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Transcriptional regulation of the human α5 nicotinic receptor subunit gene in neuronal and non-neuronal tissues

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Abstract

The human α5 nicotinic receptor subunit gene appears to be expressed in several structures of the nervous system, but also in a number of non-neuronal tissues, with maximal expressions occurring in the entire gastrointestinal tract, thymus and testis. To understand whether specific transcriptional mechanisms are involved in the tissue-specific expression of the α5 subunit in neuronal and non-neuronal cells, we isolated the 5′-regulatory region of the human gene and characterized its functional properties. We demonstrate that specific DNA elements, with positive or negative activities depending on the cell type, are responsible for the diversified expression of the α5 subunit in different tissues. We therefore conclude that the expression of the α5 subunit relies on a highly complex promoter that uses distinct regulatory elements to comply with the different functional and developmental requirements of the various tissues and organs.

Keywords: Nicotinic receptor; Neuron, human; Gene; Transcriptional regulation; Promoter

1. Introduction

Neuronal nicotinic receptors form a family of acetylcholine-gated cation channels that are expressed in the autonomic and sensory ganglia, the adrenal medulla and distinct areas of the Central Nervous System (CNS). Eleven distinct neuronal nicotinic subunits have so far been cloned and classified into two subfamilies of eight α (α2–α9) and three β subunits (β2–β4). In heterologous expression systems, α7, α8 and α9 are the subunits that can form homomeric receptors, whereas α2, α3, α4 and α6 subunits always need to be co-expressed with β2 or β4 subunits in order to generate functional channels (Gotti et al., 1997; Changeux et al., 1998, for reviews). The α5 subunit has long been considered ''orphan'' since it is unable to form functional receptors when expressed in paired subunit combinations. Indeed α5 can only be assembled with other functional α and β combinations (Conroy et al., 1992; Groot-Kormelink et al., 1998). α5 is expressed in the autonomic ganglia and in some areas of the CNS. In the autonomic ganglia it assembles with α3 in combination with β2, β4 or even both β subunits (Conroy and Berg, 1995); in the CNS, α5 can assemble with α4/β2 and α3/β2 receptor subtypes (Conroy and Berg 1998). The co-assembly of α5 has profound effects on the functional properties of the receptors: it increases their desensitization rate and Ca^{2+} permeability and modifies their sensitivity to some agonists (Ramirez-LaTorre et al., 1996; Kuryatov et al., 1997; Gerzanich et al., 1998). Besides being found in the nervous system, the α5 transcript has also been detected in a large variety of non-neuronal cell lines (Chini et al., 1992). The significance of this expression is uncertain, since no evidence of a correspondence with normal tissues has been found, which raised the suspicion that the presence of the α5 transcript in these cell lines could be aberrant. The present study shows, for the first time, that α5 mRNA is expressed in several normal human adult and fetal tissues. In order to understand the genetic mechanisms controlling the expression of α5 in neuronal and non-neuronal cells, we analyzed the transcriptional properties of the 5′-flanking region of the

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gene. The results show that, although the α5 gene is transcribed in multiple tissues and corresponding cell lines, its promoter is not of the housekeeping type, but contains elements that manifest their crucial role only in a certain cell type. This highly diversified functional organization of the genomic regulatory region is likely to comply with the different spatio-temporal requirements of α5 gene expression in various neuronal and non-neuronal cell types. All of these findings increase the interest in a nicotinic subunit that may be involved in a number of still unknown functions.

2. Materials and methods

2.1. RNA preparation and analysis

Total RNA from different cell lines was isolated using RNAfast-II (Molecular Systems, San Diego, CA) according to the manufacturer's instructions. Briefly, $\sim 10^8$ cells were collected by means of centrifugation and lysed with a solution containing guanidine salts and phenol. The RNA was extracted with chloroform, purified on RNA-binding resin and quantified using a spectrophotometer. Ten micrograms of total RNA were fractionated in a 2.2 M formaldehyde/1% agarose gel as described by Sambrook et al. (1989), transferred to a nylon membrane (Biodyne A, Pall, Pall Europe Limited, England) by means of a Turboblotter (Schleicher and Schuell, Dassel, Germany), UV cross-linked, and pre-hybridized in 0.125 M Na_2HPO_4, 1 mM EDTA, 0.25 M NaCl, 7% Sodium Dodecyl Sulfate (SDS), 10% Polyethylene glycol (PEG), 1% Bovine Serum Albumin (BSA) for at least 2 h at 65°C. Hybridization was performed in the same solution containing 10^6 cpm/ml of a ^{32}P-labelled cDNA corresponding to the region encoding the cytoplasmic domain of the α5 subunit (nucleotides +1005/+1263 according to Chini et al., 1992) for at least 12 h. The membranes were initially washed for 5 min at room temperature in 3× Sodium Sodium Citrate (SSC) buffer plus 0.1% SDS, and then washed twice in 3× SSC/0.1% SDS and twice in 1× SSC/0.1% SDS at 65°C for 30 min each (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7). The filters were exposed to Hyperfilm MP (Amersham Life Science, UK) with intensifying screens at −80°C. After stripping, the same blots were re-hybridized to a human 18S cDNA probe (nucleotides 715–794; Ambion, Austin, TX, USA) in order to check the quality of the RNAs and to normalize the previously obtained signals. The autoradiographic films were then scanned and densitometric analyses were carried out using the NIH Image program.

The human Multiple Tissue Expression Array (Clontech Lab., Palo Alto, CA, USA) was hybridized with the same α5 cDNA probe as that used for Northern blots ($\sim 2 \times 10^6$ cpm/ml) following the manufacturer's instructions. The filter was washed four times for 20 min in 2× SSC/1% SDS at 65°C and twice for 20 min in 0.1× SSC/0.5% SDS at 50°C, and then exposed to Hyperfilm MP (Amersham Life Science) with intensifying screens at −80°C. The same blot was stripped and re-hybridized with the cDNA probes encoding the cytoplasmic domains of the human α3 (Fornasari et al., 1997) and β4 nicotinic subunits (Tarroni et al., 1992).

2.2. Construction of the human α5 promoter-luciferase fusion plasmids

The restriction and modification enzymes were purchased from New England Biolabs (New England Biolabs, Beverly, MA, USA) or Promega (Promega, Madison, WI, USA). All of the Polymerase Chain Reaction (PCR) experiments were carried out using Pfu DNA Polymerase (Promega), and all of the plasmids were purified using Qiagen columns (QIAGEN, CA, USA). DNA sequencing was carried out using a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science). The oligonucleotides were obtained from Gibco-BRL (Gibco-BRL, Life Technologies, UK). All of the plasmids used in the transfection experiments were generated by subcloning different parts of the 5′-regulatory region of the α5 human nicotinic subunit gene into the pGL3-basic plasmid (Promega), upstream of the reporter gene *firefly* luciferase. The *Xho*I–*Sac*II 6.6-kb fragment (Fig. 2) lacked 20 nucleotides of the 5′-UTR between the *Sac*II site and the start codon (Chini et al., 1992).

In order to reconstitute the whole region upstream of the start codon, we used two complementary oligonucleotides that contained the missing sequence 5′-GGTCCCG-CGCGGGCGCGGGGCC-3′ as the sense and 3′CGCCA-GGGCGCGCCCGCGCCCCGGTAC-5′ as the antisense oligonucleotide. Upon annealing, two hemi-restriction sites were generated at the 5′ end (a *Sac*II hemi-site) and at the 3′ end (a *Nco*I hemi-site) of the double-stranded oligonucleotide. In order to generate the *Nco*I hemi-site, it was necessary to introduce a point mutation at the nucleotide +180 where a G was substituted by a C. This mutation did not affect the consensus for translation initiation (Kozak, 1992) and allowed us to create chimeric constructs in which the α5 5′-UTR was entirely and directly fused to the *firefly* luciferase coding sequence (in the pGL3-basic plasmid, the start codon of the *firefly* luciferase reporter gene is embedded in a *Nco*I site). We eliminated the 5′-UTR of the *firefly* luciferase gene by digesting pGL3-basic with *Hin*dIII and *Nco*I and then we carried out a three-way ligation using the digested pGL3-basic plasmid, the double-stranded oligonucleotide described above and the *Hin*dIII–*Sac*II α5 fragment. In this way, we constructed the **−1011/+180** plasmid (Figs. 2 and 4A). The **−6400/+180** construct (Figs. 2 and 4A) was obtained

by digestion of −1011/+180 with *Hin*dIII and *Xho*I and subsequent ligation with the 5.4 kb *Xho*I /*Hin*dIII fragment. Since *Sac*I sites are contained in both the pGL3-basic polylinker and the α5 regulatory region, *Sac*I digestion was used to generate the **−2261/+180** construct (Figs. 2 and 4A) from the −6400/+180 plasmid. Following the removal of the 4.2-kb upstream fragment, the linear −2261/+180 construct was self-ligated.

The **−368/+180** (Figs. 2 and 4A) was obtained by digesting the −1011/+180 construct with *Hin*dIII and *Afl*II. Following the removal of the 0.65-kb upstream fragment, the linear −368/+180 construct was blunted by means of T4-DNA-polymerase and self-ligated. The **−240/+180** construct (Figs. 2 and 4A) was derived from the −1011/+180 construct by digestion with *Sma*I (which is in the polylinker of pGL3-basic) and *Stu*I, removal of the upstream segment and self-ligation. The −240/+53 and −240/−2 constructs represent 3′ deletions of −240/+180. In these plasmids the residual part of the region specifying the 5′-UTR was fused to the DNA region specifying the 5′-UTR of the *firefly* luciferase gene. More precisely, the **−240/+53** construct (Figs. 2 and 4A) was obtained by PCR amplification, using the −1011/+180 construct as the DNA template. The upper primer was 5′-GGAGATCTGCTGTTATCTTAAG-GTGTGCAGTG-3′, which extends from nucleotide −371 to −348 and contains a *Bgl*II site at the 5′ end (underlined). The lower primer was 5′-CCCAAGCTTCCGCTC-CGCGCCACAGC-3′, which extends from nucleotide +53 to +36 and contains a *Hin*dIII site at the 5′ end (underlined). Upon digestion, the PCR product was subcloned in the *Bgl*II and *Hin*dIII sites of pGL3-basic; the plasmid was then digested with *Stu*I and *Sma*I and self-ligated. The **−240/−2** construct (Figs. 2 and 4A) was obtained following the same strategy as that used to obtain −240/+53. Only the lower primer was different, namely, 5′-GCGAAGCTTGCGGGCTCGGGACGCATGTG-3′, which extends from nucleotide −2 to −21 and contains a *Hin*dIII site at the 5′ end (underlined). The **+32/+180** construct (Figs. 2 and 4A) was obtained by PCR amplification, using as a template −240/+180. The upper primer was 5′-CCCAAGCTTCGGGAGCTGTGGCGCGGAGC-3′, which extends from nucleotides +32 to +51 and contains a *Hin*dIII site at the 5′ end (underlined). The lower primer was 5′-CTTTATGTTTTTGGCGTCTTCC-3′, which was designed from nucleotides 24 to 2 of the *firefly* luciferase coding sequence (numbering from the start codon). The PCR product was digested by means of *Hin*dIII and *Nco*I and subcloned into the same sites of the pGL3-basic vector.

SV40 (Fig. 3) corresponds to the pGL3-promoter vector from Promega, in which the *firefly* luciferase reporter gene is under the control of the SV40 promoter. All of the constructs were checked by means of restriction analysis and partial sequencing, except for the PCR products whose sequences were completely determined.

2.3. Cell lines and cultures

The SY5Y and SK-N-BE human neuroblastoma cell lines were grown in RPMI 1640 medium, 10% fetal calf serum, 50 units/ml penicillin, 50 mg/ml streptomycin, and 2 mM L-glutamine. All of the other cell lines (IMR 32, HeLa and MOLT-4) were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown according to the supplier's instructions.

2.4. Transient transfections and Firefly and Renilla luciferase assays

All of the cell lines were transiently transfected by means of lipofection (DMRIE-C from Gibco-BRL). Briefly, 5×10^4–2×10^5 cells, depending on the line, were plated onto a well of a 6-MULTIWELL™ tissue culture plate (Falcon, Franklin Lakes, NJ, USA). The cells were grown for 48 h to a confluency of 70–75%. After two washings with the medium without serum and antibiotics, 1 ml of OPTIMEM I (Gibco-BRL) was added. Immediately afterwards, 100 μl of DNA/lipofectin mixture, containing 78 fmol of a certain construct and an equimolar amount of pRL-RSV, were added and incubated for 5 h at 37°C. The pRL-RSV plasmid (Battaglioli et al., 1998) expresses the *Renilla* luciferase reporter gene under the control of the Rous Sarcoma Virus (RSV) long terminal repeat promoter and was co-transfected in each sample in order to normalize transfection efficiency. The DNA/lipofectin mixtures were prepared as follows: for each construct to transfect, 2 μl of DMRIE-C were equilibrated with 50 μl of OPTI-MEM I (Gibco-BRL) for 20 min at RT. In parallel, appropriate amounts of the two plasmids were also resuspended in 50 μl of OPTIMEM I; then, the lipofectin and DNA preparations were mixed, incubated at RT for 40 min and added to the cells. After incubation with the DNA, the cells were then washed and incubated with 2 ml of complete medium for 36 h, after which the activities of the *Firefly* luciferase and *Renilla* luciferase were determined. *Firefly* luciferase and *Renilla* luciferase activities were detected using the Dual-Luciferase Reporter Assay System (Promega) as described elsewhere (Battaglioli et al., 1998). All of the transfections were performed in triplicate, and each construct was tested in at least three independent experiments using different batches of plasmid preparations.

2.5. Analysis of the transient transfection data

For each construct, the values of *Firefly* luciferase obtained in the different experiments (expressed in relative luminescent units) were plotted against the corresponding values of *Renilla* luciferase (also expressed in relative luminescent units). Linear correlations were obtained with

correlation coefficients ranging from 0.75 to 0.99. The transcriptional activity of each construct was defined as the slope of the straight line, and expressed as the fold increase over the transcriptional activity of the promoterless plasmid pGL3-basic, or as the percentage of the activity of the $-6400/+180$ construct.

3. Results

3.1. Expression profile of the human α5 subunit in normal tissues and cell lines

In order to evaluate the expression of α5 in human cell lines we carried out Northern blot analysis by hybridizing a cDNA probe (corresponding to the region encoding the α5 cytoplasmic loop) to total RNAs purified from two distinct human neuroblastoma cell lines (SY5Yand SK-N-BE), and from two non-neuronal cell lines (MOLT-4, a human T-lymphocyte cell line and HeLa, an epithelial cell line).

Five distinct bands were detected whose size ranged from 9.5 to 1.5 kb and which matched in the two neuroblastoma cell lines (Fig. 1). Transcripts encoding α5 were also detected in HeLa and MOLT4 cells, as expected, but the expression pattern was different since non-neuronal cell lines completely lacked the transcripts A and B.

In order to understand whether the expression of α5 in non-neuronal cell lines reflects a physiological condition that also occurs in some normal tissues, we analyzed the RNAs extracted from a number of human adult or fetal organs by means of dot blot hybridization with the same α5 cDNA probe (Table 1). It is worth noting that the amount of poly-A^+ loaded on each dot by the manufacturer was previously normalized to the expression of eight different housekeeping genes, so that the differences in the expression of a certain gene in different tissues can be considered semi-quantitative. In the nervous system, weak hybridization was observed throughout the different tested areas but much more robust signals were detected in the cerebellum and thalamus. The other anatomical district in which the expression of α5 was expected was the adrenal gland, which indeed showed a modestly intense spot. The most impressive extra-neuronal localization of α5 expression was the gastrointestinal tract (Table 1), with a peak in the duodenum. A similarly intense hybridization signal was only observed in the thymus, whereas spleen and peripheral blood leukocytes were completely devoid of the α5 transcript. Very modest signals were detected in lymph nodes and bone marrow, but the appendix showed a more consistent hybridization, although it is still unclear whether this was due to the lymphoid component of this anatomic structure. The thymus is thus probably the main site for α5 expression among the different primary and secondary lymphoid organs. A consistent signal for α5 was also detected in testis, prostate, and skeletal muscle (Table 1). Analysis of α5 expression in fetal tissues revealed interesting patterns of developmental regulation (Table 1). On the basis of these data, the different organs can be classified into those that express the α5 gene in fetal and adult life with apparently minimal modifications in the overall levels of the transcript, such as the heart and the brain; those in which α5 expression is down-regulated after birth, such as the kidney, the liver and the spleen; and those in which α5 expression increases in adult life, such as the lung and the thymus. The expression of α5, therefore, appears to be differentially regulated during development according to the organ and the cell type.

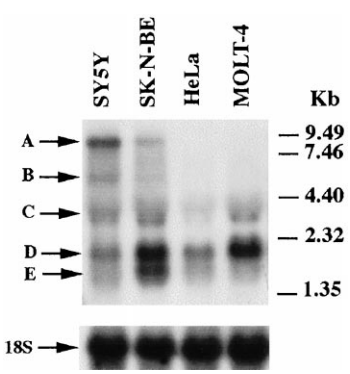

Fig. 1. Northern blot analysis of the expression of the human α5 gene in different human cell lines. A human cDNA corresponding to the region encoding the cytoplasmic domain of the α5 nicotinic subunit was hybridized to 10 μg of total RNA purified from the indicated cell lines. The cDNA identified the different transcripts that are indicated on the left by arrows and letters. The RNA molecular weight markers are indicated on the right. At the bottom, the hybridization signals obtained using a human cDNA for ribosomal RNA 18S on the same blot, after stripping of α5 probe.

Minor hybridization signals were detected in some exocrine and endocrine glands, in placenta, bladder, and the uterus. In order to investigate whether the widespread expression of α5 in neuronal and extra-neuronal human tissues was a peculiarity of this subunit, we studied the distribution of the mRNAs encoding two other nicotinic subunits, α3 and β4, which are often assembled with α5. We confirmed that α3 is expressed in some selected areas of the human CNS (Rubboli et al., 1994; Terzano et al., 1998), in the adrenal gland, and in the fetal and adult thymus (Table 1) (Mihovilovic et al., 1993). We also demonstrated, for the first time, that α3 mRNA is expressed in the entire gastrointestinal tract, even though to a lesser extent than α5. In all of these anatomical areas the two transcripts colocalized, but α3 was absent from all of the other tissues expressing α5. The expression of β4 was much more restricted than that of the other two subunits. Indeed, we could not detect any signal in the nervous system or in any other extra-neuronal tissue, with the exception of the thymus, the lymph nodes and the adrenal gland, as expected, and, surprisingly, the testis. Thus, according to these data, it seems that widespread expres-

Table 1
Multiple tissue array analyses of α5, α3 and β4 gene expression
Multiple Tissues Arrays (Clontech, CA) were hybridized with the human cDNAs encoding the cytoplasmic domain of the α5, α3 and β4 nicotinic subunits; scores were attributed as follows: +, low level; ++, intermediate level; +++, high level; ++++, very high level.

Nervous system	α5	α3	β4
Whole brain	+	−	−
Cerebral cortex	+	−	−
Frontal lobe	+	−	−
Parietal lobe	+	−	−
Occipital lobe	+	−	−
Temporal lobe	+	−	−
p.g. of cerebral cortex	+	−	−
Pons	+	−	−
Left cerebellum	++	++	−
Right cerebellum	++	++	−
Corpus callosum	+	+	−
Amygdala	+	+	−
Caudate nucleus	+	+	−
Hippocampus	+	−	−
Medulla oblongata	+	−	−
Putamen	+	−	−
Substantia nigra	+	−	−
Accumbens nuclei	+	−	−
Thalamus	++	++	−
Pituitary gland	+	+	−
Spinal cord	+	−	−
Foetal brain	+	−	−
Gastrointestinal tract			
Esophagus	++	+	−
Stomach	++	+	−
Duodenum	++++	+	−
Jejenum	+++	+	−
Ileum	+++	+	−
Ilocecum	++	+	−
Appendix	++	+	−
Ascending colon	++	+	−
Transverse colon	+++	+	−
Descending colon	+++	+	−
Rectum	++	+	−
Liver	−	−	−
Foetal liver	+	−	−
Cardiovascular system			
Heart	++	−	−
Aorta	+	−	−
Left atrium	++	−	−
Right atrium	++	−	−
Left ventricle	+	−	−
Right ventricle	++	−	−
Interventricular septum	++	−	−
Apex of the heart	++	−	−
Foetal heart	+	−	−
Glands			
Pancreas	+	−	−
Adrenal gland	+	+	+
Thyroid gland	+	−	−
Salivary gland	+	−	−
Mammary gland	+	−	−
Urogenital tract			
Bladder	+	−	−
Uterus	+	−	−
Urogenital tract			
Prostate	++	−	−
Testis	+++	−	+++
Ovary	+	−	−
Foetal kidney	++	−	−
Respiratory tract			
Trachea	+	−	−
Lung	++	−	−
Foetal lung	+	−	−
Lymphoid organs			
Spleen	−	−	−
Thymus	++++	++++	++
Peripheral blood leukocytes	−	−	−
Lymph node	+	−	++
Bone marrow	+	−	−
Fetal spleen	+	−	−
Foetal thymus	++	+++	−
Miscellanea			
Skeletal muscle	++	−	−
Placenta	+	−	−

sion in human neuronal and especially extra-neuronal tissues is not a feature common to all of the nicotinic subunits.

3.2. Functional characterization of the α5 5′-regulatory region

In order to understand the genetic mechanisms controlling the expression of α5 in neuronal and non-neuronal cells, we decided to isolate the 5′-regulatory region of the human α5 gene. To this end, we screened a human genomic library and isolated a 6.6 kb fragment that contained the region specifying the 5′-UTR of the α5 gene and the upstream regulatory region. The sequence and the structural features of the α5 5′-regulatory region will be published elsewhere. Briefly, the expression of the α5 gene was found to be driven by a multiple start site promoter with no CAAT and TATA boxes and containing several Sp1 sites, as depicted in Fig. 2. Remarkably, a set of four initiation sites was identified only in neuronal cells, downstream of the cluster of sites common to all of the tested cell lines. In order to confirm that the 6.6 kb fragment actually contained the α5 promoter and represented the 5′-regulatory region, we subcloned the 6.6 kb fragment into the pGL3-basic vector, upstream of the *Firefly* luciferase reporter gene, in order to generate the −6400/+180 construct (Figs. 2 and 4A), and performed transient transfections. The pRL-RSV plasmid, bearing the *Renilla* luciferase reporter gene under the control of the RSV promoter, was always co-transfected to correct for transfection efficiency. In order to obtain more precise quantitative information on the strength of this regulatory

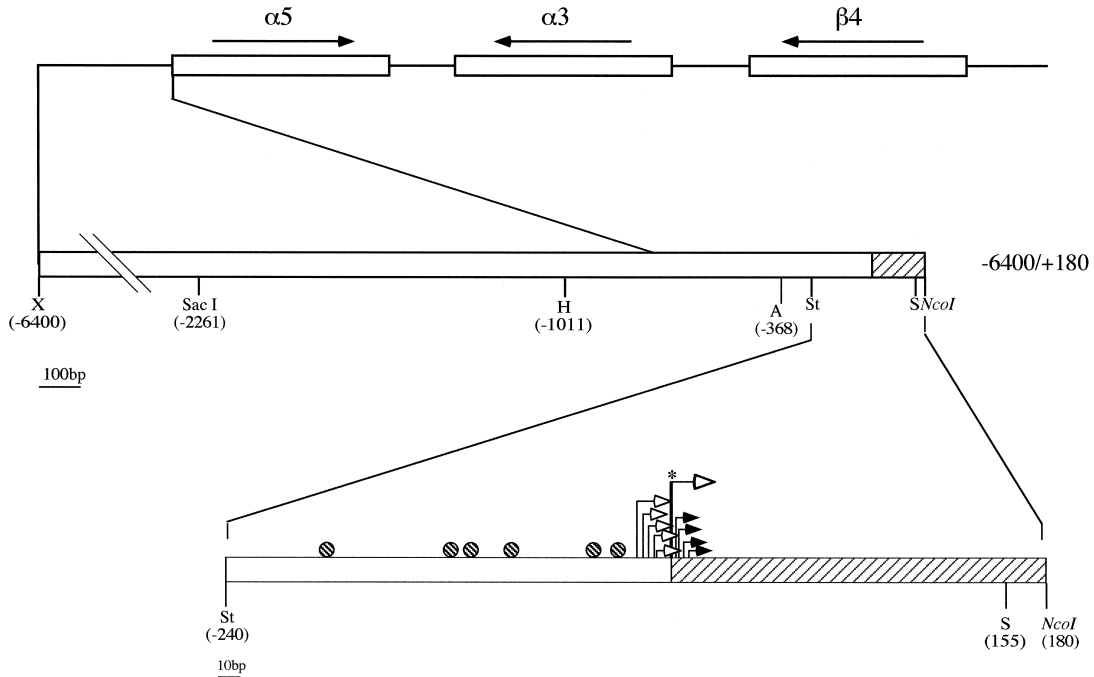

Fig. 2. Structural features of the genomic fragment corresponding to the 5′-regulatory region of the human α5 gene. Top: the human genomic cluster containing the three nicotinic subunit genes (α5, α3 and β4; Raimondi et al., 1992). The arrows indicate the direction of transcription; the first enlargement represents the 6.6-kb cloned region of the α5 gene (X, *Xho*I; H, *Hin*dIII; A, *Afl*II; S, *Sac*II; St, *Stu*I;); the striped region corresponds to the 5′-UTR specifying region of the α5 gene as revealed by primer extension and RNase protection analysis (not shown). The second enlargement represents the core promoter. The arrows identify the transcriptional start sites detected by primer extension and RNase protection assay; the white arrows represent the transcription start sites common to neuronal and non-neuronal cell lines, with the asterisk identifying the major transcription start site; the black arrows represent the neuro-specific transcription start sites. The shaded circles indicate the putative Sp1 binding sites. The relevant restriction sites used for subcloning are shown.

region in driving transcription, we performed parallel transfections with SV40, which contains the well-characterized SV40 promoter, and pGL3-basic, a promoterless vector providing the background of the experimental system. The three constructs were transfected in the four human cell lines indicated in Fig. 3.

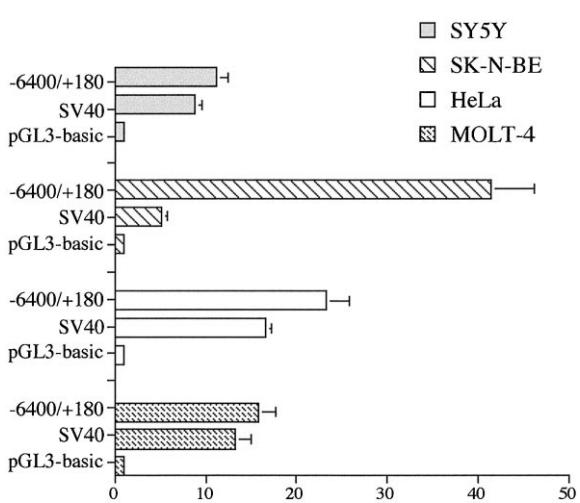

Fig. 3. Activity of the −6400/+180 α5 construct. The activity of the longest construct was analyzed in the four indicated cell lines and compared with that of pGL3-promoter and pGL3-basic plasmids. The bars indicate the transcriptional activities of the constructs, calculated as described in Materials and Methods. The values are given as fold increase over pGL3-basic activity. The standard deviation is indicated.

The −6400/+180 construct showed conspicuous transcriptional activity in all of the cell lines, but was particularly active in SK-N-BE neuroblastoma cells, in which the expression of the *firefly* luciferase reporter gene was 40 times that of background. In the other cell lines, the activity of −6400/+180 was comparable with that of SV40. These functional data proved that the 6.6-kb fragment represented at least part of the 5′-regulatory region of the α5 gene.

3.3. Molecular dissection of the 6.6-kb region and functional delineation of the α5 core promoter

In order to identify discrete DNA regions that could account for the transcriptional activity of the 6.6-kb region in different cell types, and to define the core promoter driving the expression of the α5 gene, we generated a set of 5′ and 3′ end-deleted constructs (Figs. 2 and 4A) that were analyzed by means of transient transfections in both neuronal and non-neuronal cell lines (Fig. 4B). A large deletion at the 5′ end that removed more than 4 kb led to a decrease in transcriptional activity in all of the cell lines,

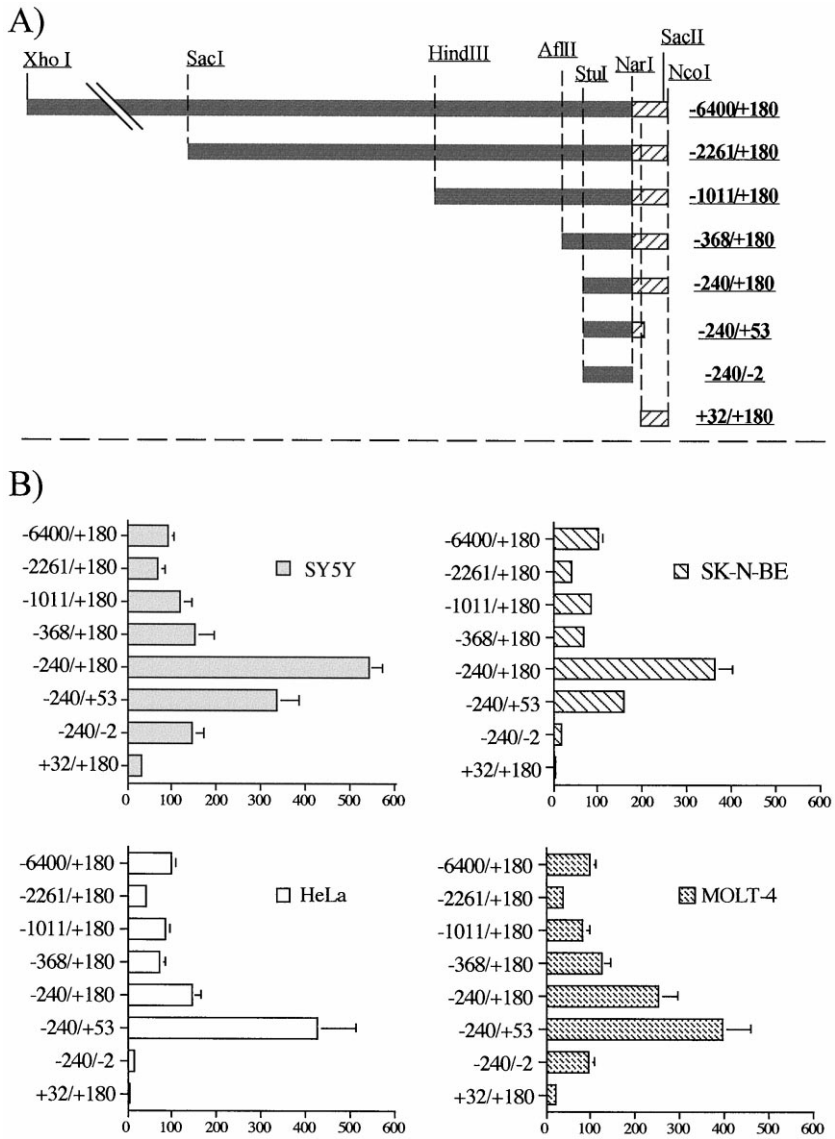

Fig. 4. Schematic representation and functional characterization of the α5 5′-regulatory region derived constructs. (A) The genomic regions cloned upstream of the *luciferase* gene to generate the constructs for the transfection assays; the related restriction sites are indicated. The numbers on the right represent the first and the last nucleotides on the genomic sequence of each fragment. The striped boxes indicate the 5′-UTR specifying region. (B) Functional characterisation of the α5 regulatory region in different human cell lines. The bars indicate the transcriptional activities of the constructs, calculated as described in Materials and Methods. The values are expressed as percentages of the −6400/+180 construct activity for each cell line. The standard deviation is indicated.

as demonstrated by comparison of the −2261/+180 construct with −6400/+180.

This decrease was only 25% in SY5Y cells, but was nearly 60% in the other three cell lines. A further deletion of approximately 1.2 kb led to the opposite effect: a doubling of transcriptional activity in all of the cell lines, made clear by transfections with the −1011/+180 construct. No relevant effects were observed in the four cell lines with the transfection of the −368/+180 construct, which represents a 5′ deletion of −1011/+180. In contrast, the removal of an additional 130 bp had a striking effect, especially in the neuronal cell lines: with the −240/+180 construct, the expression of the reporter gene in SY5Y and SK-N-BE was respectively 3.6 and 5.4 times higher than that obtained with the parental plasmid. A similar but less intense phenomenon was observed with the two non-neuronal cell lines, in which the expression of the reporter gene doubled. These data clearly indicated that a negative element, located in the 130 bp, down-regulated the downstream promoter, with preferential activity in neuronal cells. We also analyzed the contribution to the expression of the α5 gene of the 5′-UTR specifying region, by transfecting the four cell lines with the −240/+53 and −240/−2 constructs. The −240/+53 construct,

from which most of the 5′-UTR specifying region was deleted without affecting any transcription start site, produced opposite results in the neuronal and non-neuronal cell lines. The construct worked 40% and 60% less than the plasmid −240/+180 in SY5Y and SK-N-BE cells, respectively, thus indicating that the region between +54 and +180 had a positive effect on the expression of the reporter gene. In contrast, this region seemed to work as a negative element in the non-neuronal cell lines, since its removal led to an increase in the expression of the reporter gene. This increase was relatively modest in MOLT-4 cells (40%), but was three-fold in HeLa cells. We then analyzed the activity of the −240/−2 construct, a further 3′ end deletion from which the major and all of the neuron-specific transcription start sites were removed. Quite surprisingly, the effect of this deletion was very severe in HeLa cells, in which it caused a 25-fold reduction in the expression of the reporter gene, and this made the transcriptional activity of −240/−2 very similar to that of the promoterless plasmid pGL3-basic. The deletion of the −1/+53 fragment also had considerable consequences in SK-N-BE and MOLT-4 cells, with a reduction of approximately 9 and 4 times, respectively, in the expression of the *Firefly* luciferase. However, the −240/−2 construct still retained a good degree of transcriptional activity in MOLT-4 cells (approximately 15 times the activity of pGL-3 basic), and continued working efficaciously (18 times the activity of pGL-3 basic) in SY5Y, in which the effect of the deletion of the −1/+53 region was quite modest. Finally, we tested whether the part of the 5′-UTR specifying region that does not contain transcription start sites had any transcriptional activity itself by transfecting the +32/+180 construct. This construct produced the same luciferase activity as the pGL3-basic plasmid in HeLa and SK-N-BE cells, and it retained residual activity (approximately three times over the background) in SY5Y and MOLT-4 cells.

In summary, the −240/+53 construct, which only possesses the common transcription start sites and a short upstream region containing multiple putative Sp1 binding sites (Fig. 2), showed considerable transcriptional activity in all of the tested cell lines. The −240/+53 region therefore identifies the human α5 core promoter, although the strength of the promoter relies on different DNA elements, depending on the cell type.

4. Discussion

This paper describes the results of a study of the expression and transcriptional regulation of the human α5 nicotinic receptor subunit. We first analyzed the expression of the α5 gene in human cell lines and found that the two different human neuroblastoma cell lines considered expressed five distinct α5 transcripts. Interestingly, the analysis of α5 expression in two non-neuronal cell lines (HeLa and MOLT-4) suggested that two of these transcripts may be neuro-specific. The fact that the α5 gene is also expressed in non-neuronal cell lines has already been documented (Chini et al., 1992), and the extra-neuronal expression of α5 has also been described in normal non-neuronal tissues, such as the human thymus (Mihovilovic and Roses, 1993) and human keratinocytes (Grando et al., 1996). At these sites, α5 appears to be coexpressed with other neuronal nicotinic subunits, with which it forms functional receptor molecules, at least in keratinocytes (Grando et al., 1996).

We carried out a more systematic analysis of α5 expression in human tissues and organs by means of RNA dot blot studies. α5 was expressed in many but not all tissues, since adult kidney, spleen, liver, and peripheral blood leukocytes did not show any hybridization signal. Low α5 expression was detected in the adrenal gland and in all of the tested areas of the CNS, with the exceptions of the cerebellum and thalamus, which showed considerable hybridization. Interestingly, the cerebellum and the thalamus were also the areas of maximal expression of the α3 subunit. It is remarkable that no α5 expression has been detected in the cerebellum, and thalamus, or amygdala of rats (Boulter et al., 1990; Wada et al., 1990), which suggests that the distribution of the α5 subunit in the CNS seems to diverge across species. A similar situation has been described in relation to the α3 subunit in the hippocampal formation of monkey and rat brains (Cimino et al., 1992). Taken together, these findings clearly demonstrate that species-specific differences in the regional expression of neuroreceptors can exist. We did not observe any expression of β4 mRNA in the different areas of the CNS we tested. In the postnatal rat brain, it has been shown that the expression of β4 mRNA is confined to the pineal gland and to the medial habenula, two anatomical areas that were not investigated in the present study, and to the cerebellum, where the expression is much less pronounced and limited to the external germinal layer (Zoli et al., 1995). Thus, the lack of β4 expression in the human cerebellum may reflect again a species-specific difference or simply the inadequate sensitivity of our technique. The major extra-neuronal site of α5 expression was the thymus, a finding that confirms data previously published by other authors (Mihovilovic and Roses, 1993). The same authors also showed that α3, β4 and α5 transcripts are expressed by both thymocytes and cells derived from thymic epithelia (Mihovilovic et al., 1997). In the present study, we confirm that α3 and β4 are abundantly expressed in the human thymus and show that their expression as well as that of α5 are upregulated during development. Taken together, these data strongly suggest a possible role of nicotinic receptors in human T-lymphocyte biology.

The other extra-neuronal site in which the expression of α5 was substantial, diffuse and coincident with that of α3

was the gastrointestinal tract. In principle, the hybridization signals could arise from the extended neuronal network of the enteric division of the autonomic nervous system; however, it should be emphasized that we did not detect any β4 expression in the gastrointestinal tract, in spite of the fact that this subunit is believed to be a requisite participant in the formation of the nicotinic receptor subtype of the autonomic nervous system. Thus, it is possible that the α3 and α5 signals detected in the gastrointestinal tract are actually related to different anatomic structures that should be identified by in situ hybridization. The other extra-neuronal localizations of α5 expression also deserve careful analysis in order to clarify which cell type is responsible for the expression, reveal which other subunits, if any, are co-expressed, and finally to analyse the structure and function of the specific receptor subtypes at the protein level, bearing in mind that α5 may also co-assemble with still unknown nicotinic subunits and/or perform functions unrelated to an ion channel.

The fact that the α5 transcript is present in many but not all tissues, and that its expression is differentially regulated during development depending on the organ, may imply that distinct regulatory modules dictate the appropriate spatio-temporal patterns of α5 gene transcription. In order to gain further insight into these regulatory mechanisms, we isolated the 5′-regulatory region of the human α5 gene and characterized its structural and functional features. The structural data, which we will be presented elsewhere, indicate that α5 is transcribed by a GC-rich, TATA-and CAAT-less promoter, that contains a number of putative Sp1 binding sites. All of these features are typical of multiple start site promoters and are common to all of the neuronal nicotinic receptor promoters characterized so far, with the exception of the chicken β3 promoter (Hernandez et al., 1995; Fornasari et al., 1999 for a review). We then analyzed the functional properties of the human α5 5′-flanking region by transient transfections followed by luciferase assays, with the aim of identifying discrete regions with tissue-specific activity.

Deletion analysis allowed us to identify three distinct regulatory regions that showed particular activities in the different cell types. The first region was 130 bp long and was located immediately upstream of the core promoter (between nucleotides −368 and −241). This region had a negative effect on α5 expression in all of the tested cell lines, but this was much more pronounced in neuronal cells. The presence of a negative regulatory region immediately upstream of the core promoter is reminiscent of the human α3 nicotinic subunit gene, in which an *Alu* repeat sequence has negative effects on gene transcription with preferential activity in neuronal cells (Fornasari et al, 1997). A speculative view concerning these negative regulatory regions is that, by binding inducible or developmentally regulated transcription factors, they can keep down the expression of potentially harmful ion channels under circumstances in which neurons might be more vulnerable. The other two regulatory regions were identified in the DNA segment that specifies the 5′-UTR of α5 mRNA. The first was made up of the nucleotides between +54 and +180 and its deletion produced opposite effects in neuronal and non-neuronal cell lines, working as an activator of gene expression in the former and as an inhibitor in the latter (especially HeLa). This is the most striking evidence that the expression of the human α5 gene is regulated in a tissue-specific fashion and it is likely that distinct *cis*-acting elements contained in this region are responsible for its opposite regulatory effects in neuronal and non-neuronal cells. The second regulatory region was defined by nucleotides −1 and +53, and included the neuro-specific and major transcription start sites. For obvious reasons, we expected that the removal of this region would have more drastic effects on neuronal cells, but this was only partially the case: the expression of the reporter gene in SK-N-BE cells actually underwent a nine-fold decrease, but this was quite moderate in SY5Y. This discrepancy may reflect the different differentiation stage of the two cell lines (SK-N-BE cells have a less differentiated phenotype, with an epithelioid-like morphology). Deletion of the −1/+53 region had even more dramatic effects on HeLa cells, leading to a 25-fold decrease in the expression of the luciferase gene, and it also had substantial but less dramatic consequences in MOLT-4 cells. Thus, the −1/+53 region seems to play a general role in the expression of the α5 gene, but its activity seems to be maximal in the epithelial cell type. The presence of a 5′-UTR encoding region which is capable of regulating the activity of the nearby promoter is reminiscent of the downstream regulatory region (DRR) that we have described in the human α3 nicotinic subunit gene (Fornasari et al., 1997). In that case, the 5′-UTR specifying region displayed activity only in neuronal cells and not in cells that do not express the α3 transcript (as expected) or in T-lymphocyte cells (Battaglioli et al., 1998), the other important site in which α3 mRNA was identified. In the case of the α5 gene, its 5′-UTR encoding region seems to have more complex regulatory functions in both neuronal and non-neuronal cells.

In conclusion, over the past few years, evidence have been found showing that neuronal nicotinic subunits are also expressed in non-neuronal tissues and often form functional receptors: the α7 subunit has been identified in tendon and periosteum (Romano et al., 1997a), skeletal muscle (Corriveau et al., 1995; Romano et al., 1997b) and, more recently, in the lung (Sekhon et al., 1999); α4 and β2 in skeletal muscle (Sala et al., 1996), and α3, β4 and α5 in keratinocytes (Grando et al., 1996) and T-lymphocytes (Mihovilovic et al., 1997). These data suggest that any drug aimed at the nicotinic system may have multiple effects outside the CNS. In this study, we analyzed the expression of α5 mRNA in human neuronal and non-neuronal tissues, and found that the distribution of this nico-

tinic subunit in non-neuronal tissues is widespread. This very heterogeneous expression seems to be guaranteed by a highly complex promoter that uses distinct regulatory mechanisms to comply with the different functional and developmental requirements of the various tissues and organs.

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References

Battaglioli, E., Gotti, C., Terzano, S., Flora, A., Clementi, F., Fornasari, D., 1998. Expression and transcriptional regulation of the human α3 neuronal nicotinic receptor subunit in T lymphocyte cell lines. J. Neurochem. 7, 1261–1270.

Boulter, J., O'Shea-Greenfield, A., Duvoisin, R.M., Connolly, J.G., Wada, E., Jensen, A., Gardner, P.D., Ballivet, M., Deneris, E.S., McKinnon, D., Heinemann, S., Patrick, J., 1990. α3, α5, and β4: three members of the rat neuronal nicotinic acetylcholine receptor-related gene family form a gene cluster. J. Biol. Chem. 265, 4472–4482.

Changeux, J.P., Bertrand, D., Corringer, P.J., Dehaene, S., Edelstein, S., Lena, C., Le Novere, N., Marubio, L., Picciotto, M., Zoli, M., 1998. Brain nicotinic receptors: structure and regulation, role in learning and reinforcement. Brain Res. Rev. 26, 198–216.

Chini, B., Clementi, F., Hukovic, N., Sher, E., 1992. Neuronal-type α-bungarotoxin receptors and the α5-nicotinic receptor subunit gene are expressed in neuronal and non-neuronal human cell lines. Proc. Natl. Acad. Sci. U. S. A. 89, 1572–1576.

Cimino, M., Marini, P., Fornasari, D., Cattabeni, F., Clementi, F., 1992. Distribution of nicotinic receptors in cynomolgus monkey brain and ganglia: localization of α3 subunit mRNA, α-bungarotoxin and nicotine binding sites. Neuroscience 51, 77–86.

Conroy, W.G., Berg, D.K., 1995. Neurons can maintain multiple classes of nicotinic acetylcholine receptors distinguished by different subunit compositions. J. Biol. Chem. 270, 4424–4431.

Conroy, W.G., Berg, D.K., 1998. Nicotinic receptor subtypes in the developing chick brain: appearance of a species containing the α4, β2, and α5 gene products. Mol. Pharmacol. 53, 392–401.

Conroy, W.G., Vernallis, A.B., Berg, D.K., 1992. The α5 gene product assembles with multiple acetylcholine receptor subunits to form distinctive receptor subtypes in brain. Neuron 9, 679–691.

Corriveau, R.A., Romano, S.J., Conroy, W.G., Oliva, L., Berg, D.K., 1995. Expression of neuronal acetylcholine receptor genes in vertebrate skeletal muscle during development. J. Neurosci. 15, 1372–1383.

Fornasari, D., Battaglioli, E., Flora, A., Terzano, S., Clementi, F., 1997. Structural and functional characterization of the human α3 nicotinic subunit gene promoter. Mol. Pharmacol. 51, 250–261.

Fornasari, D., Battaglioli, E., Terzano, S., Clementi, F., 1999. Transcriptional regulation of neuronal nicotinic receptor subunit genes. In: Arneric, S.P., Brioni, J.D. (Eds.), Neuronal Nicotinic Receptors: Pharmacology and Therapeutic Opportunities. Wiley, New York, pp. 25–42.

Gerzanich, V., Wang, F., Kuryatov, A., Lindstrom, J., 1998. α5 Subunit alters desensitization, pharmacology, Ca^{2+} permeability and Ca^{2+} modulation of human neuronal α3 nicotinic receptors. J. Pharmacol. Exp. Ther. 286, 311–320.

Gotti, C., Fornasari, D., Clementi, F., 1997. Human neuronal nicotinic receptors. Prog. Neurobiol. 53, 199–237.

Grando, S.A., Horton, R.M., Mauro, T.M., Kist, D.A., Lee, T.X., Dahl, M.V., 1996. Activation of keratinocyte nicotinic cholinergic receptors stimulates calcium influx and enhances cell differentiation. J. Invest. Dermatol. 107, 412–418.

Groot-Kormelink, P.J., Luyten, W.H., Colquhoun, D., Sivilotti, L.G., 1998. A reporter mutation approach shows incorporation of the ''orphan'' subunit β3 into a functional nicotinic receptor. J. Biol. Chem. 273, 15317–15320.

Hernandez, M.C., Erkman, L., Matter-Sadzinski, L., Roztocil, T., Ballivet, M., Matter, J.M., 1995. Characterization of the nicotinic acetylcholine receptor β3 gene. Its regulation within the avian nervous system is effected by a promoter 143 base pairs in length. J. Biol. Chem. 270, 3224–3233.

Kozak, M., 1992. Regulation of translation in eukaryotic systems. Annu. Rev. Cell. Dev. Biol. 8, 225.

Kuryatov, A., Gerzanich, V., Nelson, M., Olale, F., Lindstrom, J., 1997. Mutation causing autosomal dominant nocturnal frontal lobe epilepsy alters Ca^{2+} permeability, conductance, and gating of human α4β2 nicotinic acetylcholine receptors. J. Neurosci. 17, 9035–9047.

Mihovilovic, M., Denning, S., Mai, Y., Whichard, L.P., Patel, D.D., Roses, A.D., 1997. Thymocytes and cultured thymic epithelial cells express transcripts encoding α3, α5 and β4 subunits of neuronal nicotinic acetylcholine receptors: preferential transcription of the α3 and β4 genes by immature CD4+8+ thymocytes. J. Neuroimmunol. 79, 176–184.

Mihovilovic, M., Roses, A.D., 1993. Expression of α3, α5, and β4 neuronal acetylcholine receptor subunit transcripts in normal and myasthenia gravis thymus. Identification of thymocytes expressing the α-3 transcripts. J. Immunol. 151, 6517–6524.

Raimondi, E., Rubboli, F., Moralli, D., Chini, B., Fornasari, D., Tarroni, P., De Carli, L., Clementi, F., 1992. Chromosomal localization and physical linkage of the genes encoding the human α3, α5, and β4 neuronal nicotinic receptor subunits. Genomics 12, 849–850.

Ramirez-Latorre, J., Yu, C.R., Qu, X., Perin, F., Karlin, A., Role, L., 1996. Functional contributions of α5 subunit to neuronal acetylcholine receptor channels. Nature 380, 347–351.

Romano, S.J., Corriveau, R.A., Schwarz, R.I., Berg, D.K., 1997a. Expression of the nicotinic receptor α7 gene in tendon and periosteum during early development. J. Neurochem. 68, 640–648.

Romano, S.J., Pugh, P.C., McIntosh, J.M., Berg, D.K., 1997b. Neuronal-type acetylcholine receptors and regulation of α7 gene expression in vertebrate skeletal muscle. J. Neurobiol 32, 69–80.

Rubboli, F., Court, J.A., Sala, C., Morris, C., Chini, B., Perry, E.K., Clementi, F., 1994. Distribution of nicotinic receptors in the human hippocampus and thalamus. Eur. J. Neurosci. 6, 1596–1604.

Sala, C., Kimura, I., Santoro, G., Kimura, M., Fumagalli, G., 1996. Expression of two neuronal nicotinic receptor subunits in innervated and denervated adult rat muscle. Neurosci. Lett. 215, 71–74.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press.

Sekhon, H.S., Jia, Y., Raab, R., Kuryatov, A., Pankow, J.F., Whitsett, J.A., Lindstrom, J., Spindel, E.R., 1999. Prenatal nicotine increases pulmonary α7 nicotinic receptor expression and alters fetal lung development in monkeys. J. Clin. Invest. 103, 637–647.

Tarroni, P., Rubboli, F., Chini, B., Zwart, R., Oortgiesen, M., Sher, E., Clementi, F., 1992. Neuronal-type nicotinic receptors in human neuroblastoma and small-cell lung carcinoma cell lines. FEBS Lett. 312, 66–70.

Terzano, S., Court, J.A., Fornasari, D., Griffiths, M., Spurden, D.P., Lloyd, S., Perry, R.H., Perry, E.K., Clementi, F., 1998. Expression of the α3 nicotinic receptor subunit mRNA in aging and Alzheimer's disease. Mol. Brain Res. 63, 72–78.

Wada, E., McKinnon, D., Heinemann, S., Patrick, J., Swanson, L.W., 1990. The distribution of mRNA encoded by a new member of the neuronal nicotinic acetylcholine receptor gene family (α5) in the rat central nervous system. Brain Res. 526, 45–53.

Zoli, M., Le Novere, N., Hill, J.A. Jr., Changeux, J.P., 1995. Developmental regulation of nicotinic Ach receptor subunit mRNAs in the rat central and peripheral nervous systems. J Neurosci 15, 1912–1939.