Linkage of the calmodulin and ubiquitin loci in *Trypanosoma cruzi*

Sul-Hee Chung and John Swindle*
Department of Microbiology and Immunology, University of Tennessee, 858 Madison Ave, Memphis, TN 38163, USA

Received March 2, 1990; Revised and Accepted July 6, 1990 EMBL accession no. X52096

ABSTRACT

We describe here the organization of the calmodulin genes of *Trypanosoma cruzi* and their linkage to the ubiquitin gene family. The nucleotide sequence of the *CalA2* gene has been determined and is 85% homologous to the protein coding sequence of the calmodulin genes of the African trypanosome, *Trypanosoma brucei*. The proteins encoded by *CalA2* and the *T. brucei* genes contain a single mismatch out of a total of 149 amino acids. The genome of *T. cruzi* contains eight calmodulin genes present at two distinct loci (*2.8* and *2.65*) each of which is linked to downstream ubiquitin genes. Within each locus two calmodulin gene families have been defined, *CalA* and *CalB*. Each calmodulin locus consist of alternating tandem arrays of the *CalA* and *CalB* genes. Both *2.8* and *2.65* calmodulin loci and their respective downstream ubiquitin genes share the same DNA coding strand. Transcription of the calmodulin genes in the epimastigote stage of *T. cruzi* results in the generation of two stable mRNAs of 1.6Kb and 1.1Kb.

INTRODUCTION

Calmodulin and ubiquitin are two of the most conserved proteins throughout eukaryotic evolution. Calmodulin through its association with calcium plays a critical role in many cellular processes (for a review see ref. 1). Similarly, ubiquitin is a multifunctional protein which can variably target proteins for degradation (1,3), become an integral part of certain nuclear (4) and cell surface proteins (5) or possibly act as a protease itself (6). Recently it has been demonstrated that free calmodulin and not the actively bound protein are in fact substrates for ubiquitination (7,8,9). Therefore one possible mechanism of regulating calmodulin levels may be through targeting of inactive protein for degradation via the ubiquitin dependent pathway (8).

The genomic organization and transcription of the calmodulin genes of the African trypanosome, *Trypanosoma brucei* has been previously reported (10,11) while the organization and expression of the ubiquitin genes of the New World trypanosome, *Trypanosoma cruzi* has also been described (12). The genomes of all *T. brucei* subspecies examined contain two chromosomal calmodulin loci each consisting of direct tandem repeats of the protein coding sequence (10). The calmodulin genes appear to be transcribed initially as a part of a longer polycistronic RNA which is processed yielding the mature messenger RNA molecules (mRNAs) (11). The ubiquitin genes of *T. cruzi* strain *CL* consist of five polyubiquitin genes and five ubiquitin-fusion genes (12). Transcription of the ubiquitin gene family results in the generation of five discrete polyubiquitin transcripts as well as ubiquitin-fusion mRNAs.

We describe here the characterization of two calmodulin loci (the *2.65* and *2.8* loci) in *T. cruzi* strain *CL* and their linkage to the ubiquitin genes. Within the two loci the calmodulin genes have been assigned as members of either the *CalA* or *CalB* gene families. The *2.65* locus contains two *CalA* genes (*CalA1* and *CalA2*) and a single *CalB* gene (*CalB1*). The *2.8* locus consist of three *CalA* genes (*CalA3*, *CalA4* and *CalA5*) and two *CalB* genes (*CalB2* and *CalB3*). Both calmodulin loci and the ubiquitin genes would be transcribed from the same DNA strand as the downstream ubiquitin genes. Transcription of the calmodulin genes results in the generation of two stable mRNAs.

MATERIALS AND METHODS

Culture conditions

T. cruzi strain CL epimastigotes were cultured in liver infusion tryptose medium (13) at 28°C. In all experiments midlog cultures were used (5×10^6 to 1×10^7cells/ml).

Construction of plasmid clones

Plasmid construction is described in the text and appropriate figure legends.

Construction of Cosmid clones

A genomic cosmid library was constructed using the vector pJB8 (14). 15ug of genomic *T. cruzi* DNA was partially digested with BamHI and size fractionated on a 0.7% agarose gel. From this gel the 25Kbp to 40Kbp fraction was isolated and ligated into the BamHI site of pJB8. Following *in vitro* packaging and infection, colonies harboring cosmids containing the ubiquitin genes and flanking regions were isolated using probes for the ubiquitin coding sequence. One cosmid COS-UbIg4 was isolated which, in addition to the ubiquitin genes, carried sequences homologous to the calmodulin gene.

* To whom correspondence should be addressed

DNA sequence analysis

The DNA sequence was determined by the dideoxy chain termination method (15). Synthetic oligonucleotides homologous to the calmodulin coding sequence and flanking regions were used as primers for elongation.

Southern hybridizations

All Southern analysis was carried out using Gene Screen Plus. Hybridizations were carried out at 42°C in 50% formamide, 5× SSC, 5× Denhardts, 1% SDS and 100 μg/ml carrier tRNA. All filters were washed twice at room temperature in 5×SSC for 30 minutes, twice at room temperature in 2× SSC, 0.1% SDS for 30 minutes, and once at 65°C in 0.1× SSC, 0.1% SDS for 20 minutes.

Isolation of RNA and Northern hybridizations

Total cellular RNA was isolated by the guanidinium/cesium chloride method (16). PolyA$^+$ RNA was isolated following three passages over oligo(dT)-cellulose (16). The flow through RNA following the third passage over oligo(dT)-cellulose was used as the polyA$^-$ fraction. RNA was size fractionated on 1.1% agarose gels containing 1.0 M formaldehyde (16). Blotting, hybridization and washing conditions were precisely as described for Southern analysis (see above)

Synthesis of ^{32}P-labeled DNA probes

Probes for Southern and Northern analysis were generated either by random priming of purified insert DNA using the Klenow fragment of *E. coli* DNA polymerase I or in polymerase chain reactions (PCR) (17) using synthetic oligonucleotides as primers. PCR generated probes were prepared as follows: reactions contain 50ng of supercoiled 3.0Kbp recombinant plasmid, 50mM KCl 10mM Tris pH 8.0, 1.5mM $MgCl_2$, 0.01% gelatin, .05mM dGTP, .05mM dATP, .05mM TTP, .012mM dCTP, 50μCi alpha-^{32}P-dCTP (Amersham 800 Ci/mmole), 2.5units of *Taq* polymerase (Perkin Elmer Cetus) and 0.1μg of each oligonucleotide primer. A total of 15 cycles were carried out for each probe. Specific cycle conditions for each primer set are given in the appropriate figure legends.

RESULTS

Characterization of the *CalA2* gene of *T. cruzi*

The *CalA2* gene was identified through analysis of the genomic ubiquitin clone pTC-UbIg. The isolation and characterization of pTC-UbIg has been reported previously (12). pTC-UbIg carried approximately 3.2Kbp of unanalysed sequence upstream of the *FUS1* and partial *PUB12.5* ubiquitin genes (figure 1). DNA sequence analysis of the cloned insert beginning near the PvuII restriction site distal to the ubiquitin genes in pTC-UbIg is shown in figure 2. A 447bp open reading frame extends from the ATG translation initiation codon at nucleotide 471 to a TAG translation termination codon at nucleotide 918 (figure 2). The potential 149 amino acid translation product was identified as calmodulin based on its near perfect homology to the reported sequence for the calmodulin protein of *T. brucei* (10). There is in fact only a single amino acid difference between the two proteins (i.e. *T. cruzi* Val_{109} instead of Ile_{109}).

The protein coding sequence of the *T. cruzi* and *T. brucei* calmodulin genes are also highly conserved having 85% homology (figure 2) (the nucleotide sequence of the translated regions of the three *T. brucei* calmodulin genes are identical [10]). The 69 nucleotide differences between the coding sequences of the two genes are limited to single base pair changes. Further, of the 69 single base changes 67 are in the third position of the codon. Outside of the coding sequence however, the DNA sequence diverges completely.

Identification of two calmodulin gene families

Southern analysis of genomic DNA revealed the presence of two distinct calmodulin gene repeats. The identification was initially made using probes specific for either the 5′ (pTC-Cal:5′) or 3′(pTC-Cal:3′) portions of the *CalA2* coding sequence (figure 3). Genomic Southern blots of BglII and AvaII restriction digest each contains bands of 1.7Kbp and 1.1Kbp which hybridize to both the 5′ and 3′ probes (figure 3, lanes 1,2,4 and 5). This result indicates that both bands of hybridization represent direct tandem repeats of the calmodulin coding sequence (the AvaII site is 17 nucleotides from the PstI restriction site which defines the 5′ and 3′ probes).

The 1.1Kbp repeat has several distinguishing features relative to the 1.7Kbp repeat. First, only the 1.1Kbp repeat hybridizes with the intergenic probe pTC-Cal:0 (figure 3, lanes 7−9). Second, DNA sequence analysis of the region immediately upstream of the translation start site for the *CalA2* gene predicts the presence of a second PstI restriction site in the intergenic region in addition to the PstI site within the coding sequence. As shown in figure 3 (lane 9) only the 1.1Kbp repeat is cut with PstI. As demonstrated in figure 4 the intergenic region of the

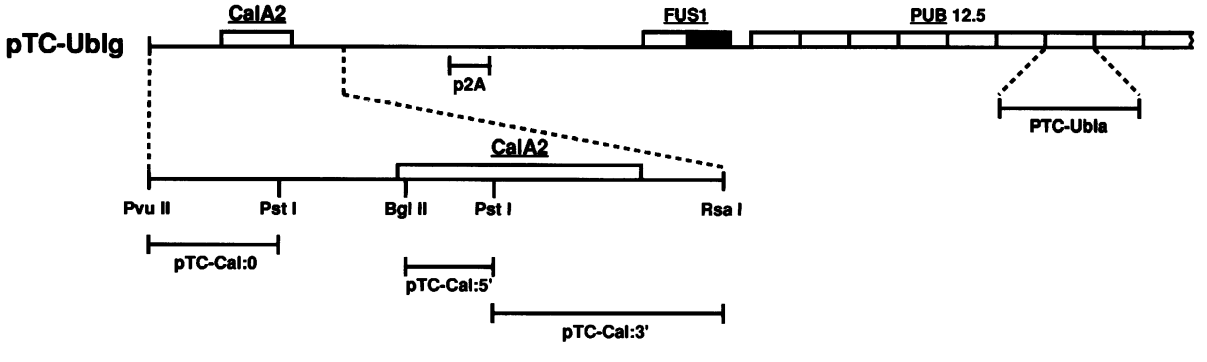


Figure 1. A description of the construction of pTC-UbIg is given in ref. 12. The expanded map immediately below the pTC-UbIg restriction map represents the region of the clone for which DNA sequence data is presented in figure 2. Subclones depicted below pTC-UbIg were generated by ligating the indicated fragments isolated from pTC-UbIg into the appropriate sites of the vector Blue Scribe (+) (Vector Cloning Systems).

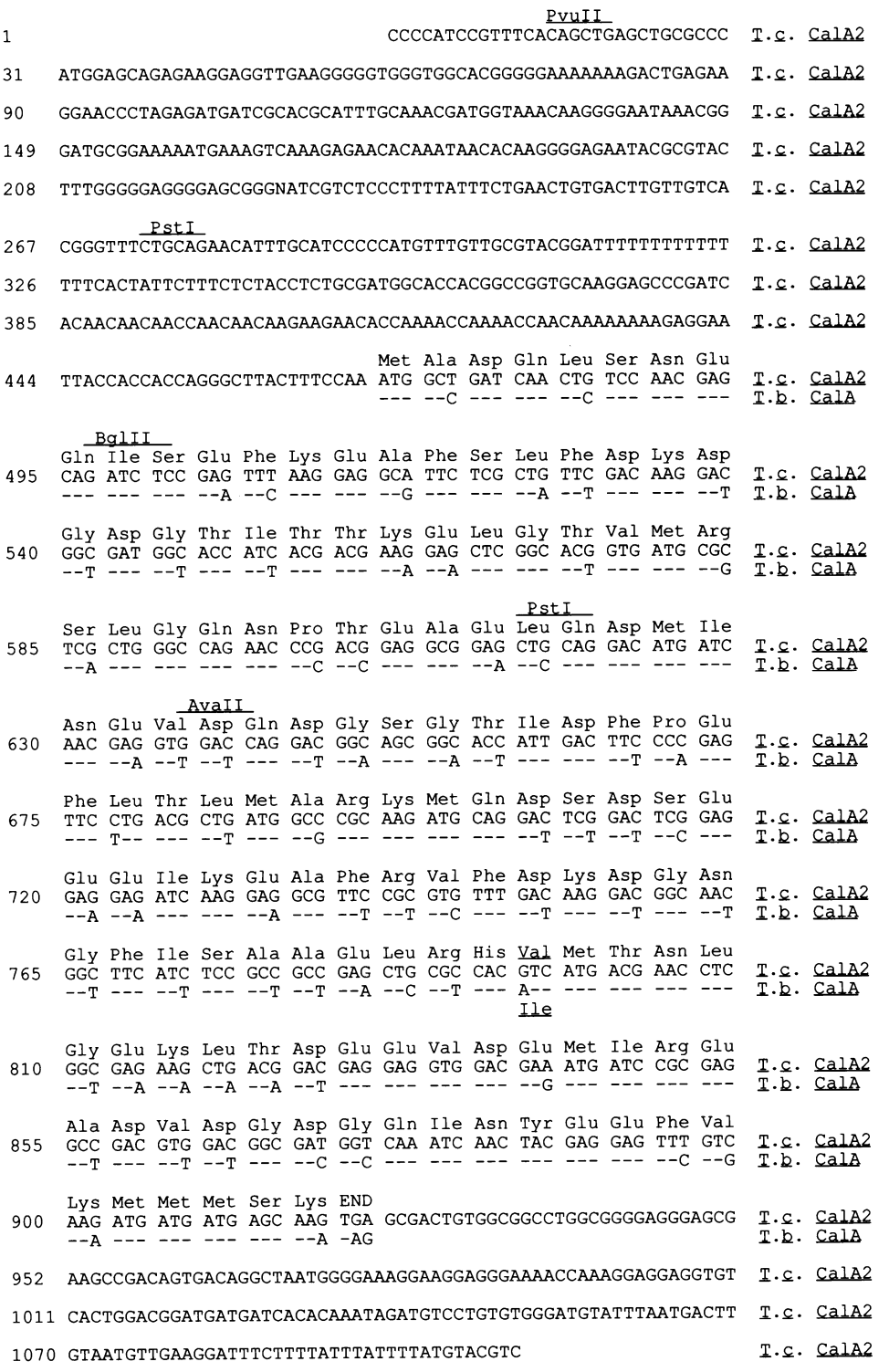


Figure 2. Comparison of the DNA and amino acid sequences of the *CalA2* calmodulin gene of *T. cruzi* with the gene and amino acid sequences of the calmodulin genes of *T. brucei* (10). The *T. cruzi* DNA sequence shown extends from immediately 5′ of the PvuII site to the RsaI site of pTC-UbIg (see legend to figure 1). Selected DNA restriction sites are represented immediately above the derived amino acid sequence. *T.c. CalA2* identifies the *T. cruzi* nucleic acid sequence. T.b. *CalA* identifies the nucleic acid sequence of the *T. brucei* gene. A dash (−) in the *T. brucei* sequence indicates nucleotide identity with the *T. cruzi* gene. The derived amino acid sequence for the *T. cruzi CalA2* is presented immediately above the *T. cruzi* DNA sequence. The underlined amino acids at position 109 illustrate the single amino acid difference between the two proteins.

1.7Kbp repeat also contains a KpnI site which is not present in the 1.1Kbp repeat. Lane 1 in figure 4A and 4B show a BglII genomic digest hybridized with either the pTC-Cal:3′ or pTC-Cal:0′ probes respectively. The 1.7Kbp and 1.1Kbp bands of hybridization are clearly evident. Lanes 2 and 4 represent genomic KpnI and KpnI plus BglII double digest respectively.

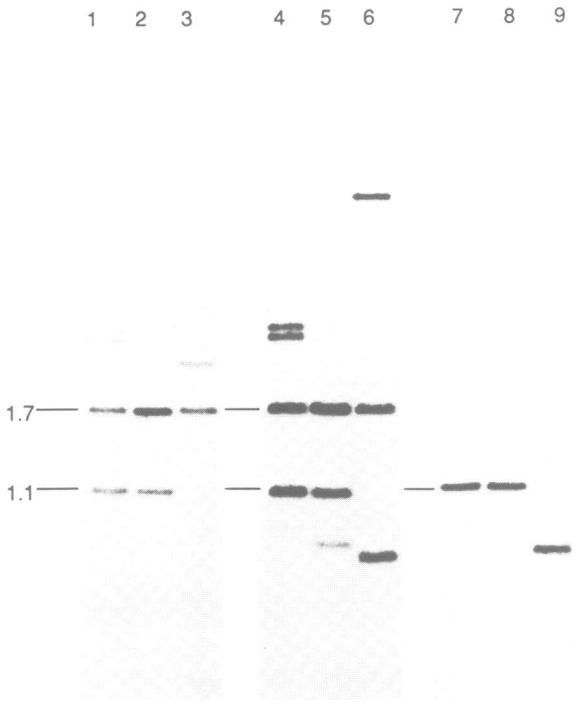
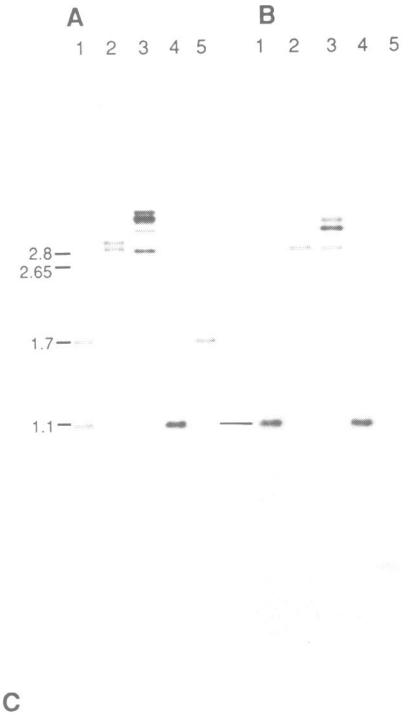


Figure 3. Genomic Southern analysis of the calmodulin genes. Each lane contains 1.0ug of genomic DNA. Lanes 1, 4 and 7, BglII. Lanes 2, 5 and 8, AvaII. Lanes 3, 6 and 9, PstI. Lanes 1,2 and 3 were probed with ^{32}P labeled pTC-Cal:5′. Lanes 4, 5 and 6 were probed with ^{32}P labeled pTC-Cal:3′. Lanes 7, 8 and 9, were probed with ^{32}P labeled pTC-Cal:0. The probes used in this analysis were generated using the polymerase chain reaction technique described in Materials and Methods. In this case each of the genomic fragments was cloned in the polylinker of the vector Blue Scribe (+) (Vector Cloning Systems) and could therefore be amplified using synthetic oligonucleotides homologous to the T_3 and T_7 promoters. Cycle conditions: Denaturation) 94°C, 1.5 minutes, Annealing) 42°C, 3.0 minutes, Polymerization) 70°C, 4.0 minutes.



C

Position of band (kb)	2.8	2.65	1.7	1.1
Area	0.25	0.28	0.90	1.01
rel. ratio of area	1	1	3	3

Figure 4. Genomic Southern analysis. Each lane in the Southern blots shown in A and B contains 1.0μg of restricted genomic DNA. The blots shown in A and B represent parellel experiments in which genomic DNA was digested with, lane 1, BglII; lane 2, KpnI; lane 3, PvuII; lane 4, BglII plus KpnI; lane 5, BglII plus PvuII. The blots shown in A and B were hybridized with the probes pTC-Cal:3′ and pTC-Cal:0 respectively. The data shown in 4C was derived from the densitimetric tracing of lane 1 of panel 4A.

The double digest results in the cleavage of the 1.7Kbp repeat while the 1.1Kbp repeat is unaffected. The data presented in figure 4 also reveal that the PvuII site predicted from the DNA sequence analysis shown in figure 2 is unique to the 1.1Kbp repeat (lanes 3 and 5). Based on these observations we have arbitrarily assigned all calmodulin genes as members of either the *CalA* or *CalB* gene families. The two gene families are distinguished by the presence of the pTC-Cal:0 sequence in the intergenic sequence downstream of the *CalB* genes.

To determine the calmodulin gene copy number the genomic Southern shown in figure 4A, lane 1 was subjected to densitometric tracing. The data from such an experiment is shown in figure 4C. The ratios of 1:1:3:3 (2.8Kbp: 2.65Kbp:1.7Kbp:1.1Kbp) suggest the cell contains a total of eight calmodulin genes. This calculation is based on the observation that the 2.8Kbp and 2.65Kbp BglII fragments are themselves single copy (see below, figure 6). By definition the number of *CalB* genes is equal to the number of sequences generating the 1.1Kbp repeat. There are therefore, three *CalB* genes and five *CalA* genes. A complete map of the calmodulin genes is presented in figure 8.

The *CalA* and *CalB* genes are tandemly linked

The genomic Southern data presented in figures 3 suggested that the two calmodulin gene families were tandemly linked. As shown in figure 3, the digestion of genomic DNA with PstI results in the generation of a unique 5′ flanking sequence which hybridizes with the pTC-Cal:5′ (lane 3) and a unique 3′ flanking sequence which is recognized by the pTC-Cal:3′ probe (lane 6, see figure 8 for a complete map of the PstI digest). These results indicate that the *CalA* and *CalB* gene families are tandemly linked or have conserved five prime and three prime flanking sequences. The experiment presented in figure 5 was designed to distinguish between these two possibilities. In this experiment two oligonucleotides were used to prime a polymerase chain reaction (PCR) amplification experiment. The first oligonucleotide, 0−2, is homologous to the noncoding strand of the pTC-Cal:0 sequence. The second oligonucleotide, CATG, is homologous to the first twenty nucleotides of the calmodulin coding sequence. The proposed organization of the *CalA* and *CalB* genes is dipicted in the line drawing in figure 5. Such an organization predicts the generation of two amplification products, one of 2.4Kbp and one of 0.7Kbp. Further, the proposed organization predicts that the larger fragment will contain a complete copy of the 1.7Kbp calmodulin repeat. Amplification of genomic DNA resulted in the generation of DNA fragments of 2.4Kbp and 0.7Kbp which hybridized with the pTC-Cal:3′ and pTC-Cal:0 probes fulfilling the prediction (figure 5 A and B, lane 1). Restriction digest of

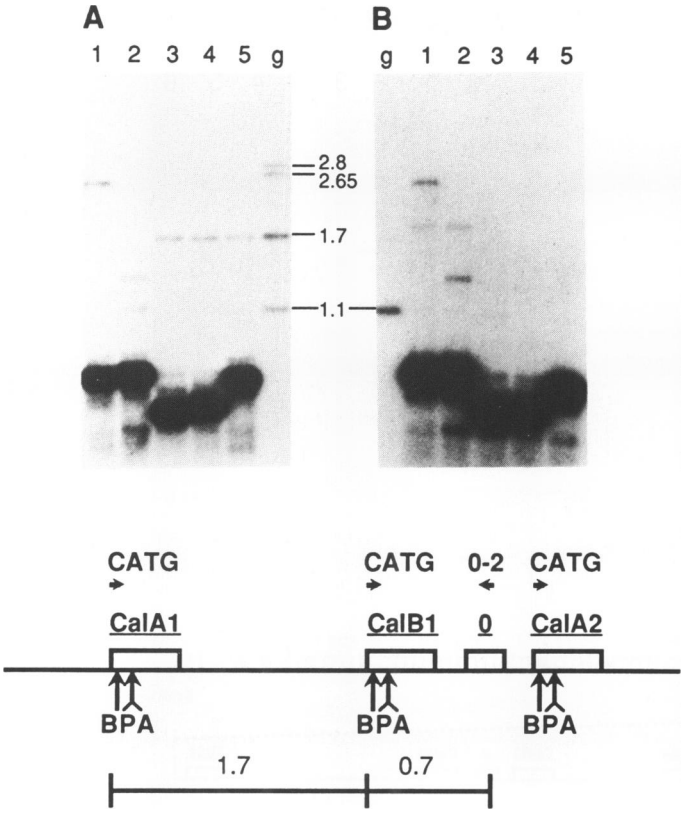


Figure 5. linkage of the *CalA* and *CalB* genes. The scheme for the PCR amplification reaction is shown in the line drawing below the Southern blots. The CATG oligonucleotide represents the first twenty nucleotides of the calmodulin coding sequence. The 0−2 oligonucleotide represents the reverse complement of nucleotides 81 through 99 of the DNA sequence shown in figure 2. Duplicate Southern blots are shown in A and B. Lane g represents a BglII restriction of 1.0μg of genomic DNA. In A and B lane 1 represents the amplification products, lanes 2,3,4 and 5 represent, KpnI, AvaII, PstI, BglII restriction digest of the amplification products respectively. The Southern blots in A and B were probed with pTC-Cal:3′ and pTC-Cal:0 respectively.

the amplification products with AvaII, PstI or BglII result in the generation of a hybridizing fragment of 1.7Kbp which co-migrates with the genomic 1.7Kbp repeat. The observation that the 1.7Kbp repeat is generated following PstI digestion identifies it as the 1.7Kbp calmodulin repeat. This identification is further supported by the observation that the 1.7Kbp fragment contains a KpnI restriction site which is also diagnostic of the 1.7Kbp calmodulin repeat (figure 5A and 5B lane 2). Finally, the 1.7Kbp fragment hybridizes with the pTC-Cal:3′ probe but not the pTC-Cal:0 probe which is diagnostic for the 1.1Kbp calmodulin repeat. The observation that the 1.1Kbp repeat fragment is not generated in these experiments indicates that the three copies of the 1.1Kbp repeat are not themselves, tandemly arranged. Taken together these data along with data presented below demonstrate that the 1.7Kbp and 1.1Kbp calmodulin repeats are tandemly arrayed one after the other on the chromosome (see map figure 8).

Genomic maps of the *2.8* and *2.65* calmodulin loci

An EcoRV restriction site polymorphism in the intergenic region separating the *FUS1* and *FUS2* genes from their respective downstream polyubiquitin genes has lead to the identification of two calmodulin loci *2.65* and *2.8* (figure 6). As shown in figure 6A, two EcoRV size fractions of 25Kbp and 9.5Kbp can be identified which contain calmodulin genes. The 25Kbp and 9.5Kbp size fractions represent the *2.8* and *2.65* loci respectively. This identification is based on the presence of the diagnostic 2.8Kbp and 2.65Kbp BglII restriction fragments in the 25Kbp and 9.5Kbp size fractions respectively (figure 6B). As shown in the following section the 2.8Kbp and 2.65Kbp BglII restriction fragments represent the DNA sequence linking the two calmodulin loci to their respective downstream ubiquitin genes (see figure 7). Additionally the data presented in figure 6B demonstrates that the *2.8* and *2.65* calmodulin loci contain both the 1.7Kbp and 1.1Kbp BglII calmodulin repeats indicating that each locus consists of a minimum of three calmodulin genes. The data described below and presented in figure 6C indicates that the *2.65* calmodulin locus represents the minimal locus in that it consists of a single 1.7Kbp repeat unit followed by a single 1.1Kbp repeat. Digestion of the genomic DNA with the restriction enzyme PvuII results in the generation of five DNA fragments of 3.6Kbp, 3.5Kbp, 3.4Kbp, 3.2Kbp and 2.8Kbp, which hybridize to the pTC-Cal:3′ calmodulin probe (figure 6C, lane 1). Two of these DNA fragments (3.5Kbp and 3.2Kbp, figure 6C lane 3) are specific for the *2.65* locus and only the 3.2Kbp DNA fragment hybridizes with the 1.1Kbp repeat intergenic probe pTC-Cal:0 (figure 6C, lane 6). As shown in figure 7 the 3.2Kbp PvuII fragment also hybridizes with the ubiquitin specific probe identifying it as the fragment which links the *2.65* calmodulin locus to the downstream ubiquitin genes. The 3.5Kbp PvuII fragment contains the KpnI restriction site diagnostic of the 1.7Kbp repeat (data not shown). Therefore, this data, together with that presented in the previous section demonstrating tandem linkage of the 1.7Kbp and 1.1Kbp repeats, indicates that the *2.65* calmoulin locus consists of a single 1.7Kbp repeat followed by a 1.1Kbp repeat.

The gene organization within the *2.8* calmodulin locus represents an elaboration of that described above for the *2.65* locus. Digestion of the 25Kbp EcoRV size fraction, containing the *2.8* calmodulin locus, with PvuII results in the generation of three DNA fragments of 3.6Kbp, 3.4Kbp and 2.8Kbp which hybridize to the calmodulin coding sequence probe pTC-Cal:3′ (figure 6C, lane 2). Two of the fragments (3.4Kbp and 2.8Kbp) also hybridize with the 1.1Kbp intergenic specific probe pTC-Cal:0, indicating that the *2.8* locus contains at least two copies of the 1.1Kbp calmodulin repeat (figure 6C, lane 5). As shown below the 3.4Kbp PvuII restriction fragment represents the sequences linking the *2.8* calmodulin locus to the downstream *FUS2* ubiquitin-fusion gene. Both the 3.6Kbp and 2.8Kbp PvuII fragments contain KpnI restriction sites diagnostic of the 1.7Kbp calmodulin repeat (data not shown). The maps resulting from these data are shown in the line drawing below figure 6 and in figure 8. The *2.8* locus consists of five calmodulin genes organized in alternating repeats of the 1.7Kbp and 1.1Kbp calmodulin repeat units.

The *2.8* and *2.65* calmodulin loci are linked to the ubiquitin genes

As stated previously, *CalA2* was initially identified approximately 2.0Kbp upstream of the first gene of the ubiquitin gene family (*FUS1*) in the genomic clone pTC-UbIg (figure 1). The following Southern results confirm that the juxtaposition of *CalA2* and the ubiquitin genes was not a cloning artifact. The DNA sequence of pTC-UbIg predicted the existence of a 3.2Kbp PvuII fragment which should hybridize to the 1.1Kbp calmodulin repeat intergenic probe pTC-Cal:0, the calmodulin coding sequence

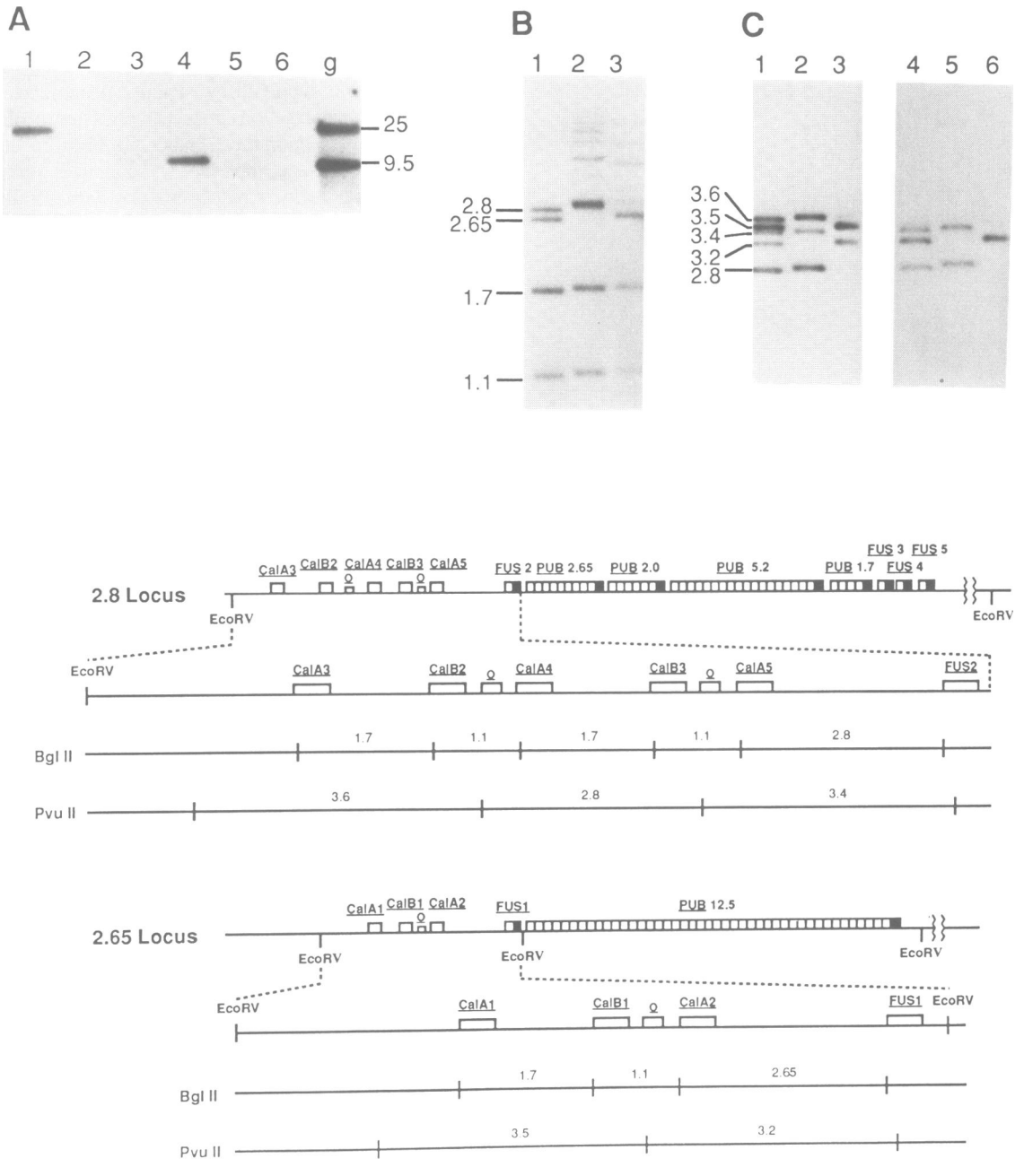


Figure 6. A) Hybridization of EcoRV size fractionated of genomic DNA with pTC-Cal:3′. 100 μg of genomic DNA was restricted with EcoRV and electrophoresised through a 0.7% agarose gel. Six slices were cut from the gel and the DNA from each was recovered by electroelution. Samples from each size fraction (lanes 1 through 6) along with a aliquot of the original genomic EcoRV digest (lane 7) were electrophoresis through a second 0.7% agarose gel and the resulting blot was probed with pTC-Cal:3′. The 25Kbp and 9.5Kbp size fractions represented in lanes 1 and 4 repectively, were used in subsequent anlaysis. B) Southern hybridization of BglII restriction digest of lane 1, 1.0μg of genomic DNA; lane 2, 25Kbp size fraction; lane 3, 9.5Kbp size fraction. Following electrophoresis the gel was blotted and probed with pTC-Cal:3′. C) Southern hybridization of PvuII restriction digest of lanes 1 and 4, 1.0 μg of genomic DNA; lanes 2 and 5, the 25Kbp size fraction; lanes 3 and 6, the 9.5Kbp size fraction. lanes 1−3 were hybridized with pTC-Cal:3′ and lanes 4−6 were hybridized with pTC-Cal:0. The genomic map generated from this data is shown in the line drawing below the Southern blots. The genomic map of the ubiquitin-fusion (*FUS*) and polyubiquitin (*PUB*) is a modification of that which was presented in ref 12.

probe pTC-Cal:3′, the *CalA2* to ubiquitin intergenic probe P2A and the ubiquitin coding sequence probe pTC-UbIa (12) (in the line drawing in figure 6 these are refered to as, 0, 3′, P2A and UbIa). The results of such a Southern blot are shown in figure 7 and confirm the expectations. Surprisingly however, two bands can be seen which hybridize to each of the four probes. The smaller 3.2Kbp PvuII band comigrates with the band generated from a PvuII digest of pTC-UbIg as expected. The larger 3.4Kbp bands comigrates with a band generated following a PvuII digest of the ubiquitin cosmid clone COS-UbIg4 (see Materials and Methods for a description of COS-UbIg4). As described above the 3.4Kbp and 3.2Kbp PvuII restriction fragments come from the *2.8* and *2.65* calmodulin loci respectively. DNA sequence analysis of the *2.8* locus indicates the difference between the *2.8*

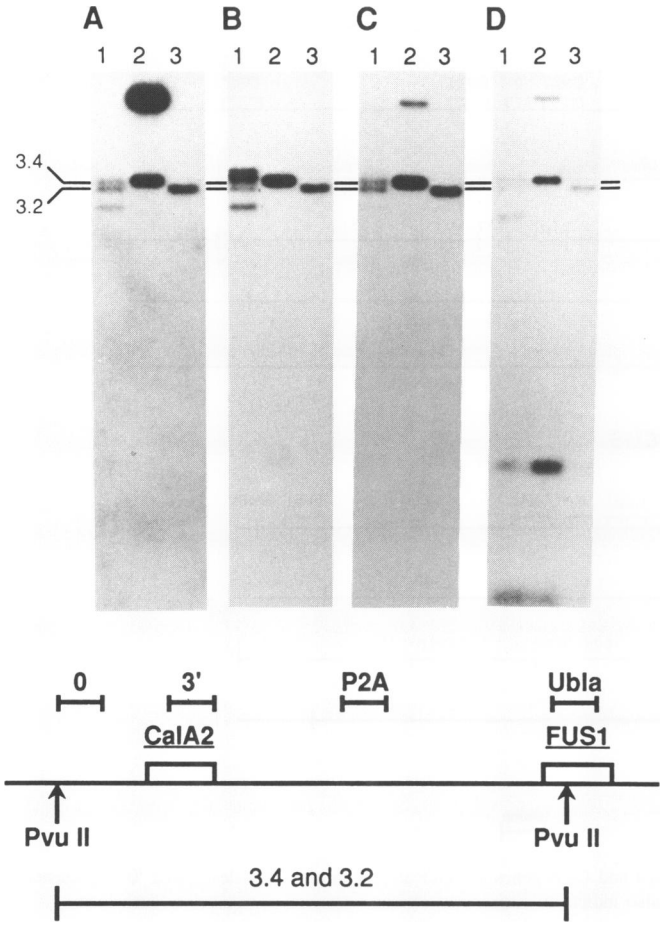


Figure 7. Genomic Southern analysis of linkage of the calmodulin and ubiquitin genes. Each panel contains PvuII restriction digest of, lane 1, 2.0μg of *T. cruzi* genomic DNA; lane 2, 1.4ng COS-UBIg4 DNA; lane 3, 1.0ng of pTC-UbIg. The ^{32}P labeled probes used in Panels A, B, C, D, were pTC-Cal:0, pTC-Cal:3', P2A and pTC-UbIa respectively. The P2A probe was generated using the PCR technique described in Materials and Methods. The synthetic oligonucleotides used to prime the PCR reaction were derived from the partial DNA sequence obtained from the untranslated region approximately 1.0Kbp down stream of *CalA2* (data not shown). A genomic map is presented immediately below the Southern blots indicating the positions of the PvuII restriction sites and the expected restriction map for each of the probes.

and *2.65* loci is due to an additional 150bp immediately downstream of the P2A sequence in the intergenic region separating *CalA5* from *FUS2* (data not shown).

Transcription of the calmodulin genes of *T. cruzi*

Transcription of the calmodulin genes during the epimastigote stage of the trypanosome life cycle results in the generation of two stable mRNAs of 1.1Kb and 1.6Kb in length (figure 9A). Both mRNAs hybridize to the pTC-Cal:3' calmodulin coding sequence probe (figure 9A and figure 9B, lane 2) as well as the pTC-Cal:5' probe (data not shown). The genes encoding the two calmodulin transcripts have not been unambigously identified. The data presented however, demonstrates that *CalA2* and/or *CalA5* are transcribed as 1.6Kb mRNAs (figure 9B, lane 3). As shown in figure 7C the P2A probe is specific for the DNA sequence linking the calmodulin and ubiquitin genes at both the *2.8* and *2.65* loci. Therefore transcription of *CalA2* and/or *CalA5* contribute to the 1.6Kb calmodulin mRNA. The 1.1Kbp calmodulin repeat specific probe pTC-Cal:0 recognizes only the 1.1Kb calmodulin mRNA (figure 9B, lane 1). The observation that transcription of the *CalA2* and/or *CalA5* genes contribute to the 1.6Kb mRNAs which do not hybridize to the pTC-Cal:0 probe suggests that the 1.1Kb mRNAs containing the pTC-Cal:0 sequence are the transcription products of the *CalB* genes. However, in the absence of cDNA sequence data we can not unambigously determine whether the pTC-Cal:0 sequence is the transcription product of the *CalB* or *CalA* genes.

DISCUSSION

The data presented describe the characterization of the calmodulin genes of *T. cruzi*. Identification of the *CalA2* gene was based on its DNA sequence homology to the calmodulin genes of *T. brucei* (10). The protein coding sequences of the calmodulin genes of the two trypanosome species are 85% homologous. The observed nucleic acid sequence homology however does not extend into either the 5' or 3' untranslated regions of the genes. The encoded 149 amino acid *T. cruzi* calmodulin protein differs at a single position compared to the *T. brucei* product (Val_{109}-vs-Ile_{109}).

The genome of *T. cruzi* contains eight calmodulin genes present at two loci designated *2.8* and *2.65*. Each locus consists of alternating 1.7Kbp and 1.1Kbp calmodulin repeats (figure 8). The calmodulin genes have been classified as either members of the *CalA* or *CalB* gene families based on two criteria. Members of the *CalA* gene family are defined as those in which the calmodulin coding sequence is followed by an intergenic region of approximately 1.2Kbp which does not hybridize with the pTC-Cal:0 intergenic probe. *CalB* genes have been defined as those in which the protein coding sequence is followed by an intergenic region of approximately 650bp which hybridizes with the pTC-Cal:0 intergenic probe.

Both the *2.8* and *2.65* calmodulin loci are linked to, and would be transcribed from the same DNA strand as the ubiquitin genes approximately 2.0Kbp downstream of each locus. Transcription of the calmodulin genes results in the generation of two stable mRNAs of 1.6Kb and 1.1Kb. The 1.1Kb transcript contains sequences which hybridize with the pTC-Cal:0 probe specific for the 1.1Kbp calmodulin repeat. However, in the absence of cDNA sequence data we can not unambigously identify which genes encode the 1.1Kbp mRNA. Transcription of *CalA2* and/or *CalA5* contribute to the 1.6Kb calmodulin mRNA population. Based on the length of the 1.6Kb *CalA2*/*CalA5* transcript its 3' end maps approximately 1.0Kbp upstream of the translation initiation codon of the *FUS1* gene. The close juxtaposition of the calmodulin loci and the ubiquitin genes raises the possibility that this region of the chromosome makes up a single large transcription unit. This possibility is supported by previous observations of apparent polycistronic transcripts in trypanosomes (11,18,19,20,21). Processing of such a polycistronic transcript would then lead to the generation of the two mature calmodulin mRNAs observed as well as the ubiquitin mRNAs.

Whether or not the *CalA* and *CalB* genes are functionally distinct has not been directly addressed by the data presented. However, the observation that two stable mRNAs exist in the cell raises the possibility the the calmodulin genes could be differentially regulated during the developmental cycle or in response to changes in the environment. A similar situation may exist in human cells which possess at least three calmodulin genes which appear to be differentially expressed (22,23,24). Although

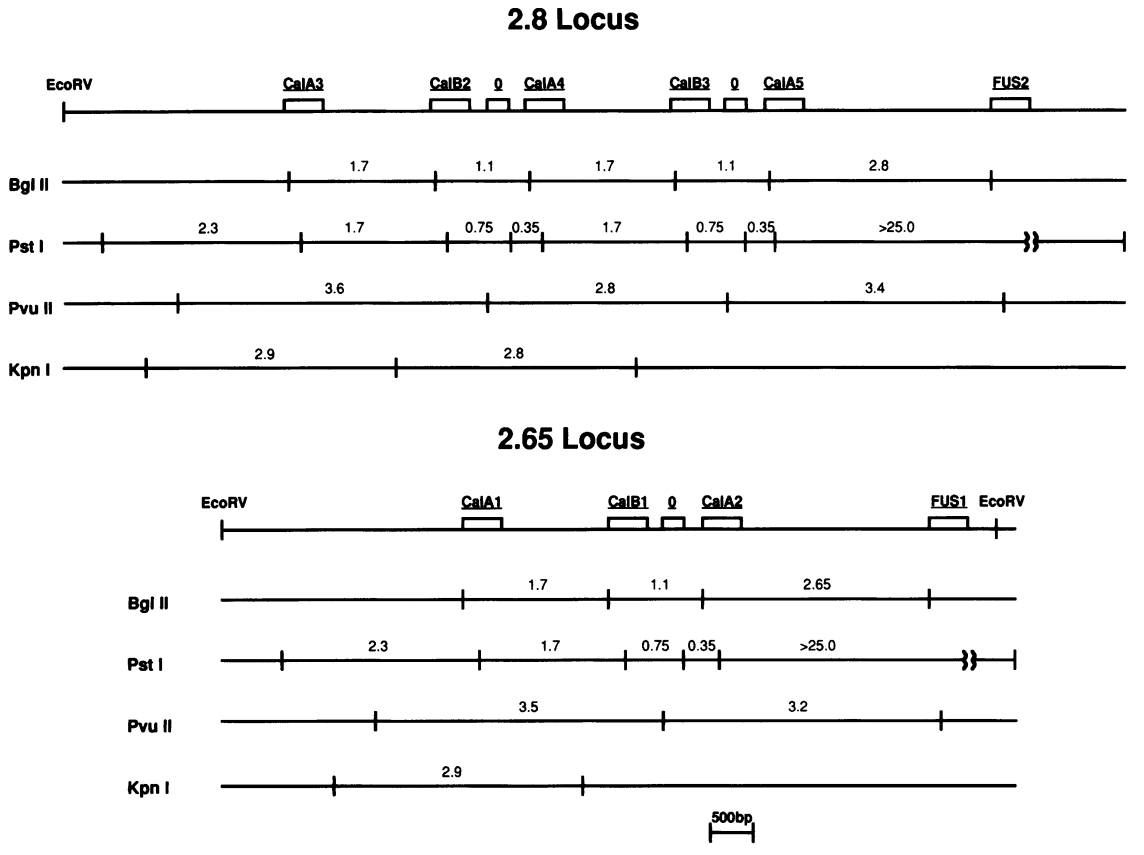


Figure 8. Genomic map of the *2.8* and *2.65* calmodulin loci. The position of the *CalA* and *CalB* genes are inidcated. The open box designated '0' represents the position of the pTC-Cal:0 sequence. The position of the *FUS1* and *FUS2* genes is also indicated. Linear restrictions maps are shown below each locus.

the nucleotide sequence of the three human genes vary significantly, each encodes an identical protein (24).

Neither promoters nor primary transcripts for protein coding genes have been identified in trypanosomes. Based on nuclear run-on experiments (18) and the occurrence of polycistronic transcripts (20) the suggestion has been made that transcription units may be far larger than single genes, possibly covering tens if not hundreds of kilobases. One possibility in such an organization is that functionally related genes are placed under the control of a common promoter. How then would calmodulin and ubiquitin fit into such a regulatory scheme? As stated previously, free calmodulin is a substrate for ubiquitination (7,9,8) and possible degradation via the ubiquitin dependent pathway. Co-regulation of calmodulin and ubiquitin expression may assure the cell of some optimal ratio of free calmodulin to ubiquitin.

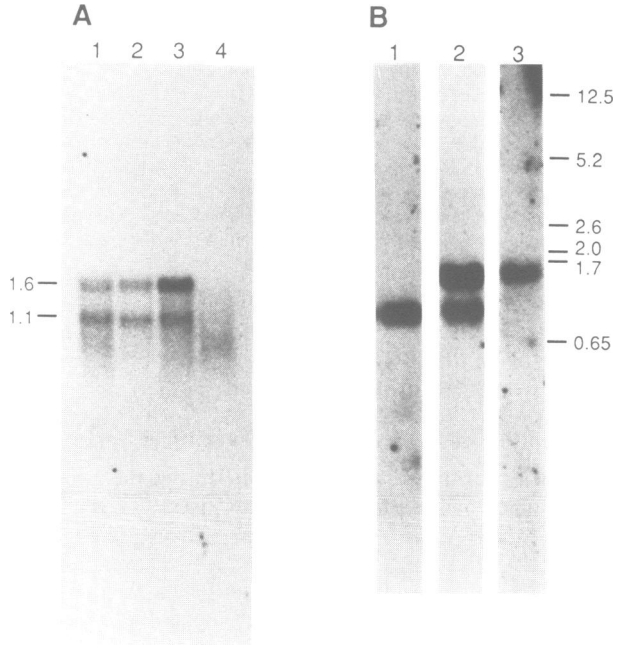


Figure 9. Northern analysis of *T. cruzi* RNA. A) Lane 1, 5.0ug total RNA, lanes 2 and 3, 0.5μg polyA$^+$ RNA, lane 4, 5.0μg polyA$^-$ RNA. The RNA in lanes 1, 2 and 4 originated from the same total RNA preparation. The polyA$^+$ RNA in lane 3 came from a separate total RNA preparation. The blot was hybridized with the calmodulin coding sequence probe pTC-Cal:3'. B) Duplicate lanes each containing 5.0μg of total *T. cruzi* RNA were hybridized with lane 1, pTC-Cal:0; lane 2, pTC-Cal:3'; lane 3, P2A. For a description of the generation of the P2A probe see the legend of figure 2 and Materials and Methods.

ACKNOWLEDGMENTS

Synthetic oligonucleotides used in this work were supplied by R. Sumrada and I. Kavori of the Molecular Resource Center of the University of Tennessee, Memphis. J.S. gratefully acknowledges the assistance of G. Togrye in the preparation of the illustrations used throughout this manuscript. The authors wish to thank R. Gains for assistance in preparation of the manuscript. This work was supported by USPHS grant AI 26578 awarded to J.S. This work was also in part supported by ACS grant NP-585 awarded to H. Eisen in whose laboratory the work was initiated.

REFERENCES

1. Means,A.R. (1988) *Recent Progress in Hormone Research*, *40*, 223–263.
2. Hershko,A. (1988) *J. Biol. Chem.*, *263*, 15237–15240.
3. Varshavsky,A., Bachmair,A., Finley,D., Gonda,D. and Wunning,I., (1988) in *Ubiquitin* (ed. Rechsteiner,M.) 287–324, Plenum, New York.
4. Bonner,W.M., Hatch,C.L., and Wu,R.S. (1988) in *Ubiquitin* (ed. Rechsteiner,M.) 157–172, Plenum, New York.
5. Seigelman,M., Bond,M.W., Gallatin,W.M., St John,T., SmithH.T., Fried,V.A. and Weissman,I.L. (1986) *Science*, *231*, 823–829.
6. Fried,V.A., Smith,H.T., Hildebrandt,E. and Weiner,K. (1987) *Proc. Natl. Acad. Sci. USA*, *84*, 3685–3689.
7. Ziegenhagen,R., Gehrke,P. and Jennissen,H.P. (1988) *FEBS Lett.*, *237*, 103–107.
8. Jennissen,H.P. and Laub,M. (1988) *Biol. Chem. Hoppe-Seyler*, *369*, 1325–1330.
9. Ziegenhagen,R. and Jennissen,H.P. (1988) *Biol. Chem. Hoppe-Seyler*, *369*, 1317–1324.
10. Tschudi,C., Young,A.S., Ruben,L., Patton,C.L. and Richards,F.F. (1985) *Proc. Natl. Acad. Sci. USA*, *82*, 3998–4002.
11. Tschudi,C. and Ullu,E. (1988) *EMBO J.*, *7*, 455–463.
12. Swindle,J., Ajioka,J., Eisen,H., Sanwal,B., Jacquemot,C., Browder,Z. and Buck,G. (1988) *EMBO J.*,*7*, 1121–1127.
13. Camargo,E.P. (1964) *Rev. Inst. Med. Trop.* Sao Paulo *6*, 93–100.
14. Bittner,M. and Vapnek,D. (1981) *Gene*, *15*, 319–329.
15. Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, *74*, 5463–5467.
16. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning, A Laborarory Mannual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Mullis,K.B. and Faloona,F. (1987) *Meth. Enzymol. 155*, 335–350.
18. Johnson,P.J., Kooter,J.M. and Borst,P. (1987) *Cell*, *51*, 273–281.
19. Gonzales,A., Lerner,T.J., Huccas,M., Sosa-Pineda,B., Norueira,N. and Lizardi,P.M. (1985) *Nuc. Acids Res.*, *13*, 5789–5804.
20. Muhich,M.L. and Boothroyd,J.C. (1988) *Mol. Cell. Biol.*, *8*, 3837–3846.
21. Imboden,M.A., Laird,P.W., Affolter,M. and Seebeck,T. (1987) *Nuc. Acids Res.*, *15*, 7357–7370.
22. Wawrzynczak,E.J. and Perham,R.N. (1984) *Biochem. Int.*, *9*, 177–185.
23. Sengupta,B., Friedberg,F. and Detera-Wadleigh,S.D. (1987) *J. Biol. Chem.*,*262*, 16663–16670.
24. Fischer,R., Koller,M., Flura,M., Mathews,S., Strehler-Page,M.A., Krebs,J., Penniston,J.,T., Carafoli,E. and Strehler,E.E. (1988) *J. Biol. Chem.*, *263*, 17055–17062.