Regulated Expression and Functional Role of the Transcription Factor CHOP (GADD153) in Erythroid Growth and Differentiation

By Margaret Coutts, Kunyuan Cui, Kerry L. Davis, Joan Cleves Keutzer, and Arthur J. Sytkowski

The hematopoietic growth factor erythropoietin (Epo) triggers changes in the expression of genes that encode important regulators of erythroid cell growth and differentiation. We now report that Epo markedly upregulates *chop* (*gadd153)* expression and that this transcription factor plays a role in erythropoiesis. Using a differential hybridization assay, we isolated a full-length cDNA of *chop* as an Epo upregulated gene in Rauscher murine erythroleukemia cells. RNase protection assays demonstrated that Epo or dimethyl sulfoxide induction increased steady-state mRNA levels 10- to 20-fold after 24 to 48 hours. Western blot analysis confirmed a marked increase in CHOP protein. Among the other *c/ebp* family members, only *c/ebp* β was also upregulated during erythroid differentiation. Among normal hematopoietic cells examined, steady-state mRNA levels were highest in erythroid cells, with levels peaking during terminal differentiation. Transient overexpression of *chop* in Rauscher cells resulted in a significant increase in Epo- or dimethyl sulfoxide (DMSO)-induced hemoglobinization, further linking *chop* upregulation to erythroid differentiation. Artificial downregulation of *chop* in normal murine bone marrow cells with antisense oligodeoxynucleotides inhibited colony-forming unit-erythroid (CFU-E)–derived colony growth in a concentration-dependent manner. Burst-forming unit-erythroid (BFU-E)–derived colony growth was not affected. Using a Far Western type of analysis, we detected several potential CHOP binding partners among the nuclear proteins of Rauscher cells. Importantly, the number and relative abundance of these proteins changed with differentiation. The results strongly suggest that CHOP plays a role in erythropoiesis, possibly through interactions with both C/EBP and non-C/EBP family members.

THE INTRACELLULAR signaling network triggered by the hematopoietic growth factor erythropoietin (Epo) modulates the expression of genes encoding important regulatory proteins. For example, the association of Epo with its receptor triggers activation of phospholipase-C-γ and protein kinase Cε.[1] This signaling pathway is required for Epo's upregulation of the transcription factor c-*myc*[2-4] and is associated with Epo's mitogenic effect. Docking of Epo with its receptor also results in the activation of the JAK/STAT pathway. JAK2 kinase associates with the receptor's cytoplasmic domains and phosphorylates STAT5.[5] The activated STAT5 dimerizes and translocates to the cell's nucleus. Erythroid differentiation also requires the downregulation of c-*myb*.[6,7] In some experimental systems, artificial downregulation of Myb protein in the absence of erythropoietin is sufficient to induce hemoglobinization.[8] Other regulatory proteins important to erythroid differentiation include GATA-1[9,10] and NF-E2.[11] These transcription factors regulate globin gene expression in a coordinate fashion; however, it is not known which signal transducers activate them. One approach to understanding how Epo-induced signaling leads to the establishment of erythroid-specific patterns of gene expression is to compare the species of mRNAs present before and after Epo treatment.

In the present study, we used a differential hybridization technique to identify Epo-regulated genes. We report that, in Rauscher murine erythroleukemia cells, both Epo and dimethyl sulfoxide (DMSO) markedly upregulate *chop (gadd153)*, a member of the *c/ebp* family of transcription factor genes.[12,13] We further show that an increase in *chop* transcript levels is associated with terminal differentiation of normal erythroid cells in vivo and that antisense *chop* oligodeoxynucleotide treatment of bone marrow cells inhibits colony-forming unit-erythroid (CFU-E) but not burst-forming unit-erythroid (BFU-E) colony growth. In addition to the possibility of CHOP-C/EBP interactions, our data also suggest that there may be other, non-C/EBP proteins in erythroid cells with which CHOP interacts. Moreover, these potential alternative binding partners change during erythroid differentiation.

CHOP was first isolated as growth arrest and DNA-damage inducible gene 153 (*gadd153*).[12,14] CHOP was also isolated on the basis of dimerization with C/EBPβ.[13] *CHOP* and the other *gadd* genes are induced by a wide variety of treatments that cause metabolic stress or cessation of mitosis. Some of these inducers are DNA alkylation, nutritional deprivation, oxidative stress, and treatments that perturb endoplasmic reticulum function.[15-18] These treatments also tend to induce cessation of mitosis. Cessation of mitosis also occurs when cells undergo terminal differentiation: in fact, direct expression of CHOP can lead to cell cycle arrest.[19] CHOP mRNA increases dramatically when dividing 3T3-L1 fibroblasts are induced to differentiate into amitotic, lipid-rich adipocytic cells[13,15,16] and during keratinocyte differentiation.[20] However, because CHOP is ubiquitously expressed (see Results), it is likely that it plays a role in the differentiation of many tissues.

From the Laboratory for Cell and Molecular Biology, Division of Hematology and Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA.

Submitted November 2, 1998; accepted January 13, 1999.

Supported by National Institutes of Health (NIH) NRSA F32 DK08986 (M.C.), NRSA F32 DK09201 (K.C.), NRSA F32 HL08563 (J.C.K.), and NIH R01 DK38841 and by US Navy Grant No. N00014-93-1-0776 (A.J.S.).

Address reprint requests to Arthur J. Sytkowski, MD, Laboratory for Cell and Molecular Biology, Division of Hematology and Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, 1 Deaconess Rd, 21-27 Burlington Bldg, Room 548, Harvard Medical School, Boston, MA 02215.

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. section 1734 solely to indicate this fact.*

0006-4971/99/9310-0012$3.00/0

Other members of the C/EBP family have been studied more extensively in terms of the role they play in differentiation. In adipocytic differentiation, the α, β, and δ C/EBP isoforms are expressed with complex kinetics.[21] These C/EBP protein isoforms may interact in specific hierarchical patterns that change as differentiation proceeds.[22] Differentiating monomyelocytic cells have their own distinct pattern of C/EBP isoform expression.[23] Similarly, C/EBP α levels fluctuate during differentiation of hepatoma cells,[24] ovarian follicles,[25] and gut epithelium,[26] as do levels of C/EBPβ levels during B-cell[27] and eosinophilic differentiation.[28] Expression of the recently described C/EBPε may be particularly important in the development of granulocytic cells.[29] CHOP is an unusual member of this family, in that it does not appear to form homodimers and it has a noncanonical DNA-contact domain.[30] Hence, its function and mechanism may be substantially different than other C/EBPs.

MATERIALS AND METHODS

Cell culture. Rauscher murine erythroleukemia cells[31,32] were cultured in Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS; heat-inactivated), 36°C, 95% air/5% CO_2. Cells were induced with recombinant human Epo (50 to 100 U/mL) or with DMSO (0.7% to 1.0%, vol/vol).

Construction and screening of the cDNA library. A Rauscher cell cDNA library was prepared by Clontech (Palo Alto, CA) using the λ DR2 vector. RNA was isolated from cells treated with Epo (50 U/mL) and cycloheximide (10 μg/mL) for 1 hour using the acid guanidinium thiocyanate method of Chomczynski and Sacchi,[33] except that lithium precipitation was not performed. The cDNA library was screened using differential plaque hybridization. Approximately 40,000 clones were plated in 10-cm dishes at 8 pfu/cm^2. Nitrocellulose filters (Millipore, Bedford, MA) were lifted off each plate in duplicate. One set of nitrocellulose lifts was hybridized to ^{32}P-labeled cDNA prepared from untreated Rauscher cells. The duplicate set was hybridized to ^{32}P-labeled cDNA prepared from cells treated with Epo (50 U/mL) and cycloheximide (10 μg/mL) for 1 hour. The cDNA was prepared using Superscript reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer's instructions. Hybridizations were performed in 50% formamide, 6× SSC, 5× Denhardt's, 0.5% sodium dodecyl sulfate (SDS), 100 μg/mL salmon sperm ssDNA at 42°C. Filters were washed three times in 1× SSC, 0.5% SDS at 60°C for 1 hour. DNA sequencing was performed using a USB DNA sequencing kit (USB, Cleveland, OH).

RNA analysis. RNase protection assays were performed essentially as directed by the Ambion RNase Protection Assay kit (Ambion, Inc, Austin, TX) except that 20 U RNase T1 and 0.5 U RNase A were used in a sample volume of 100 μL. The antisense probes protected the *chop* cDNA sequence from nucleotide 470 to 782; the glyceraldehyde 3-phosphate dehydrogenase *(gapdh)* probe protected nucleotide 1120 to 1228. Northern blots were performed on poly A$^+$ RNA isolated from 1 mg of total RNA using standard methods. Electrophoresis was through formaldehyde-agarose gels. Washes were 20 minutes each of 2× SSC, 1× SSC, and 0.1× SSC, all with 0.1% SDS at 60°C.

Expression of recombinant CHOP. For expression of CHOP protein in *Escherichia coli*, *Bam*HI and *Eco*RI restriction sites were introduced into the *chop* cDNA 5′ and 3′ ends, respectively, by polymerase chain reaction (PCR) using the following two primers, 5′-TTAAGGGATCCCAGCTGAGTCCCTG-3′ and 5′-TTCGGAATTCCTATGTGCAAGCCGA-3′. The PCR product was cloned into the *Bam*HI and *Eco*RI sites of the pTrc-His expression vector (InVitrogen, Carlsbad, CA) in the reading frame, resulting in a histidine tagged CHOP protein when expressed in *E coli*. The histidine-tagged protein was purified using Ni-NTA resin (Qiagen, Valencia, CA). Briefly, cells from 3 L of culture were collected by centrifugation and resuspended in sonication buffer (50 mmol/L sodium phosphate, 300 mmol/L NaCl, pH 8.0). The cells were disrupted by sonication, and the sonicated mixture was centrifuged at 10,000*g* for 20 minutes. The supernatant was collected, and 6 mL of 50% slurry of Ni-NTA resin, previously equilibrated in sonication buffer, was added and stirred at 4°C for 60 minutes. The resin was poured into a 1.5-cm diameter column and washed with sonication buffer followed by 50 mmol/L sodium phosphate, 300 mmol/L NaCl, pH 6.0, until the A_{280} of the effluent was less than 0.01. The proteins were eluted with a pH gradient from 6 to 3.5. The fractions containing CHOP protein were collected, and the pH was adjusted to 7.4 with phosphate buffer.

For expression of the CHOP-glutathione-S-transferase (GST) fusion protein, the *chop* PCR product was cloned into the pGEX-3x vector (Amersham Pharmacia Biotech, Inc, Piscataway, NJ). CHOP-GST protein was purified following the manufacturer's procedure (Pharmacia). Briefly, cells were centrifuged, resuspended in phosphate-buffered saline (PBS) buffer (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 1.8 mmol/L KH_2PO_4, pH 7.3), disrupted by sonication, and centrifuged. Glutathione Sepharose 4B equilibrated with PBS buffer was added to the supernatant of the sonicated mixture, and the mixture was incubated with gentle agitation at room temperature for 1 hour. The mixture was then packed into a small column and washed with PBS buffer until the A_{280} of flow-through was less than 0.01. The CHOP-GST fusion protein was eluted with 10 mmol/L reduced glutathione in 50 mmol/L Tris-HCl, pH 8.0.

Western blot analysis. Cells were harvested by centrifugation, washed once with ice-cold Dulbecco's PBS, and lysed directly in SDS sample buffer. A 10:1 ratio of sample buffer to cell pellet was used. After boiling the samples for 5 minutes, they were passed repeatedly through a 23-gauge needle and syringe to fragment the DNA. Cell lysate protein (100 μg/lane) was electrophoresed on a 13% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and was transferred electrophoretically (100 mA for 2 hours at 4°C) onto polyvinylidene fluoride (PVDF) filters (Millipore). The filters were allowed to dry after transfer and then were rewet with methanol. The blots were blocked overnight with 5% nonfat dry milk in PBS, 0.1% Tween 20.

Rabbit polyclonal antiserum against His-CHOP was prepared by Organon Teknika (Durham, NC) using purified CHOP protein as antigen. The polyclonal antibody was affinity purified using His-CHOP as the ligand immobilized on Affi-Gel 15 (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. For antibody purification, 150 mL of anti-CHOP rabbit serum diluted in an equal volume of buffer A (10 mmol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.5) was applied to the affinity column by circulation overnight. The column was washed sequentially with buffer A and buffer B (10 mmol/L Tris-HCl, 4 mol/L NaCl, pH 7.5) sequentially and the eluates were collected. The antibodies were eluted finally with 4 mol/L $MgCl_2$ followed by 4 mol/L guanidine HCl in buffer A. The elution was monitored by A_{280}. The collected fractions were dialyzed, concentrated by ultrafiltration, and stored at −20°C.

Antisense oligodeoxynucleotide experiments. To demonstrate a role for CHOP in normal erythropoiesis, loss-of-function experiments were performed. Mouse bone marrow cells were treated with *chop* antisense (GGACTCAGCGCCATGAC) or missense (CAGTACCGTCGACTCAGG) oligodeoxynucleotides (oligos; Oligos Etc, Wilsonville, OR). BFU-E– and CFU-E–derived colony growth was studied as follows. Bone marrow cells were flushed from the femurs of 7-week-old female C57BL6/J mice (Jackson Laboratories, Bar Harbor, ME) with Alpha medium (GIBCO-BRL) using a 23-gauge needle and syringe. To obtain a single-cell suspension, the cells were twice drawn through the same needle. The cells were washed twice in fresh Alpha medium, counted, and resuspended in Alpha medium/3% FBS (Hyclone Laboratories, Logan, UT) at 1×10^6 cells/mL. The cells were incubated in the absence or presence of specified concentrations of *chop* antisense or

missense oligos before plating. Further additions of oligos were made according to the schedules described below. All experiments were performed in duplicate.

For CFU-E growth, on day 0 the oligos were added to 0.6 mL of the cell suspension and 2.7 mL MethoCult M3330 medium (Stem Cell Technologies, Inc, Vancouver, British Columbia, Canada). After 4 hours of incubation at 37°C in a humidified 5% CO_2 incubator, 1.1 mL of the mixture was plated into each of two 35-mm dishes. On day 1, the cultures received an addition of oligos equal to 25% of the initial concentration. On day 2, the cultures were scored. Each 35-mm dish was placed within a gridded 60-mm dish, and the number of CFU-E–derived colonies in 2 quadrants of the dish was determined using an inverted microscope. The counts from duplicate dishes were averaged.

For BFU-E growth, on day 0 the oligos were added to 0.3 mL of the cell suspension and 3.0 mL MethoCult M3434 medium (Stem Cell Technologies) and the cells were incubated overnight at 37°C in a humidified 5% CO_2 incubator. Cell suspensions (1.1 mL each) were plated into replicate 35-mm dishes on day 1. The cultures received an addition of oligos equal to 25% of the initial concentration on day 1 and on each successive day. All dishes were scored on day 8 for BFU-E–derived colonies. The counts for duplicate dishes were averaged.

Isolation of hematopoietic cells. Populations of T cells, B cells, and erythroid cells were isolated using antibody-coated plates using standard methods.[34,35] Antibody-coated plates were prepared using polystyrene bacteriological petri plates. Antibodies were diluted to 100 μg/mL in 50 mmol/L Tris, pH 9.5. Ten milliliters was poured into 100 × 15 mm plates and allowed to incubate overnight at 4°C. Plates were then washed 4× in PBS. B cells were recovered using antimouse Ig. Nonadherent cells were then panned on plates coated with an antithymocyte antibody. Cells negative to both selections were harvested as erythroid cells.

Ten murine spleens were prepared as a single-cell suspension in RPMI 1640 with 5% FBS at 2×10^7 cells/dish. Cells were allowed to incubate for 30 minutes. Nonadherent cells were removed by decanting, and adherent cells were then washed 5 times in PBS with 1% FBS. Cells were removed by repeated pipetting with a pasteur pipette using the wash buffer. Erythroid precursors were isolated and fractionated as described.[36] Spleen cells were isolated from Epo-treated mice and washed in Hank's Balanced Salt Solution without magnesium or calcium (GIBCO). Spleens were crushed and filtered through a mesh filter to obtain a single-cell suspension. The cell suspension was sedimented through Ficoll-Hypaque (Pharmacia) to remove lymphocytes. The pellet from the Ficoll-Hypaque was then layered onto a discontinuous Percoll gradient that consisted of 6-mL layers of 45%, 65%, 70%, 77%, and 90% Percoll in magnesium- and calcium-free Hank's Balanced Salt Solution. Approximately 1×10^8 cells were loaded onto the 30-mL gradient and were subjected to centrifugation at 5,000*g* for 20 minutes at 4°C. After centrifugation, fractions of cells were collected using a peristaltic pump and then washed in DMEM. Cells were counted and characterized by microscopic examination and benzidine staining.[37] Differential counts were performed before and after the separation. Cell numbers were quantified using a hemacytometer.

Cell transfection. One day before transfection, Rauscher cells were plated at a density of 1×10^5 cells/mL in 10-cm tissue culture dishes in DMEM/10% FBS. Cells were harvested by centrifugation, suspended in DMEM without FBS at a density of 3×10^6 cells/mL, and transfected with $pSVK_3$-*chop* or $pSVK_3$ alone (20 μg DNA/transfection) by electroporation using a GenePulser II (Bio-Rad) according to the manufacturer's instructions. Cells were grown for 48 hours after transfection and analyzed. The transfection efficiency ranged from 10% to 20% in several experiments, as monitored by transfection of pCMV-β*gal* control plasmid.

Detection of CHOP binding partners. A variation on the nuclear extraction procedure of Dignam et al[38] was used. Extract preparation was performed at 4°C. Cells were harvested by centrifugation, washed in ice-cold PBS, resuspended in a small volume of PBS, and recentrifuged in microfuge tubes. Five packed-cell volumes of Dignam's buffer A were added to the pellet, which was gently resuspended by low-speed vortexing. The cells were then incubated at 0°C for 10 minutes. The cells were pelleted at low speed in a microfuge, and the supernatant was discarded. Five volumes of buffer A were added, and the cells were broken by vigorous vortexing. The nuclei were pelleted, and four original cell volumes of Dignam's buffer C were added. The nuclei were lysed with 20 strokes of a Dounce homogenizer (type B pestle). The lysate was stirred for 30 minutes at 0°C. The nuclear extract was obtained by centrifugation of the broken nuclei (10 minutes in a microfuge at 4°C). Five hundred micrograms of nuclear protein extract was loaded onto a 8-mm wide lane of a 13%/0.35% acrylamide/bis gel and electrophoresed. Molecular weights were assigned by comparison to prestained molecular weight markers (GIBCO-BRL). The proteins were transferred electrophoretically to a PVDF membrane (Millipore Immobilon P; 0.45 μm) for 90 minutes at 100 mA in 39 mmol/L glycine, 48 mmol/L Tris-HCl, 0.037% SDS, 20% methanol. After blotting, each lane was cut into two strips, with half being used to test for either control (GST) or experimental (CHOP-GST fusion) protein binding (modified from the procedure of Ferrell and Martin[39]). The PVDF strips were incubated in 7 mol/L guanidine HCl, 50 mmol/L Tris-HCl, 50 mmol/L dithiothreitol (DTT), 2 mmol/L EDTA, pH 8.0, at room temperature with gentle rocking for 1 hour. Blots were rinsed with and then incubated with 1% bovine serum albumin, 250 mmol/L KCl, 20 mmol/L potassium phosphate, 2 mmol/L DTT, 0.2 mmol/L EDTA, pH 7.4 (4°C overnight with gentle rocking). Blots were then blocked for 3 hours with 5% bovine serum albumin, 50 mmol/L KCl, 20 mmol/L potassium phosphate, 2 mmol/L DTT, 0.2 mmol/L EDTA, pH 7.9. To each blocked strip was added either GST protein or CHOP-GST fusion protein (15 μg/mL in blocking buffer), and the proteins were allowed to bind overnight. The blots were washed twice for 10 minutes in blocking buffer. Goat anti-GST antibody (Pharmacia) diluted 1/100 in blocking buffer was added and allowed to bind for 1 hour at room temperature with gentle rocking. The blots were washed twice for 10 minutes in blocking buffer. The second antibody (peroxidase-conjugated rabbit antigoat IgG, 1/1,000; Cappell-Organon Teknika 55358) was added. The blots were again allowed to incubate at room temperature for 1 hour and were washed as described before. ECL (Amersham Pharmacia Biotech, Inc) chemiluminecent detection of the bound complexes was performed according to the manufacturer's instructions.

RESULTS

Identification of Epo-regulated genes. To identify genes regulated by Epo, we performed a differential screening process. We constructed a λ phage cDNA library from Epo-induced Rauscher murine erythroleukemia cells and prepared duplicate nitrocellulose lifts. One lift was hybridized to [^{32}P]cDNA prepared from uninduced Rauscher cell mRNA. The other was hybridized to [^{32}P]cDNA prepared from cells induced with Epo (50 U/mL) in the presence of cycloheximide (10 μg/mL) for 1 hour. After washing and autoradiography, careful visual comparison of the duplicates showed members of the library that corresponded to candidate Epo-regulated genes. Approximately 40,000 clones were screened initially.

To verify the authenticity of the Epo-upregulation, the cDNA inserts of the tentative positives were isolated and used as probes in Northern blot assays of RNA prepared from Rauscher cells induced with Epo but without cycloheximide. Three of the initial tentative positives were authentically Epo-upregulated. DNA sequencing showed that one of the Epo-upregulated cDNAs bore exact identity to the transcription factor *chop*.[12,13]

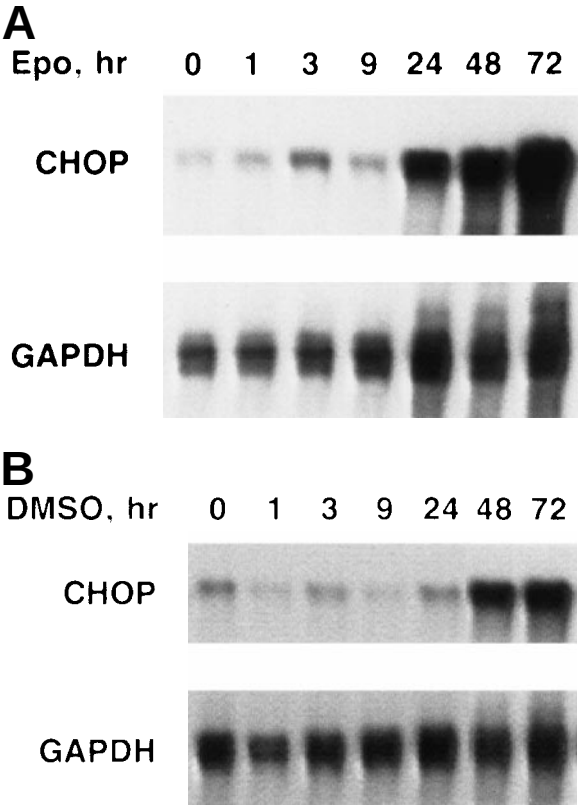

Fig 1. *chop* is upregulated during erythroid differentiation of Rauscher cells. (A) Epo induction. (B) DMSO induction. RNase protection assays for *chop* and *gapdh* (glyceraldehyde-3 phosphate dehydrogenase) were performed as described in Materials and Methods and Results.

It contained the entire coding region and complete 3′ untranslated region. The 815-bp *chop* cDNA hybridized to an mRNA of approximately 1.1 kb (not shown). The other two genes were novel and will not be discussed further.

chop *is upregulated during erythroid differentiation of Rauscher cells.* We determined the steady-state levels of *chop* mRNA in Rauscher cells after induction with Epo or DMSO using nuclease protection assays. Rauscher cells were induced with either Epo (100 U/mL) or DMSO (0.7%) for 0 to 72 hours. During this interval, the cells differentiated as evidenced by hemoglobin synthesis. Both Epo and DMSO induction resulted in a marked increase in steady-state levels of *chop* mRNA (Fig 1A and B). In some experiments, *chop* mRNA increased modestly (50% to 100%) after 1 to 3 hours of induction. However, in all experiments, *chop* mRNA increased 10- to 20-fold after 24 to 48 hours and remained at these high levels throughout the 72-hour induction. Similar results were obtained in several replicate experiments.

The Epo- or DMSO-induced upregulation of *chop* mRNA was unrelated to cell density or to nutrient depletion. The same basal level of *chop* was detected in uninduced cells (0 time) and in cells maintained in standard culture conditions for 72 hours without Epo or DMSO. Moreover, when cells were induced with Epo or DMSO but maintained at subconfluent densities (by dilution with Epo-or DMSO-supplemented media to 0.5×10^6 cells/mL), *chop* upregulation was equally striking. In contrast to results reported with adipocytic cell lines,[16] reduced glucose levels did not effect *chop* induction. Rauscher cells cultured in glucose 2 mmol/L showed the same pattern of *chop* induction as cells cultured in 10 mmol/L glucose (data not shown).

CHOP protein expression. We confirmed that CHOP protein levels increase markedly during Rauscher cell differentiation (Fig 2). Purified, bacterially expressed recombinant CHOP (rCHOP; 2.5 or 0.5 ng) or SDS-solubilized protein extracts from DMSO uninduced (0 hours) or induced (48 or 72 hours; 100 μg/lane) were subjected to SDS-PAGE and were analyzed by Western blot using affinity-purified rabbit anti-CHOP antibodies (left panel) or preimmune rabbit IgG (right panel). The anti-CHOP antibodies specifically recognized 2.5 ng but not 0.5 ng of rCHOP. Preimmune IgG did not recognize rCHOP. Both anti-CHOP and preimmune IgG cross-reacted nonspecifically with several minor protein species from Rauscher cells (lanes 0, 48, and 72). However, despite this background, only the anti-CHOP antibodies identified a prominent band in differentiating cells (left panel, 72 hours) that migrated to the same position as rCHOP. This increase in CHOP protein correlates well with the increase in CHOP mRNA (Fig 2).

Expression of c/ebp *isoforms during Rauscher cell differentiation.* In some cell types, the C/EBP isoforms are known to heterodimerize and to have changing interactions during differentiation.[23-25] Therefore, it was important to determine which *c/ebp* isoforms are expressed in erythroid cells and whether their expression changes during erythropoiesis. Northern blots were prepared with polyA$^+$ RNA from uninduced and DMSO-induced Rauscher cells and were probed with *c/ebp* α, *c/ebp* β, *c/ebp* δ, or *chop* (Fig 3A and B). The blots were probed subsequently with *gapdh* cDNA. *c/ebp* α and *c/ebp* δ mRNA levels were extremely low and did not change during differentiation. In contrast, *c/ebp* β and *chop* mRNA increased 8.5- and 9.5-fold, respectively, during induction, as determined by laser densitometry.

These blots were also used to approximate the levels of the different *c/ebp* isoforms relative to each other. In uninduced Rauscher cells, *chop* mRNA was fivefold to 10-fold more abundant than *c/ebp* β mRNA (based on the use of equal amounts of probe with equal specific activity, assaying equal amounts of polyA$^+$ mRNA). The levels of *c/ebp* α and *c/ebp* δ

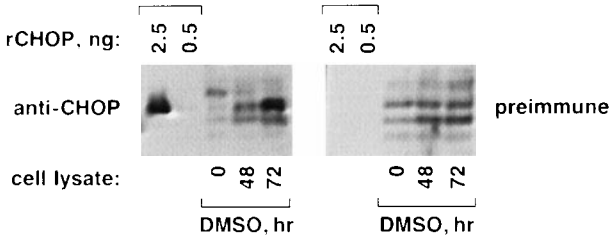

Fig 2. CHOP protein increases during erythroid differentiation of Rauscher cells. Purified recombinant CHOP protein (rCHOP) or lysates from uninduced (0 hours) or DMSO-induced (48 and 72 hours) cells were subjected to SDS-PAGE and electrophoretic transfer. They were probed with either affinity-purified anti-CHOP antibodies (left panel) or purified preimmune IgG (right panel). Note the prominent increase in CHOP protein detected after 72 hours of induction (left panel, 72 hours).

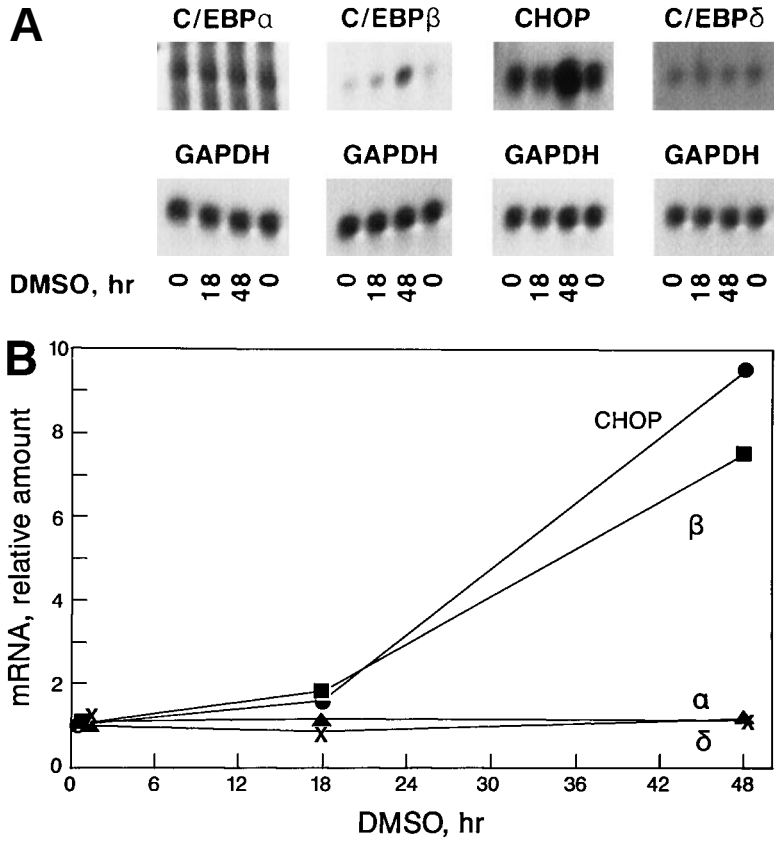

Fig 3. Expression of *c/ebp* isoforms during erythroid differentiation. (A) Autoradiograms of Northern blot analyses. (B) Densitometric analysis of data in (A) normalized to 0 hours. Note the increase in both *chop* and *c/ebp*β. In contrast, *c/ebp*α and *c/ebp*δ were unchanged.

were conspicuously lower than *chop* and *c/ebp* β. Thus, at all times during induced differentiation, *chop* steady-state mRNA levels were in large excess over the other *c/ebp*s.

Tissue distribution of chop *mRNA.* To determine whether fully differentiated cells in other tissues express *chop*, RNase protection assays were performed from a variety of adult mouse tissues (Fig 4). Although *chop* mRNA was detectable in all tissues, its abundance varied greatly. Uninduced Rauscher cells had *chop* mRNA levels 10- to 30-fold higher than most of the other tissues. Among the 14 tissues tested, only testis had conspicuously high *chop* mRNA levels, approaching that found in Rauscher cells.

CHOP *expression in normal hematopoietic cells.* Next, we compared *chop* expression in Rauscher cells with that seen in normal murine hematopoietic cells (Fig 5). Among the normal cells studied, erythroid cells isolated from the spleens of Epo-treated mice had the highest level of *chop* mRNA. This level was not as high as that seen in Rauscher cells. However, the splenic erythroid population is heterogeneous, consisting of cells at various stages of differentiation. This analysis was complicated by the rather large differences in *gapdh* mRNA seen among these various cell types despite even loading of the gels demonstrated by ethidium bromide staining. Interestingly, the *chop/gapdh* ratio seen in normal erythroid cells was as high as that seen in Rauscher cells.

Because the pronounced upregulation of *chop* in Rauscher cells correlated with induction of hemoglobinization, we measured *chop* mRNA levels in differentiating normal murine erythroid precursors. As erythroid precursors mature, they decrease in cell volume and undergo chromatin condensation

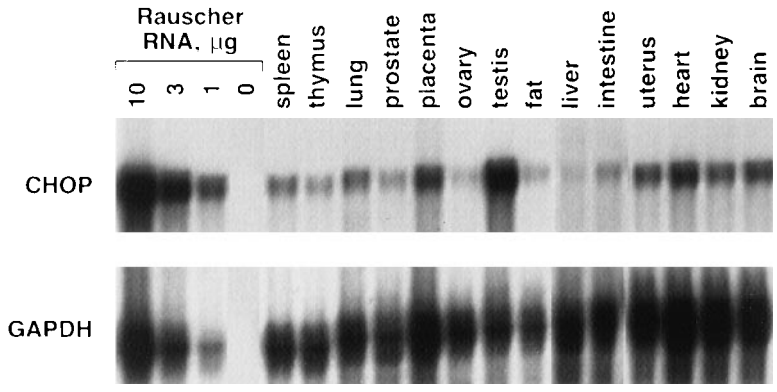

Fig 4. *chop* expression in adult mouse tissues. RNase protection assay for *chop* or *gapdh* was performed as described in the text. Ten micrograms of RNA was used for each tissue.

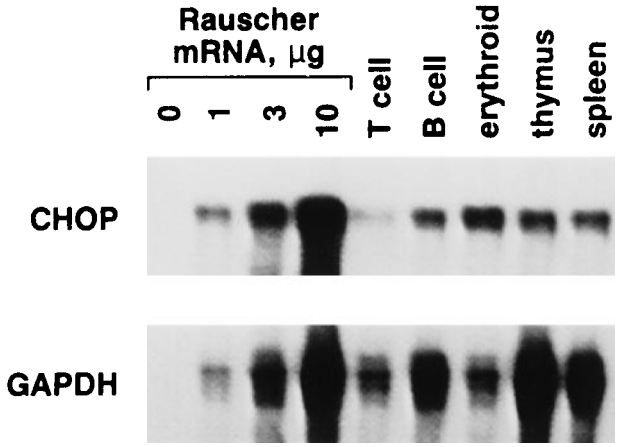

Fig 5. *chop* mRNA expression in Rauscher cells and a variety of normal murine hematopoietic cells. Splenic T, B, and erythroid cells were isolated using antibody-coated plates, as described.

and enucleation. These changes result in increasing cell density, a characteristic that can be used as the basis of a separation technique. Normal splenic erythroid precursor cells were separated on a 5-step discontinuous Percoll gradient. The cells from each were evaluated for *chop* expression by Northern blot, for their degree of hemoglobinization by benzidine staining, and for whether they had differentiated to the point that they had condensed and extruded their nucleus by microscopic examination. The least dense fraction contained the least mature population of precursors. There were only 18% hemoglobinized (Hb^+) cells and virtually all (99%) retained their nuclei. *chop* mRNA levels were relatively low in this least dense fraction. The third of the five fractions contained 90% Hb^+ cells, of which 56% retained their nuclei. Importantly, this stage of terminal differentiation was associated with a significant increase in *chop* mRNA, which is very similar to the association of *chop* upregulation and hemoglobinization of Rauscher cells. The increase was fourfold to fivefold over the least dense fraction. The two most dense fractions contained 99% Hb^+ cells

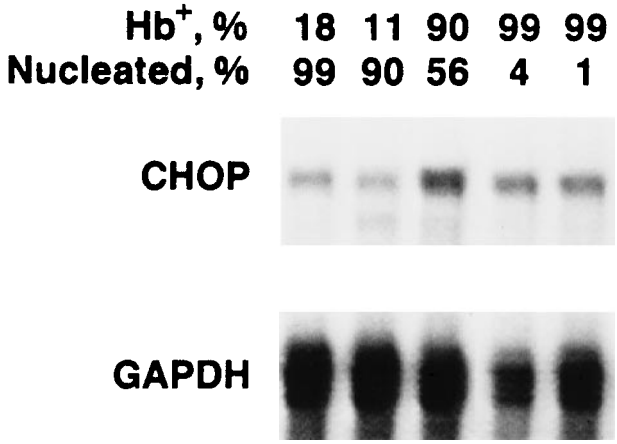

Fig 6. *chop* mRNA levels in normal murine erythroid precursors. Cells were separated by discontinuous Percoll density fractionation. Cells in each fraction were characterized for the percentage of hemoglobinized cells and the percentage of nucleated cells.

and 4% and 1% nucleated cells, respectively. *chop* mRNA began to decrease in these more mature erythroid cells (Fig 6).

Ectopic expression of chop *in Rauscher cells*. We obtained evidence of a functional role for *chop* in erythropoiesis by increasing its expression through transient transfection (Table 1). *chop* cDNA was subcloned into a $pSVK_3$ expression vector and was transfected into Rauscher cells. After 24 hours, cells were incubated in the absence or presence of Epo or DMSO for 48 hours, and differentiating cells were identified by staining for hemoglobin. Nontransfected cells induced with Epo or DMSO for 48 hours were 34% ± 4% and 49% ± 5% hemoglobinized (Hb^+), respectively. In contrast, cells transfected with and overexpressing *chop* were increased significantly to 43% ± 5% and 62% ± 5% Hb^+, respectively, strongly suggesting that increased *chop* enhanced erythroid differentiation ($P < .02$). Overexpression of CHOP protein was confirmed by Western blot analysis (not shown). Cells transfected with vector alone had responses to Epo or DMSO virtually identical to those of nontransfected cells. It is important to note that Rauscher cells are relatively resistant to transfection. Only 10% to 20% of cells were transfected on average as monitored by Gal-4 transfection. The absolute 9% to 13% increase in hemoglobinization seen in *chop*-transfected cells correlates well with the transfection efficiencies achieved.

Artificial downregulation of chop *with antisense oligodeoxynucleotides inhibits CFU-E colony growth.* Increased *chop* expression correlates with erythroid differentiation, and ectopic expression of *chop* increased Epo- and DMSO-induced differentiation of Rauscher cells. Therefore, we reasoned that downregulating *chop* levels should result in reduced differentiation. In the experiment shown in Fig 7, normal murine bone marrow cells were grown in methyl cellulose culture in the absence or presence of specified concentrations of antisense or missense *chop* oligos using conditions required for growth of CFU-E– or BFU-E–derived colonies. Further additions of oligos were made daily. CFU-E colonies were scored after 2 days, and BFU-E colonies were scored after 8 days. Antisense *chop* oligos inhibited CFU-E colony growth in a concentration-dependent manner. The maximum antisense oligo concentration used (200 μg/mL) reduced CFU-E colony numbers to 42% of control values. Importantly, missense *chop* oligo treatment had no effect on CFU-E growth, providing evidence for the specificity of the antisense effect. In other, less detailed experiments, sense *chop* oligo treatment also had no effect on CFU-E growth. In contrast to the inhibitory effect seen on CFU-E colony growth, neither antisense nor missense (nor in pilot experiments, sense) *chop* oligos inhibited BFU-E colony growth. Similar results were obtained in other experiments.

Table 1. Effect of Ectopic *chop* Expression on Epo- or DMSO-Induced Rauscher Cell Differentiation

	Hb^+ Cells (%)*	
Rauscher Cells	Epo	DMSO
Nontransfected	34 ± 4	49 ± 5
chop-transfected	43 ± 5	62 ± 5
Vector-transfected	32 ± 5	47 ± 5

*Cells were induced for 48 hours with 100 U Epo/mL or 0.7% DMSO. Hemoglobinized cells were qualified by benzidine staining. Noninduced cells exhibited less than 5% Hb^+ cells.

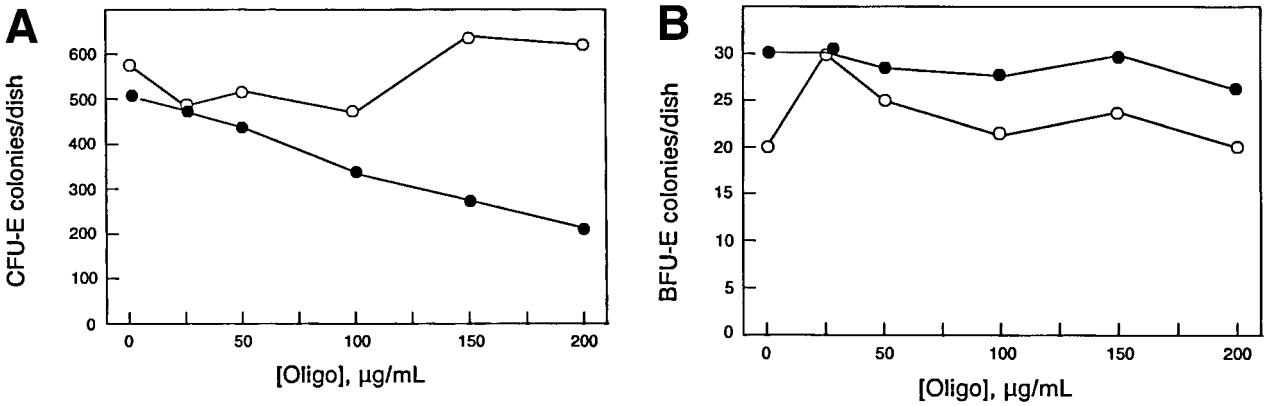

Fig 7. Effect of antisense *chop* oligodeoxynucleotides on normal murine erythropoiesis in vitro. (A) CFU-E–derived colony growth. (B) BFU-E–derived colony growth. (●) Antisense oligos; (○) missense oligos. Note the specific concentration-dependent inhibition of CFU-E colony formation by antisense *chop* oligos. Each point is the mean of duplicate determinations.

Because of technical difficulties in quantifying *chop* transcript levels in erythroid colonies grown in methyl cellulose, we used Rauscher cells grown in suspension culture to verify the action of antisense *chop* oligos (Fig 8). Rauscher cells were incubated in the absence or presence of 100 μg antisense *chop* oligo/mL for 24 hours. RNA was extracted and a Northern blot analysis was performed. Antisense *chop* oligo treatment caused a marked reduction in *chop* steady-state transcript levels. No change was observed using missense oligos (not shown).

CHOP binding partners during erythroid differentiation. Because of reports that C/EBP isoforms can interact with non-C/EBP proteins,[40-42] we used a protein-renaturation method to identify potential alternative CHOP binding partners and to determine whether they changed during erythropoiesis. Nuclear extracts from uninduced and induced cells (0.7% DMSO, 48 hours) were prepared, subjected to denaturing electrophoresis, transferred to blotting membranes, and then treated with a denaturing guanidine buffer. The denaturing buffer was washed off, and the membrane-bound proteins were allowed to renature overnight. The membranes were then probed with CHOP-GST fusion protein or GST alone (Fig 9). The CHOP-GST fusion protein interacted strongly with a number of the nuclear

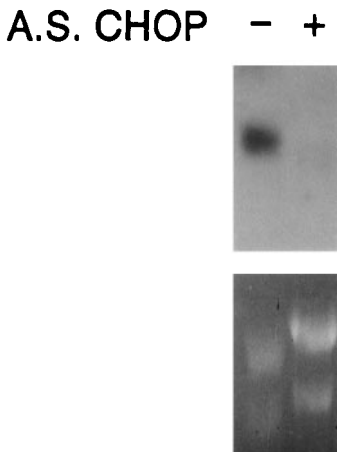

Fig 8. Northern analysis confirms downregulation of *chop* by antisense oligodeoxynucleotides.

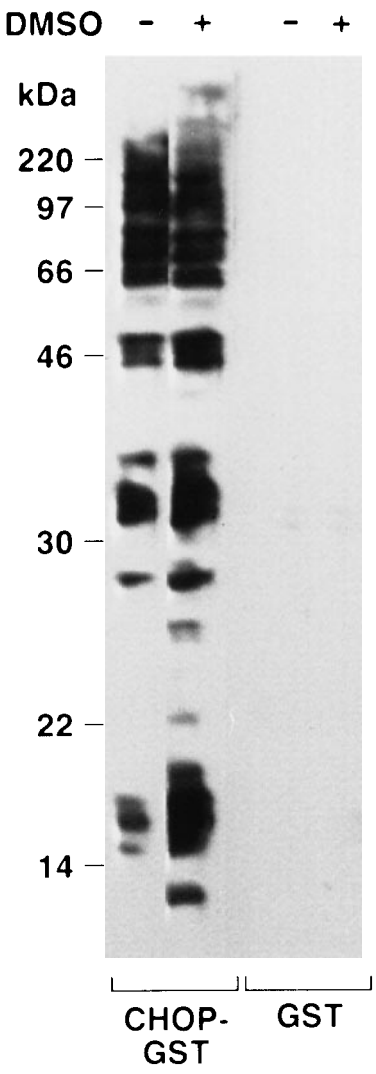

Fig 9. Far Western analysis shows numerous potential CHOP binding partners in erythroid cells and changes in them during erythropoiesis. See Results.

proteins, whereas the GST protein binding was negligible. The relatively large number of potential alternative binding partners was surprising. Nonetheless, similar observations were made in repeat experiments. Interestingly, the nuclear extract from induced cells had a number of new electrophoretically distinct species that bound CHOP-GST. In six experiments, CHOP-GST bound to proteins of 14.3, 17.5, 38.5, and 54 kD that were unique to the nuclear extracts from induced (differentiating) cells. There was also a number of CHOP binding proteins in uninduced cells, the abundance of which increased markedly during differentiation. They had molecular weights of 16, 27, 32, and 34 kD. Two proteins of approximately 66 and 95 kD, respectively, appeared to decrease in abundance during differentiation.

In addition to nuclear extracts, we also examined cytosolic fractions of both uninduced and induced cells for CHOP binding partners. There were a few proteins of very low abundance that appeared to bind CHOP-GST or GST proteins weakly. There were no differences between induced and uninduced cells (data not shown).

DISCUSSION

In this study, we have identified the transcription factor *chop* as an Epo-regulated gene and have obtained evidence that it plays a role in erythroid differentiation. *c/ebp*β was also upregulated during erythropoiesis. We found that *chop* message levels in Rauscher cells increased 10- to 20-fold after treatment with natural or chemical inducers of erythroid differentiation. Distinct signal transduction pathways are activated in response to EPO or DMSO,[2] but treatment with either inducer results in upregulation of *chop*. This suggests that inducers of erythroid differentiation converge on *chop* upregulation. We also found that *chop* message is particularly abundant in erythroleukemia cells, relative to terminally differentiated adult tissues, and that its abundance in normal erythroid cells is modulated during terminal differentiation. Furthermore, manipulation of CHOP levels perturbs normal erythropoiesis. This regulated expression is suggestive of a significant functional role for *chop* in red blood cell development.

In normal hematopoietic cells, the highest levels of CHOP were found in erythroid cells, with levels peaking during terminal differentiation. Rauscher cells have attributes of late BFU-E and early CFU-E.[43] Upon induction with Epo or DMSO, they resemble differentiating CFU-E. This later point in Rauscher cell differentiation is when *chop* levels were determined to be the highest. We demonstrated that CFU-E colony growth was inhibited by antisense *chop* treatment, whereas BFU-E colony growth was unaffected. The inhibition of CFU-E colony development by antisense *chop* is consistent with the absolute Epo requirement for CFU-E differentiation and strongly supports a role for CHOP in the terminal differentiation of erythroid cells. This finding compliments those described with *Epo* and *EpoR*-null mice, ie, there is an essential role for Epo in regulating the survival and terminal differentiation of CFU-E.[44] Because we have identified *chop* as an Epo-upregulated gene, it would be expected that its downregulation would block differentiation at the CFU-E stage, as we have shown. However, the apparent lack of antisense *chop* oligo effect on BFU-E colony numbers and morphology is less easily understood. Epo addition to BFU-E cultures can be delayed somewhat without affecting BFU-E colony development significantly. However, ultimately, Epo addition is essential for the final appearance of mature BFU-E–derived colonies. If BFU-E differentiation in vitro passes through a CFU-E stage identical to that characterized by CFU-E progenitors harvested from the bone marrow, then it would be expected that antisense *chop* oligos should inhibit terminal differentiation of this CFU-E–like cell, resulting in small and less robust BFU-E–derived colonies (even if actual colony numbers were not affected). Our results are consistent with the hypothesis that BFU-E differentiation in culture may exhibit characteristics unique to the in vitro environment. This may also include relative resistance to oligo uptake by in vitro CFU-E–like cells. Taken together, our studies of Rauscher and normal erythroid cells show that *chop* expression is most robust in terminally differentiating CFU-E progeny and that CHOP plays an important role in the regulation of erythroid differentiation.

CHOP's action is not the same in all cell types. Increased *chop* facilitates erythroid differentiation. This effect is the opposite of that seen in adipogenic differentiation, in which increased *chop* expression attenuates differentiation and interferes with the induction of *c/ebp* α and β.[45] In adipocyte systems, *chop* is induced by nutrient depletion in the culture medium and may slow down differentiation in response to metabolic stress (particularly the condition of low glucose). In contrast, *chop* expression in Rauscher cells was unaffected by altered glucose conditions; also, its induction was not related to cell density. CHOP expression can also be induced by treatments that adversely affect function of the endoplamic reticulum.[18,46]

At present, the precise mechanism of CHOP's action in erythroid differentiation is unknown, as is the mechanism by which Epo upregulates *chop*'s expression. Of note is the existence of a possible GATA-1 binding site at nucleotide −415 in the hamster *chop* gene[14] and of nucleotide −435 in the human *chop* gene.[47] The human site is a perfect, prototypical GATA site, and the hamster site conserves the GATA-1 core. Indeed, the Epo receptor itself,[48] known to be transactivated by GATA-1,[9,49] contains a rather nonconforming TTATCT sequence. Also of obvious interest (in view of Epo's *chop* upregulation concurrent with *c/ebp*β upregulation) are the numerous NF-I16 (C/EBPβ) sites in both *chop* genes.

The coordinate upregulation of both *chop* and *c/ebp*β during erythroid differentiation is suggestive of a mechanistic relationship. In this regard, it has recently been shown that C/EBPβ participates in regulating the pro-B cell-specific enhancer (PBE).[50] In this system, CHOP and C/EBPβ interact in a developmental stage-specific pattern. These factors associate and form an inactive complex in mature B-cell lines, whereas pro-B–cell lines have active complexes of C/EBP proteins, but no detectable CHOP protein. This is reminiscent of our results in erythroid cells. CHOP levels increase as the cells mature. It may well be that the CHOP-C/EBPβ has a negative regulatory function in some contexts. Earlier experiments using cell lines suggested that C/EBPβ activates transcription of the IL-6 promoter.[51] However, results from the *c/ebp*β knock-out mouse show lymphoproliferative and myeloproliferative histologies similar to mice overexpressing IL-6.[52] This in vivo result

suggests that C/EBPβ might negatively regulate the IL-6 gene. Differentiation requires restriction in developmental potential, and downregulation of the canonical CCAAT/enhancer sites may be part of this process. Global transcription that is characteristic of the undifferentiated state can be suppressed by increasing the amount of repressors such as CHOP, LIP, and, possibly, C/EBPβ. Once physiologically relevant targets of CHOP regulation are defined, the exact role of CHOP in erythroid differentiation can be addressed, ie, whether it acts as a dominant-negative regulator of C/EBP binding, directing CHOP-C/EBP heterodimers away from canonical C/EBP binding sites, or whether CHOP activates transcription from other classes of target genes.

Interactions between the different C/EBP proteins (including CHOP) may be critical to their mechanism and function during differentiation. All of the family members have a well-conserved C-terminus that includes five heptad repeats that form an amphipathic helix. Dimerization of these amphipathic helices allows formation of a leucine zipper. All of the C/EBP family members contain this motif, and most can form homodimers and heterodimers. CHOP is an unusual member of this family in that it does not form homodimers and it has a noncanonical DNA-contact domain. In some instances, CHOP acts as a dominant negative regulator of other C/EBPs.[13] Through heterodimerization, it inhibits the binding of its partner to the canonical C/EBP target sequence. In other instances, the CHOP/C/EBP heterodimer can recognize and activate noncanonical DNA binding sites.[30] Although a prefered DNA binding sequence has been defined, regulated genes have not been characterized.

The present studies point to other proteins that interact with CHOP. The Far Western assay (Fig 9) suggested that CHOP's binding partners change and become more numerous during differentiation. Other systems have shown complex and often cell-specific interactions between different C/EBP family members. There is potential for homodimerization and heterodimerization of some of the C/EBP isoforms and the possibility of regulatory interactions in trans (eg, the promoter of the *c/ebp* β gene has a C/EBPα binding site). In addition, interactions between C/EBP and non-C/EBP proteins have been documented.[40-42] In this regard, additional data obtained by us using the yeast two hybrid system confirms that erythroid cells (and, presumably, other cells) express non-C/EBP proteins that interact specifically with CHOP in vitro and in vivo[53,54] (manuscripts in preparation). Further studies in this area will help to elucidate the role of CHOP in erythropoiesis and may identify novel regulatory pathways.

Because of the association of *chop* upregulation and ER stress, Zinszner et al[55] performed targeted disruption of *chop* in murine ES cells and produced animals with a *chop* −/− phenotype.[55] Somewhat surprisingly, these mice were described as phenotypically normal and exhibited normal fertility. Unfortunately, no data regarding the hematopoietic system were reported. However, these animals, as well as embryonic fibroblasts derived from them, exhibited a defect in the development of apoptosis after treatment with agents that cause ER stress. This observation that a lack of CHOP prevents apoptosis would seem to contradict the demonstration that Epo, which increases *chop* expression, prevents apoptosis of erythroid cells. This apparent paradox mirrors the dissimilar effects of *chop* upregulation in adipogenic differentiation and in erythropoiesis and further supports the hypothesis that CHOP's role in cell growth and differentiation is dependent on the context of cell lineage and stage as well as the other binding partners available to it.

ACKNOWLEDGMENT

The authors thank Alan Friedman for the generous gift of the *c/ebp* α, β, and δ cDNAs and Cindy Miller for her tutorial on methyl cellulose culture. We also acknowledge the expert editorial work of Rosemary Panza.

REFERENCES

1. Li Y, Davis KL, Sytkowski AJ: PKC ϵ is necessary for erythropoietin's upregulation of c-*myc* and for factor-dependent DNA synthesis: Evidence for discrete signals for growth and differentiation. J Biol Chem 271:27025, 1996

2. Chern YE, Spangler R, Choi H-S, Sytkowski AJ: Erythropoietin activates the receptor in both Rauscher and Friend murine erythroleukemia cells. J Biol Chem 266:2009, 1991

3. Spangler R, Bailey SC, Sytkowski AJ: Erythropoietin increases c-*myc* mRNA by a protein kinase C-dependent pathway. J Biol Chem 266:681, 1991

4. Spangler R, Sytkowski AJ: C-Myc is an erythropoietin early response gene in normal erythroid cells: Evidence for a protein kinase C mediated signal. Blood 79:52, 1992

5. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN: JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227, 1993

6. Todokoro K, Watson R, Higo H, Amanuma H, Kuramochi S, Yanagisawa H, Ikawa Y: Downregulation of c-*myb* gene expression is a prerequisite for erythropoietin-induced erythroid differentiation. Proc Natl Acad Sci USA 85:8900, 1988

7. Valtieri M, Venturelli D, Care A, Fossati C, Pelosi E, Labbaye C, Mattia G, Gewirtz AM, Calabretta B, Peschle C: Antisense myb inhibition of purified erythroid progenitors in development and differentiation is linked to cycling activity and expression of DNA polymerase alpha. Blood 77:1181, 1991

8. Chern YE, O'Hara C, Sytkowski AJ: Induction of hemoglobin synthesis by downregulation of MYB protein with an antisense oligodeoxynucleotide. Blood 78:991, 1991

9. Chiba T, Ikawa Y, Todokoro K: GATA-1 transactivates erythropoietin receptor gene, and erythropoietin receptor-mediated signals enhance GATA-1 gene expression. Nucleic Acids Res 19:3843, 1991

10. Tsai SH, Martin DI, Zon LI, D'Andrea AD, Wong GG, Orkin SH: Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature 339:446, 1989

11. Andrews NC, Erdjument-Bromage H, Davidson MB, Tempst P, Orkin SH: Erythroid transcription factor NF-E2 is a hematopoietic-specific basic-leucine zipper protein. Nature 362:722, 1993

12. Fornace AJ Sr, Alamo I, Hollander MC: DNA damage-inducible transcripts in mammalian cells. Proc Natl Acad Sci USA 85:8800, 1988

13. Ron D, Habener JF: CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. Genes Dev 6:439, 1992

14. Fornace AJ Jr, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, Holbrook NJ: Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol Cell Biol 9:4196, 1989

15. Luethy JD, Fargnoli J, Park JS, Fornace AJ Jr, Holbrook NJ: Isolation and characterization of the hamster gadd153 gene: Activation of promoter activity by agents that damage DNA. J Biol Chem 265:1652, 1990

16. Carlson G, Fawcett TW, Bartlet JD, Bernier M, Holbrook NJ: Regulation of the C/EBP-related gene, *gadd 153*, by glucose deprivation. Mol Cell Biol 13:4736, 1993

17. Luethy JD, Holbrook NJ: Activation of gadd 153 promoted by genotoxic agents: A rapid and specific response to DNA damage. Cancer Res 52:5, 1992

18. Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjy A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D: Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153) Mol Cell Biol 16:4273, 1996

19. Baron MV, Crozat A, Tabaec A, Philipson L, Ron D: CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of GI/S arrest. Genes Dev 8:453, 1994

20. Maytin EV, Habener JF: Transcription factors C/EBPα, C/EBPβ, and CHOP (Gadd153) expressed during the differentiation program of keratinocytes in vitro and in vivo. J Invest Dermatol 110:238, 1998

21. Cao Z, Umek RM, McKnight SL: Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538, 1991

22. Yeh WC, Cao Z, Classon M, McKnight SL: Cascade regulation of terminal differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev 9:168, 1995

23. Scott, LM, Civin CI, Rorth P, Friedman AD: A novel temporal expression pattern of three C/EBP family members in differentiating myelomonocytic cells. Blood 80:1725, 1992

24. Friedman AD, Landschulz WH, McKnight SL: CCAAT/enhancer binding protein activates the promoter of serum albumin gene in clutured hepatoma cells. Genes Dev 3:1314, 1989

25. Pointkewitz Y, Enerback S, Hedin L: Expression and hormonal regulation of the CCAAT/enhancer binding protein alpha during differentiation of rat overian follicles. Endocrinology 133:2327, 1993

26. Chandrasekaran C, Gordon JI: Cell lineage-specific and differentiation-dependent patterns of CCAAT/enhancer binding protein alpha expression in the gut epithelium of normal and transgenic mice. Proc Natl Acad Sci USA 90:8871, 1993

27. Cooper CL, Berrier AL, Roman C, Calame KL: Limited expression of C/EBP family proteins during B lymphocyte development. Negative regulator Ig/EBP predominates early and activator NF-IL-6 is induced later. J Immunol 153:5049, 1994

28. Muller C, Kowenz-Leutz E, Grieser-Ade S, Graf T, Leutz A: NF-M (chicken C/EBP) induces eosinophilic differentiation and apoptosis in a hematopoietic progenitor cell line. EMBO J 14:6127, 1995

29. Morosetti R, Park DJ, Chumakov AM, Grillier I, Shiohara M, Gombart AF, Nakamaki T, Weinberg K, Koeffler HP: A novel, myeloid transcription factor, C/EBPϵ, is upregulated during granulocytic, but not monocytic, differentiation. Blood 90:2591, 1997

30. Ubeda M, Wang X-Z, Zinszner H, Wu I, Habener JF, Ron D: Stress-induced binding of the transcriptional factor CHOP to a novel DNA control element. Mol Cell Biol 16:1479, 1996

31. DeBoth NJ, Vermey M, van't Hull E, Klootwijk-van Dijke E, van Griensver LJLD, Mol LNM, Stoof RJ: A new erythroid cell line induced by Rauscher murine leukaemia virus. Nature 272:626, 1978

32. Sytkowski AJ, Salvado AJ, Smith GM, McIntyre CJ, DeBoth NJ: Erythroid differentiation of clonal Rauscher erythroleukemia cells in response to erythropoietin or dimethylsulfoxide. Science 210:74, 1980

33. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol chlorform extraction. Anal Biochem 162:156, 1987

34. Wysocki WL, Soto VL: ''Panning'' for lymphocytes: A method for cell selection. Proc Natl Acad Sci USA 75:2844, 1978

35. Mage MG: Fractionation of T cells and B cells, in Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Stroter W (eds): Current Protocols in Immunology, vol 1. New York, NY, Wiley, 1996, p 351

36. Hanspal M, Palek J: Synthesis and assembly of membrane skeletal proteins in mammalian red blood cell precursors. J Cell Biol 105:1417, 1987

37. Rifkind RA, Fibach E, Marks PA: Assay for the commitment of murine erythroleukemia cells to differentiate, in Murphy MJ (ed): In Vitro Aspects of Erythropoiesis. New York, NY, Springer-Verlag, 1978, p 266

38. Dignam JD, Lebovitz RM, Roeder RG: Accurate transcription initiation by RNA polymerase II from isolated mammalian nuclei. Nucleic Acids Res 11:1475, 1983

39. Ferrell JE, Martin GS: Thrombin stimulates the activities of multiple previously unidentified protein kinases in platelets. J Biol Chem 264:20723, 1989

40. Klampfer L, Lee TH, Hsu W, Vilcek J, Chen-Kiang S: NF-IL6 and AP-1 cooperatively modulate the activation of the TSG-6 gene by tumor necrosis factor alpha and interleukin-1. Mol Cell Biol 14:6561, 1994

41. LeClair KP, Blanar MA, Sharp PA: The p50 subunity of NFκB associates with the NF-IL6 transcription factor. Proc Natl Acad Sci USA 89:8145, 1992

42. Nagulapalli S, Pongubala JM, Atchison ML: Multiple proteins physically interact with PU.I. Transcriptional synergy with NL-IL6 beta (C/EBP delta, CRP3). J Immunol 155:4330, 1995

43. Bacon ER, Sytkowski AJ: Identification and characterization of a differentiation-specific antigen on normal and malignant murine erythroid cells. Blood 69:103, 1987

44. Wu H, Liu X, Jaenisch, Lodish HF: Generation of commited erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor. Cell 83:59, 1995

45. Batchvarova N, Wang WZ, Ron D: Inhibition of adipogenesis by the stress-induced protien CHOP (Gadd153). EMBO J 14:4654, 1995

46. Wang XZ, Ron D: Stress-induced phosphorylation and activation of the transcription factor CHOP (GADD153) by p38 MAP kinase. Science 272:1347, 1996

47. Park JS, Luethy JD, Wang MC, Farnogli J, Fornace AJ Jr, McBride OW, Holbrook NJ: Isolation, characterization and chromosomal location of the human GADD153 gene. Gene 116:259, 1992

48. Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF: Structure, function and activation of the erythropoietin receptor. Blood 81:2223, 1993

49. Zon LI, Youssoufian H, Mather C, Lodish HF, Orkin SH: Activation of the erythropoietin receptor promoter by transcription factor GATA-1. Proc Natl Acad Sci USA 88:10638, 1991

50. Saisanit S, Sun X-H: Regulation of the pro-B-cell-specific enhancer of the Id1 gene involves the C/EBP family of proteins. Mol Cell Biol 17:844, 1997

51. Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, Nakajima T, Hirano T, Kishimoto T: A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. EMBO J 9:1897, 1990

52. Screpanti I, Romani L, Musiani P, Modesti A, Fattori E, Lazzaro D, Sellitto C, Scarpa S, Bellavia D, Lattanzio G, Bistoni F, Frati L, Cortese R, Gulino A, Ciliberto G, Costantini F, Poli V: Lymphoproliferative disorder and imbalanced T-helper response in C/EBP beta-deficient mice. EMBO J 14:1932, 1995

53. Cui K, Coutts M, Stahl J, Sytkowski AJ: Interaction of CHOP and Fos transformation effector protein in erythroid cells. Blood 88:195a, 1996 (abstr, suppl 1)

54. Xu Y, Cui K, Sytkowski AJ: Interactions of CHOP, C/EBPβ and S3a/FTE in erythropoiesis. Blood 92:195a, 1998 (abstr, suppl 1)

55. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D: CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev 12:982, 1998