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RESEARCH BRIEF

Leptomonas seymouri, Trypanosoma brucei: A Method for Isolating Trypanosomatid Nuclear Factors Which Bind *T. brucei* Single-Stranded g-Rich Telomere Sequence

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FIELD, H., AND FIELD, M. C. 1996. *Leptomonas seymouri, Trypanosoma brucei:* A method for isolating trypanosomatid nuclear factors which bind *T. brucei* single-stranded g-rich telomere sequence. *Experimental Parasitology* **83,** 155–158. Sequential expression of variant surface glycoproteins (VSG) in *Trypanosoma brucei* is the basis of antigenic variation which is essential for parasite survival. Telomere distal copies of VSG genes, so-called basic copies, provide a repository of VSG sequence information for variability, but actively expressed copies are found only at subtelomeric regions of chromosomes. Of eight or so expression sites (ES) in the *T. brucei* genome, only one is active at one time. Movement of a basic copy VSG gene to an ES requires a recombination event of unknown mechanism. The properties of telomeres have been speculated to be important for control of VSG expression or basic copy mobilization, prompting us to begin to investigate telomere-binding proteins in trypanosomatids. The *T. brucei* telomere sequence is known, facilitating design of synthetic telomeric DNAs. Here we describe a method for preparation of active trypanosomatid nuclear extracts. We show that in *T. brucei* and *Leptomonas seymouri,* factors can be detected which bind a g-rich single-strand telomere sequence based on the *T. brucei* telomere. The *L. seymouri* telomere-binding factor, LST-1, dissociates in the presence of high salt to produce a core factor, LST-2, migrating similarly to the *T. brucei* telomere-binding factor TBT-1. The affinity of LST-2 and TBT-1 for DNA under high salt conditions is characteristic of telomere proteins.

INDEX DESCRIPTORS: Trypanosome; telomere; single-stranded; DNA-binding protein; g-rich; nuclear extract.

Telomeres are simple-sequence elements which cap chromosome ends. These elements begin at the double-stranded break and may extend over several kilobases. At the tip of the chromosome two or more of the repeats may be single-stranded in a 3′ overhang configuration. Two classes of specific DNA-binding proteins protect the double-stranded and single-stranded repeats (Blackburn 1991). The exact sequence of the repeat element has been determined by direct sequencing in *Trypanosoma brucei* as GGTTAG, but the structure of the end is unknown (Blackburn and Challoner 1984; Van der Ploeg *et al.* 1984). A candidate protein which binds double-stranded telomere elements has been identified in *T. brucei* (Eid and Sollner-Webb 1995). A trypanosome protein which binds single-stranded telomere overhangs has not previously been reported.

Aspects of the genetic control of the variant surface glycoproteins (VSG) of *T. brucei* may be related to their juxtaposition to telomeres. Expression sites (ES) capable of directing VSG synthesis are located close to telomeres. A single ES is selectively activated by an unknown mechanism, and it is probably relevant that expression of genes adjacent to telomeres is often repressed. Also, recombinational activity appears to be a property of telomeric apposition in a wide range of organisms. In *T. brucei,* mobilisation of VSG gene sequences from intrachromosomal basic copy (BC) sites to an ES is a recombination event. The telomere-binding proteins of *T. brucei* are worthy of study in this context, and, as a first approach to determining the identity of the telomere proteins present in trypanosomes, a nuclear extraction protocol has been identified and tested in electrophoretic mobility shift assays (EMSA).

Leptomonas seymouri and *T. brucei* appear to have telomeres of similar sequence (Blackburn and Challoner 1984; H. Field, unpublished data). We used *L. seymouri* as a test organism for nuclear extraction protocols, resulting in material giving a gel shift with telomeric sequences. The methodology was then extended to bloodstream *T. brucei.*

The following method was used for nuclear preparation

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and extraction of proteins (Roy *et al.* 1991). Briefly, cells were grown, harvested by centrifugation, snap frozen, and stored at −70°C. Cells (2.5×10^9 to 10×10^9) were resuspended in buffer A (250 m*M* sucrose, 15 m*M* Tris, pH 7.9, 140 m*M* NaCl, 2 m*M* EDTA, 0.5 m*M* EGTA, 0.15 m*M* spermidine, 1 m*M* DTT, 25 m*M* KCl, 2 m*M* $MgCl_2$, 0.4 m*M* PMSF (added fresh)). Additional protease inhibitors were added frequently: tosyllysyl chloroketone (added dry); all other inhibitors were added at 2 μg/ml: soybean trypsin inhibitor, antipain, pepstatin A, leupeptin, 1,10-phenanthroline, and chymostatin. Cells were lysed by 10 strokes of a Dounce homogeniser (Wheaton 200, with ball-shaped pestle). NP–40 was added at a final concentration of 0.5%, and a further 5 homogenisation strokes were performed. The lysate was centrifuged at 1000*g* for 8 min at 4°C. Pelleted nuclei and debris were washed in 5 ml buffer A. Nuclei were lysed in buffer B (buffer A plus 350 m*M* KCl). One pellet volume of buffer B was added, the nuclei were incubated 5 min at 4°C, and then KCl was added to 0.4 *M* final concentration. This lysate was homogenised with a further 20 strokes of the Dounce and transferred to a 1.5-ml microfuge tube. Centrifugation was for 5 min at 12,000*g* at 4°C. The supernatant was ultracentrifuged at 180,000*g* for 90 min at 4°C. The nuclear extract (supernatant) was dialysed into buffer C (20 m*M* Tris, pH 7.5, 50 m*M* KCl, 4 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 20% glycerol, 0.1 m*M* phenylmethylsulfonyl fluoride), and stored at −70°C in small aliquots.

First, *L. seymouri* extracts were tested for binding to a single-stranded oligonucleotide of *T. brucei* telomere sequence $(GGTAG)_5$. Components were added in the following order: competing oligonucleotide, then nuclear extract, then a mixture of binding buffer (25 m*M* Hepes, pH 7.5, 1 m*M* EDTA, 1 m*M* dithiothreitol, 10% glycerol), 0.5 mg/ml BSA, and 0.1 mg/ml double-stranded $d(I.C)_n$ (Boehringer-Mannheim), and finally 0.8 ng radiolabeled probe, in 20 μl

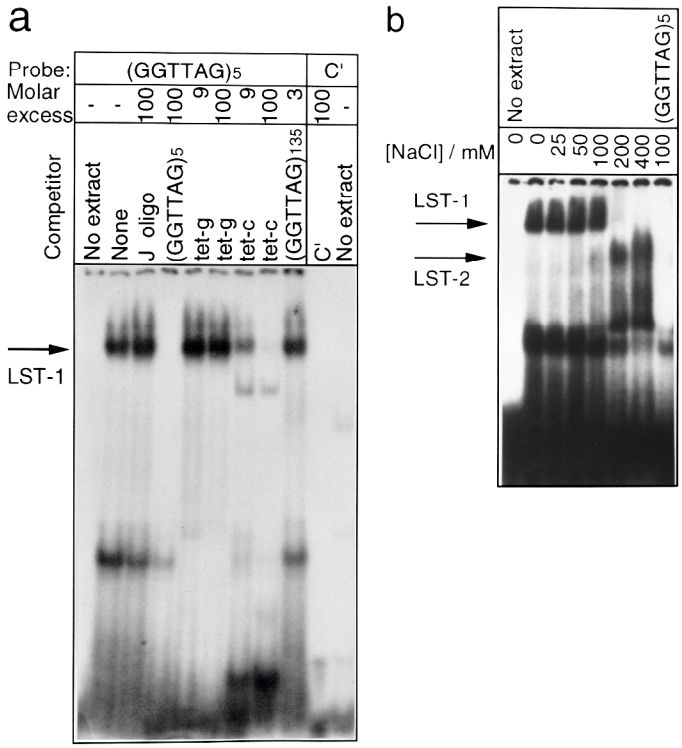

FIG. 1. (a) LST-1 defined by EMSA. Lanes contain end-labeled probes as shown, and the indicated competing DNA was added to each binding reaction at molar ratios relative to amount of probe. C′ is the complement of the probe, i.e., $5'$-$(CTAACC)_5$. Oligo J is an irrelevant single-stranded oligonucleotide ($5'$-CCAGTTCGGCCGCACCGACTCGCGGTCCAGC); tet-g is a *Tetrahymena* g-strand oligonucleotide ($5'$-$(TTGGGG)_7$CATCGATGGG); tet-c is a *Tetrahymena* c-strand which competes by binding probe ($5'$-$(CCCCAA)_7$ CCCATCGTAC); $(GGTTAG)_{135}$ consists of an array of 135 double-stranded GGTTAG telomere repeats with irrelevant ends, cut with *Eco*RI from a plasmid containing a cloned human telomere end (Zhong *et al.* 1992). (b) LST-1 disappears in the presence of high salt, when LST-2 appears. Using labeled $(GGTTAG)_5$ as probe, increasing NaCl was added to each binding reaction to give the concentrations shown. In the last lane, unlabeled $(GGTTAG)_5$ was added in a 100-fold molar excess over probe.

TABLE I
Characterisation of LST-1 and Partial Characterisation of TBT-1 by EMSA

Probe	Competitor or condition	Notes	Ratio of competitor/probe (repeats)	LST-1 present	TBT-1 present
$(GGTTAG)_5$[a]	$(GGTTAG)_5$[a]	Single-strand telomere repeats	3	(–)	NT[b]
$(GGTTAG)_5$	$(GGTTAG)_5$		9	–	(–)
$(GGTTAG)_5$	$(GGTTAG)_5$		100	–	–
$(GGTTAG)_5$	tet-g $(TTGGGG)_7$[a]	Alternative single-stranded repeats	100	+	NT
$(GGTTAG)_5$	xx$(GGTTAG)_{135}$xx[c]	Internal double-stranded telomere repeats	81	+	NT
$(GGTTAG)_5$	C′	Complement[a,d]	3	–	–
$(GGTTAG)_5$	J oligo[a]	Irrelevant single-strand	100	+	+
$(GGTTAG)_5$	NaCl	50 m*M*		+	+
$(GGTTAG)_5$	NaCl	400 m*M*		–[e] Fig. 1b	+
$(GGTTAG)_5$	Mg^{2+}	1 mM		+	+
$(GGTTAG)_5$	Nonidet P-40	0.1%		+	+
C′	None			–	–
xx$(GGTTAG)_{133}$xx[c]	None	Internal double-stranded telomere repeats		–[e]	NT

Note. Various probes (first column) were tested by EMSA for binding to trypanosomatid nuclear extracts. The presence of the band containing LST-1 or TBT-1 complexed with labeled probe is shown by a plus sign in the last two columns. A minus sign denotes the abolition of the band by the competitor or condition indicated, while a minus sign in parentheses denotes only a very weak band.

[a] Single-stranded oligonucleotide.

[b] NT not tested.

[c] Double-stranded; xx, irrelevant DNA.

[d] See Fig. 1a legend for sequence; competes by binding probe.

[e] LST-1 not seen, but other specific binding complexes are detected.

total volume (final concentrations given). After 15 min incubation at 30°C the sample was electrophoresed at <30°C on a 4.5% low-ionic-strength gel (Ausubel *et al.* 1982).

A single g-strand telomere sequence-specific bandshift was seen in *L. seymouri* extracts assayed as described above, using $(GGTTAG)_5$ as probe (Fig. 1a). This putative factor was designated LST-1 (*L. seymouri* telomere factor-1). Specificity for the *T. brucei* g-strand telomere sequence $(GGTTAG)_5$ was demonstrated by the small amount of this unlabeled oligonucleotide required to compete for binding (threefold molar excess, see Table I) and by the inability of a related sequence, the telomere sequence from *Tetrahymena,* TTGGGG, to disrupt binding (Fig. 1a). Double-stranded telomeres, the *T. brucei* c-strand, and irrelevant single- or double-stranded oligonucleotides were not bound (Table I). Interestingly, the LST-1/$(GGTTAG)_5$ complex disappears in the presence of 100–400 m*M* NaCl, and in its place appears a complex of similar but higher mobility, LST-2 (Fig. 1b). LST-2 remains bound to the telomere sequence under conditions of high ionic strength, a property of the *Oxytrichia* telomere-binding protein (Price and Cech 1989). LST-2 was displaced by excess $(GGTTAG)_5$, consistent with specific binding to single-stranded telomere g-strand. LST-2 is proposed to contain the core of LST-1, produced by dissociation of LST-1, since the amount of LST-2 increases in the presence of NaCl where LST-1 decreases. Similarly, *Oxytrichia* protein is composed of α and β subunits (Gray *et al.* 1991).

The results of all the EMSA experiments performed on *L. seymouri* extracts are summarised in Table I. LST-1 was reproducibly detected in several preparations of nuclear extract. However, no such factor was seen by EMSA using other probes, notably the double-stranded telomere repeat probe. Biochemical properties of LST-1/$(GGTTAG)_5$ complex are also indicated in Table I.

To test whether *T. brucei brucei* contains a similar protein, the method was used on parasites purified from in-

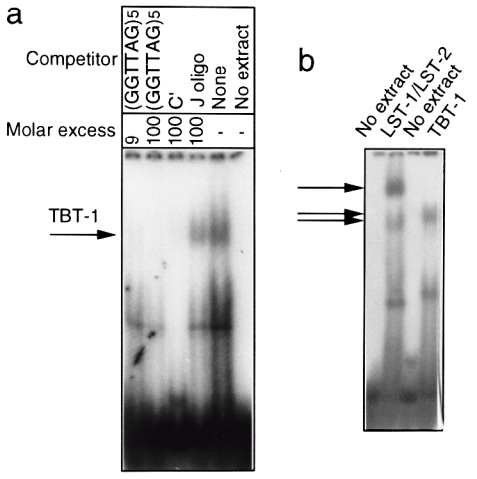

FIG. 2. (a) Definition of TBT-1. Using $(GGTTAG)_5$ as probe, nuclear extracts from bloodstream form *T. brucei* were tested in the EMSA described. TBT-1 binds to the probe, and is competed by DNAs shown, at the molar ratios (with respect to probe) given. Competing DNAs are described in the legend to Fig. 1a. (b) LST-1, LST-2, and TBT-1 can be resolved by nondenaturing PAGE. EMSAs were performed with the appropriate nuclear extracts. The sample containing both LST-1 and LST-2 was generated by including 100 m*M* NaCl in the binding reaction for EMSA.

fected rat blood on DEAE-cellulose (Field and Menon 1992). Indeed, a bandshift was observed by EMSA. Binding to the $(GGTTAG)_5$ probe by this *T. brucei* telomere-binding factor-1 (TBT-1) is characteristically competed out with low amounts of cold probe, but binding is not disrupted by irrelevant single-stranded oligonucleotide (Fig. 2a). Biochemical properties of TBT-1 as assessed by EMSA are shown in Table I. Like LST-2, it remains bound to single-stranded telomere repeats at high salt concentrations. TBT-1 appears electrophoretically distinct from LST-1 (Fig. 2b), although the presence of 100 m*M* salt may have caused the LST-2 band to migrate more rapidly; in another gel, TBT-1 apparently comigrates with LST-2 (not shown).

In conclusion, we have derived a general method for the preparation of nuclear extracts from Trypanosomatid species suitable for the detection of telomere sequence-binding activities. Factors from *L. seymouri* and the bloodstream form of *T. brucei,* which potentially bind the single-stranded telomeric g-strand, are described. The specificity and the resistance to dissociation under high salt conditions of LST-2 and TBT-1 complexes are consistent with their assignment as telomere-binding proteins. TBT-1 may complement the factor described by Eid and Sollner-Webb and protect putative 5′ (g-strand) overhangs in *T. brucei* telomeres.

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References

Ausubel et al., Eds. 1992. "Current Protocols in Molecular Biology," Unit 12.2. Wiley, New York.

Blackburn, E. H. 1991. Structure and function of telomeres. *Nature* **350,** 569–573.

Blackburn, E. H., and Challoner, P. B. 1984. Identification of a telomeric DNA sequence in *Trypanosoma brucei. Cell* **36,** 447–457.

Eid, J. E., and Sollner-Webb, B. 1995. ST-1, a 39-kilodalton protein in *Trypanosoma brucei,* exhibits a dual affinity for the duplex form of the 29-base-pair subtelomeric repeat and its c-rich strand. *Molecular and Cellular Biology* **15,** 389–397.

Field, M. C., and Menon, A. K. 1992. Biosynthesis of glycosylphosphatidyl inositol membrane protein anchors. *In* "Lipid Modifications of Proteins, A Practical Approach" (N. Hooper and A. J. Turner, Eds.). IRL Press, Oxford, UK.

Gray, J. T., Celander, D. W., Price, C. M., and Cech, T. R. 1991. Cloning and expression of genes for the *Oxytrichia* telomere-binding protein: Specific subunit interactions in the telomeric complex. *Cell* **67,** 807–814.

Price, C. M., and Cech, T. R. 1989. Properties of the telomeric DNA-binding protein from *Oxytrichia nova. Biochemistry* **28,** 769–774.

Roy, R. J., Gosselin, P., and Guérin, S. L. 1991. A short protocol for micro-purification of nuclear proteins from whole animal tissue. *BioTechniques* **11,** 770 777.

Van der Ploeg, L. H. T., Liu, A. Y. C., and Borst, P. 1984. Structure of the growing telomeres of trypanosomes. *Cell* **36,** 459–468.

Zhong, Z., Shiue, L., Kaplan, S., and de Lange, T. 1992. A mammalian factor that binds telomeric TTAGGG repeats in vitro. *Molecular and Cellular Biology* **12,** 4834–4843.

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