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The Potential Role of Ribosomal Protein S5 on Cell Cycle Arrest and Initiation of Murine Erythroleukemia Cell Differentiation

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Abstract Evidence now exists to indicate that some ribosomal proteins besides being structural components of the ribosomal subunits are involved in the regulation of cell differentiation and apoptosis. As we have shown earlier, initiation of erythroid differentiation of murine erythroleukemia (MEL) cells is associated with transcriptional inactivation of genes encoding ribosomal RNAs and ribosomal proteins S5 (RPS5) and L35a. In this study, we extended these observations and investigated whether transfection of MEL cells with *RPS5* cDNA affects the onset of initiation of erythroid maturation and their entrance in cell cycle arrest. Stably transfected MEL cloned cells (MEL-C14 and MEL-C56) were established and assessed for their capacity to produce *RPS5* RNA transcript and its translated product. The impact of *RPS5* cDNA transfection on the *RPS5* gene expression patterns and the accumulation of RPS5 protein in inducible transfected MEL cells were correlated with their ability to: (a) initiate differentiation, (b) enter cell cycle arrest at G_1/G_0 phase, and (c) modulate the level of cyclin-dependent kinases CDK2, CDK4, and CDK6. The data presented indicate that deregulation of *RPS5* gene expression (constitutive expression) affects RPS5 protein level and delays both the onset of initiation of erythroid maturation and entrance in cell cycle arrest in inducer-treated MEL cells. J. Cell. Biochem. 104: 1477–1490, 2008.

Key words: murine erythroleukemia; MEL; differentiation; ribosomal S5 protein expression; RPS5; cell cycle arrest; CDKs; transfection

The virus-transformed murine erythroleukemia (MEL, or Friend) cells have been widely used as a suitable model system for cell-restricted lineage erythroid differentiation in culture and the regulation of hemoglobin gene expression. MEL erythroid cell differentiation resembles the differentiation of early erythroid progenitor CFU-E cells into orthochromatic normoblasts [Tsiftsoglou and Wong, 1985; Marks et al., 1987; Tsiftsoglou et al., 1987, 2003a]. During differentiation of MEL cells, several genes are transcriptionally activated (e.g., genes encoding heme biosynthetic enzymes and hemoglobin), while others are progressively repressed [Tsiftsoglou et al., 2003a,b]. The group of non-globin genes that are inactivated quite early in the differentiation process include genes encoding the ribosomal RNAs (rRNAs) [Tsiftsoglou et al., 1982] and ribosomal proteins S5 (RPS5) and L35a (RPL35a) [Vizirianakis et al., 1999; Pappas et al., 2001]. Since, protein synthesis is dramatically decreased in differentiated cells while the ribosomes became fewer and fewer as the cells progress from the orthochromatic normoblast stage into reticulocytes, changes occurring in rRNA gene expression and ribosome biogenesis become part of the erythroid differentiation process [Sherton and Kabat, 1976]. Apparently, abnormalities in these processes could explain the pathogenesis of some reticulocyte disorders [Bessis et al., 1983].

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One of the fundamental questions to be resolved is how do the inducer-treated MEL cells enter the commitment to irreversible growth arrest upon erythroid differentiation [Geller et al., 1978] and which macromolecules are involved in the initiation of erythroid maturation. In an effort to address this objective, we reasoned to investigate the potential role of RPS5 on the initiation of erythroid cell differentiation. We proposed to over-express this protein via transfection of *RPS5* cDNA in order to constitutively maintain its level and then ask if this event will affect the decision of cells to enter the erythroid differentiation pathway as compared to parental MEL cells.

Here, we wish to present evidence showing that stable transfection of MEL cells with full-length *RPS5* cDNA and constitutive expression of *RPS5* gene affected cell cycle arrest at G_1/G_0 and delayed initiation of erythroid differentiation of MEL cells. Such effects were accompanied with changes in the level of both RPS5 protein and *RPS5* mRNA as well as alterations in cell cycle kinases (CDK2, CDK4, and CDK6) as suggested by earlier studies [Hsieh et al., 2000; Matushansky et al., 2000a,b, 2003; Zhu and Skoultchi, 2001]. These findings indicate that deregulation of *RPS5* protein and mRNA level by stable transfection delays the onset of erythroid differentiation presumably by affecting cell cycle arrest at G_1/G_0 and the function of CDK2, CDK4, and CDK6. A possible link between initiation of differentiation via changes in *RPS5* gene expression and cell cycle arrest is discussed.

MATERIALS AND METHODS

Chemicals and Antibodies

Dimethylsulfoxide (DMSO), hexamethylene-bis-acetamide (HMBA), benzidine dihydrocloride, vanadyl ribonucleotide complexes (VRC), and proteinase K were purchased from Sigma (St. Louis). UDP-4, a potent inducer of differentiation, was synthesized in our laboratory, as described earlier [Pappas et al., 1996]. [γ-^{32}P]-dCTP (3000 Ci/mmol) was obtained from Izotop, Institute of isotopes Co., Ltd., Budapest, whereas the DNA ^{32}P-labeling system kit was purchased from Invitrogen (Rad Prime DNA Labeling System). The rabbit anti-RPS5 polyclonal antibody (pAb) raised against C-terminal oligopeptide of RPS5 was kindly provided by Dr. Fukushi (R&D Center, BioMedical Laboratories, Matoba, Kawagoe, Saitama, Japan), The rat anti-mouse MYC mAb was purchased from Invitrogen (Paisley, UK), whereas antibodies used for the detection of CDK2, CDK4, CDK6, and GATA-1 were obtained from Cell Signaling Inc. Goat anti-mouse IgG and goat anti-rabbit IgG were obtained from Santa Cruz and agarose-G beads from Upstate.

Cells Cultures

MEL cells employed throughout this study were MEL-745PC-4A, a clone of MEL-745 cells. Cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), containing 10% (v/v) fetal bovine serum (Gibco) and antibiotics (penicillin and streptomycin, Invitrogen) (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and maintained at densities that permitted logarithmic growth.

Assessment of MEL Cell Differentiation

MEL cell cultures exposed separately to each one of different inducing agents DMSO (1.5% v/v), HMBA (5 mM), and/or UDP-4 (1 mM) in culture, as indicated in the text, were assessed for the proportion of differentiated (hemoglobin-producing) cells using benzidine–H_2O_2 solution, as previously described [Orkin et al., 1975].

Stable Transfection of MEL Cells With Full-Length Mouse *RPS5* cDNA

Exponentially growing MEL cells were transfected with a cDNA fragment encoding the full-length mouse *RPS5* cDNA (715 bp in length; designated RPS5-Myc/His) generated by PCR from the original recombinant plasmid constructed in our laboratory [Vizirianakis et al., 1999], (GenBank accession no: Y12431), by using 5′-CGgaattcGTGGTCCACGCCGAGC-3′ (sense primer) and 5′-GCggatccACGCGGTTAGACTTGGC-3′) (antisense primer). The low-case letters indicate the restriction sequences of EcoRI and BamHI, respectively, constructed to facilitate rapid PCR cloning of *RPS5* cDNA into the corresponding sites of pcDNA3.1(−) Myc/His (version A) vector (Invitrogen). The antisense primer was designed in such a way to permit cloning of the Myc/His tag domain at the carboxy-terminus of the recombinant RPS5 protein during biosynthesis within the transfected cells. This cloning strategy could also facilitate differential detection of fusion RPS5 protein from its endogenous counterpart. By inserting the amplified *RPS5* cDNA fragment

into the pcDNA3.1(−) Myc/His vector digested with EcoRI/BamHI and in sense orientation with respect to CMV promoter, the recombinant vector *pcDNA-RPS5-Myc/His* was constructed and verified by DNA sequencing. Stable transfection of MEL with the recombinant vector *pcDNA-RPS5-Myc/His* was performed by using Lipofectamine-2000^{TM} reagent (Invitrogen) and 1 μg plasmid DNA (*pcDNA-RPS5-Myc/His*) according to the accompanying manufacturer's protocol. Stably transfected cells expressing the *RPS5*-Myc/His construct were then selected phenotypically with G418, (Gibco BRL, Gaithersburg, MD, 600 μg/ml) added in the culture medium. Stably transfected MEL cells were selected out and assessed by Northern blot hybridization and/or reverse transcription-polymerase chain reaction (RT-PCR) analysis for the steady-state levels of *RPS5* RNA transcripts encoded by the endogenous *RPS5* gene as well as by the transfected *RPS5*-Myc/His cDNA. Two clones (MEL-C14 and MEL-C56) expressing both the endogenous *RPS5* gene as well as the transfected *RPS5*-Myc/His at relatively high level were used throughout this study.

Isolation of Total Cytoplasmic RNA and Northern Blot Hybridization Analysis

Control and inducer-treated cells at different times during incubation were collected by centrifugation at 150*g* for 5 min, washed three times with ice-cold PBS (pH 7.4), and processed for the isolation of total cytoplasmic RNA after cells being lysed with 0.2 ml lysis buffer [0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl (pH 6.8), 0.1% NP-40, and 10 mM VRC], as previously described [Vizirianakis and Tsiftsoglou, 2005]. The concentration of RNA was assessed spectrophotometrically at 260 nm. Purified RNA (10 μg) from each sample were electrophoretically separated on a denatured 1.0% w/v agarose gel in the presence of 2.2 M formaldehyde, transferred onto nylon membranes (Schleicher & Schuell), and hybridized with random primed [^{32}P]-labeled DNA probes for the detection of mRNAs encoded either the *RPS5* (715 bp cDNA) and/or β^{major} globin (~7.3 kb genomic DNA fragment containing the entire gene) genes, as previously shown [Vizirianakis and Tsiftsoglou, 1996]. Membranes were washed and autoradiographed using Kodak X-Omat AR film.

RT-PCR Analysis for the Detection of Exogenous and Endogenous *RPS5* RNA Transcripts Accumulated in the Cytoplasm

Cytoplasmic RNA (0.5 μg) isolated from MEL cell cultures was used for RT-PCR. The PCR experiments were performed by using the sense 5′-primer (5′-GCGGGATCCATGACTGAGTGGGAAGCA-3′ with two different sets of antisense 3′-primers specifically designed to allow the detection of either endogenous *RPS5*: 5′-GCGGAATTCTCAGCGGTTAGACTTGGC-3′, or exogenous (recombinant *RPS5*-Myc/His gene specific): 5′-TAGAAGGCACAGTCGAGG-3′, *RPS5* gene expression.

Cellular Extracts, Immunoprecipitation and Western Blot Analysis

MEL cells grown in culture were harvested, rinsed twice in ice-cold PBS, and then lysed in lysis buffer (0.5% v/v NP-40, 0.5% v/v sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonylfluoride (PMSF), 0.1 mM dithiothreitol (DTT), 10 μM aprotinin, 10 μM leupeptin) at 4°C to yield total cellular protein extracts after centrifugation at 12,000 rpm for 10 min. The protein amount was assessed by using Bradford's method. Cellular extracts containing constant protein amount (3 mg) were then precleaned for 1 h at 4°C with protein G-Sepharose beads. Cell lysates were mixed by rotation with primary antibody and protein G beads at 4°C overnight. Immunoprecipitates were washed three times with wash buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 0.5% NP-40, 0.1% bovine serum albumin, 1 mM PMSF, 0.1 mM DTT, 10 μM aprotinin, 10 μM leupeptin) and heated at 100°C in SDS sample buffer for 3 min. The samples were fractionated by SDS–PAGE, transferred onto Immobilon PVDF membranes (Millipore Corporation, Bedford, MA), and immunoblotted separately with polynoclonal antibodies RPS5 (C-terminal) (1:1,000 dilution) anti-MYC (1:2, 500), CDK2 (1:1,000), CDK4 (1;1,000), CDK6 (1:1,000), and/or GATA-1 (1:400) to detect the endogenous RPS5, recombinant RPS5-Myc/His, CDK2, CDK4, CDK6 and/or, GATA-1 protein levels, respectively.

Flow Cytometric DNA Analysis

Parental MEL and stably transfected MEL-C14 cells harvested from culture were processed for single parameter analysis of DNA content by

using the DNA-Prep Coulter® Reagents Kit obtained from Coulter Corporation (Beckman Coulter, Miami, Florida) according to the manufacturer's protocol. Linear fluorescence signals of propidium iodide (PI) were obtained by a Coulter Epics® XL MCL Flow cytometer with dye excitation by 15 mW 488 nm laser light.

RESULTS

Cloning, Transfection and Expression of Full-Length Mouse *RPS5* cDNA in MEL Cells

To explore the potential role of RPS5 in initiation of erythroid cell differentiation and cell cycle arrest at G_1/G_0 of inducer-treated highly inducible MEL cells, we stably transfected parental MEL-745PC-4A cells with the full-length mouse *RPS5* cDNA under the constitutively inducible CMV promoter of pcDNA 3.1-Myc/His recombinant eukaryotic vector (*pcDNA3.1-RPS5-Myc/His*). We designed and constructed the recombinant vector in such a way to carry the Myc/His tag sequences physically linked at the carboxy-terminus of the *RPS5* cDNA in order to facilitate the detection of the recombinant form of RPS5 in the presence of native RPS5 in the cytoplasm of transfected cells (see under Materials and Methods). MEL cells stably transfected with *pcDNA3.1-RPS5-Myc/His* construct were selected in medium containing G418 and assessed for the expression of transfected *RPS5* cDNA into RPS5ex RNA transcripts as shown by Northern blot hybridization and verified by RT-PCR analysis. Among several individual clones of transfected MEL cells tested during the first round of screening only one of them (clone C14, Fig. 1A,C) was found to express the transfected recombinant *PRS5-Myc/His* cDNA into an RNA transcript (*RPS5ex*) in addition to the expression of the endogenous *RPS5* gene (RNA transcript designated *RPS5end*) (Fig. 1A,C). In terms of size, the full-length *RPS5ex* RNA transcript or the generated RT-PCR product was of higher molecular weight than that of *RPS5end*, as expected. Northern blot hybridization and subsequent RT-PCR, confirmed the expression of the transfected *RPS5-Myc/His* cDNA only in the stably transfected MEL-C14 and not in the parental and other transfected clones (like mock-transfected cells). The relative level of the *RPS5-Myc/His* gene RNA transcript was about the same as compared to endogenous *RPS5* gene transcript.

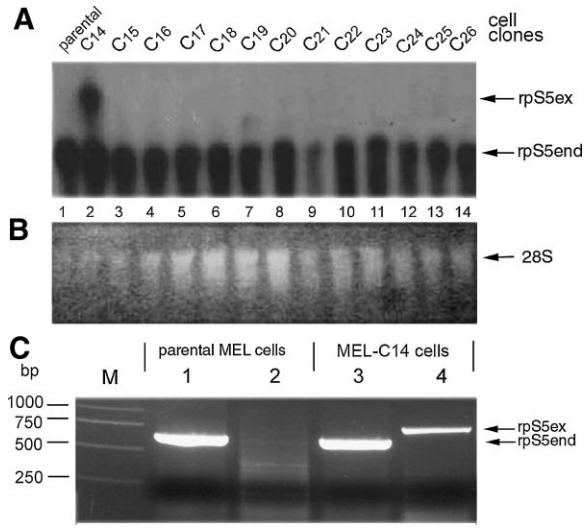

Fig. 1. Assessment of the steady-state level of RPS5 RNA transcripts in parental and stably transfected with the recombinant vector *pcDNA-RPS5-Myc/His* MEL cells. MEL-745PC-4A cells incubated in DMEM supplemented with 10% v/v FBS, and MEL-C14 cultures grown separately in the same medium supplemented with 0.6 mg/ml G418 were harvested and processed for total cytoplasmic RNA isolation. **Panel A**: Cytoplasmic RNA (10 μg) was electrophoretically separated on 1% agarose gel, transferred onto a nylon membrane, and hybridized at 65°C with [^{32}P]-labeled DNA fragments coding for mouse *RPS5* mRNA (715 bp). **Lane 1**: RNA extracted from parental MEL cells; **Lanes 2–14**: RNA extracted from the various isolated clones shown above the panel. **Panel B**: The ethidium bromide staining pattern of the isolated cytoplasmic RNA transcripts is shown, whereas the position of 28S rRNA is indicated by the arrow. **Panel C**: Total cytoplasmic RNA (0.5 μg) was analyzed by RT-PCR by using two different pairs of primers (see Materials and Methods) to assess the steady-state levels of endogenous RPS5 (lanes 1, 3) and exogenous RPS5-Myc/His RNA (lanes 2, 4) transcripts in parental MEL cells (lanes 1, 2) and in MEL-C14 clone (lanes 3, 4).

When the level of the recombinant RPS5-MYC/His protein produced in MEL-C14 cloned cells was assessed at first by Western blot analysis of cellular extracts with the use of anti-RPS5 (C-terminal) pAb or anti-MYC tag mAb, no such RPS5-MYC/His protein was detected (data not shown). These data suggested two possibilities: (a) MEL-C14 cells are unable to produce RPS5-MYC/His protein in sufficient quantity to be detected by Western blot analysis regardless to their capacity to express the *RPS5-Myc/His* cDNA into *RPS5ex* transcript; (b) The antibodies used failed to interact with the RPS5-MYC/His in cellular extracts under the conditions employed. To resolve these issues, we applied immunoprecipitation of cellular extracts derived from MEL-C14 cultured cells using either anti-RPS5 or

anti-MYC tag antibodies. These experiments confirmed that indeed MEL-C14 cells produced the RPS5-MYC/His protein, as shown in Figure 2. A second clone designated MEL-C56 was also detected during the second round of screening, as it is described later on in this study.

The Expression of Recombinant RPS5-MYC/His Protein Altered the Onset of MEL Erythroid Cell Differentiation In Vitro

To assess the impact of the expression of recombinant *RPS5-Myc/His* construct on the developmental program of MEL cells, we asked whether there was any detectable change in the kinetics of cell growth and differentiation in inducer-treated MEL-C14 cells. By comparing the kinetics of growth and differentiation of DMSO-treated parental MEL cells, stably transfected MEL-C14 cells and MEL cells transfected with the mock vector [*pcDNA3.1(−) Myc/His*], no significant change was recorded up to 96 h in culture (see Fig. 3A). However, in MEL-C14 cells grown in culture in the absence of inducer, a small decrease in their growth rate as compared to untreated parental and mock-transfected MEL cells was shown (Fig. 3E). Quantitatively, highly inducible parental MEL cells treated with DMSO for 96 h exhibited 80% benzidine-positive cells (terminally differentiated), like mock-transfected MEL cells grown under similar conditions (Fig. 3C). A slight delay was recorded in the accumulation of Bz^+ cells in the latter culture. DMSO-treated stably transfected MEL-C14 cells, however exhibited a delay of 18–24 h in the onset of differentiation, regardless if the proportion of differentiated Bz^+ cells in culture reached ~60% after 96 h (see Fig. 3C). These data indicated that stable transfection of MEL cells with *RPS5-MYC/His* delayed the onset of initiation of differentiation and reduced the proportion of terminally differentiated cells accumulated, as compared to control parental MEL cells.

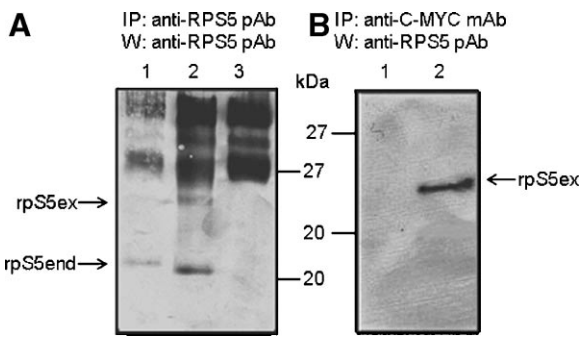

Fig. 2. Immunoprecipitation and Western blot analysis for the detection of endogenous RPS5 and recombinant RPS5-MYC/His proteins in parental and MEL-C14 cells. Highly inducible parental and MEL-C14 cells harvested from cultures, washed, lysed at 0–4°C and subsequently processed for immunoprecipitation followed by Western blot analysis. Briefly, 3 mg of cellular protein extracts derived from each culture were immunoprecipitated with either anti-RPS5 (C-terminal) pAb (**panel A**) and/or anti-MYC mAb (**panel B**), before Western blot analysis with anti-RPS5 (C-terminal) pAb to be carried out. The data obtained are shown above. **Lane 1** in panels A and B: parental MEL cells. **Lane 2** in panels A and B: MEL-C14 cells. **Lane 3** in panel A: immunoprecipitation with agarose G beads only without the addition of cellular extracts. Note that only MEL-C14 cultures produced the exogenous RPS5-MYC/His protein.

The developmental program of MEL cells has been clearly shown to operate via two discrete but closely related subprograms, one of which regulates hemoglobin production (hemoglobinization subprogram) and the other commitment to loss proliferation capacity (commitment subprogram) [Tsiftsoglou et al., 2003a,b]. Based on this concept, a more thorough kinetic analysis was carried out in cultures to demonstrate whether this delay is directly associated with one of the two subprograms. Parental MEL and MEL-C14 cells were employed in this experiment and grown continuously with DMSO for 96 h (4 days). At the end of this period, each culture was diluted with fresh medium to permit cells being able to proliferate at logarithmic phase of growth for more 72 h (3 days) in the absence of DMSO. As shown in Figure 3B, parental highly inducible MEL cells growing initially for 96 h (4 days) in the presence of DMSO, then diluted (1:10) in culture with inducer-free fresh medium and re-incubated for additional 72 h (3 days), reached the plateau phase of growth and remained at that level even after dilution. This was as expected, since it has been known that DMSO treatment causes irreversible loss of proliferation after terminal differentiation. Such DMSO-treated cell culture contained about 80% benzidine-positive cells after 96 h incubation (Fig. 3D). The proportion of hemoglobin-producing cells decreased, however, after 144 h (6 days) as expected, since the differentiated cells were able to transverse only for 4–5 divisions [Tsiftsoglou et al., 2003a], while the residual non-terminally differentiated cells (~20%) continue to divide and grow in the absence of DMSO. In the case of DMSO-treated MEL-C14 cells, a slower accumulation of Bz^+ cells occurred, while an

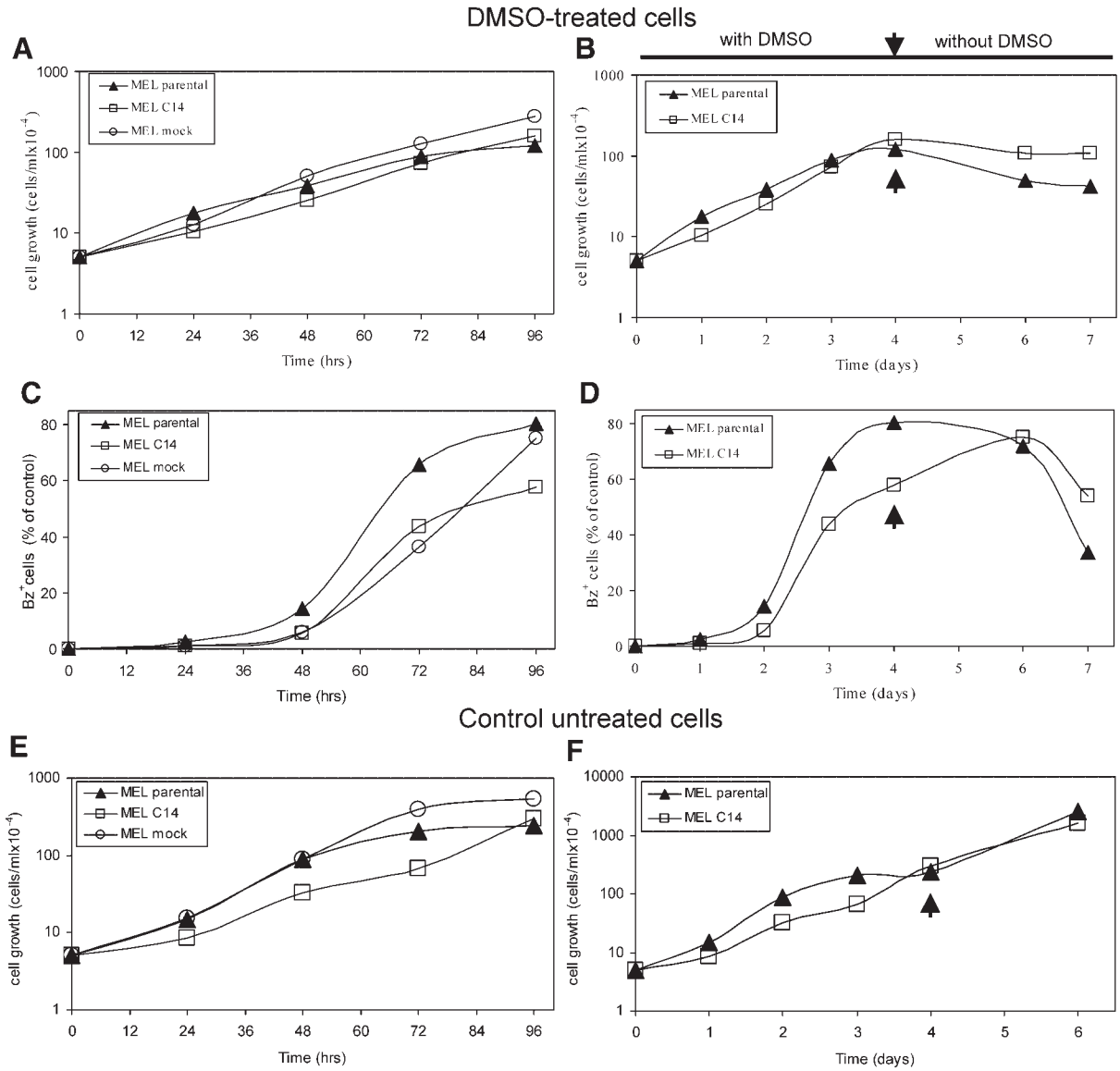

Fig. 3. Growth and differentiation kinetics of MEL-C14 cells treated in the absence or presence of DMSO. **Panels A and C**: Parental MEL cells were incubated in DMEM supplemented with 10% v/v FBS. Mock-transfected and *RPS5*-transfected MEL cells (MEL-C14) were grown in the same medium supplemented with 0.6 mg/ml G418 starting with 5×10^4 cells/ml, for 4 days in the presence of the chemical inducer DMSO (1.5% v/v). **Panels B and D**: Parental and C14 MEL cells were grown in culture as indicated above except that upon the fourth day the cultures were diluted 1:10, with the addition of fresh medium in the absence of DMSO. **Panel E**: Parental, mock-transfected and C14 MEL cells were grown in culture in the absence of inducer in conditions indicated above for panels A and C. **Panel F**: Parental and C14 MEL cells were grown as indicated in panel E except that upon the fourth day the cultures were diluted 1:10 with the addition of fresh medium. At time intervals indicated, sample was taken from each culture for the assessment of cell growth (panels A, B, E and F) and differentiation (hemoglobin-producing; Bz^+ cells) (panels C and D). The data shown represent the mean value of an experiment repeated twice.

additional 48 h (2 days) period was needed to reach the maximum level of differentiation (~80%) seen in parental MEL cells (Fig. 3D). The observations mentioned above according to Figure 3A–D were further supported when the growth rates of untreated parental, mock-transfected, and MEL-C14 cell cultures were recorded (Fig. 3E,F). A lower growth rate was observed in MEL-C14 cells grown in inducer-free medium (Fig. 3E), whereas upon dilution (1:10) of three cultures with fresh inducer-free medium the cells divide and grow, as expected. This comparative kinetic analysis indicates that stably transfected MEL-C14 cells are capable to differentiate even with a delay in the initiation of differentiation.

To rule out the possibility that the delay seen in the onset of differentiation of stably transfected MEL-C14 is not attributed specifically to DMSO but to the changes provoked by the transfection of *RPS5-Myc/His* cDNA, these cells were exposed in addition to two structurally different inducers, HMBA and UDP-4. According to the data obtained with these agents, a delay in the initiation of differentiation was also recorded (Fig. 4B,D). These results taken together indicate that stable transfection of *RPS5-Myc/His* cDNA dismantles the initiation of differentiation regardless to the nature of the chemical inducer employed. To further support this conclusion, we attempted to rule out another possibility, that this effect of transfection is not attributed to clonal cell variation. We selected another clone of stably transfected MEL cells (MEL-C56) expressing the recombinant RPS5-MYC/His protein, as mentioned above. These cells express both the exogenous *RPS5-Myc/His* transcripts to a level similar to that of MEL-C14 cells and that of endogenous *RPS5* as in parental MEL cells (Fig. 5A). The production of recombinant RPS5-MYC/His protein in the cytoplasm of MEL-C56 cells was verified by immunoprecipitation and Western blot analysis (Fig. 5B). Interestingly enough, initiation of differentiation of MEL-C56 cells upon treatment with either HMBA or DMSO, was also delayed in a manner similar to that seen for the MEL-C14 culture (Fig. 5D,F). No substantial change in the growth kinetics was observed under these conditions (Fig. 5C,E). These findings indicate that the delayed initiation of differentiation in two stably transfected MEL cell cultures (C14 and C56) results from changes induced by the transfection of *RPS5-Myc/His* rather than cell clonal variation and inducer effects (Fig. 5D,F). The *RPS5* gene expression is likely to be involved in the early stages of differentiation of MEL cells.

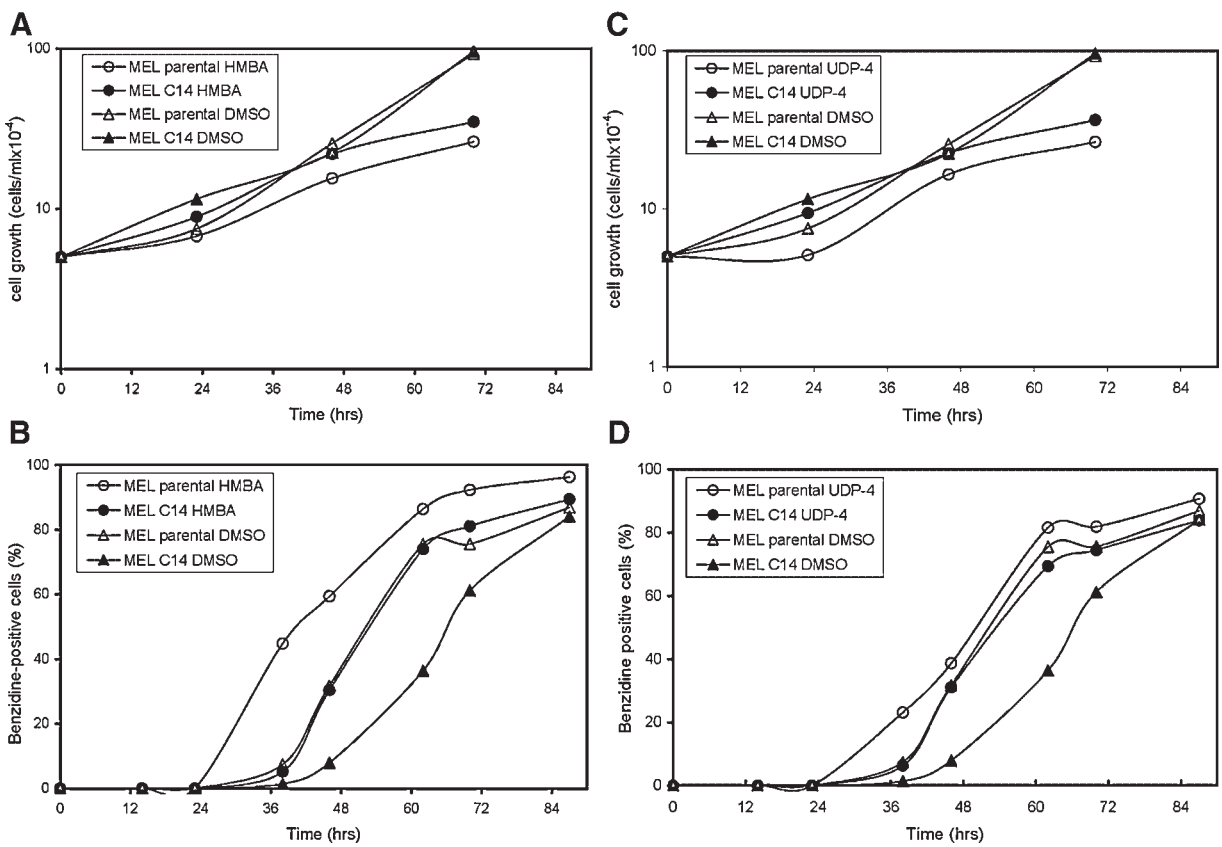

Fig. 4. Growth and differentiation kinetics of MEL-C14 treated with HMBA or UDP-4. MEL cells of various cultures were grown as shown under Figure 3 for 84 h in the presence of chemical inducer HMBA (5 mM) (**panels A and B**) and/or UDP-4 (0.25 mM) (**panels C and D**). Cells exposed to inducer DMSO (1.5% v/v) were used as control cultures. At time intervals indicated, sample was taken from each culture for the assessment of cell growth (panels A and C) and differentiation (hemoglobin-producing; Bz^+ cells) (panels B and D). The data shown represent the mean value of an experiment repeated twice.

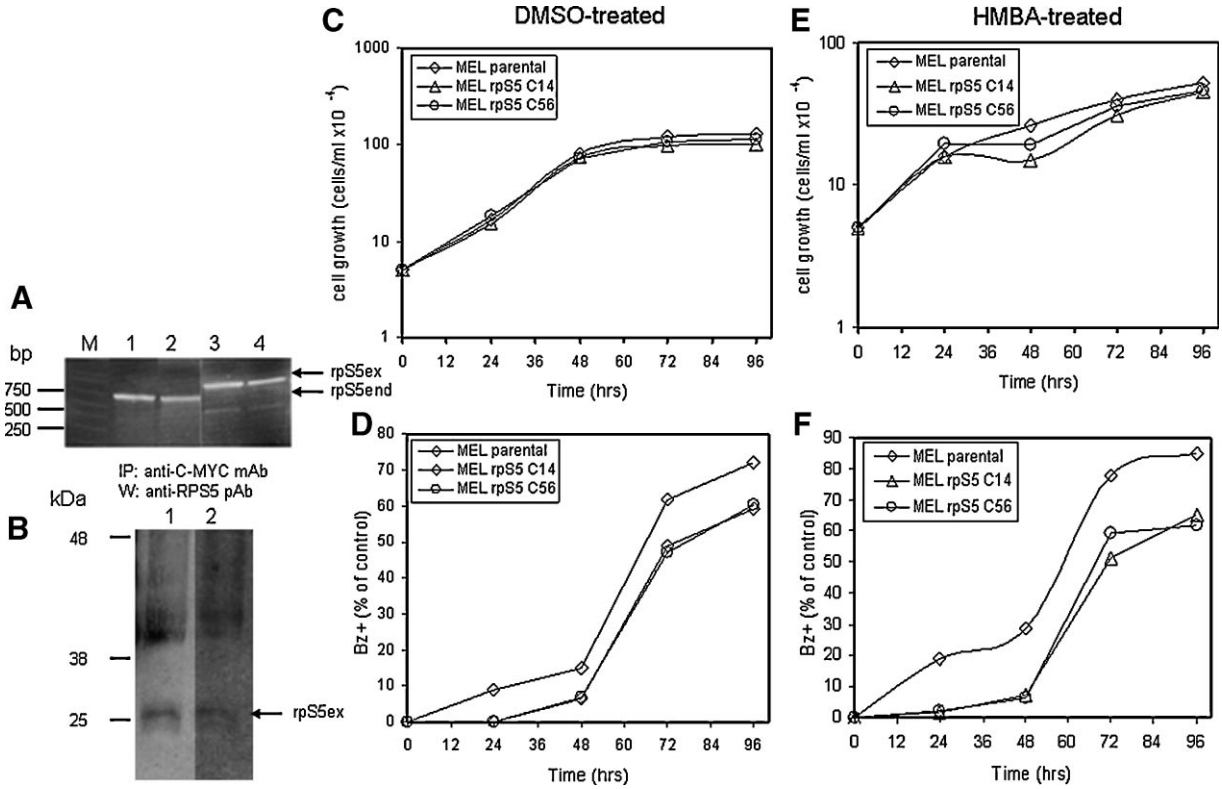

Fig. 5. Growth and differentiation kinetics as well as assessment of endogenous RPS5 and recombinant RPS5-MYC/His expression in MEL-C56 cells. Parental MEL, MEL-C14, and MEL-C56 cell cultures were grown as indicated under Figure 1. Total cytoplasmic RNA and protein extracts isolated from these cultures were assessed for endogenous RPS5 and recombinant RPS5-MYC/His expression by RT-PCR and Western blot analysis. **Panel A**: Total cytoplasmic (0.5 μg) isolated RNA was analyzed by RT-PCR by using two different pairs of primers as indicated under Materials and Methods to determine the steady-state levels of endogenous and exogenous *RPS5* mRNA in parental MEL (**lane 1**) MEL-C56 (**lanes 2 and 4**) and MEL-C14 (**lane 3**) cells. **Panel B**: Isolated protein cellular extracts (3 mg) from each culture were immunoprecipitated with anti-MYC mAb, before Western blot analysis with anti-RPS5 (C-terminal) pAb. **Lane 1**: MEL-C14 cells. **Lane 2**: MEL-C56 cells. Note that the exogenous RPS5-MYC/His protein has been detected in both cell cultures. **Panels C–F**: Parental MEL, MEL-C14, and MEL-C56 cells starting with 5×10^4 cells/ml in culture were incubated in DMEM supplemented with 10% v/v FBS (parental MEL), as well as with DMEM supplemented with 10% v/v FBS and 0.6 mg/ml G-418 (MEL-C14 and MEL-C56) for 96 h in the presence of the chemical inducer DMSO (1.5% v/v) (Panels C and D) and/or HMBA (5 mM) (Panels E and F). Every 24 h, sample was taken from each culture for the assessment of cell growth (Panels C and E) and differentiation (hemoglobin-producing; Bz^+ cells) (Panels D and F). The data shown represent the mean value of an experiment repeated twice.

Differential Cytoplasmic Accumulation of *RPS5* and β^{major} Globin RNA Transcripts in Parental MEL and MEL-C14 Cells upon Exposure to DMSO

To detect any changes occurring in the steady-state level of *RPS5* RNA transcripts during differentiation of parental MEL and MEL-C14 cells in the presence of DMSO, cytoplasmic RNA was analyzed by Northern blot hybridization using β^{major} globin gene as an internal positive control to assess MEL cell erythroid maturation. As shown in Figure 6A, the cytoplasmic accumulation of *RPS5* RNA transcripts encoded by the endogenous *RPS5* gene decreased upon induction of differentiation of parental MEL cells by DMSO, while β^{major} globin RNA transcripts were gradually accumulated in the cytoplasm of differentiating cells after 36 h exposure to DMSO (Fig. 6C), as expected by earlier studies [Vizirianakis et al., 1999]. A slight decrease in the level of endogenous *RPS5* RNA transcripts was detected in MEL-C14 cells after 96 h in culture with DMSO (Fig. 6B). It is noteworthy, however, to report that the cytoplasmic accumulation of β^{major} globin RNA transcripts in MEL-C14 cells delayed ~24 h as compared to parental MEL cells (Fig. 6C,D). Similar to parental MEL cells pattern in the cytoplasmic accumulation of RPS5 and β^{major} globin mRNAs were also detected in mock-transfected MEL cell cultures (data not shown). Moreover, the level of RPS5

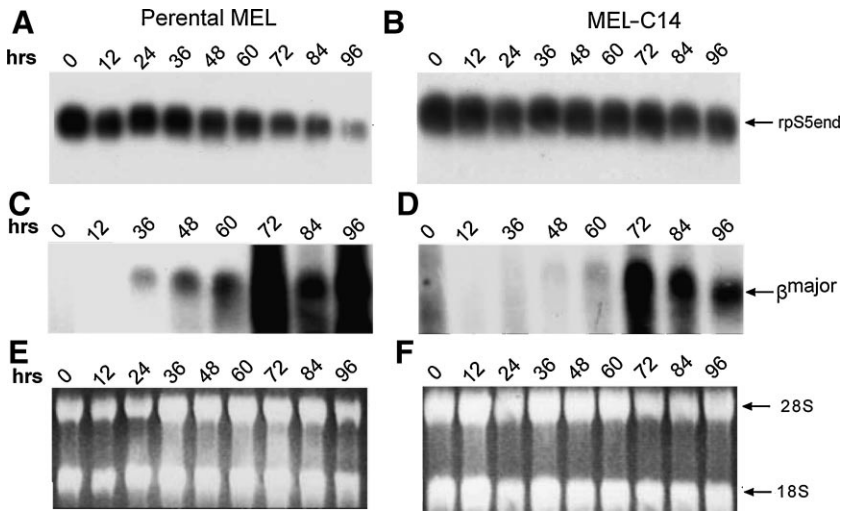

Fig. 6. Accumulation of the steady-state levels of *RPS5* and β^{major} globin RNA transcripts in parental MEL and MEL-C14 cells treated with DMSO. Parental MEL and MEL-C14 cells were incubated in DMEM supplemented with 10%v/v FBS as well as with DMEM supplemented with 10%v/v FBS and 0.6 mg/ml G418, respectively, in the presence of DMSO (1.5% v/v). At time intervals indicated (numbers above the panels), cells were removed from each culture and total cytoplasmic RNA was isolated. Cytoplasmic RNA (10 μg) from each sample was electrophoretically separated on 1% agarose gel, transferred onto a nylon membrane, and hybridized at 65°C with [^{32}P]-labeled DNA fragments encoding either for mouse RPS5 (715 bp) (**panels A and B**) or β^{major} globin genes (~7.3 kb genomic DNA fragment containing the entire gene) (**panels C and D**). **Panels E and F**: The ethidium bromide staining pattern of the isolated cytoplasmic RNA transcripts was shown, whereas the position of 28S and 18S rRNAs is indicated by the arrows.

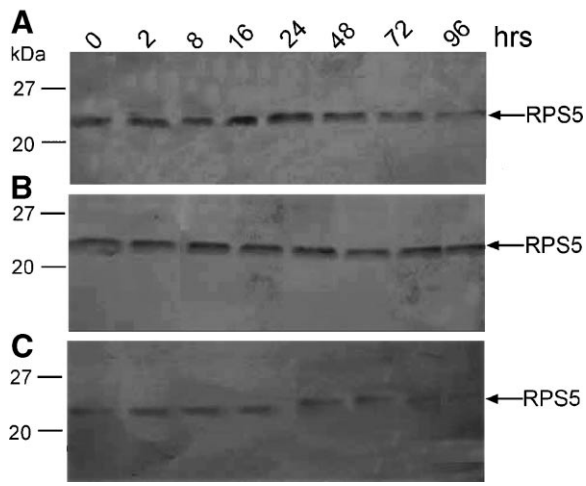

Fig. 7. Western blot analysis for the assessment of RPS5 protein level in parental, MEL-C14, and mock-transfected MEL cell cultures grown in the presence of DMSO. Parental MEL cell cultures were grown in DMEM supplemented with 10%v/v FBS in the presence of DMSO (1.5% v/v). Similarly, MEL-C14 and mock-transfected MEL cell cultures were incubated at the same conditions except that 0.6 mg/ml G418 was included. At times indicated (numbers above the panels), cells were removed from culture and total cellular protein extracts were prepared as described under Materials and Methods. Protein (30 μg) from each sample preparation was then analyzed for the assessment of RPS5 protein levels by immunoblotting. Anti-RPS5 (C-terminal) pAb (1:1,000 dilution) was used for the immunoblotting. **Panel A**: Parental MEL cells. **Panel B**: MEL-C14 cells. **Panel C**: Mock-transfected MEL cells.

protein decreased in differentiating parental and mock-transfected MEL cells after 72 h (Fig. 7A and C, respectively), while it remained constant in differentiating MEL-C14 cells (Fig. 7B). These data taken together suggest that the delay in the onset of differentiation seen in MEL-C14 cells exposed to DMSO prevented the gradual decline of endogenous *RPS5* RNA transcripts and protein to occur.

MEL-C14 Cells Undergoing Differentiation Exhibit Changes in the Level of CDKs and Delay Their Cell Cycle Arrest at G_1/G_0 Phase

By knowing that commitment to erythroid differentiation of MEL cells is correlated with prolongation of G_1 phase of cell cycle [Terada et al., 1977; Geller et al., 1978] and growth arrest at G_1/G_0 [Tsiftsoglou et al., 2003a,b], we attempted to correlate the differentiation potential of parental MEL and MEL-C14 cultures with changes in cell cycle and the expression profile of CDK2, CDK4, and CDK6. Flow cytometric analysis of cell cycle phases indicated that the majority of cells from both untreated parental and MEL-C14 cultures are actively proliferating even after 72 h, whereas no substantial difference in the percentage of cells being at different phases was shown for the

entire course of experiment (Fig. 8). In contrast, the percentage of cells entering the G_1/G_0 phase after 24 h exposure to HMBA was approximately 47.5 and 35.0% in parental MEL and MEL-C14 cell cultures, respectively (Fig. 8). At later times (48 and 72 h), such cultures did not exhibit substantial differences in the percentage of cells at G_1/G_0 phase. These data imply that a delay in the number of cells entering the G_1/G_0 phase of cell cycle occurs within the early 24 h of the initiation of MEL cell differentiation program in vitro.

Assessment of protein level of CDKs and GATA-1 (used as positive control for erythroid MEL differentiation) during the entire course of differentiation indicated that the level of CDK2 protein decreased 4–8 h following induction of differentiation in both parental and mock-transfected MEL cells (Fig. 9A and E, respectively), while it remained constant in MEL-C14 culture (Fig. 9I). Interestingly enough, two additional bands of CDK2 were detected in differentiating MEL-C14 cells, one of which was of lower molecular weight (Fig. 9I). In the case of CDK4, its level remains constant in differentiating parental (Fig. 9B) and mock-transfected (Fig. 9F) MEL cells, while decreased in MEL-C14 cells (Fig. 9J) after 16–24 h in culture. Finally, the level of CDK6 decreased in each one of the three inducer-treated cultures employed although by different time; after 24 h in parental (Fig. 9C) and mock-transfected (Fig. 9G), whereas as soon as 8 h in MEL-C14 (Fig. 9K). Under these conditions, the level of GATA-1 transcription factor remained constant (Fig. 9D,H,L). These data taken together indicate that the level of CDK6 was extensively affected in MEL-C14 cells. Whether its selective decay is directly or indirectly related to delayed differentiation of MEL-C14 cells remains to be seen.

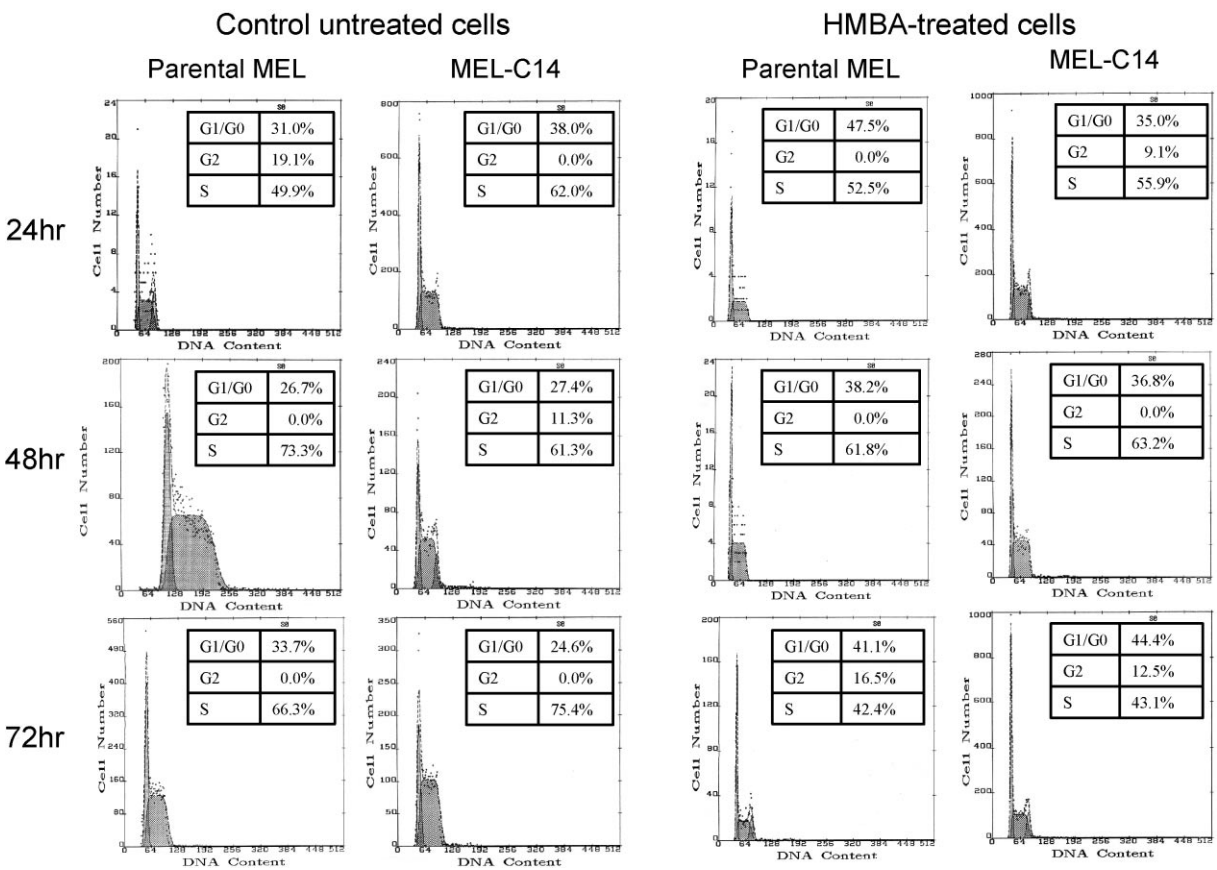

Fig. 8. Kinetics of cell cycle phase distribution of parental and MEL-C14 cells grown in the absence or presence of HMBA parental MEL and MEL-C14 cells incubated in the absence or the presence of HMBA (5 mM) harvested from culture at certain time intervals and processed for single parameter analysis of DNA content by using the DNA-Prep Coulter® Reagents Kit as indicated under Materials and Methods. In the panels shown above, the single parameter histograms of linear fluorescence signals of PI obtained are shown.

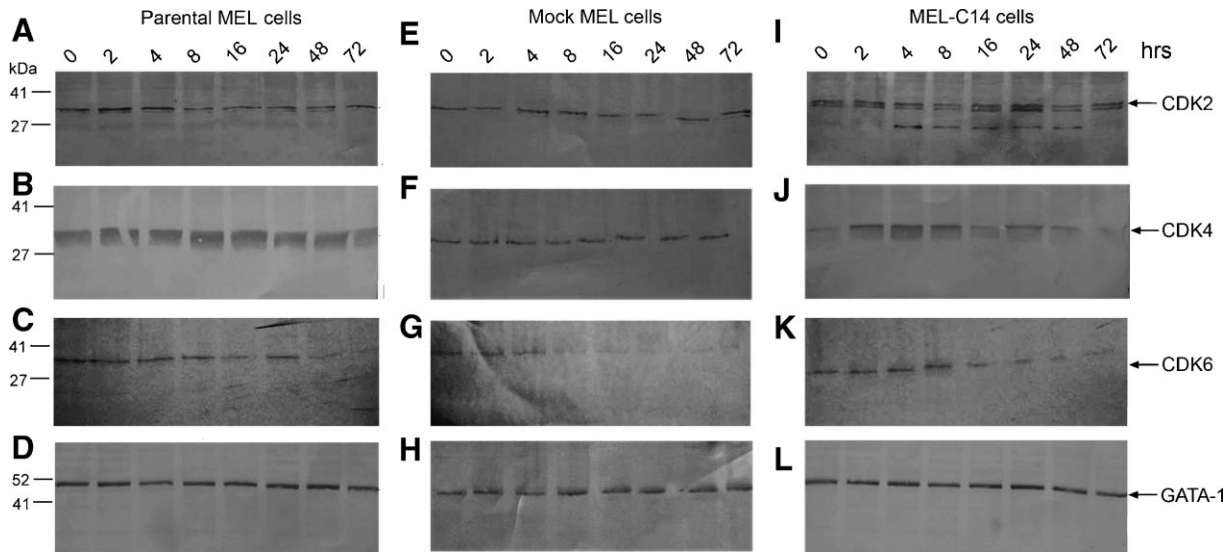

Fig. 9. Western blot analysis of the accumulation of CDK2, CDK4, and CDK6 proteins during differentiation of parental, mock, and stably transfected C14 MEL cultures exposed to DMSO. Parental MEL cells (**panels A–D**) were grown in DMEM supplemented with 10% v/v FBS in the presence of DMSO (1.5% v/v) and mock-transfected (**panels E–H**) as well as C14 (**panels I–L**) MEL cultures were grown separately in the same medium supplemented with 0.6 mg/ml G418. At times indicated (numbers above the panels), cells were harvested from culture and total cellular protein extracts were prepared as described under Materials and Methods. Protein (100 μg; panels A–C, E–G, and I–K) or 50 μg (panels D, H, and L) from each sample preparation was then analyzed for the assessment of CDK2 (panels A, E, and I), CDK4 (panels B, F, and J), and CDK6 (panels C, G, and K) protein levels by immunoblotting. Anti-CDKs antibodies were used in 1:1,000 dilution. In panels D, H, and L, the protein levels of GATA-1 were assessed by using an anti-GATA-1 antibody (1:400 dilution) and was used as a positive control of erythroid MEL cell differentiation.

DISCUSSION

Induction of MEL cell differentiation program is initiated via commitment through highly coordinated processes involving interactions of specific transcription factors with consensus DNA sequences activating and/or repressing the expression of genes [Tsiftsoglou et al., 2003a,b]. We have previously shown that MEL cell differentiation induced by chemical agents is accompanied by inactivation of the transcription of rRNAs genes, as well as significant decrease in *RPS5* and *RPL35a* gene expression as part of their developmental program [Tsiftsoglou et al., 1982; Vizirianakis et al., 1999; Pappas et al., 2001]. The latter is based on inhibitor studies. In particular, exposure of MEL cells to N^6-methyladenosine, (N^6mAdo), a blocker of commitment to erythroid maturation, did not allow repression but maintenance of active transcription of both *RPS5* and *RPL35a* genes to comparable levels as those seen in non-differentiating MEL cells. The notion that ribosomal proteins may share regulatory role in cell differentiation systems is further supported by the fact that down-regulation of ribosomal protein *RPL3* gene expression was observed upon induction of differentiation of human promyelocytic HL-60 cells [Mailhammer et al., 1992]. Furthermore, reduction in the relative level of *RPL35*, *RPL31*, *RPL27*, and *RPL21* mRNAs was recorded in both human erythroleukemia K-562 cells undergoing megakaryocytic differentiation pathway [Lin et al., 1994] and in differentiating intestinal cells [Maheshwari et al., 1993]. Moreover, targeted disruption of mouse *PRS19* gene has been found to be lethal prior to implantation by disrupting erythropoiesis and development [Matsson et al., 2004] and that RPS3 has been shown to serve as a potential receptor for hybrid polar inducers that initiate MEL cell differentiation in vitro [Webb et al., 1999]. These studies suggested extra-ribosomal functions of ribosomal proteins in growth and differentiation, in addition being involved in protein biosynthetic machinery.

In this study, we attempted to investigate the potential role of *RPS5* gene expression on the initiation of erythroid differentiation of MEL cells and cell cycle arrest. We have established two stably transfected MEL cell clones (MEL-C14 and MEL-C56) and assessed their kinetics of growth and differentiation

induced by different inducing agents. Moreover, we attempted to demonstrate whether the stable transfection of *RPS5* cDNA affects: (a) cell cycle arrest at G_1/G_0 phase as well as the expression level of CDKs that drive cell cycle, and (b) the onset of initiation of differentiation. The fact that the two stably transfected MEL-C14 and MEL-C56 clones studied were found to express recombinant RPS5 and exhibit similar delay in the initiation of differentiation upon exposure to chemical inducers, indicates that RPS5 may share an extra-ribosomal function by getting involved in erythroid cell differentiation.

Induction of commitment to MEL cell differentiation has been reported to be associated with prolongation of G_1 phase of the cell cycle [Terada et al., 1977; Geller et al., 1978] and that terminal differentiation is accompanied with arrest of the cell cycle in the G_1/G_0 phase [see for review Tsiftsoglou et al., 2003a,b]. Moreover, evidence exists to indicate that cyclin-dependent kinases (serine/threonine protein kinases) (CDK2, CDK4, and CDK6) play crucial role in cell cycle regulation of eukaryotic cells and that CDKs are involved in the initiation of commitment of MEL cells into terminal maturation [Hsieh et al., 2000; Matushansky et al., 2000a,b, 2003; Zhu and Skoultchi, 2001]. The expression level of CDK2 and CDK4 remained almost constant even at late stages of differentiation, whereas that of CDK6 declined very early before commitment of cells to terminal maturation occurs. These observations are quite interesting on light of evidence that CDK6 plays a key role in the blockade of differentiation [Matushansky et al., 2003] and the onset of the cell cycle exit in differentiated MEL cells is regulated by CDK2, CDK4, and CDKIs [Hsieh et al., 2000]. Overall, our findings indicate that the pattern of CDKs (CDK2 and CDK6 in particular) expression in differentiating MEL-C14 cultures has been altered by stable transfection of *RPS5-Myc/His* cDNA. We assume that these changes may be closely related to the delay in the initiation of differentiation.

The precise mechanism(s) and the molecules being involved in the initiation of commitment to terminal erythroid differentiation are still not known. However, evidence exists to indicate that protein molecules and RNA transcripts are involved to drive cells into commitment state of differentiation [Housman et al., 1980; Tsiftsoglou et al., 2003a]. Moreover, recent experimental data show that alteration in the intracellular level of PU.1 transcription factor via RNAi-mediated silencing has driven MEL cells into erythroid maturation and growth arrest [Papetti and Skoultch, 2007]. These observations taken together with the data presented in this study indicate that *RPS5* transcripts and maybe RPS5 protein are somehow implicated in the initiation of differentiation, since deregulation of both alters the onset of MEL cell erythroid differentiation and the entrance of cells into cell cycle arrest. Moreover, it has been shown that CDKs are also involved [Hsieh et al., 2000; Matushansky et al., 2000a,b; Zhu and Skoultchi, 2001]. Our observation that CDK6 in particular is deregulated in inducible DMSO-treated parental and MEL-C14 cells is in agreement with the data provided by Matushansky et al. [2003]. It is premature, however at this time, to support that both RPS5 expression deregulation and CDK6 activity are directly or indirectly correlated and if they have effect on the PU.1 level.

Nevertheless, the results represented provide the first evidence showing that stable transfection and constitutive expression of *RPS5-Myc/His* cDNA delays the initiation of erythroid differentiation, promotes alterations in the distribution of cells in the G_1/G_0 phase and the protein levels of CDKs. Alternatively, one can assume that constitutive expression of recombinant RPS5 somehow attenuates cell cycle decisions of differentiating MEL cells guided by CDKs, thus delaying their entrance into G_1/G_0 phase arrest and consequently their initiation to erythroid differentiation program. This is an interesting observation suggesting that commitment decisions may be coupled with limitation of proliferation potential by the combined function of specific cytoplasmic factors in accordance with external stimuli brought by inducers of differentiation. The latter, must be also seen as a possibility in light of evidence showing that the transient accumulation of MEL cells in the G_1 phase of the cell cycle upon initiation of differentiation is followed by a re-entry of cells into a proliferative state, processes that are under the control of exogenous factors present in the serum and their endogenous cytoplasmic counterparts [Mencherini et al., 1992]. The question whether the delay in the onset of erythroid differentiation is attributed to constitutive expression of *RPS5* gene among other events needs extensive investigation by applying small interfering

RNA (siRNA) technology. In fact, it has been reported that knocking down *RPL13* gene expression via siRNA led to pronounced deregulation of cancer cell growth with significant G_1 and G_2/M arrest of the cell cycle [Kobayashi et al., 2006]. A similar approach is on progress in our laboratory.

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