Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 6481–6485, September 1987
Cell Biology

Dimethyl sulfoxide-inducible cytoplasmic factor involved in erythroid differentiation in mouse erythroleukemia (Friend) cells

(hematopoiesis)

TOSHIO WATANABE AND MICHIO OISHI

Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

Communicated by Hidesaburo Hanafusa, May 29, 1987 (received for review March 27, 1987)

ABSTRACT **A previous report described an intracellular factor (differentiation-inducing factor I, or DIF-I) that seems to play a role in erythroid differentiation in mouse erythroleukemia (MEL) cells. We have detected another erythroid-inducing factor in cell-free extracts from dimethyl sulfoxide- or hexamethylenebis(acetamide)-treated MEL cells, which acts synergistically with DIF-I. The partially purified factor (termed DIF-II) triggered erythroid differentiation when introduced into undifferentiated MEL cells that had been potentiated by the induction of DIF-I. The activity in the extracts appeared in an inducible manner after addition of dimethyl sulfoxide or hexamethylenebis(acetamide), reached a maximum at 6 hr, and then rapidly decreased. The induction was inhibited by phorbol 12-myristate 13-acetate and also by cycloheximide. No induction was observed in a mutant MEL cell line defective in erythroid differentiation. These characteristics are consistent with the supposition that DIF-II is one of the putative dimethyl sulfoxide-inducible factors detected in previously reported cell-fusion and cytoplast-fusion experiments. The role of DIF-II in MEL-cell differentiation and *in vitro* differentiation in general is discussed.**

Upon exposure to a variety of agents such as dimethyl sulfoxide (Me_2SO) (1), hexamethylenebis(acetamide) (HMBA) (2), and butyric acid (3, 4), mouse erythroleukemia (MEL) cells differentiate *in vitro* to a cell type having the characteristics of erythroid cells. This system has been used as a model not only for terminal erythroid differentiation but also for *in vitro* differentiation in general. There are several reports regarding possible molecular events responsible for the *in vitro* MEL cell differentiation. These events include DNA "demethylation" processes (5, 6), single-strand breaks in chromosomes (7–9), conformational changes in chromatin structure (10, 11), changes in polyamine metabolism (12, 13), and expression or suppression of specific oncogenes (14–21).

By use of cell fusion, previous work showed the erythroid differentiation in MEL cells to be a synergistic result of at least two distinctive intracellular reactions (22); one originating from the inhibition or cessation of DNA replication and the other involving a transmembrane reaction triggered by the majority of inducing agents such as Me_2SO or HMBA. Further studies employing cytoplast fusion as well as cell fusion (23, 24) revealed the following characteristics. The former reaction is inducible but not specific to MEL cells. The latter, which is specific to MEL cells, is also inducible accompanying *de novo* protein synthesis, although the induced activity remains in the cells only transiently. The induction is inhibited by tumor-promoting phorbol esters.

More recent studies dealt with a protein factor of cytoplasmic origin that seems to be responsible for the first reaction (25). The factor can be induced in several mouse cell lines, including nonerythroid cells, following treatment of the cells with agents that affect or disturb DNA replication. Upon introduction into undifferentiated MEL cells, this factor, now designated DIF-I (differentiation-inducing factor I), triggers erythroid differentiation quite efficiently provided the cells have been potentiated by the induction of the second reaction.

Using an experimental strategy similar to the one for isolating DIF-I, we were able to demonstrate the presence of a factor in the cell-free extracts that exhibits a number of characteristics similar to those of the second factor implicated by the previous cell-fusion and cytoplast-fusion experiments. Here we report the basic characteristics of the second differentiation-inducing factor, DIF-II, for erythroid differentiation. A preliminary report of this study has appeared in abstract form (26).

MATERIALS AND METHODS

Materials. L-α-Lysophosphatidylcholine (lysolecithin), phenylmethylsulfonyl fluoride, dithiothreitol, aminopterin, hypoxanthine, and thymidine were purchased from Sigma; ouabain from Boehringer-Mannheim Yamanouchi (Tokyo); and mitomycin C from Kyowa Hakko (Tokyo). DEAE-cellulose (DE52) and Superose 12 were purchased from Whatman (Maidstone, Kent, England) and Pharmacia (Uppsala, Sweden), respectively. HMBA was a generous gift from T. Yamane (Bell Laboratories, Murray Hill, NJ) and phorbol 12-myristate 13-acetate (PMA) was a gift from M. Terada (National Cancer Center Institute, Tokyo). All the other chemicals used were reagent grade. Eagle's minimal essential medium was obtained from Nissui Seiyaku (Tokyo). Ham's nutrient mixture F-12 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma. Fetal bovine serum was obtained from Flow Laboratories (McLean, Virginia) and United Biotechnologies (Tokyo).

Cells and Cell Culture. MEL (Friend) cells (745A, DS19) and a thymidine kinase-deficient mutant (DS19 TK^-) were generously provided by M. Terada and R. A. Rifkind (Memorial Sloan–Kettering Cancer Center, New York), respectively. An ouabain-resistant DS19 line (TK^-Oua^r) was obtained after treating DS19 (TK^-) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. A MEL cell line (DR1) that is unable to differentiate in the presence of Me_2SO (or HMBA) was obtained from DS19 (TK^-Oua^r) by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. The MEL cell line 11A2, which grows in F-12/DMEM containing a low concentration (1%) of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DIF, differentiation-inducing factor; MEL, mouse erythroleukemia; Me_2SO, dimethyl sulfoxide; HMBA, hexamethylenebis(acetamide); PMA, phorbol 12-myristate 13-acetate; TK^-, thymidine kinase-deficient; Oua^r, ouabain-resistant.

fetal bovine serum and was used as a source of DIF-II, was established in this laboratory after successive adaptations of DS19 in the low-serum medium. Mouse FM3A cells (27) were supplied by D. Ayusawa (Saitama Cancer Center, Saitama, Japan). All cells except FM3A and MEL 11A2 were cultured in minimal essential medium with 12% fetal bovine serum. FM3A and MEL 11A2 cells were cultured in F-12/DMEM (1:1, vol/vol) supplemented with 1% fetal bovine serum. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Preparation of DIF-II. MEL 11A2 cells were cultured in 10-liter spinner flasks. When the cell density reached 2.5×10^6 cells per ml, the culture was diluted with fresh medium to 10^6 cells per ml. Generally, 5- to 10-liter cultures were used for the preparation of one batch of cytosol. Me_2SO was then added to a final concentration of 1.8% (vol/vol) (280 mM) and the culture was continued for 6 hr. The cells were collected by centrifugation, washed twice with phosphate-buffered saline (137 mM NaCl/2.4 mM KCl/9.6 mM Na_2HPO_4/1.1 mM KH_2PO_4) and once with TKM buffer [10 mM Tris Cl, pH 7.5/10 mM KCl/1.5 mM $Mg(OAc)_2$/0.1 mM dithiothreitol/0.1 mM phenylmethylsulfonyl fluoride], and resuspended in TKM buffer at 5×10^8 cells per ml. The cells were allowed to stand at 0°C for 15 min and were disrupted by either a Dounce or Teflon homogenizer (Potter–Elvehjem type, Iuchi level 90) for large-scale preparation. The sample was then mixed with 0.25 volume buffer S [100 mM Tris Cl, pH 7.5/1.25 M sucrose/25 mM $Mg(OAc)_2$] and centrifuged at $1200 \times g$ for 5 min to remove nuclei. The supernatant fraction was then centrifuged at $120,000 \times g$ for 90 min to separate the particulate fractions (membranes and mitochondria) from the supernatant (cytosol) fraction, which was used as the primary source of DIF-II. To detect DIF-II activity, in a typical experiment the cytosol fraction (≈550 mg protein) was diluted 1:3 with basal buffer [20 mM Tris Cl, pH 7.5/10% (vol/vol) glycerol/0.25 mM dithiothreitol] and applied to a DEAE-cellulose column (20 × 100 mm) that had been equilibrated with basal buffer. After the column was washed with 150 ml of basal buffer, DIF-II was eluted with 150 ml of basal buffer containing 50 mM NaCl. Sometimes, the column was further subjected to basal buffer solutions containing higher concentrations of NaCl. Fractions that exhibited DIF-II activity were pooled (≈8 ml), dialyzed against basal buffer for 6 hr, and concentrated severalfold in a Minicon (Amicon B-15). Protein concentrations were determined by a protein assay kit (Bio-Rad). All manipulations were carried out at 0–4°C, unless otherwise specified.

Assay for Erythroid-Inducing Activity. The erythroid-inducing activity of DIF-II was assayed as described (25, 28), with the following modification. MEL DS19 cells grown to confluence were collected by centrifugation ($500 \times g$, 5 min), washed once with phosphate-buffered saline, and resuspended in phosphate-buffered saline at 5×10^6 cells per ml. Two milliliters of the sample was then transferred to a plastic Petri dish (60-mm diameter) and irradiated (20 J/m^2) under a germicidal UV lamp (Toshiba GL15, 15-W). After centrifugation, the cells were resuspended in minimal essential medium/12% fetal bovine serum at 8×10^5 cells per ml and incubated for 15 hr at 37°C in a CO_2 incubator. The cells (total 5×10^6) were then collected by centrifugation ($500 \times g$, 10 min) and washed twice with cold phosphate-buffered saline. To the sedimented cells, 1 ml of cold (0°C) L-α-lysophosphatidylcholine solution (4.2 μg/ml in minimal essential medium) was added and the cells were thoroughly mixed using a Pasteur pipette. The cell suspension was left for 3.5 min at 0°C, and then 10 μl was quickly transferred with an automatic pipette to each well of a microplate (Falcon, 96 wells) containing 180 μl of prewarmed (37°C) minimal essential medium/12% fetal bovine serum and 20-μl samples for assay. After mixing, the cells were incubated at 37°C in a CO_2 incubator. On the fifth day, the erythroid-inducing activity was assayed by scoring benzidine-reactive cells according to the method of Orkin *et al.* (29); at least 1000 cells were scored.

RESULTS

Detection of a Me_2SO (HMBA)-Inducible Factor in Crude Extracts. Cell-free extracts (cytosol) from MEL cells that had been exposed to 1.8% (vol/vol) Me_2SO for 6 hr was first prepared under conditions that would produce a maximal level of the putative Me_2SO (or HMBA)-inducible second factor in the cells, these conditions being based upon the results obtained from previous cytoplast-fusion experiments (24). The extracts were then introduced into undifferentiated MEL cells that had been exposed to UV light (20 J/m^2), thereby maximizing the production of DIF-I, which should act synergistically with the Me_2SO-inducible factor for MEL-cell differentiation (25). For introducing the extracts into the cells, we employed a procedure to make the cells permeable to protein molecules (28), modified slightly from the original protocol successfully applied for the isolation of DIF-I (25). Although no erythroid-inducing activity was detected in the crude cytosol, an erythroid-inducing activity (assayed by hemoglobin accumulation) was detected in the eluate (50 mM NaCl) after a stepwise elution of the cytosol from a DEAE column (Fig. 1*B*). No such activity was detected in the same eluate of the cytosol prepared from control cells (no Me_2SO treatment, Fig. 1*A*). As reported previously (25), DIF-I activity, which is induced by either UV irradiation or mitomycin C, is eluted with 250 mM NaCl from a DEAE column. As shown in Fig. 2, the Me_2SO-inducible activity was not detected without UV treatment of recipient cells, suggesting that the induction of DIF-I in recipient cells is required for detection of the activity in the extracts. Fig. 2 also shows that the activity was observed only when the recipient cells were made permeable to protein molecules. Apparently, the factor must be taken up by the cells to exert the activity.

We prepared cytosol from MEL cells that had been treated with various inducing agents and inhibitors and from non-erythroid FM3A cells (a cell line derived from a mouse mammary gland tumor and used for large-scale preparation of DIF-I). The cytosol from each cell preparation was applied to a DEAE column and the erythroid-inducing activities in the 50 mM eluate were examined. In addition to Me_2SO (Fig. 3*B*), the activity was also induced by HMBA (Fig. 3*C*), another potent inducer of erythroid differentiation (2). On the other

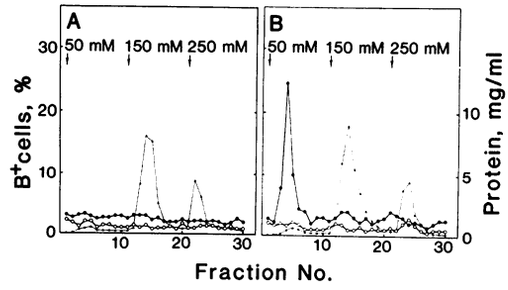

FIG. 1. Erythroid-inducing activity in cytosol subjected to DEAE column chromatography. MEL 11A2 cells were incubated for 6 hr in the absence (*A*) or presence (*B*) of 1.8% Me_2SO. The cytosol fraction (≈600 mg of protein) was applied to a DEAE-cellulose column (20 × 80 mm) and eluted in a stepwise manner with 80 ml each of 50 mM, 150 mM, and 250 mM NaCl in basal buffer. Samples (20 μl) of each fraction (8 ml) were assayed for erythroid-inducing activity on DS19 cells that had been irradiated by UV light and made permeable (●) or that had not been irradiated but had been made permeable (○). Erythroid-inducing activity is shown as percentage of benzidine-reactive (B^+) cells. Protein concentration of each fraction was measured; protein peaks occur at fractions 13–17 and 22–25.

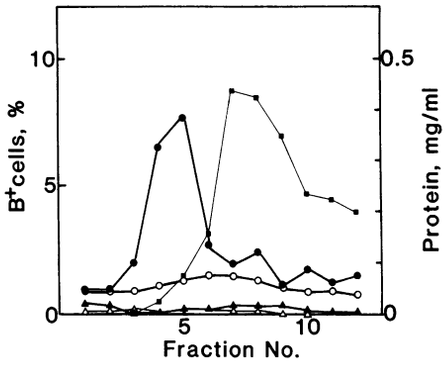

FIG. 2. Detection of erythroid-inducing activity under various assay conditions. MEL 11A2 cells were incubated for 6 hr in the presence of 1.8% Me_2SO. The cytosol fraction (≈120 mg of protein) was applied to a DEAE column (12 × 86 mm) and eluted with 50 mM NaCl in basal buffer. From each fraction (1.25 ml), samples (20 μl) were taken and assayed for erythroid-inducing activity (shown as in Fig. 1) on DS19 cells that had been irradiated by UV light and permeabilized to proteins (●), that had been irradiated but not permeabilized (○), that had not been irradiated but had been permeabilized (▲), or that had been neither irradiated nor permeabilized (△). Protein concentration of each fraction (■) is shown.

hand, no activity was detected in the eluate prepared from MEL cells that had been treated with Me_2SO in the presence of PMA, a tumor-promoting phorbol ester and specific inhibitor of erythroid differentiation of MEL cells (Fig. 3*D*). There was also no activity detected in the 50 mM eluate from cells treated with Me_2SO plus cycloheximide, an inhibitor of protein synthesis (Fig. 3*E*). No activity was induced in mouse nonerythroid FM3A cells by Me_2SO (Fig. 3*F*). These characteristics associated with induction of the activity are similar to those of the putative second intracellular factor implicated from the previous cell-fusion and cytoplast-fusion experiments: (*i*) the factor acts synergistically with DIF-I, (*ii*) the factor is induced by Me_2SO or HMBA, (*iii*) induction is inhibited by PMA, (*iv*) induction requires *de novo* protein synthesis, and (*v*) induction is specific to MEL cells. We have

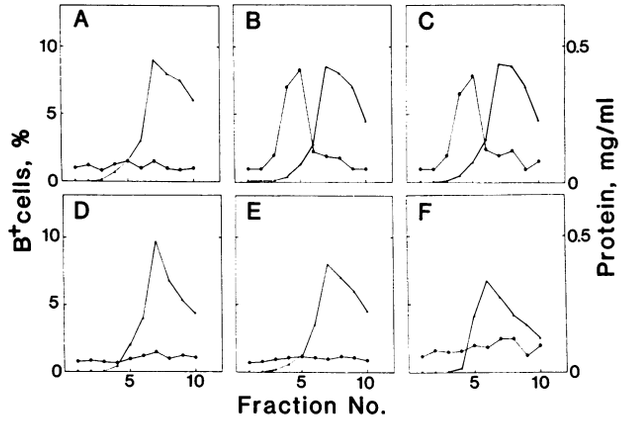

FIG. 3. Induction of erythroid-inducing activity (DIF-II) under various conditions. MEL 11A2 cells and nonerythroid FM3A cells were incubated under various conditions (see below) for 6 hr. The cytosol fraction (≈120 mg of protein) from each preparation was applied to a DEAE column (12 × 86 mm) and eluted with 50 mM NaCl in basal buffer. From each fraction (1.25 ml), samples (20 μl) were taken and assayed for erythroid-inducing activity (shown as in Fig. 1). (*A*) Untreated MEL 11A2 cells (control). (*B*) MEL 11A2 cells treated with 1.8% Me_2SO for 6 hr. (*C*) MEL 11A2 cells treated with 5 mM HMBA for 6 hr. (*D*) MEL 11A2 cells treated with 1.8% Me_2SO plus PMA at 100 ng/ml. (*E*) MEL 11A2 cells treated with 1.8% Me_2SO plus cycloheximide at 10 μg/ml. (*F*) FM3A cells treated with 1.8% Me_2SO for 6 hr. Protein concentration of each fraction was measured; protein peaks occur around fraction 7.

designated the activity differentiation-inducing factor II (DIF-II), as opposed to the factor already identified, DIF-I, which acts synergistically with DIF-II.

Induction Kinetics of DIF-II. We examined the time course for the appearance of DIF-II in the cytosol. MEL cells were exposed to 1.8% Me_2SO and, at various time intervals, the cytosol was prepared and the 50 mM NaCl eluate from the DEAE column was assayed for activity. As shown in Fig. 4, the activity apparently began to appear soon after addition of Me_2SO, reached a maximum at 6 hr of incubation, and decreased after 10 hr. It then remained at a low or nondetectable level for at least the next 30 hr. The kinetics, especially with regard to the transient nature of DIF-II, were very similar to those revealed by the cytoplast-fusion experiments on the potential level induced by Me_2SO or HMBA (24).

Lack of Induction of DIF-II in the Differentiation-Defective MEL Cell Line DR1. After mutagenesis of MEL cell line DS19 (TK^-Oua^r) with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, we isolated clones that were unable to undergo erythroid differentiation in the presence of Me_2SO or HMBA. One clone (DR1) was selected that was similar to one reported by Ohta *et al.* (30), which had exhibited almost no detectable level of erythroid induction (<0.1% benzidine-positive cells) after incubation for 120 hr with any of three typical inducing agents [Me_2SO (1.8%), HMBA (5 mM), or sodium butyrate (1 mM)]. Cell-fusion experiments with DR1 and parental MEL cells (DS19 TK^+Oua^s) indicated DR1 to be normal for DIF-I induction but defective for the Me_2SO-induced reaction (T.W., S. Nomura, and M.O., unpublished work).

We prepared cytosol from DR1 cells that had been exposed to Me_2SO for 6 hr and assayed the DIF-II activity in the 50 mM DEAE eluate. None was detected in this fraction (Fig. 5*A*). On the other hand, when cytosol was prepared from mitomycin C-treated DR1 cells and assayed for DIF-I activity after DEAE chromatography, a normal level of DIF-I activity was detected in the 250 mM eluate where DIF-I was expected to be eluted (Fig. 5*B*) (25). Thus, DR1 cells are apparently impaired in the process leading to the induction of DIF-II. This is consistent with the results of the cell-fusion experiments described above. These experiments provide further evidence that DIF-II is involved in *in vitro* erythroid differentiation by Me_2SO or HMBA.

We examined the distribution of DIF-II activity among subcellular fractions. MEL cells were incubated with 1.8% Me_2SO for 6 hr, and three subcellular fractions (nuclei, mitochondria and membrane fragments, and cytosol) were obtained by differential centrifugations. The particulate fractions were further treated with high concentrations of NaCl (0.3 M for nuclei and 0.5 M for membranes and mitochondria

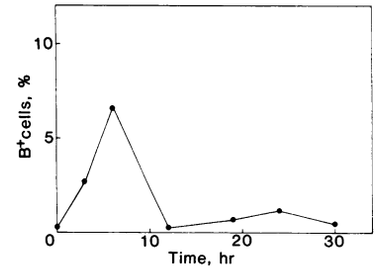

FIG. 4. Kinetics of induction of DIF-II after exposure to Me_2SO. MEL 11A2 cells were incubated in the presence of 1.8% Me_2SO for various times. Cytosol (≈70 mg of protein) was prepared, applied to a DEAE column (12 × 45 mm), and eluted with 50 mM NaCl in basal buffer. From the eluted fractions (1.25 ml), fractions 2–10 were pooled and concentrated in a Minicon (Amicon). After the protein concentration was adjusted to 1.0 mg/ml, 20-μl samples were assayed for erythroid-inducing activity, which is expressed as the percentage of benzidine-reactive (B^+) cells after subtraction of background (1.8%, B^+ cells without the samples).

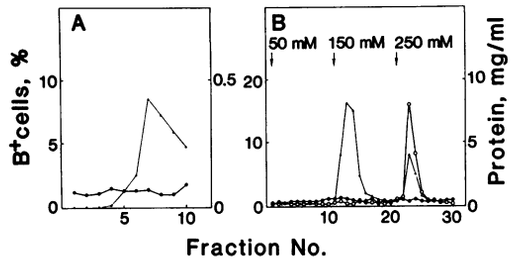

FIG. 5. DIF-II and DIF-I activities in cytosol from the differentiation-defective MEL cell line DR1. (*A*) DR1 cells were incubated in the presence of 1.8% Me_2SO for 6 hr. The cytosol fraction (≈110 mg of protein) was applied to a DEAE column (12 × 75 mm) and eluted with 50 mM NaCl in basal buffer. From each fraction (2.0 ml), 20 μl was taken for assay of erythroid-inducing activity (●), shown as percentage of benzidine-reactive (B^+) cells. Protein concentration of each fraction (▲) is shown. (*B*) DR1 cells were incubated in the presence of mitomycin C (1.0 μg/ml) for 24 hr. The cytosol fraction (≈110 mg of protein) was applied to a DEAE column (12 × 75 mm) and eluted in a stepwise manner with 20 ml each of 50 mM, 150 mM, and 250 mM NaCl in basal buffer. From each fraction (2 ml), 20 μl was assayed for DIF-I activity (○) and DIF-II activity (●). Protein concentration of each fraction was measured; protein peaks are at fraction 7 in *A* and at fractions 13 and 23 in *B*.

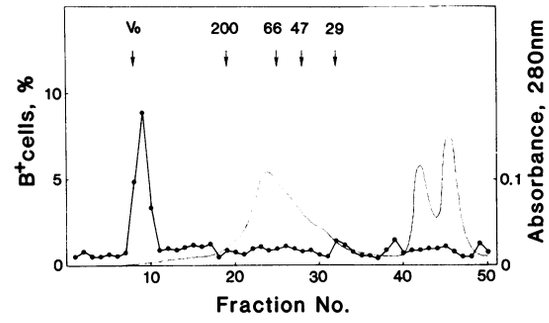

FIG. 7. Gel filtration of DIF-II. The DIF-II fraction eluted with 50 mM NaCl from a DEAE column was concentrated in a Minicon (Amicon), and 200 μl (680 μg of protein) of the sample was fractionated through Superose 12 (Pharmacia) at a flow rate of 0.5 ml/min, using basal buffer containing 50 mM NaCl. From each fraction (0.25 ml), 20 μl was assayed for erythroid-inducing activity (●). Protein concentration of each fraction was monitored automatically at 280 nm and is shown as a solid line without symbols. The molecular weight markers used to calibrate the column were β-amylase (M_r 200,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 47,000), and carbonic anhydrase (M_r 29,000). The void volume (V_o) was determined by blue dextran.

after sonication), and the activity in the extracts was examined. More than 93% of the activity was present in the cytosol fraction (data not shown), consistent with previous cytoplast-fusion experiments in which Me_2SO-induced activity was located in cytoplasts (24).

Effect of DIF-II Concentration on Erythroid Induction. Fig. 6 shows the dose–response curve for partially purified DIF-II used for the induction of erythroid cells. The cells were incubated in the presence of various concentrations of DIF-II (50 mM eluate from a DEAE column) and the percentage of benzidine-positive cells was determined 5 days later. The percentage increased as the concentration of DIF-II increased, but the activity reached a plateau at ≈50%. It can also be seen that no induction was observed, even with increased concentrations of DIF-II, in either of the cultures of MEL cells that were not made permeable to proteins or were not preirradiated with UV light.

DIF-II Is a Protein. DIF-II was partially purified from the cytosol by successive column chromatography, including stepwise DEAE-cellulose chromatography, ion-exchange FPLC (Mono Q), hydroxylapatite chromatography, and gel filtration with Superose 12 (unpublished work). The partially purified DIF-II exhibited a proteinaceous nature, the erythroid-inducing activity being completely lost when the sample was treated with trypsin (2.0 μg/ml, 5 min at 37°C) or heat (15 min at 56°C) (data not shown). Its activity was nondialyzable (data not shown) and, in contrast to DIF-I, the molecular size of DIF-II was found to be quite large. Its activity was eluted near the void volume of a Superose 12 gel-filtration column (Fig. 7). The large molecular size of DIF-II was also confirmed by glycerol gradient centrifugation in which its activity was detected at the position where proteins with a higher molecular weight than catalase (M_r 246,000) sedimented (data not shown).

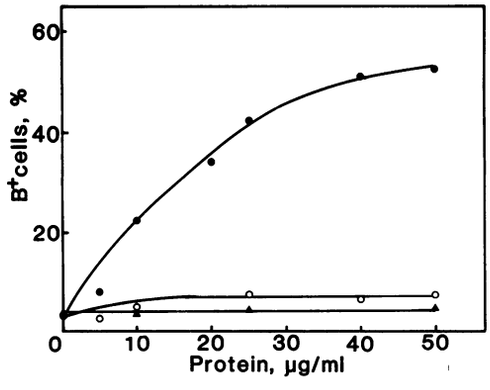

FIG. 6. Effect of DIF-II concentration on induction of erythroid cells. The DIF-II fraction, eluted with 50 mM NaCl from a DEAE column, was concentrated in a Minicon (Amicon). Three different recipient-cell preparations (see below) were exposed to various concentrations of DIF-II (abscissa) to assay for erythroid-inducing activity. ●, DS19 cells that had been irradiated by UV light and made permeable; ○, DS19 cells that had been irradiated but not made permeable; ▲, DS19 cells that had not been irradiated but had been made permeable.

DISCUSSION

This paper presents evidence for the presence of a factor responsible for *in vitro* erythroid differentiation, in the cytosol of Me_2SO (or HMBA)-treated cells. The factor, apparently a protein, induced erythroid differentiation when introduced into undifferentiated MEL cells, provided the cells had been treated with UV light, a condition necessary for inducing the factor DIF-I, previously reported (22–24). From several aspects, it is quite obvious that DIF-II is different from DIF-I. Although both are located in the cytoplasm and require *de novo* protein synthesis for induction, DIF-II is induced only transiently and only in MEL cells by Me_2SO or HMBA; its induction was inhibited by PMA and did not take place in a mutant MEL cell unable to undergo erythroid differentiation. Chromatographic behavior also distinguishes DIF-II from DIF-I.

The detection of a cytosolic factor (DIF-II) whose characteristics agree almost completely with those of the putative factor implicated from the previous cell-fusion and cytoplast-fusion experiments further supports the view that the *in vitro* terminal differentiation of MEL cells results from the synergistic action of two distinctive cellular reactions (7, 8, 22–24). In fact, we believe that exploiting this view to the full has made possible the detection and isolation of these two intracellular factors. For assay of DIF-II, we used recipient MEL cells in which one of the reactions had been fully induced; thus these cells had been sensitized to the factor(s) responsible for the other reaction. Neither DIF-II nor DIF-I

activity was detected in the extracts without such recipient-cell sensitization.

How universal is the role played by DIF-II in inducing *in vitro* cellular differentiation? Me_2SO and HMBA are the two agents most commonly used for inducing *in vitro* MEL differentiation; besides MEL cells, these agents induce differentiation in a number of other cell lines (31–34). Therefore, although the differentiation-inducing activity associated with DIF-II was examined only in MEL cells, it is reasonable to suggest that DIF-II might also have a role in the *in vitro* differentiation of other cell lines that respond to Me_2SO and HMBA.

We do not know how DIF-II triggers the erythroid differentiation or how it acts synergistically with DIF-I. In a limited number of experiments, no enzymatic activity (topoisomerase or DNase) was found to be associated with DIF-I or DIF-II. The discovery of any enzymatic activity and of any possible interaction between the two factors, along with the cloning of the genes that encode these factors, would be important for elucidating the role that these factors play in cellular differentiation.

We wish to thank Ms. M. Harada for editing the manuscript. We thank Dr. S. Nomura for valuable discussions throughout the study. This work was supported by a Grant-in-Aid for Special Project Research, Cancer-Bioscience, from the Ministry of Education, Science, and Culture of Japan.

1. Friend, C., Scher, W., Holland, J. G. & Sato, T. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 378–382.
2. Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A. & Marks, P. A. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 862–866.
3. Leder, A. & Leder, P. (1975) *Cell* **5,** 319–322.
4. Takahashi, E., Yamada, M., Saito, M., Kuboyama, M. & Ogasa, K. (1975) *Gann* **66,** 577–580.
5. Christman, J. K., Price, P., Pedrinaw, L. & Acs, G. (1977) *Eur. J. Biochem.* **81,** 53–61.
6. Razin, A., Szyf, M., Kafri, T., Roll, M., Giloh, H., Scarpa, S., Carotti, D. & Cantoni, G. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2827–2831.
7. Terada, M., Nudel, U., Fibach, E., Rifkind, R. A. & Marks, P. A. (1978) *Cancer Res.* **38,** 835–840.
8. Scher, W. & Friend, C. (1978) *Cancer Res.* **38,** 841–849.
9. McMahon, G., Alsina, J. L. & Levy, S. B. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7461–7465.
10. Luchnik, A. N. & Glaser, V. M. (1980) *Mol. Gen. Genet.* **178,** 459–463.
11. Reboulleau, C. P. & Shapiro, H. S. (1983) *Biochemistry* **22,** 4512–4517.
12. Gazitt, Y. & Friend, C. (1980) *Cancer Res.* **40,** 1727–1732.
13. Watanabe, T., Sherman, M., Shafman, T., Iwata, T. & Kufe, D. (1986) *J. Cell. Physiol.* **127,** 480–484.
14. Lachman, H. M. & Skoultchi, A. I. (1984) *Nature (London)* **310,** 592–594.
15. Lachman, H. M., Hatton, K. S., Skoultchi, A. I. & Schildkraut, C. L. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 5323–5327.
16. Ramsay, R. G., Ikeda, K., Rifkind, R. A. & Marks, P. A. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6849–6853.
17. Todokoro, K. & Ikawa, Y. (1986) *Biochem. Biophys. Res. Commun.* **135,** 1112–1118.
18. Lachman, H. M., Cheng, G. & Skoultchi, A. I. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6480–6484.
19. Coppola, J. A. & Cole, M. D. (1986) *Nature (London)* **320,** 760–763.
20. Dmitrovsky, E., Kuehl, W. M., Hollis, G. F., Kirsch, I. R., Bender, T. P. & Segal, S. (1986) *Nature (London)* **322,** 748–750.
21. Prochownik, E. V. & Kukowska, J. (1986) *Nature (London)* **322,** 848–850.
22. Nomura, S. & Oishi, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 210–214.
23. Kaneko, T., Nomura, S. & Oishi, M. (1984) *Cancer Res.* **44,** 1756–1760.
24. Watanabe, T., Nomura, S. & Oishi, M. (1985) *Exp. Cell Res.* **159,** 224–234.
25. Nomura, S., Yamagoe, S., Kamiya, T. & Oishi, M. (1986) *Cell* **44,** 663–669.
26. Watanabe, T. & Oishi, M. (1986) *J. Cell. Biol.* **103,** 495 (abstr.).
27. Nakano, N. (1966) *Tohoku J. Exp. Med.* **88,** 69–84.
28. Nomura, S., Kamiya, T. & Oishi, M. (1986) *Exp. Cell Res.* **163,** 434–444.
29. Orkin, S. H., Harosi, F. I. & Leder, P. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 98–102.
30. Ohta, Y., Tanaka, M., Terada, M., Miller, O. J., Bank, A., Marks, P. A. & Rifkind, R. A. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 1232–1236.
31. Collins, S. J., Ruscetti, F. W., Gallagher, R. E. & Gallo, R. C. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 2458–2462.
32. McBurney, M. W., Jones-Villeneuve, E. M. V., Edwards, M. K. S. & Anderson, P. J. (1982) *Nature (London)* **299,** 165–167.
33. Sachs, L. (1978) *Nature (London)* **274,** 535–539.
34. Miao, R. M., Fieldsteel, A. H. & Fodge, D. W. (1978) *Nature (London)* **274,** 271–272.