Downregulation of the mitochondrial translation system during terminal differentiation of HL-60 cells by TPA: Comparison with the cytoplasmic translation system

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Running title: Mt translation downregulation during HL-60 differentiation

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Abbreviations used:

PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; TPA, 12-O-tetradecanoyl-1-phorbol-13-acetate; EtBr, ethidium bromide; DTT, dithiothreitol; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; NP-40, nonidet P-40; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; bp, base pair; nt, nucleotide.
SUMMARY

Mitochondrial (mt) biogenesis depends on both the nuclear and mt genomes, and a coordination of these two genetic systems is necessary for proper cell functioning. Little is known about the regulatory mechanisms of mt translation or about the expression of mt translation factors. Here, we studied the expression of mt translation factors during TPA-induced terminal differentiation of HL-60 cells. For all mt translation factors investigated, mRNA expression was markedly downregulated in a coordinate and specific manner, while mRNA levels for the cytoplasmic translation factors showed only a slight reduction. An actinomycin D chase study and nuclear run-on assay revealed that the TPA-induced decrease in mt elongation factor Tu (EF-Tumt) mRNA, mainly results from decreased mRNA stability. Polysome analysis showed that there was no significant translational control of mt translation factor (EF-Tumt, ribosomal proteins L7/L12mt and S12mt) mRNA expression during differentiation. Thus, the decreased protein level of one of these mt translation factors (EF-Tumt) simply reflects its decreased mRNA level. It was also demonstrated by pulse-labeling of mt translation products, that the downregulation of mt translational activity is actually associated with downregulated mt translation factor expression during cellular differentiation. Our results illustrate that the regulatory mechanisms of mt translational activity upon terminal differentiation - in response to the growth arrest – is different to that of the cytoplasmic system, where the control of mRNA translational efficiency of major translation factors is the central mechanism for their downregulation.
INTRODUCTION

Mitochondrial (mt) biogenesis depends on both the nuclear and mitochondrial genomes (1,2). Mitochondrial DNA (mtDNA) is present in $10^3$-$10^4$ copies per cell and encodes 13 proteins, which are critical subunits of the respiratory chain complexes I, III, IV, and V, as well as two ribosomal RNAs, and 22 transfer RNAs, which are necessary for mitochondrial protein synthesis (3). Nuclear genes encode the majority of the respiratory chain subunits and all protein components necessary for maintenance and expression of mtDNA. Mitochondria play pivotal roles in eukaryotic cells in producing cellular energy and essential metabolites, as well as in controlling apoptosis by integrating numerous death signals (4). Recently, evidence has emerged that mitochondria are also implicated in the regulation of cell growth and differentiation. Inhibition of mitochondrial activity, either by deleting mtDNA (rho<sup>−</sup> cells) or by blocking translation in the organelle, has been shown to arrest or decrease proliferation in various cell lines (5-9). Mitochondrial protein synthesis inhibition is associated with the impairment of differentiation in different cell types, including mouse erythroleukemia (10) and mastocytoma cells (11), neurons (12), and human (13), avian (14) or murine myoblasts (15). The coordination of mitochondrial and nuclear genetic systems in the cell is necessary for proper mitochondrial biogenesis and cellular functioning. However, little is known either about the control of mitochondrial translation activity, or about the regulatory mechanisms governing the expression of mammalian mitochondrial translation factors.

As a first step towards understanding the integrated processes that regulate
mitochondrial translation, we studied the expression of mt translation factors during TPA-induced differentiation of HL-60 cells. The promyelocytic leukemia cell line, HL-60, is one of the best studied models of cell differentiation (16,17). This line comprises 90-95% of cells with myeloblastic/promyelocytic morphology. A variety of agents can induce differentiation of these cells either to granulocyte-like (dimethyl sulfoxide) or to monocyte/macrophage-like (TPA or 1,25-dihydroxyvitamin D3) cells (16). The effects of TPA are thought to be due, in part, to the activation of protein kinase C (18,19). Induction of differentiation by TPA in HL-60 cells is associated with activation of the stress-activated protein kinase (SAPK), mitochondrial swelling, PT (permeability transition), release of cytochrome C, activation of caspases and ultimately the induction of apoptosis (20-22).

In this report, we demonstrate downregulation of the mt translation system during terminal differentiation of HL-60 cells by TPA. In contrast to cytoplasmic translation factors, which are downregulated at the level of mRNA translation during terminal differentiation – in response to the growth arrest -, the regulation of stability and transcription, rather than translational efficiency, of mRNA is crucial for gene expression of mt translation factors. The biological significance of downregulating the mt translation system during terminal differentiation is also discussed.
EXPERIMENTAL PROCEDURES

Cells and culture conditions The HL-60 RG (Rapid Growth) derivative of the HL-60 cell line was used throughout the study (gift from Dr. Yamaguchi T., National Institute of Health Sciences). Because its DNA profiling and sensitivity to TPA-induced differentiation are almost the same as that of the parental HL-60 cell line, we chose HL-60 RG to take advantage of its rapid growth rate (normal doubling time 24 h). Cells were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Intergen) in the presence of penicillin (100 U/ml) and streptomycin (100 unit/ml). Cells were routinely passaged twice weekly to a density of 0.2-1.0 × 10^6 cells/ml and grown at 37 °C in 5% CO₂. Induction of differentiation (20 ml of 4 × 10^5 cells/ml for 150-mm plate) into monocyte/macrophage-like cells was achieved by the addition of 50 nM TPA (Sigma). After a 48-h exposure to TPA, proliferation was arrested, and almost all the cells adhered to the plastic dishes in the form of aggregates; they subsequently spread out and acquired a spindle-shaped morphology and prominent pseudopodia. This series of events was confirmed microscopically in all experiments.

RNA isolation and northern analysis Total RNA was isolated from cells using ISOGEN (Nippongene). RNA (10 to 20 μg per sample) was size fractionated on a 1% agarose-formaldehyde gel and transferred to a Hybond-N+ membrane (Amersham), and then UV cross-linked to the membrane. The membrane was prehybridized for 1 h at 68 °C in ExpressHyb (CLONTECH), and then hybridized in fresh ExpressHyb with a ^32P-labeled cDNA probe at 1-2 × 10⁶ cpm/ml and salmon sperm DNA at 20 μg/ml overnight (or for 2 nights for rare RNA species)
at 68° C. The membrane was washed with 2XSSC - 0.05% SDS at 68° C and then with 0.1X SSC - 0.1% SDS at 55° C. Membranes were analyzed by autoradiography, and quantified using a BAS5000 analyzer (Fuji Film). The radiolabeled cDNA probes for northern blots were prepared by random priming with a BcaBEST labeling kit (TAKARA). For analysis of mt transcripts (Fig.1 16S, COII and COIII), DNA templates were obtained by PCR of isolated HL-60RG total DNA. For analysis of nuclear transcripts (Fig.1 remaining samples), template DNAs were amplified either by RT-PCR using HL-60RG total RNA and oligo-dT primers, or by PCR of plasmids containing the objective sequences. Primers used for PCR are summarized in Table IA.

**mRNA turnover studies** Cells were grown to 4 X 10^5 cells/ml and the transcription inhibitor actinomycin D (ActD) (Sigma) was added to the medium at the concentration of 5 μg/ml. For differentiated cells, the ActD chase was initiated as follows: After 48 h of exposure to TPA, the culture medium was aspirated off to remove unattached cells, and replaced with a new medium containing ActD (5 μg/ml). Total RNA was isolated from cells at the indicated time points and subjected to northern analysis.

**Nuclear run-on transcription assay** Isolation of nuclei, run-on transcription, and hybridization were performed essentially as described in (23), with minor modifications. Approximately 1 x 10^8 growing (4 X 10^5 cells/ml) and differentiated (50 nM TPA, 48 h) HL-60RG cells were harvested and washed using ice-cold PBS. Before harvesting differentiated HL-60RG cells, unattached cells were carefully removed by washing twice with PBS. Cell pellets were lysed in 3.6 ml of ice-cold sucrose buffer I (0.32M sucrose, 3 mM CaCl₂, 2 mM Mg(Oac)₂, 0.1 mM
EDTA, 1 mM DTT, 0.5 % NP-40), and 3.6 ml of ice-cold sucrose buffer II (2 M sucrose, 5 mM Mg[Oac]₂, 0.1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 1 mM DTT) was added to the nuclei, before they were collected through 4 ml of sucrose buffer II in a SW41Ti tube (BECKMAN).

After centrifugation (45 min at 30,000 x g, 4 ° C), the nuclear pellet was resuspended in glycerol storage buffer (50 mM Tris-HCl [pH 8.3], 40 %[w/v] glycerol, 5 mM MgCl₂, 0.1 mM EDTA) at a concentration of 5 x 10⁷ nuclei / 200 µl, and they were snap frozen, and stored at -80 ° C. For run-on transcription, 200-µl aliquots of frozen nuclei were added to 200 µl of a reaction buffer (10 mM Tris-HCl [pH 8.3], 5 mM MgCl₂, 300 mM KCl, 5 mM DTT, 1 mM each ATP, CTP, and GTP, 40 U RNase inhibitor, 100 µCi of [³²P]UTP [800 Ci/mm; Amersham]), and incubated at 30 ° C for 30 min. The mixture was treated with 100 U of RNase-free DNase I for 10 min at 30 ° C, then further incubated with 400 µl of proteinase K solution (20 mM Tris-HCl [pH 7.5], 2 % SDS, 10 mM EDTA, 200 µg/ml proteinase K) for 30 min at 42 ° C. Following phenol/CHCl₃ extraction, transcripts were precipitated with isopropanol. The RNA was again treated with DNase I and proteinase K, phenol/CHCl₃ extracted and labeled RNA was dissolved in 50 µl TES (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.2 % SDS). Approximately 2 x 10⁷ cpm of labeled RNA is reproducibly obtained from 1 x 10⁸ growing cells, and 1.5 x 10⁷ cpm from the same amount of differentiated cells. Linearized plasmid constructs (10 µg) containing the relevant cDNA were alkali denatured and immobilized onto a Hybond-N+ membrane (Amersham). Hybridization was carried out in 2 ml of ULTRA Hyb (Ambion), with 2 x 10⁷ cpm of radiolabeled RNA and salmon sperm DNA at 100 µg /ml, over 2 nights at 50 ° C with rotation. The membrane
was washed with 2XSSC - 0.1% SDS at 50 °C, and unhybridized RNA was finally removed with 10 μg/ml RNase A in 2 x SSC (37 °C, 30 min). Membranes were analyzed using a BAS5000 analyzer (Fuji Film). We confirmed that the intensity of the actin signal was linearly obtained using 4 x 10⁶ - 2 x 10⁷ cpm of radiolabeled transcripts.

The plasmids containing β-actin and the MPO gene were prepared as follows: part of the β-actin and MPO cDNA sequences were amplified by RT-PCR of HL-60RG total RNA with the oligo dT primer and the primers described in Table IB. DNA fragments were cloned into pCR 2.1 TOPO vector (Invitrogen). pcDNA3.1/Zeo+ (Invitrogen) and hEF-Tumt.pcDNA3.1/Zeo+ were used for the detection of human EF-Tumt transcripts.

**Immunoblot assay for mitochondrial protein** Growing (4 X 10⁵ cells/ml) and differentiated HL-60RG cells were harvested and lysed in ice-cold lysis buffer (PBS containing 1% [v/v] Triton X-100 and protease inhibitor mixture [Roche]). After 30 min on ice, the lysate was centrifuged at 14,000 g for 20 min at 4°C, after which the supernatant was recovered and stored at -80 °C. Total protein concentration of the lysate was determined using the Bio-Rad protein assay; the proteins (10 to 50 μg/lane) were separated by SDS-PAGE, and subsequently transferred to nitrocellulose. The membrane was blocked with 5% nonfat dried milk in PBS-T (PBS containing 0.1% Tween 20) at 22°C for 1 h prior to incubation with a human EF-Tu/Ts mt polyclonal antibody (1:1000; anti-rabbit), or human CO II monoclonal antibody (1:1000; anti-mouse, Molecular Probes), and horseradish peroxidase-conjugated anti-rabbit (or mouse) immunoglobulin G (1:4,000; Amersham); the protein was visualized by ECL (Amersham) and
quantified by LAS-plus (Fuji film). The blots were also probed with a human monoclonal α-actin antibody (1:5000; Sigma) as a control for loading.

**Pulse labeling of mitochondrial translation products** Labeling of mt translation products was performed as described in (24). Briefly, cells were labeled for 60 min at 37°C in methionine-free DMEM containing 100 μCi/ml [35S]methionine and 100 μg/ml emetine. Cell pellets were resuspended in sonication buffer (2% SDS, 10 mM Tris-HCl [pH 6.7]), sonicated, and total cellular protein (100 μg) was loaded and separated on tricine-SDS-PAGE (16.5%T, 3%C) gels. Radiolabeled mitochondrial proteins were analyzed by autoradiography, quantified using a BAS5000 analyzer (Fuji Film).

**Polysome analysis** Fractionation of HL-60RG polysomes and isolation of RNA contained in the fractions was carried out using a modification of a published protocol (25). Approximately 3 x 10⁷ cells were used for each gradient (growing cells; 4 X 10⁵ cells/ml, differentiated cells; 50 nM TPA, 48 h). Before harvesting, cells were incubated with the medium containing 100 μg/ml cycloheximide (CH) for 5 min, and washed twice with PBS (containing CH 100 μg/ml). Cell pellets was resuspended in 1 ml lysis buffer (20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM MgCl₂, 0.04 M sucrose, 0.5% Nonidet P40, 1 mM DTT) containing 100 U of RNase inhibitor, and lysed by incubation on ice for 10 min with occasional shaking. Nuclei and cell debris were removed by centrifugation at 1,000 g for 10 min. The lysate was layered on top of a 11-ml 15-50% (w/v) sucrose gradient and centrifuged at 36,000 rpm in a Beckman SW41Ti rotor for 2 h 15 min at 4°C. Gradients were separated into 12 equal fractions using a density gradient.
fractionator (Towa Labo, model 152-001) while monitoring absorbance at 260 nm. Each fraction was treated with proteinase K, and RNA was extracted by phenol/CHCl₃, precipitated with ethanol, and analyzed for each mRNA species. L19 and β-actin mRNAs were detected by semi-quantitative RT-PCR using oligo dT primers. The number of amplification cycles in PCR was optimized to maintain the PCR reaction within the linear range. Primers used for PCR are summarized in Table IC. EF-Tumt, L7/L12mt and S12mt mRNAs were detected by northern blotting.
RESULTS AND DISCUSSION

Mt translation factor’ mRNAs are markedly downregulated in a coordinate and specific manner during TPA-induced differentiation of HL-60 cells

Despite a myriad of recent studies on gene expression during TPA-induced differentiation of HL-60 cells, using both DNA microarray (26,27), and differential display methods (28), little is known about the expression of mitochondrial (mt) translation factors. It is possible that the results obtained to date might be ambiguous due to the low levels of these mRNAs, or because some objective genes were absent from the DNA arrays.

We initially analyzed mRNA levels of several mt translation factors by northern hybridization. This revealed that differentiation of HL-60RG cells by TPA promotes coordinate reduction of mRNA level for all mt translation factors investigated (Fig. 1A). Nuclear-coded genes, *i.e.* initiation factor 2 (IF-2mt), elongation factor Tu (EF-Tu mt), elongation factor Ts (EF-Ts mt), Methionyl-tRNA transformylase (MTFmt), methionyl-tRNA synthetase (MetRSmt), ribosomal proteins S12mt and L7/L12mt, were reduced by approximately 10-20 fold, while mtDNA-coded 16 S rRNA is downregulated to a lesser extent (approximately 5-fold) (Fig. 1A, 16S rRNA). Similar results were confirmed with the HL-60 cell line (data not shown).

In contrast, we observed only a slight reduction of mRNA expression for the cytoplasmic (cyt) translation factor, ribosomal protein L19 (Fig. 1B). Similarly, others also observed, using DNA microarray technology, that mRNA levels of some cyt translation factors (such as elongation factor 1α, ribosomal proteins S27 and L27a) are not downregulated during
this differentiation process (27). Therefore, mt translation factor mRNAs appear to be specifically downregulated in a stringent manner during TPA-induced differentiation of HL-60 cells.

Nuclear respiratory factors (NRF-1 and NRF-2) govern the transcription of many, but not all, nuclear coded mt genes (29,30). mtDNA coded mRNAs are also downstream of NRFs, since the transcription of them is governed by Tfsam (mt transcription factor-a), whose transcription itself is governed by NRF-1 (31). The mRNA levels of both NRF-1 and NRF-2 were significantly decreased by TPA-induced differentiation, and thus, the mt oxidative phosphorylation-related genes cytochrome-C oxidase II (COII), cytochrome-C oxidase III (COIII) and F1-ATP synthetase subunit (F1-ATPase-) also declined, as was expected (Fig 1 C and D). Furthermore, previous studies reported that the DNA-binding activity of NRF1 is growth regulated (32), and that expression of L7/L12mt mRNA is regulated in a growth-dependent manner (33). These results suggest that most mt genes are coordinately downregulated, at least at a transcriptional level, through NRFs, either in a direct or indirect manner, in response to growth arrest that is associated with terminal differentiation. In agreement with this, another nuclear-coded mt mRNA, mt heat shock protein 70 (mt Hsp70), is also downregulated after TPA-induced differentiation of HL-60 cells (34).

*TPA-induced decrease in EF-Tunt mRNA results mainly from the decreased mRNA stability*

It is noteworthy that the extent of mRNA reduction is much more profound for mt translation factors, than for other mt-related transcripts (Fig.1 A and D). This indicates that
decreased stability, as well as the decreased transcription rate, of mt translation factor mRNA contributes to their marked downregulation during TPA-induced differentiation. To verify this possibility, nuclear run-on and actinomycin D (actD) chase assays were employed to assess the effect of TPA on transcription rate and mRNA stability, respectively. The EF-Tumt transcript, which is one of the most abundant transcripts among mt translation factors, was analyzed.

Myeloperoxidase (MPO) mRNA level is also downregulated after the TPA-induced differentiation of HL-60 cells, mainly due to its decreased transcription rate (35,36). A nuclear run-on assay indicated that EF-Tumt transcription is apparently not downregulated after exposure of HL-60RG cells to TPA (50 nM, 48 h), while MPO gene transcription is reduced to less than 20 % of the basal level (Fig. 2A). On the contrary, ActD chase studies demonstrated that EF-Tumt mRNA was significantly destabilized after the exposure to TPA (50 nM, 48h) (Fig. 2B). It is difficult to precisely evaluate mRNA stability for each condition, by simply comparing the mRNA half-life estimated by linear regression analysis. Because ActD induces the differentiation of HL-60 cells into granulocytes, and promote apoptosis (37,38), it may modulate the expression of various cytoplasmic proteins, possibly including the trans-acting proteins that govern the stability of EF-Tumt mRNA. Indeed, the apparent increase of EF-tumt mRNA level during the first 1.5 hour of ActD chase study may reflect such effect. However, taken together, the results of the nuclear run-on and ActD chase assays indicate that the TPA-induced decrease in EF-Tumt mRNA mainly reflects decreased mRNA stability.

In our preliminary observations, we also found that TPA-induced decreases in mRNA
levels of other mt translation factors – MTFmt, MetRSmt and IF-2mt – result from a combination of decreased mRNA stability and transcription, the former appearing dominant (unpublished data). Thus, we suggest that downregulation of mRNA stability is a general and crucial mechanism for the marked downregulation of mt translation factor mRNA, during the differentiation of HL-60 cells by TPA, and probably in response to growth arrest in general. Genomic sequence database searching indicates that the promoter regions of EF-Tumt, MTFmt, MetRSmt and IF-2mt carry putative NRF-binding sites, however, it remains to be elucidated whether these are functional. It is still possible that the transcription factors other than NRFs are important for the transcriptional regulation of mt translation factors. Functional analyses of their promoter regions are currently under investigation. It is interesting to note here that transcription of the mitochondrial adenine nucleotide translocator 2 (ANT2) gene is growth regulated, but that NRF is not involved in its control (39,40). In this case, the NRF-binding sites are apparently absent in its promoter region. So far, any common cis-elements that may regulate mRNA stability of mt translation factors have not been identified, although we compared their cDNA sequences. Thus, it is likely that each gene employs its own cis- and probably trans- elements respectively. Searches for such cis- and trans- acting elements in each gene are also underway.

**Downregulation of mt translation factor’s mRNA is associated with the decreased protein level, and mt translational activity**

We confirmed by immunoblot analysis that EF-Tumt protein expression is also downregulated in a manner which mirrors its mRNA levels (Fig.3A, EF-Tumt. The band
indicated with the arrow is the putative proteolytic product. A phosphatase treatment diminishes the lower band, indicating that the phosphorylated form of EF-Tumt described elsewhere (41) is preferentially in the lower band, unpublished result). The temporal modulation of protein level followed that of mRNA by approximately 50 hours, probably because the protein half-lives of the mt translation factors are much longer than those of mRNAs.

Fig 3B shows the analysis of mt translation products by pulse labeling, where only do novo synthesized mt translation products are radiolabeled. Mt translation activity drops to a level of less than 50 % of basal within 24 h, and reaches a minimal level (approximately 10 % of basal) within 48 h. Downregulated mt translational activity is actually associated with decreased levels of mt translation factors.

Because mt translation activity drops off much more quickly than the levels of EF-Tumt protein (Fig. 3A and 3B), cells may downregulate the mt genetic system by several ways, in addition to by downregulating the levels of mt translation factors. For example, the proteolysis of EF-Tumt may be involved. We reproducibly observe that the fraction of the lower band of EF-Tumt increases, and the amount of the intact EF-Tumt decreases, after TPA treatment (Fig. 3A). Other factors, such as mitochondrial polynucleotide phosphorylase (PNPase), may be also involved (discussed below).

It is interesting to note that the expression of CO II protein, an mtDNA- coded protein, remained constant up to 96 hours after TPA treatment (Fig.3A, COII), irrespective of mt translational activity being downregulated. This is probably because CO II is very stable, or
rather because it is stabilized in response to mt translation dysfunction, as is also observed in mice with a moderate reduction of mt DNA copy number (42-44). In any case, this observation suggests that the biological significance of downregulating mt translational activity may be independent of the regulation of respiratory activity, as is further discussed at the end of this report.

*Synthesis of mt translation factors is not regulated at the translational level during cellular differentiation*

The synthesis of many cytoplasmic translation factors is selectively regulated in a growth-dependent manner at the translational level. A structural hallmark, common to the mRNAs encoding many cyt translational machinery, is the presence of a 5' terminal oligopyrimidine tract (5'TOP), referred to as TOP mRNAs. The TOP motif comprises the core of the translational *cis*-regulatory element of these mRNAs (45). Terminal differentiation is associated with growth arrest, and indeed recent studies showed that TOP mRNAs are translationally downregulated during TPA-induced differentiation of HL-60 cells (28). We wondered whether decreased protein expression of mt translation factors during TPA-induced differentiation, (Fig. 3A, EF-Tumt for example), simply reflects decreased mRNA levels. According to a dbEST search, it is unlikely that most mt translation factor mRNA species are TOP mRNAs. However, it is reported that the translation of S12mt mRNA is downregulated in response to serum starvation, and its regulation is not mediated through the TOP sequence (46).

In order to examine whether mt translation factor mRNA was translationally regulated in
a manner independent of the TOP sequence, sucrose density gradient centrifugation was used to separate cell lysates into polysomal and subpolysomal (mRNP) fractions. Such gradients were prepared from HL-60RG cells before (-TPA) and after differentiation (+ 50 nM TPA, 48 h). RNA was extracted from successive fractions across the gradients and analyzed for EF-Tumt, L7/L12mt and S12mt mRNA expression by northern hybridization. These mRNAs were selected because their counterparts in the cyt translation system, *i.e.* mRNAs of EF-1α, and ribosomal proteins, are TOP mRNAs. Two control mRNAs, β-actin, which is efficiently translated in both conditions, and L19, a cytosolic ribosomal protein encoded by a typical, translationally regulated TOP mRNA were also analyzed by RT-PCR. As seen in Fig. 4, L19 mRNA shows a typical TOP mRNA behavior, being mainly polysomal in growing cells (-TPA) and mainly non-polysomal in resting cells (+ TPA). mRNA species of mt translation factors (EF-Tumt, L7/L12mt and S12mt) are efficiently translated in both conditions. These mitochondrial mRNAs were still associated with polysomal fractions even after 72 h of TPA treatment (data not shown). These results suggest that there is no translational control of mt translation factor mRNA expression during differentiation and in response to growth arrest. Thus, the decreasing protein levels of mt translation factors during differentiation would directly mirror the decreasing mRNA levels, although their temporal modulation vary depending on each protein stability. Behavior of S12mt mRNA akin to TOP mRNA in response to the serum starvation described in (46) might not reflect the cessation of cellular proliferation.

The regulatory mechanism of mt translational activity upon terminal differentiation – in
response to the growth arrest – shows a remarkable contrast with that of the cytoplasmic system. The regulation of stability and transcription, rather than translational efficiency, of mRNA species is crucial for gene expression of mt translation factors, while the control of mRNA translational efficiency is more important for the expression of cyt translation factors.

**Biological significance of downregulating mitochondrial translational activity after TPA-induced cell differentiation of HL-60 cells**

It should be emphasized that the downregulation of mt translation described in this paper is a phenomenon associated with terminal differentiation, rather than a requirement for the onset of cellular differentiation, apoptosis and differential gene expression for the new phenotype. It is reported that high mt activity appears to be associated with the preliminary steps of avian myoblast differentiation, and its induction just before the onset of terminal differentiation could characterize an irreversible engagement in terminal differentiation (47,48). In line with this, we observed that HL-60RG cells treated with thiamphenicol, a specific inhibitor of mt translation, were unable to differentiate to monocyte/macrophage-like cells in response to TPA (unpublished result). This indicates that downregulated mt translation is not a prerequisite for terminal differentiation and apoptosis. Mt translation activity in the early stages of HL-60RG differentiation is currently under investigation.

It is unclear whether downregulation of mt translation is just a result of growth arrest, or is also of biological significance after the cellular commitment to terminal differentiation. It is unlikely that cells downregulate mt translation activity to inhibit the mt respiration activity, and
to stop cell proliferation. Because rho° cells, which are devoid of mtDNA and deficient in respiratory activity, are able to proliferate. One fascinating possibility is that downregulation of mt translation activity may promote spontaneous apoptosis during terminal differentiation. In this respect, it is interesting that polynucleotide phosphorylase (PNPase) - a recently identified mt protein, probably involved in the degradation of mt transcripts (49) - is upregulated prior to growth arrest and terminal differentiation (50). The decreased mRNA levels of mt transcripts (Fig. 1 C, COII and COIII) and decreased mt translation activity (Fig. 3B) described in this report might be caused in part by enhancement of PNPase activity. Change in mt translational activity would effect the assembly of the respiratory complex, production of reactive oxygen species (ROS), maintenance of mt membrane permeability, formation of the voltage- dependent anion channel (VDAC), opening of the permeability transition pore (PT-pore), release of cytochrome C, and so on. Thus, cells may downregulate the mt genetic system to execute apoptosis during terminal differentiation.

ACKNOWLEDGEMENTS

We sincerely thank Dr. Suzuki K. (National Institute of Health Sciences, NIHS) for the critical advice and for the kind instruction on HL-60 culture, Dr. Yamaguchi T. (NIHS) for the HL-60RG cell line and for releasing research results before publication, Prof. Spremulli LL. (University of North Carolina) for bovine EF-Tu/Tsmt antibody, Prof. Morris DR. and Ms. Turcott E. (University of Washington) for kind instructions on polysome preparation and for helpful
suggestions, Prof. Loreni F. (Universita' di Roma), Dr. Krichevsky AM. (Harvard Medical School), Prof. Gotoh Y. and Dr. Masuyama N. (in our institute) for important advice, Dr. Tomita K. (in our laboratory) for critical reading of the manuscript. We also thank our previous colleague Mr. Namura M. for his professional skills supporting all our experiments. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology to NT.
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FIGURE LEGENDS

Figure 1. Mt translation factor’ mRNA is markedly downregulated in a coordinate and specific manner during the differentiation of HL-60RG cells by TPA. Total RNA (10 µg) was extracted from growing (4x10^5 cells/ml, left) and differentiated HL-60RG cells (50 nM TPA, 48 h, right), and analyzed by northern blotting. Hybridization was carried out overnight. (A) mt translation factors: EF-TuMt, EF-TuMt, IF-2mt, MTFTmt, MetRSmt, L7/L12mt, S12mt and 16S rRNA. (B) Cytoplasmic genes: 18S rRNA, for equal loading, EtBr staining; -actin, for constantly expressed gene; and L19, for the example of cyt translation factors. The results for -actin and L19 were essentially same when hybridization were carried out for 2 hours using 2 µg of total RNA. (C) nuclear transcription factors: NRF-1, NRF-2 and c-myc (c-myc is a control, which is downregulated on the cessation of cellular proliferation). The results for NRF-1 and NRF-2 were confirmed by semi-quantitative RT-PCR (data not shown). (D) mt genes involved in the oxidative phosphorylation: CO II, CO III and F1-ATPase-. Asterisks (†) indicate mtDNA-encoded genes.

Figure 2. The TPA-induced decrease in EF-TuMt mRNA results mainly from decreased mRNA stability (A) Transcriptional analysis of HL-60RG cells before (-TPA) and after (+TPA 50 nM, 48 h) differentiation by run-on transcription assays (left). Transcription rates were normalized relative to -actin RNA transcription levels, and are represented as 100 % in TPA- conditions.
(right). Black bar, +TPA; white bar, -TPA. Results are the average of two separate experiments. MPO, whose transcription is downregulated during the differentiation of HL-60 RG cells by TPA, was employed as a control. Part of the \( \beta \)-actin and MPO cDNA sequences were cloned into the TOPO vector, and the EF-Tumt coding sequence was cloned into the pcDNA3.1 vector. Signals obtained for vectors (either TOPO or pcDNA) arise from the non-specific binding of the probes to the vectors. (B) ActD chase studies in HL-60RG cells. ActD was added (5 \( \mu \)g/ml) to the medium for growing cells (4 \( \times \) 10\(^5\) cells/ml) and differentiated cells (50 nM TPA, 48 h). Total RNA (20 \( \mu \)g) was isolated from the cells at the indicated time points and subjected to northern analysis. RNA was hybridized with the EF-Tumt probe over 2 nights. Equal loading of the RNAs was confirmed by EtBr staining of the gel (left). Relative mRNA level was represented as 1 at time point 0 (right). Black circle, +TPA; open circle, -TPA. Results of two independent experiments were reproducible.

Figure 3. Downregulation of mt translation factor mRNA is associated with decreased protein level, and mt translation activity (A) Immunoblot analysis showing EF-Tumt and CO II protein levels in HL-60RG cells following treatment with TPA (50 nM). Cell lysates (50 \( \mu \)g) were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with anti-EF-Tumt, anti-CO II, and actin antibodies (left). ECL-generated images were quantified using Image Gage (Fuji Film), and the protein level of EF-Tumt or CO II was normalized against actin, and graphed over time (right). Sizes of the proteins: \( \beta \)-actin, \(~\)41 kDa; CO II, \(~\)26kDa; EF-Tumt, \(~\)47 kDa (The
band indicated with the arrow is the putative proteolytic product, see text.). (B) Mt translation products were analyzed by pulse labeling at the indicated time points. Radiolabeled proteins were separated by tricine-SDS-PAGE (left), quantified, and relative translational activity was graphed over time (right). White bar, COII; black bar, EF-Tu mt.

Fig. 4 Synthesis of mt translation factors is not regulated at the translational level during cellular differentiation. Polysome association in normal (-TPA, left) and differentiated (+TPA, 50 nM 48 h, right) HL-60RG cells of mRNAs were analyzed. Cellular extracts were fractionated by centrifugation through 15-50% SDG. Fractions were collected from the top of the gradient with continuous monitoring at 260 nm (upper). Each fraction was analyzed by RT-PCR (β-actin and L19, EtBr staining), or by northern blotting (EF-Tu mt, L7/L12mt and S12mt)(lower). For polysome analysis of differentiated HL-60RG cells, RNA was hybridized with the indicated probes over 2 nights.
Table I. Primers

[A] Primers used for the preparation of cDNA probes for northern blotting.
IF-2mt : 2020 bp
F 5'-ATGAAACGAAAGCTACTGAAGT-3', R 5'-TACATTTTAATGTAATT-3'
MTRmt : 1307 bp
F 5'-GCCCGACCTGGCTGGAGAAGTACG-3', R 5'-TTGTTGGAAGTCGAGACTG-3'
EF-Tumt : 1227 bp
F 5'-GGCCGTGGAGCCAAGAAGACTTAC-3', R 5'-CCCCCTATTATTTCTCCTC-3'
MetRSmt : 1668 bp
F 5'-GATGATGCGTTGTGAGGCGGCTAC-3', R 5'-GGTGGGCGTTCCCTTCACCAGCAAG-3'
S12mt : 341 bp
F 5'-GGCTACCTGTTCTCCATGGCTACC-3', R 5'-CTCTCTCTGCACGTTGGCCACAGTC-3'
L7/L12mt : 450 bp
F 5'-GTGGACCCCTTGATAACC-3', R 5'-CTACCTCAGAAACCACGGTGC-3'
16S rRNA : 400 bp
F 5'-ATTGGACCAATCTATACC-3', R 5'-AAGTAAGGACAGCTGAACC-3'
F1-ATPase-2 : 506 bp
F 5'-GTGAGAGCAAGCAATGAGGACTTTG-3', R 5'-GGTACCGCTGGTATATGTCAAATG-3'
CO II : 492 bp
F 5'-CTTATCCTGCCTCCTAGACC-3', R 5'-GCATTGACCAGTATAC-3'
CO III : 504 bp
F 5'-CTAACCATATACATGAGGCGG-3', R 5'-GAAGCAGATACTAGGAAATTGAGC-3'
NRF-1 : 359 bp
F 5'-TGCCAGCTGAGGTCTGGAAAT-3', R 5'-GTGGTGAGAAGGGCAGTCTGAG-3'
NRF-2 : 563 bp
F 5'-GACATTGAGAACAACCTACGGCGCC-3', R 5'-GGTGGAGGTCTATATCGTATCAGTC-3'
c-myc : 772 bp
F 5'-CACGCTCCCGCGGATCGGCCCTCA-3', R 5'-CAGAGTCTGCTGGTGTTGGCGG-3'
L19 : 324 bp
F 5'-GTTAGACCCCAATGAGACC-3', R 5'-CACATTCCCCCTACCCTC-3'

[B] Primers used for the construction of plasmids for nuclear run-on assays.
MPO : 1068 bp
F 5'-ATGGGGGCTCTCTCTCTCTCTGAGCT-3', R 5'-CATGGTGACAGGTTCCTGGCCAGG-3'
[α-actin] : 1781 bp
F 5'-GGGACAGAGACAGCTCGCCCTTGG-3', R 5'-AAGGTGTCAGCTTTTATTCAACTTG-3'

[C] Primers used for RT-PCR in polysome analysis.
L19 : 324 bp
F 5'-GTTAGACCCCAATGAGACC-3', R 5'-CACATTCCCCCTACCTC-3'
[α-actin] : 192 bp
F 5'-TCACCAACTGGGACAGCAATG-3', R 5'-GTACAGGGATAGCAGACGC-3'
Fig. 1

A

EF-Tumt  EF-Tsmt  IF-2mt

MTFmt  MetRSmt  L7/L12mt

S12mt  16S rRNA *

B

18S rRNA  β-actin  L19

C

NRF-1  NRF-2  c-myc

D

CO II *  CO III *  F1-ATPase-

TAKEUCHI & UEDA
Fig. 2

A

\[ \begin{align*}
\beta\text{-actin/TOPO} & \quad \text{-TPA} & \quad \text{+TPA} \\
MPO/TOPO & \quad \text{+TPA} & \quad \text{-TPA} \\
TOPO & \quad \text{+TPA} & \quad \text{-TPA} \\
EF-Tumt/pcDNA & \quad \text{+TPA} & \quad \text{-TPA} \\
pcDNA & \quad \text{+TPA} & \quad \text{-TPA}
\end{align*} \]

B

\[ \text{Time [h]} \quad 0 \quad 0.5 \quad 1 \quad 1.5 \quad 2 \quad 2.5 \quad 3 \]

\[ \text{Time [min]} \quad 0 \quad 10 \quad 20 \quad 40 \quad 60 \quad 120 \quad 180 \]

\[ \text{Rel. mRNA level remaining} \]

\[ \text{Time [h]} \quad 0 \quad 1 \quad 2 \quad 3 \]

TAKEUCHI & UEDA
Fig. 3

A

[Graph showing time course of actin and mitochondrial protein levels]

B

[Graph showing time course of mitochondrial protein translation activity]

TAKEUCHI & UEDA
Fig. 4

-TPA

+TPA

A260

α-actin

L19

EF-Tumt

L7/L12mt

S12mt

TAKEUCHI & UEDA
Downregulation of the mitochondrial translation system during terminal differentiation of HL-60 cells by TPA: Comparison with the cytoplasmic translation system
Nono Takeuchi and Takuya Ueda

J. Biol. Chem. published online September 2, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M307620200

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