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In vivo RNA–RNA duplexes from human α3 and α5 nicotinic receptor subunit mRNAs

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Abstract

Natural antisense transcripts, because of their potential to form double-stranded RNA (dsRNA) molecules, recently emerged as a mechanism acting on eukaryotic gene regulation at multiple levels. *CHRNA3* and *CHRNA5*, coding for α3 and α5 subunits of the neuronal nicotinic acetylcholine receptor, have been reported to overlap at their 3′ends in human and bovine genomes. In the present paper, four *CHRNA3* and three *CHRNA5* human transcripts were characterised, leading to the identification of different antisense complementary regions. Since the two genes are coexpressed in some neuronal and non-neuronal tissues, we ventured on the in vivo identification of RNA–RNA duplexes in both humans and cattle. Using an RNase protection-based approach, *CHRNA3*/*CHRNA5* duplexes were detected in human neuroblastoma SY5Y cells, but not in bovine cerebellum. A semi-quantitative analysis of overlapping transcript levels was performed by real-time RT-PCR. Possible consequences of sense-antisense interaction are discussed.
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Keywords: Natural antisense transcripts (NATs); Neuronal nicotinic acetylcholine receptor (nAChR); Double-stranded RNA; Tail-to-tail overlap; Alternative transcripts

1. Introduction

Overlapping genes frequently occur in viral and prokaryotic genomes as well as in mitochondrial DNA, where a dimensional constraint acts on genome size, contributing to the maximisation of the information content of nucleotide sequences (Wagner and Simons, 1994). Gene overlap was therefore considered extremely rare in higher eukaryotes. However, the presence of a surprisingly high number of overlapping genes (more than 8% of all genes) is now emerging in both human and mouse genomes (Yelin et al., 2003; Kiyosawa et al., 2003). The interest on overlapping genes is mainly focused on the gene pairs transcribed from opposite DNA strands, because of their potential to originate natural antisense transcripts (NATs), which can form perfect RNA–RNA duplexes (Kumar and Carmichael, 1998; Vanhee-Brossollet and Vaquero, 1998). Despite the wide range of applications of artificial antisense constructs in basic and clinical research, little is known on the functional role of NATs in regulating eukaryotic gene expression. Moreover, no generalisation concerning the mechanism of action can be drawn based on the few gene pairs that have been experimentally examined. In fact, sense–antisense duplexes

Abbreviations: bp, base pair(s); kb, kilobase(s); *CHRNA3*, cholinergic receptor nicotinic alpha subunit 3; *CHRNA5*, cholinergic receptor nicotinic alpha subunit 5; *CHRNB4*, cholinergic receptor nicotinic beta subunit 4; dsRNA, double-stranded RNA; nAChR, nicotinic acetylcholine receptor; NATs, natural antisense transcripts; UTR, untranslated region; cDNA, DNA, complementary to RNA; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase polymerase chain reaction; RPA, RNase protection assay(s); CNS, central nervous system; EST, expressed sequence tag; ARE, AU-rich element; ADAR, adenosine deaminase that acts on RNA; u, unit(s); dNTP, deoxyribonucleoside triphosphate; DNase, deoxyribonuclease; RNase, ribonuclease; SDS, sodium dodecyl sulfate.

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have been demonstrated to play a role in the control of gene expression at multiple levels, including genomic imprinting, RNA interference, translational regulation, alternative splicing, X-inactivation, RNA editing, and mRNA stability (Kumar and Carmichael, 1998; Vanhee-Brossollet and Vaquero, 1998; Ubeda et al., 1999).

Human *CHRNA3* and *CHRNA5* have been recently reported to partially overlap in a tail-to-tail configuration, sharing their 3′ ends (Duga et al., 2001). The two genes, which are encoded on opposite DNA strands and transcribed in convergent directions, are clustered on chromosome 15q24 together with *CHRNB4* (Raimondi et al., 1992). This gene cluster has been described in chicken, rodent, and bovine genomes (Couturier et al., 1990; Boulter et al., 1990; Campos-Caro et al., 1997), but the overlap has been demonstrated only for the human and bovine *CHRNA3/CHRNA5* pairs (Duga et al., 2001; Campos-Caro et al., 1997). The gene products of *CHRNA3* and *CHRNA5*, the α3 and α5 subunits of the neuronal nicotinic acetylcholine receptor (nAChR), can co-assemble, together with three β subunits (β2 or β4), to form pentameric ligand-gated ion channels (Dani, 2001).

In humans, nine alpha (α2–α10) and three beta (β2–β4) subunits are expressed in the peripheral and central nervous system (CNS). They are assembled in different combinations to give rise to a variety of receptor subtypes with specific functional and pharmacological properties (Dani, 2001). The resulting high plasticity of nAChRs accounts for the wide range of functions driven or modulated by the cholinergic activity, and reflects the variety of neurological diseases related to nAChRs dysfunction, including autosomal dominant nocturnal frontal lobe epilepsy, schizophrenia, Alzheimer's disease, and Parkinson's disease (Clementi et al., 2000).

Human *CHRNA3* and *CHRNA5* are candidates to give rise to RNA–RNA duplexes, owing to their coexpression in some areas of the CNS, in autonomic ganglia, and in non-excitable cells, including adrenomedullary chromaffin cells, thymocytes, and bronchial epithelial cells (Flora et al., 2000; Sharma and Vijayaraghavan, 2002). Since several *CHRNA3* and *CHRNA5* transcripts have been reported (Flora et al., 2000; Battaglioli et al., 1998), in the present paper we tackled a fine characterisation of human alternative transcripts, in order to identify regions of potential sense–antisense interaction. RNase protection assays (RPAs) were used to detect in vivo RNA–RNA duplexes in both human and bovine cells. Furthermore, levels of overlapping transcripts were determined by real-time RT-PCR both in humans and in cattle. Finally, some possible functional consequences of this overlap were discussed.

2. Materials and methods

2.1. Cell culture

The SY5Y human neuroblastoma cell line was cultured in RPMI 1640 medium, supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics (100 u/ml penicillin and 100 μg/ml streptomycin), according to standard procedures. Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

2.2. 3′ Rapid amplification of cDNA end (3′RACE)

3′RACEs were performed essentially as described by Simonic et al. (1997). Total RNA was extracted from SY5Y cells, using the guanidine-isothiocyanate method. An oligo(dT) extended by an anchor sequence to the 5′ end (AdT) and Enhanced Avian RT-PCR Kit (Sigma, St. Louis, MO, USA) were used to perform first-strand cDNA synthesis, starting from 1 μg of total RNA.

CHRNA3 and *CHRNA5* 3′ ends were PCR-amplified from cDNA, using specific forward primers (f1 primers in Table 1) coupled to a single 3′ primer (FR), annealing to the anchor sequence. RT-PCR reactions were performed in a 25 μl volume containing 2.5 μl of the reverse transcription reaction, 0.75 u BIOTAQ DNA Polymerase (Bioline, London, UK), 1X PCR buffer [67 mM Tris–HCl pH 8.8, 16 mM $(NH_4)_2SO_4$, and 0.01% Tween-20], 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.4 μM of each primer. Samples were subjected to 40 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s, and elongation at 72 °C for 30–120 s preceded by 5 min denaturation at 95 °C and followed by 10 min elongation at 72 °C in a PTC-100 thermal cycler (MJ-Research, Watertown, MA, USA). Subsequently, additional semi-nested PCRs, using specific nested forward primers (f2 primers in Table 1) and the FR primer, were performed, using the same conditions as above and annealing temperatures comprised between 48 and 51 °C for 33–35 cycles.

Table 1
Forward primers used for *CHRNA3* and *CHRNA5* 3′RACEs

Primer name[a]	Sequence (5′→3′)	Localisation[b]
CHRNA3		
ABCD-f1	CAAAGAGATTCAAGATGATTGG	Across exons 5 and 6, CDS
A-f2	TGCTTCCTAGCTGGAGTG	Across exons 6a and 7, 3′UTR
B-f2	CTTCCTAGAACAAAAAGCC	Across exons 6a and 8, 3′UTR
C-f2	GATTAGTATGCTATGCTATGG	Exon 6b, 3′UTR
D-f2	AGTAATGGTAGTATAAGAGGG	Exon 6b, 3′UTR
CHRNA5		
EFG-f1	TGATGTCCGTGAGGTTGTT	Across exons 5 and 6, CDS
E-f2	GAGGTTGTTGAAGATTGG	Exon 6, CDS
F-f2	ATAATCCACAGTAAAGTTCATCC	Exon 6, 3′UTR
G-f2	TAACCTATCTAAGTTCAAGCC	Exon 6, 3′UTR

[a] Primers are named (from A to G) according to the amplified transcript; numbers refer to the first PCR (1) or to the semi-nested one (2).
[b] CDS, coding sequence; UTR, untranslated region.

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