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Characterization of the genomic structure of the human neuronal nicotinic acetylcholine receptor *CHRNA5/A3/B4* gene cluster and identification of novel intragenic polymorphisms

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Abstract Genes coding for the α5, α3, and β4 subunits (*CHRNA5*, *CHRNA3*, and *CHRNB4*) of the neuronal nicotinic acetylcholine receptors (nAChRs) are clustered on chromosome 15q24. Linkage of this chromosomal region to autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE), an idiopathic partial epilepsy, was reported in one family. Moreover, mutations in other neuronal nAChR subunit genes coding for the α4 (*CHRNA4*) and the β2 (*CHRNB2*) subunits were associated with ADNFLE. Apart from the exon-intron structure of *CHRNA3*, the genomic organization of this gene cluster was unknown, making comprehensive mutational analyses impossible. The genomic structure of *CHRNA5* and *CHRNB4* is here reported. Moreover, two hitherto unknown introns were identified within the 3′ untranslated region of *CHRNA3*, causing a partial tail-to-tail overlap with *CHRNA5*. Four novel intragenic polymorphisms were identified and characterized in the cluster.

Key words *CHRNA5/A3/B4* gene cluster · Human neuronal nicotinic acetylcholine receptor (nAChR) · Genomic structure · Tail-to-tail overlap · Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) · Polymorphisms.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) are multisubunit, ligand-gated ion channels expressed in the peripheral and central nervous systems (Clementi et al. 2000). They are formed by different combinations of homologous subunits, assembled as pentameric structures giving rise to an aqueous central channel. The receptor subunits are encoded by a gene family composed of at least 12 members classified as α subunits (α2 to α10) and β subunits (β2 to β4) (Le Novere and Changeux 1995; Lustig et al. 2001). Alpha subunits, characterized by the presence of two adjacent cysteines in their extracellular domain, constitute the major component of the acetylcholine binding site, while β subunits have a mainly structural role, although they have been shown to contribute to ligand binding profiles (McGehee 1999). With the exception of α7 and α9 subunits that can assemble into homopentamers, neuronal nAChRs result from the assembly of five subunits, in a stoichiometry of two α to three β. The large number of different subunits giving rise to various combinations, including those containing more than one type of α and/or β subunit (McGehee 1999), can produce a huge variety of nAChRs, with different biophysical and pharmacological properties. However, the predominant forms are α4β2 and α4α5β2 in the central nervous system and α3β4 or α3α5β4 in the peripheral nervous system (McGehee 1999).

Qualitative or quantitative alterations of the nAChRs can cause brain diseases, including idiopathic epilepsies, schizophrenia, Alzheimer disease, and Parkinson disease (Clementi et al. 2000). To date, the pathogenetic mechanism has been elucidated only for autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE, MIM#600513, MIM*603204, and MIM#605375), a partial idiopathic epilepsy characterized by clusters of brief nocturnal motor seizures with a frontal lobe semiology (for a review, see Scheffer 2000). Mutations in two members of the nAChR gene family (*CHRNA4* and *CHRNB2*, coding for the α4 and β2 subunits, respectively) were demonstrated to be involved in this syndrome (Scheffer and Berkovic 2000; De Fusco et al. 2000; Phillips et al. 2001). All identified mutations affect amino acids that line the aqueous pore within the ion channel structure and determine an alteration of the functional properties of the receptor (De Fusco et al. 2000; Scheffer 2000; Phillips et al. 2001), supporting the concept that idiopathic epilepsies are, at least in part, ion channel disorders. However, mutations in *CHRNA4* and *CHRNB2*

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account only for a small proportion of ADNFLE cases (Tenchini et al. 1999).

Genes for additional subunits of the nAChR expressed in brain are obvious candidates for mutation in families and sporadic cases, in which no involvement of *CHRNA4* or *CHRNB2* can be demonstrated by linkage or mutational analyses. In particular, a third locus for ADNFLE was reported on 15q24, a chromosome region that contains the *CHRNA5/A3/B4* gene cluster coding for the α5, α3, and β4 subunits, respectively. However, neither the gene nor the mutation involved was identified (Phillips et al. 1998).

Although cDNA sequences for nAChR subunits have been analyzed in humans and in a number of different species, the genomic structure has been described only for *CHRNA3*, *CHRNA4*, *CHRNA7*, *CHRNA10*, and *CHRNB2*, coding for the α3, α4, α7, α10, and β2 subunits, respectively (Steinlein et al. 1996; Gault et al. 1998; Rempel et al. 1998; Lustig et al. 2001). The overall genomic organization of these genes is conserved and characterized by the presence of six exons, except for *CHRNA7*, which, both in humans and chick, consists of ten exons as the result of partial gene duplication.

In the present paper, the genomic organization of *CHRNA5* and *CHRNB4* together with the sequences of exon-intron boundaries are reported. Two novel introns interrupting the 3′ untranslated region (UTR) of *CHRNA3* were identified, and *CHRNA3* and *CHRNA5* were shown to partially overlap at their 3′ ends. Moreover, four novel polymorphisms were identified and their allele frequencies estimated in an Italian population sample.

Subjects and methods

Sample composition and DNA extraction

Clinical characteristics of analyzed ADNFLE probands were previously described (Oldani et al. 1998; Tenchini et al. 1999). All examined subjects gave informed consent in writing before blood withdrawal. Genomic DNA was extracted from blood samples using the Nucleon BACC1 kit (Amersham Pharmacia Biotech, Uppsala, Sweden). DNAs extracted from normal healthy Italian individuals of both sexes without sleep disorders were used to calculate allele frequencies of the identified polymorphisms.

Long-range polymerase chain reaction

Long-range inter-exon polymerase chain reaction (PCR) was accomplished using the Expand 20 kb plus PCR system kit (Roche Diagnostics, Monza, Italy) and a Personal Cycler (Biometra, Göttingen, Germany). Thermal conditions were 10 cycles of 92°C for 10 s, 60°–64°C for 30 s, and 68°C for 10–18 min, preceded by 2 min at 92°C and followed by 20 additional cycles with a time increment of 10 s for each cycle in the elongation step. A final incubation at 68°C for 7 min was also performed.

DNA sequencing

PCRs for the generation of templates were performed on 50–100 ng of genomic DNA in a standard 50 μl volume containing 1x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.001% gelatin), 1.5 mM $MgCl_2$, 0.4 μM of each primer, 200 μM dNTPs, and 2 U RED *Taq* DNA polymerase (Sigma, St. Louis, MO, USA) on a PTC-100 (MJ Research, Watertown, MA, USA) thermal cycler. Thermal conditions were 30–35 cycles of 95°C for 30 s, 52°–60°C for 30 s and 72°C for 20–60 s, preceded by 3 min at 95°C and followed by a final incubation at 72°C for 10 min.

Sequencing was performed on both strands, directly on purified PCR products, using the BigDye terminator kit and an automated ABI-310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Factura and Sequence Navigator softwares (Applied Biosystems) were used for mutation detection. Alignments were performed by the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/).

Primers

Primers used for inter-exon PCR were designed on the basis of known sequences of the *CHRNA5* and *CHRNB4* cDNAs (GenBank, accession numbers: NM_000745 and NM_000750) and are listed in Tables 1 and 2, together with primers used for exon amplification and mutational screening of *CHRNA5* and *CHRNB4*. Primers used to amplify *CHRNA3* exons were designed on the basis of the known genomic sequences of *CHRNA3* (GenBank, accession numbers: AJ007783-AJ007787) and can be provided on request. All primers were from Life Technologies (Inchinnan, Paisley, UK).

Detection of the short tandem repeat (STR) polymorphism

The tetranucleotide-repeat marker identified in intron 2 of *CHRNB4* (CHRNB4.IVS2.STR) was amplified using the primer pair B4STR-F (5′-AAAGATCACACCACTGCC CT-3′) and 6Fam-B4STR-R (5′-CATCCTATCTGAGAA ATCTTCC-3′) in a Personal Cycler (Biometra). Standard 20 μl-PCR reactions contained 50 ng genomic DNA, 1x PCR reaction buffer (20 mM TrisHCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.4 μM of each primer, and 0.5 U Platinum *Taq* DNA polymerase (Life Technologies). The thermal profile consisted of: an initial denaturation step of 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. A final extension of 10 min at 72°C was added after the last cycle. Amplified products were separated using an ABI-310 Genetic Analyzer; allele sizing was performed by Genescan 3.1 software (Applied Biosystems).

Detection of single-nucleotide polymorphisms (SNPs)

Screening for the T>C polymorphism identified at position −5 in intron 3 of *CHRNA3* (CHRNA3.IVS3.−5T>C) was

Table 1. Primers used to analyze human *CHRNA5* (α5 subunit of the neuronal nicotinic acetylcholine receptor gene)

	Name	Sequence (5′ → 3′)	Product size (kb)[a]	Annealing temp. (°C)
PCR amplification and partial sequencing				
Intron				
1	A5I1F	CGCTCCGCCTGCTGCTCTTGGT	11.5	60
	A5I1R	GGTGTTCCACAGGACGAACCCATC		
2–3	A5I2F	GATGGGTTCGTCCTGTGGAACACC	7.6	60
	A5I2R	CGTTTGTTGTCATTAACTGATTTT		
	A5I3F[b]	AAAATCAGTTAATGACAACAAACG		
	A5I3R	TGGTGTCCAGACAGAGTCTGAAGG		
4	A5I4F	CCTTCAGACTCTTCGTGGACACCA	1.4	60
	A5I4R	TACTGGTCCCTTCAAAACGTCCATC		
5	A5I5F	TAGAAACACATTGGAAGCTGCGCTC	2.45	63
	A5I5R	AAGTCCACAGAAACATCCGATCAAG		
Mutational screening				
Exon				
1	A5ex1F[c]	AGCTTCCACATGCGTCCCGA	0.419	58
	A5ex1R	CCCTCGCAACCAGGTTTTCC		
2	A5ex2F	TCACTGCAACCTTTGCCTCCT	0.315	58
	A5ex2R	CACACACCAGGCTCTCACTAA		
3	A5ex3F	TATGTTGAAAGGAGTAATGTGA	0.500	52
	A5ex3R	AGGGTTTTTTGTCTGTTTTGTT		
4	A5ex4F	AGGTGTTCTCTATTGGGGGT	0.410	53
	A5ex4R	TAAGCCTCTGTTTCCTCATC		
5	A5ex5F[d]	GCAGATGGACGTTTTGAAGGG	0.829	58
	A5ex5R[d]	CACGGACATCATTTTCCTTCAT		
6	A5ex6F	GAGTATAGGGTCACTTACCG	0.483	53
	A5ex6R[e]	ATTACTTCAGTCATACAGCCA		

PCR, polymerase chain reaction
[a] intron size estimated by gel electrophoresis
[b] primer used only for sequencing
[c] primer located within the 5′ UTR proximal to the ATG start codon
[d] exonic primer
[e] primer located within the 3′ UTR distal to the termination codon

performed by *Taq*I digestion of PCR products amplified using the following primers: 5′-GGAAGTAAAACCA GGCTGATTC-3′ (sense, position 28–49) and 5′-AAGG CCAGGTTTTAAGCACAGT-3′ (antisense, position 284–263) (numbering according to *CHRNA3* exon 4 sequence, GenBank accession number AJ007785). Digestion products were electrophoresed on 2.5% agarose gels. CHRNA5.IVS1.−34T>C and CHRNA5.IVS3.+211A>G polymorphisms were analyzed by direct sequencing of PCR-amplified genomic DNA.

Results

CHRNA5 and *CHRNB4* genomic structures

The exon-intron organization of *CHRNA5* and *CHRNB4* was identified by a PCR-based approach. The putative positions of exon-intron junctions were deduced by multiple alignment of several neuronal nAChR subunit genes. Primers were designed taking care to avoid the putative splicing junctions in order to amplify all introns of both genes. Introns 1, 4, and 5 of *CHRNA5* and introns 1, 4, and 5 of *CHRNB4* were individually amplified by inter-exon PCR, while introns 2 and 3 were obtained as a single fragment for each gene.

The genomic structure of both genes was defined (Fig. 1), and the sequence of all splicing junctions was obtained by partial sequencing of PCR product ends and by evaluation of fragment lengths by gel electrophoresis. The genomic size was about 25 and 17 kb for *CHRNA5* and *CHRNB4*, respectively. Both genes are composed of six exons and five introns, in agreement with the overall structure of known nAChR genes. All splice sites in both genes are in accordance with the consensus GT-AG (Shapiro and Senapathy 1987). Nucleotide sequences of *CHRNA5* and *CHRNB4* have been submitted to the EMBL nucleotide sequence database (accession numbers AJ306481-AJ306486 and AJ306454-AJ306459, respectively).

Characterization of a novel tetranucleotide-repeat marker in intron 2 of *CHRNB4*

Sequencing of *CHRNB4* intron 2 revealed the presence of a short tandem repeat (STR) consisting of a tetranucleotide $(GAAA)_n$ repeat, located about 1 kb upstream of the exon

Table 2. Primers used to analyze human *CHRNB4* (β4 subunit of the neuronal nicotinic acetylcholine receptor gene)

	Name	Sequence (5′ → 3′)	Product size (kb)[a]	Annealing temp. (°C)
PCR amplification and partial sequencing				
Intron				
1	B4I1F	TTCCCTGGTCCTTTTCTTCCTGG	6	60
	B4I1R	TGATGGAGATGAGCTGTGAGGAGC		
2–3	B4I2-3F	ACAATAACCTGATCCGCCCAGCC	4	64
	B4I2-3R	GAGCTGTTCCAGGTCAGGCGGTA		
4	B4I4F	CTGACCTGGAACAGCTCCCGCTAC	1.8	63
	B4I4R	AGACACCTCATAGGTCCCGTCGGC		
5	B4I5F	GAAGAATGACGATGAAGACCAGAG	4	60
	B4I5R	CACACAAACATGAACACCCACAG		
Mutational screening				
Exon				
1	B4ex1F[b]	GCACGAGCCGCCAGCAAACC	0.228	56
	B4ex1R	CCCCTTTCAGCCCAACCCAG		
2	B4ex2F	TCAGGTGCACAAGGGCCCAG	0.278	60
	B4ex2R	TTCCTTTGACCAATGATCGCCT		
3	B4ex3F	GGAGCTCACAGCCTTTTGCAC	0.280	60
	B4ex3R	TTGCAGGTCCACTGCCACCAA		
4	B4ex4F	TGGTGGCAGTGGACCTGCAA	0.250	60
	B4ex4R	GGATGACTCTTAGGGCTGGG		
5	B4ex5F1[c]	CGACGGGACCTATGAGGTGT	0.976	58
	B4ex5R1[c]	ACTCTGGTCTTCATCGTCATTC		
	B4ex5F2[d]	TCATCTCCAAGATCGTCGCA		
	B4ex5R2[d]	CACCATGGTGAACATGAGGT		
6	B4ex6F	GCCCCCCTAGTATGCAGTTC	0.380	60
	B4ex6R[e]	CCACAACCCAGAAAGAAGCAG		

[a] intron size estimated by gel electrophoresis
[b] primer located within the 5′ UTR proximal to the ATG start codon
[c] exonic primer
[d] primer used only for sequencing
[e] primer located within the 3′ UTR distal to the termination codon

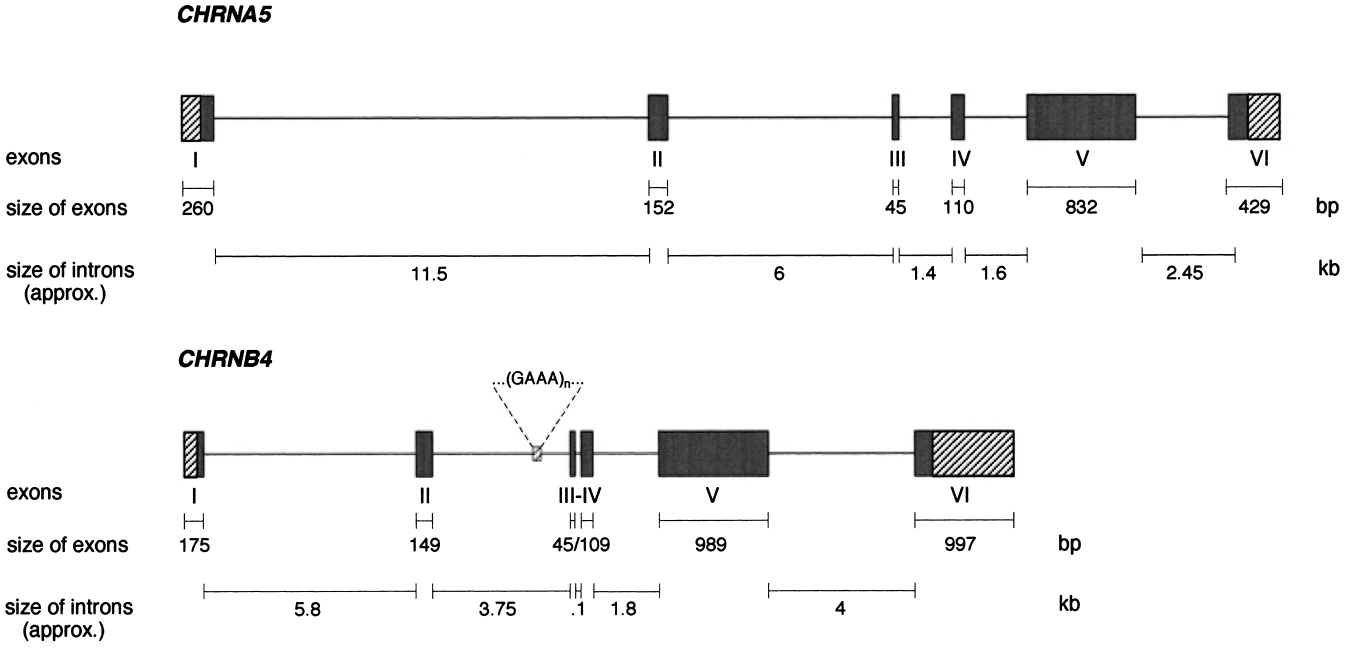

Fig. 1. Schematic representation of the genomic structure of the α5 and β4 subunits of the neuronal nicotinic acetylcholine receptor (*CHRNA5* and *CHRNB4*) genes. *Boxes* indicate exons; *horizontal lines* represent introns; *hatched boxes* indicate the 5′ and the 3′ untranslated regions. Exons and introns are drawn to different scales. The position of the novel tetranucleotide-repeat marker in intron 2 of *CHRNB4* is indicated

Table 3. Allele sizes and frequencies of the novel short tandem repeat market $(GAAA)_n$ identified in intron 2 of *CHRNB4*

Allele size (bp)	Allele frequence (%)
245	1.5
249	1
253	21.5
257	43.6
259	0.5
261	24
265	5.9
269	0.5
273	1
305	0.5

Table 4. Characterization of the three novel SNPs identified in *CHRNA3* and *CHRNA5*

SNP	Frequency of the rare allele	Heterozygosity	PIC
CHRNA3.IVS3.−5T>C	0.28	0.4032	0.3219
CHRNA5.IVS1.−34T>C	0.27	0.3942	0.3165
CHRNA5.IVS3.+211A>G	0.29	0.4118	0.3270

SNP, single-nucleotide polymorphism; *CHRNA3/A5*, α3 and α5 subunits of the neuronal nicotinic acetylcholine receptor gene; PIC, polymorphism information content

3 acceptor splice site. To verify whether this sequence represented a polymorphic site, a primer pair (B4STR-F and 6Fam-B4STR-R) flanking the repeat was designed. The reverse primer was 5′-labeled with 6-Fam. One hundred two unrelated healthy Italian individuals were genotyped by PCR amplification from genomic DNA; PCR products were analyzed using an ABI-310 Genetic Analyzer. A total of ten different alleles were detected, ranging from 245 to 305 bp. Allele lengths and frequencies of this novel *CHRNB4* marker (CHRNB4.IVS2.STR) are reported in Table 3. Heterozygosity and polymorphism information content (PIC) values are 0.7020 and 0.6549, respectively.

Mutational analysis

The search for mutations in the *CHRNA5/A3/B4* genes was performed in five unrelated ADNFLE probands. All subjects were chosen from ADNFLE families (nos. 5, 7, 12, 18, and 35) (Oldani et al. 1998; Tenchini et al. 1999) in which segregation analysis excluded linkage to *CHRNA4* and *CHRNB2* and showed compatibility with linkage to 15q24 (data not shown). Full sequencing of both strands of exonic regions and exon-intron boundaries of the three genes did not detect any mutation in the analyzed patients.

Single-nucleotide polymorphisms (SNPs) in *CHRNA3* and *CHRNA5*

As a result of sequence analysis of ADNFLE probands, three novel bi-allelic polymorphisms in the *CHRNA3* and *CHRNA5* genes were detected and designated following the recommendations of Antonarakis (1998). One SNP was identified in intron 3 of *CHRNA3* and consisted of a T to C transition at nucleotide position −5 (CHRNA3.IVS3.−5T>C). This nucleotide variation probably does not alter the corresponding acceptor splice site, since intronic position −5 can be represented by either a C or a T (Shapiro and Senapathy 1987). As this transition abolishes a *Taq*I restriction site, screening for this polymorphism was performed by PCR amplification of the relevant genomic fragment followed by *Taq*I restriction digestion in a control population of 60 unrelated healthy individuals from northern Italy. The remaining two SNPs were identified in *CHRNA5* and consisted of a T to C transition at position −34 in intron 1 (CHRNA5.IVS1.−34T>C) and an A to G transition at position +211 in intron 3 (CHRNA5.IVS3.+211A>G). As they did not alter any known restriction site, their frequency was estimated by sequencing genomic DNAs at the relevant positions of the same control individuals as above. The frequencies of the rare allele and the heterozygosity and PIC values of each polymorphism are reported in Table 4.

Identification of two novel *CHRNA3* introns

The previously reported genomic organization of *CHRNA3* consisted of six exons and five introns (Rempel et al. 1998). Amplification of exon 6 of this gene for mutational screening with the primer pair A3ex6F (5′-CTGCAACGTGT TGATTACATCTT-3′) and A3ex6R (5′-TTCATAGCCC AGGTTCTTGATC-3′) produced a 3.3 kb-fragment, approximately 2.85 kb larger than that predicted on the basis of the *CHRNA3* cDNA sequence. The possible occurrence of intervening sequences was therefore hypothesized. Direct sequencing of this 3.3 kb-fragment and subsequent alignment with the published cDNA of *CHRNA3* (Mihovilovic and Roses 1991) demonstrated the presence of two additional introns, located at cDNA positions 1576 and 1674 (numbering from the first nucleotide of the ATG start codon, according to GenBank accession number M37981) (Fig. 2). The newly identified introns 6 and 7 were shown by sequencing to span 2535 and 722 bp, respectively, extending *CHRNA3* by approximately an additional 3.2 kb (intron 6 + intron 7) at its 3′ end. Because the previously described exon 6 turns out to be interrupted by two additional introns, the *CHRNA3* exon-intron structure comprises eight exons and seven introns. The presence of an antisense Alu repeat spanning from intron 6 to intron 7 and containing the whole *CHRNA3* exon 7 was also identified by BLAST search (Fig. 2).

Tail-to-tail overlap between *CHRNA3* and *CHRNA5*

While the *CHRNA3*/*CHRNB4* intergenic region was shown to span about 4 kb in humans (Fornasari et al. 1997), the precise distance between the 3′ ends of *CHRNA5* and *CHRNA3* was unknown. Since we observed that *CHRNA3*

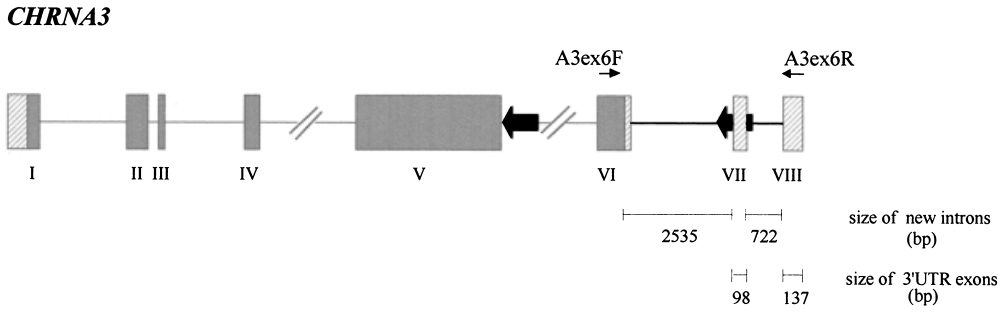

Fig. 2. Genomic structure of the α3 subunit of the neuronal nicotinic acetylcholine receptor gene (*CHRNA3*) showing the newly identified introns interrupting the 3′UTR. *Boxes* indicate exons; *horizontal lines* represent introns; the 3′UTRs are indicated by *hatched boxes*. The previously reported region of *CHRNA3* (Rempel et al. 1998) is in light gray. *Thick arrows* represent Alu sequences. A3ex6F and A3ex6R primers used to amplify the 3′ region of *CHRNA3* are indicated

extends for an additional 3.2 kb at its 3′ end and *CHRNA5* and *CHRNA3* were previously reported to be divergently transcribed (Flora et al. 2000a), the existence of a possible overlap between them was explored. To this purpose, an alignment of the 3′ regions of the two genes was performed. This analysis identified an overlapping region of 466 bp, corresponding to the whole *CHRNA5* exon 6 together with 37 bp of the 3′ end of intron 5 on one strand and to the entire *CHRNA3* exon 8 plus 329 bp of intron 7 on the opposite strand. A schematic representation of the *CHRNA5* and *CHRNA3* 3′ ends together with the sequence of the overlapping region is shown in Fig. 3. In order to confirm the existence of a partial tail-to-tail overlap between *CHRNA5* and *CHRNA3*, a PCR assay on genomic DNA using two sense primers located in the 3′ region of intron 5 of both genes was also performed. A PCR product of the expected size (about 3.3 kb) was obtained. The cDNAs of *CHRNA5* and *CHRNA3* share a 100 bp-region (corresponding to amino acids 416–448, including the first nucleotide of codon 449) of *CHRNA5* and the part immediately upstream of the polyA signal in the 3′UTR of *CHRNA3*.

In order to verify whether the genomic arrangement of *CHRNA5* and *CHRNA3* was conserved in other mammalian species, a PCR reaction on rat genomic DNA using rat-specific sense primers located in exon 6 of both genes was performed. A fragment of about 6 kb was obtained and partially sequenced at both ends. This analysis showed that the 3′UTRs of the cDNAs of *CHRNA5* (1435 bp, GenBank accession number NM_017078) and *CHRNA3* (282 bp, GenBank accession number L31621) are uninterrupted by introns, demonstrating that the two genes are not overlapped in rats. No complementarity between the two sequences was detected.

Discussion

In recent years, neuronal nAChRs have been involved in the pathogenesis of or identified as potential therapeutic targets in several neurological disorders such as idiopathic epilepsies (for a review, see Scheffer and Berkovic 2000), Alzheimer disease (for a review, see James and Nordberg 1995), Parkinson disease (Aubert et al. 1992; Schroder et al. 1995), and schizophrenia (Freedman et al. 1997). In particular, nAChR genes *CHRNA4* and *CHRNB2* have been reported to be involved in the pathogenesis of ADNFLE (Scheffer and Berkovic 2000; De Fusco et al. 2000; Phillips et al. 2001). Precise information on the genomic organization of human neuronal nAChR subunit genes as well as on the primers and conditions for PCR amplifications from genomic DNA are prerequisites for mutational screenings. Here we report detailed data on the genomic organization of *CHRNA5/A3/B4*, clustered on chromosome 15q24. Only the structure of *CHRNA3* was previously reported, although its exact genomic size had not been evaluated (Rempel et al. 1998). The exon-intron structures of *CHRNA5* and *CHRNB4* were identified and shown to be characterized by the presence of six exons and five introns, as observed for other nAChR coding genes (e.g., *CHRNA4* and *CHRNB2*) (Steinlein et al. 1996; Rempel et al. 1998). Moreover, partial sequences of the 5′ and 3′ ends of each intron were determined for both genes. During the final stage of this work, a large (about 179.4 kb) genomic clone (RP11-335K5) containing the *CHRNA5/A3/B4* cluster was deposited in GenBank (accession number AC067863). It was a draft sequence fragmented in 28 uncomplete and unordered pieces, which were reduced to ten in a very recent revision of the same clone. Alignments of the nucleotide sequences of the *CHRNA5* and *CHRNB4* intronic regions reported in the present paper with the above mentioned RP11-335K5 clone revealed a perfect identity. Moreover, three small undetermined regions in AC067863, located in *CHRNA5* introns 2 and 5, were resolved by our group as poly-thymidine stretches.

Knowledge of the exon-intron structure as well as of the splicing junctions made it possible to perform a comprehensive mutational screening in five unrelated ADNFLE patients belonging to families for which segregation analysis excluded linkage to *CHRNA4* and *CHRNB2* and for which no clear exclusion of association to the 15q24 region could be demonstrated by haplotype analysis. No mutations responsible for ADNFLE were found, indicating that these genes may not be responsible for this syndrome in the analyzed families. Nevertheless, given the genetic heterogeneity of ADNFLE (Oldani et al. 1998; Phillips et al. 1998), a

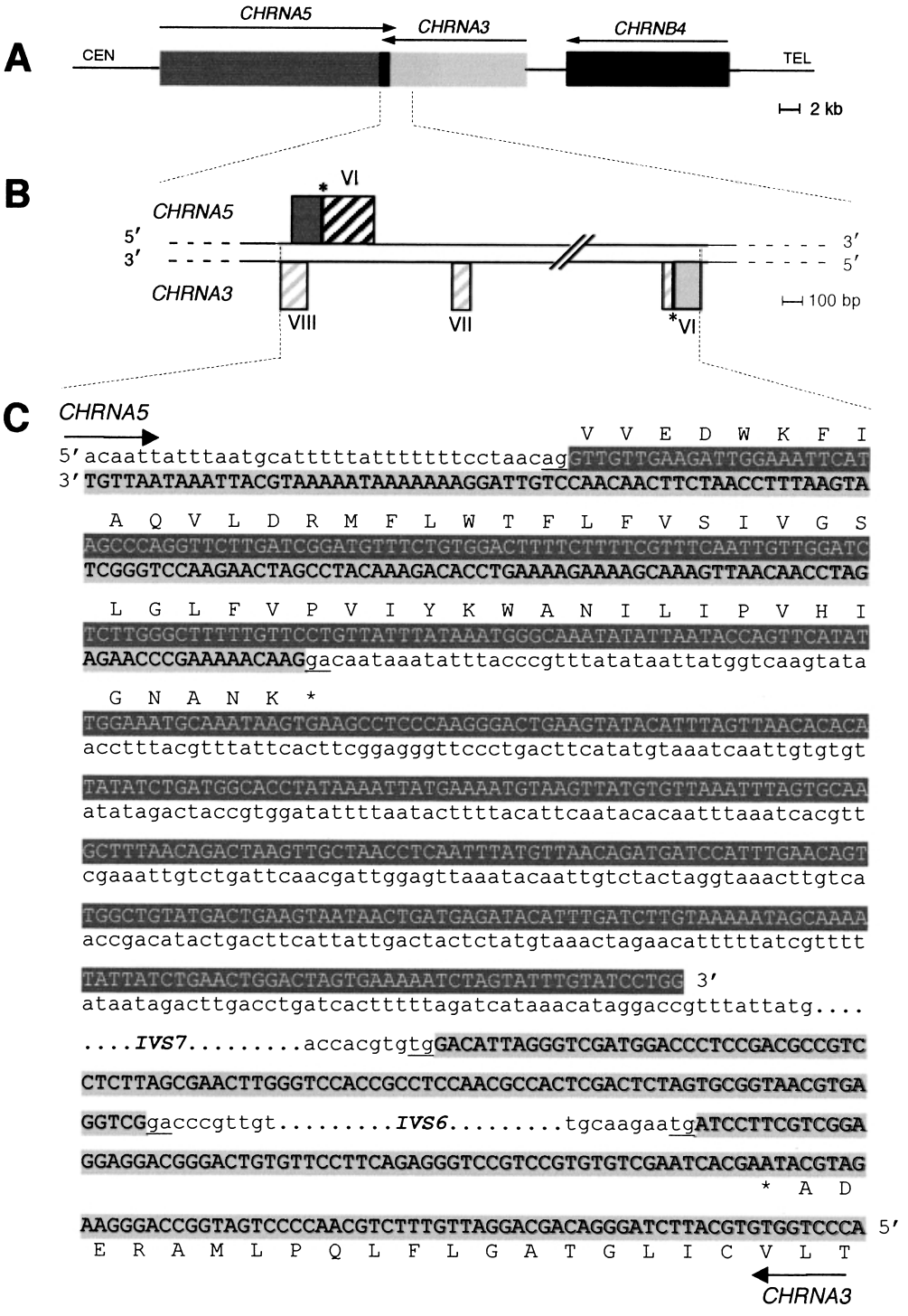

Fig. 3A–C. Genomic structure of the *CHRNA5/A3/B4* gene cluster showing the tail-to-tail overlap between *CHRNA5* and *CHRNA3*. **A** Schematic representation of the genomic arrangement of the *CHRNA5/A3/B4* gene cluster. *Arrows* indicate the direction of transcription. *CEN*, centromere; *TEL*, telomere. **B** Exon-intron structure of *CHRNA5* and *CHRNA3* 3′ regions showing their tail-to-tail overlap. *Boxes* indicate exons; *horizontal lines* represent introns; the 3′ UTRs are denoted by *hatched boxes*. *Asterisks* indicate the stop codons. **C** Nucleotide and amino acid sequences of *CHRNA5* (upper strand, exon 6) and of *CHRNA3* (lower strand, exons 6 from residue 452 to exon 8). The overlap region comprises 466 bp. Exonic sequences are *highlighted* in *light* (*CHRNA3*) and *dark gray* (*CHRNA5*). Intronic sequences are in *lowercase letters*. Consensus splice sites are *underlined*. *Asterisks* indicate the stop codons. *IVS*, intervening sequence

larger patient cohort should be analyzed in order to exclude these genes as candidates for this disease.

Four novel polymorphisms (three SNPs and one STR) were identified in the *CHRNA5/A3/B4* cluster. All were located in intronic regions. Each clustered gene is marked by at least one polymorphism. Frequencies of the identified polymorphisms were all fairly high with heterozygosity values ranging from 0.3942 to 0.7020. They therefore represent suitable markers for future association and linkage analyses to these genes.

Amplification of the *CHRNA3* 3′UTR identified two hitherto unnoticed introns, interrupting the 3′UTR. *CHRNA3* therefore displays a genomic structure (eight exons and seven introns) peculiar among the nAChR gene family. Exon 7 of *CHRNA3* is spliced out within an antisense Alu repeat which, on the basis of the nucleotides found at diagnostic positions shared by subfamily members, belongs to the Alu-Sp subfamily (Batzer et al. 1996). It is interesting to note that an antisense Alu repeat located within *CHRNA3* intron 5 was previously demonstrated to

be inserted in the *CHRNA3* mRNA by alternative splicing, giving rise to exon 5a (Mihovilovic et al. 1993). This transcriptional variant carries an in-frame stop codon and is expressed at low levels in tissues expressing the normal α3 transcript. An alignment of the newly identified Alu-Sp sequence, located across exon 7 of *CHRNA3*, with that present in intron 5 of the same gene and belonging to the closely related Alu-Sx subfamily, showed an identity of 77%. Alu elements usually contain several potential splice sites (Ohshima and Gotoh 1987). Comparison of the two transcribed *CHRNA3* Alu sequences showed that in both cases the same donor splice site was used, while the acceptor sites were different, the exon 7 acceptor site being 25 nucleotides downstream from that used for the alternative splicing of exon 5a.

BLAST searches in the human expressed sequence tag (EST) database identified *CHRNA3* ESTs lacking exon 7, indicating the occurrence of an alternative splicing of *CHRNA3* exon 7.

CHRNA3 and *CHRNA5*, which are encoded on opposite DNA strands, were shown for the first time to share a 466 bp-region at their 3′ ends. This tail-to-tail overlap produces, at the mRNA level, a 100 bp-region of complementarity between *CHRNA5* and *CHRNA3* mature transcripts. Several pairs of genes are arranged in a similar (tail-to-tail) overlapping manner (Williams and Fried 1986; Tee et al. 1995; Svaren et al. 1997; Quesada et al. 1999; Shintani et al. 1999; Ubeda et al. 1999). For some of these gene couples, such as Stat6/Nab2 (Svaren et al. 1997), CHOP/MetRS (Ubeda et al. 1999), and TC3/T5B (Williams and Fried 1986), the region of overlap is confined to a small region (about 50–100 bp) in the 3′UTR. This region contains an AUUUA sequence motif (ARE, AU-rich element), which represents a destabilizing sequence involved in the control of mRNA stability (Savant-Bhonsale and Cleveland 1992). It has been suggested that the in vivo formation of RNA–RNA duplexes involving ARE sequences could increase mRNA stability (Ubeda et al. 1999). Sequence analysis of the 100 bp-overlapping region between the *CHRNA5* and *CHRNA3* mRNAs did not evidence the presence of any ARE sequence motif.

Interestingly, *CHRNA3* is partially coexpressed with *CHRNA5* in human tissues (Flora et al. 2000b). In the human brain, areas of maximal expression of both subunits were located in the cerebellum and thalamus. In the rat brain, no *CHRNA5* expression was detected in the cerebellum, thalamus, and amygdala (Boulter et al. 1990; Wada et al. 1990); moreover, differences in the expression pattern of the α3 subunit between rat and primates have also been reported (Cimino et al. 1992). In this paper, the absence of a gene overlap between *CHRNA5* and *CHRNA3* in the rat genome was demonstrated. The presence of species-specific differences in the regional expression of neuroreceptors is consistent with the different genomic organization of the *CHRNA5/A3/B4* cluster between humans and rats.

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References

Antonarakis SE (1998) Recommendations for a nomenclature system for human gene mutations. Hum Mutat 11:1–3

Aubert I, Araujo DM, Cecyre D, Robitaille Y, Gauthier S, Quirion R (1992) Comparative alterations of nicotinic and muscarinic binding sites in Alzheimer's and Parkinson's diseases. J Neurochem 58:529–541

Batzer MA, Deininger PL, Hellmann-Blumberg U, Jurka J, Labuda D, Rubin CM, Schmid CW, Zietkiewicz E, Zuckerkandl E (1996) Standardized nomenclature for Alu repeats. J Mol Evol 42:3–6

Boulter J, O'Shea-Greenfield A, Duvoisin RM, Connolly JG, Wada E, Jensen A, Gardner PD, Ballivet M, Deneris ES, McKinnon D, Heinemann S, Patrick J (1990) Alpha 3, alpha 5, and beta 4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. J Biol Chem 265:4472–4482

Cimino M, Marini P, Fornasari D, Cattabeni F, Clementi F (1992) Distribution of nicotinic receptors in cynomolgus monkey brain and ganglia: localization of alpha 3 subunit mRNA, alpha-bungarotoxin and nicotine binding sites. Neuroscience 51:77–86

Clementi F, Fornasari D, Gotti C (2000) Neuronal nicotinic receptors, important new players in brain function. Eur J Pharmacol 393:3–10

De Fusco M, Becchetti A, Patrignani A, Annesi G, Gambardella A, Quattrone A, Ballabio A, Wanke E, Casari G (2000) The nicotinic receptor β2 subunit is mutant in nocturnal frontal lobe epilepsy. Nat Genet 26:275–276

Flora A, Schulz R, Benfante R, Battaglioli E, Terzano S, Clementi F, Fornasari D (2000a) Neuronal and extraneuronal expression and regulation of the human alpha5 nicotinic receptor subunit gene. J Neurochem 75:18–27

Flora A, Schulz R, Benfante R, Battaglioli E, Terzano S, Clementi F, Fornasari D (2000b) Transcriptional regulation of the human alpha5 nicotinic receptor subunit gene in neuronal and non-neuronal tissues. Eur J Pharmacol 393:85–95

Fornasari D, Battaglioli E, Flora A, Terzano S, Clementi F (1997) Structural and functional characterization of the human alpha3 nicotinic subunit gene promoter. Mol Pharmacol 51:250–261

Freedman R, Coon H, Myles-Worsley M, Orr-Urtreger A, Olincy A, Davis A, Polymeropoulos M, Holik J, Hopkins J, Hoff M, Rosenthal J, Waldo MC, Reimherr F, Wender P, Yaw J, Young DA, Breese CR, Adams C, Patterson D, Adler LE, Kruglyak L, Leonard S, Byerley W (1997) Linkage of a neurophysiological deficit in schizophrenia to a chromosome 15 locus. Proc Natl Acad Sci U S A 94:587–592

Gault J, Robinson M, Berger R, Drebing C, Logel J, Hopkins J, Moore T, Jacobs S, Meriwether J, Choi MJ, Kim EJ, Walton K, Buiting K, Davis A, Breese C, Freedman R, Leonard S (1998) Genomic organization and partial duplication of the human alpha7 neuronal nicotinic acetylcholine receptor gene (CHRNA7). Genomics 52:173–185

James JR, Nordberg A (1995) Genetic and environmental aspects of the role of nicotinic receptors in neurodegenerative disorders: emphasis on Alzheimer's disease and Parkinson's disease. Behav Genet 25:149–159

Le Novere N, Changeux JP (1995) Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. J Mol Evol 40:155–172

Lustig LR, Peng H, Hiel H, Yamamoto T, Fuchs PA (2001) Molecular cloning and mapping of the human nicotinic acetylcholine receptor alpha10 (CHRNA10). Genomics 73:272–283

McGehee DS (1999) Molecular diversity of neuronal nicotinic acetylcholine receptors. Ann N Y Acad Sci 868:565–577

Mihovilovic M, Roses AD (1991) Expression of mRNAs in human thymus coding for the alpha 3 subunit of a neuronal acetylcholine receptor. Exp Neurol 111:175–180

Mihovilovic M, Mai Y, Herbstreith M, Rubboli F, Tarroni P, Clementi F, Roses A (1993) Splicing of an anti-sense Alu sequence generates a coding sequence variant for the alpha-3 subunit of a neuronal acetylcholine receptor. Biochem Biophys Res Commun 197:137–144

Ohshima Y, Gotoh Y (1987) Signals for the selection of a splice site in pre-mRNA. Computer analysis of splice junction sequences and like sequences. J Mol Biol 195:247–250

Oldani A, Zucconi M, Asselta R, Modugno M, Bonati MT, Dalprà L, Malcovati M, Tenchini ML, Smirne S, Ferini-Strambi L (1998) Autosomal dominant nocturnal frontal lobe epilepsy. A video-polysomnographic and genetic appraisal of 40 patients and delineation of the epileptic syndrome. Brain 121:205–223

Phillips HA, Scheffer IE, Crossland KM, Bhatia KP, Fish DR, Marsden CD, Howell SJ, Stephenson JB, Tolmie J, Plazzi G, Eeg-Olofsson O, Singh R, Lopes-Cendes I, Andermann E, Andermann F, Berkovic SF, Mulley JC (1998) Autosomal dominant nocturnal frontal-lobe epilepsy: genetic heterogeneity and evidence for a second locus at 15q24. Am J Hum Genet 63:1108–1116

Phillips HA, Favre I, Kirkpatrick M, Zuberi SM, Goudie D, Heron SE, Scheffer IE, Sutherland GR, Berkovic SF, Bertrand D, Mulley JC (2001) CHRNB2 is the second acetylcholine receptor subunit associated with autosomal dominant nocturnal frontal lobe epilepsy. Am J Hum Genet 68:225–231

Quesada V, Ponce MR, Micol JL (1999) OTC and AUL1, two convergent and overlapping genes in the nuclear genome of *Arabidopsis thaliana*. FEBS Lett 461:101–106

Rempel N, Heyers S, Engels H, Sleegers E, Steinlein OK (1998) The structures of the human neuronal nicotinic acetylcholine receptor beta2- and alpha3-subunit genes (CHRNB2 and CHRNA3). Hum Genet 103:645–653

Savant-Bhonsale S, Cleveland DW (1992) Evidence for instability of mRNAs containing AUUUA motifs mediated through translation-dependent assembly of a > 20S degradation complex. Genes Dev 6:1927–1939

Scheffer IE (2000) Autosomal dominant nocturnal frontal lobe epilepsy. Epilepsia 41:1059–1060

Scheffer IE, Berkovic SF (2000) Genetics of the epilepsies. Curr Opin Pediatr 12:536–542

Schroder H, de Vos RA, Jansen EN, Birtsch C, Wevers A, Lobron C, Nowacki S, Schroder R, Maelicke A (1995) Gene expression of the nicotinic acetylcholine receptor alpha 4 subunit in the frontal cortex in Parkinson's disease patients. Neurosci Lett 187:173–176

Shapiro MB, Senapathy P (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res 15:7155–7174

Shintani S, O'hUigin C, Toyosawa S, Michalova V, Klein J (1999) Origin of gene overlap: the case of TCP1 and ACAT2. Genetics 152:743–754

Steinlein O, Weiland S, Stoodt J, Propping P (1996) Exon–intron structure of the human neuronal nicotinic acetylcholine receptor alpha 4 subunit (CHRNA4). Genomics 32:289–294

Svaren J, Apel ED, Simburger KS, Jenkins NA, Gilbert DJ, Copeland NA, Milbrandt J (1997) The Nab2 and Stat6 genes share a common transcription termination region. Genomics 41:33–39

Tee MK, Thomson AA, Bristow J, Miller WL (1995) Sequences promoting the transcription of the human XA gene overlapping P450c21A correctly predict the presence of a novel, adrenal-specific, truncated form of tenascin-X. Genomics 28:171–178

Tenchini ML, Duga S, Bonati MT, Asselta R, Oldani A, Zucconi M, Malcovati M, Dalprà L, Ferini-Strambi L (1999) SER252PHE and 776INS3 mutations in the CHRNA4 gene are rare in the Italian ADNFLE population. Sleep 22:637–639

Ubeda M, Schmitt-Ney M, Ferrer J, Habener JF (1999) CHOP/GADD153 and methionyl-tRNA synthetase (MetRS) genes overlap in a conserved region that controls mRNA stability. Biochem Biophys Res Commun 262:31–38

Wada E, McKinnon D, Heinemann S, Patrick J, Swanson LW (1990) The distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (alpha 5) in the rat central nervous system. Brain Res 526:45–53

Williams T, Fried M (1986) A mouse locus at which transcription from both DNA strands produces mRNAs complementary at their 3′ ends. Nature 322:275–279