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Genetics

A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia

(bone mineralization/rickets/genetic disease)

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ABSTRACT **Hypophosphatasia is an inherited disorder characterized by defective bone mineralization and a deficiency of serum and tissue liver/bone/kidney alkaline phosphatase (L/B/K ALP) activity. Clinical severity is variable, ranging from death *in utero* (due to severe rickets) to pathologic fractures first presenting in adult life. Affected siblings, however, are phenotypically similar. Severe forms of the disease are inherited in an autosomal recessive fashion; heterozygotes often show reduced serum ALP activity. The specific gene defects in hypophosphatasia are unknown but are thought to occur either at the L/B/K ALP locus or within another gene that regulates L/B/K ALP expression. We used the polymerase chain reaction to examine L/B/K ALP cDNA from a patient with a perinatal (lethal) form of the disease. We observed a guanine-to-adenine transition in nucleotide 711 of the cDNA that converts alanine-162 of the mature enzyme to threonine. The affected individual, whose parents are second cousins, is homozygous for the mutant allele. Introduction of this mutation into an otherwise normal cDNA by site-directed mutagenesis abolishes the expression of active enzyme, demonstrating that a defect in the L/B/K ALP gene results in hypophosphatasia and that the enzyme is, therefore, essential for normal skeletal mineralization.**

Alkaline phosphatases (ALPs) [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1] are cell-surface glycoproteins that hydrolyze a variety of monophosphate esters with high pH optima (1). In normal humans there are three major forms of ALP [liver/bone/kidney (L/B/K), placental, and intestinal], each encoded by a separate gene (2–6). Placental and intestinal ALPs are found predominantly in the tissues for which they are named, whereas the distribution of L/B/K ALP is widespread (1). The physiological function of ALP in most tissues is uncertain, except that the bone isoenzyme has long been thought to play a role in skeletal mineralization (1). Compelling evidence for this role is provided by hypophosphatasia, an inherited disorder of osteogenesis characterized by deficient L/B/K ALP activity in serum, tissues, and cultivated skin fibroblasts (7, 8). In contrast, placental and intestinal ALP activities are normal in affected individuals (9, 10). Other biochemical findings in hypophosphatasia include an accumulation of several phospho compounds in the serum and/or urine that are thought to be endogenous L/B/K ALP substrates. These include phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5-phosphate (11–13).

The clinical severity of hypophosphatasia is highly variable, ranging from stillbirth with almost no mineralized bone to pathologic fractures first presenting in adults. Affected siblings, however, are usually phenotypically similar. Severe forms of the disease are transmitted as an autosomal recessive trait; heterozygous carriers are usually clinically normal but often show modestly reduced serum ALP activity and increased urinary phosphoethanolamine (7).

The primary genetic defects in hypophosphatasia are unknown. The wide spectrum of clinical severity suggests a number of different mutations, each giving rise in homozygotes or double heterozygotes to a distinct clinical phenotype. Some of these mutations may involve the L/B/K ALP locus, which maps to human chromosome 1p34–36.1 (14). Indeed, genetic linkage studies, using polymorphic probes for the L/B/K ALP locus and flanking markers, are consistent with a primary abnormality in the L/B/K ALP gene in some hypophosphatasia kindreds (15, 16). However, other mutations that give rise to hypophosphatasia may involve defects in the regulation of a structurally intact L/B/K ALP gene (17).

We report the characterization of a missense mutation within the L/B/K ALP gene of an individual with perinatal (lethal) hypophosphatasia. This patient, whose parents are second cousins, is homozygous for the mutant allele. Absence of this mutation in 34 unrelated patients with various forms of hypophosphatasia is consistent with heterogeneous genetic bases for this disease.

MATERIALS AND METHODS

Family Information. The proband was the first infant born to second-cousin parents who are part of an extended inbred kindred from a small community in Nova Scotia, Canada. The family is originally of German descent. The affected patient, who died at age 3 months, exhibited the classical abnormalities associated with perinatal hypophosphatasia, including profound skeletal hypomineralization, serum ALP of 11 units/liter (normal, 168–406), and urinary phosphoethanolamine excretion of 4.32 μmol/mg of creatinine (average of three measurements) (normal, <0.33).

General Methods. Except where noted otherwise, standard methods were used for the isolation and manipulation of DNA and RNA (18). Fibroblasts from the proband were propagated from skin explants as described (19). RNA was isolated from cultured cells using the guanidinium isothiocyanate/CsCl method (20).

Isolation of L/B/K ALP cDNA from Hypophosphatasia Fibroblasts. Complementary DNA was synthesized in a 30-μl reaction mixture containing 10 μg of fibroblast total cellular RNA; 50 mM Tris·HCl (pH 8.3); 6 mM $MgCl_2$; 40 mM KCl; 10 mM dithiothreitol; 180 μg of actinomycin D per ml; 1 mM

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Abbreviations: L/B/K, liver/bone/kidney; ALP, alkaline phosphatase.

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each dATP, dGTP, dCTP, and dTTP; 5 μM oligodeoxynucleotide primer A (5'-TATGAATTCGGCTGCCGTGTGGGAAGTT-3'; the underlined region is complementary to L/B/K ALP mRNA, positions 1793–1811, see Fig. 1); 30 units of RNasin (Promega Biotec, Madison, WI); and 19 units of avian myeloblastosis virus reverse transcriptase (Promega Biotec). The mixture was incubated for 30 min at 42°C, extracted with phenol/chloroform (1:1), precipitated with ethanol, dried, and resuspended in 20 μl of water.

Half of the above cDNA was amplified by the polymerase chain reaction as described (21), using oligodeoxynucleotide primers A (above) and B (5'-ATAGAATTCATCTCTGGGCTCCAGGGA-3'; the underlined region is identical to L/B/K ALP mRNA, positions 135–153, see Fig. 1). A total of 30 rounds of amplification were performed. Each round consisted of a denaturation step (94°C for 1 min) followed by an extension step (65°C for 10 min). The first several nucleotides at the 5' ends of primers A and B contain *Eco*RI sites designed to facilitate subsequent manipulations of the amplified DNA.

The amplified DNA products were fractionated by electrophoresis in a 0.8% agarose gel and the 1693-base-pair (bp) fragment was purified by electroelution. Attempts to utilize the *Eco*RI sites at the ends of primers A and B for cloning were unsuccessful. Therefore, the 1693-bp polymerase chain-reaction product was treated with phage T4 DNA polymerase to produce blunt ends and inserted into the *Sma* I site of the plasmid vector pGEM-4 (Promega Biotec).

DNA Sequence Analysis. DNA sequence analysis was performed (22) with 7-deaza-2'-deoxyguanosine 5'-triphosphate (Boehringer Mannheim) in place of dGTP (23). L/B/K ALP cDNA constructs were sequenced by using a set of oligonucleotide primers complementary to various regions of the cDNA (sequences of these primers are available upon request).

Analysis of Mutation 711A in the Affected Pedigree. A 222-bp segment of the L/B/K ALP gene, which includes most of exon 6 and contains bp 711 of the corresponding cDNA, was amplified (21) from DNA of family members by using the primers E6-1 (5'-ACACCCCGATCTGTGGATAAA-3', complementary to the template strand of intron 5, beginning 45 bp upstream of exon 6) and O (5'-CAATGTCCCTGATGTTATGC-3', identical to the template strand, bases 822–803 of the cDNA), as shown in Fig. 2*A*. The denaturation step was at 94°C for 1 min, and the extension step was at 65°C for 3 min. The DNA was amplified for 30 cycles and 10 μl was fractionated in a 7.5% polyacrylamide minigel and electroblotted (Mini Trans-Blot, Bio-Rad Laboratories, Richmond, CA) onto Zeta-Probe membrane (Bio-Rad) according to the manufacturer's instructions. The blots were hybridized to ^{32}P-end-labeled oligodeoxynucleotide probes designed to discriminate between the normal and mutant base at cDNA position 711: 711-G (5'CCGCCTACGCCCACTCG-3' or 711-A, (5'-CCGCCTACACCCACTCG-3'), corresponding to positions 703–719 of the normal and mutant cDNAs, respectively. Blots were washed at 52°C in TMAC solution (24) (3.0 M tetramethylammonium chloride/50 mM Tris·HCl, pH 8.0/2 mM EDTA/0.1% $NaDodSO_4$) and autoradiographed for 2–4 hr at −80°C with an intensifying screen. Serum L/B/K ALP activities of various pedigree members were determined as described (25).

Construction and Analysis of the Mutant L/B/K ALP cDNA Expression Plasmid. The L/B/K ALP expression plasmid pSV2Aalp' (26) consists of the normal cDNA situated downstream of the simian virus 40 early gene promoter. A G → A transition at L/B/K ALP cDNA position 711 was created by site-directed mutagenesis (27) of a 1252-bp *Nae* I–*Ava* I fragment (bp 510–1762 of the cDNA) of pSV2Aalp' with 711-A (described above) as the mutagenic oligonucleotide. The mutagenized fragment was inserted in place of the analogous fragment in pSV2Aalp' to create the mutant cDNA plasmid pSV2Aalp' 711A. Sequence analysis of the mutagenized portion of the cDNA insert indicated the G → A transition at position 711 to be the only difference between the normal and mutant cDNA expression plasmids.

NIH 3T3 cells were transfected with circular plasmid DNA (10 μg per 10-cm dish) by the calcium phosphate coprecipitation technique (28). Cellular RNAs were isolated (20), and 15 μg of each RNA was fractionated in a 1.5% agarose gel with 2.2 M formaldehyde, transferred to Zeta-Probe membrane, and hybridized to ^{32}P-labeled L/B/K ALP cDNA (3).

To detect immunologically crossreactive material, cells were fixed with methanol/formalin (9:1) and incubated with rabbit anti-human liver ALP antiserum (1:150 dilution in 20 mM Tris·HCl, pH 7.5/500 mM NaCl/0.05% Tween 20/1% bovine serum albumin). Bound antibody was detected with a biotinylated anti-rabbit antibody and avidin-horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA). Peroxidase was visualized with 3,3'-diaminobenzidine tetrahydrochloride monohydrate and osmium tetroxide (29). ALP activity was determined in whole cell suspensions by published methods (30).

RESULTS AND DISCUSSION

We examined the L/B/K ALP genes of an individual with lethal perinatal hypophosphatasia. The parents are second cousins who are part of an extended inbred kindred from a small community in Nova Scotia. Consequently, we expected that the proband would be homozygous for the same mutant allele. Cultured fibroblasts derived from the patient were deficient in ALP activity but produced normal amounts of full-sized L/B/K ALP mRNA (data not shown). L/B/K ALP cDNA was synthesized from fibroblast RNA, and then a 1676-bp segment was amplified by using primers that flank the entire protein-coding region (Fig. 1). The amplified segment was inserted into a plasmid vector, and the sequences from three separate isolates were compared to the normal L/B/K ALP coding sequence (3, 26). Fourteen point mutations (10 transitions and 4 transversions) were unique to individual isolates but absent in the other two. We interpret these to be artifacts introduced during cDNA synthesis or amplification (31). However, one mutation, shown in Fig. 1, was present in all three isolates: a G → A transition at position 711 of the cDNA (3), hereafter referred to as mutation 711A, that causes the replacement of alanine-162 of the mature enzyme with threonine.

To detect this mutation in the affected pedigree, we amplified the corresponding region of genomic DNA from family members and hybridized the products to two allele-

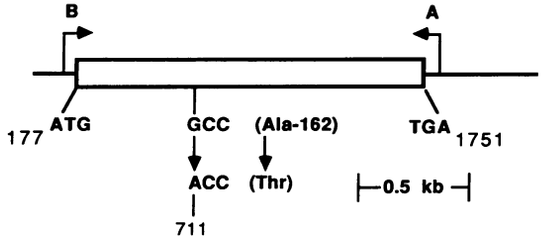

FIG. 1. Analysis of a mutant L/B/K ALP cDNA from hypophosphatasia fibroblasts. The L/B/K ALP cDNA is shown in schematic form with the translation initiation (ATG) and termination (TGA) codons numbered (3). First-strand L/B/K ALP cDNA was synthesized from fibroblast mRNA, and then the protein-coding region (open box) was amplified by use of the primers A and B. The point mutation 711A (see text) observed in the mutant L/B/K ALP cDNA is indicated. kb, Kilobase.

specific oligodeoxynucleotide probes. The probes distinguish between the normal (711G) and mutant (711A) alleles, and the hybridization pattern verifies the presence of this mutation in various individuals in the pedigree (Fig. 2). The proband is homozygous for the mutation, and both parents are heterozygous. If one classifies serum L/B/K ALP activity into two groups, above or below 70 units/liter (normal range, 68–211), then mutation 711A cosegregates with the <70 group with a maximum lod (logarithm of odds) score of 3.04 at a recombination value of 0.0 (32), indicating linkage with the trait.

To verify that mutation 711A is responsible for the deficient activity of L/B/K ALP observed in the proband, we introduced the G → A transition at position 711 into a normal L/B/K ALP cDNA by site-directed mutagenesis and tested the ability of this altered cDNA to express ALP activity (Fig. 3). NIH 3T3 cells transfected with the normal cDNA plasmid expressed ALP activity at a level about 200-fold above background. In contrast, cells transfected with the mutant plasmid failed to express L/B/K ALP activity, though they produced the corresponding mRNA as well as immunologically crossreactive material (Fig. 3). These data demonstrate that mutation 711A is sufficient to inactivate the enzyme.

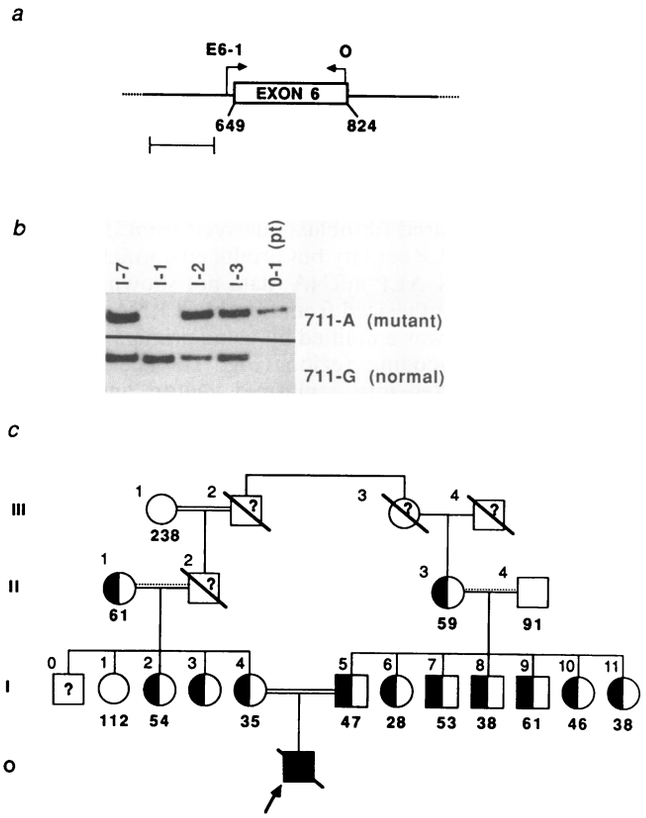

FIG. 2. Analysis of mutation 711A in the affected pedigree. (*a*) Exon 6 of the L/B/K ALP gene is shown schematically with the 5′ and 3′ boundaries numbered as described for the cDNA (3). A 222-bp segment of the L/B/K ALP gene that contains cDNA position 711 was amplified from DNA of family members by using the primers E6-1 and O. Scale bar = 100 bp. (*b*) The amplified DNA was hybridized to two allele-specific probes designed to discriminate between the normal (711-G) and mutant (711-A) alleles at cDNA position 711. A representative experiment including the DNAs of the patient (pt) and several family members is shown. (*c*) The affected pedigree. The proband is indicated by the arrow. Filled symbol, mutation 711A homozygote; half-filled symbol, mutation 711A heterozygote; open symbol, normal allele (711G) homozygote; ?, information unavailable. Dotted lines indicate probable consanguinity. Serum L/B/K ALP activity levels (units/liter; normal adult range, 68–211) are shown below each pedigree member.

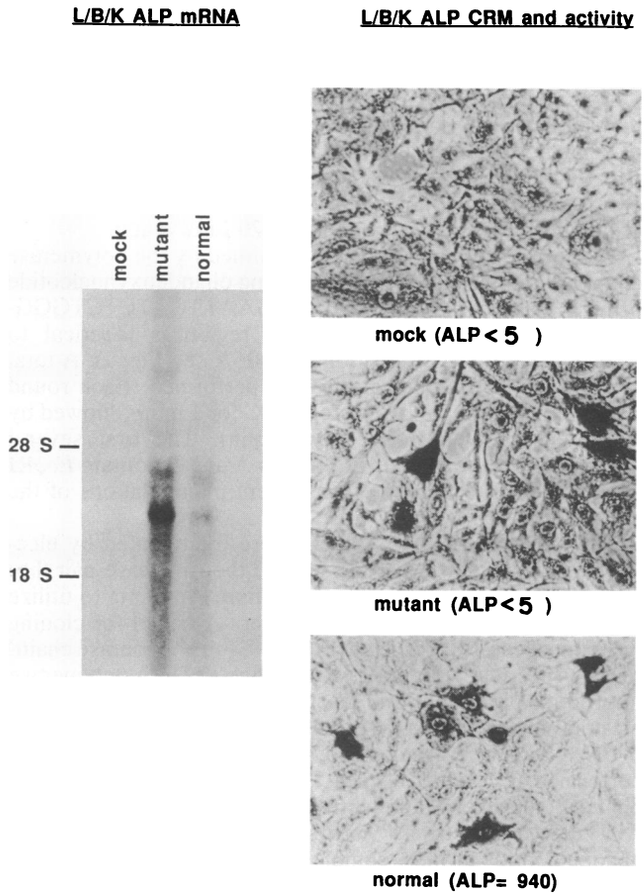

FIG. 3. Expression of the mutant and normal L/B/K ALP cDNAs. The mutant cDNA contains the G → A transition at cDNA position 711. For mock transfection, no DNA was used. The plasmids were transfected into NIH 3T3 cells and examined for transient expression of L/B/K ALP mRNA by blot hybridization (*Left*) and for immunologically crossreactive material and enzyme activity (*Right*). Positions of 28S and 18S rRNA are indicated. Micrographs show cells stained for crossreactive material (CRM) with rabbit anti-human liver ALP antiserum. Enzyme activity levels (milliunits per mg of protein) in whole-cell suspensions are shown in parentheses.

Hence, the primary defect in this hypophosphatasia patient resides at the L/B/K ALP locus.

Mutation 711A converts a CpG dinucleotide to CpA on the nontemplate strand of L/B/K ALP exon 6. Cytosine residues 5′ to guanine are hypermutable because they are subject to methylation and subsequent deamidation to thymine (33). It is, therefore, probable that mutation 711A initially arose as a CpG → TpG transition on the template strand.

Further analysis of mutation 711A is facilitated by considering the three-dimensional structure of *Escherichia coli* ALP, which has been determined by crystallographic studies (34, 35). The active pocket of the bacterial ALP consists of several components distributed throughout the polypeptide chain: three functional metal binding sites, a reactive serine that is transiently phosphorylated during the enzymatic reaction, and an arginine residue thought to stabilize the transition state during catalysis. Alignment of primary protein sequences indicates that the active sites of the bacterial and human ALPs are highly conserved (3–5).

Alanine-162 of mature L/B/K ALP is situated within a stretch of 10 amino acids that separate a group of metal ligands and the catalytically important arginine (corresponding to arginine-166 of *E. coli* ALP). It is possible that mutation 711A disrupts the spatial relationship between these two essential components of the ALP active pocket. This

alanine is present at analogous positions in human placental and human intestinal ALP (4–6), the L/B/K ALPs of rat (36, 37), mouse (38), and cow (39), and *E. coli* ALP (3). However, the corresponding position is occupied by serine in yeast ALP (40).

We were unable to detect mutation 711A in the genomic DNA of 34 unrelated individuals with the various forms of hypophosphatasia ranging from perinatal to adult. This observation is consistent with genetic heterogeneity for hypophosphatasia. Examination of the L/B/K ALP gene in different hypophosphatasia patients should define other mutant alleles that produce this disorder.

For many years, ALP has been implicated in bone formation, although the exact nature of its role is unclear (41). We have demonstrated that a mutation in the L/B/K ALP gene that abolishes enzymatic activity results in hypophosphatasia, proving that ALP is necessary for normal bone mineralization.

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