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Differentiation-dependent repression of c-*myc*, *B22*, *COX II* and *COX IV* genes in murine erythroleukemia (MEL) cells☆

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Abstract

Murine erythroleukemia (MEL) cells provide a valuable model system for uncovering the cellular and molecular mechanisms of differentiation of proerythroid cells in culture. In order to characterize genes and gene expression patterns unique for erythropoiesis, we: (i) cloned and sequenced a 226 bp cDNA encoding portion of the 3′-end *B22* subunit of mitochondrial NADH-ubiquinone oxidoreductase (complex I); (ii) assessed the steady state level of RNA transcripts encoded by *B22*, cytochrome *c* oxidase (*COX II*, *COX IV*) and c-*myc* genes in MEL cells undergoing terminal differentiation induced by dimethylsulfoxide (DMSO) and/or 2-(3-ethylureido)-6-methylpyridine; and (iii) investigated whether the gene expression patterns of *B22*, *COX IV* and c-*myc* genes seen in differentiating cells are affected by N^6-methyladenosine, an inhibitor of commitment and RNA methylation. These studies have indicated: (a) c-*myc*, *COX II* and *COX IV* genes exhibited biphasic expression pattern; a transient accumulation of c-*myc*, *COX II* and *COX IV* mRNAs was followed by a decline after 36 hr incubation with DMSO and/or 2-(3-ethylureido)-6-methylpyridine, (b) *B22* gene expression declined progressively in differentiated cells, (c) blockade of differentiation of MEL cells with N^6-methyladenosine failed to prevent the transient accumulation of c-*myc*, *COX II* and *COX IV* mRNAs, but abrogated the irreversible expression of all four genes. These findings indicated that *B22*, c-*myc*, *COX II* and *COX IV* genes are gradually repressed in terminally differentiating MEL cells presumably *via* different patterns of expression (gradual vs. biphasic). Overall, these results showed that erythroid maturation of MEL cells is accompanied by transcriptional inactivation (or repression) of at least three genes encoding mitochondrial enzyme subunits involved in cell respiration.

Keywords: MEL; Differentiation; Cytochrome *c* oxidase; NADH-ubiquinone oxidoreductase; Complex I; Complex IV; c-*myc*; *B22*; *COX II*; *COX IV*; N^6-methyladenosine; Mitochondria; Gene expression

1. Introduction

MEL cells provide a suitable model system to uncover key-regulatory mechanisms responsible for specific gene expression during neoplastic cell differentiation as well as during normal erythropoiesis [1–3]. This process resembles the normal erythropoiesis, when terminally differentiated erythroid cells upon maturation into red blood cells loose subcellular organelles, such as mitochondria, and reduce the number of ribosomes [4].

The precise mechanism(s) *via* which genes and proteins interact each other to contribute to initiation of differentiation of malignant hemopoietic (leukemic) cells are still not well understood. Detailed analysis of gene expression patterns in differentiating vs. uncommitted cells has interestingly revealed that individual cells primed with the inducers undergo unique patterns of gene expression [1,2]. Genes transcribed by RNA polymerase I (ribosomal RNA genes) are repressed quite early, while those expressed *via* RNA polymerase II behave differently. Some of them are continuously expressed along the differentiation course while others like c-*myc*, *p53*, c-*fos* exhibit minimum and maximum in expression that ends in terminally differentiated cells. Alternatively, genes encoding proteins unique to erythroid maturation processes (heme biosynthetic enzymes, globin, transferrin receptor, etc.) built their messages and products gradually [3,5]. All these programmed expression in classes of genes toward the

☆ The nucleotide sequence reported in this paper has been submitted to the Gen/EMBL Data Bank with accession number AJ300739.

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Abbreviations: MEL, murine erythroleukemia; DMSO, dimethylsulfoxide; UDP-4, 2-(3-ethylureido)-6-methylpyridine; *COX II*, cytochrome *c* oxidase subunit II; *COX IV*, cytochrome *c* oxidase subunit IV; N^6mAdo, N^6-methyladenosine; PBS, phosphate-buffered saline.

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development of the terminal differentiation phenotype indicate that some genes may "switch" on other or may repress them in terminal stages. Therefore, the basic question which are the master genes that control initiation of commitment to maturation and which are not thus essential is still open.

Transfection experiments with genes controlled by strong inducible promoters or with antisense oligos have suggested that some genes, like c-*myc* oncogene and PU-1 transcription factor, may be very pivotal in initiating or blocking differentiation [6–9]. In addition, expression of c-*myc* seems to sensitize cells to a variety of apoptotic stimuli through cytochrome *c* release from mitochondria to cytosol, while cell death is mediated with the caspase pathway [10,11]. More recently, we have observed that differentiation-dependent apoptosis (DDA) is induced in MEL cells exposed to slightly higher concentration of the inducer DMSO.[1]

In our effort to identify and study genes involved in erythroid differentiation, we cloned out and sequenced a cDNA (clone 6.2) from a lambda-ZAP cDNA expression library prepared with mRNA derived from control untreated MEL cells. Search for structural homology in DNA data banks revealed that this cloned cDNA encodes the 3′-end of the mouse mitochondrial *B22* subunit of NADH-ubiquinone oxidoreductase (complex I) containing 41aa from the C-terminus of this protein. Having this cDNA and other probes available, it was interesting to determine the expression pattern of *COX II*, *COX IV* and *B22* genes which encode mitochondrial protein subunits and correlate this with that of c-*myc* that encodes a transacting transcription factor under conditions permitting or blocking differentiation of MEL cells with selective inducers or blockers of commitment. N^6-methyladenosine (N^6mAdo), an inhibitor of both commitment and RNA methylation was employed. The data indicate that c-*myc*, *B22*, cytochrome *c* oxidase subunit II (*COX II*) and cytochrome *c* oxidase subunit IV (*COX IV*) genes are all inactivated transcriptionally in terminally differentiated MEL cells and N^6mAdo prevented this to occur *via* inhibition of commitment. Overall, these results are valuable for understanding how proerythroid cells are differentiated into mitochondria-free red blood cells during erythropoiesis.

2. Materials and methods

2.1. Cell cultures

MEL cells employed throughout this study were MEL-745PC-4A, a clone of MEL-745 cells obtained after subcloning and subsequent testing of clones derived for high degree of inducibility. All cell cultures were maintained in Dulbecco's modified Eagles medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco, Long Island, NY, USA) and antibiotics (penicillin and streptomycin, 100 μg/mL). Cells were incubated at 37° in a humidified atmosphere containing 5% CO_2 and maintained at densities that permitted logarithmic growth (8×10^4 to 1×10^6 cells/mL). Cell viability was assessed as reported elsewhere [12].

2.2. Construction and screening of a cDNA library from MEL cells

The MEL cDNA expression library was constructed from cytoplasmic polyA^+ RNA isolated from control untreated MEL cells as it has previously reported [13]. The cDNA library was screened with a polyclonal (rabbit antiserum) antibody developed against a mouse cytoplasmic 40 kDa protein (inducerphilin), that selectively binds to an inducer of MEL cells. A clone of 226 bp in size (clone 6.2) was isolated and characterized. DNA sequencing analysis for both strands was carried out according to dideoxy-chain termination method as previously shown [13].

2.3. Induction and assessment of differentiation

Cells were incubated with and without the inducing agent in the absence or presence of an inhibitor as indicated in the text. At certain time intervals during incubation, the proportion of differentiated (hemoglobin-producing cells) was assessed cytochemically with benzidine–H_2O_2 solution, as described elsewhere [14].

2.4. Assessment of steady state level of RNA transcripts by Northern blot hybridization analysis

Cytoplasmic RNA was prepared from control and drug-treated MEL cells at different time intervals during differentiation. Cells ($(1.5-6.6) \times 10^7$ cells) were harvested from culture washed three times with ice-cold phosphate-buffered saline (PBS) (pH 7.4), and collected in a tube by centrifugation (2000 *g* for 5 min at 4°). The pellet was suspended in 0.2 mL of lysis buffer (0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 6.8, 0.4% NP-40, 10 mM vanadyl ribonucleoside complex), vortexed for 10 s, overlayed on an equal volume of lysis buffer containing 24% sucrose and 0.2% NP-40 and centrifuged at 6000 *g* for 10 min (4°) in an Eppendorf microcentrifuge equipped with a swing out rotor head. The supernatant was collected, digested with proteinase K (200 μg/mL) for 30 min at 37°, extracted with phenol/chloroform, precipitated with ethanol in the presence of 0.3 M sodium acetate, dried and finally dissolved with TE buffer (10 mM Tris–HCl, pH 7.0, 1.0 mM EDTA). Samples of RNA (10 μg) were electrophoretically separated on a 1% agarose gel in the presence of 2.2 M formaldehyde, transferred onto a Hybond-N filter (Amersham, UK), and hybridized with

[1] Pappas *et al.*, in preparation.

different [^{32}P]-labeled probes, of either cloned cDNA fragment of *B22* subunit (226 bp), or β-actin (350 bp), or c-*myc* (1.7 kb), or two other mitochondrial-related genes like these encoding the subunits *COX II* (451 bp; pCOX II), and *COX IV* (700 bp; pCOX4.111) of cytochrome *c* oxidase, as reported earlier [12–15]. Filters were washed twice and then autoradiographed using Kodak XR-5 film. The pCOX4.111 and pCOX II cDNA clones were kindly donated by Eric Schon (Columbia University) and Priscilla Dannies (Yale University), respectively.

3. Results

3.1. Terminal erythroid maturation of MEL cells is accompanied by marked repression of B22 subunit gene

We cloned out and characterized a cDNA of 226 bp from a bacteriophage lambda-ZAP II cDNA expression library constructed with cytoplasmic RNA derived from MEL-745PC-4A cells and screened with a rabbit antiserum developed against the 40 kDa protein (inducerphilin) as previously described [13]. DNA sequencing and search in DNA databanks for homologous sequences revealed that this 226 bp cDNA is identical to known 3′-end of mouse mitochondrial *B22* subunit gene of NADH-ubiquinone oxidoreductase (complex I) comprising of 41aa from the C-terminus of this protein. The sequence data have been deposited into DNA data banks under accession number AJ300739 and are closely related structurally to already submitted sequences by others (accession numbers AI118218, W33821, and BE629867). Knowing that this cDNA clone encodes the *B22* subunit of NADH-ubiquinone oxidoreductase and that the mitochondrial membrane potential is altered dramatically in differentiating MEL cells as shown by our earlier studies [16], we were prompted to examine the expression pattern of *B22* gene in differentiating MEL cells. Preliminary results have shown that the amount of total cytoplasmic RNA is gradually decreased during DMSO or 2-(3-ethylureido)-6-methylpyridine (UDP-4) induced differentiation per 10^7 cells. This differentiation-dependent decline, however, occurs to a much lesser extent in cells blocked to differentiation by N^6mAdo treatment (data not shown). For these reasons we considered a constant amount of total cytoplasmic RNA from each sample to be analyzed by Northern blot hybridization instead of considering constant number of cells. Under the experimental conditions employed where more than 90% of hemoglobin-producing cells (benzidine-positive cells) accumulated in inducer-treated cultures of MEL cells after 72 hr (Table 1), progressive reduction in the accumulation of *B22* subunit RNA transcripts was observed after 24–36 hr incubation regardless to the inducer used (DMSO and/or UDP-4) (Figs. 1A and 2A). Quanti-scanning analysis of Northern blot hybridization signals revealed the pattern of repression of *B22* gene in differentiating MEL cells (Figs. 1C and 2C). Under these conditions, β-actin and GAPDH, two genes used as internal controls, exhibited down-regulation after 36–60 hr of incubation. In contrast, the β^{major} globin mRNA level increased in the cytoplasm of terminally differentiated MEL cells, as expected (data not shown) [13]. These data indicate that *B22* subunit gene is gradually repressed in MEL cells induced to differentiate by two structurally different chemical agents. In each case, this reduction occurred after 36 hr incubation when a large proportion of cells (about 60% after 48 hr) has been committed to differentiation (Table 1).

3.2. c-myc, COX II and COX IV genes exhibit biphasic expression patterns in differentiating MEL cells

Based on the observed repression of *B22* gene in differentiating MEL cells, we explored the expression pattern of two other genes encoding mitochondrial *COX* subunits. The pattern of expression of *COX II* and *COX IV* genes was

Table 1
Cell growth and differentiation of MEL cells exposed either to DMSO in the absence or presence of N^6mAdo, or to UDP-4[a]

Time (hr)	Treatment	Concentration (M)	Cell growth (cells/mL)	Benzidine-positive cells (% of control)
None	–	7.0×10^5	<1	
	UDP-4	2.5×10^{-4}	5.5×10^5	64.5
	DMSO	0.210	9.8×10^5	54.2
	DMSO + N^6mAdo	$0.210 + 5 \times 10^{-4}$	2.9×10^5	14.0
72	None	–	3.3×10^6	<1
	UDP-4	2.5×10^{-4}	9.8×10^5	94.6
	DMSO	0.210	1.6×10^6	86.0
	DMSO + N^6mAdo	$0.210 + 5 \times 10^{-4}$	8.2×10^5	25.6
96	None	–	3.4×10^6	<1
	UDP-4	2.5×10^{-4}	1.0×10^6	95.2
	DMSO	0.210	1.7×10^6	96.4
	DMSO + N^6mAdo	$0.210 + 5 \times 10^{-4}$	1.5×10^6	31.4

[a] MEL-745PC-4A cells (8×10^4 cells/mL) were incubated separately with UDP-4 or DMSO in the absence or presence of N^6mAdo at the concentrations indicated. Cell growth and the proportion of differentiated cells were determined at 48, 72 and 96 hr as previously reported [12–14].

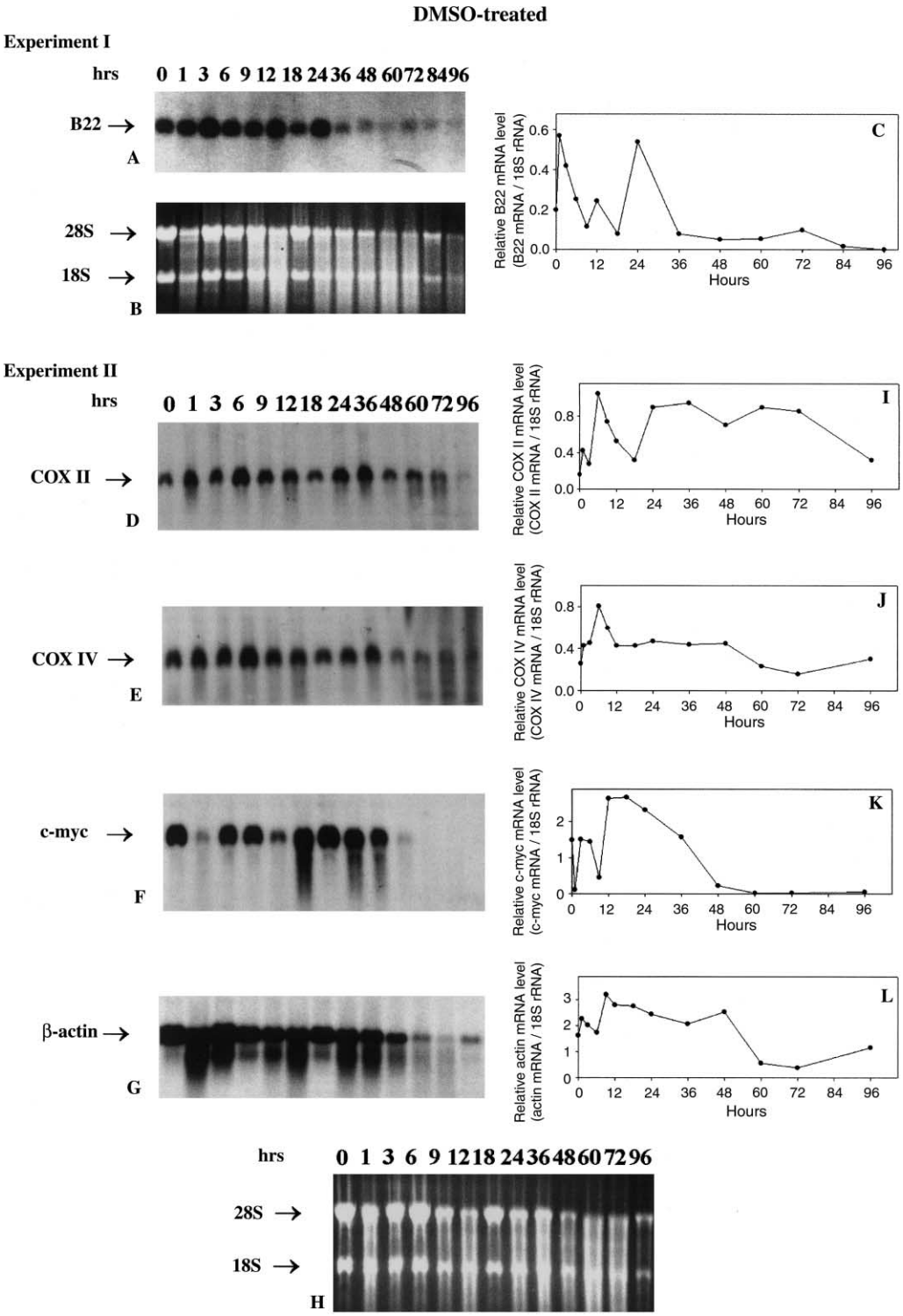

Fig. 1. Steady state accumulation of *B22*, *COX II*, *COX IV*, c-*myc* and β-actin RNA transcripts in MEL cells induced to differentiate by DMSO. Experiment I: MEL-745PC-4A cells were incubated in DMEM supplemented with 10% (v/v) FCS with DMSO (1.5% (v/v)). At times indicated (numbers above the panels), cells were removed from culture ($(1.5-6.6) \times 10^7$ cells) and total cytoplasmic RNA was isolated. Samples of cytoplasmic RNA (10 μg) were electrophoretically separated on 1% agarose gel under denaturing conditions, transferred onto a nylon membrane and hybridized at 65° with [^{32}P]-labeled cloned DNA fragment coding for mouse *B22* subunit (226 bp, clone 6.2) as described earlier [13]. The expression patterns of *B22* subunit gene in MEL cells exposed to DMSO (panel A) are shown along with the corresponding ethidium bromide pattern of isolated cytoplasmic RNAs (panel B). Quantitative scanning analysis to assess the relative amounts of *B22* subunit RNA transcripts as shown in the blot (A), was performed by using the Gel-Pro Analyzer (version 3.0, Media Cybernetics, USA). The results obtained are shown in panel C. Note that the relative mRNA level for each transcript shown in the panels is normalized by the amount of 18S rRNA (panel B) present in each lane. Experiment II: MEL-745PC-4A cells were incubated with DMSO (1.5% (v/v)) and Northern blot hybridization analysis were performed as described for experiment I. Panel D: steady state accumulation level of *COX II* subunit mRNA; panel E: steady state accumulation level of *COX IV* subunit mRNA; panel F: steady state accumulation level of c-*myc* mRNA; panel G: steady state accumulation level of β-actin mRNA; panel H: ethidium bromide pattern of isolated cytoplasmic RNAs; and in panels I–L: quanti-scanning analysis of blots shown in panels D–G, as indicated for experiment I. The Northern blot hybridization studies have been done at least twice and the data presented above are from one representative experiment.

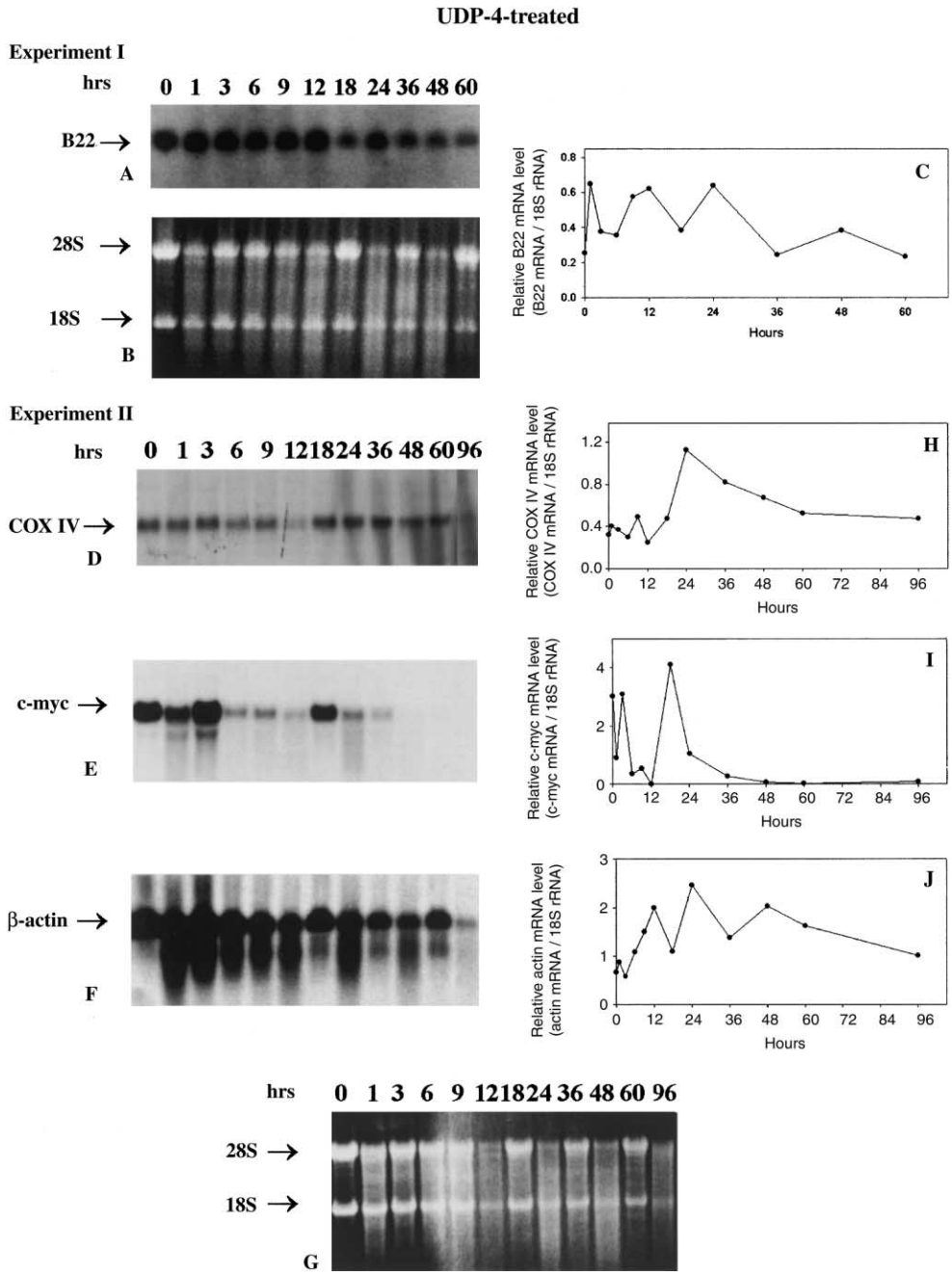

Fig. 2. Steady state accumulation of *B22*, *COX IV*, c-*myc* and β-actin RNA transcripts in MEL cells induced to differentiate by UDP-4. Experiment I: MEL-745PC-4A cells were incubated in DMEM supplemented with 10% (v/v) FCS with UDP-4 (0.25 mM) [35]. Isolation of total cytoplasmic RNA at the indicated times (numbers above the panels) and Northern blot hybridization analysis were performed as described under Fig. 1. The results obtained are indicated as follows: in panel A, the steady state accumulation level of *B22* subunit mRNA, in panel B, the ethidium bromide pattern of isolated cytoplasmic RNAs, and in panel C, the quanti-scanning analysis of blot shown in panel A, as indicated under Fig. 1. Experiment II: MEL-745PC-4A cells were incubated with UDP-4 (0.25 mM) and Northern blot hybridization analysis were performed as described for experiment I. Panel D: steady state accumulation level of *COX IV* subunit mRNA; panel E: steady state accumulation level of c-*myc* mRNA; panel F: steady state accumulation level of β-actin mRNA; panel G: ethidium bromide pattern of isolated cytoplasmic RNAs; and in panels H–J: quanti-scanning analysis of blots shown in panels D–F, as indicated for experiment I. The Northern blot hybridization studies have been done at least twice and the data presented above are from one representative experiment.

assessed in MEL cells exposed to each inducer under similar experimental conditions described above. As it is shown in Fig. 1, a transient increase in accumulation of *COX II* (panels D and I) and *COX IV* (panels E and J) RNA transcripts was observed in inducer-treated cells within the first 9 hr in culture. In particular, the *COX II* gene, was down-regulated transiently after 9 hr, then this gene was subsequently activated up to 72 hr and finally declined in DMSO-induced cells. Regarding the expression of *COX IV* gene the changes were less dramatic as compared to that of *COX II*. However, similar cytoplasmic accumulation of steady state levels of *COX IV* mRNAs was detected in

UDP-4-treated MEL cells, where an early increase during the first 24 hr was followed by a gradual decrease thereafter (Fig. 2D and H). All these changes in the expression of *COX II* and *COX IV* genes during differentiation of MEL cells were observed with parallel dramatic alterations in the accumulation of c-*myc* oncogene RNA transcripts. As it is shown in Fig. 1F and K, MEL cells exposed to DMSO exhibited a decrease in c-*myc* gene expression within 1 hr. This change was then followed by maximum and minimum accumulation resulting in a finally pronounced repression

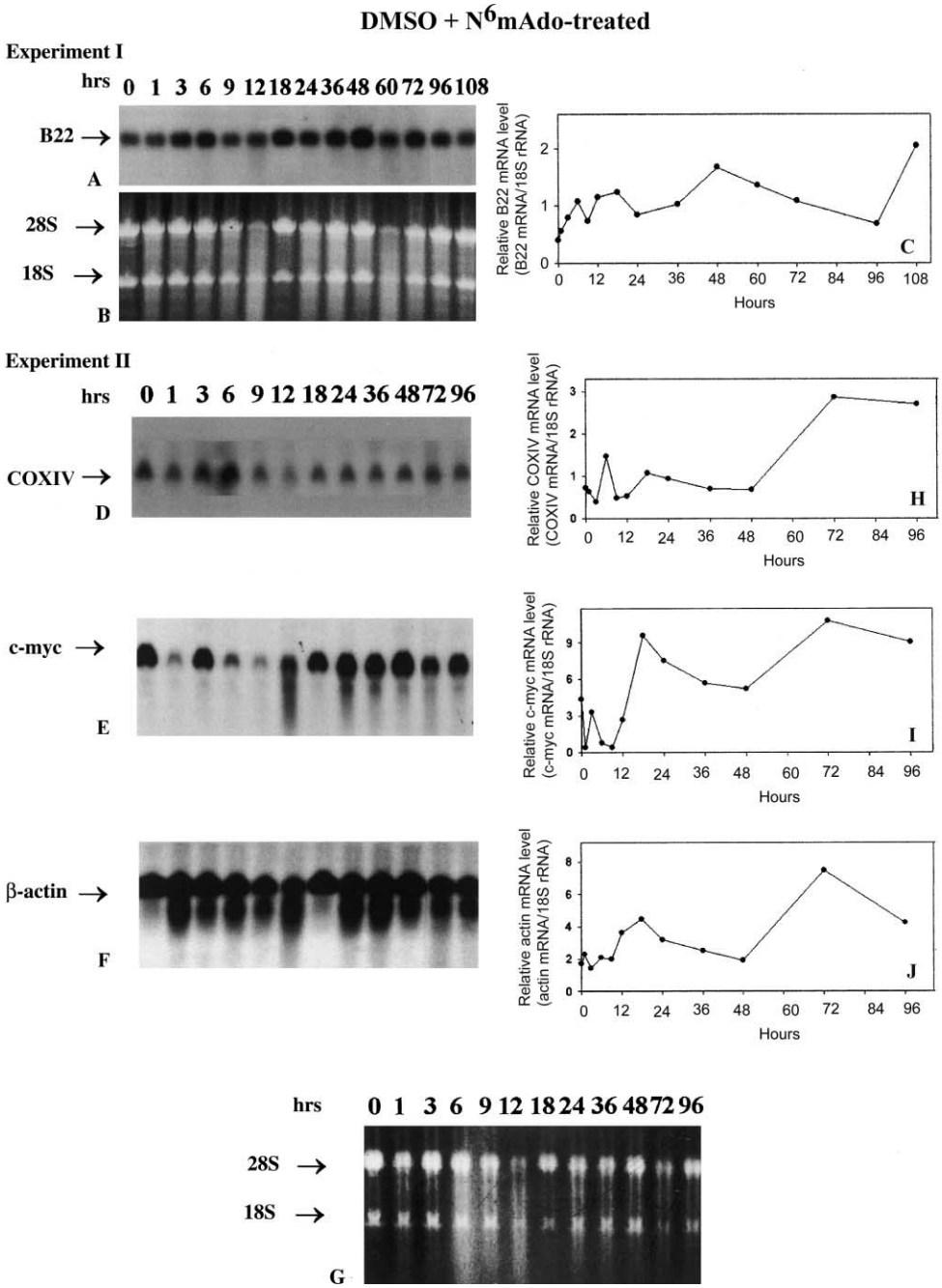

Fig. 3. Effect of N^6mAdo on the steady state accumulation of *B22*, *COX IV*, c-*myc* and β-actin RNA transcripts in differentiating MEL cells. Experiment I: MEL-745PC-4A cells were incubated in DMEM supplemented with 10% (v/v) FCS with DMSO (1.5% (v/v)) in the presence of N^6mAdo (0.5 mM) [12–14]. Isolation of total cytoplasmic RNA at the indicated times (numbers above the panels) and Northern blot hybridization analysis were performed as described under Fig. 1. The results obtained are indicated as follows: in panel A, the steady state accumulation level of *B22* subunit mRNA, in panel B, the ethidium bromide pattern of isolated cytoplasmic RNAs and in panel C, the quanti-scanning analysis of blot shown in panel A, as indicated under Fig. 1. Experiment II: MEL-745PC-4A cells were incubated with DMSO (1.5% (v/v)) in the presence of N^6mAdo (0.5 mM) and Northern blot hybridization analysis were performed as described for experiment I. Panel D: steady state accumulation level of *COX IV* subunit mRNA; panel E: steady state accumulation level of c-*myc* mRNA; panel F: steady state accumulation level of β-actin mRNA; panel G: ethidium bromide pattern of isolated cytoplasmic RNAs; and in panels H–J: quanti-scanning analysis of blots shown in panels D–F, as indicated for experiment I. The Northern blot hybridization studies have been done at least twice and the data presented above are from one representative experiment.

after 24–36 hr, as expected [17,18]. Interestingly, similar biphasic pattern of c-*myc* RNA transcripts accumulation was observed in MEL cells exposed to inducer UDP-4 (Fig. 2E and I). In these cultures, the steady state level of β-actin mRNA declined only after 48–60 hr in culture (Figs. 1G and J and 2F and L), when a large proportion of MEL cells (about 60% after 48 hr, Table 1) has been committed to differentiation, an effect reported earlier by others [17]. These findings are in agreement with earlier studies, showing a severe reduction in RNA transcripts of both cytochrome *c* oxidase *COX II* and *COX IV* subunit genes in reticulocytes during normal erythropoiesis [4], and suggest that similar mechanisms may be involved in the expression of mitochondrial function-related genes in both normal and *in vitro* induced erythroleukemia cells.

3.3. Blockade of erythroid differentiation by N^6mAdo prevented gradual repression of c-myc, B22 and COX IV genes: evidence that inactivation of these genes is part of the erythroid differentiation program of MEL cells

To further demonstrate that the decline in expression of three genes encoding vital mitochondrial proteins is indeed part of the MEL erythroid maturation process, we employed a reverse complementary approach. We exposed MEL cells simultaneously to both DMSO and N^6mAdo (an inhibitor of differentiation) [12,14], assessed the pattern of growth and differentiation and then determined the steady state level of c-*myc*, *B22* and *COX IV* RNA transcripts under these conditions. As shown in Table 1, co-treatment of MEL cells with both DMSO and N^6mAdo led to inhibition of erythroid maturation and accumulation of β^{major} globin mRNA as expected. Assessment of *B22* and *COX IV* RNA transcript levels, however, revealed that MEL cells blocked to differentiate continue to express the *B22* (Fig. 3A and C) and *COX IV* (Fig. 3D and H) genes at levels comparable to control untreated cells and much higher to that seen in differentiating cells (see Figs. 1 and 2). A similar pattern of expression was also observed for β-actin gene, a house-keeping gene used as internal control (Fig. 3F and J), as we expected [13]. In MEL cells blocked to differentiation by N^6mAdo, c-*myc* gene expression decreased within 1 hr in culture as in inducer-treated cells (Fig. 3E and I). Then c-*myc* mRNA increased as expected for 18–24 hr, but failed to decrease in contrast to that seen in differentiating cells (Figs. 1F and K and 2E and I). This indicates that blockade of differentiation kept c-*myc* gene constitutively expressed thereafter during the entire exposure period. These studies taken together indicate that suppression of mitochondrial function-related genes *B22*, *COX II* and *COX IV*, irrespectively if they being encoded by nuclear (*B22* and *COX IV*) or mitochondrial (*COX II*) genome, is consistent with the dramatic alteration in the mitochondrial membrane potential seen in differentiating cells as reported earlier [19,20]. Therefore, repression of *B22*, *COX II* and *COX IV* genes as well as of c-*myc* is part of the erythroid differentiation program that leads to loss of proliferation, inactivation of genes encoding mitochondrial proteins and eventual loss of mitochondria at the level of red blood cells.

4. Discussion

Although a lot of information has been accumulated on the developmental erythropoietic program of MEL cells, basic questions concerning the role of mitochondria in erythropoiesis are still remain. For example, it is unclear whether mitochondria play a major role in the initiation of erythroid maturation and how do the mitochondria decay along the erythropoietic maturation process when cells move from the early proerythroblast stage to reticulocytes and then to mitochondria-free red blood cells? Earlier studies have supported that changes in mitochondrial membrane potential may be related to initiation of commitment of MEL cells *via* $C\alpha^{++}$ transport, as well as to heme biosynthesis that occurs in mitochondria through the δ-aminolevulinic acid pathway [5,16,19–21]. The data presented in this study indicate the expression pattern of three genes encoding vital mitochondrial proteins during the course of erythrocytic differentiation. Northern blot hybridization analysis revealed that repression of *B22*, *COX II* and *COX IV* genes in differentiating MEL cells occurs not as fast as mitochondrial membrane depolarization. This finding suggests that these events may not be related to each other. Alternatively, one could argue that rapid depolarization of mitochondrial membrane potential contributes to the repression of *B22*, *COX II* and *COX IV* genes, later on an intriguing issue that needs further investigation.

The c-*myc* is a transcriptional regulator that controls the expression of distinct sets of target genes, thus affecting cellular differentiation and apoptosis [22–24]. The biphasic pattern of expression of c-*myc* gene in MEL cells observed upon exposure of cells to several inducers of differentiation (DMSO, HMBA, hypoxanthine, UDP-4) suggests an important role for this key-oncogene in MEL cell differentiation (Figs. 1 and 2) [17,18]. Indeed, continued expression of c-*myc* by stable transfection of MEL cells with the full length of c-*myc* cDNA impaired differentiation by inducing agents [18]. The interplay between cellular differentiation and this unique c-*myc* gene expression is further strengthened by the fact that UDP-4, an inducing agent that also triggers neuronal differentiation in RD/TE-671 cells caused similar biphasic pattern of accumulation of c-*myc* RNA transcripts in these cells as published elsewhere [25]. These data suggest that the biphasic pattern of accumulation of c-*myc* oncogene transcripts upon induction of cellular differentiation seems to be an essential element of the commitment process itself.

Experimental evidence accumulated over the last years suggests that expression of c-*myc* sensitizes cells to a

variety of apoptotic stimuli [24,26]. Although, it is not clear whether c-*myc* functions in differentiation and apoptosis are mediated by the same or different set of genes, sensitization of cells to apoptosis induced by c-*myc* seems to be mediated through cytochrome *c* release from mitochondria to cytosol, whereas other signals, such as CD96/Fas and *p53* are required to activate apoptosis through the involvement of caspases [26]. It is of great interest however that *myc*-induced apoptosis is also related to loss of mitochondrial transmembrane potential, an event seen very early in MEL cells induced to differentiate by chemical inducers [11,16]. The question whether c-*myc* gene controls the expression of mitochondrial function-related genes, like *B22*, *COX II* and *COX IV*, as c-*myc* does for genes encoding ribosomal proteins is quite challenging [22,23].

Although little is known on the role of mitochondria to hemopoietic cell differentiation, recent data indicate that inhibition of specific mitochondrial gene function, like NADH dehydrogenase and/or bcl-2, can lead to apoptosis and/or differentiation of acute myeloid leukemia cells [27–29]. Moreover, down-regulation of NADH dehydrogenase subunit 4 has been reported to occur in HL-60 cells undergoing differentiation into neutrophils but not to monocytes [30]. Whether such changes in mitochondrial gene expression in HL-60 cells result from differentiation, apoptosis and/or DDA needs further investigation.

Induction of differentiation of MEL cells by various structurally unrelated chemical inducers leads to transcriptional activation of several genes involved in hemoglobin and heme biosynthesis, while suppresses others [31]. The repression of *B22*, *COX II* and *COX IV* genes seen in differentiating MEL cells appear to be part of the maturation process that occurs independent to the inducer employed. This conclusion was further supported by the use of N^6mAdo, a specific inhibitor of differentiation, which prevented differentiation-dependent repression of *B22* and *COX IV* genes to occur. The data presented are also consistent with changes occurring in reticulocytes during normal erythropoiesis, where a marked reduction in RNA transcripts of both cytochrome *c* oxidase *COX II* and *COX IV* subunit genes was detected [4]. The different pattern of repression of *B22* and *COX* genes suggest that these genes may be regulated by different mechanisms regardless that one of these is encoded by a mitochondrial gene (*COX II*) while the others by nuclear ones (*B22*, *COX IV*). Previous studies suggested possible interactions between mitochondrial and nuclear gene products during differentiation [32,33].

The repression of three genes encoding mitochondrial function subunits (*B22*, *COX II* and *COX IV*), indicates that initiation of commitment deregulates genes critical for mitochondrial energetics. Such repression will impair protein synthesis and assembly of the NADH-ubiquinone oxidoreductase and cytochrome *c* oxidase subunits depleting ATP pools and causing cell death. Mechanistically, we have no clue how the repression of *B22*, *COX II* and *COX IV* genes has been initiated at the transcriptional level. Of course, the function of NADH-ubiquinone oxidoreductase and cytochrome *c* oxidase in mitochondria depends not only on these three genes, but to a larger number of genes (at least 41 for NADH-ubiquinone oxidoreductase and 13 for cytochrome *c* oxidase), that may be also regulated *via* commitment to erythroid maturation.

The analysis of mechanism(s) that regulate the gradual decay of mitochondrial function while heme and hemoglobin biosynthesis continue during erythropoiesis has been a long-term objective of our work. One fruitful approach to dissect these events would be to search and use agents able to block one mitochondrial function vs. the other. We already know that hemoglobin synthesis and commitment to maturation appear to be independent subprograms of erythropoiesis [34].

Acknowledgments

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