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Promoter Characterization of the Rat Na^+/I^- Symporter Gene

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The Na^+/I^- symporter is the molecule that mediates active iodide uptake in the thyroid gland. A 16.4 kb genomic DNA fragment of the rat Na^+/I^- symporter gene (rNIS) was isolated, restriction mapped and a 2 kb region immediate 5′ to the ATG site was sequenced. The transcription start site for rNIS was mapped to −98 nucleotide (nt) relative to the ATG translation initiation site. A series of 5′ genomic DNA fragments ranging from 233 bp to 8 kb were tested for promoter activity in both thyroid and non-thyroid cells. Our results indicate that the DNA regulatory elements within 8 kb of the 5′ flanking region of rNIS are not sufficient to confer thyroid-selective transcription. However, consistent with the clinical observation that NIS expression is reduced in thyroid tumors, the rNIS promoter activity is suppressed in *ret*/PTC1 transformed NIH3T3 cells, compared to non-transformed NIH3T3 cells.

The active iodide uptake by the thyroid gland, facilitated by the Na^+/I^- symporter (NIS), is essential for the synthesis of the iodine-containing thyroid hormone (1). The iodide transport system of the thyroid ensures that the radioiodide specifically reaches its target tissue. Thus, radioiodide has been used to ablate postsurgical thyroid remnants and to treat recurrent and metastatic thyroid cancer. Clinically, thyroid tumors uptake much lower levels of radioiodide compared to normal thyroid tissues, including those tumors that respond to radioiodide treatment when given at high dose (2). In agreement with this clinical observation, our initial study indicates that the expression level of human NIS is greatly reduced in thyroid tumors, compared to that of normal thyroid tissues (3). Furthermore, reduced radioiodide uptake activity in the thyroid gland of our *ret*/PTC1 transgenic mice is accompanied by down-regulation of the expression of mouse NIS (Cho, et al., manuscript in preparation). Finally, many studies showed that radioiodide uptake activity is readily lost when various oncogenes were expressed in immortalized rat thyroid cells (4-6).

To ensure that radioiodide is an effective treatment for patients with recurrent and/or metastatic thyroid cancers, it is of clinical significance to increase the expression and the activity of NIS to the greatest extent in patients with thyroid cancer prior to radioiodide therapy. The iodide uptake activity in the thyroid gland is primarily stimulated by TSH-mediated cAMP signal pathways, and inhibited by an excess of iodide (7). However, the molecular mechanism underlying these regulations is poorly understood. It has been shown that the TSH-stimulated iodide uptake is, at least in part, resulted from the transcriptional regulation of the NIS gene (8). To study the transcriptional regulation of NIS, the promoter activity of the 5′ flanking DNA fragments of rNIS was characterized.

MATERIALS AND METHODS

Cell lines. FRTL-5 rat thyroid cells (9) were grown in Coon's modified Ham's F12 medium, supplemented with 5% calf serum, and six hormone mixture containing TSH (1 mU/ml), insulin (10 μg/ml), hydrocortisone (10 nM), somatostatin (10 μg/ml), transferrin (5 μg/ml), and L-glycyl-histidyl-lysine (2 ng/ml). The Rat-1 rat embryonic fibroblast cells were maintained in DMEM (Dulbecco's Modified Eagle Media) supplemented with 10% calf serum. The PC12N21 cell line (10), a clone of the PC12 rat pheochromocytoma cells, was maintained in DMEM supplemented with 5% horse serum and 5% fetal bovine serum. All cells are incubated at 37°C in a 5% CO_2 enriched air atmosphere.

Isolation of rat NIS genomic fragment. The rNIS51 primer (5′-TGACTCGCGCTGCG-ACTCTC-3′) which was localized to the 5′ untranslated region of rat NIS cDNA was paired with a downstream primer rNISR1 (5′-CGCAGCTCTAGGTACTGGTA-3′) to perform RT-PCR on the RNA isolated from FRTL-5 cells. The 450 bp cDNA fragment was amplified, radioactively labeled and used to screen a λFixII rat genomic library. Phage DNA of one positive clone was

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isolated and the 16.4 kb insert was characterized by restriction mapping. Subsequently, a 3 kb SstI fragment which contains the immediate 5′ region of rat NIS gene was subcloned into pBluescript II vector (Stratagene, La Jolla, CA 92037). The nucleotide sequence of the 2 kb immediate 5′ flanking region of rNIS was determined by using the Sequenase Kit (USB, Cleveland, OH 44122).

Primer extension assay. The rNIS33 primer (5′-CCATGGAGACAGGTGACTCG-3′) corresponding to −16 to +4 nt relative to the ATG site was used to perform primer extension assay as described (11). The kinase labeled probe was hybridized with 10 μg total RNA isolated from FRTL-5, Rat-1 or PC12 cells at 42 °C overnight. The cDNA was synthesized with Superscript II reverse transcriptase at 42°C for 1 hr and 50°C for an additional hour. After the RNA was digested with RNase, the remaining cDNA fragments were analyzed on a 6% sequencing gel. A sequencing reaction primed with the same oligonucleotide was run in parallel to serve as a sequencing ladder.

Ribonuclease protection assay. To prepare the riboprobe for mapping the rat NIS transcription start site, a genomic fragment ranging from −352 to +4 nt relative to the ATG site was amplified by PCR, and subcloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA 92008). To evaluate the expression levels of rNIS, a cDNA fragment ranging from +1752 to +1931 nt relative to the ATG site was amplified by PCR, and subcloned into the TA cloning vector pCR2.1. To evaluate the expression levels of rat G3PDH, a cDNA fragment was amplified by PCR with primer G3PDHF1 (5′-TGAAGGTCGGTGTGAACGGATTTGG-3′) and primer G3PDHR1 (5′-AGGGAGTTGTCATATTTCTCG-3′), and subcloned into the TA cloning vector pCR2.1. The plasmid DNA constructs with inserts in desired orientation were selected. After linearizing the plasmid DNA with HindIII, the radioactive labeled antisense riboprobes were synthesized with [α-^{32}P]UTP and T7 RNA polymerase (GIBCO BRL, Gaithersburg, MD). The probes were hybridized with 10 μg total cytoplasmic RNA at 42 °C overnight, followed by digestion with the mixture of RNase T1 and RNase A. The protected riboprobe fragments were then analyzed on a 6% sequencing gel. One hundred base-pair ladder (GIBCO BRL, Gaithersburg, MD) was end labeled and run in parallel as a size marker.

5′ RACE (rapid amplification of cDNA ends). This experiment was carried out with the 5′ RACE kit from Gibco BRL. Briefly, cDNA was synthesized with rNISR1 primer and SuperscriptII reverse transcriptase, using RNA isolated from FRTL-5 cells as template. After RNase H treatment, cDNA was purified with the Glass Max DNA isolation spin cartridge (GIBCO BRL, Gaithersburg, MD). The purified cDNA was 5′ tailed with polyC and then further amplified with an anchor primer provided by the kit and an rNIS-specific nested primer rNIS31 (5′-AGTCGGGTC-CCGGCACTGCGTTGG-3′). After the identity of the amplified PCR products was confirmed by Southern blot analysis, a 250 bp fragment was re-amplified with another nested primer rNIS33 (5′-CCATG-GAGACAGGTGACTCG-3′) and the UAP primer provided by the kit. The resulting PCR product was subcloned into TA cloning vector pCR2.1 to determine its nucleotide sequence.

Plasmids. The ITPSS3/pGL2B plasmid was constructed by inserting the 3 kb SstI genomic DNA fragment of rNIS into the SstI site of the pGL2Basic vector (Promega, Madison, WI 53711). The ITPSN2/pGL2B construct was subsequently generated by removing the NcoI-SstI 1 kb fragment from ITPSS3/pGL2B, and followed by self-ligating the DNA construct after Mung-Bean nuclease treatment. The ITPSN8/pGL2B plasmid was constructed by inserting the further upstream 6 kb SstI DNA fragment into the SstI site of ITPSN2/pGL2B. To generate ITP370/pGL2B and ITP230/pGL2B, a 372 bp or a 234 bp rNIS genomic DNA fragment was amplified with primer rNIS54 (5′-CAGTTCCTTCTCCCAAGCTGCGGAGAAAGG-3′) or primer rNIS55 (5′-GAAT-TCCTTAGACTGTGAGCGG-3′) paired with primer rNIS342 (5′-ATTTATTGAACTCCGGGG-TCAGCGC-3′), respectively. The resulting PCR products were initially cloned into the TA cloning vector pCR2.1 and then subcloned into the SstI and XhoI sites of the pGL2Basic vector. The Tg/pGL2B was constructed by excising the 2 kb BamHI fragment of bovine Tg promoter from the pSK-Tg plasmid, and inserting it into the BglII site of the pGL2Basic vector.

Transfection and luciferase assay. After being cultured in media containing 5 hormone mixture (without TSH) for 5 to 7 days, FRTL-5 cells were plated on 35 mm plates (1.5×10^5 cells/plate) for two days before transfection. One day before transfection, media were replaced with fresh media containing 6 hormone mixture. For Rat-1, PC12 or NIH3T3 stable transfectant cells, 1.0×10^5 cells were plated on 35 mm plates one day prior to transfection. Media were changed 3 hr before transfection. Calcium phosphate transfection was performed using 3 μg of various luciferase reporter DNA and 0.3 μg of β-gal DNA construct for each transfection. The DNA precipitates were replaced with fresh media 10 hr later and the cells were cultured for additional 48 hr. The plates were then washed with PBS, and cells were lysed with 85 μl lysis buffer provided in the luciferase assay kit from Promega (Madison, WI 53711). For luciferase assay, 20 μl of cell lysate was used. For β-gal assay, 7 μl of lysate was tested with the Galacto-Light chemiluminescent β-galactosidase reporter assay system (Tropix, Bedford, MA 01730). The chemiluminescence was measured with the Lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany).

RESULTS AND DISCUSSION

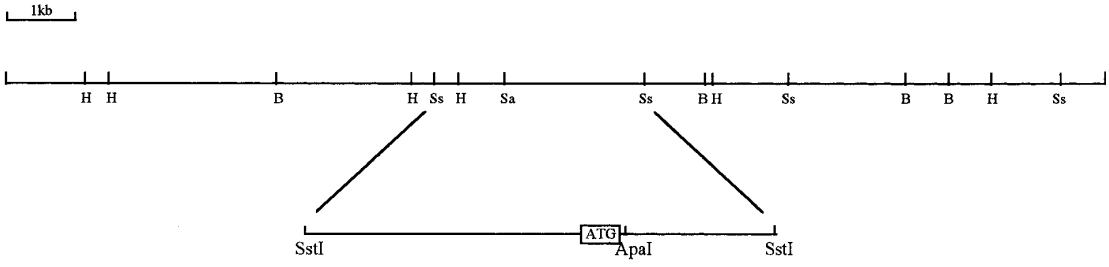
The Nucleotide Sequence of the 2 kb Immediate 5′ Flanking Region of the Rat NIS Gene Was Determined

A 16.4 kb rat genomic DNA fragment containing the 5′ flanking region of the rat NIS gene was isolated from a rat genomic DNA library using a rat NIS cDNA fragment as a probe. The restriction map of this genomic DNA fragment was determined (Fig. 1a). The 5′ non-translated region of the rNIS gene was localized within a 2 kb SstI-ApaI DNA fragment. This 2 kb DNA fragment was subjected to nucleotide sequence analysis (Fig. 1b). Comparing with the published rat cDNA nucleotide sequence (12), the genomic DNA nucleotide sequence showed a discrepancy to the first 25 bp of the reported rat NIS cDNA nucleotide sequence (Fig. 1c). We performed 5′ RACE using FRTL-5 mRNA as template to acquire the extended 5′ end cDNA. The nucleotide sequence of the extended cDNA fragment was consistent with the genomic DNA nucleotide sequence, suggesting that the first 25 bp of the reported cDNA sequence was probably resulted from a cloning artifact. Although it is unlikely, we cannot exclude the possibility of the presence of an intron, and the reported cDNA sequence was generated by differential spicing.

Based on the sequence analysis, the structure of rNIS promoter appears to be different from that of the thyroglobulin (Tg), thyroperoxidase (TPO) and thyrotropin receptor (TSHr) genes. The promoter organization of the rat Tg and TPO genes show a remarkable similarity, with three TTF-1 binding sites, one TTF-2 binding site, and one Pax-8 binding site located at similar positions within the first 170 bp of the 5′ flanking

a

rat NIS genomic fragment (16.4kb)



b

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-1949  GAGCTCTTTATCCTGCTGGCTCTAAGCCTCTGCTAGGTGGGAATGAGGCCAGCCAAACTCCGGGGACCT

-1880 TGAGCCACCAAGCTTTAGGTGAGGACTACACTGTGGATCATCATGGAAACACGGGGTTGCTTTTGTTGGG
                TTF-1      T3R-β
-1810 TGTGGCCAGCACCATCACGGTGCCTGGTTTTGAAGGAGTATAGCCTGAAGCCTCCGTGGCTGTCTTGTCA

-1740 CTGCTCTCAAGTCTCTACTGGCCACAGAGAGGAACACTGCACTTGCTTCTAGCCCCTTGCAGATATTTCC
          TTF-1              GR
-1670 ATACGTGTATGCACATAGCCCAGGGTAATTTTGAGTTTCAACCATCCAGTGCCAGCTAGAGTAAAAGCCT

-1600 GTGTCTATACTATGAAGGCCATAAGAGGGCACCGTGTCGAAGAGAAGTCTTCCCACTGAGTCCATTGGCC

-1530 CCACATCAAAGGAATGGGTGATCCCTCTGACTATGCAGTGTTTCCCTGTGCATTGTGGGGCCAGTGGCTG

-1460 CTACTTTAAAGTCATGAGGAATCTGGAAGGTTGTTCACTTCTGAGGACCTGGGTTTGTCCCTGTATTAAG
                                                        GR
-1390 ATACTGCTCCAGGGCATCGGATGCCACTGGCCTCTGCAGGCACCCGTATTCATGCAACTATCCTTCACCC

-1320 ACATACATAATTGTGGTTTTTTTGGGAGCTTGGGGAAATGATGTGTGTGACTAAGCCACTAGGAGTCAAA
                          AP-2                  AP-1
-1250 AGTGTAGGCCATTCTGGTCTACAAGGTGAGTTCCAGAACAGCCAGGGCTACACGGAGAGGCCCTATGACA

-1180 TAAAGTTGCAGTGGCATGCAACACCAGCTCCAGCCCTCCCTGGTGGCTGAGCACCGGCATGACCGCGAGT

-1110 GTCCCAGCATGGCAATCCAGCCTACCTGCCTCTGGAAGGCTCTTCCCCTCCCTCCTGTTTGTCGACCTCT
       GR
-1040 TAGCCTGCCACCTAGGCTTACGAGCTGCCCTAGTCAGGTCTCCTGACAGCACTTCCAGGCGGGCGGCGAG
                                                              Sp-1
-970  TCCATATACATCATGAGCCCACAAGGGGGCGCGTGCCTTAGTCCTACCTGCCAGCCACCAGAAACGGAGG

-900  ACTCCACAGGCAGTCAACCCTGAACACCCCAGAACCGGCCAGTTCACCAGAAAAGGGTCCCTTGAAAGTC

-830  ACAATGGGCAAACTGTTCTCACCCAGTTATGCCTGCGATAGAAGGGAGTGATGGGTGCCCAACAAACCCT
                GR
-760  CACTTCTCATCCCAGCAGTAAGGAAGCTAAGGCAGAAGGACTCACCTCCGAGGCCAGCCTGTGGGAAAGG

-690  GAAAACTGAGAAGACAGCCAGGTCAGGACAACATGCCAGCCTGAGCTGCATGAGACTTAGCCTCAAACGC
                          T3R-α                 AP-1
-620  GTGTGCACATGTACACACATCATAGGTCCAGAGTGCCCTTGTACAGGTGGGGAGTCCTAAAGCAGAAGAA
                                    GR                  AP-2
-550  AGAAATTCTCCAAGAGAACCTGAGTGCCTCCCACCGGGTTAGCTGAAGGGCAAGGTTGGACAGTTCCTTC

-480  TCCCAAGCTGCGGAGAAAGGTAGATGCTCTCTGGGGGAAGGGCTCCAGTCGCTCGCTTTTTCCTTATGGA
        TTF-1
-410  GCCCGGAAGTCTCCGAAAGTGAACCCTAGTCCGGGGTTCCTCATAACTCCGCTTCCTAAATTCCTTAGAC

-340  TGTGAGCGGAGGTCTGAGCCGTCCATAGCCTTTCCCATCTCTCCACAACCCTATACGGAACAAGCCCTAG

-270  ATGTGGGAGAAAGGGTCAGGAGACACGAGTGTTCCCCCACCCCGACTGCCCGCACCCCTGCAGGCCCGCT
                    T3R-α
-200  CGTCCCAGGACCGAAAGGGTCGGGGTCGCGCCCGCCTCCCAGGCTGGGGGCGGAGCTGCGCTGACCCCGG
                          Sp-1                  Sp-1
-130  AGTTCAATAAATCCGCAGCGCGAAAAGCGAGTGGATCGACGGCCCAGCGGTGACTCGCGCTGCGACTCTC

-60   CCACTGACCGAGAGTCCCCGACGTCCTCCGCATCCTCTCCTCACCGAGTCACCTGTCTCC ATG GAG GGT
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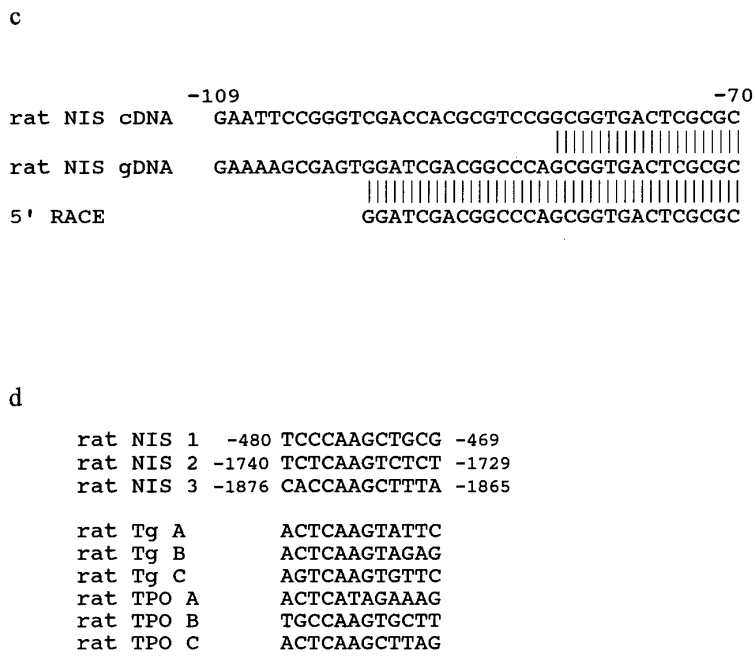


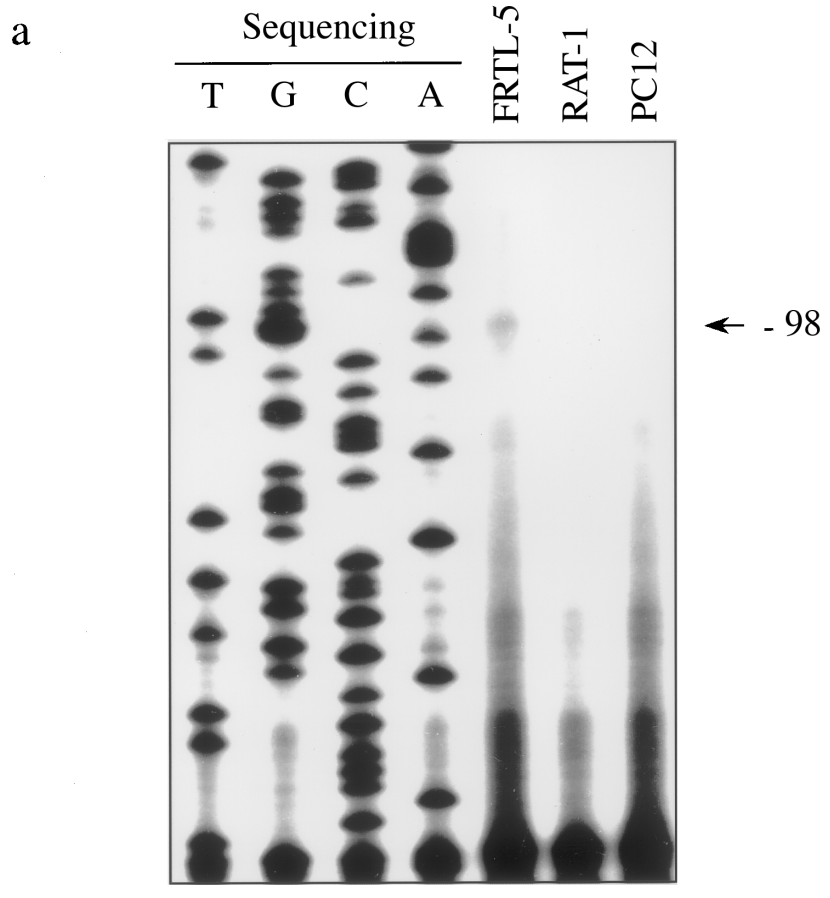
FIG. 1. Characterization of the 5′ flanking region of the rat NIS gene. (a) Restriction map of the 16.4 kb genomic DNA fragment of rat NIS. The translation initiation site (ATG) is shown. Ss, SstI; Sa, SalI; H, HindIII; B, BamHI. (b) Nucleotide sequence of the immediate 5′ flanking region of the rat NIS gene. The arrow indicates the putative thyroid specific transcription start site. The potential TATA box is double-underlined. Potential binding sites for AP-1, AP-2, GR, TTF-1, T3R-α, T3R-β, and SP1 are underlined. (c) The discrepancy between the first 25 bp of the reported rat NIS cDNA with our rat NIS genomic DNA sequence and our cDNA sequence acquired by 5′ RACE are shown. (d) The comparison of potential rat NIS TTF-1 binding sites with other known TTF-1 binding sites.

region (13). The Pax-8 binding site overlaps with the TTF-1 binding site proximal to the transcriptional start site. However, TSHr promoter shows a quite different structure in that two TTF-1 binding sites were found, yet no TTF-2 or Pax-8 binding sites was found, to be present in the 900 bp 5′ flanking region of the TSHr gene (14). In the 5′ flanking region of rNIS, three potential TTF1 sites, at −480, −1736 and −1875 nt relative to the ATG translational initiation site, were identified (Fig 1d). The TTF-1 binding site has been shown to play an important role in mediating thyroid-specific expression of Tg, TPO and TSHr genes (15-17). Thus, the functional significance of the three TTF-1 binding sites in rNIS need to be investigated. No TTF-2 binding site can be identified in rNIS 5′ flanking region. Taken together, the promoter structure of the 5′ flanking region of the NIS gene appears to be more close to that of the TSHr gene, instead of the Tg or TPO genes.

Potential binding sites for AP-1, AP-2, GR, Sp1, T3Rα, and T3Rβ were also identified in rNIS 5′ flanking region. It has been shown that glucocorticoid (binding to GR) modulate the iodide uptake activity in thyroid cells. The AP-1 activity appears to be inversely related to iodide uptake activity (5). AP-2 was demonstrated to mediate the transcriptional activation of protein kinase C and cAMP-dependent protein kinase A (18). Since no CREB site was identified, the TSH effect on rNIS expression might be mediated by AP-2. Finally, it seems logical that the iodide uptake activity in the thyroid follicular cells is regulated by the presence of T3 thyroid hormone (binding to T3Rα and T3Rβ). However, the functional significance of these binding sites in modulation of the transcriptional regulation of rat NIS need to be further characterized.

The Transcription Start Site Was Determined by Three Different Methods

Our 5′ RACE experiment resulted in three extended 5′ end cDNA fragments, starting at −98, −93 and −92 nt relative to the ATG site (Fig. 1c). Consistent with this result, primer extension assay, with a primer corresponding to −16 to +4 nt relative to the ATG site, showed an extended cDNA fragment specific to the FRTL-5 RNA, starting from −98 nt relative to the ATG site (Fig. 2a). We tentatively designate −98 to be the start site for thyroid specific transcription. Ribonuclease protection assay, with a riboprobe corresponding to −352 to +4 nt relative to the ATG translational initiation site, reveled a protected riboprobe fragment with a size consistent with other experiments (Fig. 2b). The integrity of the RNA samples used for these experiments is verified by successful detection of rat G3PDH transcripts in all three RNA samples by ribonuclease protection assay (Fig. 2c).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Sequencing
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RAT-1
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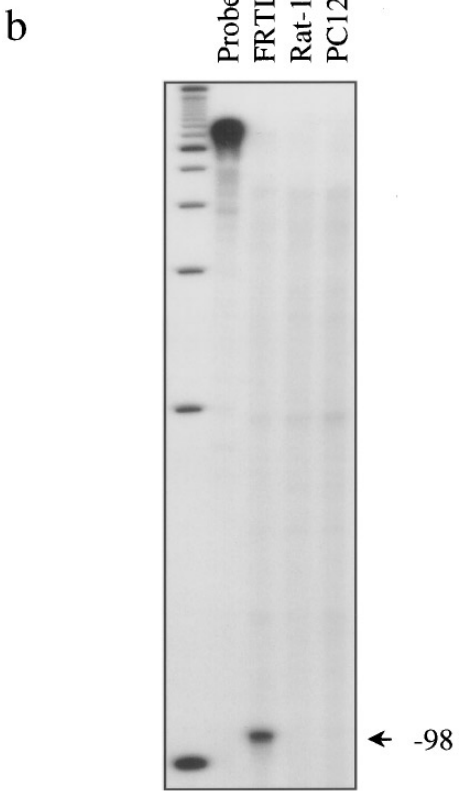
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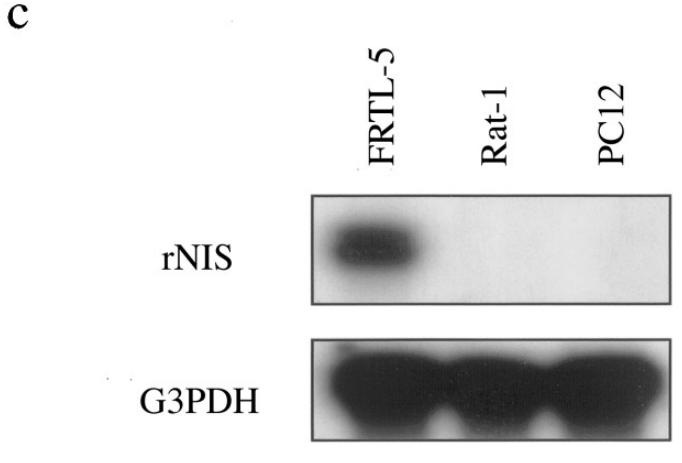
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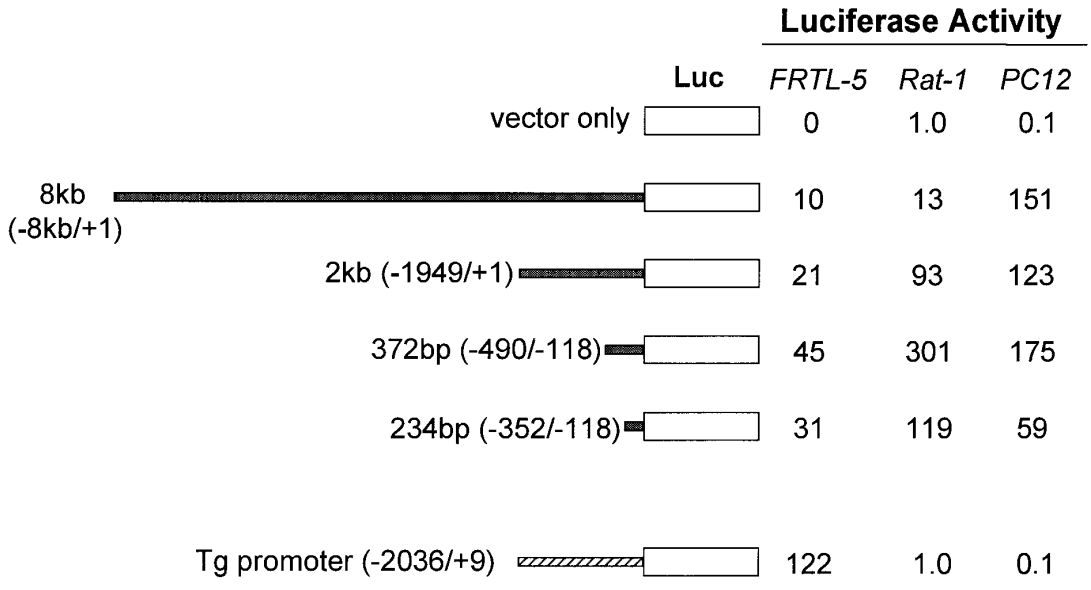

FIG. 3. Transient expression analysis of the rat NIS gene promoter activity. Luciferase reporter DNA constructs containing various lengths of the rNIS 5′ flanking region were transfected into FRTL-5, Rat-1 or PC12 cells. A β-gal construct was co-transfected with each DNA construct, to normalize the transfection efficiency. The luciferase activity of each construct is reported relative to the luciferase activity of SV40 early promoter plus SV40 enhancer of the pGL2Control DNA construct (Promega), which was arbitrarily designated as 100. The data represent three independent experiments. Duplicate transfections were performed in each experiment. All numbering of the NIS promoter DNA fragment is relative to the ATG translation initiation site. The numbering of the bovine Tg promoter is relative to the transcriptional start site (21).

Both Tg and TPO promoters have a TATA box element located at 20-30 nt upstream of the transcriptional start site (13). However, TSHr promoter does not have a TATA box. In rNIS promoter, a potential TATA box (AATAAAT) was identified at −124 to −118 nt relative to the ATG translational initiation site, which is located at 20 nt upstream of the putative rNIS transcriptional start site. Several non-canonical TATA box sequences have been reported, such as the ATAAAA in the gene for bovine elastin (19). The AATAAA box was reported to retain 30% activity of the wild type TATAAA when assayed by a reconstituted *in vitro* transcription system (20). Furthermore, comparing the 5′ flanking sequence between the genes for rat and human NIS (Ryu and Jhiang, unpublished data), we identified a stretch of 90 bp highly conserved sequence (75% identity), ranging from −199 to −110 nt relative to the ATG translational initiation site in rNIS. Thus, this 90 bp DNA fragment may contain the minimum NIS promoter. Take together, all data support that the rNIS transcription start site is located at −98 nt upstream of the ATG site.

The Rat NIS Promoter Was Mapped to −352 to −118 nt Relative to the ATG Site, and the 8 kb 5′ Flanking DNA Fragment of rNIS Is Not Sufficient to Confer Thyroid-Specific Transcription

Genomic DNA fragments with various lengths of the rat NIS 5′-flanking region were inserted in front of the promoterless luciferase reporter gene in the pGL2Basic vector to analyze the promoter activity of rNIS in three different rat cell lines, FRTL-5, Rat-1, and PC12 (Fig. 3). In the rat thyroid cell line FRTL-5 cells, the 372 bp DNA fragments (−490 to −118) appeared to have the strongest promoter activity, compared to the 8 kb and 2 kb DNA fragments. This result indicates the presence of negative DNA regulatory elements in the region upstream of the 372 bp DNA fragment. Further deletion of 138 bp from the 372 bp fragment, which resulted in a loss of a potential TTF-1 binding site, caused a significant reduction of rNIS promoter activity in FRTL-5 cells as well as two non-thyroid cell lines, the Rat-1 and PC12 cells. Therefore, the functional rele-

FIG. 2. Determination of the rat NIS gene transcriptional start site. (a) Primer extension analysis of the rNIS transcription start site. A DNA sequencing ladder, obtained using the same rNIS33 primer, was run in parallel. Arrow indicates the position of the extended cDNA fragment relative to the ATG site. (b) Ribonuclease protection analysis of the rNIS transcriptional start site. Arrow indicates the position of the putative transcriptional start site relative to the ATG site, as indicated by the size of the protected riboprobe. Radioactive labeled 100 bp ladder (GIBCO BRL) was run in parallel as a size marker. (c) Ribonuclease protection analysis of rNIS expression in three cell lines. Ribonuclease protection analysis of rat G3PDH transcript was also performed on the same RNA samples to verify the RNA integrity. FRTL-5: rat thyroid cells, Rat-1: rat fibroblasts, PC12: rat pheochromocytoma cells.

vance of this TTF-1 binding site in rNIS transcription need to be further investigated.

None of the DNA fragments investigated was sufficient to confer thyroid-specific promoter activity, since all the DNA constructs showed promoter activity not only in FRTL-5 thyroid cells but also in Rat-1 fibroblast and PC12 pheochromocytoma cells. However, RNase protection assay indicates that the endogenous rNIS promoter is inactive in Rat-1 and PC-12 cells, since rNIS transcript is undetectable in these two non-thyroid cells (Fig. 2c). A consistently higher rNIS promoter activity observed in non-thyroid cells than in FRTL-5 cells may result from poor transfection efficiency in FRTL-5 cells. To validate our promoter assay system, we included a control reporter DNA construct containing 2 kb bovine Tg promoter (21), and showed that the Tg promoter is active only in FRTL-5 thyroid cells (FRTL-5), but not in two non-thyroid cells (Rat-1 and PC12). Finally, it is noteworthy that rNIS promoter activity is much weaker than that of bovine Tg promoter in FRTL-5 cells (Fig. 3).

Among thyroid differentiation markers, the expression level of Tg appears to be most abundant in thyroid cells, compared to those of TPO, TSHr, and NIS. Consistent with this observation *in vivo,* Tg appears to have the strongest promoter activity in thyroid cells *in vitro.* As indicated by its exclusive expression in the thyroid gland *in vivo,* the thyroid-specific promoter activity of Tg has been well demonstrated among several species (13). In contrast, although NIS is predominantly expressed in thyroid, it is expressed in several non-thyroid tissues as well. Therefore, it is not surprising that the molecular mechanisms controlling NIS expression is different from that of Tg. The differences of regulatory mechanisms between NIS and Tg were also revealed in thyroid cells expressing oncogenes. It was shown that v-erbA causes loss of iodide uptake without any impairment of the other thyroid differentiated function, such as Tg, TPO and TSHr gene expression (4). Furthermore, in our thyroid-targeted *ret*/PTC1 transgenic mice, the iodide concentrating activity of the thyroid gland was significantly impaired, while no obvious change in Tg and TSHr expression was detected (Cho et al., manuscript in preparation).

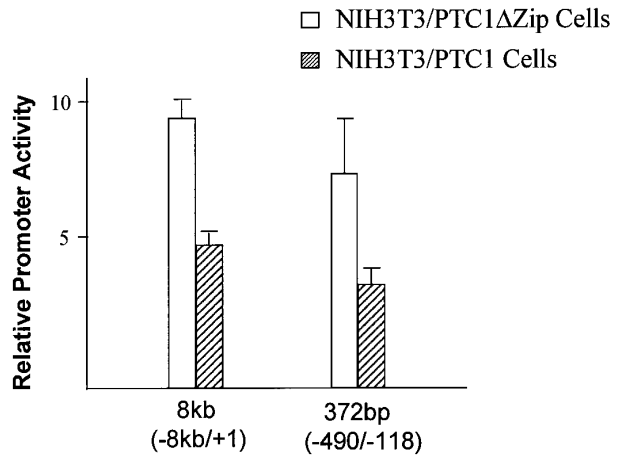

FIG. 4. The rat NIS gene promoter activity is suppressed in the PTC1 oncogene transformed cells. Luciferase reporter DNA constructs containing various lengths of the rNIS 5′ flanking region were transfected into two NIH3T3 stable transfectant, NIH3T3/PTC1 or NIH3T3/PTC1Δzip cells. A β-gal DNA construct was co-transfected with each DNA construct, to normalize the transfection efficiency. The luciferase activity of each construct is reported relative to the luciferase activity of SV40 early promoter plus SV40 enhance of the pGL2Control DNA construct (set as 100). Duplicate transfections were performed in each experiment. All numbering of the NIS promoter DNA fragment is relative to the ATG translation initiation site.

The rNIS Promoter Activity Is Reduced in PTC1 Transformed NIH3T3 Cells

When the PTC1 oncogene was transfected into thyroid cells, it causes dedifferentiation of thyroid cells, including the loss of iodide uptake activity (6). In our thyroid-targeted PTC1 transgenic mice, both iodide concentrating activity and NIS expression are significantly reduced in the mouse thyroid gland (Cho, et al., manuscript in preparation). To evaluate the effect of the PTC1 oncogene on the transcriptional regulation of rNIS, we introduced the rNIS reporter DNA constructs into the NIH3T3 stable transfectant cells which either express PTC1 or a non-functional PTC1 mutant, PTC1ΔZip. The rNIS promoter activity was found to be reduced significantly in the PTC1 transformed cells (Fig. 4). However, no effect of PTC1 on rNIS promoter activity can be demonstrated by transient co-transfection of the rNIS promoter reporter DNA construct and the PTC1 expression DNA construct into FRTL-5 and Rat-1 cells. Therefore, the suppression of rNIS expression in PTC1 transformed NIH3T3 cells may be caused by secondary effects of transformed phenotype induced by PTC1. This observation is consistent with the fact that the iodide uptake activity is significantly reduced in most thyroid tumors, compared to normal thyroid tissues.

Several Mechanisms Are Proposed to Account for Thyroid Preferential Expression of rNIS

Based on our current results, several possible mechanisms are proposed to account for thyroid preferential expression of rNIS. It is possible that, in addition to the proximal promoter we characterized, there is another upstream promoter which might be responsible for the thyroid-preferential expression of rNIS. It is also possible that additional elements located outside of the studied 8 kb genomic DNA fragment are required to inhibit

the transcription of rNIS in non-expressing tissues. In addition, DNA methylation has been shown to be an effective mechanism for transcriptional inhibition of tissue-specific genes in non-expressing cells (22). It has been shown that the DNA at the 5′ end of the human Tg gene is demethylated in thyroid, yet it is fully methylated in non-thyroid tissues (23). Thus, it is possible that the endogenous NIS gene is only expressed in thyroid and the other tissues where its DNA is demethylated, but not in the tissues where its DNA is methylated. In this study, exogenous unmethylated reporter DNA was delivered into non-thyroid cells to investigate rNIS promoter activity. Therefore, the exogenously transfected rNIS promoter is active in non-thyroid cells regardless of the methylation status of their endogenous NIS gene. Finally, chromosomal structure might also be critical for the tissue-preferential expression of NIS. The chromosome region containing the NIS gene could be in an open and active state in thyroid and in a condensed and inactive state in non-expressing tissues.

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