewhere than 3′ to the actin genes, since Southern blot hybridization with probe 6 shows that this region is not repeated in the genome. Apart from the cross-hybridization noted above, which concerns a region hybridizing to probe 3, only a single genomic DNA region could be detected, whatever the restriction endonuclease used (data not shown). We conclude that the actin gene transcription unit may encompass not only the actin gene but also two or three other sequences transcribed into stable mRNAs.

In vitro transcription. The extent of the actin gene transcription unit was also measured by probing the gene and neighboring sequences with ^{32}P-labeled RNA elongated in vitro in isolated nuclei. Transcription was detected mostly with the DNA corresponding to probes 2b and 3 but only weakly with the DNA corresponding to probe 2a (Fig. 7A; Fig. 6D shows the extent and location of these probes). The antisense DNA strand did not show any hybridization, as expected from the data of Northern blot hybridization (Fig. 6). These results confirm the observations made of steady-state transcripts and show that the sequence between −500 and −250 is not transcribed at the same rate as the actin gene, suggesting the existence of separate transcription units.

The sensitivity of transcription of the actin gene towards inhibition by α-amanitin was found to be identical to that of transcription of both the spliced leader sequence and the bulk of polyadenylated transcripts (Fig. 7B and diagram in Fig. 7C). A 50% inhibition rate is achieved with 10 μg of α-amanitin per ml in all cases.

DISCUSSION

The actin gene of *T. brucei* has been characterized. This gene is present in 2 to 4 tandemly linked copies, depending on the trypanosome strain, and seems to differ noticeably from actin-specific sequences of related trypanosomatids. The actin gene cluster is probably located on diploid chro-

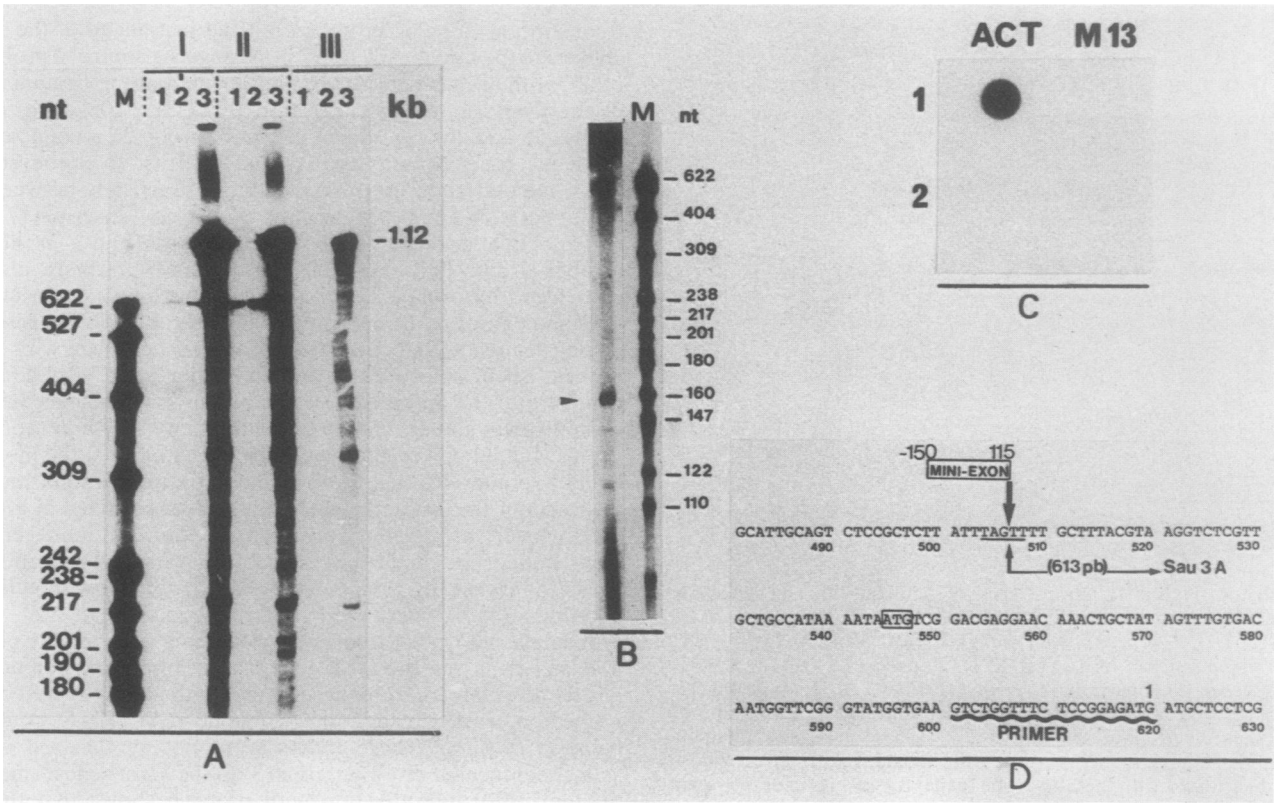

FIG. 5. Determination of 3′ splice site of the actin mRNA. (A) S1 mapping. The poly(A)$^+$ RNA (1 μg) of the clones AnTat 1.6 (I) and LiTat 1.3 (II) was hybridized with 0.7 μg of a 1.12-kb 5′-labeled fragment starting at the *Sph*I site upstream from the first actin gene copy and ending at the *Sau*3A site boxed in Fig. 2. As a control, this fragment has also been incubated under hybridization conditions but without RNA (III). After hybridization, each sample was treated with 200 (lane 1) or 40 (lane 2) U of S1 nuclease, or left without S1 nuclease (lane 3), and then electrophoresed in a 6% polyacrylamide gel. The marker is *Msp*I-digested pBR322 DNA, ^{32}P labeled by filling in the protruding ends with Klenow polymerase. (B) Primer extension. A synthetic oligonucleotide complementary to the sequence 600 to 620 (Fig. 2) was hybridized with poly(A)$^+$ RNA from AnTat 1.6 and then used as a primer for reverse transcription in the presence of [^{32}P]dCTP. The cDNA was run under the same conditions as in Fig. 5A. Its size maps the 5′ extremity of the actin mRNA about 35 nucleotides upstream from that shown in Fig. 5A. (C) The actin mRNA starts with a miniexon sequence. The template (row 1) and the coding (row 2) strands (4 μg each) of the 1.9-kb *Sal*I-*Bgl*II fragment carrying the actin gene, cloned in M13, have been spotted on nitrocellulose and hybridized with 150 μg of poly(A)$^+$ RNA from variant AnTat 1.1B. After being washed, the hybrids have been hybridized with 1 μg of a synthetic 35-mer of the miniexon, ^{32}P labeled by treating with kinase. As a control, 4 μg of wild-type M13 DNA has been incubated under the same conditions. (D) Summary of the above data. The underlined sequence marks the approximate mRNA start, as estimated by S1 mapping. The size (in nucleotides) of the cDNA synthesized by primer extension is interpreted by numbers above the sequence, starting at position 620.

mosomes from the large size class. It is transcribed to the same extent in bloodstream and procyclic forms by a polymerase moderately sensitive to α-amanitin. The main actin mRNA seems to be processed from the 5′ part of large RNA precursors, which include other stable mRNAs.

Evidence for actin in trypanosomes. Although no actin or actin-based structures have yet been detected in trypanosomes, the presence of a gene bearing strong homology with other eucaryotic actin genes, together with evidence of its transcription, leaves little doubt that actin may exist in these organisms. The actual demonstration of the presence of this protein in *T. brucei* has been recently provided by serological and biochemical detection in cytoplasmic components and by in vitro translation of the trypanosome mRNAs (P. Gehr and T. Seebeck, unpublished data).

The function of actin in trypanosomes is unclear, especially in view of the fact that it appears to be present in low concentration (T. Seebeck, unpublished). In this respect, it may be relevant to note that in closely related organisms, such as *T. congolense*, *T. vivax*, *T. mega*, or *T. cruzi*, the actin gene seems to differ remarkably from that of *T. brucei*. The resolution of this question awaits the characterization of actin-specific genes and proteins from these trypanosome species.

Probable diploidy of the actin gene. We present evidence for polymorphism of both actin gene copy number and restriction fragment length in the DNA from cloned trypanosomes. The stoichiometry of the polymorphic variants can easily be interpreted, assuming the presence of two allelic forms of an unique actin gene region. Since that region remains unique over 75 kb (P. Paindavoine, results not shown), it is unlikely that the two polymorphic versions originate from DNA duplication rather than from diploidy.

That the trypanosome genome is diploid has not yet been conclusively demonstrated, since the chromosomes do not condense at any stage of the cell cycle. Genetic exchange between trypanosomes has recently been described, but it remains to be proven that meiosis is involved (15, 29, 46). However, measurement of the DNA content versus complexity, as well as the analysis of DNA and isoenzyme patterns, strongly suggests that African trypanosomes are diploid organisms (3, 42). In particular, trypanosome housekeeping genes have been reported to exist in two allelic variants (9). Since the majority of these genes appear to be

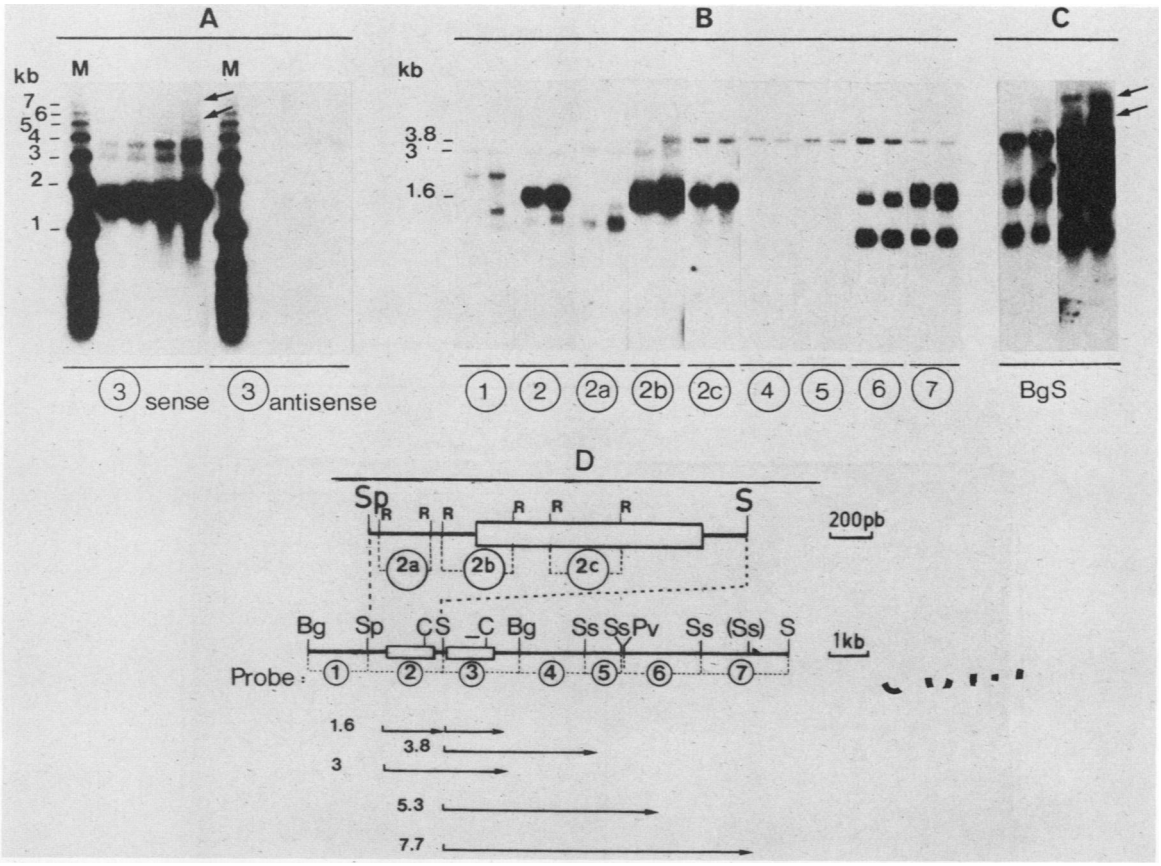

FIG. 6. Steady-state transcripts from the actin genes and environment. (A) The poly(A)$^+$ RNAs (10 μg per lane) of *T. brucei brucei* AnTat 1.1 and AnTat 1.6 bloodstream forms, AnTat 1.1-derived procyclic forms, and *T. brucei gambiense* LiTat 1.3, from left to right, have been hybridized with the M13-cloned SBg probes for sense (left) and antisense (right) transcription. The marker (M) is the 1-kb ladder (Bethesda Research Laboratories), ^{32}P labeled with the Klenow polymerase. The two arrows show the 5.3- and 7.7-kb transcripts (see text). (B) Several probes, as indicated in the map, have been hybridized with 10 μg of poly(A)$^+$ RNA from procyclic and bloodstream forms of AnTat 1.1 trypanosomes, from left to right in each pair of lanes. Probes 1, 2, 2a, and 2b are single-stranded M13 recombinants, ^{32}P labeled with the Klenow polymerase, while probes 2c, 4, 5, 6, and 7 are double-stranded fragments, ^{32}P labeled by nick translation. (C) Same as panel B, but the blots, hybridized with probe BgS (Fig. 1), are overexposed (two exposures shown) to make visible the 5.3- and 7.7-kb transcripts (arrows). (D) Map of actin gene transcription unit. The arrowed lines below the map show a possible model for the transcription pattern. R, *Rsa*I; other abbreviations are the same as for Fig. 1.

located in the large-chromosome size class, it is relevant that the actin gene copies are also in this chromosome category.

On the amplification of gene copy number. The detailed structure of the actin genes and, most importantly, the presence of the 1.5-kb *Sal*I fragment in stocks where the actin gene is amplified strongly indicate that amplification of the actin genes occurs through unequal crossing over (Fig. 8). This recombination event is likely to occur between sister chromatids, as already suggested for chromosome-internal variant-specific antigen genes (1).

The significance of the gene amplification is unclear. So far, most housekeeping genes of *T. brucei* appear to exist in multicopy clusters, such as genes for calmodulin (44), tubulin (38, 43), glyceraldehyde phosphate dehydrogenase (20), phosphoglycerate kinase (9), fructose biphosphate aldolase (4), and heat shock protein 70 (10). The only exception reported so far is the gene for triosephosphate isomerase, which is present in only one copy (41). It has been suggested that gene amplification may be a manner by which trypanosomes regulate the amount of RNA and protein to be synthesized (20). However, this explanation is difficult to invoke here, since the transcription level does not seem significantly affected by the number of gene copies.

Transcription. The transcription of the actin genes does not seem to be dependent on the stage in the parasite life cycle, since it occurs at the same rate in both bloodstream and procyclic forms. The expression of the actin genes thus seems to be constitutive.

The mature 1.6-kb steady-state mRNA is probably derived from larger precursors, in keeping with observations made about the transcription of other trypanosome genes, such as those for variant-specific antigens (5, 9, 31), phosphoglycerate kinase (9), or a protein from *T. cruzi* (11). We present evidence that the actin mRNA precursors extend far downstream from the actin-coding region and most probably encompass the sequences of other stable mRNAs. For trypanosomes, polycistronic transcription units have been proposed on several occasions (see references 2 and 45 for recent reviews). It remains to be seen whether genes of a given transcription unit are functionally or structurally related.

Although the site of the miniexon addition is clearly at 38 nucleotides upstream from the translation initiation codon, the beginning of the actin transcription unit cannot be determined. Hybridizations on Northern blots show a discontinuity in the transcription pattern close to the 5′ extrem-

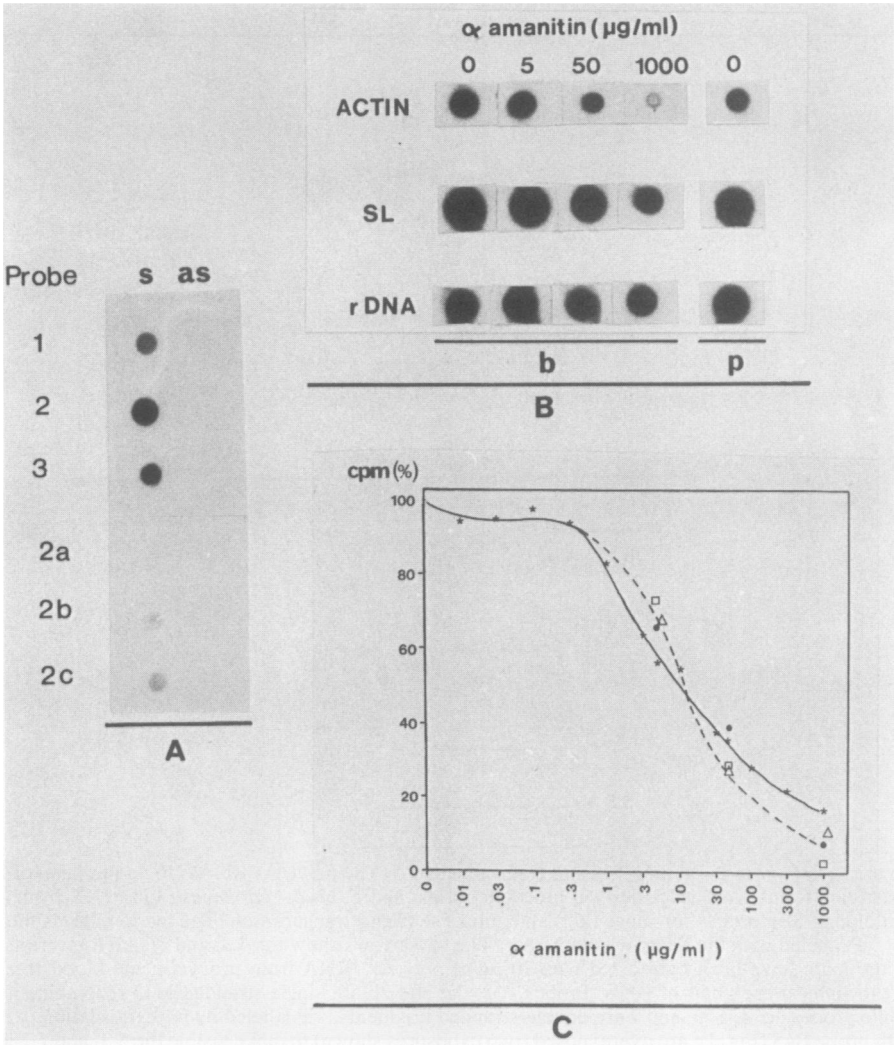

FIG. 7. In vitro transcription of the actin genes and environment. (A) Transcription level in different regions in and around the actin genes. The ^{32}P-labeled nascent RNA from AnTat 1.3 nuclei has been hybridized with 2 μg of M13-cloned DNA fragments (both sense and antisense) corresponding to probes 1, 2, and 3 (size: 1.5, 1.9, and 1.9 kb, respectively) or 2a, 2b, and 2c (each about 200 bp in length; Fig. 6D). (B) Sensitivity of actin gene transcription to α-amanitin. Dots of DNA fragments have been hybridized with the RNA synthesized in nuclei from AnTat 1.3 bloodstream forms (b) incubated with 0, 5, 50, and 1,000 μg of α-amanitin per ml or in nuclei from AnTat 1.1-derived procyclic forms (p) incubated without α-amanitin. The DNAs spotted on nitrocellulose were the BgS fragment containing the actin gene (ACTIN), a fragment from the spliced leader (SL), and a cloned sequence of genes coding for rRNA (rDNA). (C) The percent incorporation of label into total RNA (*) as a function of the concentration of α-amanitin, shown by the continuous curve. The level of incorporation was determined by scintillation counting of trichloroacetic acid precipitates. The zero concentration point of α-amanitin, representing 100% incorporation, generated 1.43×10^6 cpm. The broken curve was drawn after determining the level of radioactivity in each of the hybridizing dots; 100% incorporation is represented by 5.3×10^3 cpm for the spliced leader sequence (□), 7.4×10^2 cpm for actin (●), and 1.6×10^3 cpm for the first cDNA strand, synthesized by reverse transcription on total poly(A)$^+$ RNA from AnTat 1.1 (△).

ity of the first actin gene copy. However, such a discontinuity has also been observed ahead of variant-specific antigen genes (32), where evidence exists that transcription starts considerably farther upstream (22). By experiments involving in vitro elongation of RNA transcripts in isolated nuclei, it was found that the sequence just upstream of the actin genes is transcribed at a lower level than the genes themselves, suggesting, but not proving, the presence of a different transcription unit ahead of the actin genes. Therefore, it cannot be excluded that the actin transcription promoter is far upstream of the genes. However, some features of the proximate 5′ environment are worth mentioning, as they may play a role in transcription. The 5′ environment of the actin gene copies is perfectly conserved over 100 bp, except for the length of a tract of poly(dT), located about 30 bp upstream from the splice site (approximately position −70), which varies from 12 to 18 bp and may be interrupted by an A. Poly(dT) stretches may be involved in the control of the gene expression since they are excluded from nucleosomes in chromatin (33) and can even serve as upstream elements necessary for the transcription of several constitutively expressed genes in yeast (39). Polypyrimidine tracts are also found in about the same location upstream of other trypanosome genes (heat shock protein 70 [10], triosephosphate isomerase [41], tubulin [43]), as well as the dihydrofolate reductase-thymidylate synthase gene of *Leishmania major* (16).

Finally, we found that the RNA polymerase transcribing

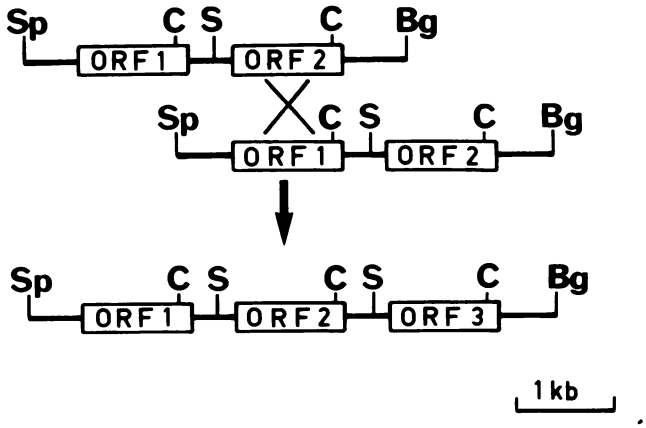

FIG. 8. Hypothetical scheme for the amplification of the number of actin gene copies. Abbreviations are defined in the legend to Fig. 2.

the actin genes exhibits a sensitivity to α-amanitin which is undistinguishable from that of the polymerase transcribing the spliced leader sequence, or, more generally, the polyadenylated RNAs. These results contrast with previous reports (17, 18) that the polymerase synthesizing mRNAs is more sensitive to α-amanitin than that transcribing the spliced leader genes. We have no explanation for this discrepancy. We have been able to detect only two and not three distinct RNA polymerase activities according to their sensitivity to α-amanitin in isolated nuclei; the enzyme transcribing the rDNA is fully resistant to the drug, whereas that transcribing the spliced leader and the actin genes is inhibited by around 10 μg/ml. These results are in full agreement with biochemical data showing the presence of two RNA polymerases in *T. brucei* (7).

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