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Suppression of Cyclin-Dependent Kinase 4 during Induced Differentiation of Erythroleukemia Cells

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Differentiation of murine erythroleukemia cells induced by hexamethylene bisacetamide (HMBA) is associated with accumulation of underphosphorylated retinoblastoma protein (pRB) and an increase in retinoblastoma (RB) gene expression. Here we show that HMBA causes a rapid decrease in the level of cyclin-dependent kinase 4 (cdk4) protein. This decrease results from decreased stability of the protein, while the rate of synthesis of the protein is not affected by HMBA. The decrease in the level of cdk4 protein is followed by suppression of the pRB kinase activity associated with cdk4. Cyclin D3, which can bind and activate cdk4, is increased in HMBA-induced cells and is found in complex with pRB and the transcription factor E2F. In uninduced cells cyclin D3 complexes with pRB and E2F are barely detected. At the later stages of differentiation, MEL cells become arrested in G_1 and cdk2 kinase activity is suppressed; this is accompanied by a decrease in the level of cyclin A and cdk2 proteins. Cells transfected with cdk4, which continue to overexpress cdk4 protein during culture with HMBA, are resistant to HMBA-induced differentiation. In contrast, overexpression of cdk2 protein does not inhibit induced differentiation. These findings suggest that suppression of cdk4 is a critical event in the pathway leading to terminal differentiation of erythroleukemia cells.

In eukaryotic cells, factors determining whether cells continue to proliferate or cease dividing and differentiate appear to operate during the G_1 phase of the cell cycle (47). Differentiation of murine erythroleukemia (MEL) cells induced by hexamethylene bisacetamide (HMBA) provides a model for investigating the molecular changes involved in generating the inducer-mediated signals to withdraw from the cell cycle and to express characteristics of the differentiated phenotype (37). In this study we provide evidence that cyclin-dependent kinase 4 (cdk4) is one critical target of the inducer-mediated signals which lead to commitment to terminal differentiation.

cdks, activated by association with cyclins, are important factors regulating cell cycle progression by phosphorylating various substrates (53). Among potential substrates, a highly likely substrate is a tumor suppressor gene product, retinoblastoma protein (pRB) (2, 22, 33, 35, 55). Introduction of pRB into pRB-deficient cells, during early G_1, inhibits the G_1-to-S transition, whereas injection of pRB during late G_1 or S can no longer inhibit DNA replication (17), suggesting that this protein is a negative regulator of the cell cycle which functions during G_1 (56). pRB is underphosphorylated in G_0/early G_1 and is progressively phosphorylated during mid- to late G_1 (5, 6, 8, 42). DNA tumor virus oncoproteins, such as simian virus 40 T antigen, adenovirus E1A, and papillomavirus E7, form complexes with pRB only in its underphosphorylated forms (7, 11, 36, 58). Complex formation seems to inactivate the growth-suppressive function of pRB, in part by blocking a binding domain of pRB for cellular proteins. The transcription factor E2F is one such cellular protein which can bind to underphosphorylated pRB, resulting in suppressed transcription of E2F-regulated genes (20, 54, 57). Among these are the genes for DNA polymerase α, dihydrofolate reductase, and thymidylate synthetase, which are required for DNA replication (45). Phosphorylation of pRB during mid- to late G_1 is accompanied by release of E2F from the E2F-pRB complex and by activation of transcription of the E2F-regulated genes.

Various combinations of cyclins and cdks interact with pRB at specific G_1 control points in mammalian cells (28, 34, 40, 43, 60). D-, E-, and A-type cyclins have been shown to regulate positively the G_1-to-S transition (3, 4, 16, 46, 49). Peak expression of cyclin E is near the G_1/S boundary (10, 29, 34) and is followed by accumulation of cyclin A in early S phase (48). When growth factor-deprived cells are stimulated to reenter the cell cycle, D-type cyclins (cyclins D1, D2, and D3) accumulate in early G_1, prior to the increase in the level of cyclin E (1, 40, 59), although different types of cycling cells display different patterns of expression of the three D-type cyclins (24, 40, 43, 44). These D-type cyclins can directly bind pRB (9, 12, 22). cdk4 associates with cyclins D1, D2, and D3, while cdk2 complexes with cyclins A, D2, D3, and E (10, 29, 38, 48, 61). A-, D-, and E-type cyclins have been demonstrated to promote hyperphosphorylation of pRB when cotransfected with pRB into pRB-deficient tumor cells, and this phosphorylation is accompanied by loss of the ability of pRB to block the G_1-to-S transition (9, 12, 21). Ectopic overexpression of cyclins D2 and D3, but not D1, has been shown to inhibit granulocyte differentiation of 32D myeloid precursor cells (23).

Phosphorylation of pRB seems to be one of the effects of the extracellular signals that induce cell cycle arrest and differentiation. In MEL cells undergoing HMBA-mediated commitment to differentiation, underphosphorylated forms of pRB accumulate, accompanied by an increasing amount of total pRB (50), which suggests that this protein may play a role in the recruitment of cells to terminal differentiation. Transforming growth factor β1 causes cell cycle arrest in G_1 accompanied by accumulation of underphosphorylated pRB (31). Transforming growth factor β1 inhibits synthesis of cdk4 (13) and prevents the activation of cyclin E/cdk2 kinase (30). Both of these changes in cdk activity could contribute to the accumulation of underphosphorylated pRB.

In this study we show that HMBA-induced differentiation of MEL cells involves a rapid decrease in the cdk4 protein level followed by suppression of pRB kinase activity associated with

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cdk4. The decrease in cdk4 protein level reflects a marked decrease in its stability, whereas its rate of synthesis is not affected. The level of cyclin D3 is increased by HMBA, and this protein forms complexes with pRB and E2F as cells are progressively committed to differentiation. Such complexes of cyclin D3 with pRB and E2F are barely detectable in uninduced MEL cells. Transfection of MEL cells with cdk4, but not cdk2, renders the cells relatively resistant to HMBA-induced differentiation. Taken together, the present data suggest that suppression of cdk4 is a critical event during induced terminal differentiation of MEL cells.

MATERIALS AND METHODS

Cell culture, elutriation, flow cytometry, and differentiation assays. MEL cells, DS19/Sc9 derived from 745A cells, were placed in culture at 10^5 cells per ml in α minimal essential medium supplemented with 10% fetal calf serum. After 36 h of incubation, cultures achieved a density of about 8×10^5 cells per ml; 6×10^8 cells were loaded onto a Beckman JE-6B elutriation rotor, and fractions were elutriated by stepwise increases in pump flow (15). For analysis of DNA content, cells were stained with propidium iodide and subjected to flow cytometry. Percentages of cells in G_1, S, and G_2/M were determined with the CELL FIT program (Becton Dickinson). Assays for commitment to differentiation and for hemoglobin content by using the benzidine stain were performed as previously described (14).

Antibodies. Anti-cyclin A antiserum was a gift from J. Pines and T. Hunter (48), and COOH terminus-specific anti-cdk2 antibodies were obtained from UBI, Lake Placid, N.Y. Anti-cyclin D2 antibodies and anti-cyclin E antibodies were raised in rabbits immunized with glutathione *S*-transferase (GST)–cyclin D2 and GST-cyclin E fusion proteins, respectively. The entire coding region of mouse cyclin D2 (CYL2) cDNA (24, 41) was ligated into the pGEX-3X plasmid vector (Pharmacia). Bacteria transformed with pGEX-2T carrying human cyclin E cDNA were provided by A. Koff (28). GST-fusion proteins were purified from the transformed bacteria by using glutathione-Sepharose (Pharmacia) as specified by the manufacturer. Antisera raised to GST-cyclins D2 and E were affinity purified by using Affi-Gel 10 (Bio-Rad) coupled to polyhistidine-tagged cyclins purified from bacteria carrying pTrcHis-cyclins. Anti-cyclin D3 antibodies were raised in rabbits immunized with a synthetic peptide, NH_2-CPSQTSTPTDVTAIHL-COOH, which corresponds to the COOH terminus of cyclin D3 (40), and then affinity purified by using Affi-Gel 10 coupled to the antigen peptide. Murine cdk4 cDNA (38) was amplified by PCR from reverse-transcribed MEL cell RNA and ligated into plasmid pTrcHis. Anti-cdk4 antibodies used for immunoblotting were raised in rabbits immunized with polyhistidine-tagged mouse cdk4 and affinity purified as described above. COOH terminus-specific anti-cdk4 antiserum used for the cdk4 kinase assay was provided by C. J. Sherr (39). Affinity-purified antibody to the same cdk4 peptide was obtained from Santa Cruz Biotechnology. The specificity of all the antibodies was tested by immunoprecipitation of cyclins and cdks which were translated in vitro from synthetic mRNAs with reticulocyte lysate (Stratagene).

Immunoblotting. Cells were lysed in Nonidet P-40 (NP-40) lysis buffer (0.5% NP-40, 50 mM Tris-HCl [pH 8.0], 0.25 M NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM PMSF, 5 μg of aprotinin per ml, 5 μg of leupeptin per ml, 10 μg of L-1-tosylamide-2-phenylmethyl chloromethyl ketone per ml). Proteins (50 μg) were analyzed on a gel with 12.5% acrylamide, transferred to nitrocellulose filters at 45 V for 16 h, and then incubated with primary antibodies. Specific proteins were visualized by using an enhanced chemiluminescence system (Amersham). pRB was detected by using monoclonal antibody pMG3-245 (Pharmingen), biotinylated anti-mouse immunoglobulin G (IgG) antibody (Vector Laboratories), and [^{125}I]streptavidin as described previously (26). The autoradiogram was scanned by using the LKB 2202 Ultrascan Densitometer.

Pulse-chase analysis. Following culture without and with 5 mM HMBA for 48 h, 2×10^7 cells were washed and resuspended in 5 ml of methionine-free medium containing 10% dialyzed fetal calf serum. After incubation for 20 min at 37°C, 2.5 mCi of [^{35}S]methionine (1,175 Ci/mmol; NEN) was added to the culture and incubation was continued for 20 min at 37°C. Cells were recovered by centrifugation and resuspended in 50 ml of fresh medium supplemented with 300 mg of nonradioactive methionine (Sigma) per liter and 10% fetal calf serum. At different times during culture up to 360 min, extracts were prepared in NP-40 lysis buffer and their protein content was measured. Protein (100 μg) was immunoprecipitated with COOH terminus-specific anti-cdk4 antiserum and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12.5% acrylamide gel. Incorporation of radioactive methionine in cdk4 protein was quantitated by densitometric analysis following fluorography.

cdk4 and cdk2 kinase assay. cdk4 kinase activity was assayed with GST-pRB as a substrate as described previously (39). GST-pRB was purified from bacteria transformed with pGEX-RB. Extracts (200 μg of proteins) were immunoprecipitated with 2 μg of affinity-purified antibody to the COOH-terminal peptide of cdk4 or normal rabbit IgG, and the immunoprecipitate was assayed for pRB kinase activity by using 0.2 μg of GST-RB as a substrate. For cdk2 kinase activity, extracts (100 μg of proteins) were immunoprecipitated with 10 μg of COOH terminus-specific anti-cdk2 antibody and assayed for kinase activity by using 1 μg of histone H1 as a substrate (25, 52). In both cdk4 and cdk2 kinase assays, preliminary studies confirmed that the rates of kinase reactions were linear for at least 30 min at 30°C and over the range of the amounts of protein extracts assayed. Therefore, the kinase reaction was performed at 30°C for 30 min and terminated by addition of SDS sample buffer and boiling. Incorporation of radioactive phosphate on the substrates was determined by SDS-PAGE with 10% acrylamide for cdk4 kinase and 12.5% acrylamide for cdk2 kinase and analysis of the dried gel on a Betascope model 603 blot analyzer (Betagen).

Analysis of protein complexes. For analysis of immune complexes by immunoblotting, extracts (1 mg of protein) prepared with NP-40 lysis buffer were immunoprecipitated with protein A-Sepharose which was coupled with anti-cyclin D3 antibodies or normal rabbit IgG by using 20 mM dimethylpimelimidate. After being washed with the lysis buffer, the immune complexes were dissociated with 100 mM glycine-HCl (pH 2.5) containing 0.05% NP-40 and subjected to immunoblotting.

Gel retardation assay. The gel retardation assay for E2F in cyclin D3 immune complexes was performed as described previously (32). Nuclear extract (750 μg of protein) was immunoprecipitated with anti-cyclin D3 antibodies in a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% glycerol, and 1% NP-40. Following adsorption onto protein A-Sepharose beads, the immune complexes were washed four times with the above buffer and once with a buffer containing 20 mM HEPES (pH 8.0), 100

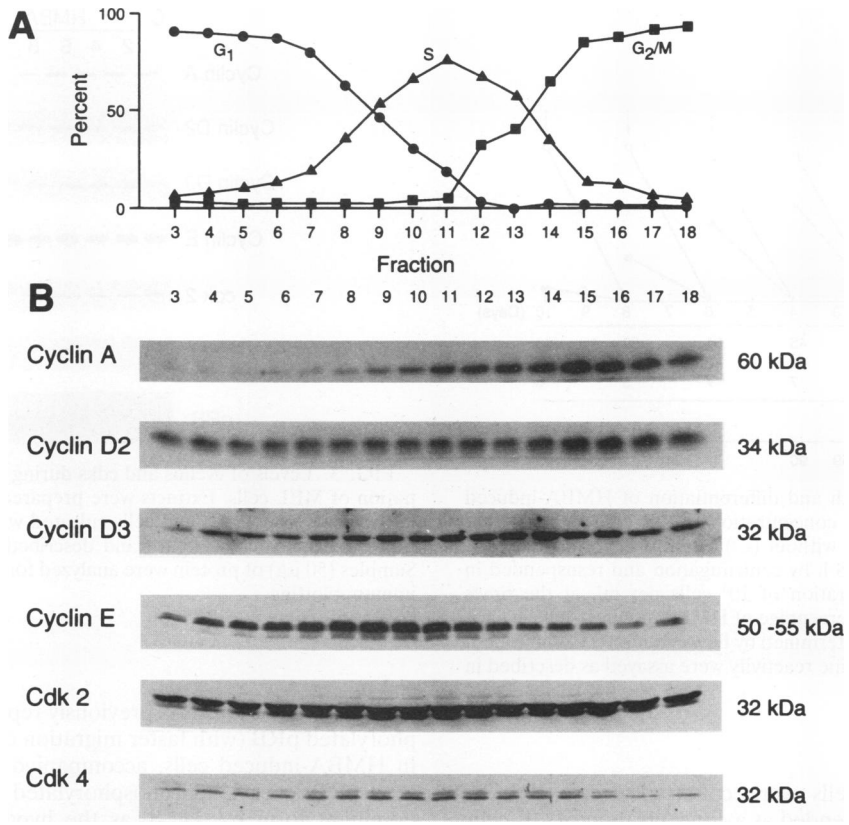

FIG. 1. Levels of cyclins and cdk proteins in MEL cells in different phases of the cell cycle. (A) Cell cycle distribution of elutriated MEL cells. Exponentially growing MEL cells were separated by elutriation, and the relative percentage of cells in G_1, S, and G_2/M was determined by flow cytometry after nuclear DNA had been stained with propidium iodide (15). (B) Immunoblots of cyclins A, D2, D3, and E, and cdk2, and cdk4. Molecular sizes of the proteins were estimated by comparing their electrophoretic migration with that of known standard proteins.

mM KCl, 0.2 mM EDTA, and 1 mM dithiothreitol. The protein complexes were disrupted by incubation on ice for 10 min with 0.8% sodium deoxycholate in a buffer containing 20 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% glycerol, 2 μg of salmon sperm DNA, and 20 μg of bovine serum albumin. NP-40 was added to 1.5%, and the samples were centrifuged. The supernatant was incubated with 0.5 ng of ^{32}P-labeled oligonucleotide E2F/E2 at room temperature for 10 min. The reaction products were separated on a 4% polyacrylamide gel. For the oligonucleotide competition experiments, a 100-fold excess of the unlabeled competitor oligonucleotides (wild type [Wt] and mutant type [Mt]) was added 10 min prior to addition of the radioactive oligonucleotide. The following double-stranded oligonucleotides were used (the sense strand is shown): E2F/E2, 5′-AGCTTGT TTTCGCGCTTAAATTTGAGAAAGGGCGCGAAACTA GTCA-3′; competitor Wt, 5′-TAGTTTTCGCGCTTAAATT TGA-3′; competitor Mt, 5′-TAGTTTTCGATATTAAATTT GA-3′.

DNA transfection. Murine cdk4 cDNA prepared from MEL cells was ligated into pRc/CMV (Invitrogen). Plasmid pRc/CMV-cdk2 was a gift from M. E. Ewen. MEL cells (4×10^6) in the logarithmic growth phase were transfected with 25 μg of plasmid by using electroporation at 200 V and 960 μF. Following a 48-h culture, neomycin-resistant clones were selected by growth for 2 weeks in medium supplemented with 1 mg of G418 (Gibco) per ml and maintained in the same medium. Clones were screened for overexpression of cdk4 or cdk2 protein by immunoblotting.

RESULTS

Cell cycle-related expression of cyclins and cdks. We first determined the changes in the levels of cyclins D2, D3, E, and A and cdk2 and cdk4 proteins at several stages of the cell cycle in uninduced MEL cells. Cyclin D1 protein was not detected in MEL cells (data not shown). Extracts were prepared from cells fractionated by centrifugal elutriation (Fig. 1A). The levels of cyclins D3, E, and A fluctuated during the cell cycle, whereas the level of cyclin D2 protein changed little, if at all (Fig. 1B). The level of cyclin E was highest at the transition from G_1- to S-enriched cell fractions. Cyclin D3 protein reached its maximum level in S-enriched cell fractions. The level of cyclin A protein increased as the proportion of S-phase cells increased, peaking in G_2/M-enriched fractions, as described previously (25). These findings with respect to cyclins E, D3, and A protein levels are consistent with observations made when using HeLa and MANCA cells (10, 29, 44, 48). On immunoblots for cdk2, two bands were detected with slightly different migration on SDS-PAGE. The protein band with the faster migration, which is presumably an active form of cdk2 phosphorylated on its Thr-160 residue (18), is most remarkable in S-enriched fractions. Like cyclin D3, the level of cdk4 protein reached its peak in S-enriched fractions.

Cell growth and differentiation in HMBA-induced MEL cells. MEL cells growing in suspension at a logarithmic rate were transferred at a concentration of 10^5 cells per ml to fresh medium without and with 5 mM HMBA (Fig. 2). To prevent density-induced growth arrest (which was observed at about 2

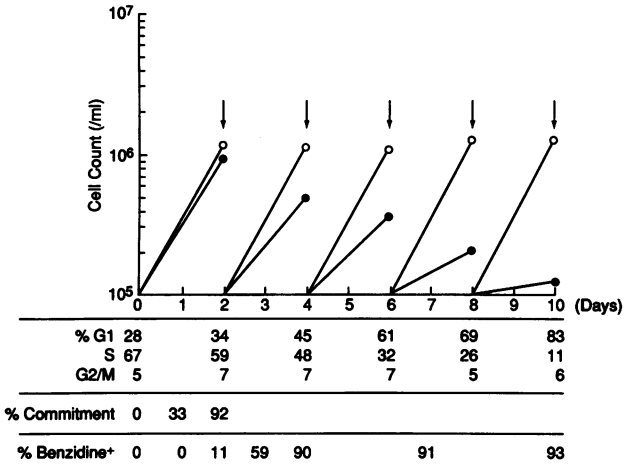

FIG. 2. Kinetics of growth and differentiation of HMBA-induced MEL cells. MEL cells at a concentration of 10^5 cells per ml were placed in suspension culture without (○) or with (●) 5 mM HMBA. Cells were collected every 48 h by centrifugation and resuspended in fresh medium at a concentration of 10^5 cells per ml, at the times indicated with arrows. The percentage of HMBA-induced cells in each phase of the cell cycle was determined by flow cytometry. Commitment to differentiation and benzidine reactivity were assayed as described in the text.

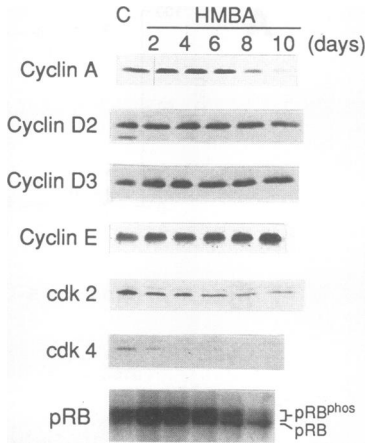

FIG. 3. Levels of cyclins and cdks during HMBA-induced differentiation of MEL cells. Extracts were prepared from uninduced cells in logarithmic growth (C) and cells cultured with 5 mM HMBA for the times indicated in the figure and described in the legend to Fig. 2. Samples (50 μg) of protein were analyzed for cyclins, cdks, and pRB by immunoblotting.

× 10^6 cells per ml), cells were collected every 48 h by centrifugation and resuspended at a concentration of 10^5 cells per ml in fresh medium without and with HMBA. The doubling time of cells in culture without HMBA was approximately 12 h and was not affected by repeated centrifugation. The doubling time of cells in culture with HMBA increased progressively after each dilution; this increase is associated with an increase in the proportion of cells in G_1. Essentially no cell growth in the presence of HMBA was observed after day 8, and at day 10 over 80% of cells were in G_1.

Commitment to terminal differentiation is defined as the irreversible capacity to express the differentiated phenotype, including hemoglobin accumulation and loss of proliferative capacity, despite removal of the inducer (14, 19). Over 90% of cells cultured with HMBA became committed by 48 h, as previously described (14). Hemoglobin accumulation was observed by 4 days in over 90% of induced cells.

Changes in levels of cyclins and cdks during HMBA-induced differentiation. We next determined the levels of the several cyclin and cdk proteins in MEL cells cultured with HMBA under the conditions described for Fig. 2. In control cells cultured without HMBA, no changes in levels of cyclin and cdk proteins were detected (data not shown). After exposure of cells to HMBA, cdk4 showed the most rapid decrease in protein level (Fig. 3). A fall in the level of cdk4 protein was detected as early as 14 h after the onset of culture with HMBA (data not shown). The level of cdk4 protein decreased to less than 5% of the control level by day 4. The level of cyclin D3 showed a threefold increase by day 4 and remained high through 10 days of observation. The level of cyclin D2 increased to a lesser extent than did that of cyclin D3. The level of cyclin E was minimally affected in cells cultured with HMBA. A decrease in the level of cyclin A was detected by day 8; the level fell to less than 10% of the control level by day 10. The level of cdk2 protein decreased during HMBA-induced differentiation, with similar kinetics to that of cyclin A protein.

The immunoblot for pRB showed changes in pRB phosphorylation and level. As previously reported (50), underphosphorylated pRB (with faster migration on the gel) accumulates in HMBA-induced cells, accompanied by an increase in the total pRB level. Underphosphorylated pRB became the predominant form by day 8, as the hyperphosphorylated form (with the slower migration on the gel) decreased in abundance.

The stability of cdk4 protein is decreased in HMBA-induced cells. Although the amount of cdk4 protein in differentiating MEL cells is markedly reduced, we did not detect a decrease in cdk4 mRNA levels (data not shown), suggesting that the decrease in the level of protein involves posttranscriptional regulation. To examine this possibility, we carried out pulse-chase analysis of cdk4 protein. MEL cells, which had been cultured with and without HMBA for 48 h, were labeled with [^{35}S]methionine for 20 min, washed, and cultured with excess unlabeled methionine for up to 6 h (Fig. 4). Immunoprecipitation of cell extracts with anti-cdk4 antibody demonstrated that similar amounts of radioactive methionine were incorporated into cdk4 protein in uninduced and HMBA-induced cells during the 20-min period of labeling, suggesting that the rates of synthesis of cdk4 protein are comparable in cells cultured without and with HMBA. The pulse-chase experiments demonstrated exponential decay of radioactive methionine in cdk4, with a cdk4 protein half-life in HMBA-induced cells of 1.8 h, compared with 5.4 h in uninduced cells. These results suggest that in HMBA-induced cells cdk4 protein is synthesized normally but is rapidly degraded.

Changes in cdk4 and cdk2 kinase activities. We next examined changes in the pRB kinase activity of cdk4 during HMBA-induced differentiation. Cell extracts were immunoprecipitated with anti-cdk4 antibodies, and the immune complex was assayed for its kinase activity by using a purified GST-pRB fusion protein as substrate (39). The pRB kinase activity immunoprecipitated with cdk4 from HMBA-induced cells was suppressed to 65% of the control level by day 4 (Fig. 5A). The activity decreased further, to 25% of the control level, by day 10.

Immunoprecipitation with normal rabbit IgG did not lead to significant kinase activity. The kinase activity of immunoprecipitated cdk2, assayed with histone H1 as the substrate,

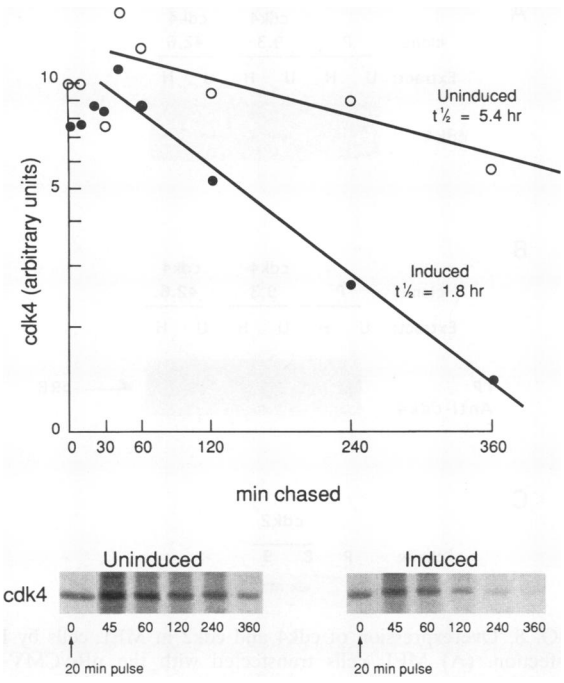

FIG. 4. Pulse-chase analysis for cdk4 protein. MEL cells, cultured without (uninduced) and with (induced) 5 mM HMBA for 48 h, were labeled with [^{35}S]methionine for 20 min. Following the period of labeling, the cells were washed and placed in culture with fresh medium supplemented with 300 mg of nonradioactive methionine per liter. Extracts were prepared after incubation for the indicated times and immunoprecipitated with anti-cdk4 antibody. cdk4 protein visualized by SDS-PAGE and fluorography is shown below the graph. Radioactivity incorporated into cdk4 was quantitated by densitometric analysis and plotted on a semilogarithmic scale.

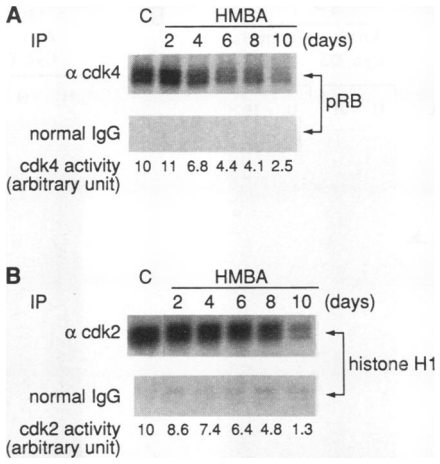

FIG. 5. Protein kinase activities associated with cdk4 and cdk2 in HMBA-induced MEL cells. (A) Extracts from uninduced cells in logarithmic growth (C) and cells cultured with 5 mM HMBA for the times indicated as described in the legend to Fig. 2 were immunoprecipitated with anti-cdk4 antibody or with normal rabbit IgG as a negative control. The immune complex was assayed for kinase activity by using pRB fused to GST as a substrate. The autoradiographic exposure time was 3 h. (B) Extracts were immunoprecipitated with anti-cdk2 antibody or with normal rabbit IgG, and the immune complex was assayed for kinase activity with histone H1 as a substrate. The autoradiographic exposure time was 1 h.

decreased gradually to 13% of the control value by day 10 (Fig. 5B).

Cyclin D3 forms complexes with pRB and E2F. It has been shown that pRB can complex with cyclin D3 in vitro (9, 12, 22). We examined whether the increased cyclin D3 and pRB levels are found in heteroprotein complexes during induction.

To determine if pRB-cyclin D3 complexes are formed during differentiation of MEL cells, extracts were prepared after 72 h of culture without and with HMBA, and the extracts were immunoprecipitated with anti-cyclin D3 antibodies. The precipitated immune complexes were analyzed by immunoblotting with anti-pRB, anti-cdk4, and anti-cyclin D3 antibodies. Extracts from HMBA-induced cells contained abundant pRB in complex with cyclin D3, whereas only trace amounts of pRB-cyclin D3 complexes were found in extracts from uninduced cells (Fig. 6). pRB in complex with cyclin D3 appears to be mainly in the underphosphorylated form, as shown by its migration on SDS-PAGE in comparison with that of pRB in whole-cell extracts. Some phosphorylated forms of pRB also appear to be complexed with cyclin D3; they migrate between the hyper- and underphosphorylated forms. pRB-cyclin D2 complexes were not detected in extracts from either uninduced or induced cells (data not shown). Less cdk4 protein was found in complex with cyclin D3 in extracts from HMBA-induced cells than in extracts from uninduced cells (Fig. 6). Since the cyclin D3 immunoprecipitate from HMBA-induced cell extracts contained about fivefold more cyclin D3 protein than does the immunoprecipitate from uninduced cell extracts (Fig. 6), it may be concluded that most of the cyclin D3 in HMBA-induced cells is not bound to cdk4.

We next asked whether the transcription factor E2F, which regulates transcription of a number of genes whose products are required for DNA replication (45), is present in complex with cyclin D3 during induced differentiation. The cyclin D3 immunoprecipitate was analyzed for E2F activity by the gel retardation assay with an oligonucleotide sequence derived from the adenovirus E2 promoter. E2F DNA-binding activity was detected in the cyclin D3 immunoprecipitate from HMBA-induced cell extracts but not in the immunoprecipitate from uninduced cell extracts (Fig. 7A). The binding of the radioactive oligonucleotide to proteins immunoprecipitated with cy-

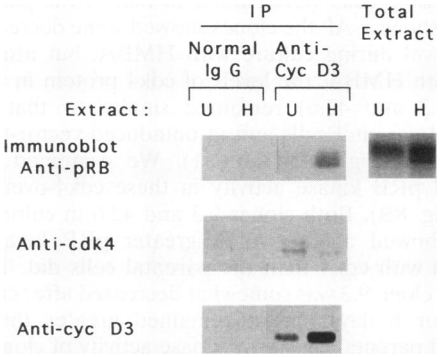

FIG. 6. pRB and cdk4 in complex with cyclin D3 in MEL cells cultured for 72 h with HMBA. Extracts prepared from uninduced (U) and HMBA-induced (H) cells were immunoprecipitated (IP) with normal rabbit IgG or anti-cyclin D3 antibodies. The immunoprecipitates were analyzed by immunoblotting with anti-pRB antibody, anti-cdk4 antibodies, and anti-cyclin D3 antibodies. Whole-cell extracts without immunoprecipitation were analyzed for pRB on the same gel.

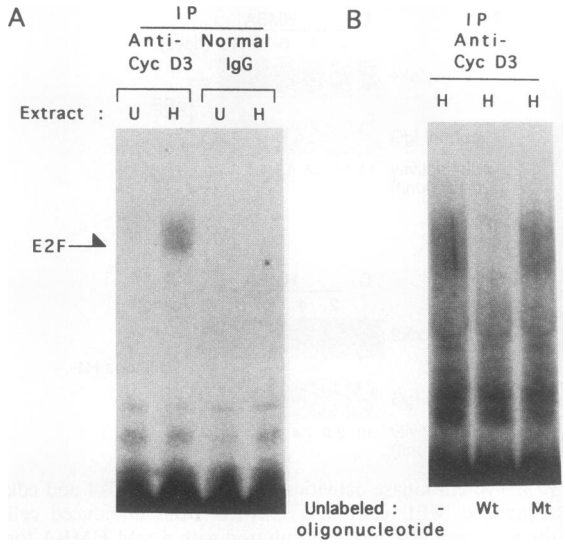

FIG. 7. Transcription factor E2F in complex with cyclin D3. (A) Nuclear extracts were prepared from uninduced (U) and HMBA-induced (H; 72 h) MEL cells. After immunoprecipitation with normal rabbit IgG or anti-cyclin D3 antibodies, the protein complexes were disrupted by treatment with sodium deoxycholate and then tested for the presence of E2F by a gel retardation assay. (B) The specificity of the E2F-DNA binding activity detected in the cyclin D3 complex (A) was tested by competition with excess of unlabeled wild-type (Wt) or mutant (Mt) oligonucleotide.

clin D3 was inhibited by a 100-fold excess of unlabeled wild-type oligonucleotide but not by a mutant oligonucleotide, consistent with the conclusion that the signal is specific for the DNA-binding activity of E2F (Fig. 7B).

Overexpression of cdk4 inhibits HMBA-induced terminal differentiation. To evaluate the significance of the suppressed level of cdk4 in HMBA-induced MEL cells, we asked whether forced expression of cdk4 by DNA transfection alters the sensitivity of MEL cells to HMBA-induced differentiation. Murine cdk4 cDNA was transfected into MEL cells by using vectors containing the cytomegalovirus promoter. Of 40 neomycin-resistant clones, 5 were found to express at least two- to threefold more cdk4 protein than the uninduced parental MEL cells did. The rates of growth of MEL cell clones overexpressing cdk4 were similar to that of the parental cells (data not shown). All the clones showed some decrease in cdk4 protein level during culture with HMBA, but after 48 h of culture with HMBA, the levels of cdk4 protein in two clones (clones 9.3 and 42.6) remained similar to that found in uninduced parental cells and in uninduced vector-transfected control cells (Fig. 8A; Table 1). We examined the cdk4-associated pRB kinase activity in these cdk4-overexpressing clones (Fig. 8B). Both clones 9.3 and 42.6 in culture without HMBA showed about twofold-greater pRB kinase activity associated with cdk4 than the parental cells did. The kinase activity of clone 9.3 was somewhat decreased after culture with HMBA for 6 days, but it remained greater than that in uninduced parental cells. The kinase activity of clone 42.6 was decreased more profoundly by culture with HMBA for 6 days. With respect to induced differentiation, clone 9.3 was highly resistant to HMBA and clone 42.6 was also resistant to a lesser extent, as measured by the proportion of cells committed to terminal cell division and by the accumulation of hemoglobin, assayed by the benzidine reaction (Table 1). The cdk4 clones in culture without HMBA showed similar distributions in the cell

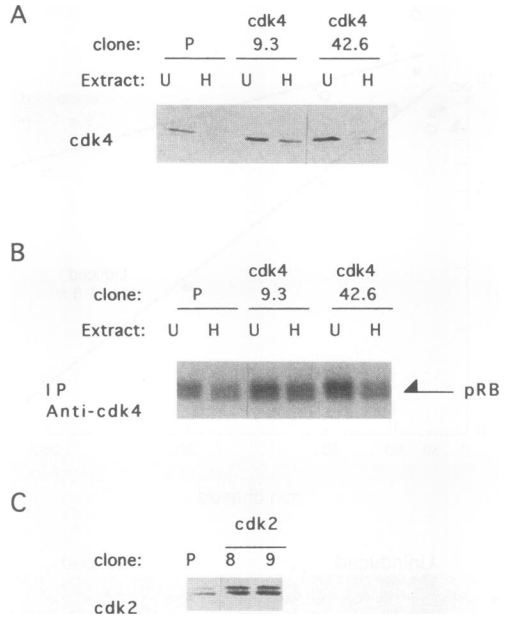

FIG. 8. Overexpression of cdk4 and cdk2 in MEL cells by DNA transfection. (A) MEL cells transfected with the pRc/CMV-cdk4 plasmid. Neomycin-resistant clones were cultured without (U) and with (H) 5 mM HMBA for 48 h, and extracts were analyzed for cdk4 protein by immunoblotting. Designations of clones correspond to those in Table 1. (B) pRB kinase activity associated with cdk4. The parental MEL cells and the cdk4 transfectant clones were cultured for 6 days without (U) or with (H) 5 mM HMBA. Cell extracts were immunoprecipitated with anti-cdk4 antibody, and the immune complex was assayed for pRB kinase activity by using pRB fused with GST as a substrate. The autoradiographic exposure time was 1.5 h. (C) MEL cells transfected with the pRc/CMV-cdk2 plasmid. Extracts of neomycin-resistant clones were analyzed for cdk2 protein by immunoblotting. Designations of clones correspond to those in Table 1.

cycle to that of the parental cells (data not shown). On day 10 of HMBA induction, 52 to 60% of clone 9.3 was found in G_1, while over 80% of the parental cells are in G_1. The HMBA resistance of these clones was stable over more than 6 months of culture. In the other three clones with cdk4 overexpression, represented by clone 5 (Table 1), the cdk4 protein levels were more profoundly suppressed by HMBA, so that they were

TABLE 1. MEL cells transfected with cdk4 and cdk2 cDNAs[a]

Clone	Level of cdk4 protein[b] after 48 h in:		% Commitment after:		% Benzidine-reactive cells after:		
	Uninduced cells	Induced cells	24 h	48 h	48 h	72 h	96 h
Parental	100	25	33	92	11	59	90
Vector control	112	23	35	95	12	65	90
cdk4 clone 9.3	257	103	2	12	0	2	5
cdk4 clone 42.6	294	90	13	47	4	18	36
cdk4 clone 5	215	22	25	88	6	45	74
cdk2 clone 8	ND[c]	ND	22	90	16	74	92
cdk2 clone 9	ND	ND	25	93	32	92	90

[a] Cells were cultured with 5 mM HMBA for the times indicated and harvested for immunoblotting, commitment assay, and benzidine staining. These data are representative of more than three independent experiments with each clone.
[b] Expressed as percentage of cdk4 protein in uninduced parental cells.
[c] ND, not determined.

comparable to the cdk4 levels in HMBA-treated parental and vector-control cells. HMBA-induced commitment to differentiation was minimally affected in these clones. These results suggest that inducer-mediated commitment to differentiation is markedly inhibited in cdk4-transfectant clones which sustain cdk4 overexpression in the presence of HMBA. Similar results were obtained by using a different plasmid vector, containing the Rous sarcoma virus promoter and cdk4 cDNA (data not shown).

We next asked whether overexpression of cdk2 protein also inhibits inducer-mediated differentiation. cdk2 cDNA in the same vector with the cytomegalovirus promoter was transfected into MEL cells. Eighteen neomycin-resistant clones were analyzed for their cdk2 protein levels, and two clones showed three- to fourfold overexpression of the protein (Fig. 8C). Both of these clones differentiated as well as parental cells upon exposure to HMBA (Table 1), indicating that overexpression of cdk2, unlike that of cdk4, does not inhibit induced differentiation. The distribution in the cell cycle in culture with or without HMBA was not affected by overexpression of cdk2 (data not shown).

DISCUSSION

The present study demonstrates that HMBA-mediated differentiation of MEL cells involves a fall in the cdk4 protein level, as a result of decreased stability of the protein. This decrease in the level of cdk4 protein is followed by suppression of cdk4-dependent pRB kinase activity. We also found that the level of cyclin D3 is increased and that this protein forms complexes with pRB and E2F. Suppression of cdk4 protein appears critical for HMBA-mediated commitment to erythroid differentiation, since forced overexpression of cdk4, by transfection of its cDNA, inhibits differentiation. Forced overexpression of cdk2 does not lead to resistance to inducer-mediated differentiation.

In HMBA-induced MEL cells, a fall in the cdk4 protein level is detectable as early as 14 h, when about 10% of cells are committed to differentiation (14). The cdk4 level falls to about 20% of the control level by 48 h, when over 90% of cells are committed. Commitment to terminal erythroid differentiation is itself a stochastic process (14, 19). One may speculate that a cell becomes committed to differentiate when its cdk4 protein level falls to a critical point and that the rate of fall of the cdk4 protein level in each cell is the stochastic function which determines the stochastic pattern of induced commitment. The HMBA-induced decrease in cdk4 protein level is due to decreased stability of the protein, and the rate of degradation is threefold greater in HMBA-induced cells than in uninduced cells. The rate of synthesis of cdk4 protein and the level of cdk4 mRNA are not affected by HMBA. The fall in cdk4 protein level is followed by suppression of cdk4-dependent pRB kinase activity and by an increase in the cyclin D3 protein level, which is due to an increased rate of synthesis of cyclin D3 (data not shown). This increased level of cyclin D3 may account for the apparent lag in the decline of cdk4-dependent pRB kinase activity relative to the fall in cdk4 protein level. Abundant cyclin D3 may efficiently bind and activate newly synthesized cdk4, even though the turnover of cdk4 is markedly increased. It should be noted that the phosphorylated form of pRB persists at the late stage of differentiation (Fig. 3), although cdk4 protein and pRB kinase activity associated with cdk4 are rapidly suppressed. This suggests that other kinases, including cdk2, whose activities are suppressed more slowly by HMBA could also be involved in phosphorylation of pRB. It is not yet known if multiple kinases catalyze phosphorylation of pRB at the same sites or if different sites of pRB are phosphorylated by different kinases.

A critical role for cdk4 suppression during HMBA-induced differentiation is supported by our observation that overexpression of cdk4 protein suppresses the recruitment of MEL cells to terminal differentiation. By comparison, overexpression of cdk2 protein does not have this effect. To evaluate if the kinase activity of cdk4 is important for its inhibitory activity on differentiation, we transfected MEL cells with a kinase-defective mutant of cdk4 bearing a mutation in the ATP-binding domain. Of 40 neomycin-resistant clones, none displayed significant overexpression of the mutant cdk4 (27), suggesting that expression of the kinase-defective mutant of cdk4 may have severe effects on the growth or survival of MEL cells. Thus, the significance of cdk4 kinase activity with regard to its inhibitory effect on differentiation remains to be clarified.

The decrease in cdk4 level and the increase in cyclin D3 level result in a sizable fraction of cyclin D3 that is not bound to cdk4 in HMBA-induced MEL cells. Cyclin D3 associates predominantly with underphosphorylated pRB (9, 12, 22). pRB is released from cyclin D3 complex when the pRB is phosphorylated by cdk4 (22). In HMBA-induced cells, depletion of cdk4 protein from cyclin D3 complexes may encourage the formation of stable complexes of cyclin D3 and underphosphorylated pRB. The transcription factor E2F is also found in complex with cyclin D3. We have observed changes in E2F complex formation in HMBA-induced MEL cells, characterized by loss of the transcriptionally active form of E2F (51); this may contribute to the withdrawal of terminally differentiated MEL cells from the cell cycle and their final arrest in G_0/G_1.

Taken together, these observations suggest that the pathway of induced differentiation involves the modulation of cyclins, cdks, transcription factors (such as E2F), and molecular intermediates such as pRB, which collectively implement a programmed phenotypic change involving restricted replication and the transcription of differentiation-specific genes.

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