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Identification of 13 New Mutations in the Vasopressin-Neurophysin II Gene in 17 Kindreds with Familial Autosomal Dominant Neurohypophyseal Diabetes Insipidus

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Summary

Familial neurohypophyseal diabetes insipidus (FNDI) is an autosomal dominant disorder characterized by progressive postnatal deficiency of arginine vasopressin as a result of mutation in the gene that encodes the hormone. To determine the extent of mutations in the coding region that produce the phenotype, we studied members of 17 unrelated kindreds with the disorder. We sequenced all 3 exons of the gene by using a rapid, direct dye-terminator method and found the causative mutation in each kindred. In four kindreds, the mutations were each identical to mutations described in other affected families. In the other 13 kindreds each mutation was unique. There were two missense mutations that altered the cleavage region of the signal peptide, seven missense mutations in exon 2, which codes for the conserved portion of the protein, one nonsense mutation in exon 2, and three nonsense mutations in exon 3. These findings, together with the clinical features of FNDI, suggest that each of the mutations exerts an effect by directing the production of a pre-prohormone that cannot be folded, processed, or degraded properly and eventually destroys vasopressinergic neurons.

Introduction

Familial neurohypophyseal diabetes insipidus (FNDI) is an uncommon, inherited disease with a uniform clinical phenotype characterized by persistent thirst, polydipsia, polyuria, and a deficiency of the antidiuretic hormone, arginine vasopressin (AVP) (Forssman 1945; Levinger and Escamilla 1955; Pender and Fraser 1955; Martin 1959; Meinders and Bijlsma 1970; Driedger and Linton 1973; Andersson et al. 1974; Baylis and Robertson 1981; Block et al. 1981; Kaplowitz et al. 1982; Blackett et al. 1983; Toth et al. 1984; Os et al. 1985; Pedersen et al. 1985). The disease is transmitted in an autosomal dominant mode and appears to be largely if not completely penetrant. In five kindreds from which autopsy data are available, the disease has been associated with degeneration of the magnocellular, neurohypophyseal neurons that normally synthesize AVP (Hanhart 1940; Gaupp 1941; Forssman 1945; Braverman et al. 1965; Green et al. 1967). Repeat studies in children from one such kindred indicate that AVP secretion is normal for the first few years of life but then degenerates rapidly and may continue to decline slowly for a decade or more (McLeod et al. 1993). Except for a normal compensatory increase in thirst and fluid intake, physiological systems, including the production of oxytocin and hormones of the anterior pituitary, appear to be unaffected. These clinical observations have led to the hypothesis that the gene responsible for FNDI is selectively expressed in vasopressin-producing neurons, where it directs the production of a mutant protein that gradually accumulates and selectively destroys these cells, thereby preventing expression of the normal allele.

Recently, FNDI has been linked to mutations in one allele of the gene that codes for vasopressin-neurophysin II (AVP-NPII), the polypeptide precursor of the hormone (Ito et al. 1991, 1993; Kovács et al. 1991; Bahnsen et al. 1992; McLeod et al. 1993; Rittig et al. 1993; Yuasa et al. 1993; Repaske et al. 1994; Rutishauser et al. 1994; Nagasaki et al. 1995). The AVP-NPII gene is located on chromosome 20 (Riddell et al. 1985) and is ~2.6 kb in length. It contains three exons that code, respectively, for (1) a signal peptide of 19 amino acids, the AVP nonapeptide, a tripeptide linker, and the first 9 amino acid residues of neurophysin; (2) the 67 residues that constitute the highly conserved central portion of neurophysin; and (3) the 17 residues at the C-terminus of neurophysin, an arginine linker, and a 39–amino acid glycosylated peptide known as "copeptin" (Land et al.

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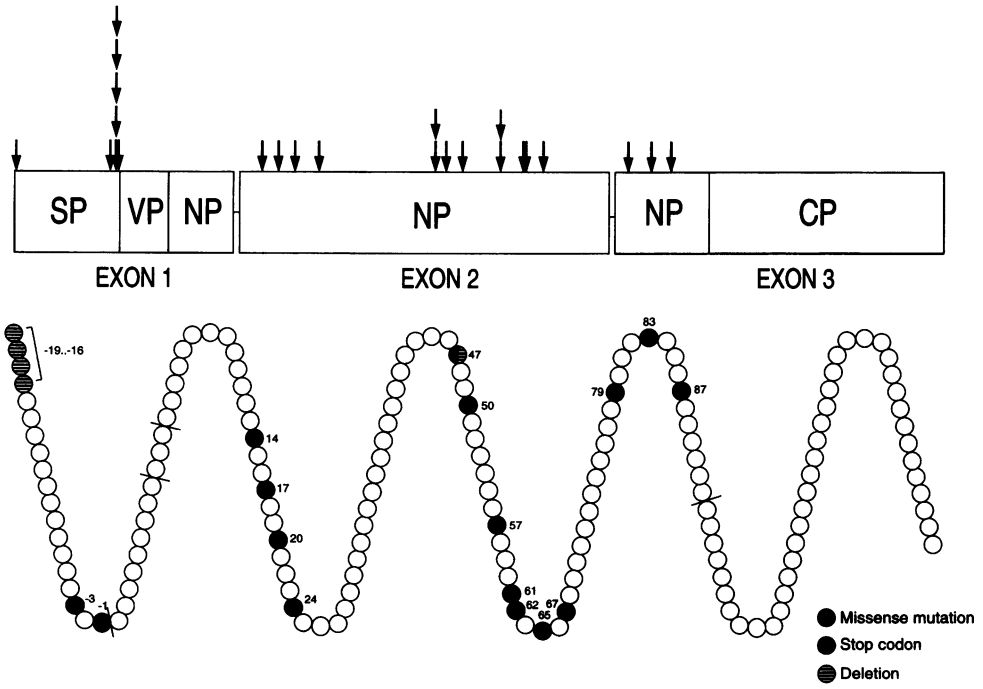

Figure 1 Schematic diagram of the coding regions of the AVP-NPII gene and the primary structure of the pre-prohormone, showing the location and type of mutations identified in FNDI.

1982; Sausville et al. 1985) (fig. 1). Eight mutations have been found at six different loci in exon 2 and two different loci in the part of exon 1 that codes for the signal peptide. One of the latter is present in three apparently unrelated kindreds from Japan, America, and Denmark. These findings are in accordance with the hypothesis that FNDI is caused by production of a mutant protein in vasopressinergic neurons and strongly suggest that only one protein, the AVP-NPII precursor, is involved. However, they also raise the question of how mutations in different parts of the gene produce a disease with such minimal variation in clinical phenotype. One unitary hypothesis consistent with most of the limited data available is that each of the mutations is "neurotoxic" because it alters or eliminates an amino acid essential for proper folding, dimerization, and subsequent cellular processing of the prohormone. To further evaluate this hypothesis, we have employed a rapid, direct-sequencing method to search for mutations in the coding region of the AVP-NPII gene in 17 additional FNDI kindreds and compared our findings to those in the other 10 kindreds as well as to previous biochemical studies of the structural requirements for normal processing, folding, and/or dimerization of the pre-prohormone.

Subjects and Methods

Subjects

We studied 60 affected (26 males), 52 unaffected (24 males), and 4 questionably affected (3 males) members from 17 kindreds in which FNDI appeared to be segregating in an autosomal dominant mode. Nine kindreds were American; three were Danish; and the remainder were Norwegian, Austrian, English, French, or Israeli. All were Caucasian except for one Afro-American kindred. Six kindreds (Hu, Jo, Pe, Ha, Kr, and Si) were examined personally by one of the authors. The remaining were diagnosed and/or referred by Peter Baylis, M.D., Royal Victoria Infirmary, Newcastle-Upon-Tyne, England (Gr); Piers Blackett, M.D., Oklahoma Medical Center, Oklahoma City (Ne); Paul Czernichow, M.D., Hôpital Robert Debrê, Paris (Na); Felix Conte, M.D., University of California Medical School, San Francisco (Ga and Ni); Reema Habyby and Curt Neely, M.D., Stanford University Medical Center, Palo Alto, CA (Hi); Maria Koebert, University of Pennsylvania Medical Center, Philadelphia (Hn); Stanley Mackowiac, M.D., Syracuse (Ly); Ingrid Os, M.D., University of Oslo, Oslo (Aa); Edith Schober, M.D., Vienna University Children's Clinic, Vienna (Ko); and Shmuel Shilo, M.D., Shaare Zedek Medical Center, Jerusalem, (Zi). In five kindreds (Ga, Ni, Hi, Ly, and Zi), the diagnosis of FNDI was based on the family history and a good response to antidiuretic therapy in one or more family members. In the other 12 kindreds, the diagnosis of FNDI was also confirmed in at least one member by assay of vasopressin during a fluid deprivation or hypertonic saline infusion test. The clinical findings in five of these kindreds (Hi, Pe, Kr, Jo, Ne, and Hu) have previously been reported at least in part (Levinger and Escamilla 1955; Blackett

Table 1

Primers and Cycle Conditions Used for PCR Amplification and Dye Terminator Sequencing of Exons 1, 2, and 3 of the AVP-NPII Gene

Exon	Primers	Sequence (5′→3′)
Exon 1[a]	E (+) (107–136)	TGG CGG CCG CGT CTC GCC TCC ACG GGA ACA
	B (−) (353–382)	GCT ATG GCT GCC CTG AGA TGG CCC ACA GTG
Exon 2[b]	C (+) (1648–1677)	TCG CTG CGT TCC CCT CCA ACC CCT CGA CTC
	G (−) (1922–1951)	CGC CCC CCC CCA GGC CCG CCC CCG CCG CGC
Exon 3[b]	H (+) (2053–2082)	CCC AGG CGC CCG TGC TCA CAC GTC CTC CCG
	D (−) (2350–2379)	CCT CTC TCC CCT TCC CTC TTC CCG CCA GAG

[a] Cycle conditions for file 1 were 96°C for 1 min, 66°C for 1 min, and 74°C for 2 min (35 cycles). For file 2, they were 74°C for 10 min. File 3, soak file 4°C.

[b] Cycle conditions for file 1 were 94°C for 5 min, 65°C for 5 min, and 74°C for 5 min (1 cycle). For file 2, they were 94°C for 1 min, 65°C for 1 min, and 74°C for 4 min (35 cycles). For file 3, they were 74°C for 10 min. File 4, soak file 4°C.

et al. 1983; Pedersen et al. 1985; Kovács et al. 1991). We also studied 42 healthy adults, 27 of whom were spouses of affected kindred members. The other 15 were healthy, unrelated Danish volunteers. The study was approved by the appropriate institutional review boards, and written, informed consent was obtained from all subjects.

Laboratory Procedures

Plasma and/or urinary vasopressin was measured by immunoassay as described elsewhere (Robertson et al. 1973; Blackett et al. 1983; Pedersen et al. 1984). DNA was extracted from the buffy coat of peripheral blood, as described by McLeod et al. (1993). Each exon of the AVP-NPII gene was amplified separately in an automated thermal cycler (Perkin Elmer Cetus) with 1 μg genomic DNA (gDNA), 15 pmol of each primer, and 2 U of recombinant *Taq* polymerase in a total volume of 100 μl of PCR buffer. The primer sequences and locations as well as the PCR cycling conditions are indicated in table 1. To amplify exon 1, Perkin Elmer PCR buffer was used (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% [w/v] gelatin). Because of the high GC content of exons 2 and 3, a PCR buffer containing 10% dimethylsulfoxide, 67 mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 10 mM 2-mercaptoethanol, and 1.0 mM $MgCl_2$ was used for amplification of these exons. To permit the sense and antisense strands to be sequenced separately, two parallel PCR amplifications were performed using 5′ biotinylated antisense primers (B, G, and D) and nonbiotinylated sense primers (E, C, and H) for the sense strands, and the opposite set of primers for the antisense strand. To isolate the single-stranded biotinylated sense and antisense strands, 15–35 μl of each PCR product was incubated with 40 μl of streptavidin-conjugated magnetic beads (Dynal A/S), denatured with 0.1 M NaOH, and washed with water and Tris-EDTA buffer.

Solid-phase sequence analysis was performed on both the sense and antisense strands by using T7DNA polymerase (Sequenase) and the nonbiotinylated primer with the automated dye-terminator method (Jensen et al. 1994; Applied Biosystems).

Restriction analysis was performed to verify the sequencing results and to screen DNA from family members who were not sequenced. For this purpose, 10–15 μl of PCR product was incubated overnight with the appropriate endonuclease, following the instructions given by the supplier. If a mutation did not create or eliminate a restriction site for a suitable endonuclease, restriction analysis was performed using an oligonucleotide-based detection assay with mismatched primers (Gregersen et al. 1991). The mismatched primer sequences and locations are indicated in table 2.

Results

DNA Sequencing and Restriction-Enzyme Digestion

Direct sequencing of the PCR amplified exons of the AVP-NPII gene revealed a mutation in one or more affected members of each of the 17 kindreds. These results are shown in table 3 and figure 1 together with those from the 10 previously reported families. In all 17 cases, the mutation was in the coding region of the gene, was evident in both the sense and antisense strands (fig. 2), and was the only mutation identified. When the PCR-amplified exon containing the mutation was digested with an appropriate restriction endonuclease (table 3), an additional fragment of the expected length was generated in every case (not shown). In four kindreds (Ko, Gr, Jo, and Na), the mutation did not create or abolish a suitable restriction site. Therefore, a modified PCR product containing an artificial

site that incorporated the mutation was generated with specially designed mismatched primers (table 2) and digested with the appropriate endonuclease (fig. 3*B*). Each of the mutations affected only one allele of the gene. In 15 of 17 samples, this heterozygosity manifested in the sequenator chromatograms as two superimposed peaks representing the normal and abnormal base (fig. 2*A*). However, in sequences from the affected members of two kindreds (Ga and Kr), only one peak representing the abnormal base was evident in both the sense and antisense strands (fig. 2*B*), even though restriction-endonuclease analysis indicated that both of these mutations were also heterozygous (fig. 3*A*). When gDNA collected from other available members of the 17 kindreds was amplified and digested with the appropriate restriction endonucleases, the expected fragment length polymorphism was observed in all 60 of those known to have FNDI but in none of their 52 unaffected, first-degree adult relatives or in any of the 40 healthy controls. Fragment-length heterozygosity was also observed in one of four children or young adults in whom the clinical phenotype was not known with reasonable certainty. Direct sequencing of the coding regions of the AVP-NPII genes in 40 healthy controls also revealed no differences with the published reference sequence (Sausville et al. 1985) except for a consistent TC inversion at base numbers 256 and 257 in exon 1. This solitary discrepancy with the reference sequence was found in all of the patients as well as the controls and also has been observed in AVP-NPII cDNA (Mohr et al. 1985) and all gDNA sequences from 17 healthy Japanese (Ito et al. 1991).

Mutations in the DNA Sequence

Almost all of the mutations found in the present study (15 of 17) are single base substitutions (table 3), usually an A or a T for a G or a C (table 4). Of these, ~40% (6 of 15) occurred in the context of a CG dinucleotide, converting it to either CA or TG. Indeed, all three of the G-to-A mutations and two of the three C-to-T mutations occurred in this context. However, even when these five CpG-related point mutations are removed, replacement of a G or C with another nucleotide is still by far the most common mutation found (8 of 13, or 62%). In addition to the single base substitutions, we also found one double mutation that converted a CG dinucleotide to GT (kindred Na) and one deletion of three bases (AGG) from a short area of repeats (CAGGAGGAGA) in exon 2 (kindred Ly). Except for the dinucleotide substitution, the types of mutations in these 17 kindreds are very similar to those identified previously in 10 other kindreds (tables 3 and 4).

Changes in the Peptide Sequence

All mutations identified in this study predict a change in the amino acid sequence of the pre-prohormone (fig. 1; tables 3 and 5). Most of them replace or create a glycine (four) or cysteine (two) in the neurophysin moiety or they replace an alanine with a threonine (two) or valine (one) in the signal peptide. Others replace a cysteine (two), proline (one), or glutamine (one) with a stop codon in the carboxy-terminal end of the second domain of the neurophysin moiety. With the possible exception of more frequent involvement of cysteine residues and stop codons, these changes are similar to previous findings (tables 3 and 5). Overall, the proportion of mutations predicting a replacement of cysteine (4 of 27, or 14.8%), glycine (7 of 27, or 25.9%), or alanine (6 of 27, or 22.2%) exceeds the frequency of each of the three amino acids in the pre-prohormone (10.3%, 11.0%, and 11.6%, respectively), even though only the latter contains the apparently hypermutable CpG dinucleotide in one of its codons. In contrast, the frequency of mutations causing replacement of a proline is similar to its frequency in the protein, even though its one and only codon also contains the CpG dinucleotide.

Comparison with Previously Described Mutations

Despite similarities in the type of base and amino acid substitutions found in all the kindreds, 13 of the 17 mutations identified in this study differ from any of the

Table 2

Mismatched Primers Used to Generate PCR Products with Artificial Restriction Sites That Incorporate the Mutations Identified in Four FNDI kindreds (Ko, Gr, Jo, and Na)

Primers	Positive (+) or Negative (−)	Position (nt)	Sequence[a] (5′→3′)
Ko-s	+	1728–1747	GCG ACC CCG GGG GCA ACG GC
Ko-as	−	1838–1857	GAC CGG CGG GGC GAC GAC AG
Gr-s	+	1809–1829	AGG CGC TGC GCT ACC AGG AAG
Gr-as	−	1950–1970	CCC CCC ACC CAC GCG TCT TCG
Jo-s	+	1715–1737	CAG TGC CTC CCC TGC AGC CCC GG
Jo-as	−	1840–1864	CTG GCC GGA CTG GAA GGG CGA CTG C
Na-s	+	2083–2105	GCA GAG AGC TGC GTG ACC GGG CC
Na-as	+	2253–2279	GCG GTG GGG GCC CCG CGG GCT CAG TAG

[a] Mismatched bases are underlined.

Table 3

Mutations of the AVP-NP II Gene Associated with FNDI in 27 Kindreds

Kindred Number	Kindred Name	Nationality	Exon	Nucleotide	Mutation	Peptide	Substitution	Restriction Enzyme	Mutation Found in			Reference
									Affected	?[a]	Unaffected	
1a		Japanese	1	279	cGc→cAc	SP_{-1}	Ala→Thr	*Pma*CI	5/5		0/3	Ito 1993
1b	Po	American	1	279	cGc→cAc	SP_{-1}	Ala→Thr	*Bst*UI/*Pml*I	15/16	1/1	0/24	McLeod 1993
1c	Ra	Danish	1	279	cGc→cAc	SP_{-1}	Ala→Thr	*Bst*UI/*Pml*I	16/16	0/2	0/25	Rittig 1993
1d	Ni	American	1	279	cGc→cAc	SP_{-1}	Ala→Thr	*Bst*UI/*Pml*I	2/2		0/0	Present report
1e	Hi	American	1	279	cGc→cAc	SP_{-1}	Ala→Thr	*Bst*UI/*Pml*I	2/2	0/1	0/0	Present report
2		Swiss	1	227	~~G~~	SP_{-19-16}	~~Met-Pro-Asp-Thr~~	*Bam*HI	4		2	Rutishauser 1994
3	Pe	Danish	1	274	tCc→tTc	SP_{-3}	Ser→Phe	*Mbo*II	5/5		0/1	Present report
4	Hn	American	1	280	gCg→gTg	SP_{-1}	Ala→Val	*Bst*UI/*Pml*I	5/5		0/5	Present report
5		Dutch	2	1740	gGc→gTc	NP_{17}	Gly→Val	*Bgl*I	4/4		0/2	Bahnsen 1992
6a		Japanese	2	1859	cGg→cAg	NP_{57}	Gly→Ser	*Msp*I/*Bgl*I	2/2		0/0	Ito 1991
6b	Ne	American	2	1859	cGg→cAg	NP_{57}	Gly→Ser	*Msp*I/*Bgl*I	1/1		0/1	Present report
7	Hu	American	2	1859	cGg→cCg	NP_{57}	Gly→Arg	*Msp*I/*Bgl*I	13/13		0/22	Present report
8		American	2	1761	cCc→cTc	NP_{24}	Pro→Leu	*Dde*I	3/3		0/4	Repaske 1994
9a		Japanese	2	1824–29	~~AGG~~	NP_{47}	~~Glu~~	*Mnl*I				Yuasa 1993
9b	Ly	American	2	1824–29	~~AGG~~	NP_{47}	~~Glu~~	*Mnl*I	1/1		0/0	Present report
10	Ko	Austrian	2	1748	cCg→cTg	NP_{20}	Arg→Cys	*Eag*I[b]	3/3		0/4	Present report
11	Gr	English	2	1830	gAg→gGg	NP_{47}	Glu→Gly	*Mbo*II[b]	3/3		0/3	Present report
12	Jo	Danish	2	1839	cTg→cCg	NP_{50}	Leu→Pro	*Pst*I[b]	5/5		0/5	Present report
13	Aa	Norwegian	2	1873	gCg→gAg	NP_{61}	Cys→stop	*Mnl*I	2/2		0/0	Present report
14	Ha	American	2	1883	gGg→gTg	NP_{65}	Gly→Cys	*Aci*I	5/5		0/3	Present report
15	Si	American	2	1872	tGc→tCc	NP_{61}	Cys→Ser	*Hga*I	3/3	0/2	0/1	Present report
16	Zi	Israeli	2	1730	cGg→cCg	NP_{14}	Gly→Arg	*Bsl*I	2/2		0/0	Present report
17		Japanese	2	1874	cGg→cTg	NP_{62}	Gly→Trp	*Dde*I	2/2		0/1	Nagasaki 1995
18		Japanese	2	1891	gCg→gAg	NP_{67}	Cys→stop	*Bbm*I	2/2		0/0	Nagasaki 1995
19	Na	French	3	2106–07	cCGa→cGTa	NP_{83}	Pro→stop	*Apa*I[b]	5/5		0/3	Present report
20	Ga	American	3	2116	cGa→cTa	NP_{87}	Glu→stop	*Mae*I	2/2	1/1	0/0	Present report
21	Kr	Danish	3	2094	gCg→gAg	Np_{79}	Cys→stop	*Bbv*I	1/1		0/4	Present report

[a] ? = questionably affected clinically.
[b] Restriction sites created by mismatched primers.

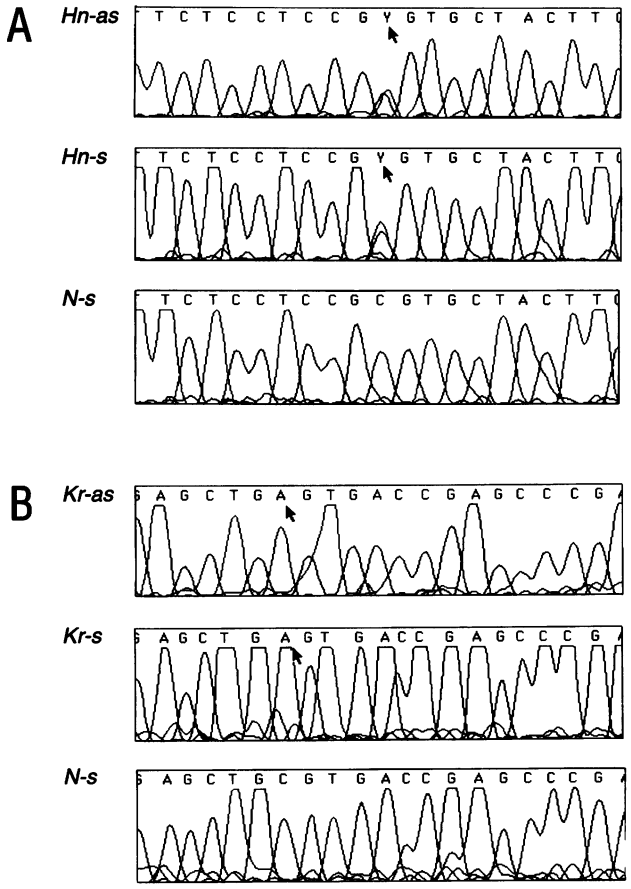

Figure 2 *A*, Section of the sequencing chromatograms obtained by bidirectional dye terminator sequencing of PCR amplified exon 1 of the AVP-NPII gene in a patient from a FNDI kindred (Hn) (Hn-s and Hn-as) and the antisense sequencing from a normal subject (N-as). A heterozygous mutation (*arrows*) is seen both in the sense and antisense chromatogram in the patient. *B*, Section of the sequencing chromatogram obtained in exon 3 in a patient from a different FNDI kindred (Kr) (Kr-s and Kr-as) and a normal subject (N-s). In this patient, only one peak corresponding to the abnormal base is observed (*arrows*).

8 mutations reported previously (table 3; fig. 1). In two cases (kindreds Hn and Hu), the differences are minor, in that they occur in the same codon and predict different amino acid substitutions at the same position in the pre-prohormone (signal peptide −1 and neurophysin 57). In the other 11 kindreds, the mutations affect novel codons not only in exon 1 (one) and exon 2 (seven) but also in exon 3 (three), an area of the gene in which a mutation has not been reported previously.

Only 4 of the 17 mutations identified in the present study are indistinguishable from one reported previously. Two of these recurrent mutations occur in exon 2 (a G-to-A substitution at base 1859 and an AGG deletion between bases 1824 and 1829). The exact location of the deletion cannot be specified, because it occurs in a context of codon repeats that would permit the same mutation to result from any one of four different trinucleotide deletions (bases 1824–1826, 1825–1827, 1826–1828, and 1827–1829). However, since both of these mutations were found in kindreds of different race and nationality (Caucasian American and Afro-American) than those in which they were found previously (Japanese), each of them probably arose independently. The other two mutations that duplicate previous findings were found at the same site (base 279 of exon 1) in two American families who have no known relationship to each other or to any of the other three kindreds (Japanese, American, and Danish) in which the same mutation has been identified previously. Thus, they may also have arisen independently, although a common progenitor for the one Danish and three American kindreds cannot be excluded.

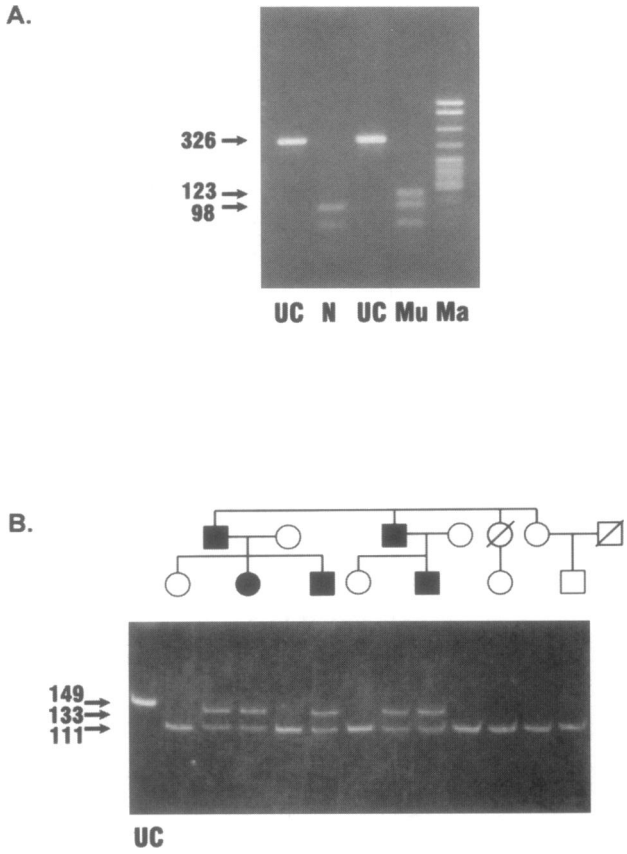

Figure 3 Agarose gel electrophoresis of the DNA fragments generated by restriction-enzyme digestion of the PCR products from affected and unaffected members of two FNDI kindreds (Kr and Jo). *A*, *Bbv*I digest of the same PCR product that yielded the sequence shown in fig. 2*B*. Note that the presence of both normal (98 bp) and mutant (123 bp) fragments in the affected subject indicates that the mutation is heterozygous. *B*, *Pst*I digests of the PCR products in which artificial control and mutation-incorporating restriction sites were introduced by amplification with the mismatched primer set shown in table 3. Note that the PCR products from all affected members (blackened symbols) yielded a fragment (133 bp) that was found in samples from any of the unaffected members (unblackened symbols). UC = uncleaved PCR product; N = unaffected subject; Mu = affected subject; and Ma = DNA marker.

Table 4

The Types of Nucleotide Substitutions Associated with FNDI

	Present Study		Total	
Mutation	Number	%	Number	%
Single base:				
G to A	3	20	7	30.4
G to T	2	13.3	4	17.4
G to C	3	20	3	13
C to T	3	20	4	17.4
C to A	2	13.3	3	13
C to G	0	0	0	0
T to C	1	6.7	1	4.3
T to G	0	0	0	0
T to A	0	0	0	0
A to G	1	6.7	1	4.3
A to C	0	0	0	0
A to T	0	0	0	0
Total	15	100	23	99.8
Dinucleotide:				
CG to CA	4	27	7	30
CG to TG	2	13	2	9
Total	6	40	9	39

When the results of this and previous studies are combined, it is apparent that the mutations of the AVP-NPII gene do not distribute randomly but tend to cluster in several areas that code for particular parts of the pre-prohormone (fig. 1; table 3). Thus, ~26% of the kindreds (7 of 27) have a mutation in one of the two trinucleotides in exon 1 that code for amino acids at the −1 and −3 position of the signal peptide. In five of these kindreds, the mutations are identical (G to A, base 279)

Table 5

Type of Change in Pre-proAVP-NPII Predicted by Mutations Associated with FNDI

Predicted Change	Present Study	Other Studies	Total
1. Deletions:			
A. Create stop codon	1	0	1
B. Delete codon	1	1	2
C. Replace start codon	0	1	1
2. Substitutions:			
A. Replace/create cysteine	2	0	2
B. Replace/create glycine	4	3	7
C. Replace alanine	3	3	6
D. Replace serine	1	0	1
E. Create/replace proline	1	1	2
3. Combinations:			
A. Both 1A and 2A	2	1	3
B. Both 2A and 2B	1	0	1
C. Both 1A and 2E	1	0	1
Total	17	10	27

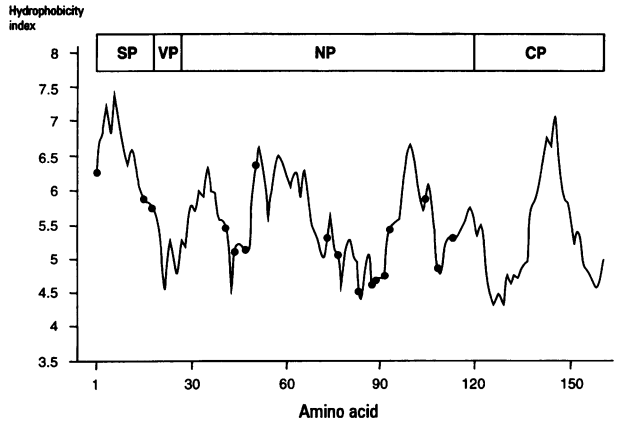

Figure 4 Hydrophobicity index of pre-proAVP-NPII showing the location of all mutations identified in FNDI. Note that the mutations tend to cluster in areas of low hydrophobicity.

and, in a sixth, the mutation affects an adjacent nucleotide in the same codon (C to T, base 280). Thus, by far the most common single mutation associated with FNDI appears to be one that results in replacement of the alanine residue at the −1 position of the signal peptide. All but one of the remaining kindreds (19 of 27) have mutations in several discrete segments of exon 2 or 3 that code for different parts of the neurophysin moiety. About half of these mutations occur in two relatively short neurophysin segments (residues 12–31 and 60–75) that show extensive internal homology. Also, all but possibly one of the mutations are located in areas of low hydrophobicity (fig. 4). Only the mutation at NP_{24} (residue 55 in the pre-prohormone), which is located in the AVP binding pocket, seems to be in a hydrophobic area. As shown by figure 5, three of the other mutations in neurophysin also involve the AVP-binding pocket, and the rest produce changes likely to alter the three-dimensional conformation of the molecule. None of the mutations identified to date occur in the parts of the gene that code for AVP or Copeptin.

Discussion

This study provides further evidence that FNDI is genetically as well as clinically homogenous. Thus, it increases from 10 to 27 the number of apparently unrelated kindreds in which the disease has been linked to a mutation in the coding region of the AVP-NPII gene. Each of these mutations is the only one found in the coding region, predicts a change in the primary structure of the pre-prohormone, and is consistently absent in unaffected, first-degree relatives as well as in healthy controls. Moreover, as expected in a disease with an autosomal dominant mode of inheritance, each mutation is present in only one allele of the gene. In 2 of the 17 kindreds in this study, the heterozygosity was evident

only on restriction-enzyme analysis (fig. 3*A*), since direct sequencing of the PCR amplified DNA showed only a single peak corresponding to the abnormal base in both the sense and antisense strands (fig. 2*B*). This occasional quirk in the method is unexplained but should be recognized as a potential source of error not only in differentiating heterozygous and homozygous mutations but also in determining whether a heterozygous mutation exists.

This study also supports a variety of other evidence that the mutations responsible for FNDI are largely if not completely penetrant (Forssman 1945; Fraser 1955; Pedersen et al. 1985; McLeod et al. 1993). Thus, in every one of the 99 adolescents or adults who could be reliably phenotyped, the mutation was associated with hormonal and/or clinical signs of neurohypophyseal DI (table 3). However, the phenotype is often completely normal in infants or young children with a mutation because the clinical and hormonal signs of FNDI usually do not develop until 1–6 years of age (McLeod et al. 1993). A few older males with a mutation also lack clinical signs of FNDI, but they invariably report severe diabetes insipidus in the past and have been found to have an underlying AVP deficiency that is at least as severe as in their overtly affected kin (Robertson et al. 1993). These pseudoremissions are not attributable to any of the recognized causes of AVP-independent urinary concentration, and their mechanism is unknown.

This study also provides additional evidence that FNDI is quite heterogenous with regard to the type or location of the mutations within the AVP-NPII gene (table 3). Thus, only 4 of the 17 mutations identified in the present study are identical to a mutation reported previously, and 3 of these 4 repeats involve an obvious hotspot immediately adjacent to the signal peptide cleavage site (fig. 1). The other 13 mutations that we have identified are novel and unique to a single kindred. Thus, they increase from 8 to 21 the number of different mutations associated with the disease and show that these mutations extend all the way from the part of exon 1 that codes for the signal peptide to the part of exon 3 that codes for the C-terminus of the neurophysin moiety (fig. 1). However, they also reveal that the mutations do not distribute randomly but tend to cluster in or adjacent to discrete areas of the gene that code for nonhydrophobic regions in the signal peptide or neurophysin moiety (fig. 4).

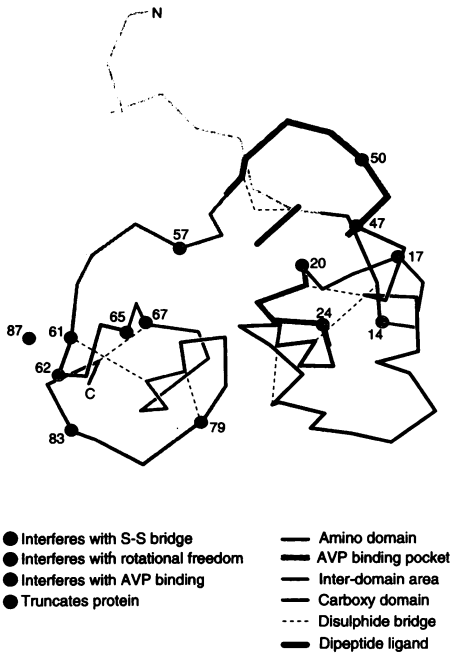

Figure 5 Three-dimensional alpha-carbon structure of neurophysin as derived from crystal structure analysis (Chen et al. 1991). The different parts of the protein are indicated by different lines. The hypothetical conformational changes predicted by the various mutations identified in FNDI are indicated by different symbols.

The pattern of mutations revealed by this study is interesting because it suggests that abnormal folding and self-association of the AVP-NPII protein may be a common pathogenic mechanism in FNDI. This defect is most obvious for the 17 different neurophysin mutations, all but 1 of which affect amino acid residues known or reasonably presumed to be essential for the prohormone to form a stable three-dimensional structure (Breslow 1993). Thus, five of them affect cysteines that form intrachain disulfide bridges necessary to stabilize the molecule; eight affect glycines or prolines that confer flexibility or rigidity in particular segments; and three alter residues that form part of an AVP-binding pocket also important for efficient folding and self-association (table 6). Only the mutation that creates a premature stop codon at position 87 in neurophysin cannot be related to this hypothesis, but it is located in a region whose role in folding and/or self-association has not yet been determined (Chen et al. 1991).

The signal peptide mutations could also impair folding of the prohormone by a different, more indirect effect. Mutagenesis experiments (Hortin and Boime 1981; Folz et al. 1988; Nagahora et al. 1988; Fikes et al. 1990; Laforet and Kendall 1991; Ito et al. 1993) as well as statistical analyses of a large number of eukaryotic signal peptides (von Heijne 1984; Prabhakaran 1990) indicate that substituting a more bulky residue for the preferred alanine or other small amino acids normally found at the −1 or −3 position of the signal peptide (table 6), impairs and/or misdirects cleavage to an alternate site one or more residues upstream. Eliminating the first four amino acids at the N-terminus of the signal peptide (−19 to −16, table 3) could have the same effect (Notwehr and Gordon 1990) because this region may be essential for proper alignment of the cleavage site with the catalytic subunit of the signal peptidase on the luminal side of the endoplasmatic reticulum (Dalbey and von Heijne 1992; Müller 1992; Shelness et al. 1993). In either case, failure to remove all of the signal peptide from the N-

Table 6

Amino Acid Residues at the Signal Peptide Cleavage Site in Most Eukaryotic Secretory Proteins, the Normal AVP-NPII Precursor and Three of the Mutated Precursors Identified in FNDI

	Position						
	−5	−4	−3	−2	−1	+1	+2
Other proteins:							
Preferred residues	PRO THR ALA SER	GLY ALA CYS GLN	VAL ALA CYS THR	TRP HIS GLN ARG	ALA CYS SER GLY	GLN CYS GLU ASP	VAL ILE CYS TYR
Side chain mass	107	101	111	170	98	137	138
SD	6	9	7	9	8	6	9
Normal AVP-NPII:							
Residues	ALA	PHE	SER	SER	ALA	CYS	TYR
Side chain mass	89	165	105	105	89	121	181
Mutant AVP-NPII:							
Residues	ALA	PHE	PHE	SER	THR VAL	CYS	TYR
Side chain mass	89	165	165	105	119 117	121	181

NOTE.—The size (mass) of the side chains at the crucial −1 and −3 positions in the normal precursor are within 1 SD of the mean found in other proteins. However, those found at the same positions in the mutated signal peptide are much larger.

terminus of the AVP moiety could sterically hinder its insertion in the binding pocket in neurophysin, an action that also is necessary for normal folding and self-association of the prohormone (Breslow 1993).

The misfolding hypothesis also predicts a series of other abnormalities that could explain the principal clinical features of the disease. Like other newly synthesized proteins destined for further processing and secretion (Helenius et al. 1992; Gaut and Hendershot 1993), pro-AVP-NPII undoubtedly must fold and self-associate correctly in the endoplasmic reticulum before it can be directed to the Golgi apparatus and secretory vesicles for further processing and storage (Halban and Irminger 1994). Misfolded protein, on the other hand, is retained in the endoplasmatic reticulum where it may bind to chaperone proteins (Rutherford and Zuker 1994; Becker and Craig 1994; Cyr et al. 1994) and undergo proteolytic degradation (Urade and Kito 1992) or accumulate as large aggregates (Marquardt and Helenius 1992) that eventually destroy the cell (Böhni et al. 1988; Wu et al. 1994). The latter could account not only for the dominance of the mutation but also for the delayed onset of the AVP deficiency (McLeod et al. 1993), the prior release into plasma of abnormally large amounts and forms of "big" AVP (Robertson 1994), and the autopsy findings of degeneration of magnocellular neurons (Hanhart 1940; Gaupp 1941; Forssman 1945; Braverman et al. 1965; Green et al. 1967).

The absence of FNDI-associated mutations in certain other parts of the AVP-NPII gene also deserves comment. Some of these "gaps" are not surprising, since they are in areas likely to be nonessential for processing. One such component is copeptin, which is not highly conserved and is not even present in the oxytocin precursor (Mohr et al. 1985). Other "gaps" may be more significant, however, because they occur in areas that probably are important for folding, self-association, and trafficking of the prohormone. One such area is the three N-terminal amino acids of AVP (Breslow 1993; Van Leeuwen et al. 1994). If this and other "gaps" in the mutation profile persist after sequencing of additional kindreds, it would suggest either that these mutations are lethal or that the "misfolding" hypothesis does not explain the pathogenesis of the disease. Thus, sequencing the AVPNPII gene in additional kindreds with FNDI may complement in vitro expression and structural studies to more rigorously test the hypothesis.

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