Ferritin Synthesis in Differentiating Friend Erythroleukemic Cells*

(Received for publication, January 12, 1987)

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We have investigated the regulation of ferritin synthesis during induction of Friend erythroleukemic cells by dimethyl sulfoxide. Northern blot analysis shows that mouse ferritin H and L mRNAs each contain approximately 1.1 kilobases. The levels of both mRNAs increase after addition of dimethyl sulfoxide in a biphasic manner. After a sharp rise in the first 6 h, the levels decline and then rise again over the next 90 h. These increases in mRNA levels reflect increased transcription of both mRNAs. Analysis of ferritin subunit synthesis surprisingly showed no corresponding increase in the rate of protein synthesis, suggesting that the additional mRNA was not in functional polysomes. These studies also indicated a novel processing of mouse ferritin H subunits. H subunits appear to be synthesized as a precursor of approximately 22,500. This form is not present in mature shells. Pulse-chase experiments indicated that the precursor is first processed to an intermediate form of 20,000 and then to the 18,000 component found in functional shells.

Ferritin is the major iron storage protein in eukaryotic cells (1). In addition to its detoxification and storage functions, recent evidence indicates that ferritin also plays an active role in regulating intracellular iron flux (2, 3). Mammalian ferritins consist of a protein shell of 24 subunits which can enclose as many as 2500 atoms of iron. Most shells are heteropolymers containing different proportions of two partially homologous subunit types, H and L (4). Tissue ferritin phenotypes are therefore regulated by the relative expression of these subunits, and this varies with tissue, developmental, or pathological state (4–6). L-rich ferritins predominate in iron storage organs, such as liver and spleen, whereas H-rich ferritins predominate in organs of low non-heme iron content such as heart and pancreas (4, 5). These distributions probably reflect functional differences in H and L ferritins. H-rich ferritins accept and release iron more readily than L-rich ferritins (7, 8). H-rich ferritin shells also turn over more rapidly than L-rich ferritin shells which, consequently perhaps, accumulate more iron (9). These metabolic differences in human ferritins suggest that the H subunit plays a key role in the intracellular traffic of iron, whereas the L subunit is better suited for long term iron storage (2).

In many cells, ferritin synthesis is regulated by iron. This regulation can occur at several levels, from transcription to shell assembly. In HeLa cells, iron acts mainly at the level of transcription and shell assembly (10), whereas in liver the immediate effect of iron is on the mobilization of ferritin mRNAs from messenger ribonucleoprotein particles to functional polysomes (11). Ferritin mRNA synthesis can also be induced with other agents such as phorbol esters or dimethyl sulfoxide which promote cell differentiation. In the differentiation of the promyelocyte cell line HL-60 into neutrophils or into macrophages, the levels of H mRNA increase more than the levels of L mRNA, but it is not known whether these changes are in transcription or in mRNA stability (12).

We have been interested in the regulation and possible role of ferritin synthesis during the development of hemoglobin synthesis. Friend cells are mouse erythroleukemic cells that resemble proerythroblasts (13). Treatment of these cells with dimethyl sulfoxide promotes their differentiation and results in the expression of genes specific to erythropoietic differentiation, such as those for globin chains, heme pathway enzymes, and membrane antigen (13–15). In this system, the activities of heme pathway enzymes are coordinately increased as early as 24 h after dimethyl sulfoxide addition. However, significant increases in heme only occur 24–48 h later (16). Several lines of evidence indicate that the increase in heme synthesis is regulated by some step in intracellular iron metabolism (17). It is known that heme iron can be obtained from ferritin. However, heme iron can also be obtained directly from an intracellular pool of iron without transiting ferritin (18). These observations, and the apparent functional differences between H and L ferritin subunits in iron metabolism, led us to examine ferritin synthesis during the differentiation of Friend cells. Our results indicate that transcription of both H and L ferritin mRNAs is induced during this differentiation. We also find an unexpected difference in the synthesis of mouse ferritins compared with other ferritins which includes a novel processing of nascent H subunits.

EXPERIMENTAL PROCEDURES

Cells—Clone 745 of mouse Friend erythroleukemia cells (13) was donated by Dr. Tambourin (INSERM U152, Paris).

Reagents—McCoy 5A medium and methionine-free Eagle’s medium were obtained from Eurobio (France). [35S]Methionine was from Amersham Corp. at a specific activity of 400 Ci/mmol. Biotinylated anti-rabbit immunoglobulin was obtained from Amersham (United Kingdom). Protein A-Sepharose was obtained from Pharmacia (Sweden).

Antibodies—Purified mouse liver ferritin and antiserum to mouse spleen ferritin were generous gifts of Dr. W. Massover (New Jersey Medical School, NJ).

Antibodies with specificities for epitopes from H or L subunits were prepared from antisera against ferritins from HeLa cells and human liver. The preparation and specificities of these antibodies have been previously described (19).

Cultures—Friend cells were grown in suspension in McCoy 5A medium supplemented with 10% fetal calf serum and 2 mM glutamine. The cultures were maintained in 5% CO2, 95% air at 37°C in a
humidified incubator. Subcultures were prepared every 3–4 days by diluting the cells to maintain logarithmic growth rate. Dimethyl sulfoxide was added 12–16 h after cell dilution to a final concentration of 1.5% (v/v). Cells were harvested by centrifugation at 800 x g for 5 min and washed twice with sterile phosphate-buffered saline (20 mM phosphate, 0.14 M NaCl, pH 7.4). The final pellet was frozen in liquid nitrogen and stored at −80 °C until used.

**Western Blot Analysis of Ferritins**—Pellets of cultured Friend cells or samples from mouse liver were homogenized in water containing 1 mM paramethylyluridate, heated to 75 °C for 10 min, cooled on ice, and centrifuged at 10,000 x g for 15 min. 50 μg of total proteins from the heat-solubilized fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9, 20) and then transferred electrophoretically to nitrocellulose (21). Uncropped sites on the membrane were blocked by incubation in 2% bovine serum albumin. Ferritin subunits were then revealed by using antibodies prepared against mouse ferritin followed by biotinylated anti-rabbit immunoglobulins and biotin-streptavidin-peroxidase complex as recommended by the manufacturer.

**In Vivo Labeling of Ferritin Subunits**—Friend cells were cultured for 3 days in McCoy medium with or without dimethyl sulfoxide. Cells (10⁷) were transferred to 10 ml of methionine-free Eagle’s medium supplemented with 10% fetal calf serum and incubated for 1 h at 37 °C. 175 μCi of [³⁵S]methionine was then added and the incubation continued at 37 °C for various time periods. At the end of the labeling period, cells were washed twice in phosphate-buffered saline and frozen in liquid nitrogen. For the chase experiments, cells were labeled for 2 h, washed twice with McCoy medium, and then incubated in the same medium for various periods.

[³⁵S]Methionine-labeled ferritin was immunoprecipitated from cleared cell lysates using anti-mouse spleen ferritin antibodies followed by protein A-Sepharose. The immunoprecipitates were analyzed by fluorography after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9).

**Northern and Dot-Blot Analysis of Mouse Ferritin Subunits and β-Globin mRNAs**—Total cellular RNA was prepared by extraction from Friend cells at various times after dimethyl sulfoxide addition and analyzed by Northern and dot-blot experiments as previously described (14). The β-globin probe was a 1.65-kilobase BamHI–EcoRI fragment from the mouse β-major globin gene (22). The ferritin probes were from essentially full-length cDNA clones for human ferritin H and L chains (2). The cDNA inserts were labeled with ³²P by nick translation to a specific activity of about 10⁶ cpm/μg (23).

**In Vitro Transcription in Isolated Nuclei**—Nuclei were isolated from Friend cells cultured for various periods of time in the presence of dimethyl sulfoxide. Nuclear transcription in vitro was performed as described (27) with modifications. Briefly, 5 x 10⁶ nuclei were incubated in 200 μl of mixture in the presence of 250 μCi of [¹⁵N]UTP (410 Ci/mmol) at 30 °C for 15 min. 15–25 x 10⁶ cpm of the ³²P-labeled primary transcripts were hybridized for 3 days to duplicate filters containing 2 μg of linearized plasmid DNA immobilized on GeneScreen (Du Pont-New England Nuclear). After washing and treating with RNase, the filters were autoradiographed. The intensities of individual hybridization dots were estimated by scanning the fluorogram with a Zeineh Soft-Lazer Scanning Densitometer. Non-specific hybridization was estimated using filters containing 2 μg of the plasmid vector pBR322 prepared in the same manner as for the ferritin H and L probes.

**RESULTS**

**Accumulation of H and L mRNAs during Dimethyl Sulfoxide Treatment**—As a first step in examining regulation of ferritin H and L synthesis during differentiation of Friend cells with dimethyl sulfoxide, we determined whether mouse H and L mRNAs were recognized by the human H and L cDNA probes. Human H and L cDNAs share about 65% homology in their coding regions but differ substantially in their noncoding regions (2). In contrast, human H cDNA is more than 80% homologous with rat L cDNA in coding regions and also has extensive homologies in noncoding regions (2). Consequently, although human H and L cDNAs do not cross-hybridize under normal stringency, human H and L probes hybridize selectively to their rat counterparts under the same conditions (11, 25). By analogy, we therefore assumed that the human H and L cDNA probes would each hybridize, respectively, to mouse H and L mRNAs. The results (Fig. 1) show that each probe recognizes different bands of mouse mRNA, each of about 1 kilobase. These sizes are similar to those found for human, rat, and rabbit ferritin mRNAs (25). Interestingly, the band identified as mouse ferritin L mRNA because of its hybridization to the human L cDNA had a slightly higher size range than that identified as mouse H mRNA.

These analyses are also of interest in terms of the relationship of sequences in ferritin H mRNA and 28 S ribosomal RNA. The 5'-noncoding region of human H ferritin cDNA has an extensive complementarity with several large repeats in human 28 S ribosomal RNA (26). As a result, 28 S RNA hybridizes strongly to probes from the 5' end of ferritin H mRNA, and this interaction complicates quantitation of ferritin H mRNA. However, the results in Fig. 1 show that the human H cDNA probe does not hybridize with mouse 28 S ribosomal RNA. This result is consistent with a similar lack of hybridization of rat and rabbit rRNA with the human H cDNA (26).

Since the H and L probes each reacted with only one major mouse species on Northern blots, we therefore estimated levels of mouse H and L mRNAs by the more quantitative dot-blot assay. The results of a typical experiment are plotted in Fig. 2, together with previously obtained data on the levels of β-globin (14) as reference for the stages of differentiation of the Friend cells. These analyses indicate that the levels of H and L mRNAs increased markedly 6 h after addition of dimethyl sulfoxide. The increase in H mRNA was about twice that in the L mRNA. After a decrease and some fluctuation, the H and L mRNAs thereafter increased to a final level of about 3-fold and 1.8-fold, respectively, over controls by the 96-h period. The reason for the fluctuation in both mRNAs is not clear. The fluctuations do not seem to be artifactual since the levels of β-globin mRNA quantitated on the same blot did not show these fluctuations (Fig. 2) and increased in characteristic fashion (14). They may, therefore, reflect differences in cell cycle. Consistent with this hypothesis, we have observed large differences in steady state levels of ferritin mRNAs in rapidly dividing uninduced cells as compared to quiescent confluent cells (not shown).

**In Vitro Nuclear Transcription**—To determine whether the
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FIG. 2. Changes in the relative levels of the H and L mRNAs and of β-globin mRNA during the dimethyl sulfoxide-mediated differentiation of cultured Friend cells. For each time point, 5 μg of total RNA and serial dilutions were spotted on Zeta-probe membranes and hybridized sequentially with the H and L probes and then with a 1.65-kilobase BamHI-EcoRI fragment from the mouse β-globin gene. Data for β-globin are taken from Ref. 14. DMSO, dimethyl sulfoxide.

increases in H and L mRNA reflected increased gene transcription or changes in turnover of mRNA, we performed in vitro transcription experiments in nuclei isolated from Friend cells at different times after dimethyl sulfoxide addition. This analysis gives a measure of the number of nascent RNA molecules whose synthesis has been initiated in vivo (24). Preliminary experiments showed that the transcription rate of total RNA remained linear for up to 20 min. In addition, recoveries of labeled ferritin transcripts were judged to be essentially complete since additional amounts of plasmid DNA on the filters did not increase the hybridization signals. The results (Table I) show that the levels of both H and L gene transcription are increased over the control level after 72 h of culture in the presence of dimethyl sulfoxide. The levels of H mRNA transcripts were approximately 4-fold higher than controls, whereas the levels of L mRNA were about 1.5-fold higher. These increases are similar to those given by quantitation of mRNA levels. Thus, we can conclude that ferritin H and L gene expression is regulated at the transcriptional level during the dimethyl sulfoxide-induced differentiation of Friend cells.

H and L Subunit Synthesis—The preceding indicates that the rate of transcription of mouse ferritin H and L mRNAs is increased during differentiation of Friend cells with dimethyl sulfoxide. These results led to an analysis of the synthesis of ferritin subunits in this process. It is known that the subunit structures of mouse ferritin differ from that in humans, horses, and rats. In iron-loaded mouse liver, the predominant ferritin subunit has an apparent molecular weight of 22,000 (28). However, with our procedures we found that the predominant subunit in this same preparation had an apparent molecular weight of 24,500. We attribute this discrepancy to different electrophoretic conditions. Since the 24,500 subunit comprises at least 80% of the total subunit population of mouse liver ferritin, it seems likely that it corresponds to the L subunit of human, horse, and rat. Similarly, it would appear that the smaller subunit (M, 18,000), which is also found in mouse liver ferritin, although to a much lesser extent, corresponds to the H subunit.

These conclusions were supported by Western blot analyses of mouse ferritins in liver and Friend erythroleukemic cells (Fig. 3). Protein staining indicates that mouse liver ferritin consists almost entirely of the larger subunit, in agreement with other analyses (28). Similar results were obtained after treating either purified mouse liver ferritin or a liver homog-
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The differentiation of Friend erythroleukemic cells by dimethyl sulfoxide offers an interesting model for examining ferritin expression during erythropoiesis. In uninduced cells, only traces of globin are produced, and the cells' requirement

denate with antibodies prepared against mouse spleen ferritins (lanes d and e). Antibodies with specificity for human L determinants recognized only the 24,500 subunit (lane c). Polyclonal and monoclonal antibodies with specificity for the human H subunit did not react with either of the mouse subunit types (not shown). In contrast to liver ferritin, the ferritin from Friend cells contained similar amounts of both subunit types as evidenced by immunochemical detection with antibodies to mouse spleen ferritin (lanes g and h). We therefore conclude from the relative distributions and immunoreactivities of the two subunits that the 24,500 subunit corresponds functionally to the H subunit, whereas the 18,000 subunit corresponds to the H subunit.

These analyses are also of interest in terms of the observed increases in synthesis of H and L mRNAs in that they show only small differences in the levels of ferritin protein in uninduced Friend cells and in cells analyzed 72 h after induction with dimethyl sulfoxide. At this point, the cytoplasmic levels of both H and L mRNAs are markedly elevated (Fig. 2). This apparently anomalous result led us to investigate the relative synthetic rates of H and L subunits in induced and noninduced cells.

Synthesis and Turnover Rate of Ferritin Subunits in Friend Cells—The biosynthesis of mouse ferritin subunits was analyzed in noninduced and induced Friend cells, and the effect of iron on the rate of ferritin synthesis in noninduced cells was also investigated. [35S]Metionine-labeled subunits were immunoprecipitated from cell extracts after 40 min, 2 h, and 4 h of labeling using the same antibodies to mouse spleen ferritin as were used for Western blot experiments. The immunoprecipitates were resolved into subunits by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and estimates of synthesis made from fluorograms by scanning densitometry. To our surprise, we found that the newly synthesized subunits differed from those in the mature shells (Fig. 4). After 40 min and 2 h of labeling, most of the radioactivity was found in subunits with apparent molecular weights of 24,500 and 22,500. After 4 h of labeling, two smaller subunits of 20,000 and 18,000 appeared. The 24,500 subunit corresponded to the larger subunit (L) previously observed in Friend cell lysates and in mouse liver homogenate (Fig. 3), but the 22,500 subunit had no counterpart in the mature ferritin shells. The 24,500 and 22,500 subunits were synthesized at similar rates in noninduced Friend cells (lanes a–c) and in noninduced Friend cells treated with iron for 1 h prior to labeling with [35S]methionine (lanes d–f). However, iron caused an approximately 2-fold increase in the overall labeling of both subunits. In the dimethyl sulfoxide-treated cells, the relative incorporation into the L subunit (Mr, 24,500) was reduced. Densitometric evaluation indicated that at each time point the relative proportion of the two subunits (24,500 over 22,500) was changed from about 1.0 in uninduced cells to 0.7 in dimethyl sulfoxide-treated cells.

A comparison of the types of subunits found in mature shells and those found by metabolic labeling suggested that the 18,000 subunit is derived from the 22,500 subunit by some post-translational processing involving the 20,000 as an intermediate form. To explore this hypothesis, we performed pulse-chase experiments in noninduced Friend cells. After 2 h of labeling, only the 24,500 and 22,500 subunits were observed (Fig. 5b). After 3 h of chase, the label now appeared in the 20,000 and 18,000 subunits (Fig. 5c). The presence of cycloheximide (100 μg/ml) during the 3-h chase period did not prevent this processing (not shown). After 4 days of chase (Fig. 5e), label was found only in the two subunits, 24,500 (L) and 18,000 (H), found in mature shells (Fig. 3). These results suggest that the H subunit is synthesized in Friend cells as a 22,500 molecular weight precursor which is processed proteolytically to an intermediate 20,900 form and then to the 18,000 form found in functional ferritin shells.

DISCUSSION

The differentiation of Friend erythroleukemic cells by dimethyl sulfoxide offers an interesting model for examining ferritin expression during erythropoiesis. In uninduced cells, only traces of globin are produced, and the cells' requirement

![Fig. 4. Biosynthesis of mouse ferritin subunits in cultured Friend cells. Noninduced Friend cells (Control cells), noninduced Friend cells treated for 1 h with iron (ferric ammonium citrate 1 mg/ml), and Friend cells cultured for 3 days in the presence of dimethyl sulfoxide (Induced cells) were labeled for 40 min (a, d, g), 2 h (b, e, h), and 4 h (c, f, i) with [35S]methionine. The figure shows a fluorograph of ferritin subunits immunoprecipitated with anti-mouse spleen ferritin antibodies. The molecular weights of the four subunits deduced from this and three other separate experiments are also indicated.]

![Fig. 5. Precursor-product relationship between the 22,500 and 18,000 subunits. The figure shows a fluorograph of ferritin subunits immunoprecipitated from noninduced Friend cells labeled for 2 h with [35S]methionine (b) and then washed and incubated in chase medium for 3 (c), 48 (d), and 96 h (e). Specificity of the immunoprecipitates was checked by omitting the first antibody (q). Lane f, 14C-labeled purified mouse liver ferritin. Lane g, 14C-labeled molecular weight markers.]

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**Discussion**

The differentiation of Friend erythroleukemic cells by dimethyl sulfoxide offers an interesting model for examining ferritin expression during erythropoiesis. In uninduced cells, only traces of globin are produced, and the cells' requirement
for iron is presumably regulated to supply the relatively small amounts of iron needed for housekeeping functions. However, as the cells differentiate to synthesize hemoglobin, their requirement for iron increases. This increased requirement is reflected in increased levels of transferrin receptors (29). Once absorbed, the iron can either go directly for heme synthesis through the low molecular weight soluble iron pool (whose identity still remains controversial), or it can be directed for temporary storage as ferritin. The proportion of incoming iron that transits ferritin increases with increasing iron intake, and the intracellular levels of ferritin may regulate this distribution (3). After the cells produce hemoglobin, ferritin is also likely to play an important role in detoxifying iron released from degradation of hemoglobin.

Our experiments indicate that the synthesis of ferritin H and L mRNAs increase during the induction period but do not reveal any large temporal differences in their production or in that of H and L subunits that would point to clear functional differences of H and L ferritins. The rates of transcription and the levels of mRNA for both H and L subunits increase essentially in parallel over the period in which the cells form hemoglobin. The increase in ferritin mRNA follows that in the mRNAs for heme pathway enzymes. This increased requirement is also likely to play an important role in detoxifying iron released from degradation of hemoglobin.

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Acknowledgments—We are particularly grateful to Drs. Sophie Vaulont and Axel Khan for their help and advice with the in vitro transcription experiments. We acknowledge valuable discussions and the generous gift from Dr. William Massover of antibodies to mouse spleen ferritin. We also thank David Belcher for technical assistance.

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