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Developmental Aspects of Uridine Addition within Mitochondrial Transcripts of *Trypanosoma brucei*

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The mitochondrial respiratory system is absent in slender bloodstream forms of *Trypanosoma brucei*, incomplete in stumpy bloodstream forms, and complete in procyclic (insect) forms. The steady-state abundance of transcripts of some mitochondrially encoded components of the respiratory system correlates with its differential expression in different life cycle stages. Recently, it was reported that uridines which are not encoded in the genome are added to cytochrome *b* and cytochrome oxidase II transcripts. We now report that the $(U)^+$ transcripts of both genes are found in procyclic forms and to some degree in stumpy forms but are absent in slender forms. The uridine additions to cytochrome oxidase II correct a frameshift in the gene and presumably allow production of a full-length protein, whereas those added to cytochrome *b* create an in-frame AUG which extends the N terminus of the predicted protein by 20 amino acids. The stage specificity of uridine additions to these transcripts thus reflects the life cycle stage during which the protein products would be used. Transcripts of MURF2, a gene of unknown function, have additional uridines in both slender and procyclic forms which create two in-frame AUGs. MURF2 transcripts additionally differ from the DNA sequence in ways which cannot be explained by uridine addition alone, implying that other processes alter these transcripts.

Production of the mitochondrial respiratory system is developmentally regulated in the protozoan parasite *Trypanosoma brucei*. The slender form in the vertebrate bloodstream lacks cytochromes and Krebs cycle enzymes and produces energy by glycolysis. The stumpy form, also in the vertebrate bloodstream, continues to produce energy by glycolysis but has some enzyme activities associated with the Krebs cycle. Procyclic forms develop upon transfer to the insect host or to suitable culture conditions. They have cytochromes and a functional Krebs cycle and create energy by aerobic respiration and the electron transport chain (for reviews, see references 5, 18, and 22).

The mitochondrial DNA of *T. brucei* is composed of thousands of 1-kilobase minicircles and 50 to 100 22-kilobase maxicircles which are catenated into a single network (5, 18, 22). The maxicircles are homogeneous in sequence and are homologous to mitochondrial genomes of other organisms. They encode mitochondrial rRNAs and components of the mitochondrial respiratory system, including apocytochrome *b* (CYb); cytochrome oxidase subunits I and II (COI and COII); and NADH dehydrogenase subunits 1, 4, and 5 (ND1, ND4, and ND5). In addition, there are two open reading frames, MURF1 and MURF2, which probably encode proteins but have no detected homology to mitochondrial genes from other organisms (2, 6, 12–14, 17).

Transcripts of all the mitochondrial genes are found in all three life cycle stages which were examined, but the abundance of specific transcripts varies between life cycle stages. All of the putative protein-coding genes except ND5 have two size classes of transcripts which differ by an apparent 150 to 200 nucleotides (nt) (7, 10, 13, 21). The larger size class of CYb, COI, and COII transcripts is more abundant in stumpy forms than slender forms and is most abundant in procyclic forms, whereas the smaller size class of transcript for many genes is most abundant in stumpy forms (7, 8, 10, 13, 21). In stocks of *T. brucei* which do not spontaneously differentiate to stumpy forms, difluoromethylornithine (DFMO) treatment of slender forms results in a stumpy morphology (1, 4, 8, 11) and a transcript abundance pattern which is very similar to that of spontaneously occurring stumpy forms (8). Polyadenylation is also differentially regulated between life cycle stages, with a larger proportion of mitochondrial transcripts being polyadenylated in procyclic forms than in slender ones (21).

Recently, it was reported that uridines which are not encoded in the genome are added, presumably either during or after transcription, to *T. brucei* COII (3) and CYb (9) transcripts. In the former case, the additions correct a frameshift in the gene, and in the latter, an AUG codon which may be the functional initiation codon (9, 9a) is created within the 5′ end of the transcript. The additions to CYb transcripts were further characterized as occurring in procyclic forms but not in slender forms (9). We now report that uridine additions to *T. brucei* CYb transcripts occur in stumpy forms, that the additions to COII transcripts are developmentally regulated in a manner similar to the CYb additions, and that MURF2 transcripts have additional uridines in both procyclic and slender forms. The additions to MURF2 transcripts create two in-frame AUGs similar to CYb transcripts. MURF2 transcripts are additionally altered, relative to the DNA sequence, in ways which uridine addition alone cannot explain. These results indicate that the process of uridine addition to transcripts is not confined to procyclic forms but also operates in both slender and stumpy bloodstream forms. They further imply that the addition of uridines to transcripts is regulated independently for each gene. The developmental regulation of uridine addition to COII and CYb transcripts correlates with abundance changes for these transcripts and with the differential expression of the respiratory system. Thus, uridine addition is another in the growing list of factors which influence the differential expression of the mitochondrial respiratory system between life cycle stages of *T. brucei*.

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MATERIALS AND METHODS

Organisms. Clone IsTaR 1 of *T. brucei brucei* (stock EATRO 164) was grown in rats and in procyclic culture and harvested as previously described (20). Stumpy bloodstream forms of this stock were produced by treatment with 2% DFMO, as previously described (8). Populations which were exposed to small amounts of DFMO retained the morphological appearance of slender forms, whereas those treated with larger amounts differentiated to ~40% stumpy forms. Slender and procyclic forms of stock STIB 388 were grown and prepared in the same manner as those of EATRO 164. STIB 388 stumpy forms were harvested from rats when the population was 80 to 90% stumpy and intermediate forms; DFMO was not used.

Oligonucleotides. The CYB-CS, CYb-D, and CYb-R oligonucleotides have been described previously (9). Briefly, CYb-CS is complementary to CYb transcripts from 4 to 57 nt 3′ of the first ATG in the CYb open reading frame (2, 14) and hybridizes to all CYb transcripts. CYb-D is complementary only to CYb transcripts lacking additional uridines, and CYb-R is complementary to those with the uridines. COII-FS is complementary to COII transcripts from 8 to 32 nt 3′ of the frameshift in the gene (12, 16). COII-D and COII-R span the frameshift region and are complementary to COII transcripts without and with the added uridines, respectively. MURF2-CS is complementary to MURF2 transcripts near the 5′ end of the MURF2 gene (nt 1885 to 1844 [16]). MURF2-R is complementary to MURF2 transcripts with additional uridines extending 5′ from nt 1823 (16). The sequences of the CYb oligonucleotides are given elsewhere (9); the sequences of the others are given as follows:

COII-FS 5′-GCAAACAAAATTATTTCATTACACC-3′
COII-D 5′-CCAGGTTCTCTACTTTTATA-3′
COII-R 5′-CCAGGTATACAATCTACTTTT-3′
MURF2-CS 5′-CTAGATCAAACCATCACAATATAAAATCATATGTTCTAATAC-3′
MURF2-R 5′-CTACAATCAAAGCACAAAAATAAAAC-3′

Northern hybridizations and RNA sequencing. Mitochondrial isolation (9) and preparation of RNA (7) were done as previously described. RNA was electrophoresed in formaldehyde gels and blotted to Nytran (Scheicher & Schuell) as previously described (7). Hybridization probes were prepared by end labeling the oligonucleotides with [^{32}P]dATP (15). Hybridization and washing conditions for the CYb oligonucleotides were followed as previously described (9). The same conditions were used for the COII oligonucleotides, but the washes were shortened to 3 to 5 min each. Primer extension was carried out as previously described (9) by using M-MLV reverse transcriptase (Bethesda Research Laboratories, Inc.) to extend hybrids in the presence of [^{32}P]dATP. RNA sequencing was performed by the dideoxy chain termination method, as previously described (9).

RESULTS

CYb uridine additions. Primer extension analysis with the CYb-CS oligonucleotide, which is complementary to the CYb transcript near its predicted 5′ end, reveals four principal primer extension products (Fig. 1A). They are not detected in the absence of primer (Fig. 1B) or with RNA from mutants devoid of mitochondrial DNA (Fig. 1A, lane Dk). Products 3 and 4 represent 5′ termini of two CYb transcripts. They differ by the presence of 34 uridines interspersed within the 5′ end of the transcript represented by product 4; these uridines are added either during or after transcription (9). Both transcripts exhibit variation in abundance among different life cycle stages; the degree of variation differs among stocks. Product 3 is most abundant in stumpy forms of both stocks examined (Fig. 1A, lanes Dl and St), while variation between slender and procyclic forms is slight. In the EATRO 164 stock, which does not spontaneously differentiate to stumpy forms, product 4 is present in small amounts in slender forms (Fig. 1A, lanes B), becomes more abundant following DFMO treatment (lanes De and Dl), and is most abundant in procyclic forms (lanes P), where it is more abundant than product 3. The increase in product 4 between populations with minimal DFMO treatment (lane De) and more extensive DFMO treatment (lane Dl) correlates with the proportion of stumpy forms in those populations. Both the spontaneous stumpy and procyclic forms of STIB 388 show increased amounts of product 4, but the amount does not exceed that of product 3. Product(s) 2, a cluster of bands, shows a pattern of abundance changes among life cycle stages which is similar to that of product 4, whereas product 1 is relatively similar in abundance in all life cycle stages. The unnumbered product which is 5 nt longer than product 1 is most abundant in stumpy forms. It is not known whether these latter three products represent transcript ends or reverse transcriptase pause sites.

The two size classes of CYb transcripts are 1,200 and 1,350 nt long (7); as discussed elsewhere (9), the 5′-end uridine additions alone cannot explain the 150-nt apparent size difference. The CYb-CS oligonucleotide, which detects all CYb transcripts, demonstrates the differential abundance of these two size classes of transcripts in different life cycle stages (Fig. 2A). In RNA from EATRO 164, the smaller size class is more abundant in procyclic than in slender forms and somewhat more abundant in stumpy forms than in either of the other stages. The larger size class is virtually undetectable in slender forms, appears in stumpy forms, and is quite abundant in procyclic forms. CYb transcripts of STIB 388 also exhibit differential abundance, with the smaller size class being most abundant in stumpy forms and the larger size class increasing from slender to stumpy forms (Fig. 2A). However, the larger transcript is less abundant in STIB 388 procyclic forms than in EATRO 164 procyclic forms (Fig. 3A). The CYb-D and CYb-R oligonucleotides are complementary to CYb transcripts without and with additional uridines, respectively. CYb-D hydridizes only to the smaller transcript class (Fig. 2B and 3B) in all life cycle stages, whereas CYb-R hybridizes to both size classes but only in stumpy forms and procyclic forms (Fig. 2C and 3C). The smaller amount of the large size class of CYb transcript in STIB 388 stumpy forms and procyclic forms is seen clearly to reflect a smaller proportion of transcripts with additional uridines than are present in EATRO 164 (compare Fig. 2A with 2C and 3A with 3C). The data from these hybridizations correlate well with the primer extension results (Fig. 1A) and

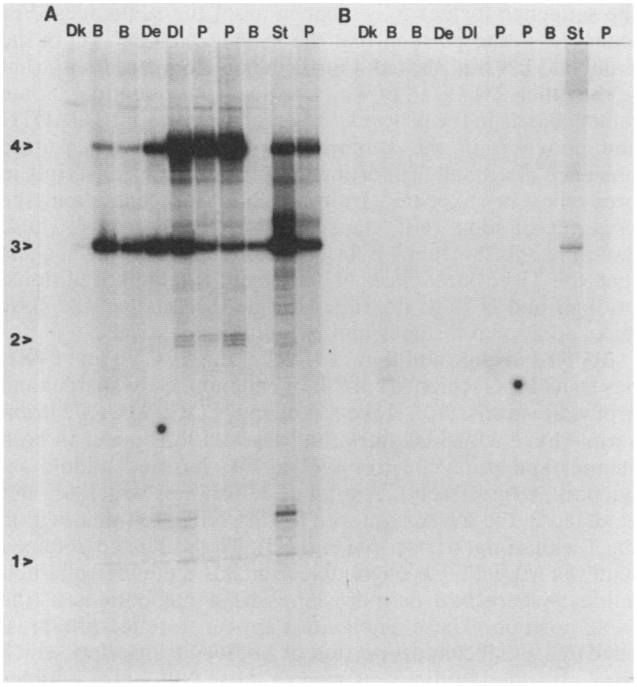


FIG. 1. Differential abundance of CYb primer extension products during the *T. brucei* life cycle. (A) Total RNA (5 μg) from different life cycle stages was hybridized to the CYb-CS oligonucleotide (50 ng) and extended by using M-MLV reverse transcriptase. Extension products 1 through 4 (9) are designated with carets. RNA for each lane is from the following sources: Dk, mutant devoid of mitochondrial DNA (19); B, slender forms; De, DFMO-treated cells which retained slender morphology; DI, DFMO-induced stumpy forms; P, procyclic forms; St, spontaneous stumpy forms. RNAs in the seven lefthand lanes are from EATRO 164, whereas RNAs in the three righthand lanes are from STIB 388. (B) RNAs from panel A were hybridized in the absence of primer and then extended as in panel A. The product seen in the St lane which appears to comigrate with product 3 is actually 2 nt smaller than product 3.

prior results (9), indicating that the conventional (DNA-like) transcript is in the smaller size class, and that the $(U)^+$ CYb transcripts are detected in both size classes, but principally in the larger size class, and that they are restricted to specific life cycle stages.

COII uridine additions. The COII gene of *T. brucei* contains a −1 frameshift (12, 16) which is corrected in the

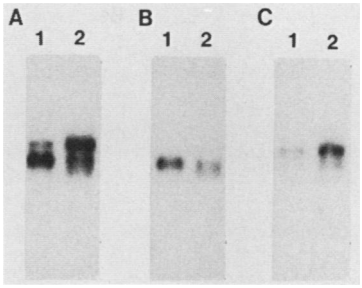


FIG. 3. Abundance of conventional and $(U)^+$ CYb transcripts in procyclic forms of different stocks. Total RNA (10 μg per lane) from STIB 388 (lanes 1) and EATRO 164 (lanes 2) procyclic forms was electrophoresed in formaldehyde gels, blotted to Nytran, and probed with end-labeled CYb-CS (A), CYb-D (B), or CYb-R (C). The larger size class of transcripts seen is 1,350 nt long, and the smaller size class is 1,200 nt long.

transcript by the addition of four uridines (3). Like CYb, COII has two size classes of transcripts of 750 and 900 nt (10). The COII-D oligonucleotide, which detects transcripts without the extra uridines, hybridizes to the smaller size class in all life cycle stages (Fig. 4A). This conventional transcript is most abundant in stumpy forms. The COII-R oligonucleotide, which detects $(U)^+$ transcripts, hybridizes to the larger size class, principally in procyclic forms (Fig. 4B). These results are consistent with earlier transcript abundance studies with hybridization probes which detected all COII transcripts (10). Those studies also showed slight hybridization to the larger size class of transcripts in stumpy forms which was not seen in these autoradiograms, presumably due to the higher background with these probes.

Sequencing studies of procyclic RNA with COII-FS, which hybridizes to the transcripts 3′ of the frameshift region, clearly show the four added uridines which restore the COII reading frame (Fig. 5, PF). RNA sequence data for slender forms was difficult to obtain because of the low levels of COII transcripts in that stage (10). The hybridization studies with COII-D (Fig. 4A) suggested that stumpy form RNA principally contains the same conventional COII RNA as do slender forms. Sequencing experiments were therefore carried out with slender form RNA, stumpy form RNA, and RNA from isolated slender form mitochondria (Fig. 5, BF, DFMO, and BFmt, respectively). Although the sequence in the region where the four uridines are added in procyclic forms is not clear in any of those three samples,

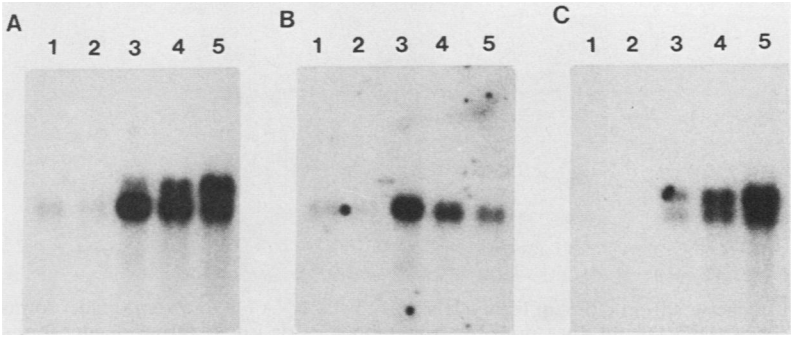


FIG. 2. Abundance of conventional and $(U)^+$ CYb transcripts during the life cycle. Total RNA (10 μg per lane) was electrophoresed in formaldehyde gels, blotted to Nytran, and probed with end-labeled CYb-CS (A), CYb-D (B), or CYb-R (C). Lanes: 1, EATRO 164 slender RNA; 2, STIB 388 slender RNA; 3, STIB 388 stumpy RNA; 4, EATRO 164 DFMO-induced stumpy form RNA; 5, EATRO 164 procyclic RNA. The larger size class of transcripts seen is 1,350 nt long, and the smaller size class is 1,200 nt long.

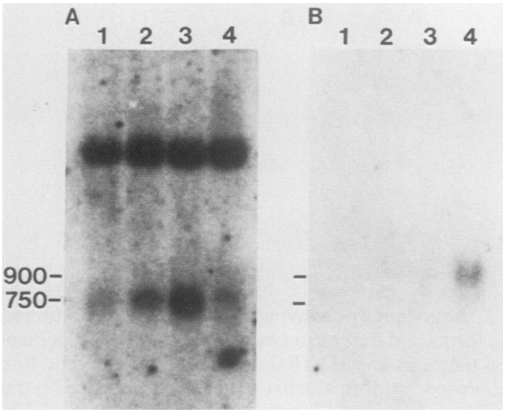


FIG. 4. Abundance of conventional and $(U)^+$ COII transcripts during the life cycle. Total RNA (10 μg per lane) was electrophoresed in formaldehyde gels, blotted to Nytran, and probed with end-labeled COII-D (A) or COII-R (B). Lanes: 1, slender form RNA; 2, RNA from DFMO-treated cells which retained slender morphology; 3, DFMO-induced stumpy form RNA; 4, procyclic RNA. The positions of the larger (900 nt) and smaller (750 nt) size classes of COII transcripts are indicated. The large abundant transcript detected by COII-D (A) probably reflects cross-hybridization to a cytoplasmic rRNA, since that transcript is detected in poly$(A)^-$ RNA and in RNA from a mutant which lacks mitochondrial DNA (data not shown).

the sequence further 5′ is clear in all of the sequences. For example, it can be seen that the sequence TGAGTG in the procyclic RNA is shifted 4 nt further 5′ compared with that of the other RNAs, all of which appear to be identical. Some faint G bands in the procyclic sequence below that TGAGTG and above it in the stumpy form sequence suggest the presence of a small proportion of conventional transcripts in procyclics, as expected from the Northern data, and the presence of some $(U)^+$ transcripts in stumpy forms. These data and the Northern hybridization data together suggest that the large size class of transcript contains additional uridines and is stage restricted, while the smaller size class lacks additional uridines and is found in all stages.

MURF2 uridine additions. MURF2, unlike CYb and COII, has transcripts which are similar in abundance in slender and procyclic forms (10). Like CYb and COII, MURF2 transcripts have added uridines, but the additions occur in both slender and procyclic forms (Fig. 6A, left and middle sequences, respectively). A total of 23 uridines which are not encoded in the DNA sequence for MURF2 are found within the 5′ end of the transcript (Fig. 6B). The sequence obtained with the MURF2-CS oligonucleotide has a number of ambiguities, where two or more lanes have nucleotides at the same position. These ambiguities appear to reflect the presence of a significant proportion of MURF2 transcripts which have no added uridines, as well as those with extra uridines

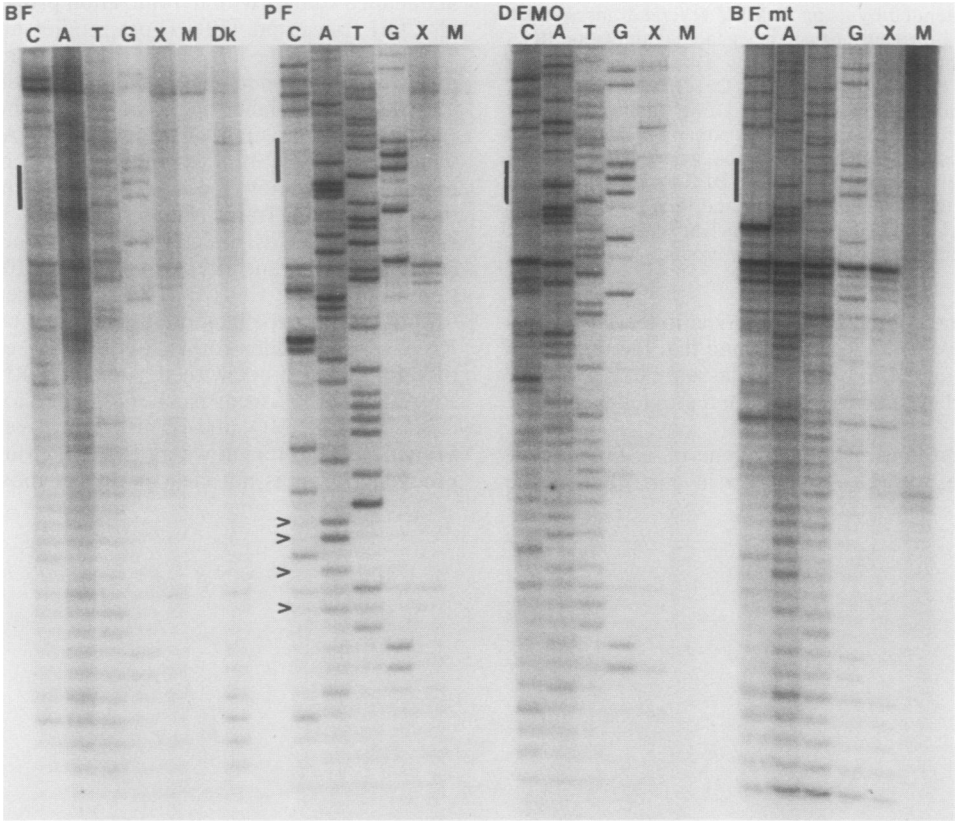
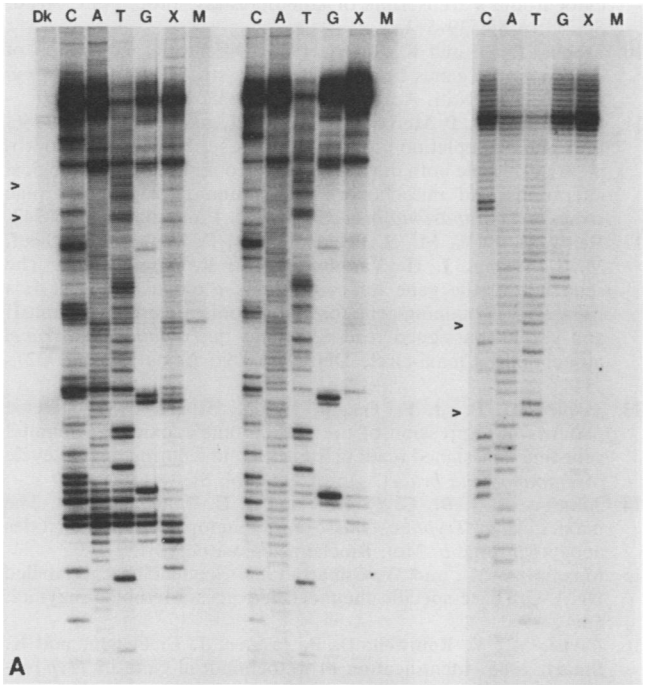


FIG. 5. Sequence of COII transcripts from different life cycle stages. Total RNA (30 μg) from slender forms (BF), procyclic forms (PF), and DFMO-induced stumpy forms (DFMO) and RNA prepared from mitochondria isolated from slender forms was hybridized to 100 ng of the COII-FS oligonucleotide and sequenced, by using M-MLV reverse transcriptase, in the presence of dideoxynucleotides and [^{32}P]dATP. Lanes X contain no dideoxynucleotides, and lanes M are controls which were hybridized without primer. Carets indicate the positions of the four added uridines in procyclic COII transcripts. Horizontal bars indicate a 6-nt sequence (3′-TGAGTG-5′) which can be seen in all the RNAs.

(see below). The complexity of the ambiguities increases approaching the 5′ end of the transcript, suggesting there may also be some transcripts which have less than 23 added uridines. Comparison of the slender bloodstream form and procyclic RNA sequences (Fig. 6A, left and middle) show that the same number of uridines are added at the same sites, although there may be a larger proportion of conventional transcripts in slender forms than in procyclic forms.

The MURF2-R oligonucleotide is complementary to the transcript with additional uridines at the 3′ end of the uridine addition region. The sequence obtained with this primer is considerably less ambiguous and reveals an in-frame AUG created by uridine addition near the 5′ end of the $(U)^+$ transcript (Fig. 6A, right sequence, 6B). In addition, an AUG codon predicted by the DNA sequence which was previously out of frame with respect to the MURF2 reading frame has been shifted to an in-frame position by the uridine additions. The shifted AUG is 9 nt upstream of the created AUG (Fig. 6A, right sequence, 6B). A primer extension product which is 22 nt shorter than the principal product is seen with the MURF2-CS oligonucleotide but not with MURF2-R (compare Fig. 6A, middle and right sequences). These data suggest that the smaller extension product represents the endpoint of the conventional MURF2 transcript. This conclusion is consistent with the sequencing ambiguities (see above) which suggested the presence of some conventional MURF2 transcripts.

In addition to the extra uridines, MURF2 transcripts appear to have other sequence alterations. Near the middle of the uridine addition region, the RNA sequence is 5′-AAUUUAGU, whereas the corresponding DNA sequence is 5′-ATTTTAAG (Fig. 6B). The change from a single A predicted by the DNA at the 5′ boundary of this sequence to AA, for example, cannot be explained by uridine addition alone. Both MURF2-CS and MURF2-R give the same RNA sequence in this region, and the sequence is the same in RNA from both slender and procyclic forms (Fig. 6A). The DNA sequence has been determined on both strands with four independent clones (16) and is identical to that determined by Hensgens, et al. (12) for this region in a different *T. brucei* stock.



B
TAGAAAGGTATATAATCTATAATGA AA G GG G ATTTTAAG A TTG GCTTTGATTG AGTCGTGTTTTTGATTTG DNA
NNNNNNNNNGAAAGGNANAUAAUCUAUAAUGAuuuuAAuGuuuGGuuGuuuuAAUUUAGuuuuAuuuUUGuGCUUUGAUUGuAGUCGUGUUUUUGAUUUG RNA

DISCUSSION

The results presented here show that uridine addition is not confined to procyclic forms, since a proportion of CYb transcripts in stumpy forms contain additional uridines. Although most COII transcripts in stumpy forms lack additional uridines, the results from RNA sequencing suggest that there is a small proportion of $(U)^+$ COII transcripts in stumpy forms. In addition, MURF2 transcripts have added uridines in both slender and procyclic forms, indicating that the enzymes or mechanisms necessary for uridine addition are present in all three life cycle stages. However, COI and ND4 transcripts, which have ATG initiation codons in their genomic sequences (12, 13), do not have uridines added within their 5′ ends in either slender or procyclic forms (J. M. Shaw, J. E. Feagin, K. Stuart, and L. Simpson, manuscript submitted). Since the addition of uridines to transcripts and the time during the life cycle at which the addition occurs are both regulated in a gene-specific manner, the addition of uridines must involve an additional level of control which confers this specificity.

The *T. brucei* MURF2 open reading frame does not begin with an ATG initiation codon, and it has been proposed that this gene uses an alternative initiation codon (12, 16). Uridine addition both creates an AUG codon and shifts another into frame within the 5′ end of MURF2 transcripts, suggesting that one of these AUGs, rather than an alternative codon, may function as the initiation codon. The fact that the MURF2 genes of the related kinetoplastids *Leishmania tarentolae* and *Crithidia fasciculata* also lack genomic ATGs but have both additional uridines and created AUGs within the 5′ ends of their transcripts (Shaw et al., submitted) supports this conclusion. The MURF2 gene has no detected homology to mitochondrial genes from other organisms except kinetoplastids (12, 16, 17), and the function of its putative protein product is unknown. Since uridines are added in both slender and procyclic forms, the protein product of MURF2 may function in both these life cycle stages. This theory correlates with the finding that MURF2 transcripts are relatively similar in abundance in these stages (10).

FIG. 6. Sequences of MURF2 transcripts. (A) Total RNA (30 μg) from slender forms (left sequence) and procyclic forms (middle sequence) was hybridized with 100 ng of the MURF2-CS oligonucleotide. Total RNA from procyclic forms was also hybridized with 100 ng of the MURF2-R oligonucleotide (right sequence). All were sequenced, by using M-MLV reverse transcriptase, in the presence of dideoxynucleotides and [^{32}P]dATP. The carets indicate the positions of the AUGs (read 5′-TAC in the sequencing ladder) which were created and shifted into frame by uridine addition. (B) The 5′-end sequence of the MURF2 $(U)^+$ transcript is shown with the DNA sequence aligned above it. Gaps are left in the DNA sequence where uridines are added and the added uridines are shown in lower case. The created and shifted in-frame AUGs are underlined. Less-abundant extension products extend up to 10 nt 5′ of the end of the transcript indicated here. The TAG triplet which begins the DNA sequence shown is the putative stop codon of the COII gene (12, 16).

The difference between the CYb and COII $(U)^+$ transcript sequences and the DNA sequences can be explained by addition of uridines alone. Unexpectedly, MURF2 $(U)^+$ transcripts have sequence alterations which suggest that processes other than uridine addition modify transcript sequences. One possibility is that the four uridines which are encoded by the DNA are eliminated, perhaps as a small intron, and three uridines are added, at a position one nucleotide 3′ of this. A second possibility is that one of the predicted uridines is removed, and four nucleotides are inverted. Or, there may be an alternate MURF2 gene. This seems somewhat less likely, since similar examples of sequence alteration involving apparent nucleotide deletions, to be detailed elsewhere (Shaw et al., submitted), have been seen for other kinetoplastid mitochondrial transcripts. Further experimentation is required to resolve these possibilities.

The MURF2 gene is located directly 3′ to the COII gene (12, 16), and the existence of low-abundance transcripts which are large enough to contain both the COII and MURF2 transcripts (10) suggests that they may be produced from the same precursor transcript. However, the 5′ end of the predominant MURF2 $(U)^+$ transcript is long enough to extend 10 nt upstream of the COII stop codon (Fig. 6B), and less-abundant primer extension products extend up to 10 nt further than that. It is possible that the 5′ terminal nucleotides are added, rather than encoded, or that nucleotide addition could create a stop codon 5′ of the existing COII stop codon. Alternatively, MURF2 and COII transcripts may be made independently from the potential precursor transcript.

Slender form RNA preparations have shown a small, variable amount of $(U)^+$ CYb RNA in primer extension experiments. One explanation of this finding is that a small proportion of CYb transcripts in each cell may have additional uridines in slender forms; this possibility is consistent with the MURF2 data showing that the process of uridine addition is operating in slender forms. An attractive alternate explanation is suggested by the fact that DFMO-treated bloodstream forms which retain slender morphology show changes in transcript abundance (8) and have $(U)^+$ CYb transcripts (Fig. 1). Thus, a small proportion of the slender population may be beginning the biochemical changes which presage procyclic forms.

Sequence alteration by uridine addition and other as yet uncertain means thus stand as the two most recently discovered modulators of mitochondrial gene expression in *T. brucei*. The uridine additions, in particular, clearly involve an additional level of control, since different transcripts have extra uridines in different life cycle stages. The other sequence alterations present a fascinating addition to the complex collection of factors influencing control of these genes. It is highly likely that mitochondrial gene expression is coordinated with the expression of nuclear genes for mitochondrial components and may further be coordinated with expression of the genes of the glycolytic pathway.

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