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Six novel mutations in the arginine vasopressin gene in 15 kindreds with autosomal dominant familial neurohypophyseal diabetes insipidus give further insight into the pathogenesis

Jane H Christensen¹, Charlotte Siggaard², Thomas J Corydon³, Luisa deSanctis⁴, Laszlo Kovacs⁵, Gary L Robertson⁶, Niels Gregersen⁷ and Søren Rittig^{*,2}

¹Pediatric Research Laboratory, Aarhus University Hospital, Skejby Sygehus, Aarhus, Denmark; ²Department of Pediatrics, Aarhus University Hospital, Skejby Sygehus, Aarhus, Denmark; ³Department of Human Genetics, University of Aarhus, Aarhus, Denmark; ⁴Department of Pediatrics, University of Torino, Torino, Italy; ⁵Comenius University Medical School, Comenius University, Bratislava, Slovakia; ⁶Department of Medicine, Northwestern University Medical School, Chicago, IL, USA; ⁷Research Unit for Molecular Medicine, Aarhus University Hospital, Skejby Sygehus, Aarhus, Denmark

Autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI) is caused by postnatal arginine vasopressin (AVP) deficiency resulting from mutations in the AVP gene encoding the AVP pro-hormone. To advance the understanding of adFNDI further, we have searched for mutations in the AVP gene in 15 unrelated kindreds in which diabetes insipidus appeared to be segregating. In nine kindreds, seven different previously described mutations were identified. In each of the other six kindreds, unique novel mutations were identified. Two of these (225A > G and 227G > A) change a nucleotide in the translation initiation codon of the signal peptide, whereas the other four (1797T > C, 1884G > A, 1907T > G, and 2112C > G) predict amino-acid substitutions in the neurophysin II moiety of the AVP prohormone, namely V67A (NP36), G96D (NP65), C104G (NP73), and C116W (NP85). Among these, the mutation predicting the V67A (NP36) substitution is remarkable. It affects a region of the neurophysin II not affected by any other mutations, produces only a minor change, and its inheritance suggests an incomplete penetrance. Our findings both confirm and further extend the mutation pattern that has emerged in adFNDI, suggesting that the mutations affect amino-acid residues known or reasonably presumed to be important for the proper folding and/or dimerization of the neurophysin II moiety of the AVP prohormone.

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*Correspondence: Dr S Rittig, Department of Pediatrics, Aarhus University Hospital, Skejby Sygehus, Brendstrupgaardsvej, DK-8200 Aarhus N, Denmark. Tel: +45 89 49 67 73; Fax: +45 89 49 60 11; E-mail: rittig@iekf.au.dk

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Introduction

Autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI) is a rare inherited disease (OMIM: 192340, 125700) that appears to be largely, if not completely, penetrant.¹ It usually presents several months to years after birth and is clinically characterized by

polyuria accompanied by persistently increased thirst and/or fluid intake.² The underlying cause is a deficient neurosecretion of the antidiuretic hormone, arginine vasopressin (AVP), which in most cases, but not all, is so severe that even an intense stimulus like hypertonic dehydration fails to elicit enough AVP to achieve urine concentration. In the few cases in which it has been studied by repetitive fluid deprivation tests, AVP secretion appears to diminish progressively during early childhood.^{3,4} Autopsy studies in a few adFNDI patients have shown atrophy of the neurohypophysis and a decreased number of magnocellular neurons in the supraoptic and paraventricular nuclei of the hypothalamus,⁴⁻⁷ suggesting that the disease is due to selective degeneration of the cells that normally produce AVP.

Until now, adFNDI has been linked to 42 different mutations in the AVP gene (GenBank: M11166).⁸⁻¹⁵ The AVP gene is located on chromosome 20 (20p13) and contains three exons encoding the AVP pre-prohormone comprising a signal peptide, AVP, neurophysin II (NP), and a C-terminal glycopeptide, copeptin.¹⁶ Most adFNDI mutations are deduced to affect amino-acid residues known or reasonably presumed to be important for the proper folding and/or dimerization of the NP moiety of the AVP prohormone.^{17,18} It has been proposed that the dominant-negative effect exerted by the mutations is due to the production of a mutant hormone precursor that fails to fold and/or dimerize properly in the endoplasmic reticulum (ER) and, as a consequence, is retained by the ER protein quality control machinery,¹⁹⁻²¹ resulting in the cytotoxic accumulation of mutant protein in the neurons.^{22,23} This hypothesis takes into account the characteristic severity of the AVP deficiency in adFNDI, the delayed and possible progressive nature of its development, and the evidence of selective degeneration of the AVP-producing neurons. It is supported, at least partly, by heterologous expression experiments in cell cultures or transgenic

animals,²²⁻³¹ but the hypothesized role of cytotoxicity in adFNDI remains unclear.

In the present study, the identification of six novel and seven different previously described mutations in the AVP gene in 15 apparently unrelated kindreds in which diabetes insipidus appeared to be segregating further advances the understanding of adFNDI.

Materials and methods

Subjects

We studied 42 members of 15 kindreds in which diabetes insipidus appeared to be segregating (Tables 1 and 2). All kindreds were Caucasian, except for one Asian (Table 1, kindred 3) and their nationalities were as indicated. The kindreds were diagnosed and/or referred either by us or by those cited elsewhere (see Acknowledgements). AdFNDI was diagnosed in one or more family members in 13 of the 15 kindreds studied. In 10 of these (Tables 1 and 2), a family history of the disease was described by a pedigree covering the indicated number of generations and in the other three, a family history of adFNDI was well known as reported by the referring physician, but not described by a pedigree. In one of the remaining two kindreds (Table 1, kindred 1), no family history of adFNDI was reported. In the other (Table 1, kindred 3), adFNDI was diagnosed but the inheritance pattern was atypical in that, the proband apparently inherited the disease through his affected mother, although allegedly neither of his maternal grandparents had a history of polyuria, and even though a brother of the maternal grandmother was also affected. In five kindreds (Table 1, kindred 6 and Table 2, kindreds 7-9 and 12), it was reported that the diagnosis of adFNDI was confirmed in at least one member by a fluid deprivation or hypertonic saline infusion test. Magnetic resonance imaging was performed on the proband of kindred 6, showing a normal adenohypophysis as well as pituitary stalk and

Table 1 Six novel mutations in the AVP gene identified in kindreds with adFNDI

Kindred number	Nationality	Number of generations	Exon	Mutation DNA level ^a	Deduced consequence protein level ^b	Peptide ^c	Restriction endonuclease	Mutation found in		
								Affected	Not studied ^d	Not affected
1	Belgian	- ^e	1	225A>G	M1_T4del	SP-19-16	<i>FokI</i>	1/1	0/0	0/0
2	Czech	4	1	227G>A	M1_T4del	SP-19-16	<i>FokI</i>	3/3	0/0	0/0
3	American	4 ^f	2	1797T>C	V67A	NP36	<i>Acil</i>	1/1	1/1	0/0
4	Swedish	2	2	1884G>A	G96D	NP65	<i>HaeIII</i>	4/4	1/1	0/0
5	Czech	5	2	1907T>G	C104G	NP73	<i>HincII</i> ^g	3/3	0/0	1/3
6	Italian	2	3	2112C>G	C116W	NP85	<i>HhaI</i>	2/2	0/0	0/0

^aNumbers refer to the gDNA sequence of the AVP gene, GenBank accession number M11166. Nomenclature according to recommendations.^{55,56}

^bNumbers refer to codons (initiator methionine = codon 1). Nomenclature according to recommendations.^{55,56}

^cNumbers refer to the position in the individual peptides originating from the AVP pre-prohormone. SP, signal peptide; NP, neurophysin II.

^dNo clinical studies performed.

^eNo family history of adFNDI reported.

^fUnusual inheritance pattern (see Materials and methods).

^gRecognition site created by an exon 2 antisense primer with mismatched nucleotide.

Table 2 Seven recurrent mutations in the AVP gene identified in kindreds with adFNDI

Kindred number	Nationality	Number of generations	Exon	Mutation DNA level ^a	Deduced consequence protein level ^b	Peptide ^c	Restriction endonuclease	Mutation found in		
								Affected	Not studied ^d	Not affected
7	German	4	1	279G>A	A19T	SP-1	<i>Bst</i> UI	2/2	0/0	0/2
8	New Zealandish	+ ^e	1	280C>T	A19V	SP-1	<i>Bst</i> UI	1/1	0/0	0/0
9	German	4	2	1757G>C	G54R	NP23	<i>Ap</i> al	2/2	0/0	0/1
10	American	+ ^e	2	1827_29delAGG	E78del	NP47	<i>M</i> nl	1/1	0/1	0/1
11	American	+ ^e	2	1827_29delAGG	E78del	NP47	<i>M</i> nl	1/1	0/0	0/0
12	Austrian	5	2	1827_29delAGG	E78del	NP47	<i>M</i> nl	4/4	0/0	0/0
13	Norwegian	4	2	1830A>G	E78G	NP47	<i>B</i> seRI	1/1	0/1	0/0
14	Czech	2	2	1873C>A	C92X	NP61	<i>M</i> nl	2/2	0/0	0/2
15	Austrian	4	2	1883G>T	G96C	NP65	<i>H</i> aellI	0/0	1/1	0/0

^{a-d} See the notes of Table 1.

^ePositive family history of adFNDI by referring physician, pedigree not available.

optical chiasma, whereas only a thin hyperintense signal of the posterior pituitary on T1-weighted images was evident. All the studies were approved by the appropriate institutional review boards.

Laboratory procedures

Samples of purified genomic DNA or whole blood were shipped for analysis to our laboratory. In the latter case, genomic DNA was extracted from the buffy coat of peripheral leukocytes either as described previously¹⁷ or by the AquaPure Genomic DNA Isolation Kit (Bio-Rad Laboratories, Hercules, CA, USA). All three exons of the AVP gene were PCR amplified separately using 31 base pair flanking primers. The primer sequences and cycle conditions were as previously described.¹⁷ The nucleotide sequences were determined by automated dye-terminator sequencing either on isolated single-stranded biotinylated sense and antisense strands using T7 DNA Polymerase (Sequenase) and the nonbiotinylated primers as described previously¹⁷ or directly on both strands using either dRhodamine or BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA Polymerase, FS (fluorescent sequencing). The sequencing products were analyzed on an ABI PRISM® 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). Mutation detection assays based on restriction endonuclease digestion of the PCR products were performed as described previously.¹⁷ The enzymes were selected to recognize specifically either the nucleotide sequence containing the mutation in question or the corresponding normal AVP gene sequence, whereby digestions generated mutation-dependent cleavage products. One mutation (1907T>G) did not involve a recognition site of any commercial available restriction endonuclease, hence the exon 2 antisense primer was exchanged with a 5'-CCCCGCCGCGCACCGTTCGTTGCAGT-3' (position 1908–1932 of the AVP gene) antisense primer with a mismatched nucleotide (underlined) to create a mutation-

dependent recognition site for the *Hinc*II restriction endonuclease (New England Biolabs, Inc., MA, USA).

Results

In order to identify the possible adFNDI-causing mutations, the three exons of the AVP gene were PCR amplified and DNA sequencing analysis was performed in one or more affected members of 15 kindreds in whom diabetes insipidus appeared to be segregating. The results are summarized in Tables 1 and 2. In all the cases, a mutation was found in the coding region of the AVP gene; the mutation in question was the only one identified, it affected only one allele, and was evident on both the sense and antisense strand. When only one affected individual was analyzed, the presence of the mutation was confirmed on a second PCR product. In order to verify the sequencing results and in some cases to screen for specific mutations in family members not subjected to DNA sequencing, PCR amplified exons were subjected to digestion with restriction endonucleases (Tables 1 and 2).

All the mutations identified predict a change in the amino-acid sequence of the AVP pre-prohormone. In each of the six kindreds (Table 1, kindreds 1–6), unique novel mutations were identified. Two of these mutations, 225A>G and 227G>A (Table 1, kindred 1 and 2, respectively), change a nucleotide in the translation initiation codon of the AVP pre-prohormone signal peptide. The four other novel mutations, 1797T>C, 1884G>A, 1907T>G, and 2112C>G (Table 1, kindreds 3, 4, 5, and 6, respectively), all predict amino-acid substitutions in the NP moiety of the AVP prohormone, namely V67A (NP36), G96D (NP65), C104G (NP73), and C116W (NP85), where the numbers in the parentheses refer to the amino-acid position in NP.

In the other nine kindreds investigated (Table 2, kindred 7–15), seven different previously described mutations were

identified. One mutation, namely 1827_29delAGG (Table 2, kindreds 10–12), was identified in three kindreds (two American and one Austrian) with no known relationship to each other or to the other two adFNDI kindreds (Japanese and American) in which the same mutation has been identified previously.^{17,32} The other six recurrent mutations were identified in only one kindred each (Table 2, kindreds 7–9 and 13–15). None of these kindreds had any known relationship to the kindreds in which the same mutations have been described previously, namely the 279G>A (A19T) mutation identified in four American, a Japanese, a Danish, a Spanish, and a Brazilian kindred,^{3,15,17,33–36} the 280C>T (A19V) mutation identified in two American, a Lebanese, a German, and a Japanese kindred,^{10,11,17,37} the 1757G>C (G54R) mutation identified in a German and an American kindred,^{13,38} the 1830A>G (E78G) mutation identified in an English kindred,¹⁷ the 1873C>A (C92X) mutation identified in a

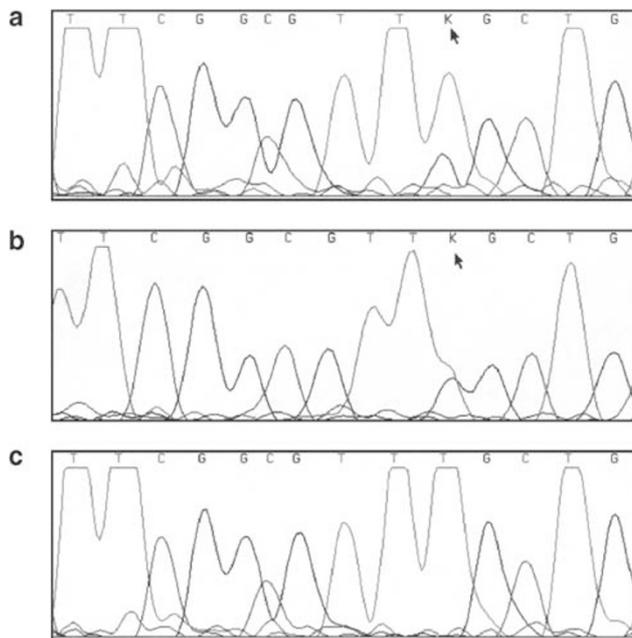


Figure 1 Automated dye-terminator sequencing of the AVP gene in members of a kindred in which adFNDI appeared to be segregating (kindred 5). All three exons of the AVP gene were PCR amplified from purified genomic DNA, and their nucleotide sequences were determined by automated dye-terminator sequencing. Panels a–c, sections of the electropherograms obtained by the sequencing of exon 2 from a clinically unaffected child at risk for inheriting adFNDI (a, sense strand and b, antisense strand) and from an unaffected member of the same kindred (c, sense strand). A 1907T>G nucleotide substitution was identified in one allele of the AVP gene in the subject at risk (arrows), in two other clinically affected family members (not shown), but not in an unaffected member of the same kindred. Thus, although still clinically unaffected, it is evident that the subject at risk for inheriting adFNDI is indeed a carrier of the mutation.

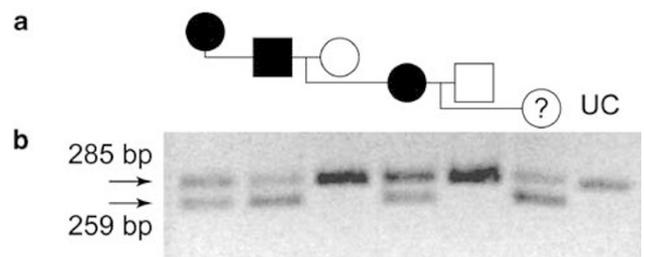


Figure 2 Restriction endonuclease digestion analysis of PCR amplified exon 2 from the AVP gene in members of a kindred in which adFNDI appeared to be segregating (kindred 5). Since the 1907T>G mutation identified in the kindred do not create or eliminate a recognition site of any commercially available restriction endonuclease, exon 2 was PCR amplified using an antisense primer carrying a mismatched nucleotide to create a mutation-dependent recognition site for the *HincII* restriction endonuclease. The digestion of the resulting PCR product (285 base pairs) with *HincII* would generate two fragments of, respectively, 26 and 259 base pairs only in the presence of the 1907T>G mutation. Panel a: a pedigree describing a part of the kindred and the occurrence of adFNDI. Clinically affected subjects are indicated by filled symbols, unaffected subjects are indicated by open symbols, and the subjects at risk for inheriting adFNDI are indicated by an open symbol with a question mark. Panel b: agarose gel electrophoresis analysis of the DNA fragments generated by *HincII* digestion of the PCR-amplified exon 2 from the family members indicated in panel a. The 259 base pair fragment was apparent in all the affected subjects, not in any unaffected members, and in the subjects at risk for inheriting adFNDI fully verifying the sequencing results. UC, uncleaved PCR amplified exon 2.

Norwegian kindred,¹⁷ and finally, the 1883G>T (G96C) mutation identified in an American kindred.¹⁷

In one kindred (Table 1, kindred 5), a mutation was identified in an unaffected 1-year-old child at risk for inheriting adFNDI (Figures 1 and 2). In three kindreds (Table 1, kindreds 3–4 and Table 2, kindred 15), a mutation was identified in, respectively, a newborn and two 2.5-year-old children without any clinical assessment, but at risk for inheriting adFNDI. In two other children, both newborn and also without any clinical assessment, no mutations were identified (Table 2, kindreds 10 and 13).

Discussion

By examining a total of 38 kindreds in which adFNDI appear to be segregating, we have now identified 24 different mutations in the AVP gene.^{1,3,9,12,17,34,39} Although the total number of unaffected subjects included in the present study was small and although no formal linkage analysis was performed to prove that the mutations identified actually cause adFNDI, their absence among 296 normal alleles from 40 healthy controls,¹⁷ 10 patients with idiopathic NDI,⁴⁰ and at least 98 healthy relatives in

kindreds with a family history of adFNDI^{8-15,17} support the assumption that each of the mutations causes adFNDI in the kindred in whom it was identified. This is even further supported by the fact that each mutation identified is the only one present in the AVP gene, they all predict a change in the amino-acid sequence of the AVP pre-prohormone, and all but one affects codons that are affected by other

mutations previously shown to cause the disease (see below and Figure 3).

One interesting finding in the present study is the probable incomplete penetrance of the mutation predicting the V67A (NP36) amino-acid substitution (Table 1, kindred 3). The unusual family history of the kindred (see Materials and methods), if correct, suggests that the

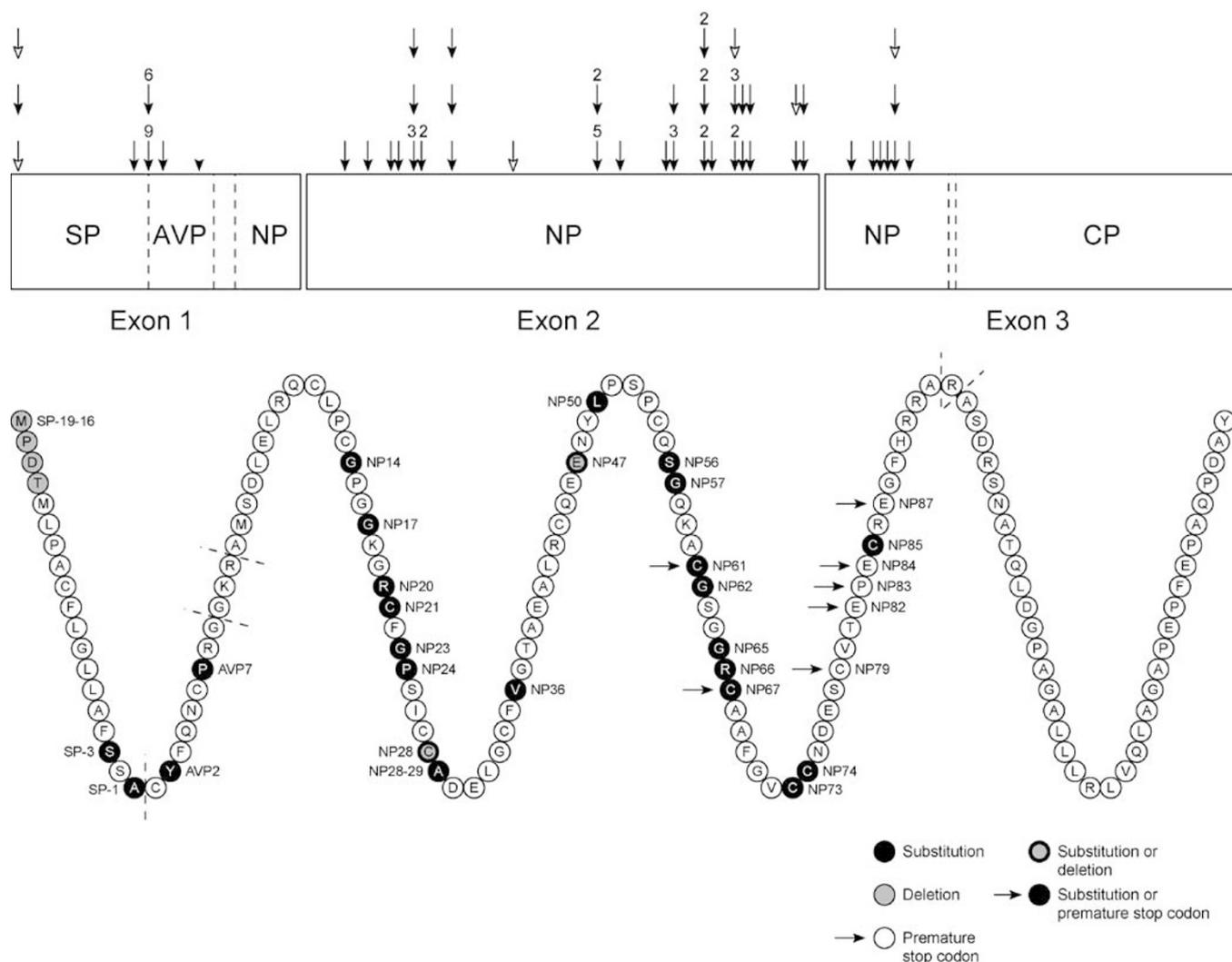


Figure 3 Schematic structure of the AVP gene (upper panel) and the AVP pre-prohormone (lower panel). The location in the AVP gene of the novel mutations associated with adFNDI identified in the present study are shown by open arrows, previously published mutations associated with adFNDI⁸⁻¹⁵ are shown by filled arrows, and a mutation associated with recessive inheritance of FNDI⁵⁷ is shown by an arrowhead. Each arrow represents a single unique mutation. Numbers above the arrows indicate the number of apparently unrelated adFNDI kindreds identified to carry the same mutation. No number above an arrow indicates that only a single adFNDI kindred has been identified to carry the specific mutation. The deduced consequence of these mutations at the protein level is shown in the AVP pre-prohormone primary structure by symbols indicating: amino-acid substitutions, filled circles; amino-acid deletions, shaded circles; premature stop codons, open circles with arrows; substitutions or deletions, shaded circles with filled frames; substitutions or premature stop codons, filled circles with arrows. Numbers refer to the amino-acid position in the individual peptides originating from the AVP pre-prohormone and the abbreviations are: SP, signal peptide; AVP, arginine vasopressin; NP, neurophysin II; and CP, copeptin. Dashed bars indicate the cleavage sites for the enzymes responsible for AVP pre-prohormone processing (also indicated in the AVP gene structure). The NP74 and NP84 mutations were originally published as NP73¹³ and NP83, respectively.¹⁰

proband inherited the mutation through his mother and maternal grandmother in whom it thus appeared to have been nonpenetrant or that his mother inherited the mutation from another male relative who was not the father of record. Since only two subjects, one affected and a newborn without clinical assessment, were studied, it is difficult to correlate the genotype and phenotype in the kindred. A more thorough clinical and genetic evaluation is indicated. The mutation is remarkable in that it affects a region of NP, not affected by any other mutations so far (Figure 3). It is predicted to produce only a minor change from a hydrophobic valine residue V67 (NP36) to the smaller, but also hydrophobic alanine residue. It can be speculated that this amino-acid substitution as a result of its location in the interface between the individual NP monomers^{18,41} and although representing only a minor change may interfere with dimerization, rather than the initial folding of the AVP prohormone. It has not been possible to examine in detail whether the clinical phenotype resulting from this mutation diverges from the classical adFNDI phenotype.

The identification of six novel and seven different recurrent mutations in the AVP gene in 15 previously unreported kindreds with diabetes insipidus in the present study both confirms and further extends the mutation pattern that has emerged from previous observations in kindreds with adFNDI (Figure 3). It suggests that mutations in the AVP gene associated with adFNDI affect amino-acid residues known or reasonably presumed to be important for proper folding and/or dimerization of the NP moiety of the AVP prohormone.^{17,18} The 225A>G and 227G>A mutations identified (Table 1, kindreds 1 and 2) change a nucleotide in the translation initiation codon of the AVP gene. The effect of a similar mutation, 227delG, identified in a Swiss adFNDI kindred⁴² also changing the translation initiation codon has been examined by *in vitro* translation experiments.²⁵ Apparently, it results in the deletion of the first four amino-acid residues of the signal peptide (M1_T4del) due to alternative initiation of translation at a second, in-frame ATG present four codons downstream. The heterologous expression of the 227delG mutation in COS-7 cells further indicates that the apparently truncated signal peptide is functional for targeting and translocation into the ER, but is markedly resistant to cleavage by signal peptidase.²⁵ Since the mutated AVP prohormone was retained in the ER and disulfide-linked aggregates were observed, it was suggested that the uncleaved signal peptide containing a cysteine residue somehow interferes with the formation of the C20–C25 disulfide bridge in the AVP moiety of the prohormone. This is likely to affect the proper folding and subsequent dimerization of individual NP molecules, since these processes seem to require the insertion of the cyclic disulfide linked N-terminal domain of the AVP moiety into the hormone-binding pocket of NP.^{41,43–48} Impaired signal peptide processing and cellular

trafficking has also been described for another adFNDI mutation,^{27,33} predicting an A19T (SP-1) amino-acid substitution of a residue known to be very critical for the signal peptidase activity.⁴⁹

The other novel mutations identified (Table 1, kindreds 4–6) are deduced to eliminate either glycine, G96D (NP65) or cysteine residues, C104G (NP73) and C116W (NP85), located in the NP domain of the AVP prohormone. Other mutations affecting these amino-acid residues have been described previously in adFNDI kindreds, namely 1883G>T (G96C) in an American kindred,¹⁷ 1884G>T (G96V) in both a German and two Japanese kindreds,^{50–52} 1908G>T (C104F) in an American kindred,¹⁴ as well as 2110T>G (C116G)⁸ and 2110T>C (C116R)⁵³ in two Dutch kindreds. They are known to be essential for the NP molecule to form a stable three-dimensional structure,⁵⁴ the glycine residues by conferring flexibility to the protein backbone and the cysteine residues by forming intrachain disulfide bridges between NP73 and NP61 as well as between NP85 and NP67. The cellular effects of the G96D (NP65) substitution are probably similar to those of the G96V substitution that has been investigated by heterologous expression in different cell lines.²⁶ It seems to cause severe impairment of the AVP prohormone processing and secretion probably due to an almost complete retention in the ER. Since the G96 residue is located in one of the eight internal β -sheets of the NP domain, it could possibly play an essential role in the proper folding of the AVP prohormone. Severe effects on the cellular handling of the AVP prohormone have also been observed for the C116G (NP85) substitution,³⁰ which is comparable to the C116W substitution deduced to result from one of the mutations identified in the present study. Since no disulfide-linked mutant AVP prohormone was detected in the cells expressing this mutation,³⁰ it was suggested that the ER retention was caused by incomplete folding of the NP moiety and not by dimerization of the AVP prohormone via its unpaired cysteine residues. Whether adFNDI mutations resulting in either elimination or addition of cysteine residues to the NP moiety of the prohormone share a common mechanism of ER retention still remains unknown.

The seven different previously described mutations identified in nine kindreds in the present study affect, respectively, the signal peptide (A19T and A19V (SP-1)), glycine residues (G54R (NP23), and G96C (NP65)), the AVP-binding pocket in NP (Δ E78 and E78G (NP47)), as well as a cysteine residue by the formation of a premature stop codon (C92X (NP61)). Although these mutations, as well as those identified previously, affect amino-acid residues throughout the AVP pre-prohormone, they do not distribute randomly but tend to cluster in specific regions especially affecting the signal peptide, the central conserved region of NP as well as in the hormone-binding pocket (Figure 3). Three of these, namely those predicting

the A19T (SP-1) and A19V (SP-1) substitutions as well as that predicting the Δ E78 (NP47) deletion are now by far the most common adFNDI mutations, identified in respectively, nine, six, and five kindreds. The E78 amino-acid residue is additionally affected by another mutation, identified in two unrelated kindreds so far, predicting an E78G (NP47) substitution. Since each of them has been identified in kindreds of different race and/or nationality and since the kindreds had no known relationship to each other, they probably arose independently. However, it is not possible to exclude a common progenitor for, for example, the mutations identified in both Caucasian American and Danish kindreds. The ratio of recurrent mutations identified in the present study 9/15 (=60%) is significantly higher than the 4/17 (=23.5%) identified in one of our previous studies.¹⁷ This could reflect the fact that the adFNDI mutation spectrum is now almost revealed.

In the present study, mutations were identified in an asymptomatic 1-year-old child (Table 1, kindred 5) and in three children under the age of 3 years without any clinical assessment (Table 1, kindreds 3–4 and Table 2, kindred 15), all at risk for inheriting adFNDI. To our knowledge, at least one of these children (Table 1, kindred 4) has developed DI symptoms meanwhile. In two other children, both newborn also without any clinical assessment, no mutations were identified (Table 2, kindreds 10 and 13). A preclinical diagnosis like this promotes early clarification concerning the disease often resulting in the relief of parental concern. Thus, although not contributing directly in clarifying the mechanism underlying the development of the disease, the identification of mutations in kindreds in which adFNDI is segregating not only seems to provide a basis for a genuine insight into the structural requirements for proper AVP prohormone folding in the ER, but also enables valuable genetic counseling.

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