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LETTER

A Transcript Map for the 2.8-Mb Region Containing the Multiple Endocrine Neoplasia Type 1 Locus

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Multiple endocrine neoplasia type 1 (MEN 1) is an inherited cancer syndrome in which affected individuals develop multiple parathyroid, enteropancreatic, and pituitary tumors. The locus for *MEN1* is tightly linked to the marker *PYGM* on chromosome 11q13, and linkage analysis places the *MEN1* gene within a 2-Mb interval flanked by the markers *D11S1883* and *D11S449*. Loss of heterozygosity studies in MEN 1 and sporadic tumors suggest that the *MEN1* gene encodes a tumor suppressor and have helped to narrow the location of the gene to a 600-kb interval between *PYGM* and *D11S449*. Focusing on this smaller *MEN1* interval, we have identified and mapped 12 transcripts to this 600-kb region. A precise ordered map of 33 transcripts, including 12 genes known to map to this region, was generated for the 2.8-Mb *D11S480–D11S913* interval. Fifteen candidate genes (of which 10 were examined exhaustively) were evaluated by Southern blot and/or dideoxy fingerprinting analysis to identify the gene harboring disease-causing mutations.

[The sequence data described in this paper have been submitted to GenBank under accession nos. EST06996, U93236, AF001540–AF001547, AF001433–AF001436, AF001891–AF001893, N55476, R19205, and W37647 (see Table 1 for listing of transcripts). The BAC clone sequences have been submitted to GenBank under accession nos. AC000134, AC000159, and AC000353.]

Multiple endocrine neoplasia type 1 (MEN 1) is an autosomal dominant disorder in which affected individuals develop variable combinations of parathyroid, anterior pituitary, endocrine pancreatic, and duodenal tumors (Metz et al. 1994). The *PYGM* (muscle glycogen phosphorylase) marker at chromosome 11q13 originally was found to be linked to MEN1, showing no recombination with the MEN1 phenotype (Larsson et al. 1988; Nakamura et al. 1989; Petty et al. 1994; Kytola et al. 1995; Smith et al. 1995; Courseaux et al. 1996). However, recombinants between the MEN1 phenotype and more centromeric and telomeric markers have narrowed the *MEN1* locus to a ~2-Mb interval flanked by the markers *D11S1883* and *D11S449* (Courseaux et al. 1996; Debelenko et al. 1997a). The *MEN1* gene is suspected to encode a classic tumor suppressor gene based on the observed loss of heterozygosity (LOH) for markers at 11q13 present in MEN 1 tumors, as well as in sporadic tumors of the types seen in fa-

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milial MEN 1 (Larsson et al. 1988; Friedman et al. 1989, 1992; Debelenko et al. 1997b; Dong et al. 1997). Tumor deletion studies have suggested that the disease locus lies just telomeric to *PYGM*, narrowing the location of the *MEN1* gene to a 600-kb region between *PYGM* and *D11S449* (Bystrom et al. 1990; Lubensky et al. 1996; Emmert-Buck et al. 1997).

At present only two genes, *ZFM1* (Toda et al. 1994) and *FAU1* (Kas et al. 1993a), have been assigned to the minimal *MEN1* interval, *PYGM–D11S449* (Courseaux et al. 1996). Both genes have been excluded as candidates for MEN 1 based on mutation analysis (Kas et al. 1993b; Courseaux et al. 1996). Here we report the isolation, mapping, characterization, and mutation analysis of 21 transcripts contained in the 1-Mb region from *PYGM* to *D11S4907*, with the most intensive effort focusing on the genes in the *PYGM* to *D11S449* interval. The transcript map, however, includes 33 transcripts spanning the 2.8-Mb interval between *D11S480* and *D11S913*.

RESULTS

Mapping of 12 Known Genes to the Contig

A high-density clone contig-based physical map was generated for the 2.8-Mb region between *D11S480* and *D11S913* (Guru et al. 1997). This clone contig was used to precisely map 12 genes known to map to this general vicinity. Four of these genes, *ZFM1* (Toda et al. 1994), *FAU1* (Kas et al. 1993a), *NOF1* (Kas et al. 1996), and *CAPN1* (calpain) (Ohno et al. 1990), mapped to the *PYGM–D11S449* region, the minimal interval for the *MEN1* gene (Fig. 1). A group of four additional genes, *PLCβ3* (Weber et al. 1994), *FKBP2* (Grimmond et al. 1995), *PNG* (Lagercrantz et al. 1996), and *VRF* (Grimmond et al. 1996), were located centromeric to *PYGM*, close to markers *D11S599* and *D11S457*. These results are consistent with the recent Fifth Chromosome 11 Workshop Report and an integrated physical map generated by the European Consortium on MEN 1 (Courseaux et al. 1996; Shows et al. 1996). An earlier report placed the group of *PLCβ3/FKBP2/PNG/VRF* on the telomeric side of *PYGM*, apparently because of the incorrect mapping of *D11S457* (Weber et al. 1994).

Two other genes, *RELA* (Deloukas et al. 1994) and *TIP60* (Kamine et al. 1996), were reported previously to map to the 11q13 region based on FISH and somatic cell hybrid mapping. Both transcripts were found to be located within the cosmid clone c132h3 in the *D11S449–D11S4907* region. Another gene, *MLK3* (Ing et al. 1994), was also mapped close to *RELA* and *TIP60*, outside the *MEN1* distal boundary defined by the marker *D11S449*.

Identification and Mapping of 21 Other Transcripts

New transcripts identified in the present investigation were identified primarily by BLAST and GRAIL analysis of genomic sequences generated for the *MEN1* region (*D11S1883–D11S4933*). The sequences originated from three different sources: (1) end sequencing of yeast artificial chromosomes (YAC, y) and bacterial [P1-derived artificial chromosome (PAC, pc), bacterial artificial chromosome (BAC, b), P1 (p), and cosmid (c)] clones (Guru et al. 1997), (2) sequencing BAC clones b137C7, b79G17, and b18H3 (GenBank accession nos. AC000134, AC000159, and AC000353, respectively), and (3) sequences available in the public database (http://mcdermott.swmed.edu/datapage/11_seq_project/) for three overlapping cosmids, cSRL 23c3/116b6/114g4 (denoted as sequence contigs C1, C2, and C3 in Fig. 1), and a cosmid cSRL32h5 that we mapped to the *PYGM–D11S449* region (Guru et al. 1997). The identified transcripts were categorized into the following four groups.

Known Human Genes Not Previously Known to Map to This Region

The BLAST analysis against National Center for Biotechnology Information (NCBI) databases identified four known genes: *GC-kinase* (Katz et al. 1994), *PP2AB56β* (McCright et al. 1996), *ARL2* (Clark et al. 1993), and *DNA polα* (Collins et al. 1993). The T7 end sequence of the BAC clone b138M20 matched *PP2AB56β*. *DNA polα* and *GC-kinase* were identified by BLAST analysis of sequence generated from BAC clones b18H3 and b137C7, respectively. *ARL2* was discovered by similar analysis of a 46-kb cosmid sequence (C2) derived from overlapping cosmids cSRL23c3/116b6/114g4, as mentioned above.

Human Homologs of Genes Known in Other Species

Three genes, *HPAST*, *HREQ*, and *Neurexin*, belong to this category. Sequence from b79G17 identified *HPAST*, whose name was based on its homology to the *Drosophila PAST-1* gene (GenBank accession no. U70135). Similarly, sequences from the end of a PAC clone pc195F17 (SP6-end) identified a gene homologous to the mouse *Requiem* (GenBank accession no. U10435) and therefore the human homolog was named *HREQ*. Both the b137C7 sequence and the end sequence of cosmid c16F4 showed homology to rat *Neurexin II-alpha* (GenBank accession no. M96376). The human homolog is being characterized.

Transcripts of Unknown Function with Expressed Sequence Tag Matches

EST matches with genomic sequence identified a total of 12 new transcripts. The ESTs were mapped back to the clones in the contig before further characterization. Independent newly identified transcripts were named with sequential Greek letters. At least two of the following criteria were required to validate a putative transcript: (1) EST matches from a minimum of two cDNA clones from different tissue sources, (2) expression of the message on Northern blots, (3) presence of a poly(A) signal followed by a track of As, and (4) comparison of the cDNA versus the genomic sequence for the presence of interrupted exons.

Two ESTs (*D11S1957E* and *D11S951E*) known to map to the 11q13 region based on a radiation hybrid map (James et al. 1994) were found to identify a single transcript of 8.5 kb, which was named *Alpha*. Transcripts *Beta, Gamma, Delta,* and *Lambda* were identified from EST hits homologous to end sequences of clones, yA87A1(left-end), b138M20(SP6-end), p47F3(SP6-end) and c72C4(T7-end), respectively. Transcripts *Mu* and *Nu* were identified based on the EST hits from the BAC clone b137C7, and *Epsilon, Zeta, Eta,* and *Theta* were defined from b18H3 sequence matches. *Iota* was identified from EST matches to the cosmid sequence contig C2; upon completion of the *Iota* cDNA sequence, the first exon was found to be in the 46-kb C2 cosmid sequence contig, and five additional exons were found in the 6.8-kb C3 sequence contig from cosmid cSRL114g4 (Fig. 1).

Transcripts of Unknown Function Derived from Software Prediction of Exons

One transcript, *Xi*, was identified solely based on exons predicted by GRAIL and FEXH. Another transcript, *Kappa*, was identified based on potential coding region similarity (BLASTX) to several kinases in the protein databases and on exon predictions by GRAIL and FEXH. Validation as bona fide transcripts was accomplished by amplification of neighboring exons from cDNA, followed by sequencing and Northern blot analysis.

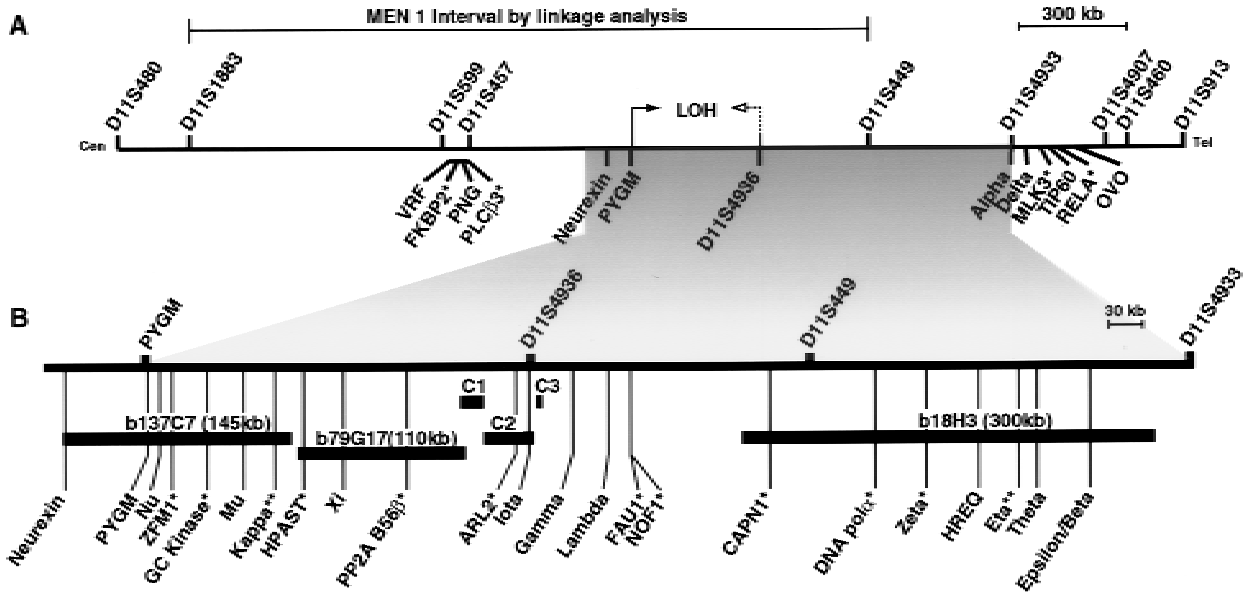

Figure 1 Transcript map of 2.8-Mb region containing the *MEN1* locus in the interval *D11S480–D11S913.* (*A*) The *MEN1* interval identified by linkage analysis spanning from *D11S1883* to *D11S449* is shown, with the region from *PYGM* to *D11S4936* marked by arrows as the minimal interval inferred from LOH data. The arrow with a dotted line represents the boundary determined from a sporadic gastrinoma. (*B*) The 1-Mb region from *PYGM* to *D11S4933* is expanded for clarity. Some relevant markers are indicated above the map and the transcripts are identified below. Sizes of BAC clones (b137C7, b79G17, and b18H3) are given. Sequence contigs from cosmids C1, C2, and C3 are 14 kb, 46 kb, and 6.8 kb, respectively. (*) Genes that have been eliminated as MEN1 candidates by mutation detection analysis. Mutation analysis is incomplete for the *Kappa* and *Eta* (**). Identification and mapping of *OVO* will be reported independently (A. Chidambaram, R. Allikmets, S.C. Chandrasekharappa, S.C. Guru, W. Modi, B. Gerrard, and M. Dean, in prep.).

Predicted exons from three b137C7 sequence contigs (Fig. 2) were found to be part of the same transcript (*Kappa*), because each of them hybridized to an 8-kb message on Northern blots, and mapped back to the same interval on the genomic clones of our contig (data not shown). The predicted exons were analyzed by PCR amplification from a leukocyte cDNA library (Fig. 2). The sequence from the five exons in sequence contig 194 were present in the PCR product amplified from the leukocyte cDNA library, precisely as predicted by GRAIL. However, three differences were observed in sequence contigs 168 and 200. GRAIL failed to predict an exon in each contig (Fig. 2, asterisk). In addition, an intron predicted by GRAIL in contig 200 was found to be part of the cDNA (Fig. 2, arrow). This very small (33-nucleotide) intron might have been predicted to be biologically unlikely.

Characterization of Transcripts

All the transcripts were analyzed for expression and size. This usually was carried out by probing one or more of three commercially available Multiple Tissue Northern (MTN) blots (Human 7760-1, HumanII 7759-1, and human endocrine blot, Human 7751-1, Clontech). The probes used varied from PCR-amplified exons as small as 120 nucleotides to complete cDNA, but in all cases, complete or nearly complete cDNA probes were eventually used for hybridization. Typical examples of the expression analysis are shown in Figure 3. A single 2.8-kb transcript was detected in all the tissues from MTN and endocrine blots with both a 220-nucleotide PCR product and a 1.8-kb cDNA probe from the *Mu* transcript. On the other hand, two transcripts, the 4.4-kb *Epsilon* and the 19-kb *Beta,* were seen with an 8.8-kb probe. However the 2.8-kb probe (Fig. 3C) detected only the 19-kb *Beta* transcript (data not shown). Both *Epsilon* and *Beta* are transcribed as intronless RNAs from the same start site, with *Epsilon* using a poly(A) signal at 4.4 kb, whereas the *Beta* transcript uses a downstream poly(A) signal. Thus, these two transcripts, originally interpreted as separate, now seem to represent polyadenylation isoforms.

Table 1 lists a number of features of 23 transcripts in the *PYGM–D11S4933* interval. There were two genes, *Gamma* and *Eta,* where expression was not visible on Northern blots. Presumably these are low abundance messages; they were considered bona fide transcripts based on EST matches, amplification from RNA, and the presence of poly(A) signal at the 3′ end. Transcript *Nu* was expressed in only 4 out of the 16 tissues tested: leukocytes, thymus, spleen, and stomach. The remaining transcripts showed roughly similar expression levels in the 8 to 20 tissues analyzed by probing Northern blots. The *Zeta* probe detected two transcripts, 2- and 2.2-kb in all the tissues tested, and sequencing of the cDNA indicated that the two transcripts differed by the use of two different poly(A) signals located 328 bases apart. The characterization of two different transcript sizes on Northern blots for *Lambda* and *Delta* has not yet been carried out.

Once a transcript was validated as bona fide and the size of the transcript was determined, cloning of the complete cDNA sequence was attempted. For three transcripts (*Zeta, Iota,* and *HREQ*), EST clones

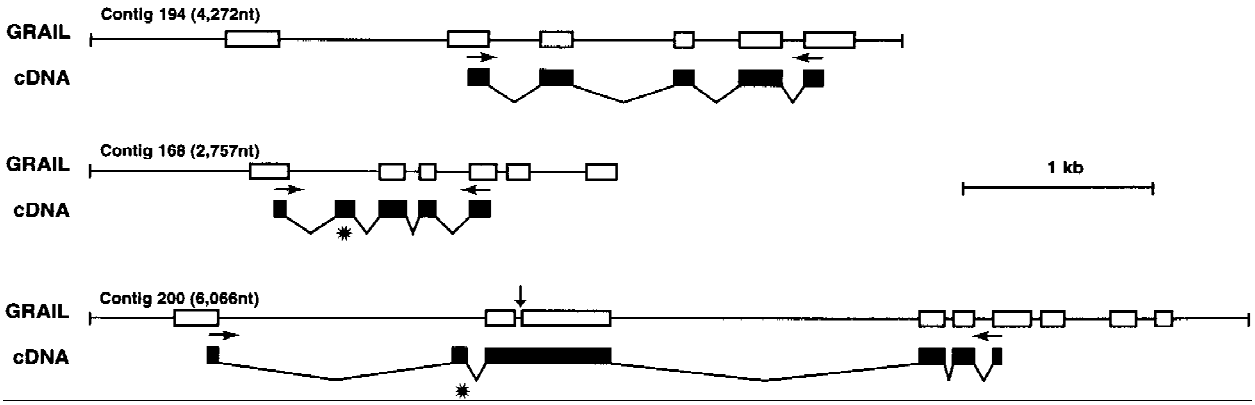

Figure 2 Comparison of GRAIL-predicted exons vs the cDNA sequence. GRAIL-predicted exons of b137C7 sequence contigs 194, 168, and 200 are shown in open boxes and compared with the corresponding exons identified by amplifying and sequencing cDNA, represented by solid boxes. (*) Two exons missed by GRAIL in contigs 168 and 200. A vertical arrow shows a 33-nucleotide intron predicted by GRAIL that is part of the exon in cDNA. Arrows indicate the primers used for amplifying the corresponding cDNA. All three contigs identified the same 8-kb transcript, denoted *Kappa,* on Northern blots.

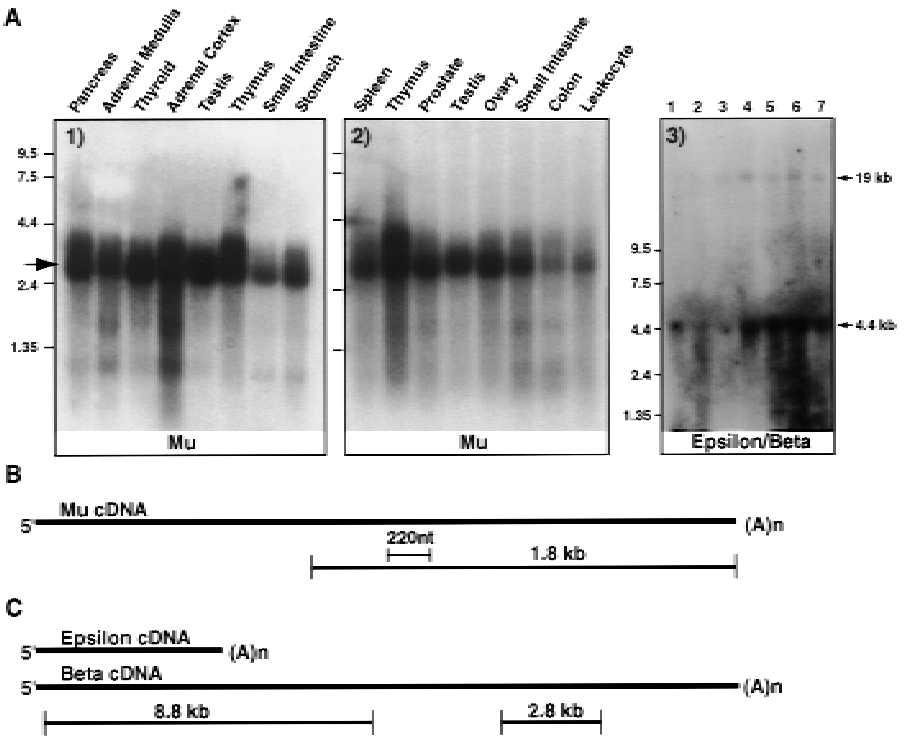

Figure 3 Representative Northern blots of new transcripts. New transcripts were analyzed for message size and expression levels by probing one or more of the three commercially available multiple tissue Northern blots. (*A, left*) Endocrine blot probed with a 1.8-kb *Mu* cDNA from the *Mu* transcript; (*middle*) blot probed with a 220-bp *Mu* cDNA. In both cases a 2.8-kb message is identified that is expressed in all tissues. (*A, right*) Blot containing total RNA from EBV-transformed lymphocytes from seven FMEN1 cases, probed with the 8.8-kb *Epsilon/Beta* probe. Two messages are seen: a more abundant 4.4-kb *Epsilon* transcript and a less abundant 19-kb message named *Beta.* (*B*) *Mu* cDNA and location of probes used for blots in *A left* and *middle.* (*C*) *Epsilon/Beta* cDNA and probes used in *A right.* Probes used in *A* are in *B* and *C.* The 2.8-kb probe in *C* independently confirmed the 19-kb *Beta* transcript, and did not detect *Epsilon* (data not shown).

in the database could be clustered into contigs covering the entire cDNA. A substantial portion of the remaining 11 transcripts, except *Kappa* and *Xi,* were assembled from overlapping ESTs from Unigene (Table 1). Efforts were then made to clone full-length cDNAs from libraries by various techniques: GENETRAPPER (GIBCO-BRL), 5′ and 3′ RACE (Clontech), PCR amplification with primers designed from GRAIL predicted exons, and PCR amplification of regions between EST clones. A single 2.8-kb cDNA clone containing the entire *Mu* transcript was isolated by the GENETRAPPER method. The cloning of a complete cDNA for another transcript, *HPAST,* was accomplished by characterizing two overlapping cDNA clones identified by EST matches, each hybridizing to the same 3.5-kb transcript. Incomplete clones for the following transcripts were recovered by at least one of the aforementioned methods: *Nu, Kappa, Xi, Theta, Epsilon/Beta, Lambda, Alpha,* and *Delta.* Also, because the expression and size of the message of *Gamma* and *Eta* could not be ascertained by Northern blot, it is not certain whether full-length cDNAs have been obtained.

The transcripts were analyzed for exon–intron structure by comparing the cDNA sequence to the available genomic sequence from the BAC or cosmid sequence contigs. For *HPAST, NOF1, Lambda,* and *MLK3,* cosmid clones containing the corresponding gene were directly sequenced by primers designed from the cDNA sequences.

Mutation Analysis of Candidate Genes

Candidate genes in the *MEN1* interval from *PYGM* to *D11S4933* were analyzed for the presence of mutations in familial MEN 1 (FMEN 1) patients by Southern blots for detecting gross structural changes and in more detail by dideoxy fingerprinting. The diagnosis of MEN 1 was based upon the presence of tumors in two of the three principal systems: parathyroid, enteropancreatic endocrine tissue, and anterior pituitary. Also, the diagnosis of FMEN 1 required at least one first-degree relative with a tumor of one or more of these systems (all participating family members gave full informed consent in a protocol approved by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Institutional Review Board). Table 1 includes a summary of the genes. Southern blot analysis was performed with *Taq*I- and *Hin*dIII-digested DNA from 32 FMEN1 affected individuals belonging to 32 different kindreds with full-length cDNA probes for 13 out of the 23 candidate genes (Table 1). No gross structural changes were observed in any of the candidate genes. In three instances (*HREQ, DNA pol*α,

Table 1. Characterization and Mutation Analysis of Transcripts Located between PYGM and D11S4933

Transcripts	GenBank ID no.	Size of mRNA (kb)	cDNA cloning	Mutation analysis of FMEN 1 patients[a]		Uni-Gene[b]	Gene Map[c]
				Southern	ddF		
Nu	EST06996	4.5	incomplete	not done	not done	yes	no
GC-kinase	U07349	2.9	GenBank	yes	yes	yes	no
Mu	U93236	2.8	complete	yes	yes[+]	yes	yes
Kappa	AF001543, AF001544	8	incomplete	not done	partial[++]	no	no
HPAST	AF001434	3.5	complete	yes	yes	yes	yes
Xi	AF001545	8	incomplete	not done	not done	no	no
PP2AB56β	L42374	2.5	GenBank	yes	yes	yes	yes
ARL2	L13687	0.9	GenBank	yes	yes	yes	no
Iota	AF001435	2.2	complete	not done	not done	yes	yes
Gamma	AF001547	no signal	1.6 kb	not done	not done	yes	yes
Lambda	AF001891	1.4/3.5*	incomplete	not done	not done	yes	no
NOF1	U39400	2.2	GenBank	yes	yes	yes	yes
DNA polα	L24559	2.2	GenBank	yes	yes	yes	yes
Zeta	AF001436	2.0/2.2*	complete	yes	yes	yes	yes
HREQ	AF001433	2.5	complete	yes	not done	yes	yes
Eta	AF001546	no signal	1.2 kb	not done	partial[+++]	yes	yes
Theta	N55476, R19205	4	incomplete	not done	not done	yes	no
Epsilon/Beta	AF001892, AF001893	4.4/19.0*	incomplete	yes	not done	yes	yes
Alpha	AF001540, AF001541, AF001542	8.5	incomplete	not done	not done	yes	yes
Delta	W37647	0.7/2.8*	incomplete	not done	not done	yes	no
MLK3	L32976	3.5	GenBank	yes	yes	yes	yes
RELA	L19067	2.5	GenBank	yes	yes	yes	yes
TIP60	U40989	2.9	GenBank	yes	not done	yes	yes

Four known genes from this region, *ZFM1* (D26120), *FAU1* (X65923), *CAPN1* (X04366), and *OVO* (A. Chidambaram, R. Allikmets, S.C. Chandrasekharappa, S.C. Guru, W. Modi, B. Gerrard, and M. Dean, in prep.), were not included here as they have previously been excluded as candidates for MEN 1. (*) Two messages on Northern blot.

[a](+)Mutation analysis by dideoxy fingerprinting identified Mu as the *MEN1* gene (Chandrasekharappa et al. 1997); (++) 22 exons excluded (+++) available 1.2 kb, excluded.

[b]Presence of EST matches with the transcript in Unigene is indicated by 'yes' and absence by 'no' (http://www.ncbi.nlm.nih.gov/Schuler/UniGene/Chr11.html).

[c]Presence of EST matches with the transcript on the Gene Map is indicated by 'yes' and absence by 'no' (http://www.ncbi.nlm.nih.gov/SCIENCE96/ResTools.html).

and *GC-kinase*), variant bands were seen that were analyzed further in normal controls and confirmed as benign restriction fragment length polymorphisms. Dideoxy fingerprinting analysis was performed for 12 candidate genes (Table 1). Genomic DNA samples from 16 different FMEN 1 kindreds and 10 normal controls were included in the analysis. For each transcript, the coding region from ATG to stop codon (if known) was screened except for *Kappa* and *Eta*. In 11 genes, no significant changes were seen that segregated with the disease. Isolated DNA samples in some of the genes showed dideoxy fingerprinting band pattern changes that were followed further by sequencing to identify the sequence change. For *GC-kinase*, two different heterozygous sequence changes were identified: exon 5 GCA to TCA (A120S) and exon 24 CGC to CAC (A579H). *NOF1* had one instance of a heterozygous G to A sequence change in exon 1, ATG to ATA (M5I). One patient had a heterozygous sequence

change in *Eta* from CAG to AAG (E159K), and one patient had AGG to AAG (R152K) in *Zeta*. These changes were further analyzed in other affected family members of the kindred, and none of them were linked with the disease phenotype. The sequence changes found in *NOF1*, *Eta*, *Zeta*, and *MLK3* are not likely to be disease-causing because these genes also lie outside the distal flanking marker *D11S449*, and therefore are excluded from the minimal 600-kb *MEN1* interval between *PYGM* and *D11S449* (Bystrom et al. 1990; Courseaux et al. 1996; Lubensky et al. 1996; Emmert-Buck et al. 1997). An additional four variants that were seen in *GC-kinase*, *Kappa*, *ARL2*, and *DNA polα*, which were also present in normal DNA samples, were found to be benign polymorphisms and therefore could be used as polymorphic markers for this region (Manickam et al. 1997). Additionally, nine dideoxy fingerprinting polymorphic band pattern changes seen only in normal controls (two each in *GC-kinase*, *HPAST*, and *PP2AB56β*, and one each in *Kappa*, *Eta*, and *Zeta*) were not followed further. Also, five ddF polymorphic band pattern changes that were equally distributed in patients and normal controls, three of which were seen in *Eta* and two in *MLK3*, were not followed further. Finally, dideoxy fingerprinting changes identified in *Mu* were found in multiple individuals affected with MEN1, allowing its assignment as the *MEN1* gene (Chandrasekharappa et al. 1997).

DISCUSSION

A map consisting of 33 transcripts was generated for the 2.8-Mb *D11S480–D11S913* interval at 11q13. Twenty-seven of these transcripts map telomeric to *PYGM*, in what appears to be a fairly gene-rich region. Four previously identified genes, *GC-kinase* (Katz et al. 1994), *PP2AB56β* (McCright et al. 1996), *ARL2* (Clark et al. 1993), and *DNA polα* (Collins et al. 1993), not known previously to map to this region, were precisely mapped. Thirteen new transcripts were isolated, including three human homologs of previously known genes, one for the *Drosophila PAST-1* gene, another for rat *Neurexin II-alpha*, and the third for mouse *Requiem*.

The gene cluster of *PLCβ3/FKBP2/PNG/VRF*, reported earlier to be telomeric to *PYGM* (Weber et al. 1994; Grimmond et al. 1995, 1996; Lagercrantz et al. 1996), now is shown to map centromeric to *PYGM*. The *FAU1* gene was also reported to be 90 kb proximal to *PYGM* (Kas et al. 1993a), but we find it to be nearly 400 kb distal to *PYGM*. These results are consistent with two other more recent efforts to generate integrated maps for this region of chromosome 11 (Courseaux et al. 1996; Shows et al. 1996).

All but two of the new transcripts were identified as EST matches with the genomic sequences (Table 1). The two exceptions were *Kappa* and *Xi*, which were identified as GRAIL-predicted exons from the two BAC clones b137C7 and b79G17, respectively. GRAIL analysis of genomic sequence contigs with good to excellent matches were characterized in detail by amplifying the exon sequences from a leukocyte cDNA library and comparing the sequences, as shown in Figure 2. Fifteen exons predicted by GRAIL were verified by amplifying from cDNA and sequencing (Fig. 2). However, two exons were missed by GRAIL. In addition, a 33-nucleotide sequence in contig 200 perceived as an intron by GRAIL was found to be part of an exon in cDNA. Though *Kappa* and *Xi* are ~8 kb transcripts, each gave a distinct tissue-specific expression pattern on MTN blots (data not shown). This along with their origin from two nonoverlapping BAC clones indicate that *Kappa* and *Xi* are independent transcripts.

Our map includes 15 transcripts mapping to the 600-kb *PYGM*–D11S449 *MEN1* interval. Previous reports include only two transcripts, *ZFM1* (Courseaux et al. 1996) and *FAU1* (Kas et al. 1993b), in this interval, both of which had been excluded by mutation analysis as candidate genes for MEN1. Another gene, *CAPN1* (Pang et al. 1996), excluded previously by mutation analysis, also maps to this region. LOH studies in MEN 1 and sporadic tumors with several new markers have subsequently resulted in narrowing the *MEN1* locus to a 300-kb *PYGM–D11S4936* region (Emmert-Buck et al. 1997), though the distal boundary has to be considered provisional because it was based on LOH in a sporadic gastrinoma. Eight transcripts (*Nu*, *GC-kinase*, *Mu*, *Kappa*, *Xi*, *HPAST*, *PP2AB56β*, and *ARL2*) lie within this new minimal *MEN1* interval and therefore were of greatest interest.

Genes excluded from the MEN1 linkage interval (*D11S1883–D11S449*) are *DNA polα*, *Zeta*, *HREQ*, *Eta*, *Theta*, *Beta/Epsilon*, *Alpha*, *Delta*, *MLK3*, *RELA*, *TIP60*, and *OVO*, all located distal to *D11S449* (Fig. 1). However, the new LOH distal boundary, *D11S4936*, excludes *Iota*, *Gamma*, *Lambda*, *FAU1*, *NOF1*, and *CAPN1*, as well. *PYGM* as a proximal boundary eliminates *PLCβ3*, *FKBP2*, *PNG*, *VRF*, and *Neurexin*. Among these genes, *PLCβ3* (de Witt et al. 1997; Weber et al. 1997) and *FKBP2* (Grimmond et al. 1995) have also been analyzed previously for mutations in MEN1 kindreds and excluded. Nine additional genes, *GC-kinase*, *HPAST*, *PP2AB56β*, *ARL2*, *NOF1*, *DNA polα*, *Zeta*, *MLK3*, and *RELA*, have been

excluded by Southern blot and dideoxy fingerprinting analysis in this study (Table 1). Since completion of the work intended for presentation in this manuscript, *MEN1* mutations were identified in one of the candidate genes (*Mu*) mapped in this transcript search. Full details of that analysis, which revealed significant alterations in 14 out of 15 FMEN 1 pedigrees, are reported elsewhere (Chandrasekharappa et al. 1997).

The present report provides a detailed map of 33 transcripts in the 2.8-Mb region on chromosome 11q13 and the mutation analysis of 15 candidate genes (of which 10 were examined exhaustively). This not only has proved valuable to identify the *MEN1* tumor suppressor gene, but is likely to provide valuable information in the future for the identification of other disease-causing genes that map to this gene-rich region. This includes Bardet–Biedl Syndrome-1 (*BBS1;* Leppert et al. 1994), insulin-dependent diabetes mellitus 4 (*IDDM4;* Davies et al. 1994), paraganglioma 2 (*PGL2;* Mariman et al. 1993), spinocerebellar ataxia type 5 (*SCA5;* Ranum et al. 1994), vitreoretinopathy neovascular inflammatory disease (*VRN1;* Stone et al. 1992a), and Best's disease (*VDM2;* Stone et al. 1992b).

METHODS

Mapping the Transcripts on the Contig

Sequence-tagged sites (STSs) were generated from gene sequences in the database, matching EST- and GRAIL-predicted exon sequences. Nearly 80 YAC, BAC, PAC, and P1 clones have been isolated and assembled into a 2.8-Mb contig based on the STS-content analysis with nearly 120 STSs (Guru et al. 1997). Cosmid clones have also been isolated and assembled into smaller contigs for this region (Guru et al. 1997). PCR was carried out with STSs representing the transcripts, in duplicates, using genomic clones (YAC, BAC, PAC, P1, and cosmid clones) as templates. The results were integrated with an STS-content map to generate an ordered map of the transcripts on the 2.8-Mb physical map.

Northern Analysis

Multiple Northern blots (human poly(A)$^+$ blots, Human 7760-1, HumanII 7759-1, and human endocrine blot, Human 7751-1, Clontech) were used to check the size and the distribution of expression in different tissues using either the full length or fragment of an EST as a probe. A Northern blot was also made containing 20 µg of total RNA isolated from Epstein–Barr virus-transformed lymphocytes from seven FMEN 1 probands. Preparation of labeled probes and hybridization conditions were as described under Southern hybridization below.

Screening cDNA Libraries by GENETRAPPER

GENETRAPPER cDNA positive selection system was used for the isolation of full-length cDNA using Human Leukocyte SuperScript cDNA library (GIBCO-BRL). In brief, a PAGE-purified oligonucleotide complementary to a segment of the target cDNA was biotinylated using biotin-14-dCTP and terminal deoxynucleotidyl transferase (TdT). A complex population of double-stranded phagemid DNA containing cDNA inserts was converted to single-stranded DNA using Gene II and Exonuclease III. Hybrids between the biotinylated oligonucleotide and single-stranded DNA formed in solution were captured on streptavidin-coated paramagnetic beads. The captured single-stranded DNA target was released from the biotinylated oligonucleotide and the cDNA clone was further enriched by using a nonbiotinylated target oligonucleotide to specifically prime conversion of the recovered single-stranded DNA target to double-stranded DNA. Repaired DNA (2 µl) was electroporated into 40 µl of ElectroMAX DH10B cells using BioRad Gene Pulser (2.5 kV in a 0.1-cm gap chamber at settings of 100 ohms and 25 µF). Positive clones were identified either by PCR or oligonucleotide hybridization and determined by digesting with *Mlu*I.

5′ and 3′ RACE

Primers were designed from the EST sequences and RT–PCR was performed on the following Mararthon cDNA sources as per the manufacturer's protocol (Clontech): pancreas, pituitary, and ovary. PCR products were cloned in the PCRII TA-cloning vector (Invitrogen). Positive clones were identified by colony hybridization to end-labeled nested oligonucleotides. Inserts of positive clones were sequenced first with vector primers and then by primer walking.

Southern Hybridization

Blood DNA (5 µg) extracted (Qiagen Genomic Tips) from 32 FMEN1 affected individuals from 32 different kindreds was digested with *Taq*I and *Hin*dIII restriction enzymes, separated on 0.8% agarose gel, and blotted to Hybond membrane (Amersham). cDNAs from *GC-kinase, Mu, HPAST, PP2AB56*β, *ARL2, NOF1, DNA pol*α, *Zeta, HREQ, Epsilon/Beta, MLK3, RELA,* and *TIP60* were radiolabeled using Random primer labeling kit (Amersham) as per the manufacturer's protocol. Labeled DNA (50–100 ng) was preannealed at 65°C (50 µl probe, 100 µg unlabeled human COT-I DNA and 50 µl 6× SSC) for 2–4 hr. These preannealed probes were hybridized to Southern or Northern blots at 65°C for 16–24 hr in hybridization solution (6× SSC, 5× Denhardt's, 0.5% SDS, and 100 µg/ml of denatured salmon sperm DNA). Filters were washed at room temperature in 2× SSC/0.1% SDS twice for 30 min, and 0.2× SSC/0.1% SDS at 65°C for 30 min. The blots were exposed to Kodak X-ray film at −70°C for 3–7 days.

Primary PCR for Dideoxy Fingerprinting

DNA was isolated from blood samples of 16 FMEN 1 probands (from 16 different kindreds) and 10 normal healthy individuals using Qiagen Genomic Tips. Genomic DNA fragments containing exons of each gene were amplified using primers designed from the genomic DNA sequence (PCR primer and dideoxy fingerprinting primer sequences can be found as supplementary material to this article at www.nhgri.nih.gov and the *Genome Research* Web site). PCR was performed in 25-µl reactions containing 100 ng DNA, 0.2 µM of each primer, 200 µM dNTPs, 10 mM Tris-HCl at pH 8.3, 50 mM KCl,

1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, and 0.5 units of AmpliTaq Gold (Perkin Elmer). DMSO was added to a final concentration of 5% for amplifying *MLK3* exons 5 and 9. Reactions were cycled in a Perkin Elmer System 9600: 92°C for 10 min to activate *Taq* Gold and then 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1–2 min depending on the size of the fragment amplified; after a final extension step at 72°C for 5 min the reactions were held at 4°C. All PCR products were checked for homogeneity by electrophoresing a 5-µl aliquot on agarose gels.

Dideoxy Fingerprinting

Primers for the dideoxy fingerprinting reaction (PCR primer and dideoxy fingerprinting primer sequences can be found as supplementary material to this article at www.nhgri.nih.gov and the *Genome Research* web site) were end-labeled with [γ-^{33}P]ATP and T4 polynucleotide kinase as per the manufacturer's protocol. Dideoxy fingerprinting was performed as per the protocol described by Sarkar et al. (1992) with modifications. The primary PCR products generated above were subjected to Sanger's dideoxy chain termination reaction using ddG (dideoxy GTP) in a 10-µl reaction containing 1 µl primary PCR template (20 ng), 0.15 µM end-labeled ddF primer, 25 µM dNTPs, 200 µM ddG, 10 mM Tris-HCl at pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (wt/vol) gelatin, and 1 unit of AmpliTaq Gold. Reactions were cycled in a Perkin Elmer System 9600: 92°C for 10 min to activate *Taq* Gold and then 35 cycles of 94°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min; after a final extension step at 72°C for 5 min the reactions were held at 4°C. To the 10-µl dieoxy fingerprinting reactions was added 40 µl of buffer containing 7 M urea, 50% formamide, bromophenol blue, and xylene cyanol. Reactions were heated at 94°C for 5 min and chilled in ice, and 5 µl was loaded on a nondenaturing gel [0.75× mutation detection enhancement (MDE) gel (FMC BioProducts) in 0.5× TBE buffer] on a sequencing apparatus. The gel was electrophoresed at a constant power of 8 watts at room temperature in a buffer system consisting of 0.5× TBE in the top reservoir and 0.8× TBE with 0.5 M sodium acetate in the bottom reservoir, until the bromophenol blue reached the bottom of the gel. The gel was removed onto Whatman paper, dried for 30 min in a sequencing gel drier, and autoradiographed overnight. One dideoxy fingerprinting primer could screen ~250 bp; if the coding region to be screened in the primary PCR product was larger, one or more additional primers were used for dideoxy fingerprinting. Samples with changes in band patterns were subjected to cycle sequencing using the same primary PCR product and the same end-labeled primer as was used in the dideoxy fingerprinting reaction. Some polymorphic changes distributed in normal controls and affecteds were also sequenced to generate markers.

Genomic Clone Sequencing

BAC clones were subjected to shotgun sequencing (Pan et al. 1994) and the data assembled using the Phred/Phrap/Consed system developed by P. Green (University of Washington, Seattle); http://chimera.biotech.washington.edu/uwgc/. All assembled contigs ≥1000 bases were analyzed using the PowerBLAST program (ftp://ncbi.nlm.nih.gov/pub/sim2/PowerBlast/), which masks low complexity sequences and repetitive elements and then performs simultaneous BLASTN and BLASTX searches, reporting the results in graphical form (Zhang and Madden 1997). Both the ''nr'' and ''est'' databases at http://ncbi.nlm.nih.gov/ were searched. Contigs were also analyzed using GRAIL (http://avalon.epm.ornl.gov/) and the FEXH and HEXON programs (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html).

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