***In vivo* UV-cross-linking hybridization: a powerful technique for isolating RNA binding proteins. Application to trypanosome mini-exon derived RNA**

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ABSTRACT

Differential gene expression in cells is achieved, in part, through direct RNA-protein interactions. Methods for the identification of RNA binding proteins require cross-linking of proteins to RNA by chemicals or ultraviolet (UV) light followed by chromatography or density-gradient centrifugation (7,11,16). We have developed a simplified method for the rapid and efficient identification of potential regulatory RNA binding proteins. In this method, irradiation of cells with UV light induces cross-links between RNA and proteins in close contact (7,11). Boiling of extracts from irradiated cells in the presence of sodium dodecyl sulfate dissociates any non-specific RNA-protein interactions (11). After analysis of the cell extracts by SDS-PAGE, followed by Western blotting onto a nitrocellulose membrane and washing of the filter, we have found that only RNA molecules that are covalently bound to proteins are retained on the filter. Hybridization of this Western blot with an appropriate nucleic acid probe allows detection of bands of RNA-protein complexes. Antisera against the binding proteins are raised by immunizing mice with a region of the nitrocellulose membrane containing the bands of RNA-protein complexes. Using this approach we have found that in African trypanosomes, mini-exon derived RNA transcripts form complexes with cytoplasmic binding proteins in different life cycle stages of the parasite. Evidence for the specificity of mini-exon derived RNA-protein interactions is shown using *in vitro* UV-cross-linking analysis in which only *in vitro* generated sense (but not antisense) mini-exon derived RNA transcripts form complexes with cytoplasmic proteins.

INTRODUCTION

The regulation of gene expression is based mainly upon the recognition of nucleic acid sequences by proteins (1,2 and refs. therein). The existence of RNA binding proteins has been proposed for many years as a mechanism by which messenger RNAs are processed and by which protein synthesis is regulated

during cell development (3–6). Because the potential number of different proteins interacting with RNAs is large (7–9), it becomes important to identify which of these make direct contact with the RNA, as some proteins may be associated with RNA via protein-protein interactions (10). To identify proteins that are tightly associated with RNAs, photochemical cross-linking of protein to RNA by ultraviolet (UV) irradiation of intact cells has proved to be a convenient method for stabilizing nucleic acid-protein complexes (7,11–13). UV light is a ‘zero length’ cross-linking agent, thought to cross-link proteins to nucleic acids at their contact points without additional elements that might cause conformational perturbations (12). Therefore, only proteins closely associated with RNA can be covalently linked by UV irradiation. The complexes formed have been shown to be resistant to both heat, detergent, and alkali (reviewed in 12). Further, it has been reported that in chemical cross-linking techniques the length of the linker may disrupt the RNA-protein complex (14) which would not be expected to be a problem with UV-cross-linking. In the absence of a cross-linking step, the RNA-protein complexes can be isolated using methods like sedimentation, density-gradient centrifugation or affinity chromatography (10,11). However, these techniques are time-consuming and also have the general disadvantage that non-specific binding of proteins to the RNA during cell fractionation may occur.

Following cross-linking of proteins to RNA by chemical or UV methods, several RNA binding proteins have been isolated using a variety of approaches such as chromatography (7,11) or the combination of chromatography and density-gradient centrifugation (15,16). Again, all these methods of detection and isolation of the complexes are cumbersome, and none of them appear to offer a simple and direct approach for detecting the presence of the transcript associated with proteins.

In this paper we report a method for the rapid identification of cytoplasmic RNA-binding proteins. In our approach, cytoplasmic extracts of UV-irradiated cells are analysed by SDS-PAGE followed by western blotting onto nitrocellulose membranes. The filters are then washed under conditions where only RNA covalently cross-linked to proteins is retained. Hybridization to the filters with specific nucleic acid probes results in the detection

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of hybridizing bands corresponding to nucleic acid-protein complexes.

We have developed this method utilizing the abundant small transcripts involved in trans-splicing during the maturation of mRNA molecules of *Trypanosoma brucei brucei* and tested whether we could detect cytoplasmic RNA-binding proteins which are specific for this transcript. In trypanosomes, formation of mature nuclear mRNA is achieved by a trans-splicing mechanism in which a 39-nucleotide sequence, the spliced leader or mini-exon, from a small abundant transcript, the mini-exon derived RNA (medRNA), is ligated to the 5' ends of pre-mRNAs (15,17-19). The identification of proteins involved in this splicing event, in particular, and RNA-associated proteins, in general, will help to elucidate mechanisms for the regulation of gene expression. Using this approach, we have found that *Trypanosoma brucei brucei* medRNA transcripts are associated with cytoplasmic proteins. Some of these proteins may be differentially expressed in different stages of the parasite life-cycle.

MATERIALS AND METHODS

Cells

Trypanosoma brucei brucei, pleomorphic clone ILTat 1.1 (20) was used in this work. The actively-dividing long slender (LS) and non-dividing short stumpy (SS) bloodstream form trypanosomes (21) were isolated from rat blood as described (22). The procyclic culture form (PCF) trypanosomes were propagated *in vitro* and isolated as described (23). Following isolation, trypanosomes were washed in phosphate-buffered saline (PBS) containing 1% glucose, collected by centrifugation, and used immediately.

In vivo UV-cross-linking and preparation of cell extracts

Fresh cells were resuspended in cold PBS (approximately 10^7 cells/ml), and 2 ml were then transferred to a circular tissue culture dish (60×15 mm; Becton Dickinson, USA) and irradiated with UV light (Transilluminator; Hoefer, USA) at 254 nm for 10 min at 4°C. After irradiation, 1 ml of cells was transferred to an 1.5 ml eppendorf tube and pelleted by centrifugation at 14000 rpm for 15 sec. The cell pellet was resuspended in 5 volumes of ice-cold homogenization buffer (250 mM sucrose, 50 mM KCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM EDTA, 50 mM Tris-HCl pH 7.4) containing 40 µg/ml each of protease inhibitors (antipain, leupeptin and E64; Cambridge Research Biochemicals) and 5 U/ml RNase Block II (Stratagene, USA) and were homogenized by pipetting up and down on ice. The nuclei were removed by centrifugation at 14000 rpm for 2.5 min at 4°C and the supernatant containing the crude cytoplasmic fraction was used immediately or stored frozen at -70°C.

SDS-PAGE and Western blot hybridization

Twenty microliters of cytoplasmic cell extracts representing approximately 10^6 cells were boiled in an equal volume of 2× SDS-PAGE sample buffer for 5 min, then separated by SDS-PAGE and electroblotted in Tris-glycine-methanol buffer onto nitrocellulose membrane as described previously (24). For large complexes, samples were separated on a horizontal 0.5% (w/v) agarose gel in SDS electrophoresis buffer and electroblotted onto a nitrocellulose membrane. The nitrocellulose membrane was then washed at room temperature for 5 min with electroblotting buffer, and for 5 min with distilled water. The filter was then

stained with amido black as described (24) and destained in 10% (v/v) acetic acid, 25% (v/v) isopropanol, 0.1% (w/v) SDS. Following destaining, the filter was prehybridized for at least 3 hours in 6.6× SSC (1× SSC = 0.15 M NaCl, 0.015 M tri-Sodium citrate, pH 7), 5× Denhardt's solution, 0.1% (w/v) SDS and 0.1% (w/v) sodium pyrophosphate pH 7.4, at the indicated temperature. This was followed by hybridization with the appropriate ³²P-labelled nucleic acid probe generated by the random priming method of Feinberg and Vogelstein (25) or by kinasing in the presence of γ-³²P-ATP (26). Hybridization was performed overnight in the prehybridization mixture. Post-hybridization washes at the indicated temperature in 0.5× SSC, 0.1% SDS were followed by autoradiography at -70°C using 'Dupont Lighting Plus' intensifying screens for 1-5 days.

In vitro UV-cross-linking of nucleic acids to proteins

For *in vitro* cross-linking of DNA to proteins, a cytoplasmic extract from approximately 10^6 non-irradiated long slender (LS) *T. brucei* cells was mixed with 2 µg of *Sau3A* I digested trypanosome genomic DNA in 400 µl of homogenization buffer and incubated at room temperature for 20 min before UV irradiation, as described. After UV treatment, 2 volumes of cold ethanol were added and the precipitate pelleted by centrifugation. The resultant dried pellet was dissolved in SDS-PAGE sample buffer and separated by electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA buffer (26). The gel was stained with ethidium bromide and nucleic acids visualized with UV light. After electrotransfer to nitrocellulose in SDS-PAGE electroblotting buffer (24), the filter was washed as described to eliminate non-cross-linked DNA. Following alkaline treatment to denature protein bound DNA and to hydrolyse contaminating RNA, the filter was neutralized, air-dried and baked at 80°C for 2 hours as described for Southern blotting (26). Prehybridization, hybridization with ³²P-labelled *Sau3A* I digested LS genomic DNA and washing conditions, at 65°C, were the same as for Western blot hybridization. Post-hybridization washes were followed by autoradiography.

For *in vitro* cross-linking of RNA to proteins, a medRNA cDNA clone in pBlueScript, pTBMED3 (27), was used to generate sense and antisense ³²P-labelled medRNA transcripts from the plasmid T7 and T3 promoters, respectively, in the presence of α-³²P-UTP. The reaction was performed according to the conditions recommended by the supplier (Stratagene, USA). 10^6 cpm (5 µl) of *in vitro* generated radioactive medRNA transcripts was mixed with 20 µl of cytoplasmic cell extracts from approximately 10^6 cells and incubated at room temperature for 20 min before exposure to UV light. Following denaturation, samples were analyzed by Western blotting. The filter was washed with distilled water to eliminate free nucleic acids, then stained with amido black prior to autoradiography.

Immunization of mice with RNA-protein complexes

After hybridization and autoradiography, a region of the nitrocellulose corresponding to the hybridizing band was excized and dissolved in 0.5 ml of DMSO in an eppendorf tube. After the nitrocellulose had completely dissolved, an equal volume of PBS was added to precipitate the dissolved filter. The precipitate was recovered by centrifugation at 14000 rpm for 1 min, washed 3 times with PBS, resuspended in the same solution and injected, without adjuvant, into the peritoneum of two BALB/c mice. After 3 subsequent inoculations with similarly prepared material, and a 21 day interval between each inoculation, the sera of the

immunized mice were collected on day 14 following final inoculation and used in immunoblot analysis with goat anti-mouse IgG peroxidase conjugate (Sigma) as the second antibody. The blots were subsequently developed and bands detected with diaminobenzidine in the presence of hydrogen peroxide (24).

RESULTS

Only nucleic acids covalently bound to proteins are detected by the UV-cross-linking hybridization (UV-CLH) technique

UV irradiation induces cross-links of nucleic acids to tightly associated proteins (7,8,11). In order to identify such nucleic acids and their associated proteins, we have developed a method which eliminates free nucleic acids and retains those that are cross-linked to proteins. The efficiency of this method was verified as follows. A cytoplasmic extract of 10^6 non-irradiated *Trypanosoma brucei* long-slender (LS) bloodstream-form (BSF) cells was mixed with 2 μ g of *Sau*3A I digested genomic DNA from LS parasites. The mixture was incubated and then UV-irradiated as described in materials and methods. After UV treatment, the sample was subjected to electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA buffer (26). For the control sample, the irradiation step was omitted. The gel was stained with ethidium bromide and visualized under UV light (Fig. 1A). Following electrotransfer of samples from the gel to the nitrocellulose, the filter was treated as described in materials and methods. Hybridization of the blot with 32 P-labelled *Sau*3A I digested LS form genomic DNA generated a strong signal with the DNA sample which was UV-cross-linked with the cell extract (Fig. 1B, lane 4). In contrast,

no hybridization signal was detected with the non-irradiated control sample (Fig 1B, lane 1). This result shows that our approach can be used to discriminate between free nucleic acids and those covalently linked to proteins. We wished to exploit this technique to identify proteins specifically bound to cytoplasmic RNAs.

In vivo and *in vitro* testing of the UV-CLH method to identify medRNA associated cytoplasmic proteins

medRNA is an abundant small transcript in trypanosomes and is involved in trans-splicing of the 39 nt sequence found at the 5' end of all mRNA transcripts (17–19). It is therefore an ideal transcript to test the efficiency of the UV-CLH method to identify RNA-specific binding proteins.

Cytoplasmic extracts from UV-irradiated actively-dividing LS and non-dividing short stumpy (SS) bloodstream-form (BSF) trypanosomes were used. Following separation by SDS-PAGE and Western blotting of the samples, the blot was stained with amido black to monitor protein transfer (Fig. 2A). Amido black staining was negative with proteinase K treated samples as expected (Fig. 2A, Prot.K: +). After amido black staining, the filter was hybridized with a 32 P-labelled probe generated from a medRNA cDNA clone, pTBMED3, which has been described previously (27). The probe hybridized to several bands corresponding to a region ranging between 80 and 200 kDa on the gel (Fig. 2B). We did not observe significant differences in the profile of hybridization after longer exposure of the blot to film (data not shown), but the equivalent bands in LS and SS form trypanosomes differed in intensity. No signal was detected with non-irradiated trypanosomes as well as with proteinase K treated (Fig. 2B, Prot.K: +) or with RNase A treated (data not shown) cytoplasmic extracts from UV-irradiated cells. These results indicate that the medRNA transcripts detected are physically bound to proteins. In similar experiments with *in vitro*

Figure 1. Only nucleic acids covalently bound to proteins are detected by the UV-CLH technique. A cytoplasmic extract of approximately 10^6 non-irradiated long slender (LS) *T. brucei* cells was mixed with 2 μ g of *Sau*3A I digested genomic DNA from LS trypanosomes, then incubated at room temperature for 20 min before UV irradiation as described. After ethanol precipitation, the dried pellet was dissolved in SDS-PAGE sample buffer and separated by electrophoresis on a 1% agarose gel in Tris-Acetate-EDTA buffer (lane 4). In the control sample, the UV treatment was omitted (lane 1). Panel A is an ethidium bromide stained gel. Lanes 2 and 3 contain size markers: *Hae* III fragments of phage ϕ X174, and *Hind* III fragments of phage lambda, respectively. In panel B, the gel was electroblotted onto nitrocellulose as described, then prehybridized in 6.6 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.1% (w/v) SDS and 0.1% (w/v) sodium pyrophosphate pH 7.4, for 3 hours at 65°C. Hybridization was performed in the prehybridization solution in the presence of 32 P-labelled *Sau*3A I digested LS form genomic DNA at 65°C. Post-hybridization washes in 0.1 $\times$ SSC, 0.1% (w/v) SDS at 65°C were followed by autoradiography.

Figure 2. *In vivo* UV-cross-linking analysis of cytoplasmic medRNA binding proteins. Trypanosomes were irradiated with UV light at 254 nm for 10 min at 4°C and lysed in a buffer containing 0.5% (v/v) NP-40 and protease inhibitors as described. Proteinase K treated (Prot.K: +) and untreated (Prot.K: -) cytoplasmic cell extracts from approximately 10^6 irradiated (UV: +) and non-irradiated (UV: -) long slender (lane LS) and short stumpy (lane SS) form trypanosomes were denatured in SDS-PAGE sample buffer at 100°C for 5 min and analysed by Western blotting. The blot was stained with amido black (Panel A) then hybridized with the clone 3 cDNA probe at 50°C to localize the complexes of medRNA-proteins (Panel B). Post-hybridization washes were in 0.5 $\times$ SSC, 0.1% SDS at 50°C. The molecular weight markers are indicated in kilodaltons (kDa).

Figure 3. *In vivo* UV-cross-linking analysis of cytoplasmic medRNA binding proteins in *T. brucei* procyclic culture forms. Cytoplasmic cell extracts from non-irradiated (UV: -) and irradiated (UV: +) trypanosomes were denatured as described and analysed by Western blotting. The blot was stained with amido black (panel A) then hybridized at 30°C with ³²P-kinase-labelled ILO 256, an oligonucleotide complementary to bases 120 to 138 of the 3' portion of medRNA. The blot was washed in 0.5× SSC, 0.1% SDS at 30°C before autoradiography (Panel B). The molecular weight markers are indicated in kilodaltons (kDa).

derived procyclic forms (PCF), hybridization was most evident in a region of approximately 70 kDa (data not shown).

We have also used a synthetic oligonucleotide probe, ILO 256, which is complementary to bases 120 to 138 of the 3' portion of medRNA. This probe hybridized strongly to a band migrating at 69 kDa in UV-irradiated PCF cytoplasmic extracts (Fig. 3B). However, the probe did not hybridize to LS and SS UV-irradiated cytoplasmic extracts (data not shown). Thus, the medRNA-protein complexes detected with the ILO 256 probe are abundant in PCF trypanosomes, but absent, or present only at undetectable levels in LS and SS BSFs of the parasite. This result suggests that protein(s) involved in this complex may be differentially expressed between BSFs and PCFs of *T. b. brucei*. The absence of hybridization of the ILO 256 probe to LS and SS extracts suggests that the 3' portion of medRNA complementary to ILO 256 may be masked by medRNA binding proteins in these stages, and thus inaccessible to the ILO 256 probe.

To verify that the bands we detected after UV-CLH are specific complexes of medRNA and proteins, *in vitro* UV-cross-linking experiments were performed using ³²P-labelled medRNA riboprobes. The medRNA cDNA clone, pTBMED3, was therefore used to generate ³²P-labelled antisense and sense riboprobes from T3 and T7 promoters of pBlueScript, respectively. The orientation of the insert was verified both by sequence analysis and by Northern blot hybridization. Only the antisense riboprobe hybridized to medRNA in Northern blot analysis (data not shown). Antisense and sense riboprobes labelled with ³²P-UTP were mixed with cytoplasmic extracts from non-irradiated cells. The mixtures were incubated at room temperature for 20 min, exposed to UV light for 10 min at 4°C, then denatured and submitted to SDS-PAGE. The gel was electroblotted onto nitrocellulose and the filter was washed and

Figure 4. *In vitro* UV-cross-linking analysis of cytoplasmic medRNA binding proteins. Clone 3 cDNA was subcloned in pBlueScript plasmid and radioactive sense and antisense medRNA transcripts were transcribed using T₇ and T₃ promoters of the vector, respectively. For each lane 20 µl of the cytoplasmic cell extracts from approximately 10⁶ long slender (LS), short stumpy (SS) and procyclic (Pro) cells were mixed with 10⁶ cpm of *in vitro* generated radioactive medRNA transcripts and incubated at room temperature for 30 min before exposure to UV light. Following denaturation, samples were analysed by western blotting. After washing with distilled water to eliminate free nucleic acids, followed by staining with amido black (panel A), the blot was autoradiographed (panel B). The molecular weight markers are indicated in kilodaltons (kDa).

stained, as described, prior to autoradiography (Fig. 4A). No signal was obtained with the antisense riboprobe. In contrast, the labelled sense medRNA generated a strong signal with at least 5 bands detectable in all samples analysed (Fig. 4B). However, two major differences appeared between the patterns of the PCF cells and those of the bloodstream forms. While the largest band seemed to be characteristic of BSFs, both LS and SS forms, the two smallest bands were mainly restricted to the PCF cells. The difference in the intensity of the bands observed between LS and SS bloodstream forms was not as obvious as in the *in vivo* experiments. This difference is probably due to the higher level of sensitivity of the *in vitro* method. The difference in the relative mobilities of complexes detected by *in vivo* UV-CLH and those detected by *in vitro* irradiation is due to the larger size of the riboprobe (about 280 bases) compared to the *in vivo* generated medRNA transcripts (140 bases) (17,18,27). The results of *in vivo* and *in vitro* experiments clearly demonstrate that the UV-CLH technique can be used to detect transcript-specific binding proteins.

Generation of antisera against medRNA binding proteins

Following the identification of transcript-specific binding proteins, it is important to have a simple and efficient system for their characterization. We tested whether it would be possible to raise antisera against the medRNA binding proteins by excising the region of the nitrocellulose containing the band shown by an arrow in figure 2B and dissolving it in DMSO. The dissolved nitrocellulose was precipitated with PBS, washed, then resuspended in PBS and injected into the peritoneum of 2 mice whose pre-immune sera did not detect any *T. brucei* proteins (Fig. 5A). After 3 inoculations with similarly prepared material, the serum of one of the immunized mice detected a single band

Figure 5. Immunodetection of a medRNA binding protein. One of the bands of medRNA-protein complexes indicated with an arrow in figure 2B was cut out and injected into a mouse. The serum of the mouse before immunization (panel A; dilution: 1/100) and after immunization (panel B; dilution: 1/200) was used to detect medRNA associated proteins in western blotting analysis of *in vivo* UV treated (UV: +) and untreated (UV: -) cytoplasmic cell extracts from long slender (lane LS) and short stumpy (Lane SS) bloodstream trypanosomes. Arrow indicates the position of a free medRNA binding protein. Lane M contains rainbow molecular weight markers (Amersham, UK).

of about 40 kDa in the cell extracts analysed (Fig. 5B), while serum of the second mouse showed no reaction. The intensity of the 40 kDa was stronger in LS than in SS forms. A very weak band of about 90 kDa was detected in some cases in the sample from UV-treated cells (data not shown). This large band may be the complex of medRNA cross-linked to the 40 kDa protein detected by the UV-CLH technique, while the band at 40 kDa could be a free medRNA binding protein. We obtained similar results with PCF cells, with the intensity of the band equivalent to the one obtained with SS forms (data not shown). These antisera gave negative results when used on a Northern blot of total *T. b. brucei* RNA. In an attempt to generate monoclonal antibodies against medRNA binding proteins, hybridomas were prepared using the spleen of the immunized mouse. In a preliminary immunoanalysis, antibodies of some cloned cells could detect several bands ranging between 30 and 45 kDa on Western blots, while some other clones allowed detection of bands of about 85–90 kDa (unpublished observations).

Large complexes can also be detected by the UV-CLH method

Although the UV-CLH method can easily detect complexes with small RNA molecules, many of the more desirable studies require a system for the identification of complexes with larger transcripts. The most abundant mRNA transcript in BSF trypanosomes is that of the major variable surface glycoprotein (VSG) (reviewed in 28,29). We therefore tested the possibility of whether the UV-CLH technique could detect complexes between VSG mRNA transcripts and cytoplasmic proteins. A nitrocellulose filter containing SDS-PAGE separated extracts of UV-irradiated bloodstream-form ILTat 1.1 trypanosomes was hybridized with a ³²P-labelled VSG cDNA probe, pILTAT 1.1 (30). This probe hybridized to two different-sized complexes in the UV-irradiated, but not the non-irradiated sample (Fig. 6A). However, the upper band was localized at the top of the stacking

Figure 6. *In vivo* UV-CLH analysis of large RNA-protein complexes. Cytoplasmic cell extracts from non-irradiated (UV: -) and irradiated (UV: +) LS trypanosomes were denatured and separated by electrophoresis on a 7.5% polyacrylamide gel (panel A) or a 0.5% (w/v) agarose gel (panel B) as described in the text. Following electroblotting, blots were hybridized with a ³²P-labelled VSG cDNA probe to localize the mRNA-protein complexes. The arrow indicates the top of the separating polyacrylamide gel in panel A. The positions of molecular weight markers are indicated in kilodaltons.

gel while the lower band migrated as far as the top of the separating gel. To improve the resolution of these large complexes, a larger pore gel was used by substituting polyacrylamide with 0.5% agarose and separated molecules transferred to nitrocellulose. Hybridization to the resultant nitrocellulose filter showed that the complexes have migrated through two-thirds of the gel, but as a broad band greater than 200 kDa (Fig. 6B), rather than two distinct bands. However, this result demonstrates that large complexes can be resolved in large pore gels, and improved resolution of different-sized complexes should be possible by altering the gel concentration.

DISCUSSION

We have developed a simple and efficient technique for establishing whether a RNA transcript associates closely with binding proteins within cells. In the first step of the *in vivo* UV-CLH technique, the complex formed by RNA and proteins is stabilized by UV cross-linking. In the second step, free nucleic acids are eliminated through SDS-PAGE and Western blotting. In the third and final step, hybridization of the Western blot with specific nucleic acid probes allows the detection of the respective transcripts linked to proteins. The fact that hybridization was only observed following UV irradiation provides evidence that the nucleic acids detected in this study are specifically and physically linked to proteins. This evidence is further strengthened by the observation that hybridization signals are not observed with proteinase K and RNase A-treated cell extracts and with non-UV-treated cells. The cross-linking is highly specific, even *in vitro*, since we do not detect an association of proteins with *in vitro*-generated antisense medRNA transcripts. We observed that some complexes are more abundant in PCFs than in BSFs, and vice versa. These differences may reflect differential expression of some medRNA binding proteins, since the same medRNA

transcripts were used in the study. Some of the PCF proteins involved in the complex detected using the ILO 256 oligonucleotide probe, complementary to bases 120 to 138 of the medRNA transcript, may bind to this portion in LS and SS bloodstream forms making the sequence inaccessible to ILO 256 for hybridization.

The complex was used to immunize mice by dissolving the region of the filter to which the probe hybridizes in DMSO and creating floccules by the addition of PBS buffer. These floccules were used to immunize mice by direct injection into the peritoneum in the absence of adjuvant. MedRNA is a small RNA of about 140-nt (17,18,27). Cross-linking of medRNA to a closely associated protein will result in an increase in the apparent molecular weight by SDS-PAGE and therefore a decrease in the relative mobility of the RNA and its binding protein. The differences between the relative mobilities of the complexes formed *in vivo* and those generated *in vitro* are due to the differences in the sizes of *in vitro* and *in vivo*-generated transcripts. A protein of 40 kDa was detected in Western blots using the serum of a mouse immunized with one of the medRNA complexes. The combined molecular weights of the medRNA transcript and the protein is approximately 90 kDa, which corresponds to the size of the complex used for immunization. In amido black stained Western blots, there were several proteins within the 90 kDa region where the filter used for immunization was sampled. Within this region, the cross-linked complex is a minor component, as there is no significant difference in amido black staining between UV-irradiated and non-UV-irradiated samples. In addition, there is only very weak detection of the complex in immunoblots of UV-irradiated extracts. Since the antiserum was specific for a 40 kDa protein, which is significantly smaller than the region used in the immunization, and since the complex forms only a small portion of the proteins used for the immunization, the RNA (of the complex) may have influenced the specificity of the immune response. The difference in intensities of the 40 kDa band between LS and SS bloodstream forms concords with the difference observed between these samples in the *in vivo* UV-CLH experiments. The apparent cross-linking efficiency *in vivo* may be related to higher levels of expression of this protein in LS cells.

The central role that medRNA plays in the expression of all trypanosome structural genes (17,18) suggests that some of the proteins associated with medRNA may be effectors involved in the post-transcriptional regulation of gene expression. Application of our technique has also allowed us to detect complexes of variant surface glycoprotein (VSG) mRNA and cytoplasmic proteins. In this instance, the replacement of polyacrylamide by agarose is required to resolve the larger RNA-protein complexes. Currently we are attempting to identify and characterize the proteins which bind to VSG and medRNA transcripts. We believe that these proteins should play an important role in the regulation of gene expression, since evidence from recent reports suggests that gene expression in African trypanosomes is mainly controlled at the level of RNA processing (31). The *in vivo* UV-CLH technique we describe here is a powerful method for identifying RNA binding proteins and has applications for studies on transcriptional and post-transcriptional control mediated by specific RNA binding proteins in general.

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