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Rapid report

Assembly of a high-resolution map of the Acadian Usher syndrome region and localization of the nuclear EF-hand acidic gene

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Abstract

Usher syndrome type 1C (USH1C) occurs in a small population of Acadian descendants from southwestern Louisiana. Linkage and linkage disequilibrium analyses localize USH1C to chromosome 11p between markers D11S1397 and D11S1888, an interval of less than 680 kb. Here, we refine the USH1C linkage to a region less than 400 kb, between genetic markers D11S1397 and D11S1890. Using 17 genetic markers from this interval, we have isolated a contiguous set of 60 bacterial artificial chromosomes (BACs) that span the USH1C critical region. Exon trapping of BAC clones from this region resulted in the recovery of an exon of the nuclear EF-hand acidic (NEFA) gene. However, DNA sequence analysis of the NEFA cDNA from lymphocytes of affected individuals provided no evidence of mutation, making structural mutations in the NEFA protein unlikely as the cellular cause of Acadian Usher syndrome.

The Usher syndromes are a group of clinically and genetically heterogeneous disorders characterized by sensorineural hearing impairment and retinitis pigmentosa [1]. Clinically, three types of Usher syndrome have been described. Patients with Type I disease have severe to profound congenital hearing impairment, vestibular dysfunction, and retinal degeneration beginning in childhood, while those with Type II disease have moderate to severe hearing impairment, normal vestibular function, and later onset of retinal degeneration [2]. The hearing loss in Type III disease is progressive and the age of onset of

Abbreviations: BAC, bacterial artificial chromosome; BLAST, basic local alignment search tool; EDTA, ethylenediaminetetraacetic acid; HIV, human immunodeficiency virus; NEFA, nuclear EF-hand acidic; PAC, P1-derived artificial chromosome; PCR, polymerase chain reaction; RT, reverse transcription; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; STS, sequence tagged site; YAC, yeast artificial chromosome

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retinal degeneration is variable [3]. Linkage studies predict at least nine different loci for Usher syndromes, and one locus, USH1B, has been identified as the gene encoding the unconventional, nonmuscle myosin type VIIa [4].

Type I Usher syndrome occurs in populations throughout the world, including a small southwestern Louisiana population descended from French-speaking emigrants exiled from Acadia (southeastern maritime Canada) by British occupancy forces in the mid-eighteenth century [5]. Two of us previously localized Acadian Usher syndrome (USH1C) to the p15.1–p14 region of chromosome 11 [1] and showed by linkage and linkage disequilibrium analyses that it lies between the genetic markers D11S861 and D11S899, a distance of approximately 2–3 centiMorgans [6]. Ayyagari et al. [7] subsequently refined the USH1C region to the interval between genetic markers D11S1397 and D11S1888. All affected individuals of Acadian ancestry have shown linkage to the USH1C locus, and no non-Acadian families have been found that show such linkage, suggesting a founder effect for Usher syndrome type 1C in the Acadian population [1,6]. Nouri et al. [8] estimated that the USH1C mutation was introduced in the Acadians approximately 15 generations ago.

Bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) have a number of properties that make them desirable for physical mapping. BACs and PACs are capable of faithful propagation of DNA fragments greater than 300 kb in size [9–12]. The basis for the stability and non-chimeric nature of the clones is the single copy number origin of the vectors, method of library construction, and bacterial host strain [9–12]. The BAC and PAC libraries are well suited to bridge the gaps in maps based on yeast artificial chromosomes (YACs) using sequence tagged sites (STSs) as anchors to provide high resolution templates for subsequent large scale DNA sequence analysis [11,12].

In this paper, we refine the candidate interval for USH1C, present a complete BAC contig of the region, localize the nuclear EF-hand acidic (NEFA) gene to the candidate region, and provide evidence that NEFA is probably not responsible for USH1C.

The families analyzed in this study were previously described by Keats et al. [6]. The 46 affected individuals have a confirmed diagnosis of Usher syndrome type I and are of Acadian ancestry. The 46 affected individuals belong to 27 nuclear families from which a total of 54 disease and 50 normal chromosomes were available for analysis. Eleven polymorphic microsatellite markers (D11S1397, D11S902, D11S4096, D11S4160, D11S921, D11S4099, D11S1890, D11S4138, D11S4130, D11S1888, D11S1310) from the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research integrated map [13,14] that are in the vicinity of the USH1C locus were used to refine the critical region by haplotype analysis. The 54 disease chromosomes had identical haplotypes from D11S902 to D11S4099. Two chromosomes had a different allele at D11S1397 and one chromosome had a different allele at D11S1890. The consistency of the haplotypes argues strongly for a founder effect with this disease locus, and the loss of disequilibrium at these genetic loci is consistent with the USH1C locus lying between D11S1397 and D11S1890, a physical distance of less than 400 kb on chromosome 11p (Fig. 1).

Individual clones were isolated from a total human genomic BAC library commercially available from Research Genetics (Releases I, II and III). This library contains approximately 221 000 independent clones (average insert size 130 kb) arranged in a microtiter format of 72 superpools representing 9-fold coverage of the human genome. The human BAC library was screened by polymerase chain reaction (PCR) amplification of 21 microsatellite markers (D11S1397, D11S902, D11S4096, D11S4160,

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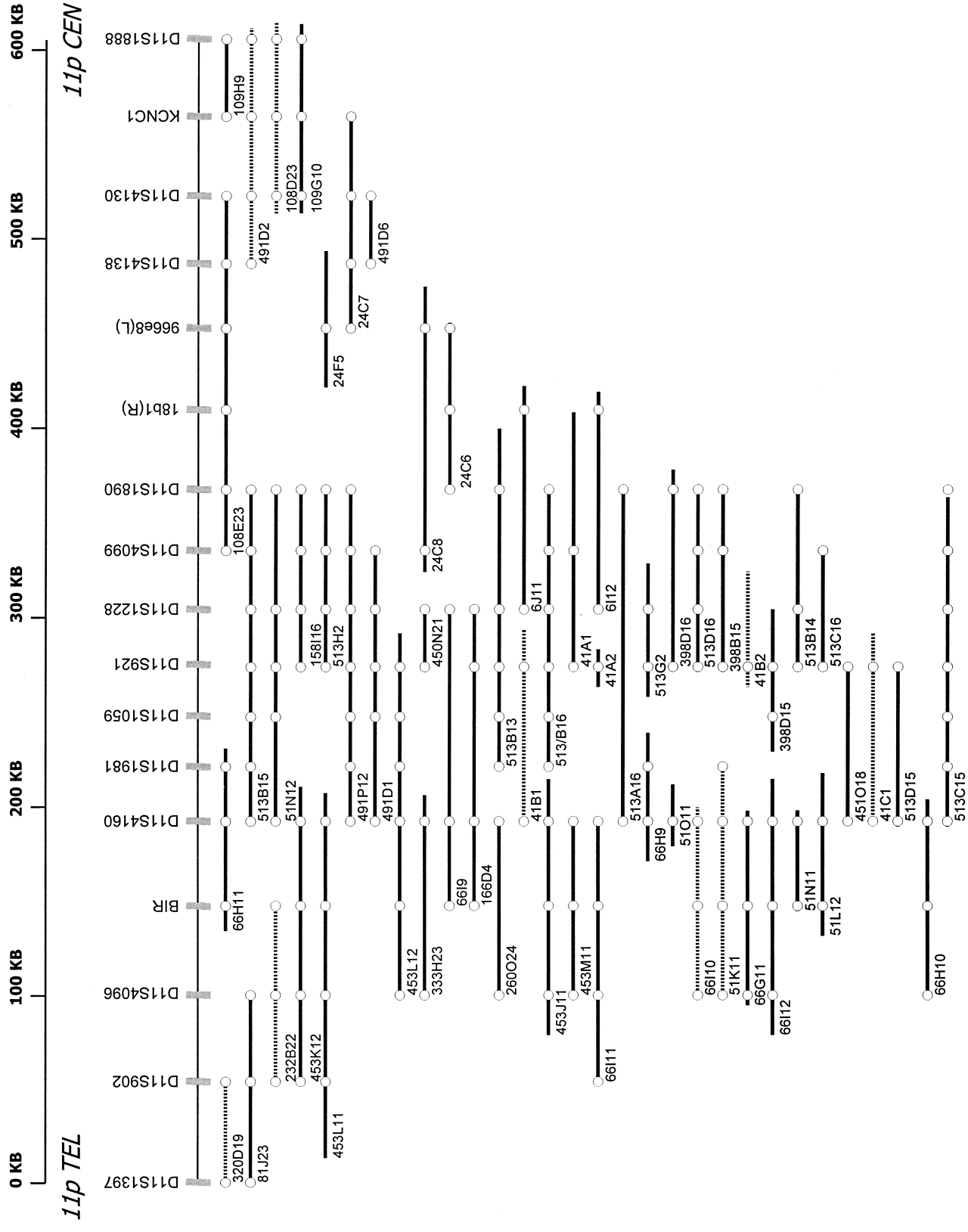
Fig. 1. A BAC contig encompassing the USH1C critical region. The region depicted here from D11S1397 to D11S1888 is covered contiguously in 60 BAC clones that span an approximate distance of 600 kb. A scale of physical distance is denoted in the top line of the figure. Genetic markers are spaced along the genetic map in the second line of the figure according to the approximate physical distance between them. BAC clones are represented by horizontal lines beneath the genetic map of the region. Individual clones are identified by plate and well addresses directly beneath and to the left of the line segment and are scaled according to size. Open circles on a clone denote positive amplification for the genetic markers denoted by gray rectangles on the genetic map. Vertically hatched horizontal lines indicate BAC clones subjected to exon trapping.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11p TEL
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D11S1397
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BIR
D11S4160
D11S1981
D11S1059
D11S921
D11S1228
D11S4099
D11S1890
18b1(R)
966a8(L)
D11S4138
D11S4130
KCNC1
D11S1888
320D19
81J23
232B22
453K12
453L11
66H11
513B15
51N12
158I16
513H2
491P12
491D1
453L12
333H23
66I9
166D4
260O24
513B13
41B1
453J11
453M11
66I11
513/B16
6J11
41A1
41A2
6I12
513A16
66H9
51O11
66I10
51K11
66G11
66I12
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398D16
513D16
398B15
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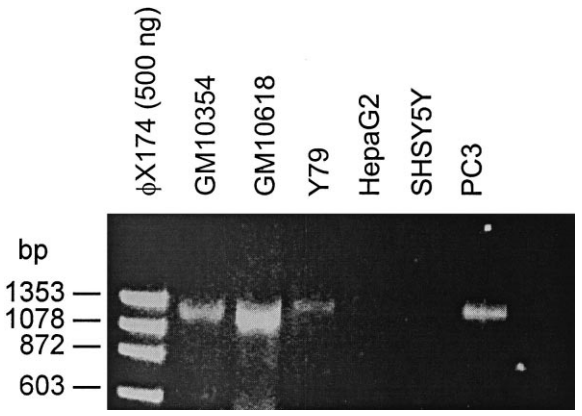

Fig. 2. Analysis of NEFA gene expression by RT-PCR. Putative NEFA cDNAs were amplified from RNA derived from various cell lines and separated by 1% agarose gel electrophoresis in the presence of 0.4 μg/ml ethidium bromide. Lanes left to right: putative NEFA cDNAs from GM10354, GM10618, Y79, HepaG2, SHSY5Y, and PC3 cell lines.

D11S1981, D11S1059, D11S921, D11S1228, D11S4099, D11S1890, D11S4138, D11S4130, D11S1888, D11S1310, and D11S2450; 18b1(R), 966e8(L), KCNC1, 239h11(L), BIR, and MYOD1) using primer sets previously described [7,13,14,15] and commercially available (Research Genetics). All amplifications were performed on 1 μg of BAC DNA in an MJ Research Thermocycler PTC200 for 35 cycles of 94°C for 1 min, 50–60°C (annealing temperatures specific to each primer set) for 1 min, and 72°C for 1 min, with an initial denaturation at 95°C for 5 min, in 25 μl reactions each containing 0.132 μM each primer, 0.2 mM dNTPs (Pharmacia); 1.5 mM Mg^{2+}; 1×RediLoad (Research Genetics), and 2.0 U of *Taq* polymerase in accompanying buffer (Perkin Elmer). The PCR products were subjected to electrophoresis on a 2% agarose gel with 0.4 μg/ml ethidium bromide to identify clones that contained each genetic marker. Seventeen microsatellite markers (D11S1397, D11S902, D11S4096, BIR, D11S4160, D11S1981, D11S1059, D11S921, D11S1228, D11S4099, D11S1890, 18b1(R), 966e8(L), D11S4138, D11S4130, KCNC1, D11S1888) successfully amplified PCR products. No clones were obtained using markers D11S1310, D11S2450, 239h11(L), and MYOD1. A total of 76 BACs were isolated using the 17 STSs, and 60 BACs were used to construct the contig across the USH1C candidate region (Fig. 1).

Small preparations of BAC DNA were made from 5 ml cultures grown overnight in 1×Luria–Bertani medium supplemented with 12.5 μg/ml chloramphenicol using a modification of the alkaline lysis miniprep procedure [16]. The standard alkaline lysis miniprep procedure was modified in the following way: 200 μl of solution I, 400 μl of solution II, and 300 μl of solution III was used; 1 volume of isopropanol was used to precipitate the DNA; and the DNA was resuspended in 30 μl of 10 mM Tris-HCl (pH 7.4) 0.1 mM ethylenediaminetetraacetate (EDTA). In order to determine the size of exogenous insert DNA *Not*I digests of BAC DNA preparations were performed in 15 μl reactions containing about 1 μg of miniprep DNA, 1×bovine serum albumin, and 2.5 U of *Not*I in accompanying buffer (New England Biolabs). Digested BACs were fractionated on 1% agarose gels in 0.5×Tris-borate-EDTA buffer using a clamped homogeneous electric field (CHEF) mapper (Bio-Rad) with 4 kb as the lower setting and 300 kb as the upper setting on autoalgorithm. BAC insert sizes were determined by comparison to Midrange Pulsed Field Gel Marker II and Lowrange Pulsed Field Gel Marker II (New England Biolabs).

Southern blots of *Eco*RI digested BAC miniprep DNA were used to confirm overlap between clones [11,17]. *Eco*RI digests of BAC DNA were performed in 40 μl reactions containing 1.5 μg DNA and 1 U of *Eco*RI in accompanying buffer (New England Biolabs). Digested BACs were fractionated on 0.8% agarose/0.5×Tris-borate EDTA gels and transferred to Hybond N membranes (Amersham) by capillary action [16]. To prepare hybridization probes from BAC DNAs, 100–200 ng of miniprep DNA was radiolabeled with [α-^{32}P]dCTP by random priming (Boehringer-Mannheim). Labeled DNA was purified using G-50 Sephadex columns. Alternatively, hybridization probes were also generated by inter-*Alu* PCR amplification of 100 ng of BAC DNA, using the primers *Alu*-5′ and *Alu*-3′ as described by Tagle and Collins [18]. Probes were prepared for hybridization as described above, except that the PCR products were purified with Microcon-100 columns (Amicon) prior to labeling [19]. Purified, labeled DNA was preannealed with both 500 μg/ml of C*ot*-1 DNA (Gibco) and 500 μg/ml of pBeloBAC11 vector DNA (Research Genetics) by boiling for 5 min and incubation at 65°C for 2 h prior to hybridization

[19]. Southern blots were prehybridized for 3 h at 65°C in 0.6 M NaCl, 0.02 M EDTA pH 8.0, 0.2 M Tris-HCl pH 8.0, 2% sodium dodecyl sulfate (SDS), and 0.1% sodium pyrophosphate, followed by hybridization for 16 h at 65°C in 50 ml of fresh prehybridization solution containing 1×10^7 cpm/ml preannealed BAC-derived probes [19]. Filters were washed twice in 2×standard saline citrate (SSC), 0.1% SDS at 65°C for 20 min, and once in 0.2×SSC, 0.1% SDS at 65°C for 20 min. Filters were then exposed to X-ray film (Kodak) for 2–3 days at −80°C.

Assembly of the BAC clones spanning the region from D11S1397 to D11S1888 encompassing the USH1C critical region was accomplished by two independent means: (a) PCR analysis of all 60 BAC clones for the presence or absence of each of the 17 genetic markers; (b) *Eco*RI restriction digests and Southern blots using whole BAC DNA as hybridization probes [17] or inter-*Alu* PCR products from the BACs as probes [19]. Hybridization experiments using *Eco*RI digested BACs confirmed overlapping regions suggested by STS analysis.

The region from D11S1397 to D11S1888 is covered contiguously in 60 BAC clones with an approximate distance of 600 kb (Fig. 1). This interval was previously reported as less than 680 kb based on the size of a single YAC (776e7) that spanned a slightly larger interval ([7]; our unpublished data). Our map represents a 10-fold average coverage of this region. Using *Not*I restriction digests of individual BAC clones the average size of the clones within the contig is approximately 105 kb. The depth of clone coverage varies from two clones at D11S1397 to 30 independent clones at D11S4160. The 60 BACs form a contig with complete coverage of the D11S1397–D11S1888 USH1C candidate region.

We compared our map with other existing maps of the region. The physical distance of our map containing D11S902, D11S4096, D11S4160, D11S921, D11S4099, D11S4138, D11S4130 and D11S1888 is 550 kb while the physical distance of this region in the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research map of human chromosome 11 is slightly larger than 400 kb [20]. However, the physical distance of the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research map may be an underestimate since it is only inferred from the genetic map. The STS order of our map (D11S1397–D11S902–BIR–D11S921–D11S1890–966e8(L)–KCNC1–D11S1888) is in agreement with a previously published YAC contig of this region [7]. The order of D11S902–D11S1981–D11S1059–D11S921–D11S1228–D11S1890 is also in agreement with the McDermott Center for Human Growth and Development chromosome 11 map [15]. A single difference in marker assignment between our map and the YAC based map of Ayyagari et al. [7] involves the placement of marker 18b1(R). We placed genetic marker 18bI(R) closer to the centromere. However, the YAC used to localize 18bI(R) in the previous map is chimeric for two different chromosomes, and this may be the cause of the discrepancy [7]. The order of genetic markers in this region shows some correspondence with a recently reported YAC and PAC contig with the following marker order D11S1890–D11S921–D11S902–MYOD1–D11S1888–D11S1310–TPH–SAA1–LDHA [21]. The difference between the previously reported genetic and physical map of this region and our map may have also resulted from the use of chimeric YACs [21]. Therefore, the contig is concordant with most of the other genetic and physical maps of this area [7,15,20].

Exon trapping was performed using a modified pSLP IV vector, kindly provided by Ken Able (University of Michigan), on nine BACs in the USH1C candidate region (320D19, 232B22, 491D2, 108D23, 41B1, 51K11, 66I10, 41B2, 41C1) (Fig. 1). BAC DNA was digested with either *Pst*I or both *Bam*HI and *Bgl*II and ligated to pSLP IV. Pooled pSLP BAC subclone DNA preparations were transfected into COS-7 cells using LipofectACE (Gibco). RNA from transfected COS-7 cultures was purified and subjected to both reverse transcription and two rounds of PCR amplification as previously described to identify putative exons [22]. Amplification products were cloned into pAMP10 using the uracil DNA glycosylase cloning system (Gibco). Colonies of pAMP-exon clones were selected (192 colonies for each BAC subcloned) and arrayed into 96-well plates for size determination by PCR amplification (1.25 U of *Taq* polymerase, 1.25 mM Mg^{2+}, 0.25 mM dNTPs), using forward primer 5′-ACGCTCTAGAGTCGACCCAGC-3′ and reverse primer 5′-CCCCTCGGGAGATCTCCAG-3′ (for 30 cycles of

94, 60, and 72°C for 1 min each on a Perkin–Elmer 9600 thermocycler). A human immunodeficiency virus (HIV) probe was used to screen clones that utilized the pSPL IV cryptic splice site. Sequence similarity analysis of putative exons was performed using the National Center for Biotechnology Information basic local alignment search tool (BLAST) algorithm [23].

Putative trapped exons from three BACs (41B1, 41B2 and 41C1) were subjected to DNA sequencing. About 20% of all pSPL-BAC subclones were determined to be either artifact or clones that utilized the cryptic splice site during their expression in COS cells. The sizes of individual trapped exons were determined following PCR amplification and varied from approximately 108 bp (vector only) to 300 bp. Subclones which amplified putative exons larger than 108 bp that did not hybridize with the HIV artifact probe were hybridized to restriction digests of the three D11S921-positive BAC clones to confirm their origin. A BLAST sequence comparison of the five confirmed exons revealed that only one showed a high degree of identity to a known gene, the human NEFA gene (about 100 bp of a trapped exon showed 100% sequence identity to a region delimited by nucleotides 487 (+267) through 586 (+366) of the previously reported NEFA coding sequence) [24]. Other exons trapped by this procedure are presently undergoing detailed analyses.

To assess the possibility that expression of NEFA is involved in the progression of a disease that causes retinitis pigmentosa, reverse transcription-PCR (RT-PCR) was performed on RNA from Y79 retinoblastoma, as well as HepaG2 hepatocarcinoma, SHSY5Y neuroblastoma, and PC3 prostate cancer cells. RNA was prepared by the guanidinium isothiocyanate-phenol-chloroform extraction method [25]. cDNA was synthesized using the Reverse Transcription System (Promega) and a specific oligonucleotide primer, NEFA 3.1 (5′-AAGTTCTGGTGGACTTCAGACT-3′), derived from the 3′-untranslated region of the NEFA cDNA sequence. Putative NEFA cDNAs were amplified by PCR using the primers NEFA 5.1 (5′-AAAATTATTTACCTGCCTGAAC-3′) from the 5′-untranslated region of the NEFA cDNA and NEFA 3.1 (0.6 μM each) under the following conditions: 3 U of Promega *Taq* polymerase in accompanying buffer, together with 3.0 mM Mg^{2+} and 0.25 mM dNTPs, in a 35-cycle reaction of 95°C for 20 s, 46.3°C for 20 s, and 72°C for 1 min, with an initial denaturation of 95°C for 2 min and a terminal extension of 72°C for 6 min. Expression and relative sizes of the NEFA cDNA were determined by agarose gel electrophoresis in the presence of 0.4 μg/ml ethidium bromide. Fig. 2 shows amplification of putative NEFA cDNAs that correspond to the 1262-bp size predicted by amplification with primers NEFA 5.1 and NEFA 3.1. Expression of the NEFA cDNA was restricted to a few cell types, including Y79 retinoblastoma.

The NEFA cDNA was also amplified from two lymphoblastoid cell lines derived from peripheral lymphocytes of patients with Acadian Usher syndrome (lines GM10618 and GM10354 of the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository). cDNAs were synthesized as described above. To facilitate cloning, the NEFA cDNA was amplified with both primer pairs NEFA 5.1a (5′-GTTTAAAATTAATTTACCTTGCCTGAAC-3′) and NEFA 3.6a (5′-GTTTACTTTAGGGTGATTTTCATCGT-3′) (as above with annealing at 45.7°C) and NEFA 5.6a (5′-GTTTAGAGAATATTTAAAAACATTGA-3′) and 3.1a (5′-GTTTAAGTTCTGGTGGACTTCAGACT-3′) (as above with annealing at 43.9°C), which generated overlapping products corresponding to 5′ and 3′ halves of the complete predicted NEFA coding region. These primers contained arbitrary 5′-ends previously described to enhance the addition of non-template, mono-adenosine overhangs by *Taq* polymerase [26] and thereby facilitate TA-cloning. Amplified NEFA halves were cloned by the TA-Cloning System (Invitrogen). Independent clones were sequenced by the Thermosequenase method (Amersham).

We also performed comprehensive sequence analysis on cloned RT-PCR material to [1] confirm the identity of RT-PCR products as NEFA, [2] confirm the published sequence of NEFA cDNA, and [3] determine the nucleotide sequence of NEFA coding sequence from affected individuals. Full-length coding sequences of putative NEFA cDNAs from each affected lymphoblastoid cell source (GM10618 and GM10354) and a single full-length sequence from Y79 cell line cDNA were obtained (GenBank accession numbers AF052642, AF052643 and AF052644,

respectively). Nucleotide analysis confirmed the identity of RT-PCR products as the NEFA cDNA. In comparing the three full-length sequences generated with the published NEFA cDNA sequence, no evidence for mutations in NEFA cDNAs from either affected patient was observed.

Linkage of the Acadian Usher Syndrome Type I locus to genetic markers in the p15.2–p14 region of chromosome 11 was the first step toward physical and fine gene mapping of USH1C. Haplotype analysis demonstrates that USH1C resides between D11S1397 and D11S1890. In an effort to identify the USH1C locus, we have assembled a contiguous and overlapping set of genomic clones derived from the candidate region on chromosome 11p. The USH1C BAC contig we report spans a 600-kb interval between D11S1397 and D11S1888 with 60 BACs. This contig exhibits an average 10-fold coverage, but some regions, such as the D11S1397 locus, have 2-fold coverage while the D11S4160 locus has 30-fold coverage. This difference in coverage of the library may result from cloning bias inherent in the construction of the BAC library, or different efficiencies of STS based PCR amplification. The physical map of chromosome 11p reported here is a high resolution BAC-based template for subsequent large-scale DNA sequence analysis and gene discovery. Since the map is based upon stable, non-chimeric, sequence-ready BAC clones, it is an improvement over the previously published YAC contig from the region [7].

Localization of the human NEFA gene to the Acadian Usher syndrome critical region between D11S4160 and D11S921 supports its analysis as a candidate gene. The human NEFA gene encodes a 420-amino acid protein [24] that belongs to the nucleobindin family [27]. The NEFA gene was originally isolated from a lambda-gt11 cDNA expression library derived from the acute lymphoblastic leukemia cell line KM3 [24]. Although its function is currently unknown, analysis of the NEFA amino acid sequence suggests that it acts as a dimeric, Ca^{2+}- and DNA-binding protein. A gene such as NEFA that encodes a DNA-binding protein is a provocative candidate for a genetically heterogeneous disease such as Usher syndrome. It immediately suggests genomic control of the expression of multiple loci, whose expression in turn may be causative or contributory to the multiple forms of the disease. However, our analysis of NEFA cDNAs suggest that the defect causing Acadian Usher syndrome is unlikely to be located within the coding sequence of NEFA. Therefore, we conclude that, aside from the possibility of a change in the expression pattern of the NEFA gene in affected tissues that has not been detected, the NEFA gene does not appear to be responsible for Acadian Usher syndrome.

The BAC contig of the USH1C region of chromosome 11p will be a valuable resource to search for additional candidate genes using exon trapping, cDNA library screening, and large scale DNA sequence analysis. The precise localization of this disease gene will eventually provide important information for use in genetic counseling, as well as major advances in our understanding of hearing and vision.

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