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Genomic and Transcriptional Linkage of the Genes for Calmodulin, EF-Hand 5 Protein, and Ubiquitin Extension Protein 52 in *Trypanosoma brucei*

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We report genomic linkage of a pair of tandem, identical ubiquitin-extension protein 52 (EP52) genes, a novel EF-hand superfamily member gene (EFH5), and the calmodulin gene cluster in *Trypanosoma brucei*. The intergenic regions of these four genes are short: about 108 bp between the calmodulin gene C and the EFH5 gene, about 111 bp between the EFH5 gene and the ubiquitin-EP52/1 gene, and about 116 bp between the ubiquitin-EP52/1 and -EP52/2 genes. RNA molecules that span these three intergenic regions have been detected by polymerase chain reaction, which suggests that the genes are transcribed in a polycistronic manner. Transcription of the calmodulin, EFH5, and ubiquitin-EP52 genes in isolated nuclei is rapidly inactivated by UV irradiation, which further strengthens the hypothesis that this cluster of three different genes is transcribed in a polycistronic manner and suggests that they are under the control of a single distant upstream promoter. These results suggest that polycistronic transcription is common in trypanosomes and will probably be found for most, if not all, protein-encoding genes. The presence of at least three housekeeping genes with different known or potential regulatory functions within a polycistronic unit suggests that regulation of transcription initiation plays an important role in the coordinated expression of housekeeping genes in trypanosomes.

In trypanosomes, the discontinuous synthesis of mRNA from nuclear genes displays at least two unusual features. (i) All of the known mRNAs have the same 39 nucleotides (nt) (termed miniexon or spliced leader) at their 5′ ends. The common miniexon sequences are acquired from an approximately 140-nt precursor RNA (2, 22, 25) by the bimolecular process of *trans*-splicing (28, 34). (ii) The precursor RNAs that are the second substrate in the *trans*-splicing reaction appear to be polycistronic molecules.

Three types of experiments have identified potential polycistronic precursor molecules in RNA extracted from trypanosomes. (i) Northern (RNA) blot analysis of the tandem 1F8 genes in *Trypanosoma cruzi* (15) and the tandem tubulin genes (26) and actin genes (1) in *T. brucei* have revealed low-abundance, higher-molecular-weight RNA molecules that represent a transcript covering two or more coding regions. (ii) Nuclease protection assays with the *T. brucei* tubulin genes (26) and the *T. brucei gambiense* calmodulin genes (37) have demonstrated the presence of RNA molecules, which are putative precursors, that span adjacent genes and the intergenic region. (iii) A kinetic analysis of RNA synthesis in trypanosomes was provided by Johnson et al. (18), who showed, by UV inactivation, that transcription of a *T. brucei* variant surface glycoprotein gene initiated approximately 60 kb upstream of the gene and yielded at least seven other stable mRNAs (23). Since this transcription unit is active only in the bloodstream form of the parasite, it is reasonable to assume a functional basis for the developmental and transcriptional linkage of the genes.

It appears that housekeeping genes in *T. brucei* are transcribed by a conventional, α-amanitin-sensitive, class II RNA polymerase (16, 21). Although transcriptional start sites have been identified for the α-amanitin-resistant variant surface glycoprotein (18, 30) and procyclic surface antigen (4, 20, 32) genes, the identification of potential transcription initiation sites and a kinetic analysis of housekeeping gene transcription is currently restricted to the identical pair of actin genes (1), which appear to be transcribed from a promoter located about 4 kb upstream of the genes. The inference of polycistronic transcription for most housekeeping genes in *T. brucei* is based on the detection of RNA molecules that span intergenic regions, not promoter identification. The data therefore do not distinguish, for the most part, between a model of polycistronic transcription of multiple genes from a common promoter and a model in which each gene has a unique promoter that gives rise to read-through transcription from within the upstream gene.

To investigate transcription of housekeeping genes in *T. brucei*, we are characterizing the expression of ubiquitin genes and of the genes upstream of ubiquitin genes. We present data that show (i) the genomic linkage of ubiquitin-extension protein 52 (UbEP52) genes, a novel EF-hand superfamily member gene (EFH5 [43]), and the calmodulin gene cluster and (ii) the presence of RNA molecules that span each intergenic region. Because these RNA molecules may represent polycistronic precursors, we provide supporting kinetic data, from nuclear run-on assays, that suggest that each gene is transcribed from a common distal promoter. The results are thus indicative of coordinated transcription of three different genes, each of which encodes a protein with potential regulatory functions.

MATERIALS AND METHODS

DNA. The genomic library constructed in the cosmid vector c2XB has been described previously (41). The genomic clones p379 (0.4-kb *Hin*dIII-*Eco*RI), pU2Bgl2

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(2.4-kb *Bgl*II), and pSac2.8 (2.8-kb *Sac*II) were constructed in pBluescript KS+ (Stratagene, La Jolla, Calif.). pL4-9 is a 0.7-kb *Bgl*II subclone from pSac2.8 in pBluescript KS+. The cDNA library was constructed from procyclic RNA and propagated in bacteriophage lambda gt10. DNA manipulations were performed by standard techniques (33). Radiolabeled probes were made by random hexamer priming (11). Sequence data analyses were performed with the DSAS program (28a) or the University of Wisconsin Genetics Computer Group package (8).

RNA. RNA for Northern blots, polymerase chain reaction (PCR) amplification of 5′ ends, and nuclease protection assays was purified by the guanidine-cesium centrifugation method (7). For the PCR assays to detect putative RNA precursor RNA, RNA was rendered DNA free by two successive treatments with RNase-free DNase (Promega Biotec) followed by centrifugation through 98% (wt/vol) cesium chloride.

For Northern blots, RNA was electrophoresed through 1.5% agarose gels containing 2.2 M formaldehyde and transferred to nylon membranes (Nytran). Washes were done at 37°C in 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and then for 20 min at 65°C in 0.1 × SSC–0.1% sodium dodecyl sulfate (SDS).

Conditions for S1 nuclease protection assays were as described by Wong et al. (43).

Oligonucleotides. The oligonucleotides used in this study were as follows: AA1045 (3′ end of calmodulin gene C [1263 to 1287]; GTTTGTGTTAAACGGTTTCATGTCT), BB559 (5′ end of EFH5 [1496 to 1518; inverse complement]; CCT TATCCTTCATACCTTTATCC), BB562 (3′ end of EFH5 [2441 to 2464]; ATCGCTTCGTCACTTCACCTTTCT), AA525 (5′ end of UbEP52/1 and UbEP52/2 [2669 to 2694 and 3359 to 3383; inverse complement]; GGTGCTATCCTTC TGGCGTGGGTGA); and AA524 (3′ end of UbEP52/1 [3115 to 3138]; CGGGTTATCTGTGAAACATGCCTC). The numbers in brackets correspond to the nucleotide numbers presented in Fig. 2.

RNA-PCR assays. The reverse transcriptase reaction was performed on 10 μg of total procyclic DNA-free RNA for 45 min at 37°C in a reaction volume of 25 μl containing 80 mM Tris-HCl (pH 8.3), 120 mM KCl, 8 mM $MgCl_2$, 20 mM dithiothreitol, 110 μM deoxynucleoside triphosphates (dNTPs), 100 ng of primer, and 14 U of reverse transcriptase (Promega Biotec). After 45 min, 10 μM dNTPs and 9 U of reverse transcriptase were added, and the reaction mixture was incubated for an additional 45 min at 37°C. A 1- to 12-μl volume of this reaction mixture was used as the template in the PCR assay.

The final buffer conditions for PCR were 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 100 μg of bovine serum albumin per ml, and 500 pmol of each primer in a total volume of 100 μl. The reaction was cycled 25 to 40 times as follows: 50°C for 1 min, 72°C for 3 min, and 94°C for 30 s. The products were electrophoresed on 1% agarose gels in 1× Tris-borate-EDTA buffer.

UV inactivation of transcription. Procyclic *T. brucei* was grown in SDM-79 medium at 27°C to a density of 10^7/ml. Samples (1×10^9 to 3×10^9 cells in a total volume of 50 ml) were irradiated in sterile petri dishes (150 by 15 mm) at a distance of 6 cm from a UV transilluminator source (302 nm) for 0.5, 1, and 2 min with agitation. After irradiation, cells were kept at room temperature in the dark for 1.5 h. The cells were sedimented, and nuclei were prepared by the Nonidet P-40 lysis method. Nuclear run-on assays were performed in a total volume of 100 μl containing 1×10^9 to 3×10^9 nuclei, 20% glycerol, 100 mM Tris-HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM spermidine, 1 mM dithiothreitol, 0.1 M KCl, 4 mM $MnCl_2$, 80 U of RNAsin (Promega Biotec), 1 mM each UTP, CTP, and ATP, 10 μM GTP, 100 μg of creatine kinase per ml, 10 mM creatine phosphate, and 250 μCi of [α^{32}P]GTP (3,000 Ci/mmol). Nascent RNA was elongated for 30 min at 30°C as described by Coquelet et al. (5). The reaction was stopped by the addition of 2 μg of RNase-free DNase I; the mixture was incubated on ice for 1 h, subjected to proteinase K (50 μg/ml) treatment and phenol extraction, and then analyzed by Bio-Gel P10 chromatography. For polymerase inhibition studies, the nuclei were preincubated in storage buffer containing α-amanitin at 1 mg/ml for 15 min at 4°C and were then pelleted and resuspended in run-on buffer containing 100 μCi of [α-^{32}P]UTP and α-amanitin at 1 mg/ml.

Labeled nascent RNA was hybridized to nylon (Nytran) filters overnight at 65°C in a hybridization buffer containing 3× SSC, 1× Denhardt's solution, 0.1% $NaPP_i$, 0.5% SDS, and 20 μg of yeast tRNA per ml. Filters were washed twice at 65°C in 2× SSC and then twice for 30 min each time at 65°C in 0.1× SSC–0.1% SDS.

The hybridization signal on slot blots was quantitated by counting the nylon strips in ScintiVerse (Fisher Scientific) in a liquid scintillation counter. Samples were corrected for background by subtraction of the Bluescript plasmid control.

Nucleotide sequence accession number. The UbEP52/1-UbEP52/2 nucleotide sequence has been submitted to the EMBL data base under the accession number X56511.

RESULTS

Cloning and characterization of the Ub-EP52 genes and upstream sequences. Hybridization of a ubiquitin probe to Southern blots of *T. brucei* genomic DNA revealed at least five regions of identity. The two strongest regions of hybridization represent alleles of a polyubiquitin gene (41, 42). This report focuses on a third ubiquitin-hybridizing region, which contains two tandem Ub-EP52 genes, located on a 2.8-kb *Sac*II fragment present in both genomic DNA and cosmid JN2. A combined restriction and transcriptional map of this locus is presented in Fig. 1.

DNA sequence analysis of this region identified four open reading frames (Fig. 2), which show significant third-base nonrandomness by the TESTCODE algorithm (12). The first open reading frame (nt 362 to 808) is 344 bp and shows complete identity with the calmodulin gene C identified by Tschudi et al. (38) in *T. brucei gambiense*. The second open reading frame (nt 1506 to 2081) is 576 bp and encodes the calmodulin-related EFH5 protein (43). Open reading frames 3 (nt 2695 to 3078) and 4 (nt 3384 to 3767) differ by a single nucleotide and represent the genes for the two UbEP52 cDNA clones described by Wong et al. (44). The two genes, which are identical for 50 bp upstream of the initiation codon and 36 bp downstream of the termination codon, are also distinguished by differences in their 3′ untranslated regions and will be referred to as UbEP52/1 and UbEP52/2.

Stable transcripts from the UbEP52, EFH5, and calmodulin genes. To identify transcripts from the ubiquitin genes and from the upstream genes, we first performed Northern blot analysis to determine the sizes of the mRNA species. The positions of mRNA termini were subsequently determined either by sequencing cDNA clones or by performing S1 nuclease protection assays.

A *T. brucei* ubiquitin coding region probe detected transcripts represented by five size classes (approximately 8.0,

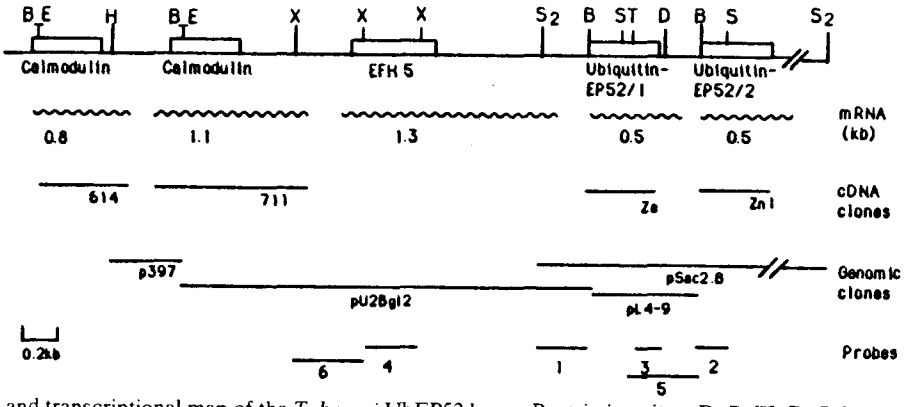

FIG. 1. Restriction and transcriptional map of the *T. brucei* UbEP52 locus. Restriction sites: B, *Bgl*II; D, *Dde*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; S_2, *Sac*II; T, *Taq*I; X, *Xmn*I. Open boxes represent the open reading frames. The sizes and locations of the RNAs from the calmodulin, EFH5, and UbEP52 genes are shown beneath the restriction map. Also shown below are the locations of cDNA clones and genomic clones used in this study. The locations of the DNA fragments used in subcloning, and as probes in Northern and Southern blots as described in the text, are indicated.

6.9, 3.8, 2.9, and 0.5 kb) in total RNA from procyclic-form *T. brucei* (Fig. 3, lane 1). A 156-bp *Taq*I-*Dde*I fragment (Fig. 1, probe 3), which is specific for the EP52 sequence of the UbEP52 genes, detected a 0.5-kb RNA (Fig. 3, lane 2), demonstrating the origin of the 0.5-kb ubiquitin transcript from this locus. DNA sequence analysis of the 3′-flanking regions show that the UbEP52/1 and UbEP52/2 open reading frames represent the genes for cDNA clones Za and Zn1 (44), respectively. From the DNA sequence of the cDNA clones Za and Zn1, we infer that the UbEP52/1-UbEP52/2 intergenic region is, at most, 188 nt. Since cDNA clone Za lacks a poly(A) tail, we cannot assume that it is full length; therefore, we have determined the 3′ end of the transcript by S1 nuclease mapping (Fig. 4). A nested set of protection products centered at about 154 nt corresponds to the 3′ end of the UbEP52/1 transcript. We therefore conclude that the intergenic region is about 116 bp. A larger product of 290 nt corresponds to protection of the insert to the *Bgl*II site within the UbEP52/2 coding region and therefore represents protection by an RNA molecule that spans the intergenic region and is potentially a precursor in polycistronic transcription of the two genes.

The 2.4-kb *Bgl*II fragment detected transcripts in three size classes (approximately 1.3, 1.1, and 0.8 kb; Fig. 3, lane 4). A 0.35-kb *Xmn*I fragment, contained within the EFH5 coding region (Fig. 1, probe 4), detected the 1.3-kb RNA (Fig. 3, lane 5), while a calmodulin probe detected the 1.1- and 0.8-kb RNA species (lane 3).

The splice acceptor site and polyadenylation sites of the EFH5 transcript have been determined previously (43) and are located at nt 1412 and 2538 to 2570 (heterogeneity due to the use of three alternative polyadenylation sites), respectively. The 5′ end of cDNA clone Za is located at nt 2686. Although clone Za lacks miniexon sequences, it is likely to use the potential splice acceptor site at nt 2682, which is within the UbEP52 conserved upstream region. From the Za cDNA sequence, we conclude that the EFH5-UbEP52/1 intergenic region is about 111 bp but not greater than 114 bp (Fig. 2).

The data of Tschudi et al. (37, 38) and the DNA sequence of cDNA clones 614 and 711 have been used to determine the termini of the calmodulin mRNAs. cDNA clone 614 is virtually indistinguishable from calmodulin genes A and B (38), and clone 711 corresponds to calmodulin gene C. The polyadenylation site of cDNA clone 711 is located at nt 1304; thus, the calmodulin C-EFH5 intergenic region is 108 bp. Preliminary experiments demonstrate that the gene upstream of the calmodulin cluster gives rise to a 3.8-kb mRNA (data not shown). Partial DNA sequence analysis of upstream sequences does not yield an identity for the upstream gene, which we shall refer to as 3.8 (see Fig. 7C).

Detection of RNA molecules that span each intergenic region. The intergenic regions described above are similar in size to or shorter than the intergenic regions between housekeeping genes for which polycistronic transcription has been postulated (10, 14, 26, 27, 37). Since polycistronic transcription of the calmodulin cluster has been inferred (37), we wished to determine whether the EFH5 gene and the UbEP52 genes are transcribed coordinately with the calmodulin genes.

S1 nuclease protection experiments have indicated the presence of RNA molecules that span the calmodulin B-calmodulin C (38, 40a), EFH5-UbEP52/1 (43), and UbEP52/1-UbEP52/2 (Fig. 4) intergenic regions. However, unexpected bands were also present in some experiments. These additional products may be explained as intermediates in the *trans*-splicing process or by allelic variation in the intergenic regions. Therefore, to unambiguously identify potential polycistronic precursor RNAs that span the intergenic regions, we used PCR. In principle, two primers are chosen; one is contained within, and is identical to, the 3′ end of the upstream mRNA, and the other is complementary to the 5′ end of the downstream mRNA. The downstream primer is used, with reverse transcriptase, to prime cDNA synthesis from total RNA. The majority of primer extension products will probably terminate at the 5′ end of the miniexon on mature mRNA. However, cDNA derived from unprocessed RNA that spans the intergenic region may subsequently be detected by PCR and will yield an amplification product of a predicted size. The identity of the PCR product is confirmed by stringent hybridization with a DNA probe from the respective intergenic region. Accurate interpretation of this assay requires three essential negative controls, since the same PCR product can be generated from contaminating genomic DNA. The first negative control (Fig. 5, lanes 2, 6, and 10) is the omission of reverse transcriptase. Absence of the predicted band indicates that the original template for reverse transcriptase was not DNA.

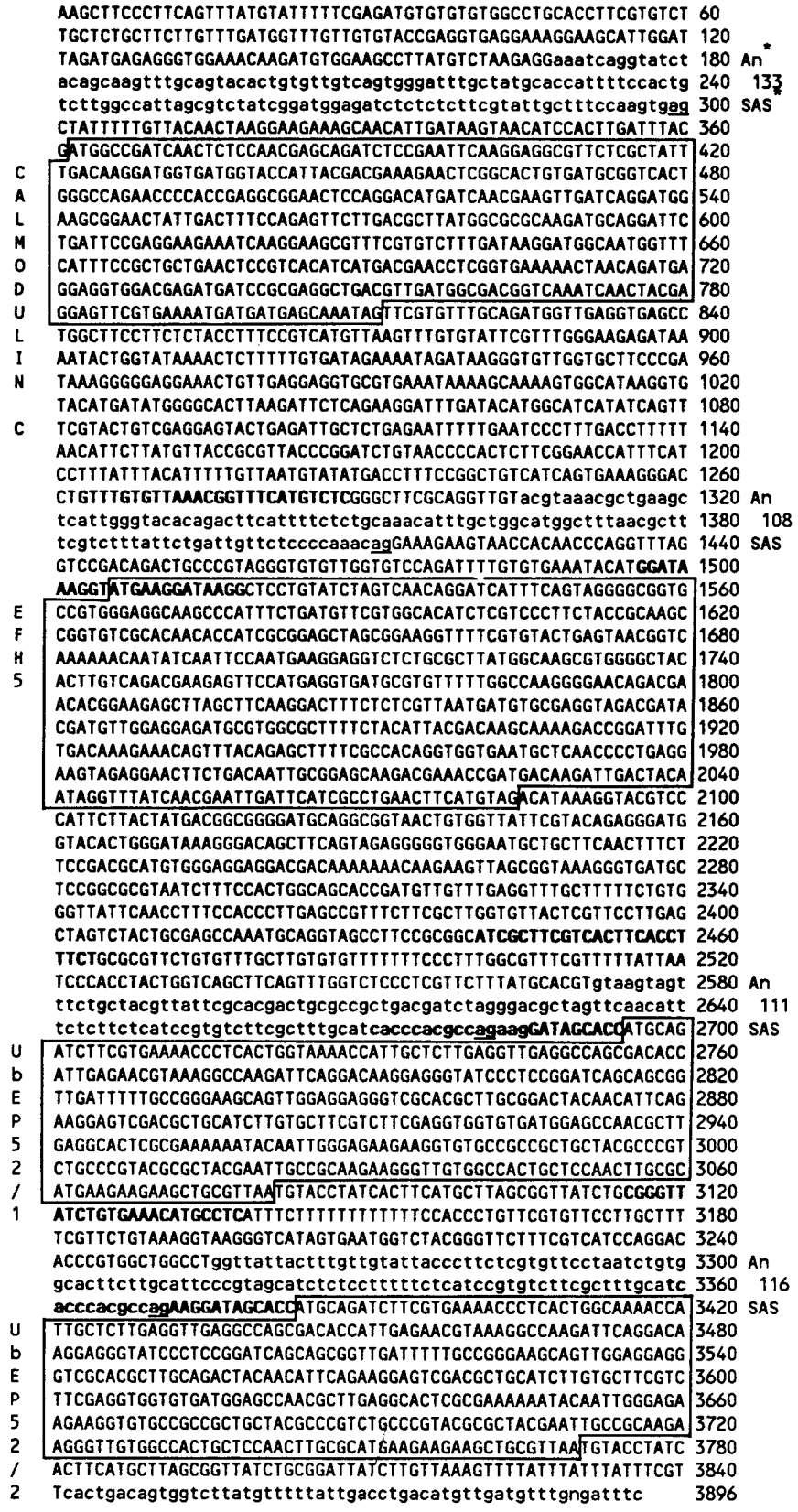

FIG. 2. DNA sequences of the calmodulin, EFH5, and UbEP52 genes. Boxed regions indicate open reading frames, uppercase letters represent stable transcripts, and lowercase letters represent intergenic regions. Conserved nucleotides flanking the UbEP52 open reading frames are nt 2645 to 2694 and 3334 to 3383 (upstream) and nt 3082 to 3117 and 3771 to 3806 (downstream of the termination codon). The length of an intergenic region (indicated at the right) is calculated as the distance between a polyadenylation site (An) and the downstream splice acceptor site (SAS; underlined "ag"). The calmodulin B-calmodulin C intergenic region (asterisks) is based on the major polyadenylation site and SAS 2 reported by Tschudi et al. (37, 38); a G-to-C transversion at position 316 eliminates the alternative SAS 1 (37). The calmodulin C-EFH5 intergenic region is calculated between cDNA clone 711, which contains a poly(A) tail, and the EFH5 SAS (43). The EFH5-UbEP52/1 intergenic region is based on the major 3'-end S1 nuclease protection product of EFH5 mRNA (43) and the UbEP52/1 SAS (predicted by virtue of the upstream conservation with UbEP52/2). The UbEP52/1-UbEP52/2 intergenic region is calculated from S1 nuclease protection of the 3' end of UbEP52/1 (Fig. 4) and cDNA clone Zn1 (44), which contains miniexon sequences. Oligonucleotides used in the PCR assay are indicated in boldface.

Although *Taq* polymerase has been reported to have reverse transcriptase activity (19, 39), the presence of a band in this control could always be attributed, by the second negative control, to contaminating DNA. The second negative control (lanes 3, 7, and 11) is treatment of the RNA with boiled RNase prior to the reverse transcriptase reaction. Absence of the predicted band confirms that the template for cDNA synthesis was RNA. The third negative control (primer only) contains all of the reagents except the template (lanes 1, 5, and 9).

To detect RNA molecules that span the UbEP52/1-UbEP52/2 intergenic region, we used total RNA from procyclic-form *T. brucei* and oligonucleotides that represent the 3' end of UbEP52/1 (AA524) and the 5' end of UbEP52/2 (AA525) in the assay. A PCR product consistent with the predicted size (269 bp) was observed in an ethidium bromide-stained gel (Fig. 5A, lane 12) and was absent in all of the negative controls (lanes 9 to 11). In this experiment, oligonucleotide AA525, which overlaps the splice acceptor site, was used to increase the likelihood of priming from potential precursor molecules. In subsequent experiments (lanes 4 and 8), we found that oligonucleotides contained completely within the coding exon primed as efficiently as from precursor RNA.

Hybridization of probe 5 (Fig. 1), which covers the UbEP52/1-UbEP52/2 intergenic region, to a Southern blot of the 269-bp PCR product (Fig. 5D) confirms the identity of the PCR product and thus indicates the presence of an RNA molecule that spans the UbEP52/1-UbEP52/2 intergenic region. The PCR result suggests that the tandem identical UbEP52 genes may be transcribed in a polycistronic fashion.

To determine whether the two UbEP52 genes are transcriptionally linked to the adjacent, upstream, nonidentical EFH5 gene, we used oligonucleotide primers BB562 (corresponding to the 3' end of the 1.3-kb EFH5 transcript) and AA525 (which is also complementary to the 5' end of UbEP52/1) in the PCR assay described above. On the basis of the genomic sequence, we predict that the presence of an RNA molecule spanning the EFH5 and UbEP52/1 mRNAs should give rise to a 254-bp product in the PCR assay. The result of the assay shows a band of the appropriate size (Fig. 5A, lane 8), which hybridizes (Fig. 5C) to the corresponding genomic DNA probe (probe 1; Fig. 1) and is absent in all of

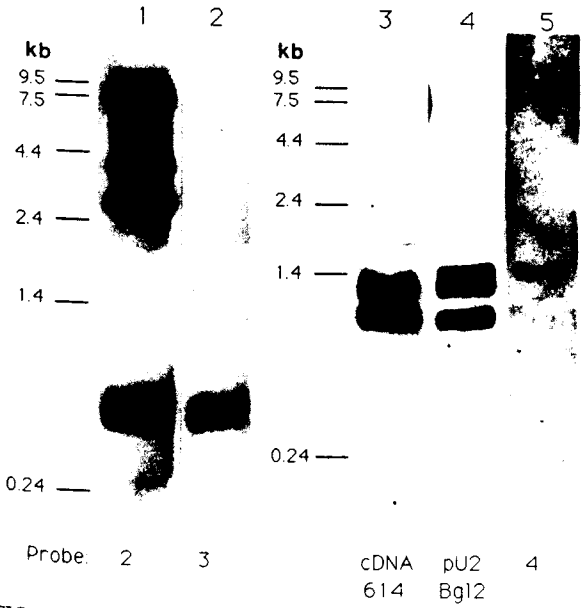

FIG. 3. Northern blot analysis of transcripts from the calmodulin, EFH5, and UbEP52 genes. Total procyclic RNA (20 μg) was probed with ubiquitin (probe 2), EP52 (probe 3), calmodulin (cDNA 614), calmodulin and EFH5 (pU2Bgl2), and EFH5 (probe 4). Faint bands of hybridization in lane 3 represent nonspecific hybridization to the LS1 and LS2 rRNA molecules. Size markers represent the RNA ladder (Bethesda Research Laboratories).

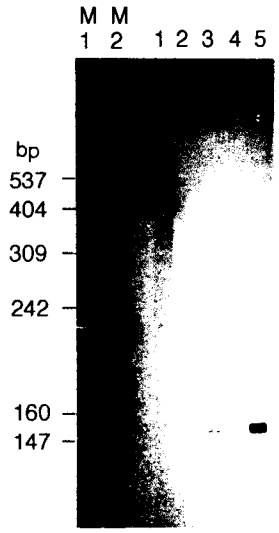

FIG. 4. S1 nuclease protection assay identifying the 3' end of UbEP52/2 mRNA and a potential polycistronic precursor RNA molecule. The protection probe (lane 1) is a 450-bp *Dde*I-*Pvu*II fragment, from pL4-9, 3' end labeled at the *Dde*I site. The probe was incubated in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 10 μg of *T. brucei* total RNA and digested with either 300 U (lanes 2 and 3) or 150 U (lanes 4 and 5) of S1 nuclease. Molecular size markers are pBR322 digested with *Hpa*II (lane M1) and pGB117 digested with *Hin*fI (lane M2).

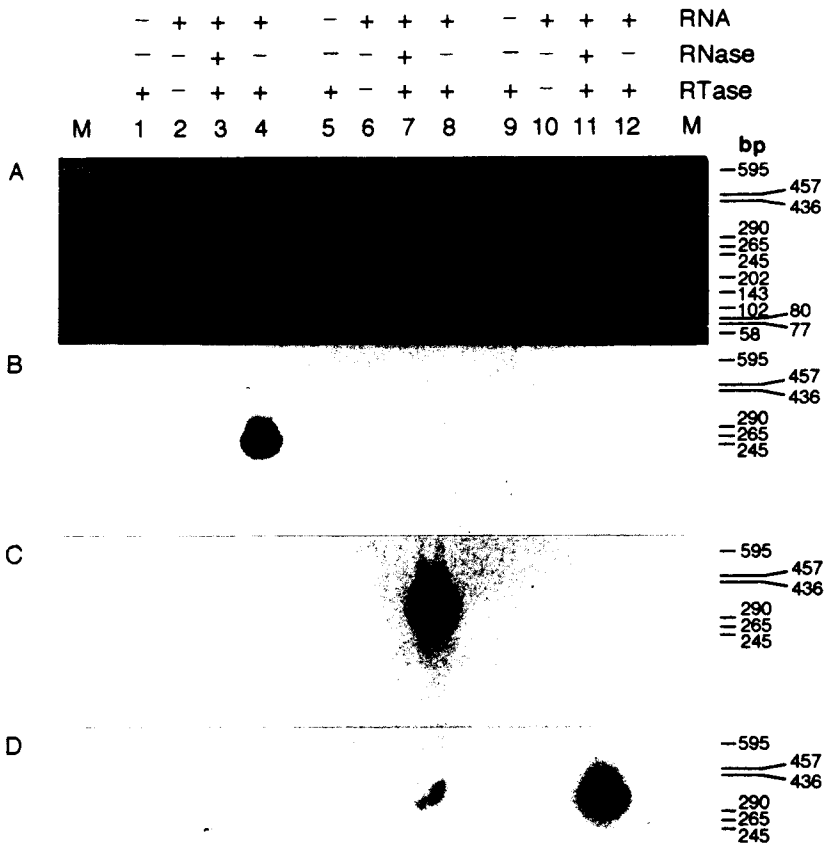

FIG. 5. PCR assay to detect RNA molecules spanning the calmodulin C-EFH5, EFH5-UbEP52/1, and UbEP52/1-UbEP52/2 intergenic regions. (A) Ethidium bromide-stained agarose gel of PCR amplification products generated from *T. brucei* procyclic RNA with primers AA1045 and BB559 (calmodulin C-EFH5; lanes 1 to 4), BB562 and AA525 (EFH5-UbEP52/1; lanes 5 to 8), and AA525 and AA524 (UbEP52/1-UbEP52/2; lanes 9 to 12). The negative controls are primers only (lanes 1, 5, and 9), omission of reverse transcriptase (RTase) (lanes 2, 6, and 10), and pretreatment of RNA with RNase (lanes 3, 7, and 11). Identities of the PCR amplification products were confirmed by hybridization of Southern blots of the gel shown in panel A with a calmodulin C-EFH5 intergenic probe (probe 6; Fig. 1) (B), an EFH5-UbEP52/1 intergenic probe (probe 1; Fig. 1) (C), and a UbEP52/1-UbEP52/2 intergenic probe (probe 5; Fig. 1) (D). The filters were washed with 0.1× SSC–0.1% SDS at 65°C. The faint cross-hybridization between the EFH5-UbEP52/1 and UbEP52/1-UbEP52/2 intergenic products is due to a common 54 nt at the 5′ end of the UbEP52/1 and UbEP52/2 genes. Lanes M, size markers.

the negative controls (lanes 5 to 7). This result suggests that the UbEP52 genes are linked polycistronically to the EFH5 gene.

A second theoretical product in the BB562/AA525 PCR reaction is a 934-bp band that would correspond to amplification of a precursor spanning EFH5 to UbEP52/2. This band is not detected, even on a longer exposure of the Southern blots. One explanation of this result is that processing is sufficiently rapid under normal conditions to preclude the observation of precursors spanning three genes. Alternatively, it is possible that the standard PCR conditions are not adequate to detect the template because of, for example, template length or different ionic requirements.

To test for transcriptional linkage of the calmodulin and EFH5 genes, the assay was repeated with oligonucleotide primers AA1045, which corresponds to the 3′ end of the calmodulin C gene, and BB559, which is complementary to the 5′ end of the EFH5 gene. A band of the predicted size (258 bp; Fig. 5A, lane 4), which hybridized (Fig. 5B) to the genomic probe (probe 6; Fig. 1), corresponds to the intergenic region and was absent in all of the negative controls (lanes 1 to 3). These data demonstrate the presence of RNA molecules spanning the calmodulin-EFH5 intergenic region. Since the tandem identical calmodulin genes have previously been implicated in polycistronic transcription (37), our data suggest that the UbEP52 gene pair and the EFH5 gene are part of the same polycistronic transcription unit as are the calmodulin genes.

When the PCR assay was performed with primers AA1045 and AA525, we did not observe a 1,429-bp band that would correspond to a precursor spanning the region between the calmodulin and UbEP52/1 genes. This result again suggests that precursors spanning three genes are not present in RNA populations that have not been enriched, for example by heat shock, for potential precursor molecules.

Transcription of the calmodulin, EFH5, and UbEP52 genes is sensitive to α-amanitin. The identity of the RNA polymerase that transcribes the calmodulin, EFH5, and UbEP52 genes was determined by labeling nascent RNA in isolated nuclei in the presence and absence of α-amanitin. Labeled RNA was subsequently hybridized to cloned DNAs that represent genes transcribed by different classes of RNA

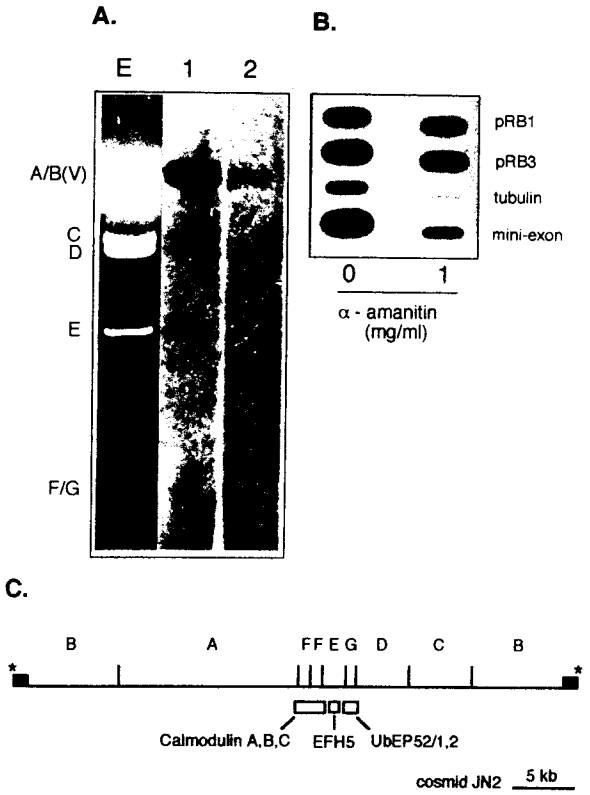

FIG. 6. Effect of α-amanitin on transcription of the calmodulin, EFH5, and UbEP52 genes. (A) Southern blot analysis. A Southern blot of cosmid JN2 digested with *Bgl*II (lane E, ethidium bromide stain) was hybridized with RNA from isolated nuclei that was labeled in either the absence (lane 1) or presence (lane 2) of 1 mg of α-amanitin per ml. (B) Slot blots of control genes hybridized with RNA from isolated nuclei that was labeled in either the absence or presence of 1 mg of α-amanitin per ml. pRB1 and pRB3 are ribosomal DNA clones. (C) Restriction map of cosmid JN2 digested with *Bgl*II. Solid boxes represent vector sequences, and asterisks represent an arbitrary site of cosmid linearization.

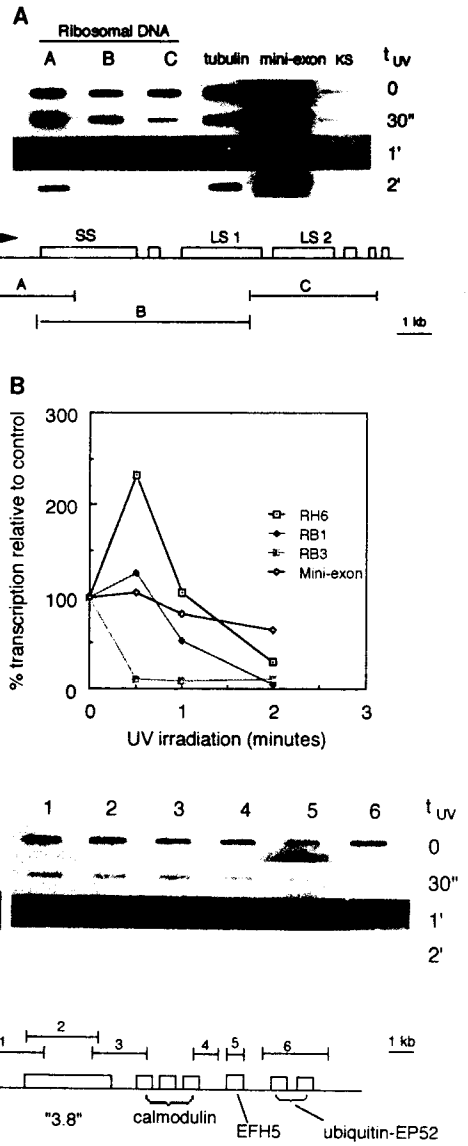

FIG. 7. Effect of UV irradiation on transcription of the calmodulin, EFH5, and UbEP52 genes in isolated nuclei. (A) Slot blot of control genes hybridized to radiolabeled nascent RNA isolated from procyclic nuclei after UV irradiation for 0, 0.5, 1, and 2 min. Ribosomal DNA samples A, B, and C represent plasmids pRH6, pRB1, and pRB3, respectively. Locations of the plasmids relative to the ribosomal gene are shown below the autoradiogram. KS represents the plasmid Bluescript vector control. Cells that received no UV radiation were mock treated to control for the effects of manipulation. (B) Quantitation of the effect of UV irradiation on transcription of the rRNA gene. Radioactivity hybridized to slots in panel A was measured by liquid scintillation counting. Values are expressed as percent relative to the value for no UV irradiation. (C) Slot blot of plasmid subclones within the JN2 cosmid hybridized to radiolabeled nascent RNA isolated from procyclic nuclei after UV irradiation for 0, 0.5, 1, and 2 min. Locations of subclones relative to the calmodulin-UbEP52 locus are shown below the autoradiogram. KS represents the plasmid vector control used for panel A.

polymerase. RNA labeled in the presence of 1 mg of α-amanitin per ml shows a reduction in hybridization intensity to a Southern blot of cosmid JN2 DNA (Fig. 6A) digested with *Bgl*II similar to that seen in slot blots of control DNA that encodes tubulin (Fig. 6B). We therefore conclude that the calmodulin, EFH5, and UbEP52 genes in *T. brucei* are transcribed by the α-amanitin-sensitive RNA polymerase II.

Transcription of the calmodulin, EFH5, and UbEP52 genes is rapidly inactivated by UV irradiation. The presence of RNA molecules that span the three intergenic regions is indicative of polycistronic transcription linking the calmodulin, EFH5, and UbEP52 genes in *T. brucei*. To provide more conclusive evidence that these genes are transcriptionally linked, we analyzed the effect of UV irradiation on nascent RNA in isolated nuclei. Because of the small sizes of the calmodulin, EFH5, and UbEP52 genes (≤1 kb), we predict that if each gene has its own promoter, RNA synthesis from each gene should be equally insensitive to UV irradiation. If the genes are transcribed from a common promoter, each gene should show a sensitivity to UV irradiation that is proportional to its distance from the promoter. As a control (Fig. 7A), we show that transcription of the rRNA gene is inactivated proportionally to its distance from the promoter and that transcription of the 140-bp miniexon gene is resistant to UV irradiation. Promoter proximal fragments of the miniexon gene and the rRNA gene (fragment A) show enhanced RNA synthesis after 30 s of UV irradiation (Fig. 7B), which is consistent with the results of

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