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Short communication

Uridine insertion/deletion RNA editing in *Leishmania tarentolae* mitochondria shows cell cycle dependence

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The mitochondrial DNA of trypanosomatid protists consists of thousands of minicircles and a lesser number of maxicircles all catenated together into a single giant network of kinetoplast DNA (kDNA), located within a disk-shaped region of the single tubular mitochondrion adjacent to the flagellum [24,25]. Replication of both minicircle and maxicircle DNA occurs fairly synchronously with replication of the nuclear DNA, yielding a double sized network that then segregates just prior to cell division into two daughter networks [26,33]. The levels of mRNA for several nuclear and kinetoplast DNA (kDNA) replication genes in *Crithidia fasciculata* have been shown to be regulated in a cell cycle specific manner [3,13,16,20,21], possibly at the level of turnover of RNA.

Transcripts of approximately 12 of the 18 mitochondrial maxicircle DNA structural genes undergo a post transcriptional modification known as uridine (U) insertion/deletion RNA editing [1,6,8,18,19,10,27–29,31]. In this process, the pre-edited mRNA transcripts of the maxicircle cryptogenes are modified by insertion and/or deletion of U's, usually within coding regions. There is evidence from in vitro editing systems from both *Trypanosoma brucei* [14,23] and *Leishmania tarentolae* [4,9] for the 'enzyme cascade' model [2], with the site specificity and number of U's inserted or deleted determined by base pairing with specific guide RNAs (gRNAs), which are encoded in both maxicircle and minicircle molecules. A single gRNA mediates the editing of one 'block' of mRNA sequence and multiple overlapping gRNAs mediate the 3′–5′ pan editing of editing 'domains' [17].

Editing is regulated during the life cycle of *T. brucei*, which involves the 'procyclic' stage in the midgut of the tsetse fly, that has a functional respiratory chain and oxidative phosphorylation, and the mammalian bloodstream stage that lacks a functional respiratory chain and oxidative phosphorylation. Several maxicircle-encoded mRNAs are edited mainly in the procyclic stage and others mainly in the bloodstream stage, whereas some transcripts are edited constitutively throughout the life cycle [5,7]. The mechanism of this regulation is not known but does not appear to involve transcriptional regulation of gRNA [15,22]. It is not known if editing is regulated during the life cycle of *Leishmania*, which involves a promastigote stage in the midgut of the sandfly vector and an intracellular amastigote stage in macrophages of the mammalian host; both stages apparently utilize oxidative phosphorylation for ATP synthesis [11,12]. The lizard *Leishmania* such as *L. tarentolae* also have a promastigote stage in the sandfly vector, but the stage in the lizard host is not well characterized.

Abbreviations: Cyb, cytochrome b; COIII, cytochrome oxidase III; gRNA, guide RNA; kDNA, kinetoplast DNA; MURF2, maxicircle unidentified reading frame 2; ND7, NADH dehydrogenase subunit 7.

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In view of the regulation of RNA editing that occurs during the life cycle of *T. brucei* and the regulation of the expression of several genes for proteins involved in DNA replication during the cell cycle of *C. fasciculata*, we decided to investigate the possibility of regulation of editing during the cell cycle in *L. tarentolae*. *L. tarentolae* was selected as an experimental system since synchronized cultures of promastigotes can be obtained using hydroxyurea and since the editing system has been investigated in some detail. Our investigations show that RNA editing does appear to vary during the cell cycle of *L. tarentolae*.

L. tarentolae (UC strain) promastigotes were grown with gentle agitation in brain heart infusion (Difco) containing 10 μg ml^{-1} hemin (Cal Biochem) at 27°C. Cells were grown to a concentration of 50×10^6 cells per ml and then incubated for 6–9 h at 27°C in medium containing 200–300 μg ml^{-1} hydroxyurea (HU). HU is known to reversibly inhibit ribonucleoside reductase and thereby block cell replication at the G1-S interphase [26]. The cells were then collected by centrifugation, washed and resuspended in medium without HU, and incubated under culture conditions.

In preliminary experiments we showed that a 6 h treatment with 300 μg ml^{-1} HU gave optimal synchronization with the *L. tarentolae* UC strain, which replicates every 6 h under our culture conditions. The extent of synchronization was monitored by assaying the percentage of dividing cells with two nuclei and/or two kinetoplasts in Giemsa-stained smears and by cell counts, as shown in Fig. 1. As found earlier [26], division of the kinetoplast in HU-synchronized cells occurred prior to the division of the nucleus, as shown by the peak of 2K-1N cells occurring prior to the peak of 2K-2N cells. A small peak of 2N-1K cells, in which nuclear division occurred prior to kinetoplast division, was also seen. A wave of cell division occurred approximately 5–6 h after release from HU inhibition and a second less synchronous wave of cell division occurred at 9–10 h.

Primer extension assays were performed as described earlier [6] on total cell RNA isolated from cells at different points in the cell cycle, using 30 μg RNA annealed with 300 000 cpm of the appropriate [γ-^{32}P]ATP-labeled oligonucleotide primer. Ethanol-precipitated pellets of the RNA and labeled oligonucleotide were resuspended in 15 μl of hybridization buffer (10 mM Tris–HCl, pH 8.3, 150 mM KCl), heated at 90°C for 5 min and annealed at 40°C for 90 min. Samples were centrifuged, and 15 μl of extension mixture (1.35 μl 1 M Tris–HCl, pH 8.3, 3.6 μl 25 mM $MgCl_2$, 0.3 μl 100 mM DTT, 3.0 μl 10 mM dNTPs (2.5 mM each), 0.15 μl 10 mg ml^{-1} actinomycin D, 6.0 μl water, 0.1 μl RNase inhibitor (Pharmacia, 30 U μ^{-1}), and 0.5 μl Superscript II Reverse transcriptase (BRL) were added to each. After incubation for 60 min at 44°C, 1.5 μl of 0.5 M EDTA and 7.0 μl of 1 M NaOH were added to each tube. Samples were then incubated at 65°C for 30 min, followed by the addition of 7 μl 1N HCl. Pellets were resuspended in 10 μl 50% formamide, 0.03% bromphenol blue, 0.03% xylene cyanole and extension products were analyzed on an 8% polyacrylamide/8 M urea gel at 1500 V for 90 min. The gel was analyzed by PhosphorImager analysis. The following oligonucleotides were used in this study. The numbers in parentheses indicate the position to which they hybridize on their respective pre-edited transcript. S-2855, 5′-CTAACACACAATAAATCAAATACACG-3′ (57–

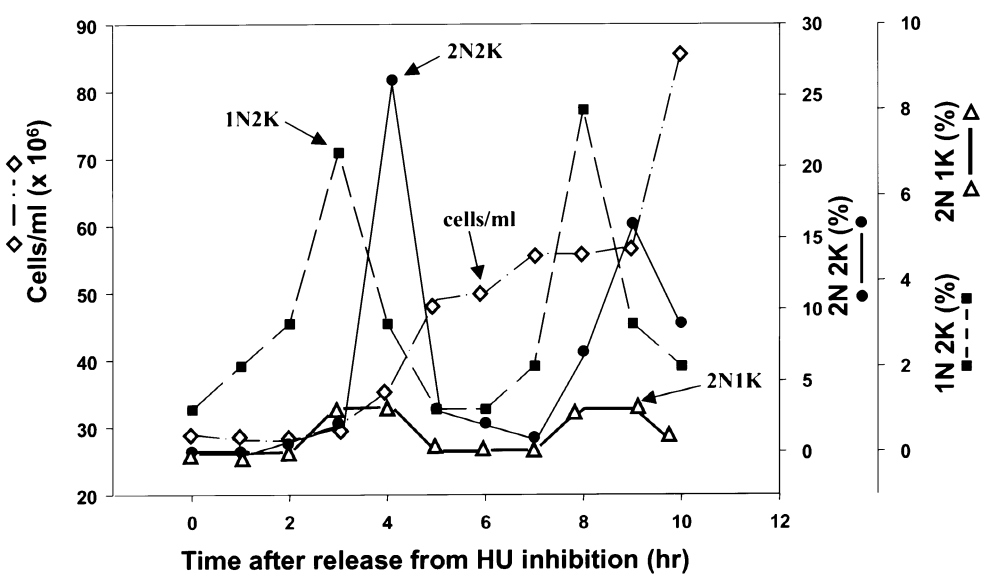

Fig. 1. Synchronization of *L. tarentolae* cells with hydroxyurea. Cells were inhibited for 6 h at 27°C with 300 μg ml^{-1} HU, and washed and incubated in fresh medium without drug.

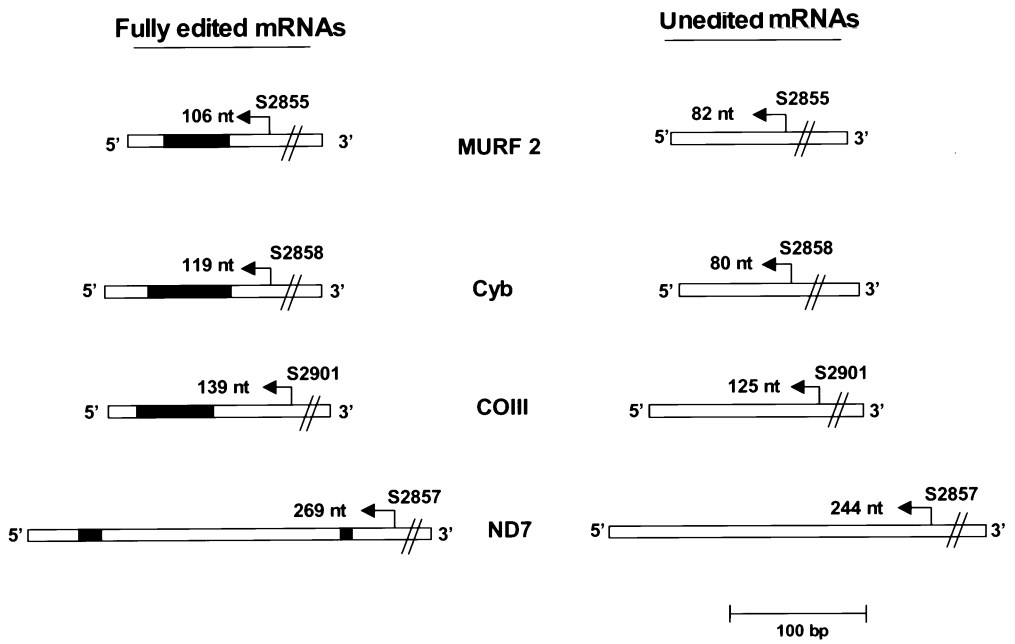

Fig. 2. Diagram of primer extension experiments. Primers hybridize to sequences just downstream of the edited region(s), which allows detection of both edited and unedited species of each gene on the same gel. The edited regions are shown in black and unedited regions in white. The localization of each primer and the expected sizes of the extension products are indicated.

82); S-2857, 5′-TACAAACTACACTTACATAATCC-AG-3′ (220–244); S-2858, 5′-CTCGTAATAAACAAC-CTGAAGT-3′ (59–80); S-2901, 5′-ATACAAAACAA-TCCACATAAAC-3′ (97–118).

As shown in the diagrams in Fig. 2, the primers in each case hybridize to unedited sequences downstream of the editing domain and should yield two major extension products, corresponding to fully edited (FE) and unedited transcripts (UE). The ratio of the edited and unedited bands yields a measure of the relative steady state abundance of fully edited versus unedited transcripts. This assay was used earlier by Missel et al. [19] to measure the effect of knockout of the mHEL61 helicase gene on RNA editing in *T. brucei*.

As shown in the primer extension results in Fig. 3A, the CO3 and Cyb transcripts yielded the expected two major extension products. A few very minor intermediate bands were visible in the Cyb lanes, which could represent partially edited RNAs [32] or RNA degradation products, but this was not investigated further. The MURF2 transcripts yielded two FE products of equal intensity differing by one nucleotide, which may be due to heterogeneity in the editing of the 5′-most site, as found earlier for the editing of the Cyb mRNA [8]. In the case of the ND7 transcripts, the expected FE and UE products were observed in addition to two minor bands which could be due to the presence of two independently edited domains, since band a is the size expected for molecules edited in Domain I and unedited in Domain II, and band b is the size expected for molecules edited in Domain II and unedited in Domain I. The independent editing of separate domains within a pan-edited RNA has a precedent in the editing of the ribosomal protein S12 mRNA in *L. tarentolae*, which was found to have three independently edited domains [18].

The FE/UE ratio was found to vary from 1.3- to 2.0-fold for all four genes during the synchronized cell cycle (Fig. 3B). The ratio peaked in S + G2 of the first cell cycle and peaked again in the same phase of the second synchronous cycle; the ratio reached a minimum value in M phase. Similar results were obtained in two independent synchronization experiments (data not shown).

Changes in the ratios of fully edited to unedited transcripts of the MURF2, Cyb and COIII genes were also seen in asynchronous cultures at different points in the growth curve. As shown in the primer extension experiments in Fig. 4A and B, the relative abundance of edited transcripts increased 5–20 fold in stationary phase cells for the different genes.

The variation in the ratio of fully edited to unedited mitochondrial mRNAs during the cell cycle and during asynchronous growth is most likely due to variation in the extent of editing. It is possible, however, that the changes are a function of different stabilities of the edited transcripts during the cell cycle. It is unlikely that the observed variation in the edited/unedited ratio in synchronized cells is an effect of hydroxyurea, since the changes occur in two waves coincident with the waves of partially synchronized cells. In addition, this

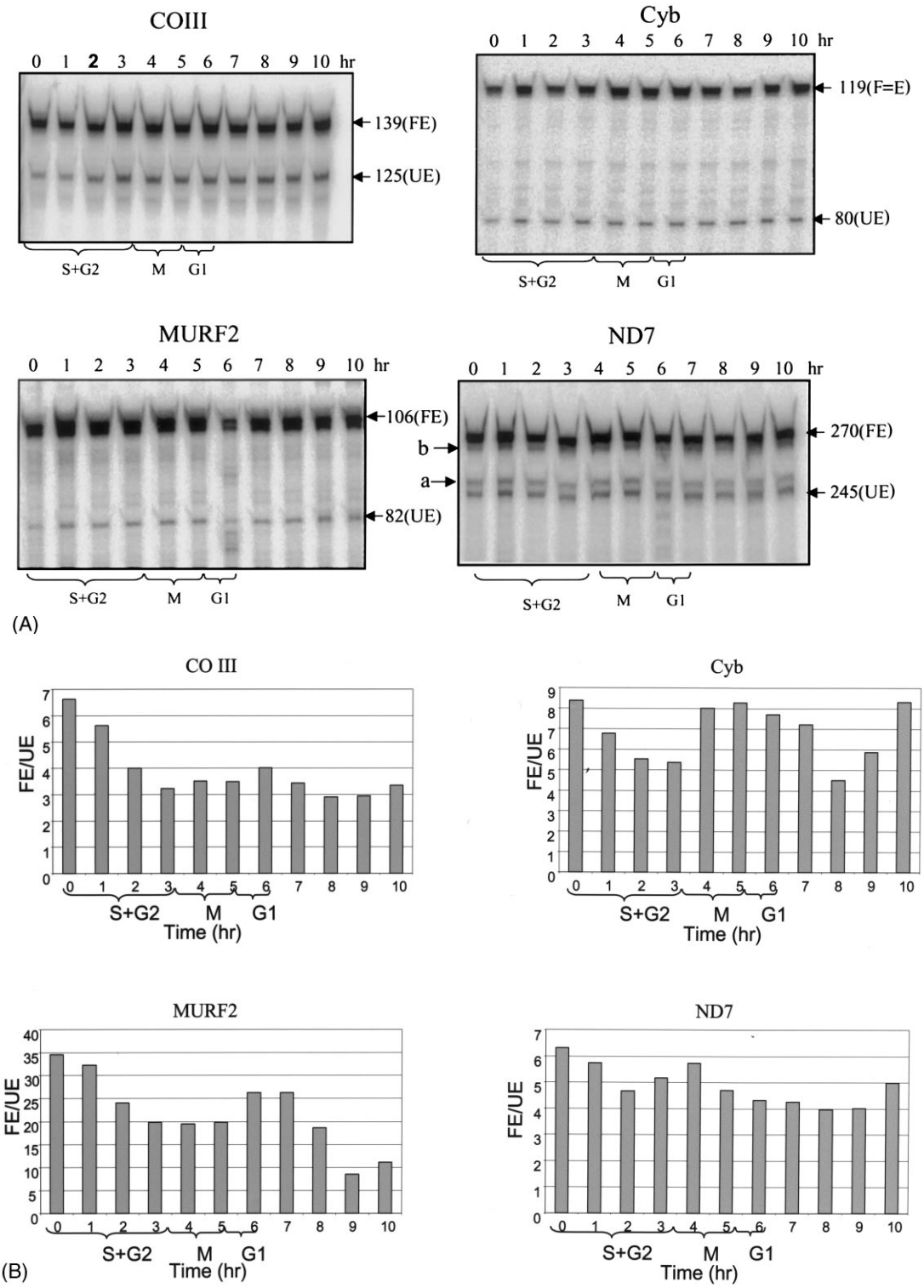

Fig. 3. Primer extension of RNA from synchronized cells. RNA was isolated from the synchronized culture shown in Fig. 1 at hourly intervals. (A) Primer extension products for fully edited (FE) (139 nt) and unedited (UE) (125 nt) transcripts of the COIII, Cyb, MURF2 and ND7 genes. In the ND7 panel, the band labeled 'a' is the product of mRNA edited only in domain I and the band labeled 'b' is the product of mRNA edited only in domain II. (B) Quantitation of the primer extension results shown in A. The ratio of the relative abundance of edited/unedited extension products is shown as a bar graph for RNA isolated from cells at different points in the cell cycle. In the MURF2 panel, the upper doublet was treated as FE. In the ND7 panel, the minor a and b bands of partially edited products were not used for the calculations.

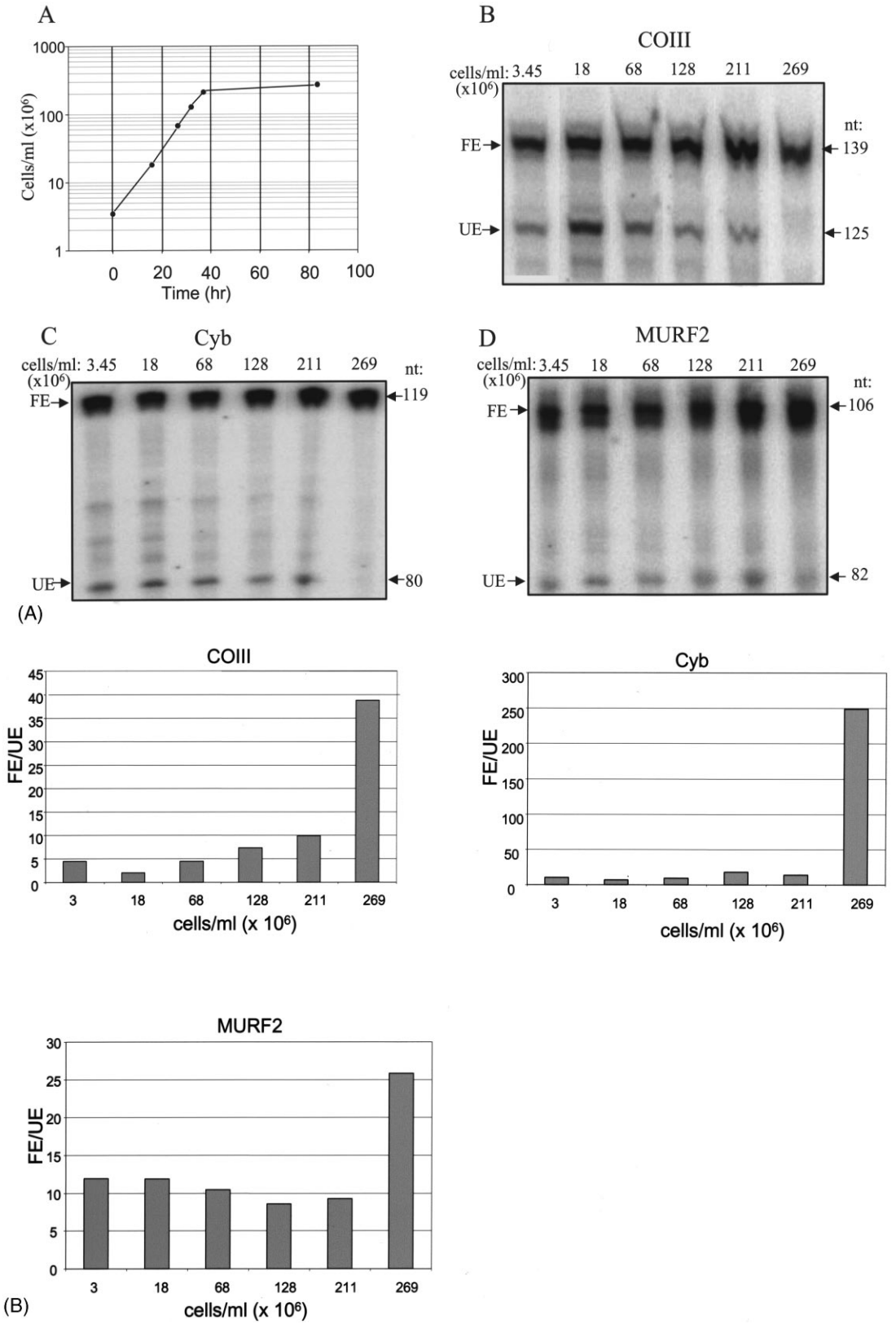

Fig. 4. Changes in the extent of edited RNAs during the growth curve of an asynchronous culture. RNA was isolated from cells at different points in the growth curve and subjected to primer extension analysis using primers specific for the COIII, Cyb, and MURF2 genes. (A) Growth curve and panels showing the primer extensions for the COIII, Cyb and MURF2 transcripts. The FE and UE products are indicated by arrows. (B) Quantitation of the primer extensions shown in Fig. 4A. The ratio of the relative abundance of edited/unedited extension products is shown as a bar graph for RNA isolated from cells at different points in the cell cycle.

method of synchronization has been used earlier with *L. tarentolae* and *C. fasciculata* without any indication of toxic effects [3,13,16,26,30].

If the variation in the ratio of edited to unedited RNAs reflects variation in the extent of editing, the question arises as to the level of this regulation. One possible mechanism to regulate editing during the cell cycle and growth curve is a differential expression of regulatory proteins for the editing process that are preferentially expressed or post-transcriptionally modified only in one stage. This phenomenon may have some relationship to the synchronicity of the nuclear and mitochondrial DNA replication phases [26,33] which appears to involve a differential expression of nuclear-encoded replication proteins due to differential turnover of mRNAs [3,13,20], but this remains to be investigated.

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