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Isolation and characterization of a cDNA clone for human ferritin heavy chain

(synthetic oligonucleotide probes/cDNA sequence/gene families)

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ABSTRACT **Ferritin, the main iron-storage protein, is composed of two partially homologous subunits, heavy (H) and light (L), with M_rs of 21,000 and 19,000, respectively. We have isolated a cDNA clone for human ferritin H chains by screening a human lymphocyte cDNA library with synthetic oligodeoxyribonucleotides. The oligonucleotide sequences were derived from two pentapeptides found in human spleen ferritin. The selected clone hybridized to both probes and selected H-chain mRNA, but not L-chain mRNA, when hybridized to HeLa cell mRNA. These results indicate that the cloned DNA codes for a H chain of human ferritin. Since the amino acid sequence derived from the cloned DNA was almost identical to the partial amino acid sequence of a minor component found in human spleen ferritin, we conclude that the minor sequence found in human spleen ferritin must be a H subunit. Genomic analysis gives a complex pattern that suggests that ferritin H chains are encoded by a multigene family or have an unusually large number of exons.**

Ferritin is the major iron storage protein in eukaryotes (reviewed in refs. 1 and 2). In addition to its role in iron metabolism, ferritin is of interest as a tumor marker (3) and as a possible regulator of myelopoiesis (4). Most of the body's ferritin is found in the liver, spleen, and bone marrow, but ferritin is probably present in all cells, where its synthesis may represent an obligatory mechanism for detoxifying iron. The molecule consists of a hollow protein shell within which up to 3,000 atoms of ferric iron can be stored (5). The shell is composed of 24 subunits, which can be of two immunologically distinct size classes, heavy (H) and light (L), of approximate M_rs 21,000 and 19,000, respectively (6). Multiple isoelectric forms of both the H and L subunits have been described, but their structural relationships are not yet known (7, 8). In most cells, H and L subunits combine in different proportions to generate families of isoferritins that differ in their relative rates of synthesis and turnover (9) and in their ability to accept and release iron (10, 11). Different tissues exhibit characteristic ferritin phenotypes, but these can change in normal and pathological states such as iron overload and malignancy (12). L-subunit-rich ferritins predominate in tissues with high levels of nonheme iron, such as liver and spleen, and increase in these and other organs on iron loading (9). H subunit-rich ferritins are found in organs with low nonheme iron contents, such as heart and pancreas, and also in fetal and malignant tissues (13).

Peptide mapping suggests that the H and L subunits have partial sequence homology (6), while cell-free translation studies indicate that the two chains are encoded by distinct mRNAs (14). In order to explore the regulation of ferritin gene expression and the structural and metabolic relationships of the H and L subunits, we have cloned ferritin mRNAs. This paper describes the identification of a cDNA clone for a H chain of human ferritin by use of synthetic oligodeoxyribonucleotides and presents an initial characterization of this clone and of the genes encoding ferritin H chains.

MATERIALS AND METHODS

Isolation of RNA. HeLa cells, grown with iron supplementation (14), were obtained from the Massachusetts Institute of Technology Cell Culture Center. Poly(A)-enriched RNA was isolated from these cells by phenol extraction and chromatography on oligo(dT)-cellulose (type II) from Collaborative Research (Waltham, MA) (15). The RNA was stored at −80°C in aqueous solutions of 1 mg/ml.

Screening with Synthetic Oligodeoxyribonucleotides. Ferritin sequences were sought in a previously described cDNA library prepared from poly(A)-enriched mRNA from mixed human lymphocytes (16). This library was constructed by standard procedures using G·C-tailing and insertion into the *Pst* I site of pAT153, a derivative of pBR322 (17). It contained about 5×10^4 recombinants with an average insert size of about 350 base pairs. Approximately 7000 colonies were replicated on GeneScreen (New England Nuclear) for colony hybridization according to the manufacturer's protocol. After baking at 80°C for 2 hr, the filters were washed for 3 days at 70°C in multiple changes of 6× NaCl/Cit (1× NaCl/Cit is 0.15 M sodium chloride/0.015 M sodium citrate) containing 1× Denhardt's solution (18), 0.1% $NaDodSO_4$, and 100 μg of denatured salmon sperm DNA per ml. The filters were then probed separately with two mixtures of ^{32}P-labeled synthetic oligodeoxyribonucleotides (see *Results* for oligodeoxynucleotide sequences). These oligonucleotides were synthesized by the phosphotriester method (19) by ChemGenes (Waltham, MA) and were labeled by us to a specific activity of 10^8 cpm/μg with [γ-^{32}P]ATP (2900 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) and polynucleotide kinase (New England Biolabs). The filters were incubated with probe at 10^6 cpm/ml in the same solution at 70°C for 1 hr. The temperature was then reduced by 3°C/hr to 25°C and held at that temperature for a further 3 hr. The filters were washed twice in the same solution, but without DNA, at 30°C and subsequently washed in this solution without DNA at 35°C and then at 40°C. The filters were autoradiographed after washing at each temperature.

DNA Preparation. Plasmid DNA was prepared by the alkaline $NaDodSO_4$ method (20) and further purified by density gradient centrifugation in cesium chloride containing ethidium bromide (21).

Hybrid Selection. The cloned DNA was used to select mRNA by hybridization according to standard procedures (22). Plasmid DNA was linearized with restriction endonuclease *Eco*RI, repurified by phenol extraction, and precipitated with ethanol. The DNA was dissolved in water at 1

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Abbreviations: H, heavy; L, light.

mg/ml, denatured by boiling in water, and rapidly cooled on ice. Five micrograms of plasmid DNA was immobilized on nitrocellulose and incubated for 3 hr at 50°C in 10 mM Pipes (pH 6.4) containing 65% formamide and 0.4 M NaCl with 50 μg of poly(A)-enriched RNA from iron-loaded HeLa cells. Bound RNA was eluted after washing and then was translated in a reticulocyte lysate system (23). A portion of the translation products was used for immune precipitation with a mixture of rabbit antibodies that recognizes both H and L subunits of HeLa ferritin as described (14).

Southern Blots. Cloned DNA was digested with *Pst* I, fractionated on 1.4% agarose gels in TBE buffer (50 mM Tris base/50 mM boric acid/1 mM EDTA), denatured, and transferred to GeneScreen according to the manufacturer's protocol. After prehybridization, replicate lanes were hybridized with the ^{32}P-labeled synthetic oligodeoxynucleotides using the same conditions described for colony hybridization.

For genomic analyses, ten micrograms of DNA (24) from lymphocytes of a normal adult woman was digested separately with *Eco*RI, *Bgl* II, and *Hin*dIII. The restriction fragments were separated by electrophoresis in 0.8% agarose gels in TEA buffer (40 mM Tris acetate/40 mM sodium acetate/1 mM EDTA, pH 8.0), denatured, and transferred to GeneScreen*Plus* (25). Genomic blots were prehybridized in 5× SET buffer (1× SET is 150 mM NaCl/20 mM Tris·HCl/10 mM Tris base/1 mM EDTA) containing 1% NaDodSO$_4$, 5× Denhardt's solution (18), 50 mM sodium phosphate (pH 6.6), and 40 μg of denatured salmon sperm DNA per ml for 1.5–3 hr. The blot was hybridized with nick-translated DNA labeled to 0.5–1.0 × 10^9 cpm/μg of DNA (26). Hybridization was 5× SET buffer containing 1% NaDodSO$_4$, 1× Denhardt's solution (18), 20 mM sodium phosphate (pH 6.6), 10% sodium dextran sulphate, and 40 μg of denatured salmon sperm DNA per ml for 16–20 hr at 68°C. The blots were washed once in 2× SET buffer containing 1% NaDodSO$_4$ and then three times in 1× SET buffer containing 0.5% NaDodSO$_4$ for 45 min each at 68°C. The stringency was increased sequentially by washing in 0.3× SET buffer containing 0.1% NaDodSO$_4$, followed by a 0.1× SET buffer containing 0.1% NaDodSO$_4$ for 45 min each. The blot was autoradiographed with a Lightning-Plus (Dupont) screen after washing at each stringency.

DNA Sequence Determination. DNA sequencing was carried out by the dideoxy method of Sanger *et al.* (27) using pBR322 primers (28) with double-stranded DNA and by M13 subcloning (29).

RESULTS

Isolation of Clones for Ferritin H Chains. We elected to identify ferritin clones by screening cDNA libraries with synthetic oligodeoxyribonucleotides. These oligonucleotide sequences were derived from amino acid sequences of human spleen ferritin. The complete sequence of a major component and the partial sequence of an unidentified minor but partially homologous component of this ferritin have recently been reported (30). Because these sequences were not obtained from separated subunits, it was not clear whether they represented different subunit types or variants of the same subunit. However, since the major sequence corresponded in size to an L subunit and since human spleen ferritin usually contains small amounts of the H subunit (6), we assumed that the major and minor sequences correspond to the L and H subunits, respectively. Since the results presented here provide substantial support for this assumption, we hereafter use this designation. From the amino acid sequences, we chose three peptides for preparation of synthetic nucleotide probes. One peptide was unique for the major sequence (designated L), one for the minor sequence (designated H), while the third was common to both sequences (designated H + L). Therefore, it was possible to use either of the unique sequence oligonucleotide probes in conjunction with the common sequence probe to screen separately for H- and L-chain clones with two probes from different regions of the same polypeptide. This paper deals only with H-chain clones.

The H-specific sequence (Lys-Met-Gly-Ala-Pro) corresponds to a partially homologous sequence (Arg-Leu-Gly-Gly-Pro) at residues 153–157 of the L sequence. The common sequence (His-Leu-Cys-Asp-Phe) is located at positions 124–128 of the L sequence. In order to minimize the complexity of these mixtures, which results from degeneracy of the genetic code, tetradecamers rather than pentadecamers were synthesized. Even then, it was necessary to synthesize mixtures containing 32 oligonucleotides for the H-specific probe and 64 oligonucleotides for the common sequence probe so that all possible coding sequences would be represented. The H-specific probe (noncoding strand) was 5′ G-G(A,G,T,C,)G-C(A,G,T,C,)C-C-C-A-T(T,C)T-T 3′. The common sequence probe (H + L) was 5′ A-A(A,G)T-C(A,G)C-A(A,G,T,C)A(A,G)(A,G)T-G 3′. These probes were end-labeled with ^{32}P and used to screen approximately 7000 colonies from the lymphocyte cDNA library, which we expected from protein synthesis patterns (31) to be relatively rich in H-chain cDNAs. Several colonies gave signals stronger than background with both probes when the filters were washed at 35°C in 6× NaCl/Cit. One colony, pDBR2, which gave the strongest signal under the most stringent conditions with the H-specific probe, was chosen for further study as a putative H-chain cDNA clone.

Southern Blot. Gel analyses of a *Pst* I digest showed that the cDNA insert of this clone contained approximately 550 base pairs. The H-specific probe hybridized strongly to this insert but not to the vector plasmid DNA (Fig. 1). The common probe also hybridized to the insert but not to the vector (not shown). The hybridization of the selected clone to two different sequences from the same protein suggested that the clone contains cDNA corresponding to the minor sequence of human spleen ferritin and, therefore, probably corresponds to ferritin H-chain sequences.

Hybrid Selection. Further support for this conclusion was obtained from hybrid selection studies with HeLa mRNA. Fig. 2 shows that the cloned DNA selected an mRNA that coded for a protein that comigrated with the H subunit of human ferritin (lane 3) and also was immunoprecipitated with antibodies to human ferritin (lane 6). Although the unfractionated HeLa mRNA also produced ferritin L chains (lane 5), no evidence of selection of ferritin L chains was obtained either by analysis of the total translation products

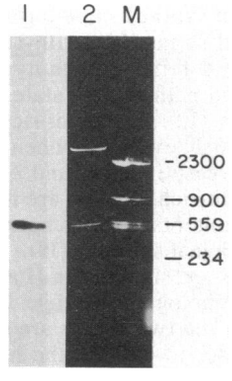

FIG. 1. Hybridization of synthetic oligodeoxyribonucleotide probes to cloned cDNA. DNA from clone pDBR2 was digested with *Pst* I, fractionated by gel electrophoresis together with marker DNA, and photographed after staining with ethidium bromide (lanes 2 and M). After transfer to GeneScreen, the blot was hybridized with the H-specific oligonucleotide probe (lane 1).

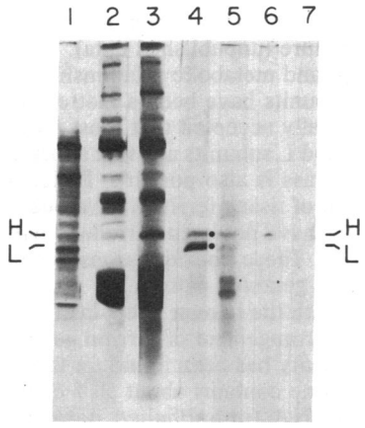

FIG. 2. Hybrid selection of ferritin H subunit mRNA by cloned cDNA. Lanes: 1, translation products from HeLa mRNA; 2, translation products selected from HeLa mRNA with a filter containing DNA from pBR322; 3, translation products selected from HeLa mRNA with a filter containing DNA from pDBR2; 4, carrier ferritin (protein stain); 5–7, immunoprecipitated products from lanes 1, 3, and 2, respectively.

from the selected mRNA (lane 3) or by immunoprecipitation (lane 6). Other bands in the immunoprecipitate of the total translation products in lane 5 are due to nonspecific contamination from endogenous rabbit globin chains and to breakdown products of human ferritin H chains (unpublished data).

Sequence Analysis. We first attempted to determine the sequence of the cDNA clone by the pBR322 primer method but had difficulty in reading through the regions of the G·C tails. Nevertheless, we obtained a partial sequence of about 170 bases from the poly(C) tail. This sequence began with a stretch of poly(A) and had a polyadenylylation sequence signal, A-A-T-A-A-A, (32) about 20 bases upstream. Therefore, we concluded that it represented the 3′ non-coding region of a messenger RNA. Although this sequence had no recognizable coding region, it contained a site for *Rsa* I about 65 bases upstream from the poly(A) tract that was used to clone the 450-base-pair fragment in M13mp8 for sequencing. This sequence (Fig. 3) has a termination codon 90 bases upstream from the *Rsa* I site. The amino acid sequence derived from about 180 bases of the coding region is compared to the published sequences of the major and minor sequences of human spleen ferritin. For this comparison we aligned these sequences to maximize homologies and used coordinates from the complete sequence of the major component, L subunit. This comparison shows that the cDNA clone contains sequences that correspond to those of the H- and (H + L)-specific probes used to select the clone (Fig. 3, line 1, underlined codons). These sequences are at the positions predicted from the amino acid sequence data. The amino acid sequence derived from the clone is very similar to that of residues 123–158 of the minor sequence of human spleen ferritin (30). The only differences are at residue 129, where we find an additional residue (isoleucine) and at residue 137 where the nucleotide sequence predicts glutamine rather than glutamic acid. This concordance of sequences, together with the hybrid selection of mRNA coding for H chains, demonstrates that the cloned DNA contains the ferritin H-chain sequence and, therefore, that the minor sequence found in human spleen ferritin is indeed from an H subunit.

Genomic Analysis. To examine the genes coding for the ferritin H chain(s), we performed Southern blot analyses of human lymphocyte DNA digested with restriction endonuclease *Eco*RI, *Bgl* II, and *Hin*dIII. These enzymes do not cut the cloned DNA and, thus, the number of fragments obtained is expected to reflect the complexity of the ferritin gene family and the number of introns. Ten to fifteen bands of genomic DNA that ranged in size from 0.5 to 20 kb hybridized to the H-chain clone in each digest (Fig. 4). Rehybridization of the same blot to a cDNA probe for the constant region of human IgM immunoglobulin gave the predicted pattern (33), showing that digestion was complete in all three cases (Fig. 4 *Right*). Therefore, these results suggest that there may be multiple genes for the 3′ end of the ferritin H-chain message because it seems unlikely that a 540-base sequence with coding and 3′ untranslated regions would contain 10–15 introns (34).

The relative intensity of most of the bands was not changed by increasing the stringency to 0.1× SET buffer at 68°C (Fig. 4 *Middle*). Thus, these bands contain sequences that are highly homologous to the cloned H subunit DNA. A few bands in each digest were relatively less intense at the higher stringency and, thus, appear to contain sequences that are partially homologous to the cloned DNA sequences.

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   123                                 130                                 140
1. CCC CAT TTG TGT GAC TTC ATT GAG ACA CAT TAC CTG AAT GAG CAG GTG AAA GCC ATC AAA GAA
2. Pro His Leu Cys Asp Phe Ile Glu Thr His Tyr Leu Asn Glu Gln Val Lys Ala Ile Lys Glu
3. Pro His Leu Cys Asp Phe Leu Glu Thr His Phe Leu Asp Glu Glu Val Lys Leu Ile Lys Lys
4. Pro His Leu Cys Asp Phe     Glu Thr His Tyr Leu Asn Glu Glu Val Lys Ala Ile Lys Glu
                           150                                 160
1. TTG GGT GAC CAC GTG ACC AAC TTG CGC AAG ATG GGA GCG CCC GAA TCT GGC TTG GCG GAA TAT
2. Leu Gly Asp His Val Thr Asn Leu Arg Lys Met Gly Ala Pro Glu Ser Gly Leu Ala Glu Tyr
3. Met Gly Asp His Leu Thr Asn Leu Arg Lys Leu Gly Gly Pro Glu Ala Gly Leu Gly Glu Tyr
4. Leu Gly Asp His Val Thr Asn Leu Arg Lys Met Gly Ala Pro Glu
                        170                                 178
1. CTC TTT GAC AAG CAC ACC CTG GGA GAC AGT GAT AAT GAA AGC TAA
2. Leu Phe Asp Lys His Thr Leu Gly Asp Ser Asp Asn Glu Ser End
3. Leu Phe Glu Arg Leu Thr Leu Lys His Asp End
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FIG. 3. Comparison of amino acid sequence of cloned DNA (pDBR2) with human ferritin sequences. The numbering of amino acid residues is according to the convention of ref. 30. Lines: 1, nucleotide sequence of pDBR2, with sequences complementary to H-specific synthetic probe (residues 153–157) and the common-sequence probe (residues 124–128) underlined; 2, amino acid sequence from 1; 3, major sequence from human spleen ferritin (30), with the sequence in common with line 2 underlined; 4, minor sequence from human spleen ferritin (30), with the sequence in common with line 2 underlined.

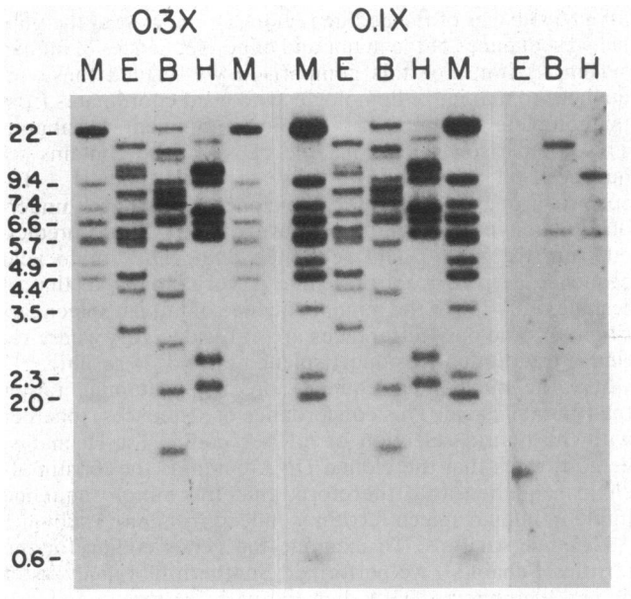

FIG. 4. Hybridization of ferritin H-chain cDNA probe to restriction fragments of human DNA. Human lymphocyte DNA was digested with *Eco*RI (lanes E), *Bgl* II (lanes B), or *Hin*dIII (lanes H) and fractionated on an agarose gel. Blotting and hybridization to nick-translated DNA from clone pDBR2 was as described and washing was at a stringency of 0.3× SET (*Left*) and 0.1× SET (*Middle*). The hybridized probe was stripped with boiling water; autoradiography showed that >90% of the hybridized probe was removed. The stripped blot was then hybridized to a clone containing a cDNA insert that codes for the C region of the IgM immunoglobulin chain (*Right*). Lanes M contain end-labeled marker DNA. Sizes are shown in kilobases.

DISCUSSION

We have obtained a human cDNA clone that selects an mRNA encoding a protein with the immunological properties of human ferritin and the size of the H subunit. The amino acid sequence derived from this clone is virtually identical to that of a minor sequence found in human spleen ferritin. Therefore, we conclude that the cloned DNA represents a human ferritin H subunit and that the minor amino acid sequence found in human spleen ferritin is that of an H subunit.

Comparison of the amino acid sequence derived from our H-chain clone with that of the L chain reveals a number of interesting features. The two sequences share about 60% homology. About 80% of the differences could have arisen from single-base changes. It is striking that the homology is dispersed throughout the entire sequenced region and that there are no insertions or deletions of amino acids except for the additional four amino acids at the COOH terminus of the H chain. These analyses are consistent with previous observations that the H and L subunits have common and unique peptides and that the human H subunit is larger than the L subunit (6). They also show conclusively that the H and L subunits do not arise from post-translational events but are clearly derived from distinct mRNAs and probably from distinct genes.

This clone and other related clones were initially identified by screening a human lymphocyte cDNA library with two synthetic oligonucleotide probes. Our task in identifying ferritin H-chain clones with these synthetic probes was complicated by the paucity of protein sequence data which precluded selection of less heterogeneous probes than the mixtures of 32 and 64 oligodeoxyribonucleotides used in this study. We have found in selecting other ferritin clones that the combined use of both mixtures is critical for minimizing the number of false positives that arise from the use of only one oligonucleotide mixture (unpublished data).

The structural and metabolic relationships of tissue ferritins and their subunits have been a matter of some controversy. It is generally accepted that most tissue ferritins are composed of H and L subunits and that a further heterogeneity within each class is also possible. Differences in amino acid composition of tissue ferritins have been reported, but these differences have not been correlated with subunit heterogeneity (1, 2). These considerations are relevant to the complexity of the genomic pattern.

Our analysis with the human H-chain clone indicates that the number and arrangement of ferritin genes is not simple. A similar complexity has been found for ferritin L chains in rats (35). Our clone contains about 50% of the sequence of the H-chain mRNA (unpublished data) but hybridizes strongly to many genomic sequences. Our stringency analyses indicate that most of these fragments contain sequences that are highly homologous to the H-chain clone. It is unlikely that the complexity we observe is due to cross hybridization with ferritin L sequences. Within the sequenced region of Fig. 3, the longest common stretch between our H-chain cDNA clone and the L chain is a hexapeptide. Thus, even if the corresponding nucleotide sequences from common peptides were entirely conserved, they would not contribute significantly to the genomic pattern under the conditions we have used. Therefore, the genomic complexity is likely to reflect sequences unique to ferritin H chains. This suggests that ferritin H-chain genes either have an unusually large number of introns or are present in multiple copies in the genome.

The biological significance of multiple genes for ferritin H chains is not clear. Some may represent pseudogenes. However, some may correspond to different isoelectric forms of the H subunit (7, 8) or to the glycosylated form of serum ferritin (36), which presumably is encoded by a distinct ferritin mRNA. Some of these forms may be derived from different genes. Alternatively, some could arise by differential splicing of multiple exons.

Since this paper was submitted, Costanzo *et al.* (37) have described a human ferritin cDNA. Their nucleotide sequence is identical to ours except at the 3′ end, where they have one less cytidine at the position corresponding to amino acid residue 171. Our sequence terminates at position 178, whereas theirs predicts a different 15 residues and terminates at 186.

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