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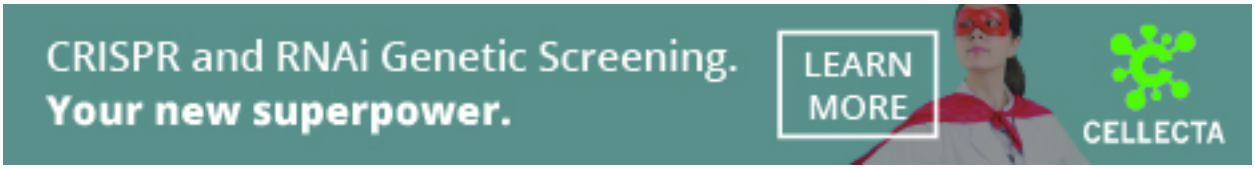

RESEARCH

Identification and Localization of the Gene for EXTL, a Third Member of the Multiple Exostoses Gene Family

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Hereditary multiple exostoses (EXT) is an autosomal dominant disorder characterized by multiple bony outgrowths from the juxtaepiphyseal region of long bones. In a small proportion of cases, these exostoses progress to malignant chondrosarcomas. Genetic linkage of this disorder has been described to three independent loci on chromosomes 8q24.1 (EXT1), 11p11-13 (EXT2), and 19p (EXT-3). The EXT1 and EXT2 genes were isolated recently and show extensive sequence homology to each other. These genes are deleted in exostoses-derived tumors, supporting the hypothesis that they encode tumor suppressors. We have identified a third gene that shows striking sequence similarity to both EXT1 and EXT2 at the nucleotide and amino acid sequence levels, and have derived its entire coding sequence. Although the mRNA transcribed from this gene is similar in size to that from EXT1 and EXT2, its pattern of expression is quite different. We have localized this gene by fluorescence in situ hybridization to metaphase chromosomes and by whole genome radiation hybrid mapping to chromosome 1p36.1 between D1S458 and D1S511, a region that frequently shows loss of heterozygosity in a variety of tumor types. This gene, EXTL (for EXT-like), is therefore a new member of the EXT gene family and is a potential candidate for several disease phenotypes.

[The sequence data described in this paper have been submitted to GenBank under accession no. U67191]

Hereditary multiple exostoses (EXT) is an autosomal dominant disorder of bone growth characterized by the presence of exostoses, bony outgrowths capped by cartilage that emerge primarily from the juxtaepiphyseal regions of the long bones (Solomon 1961). Some exostoses may be apparent at birth but continue to increase in size and number during growth until the closure of the growth plates (Hennekam 1991). These outgrowths may limit joint movement or cause pain as a result of compression of nerves. In these cases surgical intervention is often necessary, and many patients require multiple surgeries throughout the course of the disease. EXT is estimated to have a prevalence of one in 50,000 to one in 100,000 in Western populations (Hennekam 1991; Cook et al. 1993). In a few percent of cases the exostoses are transfomed to malignant chondrosarcomas or osteosarcomas, the most severe form of this disease (Hennekam 1991; Wicklund et al. 1995).

Multiple exostoses have been observed as part of the clinical profile in patients with Langer–Giedion contiguous gene syndrome, which is a deletion of 8q24.1 (Cook et al. 1993), and a recently delineated contiguous gene syndrome that is a deletion of 11p11-p13 (Bartsch et al. 1996). Consistent with these observations, linkage analysis in EXT families has revealed that this is a multigenic disorder (Cook et al. 1993), identifying genetic loci on chromosomes 8q24.1 (EXT1) (Cook et al. 1993) and 11p11-13 (EXT2) (Wu et al. 1994; Wuyts et al. 1995). Genetic linkage has also been reported to a third locus on chromosome 19p (EXT3) (Le Merrer et al. 1994). The EXT1 gene was cloned recently and shown to harbor mutations in affected members from two linked families, and to span chromosomal breakpoints identified in two multiple exostoses patients (Ahn et al. 1995).

Recently, we utilized a combination of positional cloning approaches to isolate the EXT2 gene

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(Stickens et al. 1996). A chromosome 11-specific cDNA library (Del Mastro et al. 1995) was constructed and screened with YACs encompassing the previously defined EXT2 region. A candidate gene from this library was identified from its sequence similarity to the EXT1 gene. Subsequent studies demonstrated that this gene maps precisely within the genetically defined region of 11p11-13 and within a small deletion that cosegregates with the disorder in one family. Moreover, the transcript size and expression of this candidate EXT2 gene closely resemble that of EXT1. Mutational analysis of EXT2 in a linked family identified a 4-bp deletion segregating in affected individuals, which is predicted to result in protein truncation.

Although the functions of these genes are not clear, both EXT1 and EXT2 are believed to encode tumor suppressors, based on the observation of loss of heterozygosity (LOH) of nearby markers in sporadic and exostoses-derived chondrosarcomas (Hecht et al. 1995; Raskind et al. 1995). LOH has also been found at polymorphic loci on chromosome 19p, the putative locus for EXT3, in tumors derived from patients whose disease is not linked to either EXT1 or EXT2 (Hecht et al. 1996). A separate study detected LOH of polymorphic markers linked to EXT1 and EXT2 in 44% of tumors with no LOH at 19p (Raskind et al. 1995). These observations have supported the proposal that the EXT genes fit the classic profile of tumor suppressors (Knudson 1971), wherein a mutation in one gene causes bony protrusions, and mutation or loss of the normal allele causes progression to tumors as a result of lack of tumor suppression (Ahn et al. 1995; Hecht et al. 1995; Raskind et al. 1995; Stickens et al. 1996).

At the time that we identified the EXT2 gene we also detected an additional sequence within the EST database (Lennon et al. 1996) that was similar, but not identical, to EXT1 and EXT2. We have now extended the sequence of this EST by rapid amplification of cDNA ends (RACE). Northern blot analysis indicates that this gene's transcript size is similar to EXT1 and EXT2, but its expression pattern is different. We have mapped this gene (which we have called EXTL for EXT-like) to chromosome 1p36, thus excluding it as a candidate for EXT3. Interestingly, this region of the genome is frequently deleted in a variety of tumor types and is thus believed to contain one or more tumor suppressor loci.

RESULTS

EXT1 and EXT2 cDNA sequences were searched against the expressed sequence tag (EST) database using the BLASTN and BLASTX programs (Altschul et al. 1990). Three ESTs were identified, yo78g12, yl86d04, and yf56h05, which are similar but not identical to EXT1 and EXT2. These EST sequences overlap with each other and thus appear to represent a third gene, EXTL. The EST sequences yo78g12.r1 and yf56h05.s1 represent the 5′ and 3′ ends of overlapping cDNA clones isolated from a normalized neonatal brain cDNA library (Soares et al. 1994). The DNA sequence between these two cDNA ends was derived by first amplifying human placental cDNA in the PCR with primers specific to each EST. The resulting 2-kb PCR product was cloned and sequenced, revealing an open reading frame of 852 bp before reaching a stop codon. In order to derive more 5′ coding sequences, primers were designed from this sequence and used to extend the sequence by rapid amplification of cDNA ends (RACE). The cDNA was extended an additional 1980 bp by this method, resulting in a composite sequence of 4009 bp that contains a 2031-bp open reading frame extending to a stop codon at nucleotide 2895. The conceptual translation product encoded by this cDNA shows significant similarity to both EXT1 and EXT2 proteins (Fig. 1). There are 328 (49%) identical amino acids between the EXTL coding sequence and EXT1. If one includes conservative changes, the homology reaches 72%. By comparison, the EXT1 and EXT2 proteins contain 22% identical amino acids and have 49% homology when conservative amino acid changes are included. None of the three EXT genes show significant homology to known proteins or protein motifs, although each has a clustering of hydrophobic amino acids at the amino terminus, which could comprise a leader sequence. Overall, the three predicted proteins are most similar, with fewer gaps, at the carboxyl terminus.

In order to determine the chromosomal location of EXTL, primer sets were designed from the cDNA sequence. These primers were used in the PCR to amplify DNAs from a panel of human X rodent monochromosomal somatic cell hybrids (Coriell Institute). The results indicated that this gene is located on chromosome 1. Identical results were obtained with primer sets derived from 3′ cDNA sequences (data not shown). These mapping results exclude EXTL as a candidate for EXT3, which has been genetically linked to chromosome 19p.

The chromosomal localization was confirmed and refined in two ways. First, the St. Louis yeast artificial chromosome (YAC) library (Burke et al. 1987), which had been pooled in a PCR-amplifiable format, was screened with EXTL-specific primers.

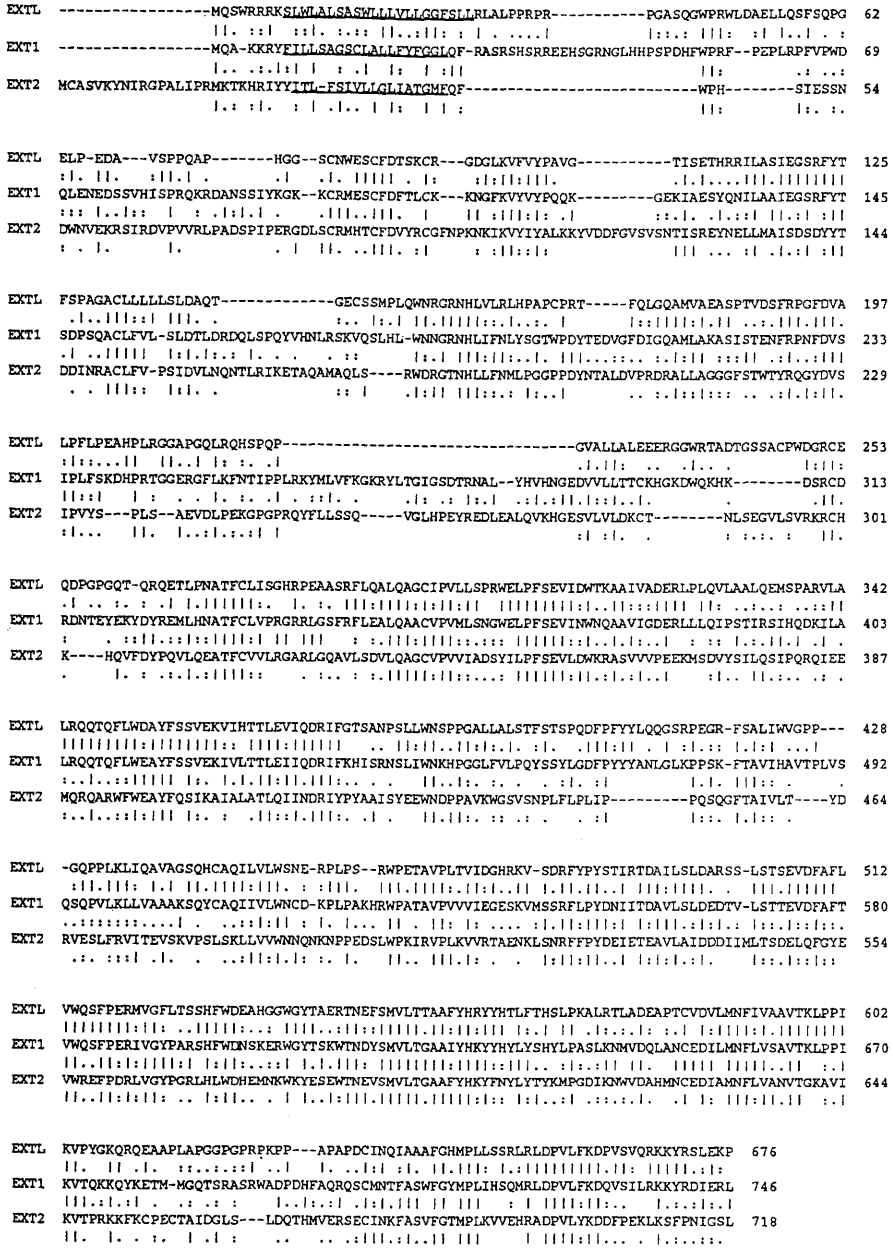

Figure 1 Amino acid alignments of EXT1, EXT2, and EXTL. The putative leader sequences are shown in bold. These alignments were generated using the GAP and MAP programs (Huang 1994; Genetics Computer Group 1995).

Two YACs were identified: B146F10, with a length of 100 kb, and B62C3, with a length of 200 kb. The YACs were purified by pulsed-field gel electrophoresis and hybridized to metaphase chromosome spreads using fluorescence in situ hybridization (FISH) (Fig. 2). This analysis positioned the YAC B146F10 on chromosome 1p36.1, confirming and sublocalizing the original mapping result. B62C3 also mapped by FISH to 1p36.1, but was chimeric, with signals also evident at several other sites in the genome (data not shown). Second, the location of EXTL was further refined by mapping in the Genebridge panel of DNAs from whole genome radiation hybrids (Research Genetics; data not shown). This analysis placed EXTL within 1.11 cR of marker WI-9053 on chromosome 1p36.1, between D1S458 and D1S511.

To determine the expression profile of this gene, a 97-bp radiolabeled cDNA fragment extending from nucleotides 1415 to 1512 was hybridized to a Northern blot of polyadenylated RNAs from a variety of human adult tissues (Fig. 3). This probe, which contains sequences that do not show appreciable homology to EXT1 or EXT2, detected a major transcript of 5.0 kb and a minor transcript of 4.2 kb in skeletal muscle and brain. Lower levels of these transcripts were also detectable in heart RNA. This profile is markedly different from the pattern of expression observed for EXT1 and EXT2, which produce similar-sized transcripts to EXTL, but which appear to be ubiquitously and abundantly expressed, as determined by Northern blots (Fig. 3).

DISCUSSION

We have identified a gene (EXTL) that has extensive sequence similarity to the EXT1 gene and less significant, but nevertheless considerable similarity to EXT2. The structures of EXTL, EXT1, and EXT2 are so similar that a priori one would predict similar functional roles, which has been demonstrated for EXT1 and EXT2 by virtue of the fact that each is associated with the same phenotype. Loss of one copy of EXT1 or EXT2 causes multiple exostoses. Thus it might be expected that loss of one copy of

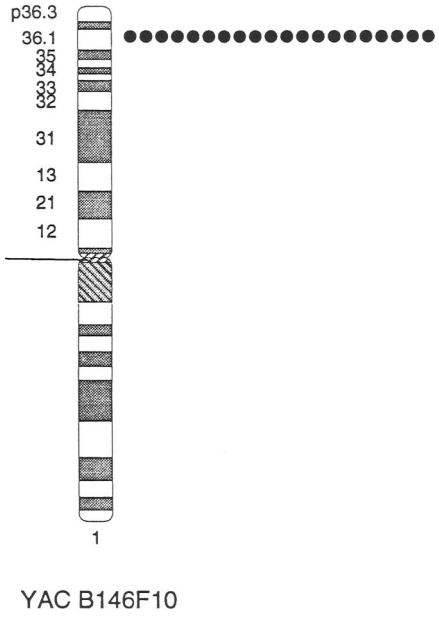

Figure 2 Localization of B146F10 by fluorescence in situ hybridization. The figure summarizes the distribution of hybridization signals observed among 10 mitotic cells. Each dot represents a labeled chromosome (usually labeled on both chromatids). Signals were observed at 1p36 in 20 out of a possible 20 instances. No specific hybridization signals were observed on other chromosomes.

EXTL would result in a similar outcome, if EXTL functions in multiple exostoses. Several studies have reported partial monosomy for 1p36–1pter associated with a variety of different phenotypes. Clinical findings in the 1p36–1pter deletion studies were quite variable and included cardiac myopathies, facial dysmorphia, and moderate to severe developmental delay (Keppler-Noreuil et al. 1995; Reish et al. 1995). Interestingly, several patients had skeletal deformities including cervical kyphosis and thoracolumbar kyphoscoliosis; however, multiple exostoses per se was not noted in these studies. The localization of EXTL to chromosome 1p36.1 excludes it as a candidate for EXT3, which has been mapped to chromosome 19p. Nevertheless, it is possible that EXTL may play a role in those cases of multiple exostoses that cannot be linked to chromosomes 8, 11, or 19.

Although there is no evidence that a multiple exostoses gene maps to 1p36, there is substantial evidence that a gene encoding a tumor suppressor lies in this region. Consistent deletions of the region have been detected in tumors derived from breast cancer (Hoggard et al. 1995; Bieche et al. 1993; Nagai et al. 1995), gastric cancer (Ezaki et al. 1996), colorectal polyps (Lothe et al. 1995), multiple endocrine neoplasia (Mulligan et al. 1993), and cervical carcinoma (Zimonjic et al. 1995). In complementary experiments, tumorigenicity was suppressed by introduction of a normal 1p35-36 region into a human colon carcinoma cell line (Tanaka et al. 1993). In addition, the most common cytogenetic abnormality observed in neuroblastoma is deletion of the short arm of chromosome 1 (White et al. 1995). If EXTL also encodes a tumor suppressor, as is thought to be the case for EXT1 and EXT2, then LOH for this region might be observed in chondrosarcomas from such individuals. It is also possible that EXTL might function as a tumor suppressor in an entirely different cell type. In this regard it is interesting to note that EXTL differs strikingly from EXT1 and EXT2 in its expression profile, as judged by Northern blot analysis. Resolving these questions will require further experimentation, including screening for the proteins that interact with EXT1, EXT2 and EXTL and searching for additional members of this gene family in man and other species.

METHODS

Primers and PCR Conditions

Oligonucleotide primers yo78g12.r1-F (TGGCCCTGTCTACTTTTTCC) and yo78g12.r1-R (CCAGATCAGGGCGCTGAAT) amplify a 97-bp cDNA fragment and a genomic DNA fragment of ~300 bp. The larger genomic product appears to contain a small intron. One hundred nanograms of cDNA or genomic DNA was added to 150 mM KCl, 2.5 mM $MgCl_2$, 25 mM Tris-HCl at pH 8.3, 1 μM each of yo78g12.r1f-F and yo78g12.r1-R primers and 1 unit Taq polymerase (Perkin Elmer Cetus) in a total volume of 25 μl. After an initial denaturation at 94°C for 1 min, 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec were carried out in a Perkin Elmer 9600 thermocycler. Primer yf56h05.s1-F (CAGTCCCTTCTTGCTGAGTTC) was used to generate first-strand cDNA by annealing 2.5 pmole to 1 μg total lymphoblastoid RNA at 65°C for 5 min. The reaction was then incubated with 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 uM each of dATP, dCTP, dGTP, and dTTP, and 200 units of SuperScript II reverse transcriptase (Gibco-BRL) for 30 min at 50°C. The reactions were heat-inactivated for 15 min at 65°C, and 2 units RNase H (Gibco-BRL) was added and incubated at 55°C for 10 min. This cDNA was then amplified in the PCR with yf56h05.s1-F and yo78g12.r1-F primers using Elongase polymerase (Gibco BRL) according to manufacturer's instructions, giving a product of ~1.9 kb.

YAC Screening

Primer set yo78g12.r1-F and yo78g12.r1-R was used to amplify PCR screening pools from a large insert YAC library using the conditions described above. Single colony positives were

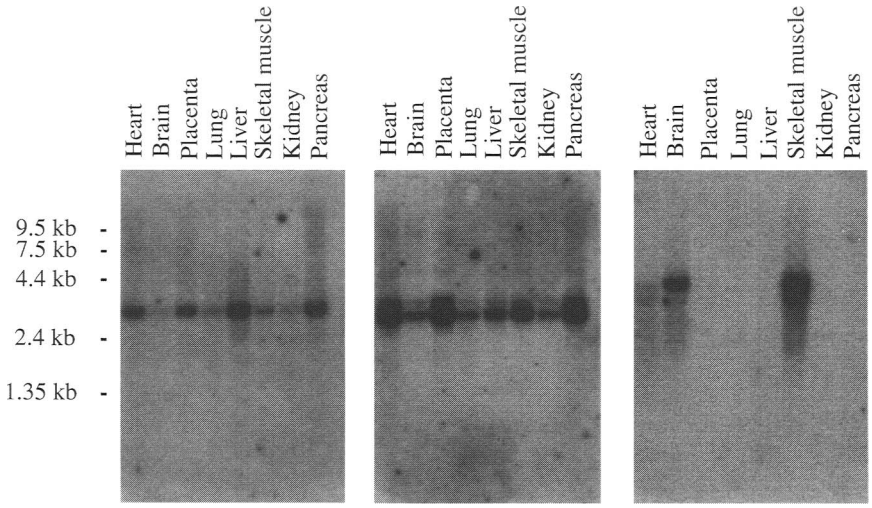

Figure 3 Northern blot analysis. The 97-bp EXTL fragment produced by amplification of brain cDNA with primers yo78g12.r1-F and yo78g12.r1-R was used to probe Northern blots of polyadenylated RNAs according to the manufacturer's instructions (Clontech). The EXT1 and EXT2 panels show the same Northern blot rehybridized with probes from these two genes, as a comparison of their expression patterns with EXTL. Lane loadings are as indicated and length standards are shown on the *left*.

identified and grown in selective (URA-, TRP-) media. Yeast chromosomes were isolated in agarose plugs. YACs B62C3 and B146F10 were separated from yeast chromosomes in low melting point gels by pulsed field gel electrophoresis (Gemmill et al. 1996).

FISH Analysis

One microliter of the melted, excised YAC band was amplified and labeled with biotin using DOP-PCR (Telenius et al. 1992) modified to include four initial rounds of low-temperature annealing and extension by Sequenase as described (Kroisel et al. 1993; Trask 1996). Labeled DNA was prehybridized in the presence of human Cot1 DNA (BRL) and hybridized to mitotic cells. Dividing cells were obtained from PHA-stimulated peripheral blood cultures released from an early-S methotrexate block in the presence of bromodeoxyuridine. Hybridization sites were labeled with fluorescein-conjugated avidin. A QFH-like banding pattern was visible after counterstaining the chromosomes with 4′, 6-diamidino-2-phenylindole (DAPI). The locations of hybridization signals were analyzed in 10 well-spread, well-banded metaphases for each YAC. Images of DAPI and fluorescein fluorescence were collected sequentially without image-shift using selective bandpass excitation filters in a computer-controlled filter wheel (Ludl) and a multiple bandpass emission filter (ChromaTechnology). Images were digitized using a Princeton air-cooled CCD camera with a Kodak KAF1400 chip operated in the 2-by-2 binning mode. Digital images were processed using Signal Analytics IPLab Spectrum v. 3.0. DAPI bands were sharpened using the built-in HAT 5x5 filter and displayed as gray values. The threshold and contrast of the FITC image was manipulated to facilitate identification of dim sites. The FITC image was then pseudocolored in green and overlaid on the DAPI image for analysis.

Northern Analysis

The 97-bp EXTL product produced by amplification of brain cDNA with the yo78g12.r1 primer set was radiolabeled and hybridized to Northern blots (Clontech) according to manufacturer's instructions. The EXT1 cDNA probe was a 102-bp PCR product from the 5′ untranslated region (UTR) and does not share any sequence homology with EXT2 or EXTL. The EXT2 cDNA probe was a 240-bp PCR product from the 3′ UTR and does not share any sequence homology with EXT1 or EXTL. Northern blots were washed two times at room temperature in 0.5% SDS, 2× SSC for 20 min each, then washed 20 min in 0.1× SSC, 0.1% SDS at 50°C. Blots were exposed to X-ray film for 3–9 days. Probes were removed by briefly boiling in 0.5% SDS prior to rehybridization with a second probe.

5′ RACE

cDNAs were extended using a 5′ RACE System for Rapid Amplification of cDNA Ends (Gibco BRL) and skeletal muscle polyadenylated RNA according to the manufacturer's instructions.

DNA Sequencing

DNAs were sequenced using a dye primer terminator cycle sequencing kit (Applied Biosystems). The cycle sequenced products were run on a 373A automated fluorescence sequencer (ABI). All sequences were analyzed using the BLAST N and BLAST X programs (Altschul et al. 1990).

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