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# Mutations of the $G_s$ $\alpha$-subunit gene in Albright hereditary osteodystrophy detected by denaturing gradient gel electrophoresis

**(pseudohypoparathyroidism/polymerase chain reaction/splice junction/heterotrimeric guanine nucleotide-binding protein)**

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ABSTRACT **Affected members of most kindreds with Albright hereditary osteodystrophy have a partial deficiency of functional $G_s$, the guanine nucleotide-binding protein that stimulates adenylyl cyclase. By use of the polymerase chain reaction to amplify genomic fragments with the attachment of a high-melting G+C-rich region (GC clamp) and analysis of these fragments by denaturing gradient gel electrophoresis, heterozygous mutations in the $G_s$ $\alpha$-subunit gene were found in two kindreds. These included a G → C substitution at the donor splice junction of intron 10 and a coding frameshift created by a single base deletion within exon 10. The findings illustrate the heterogeneity of genetic defects in Albright hereditary osteodystrophy and the usefulness of the polymerase chain reaction–denaturing gradient gel electrophoresis method to search rapidly for mutations in a large candidate gene.**

Albright hereditary osteodystrophy (AHO) is an uncommon genetic disorder characterized by a constellation of abnormal physical features including short stature, obesity, rounded facies, and skeletal defects. AHO may be expressed in members of the same kindred with or without resistance to hormones such as parathyroid hormone, thyrotropin, and gonadotropins that are coupled to stimulation of adenylyl cyclase. These forms of the disease are termed pseudohypoparathyroidism (PHP) and pseudopseudohypoparathyroidism (PPHP), respectively (1–3). The affected members (either PHP or PPHP) of most AHO kindreds demonstrate an ≈50% deficiency in all tissues of $G_s$, the heterotrimeric guanine nucleotide-binding protein (G protein) that stimulates adenylyl cyclase, as measured by functional assays utilizing $G_s$-deficient mutant S49 lymphoma cell membranes or by ADP-ribosylation via cholera toxin (4–6).

Heterotrimeric G proteins involved in signal transduction consist of $\alpha$, $\beta$, and $\gamma$ subunits, each the product of a separate gene (7–9). The $\alpha$ subunits bind guanine nucleotide and confer specificity to each G protein. Steady-state levels of $G_s$ $\alpha$-subunit ($G_s\alpha$) mRNA are reduced in the affected members of most but not all AHO kindreds (5, 10). The simplest explanation of this finding is a defect in one allele of the $G_s\alpha$ gene. Southern blotting in several cases revealed no gross deletions or rearrangements of the gene (5, 10).

The human $G_s\alpha$ gene has been sequenced and found to contain 13 exons spanning 20 kilobases (11). To screen the $G_s\alpha$ gene rapidly for mutations, the polymerase chain reaction (PCR) was used to amplify the exons and bordering intron regions from genomic DNA with an attached G+C-rich high-melting region (GC clamp) and the amplified fragments were analyzed by denaturing gradient gel electrophoresis (DGGE; ref. 12). In this report, two different mutations detected by this approach are identified.

## MATERIALS AND METHODS

**Melting-Map Analysis.** Melting maps (plots of the midpoint melting temperature as a function of position along a DNA molecule) of each genomic fragment with a GC clamp attached to either the 5′ or the 3′ end were generated using the computer algorithm of Lerman and Silverstein (13).

**Oligonucleotide Primer Synthesis.** All oligonucleotide primers for PCR and direct sequencing were synthesized on an Applied Biosystems 380B DNA synthesizer and purified by reverse-phase chromatography on "NENSorb Prep" columns (NEN/DuPont) according to the manufacturer's recommendations. The nucleotide sequences of the primers may be obtained from the authors upon request.

**PCR Amplification of Genomic DNA.** For each subject genomic DNA was isolated from either blood or cultured fibroblasts (14) and 1 $\mu$g was included in a 100-$\mu$l PCR (15) mixture containing deoxynucleoside triphosphates (200 $\mu$M each), upstream and downstream oligonucleotide primers (1.0 $\mu$M each), 0.01% (wt/vol) gelatin, 50 mM KCl, 10 mM Tris/HCl (pH 8.3), 1.5 mM $MgCl_2$, and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus). Amplification consisted of denaturation at 94°C for 5 min followed by 30 cycles consisting of annealing at 58°C for 45 sec, primer extension at 72°C for 1 min, and denaturation at 94°C for 1 min and 1 final cycle with a 3-min primer extension. The PCR products were analyzed by electrophoresis in nondenaturing 5% acrylamide gels.

**DGGE.** PCR-amplified fragments of genomic DNA were analyzed by DGGE in a Hoefer SE600 vertical apparatus as described (16). Electrophoresis was performed at 60°C, using an LKB Multitemp II thermostatic circulator. Samples (10–30 $\mu$l) were electrophoresed for 15 hr at 100 V in a 6.5% acrylamide vertical gel with a parallel gradient of denaturing conditions linearly increasing from 5% at the top to 75% at the bottom and that was maintained at 60°C [100% denaturing condition was 7 M urea and 40% (vol/vol) formamide]. Gels were stained with ethidium bromide (2 $\mu$g/ml).

**Direct DNA Sequencing.** PCR-amplified genomic fragments were sequenced using multiple oligonucleotide primers (17).

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Abbreviations: G protein, heterotrimeric guanine nucleotide-binding protein; $G_s$, G protein that stimulates adenylyl cyclase; $G_s\alpha$, $\alpha$ subunit of $G_s$; AHO, Albright hereditary osteodystrophy; PHP, pseudohypoparathyroidism; PPHP, pseudopseudohypoparathyroidism; PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis.

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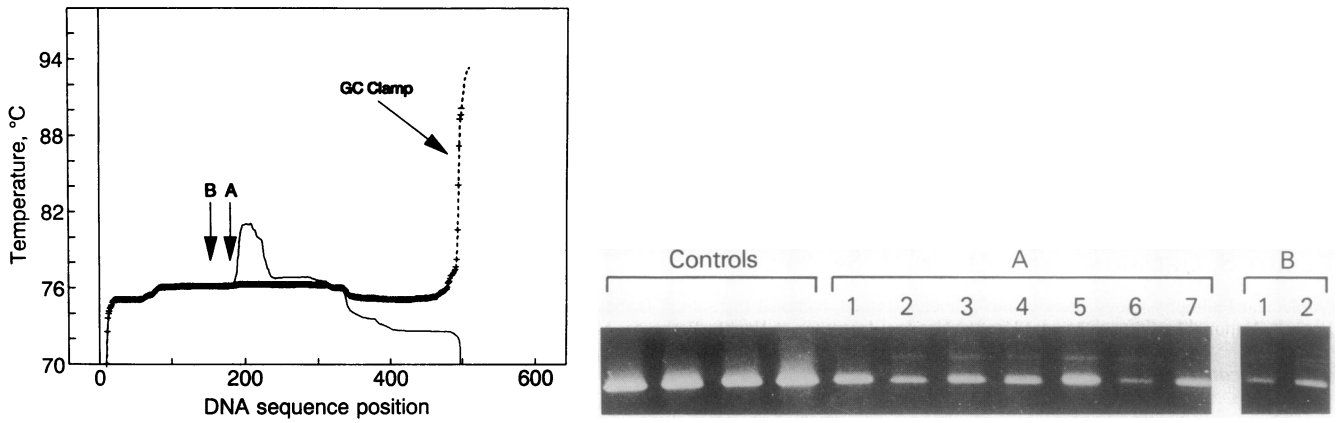


FIG. 1. (*Left*) Melting maps of the amplified genomic fragment spanning exons 10 and 11 with and without a 40-bp GC clamp attached to the 3′ end. The predicted midmelting temperature (temperature at which a base pair has a 50% probability of melting) was plotted as a function of the position of the base pair in the sequence by using the computer algorithm of Lerman and Silverstein (13). The profile without the GC clamp is shown by the thin solid line, and the positions of the mutations present in kindreds A and B are noted. A schematic of the genomic fragment is shown in Fig. 3*B*. Note that addition of the GC clamp simplifies the melting map so that the genomic sequence constitutes essentially one low-melting domain. (*Right*) DGGE analysis of the exon 10–11 genomic fragment with a GC clamp attached to the 3′ end. PCR-amplified genomic DNA with a 3′ attached GC clamp was analyzed by DGGE as described in *Materials and Methods*. The oligonucleotide primers used for PCR were 5′-AAGAATTCTTAGGGATCAGGGTCGCTGCTC-3′ (upstream primer) and 5′-CGCCCGCCGCGCCCCGCGCCCGTCCC-GCCGCCCCCGCCCCATGAACAGCCAGCAAGAGTGGA-3′ (downstream primer with GC clamp), with the underlined sequences complementary to the $G_s\alpha$ gene. The results of four control subjects, seven subjects from kindred A, and the two affected members of kindred B are shown. For kindred A the numbers above the lanes correspond to the pedigree shown in Fig. 3*A*. Compared to the patterns of control and unaffected members, which have a single band, affected members of kindred A have a pattern with one extra band and those from kindred B show three extra bands. Electrophoresis of all DNA samples in a nondenaturing 5% acrylamide gel revealed only a single band (data not shown).

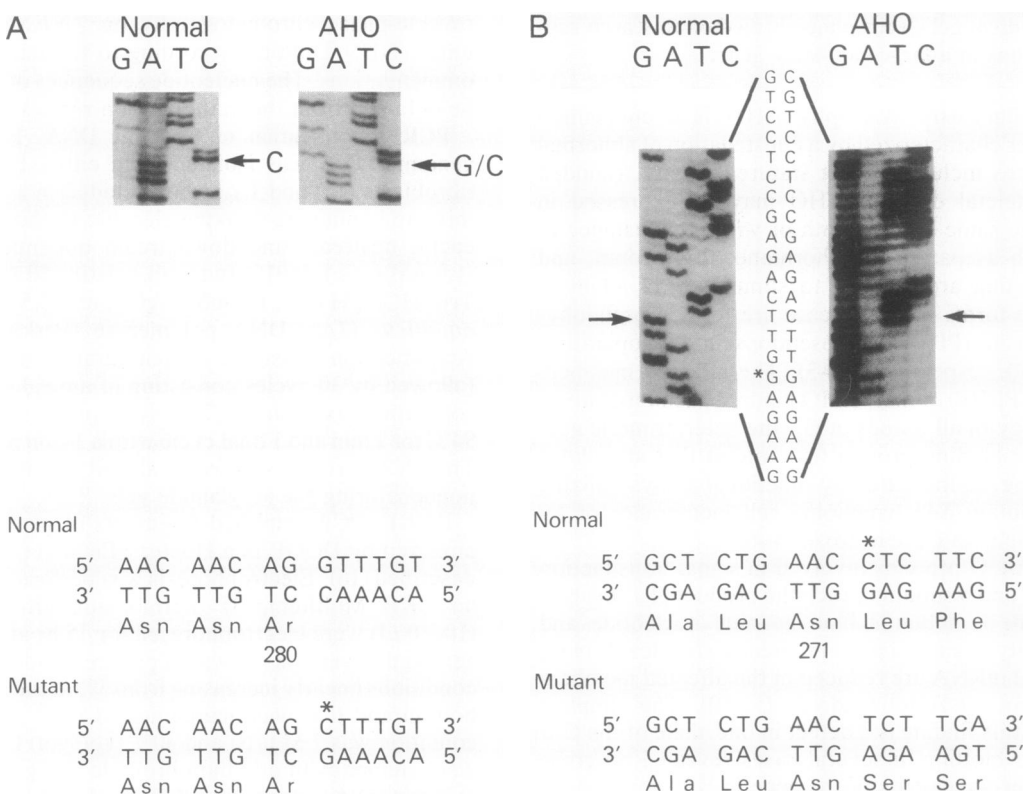


FIG. 2. (*A*) Direct sequence analysis of the amplified genomic fragment spanning exons 10 and 11 from an affected member of kindred A. Genomic DNA was amplified by PCR using the same oligonucleotides as for DGGE and was directly sequenced (17). Sequence shown is that of the complementary strand, obtained by using an oligonucleotide primer complementary to sequence within intron 10 (5′-CTTGCACGGGGTTCTTCTCTA-3′). Whereas the direct sequence of genomic DNA amplified from a control subject reveals only a C at the donor splice junction of exon 10, the corresponding sequence in an affected member reveals G and C at this position as noted. The sequences of the normal and mutant alleles in this region are shown below with the codon number (ref. 11). The mutation (asterisk) creates a new *Alu* I restriction site (AGCT). (*B*) Direct sequence analysis of the same amplified genomic fragment from an affected member of kindred B. The method and sequencing primer in this case are the same. Whereas DNA sequence of the complementary strand from a control subject through the region of exon 10 shown is unambiguous (shown in the left column), heterozygous deletion of one G in the complementary sequence (asterisk) produces a frameshift that results in a splitting by one base of all sequence beyond the mutation (right column; position of frameshift shown with arrows). This pattern was seen in the DNA sequence from both affected members and was noted when sequencing in either direction. Normal and mutant allele sequences in this region are shown below.

## RESULTS

**Screening for Mutations in the $G_s\alpha$ Gene by DGGE Analysis of PCR-Amplified Genomic DNA Fragments.** Nine exon and bordering intron regions encompassing exons 2, 3, 4–5, 6, 7–8, 9, 10–11, 12, and 13 of the $G_s\alpha$ gene were amplified by PCR from genomic DNA and the amplified fragments were analyzed by DGGE. To increase the sensitivity of DGGE, a 40-base-pair (bp) GC clamp was attached to either the 5′ or the 3′ end of each amplified fragment by including this sequence in the appropriate synthetic oligonucleotide primer 5′ to the specific complementary sequence (12). To determine whether to attach the GC clamp on the 5′ or 3′ end of each genomic fragment, the respective melting maps (13) were compared. In each case the GC clamp-attachment site that predicted the most uniform low-melting domain for the genomic sequence was the one chosen. By this method, gene regions including exons 2–13 were analyzed from subjects of four AHO kindreds.

Analysis by DGGE of a genomic fragment spanning exons 10 and 11 (Fig. 1) revealed abnormal patterns only in the affected members from two kindreds (kindreds A and B). The patterns were identical among affected members of each kindred but distinct between kindreds. Neither DGGE pattern was detected in 10 unrelated subjects, suggesting that these are not related to common polymorphisms.

DGGE analysis of PCR-amplified fragments encompassing exons 2–9, 12, and 13 detected no further mutations. Similar analysis of two other unrelated patients with AHO revealed no mutations in exon regions 2–13. Since these regions were not directly sequenced and since other portions of the gene (5′ flanking region and exon 1) were not studied, we cannot exclude additional mutations.

**Identification of a Splice Junction Mutation in Kindred A.** Direct sequencing of the amplified genomic DNA fragment containing exons 10 and 11 from affected members of kindred A revealed a heterozygous single G → C substitution of the first base of intron 10 at the donor splice junction bordering the 3′ end of exon 10 (Figs. 2*A* and 3). This single base change created a new *Alu* I restriction site, which allowed us to confirm the presence of this heterozygous mutation in all affected members and its absence in all unaffected members analyzed by *Alu* I digestion of this amplified DNA fragment (Fig. 3).

A previous report of this kindred (18) suggested that the father was the affected parent, based upon only a radiologically abnormal fifth metacarpal bone. By both clinical and laboratory examination neither parent has the florid expression of the AHO phenotype or hormonal abnormalities. The father's radiologic abnormality is most probably secondary to trauma. The mother, who bears the $G_s\alpha$ mutation, has several minor clinical features consistent with AHO, including asymmetric brachyphalangia of the first digit on one hand, radiological evidence of subcutaneous calcifications, and significant short stature relative to other members of her family. Moreover, analysis of erythrocyte membrane proteins on immunoblots probed with a $G_s\alpha$-specific antibody is consistent with the father having normal, and the mother deficient, amounts of $G_s\alpha$ membrane protein (data not shown). All four of the affected daughters have clinically evident PHP and previously determined functional $G_s$ deficiency.

**Identification of a Frameshift Mutation in Kindred B.** Direct sequencing of the amplified exon 10–11 fragment from both the mother with PPHP and daughter with PHP revealed an identical heterozygous single base deletion in exon 10 producing a frameshift of the coding region, presumably interrupting the generation of normal $G_s\alpha$ (Fig. 2*B*).

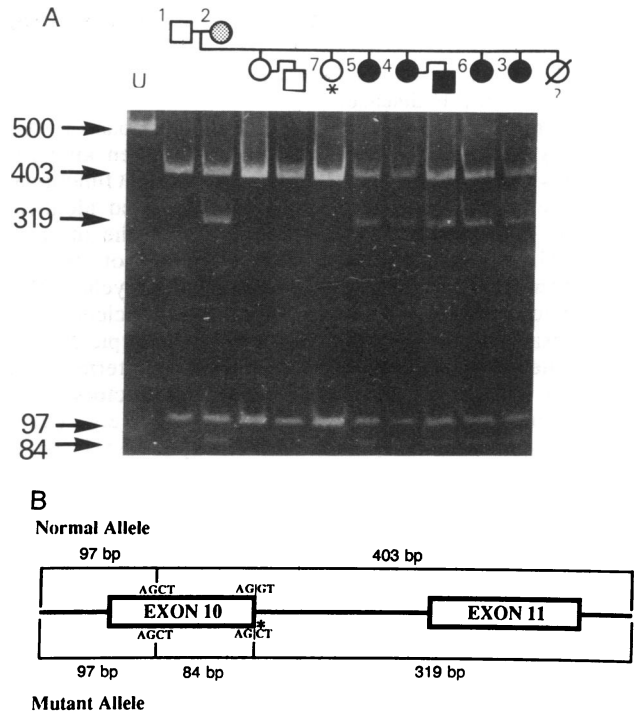


FIG. 3. (*A*) Pedigree of kindred A with *Alu* I restriction analysis of genomic DNA. The genomic fragment including exons 10 and 11 was PCR-amplified from each subject by using the same oligonucleotides as for DGGE but without the GC clamp. DNA was then isolated and concentrated in Centricon-100 filters (Amicon) and digested with *Alu* I restriction endonuclease (Bethesda Research Laboratories). The digests were electrophoresed in a nondenaturing 5% acrylamide gel. Symbols of pedigree members with PHP are blackened and that for the member with PPHP is stippled (18). The asterisk denotes an unaffected member with four unaffected sons who were not analyzed. One subject who died in infancy and whose disease status is unknown is marked with a question mark. The numbers beside members in the pedigree correlate to the DGGE analysis shown in Fig. 1 *Right*. The size of the fragments seen in the restriction analysis is depicted on the left with the 500-bp undigested fragment shown in lane U. Digestion of DNA from unaffected members results in 403- and 97-bp fragments, whereas digestion of DNA from affected members (PHP and PPHP) results in two additional fragments of 319 and 84 bp, as predicted if these subjects are heterozygous for the mutation found by direct sequencing. (*B*) Schematic of PCR-amplified genomic fragment including exons 10 and 11 and showing *Alu* I restriction sites. The 500-bp genomic fragment contains an *Alu* I site 97 bp from the 5′ end in the normal sequence of exon 10, predicting 403- and 97-bp restriction fragments in the normal allele, as shown above the diagram. As shown below the diagram, a G → C base substitution at the donor splice junction site of intron 10 creates a new *Alu* I restriction site, which would predict 319-, 97-, and 84-bp restriction fragments in the mutant allele.

## DISCUSSION

In this study two types of mutations were identified in the affected members of AHO kindreds that could account for $G_s\alpha$ deficiency. A splice junction mutation, which has been shown in other genes to result in abnormal RNA splicing (19–23), would be consistent with the decreased steady-state $G_s\alpha$ mRNA levels found in the affected members of kindred A (10). The coding frameshift mutation found in kindred B would prevent the generation of a normal full-length $G_s\alpha$ from the abnormal allele. Recently, a mutation of the initiator codon was identified in an AHO kindred with an aberrant form of $G_s\alpha$ protein and normal steady-state levels of $G_s\alpha$ mRNA (24). This mutation and the negative results obtained in two other unrelated patients indicate that mutations in the region of exon 10 will probably not be found in all patients

with AHO. The findings in all three kindreds are consistent with the dominant inheritance of AHO by a heterozygous mutation in the $G_s\alpha$ gene and illustrate the genetic heterogeneity present in this disease.

These findings define the probable molecular basis for $G_s$ deficiency in AHO. Interestingly, within a given kindred, subjects with either PPHP or PHP show equivalent functional $G_s$ deficiency (4, 5) and we now have detected identical mutations in the $G_s\alpha$ gene in both forms of the disease. Despite $G_s$ deficiency, subjects with PPHP are not resistant to hormones that act through the generation of cyclic AMP. The implication is that for most kindreds $G_s$ deficiency may be necessary but is not sufficient for full phenotypic expression of the disease (PHP). Other factors may determine the extent of disease expression in AHO. Such factors could include functional differences in other enzymes (e.g., cyclic AMP phosphodiesterase) involved in the cyclic AMP signal-transduction cascade.

DGGE, a technique that separates DNA molecules on the basis of compositional as opposed to size differences, was described over a decade ago (25). Several reports have shown its ability to detect single base mutations (26–29). Two recent developments, PCR and the attachment of GC clamps, when used in conjunction with DGGE, result in an efficient and highly sensitive method (12, 30, 31). This report demonstrates the usefulness of PCR–DGGE to rapidly screen many subjects for previously undefined mutations in a large and complex candidate gene.

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