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New insight into the transcriptional properties of the human thyrostimulin and sodium/iodide symporter genes

Thesis

presented to the Faculty of Medicine, University of Ulm
to obtain the degree of a Doctor of Human Biology
(Dr. biol. hum.)

submitted by

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2005

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Tag der Promotion: 18 November 2005

Abbreviations

ATP	adenosine 5'-triphosphate
AP-1	activator protein 1
BBP	benzyl butyl phthalate
bp	base pair
BSA	bovine serum albumin
°C	degree Celsius
ca.	approximately
CMV	cytomegalovirus
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
DBP	dibutyl phthalate
DIDP	di-isodecyl phthalate
DINP	di-isononyl phthalate
cDNA	complementary deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
DOP	di-octyl phthalate
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EF-1α	elongation factor-1-alpha
e.g.	for example
EMSA	electrophoretic mobility shift assay
fmol	femtomol
g	gram
x *g*	relative centrifugal force, RCF
GD	Graves' disease
GTP	guanosine 5'-triphosphate
h	hour
i.e.	that is
IgG	immunoglobulin
kb	kilo base

kDa	kilo dalton
l	liter
LSF	late SV40 factor
M	Molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mol	mol
mRNA	messenger ribonucleic acid
µg	microgram
µl	microliter
µM	micromolar
NF-1	nuclear factor 1
ng	nanogram
NIS	sodium/iodide symporter
NRD	negative regulatory domain
NTF-1	NIS TSH-responsive factor 1
NUE	NIS upstream enhancer
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
Pit-1	pituitary-specific factor 1
pmol	picomol
PVC	polyvinyl chloride plastics
RLU	relative light unit
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotation per minute
RT-PCR	reverse transcription polymerase chain reaction
+ SD	plus/minus standard deviation
sec	second
SV40	simian virus 40

Tg	thyroglobulin
TPA	tetradecanoylphorbol-13-acetate
TPO	thyroperoxidase
TRE	TSH-responsive element
TRH	thyroid releasing hormone
TSAb	thyroid stimulating antibody standard
TSH	thyroid stimulating hormone, thyrotropin
TSHR	thyroid stimulating hormone receptor
TTF-1	thyroid transcription factor 1
TTF-2	thyroid transcription factor 2
IU	international unit
v/v	volume/volume

1.- Introduction

1.1. Thyroid gland

1.1.1. Thyroid function

The thyroid is a master endocrine gland that plays a central role in the intermediary metabolism of virtually all tissues and is of fundamental importance for the development of the central nervous system in the fetus and the newborn. The widespread effects of the thyroid result from the biosynthesis and secretion of two rather unique hormones, triiodothyronine (T_3) and thyroxine (T_4), the only iodine-containing hormones in vertebrates.

Iodide (I^-) is an essential constituent of T_3 and T_4 so that both thyroid function as a whole and its systemic ramifications depend on an adequate supply of I^- to the gland (Carrasco, 1993). A remarkably efficient and specialised system has evolved in the thyroid that ensures that most of the ingested dietary I^- (the only source of I^-) is accumulated in the gland and thus made available for T_3 and T_4 biosynthesis. The significance of this becomes more apparent when one considers that I^- is scarce in the environment. Endemic goiter and cretinism caused primarily by insufficient dietary supply of I^- remain a major health problem in many parts of the world, affecting millions of people (Delange, 1994).

I^- uptake into thyroid follicular cells is an active transport process that occurs at the basolateral plasma membrane and is mediated by the sodium/iodide symporter (NIS), an intrinsic plasma membrane symporter protein that couples the inward "downhill" translocation of Na^+ to the inward "uphill" translocation of I^- (Taurog, 1996) (Fig. 1). I^- is then translocated from the cytoplasm across the apical plasma membrane toward the colloid in a process called I^- efflux, which has been proposed to be mediated by pendrin (a Cl^+/I^- transporter; Scott et al., 1999), and more recently, by the apical I^- transporter (AIT; Rodriguez, 2002). In a complex reaction at the cell-colloid interface, called organification of I^- and catalysed by thyroperoxidase (TPO), I^- is oxidised and incorporated into some tyrosyl residues within the thyroglobulin (Tg) molecule, leading to the subsequent coupling of iodotyrosine residues. Iodinated Tg is stored extracellularly in the colloid. In response to demand for thyroid hormones, phagolysosomal hydrolysis of endocytosed iodinated Tg ensues. T_3 and T_4 are secreted into the bloodstream, and non-secreted iodotyrosines are metabolised to tyrosine and I^-,

a reaction catalysed by the microsomal enzyme iodotyrosine dehalogenase. This process facilitates re-utilisation of the remaining I^-. All of these steps, like NIS-mediated I^- uptake, are stimulated by TSH.

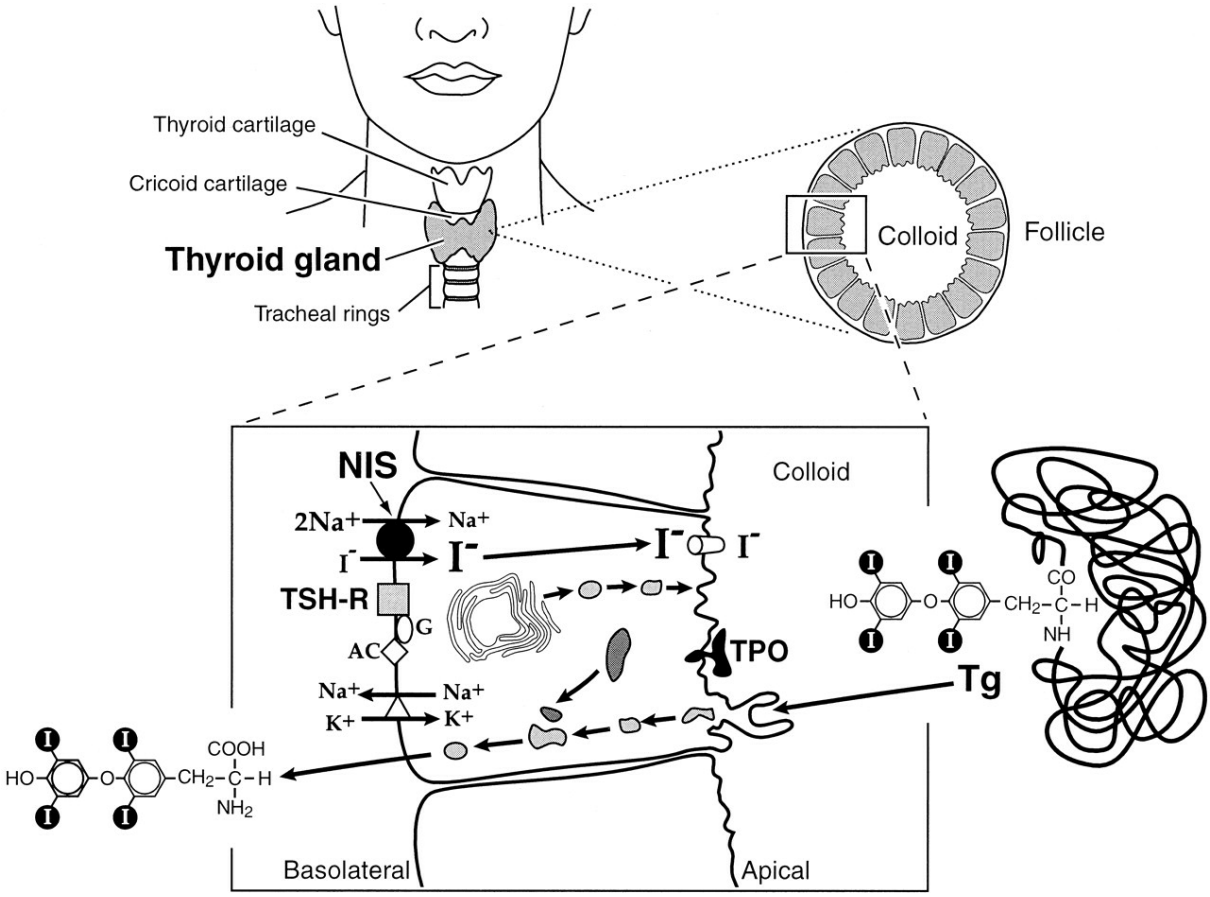


Fig. 1. Schematic representation of the biosynthetic pathway of thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) in the thyroid follicular cell. Thyroid follicles are comprised of a layer of epithelial cells surrounding the colloid. The basolateral surface of the cell is shown on the left, and the apical surface on the right. Circle, active accumulation of I^-, mediated by the Na^+/I^- symporter (NIS); triangle, Na^+/K^+ ATPase; square, thyrotropin (TSH) receptor; diamond, adenylyl cyclase; ellipse, G protein; cylinder, I^- efflux toward the colloid mediated by pendrin; TPO, thyroid peroxidase (TPO)-catalysed organification of I^-; arrows, endocytosis of iodinated thyroglobulin (Tg), followed by phagolysosomal hydrolysis of endocytosed iodinated Tg and secretion of both thyroid hormones. Modified from De La Vieja et al. (2000).

1.1.2. Graves' disease

Graves' disease (GD) is an autoimmune thyroid disease classically characterised by the presence of thyroid-stimulating antibodies. These antibodies mimic TSH by binding to the TSH receptor, thus resulting in the activation of adenylyl cyclase cascade (Kohn et al., 1983; Rees Smith et al., 1988). Consequently, this leads to the uncontrolled increase in iodine uptake, stimulation of protein synthesis and thyroid growth (Weetman and

McGregor, 1994; Rapoport et al., 1998). Graves' thyroid tissue has been shown to express elevated levels of NIS mRNA and protein (Saito et al., 1997; Ajjan et al., 1998). In addition, it is known that I^- uptake activity is increased in patients with GD (Cavalieri, 1986). Furthermore, Shoda et al. (1993) reported that GD IgG could stimulate iodide efflux from FRTL-5 rat thyroid cells.

1.1.3. Effects of endocrine disruptors on thyroid function

The diesters of benzene-1,2-dicarboxylic (phthalic) acid, the phthalates, are used as plasticisers for polyvinyl chloride plastics (PVC) in a wide range of applications, including vinyl floors, interior building surfaces, shoe soles, food wraps, cosmetics, medical devices and toys (Huber et al., 1996). Phthalates are not chemically bound to PVC and, with time and use, release from vinyl products, thus becoming regular contaminants in ambient air, drinking water and food products, all of which are potential exposure sources for the general population (Mayer et al., 1972; Sharman et al., 1994; Bauer and Herrmann, 1997).

There is scientific and public concern about potential human health risks from exposure to phthalates. These concerns stem from studies showing that a large proportion of general population are exposed to phthalates (Blount et al., 2000), as well as from animal studies consistently showing that some phthalates are developmental and reproductive toxicants (Foster et al., 1980; Agarwal et al., 1985; Mylchreest et al., 2000, 1999; Park et al., 2002).

Since the finding of Gray et al. (2000) that very high doses of some phthalates impede the sexual development of male rats, phthalates appear on a list of so-called "endocrine disrupters". The latter are defined as exogenous agents interfering with the production, release, transport, metabolism, binding, action or elimination of hormones in the body (Kavlock et al., 1996).

Among the endocrine glands, the thyroid is highly dependent on dietary uptake of iodine for normal function and thus may be particularly vulnerable to exogenous disruption (Brucker-Davis, 1998). However, the effects of phthalates on thyroid function have received little attention. Price et al. (1988) reported that three months of treatment of male rats with doses of bis-(2-ethylhexyl) phthalate (DEHP) from 50 to 1000 mg/kg per day resulted in microscopic changes in the thyroid suggesting increased activity. This was confirmed in rats treated with DEHP (50 to 2000 mg/kg) and a related compound di-octyl phthalate (DOP, 2 g/kg) for three days to nine months (Hinton et al.,

1986; Howarth et al., 2001). Here, histological changes indicated thyroid hyperactivity already visible at seven days and persisting at nine months. In addition, Wenzel et al. (2005) demonstrated a stimulation of iodide uptake by several phthalates.

1.2. Sodium/iodide symporter (NIS)

The Na^+/I^- symporter (NIS) is an integral plasma membrane glycoprotein that mediates active I^- transport into the thyroid follicular cells, the first step in thyroid hormone biosynthesis. NIS couples the energy released by the inward "downhill" translocation of Na^+ down its electrochemical gradient, generated by Na^+/K^+ ATPase, to driving the simultaneous inward "uphill" translocation of I^- against its electrochemical gradient (Carrasco, 1993). Two Na^+ are transported per each I^-. This process is competitively inhibited by thiocyanate, perchlorate and other anions (Yoshida et al., 1997).

1.2.1. Cloning of the human NIS cDNA and elucidation of the genomic organisation

The cDNA encoding hNIS was identified on the expectation that hNIS would be highly homologous to rat NIS (rNIS). Using primers to the cDNA rNIS sequence, Smanik et al. (1997) identified a cDNA clone encoding hNIS. The nucleotide sequence of hNIS reveals an open reading frame of 1929 nucleotides, which encodes a protein of 643 amino acids (Dohan et al., 2003). hNIS exhibits 84% identity and 93% similarity to rNIS. Fifteen exons encoding hNIS are interrupted by 14 introns, and the hNIS gene is mapped to chromosome 19p13 (Smanik et al., 1997).

1.2.2. Regulation of NIS protein expression

TSH is an approximately 30-kDa glycoprotein biosynthesised in the adenohypophysis by basophilic cells known as thyrotropes. TSH is the primary hormonal regulator of thyroid function overall and stimulates I^- accumulation in the thyroid (Vassart and Dumont, 1992). TRH from the hypothalamus stimulates the release of TSH, whereas T_3 and T_4 inhibit it. The majority of TSH actions are mediated by activation of adenylyl cyclase via the GTP binding protein Gαs (Laglia et al., 1996). TSH stimulation of I^- uptake is known to result from the cAMP-mediated increased biosynthesis of NIS (Weiss et al., 1984). Using high-affinity anti-NIS antibodies, Levy et al. (1997) demonstrated in rats that NIS protein expression is up-regulated by TSH *in vivo*. Consistent with these findings is a later observation by Uyttersprot et al. (1997) that the

expression of NIS mRNA in dog thyroid is dramatically up-regulated by goitrogenic treatment (i.e., treatment with propylthiouracil, which leads to elevated TSH circulating levels *in vivo*).

Other than TSH, the main factor regulating NIS activity in the thyroid has long been considered to be I^- itself (Taurog, 1996). Stated simply, high doses of I^- cause diminished thyroid function. *In vivo* studies carried out by Uyttersprot et al. (1997) showed that I^- inhibited the expression of both TPO and NIS mRNAs in dog thyroid. Eng et al. (1999) later found that both NIS mRNA and NIS protein levels in the rat thyroid-derived FRTL-5 cell line decreased significantly after either 1 or 6 days of I^- administration.

1.2.3. Transcriptional regulation of the NIS gene

Until recently, NIS was considered a thyroid-specific protein, i.e., a protein expressed exclusively in the thyroid, just like Tg, TPO and the TSHR. Although it is now clear that NIS is not a thyroid-specific protein, as it is functionally expressed in such extrathyroidal tissues as salivary glands, gastric mucosa and lactating mammary gland, the transcriptional regulation of the NIS gene in thyroid cells appears to be controlled by factors that regulate the transcription of thyroid-specific genes. The main factors regulating thyroid gland development and the expression of both the NIS gene and thyroid-specific genes are transcriptional factors TTF-1 (thyroid transcription factor 1), TTF-2 and Pax-8.

To discern the specific roles of these factors in thyroid development, several animal models lacking specific thyroid transcription factors have been generated by genetic recombination. TTF-1 null mice have no thyroid gland at all (both follicular and calcitonin-producing C cells are missing), no lung parenchyma and no pituitary (in its entirety); consequently, these mice die at birth (Kimura et al., 1996). TTF-2 null mice die within 48 h of birth; they have a cleft palate and their thyroid is either completely absent or present only as a small sublingual (i.e. ectopic) gland (DeFelice et al., 1998). When present, this ectopic gland is differentiated, as demonstrated by Tg expression. Pax-8 null mice have a very small thyroid gland devoid of follicular cells, but in these mice calcitonin production is not affected (Mansouri et al., 1998).

1.2.3.1. Analysis of the rat NIS promoter

The rat NIS (rNIS) promoter consists of two important regions: 1) a proximal promoter, reported to be TSH/cAMP regulated by a TSH-responsive element (TRE), mediated by a new protein NIS TSH-responsive factor 1 (NTF-1) (Ohmori et al., 1998). This up-regulation, however, is lower than the TSH-induced mRNA expression reported by the same group (Kogai et al., 1997). 2) The rNIS promoter also has an upstream enhancer that mediates thyroid-specific gene expression by the interaction of Pax-8 with a novel cAMP-dependent pathway (Ohno et al., 1999). The NIS upstream enhancer (NUE) stimulates transcription in a thyroid-specific and cAMP dependent manner. NUE contains the following: two Pax-8 binding sites, two TTF-1 binding sites that have no effect on rNIS transcription and a degenerate cAMP response element (CRE), which is important for NUE transcriptional activity. In NUE, both Pax-8 and the unidentified CRE-like binding factor act synergistically to obtain full TSH/cAMP-dependent transcription.

1.2.3.2. Analysis of the human NIS promoter

The human NIS (hNIS) promoter has been sequenced by three different groups. Venkataraman et al. (1998) isolated a 1.2-kb fragment of the 5'-flanking region of the hNIS gene. Significantly, they characterised the promoter in a human thyroid cell line, KAT-50, and found thyroid-specific expression between – 1044 and – 336 bp.

In contrast, Behr et al. (1998) and Ryu et al. (1998) isolated 2 kb and 1.6 kb, respectively, of the 5'-flanking region of the hNIS gene and found strong promoter activity in thyroid and non-thyroid cells within the minimal promoter. The transcriptional start site was mapped to nucleotide – 375, relative to the ATG start codon (Ryu et al., 1998).

Further, a thyroid-specific, TSH-responsive, far-upstream (– 9847 to – 8968) enhancer – highly homologous to the rat NUE – has been reported by two groups (Schmitt et al., 2002; Taki et al., 2002). It contains putative Pax-8 and TTF-1 binding sites and a CRE-like sequence. The TTF-1 binding site is not required for full activity.

NUE has been shown to be activated by TSH via the CRE (Schmitt et al., 2002; Taki et al., 2002).

1.3. Thyroid stimulating hormone (TSH)

Thyroid stimulating hormone (thyrotropin, TSH) is a 28- to 30-kDa glycoprotein produced in the thyrotropes of the anterior pituitary gland. Its synthesis and secretion are stimulated by TRH and inhibited by thyroid hormones T_3 and T_4 in a classic negative feedback loop. Differences in the molecular mass of TSH are primarily due to the heterogeneity of carbohydrate chains. Physiological roles of TSH include stimulation of differentiated thyroid functions, such as iodide uptake and organification, the release of thyroid hormones from the gland, and promotion of thyroid growth (Wondisford, 1996). It also acts as a thyrocyte survival factor and protects the cells from apoptosis (Kawakami et al., 1996). A further interesting finding is that TSH plays a critical role in ontogeny. In a mouse model with targeted disruption of the common α-subunit gene and thus devoid of circulating glycoprotein hormones, thyroid development was arrested in late gestation (Kendall et al., 1995).

1.3.1. TSH structure

TSH is a member of a pituitary and placental glycoprotein hormone family, which also includes follicle-stimulating hormone, luteinising hormone and chorionic gonadotropin. These hormones consist of two non-identical and non-covalently linked subunits termed α and β. The α-subunit is common to all members of this family and is expressed in molar excess in the pituitary to facilitate formation of the intact hormone (Kourides et al., 1984). The β-subunit is unique and confers specific biological activity to each dimeric hormone (Pierce and Parsons, 1981; Licht and Denver, 1990). Glycoprotein hormones are non-covalently linked heterodimers stabilised by a unique segment of the β-subunit termed “seat belt”, because it wraps around the α-subunit long loop. In human TSH β-subunit, three disulfide bridges form cysteine-knot motif that determines the core structure, two disulfide bridges are involved in seat-belt formation, and one links two β-hairpin loops (Fairlie et al., 1996).

1.3.2. Transcriptional regulation of the common α-subunit in thyrotropes

Expression of the gene for the common α-subunit is restricted to two cell types within the anterior pituitary gland, thyrotropes and gonadotropes. The human α-subunit promoter comprises a 118-bp region (– 224 to – 100) that is sufficient to confer both basal and cAMP-responsive transcriptional activity (Darnell and Boime, 1985; Deutsch et al., 1987; Silver et al., 1987). This region contains two sequences that closely

resemble consensus sequences of known eucaryotic promoter elements (TATAAAA between – 29 and – 23 and ATCCAAT on the opposite strand from – 82 to – 89). Within this region there is also a 36-bp cAMP-responsive element (CRE) that contains a perfect direct repeat of two 18-bp sequences, each of which contains an 8-bp palindromic element (TGACGTCA). This repeated 18-bp CRE exhibits enhancer-like properties. It functions independent of orientation, is relatively distance-independent and operates with either the α-promoter or a heterologous SV40 promoter (Delegeane et al., 1987). Although only the 8-bp palindromic core sequence has internal symmetry and is homologous to the CREs of other genes, the entire 18-bp element is required for cAMP responsiveness. Truncation of a few bases destroys the activity, even when the core remains intact.

The 18-bp CRE appears to be essential for tissue-specific expression of the α-subunit promoter. Mutations or deletions of this repeated region destroy tissue-specific activity.

The human α-subunit promoter is stimulated by TRH via the CRE (Jameson et al., 1988). Another study described a pituitary-specific protein, P-Lim, which binds to and activates the α-promoter (Bach et al., 1995).

1.3.3. Transcriptional regulation of the TSH β-subunit

The expression of the TSH β-subunit is restricted to the thyrotrope cells of the anterior pituitary. A region of about 170 bp (– 128 to + 37) is sufficient to account for cell-specific and basal expression of the human TSH β-subunit gene (Steinfelder and Wondisford, 1997).

The human β-subunit promoter is inducible by TRH, phorbol esters and the adenylyl cyclase activator forskolin via the TGGGTCA motif at – 1/+ 6 of the TSH β-gene that is similar to the consensus phorbol ester response element or the consensus CRE (Kim et al., 1993). Following stimulation, the TGGGTCA-specific factor acts together with the pituitary-specific transcription factor Pit-1 and its splice variant Pit-1T bound to sequences at – 128 to – 61 to mediate the induction of the TSH β-promoter (Haugen et al., 1993).

In addition, transcriptional factors GATA-2 and P-Lim have been shown to functionally synergise with Pit-1 to activate TSH β-promoter activity (Gordon et al., 1997; Steinfelder and Wondisford, 1997).

The expression of the human TSH β-gene is restricted to the thyrotrophs by a silencer element, which consists of multiple degenerate Oct-1 binding sites and is located in the

– 480/– 128 region (Kim et al., 1996). Since Oct-1 is expressed before the anterior pituitary development (He et al., 1989), the Oct-1-mediated silencing likely plays a role in preventing inappropriate expression of the human TSH β-gene.

1.4. Thyrostimulin

In 2002, based on GenBank searches, Nakabayashi et al. discovered two additional human glycoprotein hormone subunit-like genes and named them A2 (α2) and B5 (β5), due to their structural similarities to known subunits. Because the putative A2 subunit is likely to combine with either known or novel β subunits to yield bioactive heterodimeric hormones, a yeast two-hybrid protein-protein interaction screen was performed to identify potential dimerisation partners for A2, and, as a result, interactions between A2 and B5 were found. These putative subunits are co-localised in the anterior pituitary. Recombinant A2/B5 heterodimeric glycoproteins, purified using cation exchange and size fractionation chromatography, activated human TSH receptors, but not LH and FSH receptors, and showed high affinity to TSH receptors in a radioligand receptor assay. The heterodimer also stimulated cAMP production and thymidine incorporation by cultured thyroid cells and increased serum thyroxine levels in TSH-suppressed rats *in vivo*.

This new heterodimeric glycoprotein hormone was named as thyrostimulin based on its thyroid-stimulating activity.

1.4.1. Thyrostimulin structure

The A2 and B5 human subunits were discovered on chromosomes 11q13 and 14, respectively (Nakabayashi et al., 2002). The ORF of the A2 gene contains 106 amino acids and is encoded by three exons, whereas the ORF of the B5 gene contains 130 amino acids and is encoded by two exons interspersed by short introns.

The human A2 subunit is 35% identical at an amino acid level to the common glycoprotein α-subunit. Its pattern of expression includes the brain, pituitary, thyroid, ovary and heart. The human B5 subunit is approximately 30% identical at an amino acid level to TSH β-subunit. B5 expression is limited to brain and pituitary.

A2 and B5 share only modest amino acid similarity with the related glycoprotein hormone subunit family members, but they contain all of the cysteine residues required to form the characteristic cysteine knot structure found in these factors. The known glycoprotein hormone heterodimers are believed to be stabilised by a segment of the β-

subunit, which wraps around the α-subunit like a seat belt (Lapthorn et al., 1994). However, the B5 subunit is unique in that it lacks the disulfide bond important for seat belt formation. Indeed, the study of Nakabayashi et al. (2002) indicated that the A2/B5 heterodimer is less stable than the known glycoprotein hormones during SDS-PAGE analysis.

1.4.2. Thyrostimulin function

Although thyrostimulin was found to be a potent stimulator of thyroid cell functions *in vitro* and *in vivo*, its exact role in thyroid physiology is still unknown. TSH-independent production and secretion of T_3 and T_4 from the thyroid have been attributed to constitutive activity of the unbound TSH receptor (Ikekubo et al., 2005). However, if thyrostimulin is secreted in sufficient quantities from the pituitary, this novel hormone could account for the residual stimulation of the thyroid in patients with central hypothyroidism caused by TSH deficiency. 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.

1.5. Aim and scope of the work

In the first part of this work, the transcriptional regulation of the human sodium/iodide symporter (hNIS) gene by thyroid stimulating antibodies in patients with Graves' disease (GD) and by phthalate plasticisers will be investigated.

To demonstrate that hNIS transcriptional activation is actually responsible for the increased iodide uptake activity in patients with GD, fusion constructs of the human NIS promoter and enhancer with the luciferase gene will be transfected transiently into thyroid cell line and the resulting luciferase activity will be measured post-treatment with affinity purified GD IgG. Further, by exploiting site-directed mutagenesis, the *cis*-regulatory elements which contribute to the regulation of NIS gene expression by these IgG will be identified.

Because several phthalate plasticisers were found to stimulate iodide uptake, the role of di-isodecyl phthalate (DIDP), benzyl butyl phthalate (BBP), bis-(2-ethylhexyl) phthalate (DEHP), di-octyl phthalate (DOP), di-isononyl phthalate (DINP) and dibutyl phthalate (DBP) in the transcriptional modulation of the human NIS gene will be investigated after transient transfections and through culture of cells exposed to phthalates and subsequent RT-PCR for NIS transcript.

In the second and main part of this work, to understand the molecular mechanisms underlying thyrostimulin transcription, the promoters of its A2 and B5 subunit as well as upstream and downstream genomic sequences will be cloned and characterised. Promoter-reporter gene constructs will be transfected transiently into various cell lines of different origins and the resultant luciferase activity will be assessed, eventually under the influence of external stimuli. Through mutational and gel shift analyses, it is expected to uncover the *cis*-regulatory elements which are essential for the regulation of the A2 and B5 gene expression.

In addition, to investigate the possibility that thyrostimulin is implicated in the transcriptional regulation of the NIS gene, the medium containing the human A2 and B5 subunit proteins will be used as a stimulator of the NIS promoter and enhancer-luciferase construct.

Results from these studies will provide new insight into the transcriptional properties of the NIS gene and will give clues about the transcription of the thyrostimulin A2 and B5 subunit genes.

2.- Materials and methods

2.1. Materials

2.1.1. Enzymes and kits

TSH Rezeptor Antikoerper Konzentration assay (TRAK) (BRAHMS Diagnostics, Berlin, Germany)

HiTrap protein G HP affinity columns (Amersham Biosciences, Uppsala, Sweden)

Ultrapure dNTP set (Amersham Biosciences)

Centricon YM-100 centrifugal filter units (Millipore, Bedford, USA)

Amicon Ultra-15 centrifugal filter units (Millipore)

LightShift chemiluminescent EMSA kit (Pierce, Bonn, Germany)

Biotin 3'-end DNA labelling kit (Pierce)

NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce)

Expand long template PCR system (Roche, Mannheim, Germany)

BD Advantage 2 polymerase mix (BD Biosciences)

ExSite PCR-based site-directed mutagenesis kit (Stratagene, La Jolla, USA)

DNase I RNAse-free (Qiagen, Hilden, Germany)

QIAprep spin miniprep kit (Qiagen)

QIAfilter plasmid maxi kit (Qiagen)

QIAquick PCR purification kit (Qiagen)

QIAquick gel extraction kit (Qiagen)

RNeasy mini kit (Qiagen)

Effectene transfection reagent (Qiagen)

Restriction endonucleases (Invitrogen, Karlsruhe, Germany)

T4 DNA ligase (Invitrogen)

SuperScript II reverse transcriptase (Invitrogen)

Platinum Taq DNA polymerase (Invitrogen)

Calf intestinal alkaline phosphatase (Promega, Manheim, Germany)

2.1.2. Reporter, cloning and expression vectors

pGL3-Basic and pGL3-Promoter (Promega)

pBudCE4.1 (Invitrogen)

pCR-XL-TOPO (Invitrogen)

pCR2.1 (Invitrogen)

2.1.3. Commercial primers

GAPDH (glyceraldehydes 3-phosphate dehydrogenase) to human, rat and mouse (BD Biosciences, Heidelberg, Germany)

2.1.4. Cell lines

293	human embryonic kidney
PANC-1	human pancreatic carcinoma
MIA PaCa-2	human pancreatic carcinoma
FRTL-5	rat thyroid
PC Cl3	rat thyroid
GH3	rat somatolactotrope
HC11	mouse mammary
αT3	mouse gonadotrope
TαT1	mouse thyrotrope

293 cell line was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, No. ACC 305).

PANC-1 and MIA PaCa-2 cell lines were a kind gift from Dr. P. Michl (Universität Ulm, Ulm, Germany).

FRTL-5 cell line was a kind gift from Dr. K. Törnquist (Abo Akademi University, Turku, Finland).

PC Cl3 cell line was a kind gift from Dr. L. Nitsch (Universita degli Studi di Napoli Federico II, Naples, Italy).

GH3 cell line was a kind gift from Dr. M. Korbonits (St. Bartholomew's Hospital, London, UK).

HC11 cell line was a kind gift from Dr. N. Hynes (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland).

αT3 and TαT1 cell lines were a kind gift from Dr. P. Mellon (University of California, San Diego, USA).

2.1.5. Other materials

All plastic materials were purchased from Nunc (Wiesbaden, Germany) and Greiner-Bio One (Frickenhausen, Germany).

Matrigel was purchased from BD Biosciences (Heidelberg, Germany).

Human brain genomic DNA and human brain PCR ready first strand cDNA were purchased from BioCat (Heidelberg, Germany).

Human epidermal growth factor, forskolin, TRH, bovine TSH, insulin, hydrocortisone, transferrin, glysyl-L-histidyl-L-lysine acetate, somatostatin and 3- isobutyl-1-methylxantine (IBMX) were purchased from Sigma (Taufkirchen, Germany).

Thyroid stimulating antibody standard (TSA) was obtained from the National Institute for Biological Standards and Control (Hertfordshire, UK).

Cell culture medium RPMI 1640, Click's, DMEM and MEM were obtained from Gibco (Karlsruhe, Germany). Coon's modified Ham's F-12 medium was purchased from Biochrom (Berlin, Germany). Serum-free medium HyQSFM4HEK293 was obtained from Pierce.

The phthalate plasticisers di-isodecyl phthalate (DIDP) and di-octyl phthalate (DOP) (Fluka, Seelze, Germany), benzyl butyl phthalate (BBP), di-isononyl phthalate (DINP), dibutyl phthalate (DBP) (Sigma) and bis-(2-ethylhexyl) phthalate (DEHP) (Lancaster, Lancashire, UK) were supplied by Dr. R. H. Waring (University of Birmingham, Birmingham, UK).

The beetle luciferin (Promega) and the Bio-Rad protein assay (BioRad, Munich, Germany) were used to prepare the luciferase buffer and to determine the total protein content in cell lysates, respectively.

2.2. Methods

2.2.1. Reporter gene construction

2.2.1.1. NIS promoter and enhancer

The human NIS reporter constructs in the luciferase expression vector pGL3-Basic (Promega) – promoter (N3) and promoter and enhancer (N3 + NUE) (NIS upstream enhancer), were previously cloned in our laboratory (Behr et al., 1998; Schmitt et al., 2002). hTRE-mutated promoter and enhancer (mtN3+NUE) construct was generated by using the ExSite PCR-based site-directed mutagenesis kit (Stratagene).

Oligonucleotides used were hTREFor: 5'-TTAAAGCAGGCTGTGCAGGCTTGGA-3' and hTRERev: 5'-CACTCAAAGCCGTATTGTGCTTGAAACCTT-3'.

2.2.1.2. 5'-flanking region of the thyrostimulin A2 gene

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) using the Expand long template PCR system (Roche) with the following primers: F, 5'-CTTGCAGCTCTCTCTTCCCGTTT-3' and R, 5'-GTATTTAAAGAACTCGCCATCCCACCTG-3'. It was inserted into the pCR-XL-TOPO vector (Invitrogen) and the resulting construct was cleaved by MluI and XhoI. After separation on agarose gel and purification using the QIAquick gel extraction kit (Qiagen), the fragment that contains sequence from – 3119 to – 37 was inserted into pGL3-Basic using MluI and XhoI sites. This construct was designated – 3119/– 37.

2.2.1.3. Deletions of the 5'-flanking region of the thyrostimulin A2 gene

Progressively shorter 5' prime truncations (– 2066/– 37, – 1586/– 37, – 1046/– 37, – 506/– 37 and – 373/– 37) were generated with the Advantage 2 polymerase mix (BD Biosciences) 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' XhoI (reverse primer) and 5' MluI (forward primers) (italicised) to facilitate subsequent cloning into the MluI and XhoI 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).

2.2.1.4. Minimal promoter of the thyrostimulin A2 gene

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 XhoI (5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3') and ligated into pGL3-Basic using MluI and XhoI sites.

2.2.1.5. Deletions of the minimal promoter of the thyrostimulin A2 gene

The aforementioned 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.

2.2.1.6. Substitution mutants of the minimal promoter of the thyrostimulin A2 gene

The substitution mutants were generated by a two-step PCR procedure using overlapping inner primers with an incorporated EcoRI site that introduced a 6-bp mutation (illustrated in Fig. 2). The outer forward and reverse primers contained 5' MluI and 3' XhoI, respectively. The two products were digested with EcoRI and ligated. The purified ligation product was again subjected to PCR using the outer primers. The

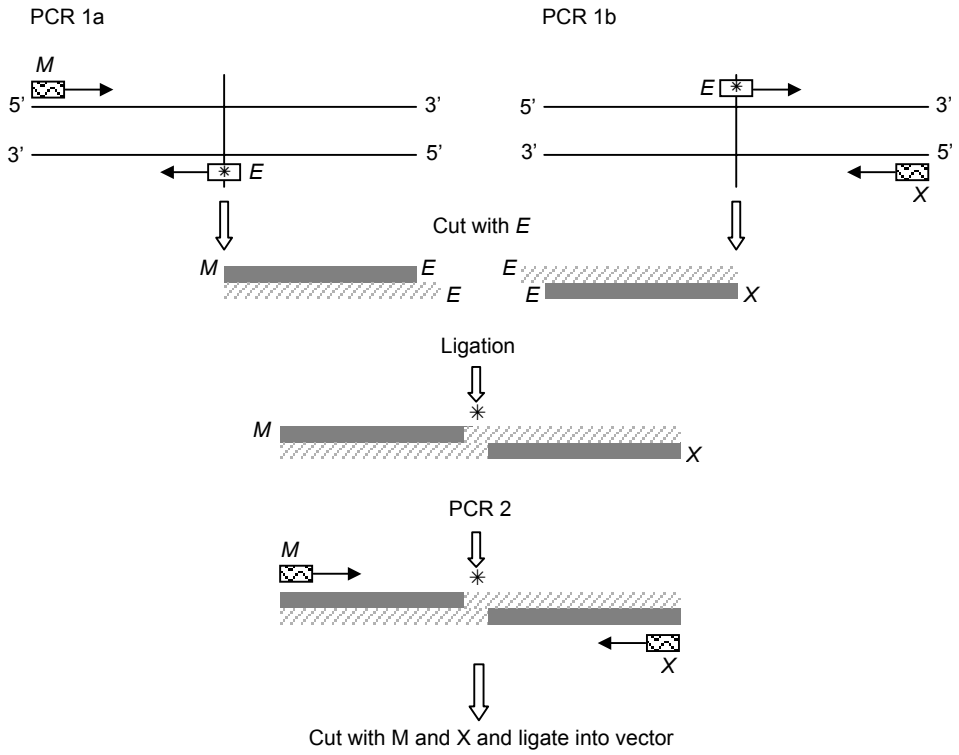


Fig. 2. Generation of substitution mutants by PCR. In separate reactions 1a and 1b, two partially overlapping fragments were synthesised by PCR. In reaction 1a, an outer primer carrying a restriction site MluI (M) and an inner primer (✳) carrying a mutation (which creates an EcoRI restriction site, E) were used. In reaction 1b, an inner primer (✳) carrying a mutation (which creates an EcoRI restriction site, E) and an outer primer carrying a restriction site XhoI (X) were used. Both PCR products were digested with EcoRI and ligated. The purified ligation product was again subjected to PCR using only the two outer primers (M and X). The resulting product was digested with MluI and XhoI and ligated into the pGL3-Basic vector.

resulting product was cleaved with MluI and XhoI and ligated into the MluI and XhoI 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.

2.2.1.7. Regions s1 and s2 of the thyrostimulin A2 gene

Because regions – 347/– 37 and – 1046/– 506 appeared to down-regulate the A2min construct, they were analysed for the presence of silencer elements. Designated s1 and s2, these regions were amplified from the – 3119/– 37 clone with primer pairs containing KpnI (F: 5'- CGAGATC*GGTACC*CTGGGGAGGGTCTGGGAATGT-3' and R: 5'- AAGAACTCGCCATCCCACCTG*GGTACC*GATCCGA-3' for s1, F: 5'- CGAGATC*GGTACC*GTTCAAGACCACCATGGGCAA-3' and R: 5'- CTTCAGGCAGGGGAGGCTGGT*GGTACC*GATCCGA-3' for s2). The products were inserted into the KpnI 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 HindIII site downstream of the SV40 and A2min promoters of pGL3-Promoter and A2min plasmids, respectively, using the same primers as above but with incorporated HindIII. These constructs were called s1-d-s-P, s1-d-as-P, s1-d-s-m, s1-d-as-m, s2-d-s-P, s2-d-as-P, s2-d-s-m and s2-d-as-m.

2.2.1.8. Exons 1 – 3 and introns 1 – 2 of the thyrostimulin A2 gene

The exons 1 – 3 and introns 1 – 2 of the thyrostimulin A2 subunit were amplified from human brain genomic DNA with the Advantage 2 polymerase mix by a two-step PCR, where an aliquot of the first PCR was used as a template in the second PCR. The resultant products were digested with the corresponding restriction enzymes and placed in sense orientation into the promoterless pGL3-Basic vector, the pGL3-Promoter vector downstream of the SV40 promoter and the A2min construct downstream of the A2min promoter (illustrated in Table 2). Finally, exon 1 and intron 2 were tested for the presence of silencer elements by the same cloning strategy as for s1 and s2 (illustrated in Table 3).

2.2.1.9. 5'-flanking region of the thyrostimulin B5 gene

DNA fragment from the human thyrostimulin B5 subunit 5'-flanking region (– 4459 to – 20, the A in the ATG initiation codon is designated as + 1) was amplified from human brain genomic DNA using the Expand long template PCR system with the following primers: F, 5'-GTTTCAGAATGCTGGTGAGGTCTCATAGTC-3' and R, 5'-GCAGCCAGATCTGCCAAACTCTGTCCTT -3'. It was inserted into the pCR-XL-TOPO vector (Invitrogen) and the resulting construct was cleaved by MluI and XhoI. After separation on agarose gel and purification using the QIAquick gel extraction kit, the fragment that contains sequence from – 4459 to – 20 was inserted into pGL3-Basic using MluI and XhoI sites. This construct was designated – 4459/– 20.

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

Mutation generated	PCR	Forward (F) and reverse (R) primers
m1	1a	F, 5'-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3' R, 5'-CTTCAGGCAGGGGAGGCTGGTTGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCCCGATCTCCAAGAGGGCGTGGC-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TGAATTCCCGATCTCCAAGAGG-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
m2	1a	F, 5'-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3' R, 5'-GGGGAGGCTGGTTTGAAAGCCGGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCAAGAGGGCGTGGCTCCAAAATG-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGGAATTCAAGAGG-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
m3	1a	F, 5'-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3' R, 5'-GGTTTGAAAGCCGATCTCCAAGGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCTGGCTCCAAAATGCTGGCAAAT-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGGAA-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
m4	1a	F, 5'-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3' R, 5'-AGCCGATCTCCAAGAGGGCGTGGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCAAATGCTGGCAAATAAAAGCCT-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
m5	1a	F, 5'-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3' R, 5'-AGAGGGCGTGGCTCCAAAATGCGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCATAAAAGCCTGGAGAGCCAGCA-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
m6	1a	F, 5'-CGAGATC*ACGCGT*GAGGCCTGGGCTGTGGGTAGCTT-3' R, 5'-TGGCTCCAAAATGCTGGCAAATGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCCTGGAGAGCCAGCACCAAGCTG-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
m7	1a	F, 5'-CGAGATC*ACGCGT*GATCGCTCTGAGGCTTCAGGCAGG-3' R, 5'-AATGCTGGCAAATAAAAGCCTGGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCCAGCACCAAGCTGGAGACCAGC-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3' R, 5'-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3'

m8	1a	F, 5’-CGAGATC*ACGCGT*GATCGCTCTGAGGCTTCAGGCAGG-3’ R, 5’-AAAAGCCTGGAGAGCCAGCACCGAATTCGATCCGA-3’
	1b	F, 5’-CGAGATCGAATTCGAGACCAGCAGGAGGCACAGGA-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
	2	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
m9	1a	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-ACCAAGCTGGAGACCAGCAGGAGAATTCGATCCGA-3’
	1b	F, 5’-CGAGATCGAATTCGGAAAACTGCAAGCCGCTCTGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
	2	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
m10	1a	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-GAGGCACAGGAAAACTGCAAGCGAATTCGATCCGA-3’
	1b	F, 5’-CGAGATCGAATTCGTTCCTGGGCCTCGGAAGTGGT-3’ R, 5’-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3’
	2	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
m11	1a	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-GGAAAACTGCAAGCCGCTCTGTGAATTCGATCCGA-3’
	1b	F, 5’-CGAGATCGAATTCGCCTCGGAAGTGGTGAGCTCGC-3’ R, 5’-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3’
	2	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
m12	1a	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CAAGCCGCTCTGTTCCTGGGCCGAATTCGATCCGA-3’
	1b	F, 5’-CGAGATCGAATTCGTGGTGAGCTCGCTGAGGCAGG-3’ R, 5’-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3’
	2	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’
m13	1a	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTGTTCCTGGGCCTCGGAAGTGGAATTCGATCCGA-3’
	1b	F, 5’-CGAGATCGAATTCTCGCTGAGGCAGGAGGAGCT-3’ R, 5’-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3’
	2	F, 5’-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3’ R, 5’-CTCGCTGAGGCAGGAGGAGCT*CTCGAG*GATCCGA-3’

m14	1a	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3' R, 5'-GGCCTCGGAAGTGGTGAGCTCGGAATTCGATCCGA-3'
	1b	F, 5'-CGAGATCGAATTCCAGGAGGAGCTCTGGGGAGGGT-3' R, 5'-TAAAGAACTCGCCATCCCACCTG*CTCGAG*GATCCGA-3'
	2	F, 5'-CGAGATC*ACGCGT*TTGAAAGCCGATCTCCAAGAGGGCGT-3' R, 5'-GCTCGGAATTCCAGGAGGAGCT*CTCGAG*GATCCGA-3'

The mutations correspond to those listed in Fig. 16 and 17. The mutated nucleotides are underlined. The introduced MluI and XhoI restriction sites are italicised. PCR 1a and 1b correspond to the two products generated by PCR 1.

Table 2. Sequences of the primers used to generate plasmids containing exon 1 – 3 and intron 1 – 2 of the thyrostimulin A2 subunit

	Vector	*pGL3-Basic*
Exon 1	Plasmid	**e1-s-B**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*ATGCCTATGGCGTCCCCTCA-3'
	R	5'-CCCAGGCTGCCACTTGCACC*AAGCTT*GATCCGA-3'
Exon 2	Plasmid	**e2-s-B**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*CCTTCAATGTGACAGTGCGA-3'
	R	5'-GCTGCACCATCAGTGGCCTG*AAGCTT*GATCCGA-3'
Exon 3	Plasmid	**e3-s-B**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*CCGCAGGTCAAAGTACAGCT-3'
	R	5'-GTCGCCTCTCTCGCTACTAG*CCATGG*GATCCGA-3'
Intron 1	Plasmid	**i1-s-B**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*GTGAGTACCTCTGGGACCGG-3'
	R	5'-GCTTGCCTATCTCCTTCTAG*AAGCTT*GATCCGA-3'
Intron 2	Plasmid	**i2-s-B**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*AAGAAGGTGAGGAGGGCCCG-3'
	R	5'-GCAGCACGGACTCCCCTCTC*AAGCTT*GATCCGA-3'

	Vector	*pGL3-Promoter*
Exon 1	Plasmid	**e1-d-s-P**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*ATGCCTATGGCGTCCCCTCA-3'
	R	5'-CAGGCTGCCACTTGCACC*CCATGG*GATCCGA-3'
Exon 2	Plasmid	**e2-d-s-P**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*CCTTCAATGTGACAGTGCGA-3'
	R	5'-CTGCACCATCAGTGGCCTG*CCATGG*GATCCGA-3'

Exon 3	Plasmid	**e3-d-s-P**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*CCGCAGGTCAAAGTACAGCT-3'
	R	5'-GTCGCCTCTCTCGCTACTAG*CCATGG*GATCCGA-3'
Intron 1	Plasmid	**i1-d-s-P**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*GTGAGTACCTCTGGGACCGG-3'
	R	5'-GCTTGCCTATCTCCTTCTAG*CCATGG*GATCCGA-3'
Intron 2	Plasmid	**i2-d-s-P**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*AAGAAGGTGAGGAGGGCCCG-3'
	R	5'-GCAGCACGGACTCCCCTCTC*CCATGG*GATCCGA-3'

	Vector	*A2min*
Exon 1	Plasmid	**e1-d-s-m**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*ATGCCTATGGCGTCCCCTCA-3'
	R	5'-CCCAGGCTGCCACTTGCACC*AAGCTT*GATCCGA-3'
Exon 2	Plasmid	**e2-d-s-m**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*CCTTCAATGTGACAGTGCGA-3'
	R	5'-GCTGCACCATCAGTGGCCTG*AAGCTT*GATCCGA-3'
Exon 3	Plasmid	**e3-d-s-m**
	Sites	HindIII-NcoI
	F	5'-CGAGATC*AAGCTT*CCGCAGGTCAAAGTACAGCT-3'
	R	5'-GTCGCCTCTCTCGCTACTAG*CCATGG*GATCCGA-3'
Intron 1	Plasmid	**i1-d-s-m**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*GTGAGTACCTCTGGGACCGG-3'
	R	5'-GCTTGCCTATCTCCTTCTAG*AAGCTT*GATCCGA-3'
Intron 2	Plasmid	**i2-d-s-m**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*AAGAAGGTGAGGAGGGCCCG-3'
	R	5'-GCAGCACGGACTCCCCTCTC*AAGCTT*GATCCGA-3'

The plasmids correspond to those diagrammed in Fig. 31 and 32. The introduced restriction sites are italicised.

Table 3. Sequences of the primers used to analyse exon 1 and intron 2 of the thyrostimulin A2 subunit for potential silencer elements

	Vector	*pGL3-Basic*
Exon 1	Plasmid	**e1-s-B**
	Sites	BglII-HindIII
	F	5'-CGAGATC*AGATCT*ATGCCTATGGCGTCCCCTCA-3'
	R	5'-CCCAGGCTGCCACTTGCACC*AAGCTT*GATCCGA-3'

	Plasmid	**e1-as-B**
	Sites	HindIII-BglII
	F	5’-CGAGATC*AAGCTT*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-CCCAGGCTGCCACTTGCACC*AGATCT*GATCCGA-3’
	Plasmid	–
	Sites	–
	F	–
	R	–
	Plasmid	–
	Sites	–
	F	–
	R	–
Intron 2	Plasmid	**i2-s-B**
	Sites	BglII-HindIII
	F	5’-CGAGATC*AGATCT*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-GCAGCACGGACTCCCCTCTC*AAGCTT*GATCCGA-3’
	Plasmid	**i2-as-B**
	Sites	HindIII-BglII
	F	5’-CGAGATC*AAGCTT*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-GCAGCACGGACTCCCCTCTC*AGATCT*GATCCGA-3’
	Plasmid	–
	Sites	–
	F	–
	R	–
	Plasmid	–
	Sites	–
	F	–
	R	–

	Vector	*pGL3-Promoter*
Exon 1	Plasmid	**e1-d-s-P**
	Sites	HindIII-NcoI
	F	5’-CGAGATC*AAGCTT*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-CAGGCTGCCACTTGCACC*CCATGG*GATCCGA-3’
	Plasmid	**e1-d-as-P**
	Sites	NcoI-HindIII
	F	5’-CGAGATC*CCATGG*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-CCCAGGCTGCCACTTGCACC*AAGCTT*GATCCGA-3’
	Plasmid	**e1-u-s-P**
	Sites	KpnI-MluI
	F	5’-CGAGATC*GGTACC*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-AGGCTGCCACTTGCACC*ACGCGT*GATCCGA-3’
	Plasmid	**e1-u-as-P**
	Sites	MluI-KpnI
	F	5’-CGAGATC*ACGCGT*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-AGGCTGCCACTTGCACC*GGTACC*GATCCGA-3’

Intron 2	Plasmid	**i2-d-s-P**
	Sites	HindIII-NcoI
	F	5’-CGAGATC*AAGCTT*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-GCAGCACGGACTCCCCTCTC*CCATGG*GATCCGA-3’
	Plasmid	**i2-d-as-P**
	Sites	NcoI-HindIII
	F	5’-CGAGATC*CCATGG*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-GCAGCACGGACTCCCCTCTC*AAGCTT*GATCCGA-3’
	Plasmid	**i2-u-s-P**
	Sites	KpnI-MluI
	F	5’-CGAGATC*GGTACC*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-AGCACGGACTCCCCTCTC*ACGCGT*GATCCGA-3’
	Plasmid	**i2-u-as-P**
	Sites	MluI-KpnI
	F	5’-CGAGATC*ACGCGT*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-AGCACGGACTCCCCTCTC*GGTACC*GATCCGA-3’

	Vector	A2min
Exon 1	Plasmid	**e1-d-s-m**
	Sites	BglII-HindIII
	F	5’-CGAGATC*AGATCT*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-CCCAGGCTGCCACTTGCACC*AAGCTT*GATCCGA-3’
	Plasmid	**e1-d-as-m**
	Sites	HindIII-BglII
	F	5’-CGAGATC*AAGCTT*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-CCCAGGCTGCCACTTGCACC*AGATCT*GATCCGA-3’
	Plasmid	**e1-u-s-m**
	Sites	KpnI-MluI
	F	5’-CGAGATC*GGTACC*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-AGGCTGCCACTTGCACC*ACGCGT*GATCCGA-3’
	Plasmid	**e1-u-as-m**
	Sites	MluI-KpnI
	F	5’-CGAGATC*ACGCGT*ATGCCTATGGCGTCCCCTCA-3’
	R	5’-AGGCTGCCACTTGCACC*GGTACC*GATCCGA-3’
Intron 2	Plasmid	**i2-d-s-m**
	Sites	BglII-HindIII
	F	5’-CGAGATC*AGATCT*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-GCAGCACGGACTCCCCTCTC*AAGCTT*GATCCGA-3’
	Plasmid	**i2-d-as-m**
	Sites	HindIII-BglII
	F	5’-CGAGATC*AAGCTT*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-GCAGCACGGACTCCCCTCTC*AGATCT*GATCCGA-3’
	Plasmid	**i2-u-s-m**
	Sites	KpnI-MluI
	F	5’-CGAGATC*GGTACC*AAGAAGGTGAGGAGGGCCCG-3’
	R	5’-AGCACGGACTCCCCTCTC*ACGCGT*GATCCGA-3’
	Plasmid	**i2-u-as-m**
	Sites	MluI-KpnI
	F	5’-CGAGATC*ACGCGT*AAGAAGGTGAGGAGGGCCCG-3’

	R	5'-AGCACGGACTCCCCTCTC*GGTACC*GATCCGA-3'

The plasmids correspond to those diagrammed in Fig. 33 and 34. The introduced restriction sites are italicised.

2.2.1.10. Deletions of the 5'-flanking region of the thyrostimulin B5 gene

All deletions were generated with the Advantage 2 polymerase mix using a PCR strategy that employed – 4459/– 20 as a template. The constructs – 4459/– 2312, – 4459/– 1716, – 4459/– 1043, – 4459/– 993 and – 4459/– 892 were made with forward primer 5'-CGAGATC*ACGCGT*GTTTCAGAATGCTGGTGAGGTCTCATAGTC-3' and reverse primers with the following sequences: 5'-AGTTCTGGTTATGCTACTCACAG*CTCGAG*GATCCGA-3', 5'-GTCTGCCCAATGTAGAACATGTG*CTCGAG*GATCCGA-3', 5'-CTACCATTCATTGTGACTTCCTG*CTCGAG*GATCCGA-3', 5'-TCAGCTCTAAAAGAAGAGTGGGC*CTCGAG*GATCCGA-3' and 5'-GTAAGGTCAGAAGTAGGAACTGT*CTCGAG*GATCCGA-3', respectively. They were designed with a 5' MluI (forward primer) and 3' XhoI (reverse primers) (italicised) to facilitate subsequent cloning into the MluI and XhoI sites of pGL3-Basic.

The fragments – 3351/– 2312, – 3351/– 1716, – 3351/– 1043, – 3351/– 993 and – 3351/– 892 were constructed using forward primer 5'-CGAGATC*ACGCGT*GCTACAAACTCTCACTCTGTCCA-3' and the same reverse primers as described above.

The fragments – 3201/– 2312, – 3201/– 1716, – 3201/– 1043, – 3201/– 993 and – 3201/– 892 were constructed using forward primer 5'-CGAGATC*ACGCGT*AACACAACATTCCAAGTAGCCTC-3' and the same reverse primers as described above.

Forward primer for constructs – 1217/– 945 and – 1217/– 803 was 5'-CGAGATC*ACGCGT*AAAGGGTTTGCCATGAGCTGCTAGT-3' and reverse primers were 5'-GTGCTTCTCCGTATTTGTAACCTCA*CTCGAG*GATCCGA-3' and 5'-AATCCACGTGAGTTCTGTCTAGTG*CTCGAG*GATCCGA-3', respectively.

2.2.2. Expression vector construction

Human thyrostimulin A2 and B5 subunit cDNAs (GenBank accession Nos. NM_130769 and NM_145171) were generated by PCR using the Advantage 2 polymerase mix and human brain first strand cDNA (BioCat) as template. Forward primer for the A2 cDNA, 5'-

CGA*AAGCTT*G**GCCACC**ATGCCTATGGCGTCCCCTCAA-3', contained a perfect Kozak consensus sequence (bold) and 5' HindIII (italicised). Reverse primer for the A2 cDNA was 5'-CTTGACATTCTGGTGGGGGAAACCTGT-3'. Similarly, forward primer for the B5 cDNA, 5'-CGA*GGTACC***GCCACC**ATGAAGCTGGCATTCCTCTTCCTTG contained a perfect Kozak consensus sequence and 5' KpnI, while the reverse primer was 5'-CCACGGAGTGTGAGACCATCTGA-3'. The resultant PCR products were ligated into the TA cloning vector pCR2.1 (Invitrogen) yielding pCR-A2 and pCR-B5.

Next, pCR-A2 was cleaved by HindIII and XbaI and, after separation on agarose gel and purification using the QIAquick gel extraction kit, the fragment containing A2 cDNA was ligated into the bi-promoter pBudCE4.1 vector (Invitrogen) using HindIII and XbaI of the CMV multiple cloning site. Likewise, pCR-B5 was digested with KpnI and the gel purified fragment containing B5 cDNA was inserted into KpnI site of the EF-1α multiple cloning site of the same vector. The selected clone with A2 and B5 cDNAs in sense orientation was named pBud-A2/B5. Prior transfection, pBud-A2/B5 was linearised using NheI unique site to increase the chances that it does not integrate in a way that disrupts the A2 and B5 genes or elements necessary for their expression.

2.2.3. Maxipreps of plasmid DNA

Plasmid DNA was purified with the Qiagen plasmid kit (Qiagen) and the DNA concentration was measured by UV spectrophotometry. Plasmids were also checked for purity, concentration, supercoiling and restriction pattern by agarose gel electrophoresis.

2.2.4. Sequencing

Double-stranded DNA sequencing of all constructs was performed by Agowa (Berlin, Germany). Data were viewed using the Chromas 1.55 program (Technelysium Pty Ltd., Queensland, Australia).

The following sequencing primers (Agowa) were used:

RVprimer3: 5'-CTAGCAAAATAGGCTGTCCC-3'

GLprimer2: 5'-CTTTATGTTTTTGGCGTCTTCCA-3'

M13 Reverse: 5'-CAGGAAACAGCTATGAC-3'

T7 promoter: 5'-CCCTATAGTGAGTCGTATTA-3'

CMV Forward: 5'-CGCAAATGGGCGGTAGGCGTG-3'

BGH Reverse : 5'-CCTCGACTGTGCCTTCTA-3'

2.2.5. IgG purification

Sera were obtained from 29 patients with active Graves' disease and from 7 normal subjects. TSHR-binding autoantibodies were measured in a commercial TRAK (TSH Rezeptor Antikoerper Konzentration) assay (BRAHMS Diagnostics).

IgG were purified using 1 ml HiTrap protein G HP affinity columns (Amersham Biosciences) according to the instructions of manufacturer. Binding buffer consisted of 20 mM sodium phosphate, pH 7.0. Elution buffer consisted of 0.1 M glycine-HCl, pH 2.7. Collection tubes were prepared by adding 200 µl 1 M Tris-HCl, pH 9.0 per ml of fraction to be collected. The column was washed with 10 column volumes of binding buffer at 1 ml/min. Sera diluted 5x with binding buffer were applied to column using a syringe fitted to the luer adaptor. The column was then washed with 10 column volumes of binding buffer. The IgG fraction was eluted with 2 column volumes of elution buffer. Centricon YM-100 concentrators (Millipore) were used for IgG desalting and dialysis against PBS.

The protein concentration was determined using the Bio-Rad protein assay (BioRad). The absorbance at 595 nm was read in a spectrophotometer (Beckman DU-640, Krefeld, Germany) and compared against a BSA standard curve.

2.2.6. Cell culture and transfection

2.2.6.1. Cell culture

293, PANC-1, MIA PaCa-2, αT3 and TαT1 cell lines were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco), 4.5 mg/ml glucose, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Gibco). TαT1 cells were seeded on Matrigel-coated plates (BD Biosciences) which facilitated adhesion. Matrigel was diluted 20-fold with DMEM before coating the plates and allowed to dry before plating cells. HC11 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 5 µg/ml insulin and 10 ng/ml recombinant human epidermal growth factor (Sigma). GH3 cell line was grown in MEM containing 15% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. FRTL-5 cell line was maintained in a 1:1 mixture of Click's (Gibco) and RPMI 1640 medium supplemented with 5% calf serum (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). Similarly, PC Cl3 cells were cultured in

Coon's modified Ham's F-12 medium (Biochrom) supplemented with 5% CS 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).

2.2.6.2. Stable transfection

2.2.6.2.1 Stable transfection with thyrostimulin

293 cells were seeded onto a 12-well plate. At ca. 70-85% confluence, 300 ng of NheI-linearised pBud-A2/B5 was transfected into each well using the Effectene transfection reagent (Qiagen). After 48 h, cells were split onto twelve 10-cm Petri dishes and grown in medium containing 150 µg/ml Zeocin (Invitrogen). After 10 days, eight isolated clones were carefully scraped and used to establish cell lines No. 1-8. These lines were maintained post-selection in medium containing 100 µg/ml Zeocin.

2.2.6.2.2. Adaptation of 293 cells to protein-free medium

The stable cell line No. 8 was seeded onto a 10-cm Petri dish and fed DMEM with FBS supplementation of 5%. After 3.5 days, the cells were passaged at ca. 70% confluence and further grown in DMEM/5% for another 3.5 days. The cells were then passed as above but FBS concentration was reduced to 2.5%. They were cultured in this medium until good cell growth was observed (2 weeks). Upon the consequent passage, the cells were fed again DMEM/2.5% and allowed to attach and spread. Following the 24 h incubation, the serum-containing medium was removed and replaced with the protein-free medium HyQ SFM4HEK293 (Pierce). The cells were passed without trypsin at a passage interval of 3.5 days.

As a control, the cell line containing a stable integration of pBudCE4.1 empty vector was established and adapted to protein-free medium.

2.2.6.3. Transient transfection and stimulation

2.2.6.3.1. Transient transfection

All cells were seeded onto 12-well plates 24 h before transfection. At ca. 70-85% confluence, 300 ng of the respective luciferase reporter construct was transfected into appropriate wells with the Effectene transfection reagent. Each reaction contained 2.4 µl

Enhancer and 6 µl Effectene reagent in a total volume of 484 µl per well. In co-transfection experiments, 300 ng of the luciferase vector and 60 ng of the expression construct were used.

Cells were collected for luciferase activity assay 48 h post-transfection.

2.2.6.3.2. Treatment of cells

To investigate the effects of TSH and Graves' IgG in reporter gene assays, FRTL-5 cells were transfected in 6H medium supplemented with 5% CS. After 24 h, the cells were cultured for additional 48 h in 5H medium (0.5% CS, no TSH) and then treated with 100 µg/mL IgG, 0.0008 IU/µl thyroid stimulating antibody standard (TSA) or 1.5 mU/ml TSH for 24 h. A phosphodiesterase inhibitor, 3- isobutyl-1-methylxantine (IBMX, 0.5 mmol/l) (Sigma) was added to the medium containing IgG, TSA or TSH (Kraiem et al., 1988; Nguyen et al., 2002). Negative control consisted of medium supplemented with 0.5 mmol/l IBMX.

The effect of phthalates was studied 24 h post-transfection by starving near confluent PC Cl3 cells for TSH and insulin in the presence of 0.2% CS (4H medium) (Costamagna et al., 2004) for 48 h. After starvation, TSH (1.5 mU/ml) and phthalates (1 mM) were added to culture medium. Luciferase activity was determined 24 h after treatment.

To analyse the effect of phthalates on NIS gene expression, PC Cl3 cells were cultured in 4H medium for 48 h and treated with TSH (1.5 mU/ml) or TSH plus phthalates (1 mM) for 24, 48 and 72 h. Stimulated and non-stimulated cells were used to isolate total RNA, followed by RT-PCR.

To examine the effect of thyrostimulin in the luciferase reporter assay, conditioned protein-free medium (2 days) from the 293 stable cell line expressing both A2 and B5 subunits was collected and concentrated 200-fold using Amicon Ultra-15 10-kb membranes (Millipore). As a control, protein-free medium and conditioned protein-free medium from the empty vector-stably transfected cell line were also concentrated 200-fold. FRTL-5 cells were transiently transfected in 6H medium. After 24 h, the cells were cultured for additional 48 h in 5H medium and then treated with 10 µl concentrated medium (containing the A2/B5 pair) per well or 1.5 mU/ml TSH for 24 h. Cells were collected for luciferase activity assay.

Transfected 293, GH3 and TαT1 cells were cultured in their respective growth medium containing either 100 nM TRH or 10 µM forskolin. Luciferase activity was determined 24 h after the treatment.

2.2.6.4. Luciferase and protein assay

Whole cell extracts were made with 200 µl/well of 1x reporter lysis buffer (Promega). After 10 min at room temperature, cells were frozen at -20°C. Thawed cells were scraped off, vortexed for 10 sec, followed by centrifugation at 13000 rpm for 1 min. The resulting supernatant was taken for the luciferase and protein assay.

50 µl of cell extract was mixed with 50 µl of luciferase assay substrate (270 µM coenzyme A, 470 µM luciferin, 530 µM ATP, 33.3 mM DTT, 0.1 mM EDTA, 2.67 mM $MgSO_4$, 1.07 mM $(MgCO_3)_4Mg(OH_2)x5H_2O$, 20 mM tricine) and analysed using the Lumat LB9501 luminometer (Berthold, Wildbad, Germany).

For normalisation of the luciferase activities the protein content was measured in each well by the BioRad protein assay. 20 µl of cell extract was mixed with 1 ml of diluted protein-dye reagent (1:5, v/v, reagent:distilled water). After 10 min at room temperature, the absorbance at 595 nm was read in a spectrophotometer.

The standard control in the luciferase and protein assays consisted of 1x reporter lysis buffer.

2.2.7. Nuclear extract preparation

Nuclear extracts from GH3, αT3 and 293 cells were made using the NE-PER kit (Pierce) as described by the manufacturer. For each preparation, ca. 40 mg of cell paste was used. All procedures were carried out at 4^oC.

Cells were washed with ice cold PBS and scraped off in 1.5 ml PBS, followed by homogenisation in an all-glass Dounce homogeniser (Wheaton pestle B, 10-15 strokes). After centrifugation for 3 min at 500 x *g*, 200 µl of ice cold reagent CER I was added to the cell pellet. The tube was vortexed vigorously on the highest setting for 15 sec to fully resuspend the pellet and allowed to stand on ice for 10 min. After that, 11 µl of ice cold CER II containing protease inhibitor cocktail was added to the tube. The cell pellet was then vortexed for 5 sec, incubated on ice for 1 min and vortexed again for 5 sec. After centrifugation for 5 min at 16000 x *g*, the insoluble pellet fraction, which contained nuclei, was resuspended in 100 µl of ice cold reagent NER (with added protease inhibitor cocktail). The pellet was vortexed for 15 sec, returned on ice and was

(A)

```
-512  GCTGGT

          LSF                          Sp-1
-506  TTGAAAGCCGATCTCCAAGAGGGCGTGGCTCCAAAATGC
      Probe 1          Probe 2

          Oct-1     TATA    TTF-1          NF-1
-467  TGGCAAATAAAAGCCTGGAGAGCCAGCACCAAGCTGGAG
                   Probe 3

                   Pax-5                GAGA
-428  ACCAGCAGGAGGCACAGGAAAACTGCAAGCCGCTCTGTT
                Probe 4              Probe 5

      Ets       Ets     c-Myb      AP-1
-389  CCTGGGCCTCGGAAGTGGTGAGCTCGCTGAGGCAGGAGG
                      Probe 6

-350  AGCT
```

(B)

```
Probe 1    GCTGGTTTGAAAGCCGATCTCCAAG
Probe 1m1  GCTGGTTGAATTCCCGATCTCCAAG
Probe 1m2  GCTGGTTTGAAAGCCGGAATTCAAG

Probe 2    ATCTCCAAGAGGGCGTGGCTCCAAAAT
Probe 2m   ATCTCCAAGGAATTCTGGCTCCAAAAT

Probe 3    AAATGCTGGCAAATAAAAGCCTGGAGAGCCAGCACC
Probe 3m1  AAATGCGAATTCATAAAAGCCTGGAGAGCCAGCACC
Probe 3m2  AAATGCTGGCAAATGAATTCCTGGAGAGCCAGCACC
Probe 3m3  AAATGCTGGCAAATAAAAGCCTGGAATTCCAGCACC

Probe 4    CCAGCAGGAGGCACAGGAAAAC
Probe 4m   CCAGCAGGAGAATTCGGAAAAC

Probe 5    GCAAGCCGCTCTGTTCCTGGGCCTCGGAAGTGGTGAGC
Probe 5m1  GCAAGCGAATTCGTTCCTGGGCCTCGGAAGTGGTGAGC
Probe 5m2  GCAAGCCGCTCTGTGAATTCGCCTCGGAAGTGGTGAGC
Probe 5m3  GCAAGCCGCTCTGTTCCTGGGCCGAATTCGTGGTGAGC

Probe 6    TCGGAAGTGGTGAGCTCGCTGAGGCAGGAGGAGCT
Probe 6m1  TCGGAAGTGGAATTCTCGCTGAGGCAGGAGGAGCT
Probe 6m2  TCGGAAGTGGTGAGCTCGGAATTCCAGGAGGAGCT
```

Fig. 3. Sequence of the wild type and mutated probes used in EMSA studies of the A2 minimal promoter. 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. The numbering of nucleotides refers to their positions relative to the ATG start codon of the A2 gene **(A)**. The mutations are indicated by m (dotted box) **(B)**.

continued to be vortexed for 15 sec every 10 min, for a total of 40 min. The resulting suspension was centrifuged for 10 min at 16000 x *g* and the supernatant fraction (nuclear extract) was immediately transferred to a pre-chilled tube and placed on ice. The samples were divided into small aliquots and stored at -80°C.

2.2.8. Electrophoretic mobility shift assay

Complementary oligonucleotides containing the wild-type or mutated sequences (Fig. 3) were labelled separately using the biotin 3' end DNA labelling kit (Pierce). The labelling reaction consisted of 100 nM unlabelled oligonucleotide, 0.5 µM biotin-N4-CTP, 0.2 U/µl terminal deoxynucleotidyl transferase (TdT) and 10 µl TdT reaction buffer in a total volume of 50 µl. The reactions were incubated at 37°C for 30 min. To stop each reaction, 2.5 µl of 0.2 M EDTA was added. Then, 50 µl of chloroform:isoamyl alcohol was added to each reaction to extract the TdT. The mixture was vortexed briefly and centrifuged for 2 min at 13000 rpm to separate the phases. The top (aqueous) phase was removed and saved.

Labelled complementary oligonucleotides were annealed by heating to 95°C in equal molar mixture of the upper and lower strands in Tris-EDTA buffer and cooled slowly to room temperature (1 h).

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) and 2 µl nuclear extract in a total volume of 20 µl. Binding buffer was 100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5. 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 sec 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.

```
+81   ATCCCAGGCTGCCACTTGCACCCCTTCAATGTGACAGTGCGAAGTGACCGCCAA
      :::::::::::::::::::::::::: :: :::::::::::::::::: :::::
+84   ATCCCAGGCTGCCACTTGCACCCCTTTAACGTGACAGTGCGAAGTGATCGCCAT
      :::::::::::::::::::::::::::::::::::: ::::: ::::: ::::
+78   ATCCCAGGCTGCCACTTGCACCCCTTCAATGTGACGGTGCGCAGTGATCGCCTC

+135  GGCACCTGCCAGGGCTCCCACGTGGCACAGGCCTGTGTGGGCCACTGTGAGTCC
      :::::::::::::::::::: ::::::::::: ::::: :: :::::::::::
+138  GGCACCTGCCAGGGCTCCCATGTGGCACAGGCGTGTGTAGGACACTGTGAGTCT
      ::::: :::::::::::::::::::::::::::::::: :: :::::::::::
+132  GGCACTTGCCAGGGCTCCCACGTGGCACAGGCCTGTGTAGGACACTGTGAGTCT

+189  AGCGCCTTCCCTTCTCGGTACTCTGTGCTGGTGGCCAGTGGTTACCGACACAAC
      :: :: :::::::::::::::::::::::::: :::::::: :: :::::::::
+192  AGTGCTTTCCCTTCTCGGTACTCTGTGCTGGTTGCCAGTGGCTATCGACACAAC
      :: :: :::::::: :::::::::::::::::::::::::::: :: :: ::::::
+186  AGTGCTTTCCCTTCCCGGTACTCTGTGCTGGTGGCCAGTGGCTATCGGCACAAC

+243  ATCACCTCCGTCTCTCAGTGCTGCACCATCAGTGGCCTGAAGAAGGTCAAAGTA
      :::::::: :::::::::::::: ::::::::  :::: :: ::::: :  ::
+246  ATCACCTCTGTCTCTCAGTGCTGTACCATCAGCAGCCTTAAAAAGGTGAGGGTG
      ::::::::   ::: ::::::::::::::::  :::: :  ::::: :  ::
+240  ATCACCTCTTCCTCCCAGTGCTGCACCATCAGCAGCCTCAGAAAGGTGAGGGTG

+297  CAGCTGCAGTGTGTGGGGAGCCGGAGGGAGGAGCTCGAGATCTTCACGGCCAGG
        :::::: :: ::::::: :: : : : :::::::::::::::::::::: :::
+300  TGGCTGCACTGCGTGGGGAACCAGCGTGGGGAGCTCGAGATCTTCACGGCTAGG
        ::::::::: ::::::: :: : : : :::::: :::::::: :: :: :::
+294  TGGCTGCAGTGCGTGGGGAACCAGCGTGGGGAGCTTGAGATCTTTACTGCAAGG

+351  GCCTGCCAGTGTGACATGTGTCGCCTCTCTCGCTACTAG human
      :::::::::::::: ::::: :: ::::: :::::::::
+354  GCCTGCCAGTGTGATATGTGCCGTCTCTCCCGCTACTAG rat
      :::::::::::::: ::::: ::  :::: :::::::::
+348  GCCTGCCAGTGTGATATGTGCCGTTTCTCCCGCTACTAG mouse
```

Fig. 4. Sequence alignment of rat and mouse A2 subunit cDNAs with the human A2 subunit cDNA. RT-PCR primers are underlined. Forward primer for the human A2 cDNA was used for amplification of the rat and mouse A2 cDNA. Numbering is relative to the ATG start codon in the human, rat and mouse A2 genes, respectively.

2.2.9. RT-PCR

2.2.9.1. Effects of phthalates on the endogenous NIS gene expression

Total RNA was isolated from PC Cl3 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) in a final volume of 20 µl. PCR amplification was performed using 2 µl cDNA, 10 pmol of each primer and 2.5 U Platinum *Taq* DNA polymerase in final volume of 50 µl, according to the protocol of manufacturer (Invitrogen). Control reactions with RNA used as template were also carried out to exclude the possibility of genomic contamination (negative control).

PCR conditions were as follows: for the rat NIS gene, denaturation at 95°C (1 min), annealing at 59°C (30 sec) and extension at 72°C (2 min) for 35 cycles; for the GAPDH gene, denaturation at 95°C (1 min), annealing at 53°C (30 sec) and extension at 72°C (2 min) for 35 cycles.

Then, 10 µl of 50 µl of the amplification products were analysed on 1.2 % Tris-borate-EDTA agarose gel containing ethidium bromide.

Primers for rat NIS (RFNIS2: 5'-GGTGATCCTGGCCCGAGGCGTCA-3' and RNIS1: 5'-CCACTGTAAGCACAGGCCAGGAA-3') were designed according to the rat NIS cDNA sequence reported by Dai et al. (1996); they produce an amplicon of 441 bp. Primers for the GAPDH gene were: 5' primer, 5'-ACCACAGTCCATGCCATCAC-3' and 3' primer, 5'-TCCACCACCCTGTTGCTGTA-3' (BD Biosciences). The amplification yielded a 452 bp DNA product.

```
+74   CTCCAGTGGGAACCTGCGCACCTTTGTGGGCTGTGCCGTGAGGGAGTTTACTTT
      :::::: :::::::: : ::: :::::::: ::::: :::::::: :: :::::
+71   CTCCAGCGGGAACCTACACACTTTTGTGGGATGTGCTGTGAGGGAATTCACTTT
      :::::::::::::::::: ::: :::::::::::::: :::::::: :: :::::
+74   CTCCAGTGGGAACCTGCACACTTTTGTGGGCTGTGCTGTGAGGGAATTCACTTT

+128  CCTGGCCAAGAAGCCAGGCTGCAGGGGCCTTCGGATCACCACGGATGCCTGCTG
        :::::::::::::::::::::::::: :::::::::::::: ::::::::::::
+125  TGTGGCCAAGAAGCCAGGCTGCAGGGGACTTCGGATCACCACAGATGCCTGCTG
      : :::::::::::::::::::::::::: :::::::::::::: ::::::::::::
+128  CATGGCCAAGAAGCCAGGCTGCAGGGGACTTCGGATCACCACAGATGCCTGCTG

+182  GGGTCGCTGTGAGACCTGGCAGAAACCCATTCTGGAACCCCCCTATATTGAAGC
      :::::::::::::::::::: ::::::::::::::::: :: ::::: :: :::::
+179  GGGTCGCTGTGAGACCTGGGAGAAACCCATTCTGGAGCCTCCCTACATAGAAGC
      ::: ::::: ::::::::: :::::::::: ::::: :: ::::: ::::::::
+182  GGGCCGCTGCGAGACCTGGGAGAAACCCATCCTGGAGCCTCCCTACATTGAAGC

+236  CCATCATCGAGTCTGTACCTACAACGAGACCAAACAGGTGACTGTCAAGCTGCC
      : :::::::::: ::::::::::: ::::::: ::::::::: :: ::::::::
+233  CTATCATCGAGTGTGTACCTACAATGAGACCAGACAGGTGACGGTGAAGCTGCC
      : :::::::::: ::::: ::::: ::::::: ::::::::: ::::::::::
+236  CTATCATCGAGTGTGTACATACAATGAGACCAGACAGGTGACAGTGAAGCTGCC
```

```
+290 CAACTGTGCCCCGGGAGTCGACCCCTTCTACACCTATCCCGTGGCCATCCGCTG
      :::::::::::: :::::::::::::::::::::::: ::  ::::  :::: ::
+287 TAACTGTGCCCCTGGAGTCGACCCCTTCTACACCTACCCTATGGCTGTCCGATG
      :::::::::::: :::::::: :: ::::::::::: ::  ::::  :::::::
+290 TAACTGTGCCCCTGGAGTCGATCCTTTCTACACCTACCCTATGGCTGTCCGATG

+344 TGACTGCGGAGCCTGCTCCACTGCCACCACGGAGTGTGAGACCATCTGA human
     ::::::::: :: :: :::::::::::::: :::::::::::::::::
+341 TGACTGCGGGGCATGTTCCACTGCCACCACTGAGTGTGAGACCATCTGA rat
     :::::: :: :: :: :::::::::::::: :::::::::::::::::
+344 TGACTGTGGGGCGTGTTCCACTGCCACCACTGAGTGTGAGACCATCTGA mouse
```

Fig. 5. Sequence alignment of rat and mouse B5 subunit cDNAs with the human B5 subunit cDNA. RT-PCR primers are underlined. Forward primer for the human B5 cDNA was used for amplification of the mouse B5 cDNA. Reverse primer for the human B5 cDNA was used for amplification of the rat and mouse B5 cDNA. Numbering is relative to the ATG start codon in the human, rat and mouse A2 genes, respectively.

2.2.9.2. Expression of the A2 and B5 genes in different cell types

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. 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) in a final volume of 20 µl. PCR amplification was performed using 2 µl cDNA, 10 pmol of each primer and 2.5 U Platinum *Taq* DNA polymerase in final volume of 50 µl, according to the protocol of manufacturer (Invitrogen).

PCR conditions were as follows: denaturation at 95°C (1 min), annealing at 62°C (30 sec) and extension at 72°C (2 min) for 35 cycles.

Then, 10 µl of 50 µl of the amplification products were analysed on 1.2 % Tris-borate-EDTA agarose gel containing ethidium bromide.

Forward primer for human, rat and mouse thyrostimulin A2 subunit was 5'-ATCCCAGGCTGCCACTTGCAC-3'. Reverse primers for human, rat and mouse A2 subunit were 5'- TGTCGCCTCTCTCGCTACTAG-3', 5'-TGCCGTCTCTCCCGCTACTAG-3' and 5'-TGCCGTTTCTCCCGCTACTAG-3', respectively. These primers produce an amplicon of 308 bp and are presented in Fig. 4.

Forward primer for human and mouse thyrostimulin B5 subunit was 5'-CTCCAGTGGGAACCTGCGCAC-3'. Forward primer for rat thyrostimulin B5 subunit was 5'-CTCCAGCGGGAACCTACACAC-3'. Reverse primer for human, rat and

mouse B5 subunit was 5'-ACGGAGTGTGAGACCATCTGA-3'. These primers produce an amplicon of 319 bp and are presented in Fig. 5.
Omission of the RT step led to loss of the PCR products.

2.2.9.3. Expression of the A2 and B5 genes in stably transfected 293 cells

The stable cell line No. 8 adapted to protein-free medium was used to isolate total RNA, followed by RT-PCR as described above. Forward primer for A2 cDNA, 5'-TAGGGAGACCCAAGCTTGGCCACCA-3', included CMV promoter of pBudCE4.1 to differentiate between the endogenous and exogenous A2. Reverse primer for A2 cDNA was 5'-CTTGACATTCTGGTGGGGGAAACCTGT-3'. These primers produce an amplicon of 479 bp. Forward and reverse primers for B5 cDNA were 5'-ATGAAGCTGGCATTCCTCTTCCTTG-3' and 5'-CCACGGAGTGTGAGACCATCTGA-3', respectively; they produce an amplicon of 392 bp.

2.2.10. Statistical analysis

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.

2.2.11. *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.

2.2.12. Universal restriction enzyme buffer

All restriction enzyme digestions were performed using potassium glutamate buffer, which consisted of 1 M potassium glutamate, 0.25 M Tris acetate (pH 7.6), 0.1 M magnesium acetate, 5 mM β-mercaptoethanol and 500 µg/ml BSA.

2.2.13. Preparation of competent cells

For the preparation of chemically competent cells, *E. coli* TOP10 cells were streaked on an LB plate and incubated for 16 h at 37°C. Next, 5 ml LB broth culture was inoculated with a single colony and shaken for 16 h at 220 rpm at 37°C. The culture was

transferred into a flask containing 50 ml LB and shaken for 4 h at 220 rpm at 37°C. The flask was then placed on ice to cool roughly to 4°C. Following centrifugation for 10 min at 5000 rpm at 4°C, the pellet was resuspended in 40 ml ice-cold 0.1 M $CaCl_2$. The cells were centrifuged again as above, resuspended in 50 ml ice-cold 0.1 M $CaCl_2$ and kept on ice for 8 h. Finally, the cells were re-centrifuged as above and resuspended in 8.6 ml ice-cold 0.1 M $CaCl_2$ mixed with 1.4 ml sterile glycerol. Aliquots were made at 4°C and stored at -80°C.

3.- Results

3.1. Modulation of the human sodium/iodide symporter (hNIS) activity by Graves' disease antibodies

3.1.1. IgG from patients with Graves' disease activate the hNUE gene transcription

IgG from 29 patients with active Graves' disease (GD) and 7 normal subjects were assayed for their ability to stimulate the hNIS promoter and enhancer. Rat thyroid FRTL-5 cells, transiently transfected with hNIS promoter- (pGL3-N3) and hNIS promoter and enhancer- (pGL3-N3+NUE) luciferase-fused constructs (Behr et al., 1998; Schmitt et al., 2002), were treated with purified GD IgG, IgG from normal subjects, thyroid stimulating antibody standard (TSAb) or TSH.

In cells transfected with pGL3-N3 no activation was observed after the treatment with 100 μg/ml GD IgG (Fig. 6).

In contrast, luciferase activity was significantly increased when pGL3-N3+NUE-transfected cells were treated with GD IgG. The activation of pGL3-N3+NUE was even higher when the cells were treated with 0.0008 IU/μl TSAb or 1.5mU/ml TSH, whereas pGL3-N3 was not stimulated by either. Furthermore, the activation of pGL3-N3+NUE by IgG from normal subjects was not significant, while pGL3-N3 was not activated.

GD IgG-induced up-regulation of the CRE mutation-containing construct pGL3-N3+mtNUE was reduced approximately 30-fold compared to that of the wild-type construct.

The mean individual pGL3-N3+NUE luciferase activities and TRAK values of 29 GD IgG are presented in Table 4.

3.1.2. A novel regulatory region is involved in the transcriptional up-regulation of the hNIS gene by TSH and Graves' disease IgG

It has been reported by Ohmori et al. (1998) that TSH/cAMP-induced up-regulation of the rat NIS (rNIS) gene expression requires a novel thyroid transcription factor interacting with the TSH-responsive element (rTRE) in the promoter region. Kogai et al. (2001) detected two possible hTRE sites with a consensus sequence GNNCGGANG in the hNIS promoter (1 and 2 base mismatch).

Based on the sequence comparison of the rat and human NIS promoters, a third putative hTRE motif located at – 699 to – 690 nt (1 base mismatch) of the hNIS promoter was

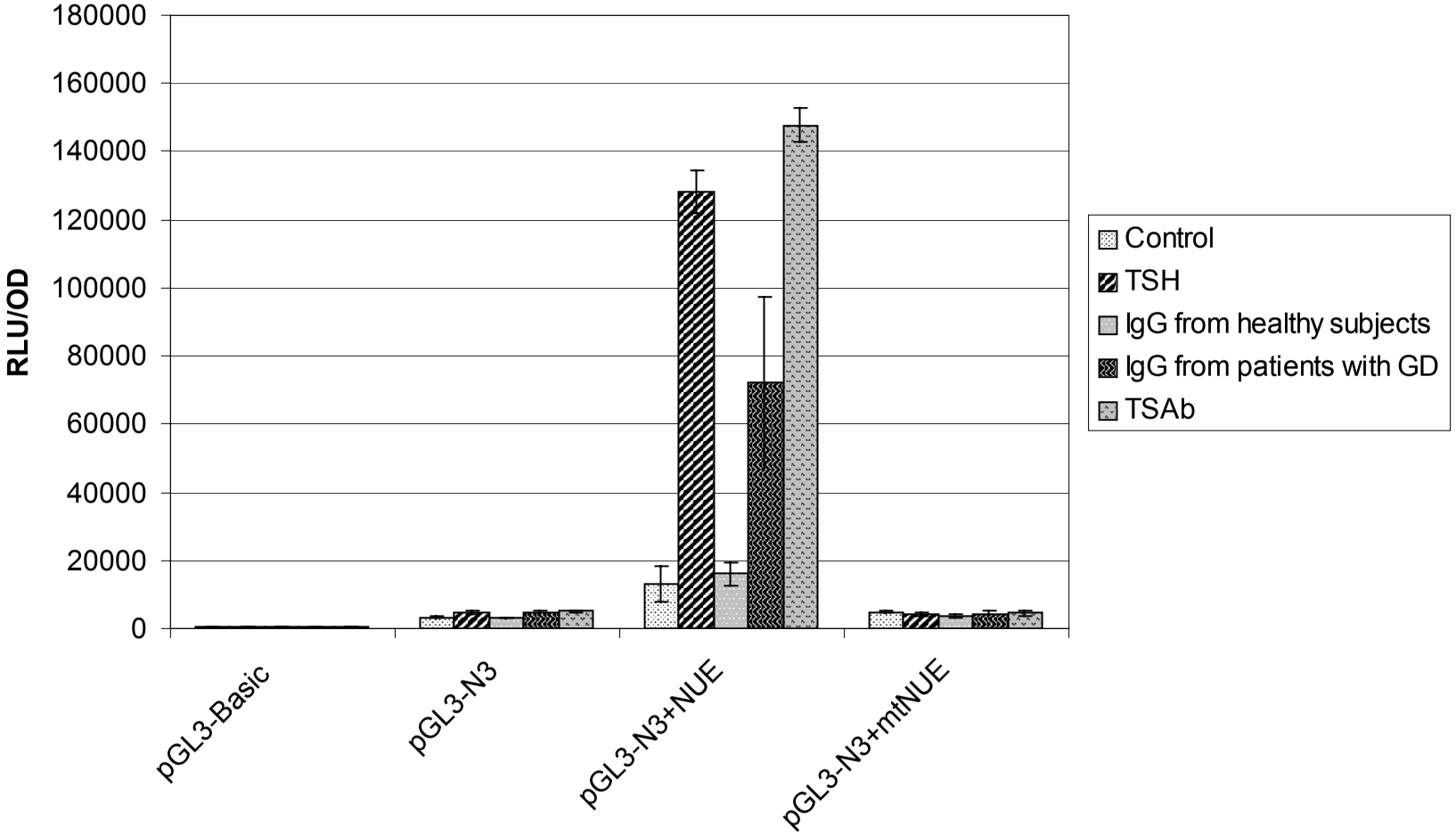


Fig. 6. Influence of thyroid stimulating antibodies on hNIS promoter and enhancer. Luciferase constructs pGL3-Basic, pGL3-N3, pGL3-N3+NUE and pGL3-N3+mtNUE were transfected into FRTL-5 cells and stimulated with 1.5 mU/ml TSH, 100 µg/ml IgG from healthy subjects (n = 7), 100 µg/ml IgG from patients with Graves' disease (GD) (n = 29) or 0.0008 IU/µl thyroid stimulating antibody standard (TSAb). IgG from 29 patients with GD or 7 healthy subjects have been tested individually and the results have then been pooled. 0.5 mmol/l IBMX was added to the medium containing IgG, TSH or TSAb. Negative control consisted of medium supplemented with 0.5 mmol/l IBMX. Luciferase activities are given in relative light units (RLU) in relation to OD and represent mean values ± SD of three experiments done in triplicate.

Table 4. pGL3-N3+NUE-stimulating GD IgG luciferase activities in FRTL-5 cells

GD patient №	pGL3-N3+NUE (mean RLU/OD ± SD)	TRAK
1	121300	40
2	120200	40
3	122700	40
4	119600	40
5	81840	13,7
6	86750	26,8
7	82790	26,2
8	88260	40
9	92090	40
10	75310	16,3
11	77880	10,7
12	76420	16,3
13	61670	13,4
14	65090	16,5
15	65830	18,5
16	69620	14,7
17	66420	16,9
18	64370	16,2
19	68830	14,7
20	62420	12,5
21	58120	40
22	54510	8,2
23	60510	8,3
24	44390	8,9
25	48830	40
26	43700	5,5
27	33410	6,9
28	38300	4,7
29	35740	4,2

For each GD patient the experiments were performed in triplicate wells and repeated three times. TRAK values represent TSHR-binding autoantibodies.

```
-461 TCCTTATGGAGCCCGGAAGTCTCCGAAAGTGAACCCTAGTCCGGGGTTC-//-
-772 CGGCTTTGAGTGCTGAAGCAGGCTGTGCAGGCTTGGATAGTGACATGCC-//-

-123 GGGGGCGGAGCTGCGCTGACCCCGGAGTTCAATAAATCCGCA -81 rat
-434 GGAGGCGGAGTCGCGGTGACCCGGGAGCCCAATAAATCTGCA -392 human
```

Fig. 7. The sequence comparison between rat (upper) and human (lower) NIS promoters. The putative TRE is shown in bold, the AP-1 motif is boxed, the GC box is italicised and the TATA-box is underlined. The numbering of nucleotides refers to their positions relative to the ATG start codon of the NIS gene.

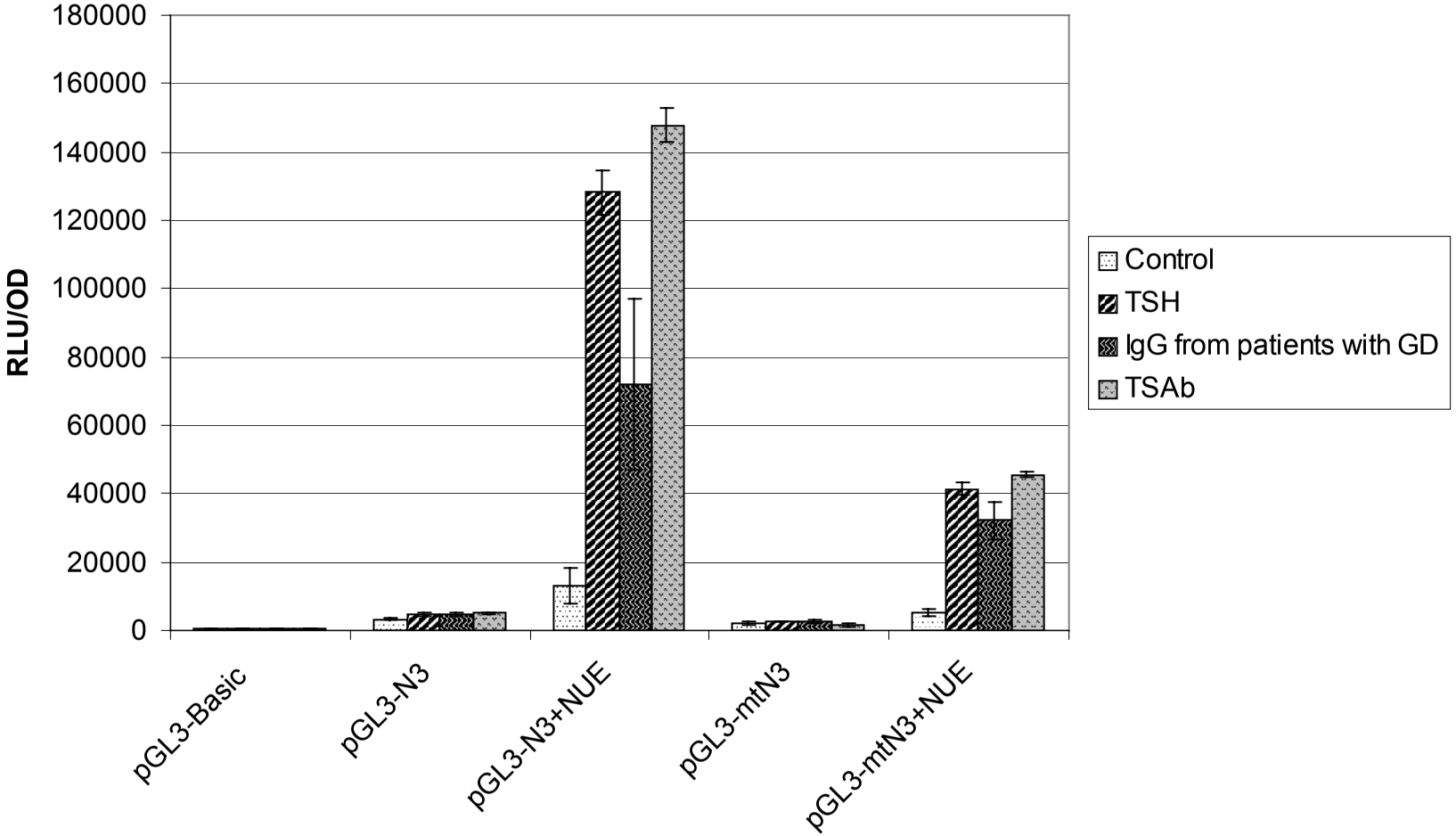
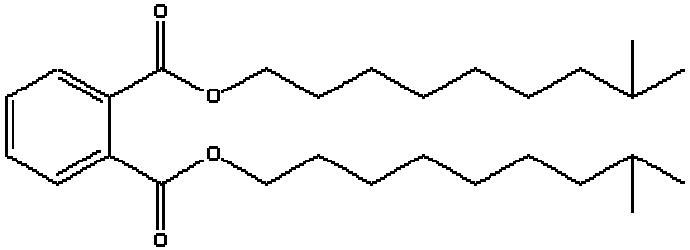
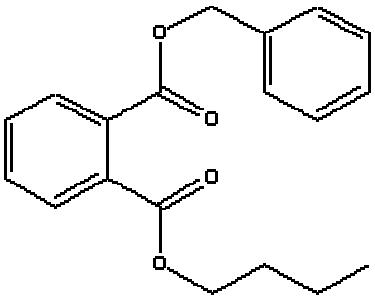


Fig. 8. Site-directed mutagenesis of the TRE. Constructs pGL3-mtN3 and pGL3-mtN3+NUE, containing two mutated nucleotides in the AP-1 sequence, were used in transfection assays in FRTL-5 cells together with wild-type constructs pGL3-N3 and pGL3-N3+NUE and pGL3-Basic. Treatment with TSH, IgG from patients with Graves' disease (GD) and thyroid stimulating antibody standard (TSAb) was performed exactly as described in Fig. 6. Luciferase activities are given in relative light units (RLU) in relation to OD and represent mean values ± SD of three experiments done in triplicate.

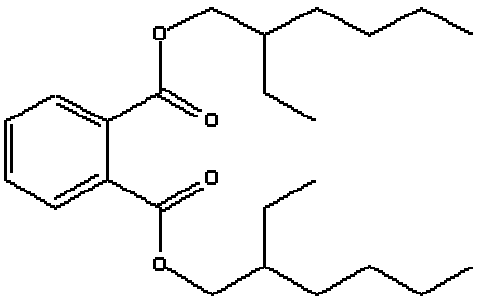
Diisodecyl phthalate (DIDP)
$C_{28}H_{46}O_4$



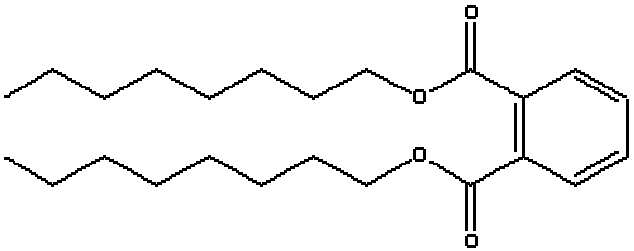
Benzyl butyl phthalate (BBP)
$C_{19}H_{20}O_4$



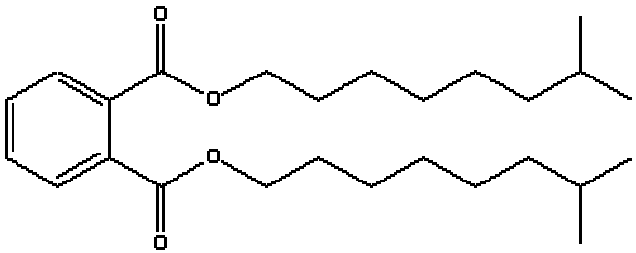
Bis(2-ethylhexyl) phthalate (BEHP)
$C_{24}H_{38}O_4$



Dioctyl phthalate (DOP)
$C_{24}H_{38}O_4$



Diisononyl phthalate (DINP)
$C_{26}H_{42}O_4$



Dibutyl phthalate (DBP)
$C_{16}H_{22}O_4$

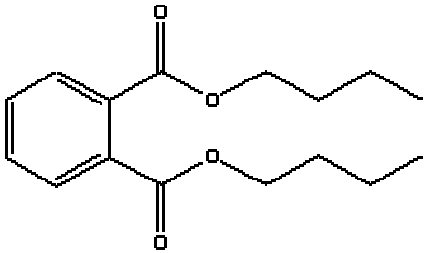


Fig. 9. Chemical formulas and structures of six major phthalates. Bis(2-ethylhexyl) phthalate (BEHP) and dioctyl phthalate (DOP) are constitutional isomers.

identified, whose positioning is very similar to that of rTRE (Fig. 7). To determine the functional relevance of this novel region, it was mutated in the pGL3-N3+NUE construct. The mutant construct was called pGL3-mtN3+NUE. When two nucleotides in hTRE were mutated, pGL3-mtN3+NUE was still activated by GD IgG, TSA or TSH but the activation was reduced approximately 3-fold compared to that of wild-type pGL3-N3+NUE (Fig. 8). No significant stimulation was found when the cells transfected with pGL3-mtN3+NUE were treated with IgG from normal subjects.

3.2. Transcriptional regulation of the human NIS promoter by phthalate plasticisers

3.2.1. Effect of the six major phthalates on the activity of hNIS promoter constructs

To study whether phthalates influence transcription of the hNIS gene, we used hNIS promoter (pGL3-N3) and hNIS promoter and enhancer (pGL3-N3+NUE) luciferase-fused constructs transiently transfected into rat thyroid PC Cl3 cells. At 24 h post-transfection, cells were cultured in 4H medium (48 h) and treated with 1.5 mU/ml TSH plus 1 mM di-isodecyl phthalate (DIDP), benzyl butyl phthalate (BBP), bis-(2-ethylhexyl) phthalate (DEHP), di-octyl phthalate (DOP), di-isononyl phthalate (DINP) or dibutyl phthalate (DBP) (Fig. 9).

After 24 h incubation, the transcriptional activity of pGL3-N3 was increased 2.5-fold by DIDP, 2.6-fold by BBP and 2.4-fold by DOP (Fig. 10). Conversely, a 0.7-fold down-regulation of pGL3-N3 by DBP was detected. No effect was observed for DEHP and DINP.

Curiously, TSH-induced activation of pGL3-N3+NUE was slightly decreased by DIDP, BBP, DEHP, DOP and DINP, whereas DBP completely abolished this activation (Fig. 11).

The controls with solvent ethanol treatment only (1%) did not significantly modulate pGL3-N3 and pGL3-N3+NUE activities.

3.2.2. Effect of the six major phthalates on NIS mRNA level

Starved PC Cl3 cells were treated with 1.5 mU/ml TSH plus 1 mM DIDP, BBP, DEHP, DOP, DINP or DBP, and endogenous rat NIS (rNIS) mRNA expression levels were analysed by RT-PCR and compared with those induced by TSH only.

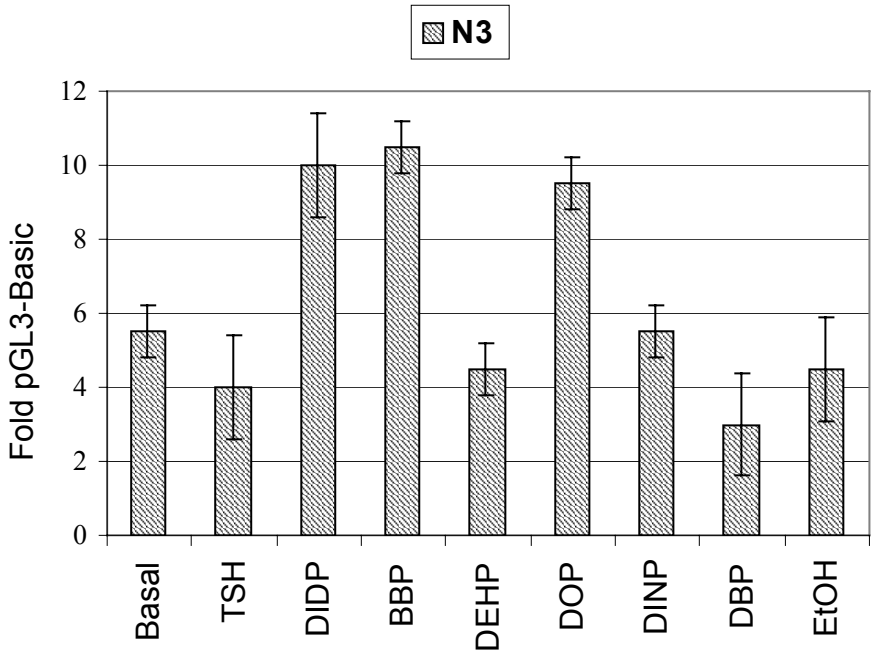


Fig. 10. Influence of DIDP, BBP, DEHP, DOP, DINP and DBP on the transcriptional activity of pGL3-N3. The luciferase construct pGL3-N3 was transfected into PC Cl3 cells, which were cultured post-starvation in 4H medium containing 1 mM DIDP, BBP, DEHP, DOP, DINP and DBP in the presence of 1.5 mU/ml TSH. The controls Basal, TSH and EtOH, received vehicle – 4H medium, 1.5 mU/ml TSH and 1% absolute ethanol, respectively. The control Basal equals the basal pGL3-N3 promoter activity. Given are the luciferase values in relation to pGL3-Basic, which represent mean values ± SD of three experiments done in triplicate.

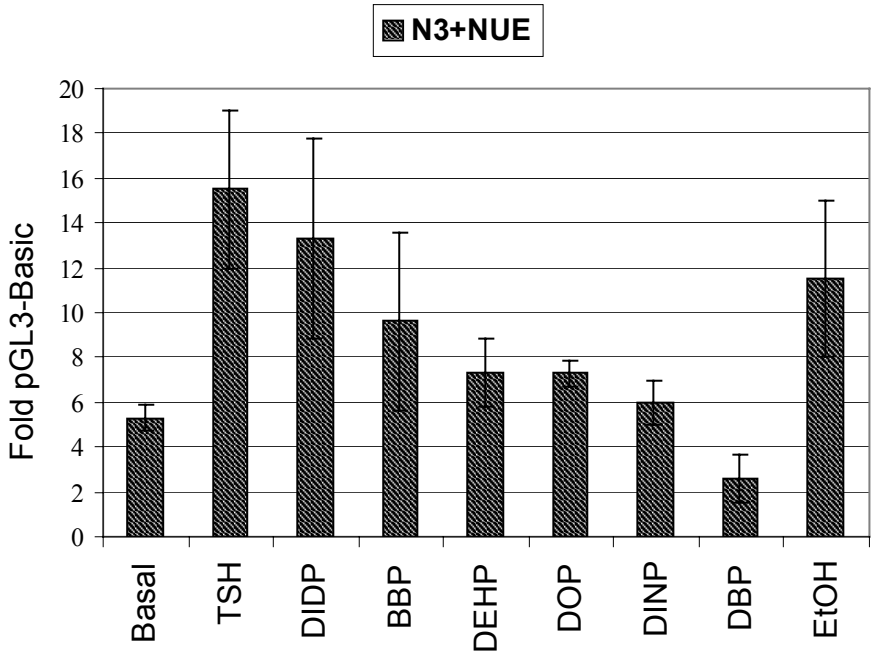


Fig. 11. Influence of DIDP, BBP, DEHP, DOP, DINP and DBP on the transcriptional activity of pGL3-N3+NUE. The experimental design and the calculation of data were the same as for Fig. 10.

As shown in Fig. 12A, after 24 h incubation none of these compounds could alter rNIS mRNA. In contrast, at 48 h mRNA levels were increased ca. 2-fold by DIDP, BBP and DOP, but remained unaffected by DEHP, DINP and DBP (Fig. 12B). The mRNA expression measured 72 h post-treatment was also unchanged (Fig. 12C).

Similarly to luciferase experiments, no significant effects were detected when the solvent ethanol was used as a control.

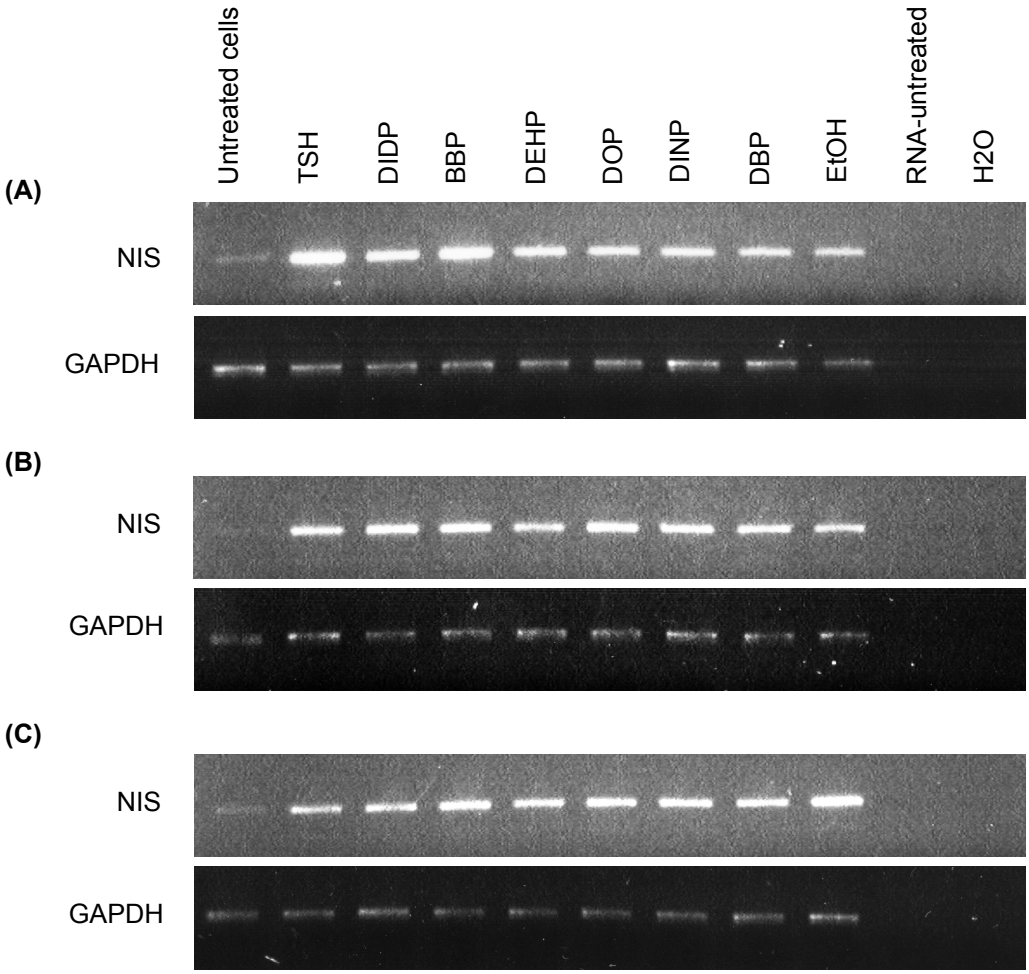


Fig. 12. Effect of DIDP, BBP, DEHP, DOP, DINP and DBP on NIS mRNA levels. PC Cl3 cells were starved and then treated with 1 mM DIDP, BBP, DEHP, DOP, DINP and DBP in the presence of 1.5 mU/ml TSH for 24 h **(A)**, 48 h **(B)** and 72 h **(C)**. Amplification of the GAPDH message levels reflected differences in cDNA loading. Control reactions with RNA from untreated cells used as template were also carried out.

3.3. Transcriptional properties of the human thyrostimulin A2 subunit

3.3.1. 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 αT3) and non-pituitary cell lines (293). 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 cells, 4.4-fold in GH3 cells and 2.9-fold in αT3 cells), indicating the presence of negative DNA regulatory elements.

The incremental 5' deletions extending to – 373 were performed as shown in Fig. 13. There was a similar overall decrease in activity between – 3119 and – 373 in all cell lines. However, when the region between – 373 and – 37 was deleted, a striking increase of the promoter activity was observed, with the strongest activation being detected for the – 506/– 347 fragment in 293 cells of 50-fold, in GH3 cells of 14.8-fold and in αT3 cells of 11.7-fold. This indicated that the – 506/– 347 fragment contains the minimal promoter.

To delineate the minimal promoter region, 5' and 3' deletions of the – 506/– 347 fragment were performed. 293, GH3 and αT3 cells were transfected with luciferase expression constructs containing each of the deleted promoters. Results of these experiments are shown in Fig. 14. Deletion of sequence between – 506 and – 489 resulted in a 5.7-fold decrease in the activity in 293 cells, 6.2-fold in GH3 cells and 3.8-fold in αT3 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 the three 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, 13.9-fold in GH3 cells and 5.3-fold in αT3 cells). Curiously, further deletion of extra 16 nt (– 373 to – 347) reduced this inhibitory effect to only 2.7-fold, 1.2-fold and 1.7-fold in 293, GH3 and αT3 cells, respectively (Fig. 14 and 15). Further deletions from – 433 to – 373 showed ca. 2 to 3-fold drop in the promoter activity in all cell lines, leading to a conclusion that the minimal promoter region for A2 gene lies between the – 506 and – 347 nt (Fig. 14). This minimal promoter was designated – 506/– 347 or A2min.

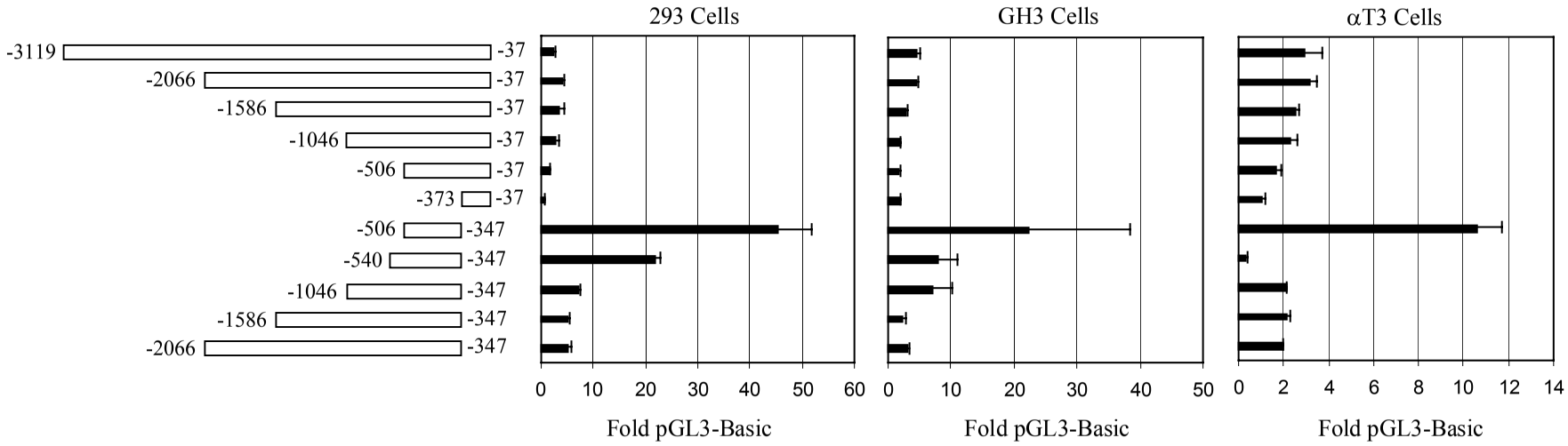


Fig. 13. Transient expression analysis of the A2 promoter activity. Luciferase reporter constructs containing various lengths of the A2 5' flanking region were transfected into 293, GH3 and αT3 cells. The constructs are represented schematically on the vertical axis. The numbering is relative to the 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 means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns.

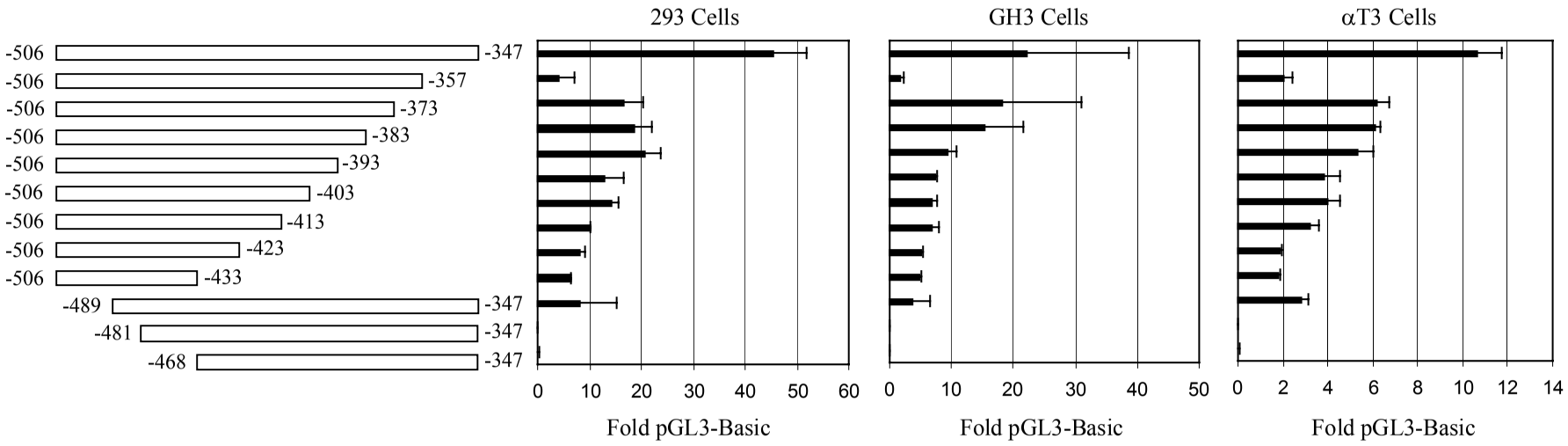


Fig. 14. Transient expression analysis of the A2 promoter deletion constructs. Luciferase reporter constructs containing various lengths of the A2 5' flanking region were transfected into 293, GH3 and αT3 cells. The constructs are represented schematically on the vertical axis. The numbering is relative 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 means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns.

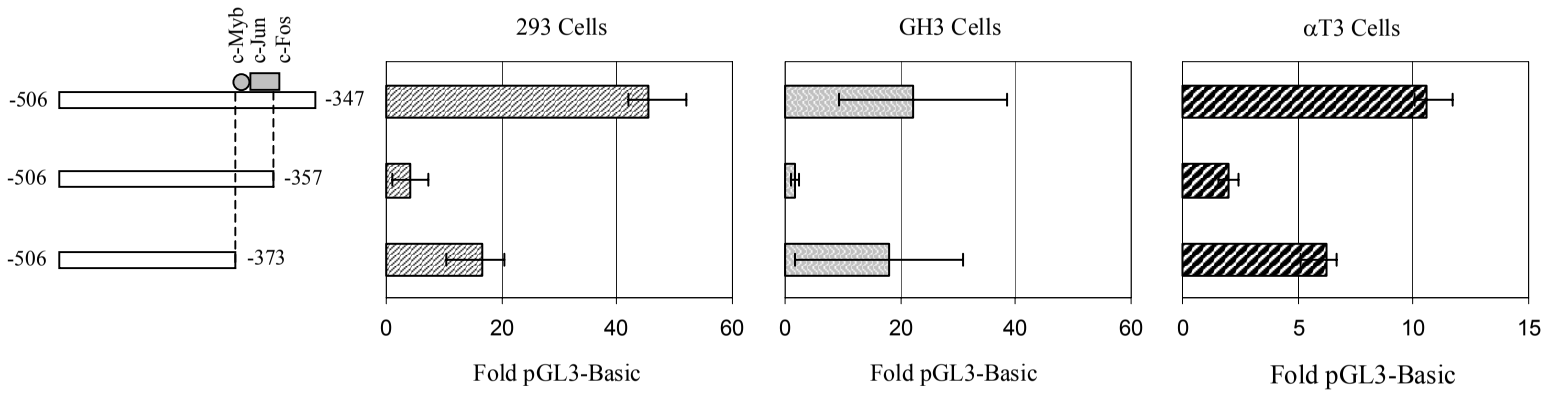


Fig. 15. Comparison of the transcriptional activities of – 506/– 347 (A2min), – 506/– 357 and – 506/– 373. Luciferase reporter constructs containing various lengths of the A2 5' flanking region were transfected into 293, GH3 and αT3 cells. On the left side, the luciferase constructs are drawn schematically with the numbering relative to the ATG start codon of the A2 gene. Putative transcription factor c-Myb is circled and c-Jun/c-Fos heterodimers are boxed. On the right side, the resulting luciferase values are expressed as fold stimulation of the activity of pGL3-Basic alone, which was set to 1. Error bars represent means ± SD.

3.3.2. Substitution mutant analysis of the A2 subunit promoter

The 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. 16). 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. 293, GH3 and αT3 cells were transfected with luciferase expression constructs containing each of the mutated promoters, and the results are shown in Fig. 17.

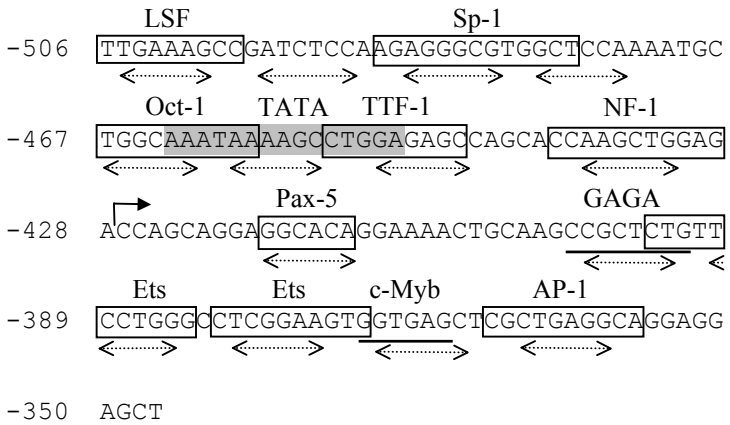


Fig. 16. Substitution mutants of the A2 minimal promoter. The location of each mutation (dotted arrow with heads pointing in opposite directions) relative to the transcription start site (bent arrow) is schematically indicated. The TATA box is shaded in grey and the predicted transcription factor binding sites are boxed or underlined. The numbering is relative to the ATG start codon of the A2 gene.

The mutation 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, of 32.7-, 49- and 10.9-fold in GH3 cells and of 18-, 4.8- and 6-fold in αT3 cells, respectively.

In contrast, the mutation of the NF-1 site (m8) lowered transcriptional activity only slightly (by 1-fold in 293 cells, by 1.7-fold in GH3 cells and by 1.2-fold in αT3 cells), suggesting that this region does not contain a stimulatory element.

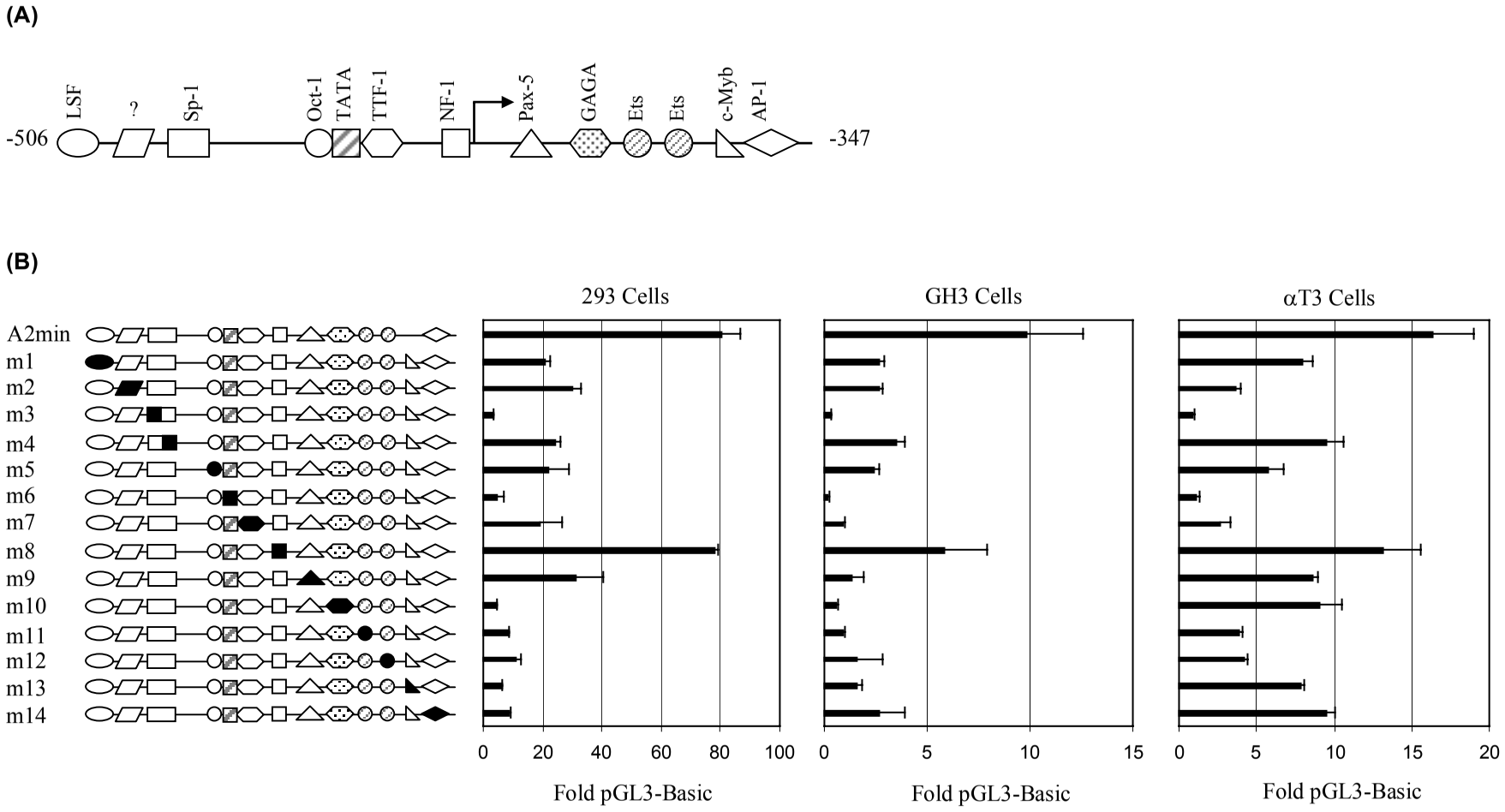


Fig. 17. Luciferase activity of the A2 minimal promoter substitution mutants. The consensus transcription factor binding sites are schematically drawn **(A)**. 293, GH3 and αT3 cells were transfected with the mutation constructs, with the mutated nucleotides marked by black boxes on the vertical axis **(B)**. The 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 means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns.

Some distinct effects were observed in each cell line among the rest of the mutations analysed. 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 lesser extent (by 16.3-, 10.9-, 6.1-, 6.1- and 3.6-fold, respectively). Of these mutations, only m11 and m12 significantly down-regulated promoter activity in αT3 cells (by 4.2- and 3.9-fold), while m10, m13 and m14 modestly reduced it (by 1.8-, 2- and 1.7-fold).

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. In αT3 cells, mutant m2 showed a decrease in transcriptional activity of 4.5-fold, whereas the activities of three other mutants were within 2-fold of the wild-type promoter activity. Strongly reduced promoter activity was observed with m9 (mutated Pax-5 site) in GH3 (of 7.5-fold) but not in 293 (of 2.5-fold) or αT3 cells (of 1.9-fold).

3.3.3. 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, EMSAs with 293, GH3 and αT3 nuclear extracts were performed. A series of double-stranded biotin-labelled oligonucleotide probes were generated by PCR amplification (Fig. 3).

The incubation of 293, GH3 and αT3 extracts with probe 1 revealed a protein-DNA complex 1a, though it was less intense in the latter two extracts (Fig. 18). An additional low-intensity complex 1b was seen in nuclear extracts from 293 cells.

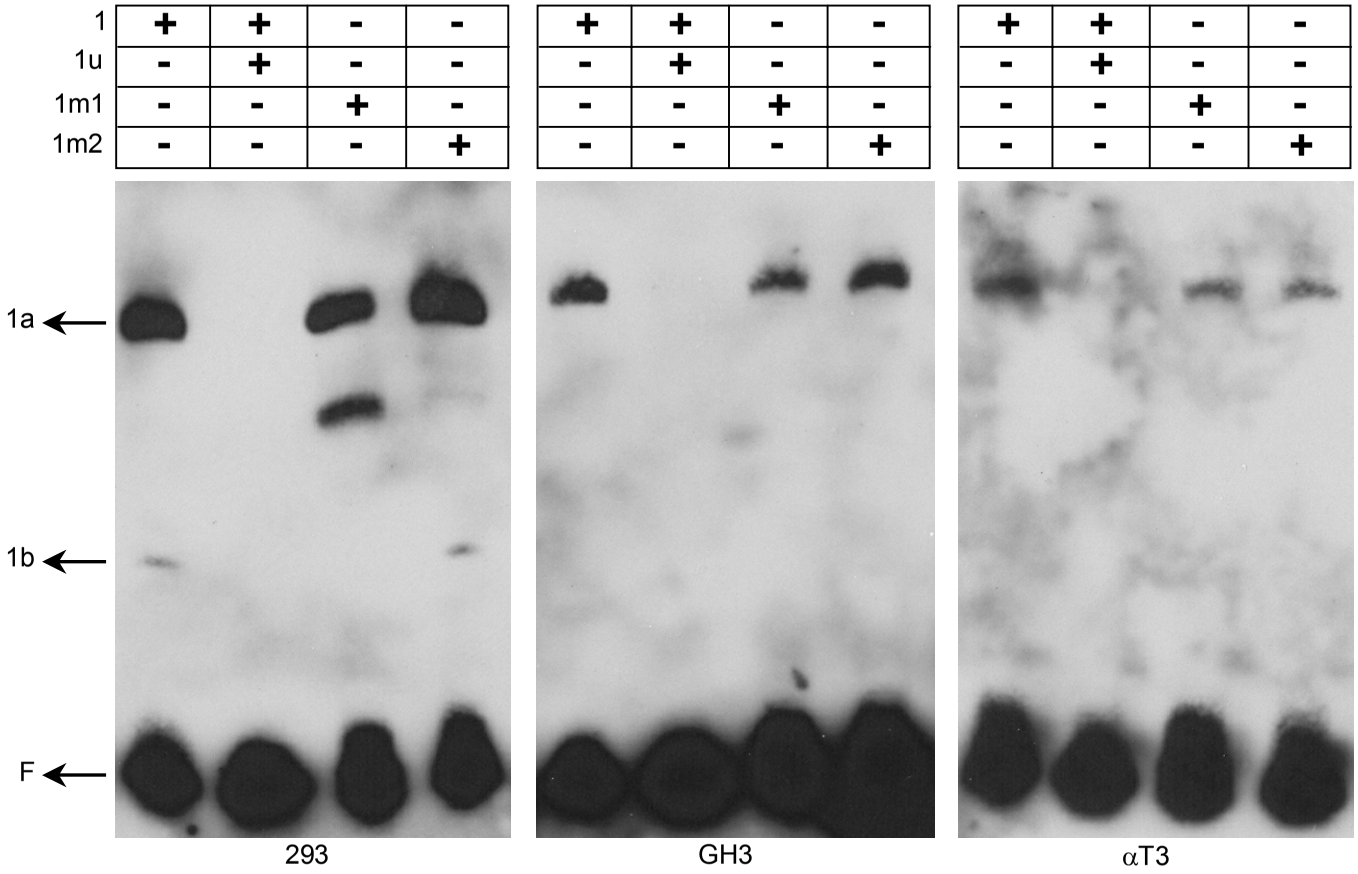


Fig. 18. EMSA with probe 1. Shown are complexes formed on the wild type and mutated probe 1 in nuclear extracts from 293, GH3 and αT3 cells. Probes numbered as in Fig. 3. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

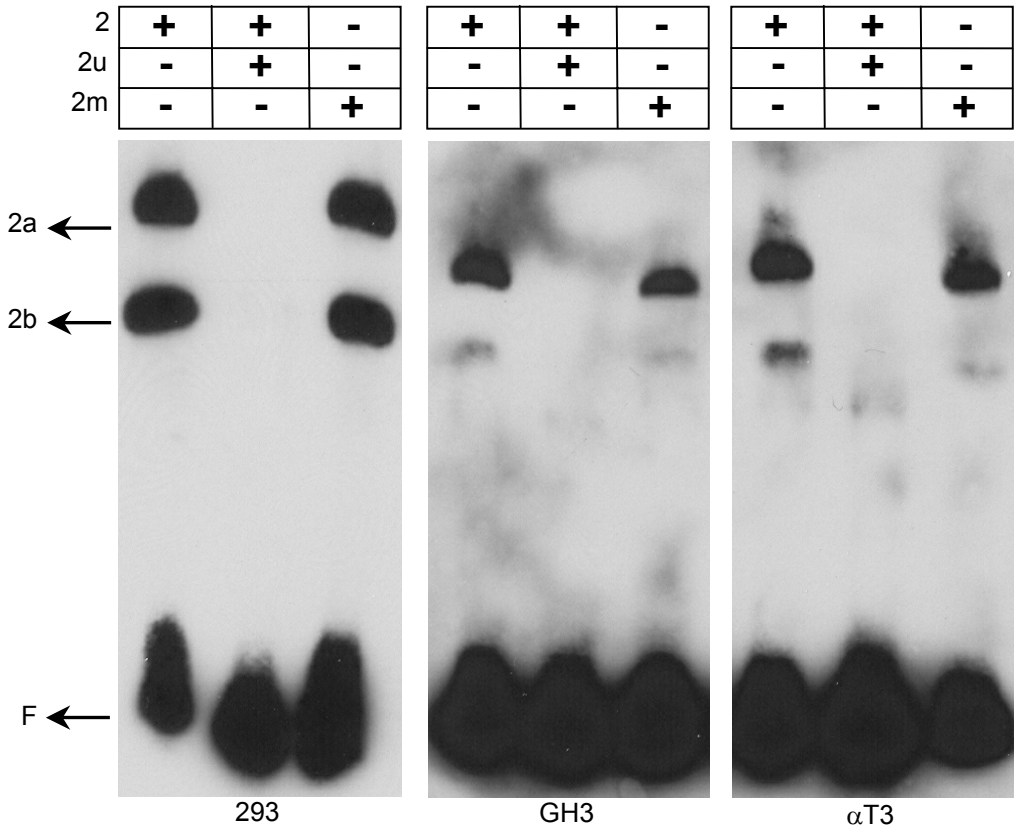


Fig. 19. EMSA with probe 2. Shown are complexes formed on the wild type and mutated probe 2 in nuclear extracts from 293, GH3 and αT3 cells. Probes numbered as in Fig. 3. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

Probe 2 formed two complexes 2a and 2b in extracts from all the three cell lines tested, however, the latter complex was much more abundant in 293 extracts (Fig. 19). Hence, it is easy to imagine that this complex is accounted for by the same unknown factor that binds to the site adjacent to LSF on probe 1 in 293 extracts (1b). It is possible that the formation of this complex, barely detectable on probe 1, may now be visible on probe 2 because of the addition of extra nucleotides that are required for its binding.

Three complexes 3a, 3b and 3c were detected when nuclear extracts from all cell lines were incubated with probe 3 (Fig. 20). Here, complexes 3a and 3b also appeared more abundant in 293 extracts.

Probe 4 yielded a single complex 4a, which, again, was more intense in extracts (Fig. 21).

The shift pattern observed with probe 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 (Fig. 22). Furthermore, complex 5b was barely detectable in

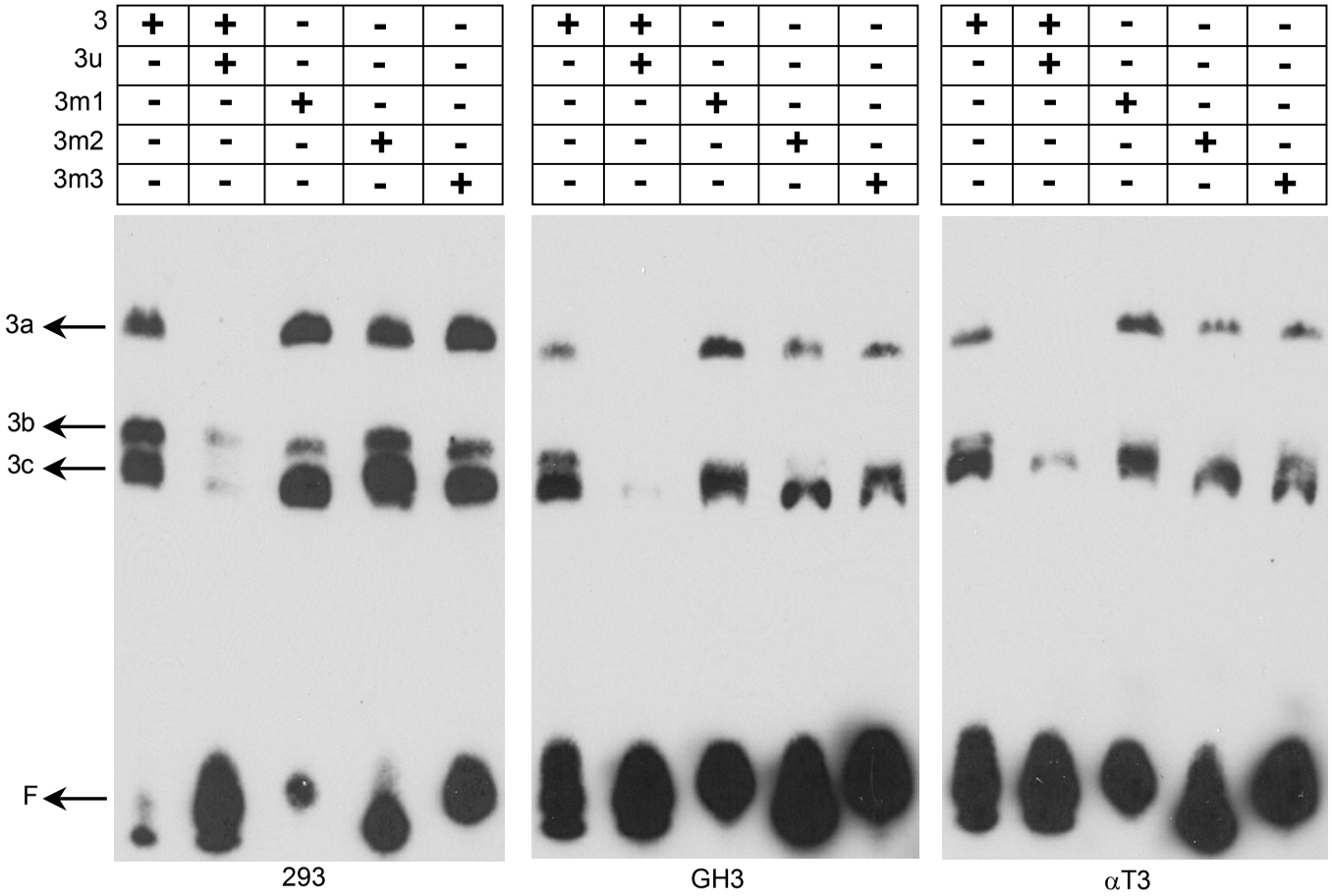


Fig. 20. EMSA with probe 3. Shown are complexes formed on the wild type and mutated probe 3 in nuclear extracts from 293, GH3 and αT3 cells. Probes numbered as in Fig. 3. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

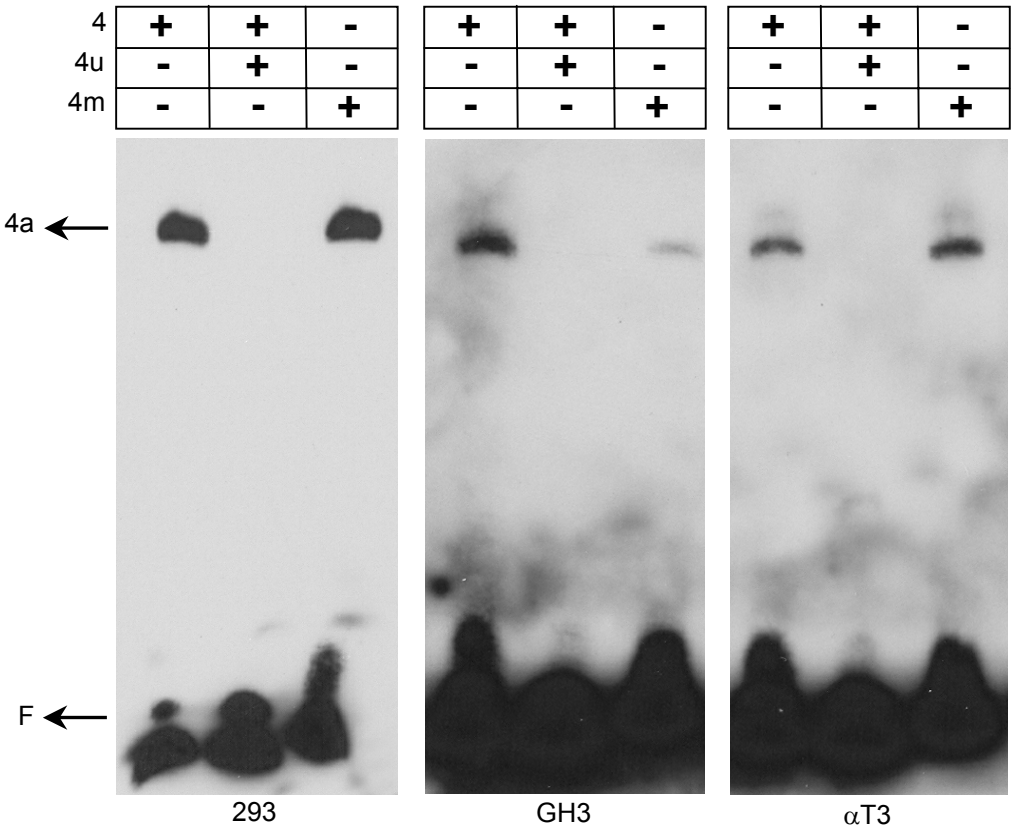


Fig. 21. EMSA with probe 4. Shown are complexes formed on the wild type and mutated probe 4 in nuclear extracts from 293, GH3 and αT3 cells. Probes numbered as in Fig. 3. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

293 and GH3 nuclear extracts, whereas it appeared abundant in αT3 extracts. In addition, complexes 5a and 5c were far more abundant in extracts from 293 cells compared to those in GH3 and αT3 extracts.

The last probe 6 resulted in the formation of three complexes 6a, 6b and 6c in 293, GH3 and αT3 extracts, with complex 6a being most intense in extracts from 293 cells (Fig. 23). By analogy with complexes 1b and 2b, discussed above, perhaps complexes 5d and 6c represent the same factor bound to the second Ets site in 293 extracts. The higher concentration of 5d could be the result of probe 5 containing the full motif for the Ets factor, in contrast to probe 6 that lacks the motif's first few bases.

In competition experiments, 200-fold excess of unlabeled probes 1 – 6 abolished all complex formation in nuclear extracts from the three cell lines examined, with an exception of 293 extracts where complexes 3b and 3c were strongly reduced (Fig. 20).

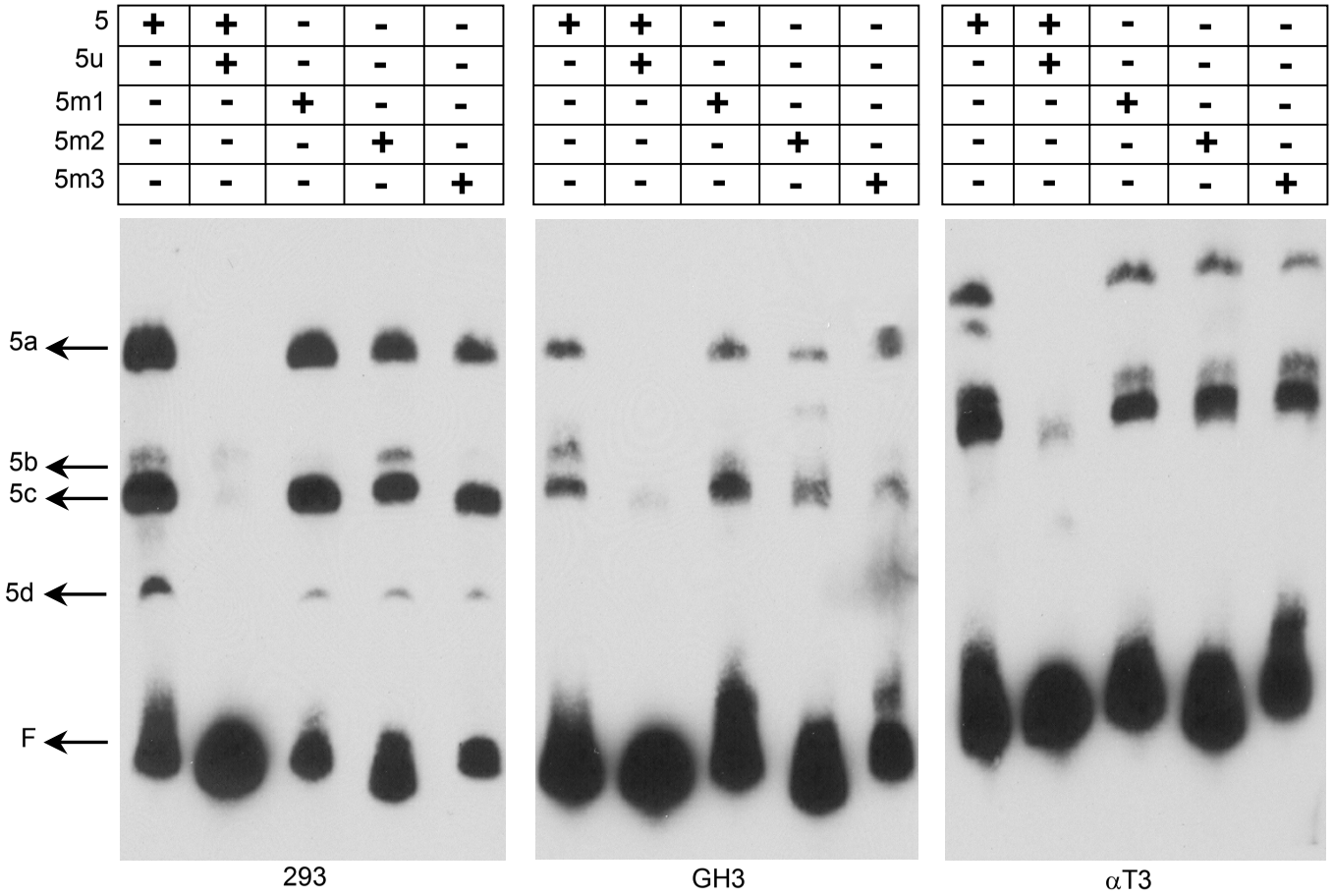


Fig. 22. EMSA with probe 5. Shown are complexes formed on the wild type and mutated probe 5 in nuclear extracts from 293, GH3 and αT3 cells. Probes numbered as in Fig. 3. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

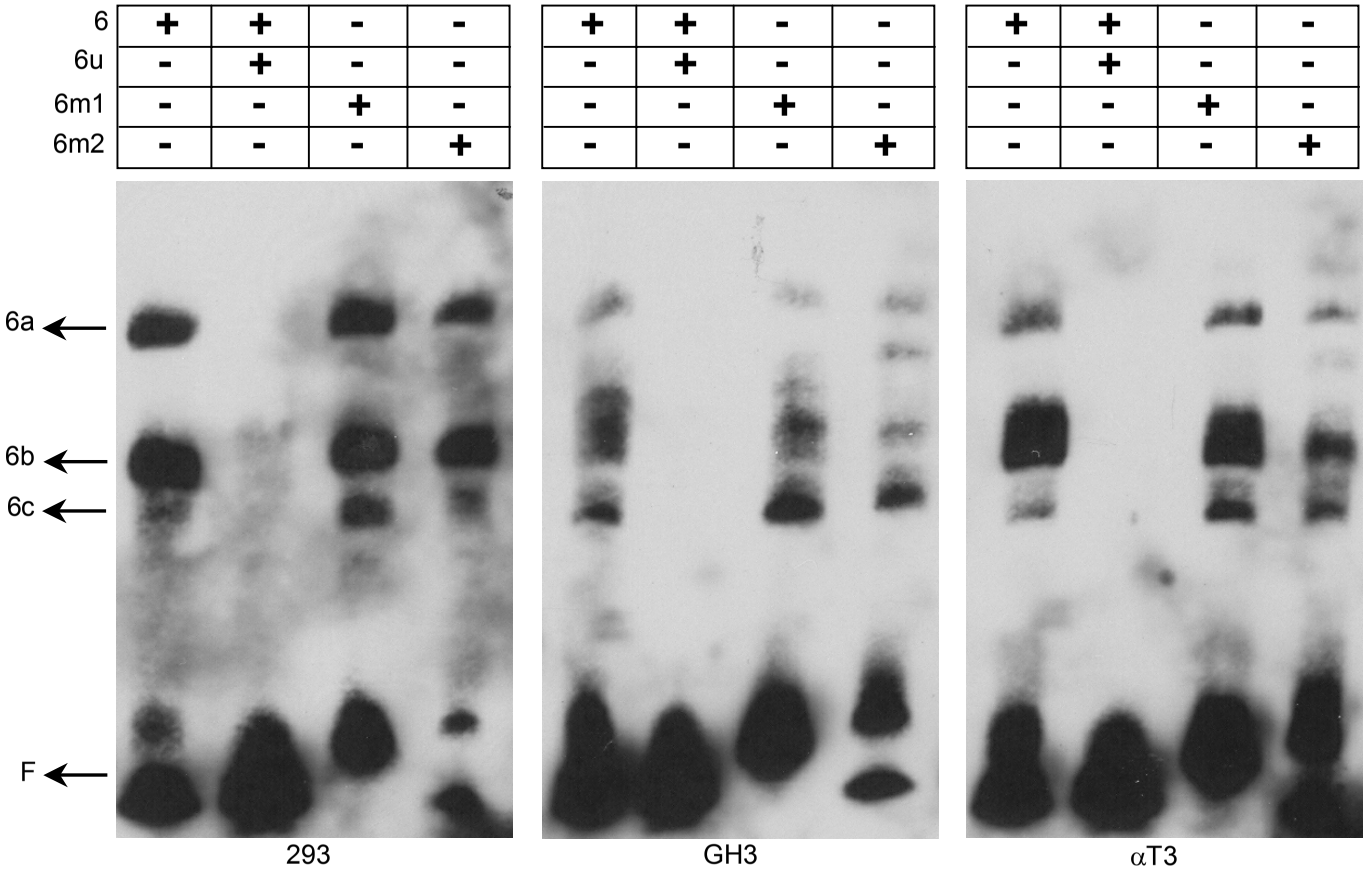


Fig. 23. EMSA with probe 6. Shown are complexes formed on the wild type and mutated probe 6 in nuclear extracts from 293, GH3 and αT3 cells. Probes numbered as in Fig. 3. Every second reaction contained unlabeled competitor (u) in 200-fold excess as diagrammed on top of each gel. The shifted complexes and free probe (F) are marked with arrows at the left.

Next, biotin-labelled probes containing mutated potential transcription factor binding sites (that eliminated promoter function in luciferase assays, Fig. 3B and 16) were prepared 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 (Fig. 22), in GH3 extracts two complexes 3b and 5b were eradicated on the corresponding probes 3m1, 3m2, 3m3, 5m1, 5m2 and 5m3 (Fig. 20 and 22) and in αT3 extracts the formation of complex 3b was completely disrupted on probes 3m1, 3m2 and 3m3 (Fig. 20).

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) (Fig. 20 and 22), complexes 2b on 2m, 4a on 4m and 6b on 6m1 and 6m2 (GH3 extracts) (Fig. 19, 21 and 23) and complexes 2b on 2m, 5b on 5m1, 5m2 and 5m3 and 6b on 6m2 (αT3 extracts) (Fig. 19, 22 and 23).

It should also be noted that probe 1m1 resulted in the appearance of a new complex in 293 extracts (Fig. 18), which is most likely attributable to the accidental generation of the fortuitous binding site for another protein. Similarly, probe 6m2 yielded an additional, although barely detectable, complex in nuclear extracts from all cell lines (Fig. 23). A weak band yielded by probe 6m2 was also noted in extracts from all three cell lines.

With regard to the remaining few complexes, their formation was unaffected on the mutated probes.

3.3.4. 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 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

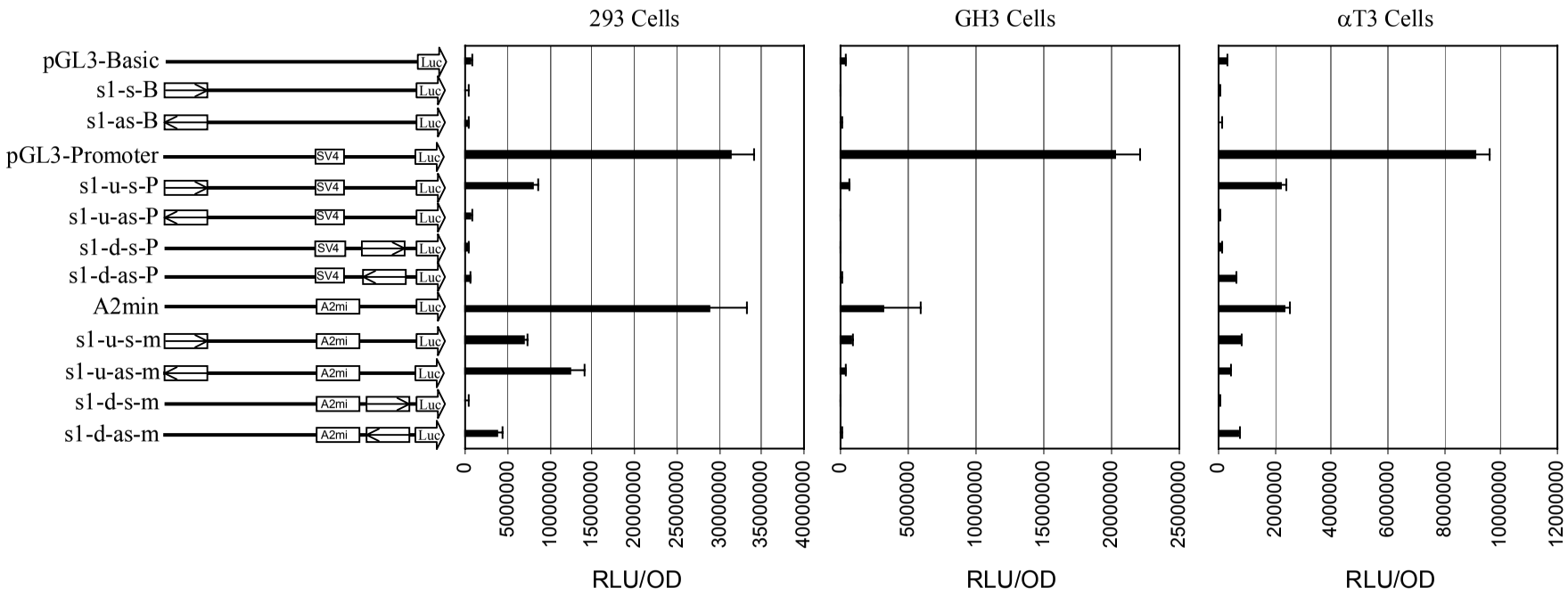


Fig. 24. Region s1 represses transcription from native and heterologous promoters in a position- and orientation-independent manner. The resulting relative luciferase values after transfection into 293, GH3 and αT3 cells represent means ± SD of three experiments done in triplicate. The absence of error 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.

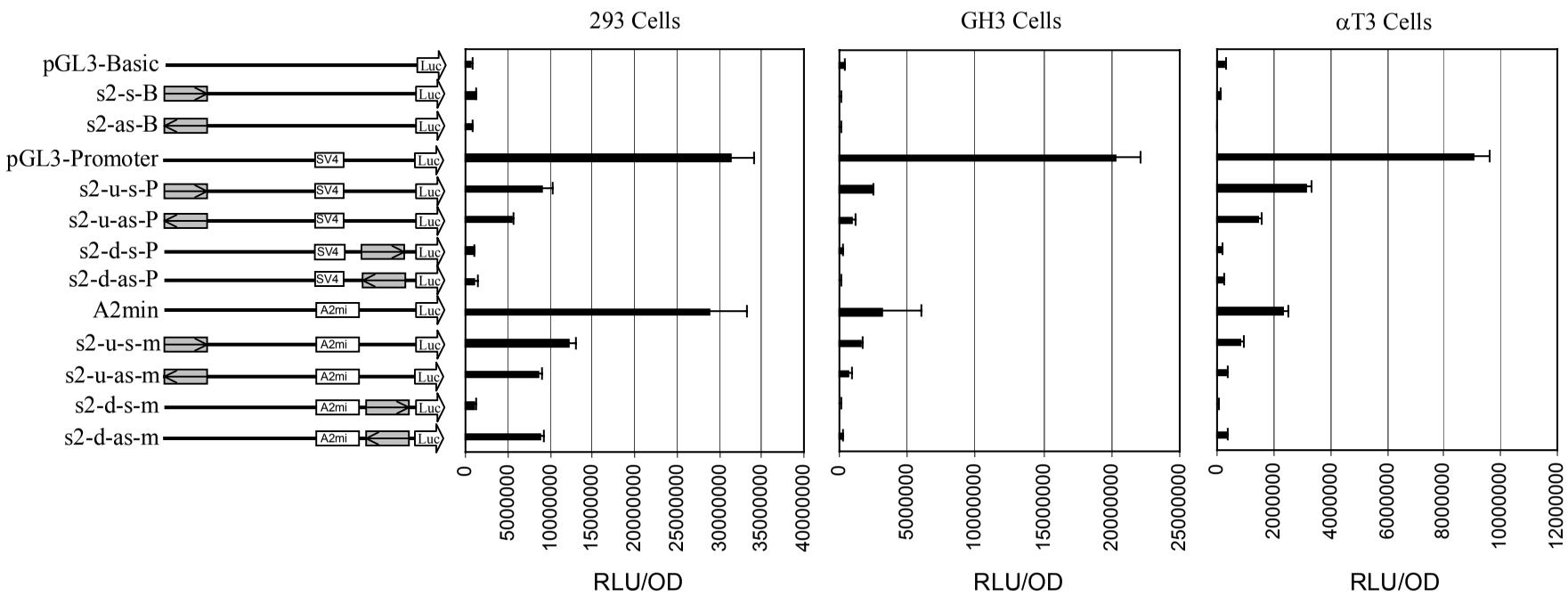


Fig. 25. Region s2 represses transcription from native and heterologous promoters in a position- and orientation-independent manner. The resulting relative luciferase values after transfection into 293, GH3 and αT3 cells represent means ± SD of three experiments done in triplicate. The absence of error 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.

transfected into pituitary (GH3 and αT3) 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 all cell lines (Fig. 24). 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 cells (4- and 2.3-fold of A2min, 4- and 44-fold of pGL3-Promoter) and αT3 cells (3- and 6-fold of A2min, 4- and 219-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 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 the three cell lines studied.

Further, it was examined how the fragment s2 altered the activity of parent reporter plasmids. No transcriptional activity was demonstrable in the three cell lines tested when s2 was fused to pGL3-Basic (s2-s-B and s2-as-B) (Fig. 25). 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, by 2- and 5-fold in GH3 cells and by 2.7- and 7-fold in αT3 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, by 8.2- and 23-fold in GH3 cells and by 2.9- and 6.4-fold in αT3 cells.

As in the case of s1, the positioning of s2 downstream of the respective promoters of the 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.

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

First, the A2 gene expression in diverse cell lines was examined by the RT-PCR analysis. As shown in Fig. 26A, 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 A2 mRNA expression.

After that, the above eight cell lines were transiently transfected with the A2min construct to elucidate cell-specific regulation of the A2 gene (Fig. 26B). A strong promoter activity was demonstrable in all cell lines, being at its highest in 293 cells.

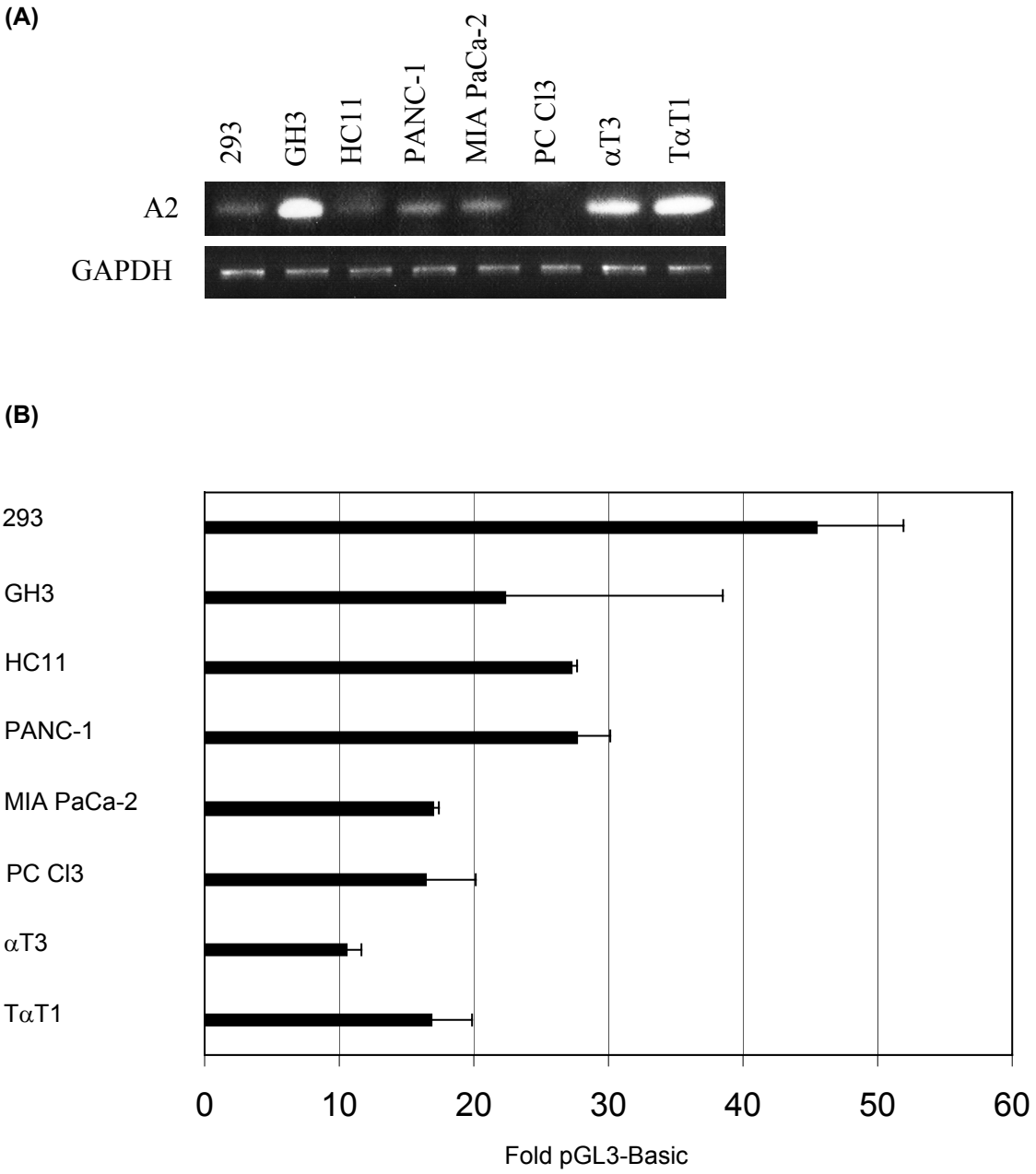


Fig. 26. Expression of the A2 gene and luciferase activity of its minimal promoter in various cell lines. (A) RT-PCR analysis of A2 transcript in 293, GH3, HC11, PANC-1, MIA PaCa-2, PC Cl3, αT3 and TαT1 cells. Amplification of the GAPDH message levels reflected differences in cDNA loading. **(B)** Transient expression analysis of the A2 minimal 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 means ± SD of three experiments done in triplicate.

3.3.6. Effect of TRH and forskolin on the A2 subunit promoter activity

In view of the positive regulation of the glycoprotein α-subunit gene expression by TRH and cAMP (Hashimoto et al., 2000), the effect of TRH and the cAMP agonist forskolin was analysed in reporter gene assays. The A2min construct was transfected into pituitary (rat somatolactotrope GH3 and mouse thyrotrope TαT1) and non-pituitary (human embryonic kidney 293) cell lines. At 48 h post-transfection, TαT1 cells were treated with 100 nM TRH, 293 cells were treated with 10 µM forskolin, whereas GH3 cells were treated with either 100 nM TRH or 10 µM forskolin. After 24 h incubation, no significant effect on the transcriptional activity of A2min was observed (Fig. 27 and 28).

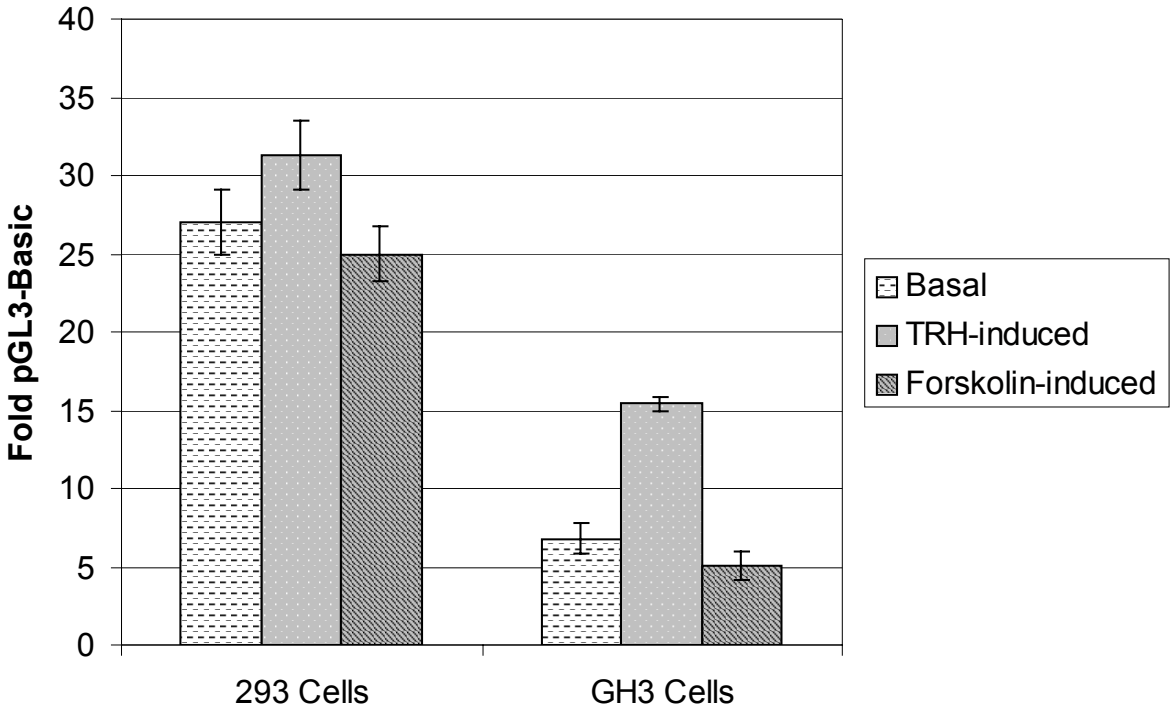


Fig. 27. Influence of TRH and forskolin on the transcriptional activity of the A2 minimal promoter. The luciferase construct A2min was transfected into 293 and GH3 cells, which were cultured in the absence (Basal) or presence of 100 nM TRH (TRH-induced) or 10 µM forskolin (Forskolin-induced). Given are the luciferase values in relation to pGL3-Basic (Basic=1), which represent mean values ± SD of three experiments done in triplicate.

3.3.7. Auto-regulation of the A2 subunit promoter

To test if thyrostimulin can modulate its own promoter, the activity of the A2min construct was analysed in transient co-transfections with the bi-promoter expression vector pBud-A2/B5, which contained A2 and B5 subunit cDNAs (Fig. 29). Compared to co-transfections with the empty expression vector pBudCE4.1 alone, pBud-A2/B5

led to ca. 2-fold decrease in 293 cells and ca. 3-fold decrease in GH3 cells (Fig. 30). These experiments indicated a negative auto-regulatory effect of thyrostimulin on its own transcription.

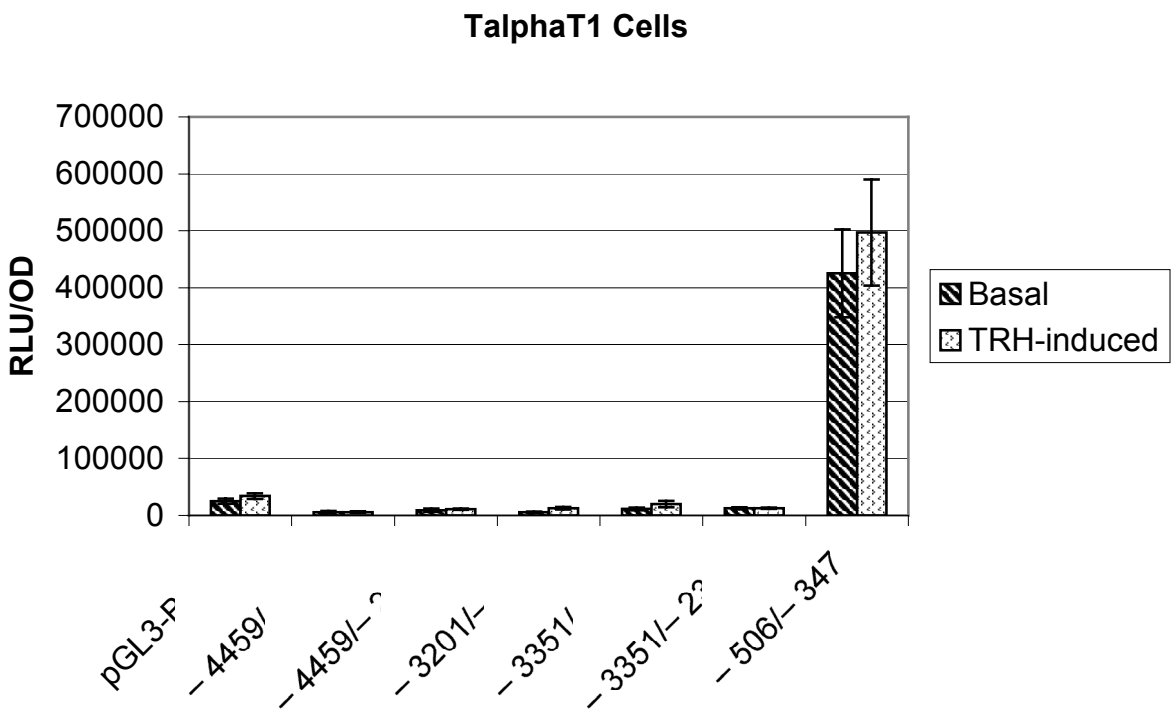


Fig. 28. Influence of TRH on the transcriptional activity of genomic DNA fragments of the B5 gene and of the A2 minimal promoter. The luciferase constructs – 4459/– 993, – 4459/– 2312, – 3201/– 993, – 3351/– 993, – 3351/– 2312, – 506/– 347 (A2min) and empty vector pGL3-Basic were transfected into TαT1 cells, which were cultured in the absence (Basal) or presence (TRH-induced) of 100 nM TRH. Luciferase activities are given in relative light units (RLU) in relation to OD and represent mean values ± SD of three experiments done in triplicate.

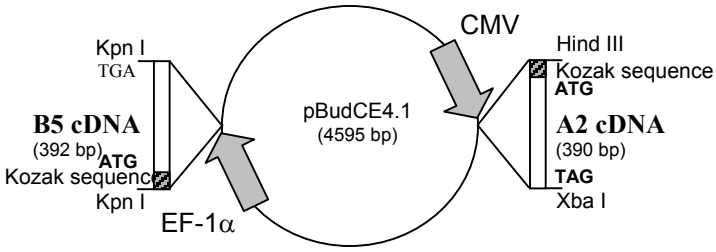


Fig. 29. Schematic representation of the expression construct pBud-A2/B5. The A2 cDNA was cloned downstream of the CMV promoter and contained a perfect Kozak sequence, 5' HindIII and 3' XbaI. The B5 cDNA was cloned downstream of the EF-1α promoter and contained a perfect Kozak sequence and KpnI on both 5' and 3' sites.

3.3.8. Characterisation of the exon- and intron-located candidate silencer elements of the A2 subunit promoter

To examine transcriptional contribution of exons 1 – 3 and introns 1 – 2 of the A2 subunit, they were first cloned in sense orientation into the promoterless pGL3-Basic vector (e1-s-B, e2-s-B, e3-s-B, i1-s-B and i2-s-B), the pGL3-Promoter vector downstream of the SV40 promoter (e1-d-s-P, e2-d-s-P, e3-d-s-P, i1-d-s-P and i2-d-s-P) and the A2min construct downstream of the A2min promoter (e1-d-s-m, e2-d-s-m, e3-d-s-m, i1-d-s-m and i2-d-s-m) and tested in transient transfection assays in 293, GH3 and αT3 cell lines. Subsequently, the luciferase activities were compared with the activities of the corresponding parent constructs. As shown in Fig. 31 and 32, the indicated inserts

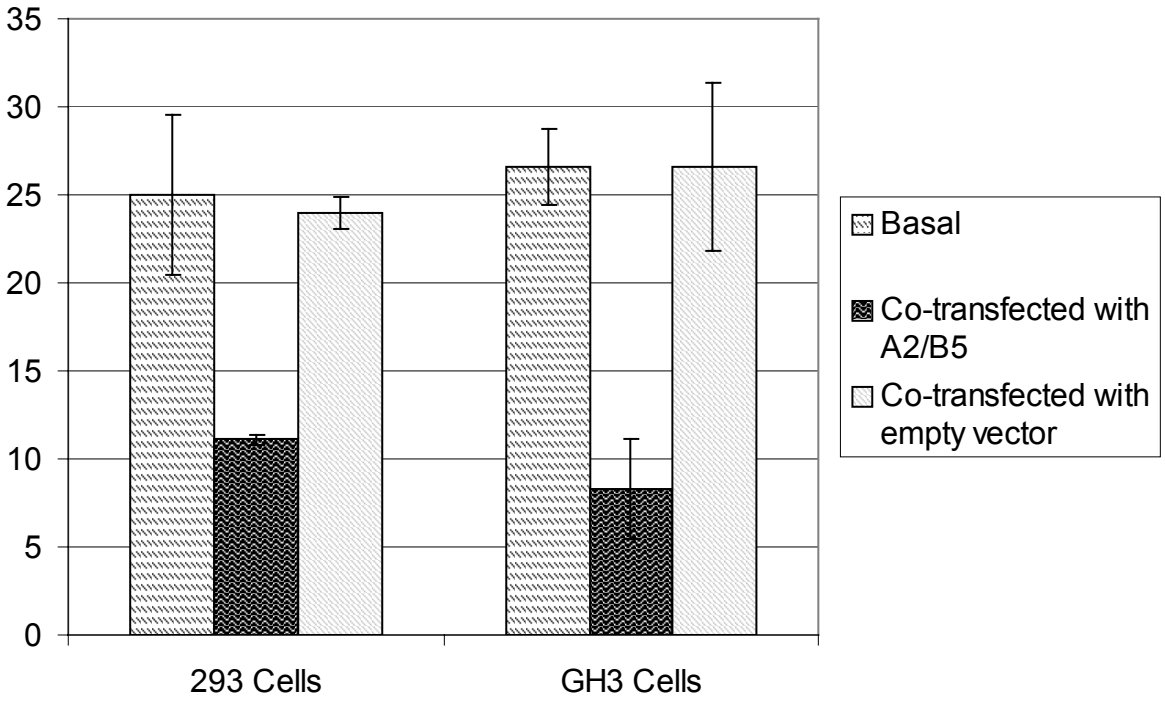


Fig. 30. Co-transfection with pBud-A2/B5. 293 and GH3 cells were transfected with the A2min construct and either the empty expression vector pBudCE4.1 or pBud-A2/B5. The resulting luciferase values, which were corrected for protein content, are expressed as fold stimulation of the activity of pGL3-Basic alone, which was set to 1. Error bars represent means ± SD.

in constructs e1-s-B, e2-s-B, e3-s-B, i1-s-B and i2-s-B had no effect on the basal activity of pGL3-Basic in all cell lines.

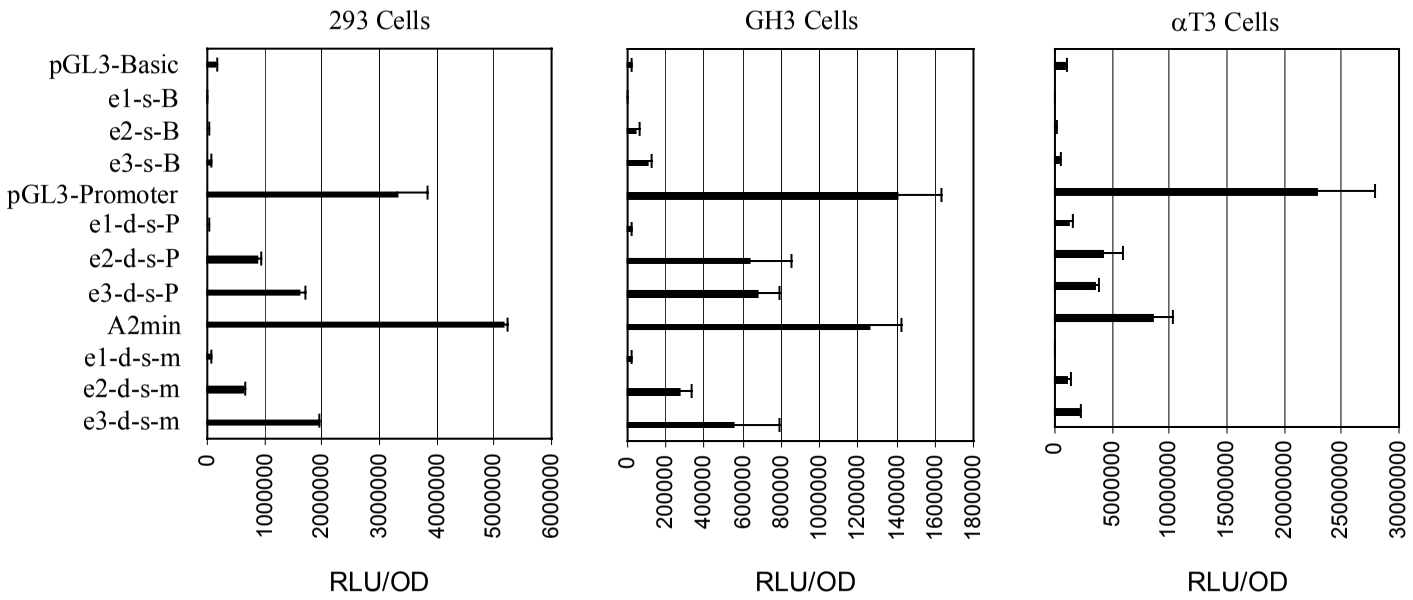


Fig. 31. Transient expression analysis of exon 1-3 (A) of the A2 gene. The resulting relative luciferase values after transfection into 293, GH3 and αT3 cells represent means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns. The constructs are presented on the vertical axis, with the A2 minimal promoter designated as A2min.

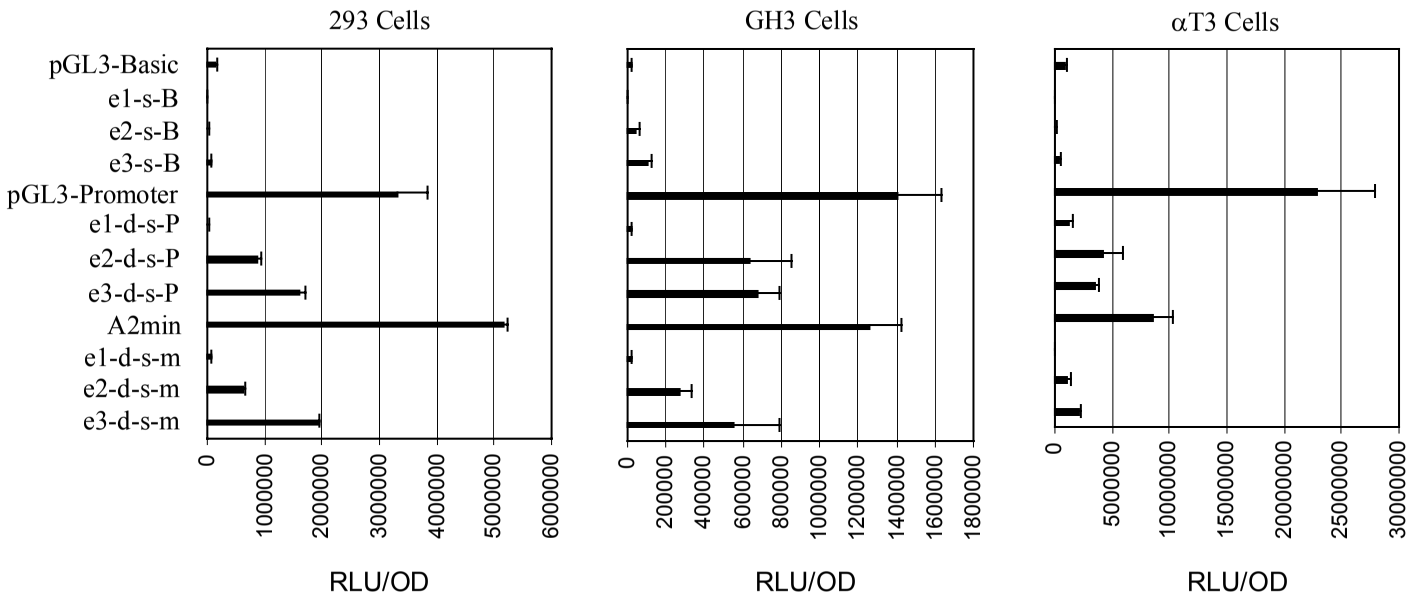


Fig. 32. Transient expression analysis of intron 1-2 (B) of the A2 gene. The resulting relative luciferase values after transfection into 293, GH3 and αT3 cells represent means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns. The constructs are presented on the vertical axis, with the A2 minimal promoter designated as A2min.

The transcription of constructs e1-d-s-P, e1-d-s-m, i2-d-s-P and i2-d-s-m was completely inhibited in the three cell lines studied, suggesting that exon 1 and intron 2 conceal silencing elements.

On the other hand, e2-d-s-P, e3-d-s-P, e2-d-s-m, e3-d-s-m, i1-d-s-P and i1-d-s-m also showed a decreased promoter activity, although to a lesser extent (within 2- and 3-fold of wild-type constructs).

Since exon 1 and intron 2 appeared to strongly down-regulate both A2min and SV40 promoters, it was necessary to confirm that they indeed contain silencer or negative regulatory elements. For this purpose, additional plasmids were constructed by positioning these two regions A, in anti-sense orientation into the pGL3-Basic (e1-as-B and i2-as-B); B, in anti-sense orientation downstream of the SV40 promoter (e1-d-as-P and i2-d-as-P) and in both orientations upstream of the SV40 promoter (e1-u-s-P, e1-u-as-P, i2-u-s-P and i2-u-as-P) into the pGL3-Promoter; B, in anti-sense orientation downstream of the A2min promoter (e1-d-as-m and i2-d-as-m) and in both orientations upstream of the A2min promoter (e1-u-s-m, e1-u-as-m, i2-u-s-m and i2-u-as-m) into the A2min construct.

The transient transfection of constructs e1-d-as-P and e1-d-as-m into 293, GH3 and αT3 cells resulted in a ca. 3-fold down-regulation of the promoter activity of these genes in the first two cell types, while in αT3 cells a decrease of only ca. 2-fold was observed (Fig. 33).

Constructs e1-u-s-P, e1-u-as-P, e1-u-s-m and e1-u-as-m exhibited significantly reduced promoter activities only in 293 cells (of 3-, 2.8-, 2- and 2.5-fold, respectively). Their down-regulation was within ca. 1-fold in GH3 and αT3 cell lines.

Fig. 34 shows results of transient transfections with constructs containing intron 2. In this case, i2-d-as-P and i2-d-as-m exhibited a marked decrease in luciferase activity only in GH3 cells (of 4.8- and 3.9-fold, respectively). No down-regulation of activity was found in αT3 cells, whereas in 293 cells it was only of 1.5-fold for i2-d-as-P and 2.3-fold for i2-d-as-m.

In 293 and GH3 cells, constructs i2-u-s-P, i2-u-as-P, i2-u-s-m and i2-u-as-m demonstrated diminished promoter activities of ca. 1.5-fold. Constructs i2-u-s-m and i2-u-as-m showed no activity decrease in αT3 cells, however, a down-regulation of ca. 2-fold was observed for i2-u-s-P and i2-u-as-P.

Both plasmids e1-as-B and i2-as-B displayed similar luciferase activities to that of pGL3-Basic.

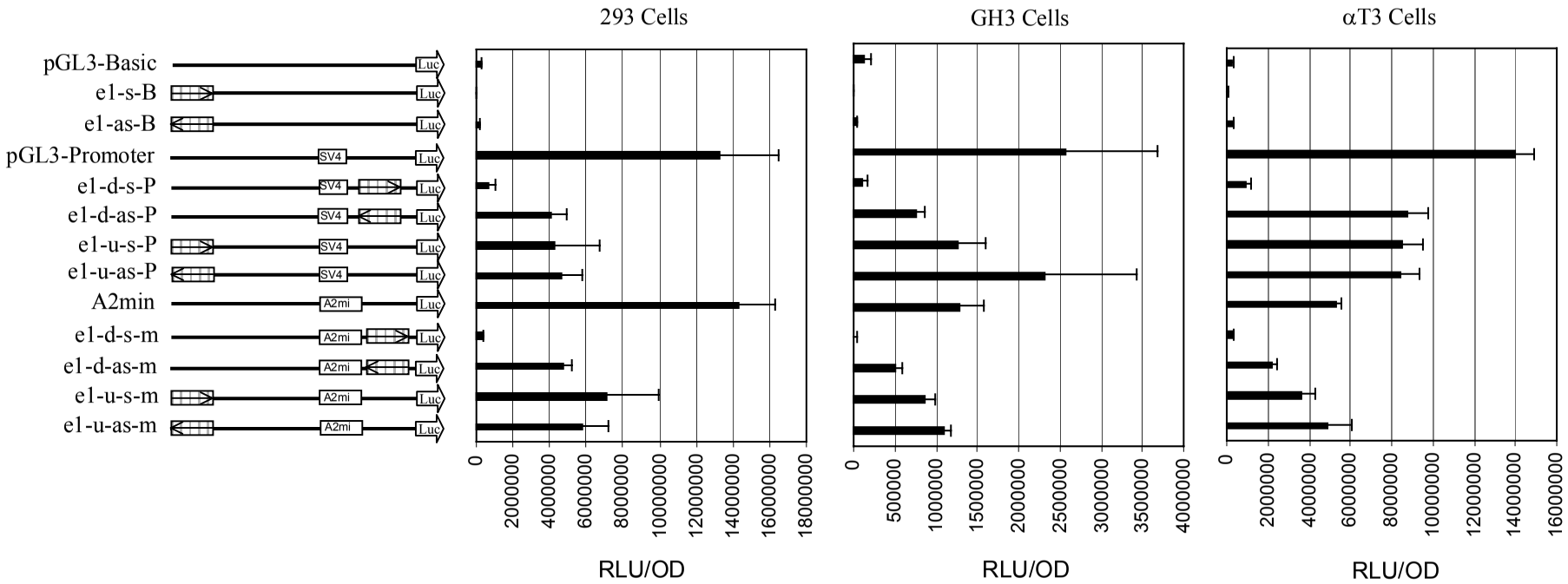


Fig. 33. Transient expression analysis of the silencing activity of exon 1. The resulting relative luciferase values after transfection into 293, GH3 and αT3 cells represent means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns. The constructs are drawn schematically on the vertical axis, with the A2 minimal promoter designated as A2min.

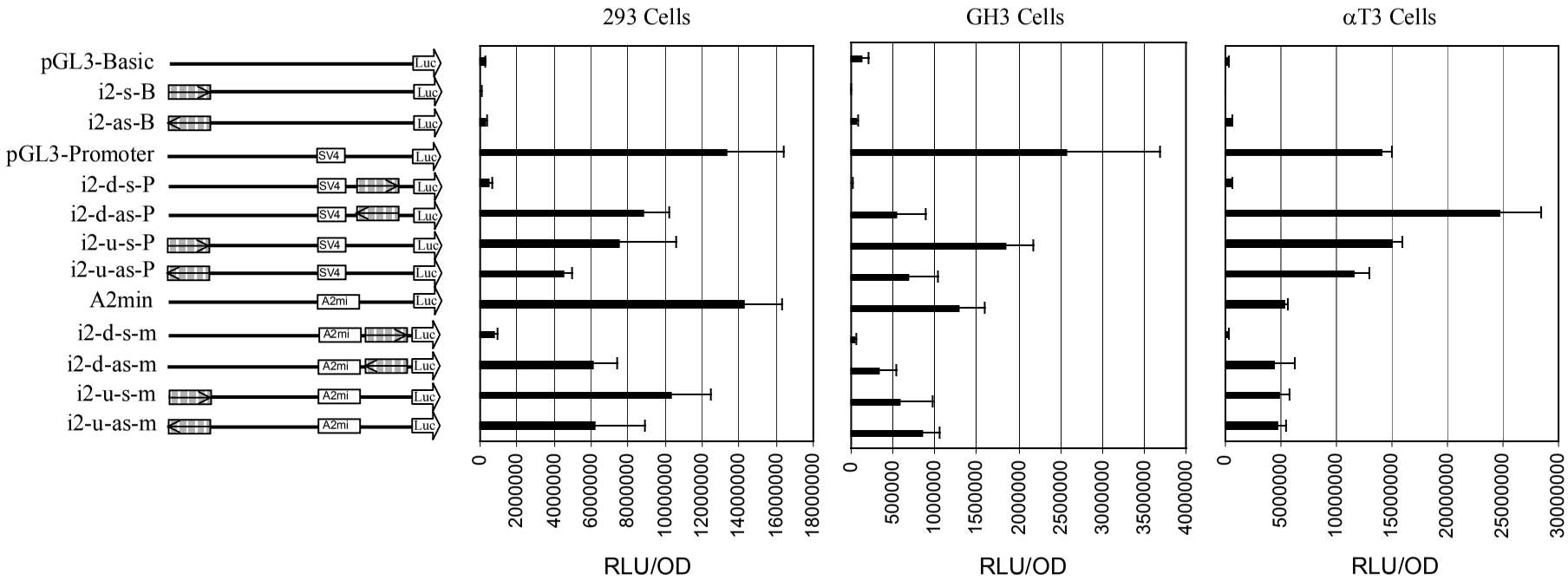


Fig. 34. Transient expression analysis of the silencing activity of intron 2. The resulting relative luciferase values after transfection into 293, GH3 and αT3 cells represent means ± SD of three experiments done in triplicate. The absence of error bars indicates that the error was too small to display in these columns. The constructs are drawn schematically on the vertical axis, with the A2 minimal promoter designated as A2min.

3.4. Transcriptional properties of the human thyrostimulin B5 subunit

3.4.1. Analysis of the 5'-flanking region of the B5 subunit gene

Luciferase constructs depicted in Fig. 35 were transiently transfected into pituitary (rat somatolactotrope GH3 and mouse thyrotrope TαT1) and non-pituitary (human embryonic kidney 293) cell lines, and the transcriptional activity was measured. None of the constructs showed significant transcriptional activity (Fig. 28, 36, 37, 38 and 39). Moreover, in the case of constructs – 4459/– 20, – 4459/– 1716, – 3351/– 1716 and – 3201/– 1716 transfected into 293 cells and all constructs transfected into GH3 and TαT1 cells, basic transcription mediated by pGL3-Basic was profoundly suppressed.

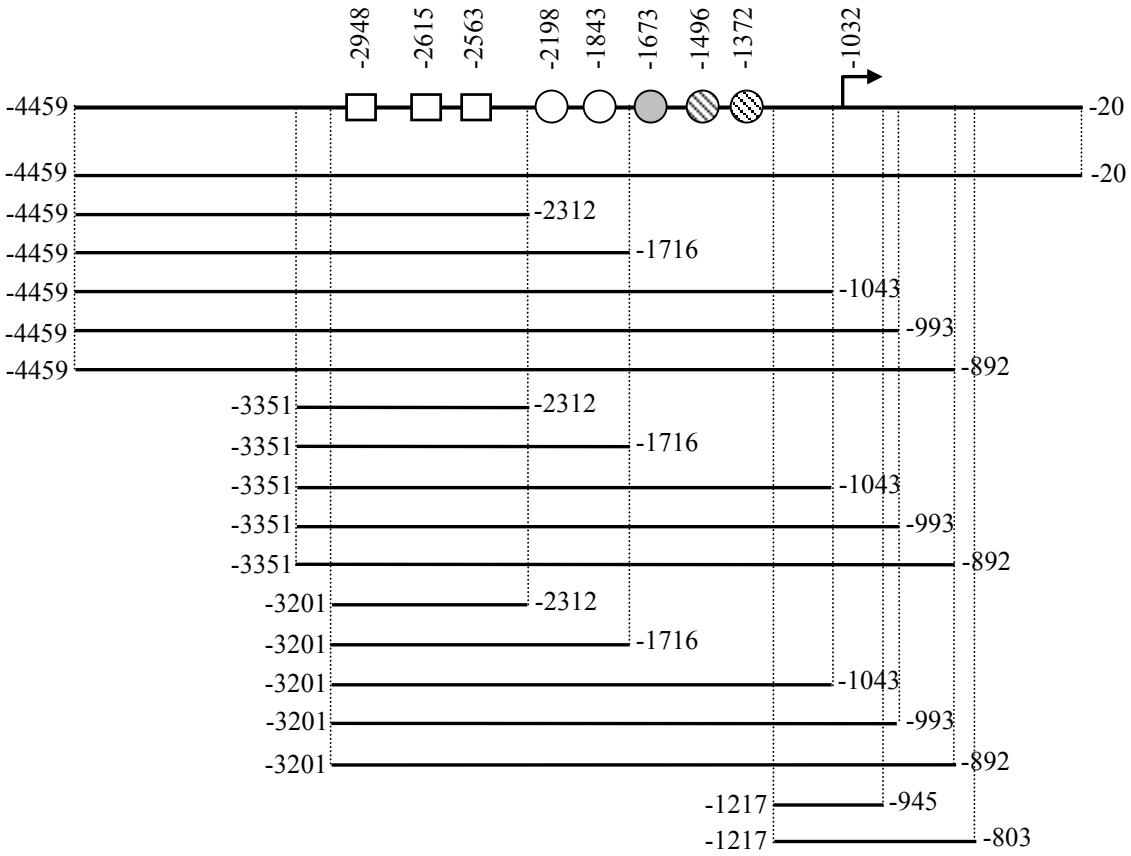


Fig. 35. Schematic representation of luciferase constructs containing genomic DNA fragments of the B5 gene. The numbering is relative to the ATG start codon of the B5 gene. The tentative transcriptional start site was mapped to –1032 nt. TATA boxes are represented by white boxes, GATA-1 motifs are represented by white circles, CBF1/RBP-Jκ motif is represented by the grey circle and CDP motifs are represented by shaded circles.

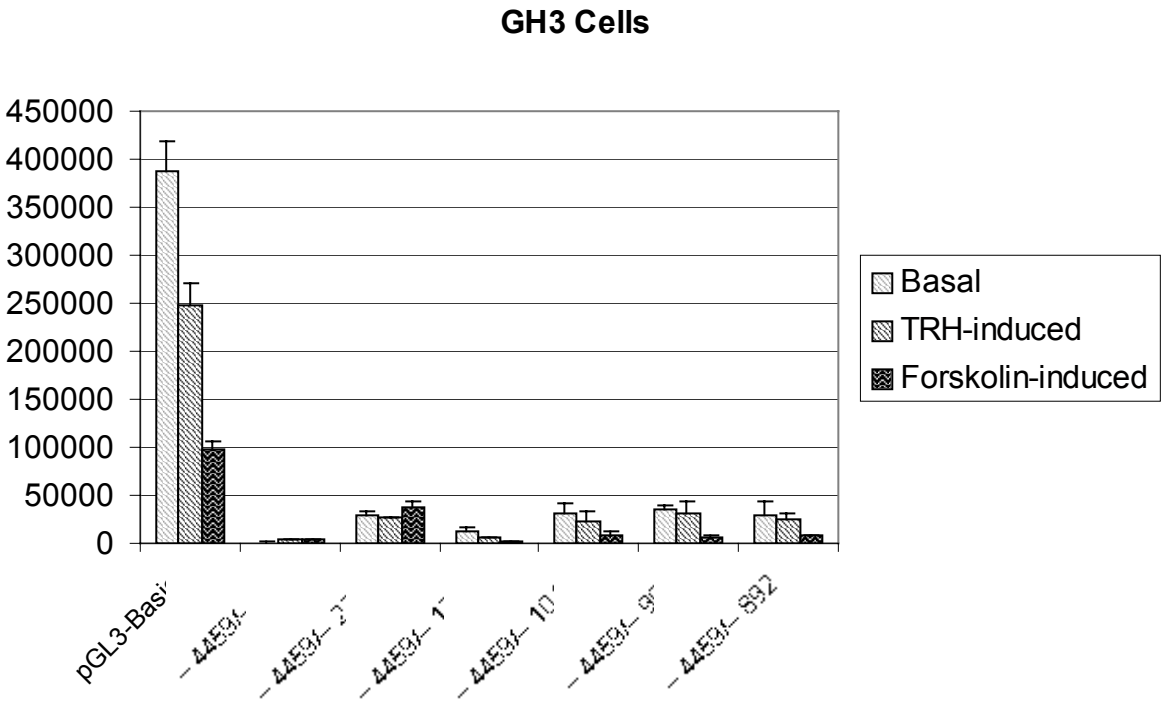


Fig. 36. Influence of TRH and forskolin on the transcriptional activity of genomic DNA fragments of the B5 gene. Luciferase constructs pGL3-Basic, – 4459/– 20, – 4459/– 2312, – 4459/– 1716, – 4459/– 1043, – 4459/– 993 and – 4459/– 892 were transfected into GH3 cells, which were cultured in the absence (Basal) or presence of 100 nM TRH (TRH-induced) or 10 μM forskolin (Forskolin-induced). Given are the luciferase values in relation to pGL3-Basic (Basic=1), which represent mean values ± SD of three experiments done in triplicate.

3.4.2. Analysis of the B5 subunit gene expression in different cell types

The B5 gene expression in diverse cell lines was examined by the RT-PCR analysis. The B5 transcript consistent with an expected size of 392 bp was not detected in any of the cell lines (data not shown).

3.4.3. Effect of TRH and forskolin on the B5 subunit transcription

In view of the positive regulation of the TSH β-subunit gene expression by TRH and cAMP (Steinfelder and Wondisford, 1997), the effect of TRH and the cAMP agonist forskolin was analysed in reporter gene assays. The constructs – 4459/– 20, – 4459/– 2312, – 4459/– 1716, – 4459/– 1043, – 4459/– 993, – 4459/– 892, – 3351/– 2312, – 3351/– 1716, – 3351/– 1043, – 3351/– 993, – 3351/– 892, – 3201/– 2312, – 3201/– 1716, – 3201/– 1043, – 3201/– 993, – 3201/– 892– 1217/– 945 and – 1217/– 803 were transfected into pituitary (rat somatolactotrope GH3 and mouse thyrotrope TαT1) and non-pituitary (human embryonic kidney 293) cell lines. At 48 h post-transfection, TαT1

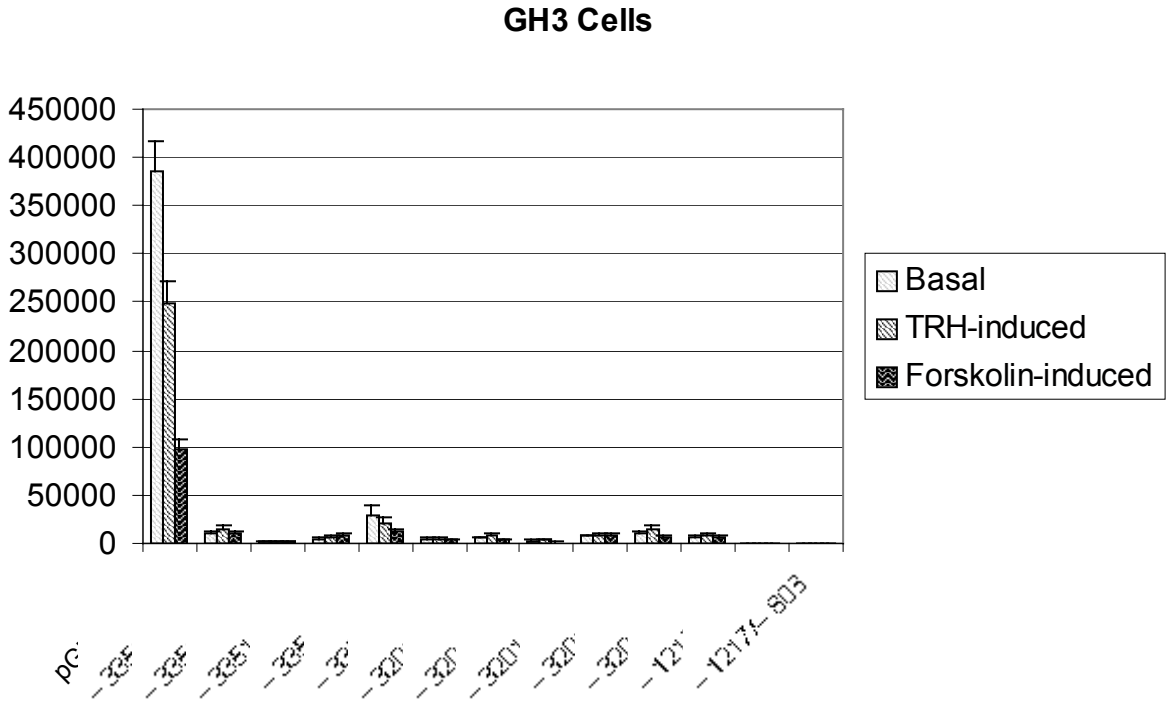


Fig. 37. Influence of TRH and forskolin on the transcriptional activity of genomic DNA fragments of the B5 gene. Luciferase constructs pGL3-Basic, – 3351/– 2312, – 3351/– 1716, – 3351/– 1043, – 3351/– 993, – 3351/– 892, – 3201/– 2312, – 3201/– 1716, – 3201/– 1043, – 3201/– 993, – 3201/– 892, – 1217/– 945 and – 1217/– 803 were transfected into GH3 cells, which were cultured in the absence (Basal) or presence of 100 nM TRH (TRH-induced) or 10 μM forskolin (Forskolin-induced). Given are the luciferase values in relation to pGL3-Basic (Basic=1), which represent mean values ± SD of three experiments done in triplicate.

cells were treated with 100 nM TRH, 293 cells were treated with 10 μM forskolin, whereas GH3 cells were treated with either 100 nM TRH or 10 μM forskolin. After 24 h incubation, no effect on the transcriptional activity of these constructs was observed (Fig. 28, 36, 37, 38 and 39).

3.5. Transcriptional regulation of the human NIS gene by thyrostimulin

Studies performed by Nakabayashi et al. (2002) have shown that the conditioned medium of 293 cells transfected with cDNAs encoding the A2 and B5 subunits was capable of stimulating cAMP production by cells expressing TSH receptors. Based on these observations, the effect of thyrostimulin was analysed in reporter gene assays. Thyrostimulin A2 and B5 subunit cDNAs were cloned into a bi-promoter expression vector (Fig. 29) and the resultant construct was stably transfected into 293 cells. A2 and B5 mRNA expression levels were accessed by RT-PCR (Fig. 40). Protein-free conditioned medium (2 days) containing recombinant proteins was collected and concentrated 200-fold.

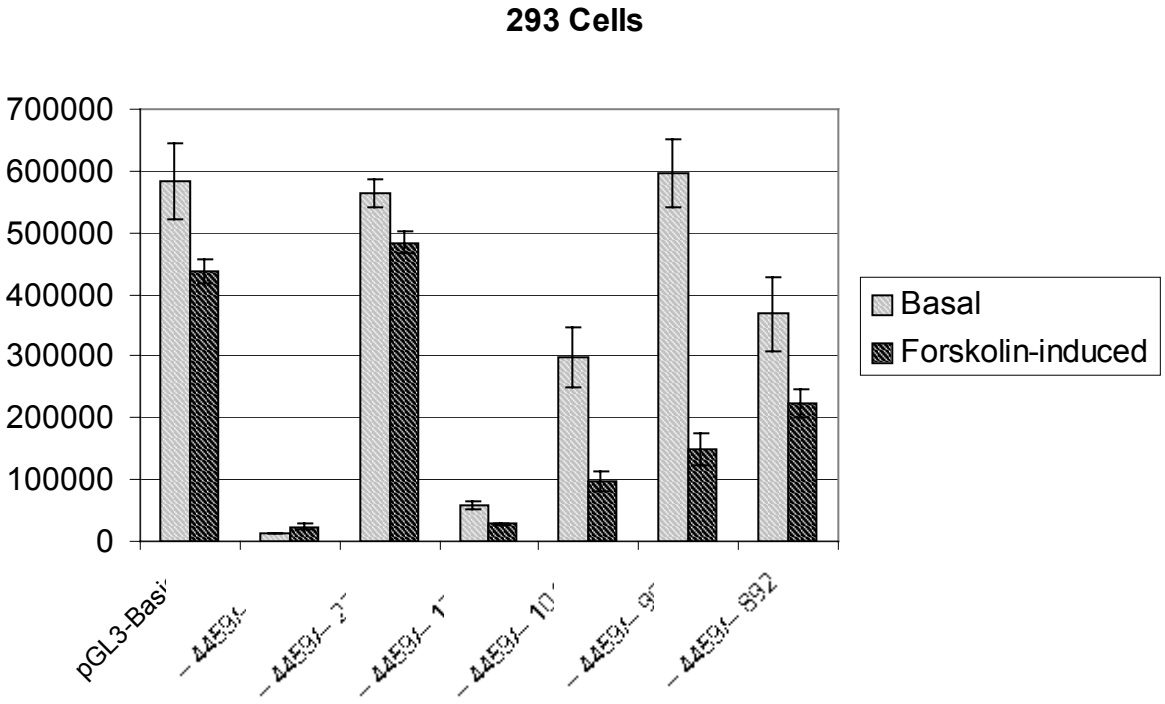


Fig. 38. Influence of forskolin on the transcriptional activity of genomic DNA fragments of the B5 gene. Luciferase constructs pGL3-Basic, – 4459/– 20, – 4459/– 2312, – 4459/– 1716, – 4459/– 1043, – 4459/– 993 and – 4459/– 892 were transfected into 293 cells, which were cultured in the absence (Basal) or presence (Forskolin-induced) of 10 µM forskolin. Given are the luciferase values in relation to pGL3-Basic (Basic=1), which represent mean values ± SD of three experiments done in triplicate.

The hNIS promoter and enhancer- (pGL3-N3+NUE) luciferase-fused construct was transiently transfected into rat thyroid FRTL-5 cells. At 24 h post-transfection, cells were cultured in 5H medium (48 h) and treated with 10 µl conditioned concentrated medium per well or 1.5 mU/ml TSH. After 24 h incubation, the transcriptional activity of pGL3-N3+NUE was increased ca. 3-fold by the conditioned protein-free medium from the 293 stable cell line expressing both A2 and B5 subunits (Fig. 41). Concentrated protein-free medium and conditioned protein-free medium from the empty vector-stably transfected cell line did not stimulate the activity of the pGL3-N3+NUE construct.

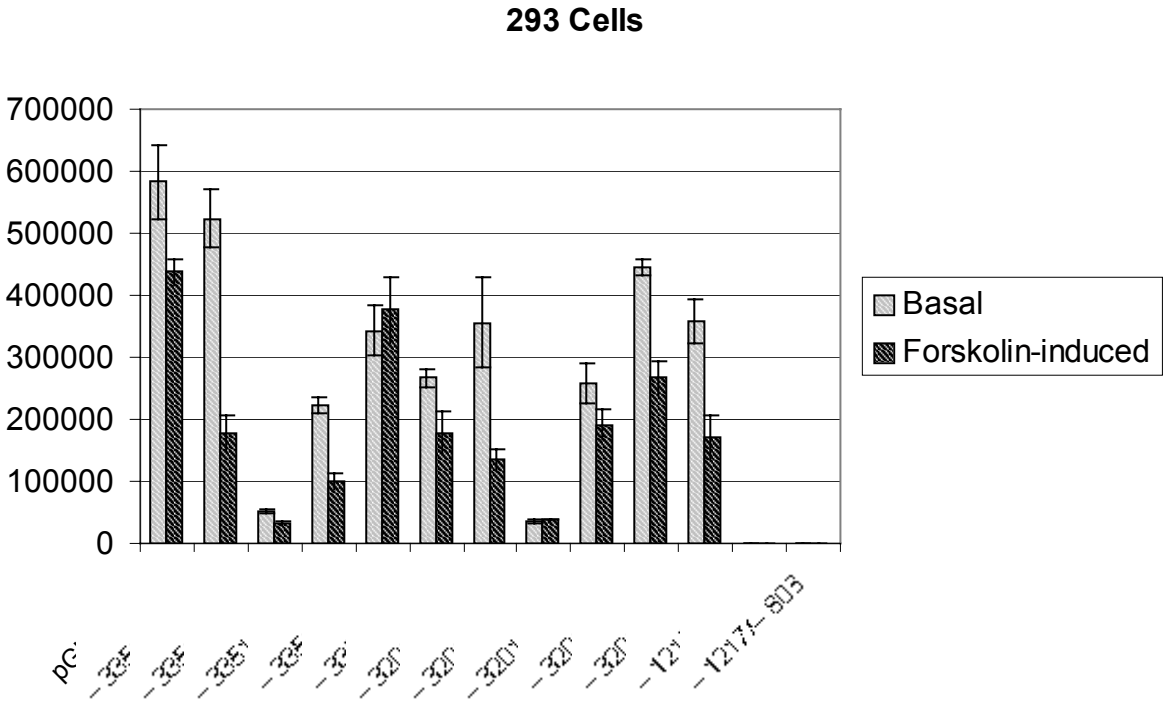


Fig. 39. Influence of forskolin on the transcriptional activity of genomic DNA fragments of the B5 gene. Luciferase constructs pGL3-Basic, – 3351/– 2312, – 3351/– 1716, – 3351/– 1043, – 3351/– 993, – 3351/– 892, – 3201/– 2312, – 3201/– 1716, – 3201/– 1043, – 3201/– 993, – 3201/– 892, –1217/– 945 and – 1217/– 803 were transfected into 293 cells, which were cultured in the absence (Basal) or presence (Forskolin-induced) of 10 μM forskolin. Given are the luciferase values in relation to pGL3-Basic (Basic=1), which represent mean values ± SD of three experiments done in triplicate.

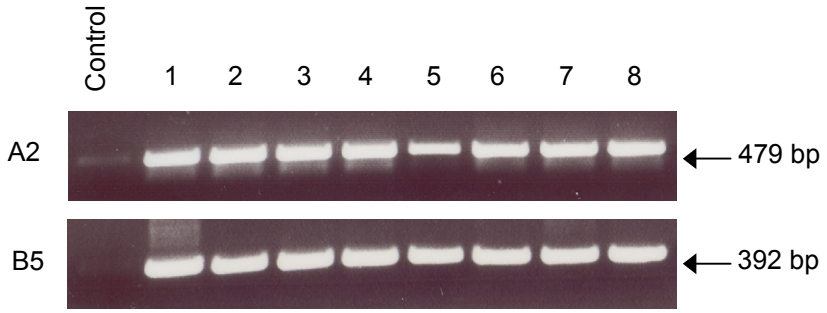


Fig. 40. Expression of A2 and B5 mRNA in stably transfected cell lines No. 1-8. Arrows indicate the position of the expected PCR products (in bp). Control represents total RNA from cells.

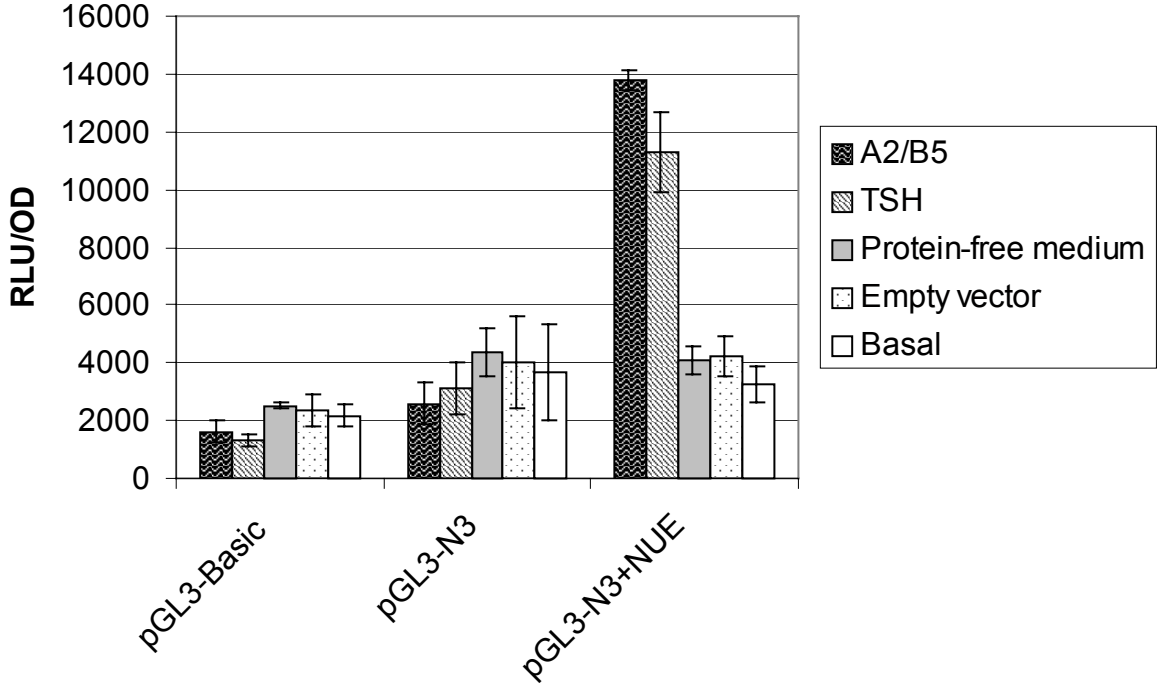


Fig. 41. Effect of thyrostimulin on the transcriptional activity of hNIS reporter genes. Luciferase constructs pGL3-Basic, pGL3-N3 and pGL3-N3+NUE were transfected into FRTL-5 cells, which were cultured in the absence (Basal) or presence of 10 µl/well concentrated medium from 293 cells expressing pBud-A2/B5 (A2/B5), 1.5 mU/ml TSH (TSH), 10 µl/well non-conditioned concentrated medium (Protein-free medium) and 10 µl/well concentrated medium from 293 cells expressing pBudCE4.1 (Empty vector). Luciferase activities are given in relative light units (RLU) in relation to OD and represent mean values ± SD of three experiments done in triplicate.

4.- Discussion

4.1. Transcriptional regulation of the human NIS gene by Graves' disease antibodies

IgG produced by B-lymphocytes infiltrating the thyroid play a major role in the development of Graves' disease (GD) (Kendall-Taylor et al., 1984; McLachlan et al., 1986). These IgG bind the TSH receptor (TSHR) and stimulate cAMP production in several thyroid systems, including human thyroid slices and membranes (Rees Smith et al., 1988; Zakarija and McKenzie, 1987). Activation of the cAMP pathway is related to increased iodide uptake and thyroid growth in GD (Jin et al., 1986; Tramontano et al., 1986; Zakarija et al., 1988).

This study is the first to present the direct evidence of GD IgG stimulation of the hNIS gene transcription. In addition, the obtained results help to elucidate the molecular mechanisms involved in the transcriptional activation of the hNIS gene by GD IgG and TSH. By testing IgG from 29 patients with active GD it was found that, similar to TSH, GD IgG up-regulate the upstream enhancer of the hNIS gene (NUE). The stimulation of NUE containing a mutation in the cAMP response element was reduced approximately 30-fold, thus suggesting that GD IgG activate NUE via this regulatory sequence. No significant stimulation of the hNIS promoter-only construct (N3) by GD IgG or TSH was detected. This observation is in accordance with the previous data on TSH stimulation of hNIS *cis*-acting elements (Schmitt et al., 2002). Furthermore, NUE-stimulating luciferase activities of 29 GD IgG were compared with their TRAK values, which represent a pool of TSHR stimulating and blocking autoantibodies. For most patients a distinct correlation between the two values was found, however, in a few patients such correlation was not observed. This is likely due to the prevalence of blocking TSHR-autoantibodies in IgG fraction of these patients.

Based on the sequence homology of hNIS and rat NIS (rNIS) promoter regions, a novel putative regulatory region was found in the hNIS promoter. It is located 232 bp upstream of the GC-box, which is a very similar positioning to the known rNIS TSH-responsive element (rTRE) (Ohmori et al., 1998). This region, which we termed hTRE, also corresponds to the binding site for the AP-1 family of proteins, which recognise either 12-O-tetradecanoylphorbol-13-acetate (TPA) or cAMP response elements (CRE) (Shaulian and Karin, 2002). Interestingly, the mutation of two nucleotides of hTRE reduced the activation of the pGL3-mtN3+NUE construct approximately 3-fold but did

not abolish it completely. This may indicate that additional nucleotides participate in the interaction of the discovered regulatory region and its nuclear factor. To summarise, hTRE might play a role in hNIS up-regulation by GD IgG and TSH, however, further studies are required to examine the nuclear factor interacting with this new putative regulatory element.

The obtained results complement our current knowledge of the pathogenesis of GD. Fig. 42 shows a schematic model of thyroid-specific pathways of transcriptional activation of the NIS gene consistent with the data presented in this work. The discovery of the novel putative regulatory element in the hNIS promoter should allow a further detailed investigation of the transcriptional regulation of the hNIS gene expression. Taken together, these results will contribute to a better understanding of the complex mechanisms regulating the thyroid iodide concentrating activity.

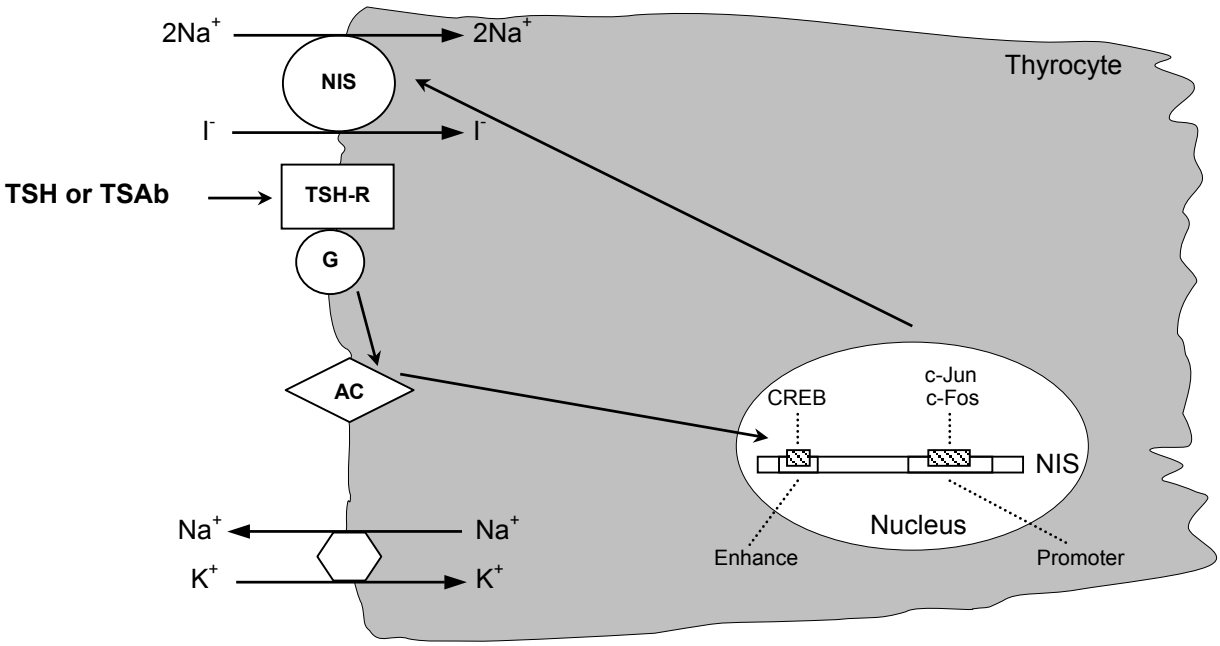


Fig. 42. Iodide transport in the thyroid follicular cell. Active accumulation of I^- is mediated by the Na^+/ I^- symporter (NIS) and is driven by the Na^+ gradient generated by the Na^+/ K^+ ATPase. All steps are stimulated by TSH from the pituitary (normal subjects) or thyroid stimulating antibodies produced by lymphocytes (TSAb, patients with Graves' disease). TSH and TSAb bind the G protein-coupled TSH receptor (TSH-R), which activates adenylyl cyclase, thereby stimulating cAMP production. This, in turn, activates the NIS gene via the cAMP response element in the enhancer and the AP-1 site (TRE) in the promoter, leading to the synthesis of NIS molecules and the consequent increase in I^- uptake.

4.2. Transcriptional regulation of the NIS gene by phthalate plasticisers

The present study corroborates the previous findings that some phthalates may cause thyroid hyperactivity (Howarth et al., 2001; Price et al., 1988; Hinton et al., 1986). Here, for the first time an increase in NIS transcriptional activity induced by the three major phthalates is demonstrated in PC Cl3 rat thyroid cell line. These cells present a very appealing set of properties that resemble those ascribed to normal differentiated thyrocytes (Medina and Santisteban, 2000), such as TSH dependence for growth and differentiated functions, iodide uptake and thyroglobulin and thyroperoxidase gene transcriptions. Here, the hNIS promoter construct (pGL3-N3) was up-regulated 2.5-fold by DIDP, 2.6-fold by BBP and 2.4-fold by DOP in the presence of TSH. Likewise, these phthalates also enhanced rNIS endogenous mRNA expression, which increased ca. 2-fold after 48 h of treatment compared with the expression level generated by TSH only. mRNA content was unchanged at 72 h, which could be due to the degradation of mRNA and/or to metabolisation of phthalate compounds as well as due to the depletion of TSH.

These results are consistent with the study where DIDP, BBP, DOP, DINP and DEHP augmented iodide uptake in FRTL-5 rat thyroid cell line (Wenzel et al., 2005). However, no effects were seen when pGL3-N3 was treated with DEHP and DINP, possibly because these two phthalates modulate the activity of NIS at the post-transcriptional level. DBP appeared to down-regulate both pGL3-N3 and pGL3-N3+NIS upstream enhancer (NUE) in this work and did not alter iodide uptake in the study of Wenzel et al. (2005). The slightly lowered TSH-induced transcriptional activities of pGL3-N3+NUE by DIDP, BBP, DOP, DINP and DEHP may be a result of the interference with important accessory factors, necessary for TSH-mediated induction of hNIS. As the key component of this pathway is cAMP, one cannot exclude that phthalates exert slight inhibitory effects on e.g. adenylyl cyclase.

To surmise, the data presented in this work suggest that the increase in thyroid iodide uptake by DIDP, BBP and DOP is a consequence of NIS transcriptional activation by these major phthalates. The detailed analysis of NIS regulatory regions responsible for the effects of phthalates could facilitate understanding of the mechanisms behind such activation. Although the demonstrated stimulation of NIS transcription by DIDP, BBP and DOP is not very strong, this finding is of great importance as humans are routinely exposed for long periods to phthalate plasticisers, the accumulation of which may contribute to thyroid hyperfunction.

4.3. Promoter cloning of the human thyrostimulin A2 subunit promoter

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, rat pituitary somatolactotrope GH3 and mouse pituitary gonadotrope αT3 cell lines, the 3082-bp fragment was found to exhibit promoter activity in all cell types. By using a series of deletion constructs this activity was confined to a rather short stretch of DNA between – 506 and – 347. It is notable that this minimal promoter (referred to as A2min) is almost the same length as the 118-bp minimal promoter region of the α-subunit gene (Jameson et al., 1988; Hashimoto et al., 2000).

A curious behaviour was also observed. 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. 2-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. 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 (Sakura et al., 1989; Kalkbrenner et al., 1990).

The tissue specificity of A2min was also accessed by testing its activity in the A2-producing mouse thyrotrope TαT1, 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, GH3 and αT3 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.

4.4. Characterisation of the transcriptional regulation of the human thyrostimulin A2 subunit promoter

Further, substitution mutations were introduced into potential binding sites for transcription factors predicted by *in silico* analysis. This mutant analysis identified three mutations (m3, m6 and m7) that resulted in an almost complete loss of promoter function in 293, GH3 and αT3 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 middle 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) transcription factor (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 and αT3 cells the additional, not yet mutated, nucleotides are involved in Pax-5 binding. Although the binding site for Pax-5 was predicted by both MatInspector and AliBaba2 software programs, it is unlikely that this factor is actually involved in the induction of the A2min promoter due to its expression being strictly confined to B-lymphocytes (Hagman et al., 2000). Rather, another unidentified protein might be binding to this site.

Additional nucleotides involved in protein-DNA binding could also explain the only slight decrease of transcriptional activity shown by constructs m10 (mutated binding site for GAGA transcription factor), m13 (mutated c-Myb site) and m14 (mutated AP-1 binding sequence) in αT3 cells, as opposed 293 and GH3 cells where a marked drop in activity was observed.

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.

Next, EMSAs were used to subject the regions containing the identified transcription factor recognition sites to further functional analysis. Nuclear extracts from 293, GH3 and αT3 cell lines were employed to bind probes comprising the above-described sites. Probe 1, encompassing the binding site for LSF, indeed formed a specific complex 1a,

whose intensity was slightly higher in 293 extracts than that in GH3 and αT3 extracts. 1a seemed unaffected by the same mutation of LSF (probe 1m1) which reduced promoter function in the mutational analysis. Thus, it is probable that the nucleotides mutated in probe 1m1 are not involved in the formation of complex 1a, that is thought to contain the potential LSF protein. This questions the role of the latter mutation in reducing promoter function, which may have resulted from the creation of a new binding site. Additional substitution mutations at the same location are necessary.

Probe 2 included the Sp1 consensus sequence. A specific complex 2a was seen in the nuclear extracts from the three cell lines examined. On the other hand, the labelled probe 2m, where the mutated half site for Sp1 resulted in the absence of promoter activity, still formed complex 2a. 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 (Goff et al., 1991; Grasser et al., 1992).

Further, three specific complexes 3a, 3b and 3c were seen bound to probe 3 in nuclear extracts from all 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 the three 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), it is easy to imagine 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 the putative Pax-5 binding site, a single specific complex 4a was formed in the extracts from the three cell lines studied. It was shown 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 and αT3 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, specific shifted complexes 5a, 5b and 5c 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). 5a, 5b and 5c were also formed in GH3 and αT3 extracts. 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).

Finally, in 293 extracts, specific complexes 6a and 6b formed on probe 6 appeared unaffected by mutations of the tentative c-Myb (6m1) and AP-1 sites (6m2). Conversely, the abundance of complex 6b was diminished by both 6m1 and 6m2 in GH3 extracts and by 6m2 in αT3 extracts. It should be noted that despite the presence of the putative phorbol ester response element (AP-1 site), both TRH and forskolin failed to induce the transcriptional activity of the A2 promoter.

To confirm the identity of the above-described complexes, gel supershift analyses and co-transfection assays with transcription factor-encoding genes are needed.

It is interesting to compare the transcriptional properties of the A2 gene with those of the common α-subunit gene, which is known to be regulated by the pituitary-specific protein P-Lim (Bach et al., 1995) and thyrotropin-releasing hormone (TRH) (Jameson et al., 1988). TRH has been shown to bind to the cAMP response element (CREs) in the promoter of the α-subunit gene. In contrast, the A2 promoter contains neither the binding site for P-Lim nor CRE and was not responsive to the treatment with TRH or forskolin in the reporter gene assays. This suggests that the regulation of the A2- and α-subunit genes diverges at least at the level of the transcription factors controlling gene expression.

4.5. Silencer elements upstream and downstream of the thyrostimulin A2 subunit promoter

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 construct in two positions, upstream and downstream of the A2min promoter, resulted in a striking decrease of promoter activity in 293, GH3 and αT3 cell lines, 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, it was inserted 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. These 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 (Fig. 43). 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

```
-347    CTGGGGAGGGTCTGGGAATGTGGACGAGGGCCCTGCAGGCCAAGATGGGGCA
                CBF1/RBP-Jκ
-295    GATGGAGGGAGGAAGATGTTTGCGCTCCAGATGGCGAAGAAAATTCCAGGGA
                     Oct-1
-243    AGGGAGAATCACTGCACAGAGGGCTGACACACAGGTCCTTTCCAGAGACAGC
                   GATA-1
-191    TGCTCACACTCACACCCATACACACACACACACACACACAAAGGCAGATACA
                                                     PEA3
-139    GGGAAAAGGCAGCACCATTCAGGCACACCTCACCTGTCAGACCAGCCAGCCC

-87     TGGCTCACTCACCTGGAATGCAGTATTTAAAGAACTCGCCATCCCACCTG
```

Fig. 43. Characterisation of the downstream s1 region of the A2 gene. Potential binding sites for CBF1/RBP-Jκ, Oct-1, GATA-1 and PEA3 are underlined. The numbering of nucleotides refers to their positions relative to the ATG start codon of the A2 gene.

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.

The same positional cloning approach was used to analyse the second region s2 and it was hence determined that this region 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 (Fig. 44). 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, it is proposed that they may contribute to the restriction of promoter activity by s2.

```
-1046  GTTCAAGACCACCATGGGCAACATGGCAAAATCCTGTCTCTACAAAAAAATA

-994   CAAAAGTTAGCTGGTCATAGTGGTGTGCCTGTGGTCCCAGCTACTCAGGAGG
                                        GATA-1
-942   CTAAGGGGGAGGATGGCTTGAGTCTGGGAGGCTGGGAGGTGGAGGTTGCAGT

-890   GAGCCGAGATAACACCACCACACTCCAGCCTAGGTGACAGAGCCAGACCCTC
             GATA-1
-838   TCAAAATAATAGTAATAAATAATAATAAAGTTAGGGGTGATCAGGCCCTGAA
                     Brn-3                   GATA-1
-786   CAGGGAAATGTTCTGTCCAAGGTCACAGAGCAAAGAGAACCCAGGTGTCCAC
                 NF-1                                GATA-1
-734   AACTCAGCCAAGAGTCTTGACCCCTGTGTCGTCAGCTGCACACACGCTGCAC

-682   ACTCCACCAGCTCCTAGGCTTCTCTCCACATCTGACTTATCGAGGCCTGGGC
                                                     NF-1
-630   TGTGGGTAGCTTTTTGGACAGAAAGAAGCAGAAAGCAGCAGGAGCTGCTCTC
                       GATA-1  Oct-1      PEA3
-578   CGAGGACCGCACCTCAAAGAGCAAGATCCCCCAGGTGGGATCGCTCTGAGGC
                                          Sp1
-526   TTCAGGCAGGGGAGGCTGGT
         NF-1       Sp1
```

Fig. 44. Characterisation of the upstream s2 region of the A2 gene. Potential binding sites for GATA-1, Brn-3, NF-1, Oct-1, PEA3 and Sp1 are underlined. The numbering of nucleotides refers to their positions relative to the ATG start codon of the A2 gene.

It is notable that in αT3 cells, the addition of nucleotides between – 540 and – 506 almost abolished the function of the A2min promoter, signalling that the elements responsible for silencing in these cells may be contained in this region. Given the evidence that the latter displays multiple Sp1 binding sites, one cannot rule out the possibility of Sp1 acting as a repressor in this case.

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.

4.6. Exonic and intronic silencer elements of the thyrostimulin A2 subunit promoter

Since positive and negative regulatory regions in exonic and intronic sequences have been identified in various genes (Shamsher et al., 2000; Xu et al., 2000; Lum and Lee, 2001; Stark et al., 2001), all exons and introns of the A2 gene were positioned in sense orientation downstream of the respective promoters of the A2min and pGL3-Promoter constructs. Surprisingly, all insertion constructs showed reduced promoter activity relative to the parental construct in 293, GH3 and αT3 cell lines. Because exon 1 and intron 2 demonstrated the most profound down-regulation of promoters, they were analysed for the presence of negative regulatory elements using the same strategy as for regions s1 and s2. Specifically, exon 1 exhibited significant silencing activity in 293 and GH3 cell lines, whilst it was not so strong in αT3 cells. *In silico* analysis revealed a motif for GATA-1, again implicating this factor in repression. Although the experiments to better characterise exon 1 are needed, this finding is exciting as until now, only a very small number of groups reported identification of exon-located silencers (Frenkel et al., 1993; Hoeben et al., 1995; Shimokawa and Fujimoto, 1996).

In addition, intron 2 was capable of moderately repressing promoter activity regardless of orientation and position, in the context of a native and heterologous promoter in 293 and GH3 cells. However, it did not affect the promoter activity in αT3 cells. Similarly to exon 1, this intron also contained a binding site for GATA-1.

The present report describes for the first time the cloning of the minimal promoter of the human thyrostimulin A2 subunit, whose regulation appears slightly different in 293, GH3 and αT3 cell types. Various nuclear levels of transcription factors might contribute to this difference. Given the evidence that thyrostimulin appears to act as a repressor of

the A2 subunit promoter activity, it is reasonable to suspect that it negatively auto-regulates its own synthesis. In addition, a clockwork of silencer elements is revealed that underscores the complexity of the control of the A2 subunit transcription. Future work will need to further characterise the regulatory elements uncovered in this study and to determine how these factors interplay with one another to regulate the A2 subunit gene.

4.7. Transcriptional properties of the human thyrostimulin B5 subunit

The 5'-flanking region of the B5 gene, which extended from – 4459 to – 20 nt relative to the ATG and contained the tentative transcription start site for the B5 gene (which was determined by identifying the 5' end of the respective mRNA [GenBank accession no. NM_145171]) and three potential TATA boxes (Fig. 45), was cloned into the promoter-less vector pGL3-Basic. Despite the fact that B5 mRNA was found to be expressed in the pituitary tissue in human (Hsu et al., 2002) and in pituitary, thyroid, oviduct and heart tissues in rat (Nakabayashi et al., 2001), none of the eight cell lines used in this study had detectable expression of the B5 subunit. Still, the 4439-bp 5'-flanking region was tested for promoter activity in 293, GH3 and TαT1 cell lines. TαT1 cells were chosen because they contain the transcription factor Pit-1 and its thyrotrope-specific isoform Pit-1T (Yusta et al., 1998), which are both necessary for the transcriptional regulation of the human TSH β-subunit gene (Steinfelder et al., 1992; Haugen et al., 1993; Kim et al., 1993; Gordon et al., 1997). Indeed, two putative binding sites for Pit-1 were found in the sequence of the B5 5'-flanking region. However, in transient transfection assays, the 4439-bp fragment markedly suppressed the basal transcription mediated by its parent construct pGL3-Basic in the three cell lines examined, suggesting the presence of silencing elements.

In silico analysis of the aforementioned region revealed a motif for the transcription repressor CBF1/RBP-Jκ, two motifs for the transcriptional repressor CDP and two motifs for GATA-1, which is also known to repress a number of genes (Nerlov et al., 2000; Li et al., 1998). In an effort to relieve the repressor effects of these factors, a series of specific deletional constructs was made and tested for activation in 293, GH3 and TαT1 cells. Nevertheless, no transcriptional activity was demonstrable for all these constructs.

Since the transcription of TSH β-subunit is stimulated by TRH and forskolin (Kim et al., 1993; Shupnik et al., 1996; Hashimoto et al., 2000), their action was also

-4480 GTTTCAGAATGCTGGTGAGGTCTCAT

-4428 AGTCCCCAAAGACTTTGCTTCTTCTCTTCTCAGACTCACATTTTTTTTTTCA

-4376 CCTCTAATATCTTCGAAATCAGGATATGCCTAACAATTGCTGGTGTGATGTA

-4324 ATTTTTTATTGGCAGCTTTTTATTTCTTAGTGGTACACATGATAGTGATGCA

-4272 TTTCCAGGGAAGGAAAGAGAAACAATAATTATTGCCTGCTCATCATGTATCA

-4220 AGCCCTGTGAGAAGTAATTTATAGACATTATTTTGTAGAATCCTCACATGAC

-4168 CCCTATGATGGAGGCATTTTCAATTTTGTAGGAAGCTGAGACTCAGGTGGGC

-4116 TACTAGACTTGTGCAAGATCATAAGTTGGCGAGTGTCAGAATCAAGATTTCA

-4064 GCACAATGTAGCTGACTTTATGGTTGTGTAACCTTCCGCACTCTCTCTCTGA

-4012 CTTGTCCTCAGCTTCTTGTGCTCATAAAATAGTTTATAAATATTTAAATTCT

-3960 GGAGCTGGGAAGGGCCTTAGGAGTCACAGGGTCCAACCCCACTGTGTTACAG

-3908 ATGAAGAAACTGAGGCTCAGAGAAGTGACTTGCTCAAGGTAGTCCCAAAGGA

-3856 GATTGTGCCACTGAGAATCAACATGGCTGCGGGAGGGAAGGGAGTGTTTCCA

-3804 GGCTCTGTCAGTGTCTCCATCTCCTGCACACAGTGTGCACCCCAGGATTTCC

-3752 CCACGTGGGACTGCCTTCCCTGGACCAGGTCTCAGTTTCTCAGTCACCCCTT

-3700 TGAGGCACTAACACTTGGCTCCTTCCTCTGGAGTCACGATCCCCCTGGAATT

-3648 CAAGATGATTGCTCACATACACAACTTCTCCTTTCTTTCTGCCCCTAATGAA

-3596 CGTATATCTTGTGCCTCACCCTCCAAGGTGACAATAAATCCATGTGAAGATG

-3544 ATCATGGAACAAAAGGAGAGAAGATTCTCCAGAGTCTATCCTTGGAGTCATT

-3492 ACTCATATGTTTCTTTGCTACCTCAAGAGAGGGACTTTAATGGTGGGAACTT

-3440 CAATGTAGATTGAAGGGAAGGTGGCAGGCACATGCACACCATGGTGCAGAGA

-3388 TATTCCGTAGGTAGCAGGGCATTAATTAGCCTTTGAAGTCACGCTACAAACT

-3336 CTCACTCTGTCCACAGAGAGGTGCGACACGGCCTAGATTGCAAAAGGTGGCA

-3284 TCCGTACACCATTCTCCTTTCCTACACCCAGGATGAAGCTGATGGGCCACTG

-3232 CCCTTTGCCCTTAGAATGATTTCAGCTGCCTTCTTTAACACAACATTCCAAG

-3180 TAGCCTCTTCCCATTAACTGTAGTTGGTTACAGGGATGAAATAGACCCATTT

-3128 GCCTTTCCCAGTGTTGTTGAAGGCTTTGGAAGTAGGCAGGTTACAAAACGAT

-3076 TGAGTTTCTATTTCTTCCAATGTAAAAATGAGAAAATAATAATTGACATAAA

-3024 GAATTGTTGTAAGGTCTAGAGATAACATATGTAAGAGCACAGCAGATTCCAT

-2972 GGTACCATAGTAGATGCTTAGTGACTATAAATTGTCGGTTTTTTTTTTTTTT
AP-1

-2922 TTTTTTTTTTTTTTTTTTTTTTTTTGAGACGGAGTCTCGCTCTGTCGCCCAG

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-2870  GCTGGAGTGCAGTGGCGGATCTCGGCTCACTGCAAGCTCCGCCTCCCGGGTT
                                         GC box
-2818  CACGCCATTCTCCTGCCTCAGCCTCCCAAGTAGCTAGGACTACAGGCGCCCG

-2766  CCACTACGCCCGGCTATTTTTTGTAGTTTTAGTAGAGACGGGGTTTCACTGT

-2714  TTTAGCCGGGATGGTCTCGATCTCCTGACCTCGTGATCCGCCCGCCTCGGCC

-2662  TCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGCGCCCGGCCAAATTGTC
                                                  Oct-1
-2610  ATTTTTATAAAGTTAATGCTGACAGGGTGGAATGCATGATGCCACATCTCTG

-2558  CATTATAAAGAACTTGGGGATTGTGAAAGAAAGATGCTCTTGGCCTTTGTAA

-2506  TCTGTCTTTATTGTGTGACTCTGCAGACACAAGTGTGTATGTTTCAGGTCTT
                Brn-4            TTF-1
-2454  AAAGGGTTGGGCAGCCGTCTTCAGAAACAGCCCAGGATGGCAGCTCACTGCC

-2402  GACTGGCCAGCTCTTGGCCAGCAAGTTAGGAAGATCAGGCCAGCTGAGCAGT

-2350  CCGTCCCCAGAGTACTCCAGTTCTGGTTATGCTACTCACAGGCTGAAGCGCT
                                   Oct-1
-2298  GAAGAAAGTCCTTTTTCCAAGCCTAGTTCAATATTCTATAAAACATGTATAA
                                   Oct-1          Pit-1
-2246  TAATTCTGCTCTAGAGAGAGGGGTGGTAAATTGATGGACTCACTGGGCATTG
                                       AP-1
-2194  TTATCTGTTTTGAAAATGAAATCTACAATGAGCAGTACATTTATTTAAAGAA
        GATA-1
-2142  CACTTTTCTTTGCAGAAAGTGGAAATGGTTTAACTTCTTTACTTTGTTTCAC
                   Ets
-2090  TAGTTAGTTAGTGTGCATCTTCAATGTCTCTGCCTACATCATTATTTACTAT
                                                  Brn-2
-2038  GATATCAAAACTCCTAAGGGCAAAGATGATGTCTGCTTCATTTACTTTTTAG
                                         Brn-2
-1986  AGCACCCAGAAAAATGCCATGAACAAGTATATTTTTAATCCACTCATATGCT

-1934  GTTATTGTAGATTGAAATATAAACTATATCCCATACTCATAAAAATATTTTG

-1882  TTCTTATTTGAATCATTTAAGACCAAAAATCTGTAAAACTTCTCTATCAGAG
                AP-1                              GATA-1
-1830  CAGCACTGAATGTCCTTACAGACAGAACAAATGCATACCTTTTGGTTTATTT
                                                   Oct-1
-1778  TAATTTTAAAACATTGATATTGACTGACTCTTTATTACAAAAGTCTGCCCAA
                              AP-1
-1726  TGTAGAACATGTGAAAAATGCTGAAAACCATATAAAGGAAATGAAAAGTTTT
                                                Oct-1
-1674  CTTTAATTCCCATGCTGGAAGATAATCACTTGGAACATCGTGGTATTTTTTC
        CBF1/RBP-Jκ
-1622  CTAAATGATTCATTTTAAGGCGTTATTAATATCATCTAATTCTTAGCTAATT
            Pit-1                Brn-4
-1570  TTAAGACTTTCGACATGTTTCTTCTCACTGTGATTTCCATCTTTTCAGGTAG
                                   Oct-1
-1518  TGATCCTGTGTTCTTTCCAGCAAGAATTATCGTTTTGTTGTTTTTCAGTGCA
                                 CDP
-1466  TTGAAACATGTTGTCGGTTAAAGGAAAGGGTTTGCCATGAGCTGCTAGTCAC
                          Ets
-1414  TTACCCCTGTTTCAAAGGAAACTTGTGGTTACCATGAAGGAAAATTTAATCC
                                                   CDP
-1362  ATCTGCCACAATGCATTTGTTCTCCTGAGAACCAGGTGGAACTACCTCTGAC

-1310  TATTTCCTTAGGATTATGCCACCCACCAAGGTAACTTTCTATTTCAGTAGGT
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-1258  GATGTTGAGAAACAAAACAACACCCTTTAAGTTAATCAGTTTTGGAAAATAG
                            CREB
-1206  AGACTGGCTTTCTATTCCATGGCTTTGATGTGTTTAGTAGTTCCTGTAAGGA
                                            Ets
-1154  TCTGCCTCTCTTTTGTTTTGATTGACAAGACTTTTCCTATATATTTGAGCAA

-1102  TCAAGCAGCCCAGTAACAGAAGGTAGAGACATTTACCCAGGGCAAACTTCTA

-1050  CCATTCATTGTGACTTCCTGAAATCTTAGTGCAAGTTTCAGCTCTAAAAGAA
                AP-1 ⇧
-998   GAGTGGGCTCCTGCAAGATTAGGTAAGTGCTTCTCCGTATTTGTAACCTCAT
                         CREB
-946   TCCAGAGGAGGGGAAGCCCAGAGAACTTGCTACAGTAAGGTCAGAAGTAGGA

-894   ACTGTGAGAATAATGAATTCAGTAGGGGGTAAAGGTCCATGAATGCCATATC

-842   CTTTATACTTCAAAATCCACGTGAGTTCTGTCTAGTG
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Fig. 45. Characterisation of the immediate 5'-flanking region of the B5 gene. The arrow indicates the tentative transcriptional start site. Three potential TATA boxes are boxed. The potential GC box is shaded in grey. Potential binding sites for AP-1, Oct-1, Brn-2, Brn-4, TTF-1, Pit-1, GATA-1, Ets, CBF1/RBP-Jκ, CDP and CREB are underlined. The numbering of nucleotides refers to their positions relative to the ATG start codon of the B5 gene.

investigated in relation to the B5 subunit. However, even though the 5'-flanking region of the latter contained multiple binding sites for AP-1 and CREB, the activities of the deletion constructs remained unaffected by treatment with TRH or forskolin.

It therefore seems reasonable to suspect that B5 promoter is tissue specific. Further supporting this proposition is the report by Kim et al. (1996) that the expression of the human TSH β gene is restricted to thyrotrophs by a strong silencer element. In that study, the TSH β-subunit promoter was active only in the TSH-secreting thyrotropic tumour TtT-97 cells. In this case, however, the availability of only non-B5-expressing cell lines could account for the failure to localise such silencer element.

4.8. A link between thyrostimulin and the NIS gene

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. The present work attempted to explore the function of thyrostimulin by accessing the responsiveness of the human NIS promoter and enhancer to conditioned serum-free medium that contained human thyrostimulin. As a result, this medium was found to activate the hNIS transcription as strongly as bovine TSH. 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. The preliminary results presented in this study raise the possibility that the thyroid of these patients was stimulated by thyrostimulin, further implicating this new hormone, albeit indirectly, in the established view of thyroid axis.

5.- Summary

The first part of this work has focused on the transcriptional regulation of the human sodium/iodide symporter (hNIS) gene by thyroid stimulating antibodies in patients with Graves' disease (GD) and by phthalate plasticisers. NIS is a protein which mediates the active transport of iodide into the thyroid follicular cells as the crucial first step for thyroid hormone biosynthesis. Because iodide uptake activity is increased in patients with GD, the interaction of the hNIS promoter and upstream enhancer (NUE) with affinity purified GD IgG was investigated. As anticipated, these IgG were found to activate hNUE and not hNIS promoter in thyroid cell line FRTL-5, acting similarly to thyroid-stimulating hormone (TSH). Thus, this finding provides direct evidence that NIS transcriptional activation is actually responsible for the elevated iodide levels in patients with GD.

Using site-directed mutagenesis, a new putative regulatory region was further located in the hNIS promoter. This region is highly homologous to rat NIS TSH-responsive element and appears to play an important role in up-regulation of NIS gene expression by GD IgG as well as by TSH.

Since the report that very high doses of phthalates, diesters of phthalic acid, impede the sexual development of male rats, these plasticisers appear on a list of so-called “endocrine disrupters”. The effects of phthalates on thyroid function are indicated by several morphological investigations, which showed that some phthalates induced thyroid hyperactivity. The most convincing, however, is the study demonstrating a stimulation of iodide uptake by several phthalates. It was therefore thought important to determine whether phthalate plasticisers modulate the transcriptional activity of the hNIS gene.

As a result, three major phthalates di-isodecyl phthalate, benzyl butyl phthalate and di-octyl phthalate significantly increased the activity of the hNIS promoter construct in thyroid cell line PC Cl3. Likewise, these phthalates also enhanced the rat NIS endogenous mRNA expression. Although the demonstrated stimulation of NIS gene transcription by DIDP, BBP and DOP is not very strong, this finding is of great importance as humans are routinely exposed for long periods to phthalate plasticisers, the accumulation of which may contribute to thyroid hyperfunction.

In the second and main part of this work, the yet unknown transcriptional control mechanisms of the novel human heterodimeric hormone termed thyrostimulin were analysed. This hormone has attracted attention because of its ability to activate the TSH

receptor. For the first time, the cloning of the minimal promoter of the human thyrostimulin A2 subunit was described, whose transcriptional behaviour appears slightly different in pituitary (GH3 and αT3) and non-pituitary (293) cell lines.

Deletional, mutational and gel shift analyses revealed several candidate regulatory elements that are essential for the regulation of the A2 gene expression. In contrast to the common glycoprotein α-subunit gene, the A2 promoter contains neither the binding site for P-Lim nor CRE and was not responsive to the treatment with TRH or forskolin in the reporter gene assays. This suggests that the regulation of the A2- and α-subunit genes diverges at least at the level of the transcription factors controlling gene expression.

In addition, classical silencer regions upstream and downstream the A2 promoter were located. Another noteworthy feature of this gene is the presence of exonic and intronic silencer elements.

The mechanism by which the human thyrostimulin B5 subunit is regulated remains uncertain. By analogy with the TSH β-subunit, the expression of the B5 gene could be restricted to certain pituitary cell types by a strong silencer element. In this case, however, the availability of only non-B5-expressing cell lines could account for the failure to localise such silencer element.

Finally, the human thyrostimulin A2 and B5 subunit cDNAs were cloned into a bi-promoter expression vector and the resultant construct was stably transfected into 293 cells. Serum-free conditioned medium containing recombinant proteins was collected and concentrated 200-fold. It is intriguing that this medium was capable of strongly stimulating the activity of the NIS promoter and enhancer-luciferase construct in FRTL-5 cells, thereby implicating thyrostimulin, albeit indirectly, in the established view of thyroid axis.

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7.- Publications

Breous, E., Wenzel, A., Loos, U., 2003. Graves' IgG activate upstream enhancer of the sodium/iodide symporter. Mol. Cell. Endocrinol. 213(1), 109-13.

Breous, E., Wenzel, A., Loos, U. The promoter of the human sodium/iodide symporter responds to certain phthalate plasticisers. Accepted in Mol. Cell. Endocrinol.

Wenzel, A., Franz, C., Breous, E., Loos, U. Modulation of iodide uptake by dialkyl phthalate plasticisers in FRTL-5 rat thyroid follicular cells. Accepted in Mol. Cell. Endocrinol.

Breous, E., Wenzel, A., Loos, U. Promoter cloning and characterisation of the transcriptional regulation of the human thyrostimulin A2 subunit. Accepted in Mol. Cell. Endocrinol.

8.- Acknowledgements

My special thanks go to my Doktorvater Prof. Dr. med. Ulrich Loos for direction and guidance.
I am indebted to Dr. Achim Wenzel for many constructive discussions, patience and friendship.
I owe my warmest thanks to Dr. Michael Schiebe who has been a constant source of support and encouragement.
I am also extremely grateful to Prof. Dr. med. Paul Dietl for his willingness to review my dissertation and to PD Dr. med. Christian Beltinger and PD Dr. med. Jan Torzewski for acting as my examiners.

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