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A Thyroid-Specific Far-Upstream Enhancer in the Human Sodium/Iodide Symporter Gene Requires Pax-8 Binding and Cyclic Adenosine 3′,5′-Monophosphate Response Element-Like Sequence Binding Proteins for Full Activity and Is Differentially Regulated in Normal and Thyroid Cancer Cells

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The sodium/iodide symporter (NIS) gene is highly expressed in the thyroid gland and is important for the diagnosis and radioiodide therapy of differentiated thyroid cancers. We investigated a human NIS (hNIS) gene 5′-far-upstream enhancer (hNUE) (−9847 to −8968). The hNUE is TSH responsive in both FRTL-5 cells and primary normal thyroid cells, but not in human papillary thyroid cancer cells (BHP cells). The hNUE enhanced expression of the basal hNIS promoter 15-fold and required both a Pax-8 binding site and a cAMP response element (CRE)-like sequence for full activity. The hNUE activated transcription in a thyroid-selective and cAMP-dependent manner, mediated by both protein kinase A (PKA)-dependent and PKA-independent pathways. Pax-8 and two CRE-like sequence binding proteins bind to the hNUE. Supershift binding assay indicated that one of the CRE-like sequence binding protein(s) was CRE-binding protein-1, activation transcription factor-1, and/or CRE modulator, and the other was an unknown factor(s) that is absent in BHP 2-7 cells. A far-upstream enhancer is important for hNIS regulation in the thyroid. Deficient CRE-like sequence binding protein(s) that bind to the hNUE in normal thyroid cells may be responsible for reduced NIS gene expression in some thyroid carcinomas. (*Molecular Endocrinology* 16: 2266–2282, 2002)

THYROID CANCER IS the most common endocrine malignancy, and radioiodide-131 is the primary treatment for patients with differentiated thyroid carcinoma (1). Radioactive iodide is transported into cancer cells via the sodium/iodide symporter (NIS) expressed on the plasma membrane and exerts a local destructive effect. TSH is the primary stimulus to NIS gene expression in the thyroid (2, 3). Endogenous or exogenous TSH is used to augment NIS gene expression and improve the efficacy of radioiodide therapy. Loss of differentiated function, including loss of iodide transport, occurs in about one-fifth of differentiated thyroid carcinomas and results in a poor prognosis (4). Several groups reported that the expression of NIS mRNA or protein is decreased in thyroid carcinomas compared with normal thyroid tissues (5–8). Increased NIS expression, however, has been shown in some thyroid cancer specimens (9, 10).

Thyroid-specific transcription factors, thyroid transcription factor-1 (TTF-1) and the paired domain-containing transcription factor Pax-8 are required for development and differentiated function of the thyroid. Recent studies with the rat NIS gene indicate that these transcription factors are involved in the stimulation of rat NIS gene expression by TSH (11, 12). TSH stimulates rat NIS proximal promoter activity through TTF-1 and NIS TSH-responsive factor-1 (NTF-1) (11, 13). TTF-1 binds between −245 and −230 of the rat NIS gene and modestly activates the rat NIS promoter in a cell-specific manner (11). NIS TSH-responsive factor-1 binds about 170 bp upstream of the TTF-1 binding site and stimulates the rat NIS promoter activity approximately 3-fold after TSH stimulation (13). Pax-8 binds two different sites in the rat NIS upstream enhancer (rNUE), which is located between −2495 and −2264 of the rat NIS gene. The rNUE contains a cAMP response element (CRE)-like sequence that mediates thyroid-specific transcription through a novel cAMP-dependent pathway (12).

Abbreviations: AP-1, Activating protein-1; ATF, activating transcription factor; CMV, cytomegalovirus; cPKA, catalytic subunit of protein kinase A; cPKAα, catalytic α-subunit of PKA; CRE, cAMP response element; CREB, CRE binding protein; CREM, CRE modulator; DEAE, diethylaminoethyl; DTT, dithiothreitol; FBS, fetal bovine serum; hNIS, human sodium/iodide symporter; hNUE, human NIS upstream enhancer; hTg, human thyroglobulin; NIS, sodium/iodide symporter; rNUE, rat NIS upstream enhancer; SV40, simian virus 40; Tg, thyroglobulin; TTF-1, thyroid transcription factor-1.

TSH up-regulates the NIS mRNA and protein levels in both human and rat thyroid cells (2, 3). However, the homology of the NIS 5′-flanking sequence up to −3000 bp between rat and human is less than 30%, suggesting differential regulation of the NIS promoter in these species. We recently reported that reduced NIS gene expression in BHP 2-7 human papillary thyroid cancer cells is partly due to absent or reduced DNA binding of transcription factors, other than TTF-1 or Pax-8, to the human NIS proximal promoter between −596 and −415 (14). Systematic 5′-deletions from −2829 bp showed that only the region −596 to −415 conferred differential regulation between normal thyroid cells and human papillary thyroid cell lines (14). In fact, a recent study indicates that TTF-1 and Pax-8 have only a modest stimulatory effect on human NIS (hNIS) gene expression compared with the robust effects of TTF-1 and Pax-8 on other thyroid-specific genes (15).

In the present study, we systematically evaluated the far-upstream sequence of the hNIS gene and found an enhancer containing both a Pax-8 binding site and a CRE-like sequence 9 kb upstream of the hNIS gene. This region shares sequence similarity with a previously described upstream enhancer in the rat NIS gene of −2495 to −2264. The hNIS upstream enhancer (hNUE) markedly stimulates NIS gene expression in FRTL-5 rat thyroid cells, but not in BHP 2-7 human papillary thyroid cancer cells. The activation of the enhancer requires both Pax-8 and several CRE-like sequence binding proteins. Nuclear extracts from BHP 2-7 cells are deficient in proteins that bind to the CRE-like sequence.

RESULTS

A hNIS Upstream Sequence Around −9 kb Enhances a Heterologous Promoter and the hNIS Proximal Promoter

We analyzed the hNIS 5′-flanking sequence to determine whether any regions conferred differential regulation in normal and thyroid cancer-derived cells. We ligated 5′-flanking region segments (~600 to 1600 bp in length) from −9847 to −2805, upstream of the heterologous simian virus 40 (SV40) promoter in luciferase reporter plasmids (Fig. 1A). Transient transfection analysis with these plasmids was performed in FRTL-5 (rat thyroid cells) and BHP 2-7 (human papillary thyroid cells), in which the expression of the endogenous hNIS gene is markedly decreased or absent (14, 16). The fragment −9847 to −8968 activated basal SV40 promoter activity (~8.5-fold) in FRTL-5 cells but not in BHP 2-7 cells (−9847/−8968-SV40 in Fig. 1B). Luciferase activity of the construct containing −8600 to −7951 was approximately 3 times higher than that of the pGL3-promoter, but was not different between the two cell lines (Fig. 1B). In addition, this −8600 to −7951 region did not significantly enhance basal SV40 promoter activity in nonthyroid cells, such as JEG-3 human choriocarcinoma cells or MCF-7 human breast cancer cells (data not shown). Insertion of the other 5′-flanking region fragments, covering −8077 to −2805, did not significantly induce basal SV40 promoter activity. We identified a potent enhancer in the region −9847 to −8968 that is active in FRTL-5 cells with high levels of endogenous NIS expression, but not in BHP 2-7 cells.

The active enhancer fragment, −9847 to −8968, was ligated upstream of the hNIS basal proximal promoter (−812 to −268) in a luciferase reporter construct (−9847/−8968-basal hNIS in Fig. 1A). Luciferase activity of the basal hNIS proximal promoter was enhanced approximately 15-fold by insertion of the −9847 to −8968 fragment (Fig. 1C).

The hNUE Activity Requires Both CRE-Like Sequence and a Putative Pax-8 Binding Site

Inspection of the sequence of the hNIS upstream enhancer region, −9847 to −8968, revealed putative Pax-8 and TTF-1 binding sites. These sequences had a high homology to the Pax-8 and TTF-1 interaction sites (PA and TB sites, respectively) described in the rNUE located −2495 to −2264 (12) (Fig. 2). The hNUE sequence contains a CRE-like sequence, TGACGCA (a deletion of T from the consensus CRE, TGACGTCA) (17) (Fig. 2). The hNUE sequence is located 9 kb upstream and, by sequence inspection, is located within an upstream gene, ribosomal protein L18a.

To investigate which elements of the enhancer are essential for full activity, we made several deletion constructs of −9847/−8968-SV40 (Fig. 3A) and transfected them into FRTL-5 cells and BHP 2-7 cells. The activity of CT-SV40, a deletion of the putative Pax-8 binding site, was markedly decreased compared with that of −9847/−8968-SV40 or PCT (containing putative Pax-8, CRE-like, and TTF-1 binding sites)-SV40 in FRTL-5 cells (Fig. 3B). No enhancer activity was found in FRTL-5 cells when both the putative Pax-8 binding site and CRE-like sequence were deleted (T-SV40 in Fig. 3B). PC-SV40, a putative TTF-1 binding site-deleted construct, had full enhancer activity, while the construct P-SV40 lacking both putative TTF-1 binding site and CRE-like sequence lost enhancer activity (Fig. 3B). The constructs containing only putative Pax-8 binding site (P-SV40) or putative TTF-1 binding site (T-SV40) did not confer enhancer activity (Fig. 3B). The construct containing only the CRE-like sequence (C-SV40) had weak enhancer activity, similar to that of CT-SV40 (Fig. 3B). In addition, reverse orientation of the fragment −9847 to −8968 markedly stimulated the SV40 promoter activity in FRTL-5 cells (−9847/−8968-Rev-SV40 in Fig. 3B), indicating that the fragment from −9847 to−8968 acts as an enhancer, independent of its orientation. On the other hand, no enhancer activity of any constructs was found in BHP 2-7 cells (data not shown). These results indicate that the hNUE acts in FRTL-5 cells, but not in BHP 2-7 cells, and requires both

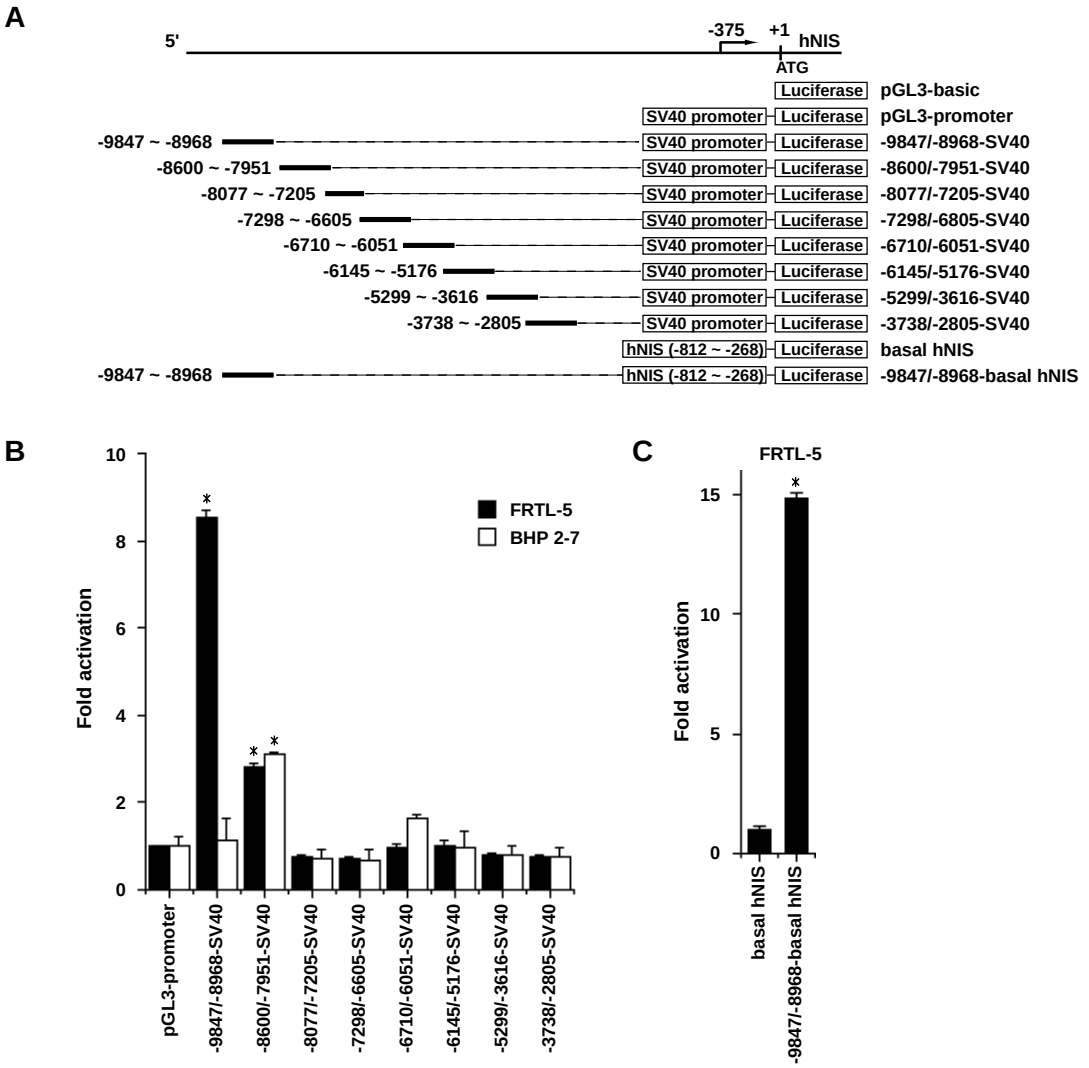


Fig. 1. Transient Transfection of the hNIS Upstream Sequence Segments −9847 to −2805 Upstream of the SV40 Promoter or the hNIS Basal Proximal Promoter in FRTL-5 Cells and BHP 2-7 Cells

A, Schematic representation of 5′-flanking region fragments from the hNIS gene inserted upstream of SV40 promoter in a pGL3-promoter vector or upstream of the hNIS basal proximal promoter (−812 to −268) controlling the luciferase gene. The first nucleotide of the hNIS translation initiation codon is referred to as +1. The nucleotide of the hNIS transcription start site is −375 (52). B, Luciferase activity is shown for each construct upstream of the SV40 promoter transiently transfected in FRTL-5 cells (maintained with TSH) and BHP 2-7 thyroid cancer cells. C, Luciferase activity of the −9847/−8968-basal hNIS was determined in FRTL-5 cells. A pRL-CMV vector was cotransfected to normalize for transfection efficiency (panels B and C). Luciferase activity of pGL3-promoter vector (panel B) or basal hNIS (panel C) was set at 1 in each cell line. Values are the mean ± SD (n = 4 to 6). *, Significant difference ($P < 0.01$) compared with each cell transfected with pGL3-promoter (panel B) or basal hNIS (panel C).

putative Pax-8 binding site and CRE-like sequence. The putative TTF-1 binding site, as reported for the rNUE, is not required for the hNUE activity.

Next, we tested the hNUE and its deletion fragments to enhance the hNIS basal proximal promoter (−812 to −268) controlling a luciferase gene (Fig. 3A) in FRTL-5 cells and BHP 2-7 cells. In FRTL-5 cells, the presence of the putative Pax-8 binding site and the CRE-like sequence enhanced the hNIS proximal promoter activity approximately 13- to 15-fold (−9847/−8968-basal hNIS, PCT-basal hNIS and PC-basal hNIS in Fig. 3C), compared with approximately 8.5-fold enhancement of the SV40 promoter (Fig. 3B). Any deletion of the putative Pax-8 binding site or the CRE-like sequence resulted in loss of the enhancer activity (CT-basal hNIS, P-basal hNIS, and C-basal hNIS in Fig. 3C). In addition, no enhancer activity of any construct was found in BHP 2-7 cells, even if the SV40 promoter was replaced by the hNIS proximal promoter (data not shown). These data demonstrate that the hNUE works more efficiently with the hNIS proximal promoter in FRTL-5 cells than with a heterologous SV40 promoter.

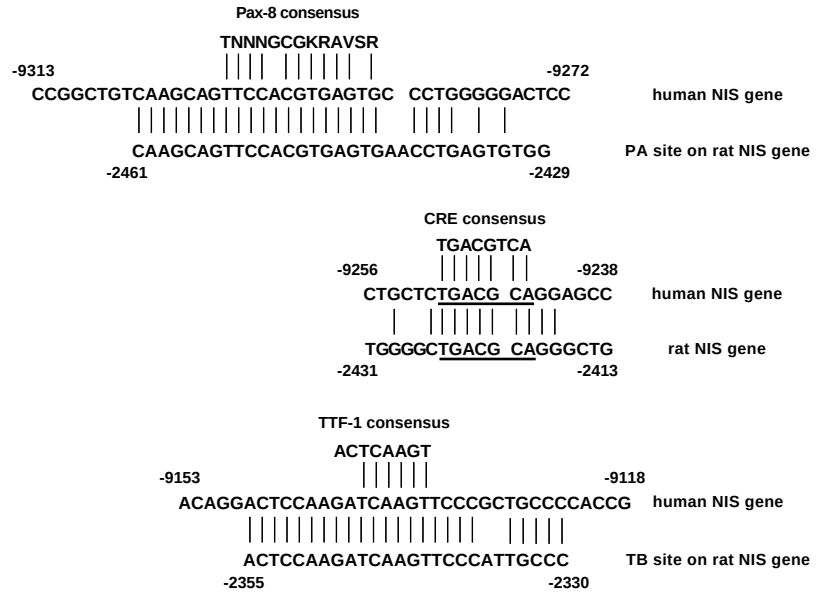


Fig. 2. The Sequence of the hNIS Upstream Region, −9847 to −8968, Contains CRE-Like Sequence and Putative Pax-8 and TTF-1 Binding Sites

The first nucleotide of the human and the rat NIS translation initiation codon is referred to as +1. The putative Pax-8 and TTF-1 binding sites on the hNIS gene shows high homology to the recombinant Pax-8 and TTF-1 interaction sites on the rNUE (PA and TB sites, respectively) (12). The CRE-like sequence on the human and rat NIS gene is *underlined*.

Effects of Mutation on the hNUE

Point mutations of the putative Pax-8, CRE-like, and putative TTF-1 domains were made in the context of the PCT (−9408 to −8968) fragment upstream of the hNIS basal proximal promoter (Fig. 4A) and tested in FRTL-5 cells. The enhancer activity was markedly decreased to approximately 15% of that of wild type when the putative Pax-8 binding site was mutated (P*CT-basal hNIS in Fig. 4B), although the luciferase activity of P*CT-basal hNIS was approximately 2 times higher than that of basal hNIS without any upstream enhancer sequence (Fig. 4B). No enhancer activity was found when the CRE-like sequence was mutated (PC*T-basal hNIS in Fig. 4B). The mutation of the putative TTF-1 binding site did not significantly affect the enhancer activity (PCT*-basal hNIS in Fig. 4B). These data indicate that both the putative Pax-8 binding site and the CRE-like sequence are required for the hNUE, consistent with the domain deletion studies (Fig. 3, B and C). TTF-1 is not involved in hNUE activity.

TSH Stimulates the hNUE through cAMP Pathway

TSH up-regulates NIS mRNA levels through a cAMP pathway (2, 3). To determine whether the hNUE is TSH responsive and/or cAMP responsive, the chimeric hNIS basal proximal promoter-luciferase constructs with (−9847/−8968-basal hNIS) or without (basal hNIS) the hNUE element were transfected into FRTL-5 cells. The cells were cultured for 7 d without TSH, and then incubated in the presence or absence of 1 mU/ml TSH or 10 μM forskolin, an agonist of adenylate cyclase, for 48 h. These conditions were designed to maximally detect TSH stimulation. Both TSH and forskolin dramatically stimulated hNUE enhancement of the hNIS proximal promoter (~24.9-fold or 17.5-fold, respectively, compared with the basal hNIS proximal promoter activity), while luciferase activity of basal hNIS was not increased by TSH or forskolin treatment (Table 1). These data indicate that hNUE responds to stimulation via the cAMP pathway and that the hNIS basal proximal promoter activity is not up-regulated by TSH or forskolin.

hNUE Activity in Long-Term Cultured Normal Human Thyroid Cells

We recently reported hNIS gene expression and iodide uptake in long-term cultured normal human thyroid cells (18). hNIS mRNA levels are up-regulated by TSH in these cells, as is seen in FRTL-5 cells (18). To assess whether the hNUE functions in human thyroid cells, the long-term cultured normal human thyroid cells were transiently transfected with basal hNIS or −9847/−8968-basal hNIS, and treated with or without 0.2 mU/ml TSH for 48 h. The hNUE significantly enhanced the hNIS proximal promoter activity in the presence of TSH (~5.6-fold) but not in the absence of TSH, indicating that the hNUE is stimulated by TSH in human thyroid cells as well as in FRTL-5 cells. The lower magnitude of TSH stimulation measured in normal human thyroid cells may be a result of TSH contained in the pituitary extracts required for cell growth.

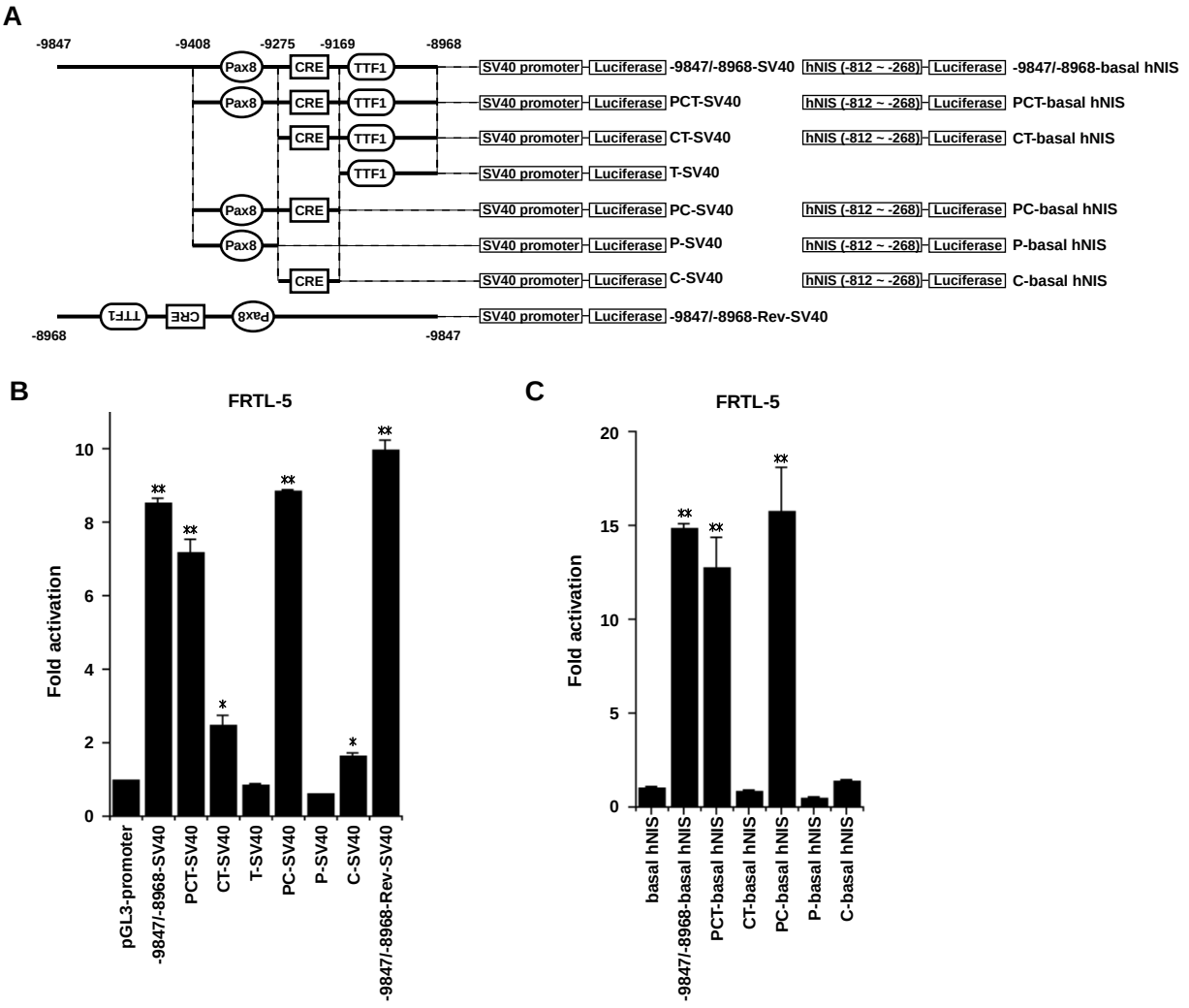


Fig. 3. hNUE Domain Deletions Upstream of the SV40 Promoter or hNIS Basal Proximal Promoter Transfected in FRTL-5 Cells

A, Schematic representation of reporter constructs used in these transfection experiments. Fragments containing hNUE sequences were inserted upstream of the SV40 promoter in pGL3-promoter vector or the hNIS basal proximal promoter (−812 to −268). B and C, Luciferase activity of SV40 (panel B) and basal hNIS (panel C) constructs in FRTL-5 cells. The constructs are named based on the presence of Pax8 (P), CRE (C), and TTF1 (T) elements. A pRL-CMV vector was cotransfected to normalize for transfection efficiency (panels B and C). Luciferase activity of pGL3-promoter vector (panel B) or basal-hNIS (panel C) was set at 1. Values are the mean ± SD (n = 4 to 6). * and **, Significant difference ($P < 0.05$ and $P < 0.01$, respectively) compared with FRTL-5 cells transfected with pGL3-promoter (panel B) or basal hNIS (panel C).

The hNUE Is Activated by Both PKA-Dependent and PKA-Independent Pathways

Chronic stimulation of FRTL-5 cells by TSH down-regulates the catalytic subunit of protein kinase A (cPKA) and results in a loss of the response of a consensus CRE-containing promoter to further cAMP stimulation (19). Thus, substitution of the cPKA after chronic TSH stimulation in FRTL-5 cells allows evaluation of whether the response of the hNUE to cAMP is PKA dependent. We transfected −9847/−8968-SV40 with or without the expression vector of the catalytic α-subunit of PKA (cPKAα) into FRTL-5 cells before the removal of TSH, and treated the cells with or without forskolin. In the absence of forskolin, the overexpression of cPKAα significantly increased the hNUE activity (up to 2.4-fold of that without cPKAα), suggesting the involvement of PKA-dependent pathway in the hNUE activation (Table 2). In the studies of the rNUE, cotransfection of cPKA increased rNUE activity approximately 7-fold without forskolin treatment, suggesting greater importance of the PKA-dependent pathway in the stimulation of the rNUE compared with that of the hNUE. On the other hand, in the presence of forskolin, luciferase activity was stimulated by the hNUE despite the absence of cPKAα (Table 2), suggesting the importance of the PKA-independent pathway in the hNUE activation.

We also used pharmacological inhibitors of the signaling pathway to determine the effects on hNUE ac-

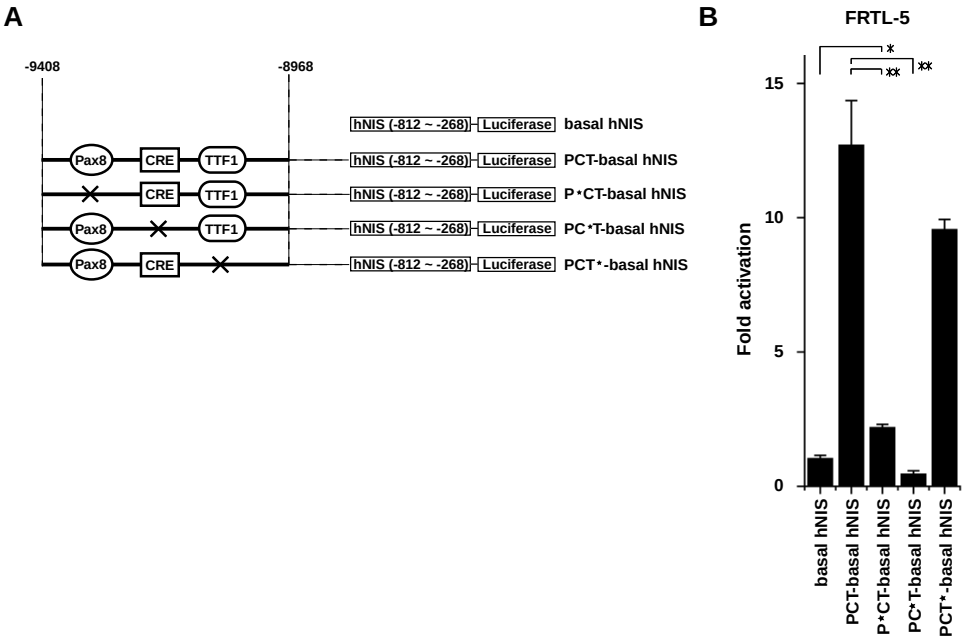


Fig. 4. hNUE Domain Mutations Upstream of the Basal hNIS Promoter Transfected in FRTL-5 Cells

A, Schematic representation of luciferase constructs used in these transfection experiments. Chimeric luciferase plasmids containing mutations of the putative Pax-8, TTF-1 binding site, or CRE-like sequence were generated by PCR using PCT-basal hNIS as a template. The mutated sequence for P*CT-basal hNIS, PC*T-basal hNIS, or PCT*-basal hNIS is shown in Fig. 7A. B, Luciferase activity of each construct in FRTL-5 cells. Luciferase activity of basal hNIS was set at 1. A pRL-CMV vector was cotransfected to normalize for transfection efficiency. Values are the mean ± SD (n = 4). *, Significant increase ($P < 0.05$) compared with FRTL-5 cells transfected with basal hNIS. **, Significant decrease ($P < 0.01$) compared with FRTL-5 cells transfected with PCT-basal hNIS.

Table 1. The hNUE Activity Is Mediated by TSH/cAMP Pathway in FRTL-5 Cells

	5H	5H + FSK	5H + TSH
pGL3-basic	1	1	1
Basal hNIS	15.08	11.15	13.24
−9847/−8968-basal hNIS	6.34	195.16[a]	330.4[a]
	(fold activation)		
−9847/−8968-Basal hNIS/basal hNIS (ratio)	0.42	17.5	24.95

FRTL-5 cells cultured for 7 d in 5H medium were transfected with each luciferase reporter construct, cultured for 24 h in 5H medium, treated with or without TSH (1 mU/ml) or forskolin (10 μM) for 48 h, and harvested, and then luciferase activity was measured. Results are normalized luciferase activity and expressed as fold activation of the value of the pGL3-basic vector. A pRL-CMV vector was cotransfected to normalize for transfection efficiency. Values are the mean for three separate experiments.

[a] Significant difference ($P < 0.01$) compared with FRTL-5 cells transfected with basal hNIS in the same condition.

tivity in FRTL-5 cells. The cells were cultured in 5H medium for 7 d and transfected with −9847/−8968-SV40 construct. Twenty-four hours after transfection, the cells were treated with signaling pathway inhibitors for 45 min before addition of forskolin, and then luciferase activity was measured 48 h after addition of forskolin. In this condition, forskolin activates both PKA-dependent and PKA-independent pathways in FRTL-5 cells. Preincubation with H-89 (10 μM), an inhibitor of PKA, inhibited forskolin-induced enhancer activity 50.3 ± 3.6%. Moreover, forskolin-induced enhancer activity was also decreased 43.2 ± 3.0% by preincubation with PD98059 (50 μM), inhibitor of upstream of ERK1/2-MAPK. Taken together, these results indicate that the hNIS gene expression is mediated by both PKA-dependent and PKA-independent pathways through the hNUE and demonstrate that ERK cascade is involved in a PKA-independent pathway for activation of the hNUE.

The hNUE Is Selective for Differentiated Thyroid Cells

To investigate whether the hNUE is cell selective, we transfected basal hNIS or −9847/−8968-basal hNIS into two BHP human papillary thyroid cell lines, BHP 7-13 that expresses Pax-8 but not TTF-1 (16, 20) and BHP 15-3 cells without Pax-8 and TTF-1 gene expression (20), and two nonthyroid cell lines, MCF-7 human breast cancer cells (which express hNIS after retinoic acid-stimulation) (21) and JEG-3 human choriocarcinoma cells. No enhancer activity was found in the BHP cells or nonthyroid cells (Table 3). These data indicate

Table 2. PKA-Dependent and -Independent Transcription of the hNIS Gene through the hNUE

		−9847/−8968-SV40
cPKAα (−)	FSK (−)	1
	FSK (+)	6.46[a]
cPKAα (+)	FSK (−)	2.43[a]
	FSK (+)	6.93[a]
		(fold activation)

FRTL-5 cells were exposed chronically to TSH and transfected with −9847/−8968-SV40 (0.1 μg) with or without cPKAα (0.1 μg of pRc/RSV-CHO PKA C-α), cultured for 48 h in 5H medium in the presence or absence of forskolin (10 μM), and harvested, and then luciferase activity was measured. A pRL-CMV vector (5 ng) was cotransfected to normalize for transfection efficiency. pcDNA3.1 empty vector (Invitrogen) was used to adjust the total amount of DNA. Results are normalized luciferase activity and expressed as fold activation of the value of −9847/−8968-SV40 without the cPKAα expression vector and forskolin treatment. Values are the mean for three separate experiments.

[a] Significant difference ($P < 0.01$) compared with FRTL-5 cells transfected with −9847/8968-SV40 without both cPKAα expression vector and forskolin treatment.

Table 3. The hNUE Activity in other BHP Cells and Nonthyroid Cells

	Basal hNIS	−9847/−8968-Basal hNIS
FRTL-5 (6H)	1	14.83[a]
BHP 7-13 [Pax-8 (+), TTF-1 (−)]	1	0.97
BHP 15-3 [Pax-8 (−), TTF-1 (−)]	1	0.52
JEG-3 (human choriocarcinoma cells)	1	0.78
MCF-7 (human breast cancer cells)	1	0.84
		(fold activation)

The cells were incubated 48 h after transfection and harvested, and luciferase activity was measured. A pRL-CMV vector was cotransfected to normalize for transfection efficiency. Results are normalized luciferase activity and expressed as fold activation of the value of basal hNIS in each cell line.

[a] Significant difference ($P < 0.01$) compared with each cell transfected with basal hNIS.

that the hNUE selectively functions in normal thyroid cells.

Effects of Overexpression of Pax-8, cPKA, and/or TTF-1 on hNUE Activity in BHP 2-7 and MCF-7 Cells

We wanted to determine whether the hNUE activity could be restored in cell lines without endogenous enhancer activity. The rNUE is synergistically activated by Pax-8 and cPKA in HeLa cells (12). We transfected −9847/−8968-SV40 with cotransfection of Pax-8, TTF-1, and/or cPKAα expression vectors. Although BHP 2-7 cells express endogenous Pax-8 (14, 16), the hNUE activity in the cells was stimulated approximately 4-fold by exogenous Pax-8, as shown in Fig. 5A. When TTF-1 was overexpressed in BHP 2-7 cells, which do not express endogenous TTF-1 (14, 16), the transcription stimulated by exogenous Pax-8 was markedly suppressed to the basal level without cotransfection (Fig. 5A). Similar results were seen with the −9847/−8968 fragment upstream of the basal hNIS promoter, although at a lower magnitude (data not shown). In addition, the repressive effect of TTF-1 on the transcription stimulated by Pax-8 in BHP 2-7 cells was dose dependent (Fig. 5B), suggesting that TTF-1 acts as a repressor of the hNUE. No significant effect of overexpression of cPKAα alone or in combination with Pax-8 and/or TTF-1 was found on the hNUE activity in BHP 2-7 cells (Fig. 5A). In MCF-7 cells, no activity of the hNUE was found even with cotransfection of any combination of expression vectors (Fig. 5A). Treatment of BHP 2-7 cells and MCF-7 cells with 10 μM forskolin after transfection to increase intracellular cAMP (16, 22) produced no significant change in the hNUE activity with any combination of expression vectors (data not shown). These results suggest that the stimulation of the hNUE by Pax-8 is thyroid specific.

To verify that the endogenous Pax-8 in BHP 2-7 cells is functional, we transfected a chimeric luciferase construct containing the human thyroglobulin (hTg) promoter and enhancer, which requires TTF-1 and Pax-8 binding for full activity (23–27). Luciferase activity in BHP 2-7 cells with overexpression of TTF-1 reached the maximal level (~100-fold compared with luciferase activity when Pax-8 and TTF-1 were not overexpressed) similar to that with overexpression of both Pax-8 and TTF-1 (Fig. 5C). In MCF-7 cells without endogenous Pax-8 or TTF-1 expression, the hTg promoter activity was enhanced approximately 46-fold by the transfection of both Pax-8 and TTF-1 expression vectors, while each expression vector did not significantly affect the hTg promoter activity (Fig. 5C). These data of the hTg promoter activity are consistent with a previous report (27) and suggest that endogenous Pax-8 in BHP 2-7 cells is functional.

The hNUE Is Promoter Selective

The hNIS gene was recently mapped to chromosome 19p (8) and the sequence of chromosome 19p13 was submitted to the NCBI database (AC005796) by the Lawrence Livermore National Laboratory. The hNUE is actually contained in the human ribosomal protein L18a gene (hRPL18a), which consists of five exons and four introns and is located upstream of the hNIS gene (Fig. 6A). To evaluate whether the hNUE also stimulates the hRPL18a basal promoter activity, chimeric hRPL18a promoter (−460 to +10 in hRPL18a)-luciferase constructs with (−9847/−8968-basal hRPL) or without (basal hRPL) the hNUE element were transfected into FRTL-5 cells, BHP 2-7 cells, and MCF-7

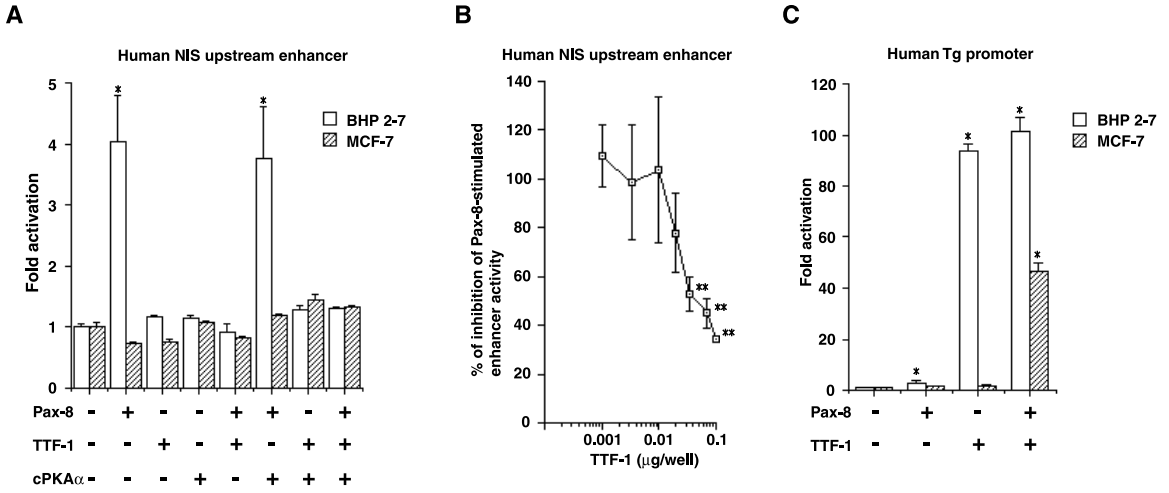


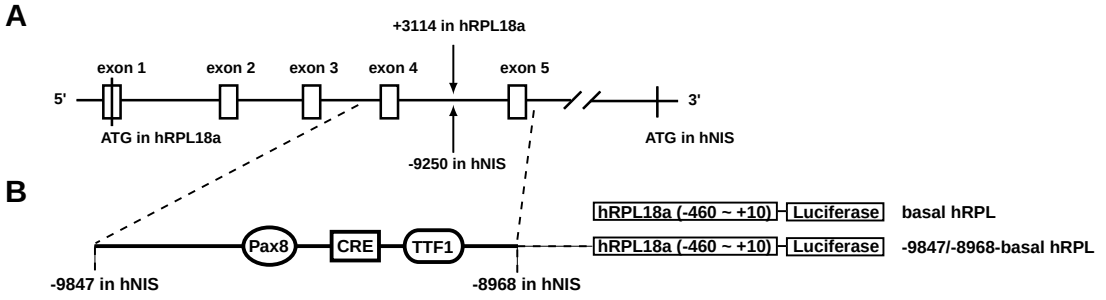
Fig. 5. Effects of Overexpression of Pax-8, TTF-1, and/or cPKAα on the hNUE in BHP 2-7 Cells and MCF-7 Cells

A, −9847/−8968-SV40 (0.1 μg) was transfected into BHP 2-7 cells or MCF-7 cells with expression vectors for Pax-8 (0.1 μg of pRc/CMV-Pax8), TTF-1 (0.1 μg of pRc/CMV-THA), and/or cPKAα (0.1 μg of pRc/RSV-CHO PKA C-α). Luciferase activity of −9847/−8968-SV40 without Pax-8, TTF-1, and cPKAα was set at 1 in each cell line. The cells were incubated for 48 h in growth medium after transfection and harvested, and luciferase activity was measured. B, Negative effects of TTF-1 on Pax-8-stimulated enhancer activity are dose dependent. Various amounts of expression vector for TTF-1 were cotransfected with −9847/−8968-SV40 (0.1 μg) and expression vectors for Pax-8 (0.1 μg) and cPKAα (0.1 μg) in BHP 2-7 cells. Luciferase activity of −9847/−8968-SV40 without TTF-1 was set at 100%. The cells were incubated for 48 h in growth medium after transfection and harvested, and luciferase activity was measured. C, Effects of overexpression of Pax-8 and/or TTF-1 on hTg promoter activity in BHP 2-7 cells and MCF-7 cells. A chimeric hTg promoter (−499 to +24)-hTg enhancer (−2705 to −2142)-luciferase construct (0.1 μg) was transfected into BHP 2-7 cells or MCF-7 cells with expression vectors for Pax-8 (0.1 μg) and/or TTF-1 (0.1 μg). Luciferase activity without cotransfection of Pax-8 and TTF-1 expression vectors was set at 1 in each cell line. The cells were incubated 48 h in growth medium after transfection and harvested, and luciferase activity was measured. pcDNA3.1 empty vector was used to adjust the total amount of DNA (panels A, B, and C). A pRL-CMV vector (5 ng) was cotransfected to normalize for transfection efficiency (panels A, B, and C). Values are the mean ± SD (n = 3). *, Significant increase ($P < 0.01$) compared with each cell transfected without Pax-8, TTF-1, and cPKAα expression vectors (panel A) or without Pax-8 and TTF-1 expression vectors (panel C). **, Significant decrease ($P < 0.01$) compared with BHP 2-7 cells transfected without TTF-1 expression vector.

cells. Although these cell lines express abundant RPL18a mRNA (Kogai, T., K. Taki, J. M. Hershman, and G. A. Brent, unpublished observation), the hNUE did not further activate the proximal hRPL18a promoter in FRTL-5 cells, in BHP 2-7 cells, or in MCF-7 cells above high basal levels (Fig. 6C). These data indicate that the hNUE is promoter selective for the hNIS gene despite its far-upstream location.

Reduced hNUE Activity in BHP 2-7 Cells Is Associated with the Lack of CRE-Like Sequence Binding Protein(s)

Our mutational analysis of the hNUE suggests that both the putative Pax-8 binding site and the CRE-like sequence are required for full enhancer activity. The BHP 2-7 cells, however, do not express the enhancer activity even though the cells express endogenous Pax-8 (14, 16). We hypothesized that the absence of factors normally bound to the CRE-like sequence is primary responsible for the loss of the enhancer activity in BHP 2-7 cells. To evaluate our hypothesis, we performed EMSAs with oligonucleotides containing a putative Pax-8 binding site (Oligo hPax8-wt), a CRE-like sequence (Oligo hCRE-wt), or a putative TTF-1 binding site (Oligo hTTF1-wt), the sequences of which are shown in Fig. 7A. Mutated oligonucleotides as shown in Fig. 7A were used as competitors. When nuclear extracts from FRTL-5 cells or BHP 2-7 cells were incubated with oligo hPax8-wt as a probe, a similar band shift pattern was observed (Fig. 7B, lanes 2 and 7). These protein-DNA complexes were competed by 100-fold excess of the unlabeled wild-type probe but not by 100-fold excess of the unlabeled mutated probe (Fig. 7B, lane 3 *vs*. 4 and lane 8 *vs*. 9). Supershift assays with anti-Pax-8 antibody indicated that the shifted band forming band P contains Pax-8 (Fig. 7B, lanes 5 and 10). Band B showing both low binding affinity and widespread binding was observed with nuclear extracts from FRTL-5 cells but not from BHP 2-7 cells, when the strong signal of band P was blocked by anti-Pax-8 antibody (Fig. 7B, lane 5 *vs*. 10). A supershifted band with anti-TTF-1 antibody was demonstrated with nuclear extracts from FRTL-5 cells but not from BHP 2-7 cells, suggesting that band B contains TTF-1 (Fig. 7B, lane 6 *vs*. 11). The nuclear extracts from FRTL-5 cells formed complexes (bands N and T) with the oligonucleotide containing the putative TTF-1 binding site (Fig. 7C, lane 2). Band N showing relatively high binding affinity was observed



C

	FRTL-5	BHP 2-7	MCF-7
pGL3-basic	1	1	1
basal hRPL	2653	622	4871
-9847/-8968-basal hRPL	2023	455	2677
	(Fold activation)		
-9847/-8968-basal hRPL / basal hRPL (ratio)	0.76	0.73	0.55

Fig. 6. The hNUE Is Promoter Selective

A, Map of the human genome (chromosome 19p) around the hNUE. The first nucleotide of the hNIS or the human ribosomal protein L18a (hRPL18a) translation initiation codon, respectively, is referred to as +1. The codon of −9250 in the hNIS gene is same as that of +3114 in the hRPL18a gene. B, Schematic representation of luciferase constructs used in these transfection experiments. The hNUE was inserted upstream of hRPL18a proximal promoter (−460 to +10) controlling the luciferase gene. FRTL-5 cells, BHP 2-7 cells, or MCF-7 cells were incubated 48 h after transfection and harvested, and luciferase activity was measured. A pRL-CMV vector was cotransfected to normalize for transfection efficiency. C, Results are normalized luciferase activity and expressed as fold activation of the value of pGL3-basic. Values are the mean of three separate experiments.

with nuclear extracts from FRTL-5 cells and BHP 2-7 cells, but band T showing low binding affinity was not observed with nuclear extracts from BHP 2-7 cells (Fig. 7C, lane 2 *vs*. 7). These bindings were sequence specific, and the putative TTF-1 binding site-mutated oligonucleotide did not compete in the formation of complexes (Fig. 7C, lane 3 *vs*. 4 and lane 8 *vs*. 9). Supershift assays with anti-TTF-1 antibody indicated that band T contains TTF-1 (Fig. 7C, lanes 6 and 11). When oligo hCRE-wt was used as a probe, multiple shifted bands were shown in both FRTL-5 cells and BHP 2-7 cells (Fig. 7D). The formation of these bands was competed by a 100-fold excess of the unlabeled wild-type probe, but not by 100-fold excess of the unlabeled mutated probe, indicating specific binding of proteins to the CRE-like sequence (Fig. 7D, lane 3 *vs*. 4 and lane 6 *vs*. 7). A specific band (band S) was observed with nuclear extracts from FRTL-5 cells but not from BHP 2-7 cells (Fig. 7D, lane 2 *vs*. 5).

Characterization of the CRE-Like Sequence of the hNUE

To evaluate the CRE-like sequence binding proteins interacting with the hNUE, we compared the pattern of the shifted bands using oligonucleotides containing the CRE-like sequence of the hNUE (Oligo hCRE-wt), the consensus CRE, TGACGTCA, from the somatostatin gene (17), and the consensus activating protein-1 (AP-1) site, TGAGTCA, a variant of the consensus CRE with deletion of one nucleotide, from the collagenase gene (28) (Fig. 8A). Band S was observed with nuclear extracts from FRTL-5 cells bound to the consensus CRE sequence as well as the oligo hCRE-wt, while the consensus AP-1 sequence did not form the band S with nuclear extracts from FRTL-5 cells (Fig. 8B, lanes 1 and 2 *vs*. 3). On the other hand, no band S was observed with nuclear extracts from BHP 2-7 cells, even if the consensus CRE was used as a probe (Fig. 8B, lanes 4, 5, and 6). The other protein-DNA complexes formed by the oligo hCRE-wt and nuclear extracts from FRTL-5 cells or BHP 2-7 cells (Ds bands) were also similar to those formed by the consensus CRE (Fig. 8B, lanes 1, 2, 4, and 5), although the consensus CRE bound significantly more protein. These results suggest that shifted bands Ds and S with the oligo hCRE-wt contain CRE-binding protein (CREB) or other proteins that bind to consensus CRE, such as activating transcription factors (ATFs). To confirm the results, we performed competition analysis of

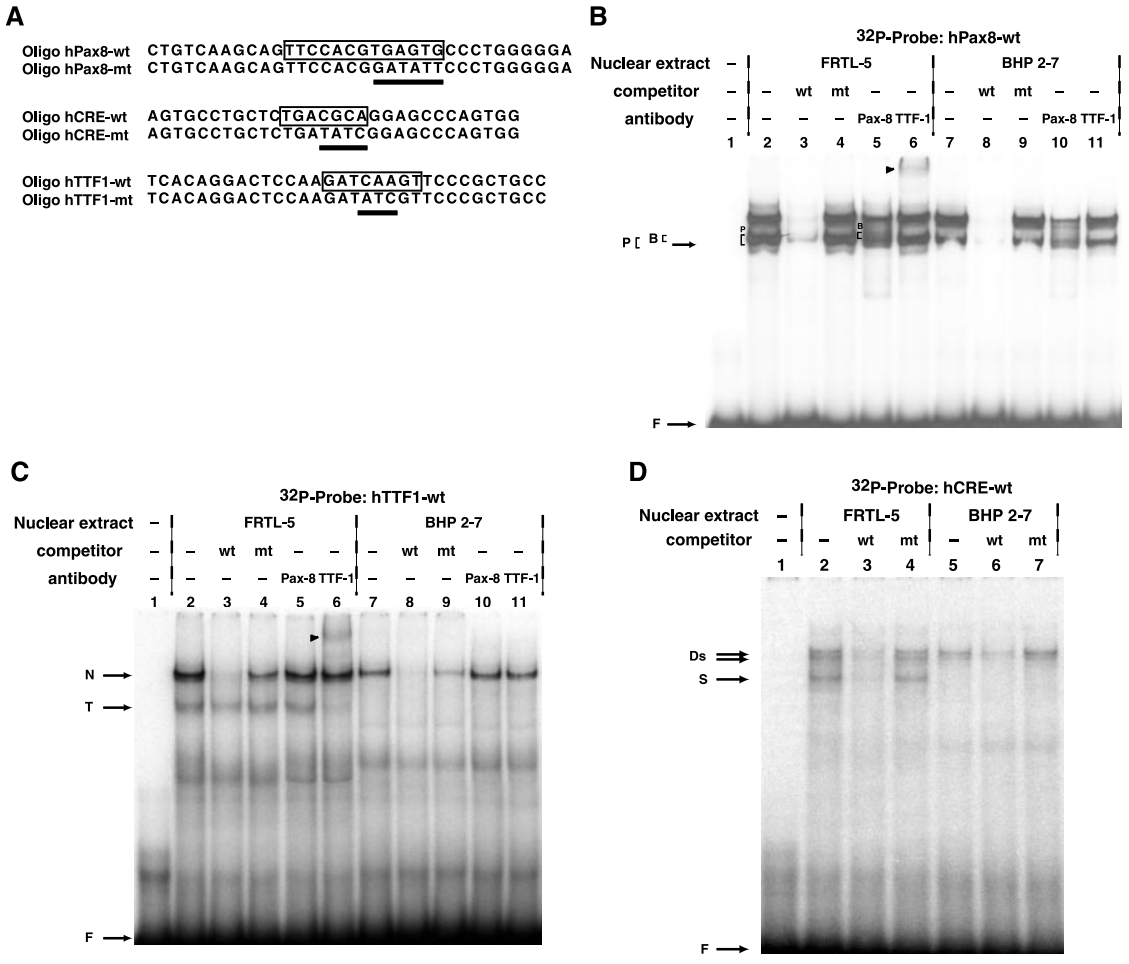


Fig. 7. Binding of Nuclear Proteins to the hNUE

Three putative binding sites, Pax-8, TTF-1 and CRE-like sequence, were investigated. A, Sequence of oligonucleotides used for luciferase reporter constructs and EMSAs. Mutated bases in the putative Pax-8 and TTF-1 binding site, and CRE-like sequence are *underlined*. B, C and D, The radiolabeled synthetic wild-type (wt) oligonucleotide was used as a probe and incubated with 4, 5, or 2 μg (panels B, C, or D, respectively) of nuclear extracts from FRTL-5 cells cultured in 6H medium or BHP 2-7 cells cultured in growth medium in the presence or absence of Pax-8 or TTF-1 antibody or competed with wt or mutated (mt) cold oligonucleotide (100-fold excess). Bands B, T, and S (panels B, C, and D, respectively) indicate a protein-DNA complex present in nuclear extracts from FRTL-5 cells, but not from BHP 2-7 cells. Bands P, N, and the double band (Ds) (panels B, C, and D, respectively) were present in nuclear extracts from FRTL-5 and BHP 2-7 cells. The supershifted band formed in the presence of anti-TTF-1 antibody is shown by *arrowhead* (panels B and C). F, Free probe, in panels B, C, and D.

the formation of bands Ds and S with consensus CRE or AP-1 oligonucleotide as a competitor (Fig. 8B). Band S formed by the oligo hCRE-wt and nuclear extracts from FRTL-5 cells was competed by 100-fold excess of the unlabeled consensus CRE probe as well as by 100-fold excess of the unlabeled oligo hCRE-wt probe (Fig. 8C, lane 1 *vs*. 4), whereas 100-fold excess of the unlabeled consensus AP-1 probe competed slightly for band S formation (Fig. 8C, lane 1 *vs*. 5). The formation of the Ds bands was blocked by 100-fold excess of the unlabeled consensus CRE probe (Fig. 8C, lanes 4 and 9), while the bands were weakly competed by 100-fold excess of the unlabeled consensus AP-1 probe (Fig. 8C, lanes 5 and 10). These observations suggest that the proteins interacting with the CRE-like sequence of the hNUE are likely to be in the CREB/ATF families.

To identify transcription factors interacting with the CRE-like sequence of hNUE, we performed supershift assay using antibody for some members of CREB/ATF families or AP-1 families: anti-CREB-1 (C-21) recognizes CREB-1 p43, ATF-1, and CRE modulator (CREM); anti-ATF-2 (C-19) is specific for ATF-2; anti-c-Jun (D) recognizes c-Jun, Jun B, and Jun D p39; and anti-c-Fos (K-25) antibody recognizes c-Fos, Fos B, Fra-1, and Fra-2. No effect of any antibody on the formation of band S was found (Fig. 8D, lanes 2, 3, 4, and 5). On the other hand, Ds bands formed with nuclear extracts from FRTL-5 cells or BHP 2-7 cells were supershifted by preincubation with anti-CREB-1 antibody, but

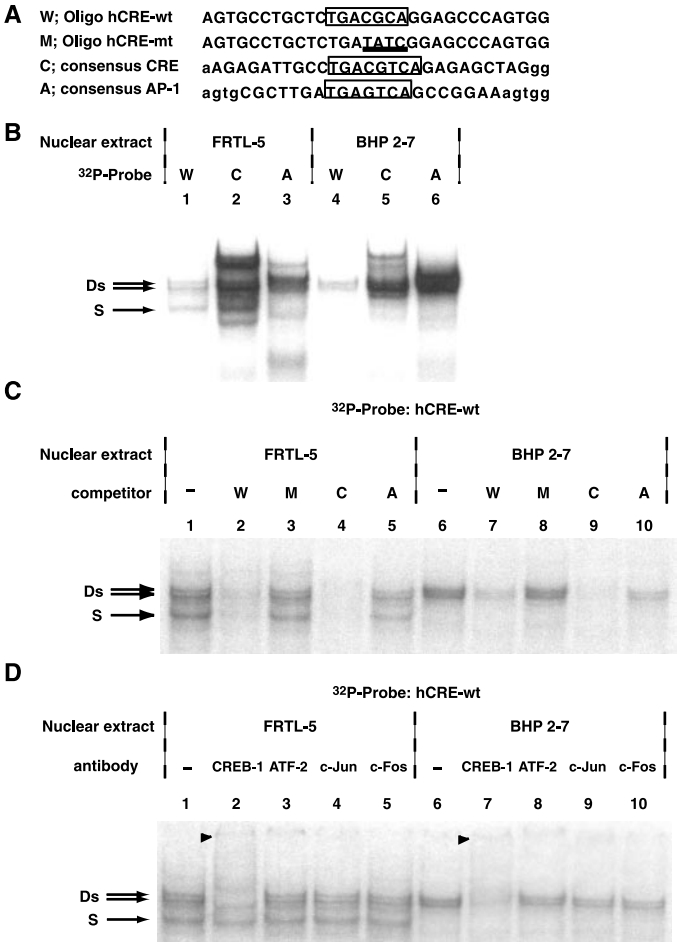


Fig. 8. Characterization of CRE-Like Sequence Binding Proteins

A, Sequence of oligonucleotides used in EMSAs. CRE-like sequence on the hNUE and consensus sequence for CRE (17) and AP-1 (28) are shown in *open squares*. Mutated bases in the CRE-like sequence are *underlined*. The size of the oligonucleotide (30 bp) was matched by adding the corresponding hNIS sequences (*lowercase letters*) to the ends of the consensus sequence for CRE and AP-1. Nuclear extracts (2 μg) from FRTL-5 cells cultured in 6H medium and BHP 2-7 cells cultured in growth medium were used for these EMSAs (panels B, C, and D). B, Comparison of the band shift pattern of the three wild-type oligonucleotides. The radiolabeled wild-type (W), consensus CRE (C), and consensus AP-1 (A) oligonucleotide were used as probes and incubated with nuclear extracts from FRTL-5 cells or BHP 2-7 cells. C, Competition analysis of the protein-DNA complexes formed by the wild-type oligonucleotide (Oligo hCRE-wt) and nuclear extracts from FRTL-5 cells or BHP 2-7 cells using unlabeled oligonucleotides containing the sequence for wild-type (W), mutated type (M), consensus CRE (C), or consensus AP-1 (A) (100-fold excess). D, Supershift analysis of the protein-DNA complex formed by the wild-type oligonucleotide (Oligo hCRE-wt) and nuclear extracts from FRTL-5 cells or BHP 2-7 cells. Band S in panels B, C, and D demonstrates a protein-DNA complex present in nuclear extracts from FRTL-5 cells but not from BHP 2-7 cells. The Ds bands in panels B, C, and D show protein-DNA complexes in nuclear extracts from each cell line. The supershifted band formed in the presence of the anti-CREB-1 (C-21) antibody is shown in lanes 2 and 7 (*arrowhead*).

not by anti-ATF-2, -c-Jun, or -c-Fos antibody (Fig. 8D, lane 2 or 7 *vs*. 3, 4, and 5 or 8, 9, and 10, respectively). No effect of anti-ATF-3 (C-19) or anti-ATF-4 (C-20) antibody on the formation of band S was found (data not shown). These results suggest that the transcription factors recognized by anti-CREB-1 (C-21) antibody bind to the CRE-like sequence of the hNUE in both FRTL-5 cells and BHP 2-7 cells and formed Ds bands. The CRE-like sequence binding protein(s) that is missing in BHP 2-7 cells is an unknown protein, likely to be in the CREB/ATF family, but different from the proteins tested in this study.

Effects of TSH/Forskolin on the Formation of the CRE-Like Sequence Binding Proteins in FRTL-5 Cells

Next, we assessed effects of TSH/forskolin on the formation of bands Ds and S using nuclear extracts from FRTL-5 cells. Ds bands were observed in nuclear extracts from FRTL-5 cells cultured without TSH for 7 d (5H cells) as well as in that from the cells cultured with TSH (6H cells) or in that from 5H cells treated with forskolin for 24 h (FSK cells) (Fig. 9, lanes 1, 2, and 3).

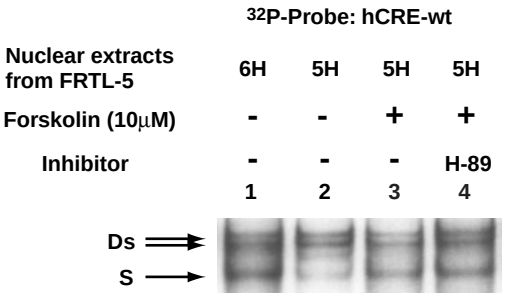


Fig. 9. Effects of TSH and Forskolin with or without PKA Inhibitor on the Formation of Bands Ds and S

The radiolabeled oligonucleotide (Oligo hCRE-wt) was incubated with nuclear extracts from FRTL-5 cells. The cells were cultured in 6H medium (6H cells) or in 5H medium for 7 d (5H cells). Treatment of 5H cells with forskolin (10 μM) was performed for 24 h. H-89 (10 μM) was preincubated for 45 min before addition of forskolin.

On the other hand, the increase of the formation of band S was observed in nuclear extracts from 6H cells and FSK cells compared with that from 5H cells (Fig. 9, lanes 1 and 3 *vs*. 2). The formation of Ds bands and forskolin-induced band S, however, was not affected significantly by the preincubation of PKA inhibitor, H-89 (Fig. 9, lane 4). These results suggest that the transcription factors forming band S are up-regulated by TSH/forskolin treatment and regulated, at least in part, by a PKA-independent pathway.

DISCUSSION

NIS gene expression in the thyroid, and the resulting iodide uptake, are up-regulated predominantly by TSH (3, 29–32). In the present study, we investigated the regulation of the hNIS gene and identified the sequence −9847 to −8968 as a potent cell type-specific enhancer, which responds to TSH or forskolin via both PKA-dependent and PKA-independent pathways.

The hNUE sequence contains a CRE-like sequence, and putative Pax-8 and TTF-1 binding sites, with a high homology to regions of the rat NIS upstream enhancer (rNUE) (−2495 to −2264) (12). Our functional analysis indicated that both the CRE-like sequence and the Pax-8 binding site, but not the TTF-1 binding site, are required for full hNUE activity. The hNUE was a potent enhancer of both the heterologous SV40 promoter and the basal hNIS promoter.

We demonstrated direct binding of Pax-8 to the hNUE. Our cotransfection study showed that exogenous Pax-8 activated the hNUE in BHP 2-7 cells, but not in nonthyroid cells. In contrast to these findings, the rNUE is activated by exogenous Pax-8 in nonthyroid cells (12). Additionally, the rNUE has two Pax-8 binding sites, rather than the single site shown in the hNUE. Other novel thyroid-specific transcription factor(s) may be involved in the differential regulation of these NIS upstream enhancers by Pax-8 in human and rat.

TTF-1 is one of the key molecules for differentiation of thyroid cells and is required for the expression of thyroid-specific genes, such as thyroglobulin (Tg) (24, 33, 34), thyroid peroxidase (35, 36), and the TSH receptor (37–39). Although the putative TTF-1 binding site in the hNUE binds TTF-1, this element is not required for full enhancer activity. The TB site in the rNUE contains a TTF-1 binding site, shown by footprint analysis with recombinant TTF-1, and is also not required for the rNUE function (12). Exogenous TTF-1 inhibited Pax-8 stimulation of hNUE. Deletion or mutation of the TTF-1 binding site, however, did not significantly influence the hNUE activity, suggesting that the negative effect of TTF-1 is not mediated by the TTF-1 binding site. The Pax-8 binding site (PA site) of the rNUE, which has high homology to the Pax-8 element of the hNUE, overlaps a TTF-1 binding site (12). Our supershift assay demonstrated that TTF-1 is recruited to the Pax-8 element of the hNUE. Thus, the competition by TTF-1 for the binding of Pax-8 to the hNUE might contribute to TTF-1 repression of Pax-8-stimulated hNUE activity.

Our functional analysis of the hNUE indicated that the CRE-like sequence is required for full hNUE activity. We further showed in this study that two consensus CRE binding proteins, bands Ds and S (Fig. 8), bind to the CRE-like sequence on the hNUE. Ds bands are probably CREB-1, ATF-1, and/or CREM, based on the antibody in our supershift assay. The antibodies tested, however, did not recognize band S. A recent study of the rNUE CRE-like sequence showed that the rat NIS CRE-like sequence binding proteins are similar to consensus AP-1 binding proteins and are recognized by anti-c-Fos, -c-Jun, or -ATF-2 antibody, but not anti-CREB or anti-ATF-1 antibody (40). The difference in the primary proteins binding to the CRE-like sequence in human and rat may be the result of sequences surrounding the CRE-like element, which are completely divergent between the hNUE and the rNUE.

In patients with thyroid cancer, reduced NIS gene expression and iodide uptake result in low efficacy of radioiodide therapy. Recently, we reported on reduced activity of the hNIS proximal promoter in BHP 2-7 cells compared with normal human thyroid cells (14). Our present study showed the absence of the hNUE activity in BHP 2-7 cells, which express functional Pax-8. Cotransfection of Pax-8, however, restored some hNUE activity in BHP 2-7 cells. The CRE-like sequence is necessary for full hNUE activity, and our EMSAs showed the absence of binding to the CRE-like sequence with nuclear extracts from BHP 2-7 cells compared with FRTL-5 cells (the band S in FRTL-5 cells). Binding of Pax-8 and CREB-1, ATF-1, and/or CREM to the hNUE was similar utilizing nuclear extracts from BHP 2-7 or FRTL-5 cells. Therefore, the absent binding of the unknown factor(s) to the CRE-like sequence on the hNUE is likely to contribute to reduced hNUE activity in BHP 2-7 cells.

Recently, novel cAMP-binding proteins have been identified that exhibit guanine nucleotide exchange activity referred to as cAMP-guanine nucleotide ex-

change factors (cAMP-GEFs) (41) or exchange proteins activated by cAMP (Epacs) (42). These novel cAMP-binding proteins can activate the small GTPases such as Rap1 (41, 42), Rap2 (43, 44), and possibly Ras (45), which are known activators of downstream kinase cascades, including MAPK (46). Very recently, several groups reported that TSH induces the activation of Rap1 in both a PKA-dependent and PKA-independent manner, and activates the MAPK cascade in FRTL-5, Wistar rat thyroid, and dog thyroid cells (47–50). The expression of both rat and human NIS genes is mediated by both PKA-dependent and PKA-independent pathways through the upstream enhancer (Ref. 12 and our data). In addition, we showed that the ERK cascade is involved in PKA-independent pathway for the activation of the hNUE. The cotransfection of the cPKA in BHP 2-7 cells did not increase the hNUE activity stimulated by Pax-8, while the exogenous Pax-8 and cPKA synergistically increase the rNUE activity in HeLa cells (12). Forskolin, 10 μM, did not increase the hNUE activity stimulated by Pax-8, even with the cotransfection of cPKA in BHP 2-7 cells, although forskolin increased cAMP accumulation in the cells (16). cAMP leads to activation of the PKA-dependent pathway, and CREB, ATF-1, or CREM is directly phosphorylated and activated by PKA. The absence of a response of the hNUE to forskolin in BHP 2-7 cells suggests that the PKA-dependent pathway is less important for hNUE activity compared with the PKA-independent pathway. Interestingly, CRE-like sequence binding protein(s) (band S in FRTL-5 cells) were absent in BHP 2-7 nuclear extracts, while CREB-1, ATF-1, and/or CREM bound to the CRE-like sequence in both FRTL-5 cells and BHP 2-7 cells. Our EMSAs showed that in FRTL-5 cells, CREB-1, ATF-1, and/or CREM were not changed significantly by TSH/forskolin. These findings are supported by previous data with Western blot analysis, which showed that total CREB protein was detected in FRTL-5 cells cultured without TSH, and no induction of total CREB protein was found after forskolin treatment in those cells even if phosphorylation of CREB reached maximum within 30 min and declined steadily over the next 4 to 6 h (19). On the other hand, the CRE-like sequence binding protein(s) (band S) was increased by TSH/forskolin. In addition, no effect of PKA inhibitor on forskolin-induced formation of band S was found. Therefore, the CRE-like sequence binding protein(s) (band S) observed in FRTL-5 cells are regulated, at least in part, downstream of the PKA-independent pathway that activates the hNUE.

The map of chromosome 19p13 showed that the hNUE sequence is within the human ribosomal protein L18a (hRPL18a) gene, located upstream of the hNIS gene. Interestingly, our transfection study demonstrated that the hNUE does not enhance the hRPL18a proximal promoter in FRTL-5 cells. The hNIS promoter (14, 51–54) or SV40 promoter (55, 56) contains both a GC box and TATA-like motif, while the hRPL18a promoter contains only a GC box. The selectivity of promoter enhancement through the hNUE, therefore, may be explained by the difference of protein-protein interaction between the transcription factors binding to the hNUE and basal transcription factors recruited to the TATA or GC box of the promoter.

In conclusion, we identified a highly active promoter-selective and tissue-specific enhancer that upregulates hNIS expression in the thyroid. The hNUE contains a Pax-8 element and CRE-like sequence, both required for full activity, and mediates the signal of TSH/cAMP by both PKA-dependent and PKA-independent mechanisms. The absence of CRE-like sequence binding protein(s) in human papillary thyroid cancer cells, demonstrated in normal thyroid cells, may be involved in the loss of the hNUE activity. Further investigation of proteins binding to a CRE-like sequence will contribute to better understanding of the pathophysiology of reduced hNIS gene expression in thyroid carcinomas.

MATERIALS AND METHODS

Cell Culture

FRTL-5 rat thyroid cells, kindly provided by Dr. L. D. Kohn (Ohio University, Athens, Ohio), were grown in Coon's modified Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 5% calf serum and a six-hormone mixture (6H) containing bovine TSH (1 mU/ml), insulin (10 μg/ml), cortisol (10 nM), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (57). All hormones were purchased from Sigma. In some experiments, FRTL-5 cells were cultured for 7 d in medium without TSH (5H) after reaching 70% confluency before use.

BHP 2-7, 7-13, and 15-3 cell lines, derived from human papillary thyroid cancer (16, 58), were grown in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate.

Normal human primary thyroid cells were prepared and maintained as previously described (14, 18, 59). Briefly, thyroid tissue, obtained from the normal, nonaffected lobe in patients with papillary carcinoma or follicular adenoma who underwent thyroidectomy, was freed from adherent connective tissue, cut in small (<1-mm diameter) pieces, and washed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution. The enzymatic digestion was performed for 2 h with a solution consisting of 20 U/ml collagenase (Sigma), 0.75 mg/ml trypsin (Invitrogen, Carlsbad, CA, 1:300), and 2% heat-inactivated dialyzed chicken serum in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution. Released cells were grown in Coon's modified Ham's F-12 medium supplemented with 5% FBS, bovine insulin (1 μg/ml), transferrin (5 μg/ml), and cortisol (10 nM). Freshly frozen bovine hypothalamus and bovine pituitary (Pel-Freez Biologicals, Rogers, AR) extracts were prepared as previously described (59) and added to a final concentration of 75 and 5 μg of protein/ml of the medium, respectively. When the primary cells were used, the growth medium was switched to induction medium that is Coon's modified Ham's F-12 medium supplemented with 0.2% FBS, insulin (1 μg/ml), transferrin (5 μg/ml), and cortisol (10 nM) with or without bovine TSH (0.2 mU/ml). The primary cells can survive in the medium without hypothalamus and pituitary extracts for only 3–4 d. Before utilization of the primary cells, Tg production was confirmed with a commercial kit (Diagnostic Products, Los Angeles, CA), according to the manufacturer's instructions (59). Use of human surgical material was approved by the institutional human subjects review board.

MCF-7 cells, a human breast cancer cell line, and JEG-3 cells, a human choriocarcinoma cell line, were obtained from

Table 4. Primers Used for PCR Amplification

Construct	Sequence (Forward Primer) (Reverse Primer)
−9847/−8968-SV40	5′-GATCGGTACCACCACTGTTTTCTGGGACCCACTG-3′ 5′-TCATACGCGTGAGCTCTGCAGTGTGCAAAG-3′
−8600/−7951-SV40	5′-GATCGGTACCTTTCAGAGTAGACACAAGTGGGCACCCGAGG-3′ 5′-GATCCCCGGGAGAGGAAAGGCTGTGTCCTTGCCCTGAGTG-3′
−8077/−7205-SV40	5′-GATCGGTACCTGGAGAGATCGTGGCTTACTGTAGCC-3′ 5′-GATCACGCGTACACCTGTAATCCCAGCACTTTGG-3′
−7298/−6605-SV40	5′-GATCGGTACCTACAGACGGGGTTTCACCATGTGGG-3′ 5′-GATCACGCGTCCCTCACTGCTCCTGTGTGTTGC-3′
−6710/−6051-SV40	5′-GATCGGTACCGTGCCATTTCTGAGAAAGAACGCCTCTGGC-3′ 5′-GATCCCCGGGAGGTAGGTGAATAGGTGCAGGCTAGTGATC-3′
−6145/−5176-SV40	5′-GATCGGTACCAGCTGAGACATGGAGGCCAATCC-3′ 5′-GATCACGCGTGGTCTTGTACCTTCTCTCAACAGCC-3′
−5299/−3616-SV40	5′-GATCGGTACCGTAACCTCTCCACTGCACTCCAGC-3′ 5′-GATCACGCGTAGACAATGTGAGCAGAATGTGCAGG-3′
−3738/−2805-SV40	5′-GATCGGTACCACAGCAGCTCACAACTTCTCACC-3′ 5′-GATCACGCGTCCTGCCGACCAATGTAAACATATGC-3′
PCT-SV40	5′-AGTAGGTACCCACAGACCGAGACATGGGT-3′ 5′-TCATACGCGTGAGCTCTGCAGTGTGCAAAG-3′
CT-SV40	5′-ATTAGGTACCTCCCCTGGAGGGAAGTGCCT-3′ 5′-TCATACGCGTGAGCTCTGCAGTGTGCAAAG-3′
T-SV40	5′-AGTAGGTACCATGCCTCCCTTCCTCACAG-3′ 5′-TCATACGCGTGAGCTCTGCAGTGTGCAAAG-3′
PC-SV40	5′-AGTAGGTACCCACAGACCGAGACATGGGT-3′ 5′-TACTACGCGTGTGAGGAAGGGAGGCATGT-3′
P-SV40	5′-AGTAGGTACCCACAGACCGAGACATGGGT-3′ 5′-TAATACGCGTAGGCACTTCCCTCCAGGGGAGT-3′
C-SV40	5′-ATTAGGTACCTCCCCTGGAGGGAAGTGCCT-3′ 5′-TACTACGCGTGTGAGGAAGGGAGGCATGT-3′
−9847/−8968-Rev-SV40	5′-TAATACGCGTCACCACTGTTTTCTGGGACCCACT-3′ 5′-ATGTGGTACCTGAGCTCTGCAGTGTGCAAAG-3′
Basal hNIS (Ref. 14)	5′-TGATCAGATCTTTGGGGGTGGTAAAGCCAG-3′ 5′-GATCAAGCTTCTCACTCTGGGTTC-3′
Basal hRPL	5′-GATCAGATCTGAGACCTCCGTAACACACCTCC-3′ 5′-GATCAAGCTTAGGCCTTCATGGCGTGCTCT-3′

American Type Culture Collection (Manassas, VA) and maintained according to the recommended conditions.

Chemicals

Forskolin was obtained from Sigma. Signaling pathway inhibitors H-89 and PD98059 were purchased from Calbiochem (San Diego, CA).

Reporter Plasmids and Expression Vectors

DNA fragments of the human NIS 5′-flanking region were obtained by PCR using genomic DNA from MCF-7 cells, which express functional NIS under retinoic acid stimulation (21), as a template. Forward primers contained a *Kpn*I site with the exception of the forward primer for −9847/−8968-SV40, which contained an *Mlu*I site; and reverse primers contained either an *Mlu*I or *Sma*I site with the exception of the reverse primer for −9847/−8968-SV40, which contained a *Kpn*I site (Table 4). The sequences of hNIS proximal promoter (−812 to −268) (14), human ribosomal protein L18a promoter (−420 to +10), and hTg promoter (−499 to +24) and hTg enhancer (−2705 to −2142) were obtained by PCR. The primers for hNIS proximal promoter and human ribosomal protein L18a promoter contained a *Bgl*II site (forward primer) or *Hin*dIII site (reverse primer) (Table 4). The amplified DNA fragments were cloned into firefly luciferase reporter gene vectors, the pGL3 basic vector (Promega Corp., Madison, WI), or the pGL3 promoter vector (Promega Corp.), and these

sequences were confirmed (Laragen, Inc., Los Angeles, CA). Mutation of the CRE-like sequence, putative Pax-8, or putative TTF-1 binding site in the luciferase reporter gene plasmid was generated by PCR as described (60) with the following primer: CRE-like sequence, 5′-AGTGCCTGCTCTGATATCGGAGCCCAGTGG-3′; putative Pax-8 binding site, 5′-CTGTCAAGCAGTTCCACGGATATTCCCTGGGGGA-3′; putative TTF-1 binding site, 5′-TCACAGGACTCCAAGATATCGTTCCCGCTGCC-3′.

A Pax-8 expression vector, pRc/cytomegalovirus (CMV)-Pax8 (61), was provided by Dr. L. D. Kohn. A TTF-1 expression vector, pRc/CMV-THA (62), was provided by Dr. R. Di Lauro (Naples, Italy). A cPKAα expression vector, pRc/RSV-CHO PKA C-α (63, 64), was provided by Dr. R. A. Maurer (Oregon Health Sciences University, Portland, OR).

Transient Expression Analysis

Cells were grown in 12-well plates to about 70% confluency. Unless otherwise noted, 0.3 μg firefly luciferase reporter construct was transiently transfected with 20 ng pRL-CMV (*Renilla* luciferase expression plasmid; Promega Corp.) to normalize the transfection efficiency. Diethylaminoethyl (DEAE)-Dextran transfection system (Promega Corp.) was used for the transient transfection study of FRTL-5 cells and normal human primary thyroid cells with minor modifications of the manufacturer's protocol. Briefly, the cells were exposed to 250 μl of a plasmid-DEAE-PBS mixture containing 62.5 μg DEAE-Dextran for 20 min at 37 C in a CO_2 incubator, 1 ml of the fresh medium was added, incubation was continued for 2.5 h at 37 C, and then the medium-plasmid-DEAE-PBS mixture was replaced with 1 ml of fresh medium. After incubation for 24 h, the medium was replaced with 1 ml of fresh medium with or without TSH as indicated in different experiments, and the cells were incubated for 24–48 h at 37 C. BHP cells, MCF-7 cells, and JEG-3 cells were transfected with Effectene transfection reagent (QIAGEN, Valencia, CA), according to the instructions of the manufacturer, and incubated for 48–72 h at 37 C in a CO_2 incubator. Firefly and *Renilla* luciferase activities were determined 48–72 h after the beginning of transfection with the Dual-Luciferase Reporter Assay System (Promega Corp.).

Nuclear Extracts

Nuclear extracts from FRTL-5 cells and from BHP 2-7 cells were prepared as described (14, 65) with minor modification. Cells were washed, scraped into PBS, pH 7.4, pelleted by centrifugation (1,000 $\times$ *g*), and suspended in 5-pellet volumes of buffer A [10 mM HEPES-KOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.1 mM EGTA; 0.5 mM dithiothreitol (DTT); 0.5 mM phenylmethylsulfonyl fluoride; 2 μg/ml pepstatin A; and 2 μg/ml leupeptin] containing 0.3 M sucrose and 2% Tween 40. After nuclei were released by gentle homogenization, the suspension was overlaid on 1.5 M sucrose in buffer A and centrifuged (15,000 $\times$ *g*) at 4 C. Pelleted nuclei were washed with buffer A containing 0.3 M sucrose, centrifuged (1,000 $\times$ *g*), and lysed in 2.5-pellet volumes of buffer B (10 mM HEPES-KOH, pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.1 mM EGTA; 10% glycerol; 0.5 mM DTT; 0.5 mM phenylmethylsulfonyl fluoride; 2 μg/ml pepstatin A; and 2 μg/ml leupeptin). Lysed nuclei were centrifuged (15,000 $\times$ *g*) at 4 C. The supernatant was used in EMSAs after the quantification of protein content by Bradford's method (Bio-Rad Laboratories, Inc., Hercules, CA).

EMSA

Protein-DNA interactions were assessed with EMSAs performed as previously described (13, 14). Briefly, synthesized double-stranded oligonucleotides (MWG Biotech, High Point, NC) were labeled with γ-^{32}P ATP (ICN Biomedicals, Inc., Irvine, CA) by T4 polynucleotide kinase and purified using Quick spin column (Roche Molecular Biochemicals, Indianapolis, IN). Nuclear extracts were incubated in 20 μl reaction volume for 20 min at room temperature in the following buffer: 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 5% glycerol, 0.1% Triton X-100, and 1 μg poly (dI-dC). Labeled probe (50,000 cpm; $\approx$10 fmol DNA) was added to the reaction and incubated for 20 min at room temperature. DNA-protein complexes were separated on 5% native polyacrylamide gels. For supershift assays, nuclear extracts were incubated with antibody in the same buffer for 20 min at room temperature before adding the labeled probe. Anti-CREB-1 (C-21), -ATF-2 (C-19), -c-Jun (D), -c-Fos (K-25), -ATF-3 (C-19), and -ATF-4 (C-20) antibodies for supershift assays were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-TTF-1 and anti-Pax-8 antibodies were provided by Dr. T. Onaya (Yamanashi Medical University, Yamanashi, Japan) (32, 66).

Statistical Analysis

Statistical comparison was determined by Student's *t* test, with significance at $P < 0.05$.

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