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Conserved transcription factor binding sites suggest an activator basal promoter and a distal inhibitor in the galanin gene promoter in mouse ES cells

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ABSTRACT

Galanin and its receptors have been shown to be expressed in undifferentiated mouse embryonic stem (ES) cells through transcriptome and proteomic analyses. Although transcriptional regulation of galanin has been extensively studied, the regulatory proteins that mediate galanin expression in mouse ES cells have not yet been determined. Through sequence alignments, we have found a high degree of similarity between mouse and human galanin upstream sequences at −146 bp/+69 bp (proximal region) and −2408 bp/−2186 bp (distal region). These regions could be recognized by ES cell nuclear proteins, and EMSA analysis suggests a specific functionality. Analysis of the proximal region (PR) using EMSA and ChIP assays showed that the CREB protein interacts with the *galanin* promoter both *in vitro* and *in vivo*. Additional EMSA analysis revealed that an SP1 consensus site mediated protein–DNA complex formation. Reporter assays showed that CREB is an activator of galanin expression and works cooperatively with SP1. Furthermore, analysis of the distal region (DR) using EMSA assays demonstrated that both HOX-F and PAX 4/6 consensus sites mediated protein–DNA complex formation, and both sites inhibited luciferase activity in reporter assays. These data together suggest that CRE and SP1 act as activators at the basal promoter, while HOX-F and PAX 4/6 act as silencers of transcription. The interplay of these transcription factors (TF) may drive regulated galanin expression in mouse ES cells.

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1. Introduction

Galanin is a highly conserved 29 (30 in human) amino acid peptide that plays a neuromodulatory role, in addition to an important trophic role, in neuronal tissues after injury and disease (Lang et al., 2007). Galanin has also been suggested to have a biological activity in progenitor or stem cells from both mesodermal and ectodermal origin (Louridas et al., 2009), and has been found through transcriptome and proteomic analyses in several lineages of undifferentiated human and mouse ES cells (Anisimov et al., 2002; Ramalho-Santos et al., 2002; Sato et al., 2003). To accomplish these functions, galanin signals through three receptors: GalR1 (Habert-Ortoli et al., 1994), GalR2 (Howard et al., 1997; Smith et al., 1997), and GalR3 (Wang et al., 1997).

Transcriptional *in vivo* and *in vitro* studies of the *galanin* gene from different organisms have made it possible to identify a plethora of regulatory elements at the basal promoter, such as NRE, AP1, STAT, CRE, SP1 and ERE, that activate galanin expression in several cell types or tissues (Kofler et al., 1996; Rökaeus et al., 1998). Corness et al. (1997) found a CRE element at the proximal promoter and a repressor element localized at −2.2 kb and −1.4 kb of the rat *galanin* gene that is active in primary sensory neurons in culture. In another study, Bacon et al. (2007) found ETS, STAT and Bicoid consensus sites localized at −1.9 kb of the mouse *galanin* gene that direct galanin expression after axotomy. Additionally, bovine galanin promoter sequences spanning from 5 kb or 131 bp were studied in human neuroblastoma SH-SY5Y cells and in transgenic mice, and the presence of silencer and enhancer sequences was revealed (Rökaeus et al., 1998). However, despite the large amount of data addressing galanin activation, functional transcriptional regulators of mouse galanin expression in ES cells have not yet been determined.

In the present study, we have found, through sequence alignment, a high degree of conservation between mouse and human galanin upstream sequences, located at −146 bp/+69 bp of the proximal region (PR) and −2408 bp/−2186 bp of the distal region (DR). In both regions, there was conservation of transcription factor (TF) binding sites including HOX-F and PAX 4/6 in the DR, and SP1 and CRE in the PR. By analyzing the proximal region, we showed through EMSA and ChIP

Abbreviations: Bp, Base pair; ChIP, *Chromatin immunoprecipitation*; DR, Distal region; EDTA, Ethylenediaminetetraacetic acid; EMSA, Electrophoretic mobility shift assay; ES, Embryonic stem cells; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; kb, Kilobase; KCl, Potassium chloride; MEF, Mouse embryonic fibroblasts; mg, Miligram; $MgCl_2$, Magnesium chloride; ml, Mililiter; mM, Milimolar; PBS, Phosphate-buffered saline; PCR, Polymerase chain reaction; PR, Proximal region; TF, Transcription factor; TBE, Tris/borate/EDTA.

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assays that CREB proteins interact with the *galanin* gene promoter *in vitro* and *in vivo*. Further EMSA analysis using SP1, HOX-F and PAX 4/6 consensus sites showed that all of these sequences also have the ability to bind the nuclear proteins of ES cells. When the cooperative role of these sites was investigated through transfection assays, we observed that SP1 and CRE, using proximal constructs, activated luciferase signal cooperatively, while using distal constructs, HOX-F and PAX 4/6 exerted an inhibitory action on the luciferase signal.

These data together suggest that CRE and SP1 at the basal promoter act as activators, while HOX-F and PAX 4/6 act as silencers of galanin expression in mouse ES cells.

2. Materials and methods

2.1. Computer analysis of the promoter region

Approximately 3 kb of the upstream sequence of the mouse (L11144.1) and human (L38575.1) *galanin* genes was obtained from GenBank (www.ncbi.nlm.nih.gov.br). Conservation between both species was assessed through the sequence alignment program FASTA VIRGINIA (http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml), and conservation of TF binding sites was assessed using online databanks MatInspector (www.genomatix.de), rVista 2.0 (http://rvista.dcode.org) and IFTI (www.ifti.org).

2.2. Cells and culture conditions

USP-1, a murine ES cell line (Sukoyan et al., 2002) was kindly provided by Dr. Lygia Pereira (Universidade de São Paulo) and cultured as follows: ES cells were maintained on a feeder layer of irradiated MEFs (murine embryonic fibroblast) in DMEM high glucose media (Invitrogen) supplemented with 15% fetal bovine serum (Hyclone), 100 mM nonessential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 100 U of penicillin/ml (Invitrogen), 100 mg/ml of streptomycin (Invitrogen), 0.55 μM β-mercaptoethanol (Sigma) and 1000 U/ml of leukemia inhibitory factor (LIF; Chemicon) on 0.2% gelatin-coated plates, at 37 °C in a humidified chamber with 5% CO_2. The medium was changed 3 h before splitting, and the cells were rinsed twice with 1X phosphate-buffered saline (PBS), treated with 0.25% trypsin/0.5 mM EDTA for 5 min and split 1:3 to be maintained in culture or used for preparation of protein nuclear extracts or transfection assays after removal of feeder cells. Feeders were removed by replating into a new gelatin-coated dish for propagation. These ES cells without MEF cells were expanded 1–2 passages in 50% MEF conditioned media and 50% fresh ES cell media.

2.3. Preparation of nuclear extract

For preparation of nuclear protein extracts, 1×10^7 ES cells without MEF cells were rinsed twice with PBS 1X and treated with 0.25% trypsin/0.5 mM EDTA. The material was centrifuged and the nuclear proteins were extracted as described by Dignam et al. (1983). Protein concentrations were determined by the Bradford method (Bradford, 1976).

2.4. Electrophoretic mobility shift assays (EMSA)

For EMSA assays, we used probes from PCR synthesized fragments and double-stranded (ds) DNA oligonucleotides, both end-labeled with [γ-32P] ATP (Amersham, Pharmacia) using T4 polynucleotide kinase (Invitrogen). The primer sequences used to generate PCR fragment probes corresponding to the PR and DR were as follows: PRsense 5′-AGACTGTGGGTGATC CTCTC-3′, PRantisense 5′-CTGGATGGTCGCTT ACTG-3′, DR sense 5′-GCTTTGTGTGCTGTGTCCATTACT-3′, DRantisense 5′-CCCATA TCACTGACAGATTCGCTCC-3′. The following primers (and their complements) were annealed to generate dsDNA oligonucleotide probes: SP1-sense 5′-CAGGAGGC GGCGCTGAGCGG-3′, CREB-sense 5′-AGCCGGTGACGCGGCAGCTCC-3′, CREB -Mut-sense 5′-AGCCGGTGAA GCGGCAGCTCC-3′, HOX-F-sense 5′-ATTACTCTA ATGGTGACCCATTTTG-3′, PAX-4/6-sense 5′-CTCCAGGCTGGGCTGCCTT-3′. Gel shift reactions were performed in a binding buffer [25 mM HEPES (pH-7.6), 30 mM KCl, 5 mM MgCl2, 5% glycerol], containing 1 μg of poly (dIdC), 7–10 μg of nuclear extract proteins and 80,000 cpm of [γ-32P]-labeled PCR dsDNA probes. The reactions occurred at 25 °C for 40 min and were subsequently resolved on a 4% or 4.5% native polyacrylamide gel when PCR fragments or oligonucleotides were utilized as probes, respectively; in 0.5 × TBE buffer (22.5 mM Tris/Borate/1 mM EDTA) for 4 h at 140 V. For competition reactions, a 100 molar excess of unlabeled competitor oligonucleotide or a mutated version of the specific consensus binding site was added 20 min before addition of the probe. For supershift experiments, 2 μg of anti-CREB1 (C-21) antibody (Santa Cruz Biotechnology Inc.) was incubated with a nuclear protein extract 60 min before addition of the specific probe. After addition of the specific probe, the incubation was continued for 40 min at 25 °C. The dried gels were exposed on Kodak MS films on intensifying screens at −70 °C for 48 h.

2.5. Chromatin immunoprecipitation (ChIP) assay

The ChIP assays with anti-CREB antibody (Santa Cruz technologies) were performed according to the online protocol provided by Abcam Company (http://www.abcam.com/ps/pdf/protocols/N-ChIP.pdf). DNA extractions from bound fractions were performed following the Abcam (www.abcam.com) protocol. The immunoprecipitated DNA was amplified for sequences containing binding sites by using the following CRE element sequence primers present in the mouse *galanin* gene promoter: ChIP-sense 5′-GGTCTGAGAC TGTGGGTGAT-3′; ChIP-antisense 5′-CTGCTGCCGCTATTTATG-3′. Quantification was evaluated by RT-qPCR analysis. Immunoprecipitation of anti-tubulin antibody (Santa Cruz Technologies) was used as a negative control.

2.6. Reporter vector design

The galanin firefly luciferase reporter constructs were made by cloning fragments corresponding to the PR and DR of the mouse galanin promoter into the firefly PGL3-Basic vector (Promega) upstream of the luciferase reporter gene. The constructs p80, p60 and p60mut were obtained by cloning PCR-amplified fragments corresponding to −78 bp/+357 bp and −60 bp/+357 bp into XhoI and HindIII restriction sites of pGL3-Basic. The primer sequences used for the PCR reactions were: p80-sense: 5′-ATAT*CTCGAG*TCGCAGGAGGCGGCGCT-3′; p60-sense: 5′ATC-T*CTCGAG*AGCCGGTGACGCGGCAG-3′. The p60mut construct contained, in the forward primer, a C to A base substitution (underlined) at the CRE binding site to generate a mutant version of this element: p60mut-sense: 5′-ATCT*CTCGAG*AGCCGGTGAAGCGGCAG-3′. Reverse primer pPR-antisense 5′-GCCCG*AAGCTT*CATCTGGAAGGA AAAGTGG-3′ was used to generate all the products. Forward primers have a *XhoI* restriction site and reverse primers have a *HindIII* restriction site. To generate the p2400 and the p2300 constructs corresponding to the DR, we first digested the p80 luciferase reporter construct with *SacI* and *XhoI* restriction enzymes. This digested construct was used for subsequent cloning of −2408 bp/−2186 bp and −2365 bp/−2186 bp PCR fragments from the mouse *galanin* gene separately. The primer sequences used for the PCR reactions were: p2400 sense: 5′-GGAAT*GAGCTC*CTTTGTGTGCTGTGTCC-3′ and p2300 sense: 5′-TGAAT*GAGC TC*TAGCTCCACGCTGGGCTG-3′. Reverse primer pDR-antisense: 5′-GGCTG*CTC GAG*CCCATATCACTGACAGAT-3′ was used to generate both products. Forward and reverse primers have *SacI* and *XhoI* restriction sites, respectively. All reporter pGL3 vector sequences were confirmed by DNA sequencing at the Automated DNA Sequencing Facility of Instituto de Biofísica Carlos Chagas Filho-UFRJ.

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