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Rapid communication

Promoter cloning and characterisation of the transcriptional regulation of the human thyrostimulin A2 subunit

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Abstract

The novel heterodimeric glycoprotein hormone thyrostimulin consists of two unique subunits, A2 and B5. To understand its yet unknown transcriptional regulation, we characterised the 3.1-kb immediate 5′-flanking region of the human A2 gene localised on chromosome 11q13. In transient transfection assays this sequence exhibited promoter activity, which could be confined to nucleotides −506 to −347 relative to the ATG start codon. Interestingly, this minimal promoter appeared to be non-tissue-specific. Deletional, mutational and gel shift analyses revealed regulatory elements that are essential for the regulation of the A2 gene expression. Another noteworthy feature of this gene is the presence of silencer elements upstream and downstream of the promoter. To surmise, our results provide an initial step toward a detailed analysis of the underlying molecular mechanisms of the human thyrostimulin gene expression.
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1. Introduction

Classically, the synthesis of thyroid-stimulating hormone (TSH) in the anterior pituitary is stimulated by thyrotropin-releasing hormone (TRH) produced in the hypothalamus. In the thyroid, the binding of TSH to the G protein-coupled TSH receptor (TSHR) facilitates the stimulation of adenylyl cyclase, thereby causing enhanced production of cAMP (Vassart and Dumont, 1992). The cAMP in turn activates further downstream signalling leading eventually to the production of thyroid hormones T_4 and T_3. However, recent progress has implicated, albeit indirectly, a new pituitary hormone, thyrostimulin, in the established view of thyroid axis.

TSH, follicle-stimulating hormone (FSH), luteinising hormone (LH) and chorionic gonadotropin (CG) are members of the glycoprotein hormone family that consist of two subunits, the α- and β-subunits. The α-subunit is common to all members of this family but the β-subunit is unique and confers specific biological activity to each heterodimeric protein (Shupnik et al., 1989). In 2002, a novel heterodimeric glycoprotein hormone, termed thyrostimulin, was discovered (Nakabayashi et al.). It contains two unique subunits A2 and B5 [recorded in GenBank as glycoprotein hormone A2 (GPHA2) and glycoprotein hormone beta 5 (GPHB5), respectively] that were identified in a homology search of the human genome. A2 is 35% identical at an amino acid level to the common glycoprotein α-subunit, whereas B5 is approximately 30% identical to TSH-β, FSH-β or LH-β. Despite such modest similarity, they contain all of the cysteine residues required to form the characteristic cysteine knot structure found in the related glycoprotein hormone family members.

The intriguing finding that purified thyrostimulin stimulated cAMP production via the TSHR in cultured thyroid cells and increased T_4 levels in TSH-suppressed rats in vivo (Nakabayashi et al., 2002), raised the question about the functional significance of this new hormone. Ikekubo et al. (2005) described two euthyroid patients with persistently undetectable levels of TSH, TSHR antibodies and thyroid stimulating antibodies but with almost normal levels of free thyroid hormone. This study suggested that an unknown factor, such as thyrostimulin, stimulated the thyroid. On the other hand, no apparent phenotype was found in mice lacking the B5 gene (Macdonald et al., 2005), indicating that the normal function of thyrostimulin remains uncharacterised.

In this paper, we continue to unravel the role of thyrostimulin by studying its yet unknown transcriptional regulation. To

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do so, we have cloned and functionally analysed the promoter of the human A2 subunit in pituitary and non-pituitary cell lines. The gene for this subunit is located on chromosome 11q13 and contains the ORF encoded by three exons. The latter extend from nucleotides 37229 to 37331, 37518 to 37696 and 37813 to 37919, with the translational start site ATG positioned at 37229 nt. Our results provide an initial step toward a detailed analysis of the underlying molecular mechanisms of the human thyrostimulin gene expression.

2. Materials and methods

2.1. Plasmid construction

DNA fragment from the human thyrostimulin A2 subunit 5′-flanking region (−3119 to −37, the A in the ATG initiation codon is designated as +1) was amplified from human brain genomic DNA (BioCat, Heidelberg, Germany) by PCR using the following primers: F, 5′-CTTGCAGCTCTCTCTTCCCGTTT-3′ and R, 5′-GTATTTAAAGAACTCGCCATCCCACCTG-3′. It was inserted into the pCR-XL-TOPO vector (Invitrogen, Karlsruhe, Germany) and the resulting construct was cleaved by *Mlu*I and *Xho*I. After separation on agarose gel and purification using the QIAquick gel extraction kit (Qiagen, Hilden, Germany), the fragment that contains sequence from −3119 to −37 was inserted into the luciferase reporter vector pGL3-Basic (Promega, Mannheim, Germany) using *Mlu*I and *Xho*I sites. This construct was designated −3119/−37.

Progressively shorter 5′ prime truncations (−2066/−37, −1586/−37, −1046/−37, −506/−37 and −373/−37) were generated using a PCR strategy that employed −3119/−37 as a template, reverse primer 5′-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3′ and forward primers with the following sequences: 5′-CGAGATC*ACGCGT*CCTGGCAAACAAGGACCCAGGT-3′, 5′-CGAGATC*ACGCGT*TTCTTGCTCAAGTGTAGTTTAGCACCATGG-3′, 5′-CGAGATC*ACGCGT*GTTCAAGACCACCATGGGCAACATG-3′, 5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′ and 5′-CGAGATC*ACGCGT*GTGAGCTCGCTGAGGCAGGAGGA-3′, respectively. They were designed with a 3′ *Xho*I (reverse primer) and 5′ MluI (forward primers) (italicised) to facilitate subsequent cloning into the *Mlu*I and *Xho*I sites of pGL3-Basic.

The fragments −540/−347, −1046/−347, −1586/−347 and −2066/−347 were constructed by the same method as described above (reverse primer: 5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′ and forward primers: 5′-CGAGATC*ACGCGT*GATCGCTCTGAGGCTTCAGGCAGG-3′, 5′-CGAGATC*ACGCGT*GTTCAAGACCACCATGGGCAACATG-3′, 5′-CGAGATC*ACGCGT*TTCTTGCTCAAGTGTAGTTTAGCACCATGG-3′ and 5′-CGAGATC*ACGCGT*CCTGGCAAACAAGGACCCAGGT-3′, respectively).

The minimal promoter (designated −506/−347 or A2min for simplicity) was cloned by PCR with −3119/−37 as a template, forward primer containing MluI (5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′) and reverse primer containing *Xho*I (5′-CTCGCTGAGGCAGGAGGAGCT-*CTCGAG*GATCCGA-3′) and ligated into pGL3-Basic using *Mlu*I and *Xho*I sites. The same strategy was exploited to prepare deletions of the A2min construct, with the latter serving as a template in PCR. The primers for 5′-deletions (−489/−347, −481/−347 and −468/−347) were as follows: reverse primer, 5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′and forward primers, 5′-CGAGATC*ACGCGT*GAGGGCGTGGCTCCAAAATGCTG-3′, 5′-CGAGATC*ACGCGT*GGCTCCAAAATGCTGGCAAT-3′ and 5′-CGAGATC*ACGCGT*TGGCAAATAAAAGCCTGGAGAGC-3′, respectively. The primers for 3′-deletions (−506/−357, −506/−373, −506/−383, −506/−393, −506/−403, −506/−413, −506/−423, −506/−433 and −506/−443) were as follows: forward primer, 5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′ and reverse primers, 5′-AAGTGGTGAGCTCGCTGAGGCA*CTCGAG*GATCCGA-3′, 5′-TGTTCCTGGGCCTCGGAAGTG*CTCGAG*GATCCGA-3′, 5′-CAAGCCGCTCTGTTCCTGGGC*CTCGAG*GATCCGA-3′, 5′-AGGAAAACTGCAAGCCGCTCT*CTCGAG*GATCCGA-3′, 5′-GCAGGAGGCACAGGAAAACTGC*CTCGAG*GATCCGA-3′, 5′-CTGGAGACCAGCAGGAGGCACA*CTCGAG*GATCCGA-3′, 5′-CAGCACCAAGCTGGAGACCAGC*CTCGAG*GATCCGA-3′, 5′-CCTGGAGAGCCAGCACCAAGCT-*CTCGAG*GATCCGA-3′ and 5′-GCTGGCAAATAAAAGCCTGGA*CTCGAG*-GATCCGA-3′, respectively.

The substitution mutants were generated by a two-step PCR procedure using overlapping inner primers with an incorporated *Eco*RI site that introduced a 6-bp mutation. The outer forward and reverse primers contained 5′ *Mlu*I and 3′ *Xho*I, respectively. The two products were digested with *Eco*RI and ligated. The purified ligation product was again subjected to PCR using the outer primers. The resulting product was cleaved with *Mlu*I and *Xho*I and ligated into the *Mlu*I and *Xho*I sites of pGL3-Basic. To generate the mutations 1–14, the A2min construct was used as a template, with forward and reverse primers listed in Table 1.

Because regions −347/−37 and −1046/−506 appeared to down-regulate the A2min plasmid, we set to analyse them for the presence of silencer elements. Designated s1 and s2, these regions were amplified from the −3119/−37 clone with primer pairs containing *Kpn*I (F: 5′-CGAGATC*GGTACC*-CTGGGGAGGGTCTGGGAATGT-3′ and R: 5′-AAGAACTCGCCATCCC-ACCTG*GGTACC*GATCCGA-3′ for s1, F: 5′-CGAGATC*GGTACC*GTTCA-AGACCACCATGGGCAA-3′ and R: 5′-CTTCAGGCAGGGGAGGCTGGT-*GGTACC*GATCCGA-3′ for s2). The products were inserted into the *Kpn*I site of pGL3-Basic (resulting in s1-s-B [sense], s1-as-B [anti-sense], s2-s-B and s2-as-B), pGL3-Promoter (s1-u-s-P [upstream of the SV40 promoter]) (Promega), s1-u-as-P, s2-u-s-P and s2-u-as-P) and A2min (s1-u-s-m [upstream of the A2min promoter]), s1-u-as-m, s2-u-s-m and s2-u-as-m). The regions s1 and s2 were also cloned into the *Hin*dIII site downstream of the SV40 and A2min promoters of pGL3-Promoter and A2min plasmids, respectively, using the same primers as above but with incorporated *Hin*dIII. These constructs were called s1-d-s-P (downstream of the SV40 promoter), s1-d-as-P, s1-d-s-m (downstream of the A2min promoter), s1-d-as-m, s2-d-s-P, s2-d-as-P, s2-d-s-m and s2-d-as-m.

All constructs were confirmed by sequence analysis.

2.2. Cell culture

The human embryonic kidney 293, human pancreatic carcinoma PANC-1 and MIA PaCa-2, mouse gonadotrope αT3 and mouse thyrotrope TαT1 cell lines were cultured in DMEM containing 10% FCS, 4.5 mg/ml glucose, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco, Karlsruhe, Germany). The TαT1 cells were seeded on Matrigel-coated plates (BD Biosciences, Heidelberg, Germany) which facilitated adhesion. Matrigel was diluted 20-fold with DMEM before coating the plates and allowed to dry before plating cells. The mouse mammary HC11 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Gibco), 5 μg/ml insulin and 10 ng/ml recombinant human epidermal growth factor (Sigma, Taufkirchen, Germany). The rat somatolactotrope GH3 cell line was grown in MEM containing 15% FCS, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco). The rat thyroid PC Cl3 cells were cultured in Coon's modified Ham's F-12 medium (Biochrom, Berlin, Germany) supplemented with 5% CS (Gibco) and a six-hormone mixture (1.5 mU/ml TSH, 10 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 3.6 ng/ml hydrocortisone and 10 ng/ml glycyl-L-histidyl-L-lysine acetate; 6H medium) (Sigma).

2.3. Transfection and luciferase assay

GH3, αT3, TαT1, 293, PC Cl3, HC11, PANC-1 or MIA PaCa-2 cells were seeded onto 12-well plates. At ca. 70–85% confluence, 300 ng of plasmids were transfected into appropriate wells with the Effectene transfection reagent (Qiagen). After 48 h, luciferase activity was determined using the Lumat LB9501 luminometer (Berthold, Wildbad, Germany). For normalisation of the luciferase activities the protein content was measured by the BioRad protein assay (BioRad, Munich, Germany). Three independent experiments, each done in triplicate, were performed and gave similar results. Figures show the mean of all experiments with error bars giving the standard deviation of the mean.

2.4. Preparation of nuclear extracts and EMSA

Nuclear extracts from 293 and GH3 cells were made using the NE-PER kit (Pierce, Bonn, Germany) as described by the manufacturer. For each preparation, ca. 40 mg of cell paste was used. All procedures were carried out at 4 °C.

Table 1
Sequences of the primers used to generate thyrostimulin A2 subunit promoter mutations

Mutation generated	PCR	Forward primer	Reverse primer
m1	1*a*	5′-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3′	5′-CTTCAGGCAGGGGAGGCTGGTTGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCCCGATCTCCAAGAGGGCGTGGC-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TGAATTCCCGATCTCCAAGAGG-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m2	1*a*	5′-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3′	5′-GGGGAGGCTGGTTTGAAAGCCGGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCAAGAGGGCGTGGCTCCAAAATG-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGGAATTCAAGAGG-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m3	1*a*	5′-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3′	5′-GGTTTGAAAGCCGATCTCCAAGGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCTGGCTCCAAAATGCTGGCAAAT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATCACGCGTTTGAAAGCCGATCTCCAAGGAA-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m4	1*a*	5′-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3′	5′-AGCCGATCTCCAAGAGGGCGTGGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCAAATGCTGGCAAATAAAAGCCT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m5	1*a*	5′-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3′	5′-AGAGGGCGTGGCTCCAAAATGCGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCATAAAAGCCTGGAGAGCCAGCA-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m6	1*a*	5′-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3′	5′-TGGCTCCAAAATGCTGGCAAATGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCCTGGAGAGCCAGCACCAAGCTG-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m7	1*a*	5′-CGAGATC*ACGCGT*GATCGCTCTGAGGCTTCAGGCAGG-3′	5′-AATGCTGGCAAATAAAAGCCTGGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCCAGCACCAAGCTGGAGACCAGC-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m8	1*a*	5′-CGAGATC*ACGCGT*GATCGCTCTGAGGCTTCAGGCAGG-3′	5′-AAAAGCCTGGAGAGCCAGCACCGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCGAGACCAGCAGGAGGCACAGGA-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m9	1*a*	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-ACCAAGCTGGAGACCAGCAGGAGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCGGAAAACTGCAAGCCGCTCTGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m10	1*a*	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-GAGGCACAGGAAAACTGCAAGCGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCGTTCCTGGGCCTCGGAAGTGGT-3′	5′-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m11	1*a*	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-GGAAAACTGCAAGCCGCTCTGTGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCGCCTCGGAAGTGGTGAGCTCGC-3′	5′-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m12	1*a*	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CAAGCCGCTCTGTTCCTGGGCCGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCGTGGTGAGCTCGCTGAGGCAGG-3′	5′-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m13	1*a*	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTGTTCCTGGGCCTCGGAAGTGGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCTCGCTGAGGCAGGAGGAGCT-3′	5′-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3′
m14	1*a*	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-GGCCTCGGAAGTGGTGAGCTCGGAATTCGATCCGA-3′
	1*b*	5′-CGAGATCGAATTCCAGGAGGAGCTCTGGGGAGGGT-3′	5′-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3′
	2	5′-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3′	5′-GCTCGGAATTCCAGGAGGAGCT*CTCGAG*GATCCGA-3′

The mutations correspond to those listed in Fig. 3. The mutated nucleotides are underlined. The introduced *Mlu*I and *Xho*I restriction sites are italicised. PCR 1a and 1b correspond to the two products generated by PCR 1.

The complementary oligonucleotides containing the wild-type or mutated sequences (Fig. 4B) were labelled separately using the biotin 3′ end DNA labelling kit (Pierce) and then annealed.

Gel shift assays were performed using the LightShift chemiluminescent EMSA kit (Pierce) according to manufacturer's instructions. The binding reaction consisted of 20 fmol labelled double-stranded oligonucleotide (wild-type or mutated, Fig. 4B) and 2 μl nuclear extract in a total volume of 20 μl. The whole reaction was run on a 6% polyacrylamide gel and electrophoretically transferred to a Biodyne B nylon membrane (Pierce). The transferred DNA was cross-linked to membrane at 120 mJ/cm^2 using a UV-light cross-linker (Biometra, Göttingen, Germany) (45 s exposure), detected by chemiluminescence and exposed to CL-Xposure X-ray film (Pierce) for 2 min. As a specific competitor, 4 pmol unlabeled wild-type oligonucleotide (200-fold excess) was used.

2.5. RT-PCR

Total RNA was isolated from GH3, αT3, TαT1, 293, PC Cl3, HC11, PANC-1 and MIA PaCa-2 cells using the QIAshredder and RNeasy mini spin column (Qiagen). A DNAse I RNAse-free treatment was performed to eliminate DNA

contamination. Single-stranded oligo(dT)-primed cDNA was generated from 250 ng total RNA using Superscript II reverse transcriptase (Invitrogen). PCR conditions were as follows: denaturation at 95 °C (1 min), annealing at 62 °C (30 s) and extension at 72 °C (2 min) for 35 cycles. Primers for human, rat and mouse thyrostimulin α-subunit were F: 5′-ATCCCAGGCTGCCACTTGCAC-3′, R: 5′-TGTCGCCTCTCTCGCTACTAG-3′, F: 5′-ATCCCAGGCTGCCAC-TTGCAC-3′, R: 5′-TGCCGTCTCTCCCGCTACTAG-3′ and F: 5′-ATCCCA-GGCTGCCACTTGCAC-3′, R: 5′-TGCCGTTTCTCCCGCTACTAG-3′, respectively; they produce an amplicon of 308 bp. Omission of the RT step led to loss of the PCR products. Primers for the GAPDH gene were: 5′ primer, 5′-ACCACAGTCCATGCCATCAC-3′ and 3′ primer, 5′-TCCACCACCCT-GTTGCTGTA-3′ (BD Biosciences). The amplification yielded a 452 bp DNA product.

2.6. In silico analysis

Analysis of the sequences for transcription factor binding sites was conducted with the program MatInspector (Quandt et al., 1995) and AliBaba2 (Grabe, 2002) using the selected matrix library (vertebrate section) and optimised thresholds.

3. Results

3.1. Cloning and deletional analysis of the A2 subunit promoter

The tentative transcription start site for the A2 gene was determined by identifying the 5′ end of the respective mRNA (GenBank accession no. NM_130769) and mapped to −427 nt relative to the ATG site. The amplified −3119/−37 genomic DNA fragment of the A2 5′-flanking region was sub-cloned in front of the luciferase reporter gene in the promoterless pGL3-Basic vector to analyse the A2 promoter activity in pituitary (GH3) and non-pituitary cell lines (293) (Fig. 1). The 3082 bp fragment, which contains the potential TATA box at −462 to −448 nt, showed only moderate promoter activity (2.5-fold in 293 and 4.4-fold in GH3 cells), indicating the presence of negative DNA regulatory elements. We performed incremental 5′ deletions extending to −373 (Fig. 1). There was a similar overall decrease in activity between −3119 and −373 in both cell lines. However, when the region between −373 and −37 was deleted, we observed a striking increase of the promoter activity, with the strongest activation being detected for the −506/−347 fragment in 293 cells of 50-fold and in GH3 cells of 14.8-fold. This indicated that the −506/−347 fragment contains the minimal promoter.

To delineate the minimal promoter region, we performed 5′ and 3′ deletions of the −506/−347 fragment. The 293 and GH3 cells were transfected with luciferase expression constructs containing each of the deleted promoters. Results of these experiments are shown in Fig. 2. Deletion of sequence between −506 and −489 resulted in a 5.7-fold decrease in the activity in 293 cells and 6.2-fold in GH3 cells. On the other hand, when the regions from −489 to −481 and from −481 to −468 were deleted, the promoter activity was almost completely abolished in both cell lines studied. From the 3′ end, deletion from −357 to −347 resulted in a strong decrease in the activity of the −506/−347 fragment (11.1-fold in 293 cells and 13.9-fold in GH3 cells). Curiously, further deletion of extra 16 nt (−373 to −347) reduced this inhibitory effect to only 2.7-fold and 1.2-fold in 293 and GH3 cells, respectively. Further deletions from −433 to −373 showed ca. two- to three-fold drop in the promoter activity in both cell lines, leading us to conclude that the minimal promoter region for A2 gene lies between the −506 and −347 nt. We designated this minimal promoter −506/−347 or A2min.

3.2. Substitution mutant analysis of the A2 subunit promoter

Our next approach was to introduce a series of 6-bp substitution mutations across the region from −506 to −347 nt. The mutations were introduced into the potential transcription factor binding sites predicted by MatInspector and AliBaba2 analysis (Fig. 3A and B). All mutants were prepared in the context of the −506/−347 minimal promoter fragment and were placed upstream of the luciferase reporter gene in pGL3-Basic. Each mutant was compared to the wild-type promoter that was transfected in parallel. The 293 and GH3 cells were transfected with luciferase expression constructs containing each of the mutated promoters, and the results are shown in Fig. 3C. The muta-

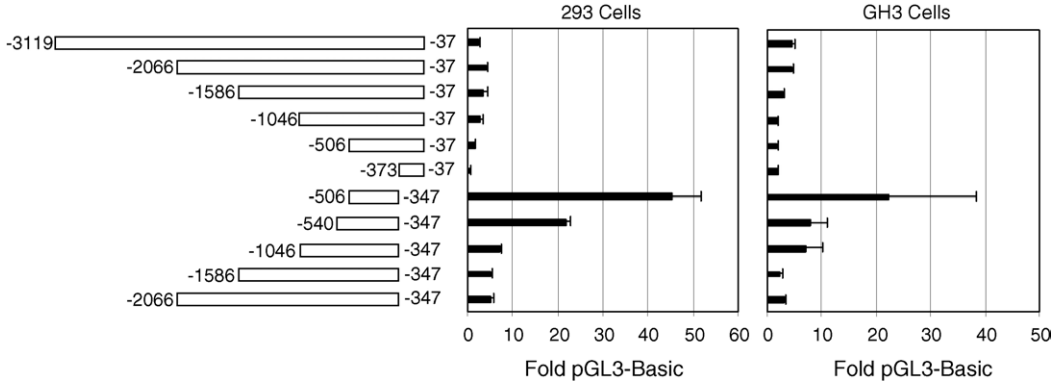


Fig. 1. Transient expression analysis of the A2 promoter activity. Luciferase reporter constructs containing various lengths of the A2 5′ flanking region were transfected into 293 and GH3 cells. The constructs are represented schematically on the vertical axis. All numbering is relative to the ATG start codon of the A2 gene. The luciferase values were expressed as fold stimulation of the parent promoter-less vector pGL3-Basic, which was set to one, and represent mean ± S.D. of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns.

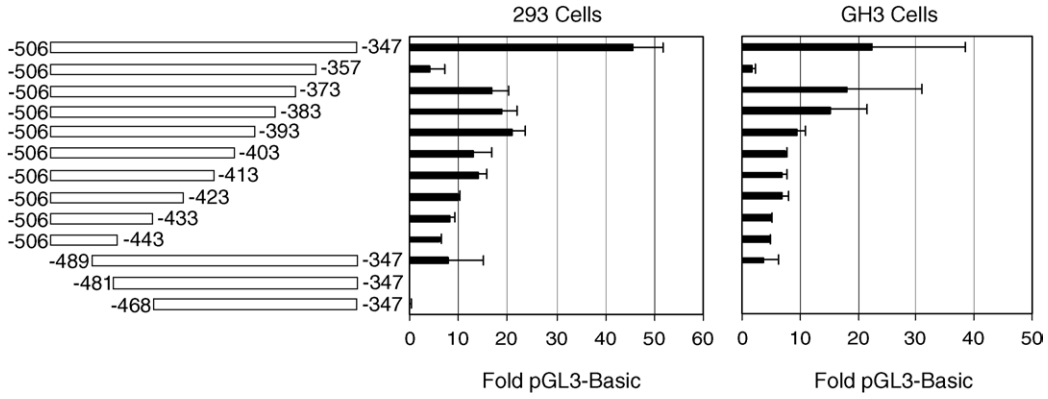


Fig. 2. Transient expression analysis of the A2 promoter deletion constructs. The experimental design and the calculation of data were the same as for Fig. 1.

tion of the first half of the putative Sp1 site (m3), the middle of the TATA box (m6) and the TTF-1 site partially overlapping the TATA box (m7) resulted in a profound decrease of activity of 25-, 16.7- and 4.3-fold in 293 cells and of 32.7-, 49- and 10.9-fold in GH3 cells, respectively. In contrast, the mutation of the NF-1 site (m8) lowered transcriptional activity only slightly (by 1-fold in 293 cells and by 1.7-fold in GH3 cells), suggesting that this region does not contain a stimulatory element. In 293 cells, five mutants m10, m11, m12, m13 and m14 (which correspond to the mutated GAGA, two Ets, c-Myb and AP-1 sites) exhibited strongly reduced promoter activities of 16.7-, 9.2-, 7.5-, 13.2- and 9-fold, respectively. The same mutations also decreased transcriptional activity in GH3 cells, although to a slightly lesser extent (by 16.3-, 10.9-, 6.1-, 6.1- and 3.6-fold, respectively). The mutations m1 (mutated LSF site), m2 (mutated unknown adjacent region), m4 (mutated second half of the Sp1 site) and m5 (mutated Oct-1 site) markedly lowered promoter activities by 3.8-, 2.6-, 3.3- and 3.6-fold in 293 cells and by 3.6-, 3.6-, 2.8- and 4-fold in GH3 cells. Strongly reduced promoter activity was observed with m9 (mutated Pax-5 site) in GH3 (of 7.5-fold) but not in 293 cells (of 2.5-fold).

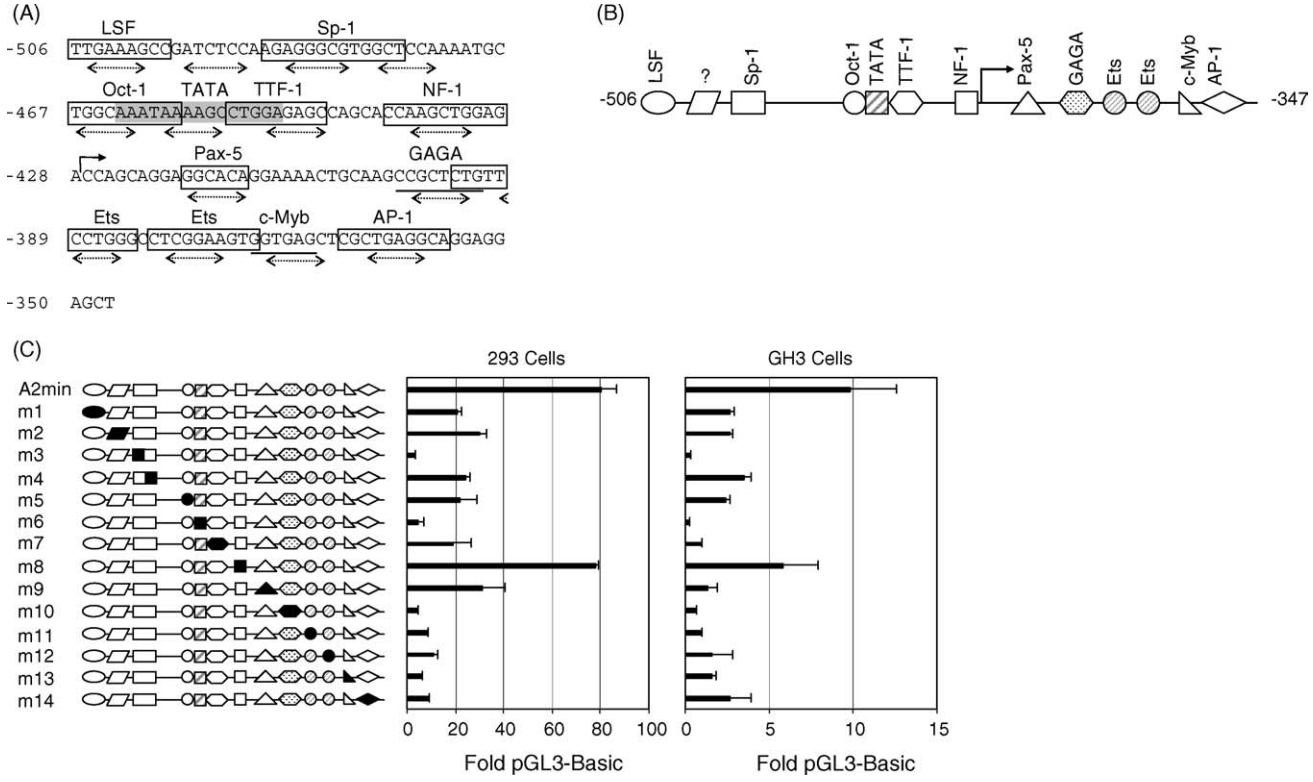


Fig. 3. Luciferase activity of the A2 minimal promoter substitution mutants. The location of each mutation (dotted arrow with heads pointing in opposite directions) relative to the transcription start site (bent arrow) is schematically indicated. The TATA box is shaded in grey and the predicted transcription factor binding sites are boxed or underlined (A). The consensus transcription factor binding sites are schematically drawn (B). 293 and GH3 cells were transfected with the mutation constructs, with the mutated nucleotides marked by black boxes on the vertical axis (C). All numbering is relative to the ATG start codon of the A2 gene. The luciferase values were expressed as fold stimulation of the parent promoter-less vector pGL3-Basic, which was set to one, and represent mean ± S.D. of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns.

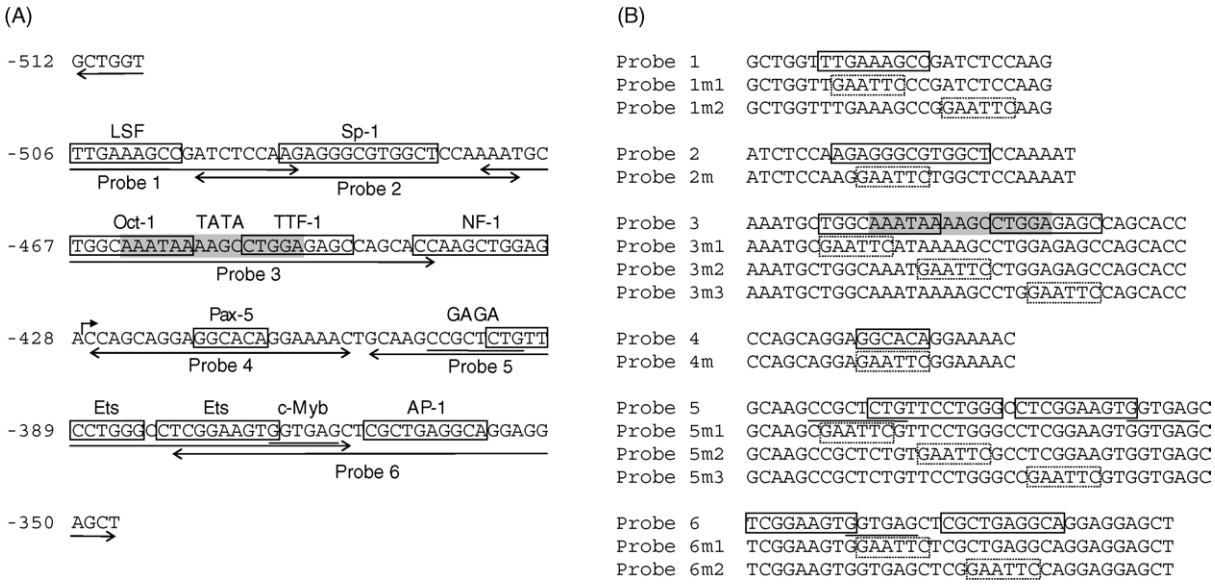


Fig. 4. Sequence of the wild type and mutated probes used in EMSA. The location of each probe (arrow with heads pointing in opposite directions) relative to the transcription start site (bent arrow) is schematically indicated. The TATA box is shaded in grey and the predicted transcription factor binding sites are boxed or underlined (A). The mutations are indicated by m (dotted box) (B).

3.3. Characterisation of the factors binding the functionally relevant sites within the A2 subunit promoter

To further assess whether the putative elements revealed by an in silico analysis and transient transfection assays were functional, we performed EMSAs with 293 and GH3 nuclear extracts. A series of double-stranded biotin-labelled oligonucleotide probes were generated by PCR amplification (Fig. 4A and B). The incubation of 293 and GH3 extracts with probe 2 revealed two protein-DNA complexes 2a and 2b, though the latter was much more intense in 293 extracts (Fig. 5). Three complexes 3a, 3b and 3c were detected when nuclear extracts from both cells types were incubated with probe 3. Here, the complexes 3a and 3b also appeared more abundant in 293 extracts. Probe 4 yielded a single complex 4a, which, again, was more intense in 293 extracts. The shift pattern observed with probe

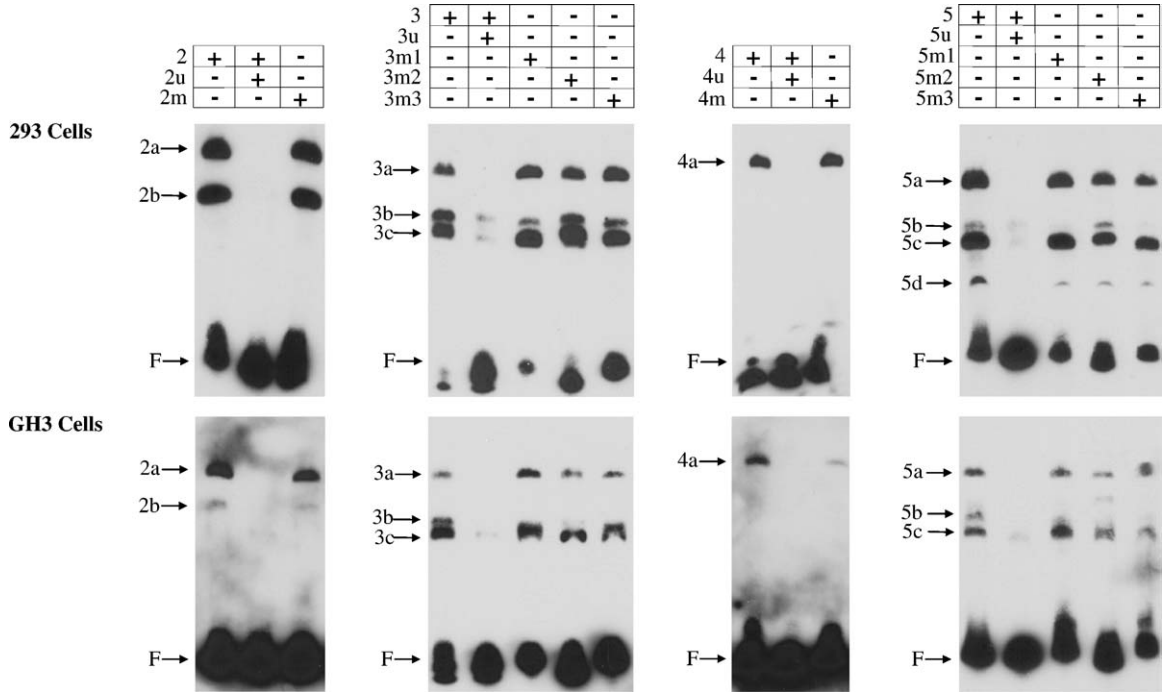


Fig. 5. EMSA shows complexes formed on the wild type and mutated probes in nuclear extracts from 293 and GH3 cells. Probes numbered as in Fig. 4. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

5 (complexes 5a, 5b, 5c and 5d) was similar to that obtained with probe 3, except that an added low-intensity complex 5d was formed in extracts from 293 cells. Furthermore, complexes 5a and 5c were far more abundant in extracts from 293 cells compared to those in GH3 extracts. One complex was formed on probe 1, whereas probe 6 resulted in the formation of three complexes in extracts from both cell lines tested (data not shown).

In competition experiments, 200-fold excess of unlabeled probes 1–6 abolished all complex formation in nuclear extracts from both cell lines, with an exception of 293 extracts where complexes 3b and 3c were strongly reduced (Fig. 5). We next prepared biotin-labelled probes containing mutated potential transcription factor binding sites (that eliminated promoter function in luciferase assays, Figs. 3A and 4B) to see if the observed complexes involve the mutated nucleotides. The equal amounts of the mutated and wild-type probes (20 fmol) were used to make sure that they possess similar specific activity. As a result, in 293 extracts the formation of complex 5b was abolished on probes 5m1 and 5m3 and in GH3 extracts two complexes 3b and 5b were eradicated on the corresponding probes 3m1, 3m2, 3m3, 5m1, 5m2 and 5m3 (Fig. 5). The abundance of the following complexes was diminished: complex 3b formed on probes 3m1 and 3m3, complex 5d on 5m1, 5m2 and 5m3 (293 extracts) and complex 4a on 4m (GH3 extracts). With regard to the remaining few complexes, we found their formation unaffected on the mutated probes (data not shown).

3.4. Characterisation of the upstream and downstream candidate silencer elements of the A2 subunit promoter

The observation that regions −347/−37 (s1, downstream of the minimal promoter) and −1046/−506 (s2, upstream of the minimal promoter) reduced the activity of the minimal promoter led us to next test them for the presence of silencer or negative regulatory elements. The fragments s1 and s2 were placed in both orientations upstream and downstream of the minimal promoter of the A2min plasmid. Similarly, the same fragments were placed in both directions upstream and downstream of the SV40 promoter of the pGL3-Promoter vector. These fragments were also cloned in both orientations into the promoterless vector pGL3-Basic. The resulting plasmids were transfected into pituitary (GH3) and non-pituitary cells (293) and their activities compared with the activity of the corresponding parent plasmids.

The fragment s1 fused to pGL3-Basic (s1-s-B [sense] and s1-as-B [anti-sense]) markedly suppressed the basal transcription mediated by pGL3-Basic in both cell lines (Fig. 6). When s1 was cloned upstream of the respective promoters of the A2min (s1-u-s-m and s1-u-as-m) and pGL3-Promoter (s1-u-s-P and s1-u-as-P), a strong inhibitory effect was observed in 293 (4- and 2.3-fold of A2min, 4- and 44-fold of pGL3-Promoter). The decrease was even stronger in GH3 cells (4- and 8-fold of A2min, 32- and 511-fold of pGL3-Promoter). Strikingly, the placement of the same fragment downstream of the respective promoters of the A2min (s1-d-s-m and s1-d-as-m) and pGL3-Promoter (s1-d-s-P and s1-d-as-P) resulted in a nearly complete abolishment of the transcription of these reporter genes in both cell lines studied.

We further examined how the fragment s2 altered the activity of parent reporter plasmids. No transcriptional activity was demonstrable in either cell line when s2 was fused to pGL3-Basic (s2-s-B and s2-as-B) (Fig. 7). The placement of s2 upstream of the minimal promoter of the A2min plasmid (s2-u-s-m and s2-u-as-m) only moderately suppressed the activity (by 2.4- and 3.3-fold in 293 cells and by 2- and 5-fold in GH3 cells). However, when s2 was cloned upstream of the SV40 promoter of the pGL3-Promoter (s2-u-s-P and s2-u-as-P), the activity was reduced more efficiently by 3.5- and 5.7-fold in 293 cells and by 8.2- and 23-fold in GH3 cells. As in the case of s1, the positioning of s2 downstream of the respective promoters of the

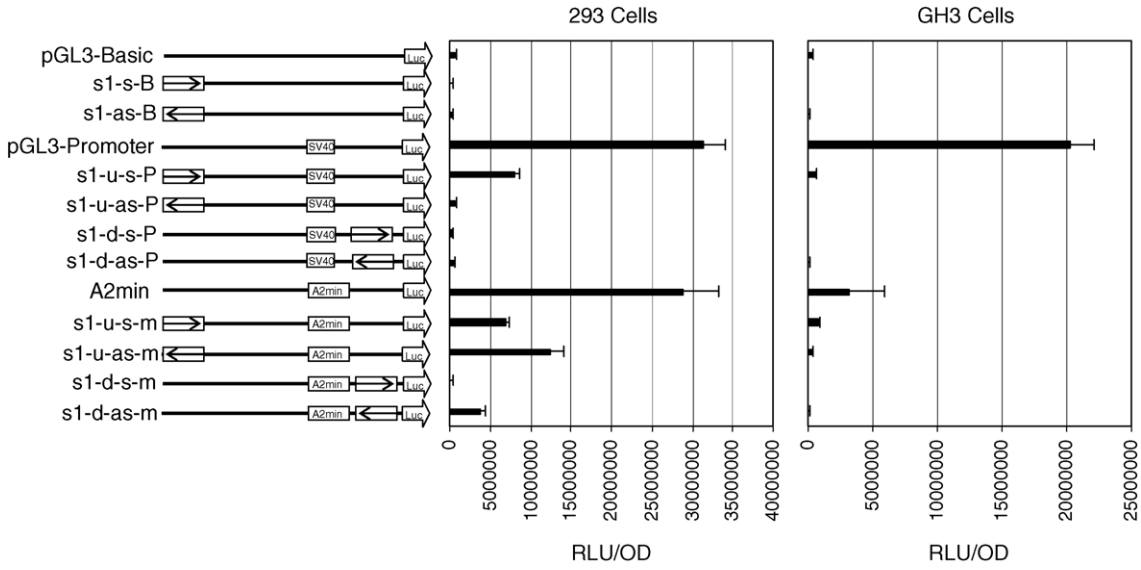


Fig. 6. Region s1 represses transcription from native and heterologous promoters in a position- and orientation-independent manner. The resulting relative luciferase values after transfection into 293 and GH3 cells represent mean ± S.D. of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns. The constructs are drawn schematically on the vertical axis, with the A2 minimal promoter designated as A2min.

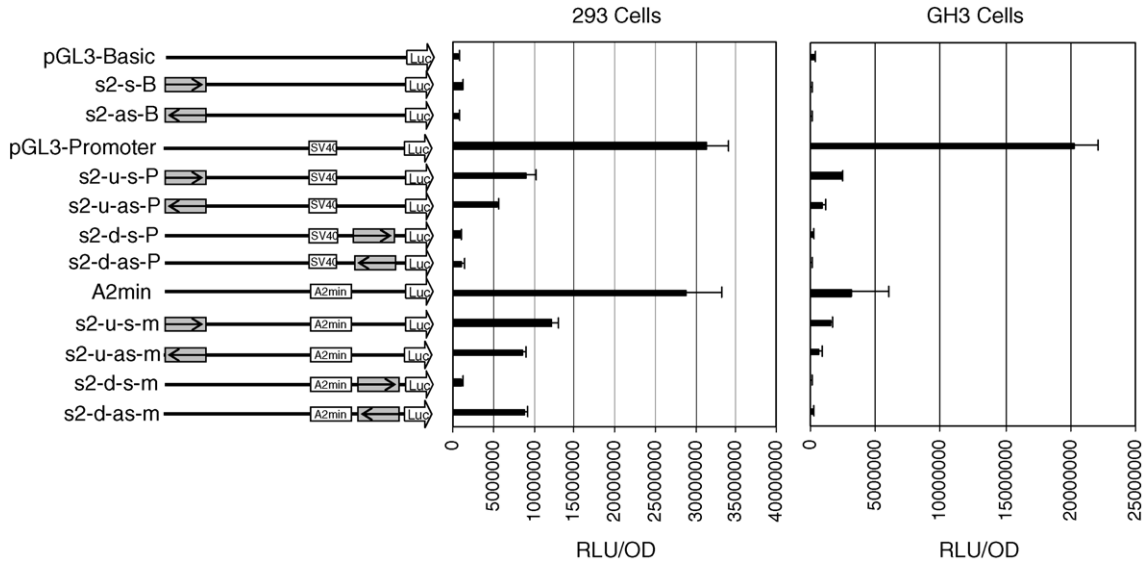


Fig. 7. Region s2 represses transcription from native and heterologous promoters in a position- and orientation-independent manner. The experimental design and the calculation of data were the same as for Fig. 6.

A2min (s2-d-s-m and s2-d-as-m) and pGL3-Promoter (s2-d-s-P and s2-d-as-P) resulted in an almost complete abrogation of the promoter activity of these genes.

3.5. Comparative analysis of the A2 subunit promoter in different cell types

We first examined the expression of A2 in diverse cell lines by the RT-PCR analysis. As shown in Fig. 8A, the A2 transcript consistent with an expected size of 308 bp was found in 293, GH3, HC11, PANC-1, MIA PaCa-2, αT3 and TαT1 cell lines, but was not detected in PC Cl3 cells. The pituitary cell lines GH3, αT3 and TαT1 showed the highest expression of A2. After that, the above eight cell lines were transiently transfected with the A2min plasmid to elucidate cell-specific regulation of the A2 gene (Fig. 8B). We were able to demonstrate strong promoter activity in all cell lines, the former being at its highest in 293 cells.

4. Discussion

Although much is currently known about the glycoprotein hormone α-subunit transcription, nothing is known about the transcriptional regulation of the thyrostimulin A2 subunit. In an effort to explore how A2 expression is regulated transcriptionally, the 5′-flanking region of the A2 gene extending from −3119 to −37 nt relative to the ATG initiation codon was fused to luciferase reporter gene in the promoter-less vector pGL3-Basic. This fragment contained the putative transcription initiation site at −427 nt relative to the ATG and an AT-rich sequence that was likely to function as a TATA box at −462 to −448 nt. Following transfection into human embryonic kidney 293 and pituitary rat somatolactotrope GH3 cell lines, the 3082-bp fragment was found to exhibit promoter activity in both cell types. By using a series of deletion constructs we were able to confine this activity to a rather short stretch of DNA between −506 and −347. We next subjected it to further deletions from both ends of the molecule. The results of this deletional analysis provided direct evidence that the region from −506 to −347 indeed represents the minimal sequence which supports full activity. For simplicity, we have termed this minimal promoter A2min. Moreover,

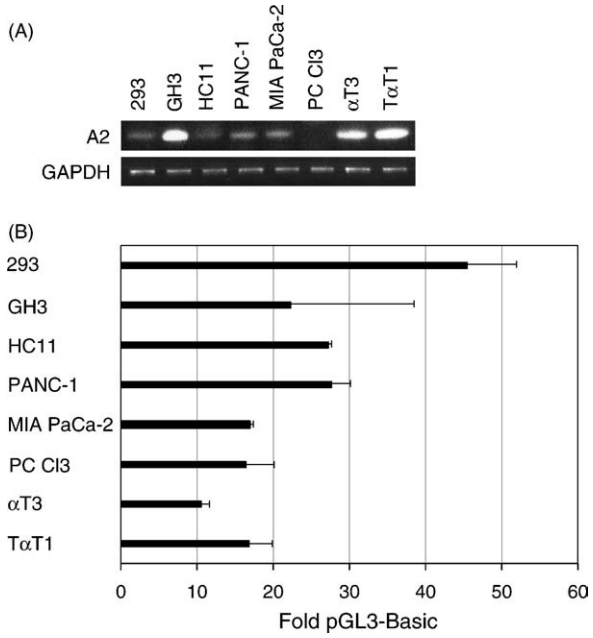


Fig. 8. Expression of A2 and luciferase activity of its minimal promoter in various cell lines. (A) RT-PCR analysis of A2 transcript in 293, GH3, HC11, PANC-1, MIA PaCa-2, PC Cl3, αT3 and TαT1 cells. Amplification of the GAPDH message levels reflected differences in cDNA loading. (B) Transient expression analysis of the A2 promoter activity in aforementioned cells. The luciferase values were expressed as fold stimulation of the parent promoter-less vector pGL3-Basic, which was set to one, and represent mean ± S.D. of three experiments done in triplicate.

deletional analysis revealed that the sequence between −506 and −468 appears essential for A2min activity. We also note a curious behaviour. In the three cell lines studied, deletion of sequence from −357 to −347 inhibited promoter activity almost completely, however, further removal of nucleotides from −373 to −357 restored this activity to ca. two-fold of wild-type. According to the in silico analysis, the region between −357 and −347 contains the half site for the transcription factor AP-1, whereas the region from −373 to −357 contains the other AP-1 half site, an adjacent c-Myb binding motif and the half site for the Ets factor. Essentially, the c-Myb transcription factor is composed of three functional domains responsible for DNA binding, transcriptional activation and negative regulation (Gonda, 1998). It seems possible, therefore, that the simplest explanation of the aforementioned behaviour is masking of the c-Myb negative regulatory domain (NRD) by the Jun and Fos proteins binding to their AP-1 site in A2min (nucleotides −506 to −347). This is further supported by the observation that the NRD contains a leucine zipper-like motif (Biedenkapp et al., 1988). Because leucine zippers generally mediate protein-protein interactions, it seems likely that the Myb leucine zipper promotes association between c-Myb and another protein which inhibits Myb function (Tavner et al., 1998). The silencing of the activity of A2min by the removal of base pairs from −357 to −347 could be caused by the disturbed binding of Jun and Fos that frees the c-Myb NRD, thereby rendering it functional. Such repression might be overcome by the deletion from −373 to −357 that eliminates the c-Myb NRD leading to increased trans-activation, consistent with previous reports that the truncation of this domain from c-Myb markedly increases its transcriptional activation capacity (Kalkbrenner et al., 1990; Sakura et al., 1989).

We also accessed the tissue specificity of A2min by testing its activity in the A2-producing mouse thyrotrope TαT1, mouse gonadotrope αT3, mouse mammary HC11, human pancreatic carcinoma PANC-1 and MIA PaCa-2 cells and the A2-non-producing rat thyroid PC Cl3 cell line, in addition to the A2-producing 293 and GH3 cells. As a result, A2min was found to be very active in transient transfections in all cell lines tested, suggesting that this promoter might be non-tissue-specific. According to the hypothesis of Cedar (1988), DNA methylation in vivo may be responsible for the transcriptional inhibition of tissue-specific genes in non-expressing cells. Hence, the endogenous A2 gene may be inactive because it is methylated in PC Cl3 cells, but the exogenous A2 promoter construct is active because it is non-methylated.

By analogy with the α-subunit gene, whose tissue-specific gene expression was conferred by an enhancer (Delegeane et al., 1987), the observed non-specificity of A2min could also be attributable to the presence of a tissue-specific enhancer around the A2 gene.

We have further introduced substitution mutations into potential binding sites for transcription factors predicted by in silico analysis. All mutations were generated in the context of A2min with the 6 bp replaced by an *Eco*RI restriction site. This mutant analysis identified three mutations that resulted in an almost complete loss of promoter function in 293 and GH3 cell lines. Here, in construct m3, the strong effect of the mutated half site for Sp1 was in accord with data obtained by employing deletional analysis, where the Sp1-containing region from −506 to −468 appeared essential. Abolition of promoter activity of m6 was caused by the mutation of the central part of the TATA box, while the near inhibition of m7 activity can be due either to the mutated TTF-1 motif overlapping with the TATA box or to the mutated last two bases of the TATA box. The promoter was down-regulated relatively strongly by mutations of the putative binding sites for the Late SV40 Factor (LSF) (m1), region adjacent to the LSF site (m2, unidentified by in silico analysis), second half of the Sp1 site (m4), Oct-1 factor (m5) and Ets factors (m11 and m12) in the above cell types. Of these factors, LSF has been suggested to play a role in the regulation of cell cycle progression and cell survival (Powell et al., 2000; Bruni et al., 2002), however, LSF-regulated genes have not yet been identified. Similarly, the Ets gene family has been implicated in a variety of biological pathways that regulate cell growth, differentiation and apoptosis, but no clear unifying theme has emerged (Sharrocks, 2001). Sp1 is a known regulator of gene expression of multiple target genes, which acts through the GC-box or GT-box elements in the promoter/enhancers (Li et al., 2004; Zhao and Meng, 2005). Protein Oct-1, which is probably contained in all proliferating eukaryotic cells, controls numerous housekeeping and tissue-specific genes. The latter include the genes for gonadoliberin (Chandran et al., 1999), prolactin (Voss et al., 1991), TSH (Pruijn et al., 1988), TTF-1 (Bingle and Gowan, 1996) and Pit-1 (Delhase et al., 1996). It therefore seems plausible that these ubiquitously expressed transcription factors may regulate A2 gene expression. In case where the Pax-5 motif was mutated (m9), the promoter activity was profoundly diminished only in GH3 cell line. It seems reasonable to suspect that in 293 cells the additional, not yet mutated, nucleotides are involved in Pax-5 binding. Since there was almost no effect on the activity when the binding motif for Nuclear Factor-1 (NF-1) was mutated, it is difficult to ascertain whether this site corresponds to authentic control element. To investigate the possibility of the NF-1 activity being redundant with the activities of the neighbouring factors, simultaneous mutations of the binding sites for the latter proteins are needed.

We used EMSAs to subject the regions containing the identified transcription factor recognition sites to further functional analysis. Nuclear extracts from 293 and GH3 cell lines were employed to bind probes comprising the above-described sites. Probe 2 encompassed the site for the region adjacent to LSF as well as the Sp1 consensus sequence. Two specific complexes 2a and 2b were seen in the nuclear extracts from the two cell lines we examined, with both shifts being in much higher concentration in 293 extracts. The complex 2a most likely contains Sp1. On the other hand, the labelled probe 2m, where the mutated half site for Sp1 resulted in the absence of promoter activity, still formed the 2a complex. One possible explanation is that the Sp1 protein retains binding affinity for its partially mutated recognition motif but fails to participate in transcriptional activation. This is supported by the study of Pfau and Taylor (1998) where the mutated ToxR protein strongly bound to DNA but failed to activate transcription from the *ctx* promoter. In addition, several mutations in the DNA-binding domain of Myb-related proteins have been

described that can bind DNA but are defective for transcriptional activation (Grasser et al., 1992; Goff et al., 1991). Further, three specific complexes 3a, 3b and 3c were seen bound to probe 3 in nuclear extracts from both cell lines. This probe includes the TATA box and the binding sites for Oct-1 and TTF-1, both of which partially overlap with the former. The intensity of 3a, 3b and 3c was the highest in 293 extracts, indicating that the proteins contained are more abundant in these cells. Interestingly, each of the individual mutations of the sites for the aforementioned factors (labelled probes 3m1, 3m2 and 3m3) appeared to abolish the formation of complex 3b in extracts of both cell lines tested (with the exception of 293 extracts, where 3b formation was eliminated by 3m1 and 3m3 and strongly reduced by 3m2). Probe 3 encompasses the nucleotides from −44 to −11 relative to the tentative transcription start site. Because the TFIID DNase I footprint often extends from −40 to +50 (Burley and Roeder, 1996), we think that the abolition of 3b formation could be caused by the TFIID binding across all probe, thereby requiring all the three sites disturbed in probes 3m1, 3m2 and 3m3. As TFIID does not contact all of the bases in the footprint (Chiang et al., 1993), it remains to be determined whether the Oct-1 and TTF-1 factors bind in a sequence-specific fashion within these "open" regions or they exert their effects indirectly, perhaps by interacting with TFIID. With regard to probe 4 that contains Pax-5 binding site, a single specific complex 4a was formed in the extracts from both cell lines studied. We were able to show that 4a was significantly diminished on the labelled probe 4m in GH3 extracts. This finding was expected because of the strongly reduced promoter activity of the construct with the mutated Pax-5 site that we noticed only in GH3 cells, making it likely that the additional nucleotides may be necessary for Pax-5 binding in 293 extracts. Probe 5 was designed to enclose the putative motif for the GAGA factor, two sites for the Ets factor and the site for c-Myb. In 293 extracts, four specific shifted complexes 5a, 5b, 5c and 5d were observed. The formation of the weak complex 5b was eradicated by the mutation of the GAGA motif (probe 5m1) and also by the mutation of the second Ets site (probe 5m3). The also low abundant complex 5d was strongly diminished on probes 5m1 and 5m3 as well as on 5m2, where the first Ets site was mutated. Alternatively, in GH3 extracts, only three complexes 5a, 5b and 5c were formed. Here, the binding of 5b was inhibited on probes 5m1, 5m2 and 5m3. Taken together, these data raise the possibility that three nuclear factors bind cooperatively to the predicted motifs for GAGA, Ets and c-Myb factors on probe 5. Out of these three factors, Ets and c-Myb were in fact found to act synergistically to activate transcription of several important hematopoietic genes (Postigo et al., 1997).

The preceding deletional studies uncovered two potential negative regulatory elements residing in regions from −347 to −37 nt (s1, located downstream of the minimal promoter) and from −1046 to −506 nt (s2, located upstream of the minimal promoter). More importantly, the placement of the region s1 in forward and reverse orientations into the A2min plasmid in two positions, upstream and downstream of the A2min promoter, resulted in a striking decrease of promoter activity in both cell lines examined, being particularly strong in GH3 cells. Thus, the s1 negative regulatory region functions in different positions and in both orientations, properties characteristic of a transcriptional silencer. To determine whether s1 can exert its effect on a heterologous promoter, we inserted it in both directions into the enhancer-less pGL3-Promoter vector upstream and downstream of the SV40 promoter. As anticipated, the resultant down-regulation was even more intense than that of A2min. Our results therefore provide direct evidence that the s1 negative regulatory region can repress the basal activity of a strong heterologous constitutive promoter and functions as a silencer in a position- and orientation-independent manner. Above all, based on the computer-assisted sequence analysis, s1 was found to contain binding sites for several transcription factors, including a site for the transcription repressor CBF1/RBP-Jκ at position −338, Oct-1 site at −282, GATA-1 site at −233 and PEA3 site at −135. Among these, the C-promoter binding factor 1 (CBF1 or RBP-Jκ) is a known mediator of transcriptional silencing (Makar et al., 2001). The ubiquitous POU homeodomain protein Oct-1 has also been shown to possess silencing activity (Kim et al., 1996). Furthermore, the erythroid transcription factor GATA-1 was found to repress various genes (Nerlov et al., 2000; Li et al., 1998), as well as a member of the Ets transcription factor family PEA3 that was shown to repress the erbB-2 oncogene (Menendez et al., 2005). It seems possible, therefore, that these elements are responsible for transcriptional repression of the A2min and SV40 promoters by s1. We used the same positional cloning approach to analyse the second region s2 and hence determined that it indeed behaves as a silencer whose action is promoter, position and orientation independent. However, the suppression of A2min and SV40 activity by s2 was shallower than that by s1. In silico analysis of this region predicted binding sites for GATA-1 at positions −963, −886, −800, −741 and −615, for Oct-1 at −608 and for the POU homeodomain factor Brn-3 at −828. Previous studies have identified three distinct Brn-3 factors, Brn-3a, Brn-3b and Brn-3c, of which Brn-3b was found to repress transcription from several promoters (Dawson et al., 1996; Latchman, 1996). Because of the repressor abilities of the abovementioned transcription factors, we propose that they may contribute to the restriction of promoter activity by s2. To sum up the role of silencers in A2min modulation, further studies are required to determine whether their entire regions are required for silencer activity or whether sub-fragments spanning parts of these regions may be sufficient for transcriptional repression.

In this paper, for the first time we describe the cloning of the minimal promoter of the human thyrostimulin A2 subunit, whose regulation appears slightly different in 293 and GH3 cell types. Various nuclear levels of transcription factors might contribute to this difference. The transcriptional control of the A2 gene is also different to that of the α-subunit, which was shown to be regulated by TRH via pathways mediated by the P-Lim and CREB transcription factors (Hashimoto et al., 2005). Indeed, TRH treatment of 293 and GH3 cells transiently transfected with the minimal promoter did not affect the transcriptional behaviour of the latter (unpublished observations). We also revealed silencer elements upstream and downstream the promoter that underscores the complexity of the control of A2 transcription. Future work will need to further characterise the

regulatory elements uncovered in our study and to determine how these factors interplay with one another to regulate the A2 subunit gene.

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References

Biedenkapp, H., Borgmeyer, U., Sippel, A.E., Klempnauer, K.H., 1988. Viral myb oncogene encodes a sequence-specific DNA-binding activity. Nature 335 (6193), 835–837.

Bingle, C.D., Gowan, S., 1996. Oct-1 interacts with conserved motifs in the human thyroid transcription factor 1 gene minimal promoter. Biochem. J. 319 (Pt 3), 669–674.

Bruni, P., Minopoli, G., Brancaccio, T., Napolitano, M., Faraonio, R., Zambrano, N., Hansen, U., Russo, T., 2002. Fe65, a ligand of the Alzheimer's beta-amyloid precursor protein, blocks cell cycle progression by down-regulating thymidylate synthase expression. J. Biol. Chem. 277 (38), 35481–35488.

Burley, S.K., Roeder, R.G., 1996. Biochemistry and structural biology of transcription factor IID (TFIID). Annu. Rev. Biochem. 65, 769–799.

Cedar, H., 1988. DNA methylation and gene activity. Cell 53 (1), 3–4.

Chandran, U.R., Warren, B.S., Baumann, C.T., Hager, G.L., DeFranco, D.B., 1999. The glucocorticoid receptor is tethered to DNA-bound Oct-1 at the mouse gonadotropin-releasing hormone distal negative glucocorticoid response element. J. Biol. Chem. 274 (4), 2372–2378.

Chiang, C.M., Ge, H., Wang, Z., Hoffmann, A., Roeder, R.G., 1993. Unique TATA-binding protein-containing complexes and cofactors involved in transcription by RNA polymerases II and III. EMBO J. 12 (7), 2749–2762.

Dawson, S.J., Morris, P.J., Latchman, D.S., 1996. A single amino acid change converts an inhibitory transcription factor into an activator. J. Biol. Chem. 271 (20), 11631–11633.

Delegeane, A.M., Ferland, L.H., Mellon, P.L., 1987. Tissue-specific enhancer of the human glycoprotein hormone alpha-subunit gene: dependence on cyclic AMP-inducible elements. Mol. Cell. Biol. 7 (11), 3994–4002.

Delhase, M., Castrillo, J.L., de la Hoya, M., Rajas, F., Hooghe-Peters, E.L., 1996. AP-1 and Oct-1 transcription factors down-regulate the expression of the human PIT1/GHF1 gene. J. Biol. Chem. 271 (50), 32349–32358.

Goff, S.A., Cone, K.C., Fromm, M.E., 1991. Identification of functional domains in the maize transcriptional activator C1: comparison of wild-type and dominant inhibitor proteins. Genes Dev. 5 (2), 298–309.

Gonda, T.J., 1998. The c-Myb oncoprotein. Int. J. Biochem. Cell. Biol. 30 (5), 547–551.

Grabe, N., 2002. AliBaba2: context specific identification of transcription factor binding sites. In Silico Biol. 2 (1), S1–S15.

Grasser, F.A., LaMontagne, K., Whittaker, L., Stohr, S., Lipsick, J.S., 1992. A highly conserved cysteine in the v-Myb DNA-binding domain is essential for transformation and transcriptional trans-activation. Oncogene 7 (5), 1005–1009.

Hashimoto, K., Yamada, M., Monden, T., Satoh, T., Wondisford, F.E., Mori, M., 2005. Thyrotropin-releasing hormone (TRH) specific interaction between amino terminus of P-Lim and CREB binding protein (CBP). Mol. Cell. Endocrinol. 229 (1–2), 11–20.

Ikekubo, K., Hino, M., Saiki, Y., Son, C., Iwakura, T., Kobayashi, H., Ishihara, T., 2005. Immeasurably low and non-TRH-stimulatable TSH associated with normal I-123 uptake in two goitrous euthyroid patients: possible existence of other thyroid-hormone regulated thyroid stimulators other than TSH. Endocr. J. 52 (1), 61–68.

Kalkbrenner, F., Guehmann, S., Moelling, K., 1990. Transcriptional activation by human c-myb and v-myb genes. Oncogene 5 (5), 657–661.

Kim, M.K., Lesoon-Wood, L.A., Weintraub, B.D., Chung, J.H., 1996. A soluble transcription factor, Oct-1, is also found in the insoluble nuclear matrix and possesses silencing activity in its alanine-rich domain. Mol. Cell. Biol. 16 (8), 4366–4377.

Latchman, D.S., 1996. Activation and repression of gene expression by POU family transcription factors. Philos. Trans. R. Soc. Lond. B Biol. Sci. 351 (1339), 511–515.

Li, J., Noguchi, C.T., Miller, W., Hardison, R., Schechter, A.N., 1998. Multiple regulatory elements in the 5′-flanking sequence of the human epsilon-globin gene. J. Biol. Chem. 273 (17), 10202–10209.

Macdonald, L.E., Wortley, K.E., Gowen, L.C., Anderson, K.D., Murray, J.D., Poueymirou, W.T., Simmons, M.V., Barber, D., Valenzuela, D.M., Economides, A.N., Wiegand, S.J., Yancopoulos, G.D., Sleeman, M.W., Murphy, A.J., 2005. Resistance to diet-induced obesity in mice globally overexpressing OGH/GPB5. Proc. Natl. Acad. Sci. U.S.A. 102 (7), 2496–2501.

Makar, K.W., Ulgiati, D., Hagman, J., Holers, V.M., 2001. A site in the complement receptor 2 (CR2/CD21) silencer is necessary for lineage specific transcriptional regulation. Int. Immunol. 13 (5), 657–664.

Menendez, J.A., Vellon, L., Lupu, R., 2005. Antitumoral actions of the anti-obesity drug orlistat (XenicalTM) in breast cancer cells: blockade of cell cycle progression, promotion of apoptotic cell death and PEA3-mediated transcriptional repression of Her2/neu (erbB-2) oncogene. Ann. Oncol. (Epub ahead of print).

Nakabayashi, K., Matsumi, H., Bhalla, A., Bae, J., Mosselman, S., Hsu, S.Y., Hsueh, A.J., 2002. Thyrostimulin, a heterodimer of two new human glycoprotein hormone subunits, activates the thyroid-stimulating hormone receptor. J. Clin. Invest. 109 (11), 1445–1452.

Nerlov, C., Querfurth, E., Kulessa, H., Graf, T., 2000. GATA-1 interacts with the myeloid PU.1 transcription factor and represses PU.1-dependent transcription. Blood 95 (8), 2543–2551.

Pfau, J.D., Taylor, R.K., 1998. Mutations in toxR and toxS that separate transcriptional activation from DNA binding at the cholera toxin gene promoter. J. Bacteriol. 180 (17), 4724–4733.

Postigo, A.A., Sheppard, A.M., Mucenski, M.L., Dean, D.C., 1997. c-Myb and Ets proteins synergize to overcome transcriptional repression by ZEB. EMBO J. 16 (13), 3924–3934.

Powell, C.M., Rudge, T.L., Zhu, Q., Johnson, L.F., Hansen, U., 2000. Inhibition of the mammalian transcription factor LSF induces S-phase-dependent apoptosis by downregulating thymidylate synthase expression. EMBO J. 19 (17), 4665–4675.

Pruijn, G.J., van Miltenburg, R.T., Claessens, J.A., van der Vliet, P.C., 1988. Interaction between the octamer-binding protein nuclear factor III and the adenovirus origin of DNA replication. J. Virol. 62 (9), 3092–3102.

Quandt, K., Frech, K., Karas, H., Wingender, E., Werner, T., 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res. 23 (23), 4878–4884.

Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T.J., Ishii, S., 1989. Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene. Proc. Natl. Acad. Sci. U.S.A. 86 (15), 5758–5762.

Sharrocks, A.D., 2001. The ETS-domain transcription factor family. Nat. Rev. Mol. Cell. Biol. 2 (11), 827–837.

Shupnik, M.A., Ridgway, E.C., Chin, W.W., 1989. Molecular biology of thyrotropin. Endocr. Rev. 10 (4), 459–475.

Tavner, F.J., Simpson, R., Tashiro, S., Favier, D., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., Macmillan, E.M., Lutwyche, J., Keough, R.A., Ishii, S., Gonda, T.J., 1998. Molecular cloning reveals that the p160 Myb-binding protein is a novel, predominantly nucleolar protein which may play a role in transactivation by Myb. Mol. Cell. Biol. 18 (2), 989–1002.

Vassart, G., Dumont, J.E., 1992. The thyrotropin receptor and the regulation of thyrocyte function and growth. Endocr. Rev. 13 (3), 596–611.

Voss, J.W., Wilson, L., Rosenfeld, M.G., 1991. POU-domain proteins Pit-1 and Oct-1 interact to form a heteromeric complex and can cooperate to induce expression of the prolactin promoter. Genes Dev. 5 (7), 1309–1320.

Zhao, C., Meng, A., 2005. Sp1-like transcription factors are regulators of embryonic development in vertebrates. Dev. Growth Differ. 47 (4), 201–211.