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An RNA Polymerase II Promoter in the *hsp70* Locus of *Trypanosoma brucei*

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To study the structure of RNA polymerase (pol) II transcription units and the influence of temperature on the regulation of gene expression in *Trypanosoma brucei*, an *hsp70* intergenic region promoter was characterized. In *T. brucei*, the *hsp70* locus contains, from 5′ to 3′, a cognate *hsp70*-related gene (gene 1) which is separated by about 6 kb of DNA from a cluster of five identical *hsp70* genes (genes 2 to 6). Transcription proceeds on the entire 23-kb locus, and polycistronic transcription occurs in *hsp70* genes 2 to 6. Transcription of *hsp70* genes 2 to 6 is only moderately sensitive to UV irradiation, indicating that it cannot be driven by a single far-upstream promoter, which suggests that promoters could be located in the region close to the *hsp70* coding region. Transient transformations demonstrated that sequences located upstream of *hsp70* gene 2 and in the intergenic region between *hsp70* genes 2 and 3 are able to direct transcription of the reporter gene, the chloramphenicol acetyltransferase (CAT) gene. The plasmid DNA driven by the *hsp70* intergenic region promoter gave CAT activity ~185-fold above the background level. This is equivalent to ~1% of that derived from a CAT plasmid driven by the procyclic acidic repetitive protein gene promoter, which is controlled by RNA pol I. The *hsp70* intergenic region promoter can drive α-amanitin-sensitive transcription at an internal position of the chromosome as well as an episome, suggesting that it is controlled by RNA pol II. However, this *hsp70* intergenic region promoter, along with the 3′ splice site and the 5′ untranslated region of the *hsp70* genes that controls the transcription of the reporter gene, cannot up-regulate the expression of the reporter gene during heat shock. This result is consistent with the previous observation that expression of the *hsp70* genes in *T. brucei* is mainly controlled at the posttranscriptional level.

Transcription of protein-coding genes and mRNA maturation in trypanosomes and other kinetoplastida involve mechanisms which are different from those in most higher eukaryotes (1, 6, 37, 47). Every mRNA in trypanosomes contains two exons, the 5′ miniexon and the main coding exon, which are transcribed from two separate genes. Through *trans* splicing, a capped 39-nucleotide transcript is joined with the main coding exon (1, 36, 43). Because of the discontinuous synthesis of mRNA, the 5′ end of the mRNA does not identify the transcription initiation site of the protein-coding genes but identifies that of the miniexon RNA. Therefore, it is still unclear where initiation of RNA polymerase (pol) II-transcribed protein-coding genes occurs.

Most genes in trypanosomes are found in multiple copies which are organized in tandem arrays and are separated from each other by short intergenic region sequences of a few hundred nucleotides (19, 38, 46). These clustered genes are usually polycistronically transcribed. These polycistronically transcribed mRNAs can encode proteins that are differentially expressed or expressed at similar levels. Through the processing events of poly(A) addition and *trans* splicing, intergenic regions are excised from the precursor RNA, resulting in the generation of the mature mRNAs. As a result of the polycistronic transcription of protein-coding genes, the differential expression of steady-state mRNAs from a single polycistronic transcript requires posttranscriptional control (14, 50). The mechanism of posttranscriptional control is not yet completely clear. However, the selection of the poly(A) addition site is specified by the location of the downstream 3′ splice acceptor site, indicating that *trans* splicing and poly(A) addition may be coupled (24).

Polycistronic transcription of genes could occur either from promoters located upstream of the tandem arrays of genes or from individual promoters located in front of each gene separately. Alternatively, the two events could occur simultaneously. Until now, only two protein-coding gene promoters have been well characterized in *Trypanosoma brucei*, the variant surface glycoprotein (VSG) gene and the procyclin, or procyclic acidic repetitive protein (PARP), gene. Transcription of these two genes is resistant to α-amanitin and presumably controlled by an RNA pol I or pol I-like enzyme (10). In addition, one DNA fragment located 4 kb upstream of the actin gene was shown to be able to direct transcription in transient transformations and is referred to as a putative actin gene promoter (3). The structures of promoters for all other protein-coding genes transcribed by the conventional α-amanitin-sensitive RNA pol II in trypanosomes have not yet been defined. A structural analysis of the PARP and VSG gene promoters demonstrated that well-defined transcription initiation sites were identified for these two polycistronic loci. The promoter of the VSG gene expression site (ES) is mapped at ~50 kb upstream of the VSG-coding sequence (2, 17, 52), while the promoter for PARP gene transcription is located at the immediate 5′ end of the coding sequence (7, 40, 42). However, it is not known whether transcription of protein-coding genes by RNA pol II is controlled by well-defined promoters.

The heat shock response and the hsp70 proteins are evolutionarily most highly conserved (34). Thus, I chose the *hsp70* genes to define the basic mechanisms for RNA pol II transcription and the mechanisms for regulated *hsp70* expression in *T. brucei*, an organism possessing a life cycle which alternates between a warm-blooded mammalian host and a cold-blooded

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insect vector, the tsetse fly. To determine the structure of transcription units that occur on the *hsp70* locus, I searched for potential promoter regions in the *hsp70* locus. In *T. brucei*, the *hsp70* locus contains six *hsp70* genes. From 5′ to 3′, a cognate *hsp70*-related gene (gene 1) is separated by ~6 kb of DNA from a cluster of five identical *hsp70* genes, genes 2 to 6 (15, 27, 29). I have previously shown that transcription proceeds on the entire 23-kb *hsp70* locus and polycistronic transcription occurs in *hsp70* genes 2 to 6 (29). Here, evidence is presented that the intergenic regions of the *hsp70* genes can function as an RNA pol II promoter, extrachromosomally or at an internal position on the chromosome, and that this *hsp70* promoter cannot maintain or up-regulate mRNA levels during heat shock. Together with a previous observation that transcription of the *hsp70* genes in *T. brucei* is not increased by an in vitro temperature shift (32, 35), this result further confirms the notion that in contrast to other eukaryotes, the differential expression of *hsp70* genes in *T. brucei* is mainly posttranscriptionally controlled.

MATERIALS AND METHODS

Trypanosomes. All trypanosomes used were originated from *T. brucei* stock 427-60. The bloodstream-form *T. brucei* variant 118 clone 1 has been described by Lee and Van der Ploeg (28). The procyclic 118 clone 1 trypanosome was established from *T. brucei* variant 118 clone 1 bloodstream form adapted to procyclic culture medium (38a). The procyclic *T. brucei* 427 RB was originally obtained from R. Brun. Bloodstream-form trypanosomes were passaged in rats and harvested by cardiac puncture. Procyclic trypanosomes were maintained in SDM 79 medium at 25°C as described by Brun and Schonenberger (8).

Plasmid constructs. The H23-CAT construct consists of sequences located upstream of the ATG of *hsp70* gene 3 to the *Hin*dIII site located at the 3′ end of the coding region of *hsp70* gene 2, fused to a previously described chloramphenicol acetyltransferase (CAT) vector, pCOCAT (40). All other H23 promoter-CAT-derived constructs contain a 5′ truncation of the promoter described for H23-CAT. This H23 intergenic region contains the poly(A) addition site of the *hsp70* gene 2 and the 3′ splice acceptor site of *hsp70* gene 3. The length of sequence upstream of the major 3′ splice site in each construct is listed in Fig. 2. In the H2-CAT construct, the promoter fragment contains sequences spanning from upstream of the ATG of *hsp70* gene 2 to the *Xba*I site located at 566 bp upstream of the 3′ splice acceptor site. The H23-H-B7 construct consists of, from 5′ to 3′, the *hsp70* intergenic region promoter as described for H23-CAT, the hygromycin phosphotransferase (*hph*) gene, the blunt-ended *Bgl*II-*Eco*RI fragment derived from the βα-tubulin intergenic region, and the blunt-ended *Hin*dIII-*Eco*RI fragment derived from the 5′-end pseudo-VSG gene of the VSG 118 ES (25). Prior to transformation, the plasmid was linearized at the *Sal*I site located in the center of the VSG ES-derived sequence. The rRNA promoter sequence in constructs RPJ-CAT and RPαsp-CAT is derived from a 518-bp *Alu* fragment spanning the 5′ end of the 18S rRNA gene.

UV inactivation of transcription. UV irradiation of the procyclic- and bloodstream-form trypanosomes was performed as previously described (20, 40). Cell suspensions were irradiated by a portable germicide lamp with an emission maximum at 254 nm. The dose rate was measured with a model J225 Blak-Ray UV meter at ~3,500 μW/cm^2. The UV dose was increased by varying the irradiation time. After irradiation, cells were spun down and nuclei were prepared by passing cells through a Stansted cell disruptor (40).

Transient DNA transformation. DNA transformation was performed with a BTX electroporator as previously described (7, 40). Ten micrograms of DNA of each construct was introduced into the procyclic *T. brucei* by electroporation. Eighteen hours postelectroporation, the cell extract was isolated by freeze-thaw. One-third of each extract was used for the CAT assay. For constructs driven by the PARP promoter, only 1 μg of plasmid DNA was used for electroporation and 1/10 of the extract was used for the CAT assay. The positive control for the CAT assay was performed with the commercially available CAT enzyme to ensure that the assay was within a linear range. The CAT assay was performed as described previously (7, 40). CAT activities were measured after incubation of cell extracts at 37°C, using 50 μM chloramphenicol, 250 μM butyl coenzyme A, and 0.1 μCi of [^{3}H]chloramphenicol followed by xylene extraction and scintillation counting. The relative CAT activity was adjusted for the amounts of DNA and extract used.

Stable DNA transformation. Linearized plasmid H23-H-B7 (10 or 20 μg) was electroporated into the procyclic 118 clone 1 trypanosomes as previously described (30). Thirty-six hours postelectroporation, hygromycin B was added to cell cultures to a concentration of 40 μg/ml. Two weeks after electroporation, the individually transformed drug-resistant trypanosomes were cloned by limiting dilution in microtiter dishes. Each well of the microtiter dishes contained 0.5 transformed cells and 2.5×10^6 wild-type trypanosomes in 400 μl of medium. The cloned trypanosome cell lines were maintained routinely in medium containing 40 μg of hygromycin B per ml.

Southern genomic blot analysis. Nuclear DNA was isolated from parasites as described by Van der Ploeg et al. (48). Following digestion with restriction endonucleases, the DNA was separated on a 0.8% agarose gel and transferred onto nitrocellulose filters. The blots were hybridized with ^{32}P-labeled probes. The final posthybridization wash was performed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) at 65°C. The hybridization intensities were quantitated by counting the nitrocellulose filters in a Betagen Betascope 603 blot analyzer. The data were analyzed by using a Betagen version 2.0 software package.

PFGE. Chromosome-size DNA was prepared as previously described (18, 49). The pulsed-field gel electrophoresis (PFGE) running conditions for each gel are described in the figure legends. The γ irradiation of chromosome blocks was performed with a Cs source as described by Ellenberger and Beverley (13).

Nascent RNA analysis. Preparation of nuclei and nuclear run-on reactions were performed as described previously (40). Hybridizations were performed for 48 h. The final posthybridization wash was performed in 0.1× SSC–0.1% SDS at 65°C. For the UV inactivation of transcription assay, hybridization signals were quantitated with a Betagen Betascope 603 blot analyzer for 20 h. Probes used for nuclear run-on assays are as follows. Subclones pEMR1 to pEMR5 were derived from the *T. brucei* rRNA transcription unit (17, 20, 40). The rRNA gene probe pR4, was described by Kooter and Borst (21). The αβ-tubulin gene probe pTb α-β T-1, was described by Thomashow (44). The VSG 118 clone 1 ES-derived clones (a to f) were described by Lee (25). The VSG 118 cDNA TcV118-2 probe was described by Bernards et al. (4). The ES-associated gene 1 (ESAG1) clone was described by Cully et al. (11). The VSG 118 ES promoter clone was described by Gottesdiener et al. (17). The 5S rRNA gene probe was described by Lenardo et al. (33). The miniexon gene clone pCL103 was described by Laird et al. (23). The PARP cDNA was described by Rudenko et al. (39). Tb-17, Tb-29, and CRAM represent the Tb-17 cDNA (51), the Tb-29 cDNA (32), and the CRAM cDNA (26). The *hsp70* coding region probe and the *hsp70* intergenic region probe were as previously described (15, 29).

DNA sequence analysis of the 5′ end of the *hph* mRNA. The cDNAs spanning the 5′ end of the *hph* transcript were obtained by primer extension with a 21-mer oligonucleotide (CTCCCGAGAGCTGCATCAGGT) complementary to the 5′ coding region of the *hph* gene. The cDNAs were amplified by PCR, using the *hph* primer and a miniexon 21-mer sense-strand primer (CACAGTTTCTGTAC TATCTTG). The nucleotide sequence of amplified DNA was determined by the dideoxy-chain termination method (41).

Northern (RNA) blot analysis. Total RNAs were isolated from trypanosomes by guanidine thiocyanate lysis and purified by centrifugation through CsCl cushions (9). RNA samples were separated in a 1% formaldehyde-agarose gel and transferred to nitrocellulose filters. The blots were hybridized with ^{32}P-labeled probes. The final posthybridization wash was performed in 0.1× SSC–0.1% SDS at 65°C.

RESULTS

Localization of the *hsp70* gene promoter by UV inactivation of transcription. The transcriptional mapping by UV inactivation and nuclear run-on assay has been previously applied to identify the VSG gene promoter and the PARP gene promoter (17, 20, 22, 40). This method is based on the fact that RNA pol is not able to traverse pyrimidine dimers generated in the DNA template by UV irradiation (5, 16). Therefore, the longer a transcription unit is, the more sensitive it is to UV irradiation. Thus, whether a single far-upstream promoter controls the transcription of the entire *hsp70* locus or the array of *hsp70* genes 2 to 6 is transcribed by a promoter or promoters adjacent to the coding region can be distinguished by the sensitivity of transcription to UV irradiation. Figure 1 shows the UV inactivation of transcription in bloodstream-form (Fig. 1A) and procyclic-form (Fig. 1B) trypanosomes. The miniexon genes and five rRNA gene sequences located at increasing distances from their promoters (pEMR1 spans the rRNA gene promoter region; the average distances of pEMR2, -2.5, -3, and -5 to their promoters are ~1.95, 2.95, 4.3, and 8.7 kb, respectively) were used as controls for the UV inactivation mapping. Transcription of rRNA sequences that progressively extend toward the 3′ end of the rRNA genes is more rapidly diminished by UV irradiation. Transcription of the rRNA promoter (pEMR1), the VSG gene promoter, and the miniexon gene is highly resistant to UV irradiation. The sensitivity of transcription of *hsp70* genes 2 to 6 to UV irradiation was measured by using the

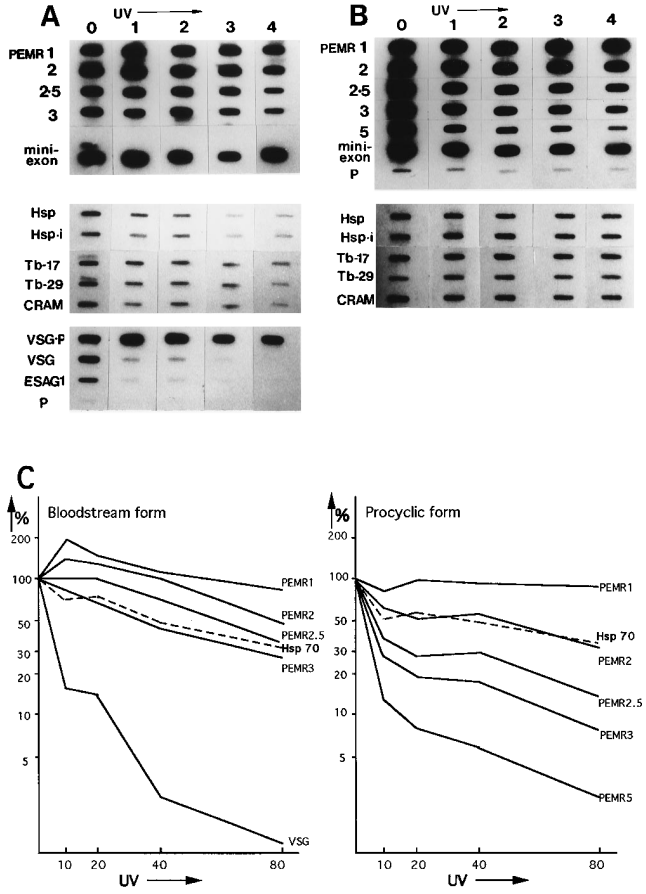

FIG. 1. Transcriptional mapping by UV inactivation. Trypanosomes were exposed to increasing doses of UV light, and their nuclei were isolated for nuclear run-on assays to measure the distribution of nascent RNA. Arrows indicate the direction of increasing doses of UV light. The UV doses applied to the bloodstream-form trypanosome variant 118 clone 1 (A) were 10^3 (lane 1), 2×10^3 (lane 2), 4×10^3 (lane 3), and 8×10^3 (lane 4) ergs/mm^2; the UV doses applied to the procyclic-form trypanosome 427-RB (B) were 1.5×10^3 (lane 1), 2.75×10^3 (lane 2), 4×10^3 (lane 3), and 8×10^3 (lane 4) ergs/mm^2. Lanes 0 represent trypanosomes exposed to no UV irradiation. The top panels show slot blots of different control genes: pEMR1, -2, -2.5, -3, and -5 are clones spanning the ribosomal DNA transcription unit (17, 20, 40). Mini-exon, miniexon genes (23); P, the plasmid vector control (pUC18). The bottom right panel shows the VSG gene ES-derived clones: VSG·P, VSG gene promoter sequence (17); VSG, VSG 118 coding region (4); ESAG1 (11); and P, the plasmid vector control. The middle panels show RNA pol II-transcribed genes: Hsp, the *hsp70* coding region (15, 29); Hsp·i, *hsp70* intergenic region (29); Tb-17 (51); Tb-29 (31); and CRAM (26). (C) Quantitation of the UV inactivation of *hsp70* gene transcription, plotted on semilogarithmic graphs of the relative transcriptional efficiency ([signal after UV dose/signal with no UV] × 100) plotted against the UV dose (ergs [10^2]/mm^2).

hsp70 coding sequence as a probe, this UV sensitivity was between those of pEMR2.5 and pEMR3 in bloodstream-form trypanosomes and was comparable to that of pEMR2 in procyclic trypanosomes (Fig. 1). This result demonstrated that transcription of the *hsp70* coding sequences is only moderately sensitive to UV irradiation. This sensitivity indicates that promoters for *hsp70* gene transcription are located slightly upstream of the *hsp70* coding sequence and is not compatible with a single far-upstream promoter. This differs from transcription of the VSG gene and ESAG1, which is immediately abolished by a low dose of UV irradiation (VSG and ESAG1 are located ~50 and 25 kb, respectively, downstream of the promoter [17, 20, 22]). As controls, the sensitivities to UV irradiation of three other protein-coding genes, Tb-17 (51), Tb-29 (31), and CRAM (encoding a cysteine-rich acidic integral membrane protein [26]), were measured. Tb-29 and CRAM are single-copy genes, and the Tb-17 gene family spans ~23 kb in the *T. brucei* genome. These genes also showed a moderate sensitivity to UV irradiation. These results suggest that promoters could be located in an area adjacent to these RNA pol II-transcribed protein-coding genes. This hypothesis was tested by assaying the ability of sequences located upstream of *hsp70* gene 2 and the intergenic region of *hsp70* genes 2 and 3 to direct transcription of the CAT gene in a transient DNA transformation assay with the procyclic form of *T. brucei.*

Strength of the *hsp70* promoter(s) in transient transformation of procyclic *T. brucei*. Sequences derived from upstream of *hsp70* gene 2 and the entire intergenic region of *hsp70* genes 2 and 3 were linked to the CAT gene. These constructs were then introduced into procyclic trypanosomes. Promoter activity was measured by assaying the transiently expressed CAT activity (Fig. 2). A CAT vector containing no promoter (pCOCAT), which gives no activity or background activity, was used as a negative control. The CAT activity derived from each construct was the average of three assays and calculated relative to the background activity derived from pCOCAT. Construct H23-CAT, containing sequences spanning from the *Hin*dIII site in the 3′ end of gene 2 to the immediate 5′ end of the ATG codon in gene 3, produced an activity which is ~185-fold higher than the background activity. Bnsp-CAT, driven by the PARP promoter and its 3′ splice site, produced an activity which is ~18,550-fold higher than the background activity and ~100-fold higher than that derived from H23-CAT. Shortening the 5′ end of the intergenic region sequence of *hsp70* genes 2 and 3 to 464, 366, and 188 bp upstream of the 3′ splice site reduced the CAT activity to ~130-fold higher than the background activity (H23Hp-CAT, H23A-CAT, and H23S-CAT, respectively). Shortening this region to 89 bp (H23P-CAT) reduced the CAT activity to ~80-fold higher than the background activity. The sequence containing only the 3′ splice site and the 5′ untranslated region of *hsp70* (construct H23J-CAT) gave no obvious promoter activity.

To show that construct H23J-CAT still retained a functional 3′ splice site, a polycistronic construct, PARPNJ-CAT, was prepared; in this construct, a transcription unit of the PARP promoter and PARP 3′ splice site driving the neomycin resistance gene (PARP-NEO) was linked to the 5′ end of H23J-CAT. Transcription of the CAT gene by this construct is driven by the PARP promoter located far upstream, and *trans* splicing of the CAT gene uses the 3′ splice site of *hsp70* genes 2 and 3. This construct produced an activity equivalent to that obtained from the PARP promoter in construct Bnsp-CAT (Fig. 2). The function of the *hsp70* 3′ splice site in construct H23J-CAT was further confirmed with construct RPJ-CAT, which contained the rRNA promoter directly linked to H23J-CAT. RPJ-CAT produced a CAT activity ~42,850-fold higher than the background activity, which is slightly higher than that produced by construct RPαsp-CAT, which contains the CAT gene driven by the rRNA promoter and the 3′ splice site of the α-PARP gene. Furthermore, reverse transcription-PCR analysis of CAT mRNA derived from PARPNJ-CAT and H23P-CAT confirmed that the *hsp70* 3′ splice site was used for the generation of mature CAT mRNA produced by these constructs (data not shown).

To show the sequence specificity of the *hsp70* promoter, a ~500-bp fragment spanning the 3′ coding region and 3′ untranslated region of the β-tubulin gene was linked to H23J-CAT, generating AJ-CAT. This construct produced an activity

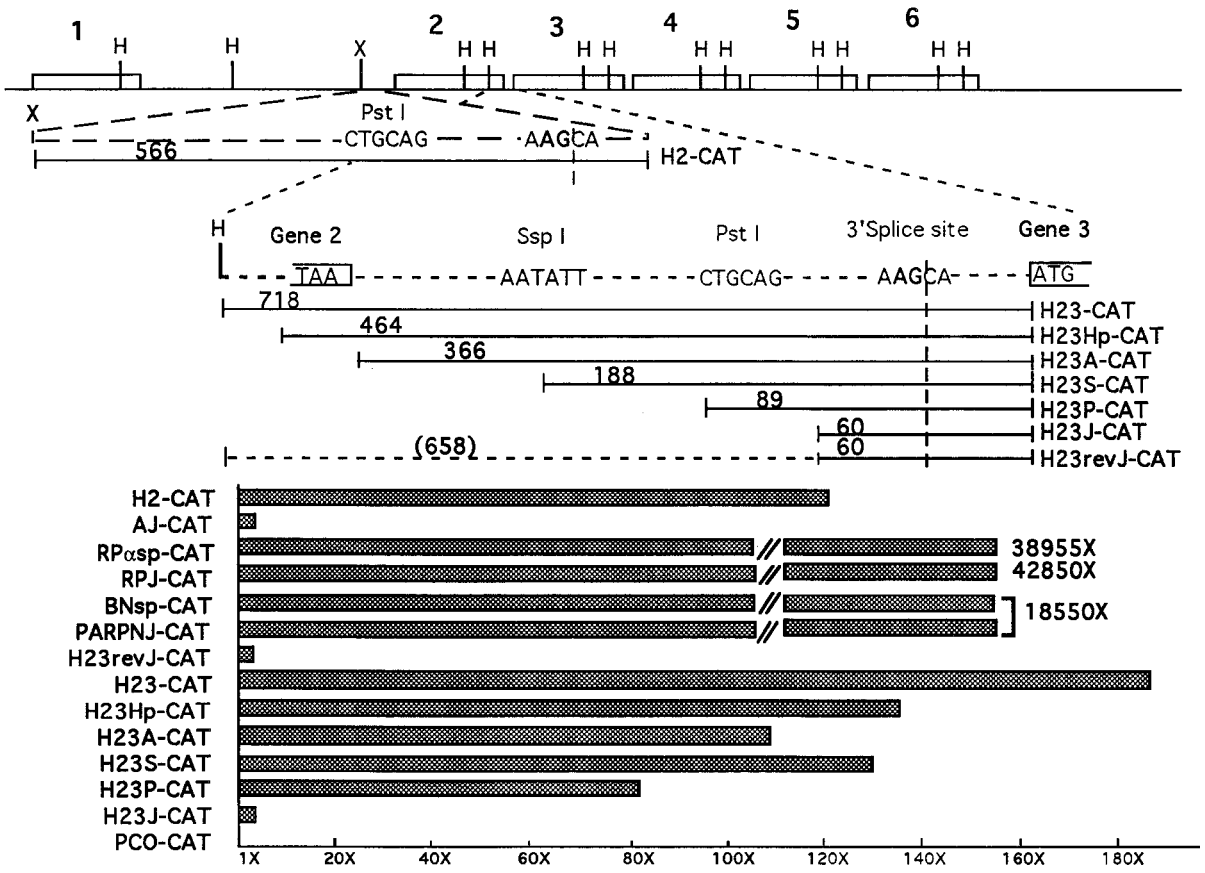

FIG. 2. Transient transformation and CAT activities. Procyclic-form trypanosomes (4×10^7) were electroporated with 10 μg of each *hsp70* promoter-derived construct and 1 μg of each PARP promoter-derived construct. Eighteen hours after transformation, whole cell extracts were prepared for CAT assay. To ensure that all CAT activities were in the linear range of the enzyme assay, various dilutions of purified *Escherichia coli* CAT enzyme (Pharmacia) were assayed in parallel with the trypanosome extracts. In a typical assay, H23CAT plasmid generates a CAT activity of ~94,000 cpm, compared with a background of 500 cpm for the CAT gene alone (pCOCAT). The top diagram shows the structure of the *hsp70* locus and the sequences derived from the *hsp70* locus in each construct. 1 to 6 indicate *hsp70* genes 1 to 6, respectively. H, *Hin*dIII; X, *Xba*I. The number above the line for each construct represents the length of the sequence upstream of the 3′ splice site. The bar graph demonstrates the relative CAT activity generated by each construct.

comparable to that of H23J-CAT, a background level of activity (Fig. 2). Additionally, when the 658-bp DNA fragment located 60 bp upstream of the *hsp70* 3′ splice site was linked to H23J-CAT in the reverse orientation (H23revJ-CAT), no significant CAT activity was observed. In summary, the transient transformation experiments demonstrated that the intergenic region of *hsp70* genes 2 and 3 can function as a promoter.

Since the 5′ flanking sequence of *hsp70* gene 2 is identical to the intergenic region sequence of genes 2 to 6 (up to 73 bp upstream of the major 3′ splice site [29]), a DNA fragment containing sequences from upstream of the ATG of gene 2 to 566 bp upstream of the 3′ splice site was tested for promoter activity, as indicated by H2-CAT. The H2-CAT construct produced an activity which is ~120-fold higher than the background activity. In summary, I have shown, by transient transformation, that promoters could be present in front of *hsp70* gene 2 and in the intergenic region of *hsp70* genes 2 to 6. Together with the previous observation that polycistronic transcription occurs in *hsp70* genes 2 to 6, the identification of putative promoters indicated that overlapping transcription units could also occur in *hsp70* genes 2 to 6.

Stably transformed procyclic trypanosome cell lines generated by a construct driven by the *hsp70* intergenic region promoter. To further confirm the promoter function of the *hsp70* intergenic region and analyze its mode of transcription, stably transformed trypanosome cell lines were established by using the *hph* gene driven by the *hsp70* intergenic region promoter (construct H23-H-B7). The promoter function was tested in a transcriptionally silent region in the VSG 118 ES and in extrachromosomal molecules as previously described (25). The result demonstrated that the *hsp70* intergenic region promoter can function at an internal position of the chromosome and at an episome (see below).

The H23-H-B7 construct contains the *hsp70* intergenic region promoter (H23) followed by the *hph* gene, βα-tubulin intergenic region sequence providing for a poly(A) addition site, and a targeting sequence derived from the VSG 118 ES (Fig. 3) (25). The targeting sequence spans the downstream region of the large 70-bp array and the 5′ end of the pseudo-VSG 118 gene of the VSG 118 ES (25). The linearized H23-H-B7 construct was introduced into the procyclic 118 clone 1 trypanosomes. Southern blot analysis of chromosome-size DNA or restriction enzyme-digested genomic DNA revealed two types of transformed cell lines that were generated with the H23-H-B7 construct. Type 1 cell lines contained one to four copies of the H23-H-B7 construct integrated into the VSG 118 ES. A representative line (H23H.A), containing four tandemly linked H23-H-B7 sequences at the VSG ES, was analyzed in detail. By genomic Southern analysis, the physical

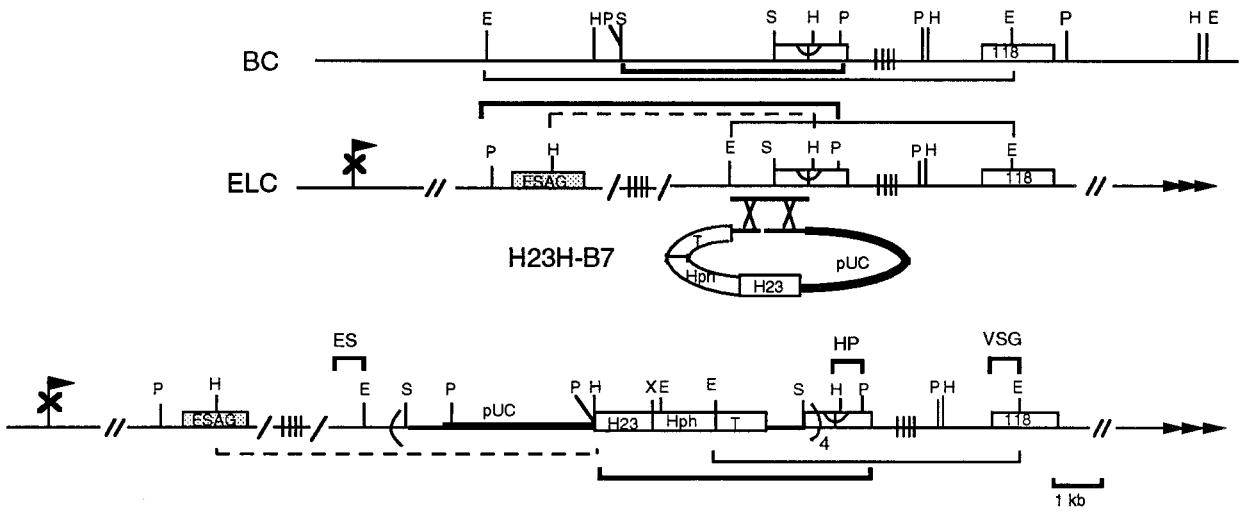

FIG. 3. Physical maps of the VSG 118 loci of *T. brucei* variant 118 clone 1 and the H23-H-B7 construct and the structure of the *hph* locus in trypanosome cell line H23H.A. Top, structure of the basic-copy (BC) VSG gene locus. Middle, structure of the VSG 118 ES (17, 28) and plasmid H23-H-B7. Bottom, structure of the *hph*-VSG 118 ELC locus. Blocks represent different VSG genes. The *Eco*RI-*Hin*dIII fragment, underlined in ELC, was used as a targeting sequence. H23, the *hsp70* intergenic region promoter and the *hsp70* 3′ splice acceptor site (29); Hph, hygromycin resistance gene; T, tubulin intergenic region sequence (44); pUC, vector pUC18; 118, VSG 118 gene (28); ψ, the pseudo-VSG 118 gene (28); ESAG, expression site-associated genes (11); double slashes, a distance of 10 to 20 kb; triple arrows, telomere repeats; flag with a cross, the VSG promoter of the inactive VSG 118 ES; four closely arranged vertical lines, 70-bp repeats. The number 4 indicates that four copies of a tandemly arranged input plasmid were integrated into the target site in the analyzed cell line. Restriction enzyme sites: E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; S, *Sal*I; X, *Xba*I. Probes used in the Southern analysis are indicated above the map: ES, expression site-derived probe; HP, the *Hin*dIII-*Pvu*II fragment spanning the 3′ end of the pseudo-VSG 118 gene; VSG, the 5′ coding region of the VSG 118 gene. The thick, thin, and dashed lines indicate fragments detected by probes HP, VSG, and ES, respectively.

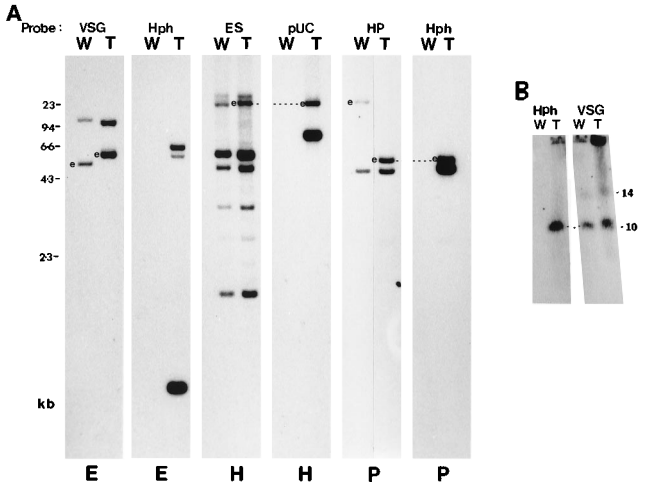

FIG. 4. Structure of the *hph* locus in trypanosome cell line H23H.A. (A) Southern Blot analysis. Genomic DNAs from wild-type trypanosomes (W) and H23H.A trypanosomes (T) were digested with restriction enzymes, separated in a 0.8% agarose gel, and transferred onto nitrocellulose filters. The final posthybridization wash was performed in 0.1× SSC–0.1% SDS at 65°C. Dotted lines align the fragments generated by the integration event. e, VSG ES-associated fragment. The probe used in each panel is indicated at the top. VSG, ES, and HP are described in the legend to Fig. 3. The Hph is the *hph* coding region probe. pUC is pUC18. E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II. (B) Chromosomal location of the *hph* gene in the transformed trypanosomes. PFGE and chromosome assignments in *T. brucei* were performed as previously described (18, 49). Chromosome blocks of H23H.A trypanosomes (lanes T) and wild-type trypanosomes (W) were separated in a 1% agarose gel for 7 days at 3 V cm^{-1} and a pulse frequency of 3,000 sec. Parallel PFGE blots were hybridized with the Hph probe and the VSG probe. The final posthybridization wash was performed in 0.1 × SSC–0.1% SDS at 65°C. 10 and 14 indicate chromosome bands 10 and 14, respectively, as described previously (18, 49).

map of the H23-*hph* locus in the H23H.A cell line was generated (Fig. 4A).

Genomic blots containing restriction enzyme-digested genomic DNA from wild-type trypanosomes and H23H.A trypanosomes were hybridized with various probes (Fig. 4A). The altered sizes of the VSG gene ES-derived fragments in the H23H.A genomic DNA are attributed to the integration event that occurred downstream of the large 70-bp array and upstream of the pseudo-VSG gene in the VSG 118 ES. For example, in the *Eco*RI-digested DNA, the 4.8-kb *Eco*RI fragment of the VSG ES-linked copy (ELC) detected by the VSG 118 probe in the wild-type trypanosomes is not detected in the H23H.A trypanosomes, but a new fragment of 5.4 kb was visualized. When the ES probe (an ES-derived fragment) was used instead of the 15-kb *Hin*dIII ELC fragment observed in the wild type, a 18-kb *Hin*dIII fragment was detected in the H23H.A cell line. This 18-kb *Hin*dIII fragment was also visualized by the pUC18 probe. Similarly, when the 3′ end of the pseudo-VSG gene was used as a probe (probe HP), the ~23-kb *Pvu*II ELC observed in the wild-type trypanosomes could not be seen in the H23H.A trypanosomes. Instead, a 5.5-kb *Pvu*II fragment, which was also detected by the *hph* probe, was observed in the H23H.A trypanosomes. The *hph* and pUC18 hybridizations also revealed an additional fragment(s) of a size predicted for the input plasmid with a higher intensity in the transformed trypanosomes. This fragment is presumably derived from the integration of multiple tandemly arranged input plasmids as previously described (30).

The chromosomal location of the *hph* gene in cell line H23H.A was determined by PFGE analysis (Fig. 4B). The *hph* gene was detected at chromosome band 10 (Fig. 4B, Hph), and this chromosome is also detected by the VSG 118 gene probe (Fig. 4B, VSG). Chromosome 14, which is also visualized with the VSG probe, contains the basic-copy VSG gene locus. By measuring the relative intensity of the *hph*-hybridizing bands in the H23H.A DNA, it was concluded that four copies of tan-

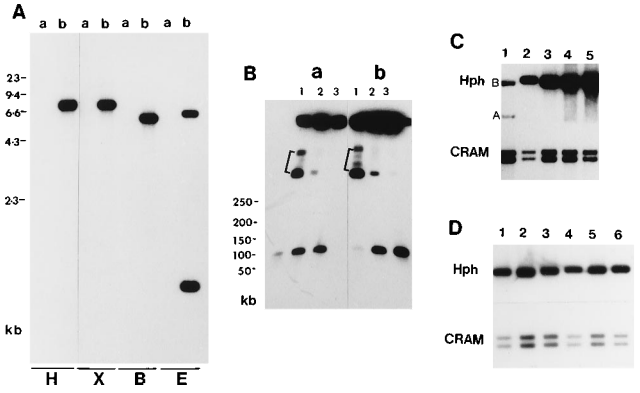

FIG. 5. Structure of the *hph* locus in the transformed trypanosome cell line H23H.B. (A) Southern genomic blot analysis. Genomic DNAs derived from wild-type trypanosomes (lanes a) and H23H.B (lanes b) were digested separately with restriction enzymes *Hin*dIII (H), *Xba*I (X), *Bgl*II (B), and *Eco*RI (E), size separated in a 0.8% agarose gel, and then transferred onto nitrocellulose filters. The genomic blots were probed with the Hph probe. (B) Chromosomal location of the input plasmid in cell line H23H.B by γ-irradiation analysis. Chromosome blocks of H23H.B trypanosomes were irradiated with various amounts of γ radiation and then separated in 1% pulsed-field gels for 26 h at 16 V cm^{-1} and a 28-s pulse frequency. In lanes 1, 2, and 3, the blocks were irradiated with doses of 0, 20, and 50 krad, respectively. a, chromosome-size DNA derived from H23H.B trypanosomes that were maintained in medium containing 40 μg of hygromycin B per ml; b, samples derived from H23H.B cells that were maintained in medium containing 400 μg of hygromycin B per ml for ~60 generations. The blots were hybridized with the Hph probe. Brackets indicate DNAs located in an area where no chromosomal DNA can be observed. (C) Determination of the copy number of H23-H-B7 in H23H.B trypanosomes. Genomic DNAs from H23H.A trypanosomes (lane 1), H23H.B trypanosomes that were maintained in medium containing 40 μg of hygromycin B per ml (lanes 2 and 3), and H23H.B trypanosomes that were maintained in medium containing 400 μg of hygromycin B per ml for ~40 and ~60 generations (lanes 4 and 5, respectively) were digested with *Hin*dIII, separated in a 0.8% agarose gel, and transferred onto a nitrocellulose filter. The filter was sliced into two panels. The top panel, containing the digested genomic DNA larger than 4 kb, was hybridized with the Hph probe; the lower panel, containing the digested genomic DNA smaller than 4 kb, was hybridized with the CRAM probe (the size of the *Hin*dIII fragment encoding CRAM is ~3 kb). A and B indicate the single-copy *hph*-hybridizing band and the three copies of the *hph*-hybridizing band with the size of the original input plasmid in H23H.A. (D) Quantitation of the H23-H-B7 plasmid in H23H.B trypanosomes maintained in medium without hygromycin B. Lane 1 to 6 represent genomic DNA isolated from H23H.B grown in the absence of drug selection for 0, 11, 15, 19, and 23 days, respectively. The top and bottom panels were hybridized with the Hph and CRAM probes, respectively. The final posthybridization wash was performed at 65°C in 0.1× SSC. Hybridization signals were quantitated in a Betagen Betascope 603 blot analyzer.

demly arranged H23-H-B7 were integrated into the target site at the VSG 118 ES of the procyclic 118 clone 1 trypanosomes (see below).

In the second type of cell line generated with the H23-H-B7 construct, no obvious integration events could be identified. The status of the H23-H-B7 plasmid in one representative line (H23H.B) was analyzed in detail. In the genomic DNA derived from H23H.B cell line, only DNA fragments of a size for the input plasmid could be detected, and additional fragments predicted for integration events were not observed (Fig. 5A). This result indicated that most likely the plasmid DNA is extrachromosomally located in the H23H.B cell line, as previously described (25). This event was further confirmed by PFGE and γ-irradiation analysis (Fig. 5B). Circular DNA molecules that receive a single double-stranded break by γ irradiation will be converted to a linear molecule, while linear DNA molecules will be randomly cleaved by γ irradiation, generating a background smear in the gel. The PFGE analysis showed that the majority of plasmid DNA in H23H.B cells is located in the slots and in an aberrant area where no chromosome assignment can be obtained (Fig. 5B, brackets). Following the γ irradiation, a discrete band in the pulsed-field gel at a size of ~100 kb was detected with the Hph probe. Increasing the radiation dose did not change the banding pattern, but it increased the intensity of the 100-kb band and decreased the amount DNA retained in an aberrant area (Fig. 5B, lanes 2 and 3). This result indicated that the plasmid DNA molecules are present as extrachromosomal circles (episomes). The copy number of the input plasmid and the 100-kb episome in H23H.B cell line was determined. Figure 5C demonstrates the relative hybridization intensities of the *hph* bands in the H23H.A (lane 1) and H23H.B (lanes 2 to 5) cell lines. CRAM gene hybridization was used as an internal control to adjust for different amounts of DNA loaded in the lanes. The hybridization was performed in the presence of an excess amount of probe, and the amount of radioactivity in each band was quantitated with a Betascope analyzer. A and B represent a single copy of the polymorphic *hph* fragment generated by the integration event and the other three copies of the *hph* fragment of a size predicted for the original input plasmid, respectively, in H23H.A trypanosomes (Fig. 5C, lane 1). On the basis of the amount of *hph* hybridization, it was estimated that there were ~55 copies of input plasmid, equivalent to 3.5 copies of the 100-kb episome, in each H23H.B cell (Fig. 5C lanes 2 and 3). The copy number of the episome can be increased by increasing the amount of hygromycin B present in the culture medium. Lanes 4 and 5 of Fig. 5C show the hybridization of DNA from the H23H.B cell line that had been maintained in medium containing 400 μg of hygromycin B per ml for 20 and 30 days (~40 and 60 generations, respectively). Under the high concentration of drug selection, the copy number of the input plasmid increased to ~72 (lane 4) and ~85 (lane 5) per cell. However, the size of episomes in the trypanosomes selected with a high dose of drug was maintained as 100 kb, as shown in panel b of Fig. 5B. The stability of the episome was also determined in the absence of the hygromycin B selection. Figure 5D demonstrates the amount of the *hph* hybridization detected in the genomic DNA from H23H.B trypanosomes that were maintained in medium without hygromycin B for up to 23 days (~46 generations). No loss of the episome was observed in H23H.B cells grown in the absence of drug selection. This result indicates that these episomes can be properly propagated and segregated in trypanosomes.

Transcriptional analysis of the *hph* gene driven by the *hsp70* intergenic region promoter. To analyze the mode of transcription events that occur in the H23-*hph* locus, the nascent RNA transcription in wild-type trypanosomes was compared with that in cell lines transformed with the H23-H-B7 construct by nuclear run-on assays (Fig. 6). Transcription of the *hph* gene is observed in the transformed cell line H23H.A but not in wild-type trypanosomes (Fig. 6A). More importantly, *hph* transcription is abolished by the presence of α-amanitin, as is transcription of the endogenous *hsp70* gene and the tubulin gene, whereas transcription of the rRNA genes, the PARP gene, and the VSG promoter sequences is resistant to α-amanitin. This result demonstrates that the *hph* gene driven by the H23 promoter is transcribed by RNA pol II. In cell line H23H.A, no transcription was detected in the region upstream of the integration site, as indicated by probe a. Transcription derived from plasmid H23-H-B7 proceeds to 2.5 kb downstream of the integration site and stops in front of the VSG 118 gene (Fig. 6). No transcription was observed in the 800-bp *Hin*dIII-*Pst*I fragment located upstream of the VSG 118 coding region (probe d). The exact mechanism that operates transcription termination of the H23-H-B7 transcription complex at the VSG 118 ES is not clear. However, this transcription termination event

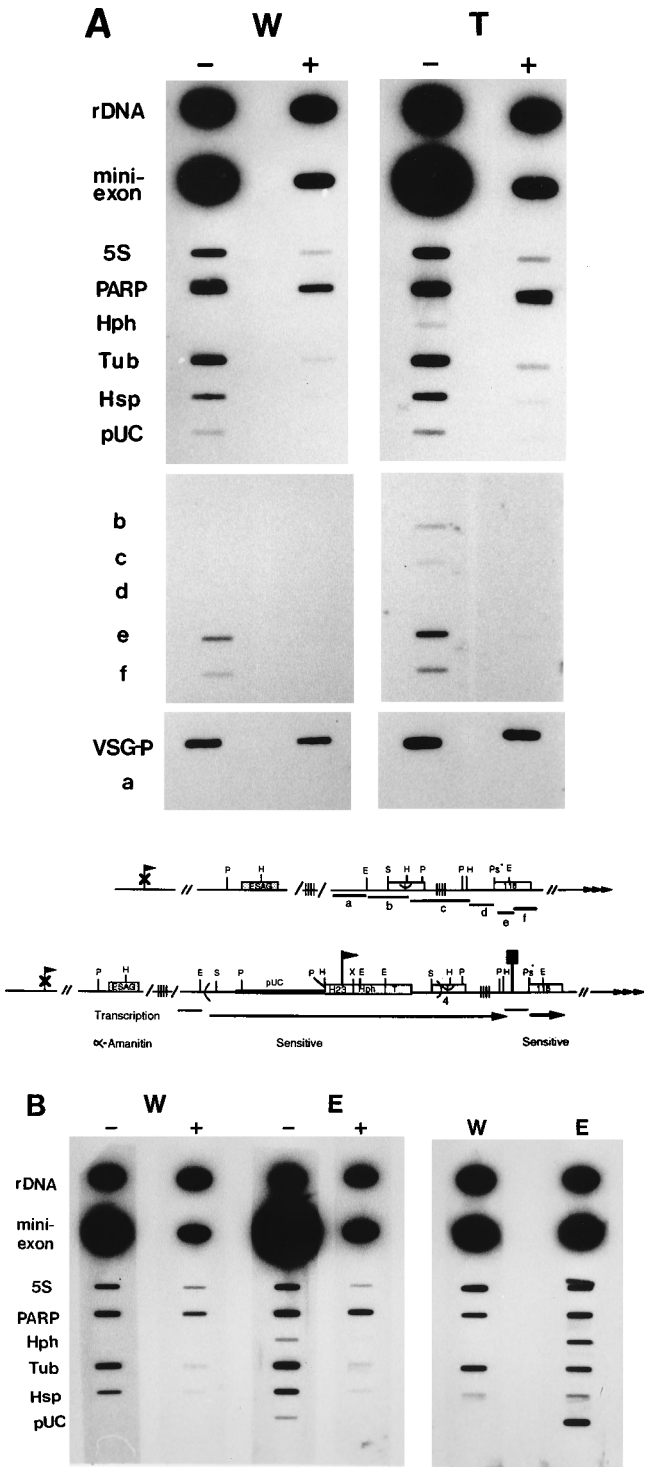

FIG. 6. Nascent RNA analysis of transcription in the *hph* locus in cell lines transformed with the H23-H-B7 construct. (A) Comparison of the nascent RNA transcription in wild-type trypanosomes and the H23H.A cell line. Preparation of nuclei and the nuclear run-on reaction were performed as described previously (40). The reaction was performed without (−) or with (+) α-amanitin added to a final concentration of 1 mg/ml. Nascent RNAs isolated from the wild-type 118 clone 1 procyclic trypanosomes (W) and the H23H.A trypanosomes (T) were hybridized with slot blots containing various probes. Probes used are DNA fragments purified from the *T. brucei* ribosomal transcription unit clone pR4 (rDNA) (21); the miniexon clone, pCL103 (mini-exon) (23); 5S, a 5S rRNA clone (33); a PARP coding region clone CPT4 (PARP) (39); the coding region of *hph* (Hph); the coding region of the tubulin gene (Tub) (44); the coding region of the *hsp70* gene (Hsp); and plasmid pUC18 (pUC). Probes a to f are derived from the VSG 118 ES as indicated below the physical map. The VSG-P represents a 1.3-kb *Hin*dIII-*Pst*I fragment derived from the VSG 118 ES promoter probe 1 as described by Gottesdiener et al. (17). The final posthybridization wash was performed in 0.1× SSC–0.1% SDS at 65°C. The transcriptional map of the VSG 118 ES in the H23H.A trypanosome line is shown at the bottom. The large flag indicates the *hsp70* intergenic region promoter. The flags with crosses represent the inactivated VSG ES promoter. The bar with a black square indicates the region where no transcription was observed. The lines with arrowheads and plain thin lines underneath the map indicate the transcriptionally active region and silent region, respectively. Ps* represents the presence of a *Pst*I site at the indicated location; other *Pst*I sites in this locus are not marked. Other symbols and abbreviation are described in the legend to Fig. 3. (B) Comparison of nascent RNA transcription in wild-type trypanosomes and the H23H.B cell line. Nascent RNAs were isolated from nuclei of wild-type trypanosome 118 clone 1 (W) and H23H.B trypanosomes (E). The nuclear run-on reactions were performed without (−) and with (+) α-amanitin of a concentration of 1 mg/ml. Probes are as described for panel A. In the left-hand panel, the H23H.B trypanosomes were maintained in medium containing 40 μg of hygromycin B per ml. In the right-hand panel, the H23H.B trypanosomes were maintained in medium containing 400 μg of hygromycin per ml for ~60 generations and contained a higher number of the 100-kb episome. The right-hand panel shows the nuclear run-on reaction performed in the absence of α-amanitin.

was also observed when the α-amanitin-resistant transcription unit driven by the PARP promoter was placed at this location (25). As previously documented, a low level of α-amanitin-sensitive transcription was observed in the VSG 118 coding region (25). The transcriptional efficiency of the *hph* gene in the H23H.A cell line was ~76% of that of the endogenous *hsp70* genes, calculated on the basis of per copy number and per unit length of the probe. The H23H.A cell line contains 4 copies of the *hph* gene, 10 copies of identical *hsp70* genes (genes 2 to 6; 5 copies per allele), and 2 copies of *hsp70*-related gene 1.

Figure 6B demonstrates a comparison of nascent RNA transcription in wild-type trypanosomes and in the H23H.B cell line that contained *hph* episomes. As in the case of transcription at an internal position of the chromosome, transcription of the episomal *hph* gene driven by the H23 promoter is also sensitive to the presence of α-amanitin. The amount of *hph* gene transcription was increased in H23H.B cells that were maintained in medium containing a higher concentration of drug, as shown in Fig. 6B (right panel). A significant amount of transcription of the plasmid vector (pUC18) was observed in both types of cell lines, indicating that efficient read-through may occur when RNA pol II operates.

In summary, the *hsp70* intergenic region sequence can function as an RNA pol II promoter at an internal position of the chromosome and at an episome. Further analysis of the *hph* cDNA showed that the 39-nucleotide miniexon had been *trans* spliced onto the 5′ end of the *hph* mRNA by means of the 3′ splice acceptor site located in the 5′ end of the *hsp70* coding region (Fig. 7).

Influence of temperature on expression of the *hph* gene driven by the *hsp70* intergenic region promoter. Expression of the *hph* gene following heat shock in the H23H.B cell line was measured by Northern blot analysis (Fig. 8). Steady-state RNAs were isolated from H23H.B cells that had been heat shocked at 41°C for 30, 60, and 180 min. The amount of mature *hph* mRNA in heat-shocked cells was drastically decreased in response to heat shock (Fig. 8A), while expression of endogenous *hsp70* genes was maintained at a high level even after heat shock at 41°C for 3 h (Fig. 8C). A reduction of the steady-state mRNA upon heat shocks was also observed for β-tubulin genes (Fig. 8D). This heat shock-induced reduction was observed in the expression of many protein-coding genes, including the PARP gene (data not shown). This result suggests that sequences of the *hsp70* intergenic region promoter

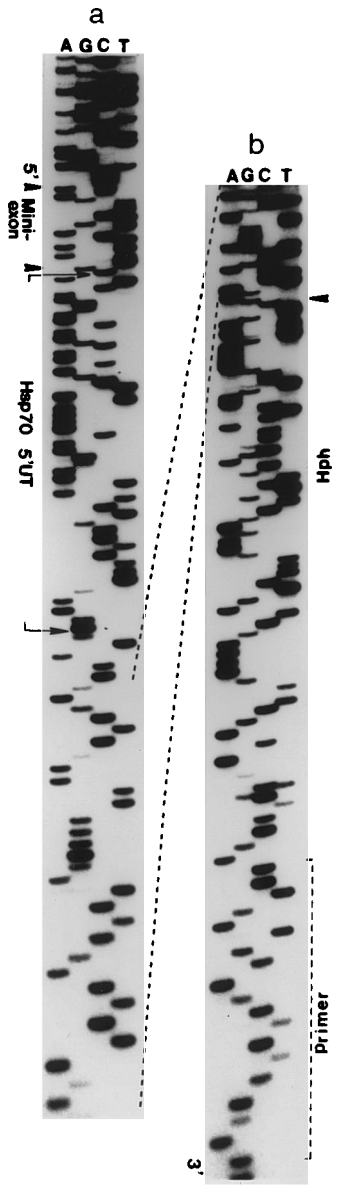

FIG. 7. Nucleotide sequence at the 5′ end of the *hph* mRNA. The sequences of cDNAs spanning the 5′ end of the *hph* transcripts were analyzed by the dideoxy-chain termination method (41). Arrowheads separate, from 5′ to 3′, the miniexon sequence, the *hsp70* 5′-end-derived sequence that immediately follows the major 3′ splice acceptor site, plasmid linker sequences, and *hph*-derived sequences. The location of the oligonucleotide sequence, complementary to the *hph* sequence, used in primer extension is indicated (primer). 5′UT, 5′ untranslated region.

and the 5′ untranslated region of the *hsp70* gene do not upregulate or maintain expression of the *hph* gene upon heat shock. Similar results were obtained with the H23H.A cell line, which contained integrated constructs (data not shown). Furthermore, by nuclear run-on assays, it was confirmed that transcriptional induction by heat shock did not occur in the H23-*hph* locus in the H23H.B cell line (data not shown). This result is consistent with my previous observation that heat shock does not increase the transcriptional efficiency of the *hsp70* genes of *T. brucei* (31).

Whereas mature *hph* mRNA levels were decreased after heat shock, the levels of large *hph* precursor mRNAs were increased. The largest *hph* mRNA observed in heat shock cells is also detected by the 5′ pseudo-VSG gene probe, located downstream of the *hph* gene in the H23-H-B7 construct, and the plasmid vector probe (Fig. 8B and data not shown). The structure of this largest *hph* mRNA is not yet completely characterized, but it is of a size approximately equivalent to that of input plasmid (indicated by arrows). It is possible that this largest *hph* mRNA may be generated by using the processing signals [poly(A) addition and *trans* splicing] present in the H23 promoter fragment. Additionally, all sizes of the *hph* mRNA detected are present in the poly(A)$^+$ fraction and absent in the poly(A)$^-$ fraction (Fig. 8E). This result indicated that the elevated temperature may have inhibited the efficiency of RNA processing, thereby generating a high level of large *hph* precursor mRNAs; the unprocessed precursor RNA may be unstable and rapidly degraded. This observation is consistent with the notion previously described by Muhich and Boothroyd (35) that the processing machinery responsible for the maturation of tubulin mRNA precursors in *T. brucei* can be disrupted by heat shock, resulting in an accumulation of polycistronic RNA species.

DISCUSSION

An unanswered question regarding trypanosomes is the mechanism of protein-coding gene transcription by RNA pol II. *trans* splicing, polycistronic transcription, and tandem organization of gene arrays have hampered the identification of RNA pol II promoters. Evidence presented here indicates that promoters could be present in front of each copy of *hsp70* genes 2 to 6 in *T. brucei*. First, transcriptional mapping by UV inactivation eliminated the possibility that a single far-upstream promoter controls the transcription of the *hsp70* genes. This result, rather, indicated that promoters could be located in front of the *hsp70* genes. Second, transient transformation assays demonstrated that sequences immediately upstream of *hsp70* gene 2 and in front of *hsp70* gene 3 are able to drive transcription. Finally, in two types of stably transformed trypanosomes, it was demonstrated that transcription driven by the *hsp70* intergenic region is sensitive to α-amanitin. These results suggested that the *hsp70* intergenic region can function as an RNA pol II promoter. Since *hsp70* genes 2 to 6 are polycistronically transcribed (29) and promoters could be located in front of *hsp70* gene 2 and in the intergenic region, it is possible that multiple transcription units occur simultaneously, thus generating overlapping transcription units on the *hsp70* locus of *T. brucei.*

The moderate sensitivity of transcription to UV irradiation was also observed for several other RNA pol II-transcribed protein-coding genes, including genes that are present as a single copy or a tandem array of multiple copies in the genome of *T. brucei*. This result indicated that transcription of these genes cannot be driven by a single far-upstream promoter, as is the case for the VSG gene. I have quantitated the data for UV inactivation of transcription and estimated the average distance between the *hsp70* promoters and the coding region, on the basis of the classical kinetics model applied to the mapping of the rRNA gene promoter as described by Johnson et al. (20). Because polycistronic transcription proceeds on *hsp70* genes 2 to 6, the UV sensitivity of *hsp70* gene transcription presumably results from the cumulative effects of different UV sensitivities of the distal and proximal *hsp70* genes. A distance of 1 to 4 kb upstream of the coding sequence was reproducibly measured for the location of the *hsp70* promoters. Since the *hsp70* locus, spanning 12.5 kb, contains five copies of a reiterated coding sequence unit, with a size of 2.5 kb, I tentatively

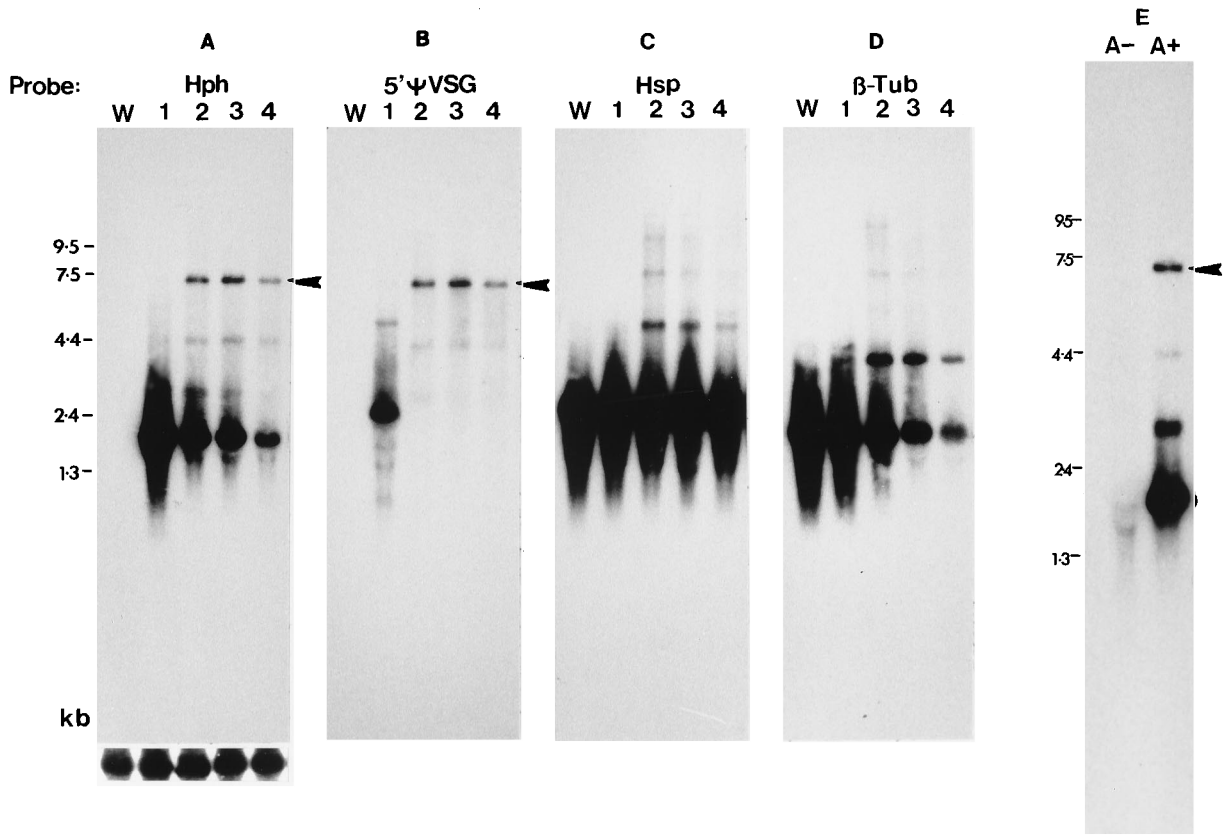

FIG. 8. Expression of the *hph* gene driven by the *hsp70* intergenic region promoter in response to an elevated temperature. The RNA samples were isolated from log-phase H23H.B trypanosomes that were maintained at different temperatures for various periods of time. Lanes W and 1 represents total RNAs isolated from the wild-type and H23H.B trypanosomes, respectively, that had been maintained at a normal growing temperature, 23 to 25°C. Lanes 2, 3, and 4 represent total RNAs derived from H23H.B trypanosomes incubated at 41°C for 30, 60, and 180 min, respectively. RNA samples were separated on a 1% formaldehyde-agarose gel and transferred onto nitrocellulose filters. The filters were sequentially hybridized with different ^{32}P-labeled probes as indicated at the top. The signal was stripped from the filter before each rehybridization. The final posthybridization wash was performed at 65°C in 0.1× SSC–0.1% SDS. The small segment below panel a represents the hybridization with the 28S rRNA gene probe, demonstrating that equal amounts of total RNA were loaded in all lanes. Hph and Hsp are as described in the legend to Fig. 3. Other probes: β-Tub, the *Hin*dIII-*Eco*RI fragment of the β-tubulin coding region (44); 5′ψVSG, the *Hin*dIII-*Eco*RI fragment derived from the 5′ end of the pseudo-VSG 118 gene. A− and A+ indicate poly(A)$^-$ and poly(A)$^+$ RNAs, respectively. Arrowheads indicate the RNA of a size equivalent to the full size of the H23-H-B7 construct.

concluded that the *hsp70* promoter is located either immediately upstream of gene 2 or in front of each gene separately. Similar results were obtained for many other protein-coding genes, as exemplified by the Tb-17, Tb-29, and CRAM genes. In an additional example, previously reported by Wong et al. (50), transcription of tubulin genes also shows a moderate sensitivity to UV irradiation. Wong et al. interpreted this finding to indicate a transcription start site existing proximal to the first tubulin gene in the cluster. These results suggest the notion that the presence of immediately adjacent promoters may be a common feature of a subset of RNA pol II transcription units. Transcription of the calmodulin EFH5 and ubiquitin EP-52 genes was shown to be rapidly inactivated by UV irradiation, suggesting that this cluster of genes is under the control of a single distant upstream promoter (50). The promoter of the actin genes was mapped to a region located ~4 kb upstream from the genes and shown to be able to drive transcription in transiently transformed trypanosomes (3). However, it is not clear what the strength of the actin promoter is in comparison with that of the PARP promoter, whether this promoter can be functional in stably transformed trypanosomes, and whether transcription derived from this actin promoter is controlled by the α-amanitin-sensitive RNA pol II. Further characterization of promoters driving transcription of these protein-coding genes will be essential for our understanding of the structure of RNA pol II transcription units.

The transient transformation assay demonstrated that the promoter strength of the *hsp70* intergenic region sequence is ~1% of that produced by the PARP promoter, which is presumably transcribed by RNA pol I or a pol I-like enzyme (10). Deletion mutation or replacement of the *hsp70* intergenic region promoter with an unrelated DNA fragment abolished the promoter activity, indicating that this activity is sequence specific. 5′ deletion of the *hsp70* intergenic region to 89 bp upstream of the 3′ splice site reduced the promoter activity to ~43% of that produced by the full-length *hsp70* intergenic region sequence. However, 5′ deletion to 60 bp upstream of the 3′ splice site abolished 98% of the promoter activity but retained the functional 3′ splice site. This result suggests that multiple functional domains may be present in the *hsp70* intergenic region promoter. Further analysis by linker scanning mutagenesis will be required to determine the functional domains of this *hsp70* intergenic region promoter. Transcription driven by the *hsp70* intergenic region promoter is sensitive to α-amanitin, as predicted for RNA pol II transcription. This mode of transcription occurs at an internal position of the chromosome or at an extrachromosomal molecule. Because of the low level of unspliced precursor RNA present in the

steady-state RNA, the transcription initiation site has not yet been unambiguously and accurately determined (data not shown). Thus, the structure of the *hsp70* intergenic region promoter remains to be characterized, and the question of whether a TATA box may play a role in the promoter element of this promoter remains unanswered.

CurottoDeLafaille et al. have shown that a polypyrimidine tract and 3′ splice acceptor site linked to the CAT gene were sufficient to produce mRNA resulting in a significant CAT activity in *Leishmania* sp. (12). Therefore, the presence of weak promoters in the intergenic region of protein coding genes may not be visualized in this organism. Unlike the case for *Leishmania* sp., a polypyrimidine tract and 3′ splice acceptor site linked to the CAT gene give no significant CAT activity in *T. brucei*, as exemplified by the polypyrimidine tract and 3′ splice acceptor site derived from the PARP gene (7) and the *hsp70* gene, respectively. Thus, sequences of a promoter function can be assayed by directing the CAT gene expression in transiently transformed *T. brucei.*

Transcription of the *hsp70* genes in other eukaryotes was shown to be transcriptionally induced by elevated temperatures. This transcriptional induction results from the binding of heat shock transcription factors to the conserved 14-nucleotide sequence of a heat shock transcription factor-binding element in the *hsp* gene promoters, leading to the activation of *hsp* genes (34, 45). Unlike the case for other eukaryotes, heat shock does not induce increased transcription of the *hsp70* genes in *T. brucei* (31, 35). Therefore, the regulated expression of the *hsp70* gene upon heat shock or differentiation is mainly posttranscriptionally controlled. The results presented here demonstrate that the *hsp70* intergenic region promoter and its 3′ splice site and 5′ untranslated region that controls the transcription of the *hph* gene cannot up-regulate or maintain expression of the *hph* gene during heat shock. Whether other regions of the *hsp70* gene, such as the 3′ untranslated region and the *hsp70* coding region, may affect the abundance of the *hsp70* mRNA during heat shock remains to be investigated.

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