Murine Erythroleukemia Cell Variants: Isolation of Cells That Have Amplified the Dihydrofolate Reductase Gene and Retained the Ability To Be Induced To Differentiate†

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ABSTRACT: A series of murine erythroleukemia cell (MELC) variants was generated by selection for the ability to grow in increasing concentrations of the folate antagonist methotrexate (MTX). Growth of the parental MELC strain DS-19 was completely inhibited by 0.1 μM MTX. We isolated cells able to grow in 5, 40, 200, 400, and 800 μM MTX. Growth rates and yields were essentially the same in the presence or absence of the selective dose of MTX for all variants. MTX resistance was not the result of a transport defect. Dihydrofolate reductase (DHFR) from our variants and DS-19 was inhibited to the same extent by MTX. Variants had increased dihydrofolate reductase activities. The specific activity of DHFR was proportional to the selective concentration of MTX employed to isolate a given variant. DNA dot blotting established that the cloned variant (MR400-3) had a 160-fold increase in DHFR gene copy number relative to the parental strain (DS-19). Hybridization studies performed in situ established the presence of amplified DHFR genes on the chromosomes of the MTX-resistant but not the MTX-sensitive (parental) cells. Quantitation of DHFR mRNA by cytoplasmic dot blotting established that the amplified DHFR gene expression was proportional to gene copy number. Thus, MTX resistance was due to amplification of the DHFR gene. The variants retained the ability to be induced to differentiate in response to dimethyl sulfoxide and hexamethylenebis(acetamide) as evaluated by the criteria of globin mRNA accumulation, hemoglobin accumulation, cell volume decreases, and terminal cell division. Induced differentiation also resulted in a decrease of DHFR mRNA levels of up to 100-fold relative to uninduced matched control cells. These decreases of DHFR mRNA content were readily apparent by 48 h of exposure to inducer. We have thus established a series of MELC variants in which the expression, i.e., downregulation, of a nonstructural housekeeping gene can be studied during induced terminal erythroid differentiation.

Chemically induced differentiation of murine erythroleukemia cells (MELC)[1] results in expression of the erythrocyte phenotype (Friend et al., 1971; Gusella et al., 1976, 1982; Marks & Rifkind, 1978). We previously proposed that induced terminal erythroid differentiation has two facets: one involves expression of previously quiescent, erythrocyte-specific functions, e.g., hemoglobin accumulation; the second is manifested as terminal cell division and loss of neoplasticity (Corin et al., 1984). It was also suggested that terminal cell division might result from the concerted down regulation of a large number of housekeeping functions, e.g., folate utilization (Corin et al., 1984). A great deal of information has emerged regarding the molecular biology of the induction of erythrocyte-specific functions during induced differentiation of MELC (Hofer et al., 1982; Sheffrey et al., 1983; Gusella et al., 1983). Conversely, similar information regarding the terminal cell division aspect of differentiation is scant (Shen et al., 1983). Since nonstructural housekeeping genes are often single-copy genes expressed at low levels (Lewin, 1980), the study of their downregulation might present a logistical problem.

To study the regulation of a nonstructural housekeeping function during induced differentiation of MELC, i.e., the DHFR gene, we have stepwise selected for a series of methotrexate-(MTX-) resistant MELC variants. The rationale for this study was that by increasing the DHFR copy number studies of the enzyme and gene during differentiation of MELC would be technically feasible. A similar approach has demonstrated for other murine cells that in DHFR gene amplified cells the expression of amplified genes is subject to regulation by growth (Gudewicz et al., 1981; Hendrickson et al., 1980; Johnson et al., 1978; Kaufman & Sharp, 1983; Leys & Kellems, 1981; Schimke, 1981), cAMP (Kellems et al., 1978; Wu et al., 1982), and viral infection (Fearson et al., 1966a,b; Kellems et al., 1976). This paper documents the isolation of a series of DHFR gene amplified variants that can be chemically induced to terminal erythroid differentiation. Regulation of DHFR gene expression at the level of DHFR mRNA accumulation, during induced differentiation, is also demonstrated.

Experimental Procedures

Cells and Culture Methods. MELC strain DS-19 was employed and was the parent for the MTX-resistant MELC that were generated. Growth, harvesting, and benzidine staining of cells was as previously described (Corin et al., 1984). MTX-resistant MELC were produced by stepwise selection for cells that grew in MTX. DS-19 cells in batch culture were exposed to a less than maximally inhibitory concentration of MTX (5 nM). Cells were maintained in the

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[1] Abbreviations: MELC, murine erythroleukemia cells; HMBA, hexamethylenebis(acetamide); Me_2SO, dimethyl sulfoxide; DHFR, dihydrofolate reductase; SSC, 0.15 M NaCl/0.015 M sodium citrate; MTX, methotrexate.

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selective concentration of MTX until approximately two to five passes without lag times were achieved. Cells were then passed to medium containing 2–4-fold higher concentrations of MTX. Cells were maintained at MTX concentrations of 5, 40, 200, 400, and 800 μM. At 5, 40, and 400 μM MTX, a number of clones were derived. Cells were cloned by inoculating 0.2 mL of medium with one cell per 2 mL into wells of microtiter plates. The nomenclature adopted was such that the numbers appearing after MR are the micromolar selective concentration of MTX in which growth occurred in a given line. The number after the dash is the clone designation; e.g., MR5-1 is clone number one for cells able to grow in 5 μM MTX. Cell stocks were always maintained at the selective concentration of MTX. Cells from each clone were frozen in liquid nitrogen. Although the phenotypes studied were stable, cells were not employed after 50 passes.

Cell Volume Measurement. Cell volume was measured with a Coulter Channelyzer (Coulter Electronics, Hilaleah, FL).

Transport Assay. Initial rates of [^{3}H]methotrexate transport were determined at 24 °C at a final MTX concentration of 1 μM as previously described (Corin et al., 1984).

Dihydrofolate Reductase. Cell-free extracts were prepared (Reem & Friend, 1976), and DHFR (EC 1.5.1.3) activity was assayed (Matews et al., 1963) as previously described.

In Situ Hybridization. The probe used in this study was pDHFR11, cDNA derived from the mouse DHFR gene (Nunberg et al., 1980), and was generously provided by Dr. Robert T. Schimke. Metaphase spreads were accumulated by colcemid arrest and slides prepared by standard methodology.

The probe was labeled with ^{125}I-dCTP (Amersham Corp.) and the hybridization done essentially as previously described (Gerhard et al., 1981). The only significant difference was that a 0.1× SSC wash at 55 °C for 15 min was included to ensure the specificity of the hybrids. The slides were coated with 1:1 dilution of NTB2 autoradiographic emulsion (Kodak Corp.) in water, exposed for 1–5 days, and developed by standard methodology. After development, the slides were stained with Wright's stain (Chandler & Yunis, 1978).

DHFR Gene Copy Number. DNA was extracted from DS-19 and MR400-3 cells as previously described (Etkind et al., 1982). For copy number determinations, a known amount of DNA from each cell type was adjusted to 0.5 N NaOH, incubated for 30 min at 25 °C, adjusted to 10× SSC, and applied to Zetapor membrane filters with a BRL slot blotting apparatus. The slot blots were processed and hybridized with ^{32}P-labeled pDHFR11 as suggested by the manufacturer. After being washed, the membranes were exposed to Kodak XAR 5 film for 15 h and scanned on a densitometer to quantitate the hybridization level.

DHFR mRNA, Globin mRNA, and Actin mRNA Accumulation. The cytoplasmic dot blot method of White and Bancroft (1982) was employed to measure DHFR and globin mRNA levels in murine β-globin probe with the vector pCR1 being employed. The globin probe was kindly provided by Dr. M. Sheffery. For actin mRNA, ^{32}P-labeled hamster probe pAct1, a cytoplasmic actin cDNA, was employed. The insert of the actin probe was purified by gel electrophoresis after *Pst*I digestion of pAct1. The actin probe was kindly provided by Dr. P. Soriano. The probes were nick-translated and hybridized by standard procedures.

Results and Discussion

We previously reported that 0.1 μM MTX produced maximal growth inhibition of the parental MELC strain DS-19 (Corin et al., 1984). Since our goal was to develop MELC variants with amplified genes, a stepwise selection procedure was employed (Schimke, 1984). A series of MTX-resistant MELC were developed by utilization of this protocol. The growth of five such variants in the presence or absence of the selective concentration of MTX is shown in Figure 1. Growth of the parental strain DS-19 in the presence and absence of 0.1 μM MTX is also shown (Figure 1). The generation times did increase with increasing concentrations of MTX. This finding might indicate that resistance required expenditure of biosynthetic capabilities, e.g., amplification of the DHFR gene.

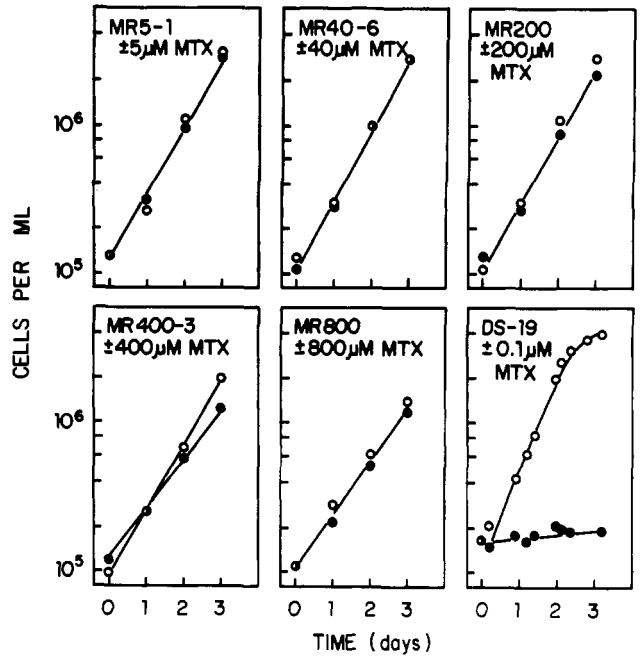

FIGURE 1: Growth of methotrexate-resistant MELC. Cells were washed free of MTX by centrifugation, inoculated at the indicated densities, and counted at the indicated times. Growth was in the absence (○) or the presence (●) of the selective concentration of MTX.

There are several mechanisms by which cells may become resistant to the cytocidal action of MTX (Schimke, 1984). These include loss of the ability to transport MTX (Sirotnak et al., 1981), a mutation in the target enzyme, DHFR, such that it binds MTX with a lower affinity than the parental enzyme (Flintoff et al., 1976; Haber et al., 1981), overproduction of DHFR (Alt et al., 1976; Bostock & Tyler-Smith, 1981; Melera et al., 1980), or some combination of the aforementioned mechanisms. Several of the MELC variants (MR5-1, MR200, MR400-3) were assayed for the ability to accumulate [^{3}H]MTX. The variants transported MTX at a rate similar to that exhibited by the parental strain (not shown). Thus, resistance probably does not result from a transport defect. MTX was able to inhibit DHFR in cell-free extracts of DS-19 and MR400-3 cells similarly with complete inhibition at 0.1 μM MTX (not shown). This result indicates that MR400-3 resistance does not result from a mutant DHFR that is insensitive to inhibition by MTX. As cells acquired the ability to grow in MTX, there was a corresponding increase in the specific activity of DHFR (not shown). It was concluded that MTX resistance of MELC variants results from increased levels of DHFR. Although the variants may have DHFR's with altered kinetic properties with respect to substrates, such changes would not account for resistance in MR400-3 since at saturating levels of substrates (assay conditions) the enzyme was potently inhibited by MTX.

To determine whether the increased DHFR activity in our MELC variants was the result of gene amplification, DHFR gene copy number was determined for DS-19 and MR400-3

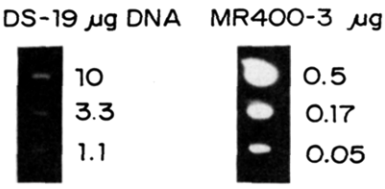

FIGURE 2: Quantitation of amplified DHFR genes in parental (DS-19) and variant (MR400-3) MELC. DNA was prepared from parental (DS-19) and variant (MR400-3) cells as previously described (Etkind et al., 1982). Aliquots of DNA were denatured in 0.5 N NaOH and adjusted with 10× SSC, and the indicated quantities of DNA were bound to Zetaphor membrane filters with a BRL slot blotting manifold. The blots were annealed with ^{32}P-labeled pDHFR11, and the signal was quantitated by scanning of an X-ray film exposed to the blot. A printed negative of the X-ray film is shown.

cells. DNA was extracted from cells, dotted on Zetaphor membranes, and hybridized with ^{32}P-labeled pDHFR11 (Figure 2). As shown, there was more hybridization of the probe to DNA from the variant cells. Densitometric scanning of X-ray films of the dots indicated that MR400-3 cells had a 160-fold increase in DHFR copy number. When cells were grown for 19 generations in the absence of selective pressure, the DHFR gene dosage remained high (not shown). This result might indicate the presence of stably amplified DHFR genes, i.e., chomosomally localized as opposed to extrachromosomal. The DNA was subjected to restriction enzyme digestion with a variety of enzymes (*Eco*RI, *Bam*HI), blotted to Zetaphor membranes, and hybridized with ^{32}P-labeled pDHFR11. The restriction digests for DS-19 and MR400-3 were identical (not shown). Thus, amplification did not alter the structure of the DHFR gene as determined by these criteria. In addition, the fragment restriction patterns observed were similar to, although not identical with, those seen with other murine DHFR genes (Nunberg et al., 1980). We believe this to be due to genetic variation between mouse strains.

To determine the localization of the amplified DHFR genes, in situ hybridization studies were performed with cells by use of the ^{125}I-labeled probe pDHFR11. The distribution of silver grains over DS-19, MR40-6, and MR400-3 interphase nuclei after a 1-day autoradiographic exposure time showed a striking difference. The average number of silver grains per interphase nucleus rose from 1.2 for DS-19 to 4.9 for MR40-6 and 20.3 for MR400-3, reflecting an increase in the DHFR gene copy number. Amplification of murine DHFR genes is initially characterized by the production of double minute chromosomes carrying the amplified genes (Kaufman et al., 1979). The MTX-resistant MELC variants may be atypical in that the bulk of the amplified genes were chromosomal. However, we believe our findings reflect long-term selection pressure favoring chromosomal amplification of the DHFR gene (Schimke, 1984). Figure 3 compares several typical metaphase cells from DS-19 (panel A) and MR400-3 (panels B and C). DS-19 was labeled at the background level; i.e., no consistent concentration of silver grains was observed at this exposure time as expected for a single- or low-copy gene. MR400-3 showed clusters of silver grains (arrows) concentrated near the telomeres of two large acrocentric chromosomes. This general labeling pattern was observed for all variants, although the number of grains associated with the amplified DHFR regions was lower for the variants selected at lower MTX concentrations. Some cells, especially the uncloned MR200 variants, showed a more variable labeling pattern (not shown). Other chromosomes and occasional double minutes were found to contain amplified DHFR genes. However, the bulk of metaphases were labeled at the chromosomal sites shown in Figure 3. The banding pattern with Wright's stain and size of the

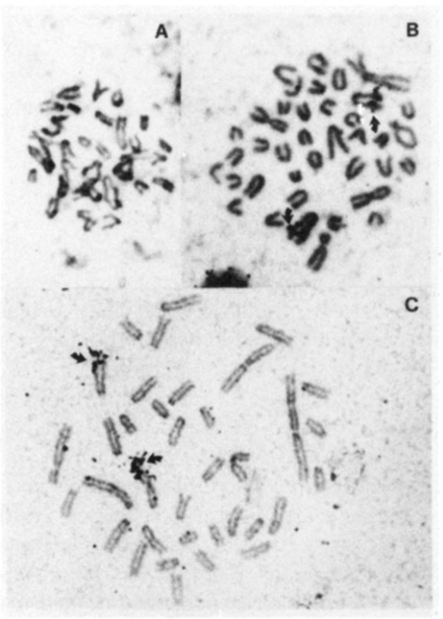

FIGURE 3: Localization of amplified DHFR genes in methotrexate-resistant MELC. Hybridization of ^{125}I-labeled pDHFR11 to MELC chromosomes. Plate A shows a metaphase cell of DS-19, the parental MELC, hybridized with ^{125}I-labeled pDHFR11 (sp act. approximately 10^8 dpm/μg) and exposed to autoradiographic emulsion for 5 days. No silver grains are seen over these chromosomes. Plates B and C show two metaphases from the MTX-resistant MELC clone MR400-3 hybridized by the same manner as the DS-19 cells in plate A. Both cells contain a pair of large acrocentric chromosomes marked by clusters of silver grains (arrows) near the telomere. This labeled site was observed in the majority of cells from MTX-resistant lines.

chromosome with the amplified DHFR genes is consistant with those of chromosome 2. In the murine leukemic cell line LY5178, chromosomally localized amplified DHFR genes had been shown to be present on chromosome number 2 (Dolnick et al., 1979).

Clone MR400-3 was chosen for characterization with respect to differentiation. Figure 4A shows dose responses to hexamethylenebis(acetamide) (HMBA), dimethyl sulfoxide (Me_2SO), and butyrate for the production of benzidine-reactive (hemoglobin-containing) cells. All three agents are inducers of hemoglobin accumulation for the parental strain DS-19 (Marks & Rifkind, 1978) as well as the variant MR400-3 (Figure 4A). The kinetics of hemoglobin accumulation are shown in Figure 4B. The kinetics are slower than for DS-19 cells, but by 6 days, greater than 90% of the cells become benzidine reactive [see Figure 3 in Corin et al. (1984) for comparison]. The slower kinetics might be accounted for by the longer generation time for MR400-3 cells (18 h) as compared to DS-19 cells (11.5 h). The concentration of HMBA (2.5 mM) used in this experiment was somewhat less than optimal (Figure 5A). Thus, the responsiveness of MR400-3 cells seems to be extensive and uniform with respect to induction of hemoglobin-containing cells. Exposure of MR400-3 cells to HMBA (2.5 mM) or Me_2SO (1.5%) for 24 h resulted in reduction of cell volume when compared to control cells not exposed to inducers (not shown). Such volume changes are characteristic of induced differentiation of MELC (Loritz et al., 1977). Figure 4C demonstrates that clone MR400-3 terminally divides in response to HMBA. This is evidenced by the inability of growing cells to maintain exponential growth upon dilution to low cell densities in the presence of HMBA. Similar results were obtained when Me_2SO (1.5% v/v) was the inducer (not shown). Terminal cell division did not result from toxicity of HMBA or Me_2SO since cells terminally divided if the inducer was removed after 4 days (not shown).

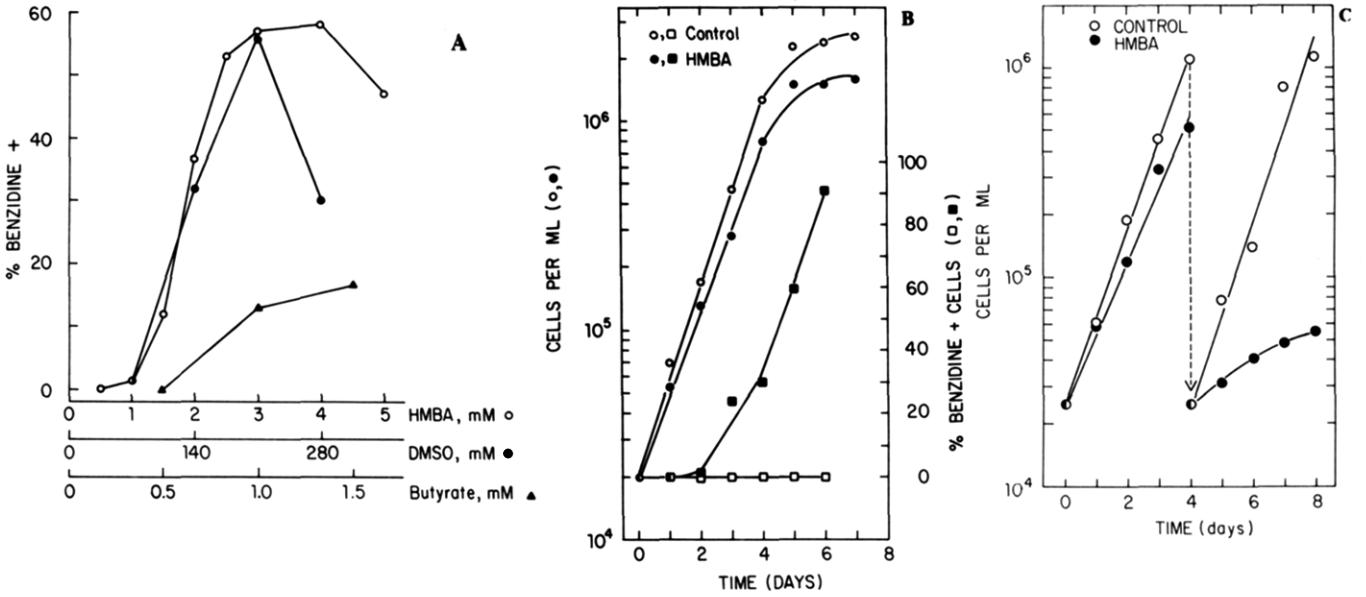

FIGURE 4: Differentiation of MTX-resistant MELC clone MR400-3. (A) Dose responses to inducers. MR400-3 cells were washed free of MTX and inoculated into MTX-free medium at a final density of 1×10^5 cells/mL. Cells were benzidine-stained after 5 days of growth in the indicated concentrations of HMBA (○), Me_2SO (●), and butyrate (▲). Similar results were obtained when the experiment was performed in the presence of 400 μM MTX. (B) Kinetics of growth and induction of hemoglobin-containing cells. Cells were prepared as in (A) and inoculated into MTX-free medium in the absence (○, □) or presence (●, ■) of 2.5 mM HMBA, counted, and benzidine-stained at the indicated times. Similar results were obtained when the experiments were performed in the presence of 400 μM MTX. (C) Terminal cell division. Cells were washed free of MTX and inoculated into MTX-free medium in the absence (○) or presence (●) of 2.5 mM HMBA. Similar results were obtained when the experiment was performed in medium containing 400 μM MTX.

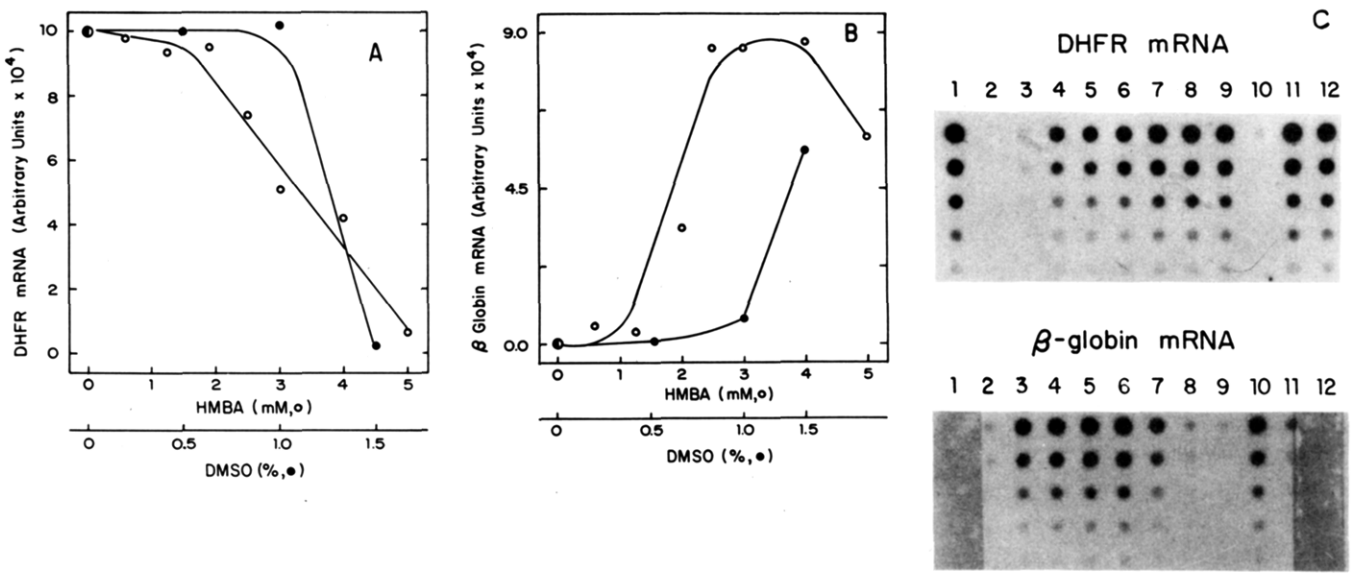

FIGURE 5: Cells were grown with the indicated concentration of inducer for 4 days. Cells were counted daily and diluted to a density of 10^5 cells/mL each day. Cells were harvested, and MRNA was prepared, blotted onto nitrocellulose with a BRL dot blotting manifold, and hybridized to ^{32}P-labeled cDNA probes to murine DHFR or β-globin (methods). Blots were exposed to X-ray film and scanned on a Schoefel SD-3000 densitometer. (A) Dose responses of MR400-3 cells to Me_2SO or HMBA for cytoplasmic levels of DHFR mRNA. (B) Dose response of MR400-3 cells to Me_2SO or HMBA for cytoplasmic levels of β-globin mRNA. (C) Cytoplasmic dot blots hybridized to ^{32}P-labeled cDNA probes to DHFR or β-globin, as indicated. Each lane contains the extract from equal numbers of cells for the following cell types under the following conditions: (1) MR400-3 cells, no inducer; (2) DS-19 cells, no inducer; (3) MR400-3 cells, 5 mM HMBA; (4) MR400-3 cells, 4 mM HMBA; (5) MR400-3 cells, 3 mM HMBA; (6) MR400-3 cells, 2.5 mM HMBA; (7) MR400-3 cells, 1.875 mM HMBA; (8) MR400-3 cells, 1.25 mM HMBA; (9) MR400-3 cells, 0.625 mM HMBA; (10) MR400-3 cells, 1.5% (v/v) Me_2SO; (11) MR400-3 cells, 1.0% (v/v) Me_2SO; (12) MR400-3 cells, 0.5% (v/v) Me_2SO. The first lane in each well received the extract from 50 000 cells, and serial 2-fold dilutions were made.

Additionally, HMBA- and Me_2SO-treated cells were able to exclude the vital dye trypan blue to the same extent (greater than 95%) as control cells throughout the terminal cell division experiment (not shown). DS-19 has been shown to exhibit similar behavior in this terminal cell division assay (Marks et al., 1983).

The levels of mRNA for β-globin and DHFR were measured during differentiation of the MR400-3 cell. MR400-3 cells express DHFR mRNA (lane 1, Figure 5C). Under the same conditions, DHFR mRNA could be detected in DS-19 cells at levels at least 100-fold less than in MR400-3 cells. Figure 5A,B shows dose responses of MR400-3 cells to Me_2SO and HMBA. The levels of DHFR mRNA (Figure 5A) and β-globin (Figure 5B) were measured. With increasing doses of inducer, there was a corresponding decrease in the cell content of DHFR mRNA (Figure 5A). The reverse was seen

for cell content of β-globin mRNA (Figure 5B). It is interesting to note that Me_2SO (1.5% v/v) yielded the optimal effect in that there was a minimum of a 100-fold decrease of detectable DHFR mRNA under these conditions (Figure 5A). This was also the optimal dose of inducer for accumulation of benzidine-reactive cells (Figure 4A). HMBA was also effective in reducing the DHFR mRNA content (Figure 5A). However, the optimal dose of HMBA for hemoglobin accumulation was not as effective as Me_2SO for reduction of the DHFR mRNA content; i.e., at 3 mM HMBA, benzidine staining (Figure 4A) and globin mRNA content (Figure 5B) were maximal, yet the DHFR level had decreased by approximately 40% relative to the control signal. The optimal dose of Me_2SO for production of benzidine-staining cells and accumulation of globin mRNA resulted in approximately a 95% reduction in DHFR mRNA levels relative to uninduced controls. For this experiment, cells were harvested at day four after addition of the inducers. Every day prior to harvest, cells were diluted in appropriate fresh medium to 100 000/mL to maintain the cells at exponential cell densities. Thus, the observation of decreased DHFR mRNA content probably was a differentiation effect and not a growth-phase effect. This was also not a toxic effect since viability was not affected as determined by the ability to exclude trypan blue (not shown). The data in Figure 5B demonstrate that these cells were able to produce mRNA under induction conditions. This blot was made from the same cells as those used in Figure 5A, but the mRNA was hybridized to ^{32}P-labeled cDNA to the murine β-globin gene. As shown, HMBA and Me_2SO were both able to induce accumulation of globin mRNA in a dose-dependent fashion (Figure 5B). The data shown in Figure 5 were from an experiment performed in the absence of MTX. Nearly identical results were obtained when the experiment was performed in the presence of 400 μM MTX (not shown). This result might seem paradoxical; i.e., how do cells survive in the presence of high doses of MTX with the concomitant down regulation of DHFR? However, MTX is only toxic to growing cells, and the decrease of DHFR referred to was on day four, a time at which cells have ceased exponential growth (Figure 4C). By 48 h there were substantial decreases in levels of mRNA coding for DHFR (not shown).

The normalization of DHFR mRNA levels for uninduced vs. induced cells might present a tricky issue since induced cells exhibit overall decreases of macromolecular synthesis and cell volume (Sherton & Kabat, 1976; Loritz et al., 1977). We therefore presented our data normalized to cell number. This paper presents data concerning a nonstructural gene that is normally expressed at low levels in uninduced parental MELC (Figure 5C) as well as other murine cell types (Alt et al., 1976). There have been two reports concerning expression of actin, a structural housekeeping gene, during induced differentiation of MELC (Nagi et al., 1984; Lachman & Skoultchi, 1984). It is unclear why the two laboratories obtained conflicting results regarding actin expression; i.e., Nagi et al. (1984) found no change in actin expression with differentiation whereas Lachman and Skoultchi (1984) found decreases of actin expression. Although both laboratories employed nonvariant strains of MELC, the manner of cell handeling, e.g., frequency and conditions for cell passage, may have been sufficiently different to generate cells with different phenotypic expressions of the actin gene during differentiation. Thus, the differences may be due to strain variation. Since our cells, MR400-3, are clearly variants, it would be difficult to evaluate actin expression in MR400-3 cells relative to that of the parental-type MELC employed in these previous studies (Nagi et al., 1984; Lachman & Skoultchi, 1984). With these qualifications in mind, we examined the levels of actin mRNA in MR400-3 cells for uninduced and induced cells. After 4 days of exposure to Me_2SO or HMBA, both at optimal inducing concentrations of 1.5% (v/v) and 3 mM, respectively, there was not a substantial decrease of actin mRNA levels in MR400-3 cells whereas there was a decrease of DHFR mRNA (not shown). Our results are similar to those of Nagi et al. (1984); however, our results do not contradict those of Lachman and Skoultchi et al. (1984) since there may be strain variation with respect to expression of the actin gene during induced differentiation. Whatever the reason for these differing results, we feel they have little bearing upon our arguments concerning DHFR expression. This follows since DHFR, unlike actin, is a nonstructural gene that is expressed at low levels in parental cells.

In conclusion, we have selected for MTX-resistant MELC that are resistant by virtue of amplification of the DHFR gene. Furthermore, the cells retain the capacity to be induced to terminal erythroid differentiation. To our knowledge, this is the first report of a DHFR gene amplification in a cell that can be induced to terminally divide. Differentiation results in decreased levels of DHFR mRNA. It was recently reported that induced differentiation of HL-60 cells resulted in a decrease of RNA coding for DHFR (Nienhuis et al., 1985). Thus, the MELC variants described provide a unique tool to study the regulation of a nonstructural housekeeping gene during MELC differentiation. It is now possible to study irreversible growth regulation during terminal cell division of a nonstructural housekeeping gene, i.e., DHFR.

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Extended X-ray Absorption Fine Structure Studies of Zn_2Fe_2 Hybrid Hemoglobins: Absence of Heme Bond Length Changes in Half-Ligated Species†

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Appendix

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ABSTRACT: Metal hybrid hemoglobins, in which Zn(II) replaces Fe(II), have been structurally characterized by extended X-ray absorption structure (EAFS) studies. Since Zn and Fe have very different K absorption edge energies, the structures of the ligated (Fe) and unligated (Zn) sites could be examined independently within a single molecule that mimics an intermediate ligation state. The observed EXAFS spectra and associated structural parameters are compared among the ligand free $(\alpha Zn)_2(\beta Zn)_2$, half-ligated $(\alpha FeCO)_2(\beta Zn)_2$ and $(\alpha Zn)_2(\beta FeCO)_2$, and fully ligated $(\alpha FeCO)_2(\beta FeCO)_2$ systems.

Hemoglobin has long served as a paradigm for cooperative ligand binding in proteins (Antonini et al., 1971, Edelstein, 1975). However, the detailed mechanism of hemoglobin cooperativity remains controversial. A number of specific mechanisms have been proposed (Monod et al., 1965; Koshland et al., 1965; Gelin & Karplus, 1977; Warshel, 1977; Perutz, 1976). Perhaps the best known model is the "tension" or restraint model of Hoard and Perutz (Hoard, 1971; Perutz, 1976). In this model, the protein can adopt two quaternary structures corresponding to the MWC model (Monod et al., 1965). In the ligand-free (T) structure, this model suggests that the tension is localized in the $Fe-N_{Im}$ bond because the Fe atom is forced out of the plane of the heme while the protein restrains the axial imidazole ligand from its normal binding position. As ligands are successively added to the protein, the specific interactions that stabilize the T quaternary structure

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