0888-8809/04/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 18(11):2817–2829

doi: 10.1210/me.2004-0020

The CRE-Like Element Inside the 5′-Upstream Region of the Rat Sodium/Iodide Symporter Gene Interacts with Diverse Classes of b-Zip Molecules that Regulate Transcriptional Activities through Strong Synergy with Pax-8

J. T. CHUN, V. DI DATO, B. D'ANDREA, M. ZANNINI, AND R. DI LAURO

Department of Biochemistry and Molecular Biology (J.T.C., V.D.D., R.D.L.), Stazione Zoologica 'Anton Dohrn,' Villa Comunale, 80121 Napoli, Italy; and Istituto di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche and Dept. di Biologia e Patologia Cellulare e Molecolare (B.D'A., M.Z.), Universita' di Napoli Federico II, 80131 Napoli, Italy

We previously demonstrated that transcription of the rat sodium/iodide symporter (NIS) gene is regulated by NUE, an upstream enhancer located between nucleotides −2264 and −2495 of the 5′-flanking region. To elucidate the mechanism of TSH/cAMP-mediated regulation of NIS gene expression, we have characterized the putative cAMP response element (CRE)/activator protein (AP)-1 site (termed NUC) that is closely located between the two Pax-8 (paired box domain transcription factor-8) binding sites within NUE. In two different approaches using either gel supershift analyses or dominant-negative inhibitors of b-Zip molecules, we have shown that NUC can be recognized by several members of the AP-1 and CREB family transcription factors that modulate the transcriptional activity of NUE. Using tethered dimers of b-Zip molecules, we have also demonstrated that specific homo- or heterodimers of AP-1 can synergistically stimulate NUE activity in concert with Pax-8. To demonstrate further that NUC is a *bona fide* CRE, we made an artificial promoter with the five-time tandem repeat of this sequence (5xNUC). In comparison to the canonical CRE (5xCRE), 5xNUC manifested greater transcriptional activity and broader response to cAMP signaling. Hence, we postulate that the significance of this evolutionally conserved CRE-like site may lie in its broader cell type specificity. (*Molecular Endocrinology* 18: 2817–2829, 2004)

TSH IS REQUIRED for differentiation and proliferation of thyroid cells. TSH stimulates expression of thyroid-enriched genes, *e.g*. thyroglobulin (Tg), TSH receptor (TSHR), thyroid peroxidase (TPO), and sodium/iodide symporter (NIS). The effects of TSH on thyroid cells are mostly mediated by cAMP, a second-messenger molecule that is immediately induced by TSH. In cultured thyroid cells, cAMP can substitute TSH and stimulate expression of these thyroid-enriched genes. However, the molecular mechanism of cAMP-dependent gene expression in thyroid is still largely unknown (1–4).

Abbreviations: AP, Activator protein; ATF-2, activating transcription factor-2; CAT, chloramphenicol acetyl transferase; C/EBP, CCAAT/enhancer binding protein; CMV, cytomegalovirus; CRE, 3′, 5′-cyclic adenosine monophosphate response element; 4H, four-hormone media; NIS, sodium/iodide symporter; N17, dominant-negative mutant of human Rap1A; NUC, NIS upstream cAMP response element; NUE, NIS upstream enhancer; Pax-8, paired box domain transcription factor-8; PKA, protein kinase A; SI, synergy index; Tg, thyroglobulin; TPO, thyroid peroxidase; TSHR, TSH receptor; TTF-1, thyroid transcription factor-1.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

The most characterized mechanism of cAMP-dependent gene expression is through CRE (cAMP response element), a small palindromic *cis*-acting element in the regulatory region of the gene. Thus, the genes responding to cAMP usually contain one or more CRE sequences near their promoters. CRE recruits transcription factors like CREB, which can be activated by cAMP-dependent protein kinase A (PKA) and other kinases (5–8). However, other mechanisms may be also at work to conduct cAMP signaling and regulate gene expression. With the exception of TSHR, for instance, the other three cAMP-responsive thyroid-enriched genes (*e.g*. Tg, TPO, and NIS) do not contain the canonical CRE (4, 9). Hence, it has been suggested that cAMP-dependent expression of these genes might be mediated in some indirect mechanisms that involve modulation of other transcription factors such as TTF-1 (thyroid transcription factor-1) or Pax-8 (10, 11).

In our previous study, we characterized the 5′-upstream region of the rat NIS gene, and demonstrated that the 232-bp fragment located between −2264 and −2495 nucleotides can recapitulate the most relevant features of NIS gene regulation, *i.e.* thyroid cell-specific transcription and strong response to cAMP (12).

This enhancer element (NIS upstream enhancer; NUE) contains a CRE-like site (TGACGCA) that deviates from the canonical CRE (TGACGTCA) or activator protein (AP)-1 (TGA$^{C}_{G}$TCA) sequences by only one nucleotide. This CRE-like sequence element, which we call NUC (NIS upstream cAMP response element), is quite unique in several aspects. Firstly, although CRE is usually located within 150 bp upstream from the promoter region (13, 14), NUC is located over 2.5 kb away from the transcription start site. Secondly, although chronic exposure of cells to cAMP induces a refractory phase, where cells do not respond further to cAMP (15, 16), the 232-bp enhancer unit containing NUC can respond to cAMP in FRTL-5 cells during the refractory phase (12). Finally, the sequence of NUC (TGACGCA) is conserved in the rat and human genomes and is immediately flanked by binding sites of thyroid- enriched transcription factors, *e.g.* Pax-8 and TTF-1 (12, 17, 18). Previous work demonstrated that Pax-8, and not TTF-1, plays a direct role in modulating the transcriptional activities of NUE (12).

In this article, we have demonstrated that NUC is a functional CRE that produces cAMP-dependent gene expression. We have characterized the NUC site not only in the natural context within the rat NIS upstream enhancer, but also in an artificial promoter containing five-time tandem repeat of the NUC sequences. Using gel mobility supershift assay and dominant-negative inhibitors of specific b-Zip molecules, we have shown that NUC can specifically interact with CREB and several other b-Zip molecules. In explanation of our observation that cAMP-dependent transcriptional activity of NUE requires integrity of NUC and at least one Pax-8 binding site, we have also demonstrated that specific AP-1 dimers can create a strong synergy with Pax-8 in our heterologous reporter gene assays.

RESULTS

NUC Plays a Pivotal Role in Mediating cAMP-Responsive Transcriptional Activation, but It Requires Cooperation of at Least One of the Two Adjacent Pax-8 Binding Sites

NUE contains at least three crucial sites where mutagenesis of the sequence leads to severe loss of enhancer activities in FRTL-5 cells (12). One of them is NUC (NISB), and the other two are overlapping binding sites of Pax-8/TTF-1 (NISA) and the second binding site for Pax-8 (NISC). To determine the sequence element that is directly responsible for cAMP-mediated stimulation of the enhancer activity, we introduced site-directed mutations to the pNISTKLUC3 plasmid (Fig. 1A), and examined their abilities to respond to cAMP. To this end, transiently transfected FRTL-5 cells were arrested in basal media (0.2% serum) for 72 h and then recovered in 4-hormone media (without insulin and TSH) for additional 72 h in the presence or absence of the cAMP-releasing agent forskolin. In the absence of forskolin, the wild-type and the three mutant NUE constructs displayed similar basal level of reporter gene activities (Fig. 1B). When forskolin was added, however, the plasmid with mutations at the NUC site manifested a significant loss of cAMP responsiveness, whereas the wild-type plasmid displayed 10-fold increase of reporter gene activities, similar to the changes of the endogenous NIS mRNA levels monitored in the same experimental conditions (Fig. 1, B and C). In contrast, mutagenesis of one of the two Pax-8 sites (NISA or NISC) did not impair cAMP-

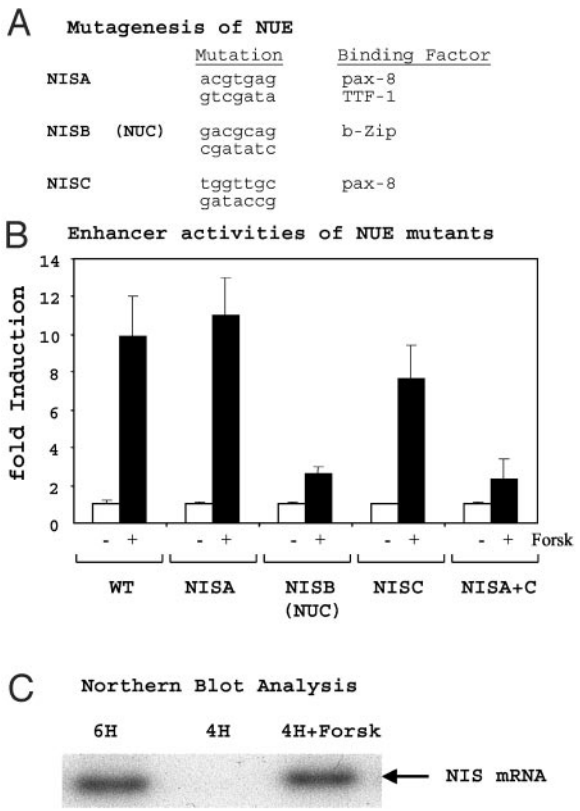


Fig. 1. cAMP-Dependent Stimulation of NUE Transcriptional Activity Requires NUC and One of the Pax-8 Sites

A, pNISTKLUC3 was mutated at the three major regulatory sites, NISA (corresponding to 2444–2450 bp upstream to the first codon), NISB (NUC, −2424 to −2418 bp), and NISC (−2399 to −2405 bp). The mutated sequences were indicated below the wild-type sequences for each case. B, Transiently transfected FRTL-5 cells were arrested in basal media (0.2% calf serum) for 72 h and subsequently maintained in 4H (without insulin and TSH) for additional 72 h in the absence (−) or presence (+) of 10 μM forskolin. As a control for transfection efficiency, luciferase activity was normalized with CAT activities of the internal control vector. *Error bars*, SD from three independent experiments. C, Northern blot analysis of the endogenous NIS mRNA in FRTL-5 cells under the same experimental conditions as transfection assays. Five micrograms of total RNA were resolved in 1% agarose gels and hybridized to the rat NIS cDNA probe as described previously (12). Definition of culture media: 6H, complete media with six hormones, as described in *Material and Methods* section; 4H, same as 6H media without insulin and TSH.

mediated stimulation of NUE activities. When both Pax-8 sites were modified, however, the ability to respond to cAMP was also severely impaired (Fig. 1B; NISA + C). Hence, although NUC plays a central role in mediating cAMP-induced stimulation of enhancer activity, it requires cooperation of at least one of the Pax-8 binding sites.

Transcriptional Activity of NUE Is Suppressed by Specific Dominant-Negative Inhibitors of b-Zip Transcription Factors

NUC bears sequence similarity to both AP-1 and CRE consensus sequences. Transcription factors binding to AP-1 or CRE are known as members of the b-Zip family. These molecules are characterized by a stretch of basic amino acids and a leucine zipper structure at the C terminus, which are required for DNA binding and dimerization, respectively (19). To demonstrate that transcriptional activity of NUE is under the regulation of b-Zip transcription factors, we used specific dominant-negative inhibitors of b-Zip. These molecules can dimerize with the target b-Zip with high affinity, but the resulting dimer cannot bind to DNA because of the stretch of negatively charged amino acid residues (20). Hence, dominant-negative inhibitors can specifically sequester the target b-Zip molecules and prevent them from binding to DNA (Fig. 2A). When these dominant-negative inhibitors were used for the cotransfection assay in FRTL-5 cells, we observed that dominant-negative inhibitors of CREB, Fos, JunD, and ATF-2 (activating transcription factor-2) consistently repress the NUE enhancer activities by 25–40% (Fig. 2B). In contrast, dominant-negative inhibitors for ATF-4 and CCAAT/enhancer binding protein (C/EBP) produced an increase of enhancer activities, whereas ATF-3 and A1-Zip made no difference. These results indicate that the transcriptional activity of NUE is specifically stimulated by CREB, ATF-2, Fos, and JunD, whereas other b-Zip molecules such as ATF-4 and C/EBP have repressive effects.

In control experiments, the dominant-negative b-Zip molecules had no effect on the reporter gene activities of the mutated pNISTKLUC3 plasmid with site-directed modification at the NUC motif sequence (Fig. 2C). Hence, the effect of dominant-negative b-Zip molecules on pNISTKLUC3 is specifically mediated by NUC, and not by other sequence elements.

Tethered b-Zip Dimers Can Stimulate Transcription Activity of NUE by Forming Strong Synergy with Pax-8

In an inverse approach to demonstrate that transcription activity of NUE is under the regulation of b-Zip, we used tethered b-Zip in which AP-1 monomers were joined by a polypeptide tether to force specific pairing (Fig. 3A). Considering the possibility that a given b-Zip can form multiple forms of dimers, this approach provides an advantage to study the effect of the exact homo- or heterodimers of b-Zip (21). To study the cooperation of Pax-8 and the various forms of tethered b-Zip dimers, NUE reporter gene (pNISTKLUC3) and the tethered b-Zip plasmids were cotransfected into HeLa cells in the presence or absence of exogenous Pax-8 (Fig. 3B). When Pax-8 was transfected into HeLa cells without exogenous tethered b-Zip, it stimulated NUE transcription over 3-fold. In the absence of exogenous Pax-8, the tethered b-Zip manifested either strong stimulation (JunD:Fra2, CREB:CREB), moderate stimulation (JunB:Fra2, JunB:ATF2, and JunD:ATF2), or slight repression (c-Jun:JunD). On the other hand, other tethered b-Zip homo- or heterodimers showed marginal effect by themselves (cJun:Fra2, cJun:cJun, JunD:JunD, and cJun:ATF2). However, in the presence of Pax-8, most tethered AP-1 (except cJun:JunD) displayed strong synergy with Pax-8 because the reporter gene activities were much greater than the sum of the inductions elicited by Pax-8 or tethered AP-1 alone. For the sake of quantitative comparison, we have defined Synergy Index (SI) as the combinatorial effect of the Pax-8 and a given tethered b-Zip dimer divided by the sum of the individual effects by the two (fold induction). The strongest synergy with Pax-8 was observed with cJun:Fra2 and JunD:JunD, where SI values reached 9.6 and 8.8, respectively (Fig. 3B). In contrast, despite the fact that CREB:CREB homodimer can stimulate NUE activity by 4-fold by itself, its combinatorial effect together with Pax-8 was rather additive rather than synergistic (SI = 1.2).

In control experiments, the tethered b-Zip molecules had no effect on the reporter gene activities of the mutated pNISTKLUC3 plasmid with site-directed modification at the NUC motif sequence (Fig. 3C). Hence, the effect of tethered b-Zip molecules on pNISTKLUC3 is specifically mediated by NUC, and not by other sequence elements.

Direct Identification of Transcription Factors Binding to NUC by Gel Mobility Supershift Assay

To demonstrate that b-Zip transcription factors exert their effects on NUE by direct interaction with the target site NUC, we performed supershift assays. The 28-bp oligonucleotide probe containing the NUC sequence displayed a complex binding pattern, which were arbitrarily labeled D, W, L1, U, and L2 (control lane, No Ab) in Fig. 4. All of these characteristic mobility shift patterns except the U band represent specific binding to the CRE-like core motif sequence, as mutation of the NUC sequence in the same probe led to elimination of all bands except U (data not shown). In the presence of the specific antibodies, supershift patterns were observed for some of those b-Zip molecules that modulated NUE activities in the transfection experiments using dominant-negative plasmid. ATF-2 antibody specifically supershifted the D bands, suggesting that ATF-2 is the main component of the D complex (Fig. 4). In comparison to that, c-Jun antibody

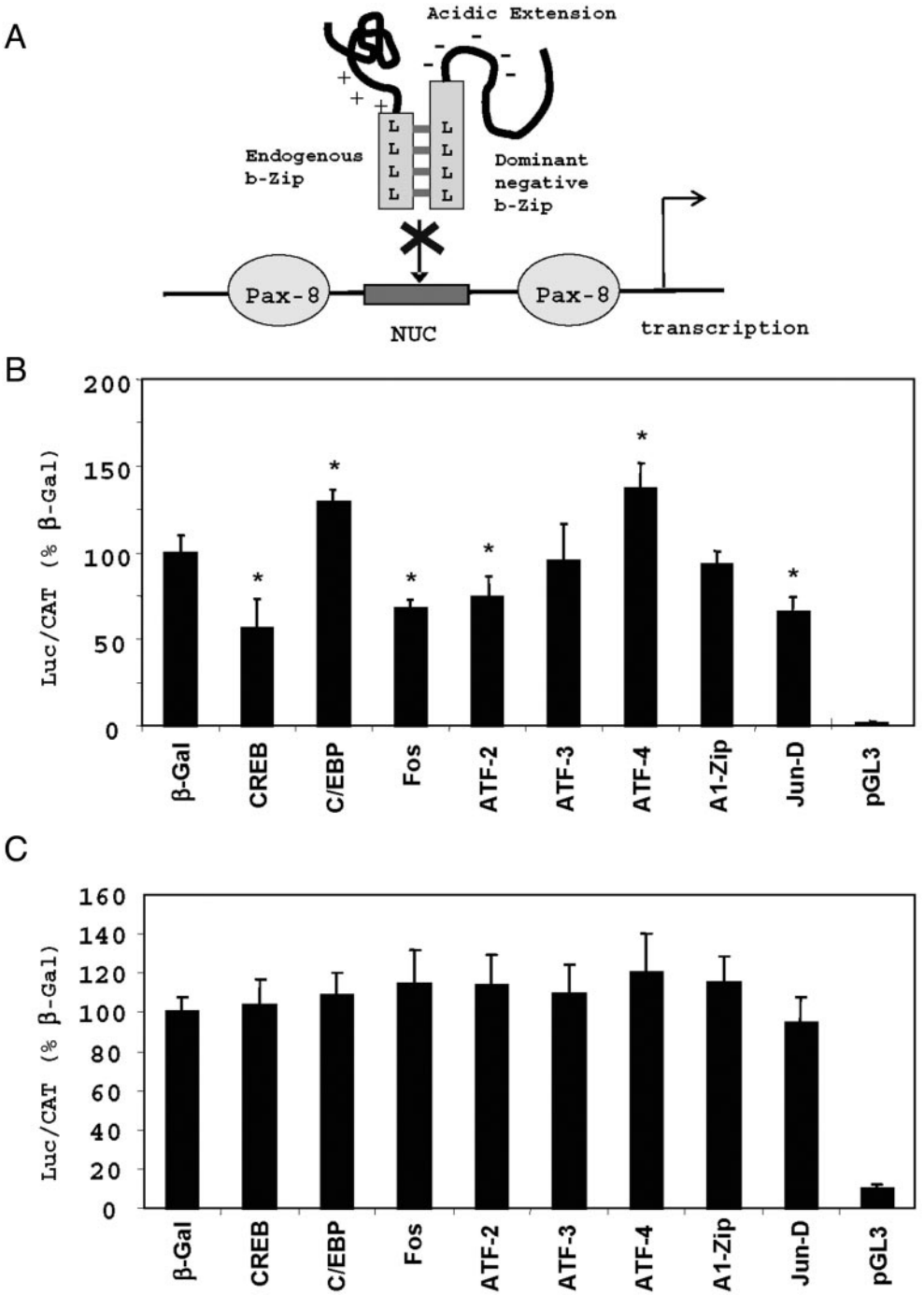
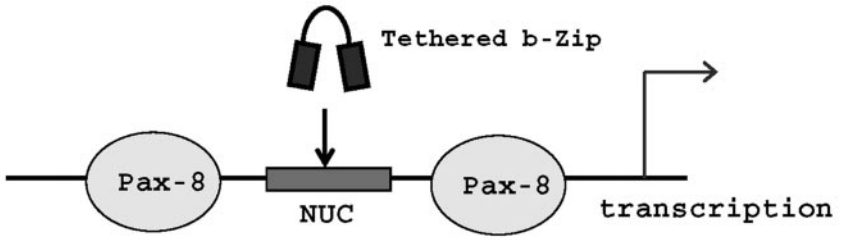


Fig. 2. Effects of Dominant-Negative Inhibitors of b-Zip Molecules on Transcriptional Activity of NUE

A, Dominant-negative inhibitors of b-Zip molecules can form a stable dimer with the target molecule, and thereby prevent it from binding to DNA. B, FRTL-5 cells were cotransfected with pNISTKLUC3 (3 μg), pCMV-CAT internal control (1 μg), and the plasmid overexpressing specific dominant-negative inhibitor of b-Zip (2 μg). Transfected cells were incubated in 6H media for 72 h, and lysed for luciferase and CAT (internal control) assays. C, Control experiments with the mutated pNISTKLUC3 modified at NUC core motif. Normalized data were plotted in reference to the control value (the empty cloning vector for b-Zip was used as effector). *Error bars*, SD from three independent experiments.

produced two similarly supershifted complexes, but with only slight reduction of the D complex. However, the concomitant disappearance of the D band is obvious when considering that the relative intensity of D complex in the c-Jun lane is much reduced with respect to W, in comparison with the situation with the

A



B

bZip dimer	Co-transfectant		Synergy
	β–Gal	Pax-8	Index
control (β-Gal)	1.00 ± 0.25	3.25 ± 0.43	-
JunB:Fra2	2.36 ± 0.91	36.07 ± 2.45	6.4
cJun:Fra2	1.70 ± 0.49	47.28 ± 7.69	9.6
cJun:cJun	0.85 ± 0.14	21.72 ± 4.25	5.3
JunD:Fra2	14.95 ± 1.88	101.32 ± 9.31	5.5
cJun:JunD	0.55 ± 0.02	4.32 ± 0.99	1.1
JunB:ATF2	3.15 ± 0.65	28.15 ± 1.01	4.4
JunD:ATF2	2.96 ± 0.16	33.03 ± 7.25	4.6
JunD:JunD	1.00 ± 0.10	37.54 ± 0.52	8.8
cJun:ATF2	1.49 ± 0.26	28.80 ± 2.46	6.1
CREB:CREB	4.33 ± 0.59	9.37 ± 1.93	1.2
pGL3	0.05 ± 0.01	0.09 ± 0.04	-

C

bZip dimer	Co-transfectant	
	β–Gal	Pax-8
β-Gal	1.00 ± 0.07	1.43 ± 0.39
JunB:Fra2	0.94 ± 0.15	1.03 ± 0.13
cJun:Fra2	0.96 ± 0.19	1.07 ± 0.15
cJun:cJun	1.04 ± 0.12	1.07 ± 0.14
JunD:Fra2	1.18 ± 0.32	1.21 ± 0.18
cJun:JunD	1.02 ± 0.10	1.92 ± 0.35
JunB:ATF2	1.08 ± 0.11	1.30 ± 0.19
JunD:ATF2	0.97 ± 0.09	1.43 ± 0.15
JunD:JunD	0.97 ± 0.18	1.50 ± 0.35
cJun:ATF2	0.90 ± 0.07	1.42 ± 0.39
CREB:CREB	1.20 ± 0.13	2.13 ± 0.61
pGL3	0.04 ± 0.01	0.19 ± 0.10

Fig. 3. NUE Transcriptional Activity Is Regulated by Synergy between Pax-8 and b-Zip Dimers

A, Tethered b-Zip dimers with specific pairing were designed to interact with NUC, which is situated between the two Pax-8 sites within NUE. B, HeLa cells were cotransfected with pNISTKLUC3 reporter (3 μg), internal control pCMV-CAT (0.5 μg), and plasmid expressing tethered b-Zip dimer (2 μg). To evaluate the activities of tethered b-Zip in the presence or absence of exogenous Pax-8, either Pax-8 or β-Gal-expressing plasmids (2 μg) were added as cotransfectant. The values in the column of β-Gal represent the effect of tethered b-Zip alone, whereas the values in the column of Pax-8 represent the combinatorial effect of Pax-8 and tethered b-Zip. The total amount of transfected DNA was equalized by pCMV-β-Gal. Transfected cells were harvested in 48 h for luciferase and CAT assays, and the normalized activity was presented as fold induction in reference to the control value (cotransfection with only β-Gal). SI was arbitrarily defined to evaluate the synergism between Pax-8 and the tethered b-Zip (SI = the combinatorial effect in fold induction divided by the sum of the individual effects). C, Control experiments with mutated pNISTKLUC plasmid modified at the NUC motif.

control lane (No Ab). On the other hand, CREB-1 antibody specifically inhibited the formation of the L1 band, identifying CREB-1 as the major component of the L1 complex. Similarly, c-Fos antibody specifically but partially inhibited the formation of the W band, suggesting that the W band might be composed of

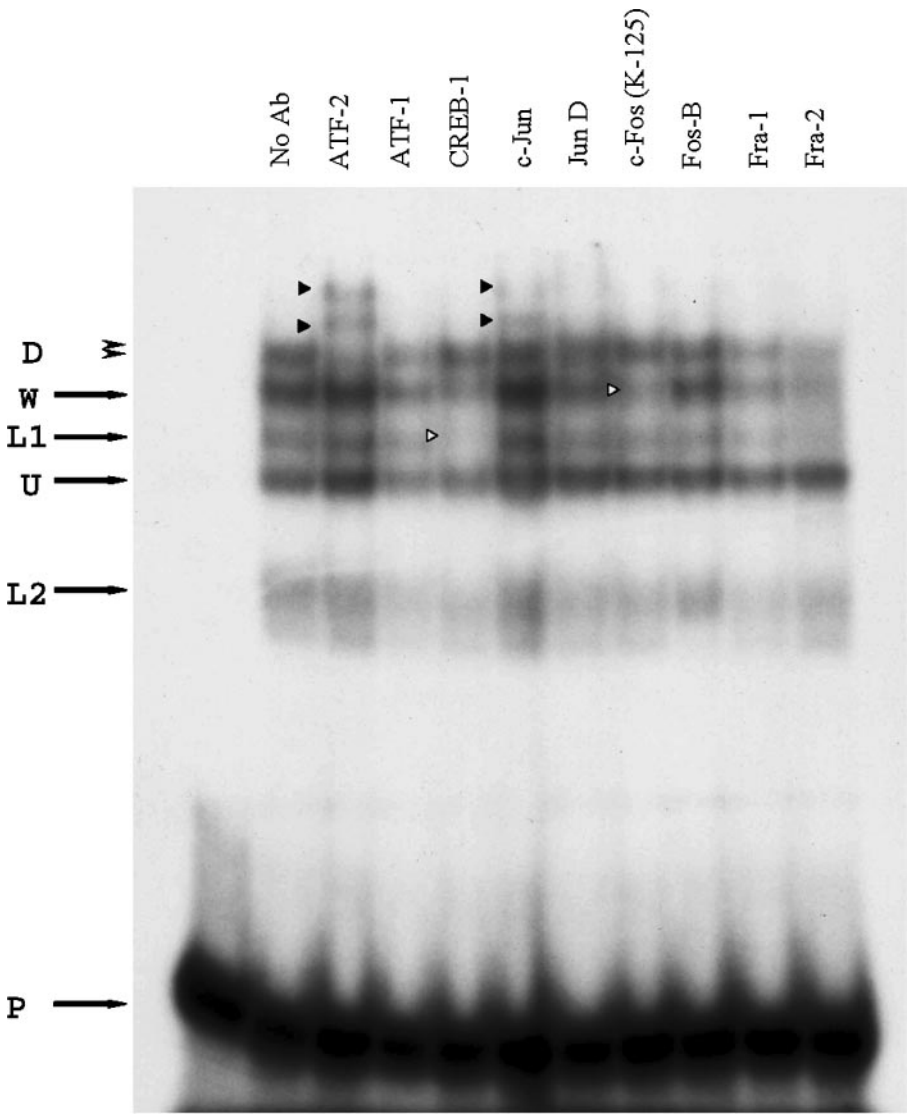


Fig. 4. Direct Identification of Transcription Factors Binding to NUC by Gel Mobility Supershift Analysis

Four micrograms of specific antibodies were added to the reaction mixture as described in *Materials and Methods*. After 20 min incubation at room temperature, the reaction mixture was resolved in 5% polyacrylamide gels and processed for autoradiography. P, Unbound probe; D, retardation complex with doublet bands; W, a wide-spread retardation complex; L1 and L2, retardation complexes with lower masses; U, ubiquitous band. Supershifted or inhibited protein-DNA complexes were marked with *black* and *white arrowheads*, respectively.

other factors as well. Finally, ATF-1, FosB, Fra1, Fra2, and JunD antibodies did not produce evident supershift patterns in the given experimental conditions.

cAMP Induces Binding Activities to the NUC Site

TSH/cAMP increases NIS expression and transcriptional activities of NUE. Because activation of CREB and AP-1 transcription factors by phosphorylation does not increase their DNA binding affinities in most cases (22), modulation of transcriptional activities of these factors by cAMP signaling pathways may not be visualized by changes in the binding patterns at NUC, unless such proteins are newly synthesized by cAMP signaling. To test whether cAMP signaling causes changes of binding patterns at NUC, we performed gel shift assays using FRTL-5 cells that were maintained in the basal media (0.2% serum) for 72 h and subsequently in the four-hormone media (4H) in the presence or absence of 10 μM forskolin for additional 72 h. As shown in Fig. 5A, starved cells do not form the doublet complexes (D) and the L1 band in the absence of forskolin. However, cells treated with forskolin for 72 h regained binding activities that form the L1 and, to a lesser extent, D complexes (Fig. 5A). The loss of NUC binding activities forming D and L1 bands after cell starvation is not caused by general down-regulation of transcription factors, as judged by the incre-

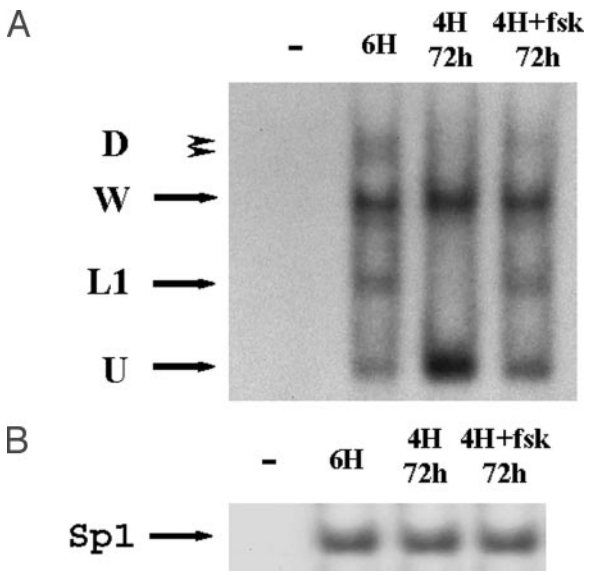


Fig. 5. cAMP Increases NUC Binding Activities in Complex D and L1

FRTL-5 cells were maintained in the basal medium (0.2% serum) for 3 d, and transferred to 4H (without insulin and TSH) for additional 3 d (72 h) in the presence or absence of 10 μM forskolin. Cell extracts were collected and analyzed by gel shift as described in *Materials and Methods*. A, Gel shift patterns with the double strand NUC probe. B, Gel shift pattern obtained with the Sp1 probe (5′-ATTCGATC-GGGGCGGGGCGAG-3′: core motif *underlined*) using the same cell extracts. Definition of culture media: 6H, complete media with six hormones, as described in *Materials and Methods*; 4H, same as 6H media without insulin and TSH.

ment of the U band and the unchanged binding activities of Sp1 in the absence of forskolin (Fig. 5B).

The Artificial Promoter with Five-Time Tandem Repeat of NUC Displays Enhancer Activities and Responds to cAMP with Broader Cell Specificity in Comparison to the Equivalent 5xCRE Promoter

To characterize further the intrinsic nature of NUC, we constructed an artificial promoter with a five-time tandem repeat of the NUC sequence (Fig. 6A). This plasmid (p5xNUC-CAT) displayed enhancer activities in both thyroid and nonthyroid cells because the reporter gene activity (CAT; chloramphenicol acetyl transferase) was much higher than that of the vacant cloning vector (pE1b-CAT) in all cell types tested (Fig. 6B). In all cases, p5xNUC-CAT manifested slightly higher enhancer activities in comparison to the similarly constructed plasmid using the canonical CRE sequence (p5xCRE-CAT). Because the transcriptional activities were completely suppressed by the mutagenesis of the core motif sequences, the transcriptional activities of the NUC and CRE tandem repeat were attributed to the core motif itself and not to the flanking sequences (data not shown).

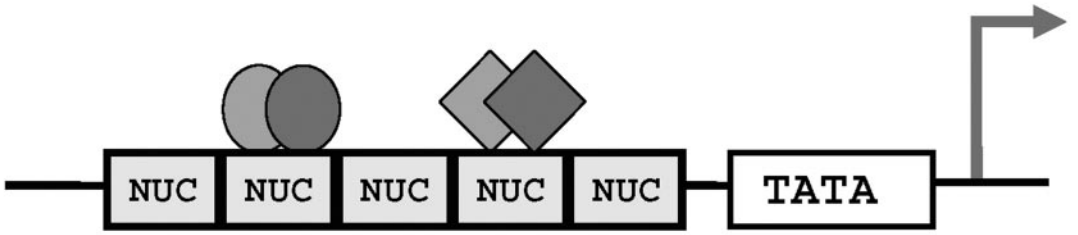
NUE activity is stimulated by cAMP signaling in FRTL-5 cells during the refractory phase in which the cells are depleted of PKA catalytic subunits by chronic exposure to TSH/cAMP. In the same condition, however, the somatostatin promoter containing a canonical CRE did not respond to cAMP in FRTL-5 cells (12, 15). To test whether NUC can recapitulate such PKA-independent transcriptional activities manifested by NUE, we carried out the reporter gene assays using p5xNUC-CAT in the FRTL-5 cells at the refractory phase (without starvation of the cells). However, forskolin was able to stimulate the transcriptional activities not only for 5xNUC but also for 5xCRE in FRTL-5 cells during the refractory phase. Hence, the ability of NUE to respond to cAMP in a PKA-independent pathway was not due to the intrinsic nature of NUC but should be attributed to the unique combinatorial synergism of the entire transcriptional unit involving NUE.

The major difference between 5xNUC and 5xCRE enhancer activities was rather observed in their cell type specificity. Although the transcriptional activity of 5xNUC was stimulated by forskolin and PKA in all cell types tested, 5xCRE displayed only marginal induction in HEK-293 and P19 cells by forskolin or PKA (Fig. 6B). Hence, NUC might have certain advantages over the canonical CRE in a sense that NUC can be activated by cAMP signaling pathways in much broader cellular milieu.

cAMP-Dependent Stimulation of NUE Activity Is Repressed by a Dominant-Negative Mutant of Rap1A

As mentioned above, cAMP induces NUE activities in both PKA-dependent and -independent pathways (12). Recent studies suggested that cAMP not only activates PKA but also directly binds to a guanine nucleotide exchange factor Epac, which in turn specifically activates a Ras-related molecule Rap1 (23, 24). Because Rap1 is readily activated by TSH in thyroid cells (25) and can function in a PKA-independent way in several other systems (23, 26), we reasoned that Rap1 may contribute to the PKA-independent stimulation of NUE activities in FRTL-5 cells. Rap1 can activate a transcription factor elk-1 through B-Raf and MAPK pathway, which subsequently induce expression of certain genes responsible for neuronal phenotype in PC-12 cells (27). If similar events take place in thyroid cells, it is expected that overexpression of the activated form of Rap1 can induce the NIS gene expression in the absence of cAMP. However, expression of either active form (V12) or wild type of Rap1 was not able to stimulate the NUE enhancer activities in the absence of forskolin (Fig. 7A). Similarly, expression of Epac did not stimulate NUE activities in FRTL-5 cells in the absence of forskolin either by itself or in combination with Rap1A (data not shown). However, a dominant-negative mutant (N17) of human Rap1A inhibited the cAMP-mediated stimulation of NUE activity by 45% (Fig. 7A). These results suggest that Rap1A may be necessary but not sufficient for cAMP-mediated induction of NUE activities. Curiously, in the same

A



(E1b promoter + CAT)

B

Cells	Plasmid	Normalized Activity (x E1b-CAT)	Forskolin (10 μM)	**Induction (fold)**	cPKA	**Induction (fold)**
Rat-2	5xNUC	17.3 ± 2.5	137 ± 34	**7.9**	67.5 ± 11	**3.9**
	5xCRE	12.7 ± 1.7	183 ± 46	**14.4**	76.2 ± 11	**6.0**
HEK-293	5xNUC	71.2 ± 16	214 ± 36	**3.0**	256 ± 4.3	**3.6**
	5xCRE	57.2 ± 18	85.8 ± 11	**1.5**	80.1 ± 4.6	**1.4**
HeLa	5xNUC	40.8 ± 7.7	110 ± 2.0	**2.7**	192 ± 61	**4.7**
	5xCRE	6.90 ± 3.6	14.5 ± 0.48	**2.1**	61.4 ± 30	**8.9**
FRTL-5	5xNUC	60.2 ± 8.9	584 ± 72	**9.7**	247 ± 72	**4.1**
	5xCRE	31.5 ± 4.9	1370 ± 270	**43.5**	142 ± 6.3	**4.5**
P19	5xNUC	5.80 ± 1.9	22.0 ± 4.6	**3.8**	42.9 ± 16.2	**7.4**
	5xCRE	1.6 ± 0.27	2.56 ± 0.64	**1.6**	9.17 ± 4.9	**5.7**

Fig. 6. The Artificial Promoter Containing a Tandem Repeat of NUC Sequences Responds to Forskolin and PKA in All Cell Types Tested

A, Plasmid vector containing E1b promoter and 5-time tandem repeat of NUC or the canonical CRE sequence motif was prepared as described in *Materials and Methods*. B, Cells were transiently transfected with p5xNUC-CAT or p5xCRE-CAT plasmids (4 μg) in the presence or absence of forskolin (10 μM) or PKA catalytic subunit-expressing plasmid (2 μg). After 48 h, reporter gene activities (CAT) were normalized with the internal control vector, and presented in reference to the background values with the vacant vector pE1b-CAT (mean ± SD). Fold induction values in *bold* represent the effect of forskolin or PKA in comparison to controls (cells without PKA or forskolin treatment). For FRTL-5 cells, transfected cells were not starved in 0.2% serum to see the effect of cAMP signaling during the refractory phase.

experiment with 5xNUC, the N17 dominant-negative inhibitor of Rap1A did not repress the cAMP-dependent stimulation of transcriptional activity (Fig. 7B), suggesting that the downstream effects of Rap1A after cAMP signaling may be conducted to NUE, but not directly through the factors binding to NUC.

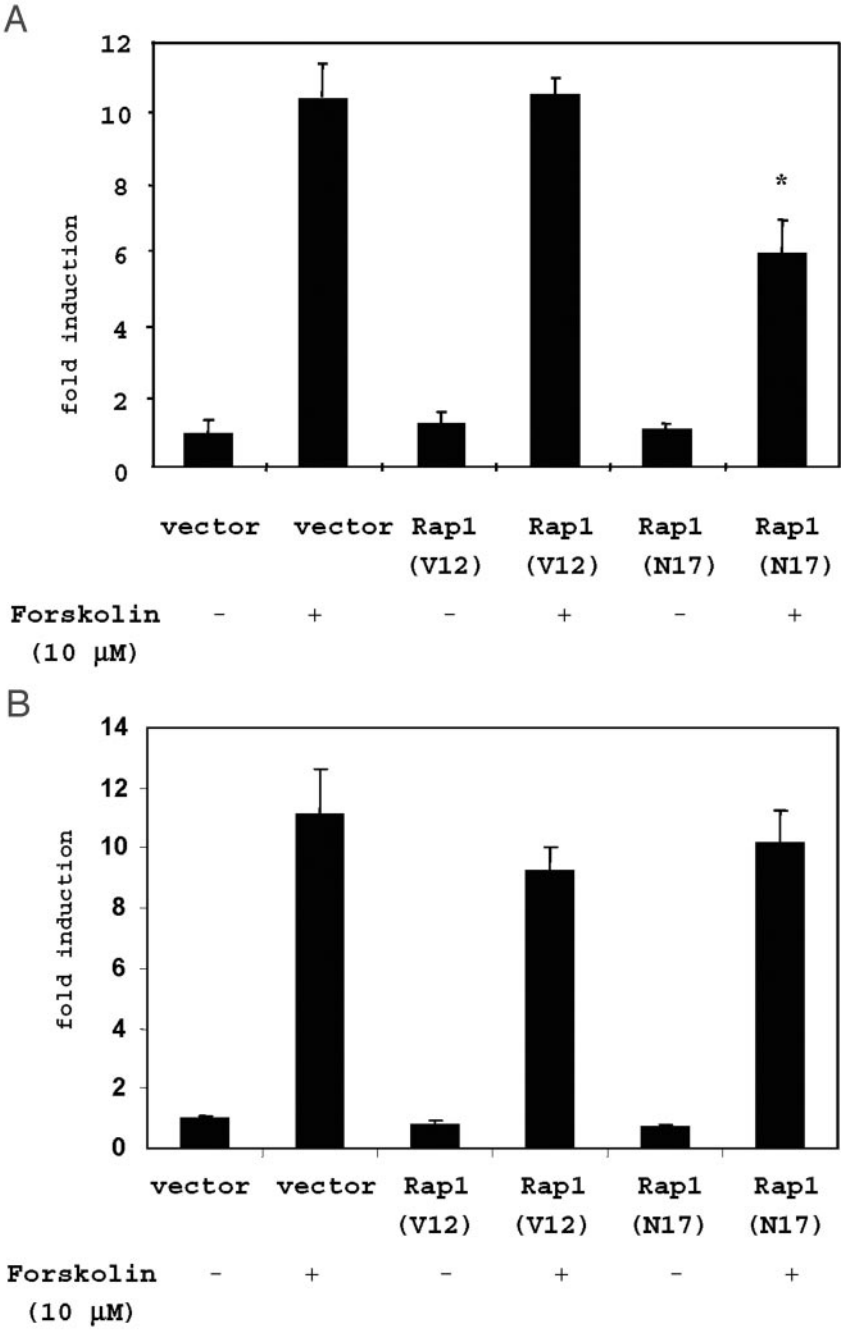


Fig. 7. Dominant-Negative Mutant of Rap1A (N17) Inhibits cAMP-Dependent Stimulation of NUE Transcriptional Activity

A, CMV promoter-based plasmids containing wild-type (WT), activated (V12), or dominant-negative (N17) forms of human Rap1A (2 μg) were cotransfected with pNISTKLUC3 reporter gene (3 μg) in FRTL-5 cells. Normalized values were presented in reference to the control values (reporter gene activities without forskolin or Rap1 plasmid). Transfected cells were maintained in starvation media (0.2% calf serum) for 72 h and subsequently recovered in 4H containing 5% calf serum for additional 72 h in the absence (−) or presence (+) of forskolin (10 μM). *Error bars*, SD from four independent experiments (*, $P < 0.01$ compared with the values with β-Gal + forskolin). B, Cotransfection assay was performed with the artificial promoter containing 5xNUC (4 μg) in the same experimental conditions. *Error bars*: SD from four independent experiments.

DISCUSSION

NIS is a membrane protein that is responsible for active transport of iodide in thyroid, gastric mucosa, and lactating mammary glands (28, 29). Iodide uptake in thyroid follicular cells is an essential step for biosynthesis of thyroid hormones, and this process is up-regulated by TSH through either posttranslational

modification of NIS (30, 31) or NIS mRNA induction in a cycloheximide-sensitive manner (32). Despite the molecular cloning of NIS promoter and its 5′-flanking regions, however, detailed mechanism by which NIS gene expression is regulated by TSH and other cell-specific cues is largely unknown (12, 33–36). The proximal 5′-flanking region of the rat NIS gene contains binding sites for TTF-1 and other transcriptional factors (34, 37), but the complete activation by TSH requires the further upstream regions (12, 32, 37).

In thyroid, the regulatory region that confers the strongest cAMP response was mapped to NUE, the 232-bp fragment located between −2264 and −2495 nucleotides (12). This region is clustered with several binding sites for TTF-1 and Pax-8, as well as a CRE-like site NUC. In this study, we have demonstrated that NUC can recruit both Fos/Jun and CREB/ATF subfamilies of b-ZIP transcription factors. Using gel supershift assays, we have identified transcription factors binding to NUC in FRTL-5 cells, *e.g.* c-Jun, c-Fos, ATF-2, and CREB-1 (also called CRE-BP1 or HB16). On the other hand, ATF-1, Fra1, Fra2, FosB, and Jun-D antibodies had no evident effects on the NUC-binding patterns in FRTL-5 cells (Fig. 4). However, it bears emphasis that the negative results do not prove that these factors (ATF-1, Fra1, Fra2, FosB, and JunD) do not bind to NUC. The negative results might simply reflect insufficient efficacy of the antibodies or low prevalence of such transcription factors in the cells at the steady state. In other independent experiments, we have observed Fra2 and FosB binding to NUC (data not shown).

NUC can bind to a variety of b-Zip molecules for three reasons: 1) the NUC core motif bears high similarities to both CRE and AP-1 consensus sequences; 2) although CREB/ATF and Fos/Jun subfamilies of b-Zip molecules preferentially bind to CRE and AP-1 sites, respectively, they also show some extent of cross-over binding specificity (38, 39); 3) certain b-Zip transcription factors can form heterodimers with each other even across the subfamily boundary. However, b-Zip heterodimerization is not promiscuous and appears to follow specific pairing rules (7, 8, 40–42).

NUC is the only site bearing CRE/AP-1 motif in NUE. Because transcriptional activity of NUE is nearly eliminated by mutation of NUC (12), transcription factors binding to NUC obviously play a pivotal role in this enhancer unit. We showed that dominant-negative inhibitors of those b-Zip molecules that bind to NUC can modulate the transcriptional activities of NUE (Fig. 2). From these results, it can be inferred that CREB-1, Jun, Fos, and ATF-2 stimulate NUE, whereas C/EBP and CREB-2 (ATF-4) work as repressors on this enhancer as in several other genes (43–45). However, all these dominant-negative inhibitors for CREB, Jun, Fos, and ATF-2 displayed moderate repression of NUE activities (the strongest effect was observed with A-CREB: 45% repression), rather than total elimination (Fig. 2). Even when both CREB and AP-1 were sequestered altogether, we were not able to produce total inhibition of NUE transcription activities (which is published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org; supplemental data item 1, table). Because the potency of these dominant negatives was proved in other systems (20), such partial repression seems to reflect the fact that NUC interact with multiple b-Zip families as shown in the supershift assay (Fig. 4), and that these multiple factors contribute to transcriptional activities in parallel. Alternatively, we cannot rule out the possibility that a novel class of transcription factor may interact with NUC.

A few recent studies indicated that the CRE/AP-1 like elements can manifest changes of protein binding activity after cAMP signaling. In PC12 cells, cAMP signaling increases Fos binding activities to the CRE/AP-1 site (TGCGTCA) in dopamine β-hydroxylase gene (46). On the other hand, CRE-like sequence within human NUE recruits increased amount of binding factors after cAMP signaling (18). In this study, we observed that L1 and D bands are induced by 72 h treatment of forskolin (Fig. 5). Whether the b-Zip molecules constituting these two complexes (*e.g.* ATF-2, c-Jun, and CREB-1) are responsible for NIS induction would be an interesting subject for future studies.

Besides NUC, cAMP-dependent transcriptional activation of NUE requires participation of at least one of the two Pax-8 binding sites (Fig. 1). Indeed, using tethered b-Zip dimers with forced pairing, we have demonstrated that Pax-8 can synergize with specific AP-1 dimers, but not with CREB (Fig. 3). Because Pax-8 protein is expressed in tissue-specific manner and is induced by cAMP in thyroid cells (47–50), such a synergistic mechanism involving Pax-8 and b-Zip can partially explain how transcriptional activities of NUE can be induced by cAMP in a cell type-specific manner. Although Pax-8 and the b-Zip molecules binding to the adjacent sites in NUE create strong synergy, such cooperation does not seem to involve direct protein-to-protein interactions between Pax-8 and b-Zip molecules. Our pull-down experiment of FRTL-5 cell nuclear extracts with Pax-8-conjugated Sephadex detected TTF-1 as observed previously (51) but failed to detect CREB or other AP-1 molecules (supplemental data item 2, figure).

CRE-like sequences containing asymmetric dyad occur in a number of genes, and they display slightly different binding characteristics toward CREB in comparison with the canonical CRE. For example, whereas phosphorylation of CREB does not alter its binding affinity to canonical CRE, some asymmetric CRE-like sequence binds more efficiently to phosphorylated CREB (52). NUC, an asymmetric CRE-like, binds to CREB with lower affinity in comparison to the canonical CRE, but phosphorylation of CREB does not significantly change the binding affinity to NUC (data not shown). Thus, with respect to the binding characteristics, NUC is rather similar to canonical CRE than to the other asymmetric CRE. However, CRE probe can

produce subtly different supershift patterns in comparison to NUC (supplemental data item 3, figure).

Although PKA is the most characterized downstream effector of cAMP, other molecules transducing cAMP signals without PKA have been discovered recently, *e.g*. cyclic nucleotide-gated channels, Rap1, and Ras (23, 53, 54). Recently, it was demonstrated that stably transfected Wistar rat thyroid cells overexpressing N17-dominant-negative inhibitor of Rap1A represses NIS expression in the presence of TSH/cAMP signaling (55). However, the active form of Rap1A did not induce NIS expression in the absence of TSH/cAMP signaling. Hence, our results with NUE and Rap1A (Fig. 7) exactly parallel their observation with the endogenous NIS protein in those stable cell lines. On the other hand, N17 dominant-negative inhibitor of Rap1A represses NUE but not 5xNUC transcriptional activities in the presence of cAMP signaling (Fig. 7). Hence, the *cis*-acting element that conducts the downstream effect of Rap1A is likely to be outside NUC, *e.g*. Pax-8, TTF-1, or other binding sites.

In summary, we have identified the functional transcription factors that bind to NUC and modulate transcriptional activities of NUE. Our results in supershift assay and transient transfection experiments indicated that the NUC site can conduct diverse class of cell signals. This apparent redundancy makes it possible for cAMP and other cell signals to reach NUE through multiple routes during development and in certain physiological conditions. For instance, in contrast to 5xCRE, the artificial promoter containing 5xNUC can respond to cAMP not only in differentiated cell lines, but also in undifferentiated embryonal cell lines such as P19 cells. More importantly, we have also demonstrated that Pax-8 can synergize with b-Zip molecules at NUC site and stimulate NUE transcriptional activities. Hence, future studies on the regulatory mechanism of this synergism are likely to provide more insights into the transcriptional regulation of the NIS gene.

MATERIALS AND METHODS

Plasmid

The reporter gene constructs containing the TK promoter and NUE (pNISTKLUC3) were prepared in the previous study (12). To make a plasmid with isolated NIS CRE/AP-1 sites and a reporter gene (p5xNUC-CAT), oligonucleotides containing five time tandem repeat of the NUC sequence element (5′-TGGGGCTGACGCAGGGC-3′; core motif *underlined*) were chemically synthesized and annealed to the antisense oligonucleotides, and subsequently ligated to pE1b-CAT. The control plasmid with five time repeat of the canonical CRE sequence (5′-GATTGCCTGACGTCAGAG-3′: core sequence *underlined*) was constructed in the same way. The expression vectors for Rap1 and Epac were made by PCR amplification of human Krev-1/Rap1A (Dr. H. Kitayama, Kyoto University, Kyoto, Japan) and Epac cDNA (Dr. J. L. Bos, Utrecht University, Utrecht, The Netherlands) and subsequent ligation to a cytomegalovirus (CMV)-based expression vector. Mutagenesis of target sequences on the plasmid was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All expression vectors were verified by DNA sequencing and the Western blot analysis of the lysates from the transfected cells (HeLa) to ensure proper expression. The CMV-based expression vectors for dominant-negative b-Zip molecules were generously provided by Dr. Charles Vinson at the National Institutes of Health (Bethesda, MD) (20). Tethered AP-1 plasmids were kindly provided by Dr. Moshe Yaniv at the Pasteur Institute (Paris, France). Tethered CREB:CREB plasmid was constructed using the same strategy as specified in Bakiri *et al.* (21).

Cells and Transient Transfection Assay

The FRTL-5 cell line was maintained in F12 media containing 5% calf serum and six hormones (3.6 ng/ml cortisol, 5μg/ml transferrin, 10 ng/ml glycyl-L-histydil-L-lysine acetate, 10 ng/ml somatostatin, 10 μg/ml insulin, and 1 mU/ml bovine TSH) and plated on 60-mm plastic dishes 2 d before transfection. Transfection experiments were performed with calcium phosphate method after the previously described protocols (12). The transcriptional activities of the test plasmid were monitored with reporter gene activities 3d after transfection, and the data were normalized with activities of internal control plasmid (pCMV-CAT, pCMV-Luc, or pTK-RenillaLuc). For assays of NUE transcriptional activity, 3 μg of pNISTKLUC3 and 1 μg of pCMV-CAT were used for each plate (60 mm). For assays of p5xNUC-CAT and p5xCRE-CAT activities, the amount of the test plasmid was set to 4 μg. In cotransfection assay, 2 μg of effector plasmid (b-Zip or Rap1 plasmid) was used per plate unless specified otherwise, and the total amount of transfectant DNA was adjusted to equal for each plate using pCMV-GAL plasmid. To study cAMP effect, cells were maintained in starvation media (0.2% calf serum) for 3 d after transfection, and for additional 3 d in 4H in the presence or absence of forskolin (10 μM) before analysis. Data were pooled from at least three independent experiments with triplicate samples, and statistical analysis was performed with ANOVA. Transfection assays for HeLa cells and HEK 293 cells followed a similar procedure, except that the cells were plated 1d before transfection.

Gel Mobility Shift

Oligonucleotides were elected from the NUC site (5′-AGTGTGGGGCTGACGCAGGGCTGCAGGG-3′: core motif *underlined*), and the nuclear extracts were prepared from FRTL-5 cells after the previously described method (56). The chemically synthesized oligonucleotides were labeled with ^{32}P using polynucleotide kinase and annealed to the antisense complementary sequences. The end-labeled double-strand oligonucleotide probes (80,000 cpm) were mixed with FRTL-5 cell extract (7 μg) in 20 μl of reaction buffer [reaction buffer: 10 mM Tris (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 50 μg/ml poly(deoxyinosine-deoxycytosine), 5 μg/ml BSA, 13% glycerol] and incubated at room temperature for 20 min before loading on the 5% polyacrylamide gel in 0.5× TBE buffer at 200 V. For supershift assay, 4 μg of specific antibodies (Santa Cruz Biotechnology) were added to the reaction mixture and further incubated at room temperature for additional 20 min. After electrophoresis in a cold room, gels were dried and processed for autoradiography on Kodak (Rochester, NY) OMAX films for 24 h.

Acknowledgments

We are grateful to Dr. C. Vinson for his generous gift of the plasmids overexpressing dominant-negative inhibitors of b-Zip, and to Dr. M. Yaniv for kindly sharing his plasmids overexpressing tethered AP-1 dimers. We would also like to express our gratitude to Dr. P. Verde (IGB, Naples, Italy) for

useful advice and discussions and to Dr. E. Biffali and his staff at the Stazione Zoologica for the technical support.

Received January 16, 2004. Accepted July 30, 2004.

Address all correspondence and requests for reprints to: Prof. Roberto Di Lauro, Department of Biochemistry and Molecular Biology, Stazione Zoologica 'Anton Dohrn,' Villa Comunale, 80121 Napoli, Italy. E-mail: rdilauro@unina.it.

This work was supported in part by Telethon Grant GP0208Y01, Associazione Italiana per la Ricerca sul Cancro grants (to R.D.L. and M.Z), and Ministero dell'Universita' e della Ricerca Scientifica e Tecnologica Grant "I geni cell'uomo'' cluster 01.

REFERENCES

1. Avvedimento VE, Tramontano D, Ursini MV, Monticelli A, Di Lauro R 1984 The level of thyroglobulin mRNA is regulated by TSH both in vitro and in vivo. Biochem Biophys Res Commun 122:472–477
2. Magnusson RP, Rapoport B 1985 Modulation of differentiated function in cultured thyroid cells: thyrotropin control of thyroid peroxidase activity. Endocrinology 116: 1493–1500
3. Dumont JE, Lamy F, Roger P, Maenhaut C 1992 Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other factors. Physiol Rev 72:667–697
4. De La Vieja A, Dohan O, Levy O, Carrasco N 2000 Molecular analysis of the sodium/iodide symporter: impact on thyroid and extrathyroid pathophysiology. Physiol Rev 80:1083–1105
5. Yamamoto KK, Gonzalez GA, Biggs 3rd WH, Montminy MR 1988 Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature 334: 494–498
6. Gonzalez GA, Montminy MR 1989 Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680
7. Lalli E, Sassone-Corsi P 1994 Signal transduction and gene regulation: the nuclear response to cAMP. J Biol Chem 269:17359–17362
8. Shaywitz AJ, Greenberg ME 1999 CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 68:821–861
9. Ikuyama S, Shimura H, Hoeffler JP, Kohn LD 1992 Role of the cyclic adenosine 3′,5′-monophosphate response element in efficient expression of the rat thyrotropin receptor promoter. Mol Endocrinol 6:1701–1715
10. Kambe F, Seo H 1996 mediation of the hormone- and serum-dependent regulation of thyroglobulin gene expression by thyroid transcription factors in rat thyroid FRTL-5 cells. J Endocrinol 150:287–298
11. Poleev A, Oklandova O, Musti AM, Schneider D, Royer-Pokora B, Plachov D 1997 Determination of functional domains of the human transcription factor pax8 responsible for its nuclear localization and transactivating potential. Eur J Biochem 247:860–869
12. Ohno M, Zannini M, Levy O, Carrasco N, Di Lauro, R 1999 The paired-domain transcription factor Pax8 binds to the upstream enhancer of the rat sodium/iodide symporter gene and participates in both thyroid-specific and cyclic-AMP-dependent transcription. Mol Cell Biol 19: 2051–2060
13. Montminy MR, Gonzalez GA, Yamamoto KK 1990 Regulation of cAMP-inducible genes by CREB. Trends Neurosci 13:184–188
14. Montminy M 1997 Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807–822
15. Armstrong R, Wen W, Meinkoth J, Taylor S, Montminy M 1995 A refractory phase in cyclic AMP-responsive transcription requires down regulation of protein kinase A. Mol Cell Biol 15:1826–1832
16. Lalli E, Sassone-Corsi P 1995 Thyroid-stimulating hormone (TSH)-directed induction of the CREM gene in the thyroid gland participates in the long-term desensitization of the TSH receptor. Proc Natl Acad Sci USA 92: 9633–9637
17. Schmitt TL, Espinoza CR, Loos U 2002 Characterization of a thyroid-specific and cyclic adenosine monophosphate-responsive enhancer far upstream from the human sodium iodide symporter gene. Thyroid 12:273–279
18. Taki K, Kogai T, Kanamoto Y, Hershman JM, Brent GA 2002 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. Mol Endocrinol 16:2266–2282
19. Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M 2002 Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol 22: 6321–6335
20. Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C 1998 A dominant negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol 18:967–977
21. Bakiri L, Matsuo K, Wisniewska M, Wagner EF, Yaniv M 2002 Promoter specificity and biological activity of tethered AP-1 dimers. Mol Cell Biol 22:4952–4964
22. Karin M 1995 The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270:16483–16486
23. De Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL 1998 Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396:474–477
24. Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM 1998 A family of cAMP-binding proteins that directly activate Rap1. Science 282:2275–2279
25. Dremier S, Vandeput F, Zwartkruis FJ, Bos JL, Dumont JE, Maenhaut C 2000 Activation of the small G protein Rap1 in dog thyroid cells by both cAMP-dependent and -independent pathways. Biochem Biophys Res Commun 267:7–11
26. Boussiotis VA, Freeman GJ, Berezovskaya A, Barber DL, Nadler LM 1997 Maintenance of human T cell anergy: blocking of IL-2 gene transcription by activated Rap1. Science 278:124–128
27. Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJS 1997 cAMP activates MAP kinase and Elk-1 through a B-Raf and Rap-1 dependent pathway. Cell 89:73–82
28. Dai G, Levy O, Carrasco N 1996 Cloning and characterization of the thyroid iodide transporter. Nature 379: 458–460
29. Tazebay UH, Wapnir IL, Levy O, Dohan O, Zuckier LS, Zhao QH, Deng HF, Amenta PS, Fineberg S, Pestell RG, Carrasco N 2000 The mammary gland iodide transporter is expressed during lactation and in breast cancer. Nat Med 6:871–878
30. Levy O, Dai G, Riedel C, Ginter CS, Paul EM, Lebowitz AN, Carrasco N 1997 Characterization of the thyroid Na+/I− symporter with an anti-COOH terminus antibody. Proc Natl Acad Sci USA 94:5568–5573
31. Paire A, Bernier-Valentin F, Selmi-Ruby S, Rousset B 1997 Characterization of the rat thyroid iodide transporter using anti-peptide antibodies. Relationship between its expression and activity. J Biol Chem 272: 18245–18249
32. Kogai T, Endo T, Saito T, Miyazaki A, Kawaguchi A, Onaya T 1997 Regulation by thyroid-stimulating hormone of sodium/iodide symporter gene expression and

protein levels in FRTL-5 cells. Endocrinology 138: 2227–2232
33. Tong Q, Ryu KY, Jhiang SM 1997 Promoter characterization of the rat Na+/I− symporter gene. Biochem Biophys Res Commun 239:34–41
34. Endo T, Kaneshige M, Nakazato M, Ohmori M, Harii N, Onaya T 1997 Thyroid transcription factor-1 activates the promoter activity of rat thyroid Na+/I− symporter gene. Mol Endocrinol 11:1747–1755
35. Behr M, Schmitt TL, Espinoza CR, Loos U 1998 Cloning of a functional promoter of the human sodium/iodide-symporter gene. Biochem J 331:359–363
36. Venkataraman GM, Yatin M, Ain KB 1998 Cloning of the human sodium-iodide symporter promoter and characterization in a differentiated human thyroid cell line, KAT-50. Thyroid 8:63–69
37. Ohmori M, Endo T, Harii N, Onaya T 1998 A novel thyroid transcription factor is essential for thyrotropin-induced up-regulation of Na+/I− symporter gene expression. Mol Endocrinol 12:727–736
38. Masquilier D, Sassone-Corsi P 1992 Transcriptional cross-talk: nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun. J Biol Chem 267:22460–22466
39. Rutberg SE, Adams TL, Olive M, Alexander N, Vinson C, Yuspa SH 1999 CRE DNA binding proteins bind to the AP-1 target sequence and suppress AP-1 transcriptional activity in mouse keratinocytes. Oncogene 18: 1569–1579
40. Hai TW, Liu F, Coukos WJ, Green MR 1989 Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev [Erratum 4:682 (1990)] 3:2083–2090
41. Hai T, Curran T 1991 Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity. Proc Natl Acad Sci USA 88: 3720–3724
42. Hoeffler JP, Lustbader JW, Chen CY 1991 Identification of multiple nuclear factors that interact with cyclic adenosine 3′,5′-monophosphate response element-binding protein and activating transcription factor-2 by protein-protein interactions. Mol Endocrinol 5:256–266
43. Karpinski BA, Morle GD, Huggenvik J, Uhler MD, Leiden JM 1992 Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element. Proc Natl Acad Sci USA 89:4820–4824
44. Shimizu M, Nomura Y, Suzuki H, Ichikawa E, Takeuchi A, Suzuki M, Nakamura T, Nakajima T, Oda K 1998 Activation of the rat cyclin A promoter by ATF and Jun family members and its suppression by ATF-4. Exp Cell Res 239:93–103
45. Lekstrom-Himes J, Xanthopoulos KG 1998 Biological role of the CCAAT/enhancer-binding protein family of transcription factors. J Biol Chem 273:28545–28548
46. Swanson D, Zellmer E, Lewis EJ 1998 AP1 proteins mediate the cAMP response of the dopamine b-hydroxylase gene. J Biol Chem 273:24065–24074
47. Van Renterghem P, Vassart G, Christophe D 1996 Pax 8 expression in primary cultured dog thyrocyte is increased by cyclic AMP. Biochim Biophys Acta 1307:97–103
48. Fabbro D, Pellizzari L, Mercuri F, Tell G, Damante G 1998 Pax-8 protein levels regulate thyroglobulin gene expression. J Mol Endocrinol 21:347–354
49. Medina DL, Suzuki K, Pietrarelli M, Okajima F, Kohn LD, Santisteban P 2000 Role of insulin and serum on thyrotropin regulation of thyroid transcription factor-1 and pax-8 genes expression in FRTL-5 thyroid cells. Thyroid 10:295–303
50. Mascia A, Nitsch L, Di Lauro R, Zannini M 2002 Hormonal control of the transcription factor Pax8 and its role in the regulation of thyroglobulin gene expression in thyroid cells. J Endocrinol 172:163–176
51. Di Palma T, Nitsch R, Mascia A, Nitsch L, Di Lauro R, Zannini M 2003 The paired domain-containing factor Pax8 and the homeodomain-containing factor TTF-1 directly interact and synergistically activate transcription. J Biol Chem 278:3395–3402
52. Nichols M, Weih F, Schmid W, DeVack C, Kowenz-Leutz E, Luckow B, Boshart M, Schutz G 1992 Phosphorylation of CREB affects its binding to high and low affinity sites: implications for cAMP induced gene transcription. EMBO J 11:3337–3346
53. Tsygankova OM, Kupperman E, Wen W, Meinkoth JL 2000 Cyclic AMP activates Ras. Oncogene 19: 3609–3615
54. Kaupp UB, Seifert R 2002 Cyclic nucleotide-gated ion channels. Physiol Rev 82:769–824
55. Tsygankova OM, Saavedra A, Rebhun JF, Quilliam LA, Meinkoth JL 2001 Coordinated regulation of Rap1 and thyroid differentiation by cyclic AMP and protein kinase A. Mol Cell Biol 21:1921–1929
56. Civitareale D, Lonigro R, Sinclair AJ, Di Lauro R 1989 A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. EMBO J 8:2537–2542

Molecular Endocrinology **is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**