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Heterogeneous AVPR2 Gene Mutations in Congenital Nephrogenic Diabetes Insipidus

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Summary

Mutations in the AVPR2 gene encoding the receptor for arginine vasopressin in the kidney (V2 ADHR) have been reported in patients with congenital nephrogenic diabetes insipidus, a predominantly X-linked disorder of water homeostasis. We have used restriction-enzyme analysis and direct DNA sequencing of genomic PCR product to evaluate the AVPR2 gene in 11 unrelated affected males. Each patient has a different DNA sequence variation, and only one matches a previously reported mutation. Cosegregation of the variations with nephrogenic diabetes insipidus was demonstrated for two families, and a de novo mutation was documented in two additional cases. Carrier detection was accomplished in one family. All the variations predict frameshifts, truncations, or nonconservative amino acid substitutions in evolutionarily conserved positions in the V2 ADHR and related receptors. Of interest, a 28-bp deletion is found in one patient, while another, unrelated patient has a tandem duplication of the same 28-bp segment, suggesting that both resulted from the same unusual unequal crossing-over mechanism facilitated by 9-mer direct sequence repeats. Since the V2 ADHR is a member of the seven-transmembrane-domain, G-protein–coupled receptor superfamily, the loss-of-function mutations from this study and others provide important clues to the structure-function relationship of this and related receptors.

Introduction

Nephrogenic diabetes insipidus (NDI) is a potentially fatal human disorder characterized by insensitivity of the renal concentrating system to the water-sparing effects of the antidiuretic hormone arginine vasopressin (ADH or AVP) secreted by the posterior pituitary. Individuals affected with NDI have high circulating levels of ADH but do not concentrate their urine (Reeves and Andrioli 1989). To maintain water homeostasis, they must consume three to five times the usual daily intake of water. Failure to do so results in hypernatremia and dehydration, and, in infants, it can precipitate permanent brain damage or even death.

NDI may be acquired, usually because of drug toxicity or systemic disease, or it may be inherited. The latter form of the disease, congenital NDI (CNDI), presents in infancy and is usually inherited in an X-linked fashion with variable, incomplete expression in females. Because of the failure to respond to high levels of circulating ADH, the receptor for ADH expressed in the kidney, the V2 ADH receptor (V2 ADHR), was long considered a prime candidate for the defective step in ADH-mediated response in patients with CNDI. In 1990, Jans et al. (1990) localized a gene conferring V2-like binding activity to the same region of the X chromosome (Xq28) as that of the NDI locus, providing independent support for this theory.

Recently, rat and human cDNAs encoding a putative receptor molecule were cloned on the basis of their ability to confer to cells in which they were expressed properties consistent with the V2 ADHR (Birnbaumer et al. 1992; Lolait et al. 1992). Seibold et al. (1992) confirmed localization of the cloned receptor-encoding gene, designated "AVPR2," to Xq28 by PCR amplification from hamster/human somatic cell hybrids. Mutations in the AVPR2 gene were then described in several patients with CNDI (Pan et al. 1992; Rosenthal et al. 1992; van den Ouweland et al. 1992; Holtzman et al. 1993; Merendino et al. 1993), indicating that disruption of this gene may cause CNDI.

The base sequence of the cloned cDNA predicts a protein with seven hydrophobic segments, suggesting membership in the superfamily of seven-transmembrane-domain receptors that transduce hormonal signals via an interaction with heterotrimeric guanine nucleotide–binding proteins (G-proteins). Since G-protein–mediated stimulation of adenylate cyclase was previously documented for the V2 ADHR, it is reasonable to assume a typical seven-transmembrane-domain structural model for this molecule. An analysis of receptor mutations that have been naturally selected for loss of function can provide useful information about the structure-function relationship of this receptor and the large superfamily of similar receptors. Several of these receptors have recently been shown to be mutated in human diseases (reviewed in Clapham 1993).

We describe 11 families in which a mutation in the

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AVPR2 gene is associated with CNDI, and we discuss the predicted changes in the primary peptide sequence in the context of our current view of receptor structure. We also report the unusual finding of reciprocal deletion and duplication mutations affecting the same gene segment and associated with short flanking direct repeats. This finding suggests the presence of a relative hot spot for mutation in this gene and supports close-range intragenic unequal crossing-over as a likely mutation mechanism.

Patients and Methods

Patients

Eleven unrelated probands with CNDI were studied. Seven had a family history of NDI, which ranged from two to five affected male relatives. In two of the four patients with no family history, mother and/or grandmother had a history of excessive water intake. All probands were male, and all were of European ancestry. Each had the onset of symptoms in infancy and was unable to concentrate his urine when deprived of water. At least one affected male in each family had been tested and had failed to respond to an exogenously administered ADH agonist with antidiuresis, a test commonly used to distinguish nephrogenic diabetes insipidus from the central form where symptoms are due to lack of ADH secretion. There were no consistent differences among the study patients in the degree or form of clinical manifestation. Both the age at onset of symptoms referable to dehydration and the presence or absence of mental retardation or developmental delay are given in table 1.

Methods

Oligonucleotides used in PCR and DNA sequencing.—The synthesizer is from Genetic Systems. The sequences (5′-3′) are BW100: TGCTCCTCAGGCAGAGGCTGA; BW101, CTCAGTCTTGGCCACAGCTGC; BW102, GTGGCCAAGACTGTGAGGATG; BW103, AGGGATTAGAAAGGCGGAGACA; BW104, CTCCATAGTCTTTGTGGCTGT; BW106, CCTGTGTCGGGCCGTGAAGTA; BW107, CCAATGAAGACGTGTATGGGT; BW108, CGCCTGCCAGGTGCTCATCTT; BW109, GCGGTGGTGCAGGACTCATCT; BW110, GGGCCTTCTCGCTCCTTCTCA; and BW115, CCCTGCACAGCACCCTCTCTA (intron A).

DNA purification and PCR.—High-molecular-weight genomic DNA was isolated from peripheral blood leukocytes by proteinase K/SDS digestion, phenol extraction, and ethanol precipitation and was quantified by measuring absorbance at 260 nm. Human-hamster somatic cell hybrid cell line DNA samples were a gift from R. Scott Hansen and Stanley Gartler, Department of Genetics, University of Washington. Cell lines were GM06318B (Human Genetic Mutant Cell Repository, Camden, NJ), CHOYH21 (Rosenstraus and Chasin 1975), and WAVR4dF9-4a17 (Kozak et al. 1977). For PCR, 0.5 μg of genomic DNA was used as template in a 100-μl reaction with standard buffer (Perkin-Elmer), 10 pmol of each oligonucleotide primer, and 1.87 U of Amplitaq heat-stable DNA polymerase (Perkin-Elmer). Addition of the oligonucleotides to the reaction was withheld until it had been brought to 95°C, just prior to cycling. Thermal cycling parameters were 1 min at 95°C, 1.5 min at 54°C, and 4 min at 72°C, for 35 cycles, followed by 7 min at 72°C and then soaking at 10°C.

The authenticity of the 2.0-kb PCR product was supported by positive hybridization with a third AVPR2 cDNA oligonucleotide, by amplification from internal primers, and by showing amplification in human-hamster hybrids only when the human X chromosome was present. Direct sequencing confirmed AVPR2 identity.

Agarose gel electrophoresis and Southern blotting.—Both electrophoresis in 1% agarose in Tris-acetate EDTA (TAE) buffer and Southern blotting to nitrocellulose were performed according to the protocol of Sambrook et al. (1989). The blot was baked for 2 h at 80°C and prehybridized for 6 h at 42°C in 8 ml of 6 × SSPE (20 × SSPE = 3 M NaCl, 200 mM NaH_2PO_4, 20 M EDTA), 5 × Denhardt's, 0.1% SDS, and 100 μg of salmon sperm DNA/ml. Oligonucleotide BW101 (20 pmol) was labeled at the 5′ end by incubation for 45 min at 37°C in 100 mM Tris-Cl pH 8, 10 mM $MgCl_2$, 10 mM DTT, 50 μCi of [γ-^{32}P]-ATP (3,000 mCi/mmol; NEN), and ~5 U of T4 polynucleotide kinase (New England Biolabs). After addition of 1 μl of 0.5 M EDTA to stop the reaction, half was added to the prehybridization solution, and the blot incubated overnight at 42°C. The blot was washed in 5 × SSPE, 0.1% SDS, and 0.1% sodium pyrophosphate at 42°C twice, prior to autoradiography. Signal from hybridization with nonspecific DNA on the blot (e.g., marker lanes) was nil.

HhaI digestion and PAGE.—The PCR products were purified by phenol/chloroform and chloroform extraction, followed by ethanol precipitation. One-fifth of the original reaction was digested for 3–6 h in a 10-μl volume at 37°C with *Hha*I enzyme (BRL) in the recommended buffer. The digestion products were separated by electrophoresis on a 0.8-mm-thick 6% polyacrylamide gel in Tris-borate EDTA (Sambrook et al. 1989), were stained with ethidium bromide, and were photographed with UV transillumination. Fragment lengths were estimated by comparison with a marker preparation run on each gel (1 kb ladder; BRL).

Direct sequencing of PCR products.—PCR was performed as above, except that the extension time was 2 min and that three shorter, overlapping products were generated with primer pairs BW100/BW107, BW104/BW101, and BW108/BW103. One hundred microliters of PCR reaction was ethanol precipitated, redissolved in TAE buffer (Sambrook et al. 1989), and electrophoresed in 1% NuSieve agarose (FMC Bioproducts) and 0.5 μg of ethidium bromide/ml, in TAE buffer. Ten microliters of the melted

Table 1

Seattle AVPR2 Mutations Associated with CNDI

Patient	Mutation[a]	Predicted Consequence[b]	Location in V2 ADHR	Family History of Affected Males	Age at First Symptoms	MR or DD[c]
86-144 (family 3)	Deletion of 28 bases (98–125)	Frameshift at amino acid 9; TER after 18 amino acids	N-terminal extracellular domain	No New mutation[d]	8 wk	No
87-149 (family 2)	Tandem duplication of 28 bases (98–125)	Frameshift at amino acid 19; TER after 181 amino acids	N-terminal extracellular domain	Yes	3–4 wk	No
93-141	$T^{229}\rightarrow G$	$Leu^{53}\rightarrow Arg$	Transmembrane I	No, but sister affected	<3 mo	No
86-135	$T^{319}\rightarrow C$	$Leu^{83}\rightarrow Pro$	Transmembrane II	Yes	1 wk	No
92-233	$C^{355}\rightarrow T$	$Pro^{95}\rightarrow Leu$	Junction of transmembrane II and extracellular loop I	None known[d] (adopted)	8 mo	No
93-169	$C^{571}\rightarrow T$	$Ser^{167}\rightarrow Leu$	Transmembrane IV	No[e]	Soon after birth	DD
88-082	$A^{685}\rightarrow G$[f]	$Tyr^{205}\rightarrow Cys$	C-terminal end of extracellular loop II	Yes	Soon after birth	No
88-004	$G^{795}\rightarrow T$	$Glu^{242}\rightarrow$TER; truncated peptide	Cytoplasmic loop III	Yes	Soon after birth	MR
87-203 (family 1)	Deletion of A^{834}	Frameshift at amino acid 255; TER after 15 amino acids	Cytoplasmic loop III	Yes	4 wk	No
92-167	agGGG→ggGGG (mutated splice acceptor for intron B)	Aberrant splice at amino acid 304	Extracellular loop III	No New mutation[f]	2 wk	No
87-151	$T^{1038}\rightarrow A$	$Trp^{323}\rightarrow Arg$	Transmembrane VII	Yes	Soon after birth	No

[a] Base numbering as in GenBank accession number Z11687, submitted by Birnbaumer et al. (1992).
[b] Amino acid numbering as in reported coding sequence in same GenBank entry.
[c] MR = mental retardation; and DD = developmental delay.
[d] Demonstrated by absence of mutation in mother's DNA.
[e] Biological mother or maternal grandmother is reported to have had excessive thirst; DNA not available.
[f] Same as Van den Ouweland's (1992) patient 134.

gel slice containing the desired PCR product and 5 μl of 2 pmol sequencing primer/μl were boiled for 2–8 min and annealed at 37°C for 5 min. Sequencing reactions were carried out by a modification of the Sequenase V2.0 kit instructions (USB). A 9.2-μl portion of labeling reaction mix (0.7 μl of dITP labeling mix [diluted 1:15], 2 μl of 0.1 M DTT, 2 μl of 5 × Sequenase buffer, 2 μl of [α-^{32}P]-dATP [10 μCi/μl; NEN], and 2.5 μl of Sequenase V2.0 enzyme [diluted 1:8 in glycerol dilution buffer]) was mixed with the annealed template and primer and incubated at 37°C for 30 s. Four microliters of this was mixed with 2.5 μl of each dITP termination mix and incubated for an additional 5 min at 37°C, and the reaction was stopped by addition of 4 μl of Stop buffer. Stopped reactions were heated to 95°C and were run on a 0.4–1.2-mm-thick wedge gel of 8% polyacrylamide/8 M urea in glycerol-tolerant buffer (USB) at 55°C. The gel was fixed in 10% acetic acid/10% methanol or water, was dried, and was autoradiographed for 1–5 d. A normal, unrelated male was sequenced and was run alongside each unknown to facilitate mutation detection, and the complete sequence of each unknown was read into the computer and was compared with the published sequence (Birnbaumer et al. 1992). DNA sequence analysis was performed using GenePro software for PC (Riverside Scientific), and multiple protein alignments were performed using GeneWorks software for the Apple MacIntosh (Intelligenetics).

Results

A Screen for AVPR2 Gene Alterations in, and Segregation with, CNDI

The AVPR2 genes from seven patients were initially screened for size variations after PCR amplification from

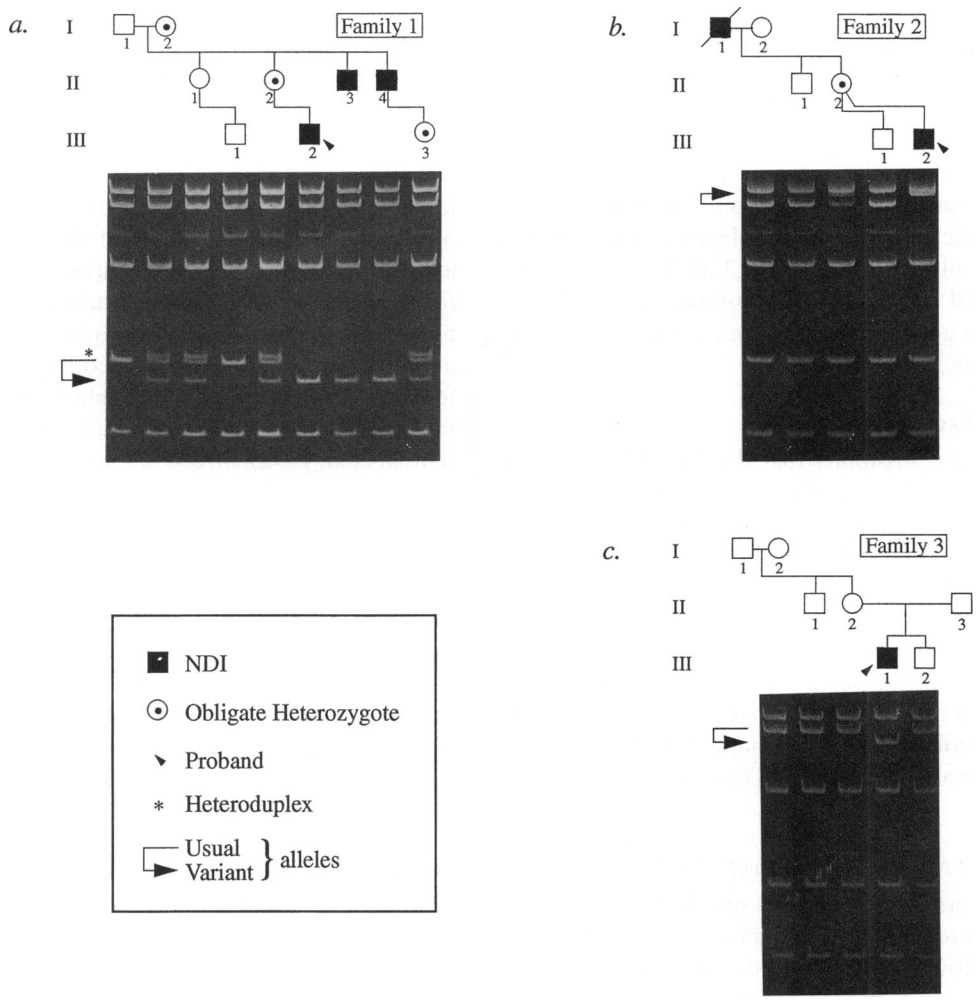

Figure 1 *Hha*I restriction fragments from the amplified AVPR2 gene in three families with CNDI. Usual fragment sizes (from top) are 593, 554, 416, 281, and 198 bp in length. The occasional faint band at ~450 bp is an artifact of PCR. In panel *a*, the band marked with an asterisk (*) represents a heteroduplex of complementary Watson-Crick strands of normal and variant DNA molecules created during amplification.

genomic DNA by using oligonucleotide primers (BW100 and BW103) based on the 5′ leader and the 3′ UTRs of the human AVPR2 cDNA sequence (Birnbaumer et al. 1992). This generated an amplification product of ~2.0 kb. Prolonged electrophoresis of the intact products on an agarose gel revealed subtle mobility differences in three of the seven patients, suggesting the presence of two small deletions and one insertion (data not shown). To assess these size differences further, we digested the amplification product with the restriction enzyme *Hha*I. In normal males, this generates a ladder of five bands upon separation by electrophoresis on a 6% polyacrylamide nondenaturing gel (fig. 1). In this assay, an alteration in band migration could reflect a mutation creating or eliminating a *Hha*I restriction site, the insertion or deletion of one or more bases, or, much less likely, a change in base composition.

In the three affected males with subtle variations in the mobility of the intact PCR product, individual variations from the usual *Hha*I fragment sizes were evident (indicated by arrows in fig. 1). For two of these probands with a family history of NDI (families 1 and 2), the variations were present in the DNA of family members, in concordance with the known presence of the CNDI allele; that is, affected males had only the variant AVPR2 allele, unaffected males and noncarrier females had only the usual allele, and obligate heterozygotes for CNDI had both the variant and the usual alleles (fig. 1*a* and *b*).

Individual II.1 in family 1 has a 50% a priori chance of having inherited the CNDI mutation. She has a band pattern consistent with heterozygosity for the AVPR2 variation (fig. 1*a*). By an informative linkage analysis performed earlier, she was also shown to have inherited the allele of the CNDI-linked polymorphic locus DXS52 (Kambouris et al. 1988; Knoers et al. 1988) associated with NDI in her family and was thus assigned probable carrier status for CNDI (data not shown). Hence, the AVPR2 variations

segregate with the CNDI gene in families 1 and 2 and can be used to identify gene carriers.

The proband of family 3 had no family history of NDI, and his mother had no complaint of polyuria or polydipsia. As seen in figure 1*c*, the affected proband has only a variant AVPR2 allele. Since we know that the AVPR2 PCR product originates from the X chromosome, he must have inherited that allele from his mother. His mother, however, demonstrates only the usual AVPR2 allele. The new appearance of NDI in this family is thus associated with the acquisition of a genetic variation, i.e., a new mutation, in the AVPR2 gene.

Polymorphism Excluded

To examine the possibility that the AVPR2 gene variations seen in three of seven affected males represent polymorphism within the general population and not mutations associated with CNDI, we assayed the AVPR2 genes of 24 males and 18 females who were unrelated, of European ancestry, and ascertained randomly with respect to genetic disease. None of these individuals, representing 60 independent X chromosomes in toto, showed any detectable variation in the size of the *Hha*I fragments (data not shown). The probability of this happening by chance is <.001 (Fisher exact test, one tail; Diem and Seldrup 1982, p. 227).

Different AVPR2 Mutations in Each of 11 Affected Males

The identification of alterations of AVPR2 gene size in some patients confirmed that mutations in this gene can cause CNDI. However, four of the original seven patients and four new patients showed no variations in size, so their AVPR2 coding regions were evaluated by direct DNA sequencing of PCR product. (The complete sequences of the two introns that interrupt the AVPR2 coding region have also been determined. The genomic sequence of exons plus introns appears under GenBank accession number U04357.) The sequences of the three alleles with altered size were also determined. The entire coding region was sequenced to avoid missing multiple sequence variations in the same allele, since this had been found in one case reported elsewhere (Pan et al. 1992).

Ten of the variant base sequences are illustrated in figures 2 and 3. Both the location of each mutation in the seven-transmembrane-domain structural model and the predicted consequences in translation are summarized in table 1. Seven are single base substitutions, one a deletion of a single base that creates a new *Hha*I restriction site, one a splice acceptor site mutation at the 5′ end of the third exon, one an oligonucleotide deletion, and one an oligonucleotide duplication.

The DNA sequence changes responsible for the gene size variations found in families 2 and 3 are shown in figure 3. The proband in family 3 (86-144) has a deletion of 28 bp at the 5′ end of exon II. The deletion encompasses one of three 9-bp direct repeats that flank this segment, as well as the intervening 19 bp between two of the repeats. Interestingly, the proband of family 2 (87-149) has a tandem duplication of the same 28-bp segment.

Like the proband of family 3, in whom a new mutation was demonstrated (fig. 1), another patient (92-167) had no family history of NDI, and his mother had no complaints of unusual thirst. Sequencing of the mother's AVPR2 alleles showed that she is not a carrier (data not shown). As for family 3, a new mutation causing CNDI has occurred between the proband and his mother. No patient lacked a unique variation in the AVPR2 coding region, and, except for the DNA sequence polymorphism in the third position of codon 309, described elsewhere (Pan et al. 1992; Rosenthal et al. 1992), no second mutations were found.

AVPR2 Mutations and a Common Intragenic Population Polymorphism

We confirmed the presence of a previously described, functionally silent, two-allele polymorphism generated by a single base substitution (A↔G) in the third position of a leucine codon at position 309 of the V2 ADHR polypeptide (Pan et al. 1992; Rosenthal et al. 1992). Eight of the mutations reported here were associated with the "A" allele, three with the "G" allele (data not shown). Another coding-region DNA polymorphism reported by Pan et al. (1992) and resulting in a Met-for-Val substitution at position 88 (Y. Pan, personal communication) was not found in any of our probands. Thus, mutations have occurred on the background of both the "A" and "G" polymorphic alleles in AVPR2.

Discussion

CNDI is a classic example of a physiologic disorder caused by end-organ resistance to hormone. A defect in the renal receptor for ADH has long been the most logical candidate etiology. With the cloning of the V2 ADHR cDNA, direct examination of this hypothesis was made possible, and it is now clear that the majority of CNDI patients have mutations in the AVPR2 gene encoding the receptor. At least one such mutant receptor has been shown to bind hormone but to fail to stimulate adenylate cyclase, providing a molecular explanation for the presumed signaling defect (Rosenthal et al. 1993). Several other human diseases have been shown to result from mutation in other G-protein–coupled receptors, including rhodopsin in autosomal dominant (Dryja et al. 1990, and many more) and autosomal recessive (Rosenfeld et al. 1992) retinitis pigmentosa, the thyrotropin receptor in thyroid adenoma (Parma et al. 1993), leutinizing-hormone receptor in familial male precocious puberty (Kremer et al. 1993; Shenker et al. 1993), adrenocorticotropic hormone receptor in glucocorticoid deficiency (Clark et al. 1993; Tsigos et al. 1993), and the Ca^{2+}-sensing receptor in famil-

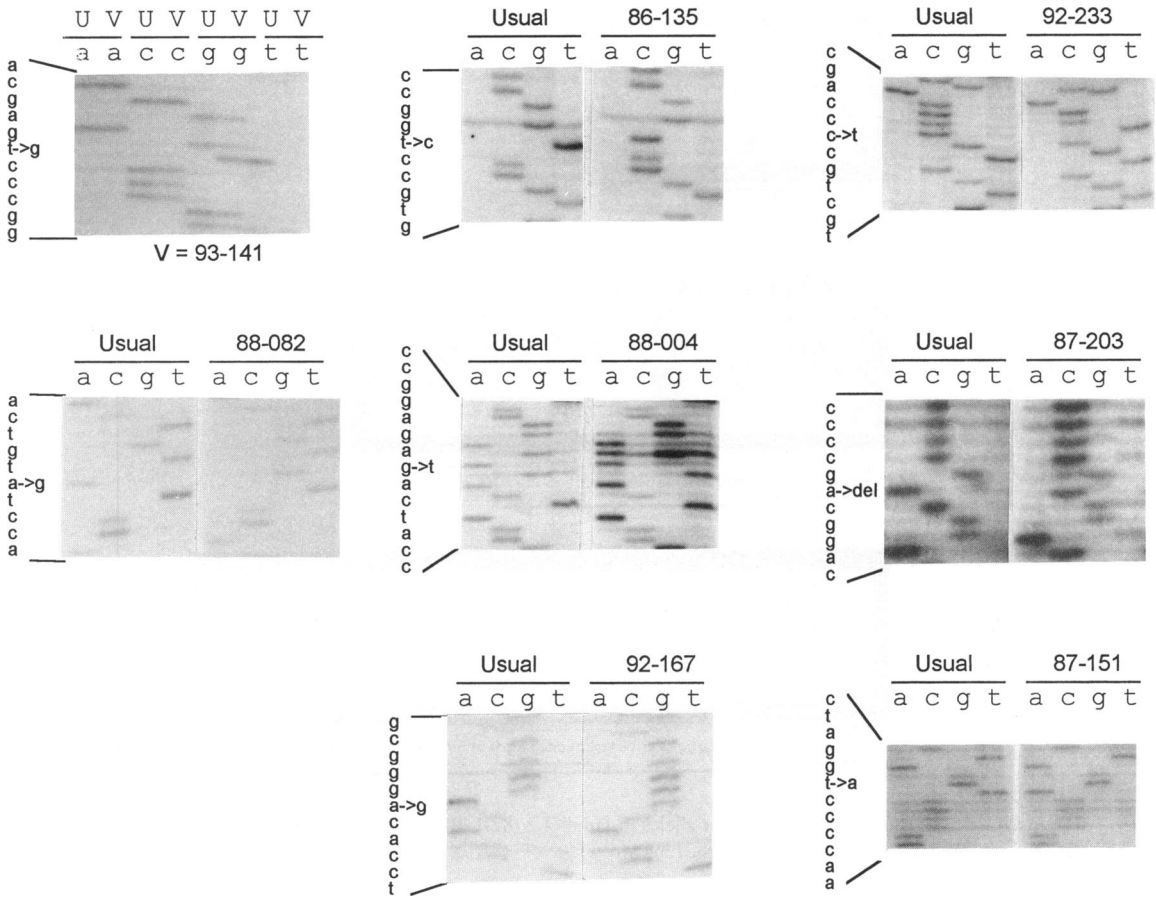

Figure 2 DNA sequence variants in the AVPR2 gene associated with CNDI. U = usual sequence; and V = variant sequence. Five-digit numbers identify the proband.

ial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism (Pollak et al. 1993). Some of the mutations constitutively activate, while others disable, receptor signal transduction. A substantial literature of experimental mutations has also developed that defines domains in other cognates of the seven-transmembrane-domain–receptor superfamily, particularly the biogenic amine receptors and opsins (reviewed in Dohlman et al. 1991; Nathans 1992; Wess 1993). The addition of a system that selects for naturally occurring, functionally significant mutations that can be examined in vitro may allow further delineation of receptor domains and properties. The study of the AVPR2 mutations causing CNDI provides such a system.

In this study we amplified the human AVPR2 gene; applied a semisensitive screening technique to detect variations in this gene among patients with CNDI that are not seen among unrelated, asymptomatic individuals; and showed that the presence of the gene variations segregates with the known presence of CNDI alleles in families. We next sequenced the AVPR2 alleles from a total of 11 probands with CNDI, finding a new sequence variation in each. This represents the largest published cohort of AVPR2 alleles associated with CNDI. This study, taken together with those reported by others, provides an opportunity to profile the frequency and types of mutations. It is also the largest collection of naturally occurring mutations in mammalian G-protein–coupled receptors that have been selected for loss of function.

V2 ADHR Structure-Function Implications

The current data expand the number of reported mutations in AVPR2 associated with CNDI, from 18 to 28 (our patient 88-082 has a mutation identical to that of patient 134 of van den Ouweland et al. [1992]; we do not know whether the two patients have common ancestry, but to our knowledge they were ascertained independently on different continents). These mutations represent a subset of naturally occurring random mutations selected for loss of receptor function. They thus provide unbiased pointers to amino acid residues and domains critical for proper membrane insertion or for ligand binding or signal transduction leading to urinary concentration.

Several generalizations about the receptor molecule can be made by inspecting these cumulative data (fig. 4). First,

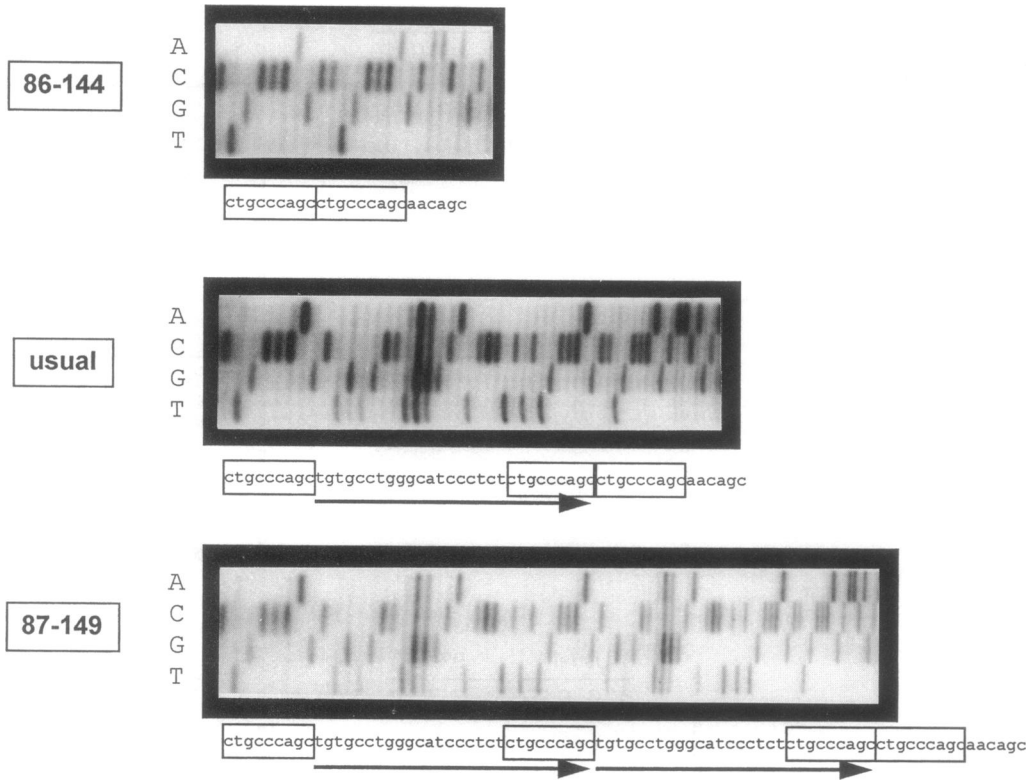

Figure 3 DNA sequence of the AVPR2 size-variant alleles in families 2 (87-149) and 3 (86-144) from fig. 1. Direct repeats are indicated by boxes, and the deleted/duplicated segment is indicated by the long arrows.

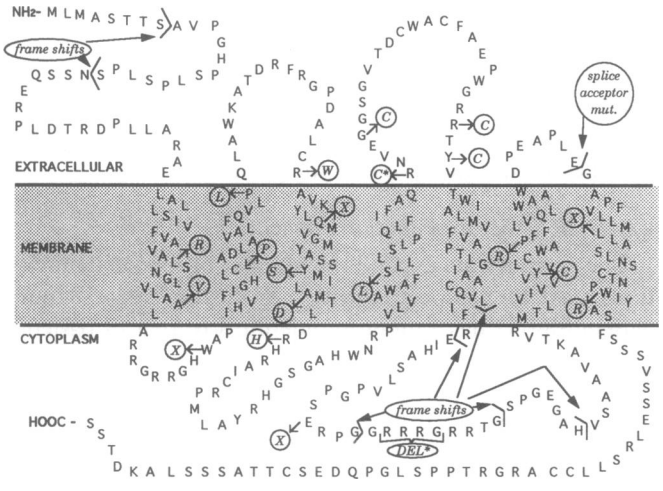

Figure 4 Seven-transmembrane-domain model of the V2 ADHR polypeptide, based on computer hydrophobicity analysis of the amino acid sequence. Mutations associated with CNDI are annotated in circles and ovals. Sources of data are Pan et al. (1992), Rosenthal et al. (1992), van den Ouweland et al. (1992), Bichet et al. (1993), de Marco et al. (1993), Holtzman et al. (1993), Merendino et al. (1993), Pang et al. (1993), and the present report.

the frameshift, nonsense, and splice mutants can be expected to disrupt the formation of a complete polypeptide and thus of one or more transmembrane segments, disturbing the overall seven-transmembrane-domain motif so highly conserved in evolution. Second, point mutations resulting in amino acid substitutions cluster significantly in or near the putative transmembrane domains. Since a minority of these result from C-to-T or G-to-A nucleotide transitions, which are more frequent in C-G–rich regions, this clustering is not due to a relatively increased C-G content in the nucleotide sequence coding for these domains. Third, the substitution mutations are generally nonconservative in nature and occur at positions that are usually identical or conserved among closely related receptors (table 2).

These results emphasize the functional importance of the structure of the V2 ADHR hydrophobic transmembrane domains and mirror the generally greater degree of evolutionary conservation in these segments among all G-protein–coupled receptors (Savarese and Fraser 1992). The fine structure of the transmembrane domains is critical for ligand binding in rhodopsin and in the receptors for biogenic amines (reviewed in Savarese and Fraser 1992). Though AVP is a somewhat larger ligand, some binding affinity and perhaps specificity may be conferred by resi-

Table 2

Conservation of Amino Acid Residues at Positions Altered in CNDI

Aligned Receptor[a]	Position in Human V2 ADHR Amino Acid Sequence																	
	53	61	83	88	95	113	128	132	137	167	181	185	203	205	247–250	280	286	323
Human V2 ADHR (usual)	L	A	L	V	P	R	Y	A	R	S	R	G	R	Y	RRRG	Y	P	W
Rat V2 ADHR	L	A	L	V	P	R	Y	A	R	S	R	N	R	Y	PQRA	Y	P	W
Pig V2 ADHR	L	A	L	V	P	R	Y	A	R	S	R	D	R	Y	HRGG	Y	P	W
Rat V1 ADHR	L	A	L	V	P	R	Y	V	R	S	I	N	R	Y	...[b]	Y	P	W
Human oxytocin receptor	S	A	I	V	P	R	Y	L	R	C	R	D	K	Y	...[b]	F	P	W
Pig oxytoxin receptor	S	A	I	V	P	R	Y	L	R	C	R	D	K	Y	...[b]	F	P	W
Human V2 AHDR in CNDI[c]	R	V	P	M[d]	L	W	S	D	H	L	C[e]	C	C	C	Deleted[e]	C	R	R

[a] The sequences of all receptors were obtained from GenBank.
[b] No similarity in alignment.
[c] References are the same as for fig. 4.
[d] Reported to be a polymorphism not associated with CNDI (Pan et al. 1992).
[e] Both mutations are in the same allele (Pan et al. 1992).

dues in the hydrophobic segments. Nonconservative substitutions in these segments may also disrupt membrane insertion. These predictions are currently being tested.

Last, no single region of the primary amino acid sequence has a significant clustering of mutations. This suggests either that multiple domains with differentiable functions are affected or that multiple segments of the polypeptide chain contribute to the formation of functional spatial domains in the properly folded, membrane-inserted receptor. Evidence from the targeted mutation studies of the adrenergic receptors supports the latter hypothesis (reviewed in Savarese and Fraser 1992). The distinction between these possibilities for the V2 ADHR can be made by testing the mutant receptors for ligand binding, G-protein association and activation, signal-transducing capability, and membrane insertion. Rosenthal et al. (1993) analyzed one CNDI mutant receptor in this way, showing that it bound vasopressin normally but failed to stimulate adenylate cyclase. It remains to be seen whether the lack of substitution mutations observed in the amino-terminal extracellular domain and in the carboxy-terminal cytoplasmic "tail" predicts functional insignificance.

Mutation Mechanism

The majority of the CNDI-associated alleles—8 of 11 in this report, plus 14 of 18 in others (Pan et al. 1992; Rosenthal et al. 1992; van den Ouweland et al. 1992; Bichet et al. 1993; De Marco et al. 1993; Holtzman et al. 1993; Merendino et al. 1993; Pang et al. 1993)—differ from the usual sequence by a single base substitution. Thirteen of the cumulative 21 substitutions are transitions; 9 replace C with T (or G with A), and, of these, 5 occur at CG dinucleotides. These frequencies, including the excess of transitions, agree with previous findings in other genes (Koeberl et al. 1990).

Perhaps the most intriguing and unusual genetic feature of our data is the simultaneous discovery of two mutations that affect the same sequence in reciprocal ways. Patient 86-144 has a deletion of 28 bases, and patient 87-149 has a tandem duplication of the same 28 bases. Interestingly, the deleted or duplicated segment comprises 19 bases plus one of two flanking 9-base direct repeats. A third repeat lies immediately downstream (fig. 3).

Short deletions of two to eight bases in the region of direct repeats have been reported in several human diseases (Efstratiadis et al. 1980; Fukuhara et al. 1990; Kornreich et al. 1990; Mita et al. 1990; Krawczak and Cooper 1991; Tanoue et al. 1991; Carre-Eusebe et al. 1992; Richards et al. 1992; Wajcman et al. 1992). Reports of tandem duplications such as the one seen here are few. To our knowledge, a deletion and duplication affecting the same intragenic segment has been reported only once before: the β-globin variants causing unstable hemoglobins Gunn Hill and Koriyama represent a reciprocal deletion and duplication, respectively (Bradley et al. 1967; Kawata et al. 1988), of seven bases plus one of two eight-base flanking direct repeats. Ours is the first report of both reciprocal products found in a single series of patients.

We know that the mutations in 86-144 and 87-149 did not arise from the same ancestral event, because the former was shown to be a new mutation in the proband. Thus, their codiscovery is by chance. It is nonetheless apparent that the nature of the wild-type sequence may in some way promote mutation events that can give rise to reciprocal products. Indeed, it is interesting that the only other multibase deletion reported in AVPR2 also occurred precisely at a pair of direct repeats (CGCCG) and resulted in the loss of one whole repeat and the seven intervening bases (see patient 5 of Pan et al. 1992).

Because both reciprocal mutations were found in our

patients, we believe unequal crossing-over mediated by illegitimate homologous pairing of the first repeat with the second is a likely mutation mechanism. This is analogous to the mechanism thought to explain deletion or duplication events affecting adjacent genes with high sequence similarity, as in the β-globin gene cluster variants causing Hb Lepore and anti-Lepore (Weatherall 1994) or the X-linked color-vision genes (Deeb et al. 1992). Most discussions of unequal crossing-over suggest that it occurs only between adjacent gene segments with >1 kb of similar sequence. However, identities as small as 14 bp have been shown to mediate homologous recombination in eukaryotic cells (Rubnitz and Subramani 1984).

A number of other mutation mechanisms have been proposed to explain the genesis of deletions associated with direct repeats (Streisinger et al. 1966; Efstratiadis et al. 1980; Mita et al. 1990; Krawczak and Cooper 1991), but none can simultaneously explain reciprocal duplications. Similarly, those modified models that do explain duplications cannot explain deletions. While postulating a combination of mechanisms might suffice, we find parsimony in proposing the single mechanism of unequal crossing-over to explain both reciprocal mutations seen in our two patients. Since unequal crossing-over events are reciprocal, one might expect to detect duplications as commonly as deletions; however, reports of tandem duplication are less frequent in the human mutation literature than are reports of repeat-mediated deletion. This apparent discrepancy could be due to an inherent instability of tandem duplications, to admixture with other mechanisms that result in deletion only, and/or to a bias toward clinical ascertainment of deletions.

Another feature of the precise form of deletion or duplication described here is that, unlike typical illegitimate recombination or frameshift events, no new joints are formed in the DNA sequence. One technical consequence of this is that sequence-analysis strategies that depend on hybridization to arrays of short defined oligonucleotides would not differentiate the duplication mutations from normal sequence, since each sequence pattern is present and no new pattern is created.

It is also interesting that the multiple tandem repeats of simple sequence motifs found throughout the genome are often highly polymorphic, the different alleles varying only in the number of repeats present (Kuhl and Caskey 1993). This variation could be viewed as a special case of deletion and duplication mediated by "flanking" direct repeats. It is thus tempting to speculate that the events responsible for the deletion and duplication in our CNDI patients and those giving rise to variability in tandem repeat number may be mechanistically related.

New Mutation

Four of our probands had no family history of CNDI. The mothers and/or grandmothers of two reported unusual thirst and mild polyuria. While the presence or absence of symptoms in a female is, in the absence of further testing, an unreliable measure of carrier status, those whose mothers were asymptomatic were found to have new mutations; that is, the son's AVPR2 allele had a mutation not present in either allele of the mother. A moderate frequency of new mutations is expected in X-linked disorders with reduced genetic fitness (Haldane 1935), and a new mutation in the AVPR2 gene causing CNDI has been reported elsewhere (Pan et al. 1992). The occurrence of new mutations associated with the new appearance of CNDI in a family provides direct evidence that AVPR2 mutations can cause CNDI.

Taken together with other series in the literature (Pan et al. 1992; Rosenthal et al. 1992; van den Ouweland et al. 1992; Bichet et al. 1993; De Marco et al. 1993; Holtzman et al. 1993; Merendino et al. 1993; Pang et al. 1993), these data indicate that ~90% of cases ascertained under the biases inherent in our medical diagnosis and referral system will have a positive family history for affected males or mildly symptomatic females—and that ~10% of patients will have a new mutation. Haldane (1935) calculated that new mutations occur in one-third of cases of completely ascertained X-linked recessive disorders where the genetic fitness of the affected males is zero. Since the genetic fitness of CNDI is reduced but is not zero, and since some ascertainment bias in favor of families with more than one affected male may be operating, our experimentally determined incidence of new mutations is consistent with Haldane's prediction. When the biases are ignored and Haldane's principles are used, a new mutation frequency of .1 would correspond to a genetic fitness of .7 for affected males with CNDI.

Locus Heterogeneity

Since multiple gene products are involved in the vasopressin-mediated signal transduction resulting in urinary concentration, several genetic forms of hereditary NDI could theoretically exist. Autosomal dominant and autosomal recessive models for NDI exist in mice (Homma et al. 1991) and chickens (Braun and Stallone 1989), respectively.

In humans, three reports describe families with more than one member affected with NDI where solid genetic reasoning indicates that the disorder is not inherited with the AVPR2 locus. Langley et al. (1991) reported two affected sisters born to parents who were first cousins. The father was not affected, and the sisters inherited from their mother different alleles of a polymorphic marker tightly linked to AVPR2. Pan et al. (1992) found no AVPR2 coding-region mutation in two affected brothers who had inherited different AVPR2 alleles from their mother, on the basis of analysis of a rare intragenic polymorphism (V88M). (Interestingly, this substitution polymorphism, like the mutants, occurs at a position that is invariant

among the closely related cyclic-peptide receptors [see table 2].) Last, Moses et al. (1988) reported father-to-son transmission of NDI. In this case, the father had defective hemostatic responses to dDAVP, a V2 ADHR–specific phenomenon.

We found a unique mutation in the AVPR2 gene in each of the 11 patients whom we have tested. No CNDI patient had the published normal AVPR2 sequence. We have directly excluded population polymorphism as an explanation for the variations in three instances. Pan et al. (1992) reported finding only the V88M and the Leu309 polymorphisms among 50 individuals screened with SSCP. Furthermore, the probability of detecting a new and unique polymorphism in each of 11 consecutive patients, without detecting any repeats or normal alleles, is exceedingly small. We conclude that the variations detected in our sample represent mutations that cause CNDI. Hence, the fraction of all examined CNDI cases not attributable to mutations in the AVPR2 gene is probably small (estimate 5%) but apparently not zero. It is unclear why our cohort included only patients with an AVPR2 mutation, but low frequency of nonlinked cases and differences in diagnostic and ascertainment criteria are possible explanations.

These findings suggest caution in the counseling of families where a clear X-linked pattern of inheritance has not been established, and it supports a role for AVPR2 DNA testing—or, potentially, V2 ADHR functional testing—in the clinical setting. Further examination of the non–AVPR2-linked families will presumably both identify subtle differences that allow clinical differentiation of the different forms and yield useful molecular insights into the renal concentrating system.

*Hha*I Analysis

We initially used the visualization of electrophoretically separated *Hha*I restriction fragments of a PCR product as a means to identify families with AVPR2 gene mutations, before any had been published by other investigators. It allowed rapid and simple analysis of family members in those cases where an alteration was detected. As used here, this method failed to detect several point mutations; however, the potential exists to improve the sensitivity by altering the electrophoresis conditions—as in SSCP, for example. The simultaneous analysis of multiple small fragments representing the entire coding region, as in our *Hha*I digestions, is comparable to multiplex PCR techniques used for other genes and is of distinct practical advantage in mutation-detection strategies. Nonetheless, until full mutation detection is demonstrated using a less laborious procedure, sequencing of the coding region remains the most sensitive approach.

During the preparation of the manuscript of this report, we became aware of an additional proband with CNDI who was found to have the same 28-bp duplication as does our patient 87-149 (fig. 3) (E. Holtzman and F. Kolakowski, personal communication, 1993). Since the origin of the two independently ascertained mutations has not been documented, it is not clear whether they represent repeated mutation or a single event that we have not immediately linked genealogically. In the family of our patient, the maternal grandmother had two affected sons (the first known cases in the family) and three carrier daughters, so she must have been a carrier; however, she also had six unaffected brothers and six sisters who were not known to be carriers, suggesting that the mutation may have arisen first in her. Hence, it is possible that three independent mutation events—one deletion and two duplications—have occurred at this sequence element in AVPR2. This further supports the contention that short direct repeats can be recombinogenic in humans and that this particular AVPR2 element may be a hot spot for recurrent mutations causing CNDI.

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