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Research note

Transcriptional and post-transcriptional *in organello* labelling of *Trypanosoma brucei* mitochondrial RNA

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Abstract

In organello labelling of *Trypanosoma brucei* mitochondrial (mt) RNA was characterised with respect to nucleotide requirements and drug sensitivity. Mitochondrial transcriptional activity is maximal in the presence of all ribonucleoside-triphosphate NTPs, and can be inhibited by UTP depletion. Mitochondrial transcription can also be partially inhibited by actinomycin D (actD) or ethidium bromide (EtBr). Post-transcriptional UTP incorporation is insensitive to actinomycin D or ethidium bromide. Proteins were identified that interact with transcriptional and post-transcriptionally labelled RNAs, and confirm the in vitro RNA-binding properties discovered for a number of *T. brucei* mt proteins. These experiments reveal new strategies for studying mt transcription and processing in *T. brucei* mitochondria.

Keywords: *In organello* labelling; Mitochondria; RNA; Trypanosome

In organello metabolic labelling experiments have been a popular approach to examine mt gene expression in *Trypanosoma brucei*. The *in organello* system faithfully represents mt gene expression, as isolated mitochondria are capable of transcription [1–4], RNA editing [4], transfer RNA maturation [5], RNA degradation [1,6], and 3′ poly(U) tail addition to ribosomal RNAs (rRNAs) and guide RNAs (gRNAs) [3,7–9]. To expand the utility of the *T. brucei in organello* metabolic labelling system, the nucleotide requirements and drug sensitivity of transcriptional and post-transcriptional *in organello* RNA labelling were precisely determined.

In organello T. brucei mt transcription was initially characterised by Harris et al. [4]. Mitochondrial transcriptional activity was measured by labelling purified mt vesicles with [α-^{32}P]GTP in the presence of unlabelled CTP, UTP, and ATP and quantifying radiolabelled RNA. This incorporation activity was shown to represent *bona fide* mt transcription as the pulse-labelled RNAs were able to hybridise to mt DNA fragments [4]. In this report, the nucleotide requirements for *in organello* transcription were precisely determined. These experiments were performed in order to define conditions that allow maximal transcriptional labelling of mt RNA, and also to define conditions that inhibit transcriptional labelling of mt RNA. Inhibition of transcriptional RNA labelling is useful in the study of RNA stability and other post-transcriptional gene expression pathways.

Transcriptional activity was maximal in the presence of all four ribonucleosidetriphosphates (NTPs) and this activity was designated 100% (Fig. 1). Transcription under these conditions is characterised by heterogeneously sized RNAs ranging from approximately 100 to 1000 nucleotides (Fig. 2, Lane 1) [3,4]. A low level of mature 9S and 12S rRNAs was discernible. When CTP or ATP was omitted from the reaction, transcriptional activity was reduced to 31 or 51%, re-

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spectively, of levels observed in the presence of all four NTPs (Fig. 1). Since transcriptional activity was not completely eliminated in the absence of exogenous CTP or ATP, isolated mitochondria must contain pools of CTP and ATP that are able to support transcription. In contrast to what was observed in reactions lacking CTP or ATP, when only UTP was omitted from the reaction, transcriptional activity was almost completely abolished, exhibiting only 6% activity (Fig. 1). The lack of substantial transcriptional activity in the absence of added UTP presumably reflects a large UTP requirement for the synthesis of uridine-rich transcripts, as the uridine content of many mt RNAs exceeds 45% [10]. In addition, the mt UTP pool is probably quite low due to its continual depletion by post-transcriptional processes such as RNA editing and poly(U) tail addition to gRNAs and rRNAs. Alternatively, UTP may be preferentially lost from the mitochondria during preparation. The effects of nucleotide depletion on transcriptional activity were highly reproducible, even with different mt vesicle preparations. However, these results differ from those of Harris et al., who reported that transcriptional activity was abolished in the absence of either UTP or CTP or ATP [4]. The addition of UTP alone was not sufficient for robust transcription, however, as only 23% activity was observed under these conditions (Fig. 1, 0.1 mM-UTP). In summary, these results

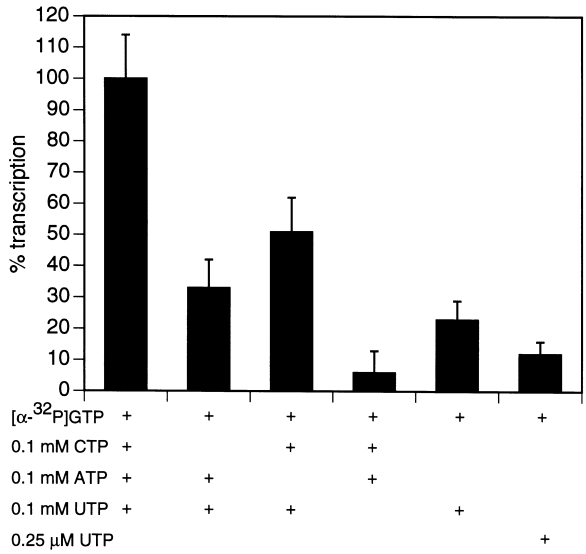

Fig. 1. Nucleotide requirements for *T. brucei* mitochondrial transcription *in organello*. *T. brucei brucei* clone IsTaR1 from stock EATRO 164 [19] was grown in SDM-79 media supplemented with 10% fetal bovine serum [20]. Mt vesicles were isolated, resuspended in transcription buffer containing 0.1 mM-ATP at a concentration of 1 mg/ml protein, and incubated at 30 min at 27°C to partially deplete nucleotide pools [4]. Vesicles were subsequently labelled with [α-^{32}P]GTP (400 Ci/mmol) at a concentration of 100 μCi/ml in the presence of different combinations of unlabelled CTP, UTP, and ATP at the concentrations indicated in the figure for 30 min at 27°C. Subsequently, reactions were terminated by treatment with SDS/proteinase K, and total RNA was isolated after phenol:chloroform extraction. RNAs were spotted onto DE81 paper (Whatman), washed 3× for 2 min with 0.5 M-Na_2HPO_4, rinsed sequentially with H_2O and 95% ethanol, dried, and analysed by scintillation counting. Each bar is the average of at least six separate experiments representing at least two different mt vesicle preparations. Error bars represent one standard deviation. Activity in the presence of all four NTPs was designated 100%.

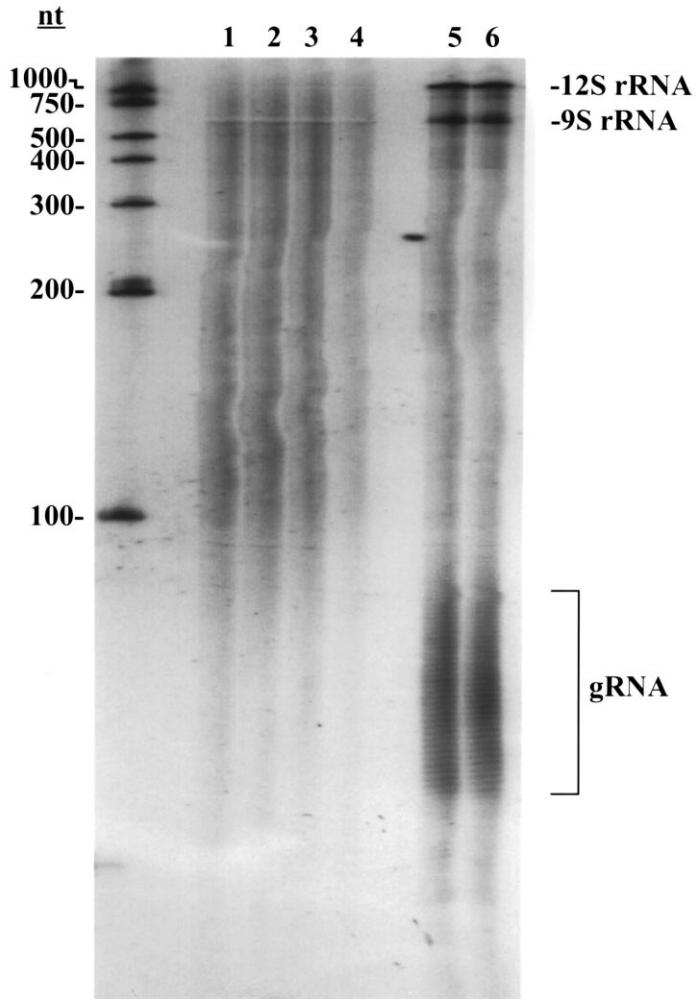

Fig. 2. Gel electrophoresis analysis of *in organello* labelled RNAs. Isolated mt vesicles (treated as in the Fig. 1 legend) were pulsed with [α-^{32}P]GTP (400 Ci/mmol) at a concentration of 100 μCi/ml in the presence of unlabelled 0.1 mM-CTP, 0.1 mM-UTP, and 0.1 mM-ATP for 5 min at 27°C to measure transcription (Lanes 1–4). Vesicles were labelled with [α-^{32}P]UTP (400 Ci/mmol) at a concentration of 100 μCi/ml for 5 min at 27°C in the absence of the remaining three unlabelled NTPs to measure post-transcriptional UTP incorporation activity (Lanes 5 and 6). Increasing amounts of EtBr were added 10 min prior to the addition of radiolabelled nucleotide. RNA was isolated, separated on a 6% acrylamide/7 M-urea gel, and detected by autoradiography. Lane 1, transcription assay without EtBr; Lane 2, transcription assay with 0.5 μg/ml EtBr; Lane 3, transcription assay with 5 μg/ml EtBr; Lane 4, transcription assay with 20 μg/ml EtBr; Lane 5, post-transcriptional UTP incorporation assay without EtBr; Lane 6, post-transcriptional UTP incorporation assay with 20 μg/ml EtBr.

demonstrate that UTP limitation is a highly efficient method to inhibit *T. brucei* mt transcription *in organello*.

Transcriptional inhibitors have been a valuable tool for studying transcription, RNA stability, and other processes including translation in both cytoplasmic and mt systems. Since transcriptional inhibitors would be a valuable tool for studying mt gene expression in *T. brucei*, the sensitivity of *T. brucei* mt transcription to actD and the classical mt transcriptional inhibitor EtBr was evaluated. Mitochondrial vesicles were pretreated with increasing amounts of actD or EtBr before labelling with [α-^{32}P]GTP in the presence of unlabelled UTP, CTP, and ATP. Both EtBr and actD inhibited mt transcription in a dose-dependent manner resulting in 69 and 59% inhibition, respectively, at the highest concentrations tested (Figs. 2 and 3). Complete inhibition of transcriptional activity was never observed. The fact that EtBr did not completely inhibit transcription was surprising, since much lower concentrations of this drug have been shown to completely inhibit transcription in yeast mitochondria [11], rat liver mitochondria [12], and human mitochondria [13]. The fact that actD was not able to completely eliminate transcription in our system may be due to the inherent impermeability of this drug to mitochondria membranes as observed for yeast mitochondria [11] and rat liver mitochondria [12]. Interestingly, human mitochondrial membranes are apparently permeable to actD [13]. Experiments using greater concentrations of EtBr and actD were not performed, since the highest concentrations tested are 5–40 times greater than the effective concentrations used in other systems. The lack of complete transcriptional inhibition by actD and EtBr is best explained by DNA-independent incorporation of [α-^{32}P]GTP into mt RNA, or by the inherent insensitivity of mitochondrial transcription to DNA intercalating agents. Transcriptional activity was insensitive to 200 μg/ml rifampicin (data not shown), presumably reflecting the inherent insensitivity of mt transcription to this drug, as described for yeast [11]. Nonetheless, EtBr and actD can be used to strongly reduce, although not completely eliminate, transcriptional labelling of mt RNAs in this system. Our laboratory has since used these drugs to analyse potential coupling between *T. brucei* mt transcription and UTP-dependent RNA turnover [6].

The sensitivity of post-transcriptional UTP incorporation to actD and EtBr was also evaluated since this activity has been used to study both RNA editing [4], and poly(U) tail addition to gRNAs and rRNAs in *T. brucei* mitochondria [3,4,7–9]. Mitochondrial vesicles were pretreated with EtBr or actD before labelling with [α-^{32}P]UTP in the absence of any other NTPs. Radiolabelled RNA was isolated and quantified. These labelling conditions employ [α-^{32}P]UTP at a concentration of 0.25 μM-UTP. At this UTP concentration, there is only 12% of the transcriptional activity observed in the presence of all four NTPs (Fig. 1). As previously determined by RNA sequencing [7] and gel electrophoresis and hybridisation analysis [3,4], under these conditions, [α-^{32}P]UTP is predominately incorporated post-transcriptionally into the 9S rRNA, 12S rRNA, and gRNA, presumably due to labelling of their 3′ poly(U) tails (Fig. 2, Lane 5). Heterogeneously sized RNAs ranging from less than 100 nucleotides to approximately 1000 nucleotides were also labelled and may be the result of RNA editing or residual tran-

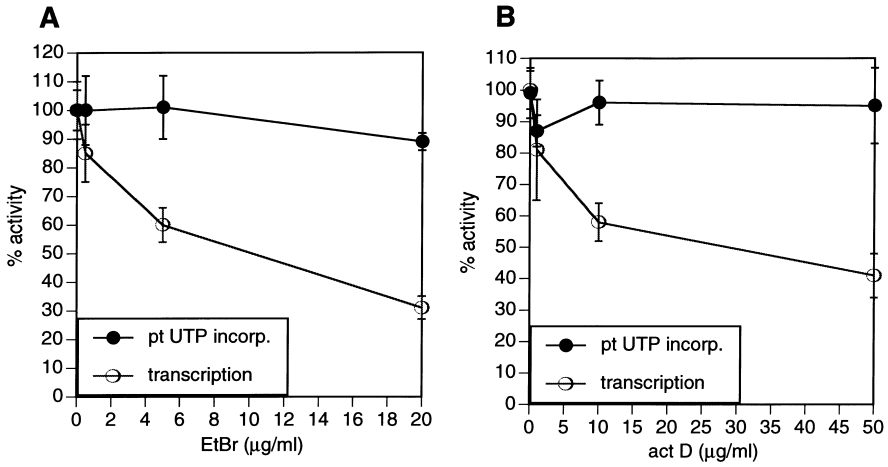

Fig. 3. Sensitivity of i*n organello* mt transcription and post-transcriptional UTP incorporation activity to ActD and EtBr. Mitochondrial vesicles were assayed for transcription or post-transcriptional UTP incorporation (pt UTP incorp.) as described in the Fig. 2 legend. Increasing amounts of EtBr (A) or actD (B) were added 10 min prior to the addition of radiolabelled nucleotide. Incorporation of radioisotope into RNA was determined as described in the Fig. 1 legend. Each point is the average of at least six separate experiments representing at least two different mt vesicle preparations. Error bars represent one standard deviation. Activity in the absence of drug treatment was set at 100%.

scription [4]. As expected, neither actD nor EtBr had a significant inhibitory effect on post-transcriptional UTP incorporation (Figs. 2 and 3), as post-transcriptional UTP incorporation is not predicted to be dependent upon a mt DNA template.

Having defined criteria for discriminating between transcriptional and post-transcriptional RNA synthesis in mt vesicles, we determined if this system could be used to provide information about proteins involved in mt transcription and post-transcriptional RNA processing. Proteins that bind to transcriptionally synthesised and post-transcriptionally synthesised RNAs were identified by u.v. cross-linking [14]. Mitochondrial vesicles were labelled with [α-^{32}P]UTP either in the presence of unlabelled CTP, GTP, and ATP to allow maximal transcription, or in the absence of unlabelled nucleotides to promote post-transcriptional labelling of primarily rRNA and gRNA. Under transcriptional labelling conditions, three proteins of 9, 16 and 40 kDa were predominately observed by u.v. cross-linking (Fig. 4, Lane 1). Interestingly, the 9 and 40 kDa bands were not readily detected under post-transcriptional labelling conditions, suggesting that these interactions are dependent upon active transcription (Fig. 4, Lane 2). Under post-transcriptional labelling conditions, five proteins of 16, 25, 55, 90 and >200 kDa were intensely labelled (Fig. 4, Lane 2). These bands could also be detected under transcriptional labelling conditions with longer autoradiograph exposure times, most likely due to active post-transcriptional labelling under these conditions (data not shown). The sizes of the proteins identified in intact organelles under post-transcriptional labelling conditions correspond remarkably well to the sizes of proteins that were previously identified as gRNA binding proteins by u.v. cross-linking in vitro [14,15]. Genes encoding three proteins with in vitro gRNA binding activity have been cloned from *T. brucei*. These proteins, RBP16 [16], gBP21 [17], TBRGG1 [18], likely correspond to the 16, 25 and 90 kDa bands in Fig. 4 (Lane 2). RBP16 was previously identified in our laboratory and was shown via immunoprecipitation to associate with endogenous gRNA and rRNA in mt lysates [16]. The immunoprecipitation of RBP16 from u.v. cross-linked vesicles demonstrates that RBP16 binds post-transcriptionally synthesised RNA within the intact mt vesicle (Fig. 4, Lane 4). A small amount of RBP16 was also immunoprecipitated from mitochondria incubated under transcriptional labelling conditions (Fig. 4, Lane 3). This is likely the product of concurrent posttranscriptional labelling of RNA under these conditions, since RBP16 preferrentially binds to the posttranscriptionally added poly(U) tail of gRNAs in vitro [16] and in vivo (16 and Fig. 4,

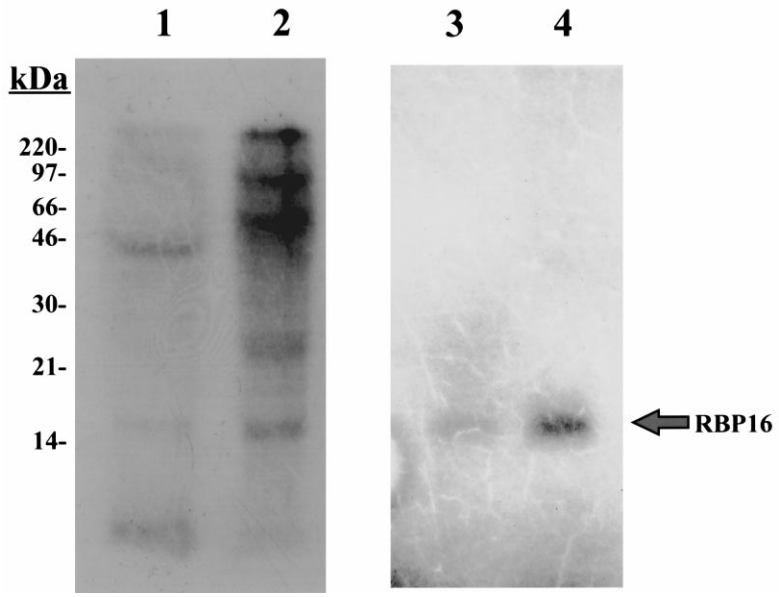

Fig. 4. Detection of RNA-binding proteins within mt vesicles. Mitochondrial vesicles were labelled with either [α-^{32}P]UTP (400 Ci/mmol) at a concentration of 100 μCi/ml in the presence 0.1 mM-unlabelled CTP, 0.1 mM-GTP, and 0.1 mM-ATP to allow transcription (Lanes 1 and 3), or with [α-^{32}P]UTP (400 Ci/mmol) at a concentration of 100 μCi/ml alone to promote post-transcriptional labelling of RNAs (Lanes 2 and 4). Labelled vesicles were u.v. cross-linked [11], and RNA was digested with RNase A. Total proteins were precipitated with 3% TCA (Lanes 1 and 2), or RBP16 was immunoprecipitated with anti-RBP16 antibodies [13] (Lanes 3 and 4). Labelled proteins were separated by 15% SDS–PAGE, and detected by autoradiography. Lane 1, total u.v. cross-linked proteins under transcriptional labelling conditions; Lane 2, total u.v. cross-linked proteins under post-transcriptional labelling conditions; Lane 3, immunoprecipitation of RBP16 from u.v. cross-linked vesicles labelled under transcriptional conditions; Lane 4, immunoprecipitation of RBP16 from u.v. cross-linked vesicles labelled under post-transcriptional conditions.

Lane 4). However, it cannot be ruled out that RBP16 also binds to newly transcribed RNA.

These experiments provide criteria to accurately differentiate between transcriptional and post-transcriptional *in organello* labelling of *T. brucei* mt RNAs. These tools will be useful in dissecting the pathways of RNA synthesis and RNA processing in *T. brucei* mitochondria.

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