Human ferritin gene is assigned to chromosome 19

(iron/hemochromatosis/radioimmunoassay/hamster–human hybrids)

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ABSTRACT  Ferritin is the intracellular iron storage protein. Tissue ferritin stores are markedly increased in hemochromatosis, a disease of iron overload that has been linked to chromosome 6. In order to provide further information concerning the genetics of ferritin synthesis and to determine if the structural gene for ferritin was on chromosome 6, studies were performed to identify the human chromosome that contains the ferritin gene. Ferritin immunoassays were performed on extracts of Chinese hamster ovary somatic cells that were hybridized with human lymphocytes and fibroblasts and contained various human chromosomes in different combinations. None of the 13 cell lines that lacked immunoreactive human ferritin contained chromosome 19, and all 9 of the cell lines that produced human ferritin contained chromosome 19. No other human chromosome shared this association with human ferritin. In studies of subclones of ferritin-positive cell lines, immunoreactive ferritin consistently segregated only with chromosome 19. Immunoprecipitation studies performed on cells that had been incubated with 59Fe-containing transferrin indicated that chromosome 19-containing cells incorporated iron into intact and functional molecules of human ferritin. The necessary and exclusive association of chromosome 19 with human ferritin indicates that a defect in the structural gene for human ferritin cannot account for the abnormalities of hemochromatosis. Moreover, this hamster–human hybrid cell system should prove useful in further studies of regulation of ferritin concentration and composition.

Ferritin is the major intracellular iron storage protein of animals and unicellular organisms (1). It has the shape of a hollow sphere that permits the entry of variable amounts of iron for storage in the form of ferric hydroxide phosphate complexes (1). The apoprotein shell is composed of 24 subunits that are generally believed to be of two different types on the basis of differences in molecular weight and electrophoretic mobility. The H (heavy) subunit has a molecular weight of 21,000 and has a relatively acidic electrophoretic mobility when compared to the 19,000 molecular weight L (light) subunit (2). Although there is a great deal of homology in the amino acid compositions of H and L subunits, differences in immunologic specificity suggest that the subunits are not encoded by the same genetic material (3). It has been hypothesized that variation in the proportion of H and L subunits is primarily responsible for the electrophoretic and immunologic spectrum of different isoferritins obtained from both malignant and nonmalignant tissues, with modification in glycosylation of the protein playing a role as well (2, 4–7). With few exceptions, isoferritins contain a mixture of both H and L subunits, and all tissues appear to contain some form of ferritin, a reflection of the biological need for protection from accumulation of toxic amounts of free intracellular iron (2).

Tissue ferritin stores are markedly increased in the iron overload disease hemochromatosis. Population studies in affected patients and their families have indicated that the gene for hemochromatosis is closely linked to the HLA locus on chromosome 6 (8–10). Studies of mouse cells suggest that the gene for transferrin, the iron-transport protein, is encoded by genetic material on chromosome 3 (11). We and others have recently shown that the gene for the human transferrin receptor is also on chromosome 3 (unpublished data and ref. 12). Thus, on the basis of this information, it is unlikely that defects in the structural genes for either of the two proteins responsible for iron transport, transferrin and the transferrin receptor, accounts for the abnormalities found in hemochromatosis. In order to determine if the ferritin gene is linked to chromosome 6 and might therefore play a role in the abnormalities of hemochromatosis, and to provide further information concerning the genetics of ferritin synthesis, this study was undertaken to identify the human chromosome or chromosomes that contain(s) the ferritin gene(s). Studies were performed on Chinese hamster–human hybrid cell lines containing various combinations of human chromosomes.

MATERIALS AND METHODS

Cells and Cell Culture. Chinese hamster ovary somatic cells (CHO) were hybridized by using either polyethylene glycol 1,000 or Sendai virus with human lymphocytes and human fibroblasts as described (13). CHO–human hybrid cells were maintained in 75-cm2 flasks in F-12 or F-12D medium (14), supplemented with 5% fetal calf serum, penicillin at 125 units/ml, and streptomycin at 125 μg/ml. Cells were harvested with 0.1% trypsin/EDTA and washed three times at 4°C with 3 ml of Earle’s balanced salt solution. The final pellet was resuspended in 0.5 ml of Earle’s balanced salt solution and cell counts were performed in duplicate.

Preparation of Cell Extracts. Triton X-100 was added to each 0.5 ml of cell suspension to make a final Triton concentration of 0.1%. The cells were sonicated with a microprobe cell sonicator at 4°C and kept at 4°C overnight. The following day the mixture was centrifuged at 22,000 × g for 30 min to remove the small particulate fraction. The supernatants were stored at 4°C and assayed for ferritin within 24 hr of preparation.

Radioimmunoassay (RIA) for Human Ferritin. Cell extracts were assayed for human ferritin by using reagents from a commercially available kit supplied by Amersham. This RIA employed radiolabeled human spleen ferritin (HSF) and a double antibody immunoprecipitation technique. Each sample (100 μl) was incubated with 200 μl of 125I-labeled ferritin solution and 50 μl of sheep anti-ferritin antiserum at 37°C for 1 hr. Donkey anti-sheep IgG (500 μl) was then incubated with each

Abbreviations: H and L subunits, heavy and light subunits of ferritin; RIA, radioimmunoassay; HSF, human spleen ferritin; CHF, Chinese hamster ferritin.
sample at room temperature for 10 min followed by centrifugation at 22°C for 30 min at 1,500 X g. The supernatants were decanted, liquid was removed from the pellet by blotting, and then radioactivity was measured in a Beckman 6000 gamma counter. A standard curve was prepared from samples with known spleen ferritin concentrations.

**Ferritin Purification and Iodination.** Ferritin was purified from human placenta and from Chinese hamster livers and spleens by sequential precipitation with heat, acetic acid (pH 4.8), and ammonium sulfate followed by chromatography on Sephacry G-200 as described by Munro and Linder (15). Homogeneity was demonstrated by electrophoresis on 7% polyacrylamide gels that were stained for protein with Coomassie blue and for iron with Prussian blue. Protein concentration was determined by the method of Lowry et al. with bovine serum albumin as a standard (16). Chinese hamster ferritin (CHF) was iodinated by the chloramine-T method (17), using 30 μg of protein and 0.5 μCi (1 Ci = 3.7 × 10¹⁰ becquerels) of Na¹²⁵I (New England Nuclear). The specific activity of iodinated Chinese hamster ferritin was 2,000 cpm/μg. Additionally, rabbits were immunized with either purified HSF (Calbiochem) or purified CHF and specific antisera was collected as described (18).

**RIA for CHF.** The RIA developed for CHF utilized a simple single-antibody technique. A description of this assay and a similar assay that we have recently developed for human ferritin will be described in more detail elsewhere. Tubes in the RIA contained 700 μl, consisting of 580 μl of 10 mM KPO₄ (pH 7.5), 105 mM NaCl containing various amounts of purified CHF (2-100 ng) to determine a standard curve or solubilized cells, 0.1 μl of antisera, 99.9 μl of control rabbit serum, and 20 μl of ¹²⁵I-labeled CHF. The reaction mixture was incubated overnight on a shaker platform at 4°C, 300 μl of 14.2% (wt/vol) polyethylene glycol 6,000 in 0.5 M KPO₄ (pH 7.5) was added, and each sample was incubated for 10 min at 4°C followed by centrifugation at 22,000 X g at 4°C for 30 min. The entire supernatant was aspirated and the ¹²⁵I radioactivity in the pellet was measured in a Beckman 6000 gamma counter. Under the conditions of this assay 80% of the ¹²⁵I-labeled CHF precipitated in the absence of unlabeled ferritin. This value fell significantly and progressively to 30% as the amount of unlabeled ferritin was increased from 2 to 100 ng of protein. A standard curve was constructed for each assay by using known amounts of purified CHF in this range.

**Chromosome Analysis.** Cells grown on 60-mm² plates were harvested, centrifuged, resuspended in water, and subjected to freeze-thawing three times. After the final thaw, the cells were again centrifuged and 10 μl of the supernatant was applied to a cellulose acetate strip for electrophoresis followed by staining for particular marker enzymes associated with each chromosome as described (13, 19). The marker enzyme used for chromosome 19 was glucose-phosphate isomerase (13). The presence of the human isoenzyme in addition to the Chinese hamster enzyme indicated the presence of the associated human chromosome. Human chromosomes in cell lines were also analyzed by standard karyotype analysis (20, 21).

**Studies of ⁵⁹Fe Incorporation into CHO Hybrids.** Human transferrin (Sigma) was labeled by using ⁵⁹FeCl₃ (Amersham) according to a minor modification of the method of Bates and Schlachch (22). The ⁵⁹Fe-labeled transferrin had a specific activity of 1.3 cpm/μg. In these studies, CHO hybrid cells were grown in 175-cm² flasks containing 20 ml of F-12D medium. When these cells were still subconfluent the medium was decanted, and 20 ml of fresh medium containing 200 μl of ⁵⁹Fe-containing transferrin (15,000 cpm) was added to each flask. After 48 hr the cells were harvested and washed three times in Earle's balanced salt solution, radioactivity was measured, the

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Immunoactive protein, ng/10⁶ cells</th>
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<tr>
<td>CHO cells (Chinese hamster ovary somatic cells)</td>
<td>&lt;1</td>
</tr>
<tr>
<td>WI-38 cells (human embryonic lung fibroblasts)</td>
<td>66</td>
</tr>
<tr>
<td>K562 cells (human leukemia)</td>
<td>31</td>
</tr>
<tr>
<td>HeLa cells (H subunits of ferritin)</td>
<td>9</td>
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* Measured by RIA for human ferritin.

Table 1. Measurement of human ferritin (immunoreactive protein) in different cells

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Lowry assay</th>
<th>RIA*</th>
</tr>
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<tbody>
<tr>
<td>Human spleen ferritin (L subunits)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Human placental ferritin (H subunits)</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>Chinese hamster ferritin</td>
<td>1,000</td>
<td>&lt;1</td>
</tr>
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* RIA for human ferritin.

cells were disrupted by ultrasonication in 0.1% Triton X-100, and the solution was applied to a 1.5 × 6 cm Sephadex G-200 column that was equilibrated in 10 mM Tris, pH 8.0/150 mM NaCl/20 μg of bovine serum albumin per ml. The equilibration buffer was used for elution, and 1-ml fractions were collected and their radioactivities were measured. RIAs for human ferritin were performed on individual fractions.

**Immunoprecipitation Studies.** Because our studies had indicated that about 90% of the intracellular radioactive iron was incorporated into ferritin, immunoprecipitation studies utilized solubilized whole cell preparations. Aliquots of solubilized cells containing ⁵⁹Fe-sequestering ferritin in 400 μl of 10 mM KPO₄, pH 7.4/150 mM NaCl were incubated overnight at 4°C with 0, 0.1, 1.0, and 10 μl of specific antisera to either CHF or HSF in different amounts of control rabbit serum to make the total volume of serum 100 μl in all assays. The mixture containing 14.2% polyethylene glycol 6,000 was then added to make a final concentration of 4.26% and, after a 10-min incubation at 4°C, each sample was centrifuged at 22,000 × g for 30 min at 4°C. The supernatant was aspirated and the ⁵⁹Fe radioactivity in both the pellet and the supernatant was measured.

**RESULTS**

Specificity of Human Ferritin RIA. Table 1 shows that, by using the RIA for human ferritin, ferritin could be measured in three different human tissue culture cell lines, including HeLa cells, which contain an isoferritin that is composed almost exclusively of heavy subunits (2). No human ferritin could be detected in CHO cells that lacked human chromosomes. Table 2 shows results of assays of ferritin purified from human spleen, human placenta, and Chinese hamster liver and spleen. Because the RIA utilized antibody directed against HSF, as expected, HSF, which has a predominance of light subunits, was measured with somewhat greater sensitivity than placental ferritin, which is predominantly composed of heavy subunits. However, Table 2 also shows that as much as 1,000 ng of purified CHF could not be detected in this assay. Therefore it was concluded that this assay could specifically measure a broad spectrum of human isoferritins without detecting any CHF present in the CHO hybrid cells.

**Segregation of Human Ferritin with Chromosome 19.** Twenty-three cell lines, which contained a spectrum of all 22 autosomal human chromosomes, were tested. As shown in Fig. 1, there was no detectable human ferritin (<1 ng per 10⁶ cells).
in any of the 13 cell lines that lacked chromosome 19 as measured by both isozyme and karyotype analysis. According to isozyme analysis, all other chromosomes except chromosome 1 and chromosome 19 were present in at least one of the ferritin-negative cell lines. In contrast, all nine cell lines that did contain chromosome 19 had measurable human ferritin in amounts between 6 and 34 ng per 10^6 cells. Only two of these cell lines contained chromosome 1, and chromosome 19 was the only chromosome that was common to all of the ferritin-positive cell lines.

The RIA for CHF was performed on 10 of the cell lines, and we found that all hybrid cell lines tested contained similar amounts of measurable immunoreactive CHF (27–62 ng per 10^6 cells). Additionally, the four cell lines assayed that contained human ferritin generally had lower values for CHF than cell lines with no measurable human ferritin (see below).

To further demonstrate that chromosome 19 has a necessary and exclusive association with human ferritin, subclones were selected from two parental cell lines that contained chromosome 19 and immunoreactive human ferritin. Fig. 2 shows that the four subclones of cell line 822-5a that lost chromosome 19 lost all detectable human ferritin, whereas the subclone (916-10) that retained chromosome 19 also retained human ferritin in an amount comparable to that found in the parent cell line. Furthermore, a subclone that had lost only chromosome 19 was made from cell line 822-56, which originally contained immunoreactive ferritin. The exclusive loss of chromosome 19 from the subclone was associated with the complete loss of detectable immunoreactive human ferritin.

**Fig. 1.** Chromosome analysis and immunoreactive human ferritin in CHO hybrids. Individual chromosomes present in each cell line as determined by both isozyme and karyotype analysis are indicated by shaded areas under appropriate numbers. ND, not determined. Concentration of human ferritin was determined by RIA.

**Fig. 2.** Segregation of human ferritin with human chromosome 19. Results of chromosome analysis and human ferritin RIA in subclones made from single cells of ferritin-positive cell lines. Parent cell lines contained chromosomes as indicated by shaded areas.

**Fig. 3.** Sephadex G-200 chromatography of CHO hybrids incubated with 59Fe-containing transferrin. After incubation with 59Fe-containing transferrin, extracts of cells lacking (Upper) and containing (Lower) chromosome 19 were applied to the column. 59Fe radioactivity (•) and immunoreactive human ferritin (○) were measured in individual fractions. V_e, elution volume; V_o, void volume.
radioactivity. The immunoreactive protein assayed in this peak represented more than 80% of the total immunoreactive protein that was applied to the column, indicating that the human ferritin synthesized by these cells was not single subunits but intact ferritin. As expected, no immunoreactive human ferritin could be detected in any of the fractions of cell line 822-48a (lacking chromosome 19).

**Immunoprecipitation Studies.** The curves in Fig. 4 are based on 80% of the radioactivity representing the maximum of immunoprecipitable radioactivity (100% of radioactivity specifically precipitated) and 30% of the radioactivity representing nonspecifically precipitated radioactivity without antiserum (0% of radioactivity specifically precipitated). As shown in Fig. 4 Left, 0.1 μl of antiserum specifically precipitated 93% of the radioactivity in WI-38 fibroblasts, which contain only human ferritin, but less than 5% of the radioactivity in 822-48a cells, which lack immunoreactive human ferritin and contain only CHF. Therefore, it was concluded that 0.1 μl of anti-human ferritin antiserum specifically precipitated 59Fe-labeled human ferritin but not 59Fe-labeled CHF. In other experiments (Fig. 4 Right), 1.0 μl of anti-CHF antiserum specifically precipitated 59Fe-labeled CHF (74% of 59Fe radioactivity in 822-48a cells) but not human ferritin as indicated by specific precipitation of less than 1% of the radioactivity in WI-38 cells.

Fig. 4 also shows that much of the precipitable 59Fe radioactivity in the 822-19b3 cells, which contain chromosome 19, was precipitated with dilute anti-HSF, indicating that the intact human ferritin synthesized by these cells also has the functional ability to incorporate iron. Fig. 4 Left shows that significantly less radioactivity was precipitated with dilute anti-HSF in 822-19b3 cells than in WI-38 fibroblasts, which contain only human ferritin, suggesting that some of the 59Fe in the 822-19b3 cells was incorporated into CHF. The finding that 15% of the radioactivity in these cells was precipitated with dilute anti-CHF antiserum (Fig. 4 Right) provided further evidence that, in this cell line, radioactive iron was incorporated into both CHF and human ferritin. Thus, although results cannot be quantitated exactly with this method, they indicate that 822-19b3 cells contained intact molecules of both Chinese hamster and human ferritin, both of which have the functional ability to incorporate iron. Also, the results are consistent with quantitative RIAs in that the 822-19b3 clone, which contained 34 ng of human ferritin per 106 cells, had less immunoreactive CHF (27 ng per 106 cells) than did the 822-48a clone (50 ng per 106 cells) that lacked detectable human ferritin.

Studies identical to those described above were also performed with cell line 822-5a, a clone containing not only chromosome 19 but also other chromosomes different from those found in 822-19b3 (Fig. 1). The results of these studies also demonstrated that the immunoreactive human ferritin in this cell line was a single high molecular weight species and immunoprecipitation studies also indicated that the intact human ferritin molecule synthesized by these cells incorporated 59Fe.

**DISCUSSION**

In these studies of CHO-human hybrid cells, human chromosome 19 was the only chromosome that was present in all cell lines that contained immunoreactive human ferritin and absent from all cell lines that lacked immunoreactive human ferritin. The necessary and exclusive association of chromosome 19 with human ferritin production in CHO hybrid cells was further substantiated by the studies of subclones of ferritin-positive cell lines, which indicated that the exclusive loss of chromosome 19 from a subclone predicted the loss of ferritin, whereas the retention of chromosome 19 in a subclone resulted in persistence of detectable human ferritin. Chromosome 6, which is associated with hereditary hemochromatosis, was present in some ferritin-positive cell lines as well as some that were negative. Also, the presence or absence of chromosome 6 in ferritin-positive cell lines did not appear to correlate with the intracellular ferritin concentration in the cell lines that contained human ferritin.

The possibility was considered that subunits of ferritin were being measured in the CHO hybrid cells. Because ferritin subunits cannot incorporate iron, the studies of 59Fe incorporation into ferritin from 59Fe-carrying transferrin were undertaken to demonstrate that the CHO hybrid cells contained intact ferritin molecules. The finding that most of the incorporated 59Fe radioactivity eluted from Sephadex G-200 with 125I-labeled ferritin and the peak of immunoreactive ferritin strongly suggested that the 59Fe was associated with intact ferritin. The immunoprecipitation studies of the high molecular weight 59Fe-containing material confirmed that chromosome 19-containing cells incorporated iron into intact human ferritin molecules. The failure of all of the 59Fe radioactivity to precipitate with dilutions of antiserum specific for human ferritin suggested that some of the radioactive iron was incorporated into CHF present in the 822-19b3 cells. This finding was consistent with both detection of immunoreactive CHF in these cells and precipitation of an intermediate amount of 59Fe with diluted anti-CHF antiserum.

Studies of ferritin synthesis in cell-free systems have suggested that the H and L subunits of ferritin have different mRNA molecules, indicating that the subunits might have different genes, which could conceivably be found on separate chromosomes (23). However, our findings indicate that chromosome 19 contains the genes for both subunits of ferritin, because this chromosome is exclusively associated with the production of intact ferritin molecules, which rarely consist of just one type of subunit (2). Also, the RIA for human ferritin is sensitive enough to measure human isoferritins with either a predominant L subunits (HeLa cells) or a predominance of H subunits (spleen). Thus, if the H and L genes were on separate chromosomes and each type of subunit assembled into homogeneous intact ferritin molecules, it should still have been pos-
Genetics: Caskey et al.


sible to measure ferritin associated with two chromosomes rather than one. Thus, although it is possible that the hybrid cell lines produce a single subunit of human ferritin that may form protein hybrids with subunits of CHF, the results above strongly suggest that the genetic material for both ferritin subunits is contained on chromosome 19. Further studies, measuring human ferritin synthesis by hybrid lines containing various human chromosomes may better elucidate factors responsible for differences in ferritin composition.

The finding that the genetic material for ferritin is associated with chromosome 19 rather than chromosome 6 makes it extremely unlikely that a defect in the structural gene(s) for ferritin accounts for the abnormalities found in hemochromatosis. However, it is still conceivable that a gene on chromosome 6 or another chromosome regulates ferritin synthesis or differences in ferritin structure (isoferritins). Initial studies performed on two cell lines containing chromosome 19 indicate that, when the cells are grown in iron-deficient medium, the CHF concentration appears to "down regulate" (2, 24), whereas the human ferritin concentration does not change. Therefore, further studies of hybrid cell lines containing various human chromosomes may prove useful in determining factors that play a role in regulation of ferritin synthesis or differences in composition of isoferritins.

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