Differentiation of Murine Erythroleukemia Cells Results in the Rapid Repression of Vimentin Gene Expression

JOHN NGAI, YASSEMI G. CAPETANAKI, and ELIAS LAZARIDES
Division of Biology, California Institute of Technology, Pasadena, California 91125

ABSTRACT We show that vimentin filaments are present in undifferentiated Friend murine erythroleukemia cells, but are lost progressively to undetectable levels by 96 h of dimethyl sulfoxide-mediated differentiation. The amount of newly synthesized cytoskeletal vimentin is decreased dramatically by 24 h of induction, and is paralleled by a rapid loss of vimentin mRNA (~25-fold reduction at 96 h). Hence, disappearance of vimentin filaments in these cells appears to be regulated at the level of vimentin mRNA abundance. On the other hand, the levels of actin synthesis and actin mRNA remain essentially unchanged. The kinetics of vimentin mRNA reduction during dimethyl sulfoxide-mediated differentiation, and the levels of vimentin mRNA observed in the presence of hexamethylene-bisacetamide or hemin as inducers suggest that the cessation of vimentin expression precedes, but may be associated with commitment to terminal differentiation. Our results demonstrate the dynamic regulation of vimentin expression in mammalian erythropoiesis.

Elucidation of the events occurring in mammalian erythropoiesis has been facilitated by the study of murine erythroleukemia cell differentiation in vitro. Friend murine erythroleukemia (MEL)[1] cells originally were derived from Friend leukemia virus-infected tumor implants and proliferate rapidly in culture (13). Exposure to a variety of chemical agents, such as dimethyl sulfoxide (DMSO), hexamethylene-bisacetamide (HMBA), or butyric acid induces MEL cells to differentiate (14, 29, 48; for a review see reference 35). Among the characteristic phenomena observed during terminal differentiation of MEL cells are a maturation from a basophilic erythroblastic appearance to an orthochromatophilic normoblastic phenotype (14), a loss of proliferative capacity (12, 21), alterations in purine metabolism (46), increases in iron uptake and heme synthesis (14), an elevation of heme synthetic enzyme activities (10, 54), the induction of globin mRNAs (43, 46, 50), and the accumulation of hemoglobin (14). The tremendous induction of hemoglobin synthesis and accumulation in chemically-induced MEL cells, a distinctive feature of erythroid terminal differentiation in vivo, is effected by transcriptional activation of globin genes (2, 34, 44); the relative accumulation of globin mRNAs also may be modulated by the stabilization of these messages and/or by the destabilization of nonglobin mRNAs (2, 32, 56, 60).

Studies from this laboratory have demonstrated that vimentin is the major intermediate filament subunit in chicken erythrocytes (19). We have shown further that during chicken embryonic development, the expression of vimentin in circulating erythroid cells is regulated at the level of transcription and/or mRNA stabilization (5). An approximately 50-fold increase in the accumulation of vimentin mRNA is observed between 4-d primitive series cells and 15-d definitive series cells, and this increase appears to underlie similar changes at the protein level (5). In contrast, Dellagi et al. (7) have shown by immunofluorescence microscopy that vimentin is lost during human erythropoiesis in vivo. However, the loss of vimentin could not be correlated with a particular stage of erythroid development, as the presence of vimentin was variable in both early and late erythroblastic cells.

To define the mechanisms that regulate vimentin expression during mammalian erythropoiesis, we have examined the pattern of expression of this intermediate filament subunit in differentiating MEL cells. We have found that vimentin filaments disappear from the cytoplasm of DMSO-treated MEL cells, and that this disappearance results from a rapid decrease in vimentin synthesis. Furthermore, we demonstrate that the observed change in vimentin synthesis during induction is caused by a rapid and dramatic decrease in the amounts of steady state vimentin mRNA, indicating that vimentin filament expression in differentiating MEL cells is regulated primarily at the mRNA level. Our data suggest that the cessation of vimentin filament expression is an essential com-

[1] *Abbreviations used in this paper:* DMSO, dimethyl sulfoxide; HMBA, hexamethylene-bisacetamide; MEL, Friend murine erythroleukemia; TX-100, Triton X = 100.

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ponent of the terminal differentiation program in mammalian erythropoiesis.

MATERIALS AND METHODS

Cell Culture: Friend murine erythroleukemia cells were obtained from Dr. Barbara J. Wold (California Institute of Technology, Pasadena, CA) and were maintained in Dulbecco's modified Eagle medium (DME) supplemented with 15% fetal calf serum and penicillin/streptomycin at 37°C in an atmosphere of 95% air/5% CO_2. Cells were passaged every third day at dilutions of 1:100. For induction experiments, cultures were initiated at densities ranging from 5 × 10^4/ml to 2 × 10^5/ml, and incubated in the presence of 1.8% DMSO, 4 mM HMBA, or 75 μM bovine hemin (from a 10-mM stock solution, prepared as described by Ross and Sautner [51]) for the times indicated in each of the experiments. Inducers were added to culture media prior to the addition of cells. Cells from control cultures were harvested after 2–3 d. In general, cells were harvested while they were still in logarithmic growth (although cells induced in DMSO or HMBA for 96 h had just begun to reach plateau densities, as expected at this stage of MEL cell terminal differentiation [12, 21]). Growth kinetics were derived by estimation using a hemacytometer, and cell viability was confirmed by trypan blue exclusion.

Immunofluorescence Microscopy: Vimentin-containing intermediate filaments were visualized using a rabbit anti-chicken vimentin antiserum (17). Although this antiserum displays weaker reactivity with mammalian vimentin than with chicken vimentin, immunoprecipitation of [^{35}S]labeled MEL cell proteins (method B of reference 4) indicates that vimentin is specifically recognized in this system (data not shown).

MEL cells from different periods of induction were washed twice with Tris-buffered saline (140 mM NaCl, 5 mM KCl, 10 mM Tris-Cl, pH 7.5), resuspended in Tris-buffered saline, and allowed to settle onto Alcian Blue-treated coverslips at 4°C (19). Adherent cells were fixed for 2 min at room temperature in 2% formaldehyde, 140 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 20 mM PIPES, pH 7.0, and rinsed and permeabilized in Tris-buffered saline plus 0.5% Triton X-100 (TX-100) and 1 mM EDTA. Cells were reacted first with the rabbit anti-chicken vimentin antiserum at 1:40 dilution, and then with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG diluted 1:150 (Miles-Yeda Ltd., Rehovot, Israel). Coverslips were mounted in Tris-buffered saline and viewed in a Leitz phase/epifluorescence microscope using a 63 × objective.

Metabolic Labeling with [^{35}S]Methionine and Two-dimensional Gel Electrophoresis: For each time point examined, 2 × 10^6 cells were pelleted by centrifugation and washed in 5 ml Dulbecco's modified Eagle medium depleted of methionine and supplemented with 15% dialyzed fetal calf serum, with or without 1.8% DMSO. Cells were resuspended in 5 ml of this medium plus ~100 μCi [^{35}S]methionine (Amersham Corp., Arlington Heights, IL, ca. 1,100–1,200 Ci/mmol) and incubated at 37°C for 1 h. After labeling, cells were placed on ice, washed with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution (EBSS), and resuspended in 100 μl 100 mM KCl, 300 mM sucrose, 20 mM PIPES, 5 mM Mg acetate$_2$, 5 mM EGTA, 0.5% TX-100, pH 6.8 (cytoskeleton buffer; 39). Lysis was allowed to proceed for 5 min at 0°C, and insoluble "cytoskeletal" fractions were pelleted by centrifugation. After removal of detergent-soluble phases, pellets were resuspended in cytoskeleton buffer plus 2.5% 2-mercaptoethanol, saturated with urea. The TX-100-insoluble residues are defined operationally as cytoskeletal fractions. TX-100 fractionation is necessary for two-dimensional gel analysis of mammalian vimentin, as in our gel system the large quantities of tubulin (mostly soluble under these lysis conditions) obscures the identification of vimentin. TX-100 insoluble ^{35}S-labeled vimentin therefore represents newly synthesized and newly assembled cytoskeletal vimentin (3, 39).

Protein-incorporated ^{35}S-radioactivity was determined from total lysates as well as from TX-100 soluble and insoluble fractions. Briefly, aliquots were incubated in 1 N NaOH, 4 mg/ml methionine, and 0.4 mg/ml BSA at 37°C for 10 min to hydrolyze methionyl-tRNA complexes, precipitated with trichloroacetic acid, and collected on Whatman GF/C filters (Whatman Laboratory Products, Inc., Clifton, NJ). Radioactivity was determined by liquid scintillation counting in Aquasol-2 (New England Nuclear, Boston, MA). Typically 12–14% of protein-incorporated ^{35}S-radioactivity was found in the cytoskeletal fraction. For electrophoretic analysis, the amounts of sample used were normalized to total cell lysate protein ^{35}S-radioactivity.

Two-dimensional gel electrophoresis was performed as described by O'Farrell (42) and modified by Hubbard and Lazarides (23). Resolving gels in the second dimension contained 12.5% acrylamide and 0.1% bisacrylamide. After electrophoresis, gels were fixed, impregnated with Enhance (New England Nuclear), and subjected to fluorography using preflashed Kodak XAR-5 film (26). ^{35}S-labeled vimentin was identified on the basis of its co-migration with purified bovine lens vimentin (40).

RNA Isolation: Cells were harvested by centrifugation at appropriate times, washed twice with Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution, quickly frozen in liquid N_2, and stored at −80°C. Total cellular RNA was isolated by the method of Chirgwin et al. (6). Frozen cell pellets (each containing up to ~4–5 × 10^8 cells) were thawed into 27 ml 5 M guanidinium thiocyanate, 50 mM Tris-Cl, pH 7.5, 50 mM EDTA, and 5% 2-mercaptoethanol. The resulting viscous DNA was sheared by at least 10 strokes in a Dounce homogenizer (with a tightly fitting pestle), and by 5 additional strokes after the addition of 3 ml 30% sodium lauryl sarcosine. Homogenates were layered over 8 ml cushions of 5.7 M CsCl, 1 mM EDTA, pH 8.0, and RNA was pelleted by centrifugation for 20–24 h at 39,000 rpm in a Beckman 60 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) at 20°C. Pellets were resuspended in water, precipitated twice with ethanol, washed once with 70% ethanol, and dried. The recovery of RNA by this method is quantitative if care is taken to shear sufficiently the cellular DNA (which traps RNA); we find that recovery of RNA from cells at a given stage of induction is constant irrespective of cell number (between ~1.5 × 10^8 and ~4.5 × 10^8 cells/30 ml homogenization buffer). Poly(A)$^+$-enriched RNA was isolated by two rounds of oligo(dT)-cellulose chromatography (1). Within an induction experiment, the conditions of oligo(dT)-cellulose chromatography for each sample were maintained as identical as possible. In this manner, a constant fraction of total RNA was recovered as poly(A)$^+$ RNA (generally 2% recovery), regardless of the stage of MEL cell differentiation.

RNA Electrophoresis, Blotting to Nitrocellulose, and Hybridization: RNA was separated on 1.1% agarose gels under denaturing conditions in the presence of formaldehyde (30), essentially as described by Goldberg (16). Following electrophoresis, gels were soaked in 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM Na-citrate) and transferred to nitrocellulose in 10 × SSC according to the method of Southern (58). Filters were washed briefly in 2 × SSC, air dried, and baked in vacuo at 80°C for 2–3 h.

^{32}P-labeled DNA probes were utilized to detect specific filter-bound RNA sequences. For the detection of vimentin RNA, the ~0.5 kilobase (kb) insert from p5C5, a chicken vimentin cDNA plasmid (5) was isolated (62) and nick-translated (49) using α^{32}P-labeled deoxynucleoside triphosphates (ca. 800 Ci/mmol, New England Nuclear). Detection of β-globin RNA sequences was facilitated by nick-translation of pMB-βG2, a recombinant mouse β^{major}-globin genomic plasmid (25), whereas actin sequences were identified using a ^{32}P-labeled actin cDNA plasmid derived from chicken smooth muscle. This actin cDNA plasmid (containing a 1.9 kb-insert) was isolated by screening a chicken gizzard cDNA library with a sea urchin actin probe (55). Its identity as an actin cDNA was confirmed by positive hybrid-selected translation (data not shown).

Filter-bound RNA was hybridized to 5 × 10^5 cpm/ml nick-translated probe at 42–44°C for 20–24 h, as described by Wahl et al. (61) in the absence of dextran sulfate. Filters then were washed twice in 2 × SSC, 0.2% SDS at room temperature for 20–30 min each, and four times in 0.1 × SSC, 0.2% SDS at 42–44°C for 30 min each, and air dried. Blots were exposed to preflashed Kodak XAR-5 film with DuPont Lightning-Plus intensifying screens.

Quantitation of RNA Blot Hybridizations: The relative levels of specific RNA species during MEL cell differentiation were quantitated by determining densities of autoradiographic signals from RNA blot hybridizations. For this technique to be reliable and effective, however, a number of variables were controlled. First, for every experiment requiring quantitation, the RNA sample containing the highest amount of the sequence of interest was diluted and included in the same gel containing the other experimental samples. For example, vimentin mRNA quantitation was standardized by using control RNA diluted to 1.0-, 0.5-, 0.25-, 0.1-, and 0.05-equivalents. For actin mRNA, control RNA was diluted to 1.0-, 0.8-, 0.6-, and 0.4-equivalents, whereas for globin mRNA quantitation, RNA from cells induced for 96 h in DMSO was diluted to 1.0-, 0.5-, 0.25-, 0.1-, and 0.05-equivalents. Hence, concentration standards were treated identically as the experimental samples were through electrophoresis, transfer, hybridization, washes, exposure, and photographic development.

Second, deviations from linearity of film response to signal were taken into account. The inclusion of standards with each blot to be quantitated controlled for reciprocity failure at low intensity levels. However, hybridization bands that expose the film to or beyond saturation cannot be quantitated. The effects of nonlinear film response were minimized by preflashing film to an optical density of 0.15 at 540 nm (26), and by exposing films within linear range for several periods of duration. Autoradiograms were scanned with a densitometer, and peak areas were determined using a computer digitizer.

Fig. 1 shows an example of a standardization for vimentin mRNA. The autoradiogram of Fig. 1*A* was from the same blot (and same film) as the one of Fig. 4*A*. The graph in Fig. 1*B* shows the relationship between peak area and relative level of vimentin mRNA. Although the overall response is not linear, this type of curve is typical.

RESULTS

Disappearance of Vimentin Filaments from DMSO-induced MEL Cells

Indirect immunofluorescence microscopy using an antivimentin antiserum revealed that vimentin filaments are lost from differentiating MEL cells. Cells were cultured in control medium or for 24, 36, 48, and 72 h in the presence of 1.8% DMSO and prepared for immunofluorescence microscopy. Representative micrographs are shown in Fig. 2. MEL cells grown in control medium exhibit cytoplasmic vimentin filaments. Upon incubation with DMSO, however, vimentin-specific fluorescence progressively diminishes. After 24 h in DMSO, vimentin filaments are still visible in most cells, but their presence is more difficult to detect than in control cells. Longer periods of DMSO induction result in a further diminution of detectable vimentin, and filaments generally are not visible after 48 h of incubation. Diffuse cytoplasmic fluorescence is also observed in all cells; this is due to nonspecific staining, as it is observed when preimmune serum is used as the primary antibody (data not shown), and is present irrespective of the cellular vimentin content (see below).

The loss of vimentin filaments in differentiating MEL cell cultures appears to be uniform; for a given time point, distinct subpopulations of vimentin-positive and vimentin-negative cells are not seen. The presence of apparently vimentin-negative cells among positive cells (Fig. 2) is due primarily to the positions of cells outside the plane of focus. However, visualization of cells exposed to DMSO for 48 or 72 h occasionally reveals a cell displaying vimentin filaments (see Fig. 2). These are rare cases (<1%), and most likely represent cells that did not respond to the inducer. By this qualitative assay, the disappearance of detectable vimentin filaments occurs in differentiating MEL cells between 24 and 48 h of DMSO-mediated induction, a time that corresponds to the onset of significant hemoglobin accumulation (31, 36; see also below).

Analysis of Newly Synthesized Cytoskeletal Vimentin in Differentiating MEL Cells

MEL cells differentiating in response to DMSO rapidly and extensively reduce the synthesis of vimentin, and consequently cease the de novo assembly of vimentin filaments. Although ^{35}S-labeled vimentin is present in cytoskeletons from control MEL cells (Fig. 3*A*), after 24 h of induction newly synthesized cytoskeletal vimentin is barely detectable (Fig. 3*B*), and at 36 and 72 h of culture in DMSO (Fig. 3, *C* and *D*), remains at or below detection limits. Since de novo assembly of vimentin filaments ceases rapidly, the disappearance of filaments in MEL cell differentiation may result from dilution by cell division (see Discussion).

Immunoprecipitation of newly synthesized cytoskeletal vimentin and subsequent one-dimensional gel analysis also yields a pattern similar to that seen in Fig. 3; we observe a slight decrease in ^{35}S-labeled vimentin after 12 h of induction, and at 24 h and all subsequent time points vimentin was nearly or completely absent (data not shown). Examination of ^{35}S-labeled vimentin from total cell lysates by immunoprecipitation revealed that vimentin synthesis is diminished at 24 h of incubation in DMSO and thereafter (data not shown). Hence, the reduction in newly synthesized cytoskeletal vimentin parallels the rapid and extensive repression of vimentin synthesis. These observations are consistent with previous studies from this laboratory, which demonstrated that vimentin assembly in chicken erythroid cells occurs rapidly and post-translationally from a saturable pool, but with assembly appearing not to be regulated at this level (3, 39).

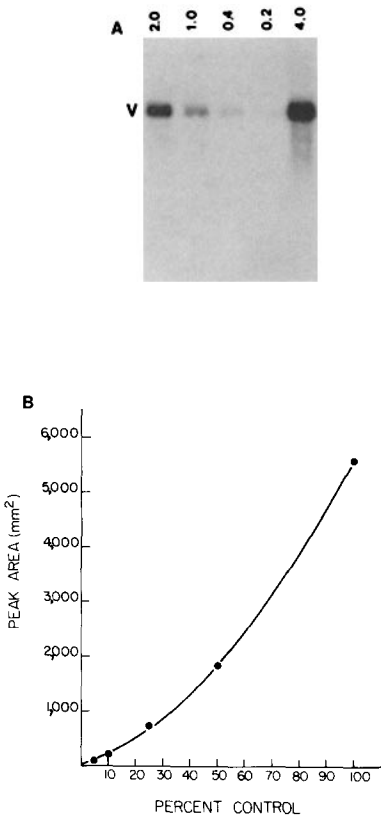

FIGURE 1 Standardization of vimentin RNA hybridization. (*A*) Autoradiogram of an RNA blot containing 4.0, 2.0, 1.0, 0.4, and 0.2 μg control MEL cell poly(A)$^+$ RNA, hybridized to the vimentin cDNA probe. (*B*) Quantitation of the blot in *A*. Vimentin mRNA peaks were scanned and integrated with a computer digitizer. The data are expressed as peak area vs. percent control, with 100% corresponding to 4 μg poly(A)$^+$ RNA.

The data in Fig. 3 indicate that the reduction in newly synthesized cytoskeletal vimentin (and consequently the reduction in newly synthesized total cellular vimentin) is specific, even though overall protein synthesis is reduced in differentiating MEL cells (57). Several other ^{35}S-labeled proteins are present in fairly constant amounts throughout the 72-h induction period, most notably actin. ^{35}S-labeled actin was also maintained at constant levels in total cell lysates (data not shown).

Rapid Reduction in Vimentin mRNA Levels During the Maturation of MEL Cells

The data presented thus far show that the disappearance of vimentin filaments in differentiating MEL cells is mediated by a decrease in vimentin synthesis. To analyze steady state vimentin mRNA levels during induction, we performed quantitative RNA blot analysis of poly(A)$^+$ RNA derived from cells undergoing DMSO-induced differentiation, using a chicken vimentin cDNA as probe. This cDNA probe, designated p5C5, corresponds to the 5′ region of the chicken vimentin gene (5). When p5C5 was hybridized to RNA blots

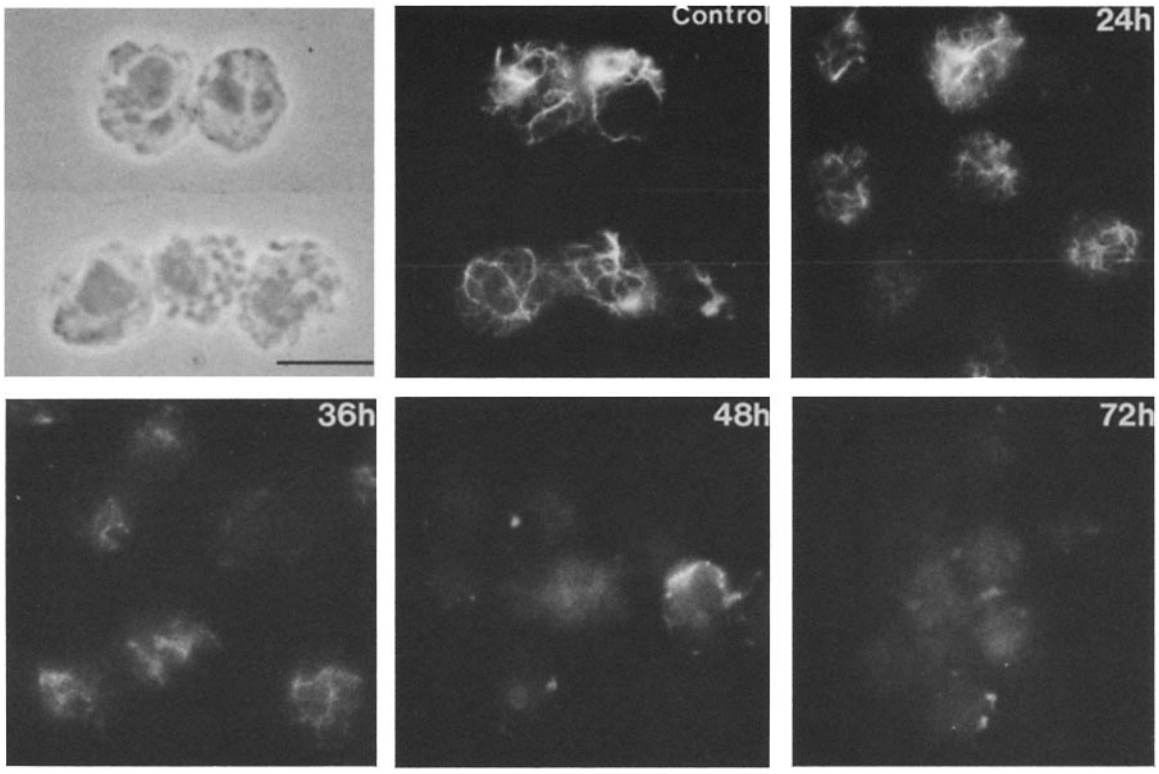

FIGURE 2 Disappearance of vimentin filaments during DMSO-induced MEL cell differentiation. Vimentin filaments in MEL cells were visualized by indirect immunofluorescence microscopy using an antivimentin antiserum. The left-most panel of the top row is a phase contrast image of the same field shown in the control fluorescence panel. Cells were examined at 24, 36, 48, and 72 h of induction in 1.8% DMSO, as indicated. Note the presence of a rare vimentin-positive cell at 48 h of differentiation (see text). Bar, 10 μm.

containing poly(A)$^+$ RNA from total 1-d-old neonatal mice, only one species with an approximate molecular length of 2.1 kb was detected (data not shown), in agreement with the data of others for the mature mammalian vimentin mRNA (8, 45).

MEL cells were cultured in the presence of 1.8% DMSO and were harvested after 12, 24, 36, 48, 72, and 96 h of induction; untreated cells were maintained in control medium. Poly(A)$^+$ RNA was isolated from each sample and analyzed by RNA blot hybridization using p5C5 as probe. An autoradiogram of a representative RNA blot is shown in Fig. 4*A*. A 2.1-kb RNA band corresponding to vimentin mRNA is clearly detected in poly(A)$^+$ RNA from control MEL cells. After 12 h of culture in DMSO, this band is somewhat reduced, and declines rapidly between 12 and 96 h of induction. Fig. 4*B*, which represents data derived from two independent induction experiments, shows the quantitation of relative vimentin mRNA levels through 96 h of DMSO induction. After 12 h of exposure to inducer, vimentin mRNA is ~70% of the control level, and falls rapidly thereafter to approximately 30% at 24 h, 10% at 48 h, and 4% at 96 h of differentiation. The results demonstrate that vimentin expression in differentiating MEL cells is regulated by the steady state level of vimentin mRNA.

The data of Fig. 4 show relative vimentin mRNA levels normalized to total cellular RNA, since the fraction of poly(A)$^+$ RNA that we recover from total RNA is constant throughout the induction period (see Materials and Methods). However, the amount of total RNA recovered per cell decreases as differentiation of MEL cells progresses. From control cultures, we obtain ~1.5–1.8 mg RNA/10^8 cells, and this value declines steadily to ~0.4–0.5 mg RNA/10^8 cells after 96 h of culture with DMSO, consistent with the results of others (33, 57). Hence, the kinetics of vimentin mRNA reduction per cell is actually more rapid than shown in Fig. 4*B*, and the overall extent of this loss is greater (~1% control levels per cell at 96 h of induction). The ~100-fold reduction in vimentin mRNA levels per cell therefore results from both a specific decrease in vimentin mRNA and a general decline in total cellular RNA.

Quantitation of Actin and β-Globin mRNA Levels in Differentiating MEL Cells

To characterize further the specific reduction in vimentin mRNA during DMSO-induced differentiation, we performed as controls quantitative RNA blots using probes for actin and β-globin. As in Fig. 4*A*, equivalent amounts of poly(A)$^+$ RNA from different time points of DMSO induction were electrophoresed and transferred to nitrocellulose. Fig. 5*A* is an autoradiogram of a blot hybridized to a ^{32}P-labeled chicken actin cDNA, and the quantitation of this blot is shown in Fig. 5*B*. From Fig. 5, *A* and *B*, it is clear that the abundances of actin mRNA in poly(A)$^+$ RNA from differentiating MEL cells remain essentially constant through 96 h of culture in the presence of DMSO. The results show that the constant level of actin expression in MEL cells is regulated at the mRNA level.

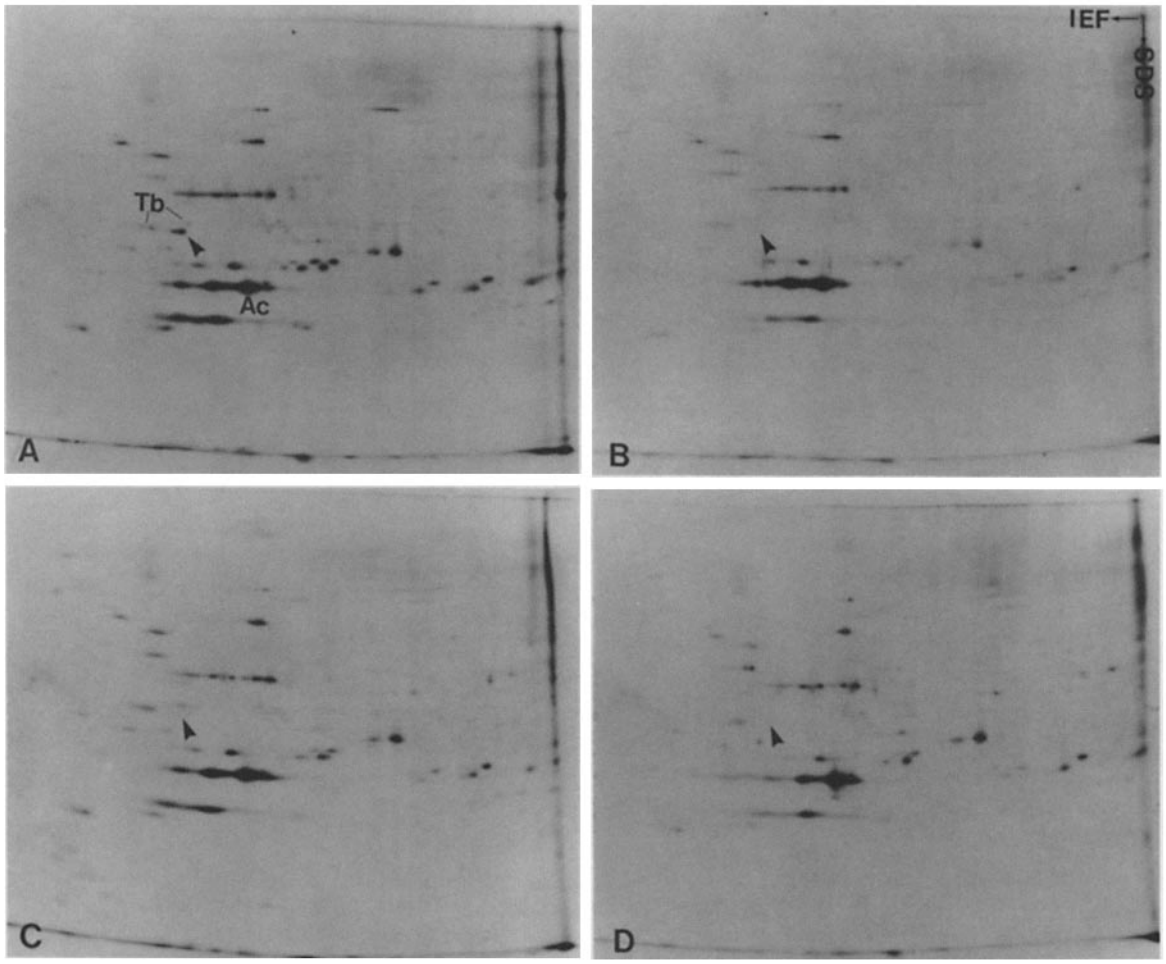

FIGURE 3 Two dimensional gel analysis of newly synthesized cytoskeletal vimentin during MEL cell differentiation. Control cells and cells cultured in the presence of 1.8% DMSO for 24, 36, or 72 h were pulse-labeled for 1 h with [^{35}S]methionine. Cytoskeletal fractions were prepared and analyzed by two-dimensional gel electrophoresis and fluorography (see Materials and Methods). (*A*) control; (*B*) 24 h; (*C*) 36 h; and (*D*) 72 h. Arrowheads demarcate vimentin, as determined by its co-migration with a purified bovine vimentin standard. *Tb*, tubulins; *Ac*, actin.

The kinetics of globin mRNA induction under our culture conditions was assessed by hybridizing RNA blots with a ^{32}P-labeled recombinant plasmid containing mouse genomic β^{major}-globin sequences (25). The results of such experiments are presented in Fig. 5, *C* and *D*. After a ~24-h lag period, a significant increase in β-globin mRNA is observed between 24 and 36 h of culture in the presence of DMSO, and there is an overall ~20-fold increase of β-globin mRNA over the basal level at 96 h of induction. The pattern of β-globin mRNA induction observed in this study is similar to the patterns observed by other investigators (32–34, 41, 51). The amount of β-globin mRNA in control MEL cells is reproducibly higher than in cells incubated for 12 or 24 h in DMSO, and most likely is due to the accumulation (after 2–3 d of culture) of a low percentage of spontaneously differentiating cells in our control cultures (14; see also reference 35). At 48 h of DMSO-stimulated differentiation, β-globin mRNA increases ~5-fold over basal levels (see Fig. 5*D*), whereas vimentin mRNA already has fallen to approximately one-tenth of control amounts (Fig. 4*B*). The decrease in vimentin mRNA levels therefore precedes the major accumulation of β-globin mRNA.

Effects of HMBA and Hemin on Vimentin mRNA Levels in MEL Cells

A variety of chemical agents other than DMSO can induce MEL cells to differentiate. For example, HMBA is a particularly potent inducer, and causes terminal differentiation of MEL cells, as does DMSO (48). On the other hand, incubation of MEL cells with hemin causes the rapid accumulation of globin mRNA, but does not induce other changes characteristic of terminal differentiation (22, 33, 51). We therefore tested the effects of these two agents on the levels of vimentin mRNA in MEL cells.

After 96 h of exposure to 4 mM HMBA, MEL cells exhibit an extensive reduction in vimentin mRNA, as shown by RNA blot analysis (Fig. 6*A*). The levels at 96 h of HMBA-mediated induction are similar to those seen with 1.8% DMSO after 72 h. Fig. 6*C* is a control, showing the large induction at 96 h of β-globin mRNA in response to HMBA. Hence, MEL cells dramatically decrease the steady state level of vimentin mRNA during both HMBA- and DMSO-mediated differentiation.

Incubation of MEL cells with 75 μM hemin for 72 h causes

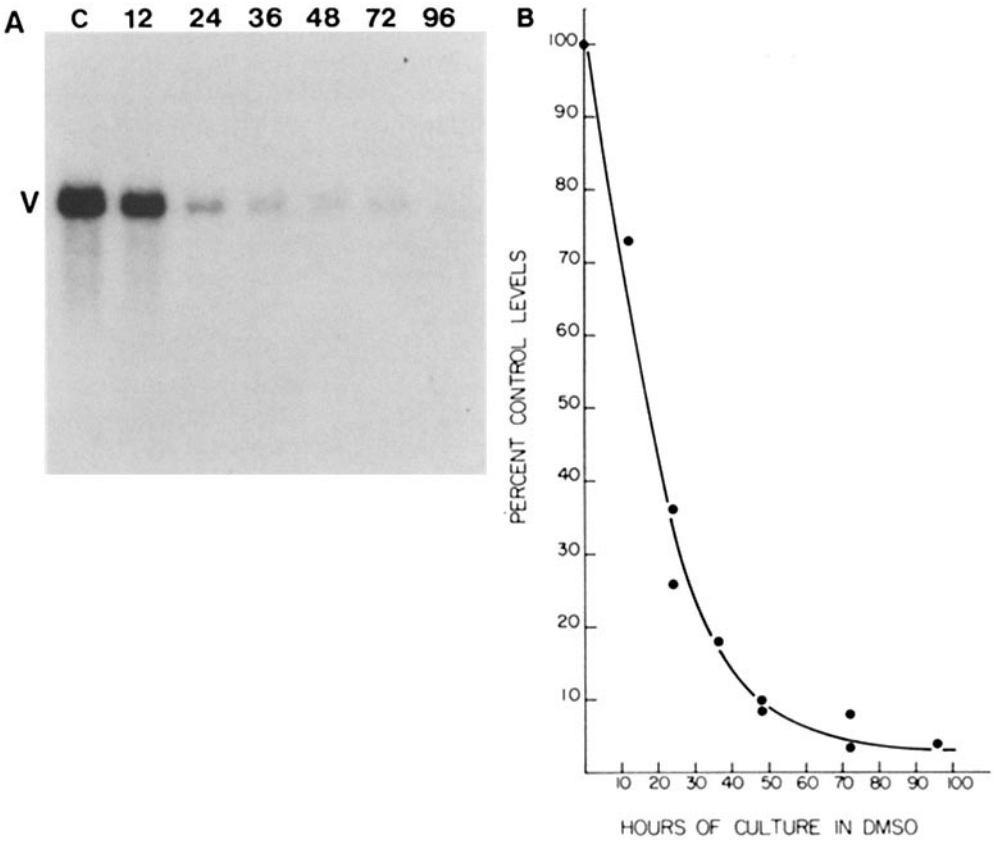

FIGURE 4 Analysis of vimentin mRNA levels during MEL cell differentiation. 4 μg of poly(A)$^+$ RNA from control cells or cells cultured in 1.8% DMSO for 12, 24, 36, 48, 72, and 96 h were electrophoresed in the presence of formaldehyde, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled chicken vimentin cDNA probe. (*A*) Autoradiogram of a representative blot. C denotes poly(A)$^+$ RNA from control cells; numbers indicate hours of culture in the presence of 1.8% DMSO from which poly(A)$^+$ RNA was derived. *V* marks the position of the 2.1-kb mouse vimentin mRNA. (*B*) Quantitation of vimentin mRNA levels during induction. The data in this graph were derived from two independent experiments from blots using either 4.0 or 2.2 μg RNA per lane, and vimentin mRNA levels were quantitated as described in Materials and Methods. The values correspond to relative quantities of vimentin mRNA normalized to cellular poly(A)$^+$ RNA content (see text), as compared with the control cell vimentin mRNA level.

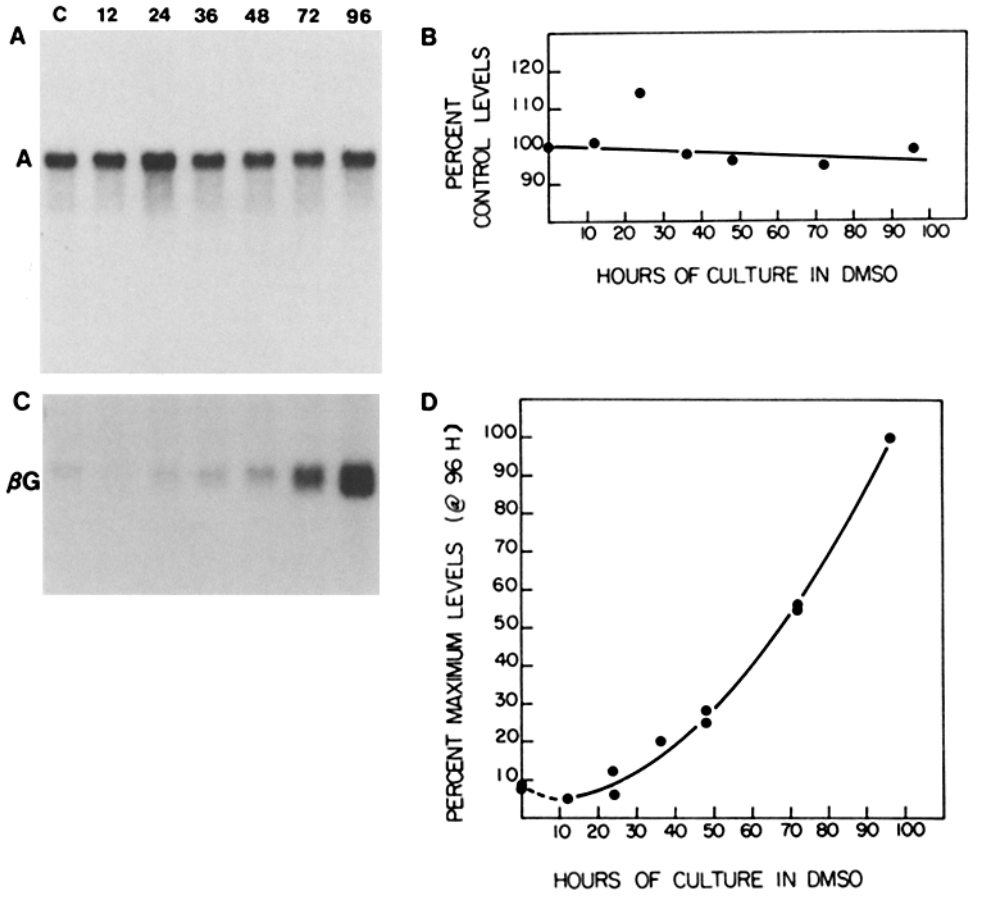

FIGURE 5 Actin and β-globin mRNA in differentiating MEL cells. Poly(A)$^+$ RNA from MEL cells was prepared for quantitative RNA blot hybridization as described in Materials and Methods. (*A*) Autoradiogram of a blot containing 3 μg of each poly(A)$^+$ RNA, which was hybridized to a chicken actin cDNA probe. (*B*) Quantitation of actin mRNA levels from the RNA blot shown in *A*. (*C*) Autoradiogram of a blot (4 μg poly(A)$^+$ RNA per lane) hybridized to a mouse β^{major}-globin probe. (*D*) Quantitation of β-globin mRNA levels during MEL cell differentiation, expressed as percentages of the level observed at 96 h of DMSO-mediated differentiation. The data in this graph were derived from two independent experiments. *C*, control cell poly(A)$^+$ RNA; *12, 24, 36, 48, 72, 96*, hours of culture in 1.8% DMSO. *A*, actin mRNA; *βG*, β-globin mRNA.

a less extensive change in vimentin mRNA, as compared with the effects of HMBA or DMSO (Fig. 6 *B*). Quantitation of an RNA blot similar to the one shown in Fig. 6 *B* indicates that vimentin mRNA is reduced to ~50% of control amounts. Fig. 6 *D* demonstrates a ~4–5-fold increase in β-globin mRNA in response to hemin, as reported by others (33, 51). By

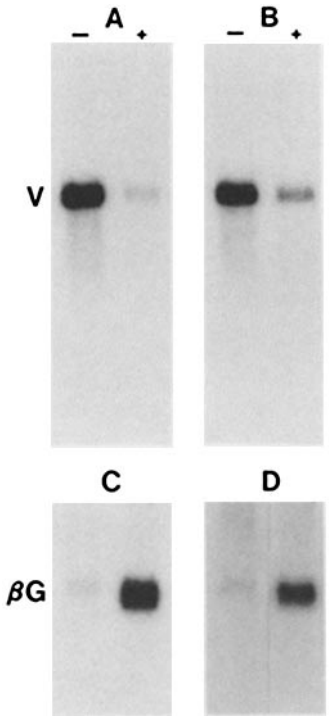

FIGURE 6 Effects of HMBA and hemin on MEL cell vimentin and β-globin mRNA levels. (*A*) Autoradiogram of an RNA blot containing poly(A)$^+$ RNA (4 μg each lane) from cells cultured in the absence (−) or presence (+) of 4 mM HMBA for 96 h, hybridized to the vimentin cDNA probe. (*B*) RNA blot analysis of vimentin mRNA from cells cultured in the absence (−) or presence (+) of hemin for 72 h (4 μg per lane). The hybridization seen with RNA from hemin-treated cells represents ~50% of control vimentin mRNA levels (see text). (*C*) RNA blot as in *A*, hybridized to the β-globin-specific probe. (*D*) Detection of β-globin mRNA in a blot similar to the one in *B*, except that 2.2 μg poly(A)$^+$ RNA was used per lane, V, vimentin mRNA; βG, β-globin mRNA.

immunofluorescence microscopy, nearly all cells cultured in the presence of 75 μM hemin for 3 d possess vimentin filaments (data not shown), indicating that most, if not all cells are responding similarly to this inducer with regard to vimentin expression. The response observed with hemin suggests that the more extensive reduction in vimentin mRNA observed in DMSO- and HMBA-treated cells is an event related to terminal differentiation (see Discussion).

DISCUSSION

Disappearance of Vimentin Filaments in Differentiating MEL Cells is Regulated at the mRNA Level

In this study we demonstrate that vimentin filaments disappear from differentiating MEL cells, and this disappearance is preceded by a rapid cessation of vimentin synthesis and assembly into the cytoskeleton (see below). Moreover, the level of vimentin synthesis is regulated primarily by vimentin mRNA levels, as the pattern of newly synthesized cytoskeletal vimentin during differentiation is paralleled by a similarly dramatic decrease in vimentin mRNA. The expression of a number of other proteins, most notably actin, is constant during chemically-induced MEL cell differentiation, indicating that the rapid and extensive repression of vimentin mRNA levels is a result of the specific regulation of vimentin gene expression.

Vimentin mRNA is reduced ~3-fold after 24 h of culture in 1.8% DMSO, and ~25-fold after 96 h (Fig. 4). Normalization of these values to cell number reveals that vimentin mRNA decreases to ~1% control levels per cell at 96 h of induction. This reduction results in a rapid decrease in newly synthesized cytoskeletal vimentin (Fig. 3). However, by immunofluorescence microscopy vimentin filaments are still detectable (with weaker fluorescence intensity) through 36 h of differentiation (Fig. 2). Vimentin filaments have been shown to be stable cellular constituents in other systems (3, 38). For example, in Ehrlich ascites tumor cells, the half-life of vimentin is similar in magnitude to the population doubling time (38); the decay of vimentin therefore is very slow compared with the mitotic rate of these cells. As the doubling time of MEL cells under our conditions of DMSO induction is 14–16 h, our data suggest that the loss of filaments is facilitated by a rapid turn-off of vimentin synthesis with continual dilution of existing filaments by cell division. It is also possible that the reduction in vimentin filaments during differentiation is accelerated by an increased rate of vimentin turnover.

Regulation of Vimentin Gene Expression

Our data demonstrate a dramatic repression of vimentin mRNA levels during MEL cell maturation. However, we do not know as yet the relative contributions toward this phenomenon by changes in vimentin gene transcription and post-transcriptional processing and turnover. Reduced transcription may result from both specific repression of the vimentin gene and an overall reduction of RNA synthesis, as the synthesis and accumulation of RNA in DMSO-treated MEL cells decreases during differentiation (57). In vitro nuclear transcription studies (20, 37) and/or pulse-labeling in vivo, with subsequent detection of newly synthesized vimentin transcripts are needed to demonstrate directly transcriptional regulation of the vimentin gene in this system. These studies also may detect any significant changes in transport of mature vimentin mRNA from the nucleus to the cytoplasm. The turnover rate of vimentin mRNA during inducer-mediated differentiation of MEL cells also may be increased, thereby accelerating the removal of vimentin mRNA from differentiating cells. Pulse-chase studies should elucidate the kinetics of vimentin mRNA turnover during MEL cell differentiation.

We have shown previously a tremendous induction of vimentin mRNA accumulation during chicken embryonic erythropoiesis (5). In chicken erythroid cells, two vimentin mRNA species of 2.0 and 2.3 kb are expressed at low levels in 4-d primitive series cells, and a specific 40–50-fold higher level of the 2.0-kb mRNA is observed by 15 d of development in definitive series cells. The increase in steady state vimentin mRNA during chicken embryonic erythropoiesis underlies similar changes observed at the protein level (5). Hence, although vimentin expression in chicken erythropoiesis in vivo and mammalian erythropoiesis in vitro are both regulated transcriptionally and/or post-transcriptionally, in the nucleated erythroid cells of chickens the regulation is positive, whereas in differentiating MEL cells the regulation is negative. In both cases, however, the change in magnitude of steady state vimentin mRNA is large (~50- to 100-fold). Such a comparison of vimentin mRNA levels during avian and mammalian erythropoiesis illustrates that vimentin gene expression can be dynamically regulated in both positive and negative fashions. The contrast between these two systems further suggests that the regulation of vimentin expression is an essential feature of each of the respective terminal differentiation programs, and the differences observed indeed may reflect the functional requirements of these cells (see below).

Relationship of Vimentin mRNA Repression to Commitment of MEL Cells to Terminal Differentiation

After a defined period in inducer, MEL cells acquire the capacity to continue in their maturation program in the

absence of chemical inducer; this characteristic represents commitment to terminal differentiation (12, 21). In the presence of DMSO, the major onset of MEL cell commitment appears to occur after 20–24 h of induction, at which time ~30% of the cells are committed; the appearance of committed cells increases rapidly thereafter, and at 48 h of induction reaches a plateau level of ~90% (21). Qualitatively similar results for DMSO-induced differentiation and commitment have been reported by others (41). By comparison, vimentin mRNA levels are reduced rapidly very early in DMSO-mediated induction; a significant decrease (to ~70% control levels) is observed at 12 h, the earliest time point examined (Fig. 4*B*). The kinetics of this repression indicates that the decline in vimentin mRNA precedes commitment of MEL cells to terminally differentiate, as vimentin mRNA levels are reduced ~3-fold by 24 h (Fig. 4*B*), corresponding to a time when a detectable fraction of committed cells is only just beginning to appear in the population (21, 41).

Two lines of evidence suggest that the decrease in vimentin mRNA expression in MEL cells is an event associated with terminal differentiation, even though this reduction temporally appears to precede commitment. First, HMBA, an agent that induces commitment and terminal differentiation (11), also causes a large repression of vimentin mRNA (Fig. 5*A*). Second, exposure to hemin for 72 h results in a comparatively smaller change in vimentin mRNA abundance (~50% of control, see Fig. 5*B*). MEL cells treated with hemin display a rapid (within ~6 h) increase in globin mRNA (22, 41, 51), but do not show changes characteristic of terminal differentiation (22), such as limited proliferative capacity (i.e., commitment; 12, 21), induction of cytidine deaminase activity (46), or induction of the chromatin-associated protein, IP_{25} (24). Our observations show that the precipitous decline in vimentin mRNA is an early event in MEL cell differentiation, and this event precedes, but may be associated with commitment to terminal differentiation. A conclusive answer to this subject awaits further experimentation with inhibitors of MEL cell differentiation (see reference 35) and the study of variant MEL cell lines resistant or hypersensitive to induction (for example, see reference 52).

Inducer-mediated MEL Cell Differentiation as a Model System for Vimentin Expression in Mammalian Erythropoiesis

Studies of human hematopoiesis in vivo have shown that vimentin expression is lost during erythropoiesis, although the stage at which this event occurs could not be ascertained (7). Furthermore, the loss of vimentin during the erythroblastic stages of erythropoiesis is maintained in the mature circulating erythrocyte, and no other intermediate filament subunits are detected (7). Intermediate filaments have never been described in the mature mammalian erythrocyte. Examination of the regulatory mechanisms involved in vimentin gene expression during MEL cell differentiation should facilitate the understanding of this phenomenon in vivo.

Since the function of intermediate filaments has not been determined directly (27, 28), the significance of the repression of vimentin expression in mammalian erythropoiesis is unclear. However, electron microscopic examination of nucleated chicken erythrocytes reveals a transcytoplasmic network of vimentin filaments that appears to anchor the centrally located nucleus (18). We speculate that the loss of vimentin filaments during mammalian erythropoiesis facilitates enucleation, as the presence of intermediate filaments at this late stage of development physically may hinder this process (for example, see reference 47). The repression of vimentin synthesis early in MEL cell differentiation may reflect the necessity in vivo of allowing a sufficient number of cell divisions to dilute existing filaments.

Generalization of MEL Cell Regulation of Vimentin Expression to Other Terminally Differentiating Cells

We have proposed previously that vimentin expression in terminally differentiating cells is regulated at the level of mRNA abundance (5). For example, neurons substitute vimentin completely with the neuron-specific intermediate filament protein during their differentiation (59). In other cell types, vimentin is replaced only partially by the cell-specific intermediate filament protein, such as desmin in muscle differentiation (15), and may be co-expressed with a cell type-specific intermediate filament subunit in the mature cell (e.g., chicken skeletal muscle: desmin [17], rat and chicken astrocytes: glial fibrillary acidic protein [59, 63], some mouse retinal neurons: neurofilament protein [9]). Our observation that the loss of vimentin filaments in differentiating MEL cells is regulated primarily by mRNA abundance suggests that the complete or partial repression of vimentin expression in other differentiating cell types is also controlled at the mRNA level.

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