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Regulation of the connexin 43 promoter in the brook trout testis: Role of the thyroid hormones and cAMP ☆

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ABSTRACT

Gap junctions are critical for spermatogenesis. They are composed of integral proteins, the connexins. In mammals, a loss of Cx43 expression results in the inhibition of spermatogenesis. We have shown that Cx43 is expressed in the Sertoli cells of rainbow trout and that cAMP and triiodothyronine (T_3) regulate testicular *Cx43* expression in brook trout testis. The objective of this study was to determine if cAMP and T_3 act at the level of the *cx43* promoter to regulate its expression. A 607 bp 5′ flanking sequence of the *cx43* promoter was obtained by Genome Walking. A TATA box was predicted to be located between positions −36 and −30 relative to the transcriptional initiation site. 5′-Rapid amplification of cDNA ends indicated a single transcriptional start site. Single C/EBP (−164 to −156) and *tr-beta* (−112 to −107) response elements were identified and electrophoretic mobility shift assays indicated the presence of competitive protein binding sites at each region. Immortalized rainbow trout gonadal cell line (RTG-2) which express *cx43* and *tr-beta* transcripts were transfected with a vector containing the Cx43 promoter inserted into a luciferase expression vector. Transactivation of the reporter genes was stimulated by either cAMP or T_3. Sequential deletion and point mutations in either the C/EBP or *tr-beta* response element indicated that T_3 but not cAMP directly induced luciferase transactivation of the luciferase gene by acting on different sites of the Cx43 promoter. Together, these data indicate that T_3 stimulates *cx43* expression via direct regulation of gene transcription.

1. Introduction

Spermatogenesis is a process that requires complex cell–cell interactions. Gap junctions, which are low-resistance channels, allow the passage of small molecular weight metabolites (<1 kDa) such as cAMP, inositol, and ions (Ca^{2+}, H^+, Cl^-) between neighboring cells, thereby allowing direct intercellular communication between adjacent cells. Gap junctions are composed of hexomeric connexons which are themselves composed of connexins (Cxs) which are differentially expressed in different tissues. Certain cells express a single Cx while others can express several (Beyer et al., 1990). There are few studies on Cxs in fish. A recent study on the zebrafish genome revealed the presence of 37 different Cxs (Eastman et al., 2006). Previous studies done in our laboratory have identified four different Cxs in both rainbow and brook trout testes: Cx43, Cx43.4, Cx30, and Cx31 (de Montgolfier et al., 2007, 2008). Each of the four testicular Cxs is expressed in different cell types. Cx43 (also known as gap junction protein A1, GjA1) was localized along the plasma membrane of Sertoli cells in both rainbow and brook trout testes, as is the case in mammals (Risley et al., 1992; Pointis and Segretain, 2005; de Montgolfier et al., 2007).

In brook trout, 11-ketotestosterone (11-KT) does not appear to be an important regulator of testicular *cx43* (de Montgolfier et al., 2009). However, cAMP and triiodothyronine (T_3) can modify *cx43* mRNA levels, suggesting that FSH, acting through cAMP, and thyroid hormones may be implicated in regulating *cx43* levels in brook trout testis (de Montgolfier et al., 2009). cAMP has been implicated in the regulation of gap junctions in various cell types (Dowling-Warriner and Trosko, 2000; Romanello et al., 2001; TenBroek et al., 2001; Yao et al., 2006).

Thyroid hormones (THs) are involved in fish reproduction (Cyr and Eales, 1996) and thyroid inhibitors have been reported to inhibit spermatogenesis (Swapna et al., 2006). In brook trout, two isoforms of the thyroid hormone receptors (TRs), *tr-alpha* and *tr-beta*, are present in the testis (de Montgolfier et al., 2009). Furthermore, incubation of testicular fragments with varying concentrations of T_3 is correlated with increased levels of *cx43* mRNA levels. Studies in mammals have also shown that THs can modify testicular Cx43 levels in rat as well as in a murine Sertoli cell line (St-Pierre et al., 2003; Gilleron et al., 2006). Moreover, THs are known to be important regulators of Sertoli cell differentiation (Buzzard et al., 2003;

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Holsberger et al., 2003; Holsberger and Cooke, 2005), a process that is dependent on *Cx43* (Roscoe et al., 2001; Brehm et al., 2007; Sridharan et al., 2007).

While we have previously shown that testicular mRNA levels of *cx43* are dependent on both cAMP and T_3, there is no evidence to indicate whether or not this regulation occurs at the transcriptional level or via other means. In order to determine if cAMP and/or T_3 regulate the transactivation of the brook trout Cx43 (btCx43) gene, we intend to characterize the btCx43 promoter and, using *in vitro* approaches, assess whether or not cAMP and T_3 can directly stimulate the transcription of the btCx43 gene.

2. Materials and methods

2.1. Animals

One-year-old male brook trout with testes at stages 2 and 3 of development were sampled at the Station aquicole de Pointe-au-Père (Rimouski, Québec). Fish were held indoors in 500 L circular tanks under natural photoperiod and water temperature. At the time of sampling, fish were anesthetized in 3-aminobenzoic-ethyl-ester-acid (MS-222; 0.16 g/L) with constant aeration. Fish were weighed, measured, and sexed. All animal procedures were approved by the University Animal Care Committee.

2.2. mRNA extraction and reverse transcription

Total RNA was isolated from brook trout testes and rainbow trout gonadal cells (RTG-2; American Type Culture Collection, Manassas, VA; CCL 55) using the Absolutely RNA® Miniprep kit (Stratagene, La Jolla, CA). The isolated RNA was treated with DNase (1 U/μg of RNA; deoxyribonuclease I, amplification grade; Canadian Life Technologies, Burlington, ON) to remove any contaminating genomic DNA. The resulting RNA was reverse transcribed using oligod(T)16–18 primers (GE Health Sciences, Baie D'Urfe, QC) and M-MLV reverse transcriptase (Canadian Life Technologies) according to the supplier's instructions.

2.3. RT-PCR

cDNA templates (250 ng) were amplified for *cx43* using gene-specific primers (Forward 5′-ATGGGTGACTGGAGYKYCYTRG; Reverse 5′-ACCACCARCATRAAGAYRATGAAG). PCR amplifications were done using 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and elongation at 72 °C for 30 s. Amplicons were separated on a 2% agarose gel and visualized by ethidium bromide staining with a predicted size of 649 bp.

2.4. Rapid amplification of cDNA 5′ ends (RACE)

Total RNA was isolated from brook trout testes at stages 2 and 3 of spermatogenesis using the Absolutely RNA® Miniprep kit (Stratagene). The isolated RNA was treated with DNase (1 U/μg of RNA; deoxyribonuclease I, amplification grade; Canadian Life Technologies) to remove contaminating genomic DNA. A 1 μg aliquot of total RNA was used for first-strand cDNA synthesis and amplified using the BD SMART™ RACE cDNA Amplification kit (BD Biosciences, San Jose, CA). The amplifications were done by touchdown PCR using 5 cycles of denaturation at 94 °C for 30 s, annealing at 72 °C for 3 min; 5 cycles of denaturation at 94 °C for 30 s, annealing at 70 °C for 30 s, and elongation at 72 °C for 3 min; and 25 cycles of denaturation at 94 °C for 30 s, annealing at 68 °C for 30 s, and elongation at 72 °C for 3 min. The RACE products were separated on a 0.5% agarose gel and visualized by ethidium bromide staining. RT-PCR products were extracted from the agarose gel (QIAEX II Gel Extraction kit; Qiagen, Mississauga, ON, Canada) and sequenced using an automated sequencer (Genetic analyzer 3130, Applied Biosystems, Foster City, CA, USA). Cx43R1 (5′-CGATGTAGGTTCTCAAGAGGGCTCC) and Cx43RC1 (5′-CGT GGA GTA AGC CTG AAC CTT GT) were used as gene-specific primers in two separate reactions which were designed to amplify products with a difference of 361 bp. RACE reactions with buffer alone were used as negative controls and the amplification of human placental transferrin was used as positive control. The sequences of the resulting amplicons were compared to other known Cxs using BLAST homology comparisons (GenBank, National Center for Biotechnology Information, Bethesda, MD).

2.5. Isolation of the 5′ flanking region of the brook trout cx43 gene

The 5′ flanking region of the btCx43 gene was isolated by Genome Walking using the Genome Walker kit (Clontech, Palo Alto, CA). Genomic DNA was digested with four different enzymes (DraI, EcoRV, PvuII, and StuI). The BD GenomeWalker Adaptor was linked to the 5′ ends of each DNA fragment in the four libraries with T4 DNA ligase. PCRs were done using gene-specific primers; the resulting products were resolved on a 0.5% agarose gel and visualized by ethidium bromide staining. The PCR products were then extracted from the gel (QIAEX II Gel Extraction kit; Qiagen, Mississauga, ON, Canada) and cloned into the pCRII-Topo vector. The resulting clones were sent for automated sequencing by a commercial facility (DNA Landmarks, Ste-Julie, QC).

2.6. Extraction of nuclear proteins

Nuclear proteins from brook trout testis (pool of three animals) were extracted using a nuclear protein extraction kit (Nuclear Extraction kit; Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Protein concentrations were quantified using the Bio-Rad protein assay reagents (Bio-Rad Laboratories, Mississauga, ON).

2.7. Electrophoresis mobility shift assays (EMSA)

Electrophoretic mobility shift assays (EMSA) were done according to the methods described in previous studies (Plante et al., 2002, 2007; Dufresne and Cyr, 2007). Nuclear protein extracts from brook trout testes were prepared as described above. Two double-stranded oligonucleotides spanning the region of interest (positions −173 to −143 (BtC/EBP Forward 5′-ATT-GCAAAA**ATCAGAAAT**TTTCTGTCCAGA; binding site in bold) and −123 to −93 (BtT3R-beta Forward (CCATGCATTTG**TTAC TT**CTAGGTTAGACTA) relative to the translation initiation site) and oligonucleotides in which a mutation (underline bold) was inserted into the C/EBP (mBtC/EBP Forward 5′-ATT-GCAAAA**CGCACTCCG**TTTCTGTCCAGA) and T3R (mBtT3R Forward 5′-CCATGCATTTG**GGCAGG**CTAGGTTAGACTA) were synthesized (Invitrogen Inc., Burlington, ON). Brook trout *cx43* promoter sequences (10 pmol, sense oligonucleotides) were incubated with T4 polynucleotide kinase (Invitrogen) and [γ^{-32}P] ATP, for 30 min at 37 °C using 5 U of T4 polynucleotide kinase and reaction buffer (50 μl final volume; GE Healthcare, Bair D'Urfe, QC). Following the incubation, the labeled oligonucleotide was heated at 65.8 °C for 10 min and incubated for 30 min at room temperature in the presence of preheated 2.5 M excess non-sense DNA. The double-stranded oligonucleotide was purified on a Sephadex G-50 column (Nick Column; GE Healthcare), and the radioactive content was determined to ensure a specific activity of at least 5000 cpm/fmol. Labeled double-stranded DNA (30 fmol) was incubated for 30 min at room temperature with 30 μg of nuclear protein (a pool of three individuals) in EMSA incubation buffer (4% glycerol, 10 mM $MgCl_2$,