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Genetics

Creation of AUG initiation codons by addition of uridines within cytochrome *b* transcripts of kinetoplastids

(mitochondrial transcripts/RNA editing/*Trypanosoma brucei*/*Leishmania tarentolae*/*Crithidia fasciculata*)

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ABSTRACT **The cytochrome *b* gene of *Trypanosoma brucei* has an ATG codon near its 5′ end but the cytochrome *b* genes of the related kinetoplastids *Leishmania tarentolae* and *Crithidia fasciculata* lack an ATG. Recent results have shown that 34 uridines that are not encoded in the genome are added within the 5′ end of *T. brucei* cytochrome *b* transcripts during or after transcription. These additions create an AUG in the transcript that is 20 amino acids upstream of the AUG predicted from the genomic sequence. We report here that the cytochrome *b* transcripts of *L. tarentolae* and *C. fasciculata* also contain added uridines within their 5′ ends. The additions occur in similar numbers and positions and an in frame AUG is created at a similar site in all three species. These data strongly suggest that the created AUG functions as the initiation codon for cytochrome *b* in these species. Since some other kinetoplastid mitochondrial genes also lack conventional initiation codons, creation of initiation codons may be an important function of uridine addition.**

The mitochondrial genome of kinetoplastid flagellates consists of two types of molecules, minicircles and maxicircles, that are catenated into a single network (for reviews, see refs. 1–3). Each network contains thousands of minicircles that are 0.7–1.0 kilobase (kb), depending on species (1); their function is unknown, although they do have small transcripts (4). Each network also contains 25–100 maxicircles that are 20–38 kb, depending on species (1), and are homologous to mitochondrial genomes of other organisms. Maxicircles of *Trypanosoma brucei*, *Leishmania tarentolae*, and *Crithidia fasciculata* encode mitochondrial rRNAs and components of the mitochondrial respiratory system, including apocytochrome *b*, cytochrome oxidase subunits I, II, and III (subunit III is absent in *T. brucei*), and NADH dehydrogenase subunits 1, 4, and 5 (5–17).

Recently it has been shown that nucleotides that are not encoded in the genomic sequence are added within some maxicircle transcripts, presumably either during or after transcription. In one case, four uridines are added to the *T. brucei* (18), *C. fasciculata* (18), and *L. tarentolae* (J.M.S. and L.S., unpublished data) cytochrome oxidase II transcripts. This addition corrects a frameshift that exists in the genomic sequence of all three species. In another case, 34 uridines are added within the 5′ sequence of the *T. brucei* cytochrome *b* transcript (19). The uridine additions to cytochrome oxidase II (J.E.F. and K.S., unpublished data) and cytochrome *b* (19) transcripts of *T. brucei* are developmentally regulated, occurring in procyclic (insect) forms, which have a mitochondrial respiratory system, and not in slender (vertebrate bloodstream) forms, which lack this respiratory system.

The uridine additions to *T. brucei* cytochrome *b* transcripts result in the creation of an in frame AUG codon 20 amino acids upstream from an AUG (ATG) in the genomic sequence (19) that had been proposed to act as the initiation codon for this protein (6, 7). These data suggest that this created AUG may function as the initiating methionine codon for the translation of cytochrome *b*. The existing ATG in the *T. brucei* cytochrome *b* gene is not conserved in the *L. tarentolae* (9) or *C. fasciculata* (17) cytochrome *b* genes, which, like a number of other maxicircle genes, lack conventional ATG initiation codons and consequently have been suggested to use alternative initiation codons (9, 17). Thus, a created AUG in cytochrome *b* transcripts of these two species might act as a functional initiation codon. Furthermore, a comparison of the uridine additions to the cytochrome *b* transcripts of *L. tarentolae* and *C. fasciculata* and the predicted amino acid sequences may assist in the assessment of which of the *T. brucei* AUGs is the functional initiation codon.

We report here that uridines that are not encoded by the cytochrome *b* gene are added within the 5′ end of cytochrome *b* transcripts in *L. tarentolae* and *C. fasciculata*. The number and position of the added uridines is very similar between these species and *T. brucei*, and an upstream in frame AUG codon is created at a similar site in all three species. These data strongly suggest that the created AUGs serve as the initiation codons for cytochrome *b* in *L. tarentolae* and *C. fasciculata*. The similarity between the three species suggests that the created AUG may also function as an initiation codon in procyclic form *T. brucei*.

MATERIALS AND METHODS

Strains and Culture Conditions. Clones of *L. tarentolae* (University of California strain) and *C. fasciculata* (Cf-c1) were grown in brain heart infusion medium supplemented with 10–20 μg of hemin per ml, as described (20). Clone IsTaR 1 of *T. b. brucei* (stock EATRO 164) was grown in procyclic culture as described (21). Cells of all three species were harvested at late logarithmic or stationary phase and either were used immediately or were frozen in liquid nitrogen and stored at −80°C. Mitochondria were isolated from freshly harvested late logarithmic phase cells, as described (22).

Oligonucleotide Primers and RNA Transfer Hybridization. The LtCYb-CS synthetic oligonucleotide is 41 nucleotides long and is complementary to the RNA sequence predicted from the DNA sequence at the 5′ end of the *L. tarentolae* cytochrome *b* open reading frame (nucleotides 3223–3263 in ref. 9). This corresponds approximately to the start of (predicted) amino acid sequence homology between the *T.*

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brucei and *L. tarentolae* cytochrome *b* genes. The LtCYb-D and LtCYb-R oligonucleotides are complementary to the *L. tarentolae* cytochrome *b* genomic sequence and to the transcript with additional uridines, respectively. The 5′ 12 nucleotides are identical between them and the 5′ CC dinucleotide in these oligonucleotides is the 3′ CC dinucleotide of LtCYb-CS. The sequences are given below. The nucleotides in LtCYb-R that are complementary to added uridines in the transcript are shown in lowercase.

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LtCYb-CS  5′ CCTAAACTAAAACCTACACCATATACTCGTAATAAACAACC 3′
LtCYb-D   5′ CCTGAAGTTAAAGCCTTTTCTTTTCTC 3′
LtCYb-R   5′ CCTGAAGTTAAAaGaCaaCaTaaaTTTC 3′
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Gel electrophoresis, blotting, and hybridization were performed as described elsewhere (19) with oligonucleotides that were end-labeled with [^{32}P]dATP using T4 polynucleotide kinase as probes.

RNA Isolation and Sequencing. Total RNA was isolated as described elsewhere (23), hybridized to oligonucleotide primers, and sequenced, using Moloney murine leukemia virus reverse transcriptase, by the dideoxy chain-termination method in the presence of [^{32}P]dATP, as described (19), with one modification: the concentration of the deoxynucleotide corresponding to the dideoxynucleotide in each mix was lowered from 50 μM to 5 μM. Poly(A)$^+$ RNA was prepared from an isolated kinetoplast-mitochondrial fraction, as described (22, 24). *L. tarentolae* poly(A)$^+$ kinetoplast RNA was hybridized to 5′ end-labeled oligonucleotide primers and dideoxy chain-termination sequencing was performed using avian myeloblastosis virus reverse transcriptase as described by Barta *et al.* (25) and modified by D.-H. Hsu, A. Miller, and E. Sercarz (personal communication).

RESULTS

The sequence of the cytochrome *b* transcript of *L. tarentolae* was determined by dideoxy chain-termination sequencing using the LtCYb-CS oligonucleotide primer, which is complementary to the transcript near its 5′ end (see *Materials and Methods*). The same sequence was obtained by using poly(A)$^+$ RNA prepared from isolated mitochondria (Fig. 1 *A* and *B*) and total RNA (data not shown). The sequence reveals that the transcript contains 39 nucleotides, all of which are uridines, which are not predicted by the DNA sequence of the *L. tarentolae* cytochrome *b* gene (9). Sequence obtained with LtCYb-R, a primer complementary to the transcript sequence with additional uridines (Fig. 1*C*), confirms the LtCYb-CS-primed sequence. A block to elongation that occurs 39 nucleotides 3′ of the principal extension product in the LtCYb-CS-primed sequence is absent when the LtCYb-R primer is used (compare Fig. 1 *A* and *B* with *C*). This may reflect the presence of cytochrome *b* transcripts that lack the added uridines. A region of ambiguities resulting from blocks to elongation or compression artifacts in the mitochondrial poly(A)$^+$ sequences (Fig. 1 *A–C*) was clarified in experiments sequencing total RNA (Fig. 2). Most of these ambiguous positions represent added uridines. Although the LtCYb-CS primer differs from the sequence of the *C. fasciculata* cytochrome *b* gene (17) at 7 of 41 nucleotides, it was used to determine a partial sequence of the 5′ end of the *C. fasciculata* cytochrome *b* transcript. This sequence indicates that uridines that are not encoded in the genome are added to cytochrome *b* transcripts in similar numbers and positions in both organisms (Fig. 3).

A clear comparison of the cytochrome *b* RNA sequences of the *L. tarentolae*, *C. fasciculata*, and *T. brucei* was possible by using the LtCYb-R primer, which has one and two mismatches, respectively, from the *C. fasciculata* and *T. brucei* RNA sequences. The cytochrome *b* RNA sequences of these species are quite similar with respect to the number and location of additional uridines, until sequence similarity ends 4 nucleotides upstream of the 5′-proximal added uridine (Fig. 2). Strikingly, an in frame AUG is created by uridine addition in all three species near the 5′ point of sequence divergence. Comparison of these sequencing ladders with those obtained by using mitochondrial poly(A)$^+$ RNA (Fig. 1 *A–C*) shows that the 3′ cytidine in each pair of cytidines in Fig. 2 is artifactual, permitting resolution of ambiguities in sequences from all three species. A few blocked positions are found about 15 nucleotides from the bottom of the LtCYb-R sequencing ladder (Fig. 2). These positions can be interpreted based on cDNA sequences (19). It is not known whether they represent transcript ends or reverse transcriptase pause points. Alignment of the uridine addition region of the cytochrome *b* transcripts points out the highly conserved character of the sequences (Fig. 3). Discounting three extra uridines in the *L. tarentolae* and *C. fasciculata* sequences near the created AUG, there are only five mismatches in the RNA sequences between the three

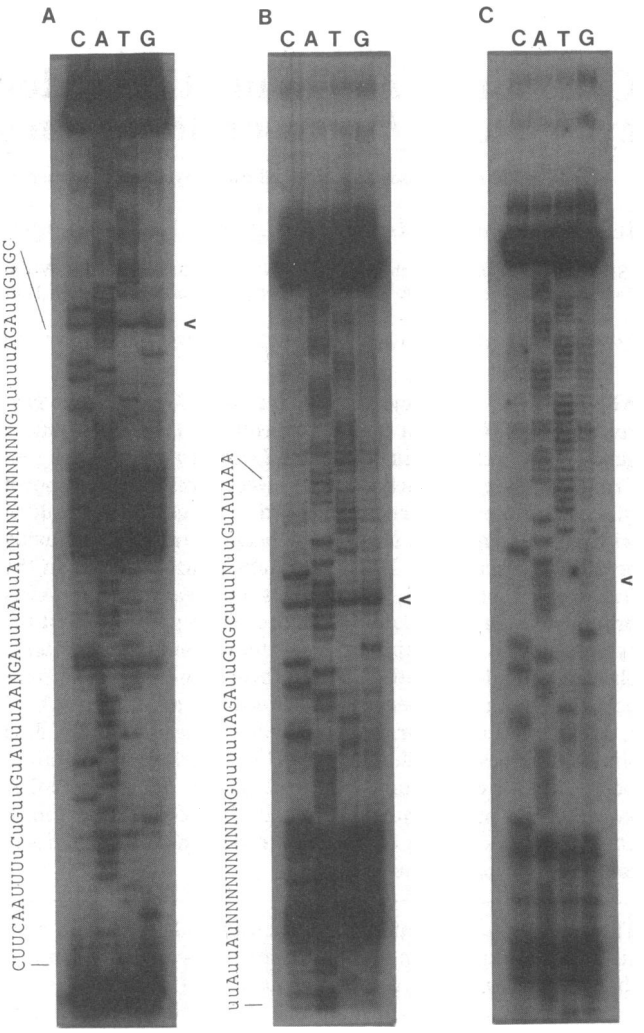

FIG. 1. Sequence of *L. tarentolae* cytochrome *b* transcripts from isolated mitochondrial RNA. Dideoxy chain-termination sequencing was performed by using 4 μg of *L. tarentolae* poly(A)$^+$ mitochondrial RNA and 2.5 ng of 5′ end-labeled [^{32}P] LtCYb-CS (*A* and *B*) or LtCYb-R (*C*) primer. Autoradiograms of a short (*A*) and long (*B*) run are shown for LtCYb-CS. The sequence of the RNA template is shown beside *A* and *B*, with added uridines indicated in lowercase. Carets to the right of *A* and *B* indicate the product corresponding to the transcript without additional uridines; the caret to the right of *C* shows where it would appear if it were detected by the CYb-R oligonucleotide.

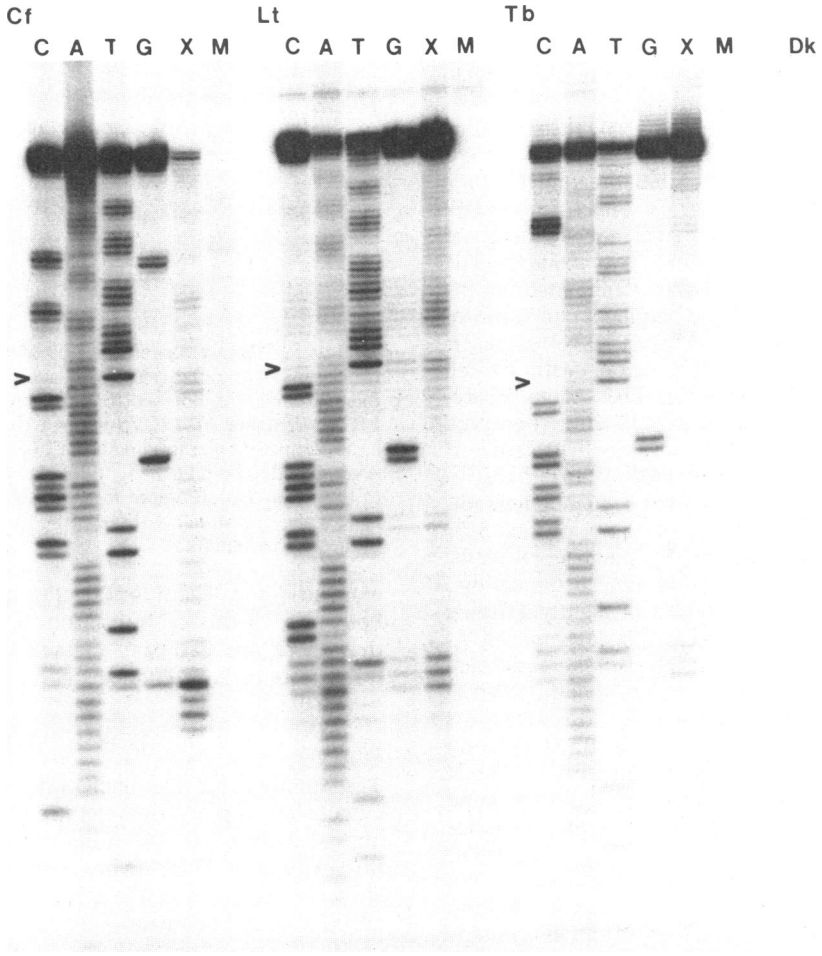

FIG. 2. Sequence of edited cytochrome *b* transcripts from three kinetoplastid species. Dideoxy chain-termination sequencing was performed by using 30 μg of total RNA from *C. fasciculata* (Cf), *L. tarentolae* (Lt), and *T. brucei* (Tb) and 50 ng of LtCYb-R primer. [^{32}P]dATP was used in the elongation reactions. Dideoxynucleotides were omitted in lanes X and primer was omitted in lanes M. The location of the created AUG (read TAC in the gel) in each sequence is marked with a caret.

species in the 60 nucleotides immediately 5′ of the genomic AUG in *T. brucei*. This level of nucleotide similarity (92%) is consistent with the overall level of 84% nucleotide similarity between the *T. brucei* and *L. tarentolae* cytochrome *b* genes.

T. brucei has 34 additional uridines in 13 sites, *C. fasciculata* has 39 uridines in 14 sites, and *L. tarentolae* has 39 uridines in 15 sites. The few differences in uridine positions between the species are largely restricted to the boundaries of the uridine addition region. *C. fasciculata* has one and *L. tarentolae* has two added uridines at the 3′ boundary that are encoded as thymidines in the *T. brucei* genome. *L. tarentolae* and *C. fasciculata* have three more uridines than does *T. brucei* immediately 3′ to the created AUG and *C. fasciculata* has an extra uridine 5′ of the AUG that is not present in the

Trypanosoma brucei

GTTAAGAATAATGGTTATAAATTTTATATAAA A G CG G AGA A A A A AGAAA G G GTCTTTTAATGTCA
NNNAANAAUAAUGGNUAUAAAUUUUAUAUAAAuAuGuuuCGuuGuAGAuuuuuAuuAuuuuuuuuuAuuAuuuAGAAAuuuGuGuuGUCUUUUAAUGUCA

Leishmania tarentolae

ATAAATTTAATTTAAATTTTAAATAATTATAAA A G CG G AGA G A A A AGAAA A G G C TTTAACTTCA
NNNNAUUUAAUUUAAAUUUUAAAUAAUUAUAAAuAuGuuuuuuCGuGuuAGAuuuuuGuuAuuuuuuuuuAuuAuuuAGAAAuuuAuGuuGuCuUUUAACUUCA
*** ** * *

Crithidia fasciculata

TCTATTTTTAATTTAAAGCTTAAAGTTAAA A G CG G AGA A A G G CTTTTAACGTCA
NNNNUUUUUAAUUUAAAGCUUAAAGUUAAAuuAuGuuuuuuCGuGuuAGAuuuuuAuuAnuuuuuuuGuuAuuuAGAAAuuuAuGuuGuCUUUUAACGUCA
* *** ** *

FIG. 3. Comparison of cytochrome *b* DNA and RNA sequences from three kinetoplastid species. The 5′ end sequences determined for *T. brucei*, *L. tarentolae*, and *C. fasciculata* cytochrome *b* transcripts are given with the corresponding DNA sequences aligned above each RNA sequence, with gaps where uridines are added to the transcripts. Added nucleotides are shown in lowercase. All of the sequences are aligned relative to the TCA serine codon (far right), which marks the beginning of the major DNA sequence homology between the three species. The created in frame AUGs and the *T. brucei* genomic AUG are underlined and differences in the number and/or position of extra uridines in *L. tarentolae* or *C. fasciculata* relative to *T. brucei* are marked with asterisks. The *T. brucei* sequence differs slightly from that previously published (19) due to resolution of ambiguities in the present work.

other two species. It may prove significant that in all three species, one (*T. brucei* and *L. tarentolae*) or two (*C. fasciculata*) uridines are added immediately 5′ of the created AUG.

The predicted N-terminal amino acid sequences resulting from translation of the cytochrome *b* RNA sequences starting at the created AUG are also quite similar among the three kinetoplastid species examined (Fig. 4). The *L. tarentolae* and *C. fasciculata* amino acid sequences are identical and differ from the *T. brucei* sequence at only two positions. The predicted sequence is very hydrophobic. Comparison of the kinetoplastid N-terminal amino acid sequences to those of the cytochrome *b* proteins of other organisms shows conservation of length of the N-terminal region, with limited conservation of amino acid sequence. The sequence predicted by the uridine addition region has 10–20% conserved amino acids, relative to other cytochrome *b* proteins. If conservative replacements are included, this rises to 25–30%. By comparison, the amino acid sequence immediately downstream of the additions is comprised of 50% conserved and conservatively replaced amino acids (Fig. 4).

T. brucei has three classes of cytochrome *b* transcripts: one matches the DNA sequence and two, of slightly differing sizes, contain additional uridines (19). To determine if *L. tarentolae* has cytochrome *b* transcripts lacking additional uridines, we hybridized LtCYb-CS, LtCYb-R, and LtCYb-D to blots of *L. tarentolae* total RNA (Fig. 5). LtCYb-CS hybridizes strongly to an ≈1300-nucleotide transcript and hybridizes slightly to a 1200-nucleotide transcript. LtCYb-R hybridizes to the same size of transcripts at about the same relative intensity. LtCYb-D, which matches the DNA sequence in the region where nonencoded uridines are present in the predominant RNA sequence, hybridizes only to the less abundant 1200-nucleotide transcript, suggesting the presence of some transcripts lacking the additional uridines. There is little possibility that these results represent cross-hybridization between edited (i.e., with added uridines) and conventional (without extra uridines) sequences, despite substantial homology between LtCYb-D and LtCYb-R. If significant cross-hybridization were occurring, the LtCYb-D probe would detect principally the larger transcript, based on its abundance. Since it does not, the hybridization conditions employed are sufficiently stringent to differentiate between the sequences recognized by the oligonucleotides. Furthermore, the presence of an extension product that is 39 nucleotides smaller than the principal product (Fig. 1 *A* and *B*) and that is not detected when sequencing with the LtCYb-R primer (Figs. 1*C* and 2) supports the existence of a class of transcripts lacking the extra uridines. The hybridization of the LtCYb-R probe to both size classes of transcripts presumably also indicates that there are two size classes of edited cytochrome *b* transcripts in *L. tarentolae*.

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                                        ▼
T. brucei          MF RCRFLLFFLLFRNLCCLLMSGCLYRIYGVGFSLGFFIALQIICGC
L. tarentolae      MFFRVRFLLFFLLFRNLCCLLTSGCLLRVYGVGFSLGFFICMQIICGV
C. fasciculata     MFFRVRFLLFFLLFRNLCCLLTSGCLLRVYGVGFSLGFFICIQIICGV
                   *  *  ** *  * *** *  *  *  ** *  *** *
H. sapiens         MTPMRKINPLMKLINHSFIDLPTPSNISAWWNFGSLLGACLILQITTGI
                      *  ** ** *  * *** *  *  *  ** *  **** *
M. musculus        MTNMRKTHPLFKIINHSFIDLPAPSNISSWWNFGSLLGVCLMVQIITGI
                   * **  *  **  *  ** *  * **  **  * **** **
S. cerevisiae      MAFRKSNVYLSLVNSYIIDSPQPSSINYWWNMGSLLGLCLVIQIVTGI
                   *  *  **  *  ** * **  **  * **** **
A. nidulans        MRILKSHPLLKIVNSYIIDSPQPANLSYLWNFGSLLALCLGIQIVTGV
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FIG. 4. Comparison ot cytochrome *b* predicted N-terminal amino acid sequences among species. The N-terminal amino acid sequences predicted from the edited cytochrome *b* transcripts of the three kinetoplastid species are aligned, with a gap in the *T. brucei* sequence where added uridines create phenylalanine codons in the other two species. Sequences upstream of the vertical arrow are predicted by the uridine addition region. N-terminal amino acid sequences from four other species (*Homo sapiens, Mus musculus, Saccharomyces cerevisiae*, and *Aspergillus ornatus*) are shown below the kinetoplastids, aligned according to downstream homologies (6, 9). Asterisks mark amino acids that are conserved or conservatively replaced in the other sequences relative to the kinetoplastids.

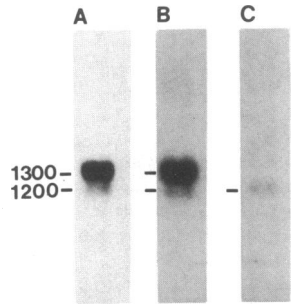

FIG. 5. RNA transfer analysis of *L. tarentolae* cytochrome *b* transcripts. Ten micrograms per lane of total RNA from *L. tarentolae* was electrophoresed, blotted, and hybridized to end-labeled LtCYb-CS (lane A), LtCYb-R (lane B), or LtCYb-D (lane C). The larger transcript is estimated to be 1300 nucleotides and the smaller is estimated to be 1200 nucleotides. Previous estimates gave the size of the major *L. tarentolae* transcript as 1200 nucleotides (26); the discrepancy presumably reflects the use of different size standards in these experiments.

DISCUSSION

The similarity of the nucleotide sequence of the altered cytochrome *b* transcripts between the three species is striking. *T. brucei* has 34 additional uridines in 13 sites, *C. fasciculata* has 39 in 14 sites, and *L. tarentolae* has 39 in 15 sites. The location of the sites and the number of uridines added at each site are strongly conserved. An AUG codon is created at approximately the same position in transcripts from all three species and the nucleotide sequence similarity between the three ceases within a few nucleotides 5′ of the created AUG. These data suggest strongly that the created AUGs in cytochrome *b* transcripts of *L. tarentolae* and *C. fasciculata*, both of which lack conventional initiation codons in the genomic sequences of this gene, serve as the initiation codons. Furthermore, the data suggest that a created AUG may also be used as an initiation codon for cytochrome *b* in procyclic form *T. brucei*, despite the presence of a downstream in frame ATG in the *T. brucei* cytochrome *b* genomic sequence.

Kinetoplastid protozoa have a number of mitochondrial genes that lack conventional initiation codons and, in these cases, isoleucine and leucine codons have been proposed to serve as alternative initiation codons (8–10, 14, 17). Sequence data to be presented in detail elsewhere indicate a correlation between uridine addition and the lack of a conventional initiation codon. Mitochondrial genes with genomic ATGs, such as cytochrome oxidase I and II, lack uridine additions within the 5′ ends of their transcripts (except for *T. brucei* cytochrome *b*), whereas those genes that lack ATGs, such as MURF2 and COIII, do have such additions. Although the creation of an AUG by uridine addition has thus far been confirmed only for cytochrome *b* and MURF2 (unpublished data) transcripts, it is possible that the creation of AUG initiation codons by uridine addition is a general phenomenon.

The existence of two AUG codons, one created and one genomic, for *T. brucei* cytochrome *b* transcripts raises the intriguing possibility that both may be functional. If both AUGs are utilized as initiation codons, two proteins, differing at the N-terminal end, would be produced. The developmental regulation of uridine addition to cytochrome *b* transcripts in *T. brucei* (ref. 19; J.E.F. and K.S., unpublished

data) suggests that production of the proteins would be under developmental control as well. Confirmation of the use of the created AUGs of all three species as initiation codons and examination of the possibility of multiple cytochrome *b* proteins in *T. brucei* await direct determination of the N-terminal sequence of the proteins.

The presence of classes of cytochrome *b* transcripts with and without additional uridines in *L. tarentolae* is highly reminiscent of the situation in *T. brucei*. However, in *T. brucei* the abundance of edited and conventional cytochrome *b* transcripts is developmentally regulated and correlates with the differential production of the mitochondrial respiratory system (ref. 19; J.E.F. and K.S., unpublished data). Leishmania are not thought to undergo similar life cycle developmental changes in expression of the mitochondrial respiratory system. The edited and conventional smaller transcripts of *L. tarentolae* are less abundant relative to the larger edited transcript than is the case for the corresponding *T. brucei* transcripts. It is possible that the smaller transcripts in both species are intermediates in the process of uridine addition and that their abundances reflect the efficiency of that process.

Although we have not performed experiments to exclude the possibility that the edited cytochrome *b* transcripts of *L. tarentolae* and *C. fasciculata* are transcribed from alternate genes, such experiments have been carried out for the edited cytochrome *b* (19) and cytochrome oxidase II (18) transcripts of *T. brucei* and no alternate genes were detected. It seems likely therefore that uridines are added to these transcripts during or after transcription. The similarity in number and position of the added uridines in the cytochrome *b* transcripts suggests that the process of uridine addition and the means for conferring specificity are similar among these species.

The addition of uridines to transcripts has been shown to correct frameshifts in the genomic sequence (ref. 18; J.M.S. and L.S., unpublished data) and to create initiation codons (ref. 19; J.E.F. and K.S., unpublished data). The data presented here argue strongly that the created AUGs are functional. Extra uridines have also been detected within the 3′ untranslated region and poly(A) tails of two transcripts (18, 19); their function at those locations is unclear. Uridine addition appears to affect a substantial proportion of kinetoplastid mitochondrial transcripts and has a principal role in creating translatable transcripts. Determination of the mechanism by which uridines are added to transcripts will assist significantly our understanding of the control of gene expression in kinetoplastid mitochondria.

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