

Complete Characterization of the 3' Region of the Human and Mouse Hereditary Hemochromatosis HFE Gene and Detection of Novel Splicing Forms

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ABSTRACT: The human HFE gene was identified in 1996 as the gene whose mutations are responsible for hereditary hemochromatosis in most patients. Expression analysis by Northern blot indicated that the gene was approximately 4.1 kb in length. However, the cDNA reported was only 2716 bp. These results implied that at least 1.4 kb of the mRNA remained to be identified. In the present study, we detected several 3' EST clones while screening the genomic region of the gene in search of potential additional HFE mRNA sequences. Subsequent sequencing of these EST clones and RT-PCR experiments revealed that exon 7 of the HFE gene has, in fact, a length of 1944 bp and it presents two polyadenylation signals. The new human HFE exon 7 region has been screened in non-C282Y HH patients in search for new putative mutations. Mouse 3' RACE experiments also further extend the previously reported mouse HFE exon 6 sequence. Additionally, we report two novel end forms of the human HFE gene detected by 3' RACE experiments and several novel splicing forms identified in the HepG2 cell line. © 2001 Academic Press

Key Words: iron; hereditary hemochromatosis; HFE gene.

INTRODUCTION

Hereditary hemochromatosis (HH) is an autosomal recessive disease involving abnormally high intestinal iron absorption that leads to iron deposition in cells and subsequent dysfunction in several organs (1). This disease has been reported as the most frequent recessive disorder, with an estimated carrier frequency in Europe of 1 in 10 (2). In 1996, a single mutation (C282Y) in the HFE gene was identified as the main cause of this disease (3).

The HFE protein binds to β_2 -microglobulin and this binding is required for the transport of the HFE protein to the membrane (4). The C282Y

mutation prevents the binding of the HFE protein to β_2 -microglobulin, and thus the HFE protein cannot reach the membrane (4, 5). In the membrane the HFE protein complexes with the transferrin receptor lowering the affinity of the transferrin receptor for transferrin (6). A second missense mutation (H63D) has also been described and the HFE protein carrying this mutation lacks the ability to reduce the affinity of the transferrin receptor for transferrin (6). However, these two mutations do not account for all of the HH cases and it has been suggested that the HH patients lacking the C282Y mutation could have a mutation in a regulatory region of the HFE gene (7).

The first Northern blot analysis reported indi-

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Abbreviations used: HH, hereditary hemochromatosis; EST, expressed sequence tags; RACE, rapid amplification of cDNA ends.

Nucleotide sequences reported in this paper have been submitted to the GenBank database and have the following accession numbers: AJ249335, AJ249336, AJ249337, AJ250635, AJ249338, AJ298838, AJ298839, AJ298840, and AJ404378.

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cated that the human HFE gene is expressed at low levels in most tissues and its length is 4.0 kb (3). However, the cDNA reported for this gene was only 2716 bp (GenBank U60319). This implied that at least 1.4 kb of the gene remained to be identified. Alternative splicing processes have been reported for the HFE gene (8–10) although none of the splicing forms further extended the initially reported cDNA sequence. In this paper we report the identification of additional novel 3' sequences that further extend the previously reported human and mouse HFE mRNAs, as well as the identification of novel splicing forms and end forms of the human HFE gene.

MATERIALS AND METHODS

RNA Isolation

Total cellular RNA was isolated from several human tissues, the HepG2 cell line and mouse liver using the guanidine/phenol solution (Tri-pure, Roche) according to the manufacturer's protocol.

RT-PCR Experiments

Five micrograms of total RNA from different tissues and from the HepG2 cell line were retro-transcribed with 200 units of SuperScript II RT RNase H⁻ enzyme (Gibco BRL, Gaithersburg, MD) at 42°C during 1 h using an oligo dT primer (5'-(₃₁)TVN-3'). After a 15-min denaturation at 70°C, Rnase H treatment was performed at 37°C for 20 min. Two microliters of the cDNA template were subsequently used for the PCRs.

Splicing variants from the HepG2 cell line were found using primers Ex1U, 5'-CTAAA-GTTCTGAAAGACCTGTTGC-3' and Ex6L, 5'-GAAGGCTAAAATCAAGGAGTTCGTC-3' in the PCR. Amplification was done with 1 unit of *Taq* polymerase, 1.5 mM MgCl₂, and 500 mM dNTPs for 40 cycles with an annealing temperature of 55°C.

Primers used to connect the 3' EST (expressed sequence tag) sequences to the HFE gene were the following: in exon 6, primer Ex6U, 5'-GATA-AAAGCACTTACTTCGTGTCC-3' and in the

EST sequence, primer ESTL, 5'-GAATGGTTT-TGCCAGAGGTGAATA-3. The primers were designed so that the region to be amplified would include intron 6 in order to discriminate true cDNA amplification products from potential contaminant genomic sequences. Twenty-five nanograms of genomic DNA from blood were used as a genomic control. PCRs were done with 1 unit of *Taq* polymerase, 1.5 mM MgCl₂, 500 nM dNTPs, and 1.5 M betaine for 35 cycles, with an annealing temperature of 60°C. Betaine addition to PCR step is needed to amplify the genomic band in the RNA retrotranscription lanes. PCR products were run in a 1% agarose gel and visualized with ethidium bromide.

Bands corresponding to the PCR amplification products were excised from agarose gel, purified and cloned in pSTBluescript II vector or in pST-Blue-1 Acceptor vector (Novagen), grown and finally sequenced using T7 and SP6 primers in a LICOR 4000L sequencer.

Isolation and Characterization of New Sequences in the Human HFE 3' Region

While screening the EST database with the U91328 genomic sequence we identified three EST clones (AI760080, AI290427, and AI278822) in the 3' region of the human HFE gene. The tissue source of the EST clones AI290427 and AI278822 was the colon while for the EST clone AI760080 it was the kidney. The EST clones AI760080 and AI290427 were purchased, grown and the insert was subsequently fully sequenced using T7 and T3 primers in a LICOR 4000L sequencer.

Northern Blot Analysis

Twenty to 40 µg of total cellular RNA was electrophoresed in 1.2% agarose/2.2 M formaldehyde gels. The RNA was transferred to nylon membranes (Nytran Supercharge, Schleider & Schuell) and immobilized by crosslinking. Blots were prehybridized with Ultrahyb (Ambion, Inc.) for 1 h at 42°C and then hybridized overnight with ³²P-labeled DNA probes (Rediprime II kit, Amersham). The probes corresponded to the linearized inserts of the EST clone W21141 (exons 6

and 7 and partially exon 5) and the 3' EST Clones AI290427 and AI760080. Hybridizations with the probe W21141 were subsequently washed in $0.1 \times$ SSC/0.1% SDS at room temperature for 20 min, twice at 37°C and twice at 65°C. Hybridizations with the AI290427 and AI760080 probes were subsequently washed in $2 \times$ SSC/0.1% SDS at room temperature for 20 min, twice at 37°C and twice at 42°C. All blots were exposed for 6 days with Phosphoimager screens and visualized using Phosphoimager equipment (Bio-Rad Laboratories, Hercules, CA).

3' RACE Experiments

The Smart RACE cDNA Amplification kit (Clontech, PA) was used to perform 3' RACE. Five micrograms of human or mouse liver RNA were retrotranscribed using 200 units of Rnase H⁻ Superscript II retrotranscriptase (Gibco BRL, Gaithersburg, MD). The following primers were used in the PCR step: human primers, race1, 5'-TGGTGCCTTCATTTGGGATGCTACT-3'; mouse primer race2, 5'-CAAAGACTTGGAGGGGGCACACT-3'. PCR was done using 1 μ l of 50 \times Advantage 2 Polymerase Mix (Clontech, PA). PCR bands were cut from the agarose gels, purified, ligated to pSTBlue-1 Acceptor vector (Novagen, Madison, WI) and sequenced in a LICOR 4000L sequencer using T7 and SP6 primers.

Sequencing Analysis of the Newly Identified Human HFE 3' mRNA Region

Four previously described non-C282Y hereditary hemochromatosis patients were included in the study (11). Several sets of primers were designed to amplify the newly detected HFE exon 7 sequences from genomic DNA in three overlapping fragments. The primers designed were the followings: ex7.1U, 5'-AT-TCTGGGAAATCAGTTCAC-3', ex7.1L, 5'-CTA-CAATGAGCGTAAATCAC-3', ex7.2U, 5'-CAG-GTGCTTCAGGATAACCATA-3', ex7.2L, 5'-GA-ATGGTTTTGCCAGAGGTGAATA-3', ex7.3U, 5'-ACATTGTCGTCTAAGTTGTAAG-3', ex7.3L, 5'-AGATTCAGAACACAGCCTAATA-3'. PCR amplification products were excised from the aga-

rose gel, purified and sequenced using the rhodamine terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems Inc.). The sequencing products were run in a 373 ABI PRISM or in an ABI PRISM 310 genetic analyzer sequencer. The sequences obtained were subsequently analyzed using the software Seqman (DNASTAR software package) in order to identify the presence of potential mutations.

RESULTS

Novel 3' UTR Sequence Extending Exon 7 of the Human HFE Gene

A BLAST search with the 3' genomic region of the HFE gene (U91328) versus the EST database revealed the presence of three EST clones (Fig. 1; AI760080, AI290427, AI278822). These EST clones were located 648–1054 bp downstream from the previously reported exon 7 (Fig. 1). The tissue source for the EST clones AI290427 and AI278822 was the colon while for the EST clone AI760080 it was the kidney. The EST clones AI760080 and AI290427 were totally sequenced and aligned to obtain a 1246-bp continuous sequence not linked to the previously reported exon 7 (Fig. 1). This EST sequence contig contains a consensus for the polyadenylation signal (ATTAAA) 15 bp upstream from the EST 3' end. Two primers were subsequently designed, one in exon 6 and the other one in the newly discovered 3' sequence, in order to perform RT-PCR experiments to determine whether this novel EST contig sequence was, in fact, part of the HFE gene (Fig. 1, primers Ex6U and ESTL). Two bands were detected in several tissues (Fig. 2A), the upper band corresponding to the genomic sequence including the intron 6 (2360 bp) and the lower band corresponding to the cDNA sequence (1205 bp). The 1205-bp bands from kidney and spleen were cloned and sequenced. The result obtained confirmed that the previously reported 432-bp exon 7 should, in fact, be 1944 bp (RT-PCR plus EST sequence, GenBank AJ404378). The new exon 7 sequence has two polyadenylation signals and it is now the longest exon reported for the HFE gene (Fig. 1).

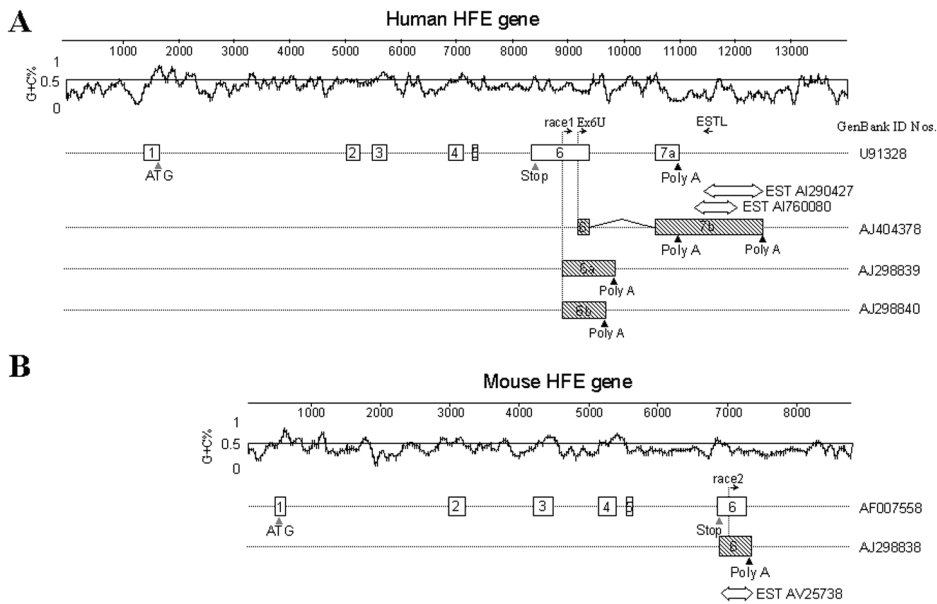


FIG. 1. Human (A) and mouse (B) genomic HFE gene diagrams. Previously reported exons are indicated with the open boxes. Hatched boxes represent the new forms described in this work. The G + C content charts are shown below the scale of the human and mouse genes. The ATG codon, the Stop codon and polyadenylation sites are indicated with a triangle. The RT-PCR (Ex6U and ESTL) and RACE (race1 and race2) primers are represented with small arrows. The EST sequences are shown as opened arrows. The previously reported genomic human and mouse HFE gene structures are drawn according to GenBank sequences U91328 and AF007558, respectively. GenBank accession numbers of the newly identified sequences are indicated at the right.

Northern Blot Analysis

Northern blot analysis performed with 20 μ g of RNA from several tissues using the HFE EST

clone W21141 as the probe (1642 bp, exons 7 and 6 and partially exon 5 of human HFE gene) revealed a band of 4.2 kb (range 4.0–4.3 kb) and an additional faint band of 1.6 kb (Fig. 3A). Northern

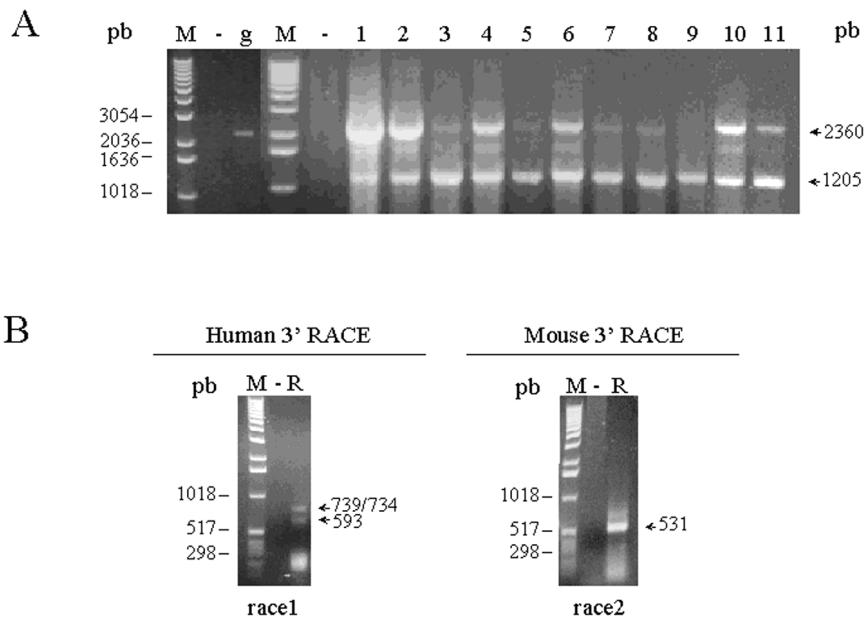


FIG. 2. RT-PCR (A) and RACE (B) experiments in the HFE gene. (A) RT-PCR using primers Ex6U and ESTL (see Fig. 1) in several tissues. M, 1 kb size marker; “–,” no RNA negative control; “g,” genomic DNA positive control; 1, spleen; 2, testis; 3, liver; 4, muscle; 5, small intestine; 6, large intestine; 7, pancreas; 8, lung; 9, leukocytes; 10, kidney and 11, HepG2 cell line. (B) 3’ RACE experiments with human and mouse RNA using primers race1 and race2 (see Fig. 1). M, 1 kb size marker; “–,” negative control; R, RACE products. The arrows indicate the PCR amplification products and their corresponding sizes.

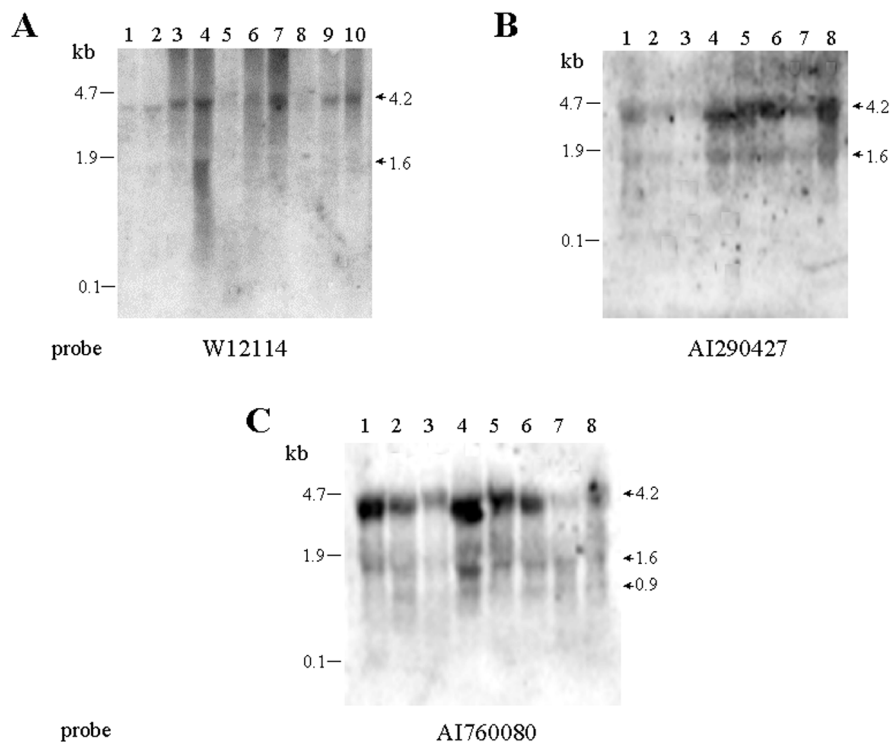


FIG. 3. Northern blot hybridizations with the HFE probes W21141 (A), AI290427 (B) and AI760080 (C). The arrows indicate the size of the identified mRNAs. Lanes in A: 1, HepG2; 2, HepG2 TPA treated; 3, kidney; 4, lung; 5, small intestine; 6, large intestine; 7, testis; 8, leukocytes; 9, pancreas and 10, spleen. Lanes in B and C: 1, lung; 2, small intestine; 3, large intestine; 4, liver; 5, spleen; 6, kidney; 7, pancreas and 8, testis.

blot analysis using the EST Clones AI760080 and AI290427 as probes corresponding to the novel 3' HFE sequences (776 and 1074 bp, respectively) were done with 40 μ g of RNA and revealed the same two bands detected with the HFE W21141 probe (4.2 and 1.6 kb) (Fig. 3B). An additional faint band of 0.9 kb was also detected in Northern blot done using the AI760080 probe. Therefore, the 4.2-kb band present in the Northern blots is consistent with the inclusion of the novel detected cDNA sequences as part of the HFE mRNA. The 1.6- and 0.9-kb bands could also correspond to some of the splicing variants detected.

RACE Experiments Detect Novel Human HFE End Forms

The 3' RACE experiment using the primer race1 located in exon 6 (Fig. 1, race1) resulted in the detection of two novel human HFE end forms (Fig. 1; 6a and 6b). These two novel HFE 3' end forms extend into intron 6 and each has its own polyadenylation consensus signal. The 6a form

includes 427 bp of intron 6 (GenBank AJ298839) and is coamplified in RACE experiment with the previously described 7a form (Fig. 2B, 739–734 bands). The 6b form is shorter, corresponding to the lower band of the 3' RACE experiments and includes 286 bp of intron 6 (GenBank AJ298840). We have also detected three EST clones (AI358948, AI160732, and AI079088), all from glioblastoma, that end at the same point as this 6b form (not shown). These EST clones corroborate that the 6b form is indeed a novel HFE end form. The 6b form has two putative AATAAA consensus sequences that could act as polyadenylation signals (see the AJ298840 sequence).

Identification of Splicing Forms in HFE Gene in the HepG2 Cell Line

RT-PCR and sequencing experiments using primers located in exons 1 and 6 resulted in the detection of 5 splicing variants of the HFE gene, in addition to the most abundant nondeleted form (Fig. 4). The first splice variant (GenBank

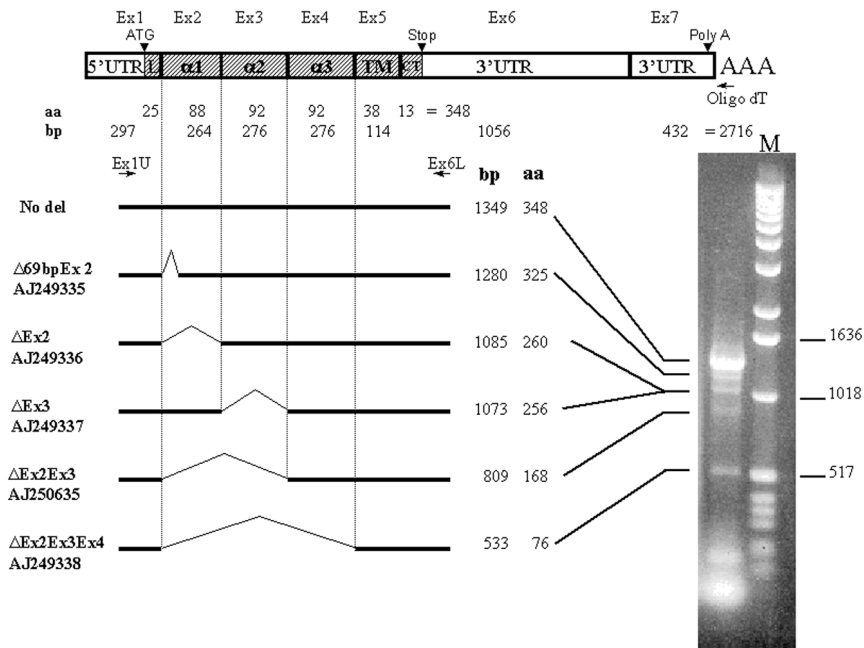


FIG. 4. Splicing forms of HFE gene in the HepG2 cell line. The initially reported HFE cDNA (U60319) structure is represented at the top. Hatched boxes represent the coding HFE region. The ATG, the Stop codon and the polyadenylation site are indicated. Abbreviations inside the exons: L, peptide leader; $\alpha 1$, $\alpha 2$ and $\alpha 3$, extracellular $\alpha 1$, $\alpha 2$, $\alpha 3$ domains; TM, transmembrane domain; CT, cytoplasmic tail; 5' UTR and 3' UTR, 5' and 3' untranslated regions, respectively. The length in base pairs (bp) and the amino acids (aa) of the splicing forms and the presently reported HFE cDNAs are shown. At the right, a photograph of the RT-PCR experiment is shown indicating the correspondence between the splicing forms detected after sequencing (left) and the PCR amplification products (lane 1 from the photograph). M, 1-kb size marker. The primers used for the RT (oligo dT) and PCR (Ex1U and Ex6L) are indicated with arrows. The GenBank accession numbers of each splicing form are indicated at the left.

AJ249335) has a length of 1280 bp and presents an in-frame deletion of the first 69 bp of exon 2 due to selection of another cryptic acceptor site situated inside exon 2 (ttcctgtttgaag/C). In the PCR amplification product around 1085 bp two splicing forms were detected, one that has skipped exon 2 (1085 bp) and another that has skipped exon 3 (1073 bp) (GenBank AJ249336 and AJ249337, respectively). The 809-bp PCR amplification fragment represents the deletion of both exons 2 and 3 (GenBank AJ250635). The shorter band identified has a size of 533 bp and lacks exons 2, 3, and 4 (GenBank AJ249338). All these splicing forms do not change the reading frame of HFE protein and only result in deletion of the sequence corresponding to the entire exons. Additionally in some of the splicing forms, a single amino acid change also occurs in the new exon-exon junction. For instance, the splicing variant del69bpEx2 results in a 325 aa protein where the amino acid alanine in position 49 of the HFE

complete protein (348 bp) changes to proline. The splicing variant with a complete deletion of exon 2 results in a 325 aa protein where the glutamate 114 changes to glutamine. The splicing variant with a complete deletion of the exon 3 only results in a 260-aa protein with no additional amino acid changes. The splicing variant with a complete deletion of exons 2 and 3 results in a 256-aa protein where the valine 206 changes to leucine. The splicing variant with a complete deletion of exons 2, 3, and 4 results in a 76-aa protein where the glutamate 298 changes to glutamine.

Novel 3' UTR Sequence in the Mouse HFE Gene

The 3' RACE experiments were performed using mouse liver RNA from its last exon (Fig. 1B, exon 6). We found that the detected product extended the 3' sequence by an additional 167 bp compared to the previously reported cDNA (Gen-

Bank NM010424) (12). This newly identified mRNA sequence contains a AATAAA polyadenylation consensus signal (Fig. 1B; GenBank AJ298838). A BLAST search using the new identified 3' sequence versus the EST database detected several 3' EST clones. The longest of the clones detected (AV257380) came from an adult male testis library and allowed the elongation of an additional 24 bp of the mouse HFE exon 6 sequence. Therefore, the mouse exon 6 sequence has been extended by a total of 191 bp, its total extension being 602 bp (Fig. 1B, GenBank AJ298838).

Screening the Novel Exon 7 Sequence in Non-C282Y HH Patients

We performed sequencing analysis of the new extended HFE gene exon 7 in 4 previously reported HH patients that do not carry the main C282Y HFE mutation (11). A C/T polymorphism was found at position 760 bp of the exon 7, but no other mutations were found.

DISCUSSION

In this study we report five splicing forms of the HFE gene in the HepG2 cell line (AJ249335, AJ249336, AJ249337, AJ250635, and AJ249338), the identification of two novel human HFE gene end forms (AJ298839 and AJ298840) and we demonstrate the existence of extended 3' mRNA sequences in the human and mouse HFE genes (AJ404378 and AJ298838).

The human HFE gene was discovered in 1996 and a 2.7-kb cDNA was described (U60319) (3). In this initial work the HFE mRNA was reported as having 4.0 kb by Northern blot analysis (3). Minor 5.7- and 2.4-kb bands were also observed by the same authors (13). In a recent study five bands of 6.1, 4.1, 1.6, 1.3, and 0.9 kb have been described for the human HFE gene (9). Our Northern blot analysis has revealed two consistent bands of 4.2 kb and 1.6 kb using three different probes (W21141 including exon 5, 6, and 7a, AI290427 and AI760080 including the newly identified extended exon 7b; Fig. 3). An additional band of 0.9 kb was only observed when

using the AI760080 probe. We are currently extending our RT-PCR experiments to determine what HFE mRNA regions could be associated with these 1.6- and 0.9-kb detected forms. Assuming that the described 4.0–4.1 kb Northern bands detected in previously studies corresponds to our 4.2-kb band, there is a discrepancy in length between Northern blots and the previously reported HFE cDNA sequence (U60319) of at least 1.3–1.5 kb (3). This fact led us to screen the genomic HFE region in search of potential EST clones that could further extend the HFE cDNA sequence. Contrary to some reports proposing that the missing HFE mRNA sequence could be located in the 5' region (9, 13), we demonstrate the presence of a substantial HFE mRNA sequence in the 3' region of the HFE gene (Fig. 1). The first clue toward the identification of this additional 3' mRNA sequence was the detection of two EST clones located in the 3' HFE genomic region. The new sequenced EST contig clone did not link with the previously described exon 7. Therefore, subsequent RT-PCR experiments were designed to determine if this new sequence represented a new exon or rather corresponded to an extension of the previously reported exon 7. The sequencing of the RT-PCR products revealed a sequence that excludes intron 6 and that is continuous between the previously reported exon 7 and the novel EST contig sequence. Thus, a combination of strategies using BLAST searches versus the EST database, RT-PCR and sequencing experiments, resulted in the extension of the human HFE exon 7 in a total of 1512 bp. Adding this extra 1512 bp to the previously described 2716-bp cDNA sequence we have a 4228 bp cDNA that could explain our reported 4.2-kb Northern blot band. The 4.2-kb Northern band was also detected with probes specific for this newly detected mRNA sequence (Figs. 3B and 3C), thus this result is consistent with the inclusion of the novel detected mRNA sequence as part of the HFE mRNA. The failure of the detection of this 3' region using 3' probes in Northern blots by other groups (9) could be explained by the detection of the new 3' region requiring washing at a lower stringency, since we found that all this region is very AT rich (Fig. 1, see G + C content).

In conclusion, the human HFE exon 7 is now the longest HFE gene exon with a total of 1944 bp and it has two polyadenylation sites (Fig. 1). Therefore, we have found that alternative poly(A) addition utilization is another mechanism through which the HFE gene results in several transcripts, besides alternative splicing. However, in spite of our results we cannot exclude that there may still exist undiscovered 5' HFE mRNA sequences that could belong to some splicing forms or to the 5.7-6.1-kb band detected in other studies (9, 13).

Surprisingly, the 3' RACE strategy also revealed two novel human HFE end forms (6a and 6b). These two novel forms lack exon 7 and may represent minor forms of the HFE gene. Further studies are needed to determine whether different end forms of HFE gene have a functional role. The 3' RACE experiments using a primer located in the 7a form have failed to detect the 7b form (not shown) probably due to the preferential amplification of short fragments in the PCR. Moreover, the high AT content of the new sequence (66.1%) could have also hindered the PCR extension.

The mouse HFE gene sequence has been reported in two papers (12, 14) and both concluded that the mRNA has a size of 1.5 kb. The mouse HFE mRNA GenBank deposited sequence (NM010424) includes an exon 6 of 411 bp with no polyadenylation site reported. Using the 3' RACE experiments and the strategy involving the identification of EST sequences we extended the mouse HFE exon 6 by an additional 191 bp, so that it has a length of 620 bp, and its polyadenylation site has been identified (Fig. 1B; AJ298838).

All newly identified human and mouse HFE sequences found (human 6a, 6b and 7b forms and mouse extended exon 6 sequences) correspond to the 3' UTR region. Some recent papers have reported that the HFE gene undergoes alternative splicing processes (8–10). Our data agree with these results since we have reported five different splicing forms obtained from the HepG2 cell line. The forms presenting the exon 2 and the exon 3 deletions, have already been described (8, 9), while the other forms are first reported in the present study (Fig. 4). These novel forms could be specific for the HepG2 cell line. However, we did

not detect the previously reported soluble form skipping exon 5 in the HepG2 cell line (10). Further studies should be done to determine if these novel HFE mRNA splicing forms result in shorter HFE protein forms with a functional role.

Since the discovery of the human HFE gene intensive research concerning its mutations and function has been done. Several studies in different populations have detected new mutations in the HFE gene in patients without the common C282Y and H63D mutations (15, 16). However, this search for mutations in the HFE gene was done only using the available mRNA sequence of 2716 bp in length. Obviously, the identification of new sequences belonging to the transcribed region of the HFE gene implies the potential existence of new mutations in this previously unscreened mRNA region. It has been reported that mutations in the 3' UTR of other genes could destabilize the mRNA so that protein levels could be affected. Despite the fact that the screening of this region in our four non-C282Y HH patients did not detect any mutation, the possibility is now open to screen for potential novel mutations in this novel 3' region, perhaps in larger groups of HH C282Y-negative patients.

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