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Insertion of an Alu Sequence in the Ca^{2+}-Sensing Receptor Gene in Familial Hypocalciuric Hypercalcemia and Neonatal Severe Hyperparathyroidism

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Summary

Missense mutations in the calcium-sensing receptor (CaR) gene have previously been identified in patients with familial hypocalciuric hypercalcemia (FHH) and neonatal severe hyperparathyroidism (NSHPT). We studied family members of a Nova Scotian deme expressing both FHH and NSHPT and found, by PCR amplification of CaR gene exons, that FHH individuals were heterozygous and NSHPT individuals were homozygous for an abnormally large exon 7. This is due to an insertion at codon 877 of an Alu-repetitive element of the predicted-variant/human-specific-1 subfamily. It is in the opposite orientation to the CaR gene and contains an exceptionally long poly(A) tract. Stop signals are introduced in all reading frames within the Alu sequence, leading to a predicted shortened mutant CaR protein. The loss of the majority of the CaR carboxyl-terminal intracellular domain would dramatically impair its signal transduction capability. Identification of the specific mutation responsible for the FHH/NSHPT phenotype in this community will allow rapid testing of at-risk individuals.

Introduction

Familial hypocalciuric hypercalcemia (FHH) is an autosomal dominant disorder characterized by modest elevation of serum calcium concentration, relative hypocalciuria, and inappropriately normal parathyroid hormone (PTH) levels (Foley et al. 1972; Marx et al. 1981; Law and Heath 1985). The condition is inherited as an autosomal dominant trait and can be diagnosed soon after birth. While the penetrance of FHH appears to be $\geq$90%, affected individuals exhibit virtually none of the morbidity associated with hypercalcemia. Physiological and biochemical studies on individuals with FHH demonstrate abnormal responses of both kidney and parathyroid gland in sensing blood calcium levels. The parathyroid gland has a special ability to "sense" extracellular calcium concentrations. The mechanism whereby this is achieved recently became clearer, when a calcium-sensing receptor (CaR), expressed in both parathyroid and kidney, was cloned and characterized (Brown et al. 1993). This calcium receptor is a member of the G-protein-coupled receptor family and responds to increased levels of extracellular calcium by triggering a phospholipase C pathway and elevating intracellular calcium levels. This, in turn, inhibits secretion of PTH from the parathyroid gland.

The CaR gene has been mapped (Pollak et al. 1993) to chromosome 3, the same chromosome to which the FHH disease locus was previously localized (Chou et al. 1992). In most FHH families studied to date, linkage to chromosome 3q predominates, although, in one family, linkage to 19p rather than 3q was demonstrated (Heath et al. 1993). In another family, the FHH locus was not linked to either chromosome 3, 19, or 11 (Trump et al. 1993). Thus, the disease exhibits genetic heterogeneity. While the inheritance of a single copy of a mutated gene causes FHH, homozygous individuals who inherit two inactive gene copies may have neonatal severe primary hyperparathyroidism (NSHPT) (Pollak et al. 1994*b*). NSHPT (Pratt et al. 1947) is characterized by marked hypercalcemia, skeletal demineralization, and parathyroid hyperplasia and, without parathyroidectomy, is usually fatal (Goldbloom et al. 1972), although some cases of less severely affected neonates have been successfully managed medically (Marx et al. 1982).

Recently, several different missense mutations in the CaR gene have been identified in FHH and NSHPT patients (Pollak et al. 1993; Heath et al. 1994; Pearce et al. 1994). We sought mutations in the CaR gene in two families, living in close proximity in Nova Scotia, having some members affected with FHH and some with NSHPT. We found that all affected members of both families had an insertion of an Alu-repetitive element within exon 7 of the CaR gene.

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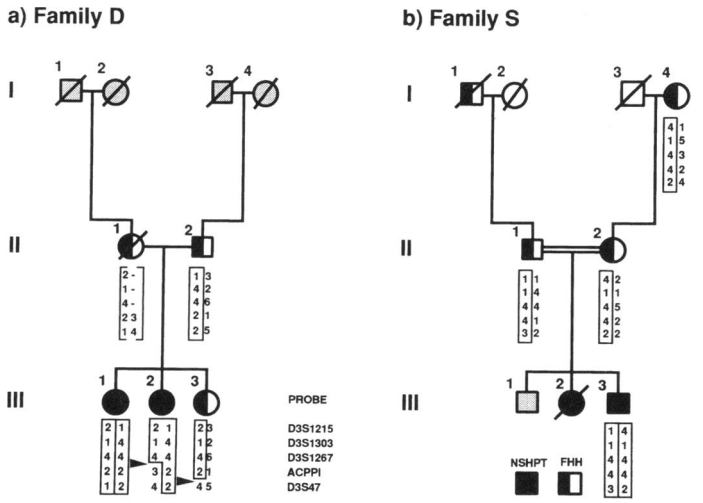

Figure 1 Pedigrees of the two FHH/NSHPT families. The genotypes for five microsatellite markers on chromosome 3q11.2-24 were determined. Markers are ordered according to their position on chromosome 3q, from centromere to telomere. In all affected individuals, the disease locus segregated with the boxed haplotypes. In family D, recombination events are indicated by the arrows.

Subjects and Methods

Families

We studied 15 members of two consanguineous family groups from Nova Scotia. Both family D (Goldbloom et al. 1972) and family S (Pratt et al. 1947; Cole et al. 1990) have been reported before. Only family S has been previously haplotyped (Pollak et al. 1994*b*) (with markers D3S1303, D3S1267, and D3S1269); there is no correspondence between the specific haplotype numbers in that report and those given here.

Genotyping

Genomic DNA was isolated from blood samples, as previously described (Ausabel et al. 1987). Genotypes were defined with five polymorphic microsatellite markers on chromosome 3q, 3cen-q24: D3S1215, D3S1303, D3S1267, ACPP, and D3S47 (Naylor et al. 1994). Analysis was performed by PCR. Oligonucleotide primers were end-labeled for 30 min at 37°C in a 10-μl reaction volume containing 10 μCi [^{32}P]ATP, 50 pmol primer DNA, 1 × kinase buffer (50 mM Tris Ph 7.6, 10 mM $MgCl_2$, 5 mM DTT, 100 mM spermidine, 100 μM EDTA), and 10 U T4 polynucleotide kinase (Pharmacia Biotech). The reaction was stopped by heating at 80°C for 10 min. Labeled primers were used directly or were stored at −20°C until use. Two hundred nanograms of each individual's genomic DNA were used as template in a 14-μl PCR containing 11 pmol of PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin), 200 μM of each deoxynucleotide triphosphate, and 0.5 units of Taq polymerase (Perkin-Elmer-Cetus). The PCR consisted of the following conditions: 5 min at 98°C, followed by 30 cycles of 20 s at 94°C, 30 s at 58°C–62°C (varied for each set of primers), 30 s at 72°C, and a final elongation step at 72°C for 10 min, using a GeneAmp PCR system thermocycler model 9600 (Perkin-Elmer-Cetus). After amplification, samples were mixed with an equal volume of stop solution (97.5% deionized formamide, 0.3% Bromophenol Blue, 0.3% Xylene Cyanol FF, 10 mM EDTA) and were electrophoresed on 6% denaturing polyacrylamide sequencing gels (Long Ranger; J. T. Baker).

Mutation Analysis

We analyzed DNA from at least one individual affected with FHH, one with NSHPT, and one unaffected individual from each family. PCR amplification of exons 4 and 7 of the calcium receptor gene were performed with the following primers: 4AF, 5′-ACTCATTCACCATGTTCTTGGTTCT-3′; 4AR, 5′-GAATTCCCGGAAGCCTGGGATCTGC-3′; 4BF, 5′-GCCATGCCTCAGTACTTCCACCTGG-3′; 4BR, 5′-CCCAACTCTGCTTTATTATACAGCA-3′; 7AF, 5′-AAGTGCCCAGATGACTTCTGGTCCA-3′; 7CR, 5′-CCATGGCGTTCTTCTGAGGCTCATC-3′; 7BF, 5′-TCGAGCTACCGCAACCACGAGCTGGA-3′; 7BR, 5′-GGATCCCGTGGAGCCTCCAAGGCTG-3′. Primers 4AF–7CR and 7BR are the same as those described by Pollak et al. 1993. Primer 7BF is the sequence of the bovine-parathyroid calcium-receptor cDNA (Brown et al. 1993) from nt 2248–2272. Amplified products were resolved on 1% agarose gels (1 × Tris-borate EDTA pH 8.0) and, if required, were subcloned into the pCRII TA cloning vector (Invitrogen Corp). Plasmid DNA was extracted using the plasmid midi protocol (Qiagen). Dideoxynucleotide sequencing, using the T7 Sequencing kit (Pharmacia, LKB) and [^{35}S]dATP (1,200 Ci/mmol; Amersham), was carried out on midiprep DNA from at least five positive colonies for each FHH, NSHPT, and normal individual. Sequenc-

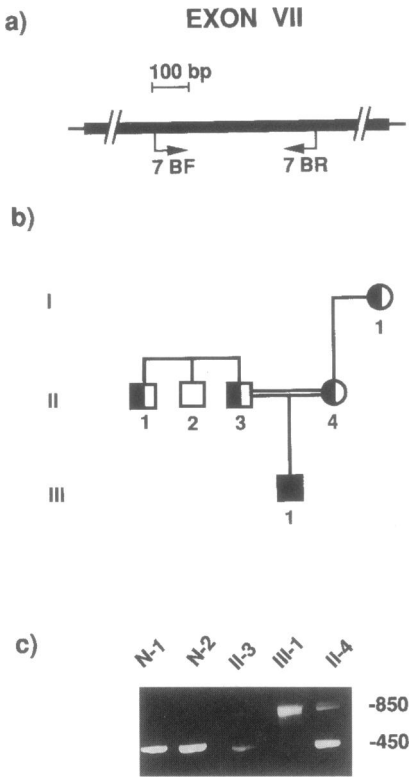

Figure 2 *a,* Exon 7 of the CaR gene showing position of the primers used for amplification from genomic DNA. Flanking intronic sequence is indicated by the thinner line. *b,* Pedigree of family S. *c,* PCR-amplified products obtained by using primers 7BF and 7BR and separated on a 2% agarose gel. Both parents (II-3 and II-4) having FHH are heterozygous with a normal sized fragment of 450 bp (the only product obtained from unrelated normal individuals N-1 and N-2) and a larger fragment of 850 bp. Their son (III-1) having NSHPT was homozygous for the larger fragment of 850 bp. A similar pattern was observed when primers 7AF and 7CR were used with both parents being heterozygous for a normal fragment and a fragment larger by 400 bp and their son being homozygous for the larger fragment (data not shown).

ing reactions were performed using T7 and SP6 primers and INS-F, 5′-TGATCGCCATCCTGGCGGCCAGCTT-3′; INS-R, 5′-GCGGCCACCTTGAAAG-3′; and INS-RC, 5′-AGATCGAGACCATCCT-3′. Primers INS-F and INS-R are part of the normal sequence of exon 7, whereas INS-RC is within the Alu repeat sequence. In order to identify positively those at-risk individuals carrying the mutation, their DNA was amplified by using primer 7NT, 5′-TGAGACGGAGTCTCGCTCTGTCGC-3′, which is the sequence immediately 3′ to the poly(T) tract in the Alu insert sequence and primer 7BR (described above). The amplified fragment of 374 bp was digested with *Dde*I (New England Biolabs) to generate fragments of 278 bp and 96 bp.

Results

Haplotype Analysis

Because this disease exhibits genetic heterogeneity, haplotype analysis was first carried out by using polymorphic microsatellite markers (Weber and May 1989; Naylor et al. 1994) in the form of di- or tetranucleotide repeats for a region of chromosome 3q (D3S1215, D3S1303, D3S1267, ACPP, D3S47). Genotyping was performed as described in Subjects and Methods. Genomic DNA was analyzed from nine members of family S and from six members of family D (fig. 1 and data not shown). Within each family, the disease locus cosegregated with the same 3q11.2-24 haplotype. This confirmed and extended an earlier report on family S (Pollak et al. 1994*b*).

Mutation Analysis

Since the mutations in the CaR gene (Capuano et al. 1994) reported initially were found in exons 4 and 7 (originally called exons 3 and 6, respectively [Pollak et al. 1993]), we first PCR amplified these exons. Exon 4 was of normal size in both families (fig. 2). However, amplification of exon 7 revealed the presence of two fragments of different sizes (450 bp and 850 bp). All unaffected individuals were homozygous for the band of normal size, whereas FHH individuals were heterozygous with one normally sized product and one of larger size. NSHPT individuals were homozygous for the larger fragment (fig. 2). Use of several different primers sets confirmed this result (data not shown). Identical results were obtained with DNA from both family D and family S members.

Exon 7 Contains an Insertion of an Alu Sequence

The large PCR product from one affected family D member and one affected family S member were subcloned into a plasmid vector. Nucleotide sequence analysis showed that the inserted sequence is an Alu-repetitive element of ~383 bp in length. This Alu element contains an exceptionally long poly (A) tract of 92–94 bp. The Alu element is inserted in the opposite (antisense) orientation, relative to that of the CaR gene, after the first C residue of codon 877 (ACC)–encoding threonine (fig. 3). A direct repeat of a 15-bp sequence within the CaR gene, located just 3′ to the insertion point, was found at the far 5′ end of the inserted sequence itself. This suggests retrotransposition as the mode of insertion.

All Affected Individuals from Both Families Have the Mutation

A primer set was designed with one primer sequence within the Alu-repetitive sequence itself and the other within the normal CaR gene sequence flanking the insert (fig. 4). DNA from normal individuals will not amplify with these primers; however, in individuals with either FHH or NSHPT, a PCR product of 374 bp is obtained. Restriction-enzyme analysis at a diagnostic *Dde*I site results in two fragments of 278 bp and 96 bp. As shown in figure 4, DNA from known affected individuals in families D and S amplified with this primer set, whereas

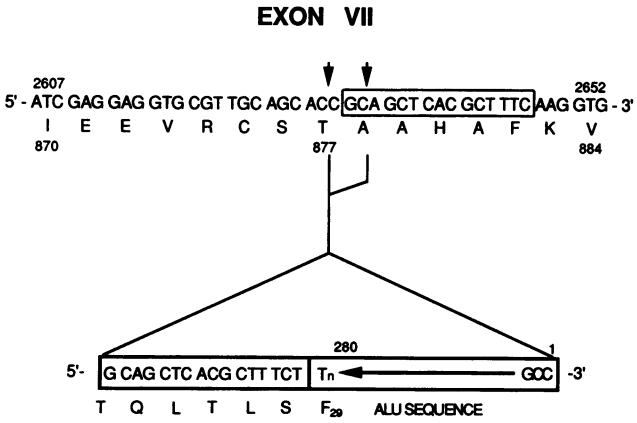

Figure 3 Diagram of the Alu insertion in exon 7 of the CaR gene. Partial sequence of exon 7, between nt 2607 and 2652, is shown. The vertical arrows indicate the point of insertion at codon 877 encoding threonine and loss of 3 nt of normal CaR gene sequence. The Alu sequence is flanked by 15-bp–perfect direct repeats, one of which is within the normal CaR gene 3′ to the insertion, and the other is at the far 5′ end of the inserted sequence. The horizontal arrow indicates the antisense orientation of the Alu element with respect to the orientation of the host gene. Thus, the 3′-most nucleotide of the insertion is position one of the Alu sequence, which extends to nt 280 and is followed by the long poly(A/T) tract.

neither PCR products from normal family member DNA nor DNA of unrelated normal individuals were obtained.

The Alu-Repetitive Element is of the Predicted-Variant/Human-Specific-1 (PV/HS-1) Subfamily

Comparison of the CaR-Alu-repetitive element with other Alu sequences showed that this sequence is a member of the PV/HS-1 subfamily (Batzer et al. 1991). A comparison of the CaR sequence with the Alu consensus sequence and the HS consensus sequence is shown in figure 5. Relative to the HS sequence, the CaR sequence has only two nucleotide substitutions and a single nucleotide deletion. The CaR sequence is very similar to HS subfamily sequences described as insertions in the NF1 (Wallace et al. 1991), cholinesterase (Muratani et al. 1991), and factor IX (Vidaud et al. 1993) genes, as well as one in close proximity to the Huntington disease gene (Goldberg et al. 1993). The CaR sequence has four nucleotide substitutions and one nucleotide deletion, relative to the NF1 sequence (fig. 5).

Predicted Structural Consequences of the Mutation

From the present sequence analysis it is not possible to assign the length of the long poly (A) tract definitively. However, regardless of its length, the consequences of insertion of the Alu sequence can be predicted to be the following: Normal CaR sequence will stop at amino acid 877—the normal length of the protein being 1,085 amino acids—and will be followed by the sequence Gln-Leu-Thr-Leu-Ser and a tract of 29 phenylalanine residues encoded by the poly (T) tract (see fig. 3). Then, depending on the reading frame, a variable-length–amino acid sequence will be found terminating at either amino acid 911, 947, or 962.

Discussion

Recently, inactivating mutations in the CaR gene were implicated as a cause of FHH and NSHPT (Pollak et al. 1993). Several different missense mutations and at least one nonsense mutation have now been found scattered (Pollak et al. 1993; Heath et al. 1994; Pearce et al. 1994) throughout the CaR gene, with preferential localization to exons 4 and 7. Exon 4 encodes part of the NH_2-terminal extracellular domain, which is thought to be involved in calcium binding, and mutations in this region would be expected to interfere with this function. Exon 7 encodes all seven putative transmembrane-spanning regions, the extracellular and intracellular loops, and the COOH-terminal intracellular tail. In one family with FHH, an R796W mutation lying within the portion coding the third intracellular loop of the CaR was found. When the mutated receptor cRNA was injected into *Xenopus laevis* oocytes, markedly attenuated responses to increases in perfusate Ca^{2+} were observed (Pollak et al. 1993). In addition, two activating mutations have been described recently. One, E128A, segregates with the affected status in a family with autosomal dominant hypocalcemia (Pollak et al. 1994*a*); the other, Q246R, segregates with the disease status in a family with autosomal dominant hypoparathyroidism (Finegold et al. 1994; Perry et al. 1994).

In the present study, we describe a novel type of inactivating mutation for the CaR gene, that of an insertion in exon 7 that disrupts the normal amino acid sequence after codon 877. This insertion is an Alu element. The Alu repeats comprise about 6% of the human genome and represent the most abundant family of SINEs (short interspersed elements) (Jelinek and Schmid 1982). A typical Alu-sequence element is an ~300-bp-long imperfect dimer with two sections separated by an A-rich region, a poly (A) tract of variable length, and flanking direct repeats (Deininger 1989). Alu sequences are normally transcribed by RNA polymerase III, and the sequence similarity between 7SL RNA and Alu elements suggests that the latter are derived from this RNA (Jurka and Smith 1988). The 7SL RNA is a part of the signal recognition particle that is essential for the movement of secretory polypeptides through the endoplasmic reticulum. It is believed that retrotransposition by nonviral retroposons is a mechanism for scattering Alu repeats throughout primate genomes (Weiner 1986). At the insertion target site, sequence duplication results in direct repeats flanking each Alu element. The flanking repeats vary in size—usually 9–21 bp—and they are different for each individual Alu element. Alu elements are pri-

mate specific and, on the basis of sequence similarity, they have been divided into different subfamilies that probably appeared at different evolutionary times (Weiner 1986; Jurka and Milosavljevic 1991). Individual Alu sequences differ from the consensus, as a result of single nucleotide substitutions and insertions and deletions. The CaR-Alu element is a member of the subfamily known as "PV" (Shen et al. 1991) or "HS" (Matera et al. 1991) that is one of the most recently

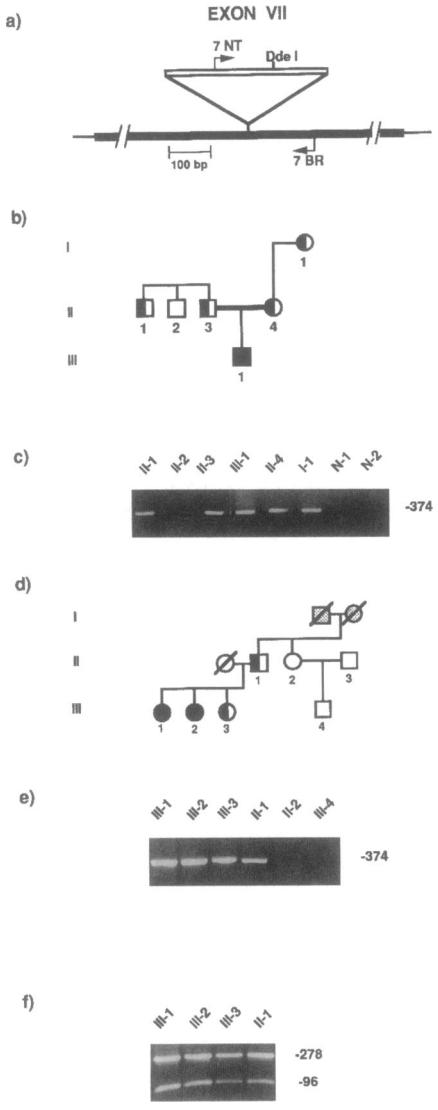

Figure 4 *a*, Schematic representation of exon 7 of the calcium-receptor gene (solid rectangle) and the insertion (open rectangle). Primer 7NT located within the insertion and primer 7BR located in the normal exon 7 sequence were used for amplification. *b*, Pedigree of family S. *c*, Agarose gel electrophoresis showing the amplified product of 374 bp obtained from genomic DNA of FHH and NSHPT individuals of family S with absent amplification from DNA of normal family members and unrelated normal individuals (N-1 and N-2). *d*, Pedigree of family D. *e*, Same result obtained in family D as in family S. When the amplified product was cut with restriction enzyme *DdeI*, the expected fragments of 278 bp and 96 bp were obtained (see panel *a*).

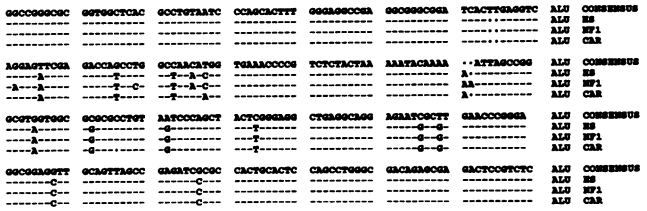

Figure 5 The CaR-Alu sequence compared with those of the Alu consensus, the Alu HS (which is the most recently inserted subfamily of Alu repeats), and a de novo inserted Alu element in the NF1 gene. The CaR-Alu insertion differs from Alu HS at only 3 nt (a substitution of C for A at position 96, a substitution of A for C at position 98, and a nucleotide deletion at position 157). The poly(A) tract that follows the consensus sequence is not shown.

inserted Alu families. The presence of five diagnostic nucleotide substitutions and a pure polyadenine tail distinguish this Alu family from other groups. Alu-repetitive sequences are detected mostly within introns or within the 5′- and 3′-noncoding regions of genes. The presence of an Alu sequence in a coding region is a rare event (Makalowski et al. 1994), since such changes in coding sequences are under strong negative selection.

The mutant CaR-Alu element contains an exceptionally long poly (A) tract of >90 bp. The exact length has not been definitively assigned, because of the inherent difficulties in PCR amplifying and sequencing such DNA. In addition, poly(A) tracts in some Alu repeats have been shown to be polymorphic, differing in length in different members of the same family (Economou et al. 1990). Regardless of the precise structure, the insertion yields a truncated protein of 911–962 amino acids in length, with loss of the normal CaR sequence of 878–1,085 amino acids. Loss of the majority of the carboxyl-terminal intracellular domain would be predicted to lead to impairment in the signal transduction capabilities of the receptor, possibly by loss of the appropriate coupling with a G protein. Alternatively, by analogy to rhodopsin and the β-adrenergic receptor, the carboxyl-terminal tail could be involved with turning off the signal by phosphorylation, and loss of this region could lead to a permanently activated receptor. However, the phenotype is clearly not that of an activating mutation. It is also possible that the insertion destabilizes the protein or alters its subcellular localization. There are several documented examples of Alu sequences incorporated into mRNA that is translated into protein (Lundwall et al. 1985; Caras et al. 1987; Brownell et al. 1989). In all described cases, only short incomplete Alu-like sequences—≤50 amino acids—were inserted and translated but not the complete Alu element, including the poly (A) tract. It has been speculated (Brownell et al. 1989) that antisense Alu elements found within exons are not translated, because of their complementarity to 7SL RNA. The RNA would hybridize with the antisense Alu sequence,

thereby inhibiting the elongation process and resulting in a truncated form of the protein.

The genotype predicts a truncated, nonfunctional protein that dissociates key tissue responses—parathyroid gland secretion, for example—from their stimulus elements, namely, ambient calcium concentrations. This prediction is consistent with the observed phenotypes, both in vitro (Marx et al. 1986) and in the patients themselves (Cole et al. 1990). Both families under study can claim a common ancestry that dates back at least 11 generations, to settlement of the area by a few New England fishing families in the mid-1700s. Although both families share common ancestral names, historical events such as unrecorded adoptions of individuals orphaned by local tragedies make any pedigree reconstruction suspect. This situation is not unlike the genetics of nephrogenic diabetes insipidus in the Maritime provinces of Canada (Bichet et al. 1992, 1993). Molecular analysis can confirm suspected common origins but may also reveal occult heterogeneity. Although we can predict that a third affected family (Marx et al. 1986) also living within a short distance of the two families described here will carry the same mutation, only molecular analysis will provide the definitive answer. However, the availability of rapid mutational testing should be of direct benefit to all families in the region of Nova Scotia who are shown to be at risk, by virtue of simple biochemical testing for an elevated serum calcium and/or a reduced urinary calcium excretion index.

The age of the insertion is not known, but we can speculate that it goes back at least as far as the first known generation of the S pedigree. Moreover, any attempt to estimate, at present, the size of the deme would ignore the effects of genetic drift associated with subsequent outmigration from the original settlement. This possibility can be explored by more widespread mutational analysis, to include families more recently settled elsewhere in North America.

In summary, we describe a mutation that deletes much of the carboxyl-terminal region of the CaR gene. The finding that FHH patients have one copy and NSHPT patients have two copies of the mutated allele confirms the gene dosage effect described previously (Pollak et al. 1993, 1994*b*). Structure-function studies of mutations in the CaR gene will lead to a better understanding of normal CaR function, as well as of the role of the receptor in the pathogenesis of FHH and NSHPT.

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