Structure and Expression of Ferritin Genes in a Human Promyelocytic Cell Line That Differentiates In Vitro

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HL-60 is a human promyelocytic cell line with the capability of differentiating in vitro to give neutrophils, macrophages, or eosinophils. We screened libraries of HL-60 cDNA clones representing different time points during these differentiation processes to isolate clones corresponding to mRNAs whose expression is regulated during terminal differentiation. Upon sequencing this group of regulated clones, one clone encoding the heavy subunit and two clones encoding the light subunit of human ferritin were identified by reference to published amino acid sequences. Southern blot analyses showed that these clones are encoded by distinct multigene families. These clones identify two mRNAs whose ratios vary in a complex manner during both neutrophil and macrophage differentiation.

Ferritins are a heterogeneous family of ubiquitous proteins which function to sequester and detoxify intracellular iron in eucaryotic cells. Ferritins have also been proposed to have a role as regulators of cellular differentiation.

Each molecule of the isoferritin family contains 24 subunits of two types, heavy (H) and light (L). The variability arises because the two subunit types can combine in widely varying ratios which depend upon tissue type and physiological role (1). For example, the H/L ratio is about 2/3 in liver isoferritins and about 4/1 or 5/1 in heart isoferritins (4). The H/L subunit ratio characteristic of a particular tissue may be altered during normal cellular differentiation or in pathological conditions such as iron overload or neoplasia (7, 15, 34). The functional significance of such changes in H/L subunit ratio is only beginning to be understood. There is some evidence that it is the L subunit-rich isoferritins which may be most effective in iron storage roles: iron challenge induces preferential synthesis of L subunits in both human (14) and rat (4) cells, and isoferritins with a low H/L subunit ratio show preferential loading and the longest lifetime in iron-challenged cells of the rat (4). In human cells, however, the evidence is contradictory. It is the H subunit-rich isoferritins which show the heaviest iron loading in human liver (3), but iron challenge of human monocytes preferentially stimulates L subunit synthesis (14) just as was noted for the rat. In addition, it is a glycosylated serum isoferritin with a high H/L subunit ratio (acidic isoferritin) which has been found to be associated with an inhibitory activity on normal myelopoiesis (7, 27).

The HL-60 human promyelocytic cell line used in our experiments was chosen because it possesses an unusual ability to undergo multipotent differentiation in vitro. Treatment with appropriate inducers can cause HL-60 cells to undergo commitment and terminal differentiation yielding cells with morphological and other characteristics of neutrophils (11), macrophages (29), or eosinophils (17). Study of

HL-60-derived neutrophils and macrophages has revealed that these differentiations are accompanied by the appearance of several enzymatic activities characteristic of normal neutrophils and macrophages (16). As reported by Collins et al. (11), terminally differentiated HL-60 cells also display many of the complex biological behaviors of their normal counterparts in the human body. Further evidence that HL-60 cells are able to make important commitment decisions comes from two-dimensional gel electrophoretic studies which indicate drastic changes in the levels of hundreds of proteins, and also from demonstrations of striking alterations in the abundance of many mRNAs during HL-60 differentiation (10; Davis et al., manuscript in preparation). Our laboratory is actively engaged in studying this cell line because its ability to differentiate provides an unusual opportunity to utilize the powerful techniques of site-directed mutagenesis to examine the mechanisms of gene regulation during the processes of cellular commitment and differentiation.

Here we report the isolation and characterization of cDNA clones corresponding to both the H and L subunits of human ferritin. We present evidence that these cDNA clones identify unique mRNAs which are encoded by distinct multigene families of different sizes and that the level of the mRNAs is regulated in a complex manner during HL-60 cellular differentiation. The results show that this human leukemic cell line provides a useful model system for studying the regulation of a class of genes whose expression can be modulated either by reversible (iron induction) or irreversible (cellular differentiation) events.

MATERIALS AND METHODS

Isolation and sequence analysis of human ferritin clones. The cDNA clones HL217, HL227, and HL1213 were isolated from an HL-60 neutrophil library which will be described elsewhere (Davis et al., in preparation). These clones together with 50 other clones were selected by differential hybridization procedures which showed that the corresponding mRNAs were "strongly regulated" during HL-60 neutrophil differentiation (Davis et al., in preparation).

Sequence analysis was by the dideoxy chain termination method (31). For this method, DNAs cleaved at the restric-

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FIG. 1. Partial restriction maps and sequencing strategies. Both the H and the L subunit clones were obtained from an HL-60 neutrophil cDNA library. Restriction sites at which regions of each cDNA were subcloned into the replicative form of M13 mp10, mp11, and mp19 or pSDL13 are shown. Solid lines and arrows indicate the regions and directions of sequence data obtained from these subclones by the dideoxy chain termination method (31). The brackets at the bottom of the two diagrams indicate the sequence contained in each HL-60 clone. bp, Base pairs.

tion sites shown (Fig. 1) were subcloned into the replicative form of bacteriophage M13 vector mp10, mp11, or mp19 (25, 26) or into the miniplasmid pSDL13 (gift from Brian Seed).

The nucleic acid sequences and their predicted amino acid sequences were compared with the sequences in the Genbank and the National Biomedical Research Foundation databases, respectively, utilizing the programs of Wilbur and Lipman (35). Clones HL217 (782 nucleotides), HL227 (571 nucleotides), and HL1213 (548 nucleotides) displayed significant homologies to the amino acid sequence of horse spleen apoferritin (19). Clones HL227 and HL1213, which displayed the highest homology in this search, have a shared region of 489 nucleotides (Fig. 1). The clone HL217 corresponds to a distinct mRNA which shows only 62% coding-region homology with the HL227 and HL1213 clones. No homology was detected between the 3'-noncoding regions of the two types of ferritin mRNAs. Amino acid sequences have recently become available for both the human ferritin H (13) and L (37) subunits, and comparison with this data identified clone HL217 as an H subunit mRNA copy and clones HL227 and HL1213 as L subunit mRNA copies.

Cell culture and RNA preparation. HL-60 cells were induced to differentiate into neutrophil-like cells (by dimethyl sulfoxide [DMSO] plus Amiloride treatment) or into macrophage-like cells (by 12-*O*-tetradecanoylphorbol-13-acetate treatment) as described elsewhere (29; Davis et al., in preparation). Treatment for 3 days with DMSO plus amiloride results in cell differentiation comparable to that achieved by treatment for 8 days with DMSO alone (8). Cytoplasmic poly(A)⁺ RNA was prepared from HL-60 cells at various stages of differentiation by the hot-phenol extraction method followed by one cycle of oligo(dT)-cellulose chromatography (23). Vanadyl ribonucleoside complexes were included to inhibit RNases (2). Adherent HL-60 macrophages were isolated by scraping monolayers which were then resuspended in ice-cold phosphate-buffered saline.

Northern blot analysis. Northern blots were prepared by a variation of the method described by McMaster and Carmichael (24) and Maniatis et al. (23). RNA samples were denatured by glyoxalation, electrophoresed on a 1% agarose gel, and then transferred to a reusable nylon membrane

(Zetapor, catalog P1200-01-045 SP; AMF-Cuno, Meriden, Conn.) by blotting overnight with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was dried and then baked under vacuum at 80°C for 2 h. Before probing, the membrane was washed in 0.1× SSC at 65°C for 1 h. ³²P-labeled probes were made by random priming pBR322-based plasmids (32). After prehybridization (in 50% formamide–5× Denhardt buffer–0.1% sodium dodecyl sulfate–100 μg of salmon sperm DNA per ml–5× SSC at 42°C for 18 h) and hybridization (approximately 10⁷ cpm in a total volume of 10 ml of 50% formamide–5× SSC–2× Denhardt buffer–100 μg of salmon sperm DNA per ml–10% dextran sulfate at 42°C for 18 h), the blot was washed (twice in 2× SSC at room temperature for 5 min, twice in 2× SSC–1% sodium dodecyl sulfate at 65°C for 30 min, and twice in 0.1× SSC at room temperature for 30 min). To reuse the blot, the probe from the previous probing was stripped from the blot by washing twice in 0.1× SSC at 70°C for 1 h. We found such Northern blots to be reusable about seven to eight times.

Autoradiography was carried out for 0.5 h to 1 week at –70°C, using preflashed Kodak X-Omat XRP film and Cronex enhancing screens. Relative band intensities were estimated by using multiple exposures, in the linear range of the film's sensitivity, and comparing with standard bands of known relative intensity.

Southern blot analysis. Southern blots were prepared and probed by the method of Gatti et al. (18). HL217-1 and HL217-2 are nonoverlapping subclones derived from cleavage at the internal *Pst*I site of the HL217 (H subunit cDNA) insert (Fig. 1). These subclones, as well as the HL227 (L subunit cDNA) clone, were carried in the single-stranded bacteriophage vector M13 mp11 when used as probes. The probes were labeled with ³²P by the extension of a hybridization primer (18, 20).

RESULTS

Identification and sequence analysis of ferritin cDNA clones.

Differential screening was used to identify a large number of clones corresponding to mRNAs strongly regulated during HL-60 differentiation. Among the first 16 of these clones to be sequenced, 3 were identified as ferritin gene copies by homology with known protein sequences, as described in Materials and Methods. Figure 1 shows the strategies used for nucleotide sequence analysis of the H and L subunit clones, and the nucleotide sequence and predicted amino acid sequence of the H subunit clone HL217 is displayed in Fig. 2. This clone contains the entire coding region for the ferritin H subunit, along with 26 nucleotides of 5'-noncoding sequence, 161 nucleotides of 3'-noncoding sequence, and 43 nucleotides of poly(A). The coding region predicts a protein of 183 amino acids and 21,217 molecular weight. The nucleotide sequence of HL217 contains an additional cytosine residue (arrow at nucleotide 552 in Fig. 2) not present in the H subunit sequence reported from liver by Costanzo et al. (13). The published liver sequence diverges from our HL-60 sequence at Leu-176 and predicts a protein of 190 amino acids and 22,294 molecular weight.

Figure 3A shows the partial nucleotide sequence and predicted amino acid sequence for the HL-60 ferritin L subunit mRNA (as derived from clones HL227 and HL1213). The sequence begins at the nucleotides encoding amino acid 32 of the mature protein and extends to the poly(A)⁺ end of the mRNA, including 146 nucleotides of 3'-noncoding sequence and 55 nucleotides of poly(A). The translated amino acid sequence matches that derived by direct protein sequencing of the major ferritin subunit in human spleen (37) at

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                                Met Thr Thr Ala Ser Thr Ser Gln
1  GCCGCCGCTCTCCTTAGTCGCCGCC ATG ACG ACC GCG TCC ACC TCG CAG

    Val Arg Gln Asn Tyr His Gln Asp Ser Glu Ala Ala Ile Asn Arg
51 GTG CGC CAG AAC TAC CAC CAG GAC TCA GAG GCC GCC ATC AAC CGC

    Gln Ile Asn Leu Glu Leu Tyr Ala Ser Tyr Val Tyr Leu Ser Met
96 CAG ATC AAC CTG GAG CTC TAC GCC TCC TAC GTT TAC CTG TCC ATG

    Ser Tyr Tyr Phe Asp Arg Asp Asp Val Ala Leu Lys Asn Phe Ala
141 TCT TAC TAC TTT GAC CGC GAT GAT GTG GCT TTG AAG AAC TTT GCC

    Lys Tyr Phe Leu His Gln Ser His Glu Glu Arg Glu His Ala Glu
186 AAA TAC TTT CTT CAC CAA TCT CAT GAG GAG AGG GAA CAT GCT GAG

    Lys Leu Met Lys Leu Gln Asn Gln Arg Gly Gly Arg Ile Phe Leu
231 AAA CTG ATG AAG CTG CAG AAC CAA CGA GGT GGC CGA ATC TTC CTT

    Gln Asp Ile Lys Lys Pro Asp Cys Asp Asp Trp Glu Ser Gly Leu
276 CAG GAT ATC AAG AAA CCA GAC TGT GAT GAC TGG GAG AGC GGG CTG

    Asn Ala Met Glu Cys Ala Leu His Leu Glu Lys Asn Val Asn Glu
321 AAT GCA ATG GAG TGT GCA TTA CAT TTG GAA AAA AAT GTG AAT CAG

    Ser Leu Leu Glu Leu His Lys Leu Ala Thr Asp Lys Asn Asp Pro
366 TCA CTA CTG GAA CTG CAC AAA CTG GCC ACT GAC AAA AAT GAC CCC

    His Leu Cys Asp Phe Ile Glu Thr His Tyr Leu Asn Glu Gln Val
411 CAT TTG TGT GAC TTC ATT GAG ACA CAT TAC CTG AAT GAG CAG GTG

    Lys Ala Ile Lys Glu Leu Gly Asp His Val Thr Asn Leu Arg Lys
456 AAA GCC ATC AAA GAA TTG GGT GAC CAC GTG ACC AAC TTG CGC AAG

    Met Gly Ala Pro Glu Ser Gly Leu Ala Glu Tyr Leu Phe Asp Lys
501 ATG GGA GCG CCC GAA TCT GGC TTG GCG GAA TAT CTC TTT GAC AAG

    His Thr Leu Gly Asp Ser Asp Asn Glu Ser
546 CAC ACC CTG GGA GAC AGT GAT AAT GAA AGC TAA GCCTCGGGCTAATTT
    ↑
594 CCCATAGCCGTGGGGTGACTTCCTGGTCACCAAGGCAGTGCATGCATGTTGGGGTTTCC
653 TTTACCTTTTCTATAAGTTGTACCAAAACATCCACTTAAGTTCTTTGATTTGTACCATT
712 CTTCAAATAAAGAAATTTGGTACCCA43

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FIG. 2. Nucleotide sequence of the HL-60 ferritin H subunit clone HL217. Arrow indicates the site of an additional cytosine not found in a previously reported clone (see text). Underlining indicates both the termination codon TAA which would provide termination in this sequence and the codon TAG which would terminate translation in the previously published sequence (13). Stretches of oligo(dG-dC) introduced during cloning (18 and 10 nucleotides) are omitted from the diagram.

all but four positions: 39, 40, 152, and 153. Wustefeld and Crichton (37) indicated uncertainty in their protein sequence at each of these positions.

In Fig. 3A, the nucleotide sequence of the human L subunit (HL227) is aligned with that for the homologous portion of a rat L subunit cDNA clone. These sequences were aligned by omitting the unique octapeptide sequence found, so far, only in rat L subunits (22). The position of this octapeptide is indicated by the asterisk at nucleotide 381. The coding sequences of the rat and human L subunits, thus aligned, are 84% homologous at the nucleic acid level, with the majority of differences between them being silent substitutions. By contrast, the HL-60 ferritin H and L subunit coding sequences are only 62% homologous at the nucleic acid level, with only 27% of the differences being silent substitutions.

The 3'-noncoding regions of the rat and human ferritin L subunits display no significant sequence homology when aligned as in Fig. 3A. However, there are several segments within this 3' region which are conserved between rats and humans (Fig. 3B). In addition to the conserved nucleotide sequences, both the human and rat L subunit 3'-noncoding regions contain regions of significant twofold symmetry. In the human L subunit mRNA such a region is centered around the cytosine residue occurring 32 nucleotides

after the termination signal and has the sequence ACTTCTGAAGGG(C)CCCTTGCAAAGT (underlining is used to indicate the symmetry). The rat L subunit mRNA has a region of symmetry centered around the thymidine residue occurring 23 nucleotides after the termination codon. Such regions of symmetry are frequently noted as sites of interactions between regulatory proteins and DNA in prokaryotic model systems (28).

Genomic organization of ferritin genes. Boyd et al. (15) have reported that a human H subunit cDNA clone hybridized an extensive series of bands on a Southern blot of genomic DNA. Since their experiments were carried out with a single probe they do not set a limit on the number of gene copies; the multiple bands seen in the Southern blot could each correspond to a separate member of a large gene family, or alternatively, if the ferritin genes contain many intervening sequences, multiple bands could result from each gene. To distinguish between these possibilities and obtain a minimum estimate of the size of the gene family, we hybridized blots of human genomic DNA digested with four different enzymes using two probes corresponding to nonoverlapping parts of the H subunit cDNA clone HL217. We also probed the same blot with the L subunit cDNA clone, HL227, to see whether it hybridized to the same gene family. Probing the same blot three times in succession,

FIG. 3. (A) Partial nucleotide sequence of the HL-60 ferritin L subunit derived from clones HL227 and HL1213. The top line indicates the amino acid sequence of the HL-60 L subunit of ferritin as predicted from the nucleotide sequence. The second line indicates the nucleotide sequence derived from the complete sequencing of clones HL227 and HL1213. Clone HL227 covers the first 571 nucleotides of this sequence, while clone HL1213 includes the last 548 nucleotides. They are identical in the 489 nucleotides of overlap. The third line indicates positions at which the nucleotide sequence of a rat L subunit cDNA clone differs from the HL-60 L subunit sequence in the coding region. Underlining in the 3'-noncoding sequence indicates three regions (a, b, and c) of homology between the rat and human sequences. These homologous regions are detailed in panel B. The stretches of oligo(dG-dC) introduced in the cloning procedures (16 and 23 nucleotides for HL227, 11 and 15 nucleotides for HL1213) are omitted from the figure. (B) Regions of local homology in the 3'-noncoding regions of the human and rat ferritin L subunit. The upper line of each sequence is the human nucleotide sequence, while the lower line is that of the rat. Colons indicate nucleotide identity. These sequences were aligned for maximal homology using the SEQH computer program (21). X indicates the end of the available rat sequence. The positions of homologies are indicated in panel A by the lower-case letters a, b, and c next to the first nucleotide in each homologous region. The sequence homologies shown agree at 25 of 30, 15 of 17, and 10 of 10 positions, respectively.

using a different probe each time, enabled us to precisely compare the positions of the bands labeled with the different probes. The results are shown in Fig. 4.

The data indicate that the H and L subunits show no cross-hybridization and are always found on different restriction fragments. Many of the bands on the Southern blot show homology with both of the subclones of the H subunit

gene. Since these subclones are not overlapping, each band which is labeled by both of these probes indicates the presence of a distinct gene copy, and our data indicate that at least nine H subunit gene copies are present (Fig. 4; see below). By contrast, the ferritin L subunit sequence hybridizes to only four to five bands, indicating a smaller gene family (Fig. 4).

FIG. 4. Three sequential hybridizations to the same genomic Southern blot with probes HL217-1, then HL227, and then HL217-2. Total human DNA was isolated from HL-60 cells, 10 μ g of which was cleaved with restriction enzymes and loaded for each lane. E, *Eco*RI; M, *Msp*I; B, *Bam*HI; T, *Taq*I. The arrows to the right of the lanes in the left and the middle autoradiographs indicate the locations of bands labeled with HL217-1 but not HL217-2, or vice versa. The bars to the left of each lane in the middle autoradiograph indicate the positions of all bands labeled by the HL227 probe. These marks (arrows and bars) were positioned by direct overlaying of the original autoradiographs and indicate that the H and L subfamilies do not cross-hybridize. Asterisks (to the right of the lanes) indicate those bands which we have found to be polymorphic in a comparison of DNAs from many subjects (Gatti et al., in preparation). kb, Kilobases.

Developmental regulation of expression of ferritin subunits. It has been reported that the ratio of H to L subunit protein concentrations reflects the stage of promyelocytic differentiation (14). This H/L ratio in undifferentiated HL-60 cells is 0.4 or 0.5. Measurements of the H/L ratio in granulocytes and macrophages (monocytes) have varied a great deal (from 1.1 to 65.7 in different laboratories and with different clinical materials [14]), but studies uniformly agree that there is a substantial increase in H/L ratio during these terminal differentiation processes. Translational regulation has been proposed to play a major role in ferritin regulation in response to iron induction (38), and we have shown that translational regulation is of major importance in the regulation of synthesis of tubulins (30) and other proteins (Davis et al., in preparation) during HL-60 cell differentiation. Therefore, we wished to determine whether this change in the ratio of ferritin subunits might be effected in part by changes in mRNA ratios. To investigate this possibility, a Northern blot containing mRNAs isolated from HL-60 cells at various stages of the differentiation process was probed with the cDNA clones of the human ferritin H and L subunits. The HL217 and HL1213 probes each hybridized to a single prominent mRNA species in undifferentiated HL-60 cells. We estimate the L subunit mRNA to be approximately 1,000 nucleotides in length and the H subunit mRNA to be about 1,100 nucleotides.

The H/L ratio increases as differentiation progresses to either neutrophil or macrophage differentiation (Fig. 5). The absolute level of the H subunit mRNA showed a net increase during both neutrophil (25-fold) and macrophage (40-fold)

differentiations (Fig. 5a and b). During neutrophil differentiation, the level of the H subunit mRNA initially decreased by 5-fold at day 1 but increased by more than 100-fold on day 3. During macrophage differentiation, the level of the H subunit mRNA increased from day 0 to day 1, reached its maximum after 2 days, and then decreased to a level 10-fold above the day 0 level on day 3.

The regulation of mRNA concentration for the L subunit is also presented in Fig. 5a and b. During neutrophil differentiation the L subunit mRNA level decreased by about eightfold from day 0 to day 1 and then increased to a level threefold over the initial (day 0) level on day 3. During macrophage differentiation, a 5-fold increase of the L subunit level was observed from day 0 to day 2, and yet on day 3 a dramatic decrease was observed (a 20-fold reduction from the level on day 2), accounting for the overall increase in ratio of H to L subunit mRNA (Fig. 5c).

DISCUSSION

The fact that we obtained 3 ferritin-specific cDNA clones out of an initial group of 16 regulated clones chosen for detailed study suggests that the ferritins are coded by mRNA species which are abundant in DMSO-induced HL-60 cells. This was confirmed by the intensity of hybridization to Northern blots (Fig. 5a).

The ferritin H subunit cDNA clone HL217 differs by one nucleotide from a published H subunit cDNA clone sequence from liver (13). Because of an additional cytosine residue at nucleotide 552, and the resulting frame shift, the last eight amino acids and the termination signal encoded by

the HL217 sequence differ completely from the corresponding 16 amino acids at the longer carboxyl-terminal sequence predicted by Costanzo et al. (13). Our sequence in this region was determined three times on different subclones and agrees with a 170-nucleotide partial sequence containing this region that was published by Boyd et al. (5). Therefore, it seems most likely that the difference between Costanzo's data and our own represents either an interesting allelic variation, which would result in significant alteration of the expressed gene product, or an artifact of either the cloning or sequencing of the H subunit clone from the liver.

Our Southern blot data suggest that there are at least nine members of the H subunit gene family in the human genome (Fig. 4). A single extended gene, with numerous large intervening sequences, can give many bands on a Southern blot; however, only one of these bands per gene would be expected to hybridize to both probes HL217-1 and HL217-2. Therefore, the use of such nonoverlapping probes provides a useful way of estimating the minimum number of genes in the ferritin H subunit gene family. The possibility that carry-over of material from one probing to the next could influence these results is excluded by the right panel of Fig. 4, which shows no evidence of such carry-over in a probing with HL227 between the HL217-1 and HL217-2 probeings.

An additional *EcoRI* band at 4.0 kilobases (Fig. 4), which hybridizes only the HL217-1 probe and not the HL217-2 probe, presumably signifies that one member of the gene family contains an *EcoRI* site within the region covered by the HL217-1 probe. However, neither HL217-1 nor HL217-2 contains cleavage sites for *EcoRI* or for the other three enzymes used here; thus, such cleavages indicate either the presence of intervening sequences or sequence variations between members of the gene family.

In contrast to the results with *EcoRI*, the *BamHI* digests show only five bands labeled by both the HL217-1 and HL217-2 probes. This suggests that multiple genes could be clustered in some of the *BamHI* fragments, especially those heavily labeled *BamHI* bands greater than 10 kilobases in size. An alternative possibility is that a greater number of large *BamHI* fragments are labeled but are unresolved by the electrophoretic separation.

We show that the ferritin L subunit probe hybridizes with four to five bands on each digest, showing strong homology with only two to three of these. This contrasts with the results of Brown et al. (6), who showed that the rat genome contains a large gene family of approximately 18 members. To distinguish whether this discrepancy might be due to either stringency conditions or species differences, we tested the hybridization of the human probes to mouse DNA. We found that the L subunit gene family is much smaller in the human than the mouse. In contrast, the ferritin H subunit gene family is larger in the human than in the mouse; a single

FIG. 5. (a) Northern blot analysis of ferritin mRNAs. Poly(A)⁺ RNA (10 µg) was collected at various stages of differentiation and analyzed. The relative amounts of H and L subunit mRNAs were measured after 0, 1, 2, and 3 days of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment and 1, 2, and 3 days of DMSO plus amiloride (A) treatment. kb, Kilobases. (b) Results shown in panel a are plotted. Each set of curves is normalized as a percentage of the highest signal observed (2-day 12-*O*-tetradecanoylphorbol-13-acetate treatment). (c) Relative changes of H-to-L subunit mRNA ratio during HL-60 cell differentiation. The data in panel b were used to compute the ratios, normalizing to give a ratio of 0.4 for the day 0 time point based upon the H/L protein subunit ratio of Dorner et al. (14).

hybridizing mouse DNA fragment was observed when *EcoRI*, *MspI*, *BamHI*, or *TaqI* digests were probed with the human H subunit ferritin probe under the same hybridization conditions (data not shown). The striking variability in gene number between mice and humans is not understood. The majority of the genes in the large families may be retrotranscribed pseudogenes, in which case it would be interesting to explain why the H subunit family is more amplified in humans while the L subunit family is amplified in the mouse. The extreme heterogeneity of isoferritins isolated from different tissues could result in part from the expression of different genes in different tissues. Active H and L ferritin genes have already been localized to chromosomes 19 and 11, respectively (9, 36), while the other gene copies appear to be dispersed throughout the genome (36; R. A. Gatti et al., manuscript in preparation).

Detailed comparison of the human and rat clones for ferritin L subunit genes reveals interesting sequence conservation in the 3'-noncoding regions. Preservation of the 30-, 17-, and 10-nucleotide sequences indicated in Fig. 3B is especially dramatic since these sequences have persisted over long evolutionary time despite the shuffling of their order from c-b-a in the human to b-c-a in the rat (Fig. 3A). These conserved regions contain four occurrences of the sequence AGCPyT in addition to one related sequence, AGCCA. As noted in Results, both the human and rat mRNAs contain regions of twofold symmetry in the 3'-noncoding regions. These features may be of regulatory importance.

The availability of cloned probes corresponding to the H and L subunits of human ferritin now makes it possible to investigate the transcriptional regulation of these genes. Previous studies have shown that the absolute amount of ferritin, or the ratio of subunits within ferritin, varies in a tissue-specific manner both during normal development and in pathological conditions (15). In some situations, especially that of ferritin synthesis in response to iron challenge, this regulation has been ascribed to control at the level of translation (38). Our cloning of both the H and L subunit mRNAs has permitted the first direct measurements of H/L mRNA ratios by Northern blot techniques. The results (Fig. 5c) show that changes in mRNA ratios are dramatic. The 16-fold change in mRNA ratio observed is adequate to explain the high H/L protein subunit ratio in macrophages without invoking translational regulation during macrophage or neutrophil terminal differentiation. According to this view, the differences in H/L protein subunit ratio between different tissues are due to differences in mRNA ratios, while translational regulation turns on synthesis of the proteins according to the need to sequester iron.

These results with the HL-60 cell line are also of interest in light of the fact that ferritins seem to be able to act as cytokines, having a regulatory effect on myelopoiesis. The relationship between serum ferritin levels and cellular ferritin synthesis is not clear; however, normal granulocytes synthesize ferritins of high H-to-L ratios (14) and HL-60 cells increase their H-to-L ferritin mRNA ratio as they differentiate toward neutrophilic granulocytes. As ferritins of high H/L ratio (acidic isoferritins) have been implicated as an inhibitor of myelopoiesis in leukemia patients (7, 28), the increase of H/L ratio of ferritin in DMSO-induced HL-60 cells may be related to a normal feedback regulation system for myelopoiesis mediated by acidic isoferritins.

Regulation of H and L subunit ferritin mRNAs during terminal differentiation of HL-60 cells appears to be complex. First, there are dramatic changes in the H/L ratio (Fig.

5c). These seem to occur in three distinct phases during macrophage differentiation: a seven- to eightfold increase on day 1 of differentiation, maintenance of a relatively constant ratio during day 2, and a further five- to sixfold increase on day 3 of differentiation, coinciding with a dramatic reduction of L subunit mRNA levels. The absolute levels of these mRNAs also show complex changes (Fig. 5b). These results suggest that there may be different regulatory events interacting in a complex way during these terminal differentiation processes. The techniques of site-directed mutagenesis would be helpful in determining the molecular details of this regulation. Our initial investigations in this area have indicated that chromosomal clones containing ferritin genes can be reintroduced into HL-60 cells and that the expression of the exogenous genes is regulated normally during HL-60 cellular differentiation (12).

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ADDENDUM

Recently, Dorner et al. have reported the sequence of another human L subunit ferritin cDNA from U937 cells (Dorner et al., Proc. Natl. Acad. Sci. USA 82:3139-3143, 1985). It differs from the sequence reported here in amino acid sequence. The guanine (G)-208 in the HL-60 cDNA sequence is replaced by an adenine (A) in the sequence reported by Dorner et al., resulting in an amino acid change from Ala to Phe. In addition, there are four nucleic acid changes which are either silent mutations or changes in the 3'-noncoding region of the mRNA. They are: a substitution (T to C) at position 67, deletion of a C residue at position 502, and nucleotide insertions of a C between positions 489 and 490 and a G between 514 and 515 (all the numberings refer to the sequence in the present paper). The report by Dorner et al. may indicate a second functional L subunit gene or an allelic variation. Drysdale and his collaborators have also cloned H and L subunit cDNAs (personal communication), but we have not had the opportunity for comparison of their sequences with our own. A preliminary report of our results has been published elsewhere (33).

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