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Isolation of Novel Non-HLA Gene Fragments from the Hemochromatosis Region (6p21.3) by cDNA Hybridization Selection

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Summary

It has previously been shown that cDNA hybridization selection can identify and recover novel genes from large cloned genomic DNA such as cosmids or YACs. In an effort to identify candidate genes for hemochromatosis, this technique was applied to a 320-kb YAC containing the HLA-A gene. A short fragment cDNA library derived from human duodenum was selected with the YAC DNA. Ten novel gene fragments were isolated, characterized, and localized on the physical map of the YAC.

Introduction

Hemochromatosis is a common autosomal recessive disease characterized by chronic dietary iron overload. The disease prevalence is estimated to be 2–5/1,000 in the Caucasian population, with a corresponding carrier frequency of .045–.071 (McKusick et al. 1992). Presymptomatic diagnosis is difficult, and when hemochromatosis is left untreated it may lead to liver cirrhosis, diabetes mellitus, congestive heart failure, and hepatocellular carcinoma (Niederau et al. 1985; Bassett et al. 1988). Early intervention with phlebotomy is an inexpensive and readily available treatment that can prevent these sequelae. The isolation of the gene responsible for hemochromatosis would be valuable for presymptomatic diagnosis.

Positional cloning has proved successful in identifying the genes responsible for many human diseases, including Duchenne muscular dystrophy (Monaco et al. 1986), cystic fibrosis (Rommens et al. 1989), familial adenomatous polyposis coli (Kinzler et al. 1991; Nishisho et al. 1991), Huntington disease (Huntington's Disease Collaborative Research Group 1993), and others (reviewed by Collins 1992). Hemochromatosis is tightly linked to the HLA-A locus on chromosome 6p21.3, with a cumulative LOD score of >60 at $\theta = .01$, and is in linkage disequilibrium with the HLA-A3 allele (reviewed by Simon et al. 1988). For these reasons, the HLA-A region is a rational starting point for positional cloning of the hemochromatosis gene.

Several techniques have been developed to facilitate the isolation of genes encoded by large genomic fragments such as YAC contigs; these techniques include regional identification of evolutionarily conserved sequences (Monaco et al. 1986) and HTF islands (Bird 1986), direct screening of cDNA libraries with cosmid or YAC clones (Elvin et al. 1990), searching for known nucleotide motifs (Call et al. 1990), and exon trapping (Duyk et al. 1990) or amplification (Buckler et al. 1991). Although each method has successfully identified coding regions in genomic sequence, some are tedious, and others potentially either suffer from low sensitivity or limit the size of the target chromosomal region to be screened.

Recently, a method of cDNA hybridization selection that identifies expressed sequences encoded within a target genomic clone has been described (Lovett et al. 1991; Parimoo et al. 1991). In order to identify gene candidates for hemochromatosis, we applied the cDNA selection technique to a 320-kb YAC containing the HLA-A gene, to isolate transcribed sequences encoded on the YAC. A short-fragment cDNA library derived from normal human duodenum was chosen for

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use because iron absorption occurs almost exclusively in this segment of the small intestine.

Material and Methods

YACs

YAC B30H3 contains a 320-kb segment of human genomic DNA from the major histocompatibility (MHC) class I region including the HLA-A gene (Bronson et al. 1991). It is from the Washington University library and was provided by Dr. David Chaplin. AB1380 is the yeast host strain. YAC 338, a 720-kb CEPH library YAC that reportedly contains HLA-A and -G, was a gift from Dr. Hadi Abderrahim (CEPH).

cDNA Library

A short fragment cDNA library was prepared from normal human duodenal tissue by using random hexamer primers as described elsewhere (Patanjali et al. 1991).

cDNA Hybridization Selection

The cDNA selection was done as described elsewhere (Parimoo et al. 1991) and by the detailed protocol outlined by Parimoo et al. (1993). Briefly, 20 ng of gel purified YAC B30H3 DNA was immobilized onto a 2.5-mm^2 nylon disk (Hybond; Amersham) and was preannealed with quenching agents consisting of yeast DNA, rRNA, DNA from a genomic chromosome 15 library, and repetitive DNA from a chromosome X library. The nylon disk was then hybridized to 600 ng of denatured PCR-amplified short-fragment human duodenum cDNA library at 65°C for 36 h. Nonspecific cDNAs were removed in serial washes of decreasing salt concentrations (from 2 × SSC/0.1% SDS to 0.1 × SSC/0.1% SDS) at 65°C for 20 min each. The specifically hybridized cDNAs were eluted and amplified using primers from the λgt-10 vector arms (5′-CCACCTTTTGAGCAAGTTCAG-3′, and 5′-GAGGTGGCTTATGAGTATTTC-3′). A second round of selection was performed under the same conditions with another nylon disk with the immobilized YAC and 20 ng of the amplified cDNAs from the first round of hybridization selection. The final eluted cDNAs were PCR amplified, gel purified, size selected (300–700 bp), digested with *Eco*RI, ligated into the λgt-10 vector arms, and packaged with Gigapack Gold (Stratagene).

Dot Blots

Novel clones from the selected library were PCR amplified and spotted onto a nylon membrane (0.2 μg/well) in a 12 × 8 dot blot array. For controls, several rRNA, repetitive, and HLA class I sequences were also included on the filter. The dot blots were sequentially hybridized to a number of the selected cDNA clones as described below.

Sequencing

PCR-amplified cDNA clones were sequenced in one direction by using an innermost nested primer (5′-ACGGTGGTTAACTCCAAGCTG-3′) from the λgt-10 vector arm (Parimoo et al. 1991). Sequencing was performed by cycle sequencing of double-stranded DNA (dsDNA Cycle Sequencing System; BRL) according to the manufacturer's recommendation. The products were resolved on a 6% polyacrylamide/urea sequencing gel.

Database Searches

Sequence analysis and database searches were performed using the GCG programs, FASTA and Wordsearch (from the Genetics Computing Group, 1991), and the Blast network service at the NCBI (Altschul et al. 1990).

Cell Lines

R66 is a radiation-reduced hybrid cell line containing an intact fragment of human 6p21.3, which includes the HLA region, on a hamster background (Zoghbi et al. 1991; Gruen et al. 1992). JY is a human lymphoblastoid cell line (JY LCL) containing HLA haplotypes: A2,2; B7,7; C-,-; and DR4,W6 (Van Seventer et al. 1988). The growth conditions for R66 and JY have been described elsewhere (Gruen et al. 1992). CHO-K is a Chinese hamster ovary cell line (ATCC CCL 61; American Type Culture Collection, Rockville, MD). Hep G2 is a human hepatocellular carcinoma cell line (ATCC HB 8065). Hutu 80 is a human duodenum carcinoma cell line (ATCC HTB 40). CHO-K, Hep G2, and Hutu 80 were grown as recommended by ATCC.

DNA Blot

Genomic DNA was prepared from YAC B30H3, host yeast AB1380, JY LCL, R66, and CHO-K (Grunebaum et al. 1984). Five micrograms of DNA were digested with *Eco*RI (NEB) according to the manufacturer's recommendation, were separated on a 1% agarose gel, and were transferred to a nylon membrane (Hybond; Amersham) (Southern 1975).

RNA Blot

Total RNA was prepared from JY LCL, human duodenum and ileum, Hep G2, and Hutu 80 cell lines ac-

Table 1

Enrichment of YAC-specific Clones in the Selected Library

Probe	Encoded on YAC B30H3	Starting Library (%)	Selected Library (%)
Class I (HLA)	+	.075	12.5
Ribosomal cDNA	−	32.0	2.5
γ-Actin	−	.8	.1
VLG 51	+	.002	20

NOTE.—The starting short-fragment cDNA library and the selected library were each screened with the clone VLG51 and probes that identify HLA class I genes, ribosomal cDNA, and γ-actin cDNA. Enrichment of the selected library is demonstrated by a >100-fold increase in MHC class I cDNAs, a >10-fold decrease in ribosomal cDNA, an 8-fold decrease in γ-actin, and a $>10^4$-fold increase in the novel clone VLG51, after two rounds of selection.

cording to the method described by Chomezynski and Sacchi (1987). Poly(A)$^+$ RNA was prepared as described by Aviv and Leder (1972). Five micrograms of poly(A)$^+$ RNA were separated at 10 V/cm on a 1% agarose gel with 2.2% formaldehyde and were transferred to a nylon membrane (Hybond; Amersham) (Maniatis et al. 1982).

Pulsed-Field Gel Electrophoresis (PFGE)

Preparation of high-molecular-weight YAC DNA and electrophoresis conditions have been described elsewhere (Gruen et al. 1992).

Probes and Hybridization Conditions

The following probes were used: LN11A, a cDNA probe from HLA-H, which cross-hybridizes with several MHC class I genes (Biro et al. 1983); ribosomal cDNA (Wilson et al. 1978); γ-actin cDNA (Gunning et al. 1983); and PCR-amplified short-fragment cDNA clones from the selected library. Hybridization conditions and oligo-labeling of probes have been described elsewhere (Gruen et al. 1992). Repetitive sequences were directly competed by prehybridizing 40 ng of each probe with 350 μg of sheared human placental DNA in a 100-μl volume for 1–2 h at 65°C (Sealey et al. 1985).

Results

Enrichment of the Selected Library

Short-fragment cDNAs representing expressed sequences in a target genomic region were isolated by a method of cDNA hybridization selection in which a normal human duodenum cDNA library was hybridized to a 320-kb, HLA-A-containing YAC. The selected fragments were cloned into λgt-10 to form a selected library that contained 1×10^6 plaque-forming units. To determine the efficacy of the selection, the starting cDNA library and the selected library were compared for content of a number of human probes. Phage filters of each library were screened sequentially with the following: LN11A, a probe that identifies HLA class I genes; probes of genes not encoded on the YAC, rRNA, and γ-actin cDNAs; and VLG51, one of the selected cDNA clones. The selected library contained a >100-fold increase in the number of LN11A-positive clones, a $>10^4$-fold increase in VLG51-positive clones, a >10-fold decrease in ribosomal-positive clones, and an 8-fold decrease in γ-actin–positive clones, when compared with the starting library (table 1). These results indicated that the selected library was enriched for sequences encoded on the YAC.

Representation of the HLA-A Gene in the Selected Library

To examine a possible bias in the selection for the 5′ or 3′ region of genes, clones containing HLA-A sequence were analyzed and compared with HLA-A published sequence. In eight clones containing HLA-A, sequence identical to exons 2–5 and 7 and intron 3 were recovered, accounting for most of the gene (fig. 1). Therefore, most HLA-A exons were represented by clones in the selected library. The clone containing intron 3 most likely represents an incompletely spliced mRNA.

Sequence Analysis of 92 Selected Clones

Sequence data from 92 isolated phage clones were obtained and compared with each other as well as with

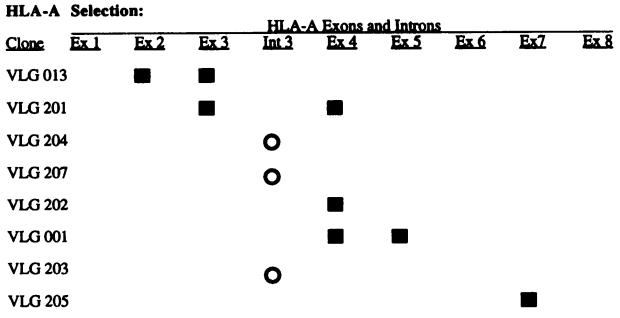

Figure 1 Distribution of HLA-A exons among selected clones. Sequence analysis of nine selected cDNA clones containing class I identity shows that they were well distributed across the 5′, middle, and 3′ regions of the HLA-A gene. Each clone is diagramed under its corresponding exon (■) or intron (O).

Table 2

Analysis Based on Sequence Information of 92 Clones from the Selected Library

Selected Clone	No.
Background coselection:	
rRNA	4
Line 1 repeats	5
Mitochondria	2
Thymosin	1
Subtotal	12 (13%)
Novel sequences	70 (76%)
HLA-A	10 (11%)
Total sequenced	92 (100%)

the GenBank and EMBL databases. The sequence comparisons served both to identify clones containing HLA-A sequence, other known sequences, and novel clones and to segregate the novel clones into groups that share sequence identity (table 2). Seventy clones (76%) had no sequence homology to entries in GenBank or EMBL. Ten clones (11%) contained sequence identical to that of HLA-A. Twelve (13%) were coselected clones containing sequence, such as rRNA, line 1 repeat, human mitochondria, and β-thymosin, not encoded on the YAC. These represented background carryover of clones present in great abundance in the starting library and were further exaggerated by PCR amplification.

Grouping the Novel Clones into Clusters and Singlets

The 70 novel clones were sequenced and compared with each other. Six of the 70 clones contained sequence that was identified only once among the analyzed clones, and these were called "singlets" (only three of these are included in this report). The remaining 64 clones segregated into eight clusters (A–F, I, and K). Each cluster consisted of a group of cDNA clones that either were identical or shared overlapping sequence. Sequential hybridizations of dot blot arrays with a representative clone from each cluster and singlet confirmed the group assignment determined by sequence analysis.

Physical Map of Clones on YAC B30H3

High-resolution physical mapping of 10 novel clones —VLG51 (D), VLG7 (B), VLG28 (E), VLG63 (F), VLG10 (C), VLG52 (K), VLG9 (A), VLG2 (I), VLG20, and VLG35—on PFGE blots (fig. 2) localized them to their cognate genomic sequences on YAC B30H3. The

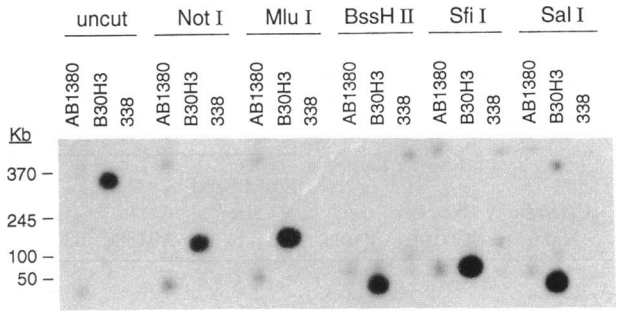

Figure 2 Representative PFGE blot. DNA from host yeast AB1380, B30H3, and 338 (a CEPH library YAC that overlaps B30H3) were digested with rare-cutting restriction enzymes, separated by PFGE, transferred to a nylon membrane, and hybridized with VLG51 (clone from cluster D). Restriction enzymes are noted above the lanes.

novel clones isolated were distributed throughout the YAC (fig. 3). They localized to six distinct restriction fragments. The relative position of clones that shared the same restriction fragment could not be determined.

Southern Blot Analysis

To further verify that the novel clones were derived from YAC B30H3, three of the singlets and a representative clone from each of the eight clusters were hybridized to a Southern blot containing *Eco*RI-digested DNA from YAC B30H3, JY LCL, and the R66 radiation-reduced hybrid cell line. All the clones tested hybridized to YAC B30H3 and identified restriction fragments of similar size in human and radiation-reduced hybrid genomic DNA, confirming that the novel clones had corresponding sequence in the YAC, in the human genome, and on 6p21.3. Each clone produced a unique

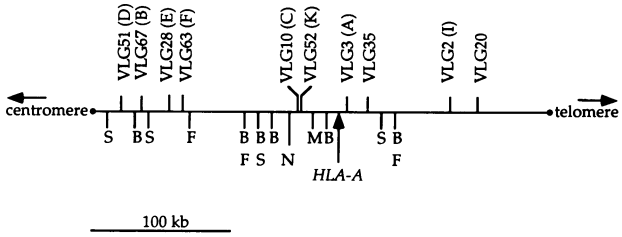

Figure 3 Physical map of the novel gene fragments on YAC B30H3, generated by PFGE. Represented above the line are the positions of the gene fragments relative to the HLA-A gene, centromere, and telomere. Below the line are the positions of the restriction sites for *Not*I (N), *Mlu*I (M), *Bss*HII (B), *Sal*I (S), and *Sfi*I (F). The relative position of each gene fragment on a given restriction fragment is not known.

Table 3

Results of Hybridization of Selected cDNA Clones from Each Cluster and Singlet to DNA and RNA Blots

			DNA Blot[a]						Poly(A)$^+$ RNA Blot[a]			Hep G2 or Hutu 80 RNA Blot[a]			
Cluster Name	No. of Clones	Probe	*Eco*RI Band Size (kb)	AB1380	B30H3	JY	R66	CHO-K	Band Size (kb)	JY	Ileum	Band Size (kb)	Poly(A)$^+$	Poly(A)$^-$	Total
A	9	VLG3	23	−	+	+	+	−	2	+	+	2	−	+	+
			4	−	+	+	+	−	1	+	−	1	−	−	−
B	14	VLG7	7	−	+	+	+	−		−	−	2.2	+	−	−
			2.3	−	+	+	+	−							
C	12	VLG10	2.3	−	+	+	−	−	2	+	+	2	+	−	−
D	14	VLG51	3.3	−	+	+	+	−	4	+	+	4	+	−	−
									2.4	+	+				
E	8	VLG28	1.9	−	+	+	+	−	1	+	+	1	+	+	+
												.5	+	+	+
F	3	VLG63	2	−	+	+	+	−	2.5	+	+	2.2	+	+	+
												1	+	−	−
I	2	VLG2	3.8	−	+	+	+	−		−	−	2.2	−	±	±
K	2	VLG52	21	−	−	−	±	±	2	+	+	2	+	−	−
			5	−	+	+	+	−				1	+	−	−
			2.3	−	+	+	−	−							
Singlet		VLG20	20	−	+	+	+	−		−	−	2.2	+	−	−
			8	−	+	+	−	−							
Singlet		VLG35	15	−	+	sm	sm	sm	2.2	+	+	4.4	+	−	−
												2.4	+	−	−
Singlet		VLG84	3	−	+	−	sm	−		−	−	2.2	+	±	±
												1	+	±	±

[a] − = no visible band; + = presence of a strong band; ± = presence of a faint band; and sm = smear.

banding pattern on Southern hybridization. The results are summarized in table 3. A representative Southern blot hybridized with VLG51 is shown in figure 4. Seven of the cDNA probes yielded a single *Eco*RI fragment in the YAC and genomic DNA, which suggested that they represent single-copy genes. Clusters A, B, and K and singlet 20 resulted in more than one *Eco*RI restriction fragment, which suggested either the presence of an *Eco*RI site in the gene or that they belonged to a family of duplicated genes or pseudogenes.

Northern Blot Analysis

The same 11 clones, three singlets, and a representative clone from each cluster were hybridized to northern blots containing (*a*) poly(A)$^+$ RNA from JY LCL and human small intestine and (*b*) RNA from either Hep G2 or Hutu 80 cell lines. The results are presented in table 3. Representative northern blots hybridized with VLG51 are shown in figure 4. Five cDNA clones hybridized to a single band presumably representing a single mRNA species. Six clones identified two or more sized transcripts, possibly because of differential splicing of mRNA, cross-hybridization to another mRNA species, or the isolation of a chimeric clone from the starting library. Clone VLG3 from cluster A showed no detectable band on poly(A)$^+$ RNA but showed strong bands on total and poly(A)$^-$ RNA. An explanation for the absence of signal in the Poly(A)$^+$ RNA lane may be that this gene either lacks a poly(A)$^+$ tail or has a very short poly(A)$^+$ tail and therefore was not captured on the oligo-dT cellulose during the purification step.

Nine different northern banding patterns produced by the 11 clones suggested that they represented at least nine different genes. Although cluster B and singlet VLG20 produced similar northern blot patterns, physical mapping of these two clones indicated that they were separated by ≥180 kb of genomic DNA that contained intervening genes. This suggested that cluster B and VLG20 originated from two different genes that coincidentally identified RNA transcripts of similar size. Therefore, the number of non-HLA genes encoded on YAC B30H3 and isolated from the duode-

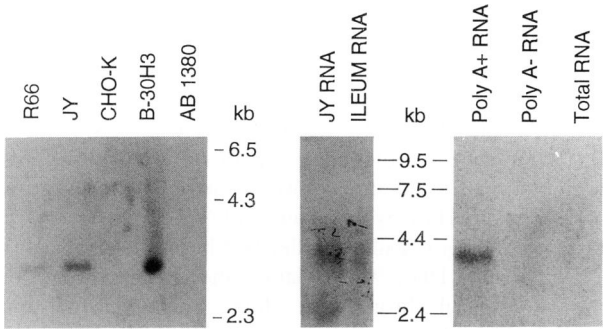

Figure 4 *Left,* Southern blot containing *Eco*RI-digested genomic DNA from R66, JY, CHO-K, B30H3, and AB1380. *Middle and right,* Northern blots; the middle panel contains poly A^+ RNA from JY and small intestine, and the right panel contains poly$(A)^+$, poly $(A)^-$, and total RNA from the cell line Hep G2. All three blots were hybridized with VLG51, a clone from cluster D.

num cDNA library is likely to be ≥10. This does not represent an exhaustive search of all the coding sequences on YAC B30H3, because only one cDNA library was selected and only 92 clones from the resulting selected library were analyzed.

Discussion

Efficacy and Limitations of cDNA Hybridization Selection

We report the use of a cDNA selection method to isolate 10 novel cDNA clones representing fragments of genes from the HLA-A region. This cDNA selection method is rapid. Once all the materials are prepared, the selected short-fragment cDNA library is generated in <2 wk. Enrichment of the selected library by clones containing sequence encoded on the YAC was demonstrated. Most regions of a given gene can be well represented, as demonstrated by the analysis of the HLA-A-containing clones. Sequences encoded on a target genomic DNA segment can be identified and isolated as long as they are present in the cDNA library used for the selection. It is a sensitive method for identifying low-abundance genes as demonstrated by northern blot analysis using poly$(A)^+$ RNA, but the absolute sensitivity of this method to identify very rare genes is unknown and would require the characterization of a large number of clones. Sensitivity may be enhanced by using normalized libraries in the selection process (Patanjali et al. 1991). A possible limitation to this approach is that in order for a transcript to be selected it must be present in the starting cDNA library. This can be overcome by performing selection with pools of cDNA libraries from several tissue types and developmental stages, which will then contain a broad representation of mRNA. Another limitation is that pseudogenes present in the target genomic DNA would also select cDNA clones and cannot be differentiated from the expressed gene.

Advantage over Other Methods

cDNA selection has advantages over other methods that have been used to identify coding sequences from a large genomic area. Labeled large genomic fragments used as probes to screen cDNA libraries result in high background and low sensitivity because of poor specific activity of individual coding sequences. HTF-island mapping is biased toward the identification of ubiquitously expressed mRNAs such as housekeeping genes, whereas cDNA selection detects 5′ and 3′ exons, as well as genes that lack HTF islands. To isolate coding sequences, cDNA selection does not rely on either sequence identification or the presence of a motif; therefore, novel sequences that do not contain previously described motifs can be identified. Exon trapping will not detect genes that do not contain introns, and it is prejudiced by splicing events in the packaging cell line that may be temporally regulated or tissue specific. cDNA selection is insensitive to the number and size of the introns, cryptic splice sites, or signals in intergenic DNA that resemble splice sites.

Others have described similar methods, which have in common the PCR rescue of selected cDNAs after specific hybridization of a PCR-amplified cDNA library to a genomic DNA target. Morgan et al. (1992) demonstrated a 10^3–10^6-fold enrichment, and Korn et al. (1992) identified a $5–8 \times 10^5$-fold enrichment, of their control gene and newly identified transcripts, after two rounds of selection. In our study a $>10^4$-fold enrichment of VLG51 was demonstrated after two rounds of selection. Enrichment was further demonstrated by the LN11A, rRNA, and γ-actin hybridizations summarized in table 1. The apparent lower level of enrichment of class I clones may be due to cross-hybridization of LN11A to other class I genes and pseudogenes present in the starting library, thereby increasing the apparent abundance of HLA-A in the starting library and minimizing the overall enrichment in the selected library. The selected library should contain reduced numbers of ribosomal and γ-actin clones, because (*a*) ribosomal sequences were used as blocking agents prior to the selection hybridization and (*b*) γ-actin is not encoded on B30H3.

Gene Density in the HLA-A Region

Application of this cDNA selection method toward the identification of the gene responsible for hemochromatosis has yielded novel gene fragments representing as many as 10 candidate genes encoded by YAC B30H3. Kahloun et al. (1992) identified seven new genes from the HLA-A region by screening a cDNA library with radiolabeled, digested YAC B30 DNA. Some of the genes described by them may be common to the gene fragments presented in the present report. In addition, the HLA-A gene and at least four HLA pseudogenes are encoded on this YAC (Geraghty et al. 1992). Therefore, there is a minimum of 11 genes on this 320 kb of genomic DNA, which represents a density of approximately one gene per 30 kb. This density is comparable to that found in the MHC class II (Ragoussis et al. 1991) and class III regions (Sargent et al. 1989), which are estimated to contain one gene per 20–50 kb of genomic DNA.

The novel gene fragments isolated are useful as single-copy probes for northern and Southern blot analysis, for screening larger-insert cDNA libraries, and for genetic mapping. The possible relevance of each of these clones to the hemochromatosis gene is being investigated.

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