Human sodium–iodide symporter (hNIS) gene expression is inhibited by a trans-active transcriptional repressor, NIS-repressor, containing PARP-1 in thyroid cancer cells

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Abstract

Radioiodine remains the only tumoricidal therapy for disseminated thyroid carcinomas; however, dedifferentiated tumors lose the expression of human sodium–iodide symporter (hNIS) gene, and cannot respond to this treatment. Previous studies suggested that a trans-active protein factor (NIS-repressor) represses endogenous hNIS transcription, likely contributing to the loss of radioiodine uptake, and defined the NIS-repressor binding site (NRBS) in the proximal hNIS promoter. Using electrophoretic mobility shift assay (EMSA), we found evidence of NIS-repressor in the nuclear extract from KAK-1 cells, and confirmed this result using nuclear extracts prepared from multiple verified thyroid cell lines. Luciferase reporter assays of hNIS promoter constructs and EMSA were used to define two core sequences, NRBS-P and NRBS-D, in the hNIS promoter as the binding sites for NIS-repressor. Electrophoretic analysis of KAK-1 nuclear extract proteins cross-linked with NRBS-P suggests that NIS-repressor is a protein complex. Analysis of KAK-1 nuclear extract proteins bound to NRBS-P, via liquid chromatography coupled with tandem mass spectroscopy, demonstrated poly(ADP-ribose) polymerase-1 (PARP-1) as a NIS-repressor component. Pharmacological inhibition of PARP-1 enzymatic activity using PJ34 stimulated both the luciferase reporter activity driven by hNIS promoter and the endogenous hNIS mRNA level. Supershift studies suggest that thyroid transcription factor 2 (TTF-2) is also associated with the NIS-repressor complex. NIS-repressor, including its PARP-1 component, presents a potential therapeutic target to restore radioiodine uptake in dedifferentiated thyroid carcinomas.

Introduction

Human sodium–iodide symporter (hNIS) is a transmembrane protein enabling thyrocytes, both benign and malignant, to concentrate iodide, permitting radioiodine to be a unique systemic cytotoxic therapy for metastatic tumors. Unfortunately, when hNIS expression is lost in dedifferentiated thyroid carcinomas, there are no effective systemic cytotoxic agents (Ain 2000). Restoration of hNIS expression in such tumors could restore the effectiveness of radioiodine treatment.

Potential causes for the loss of hNIS activity include hNIS gene mutation (Pohlenz & Refetoff 1999), loss of hNIS gene transcription, defective posttranslational processing, and failure to traffic hNIS protein to cell membranes (Pohlenz et al. 2000). There is no evidence for hNIS gene mutations in thyroid carcinomas. We previously demonstrated that methylation of CpG islands in hNIS promoter can inhibit gene
transcription, and that it is reversible with DNA methyltransferase inhibitors (5-azacytidine, AzaC) and histone deacetylase inhibitors (sodium butyrate), suggesting epigenetic loss of hNIS gene expression in thyroid cancer (Venkataraman et al. 1999). Further investigations revealed evidence for an alternative mechanism for the loss of hNIS transcription, suggesting the presence of a trans-acting repressor of hNIS transcription, termed NIS-repressor (Li et al. 2007).

Multiple cellular and nuclear factors are reported to be important for NIS transcription, including thyrotropin (TSH)/receptor (Riedel et al. 2001), thyroid transcription factor 1 (TTF-1) (Schmitt et al. 2001), and Pax8 (Pasca di Magliano et al. 2000), but there are no clear examples of repressing transcription factors for NIS transcription in thyroid cells or thyroid carcinomas. We showed NIS-repressor as a trans-acting protein binding to a specific region of the proximal hNIS promoter, NIS-repressor binding site (NRBS-P); however, its composition was not known. In the present study, we undertook to further characterize NIS-repressor and investigate the identities of its components. This involved defining NRBS-P to a narrower region of hNIS promoter and utilizing it to probe the nuclear extract, and analyzing the probe-bound proteins with liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) to characterize NIS-repressor components. The mass spectrometry analysis data demonstrated human poly(ADP-ribose) polymerase-1 (PARP-1) to be a likely component of the NIS-repressor protein complex. Pharmacological inhibition of PARP-1 activity with PJ34 stimulated endogenous NIS-repressor protein complex. Further study revealed another upstream NRBS, termed NRBS-D, suggesting multiple sites of NIS-repressor action, and also provided suggestive evidence that TTF-2 might be involved in NIS-repressor complex.

Materials and methods

Cell lines, reagents, and culture conditions

KAK-1 cells were cultured (Ain & Taylor 1994) in our laboratory, and they have been widely used for more than a dozen years; however, recent short tandem repeat (STR) genotyping revealed cross-contamination of this cell line demonstrating an STR profile matching that of the HT-29, a colon cancer-derived cell line (Schweppe et al. 2008). Much of this work had been performed prior to the discovery of this cross-contamination. Demonstration of hNIS expression in human colon carcinoma (Wapnir et al. 2003) suggests utility of data obtained with this cell line for hNIS studies, as long as it is re-verified using bona fide thyroidal cell lines. Accordingly, we re-verified key experiments using STR-verified cell lines: Cal-62 (anaplastic thyroid cancer) as obtained from Bryan Haugen (University of Colorado), KAT-18 (anaplastic thyroid cancer, established in our laboratory), and SW1736 (anaplastic thyroid cancer, developed by Leibowitz and McCombs III at the Scott and White Memorial Hospital, Temple, TX, USA, in 1977) provided by Nils-Erik Heldin (Uppsala University, Uppsala, Sweden). Additional studies were done using a primary anaplastic thyroid carcinoma culture obtained from a surgical tissue sample using established methodology (Ain & Taylor 1994). All cell lines were cultured in phenol red-free RPMI 1640 (10% fetal bovine serum (FBS), 100 nM sodium selenite, 5 μIU/ml human TSH, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate; 37 °C, 5% CO₂). PJ34 (CalBiochem/EMD Biosciences, Inc., La Jolla, CA, USA) was dissolved in distilled water at 15 mM as a stock solution.

Total RNA isolation and quantitative reverse transcription-PCR

Total RNA was isolated (TRIzol, Invitrogen Corp.), genomic DNA was removed (DNA-free kit; Ambion Inc., Austin, TX, USA), and cDNA was synthesized from 2 μg total RNA (Advantage reverse transcription (RT)-for-PCR kit; BD Biosciences Clontech) with random hexamer primers. TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and Assay-on-Demand Gene Expression Products (Hs00166567_m1 for hNIS mRNA and Hs00243335_m1 (Applied Biosystems), was used to quantify the Pax8 level in qRT-PCR studies. The same procedure was followed using 18s-R (5'-ccctcagctctcttcgctc-3') and NIS-R (5'-ccctcagctctctctg-3'), and was then ligated into pCR2.1 vector (Invitrogen). The same procedure was followed using 18s-F (5'-ctggggtctctggatgctc-3') and NIS-F (5'-ccctcagctctctctg-3'), and was then amplified using PARP-1 (5'-tcgctggtcctca-3') and NIS-R (5'-ccctcagctctctctg-3'), and was then ligated into pCR2.1 vector (Invitrogen). The same procedure was followed using 18s-F (5'-ctggggtcctctcgtc-3') and NIS-R (5'-ccctcagctctctctg-3') for 18s rRNA quantitative PCR (qPCR) standard. Plasmid DNA preparations were quantitated, diluted, and used as qPCR standards. The same procedure was followed using the primer pair (Pax8-F: 5'-gcctggtcctctcgtc-3' and Pax8-R: 5'-ccctcagctctctctg-3') to obtain Pax8 standard. The TaqMan assay, Hs00243335_m1 (Applied Biosystems), was used to quantify the Pax8 level in qRT-PCR studies.
Analysis of hNIS protein expression and iodide uptake activity

I$^{125}$ uptake assays were performed according to the method of Petrich et al. (2002). Briefly, cells were plated in triplicate in six-well plates and grown to 90% confluency. Cells were washed twice with 2 ml of Hank’s balanced salt solution (HBSS, Gibco, Cat. no. 14025), and then 2 ml of Solution A (HBSS, 10 mM HEPES, pH 7.3, and 1 mM NaCl) with 2 μCi NaI$^{125}$ or Solution B (Solution A plus 300 μM NaClO₄) with 2 μCi NaI$^{125}$ were added, and incubated at 37 °C for 1 h. Each well was washed twice with 2 ml of cold Solution A or Solution B respectively, and then 2 ml of Solution C (1% Triton X-100 and 0.1 M NaOH) were added to each well to digest the cells, and radioactivity was measured on a γ-counter. The NIS-specific radiiodine uptake was calculated by subtracting counts from the wells with Solution B from those from the wells with Solution A. Western blotting experiments were carried out according to the standard protocol (Ausubel 1995) using mouse anti-hNIS monoclonal antibody (FP5A; Thermo Fisher Scientific, Fremont, CA, USA).

hNIS promoter luciferase reporter constructs containing site-directed mutations in hNIS promoter

Site-directed mutation primers, Muta-Fx and Muta-Rx (x = 1–17 and 19–23), and primers F-4.5 (5′-gagtaacct-gagcagttcctcccaag-3′, −953 to −934 relative to ‘A’ in hNIS translation initiation codon, KpnI site is underlined) and Luc-R1 (5′-agaacggctggagctctgtg-ggggcttac-3′, −1 to −22, HindIII site is underlined) were synthesized. Supplementary Table 1, see section on supplementary data given at the end of this article, lists the sequences of mutation oligos. F1-pGL3-basic plasmid from our previous work (Li et al. 2007) was used as a template. Primer pairs F-4.5 and Muta-Rx amplified 22 different 3′-regions, all starting from F-4.5, while Luc-R1 and Muta-Fx amplified 22 different 3′-regions, all ending at Luc-R1. Fragments were gel-purified, and then 22 ligation reactions were carried out to link F-4.5/Muta-Rx fragment with Luc-R1/Muta-Fx fragment for each site separately using T4 DNA ligase. For amplification of 22 promoter mutation constructs, starting from primer F-4.5 and ending at Luc-R1, 22 ligation products as templates and F-4.5 and R1 as the primer pair were used. Fragments were gel-purified, double digested with HindIII and Kpnl, and separately ligated into pGL3-basic vector (Promega), making 22 constructs containing single site-directed mutations (F-4.5/Muta-x-pGL3-basic). A construct containing DNA amplified from the F1-pGL3-basic plasmid using F-4.5 and R1 primers functioned as the control plasmid (F-4.5-pGL3-basic). All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The DNA sequences covering −724 to −534 bp from the translation start site is indicated. Numbers above the sequence correspond to the mutations used in the transfection assays reported in (B) with the square brackets enclosing the original promoter sequences. Poly-adenosine sequences are substituted for the bracketed original sequences. In (B), the normalized luciferase activities from the 22 mutated hNIS promoter constructs relative to the F-4.5-pGL3-basic vector are depicted, and mutation 10 leading to the greatest decrease in response to cyclohexamide (CHX) treatment is shown. In (C), electrophoretic mobility shift assay (EMSA) results using radiolabeled Probe A or Probe B to probe the nuclear extract from KAK-1 cells cultured under basal conditions are shown. Lanes contain Probe A only (lane 1) and Probe A mixed with 4.5 μg (lane 2) or 18 μg (lane 3) KAK-1 nuclear extract. Lane 4 is the same as lane 3 plus 20× excess unlabeled Probe A; lanes 5 and 6 are the same as lane 3 with 2× and 3× sonicated salmon sperm DNA respectively to inhibit nonspecific protein–probe binding. Lanes 7 and 8 are the same as lane 3 with 2× and 3× poly dI:dC respectively for further inhibition of nonspecific protein–probe binding. Lane 9 contains Probe B only. Probe B is mixed with 4.5 μg (lane 10) or 18 μg (lane 11) KAK-1 nuclear extract. Lane 12 is the same as lane 11 plus 20× excess unlabeled Probe B. The arrows point to Probe A-specific bands. No probe-specific bands were seen for Probe B.

Figure 1 The NRBS-P is further refined by transfections of mutant sequences coupled to a luciferase reporter. In (A), the hNIS promoter DNA sequence region from −724 to −534 bp from the translation start site is indicated. Numbers above the sequence correspond to the mutations used in the transfection assays reported in (B) with the square brackets enclosing the original promoter sequences. Poly-adenosine sequences are substituted for the bracketed original sequences. In (B), the normalized luciferase activities from the 22 mutated hNIS promoter constructs relative to the F-4.5-pGL3-basic vector are depicted, and mutation 10 leading to the greatest decrease in response to cyclohexamide (CHX) treatment is shown. In (C), electrophoretic mobility shift assay (EMSA) results using radiolabeled Probe A or Probe B to probe the nuclear extract from KAK-1 cells cultured under basal conditions are shown. Lanes contain Probe A only (lane 1) and Probe A mixed with 4.5 μg (lane 2) or 18 μg (lane 3) KAK-1 nuclear extract. Lane 4 is the same as lane 3 plus 20× excess unlabeled Probe A; lanes 5 and 6 are the same as lane 3 with 2× and 3× sonicated salmon sperm DNA respectively to inhibit nonspecific protein–probe binding. Lanes 7 and 8 are the same as lane 3 with 2× and 3× poly dI:dC respectively for further inhibition of nonspecific protein–probe binding. Lane 9 contains Probe B only. Probe B is mixed with 4.5 μg (lane 10) or 18 μg (lane 11) KAK-1 nuclear extract. Lane 12 is the same as lane 11 plus 20× excess unlabeled Probe B. The arrows point to Probe A-specific bands. No probe-specific bands were seen for Probe B.
are enclosed within the square brackets and sequences that are given in lower case below each bracket indicate the mutated sequences).

NRBS-P-deletion luciferase reporter plasmid (F4Δ-pGL3-basic) and double-deletion (NRBS-P and NRBS-D) luciferase reporter plasmid (F4ΔΔ-pGL3-basic)

The 5′- and 3′-fragments were amplified using F1-pGL3-basic template with Luc-F4/−668R (5′-tgtagctgtaggcaacagctgctc-3′/5′-aggatctggcaagcgtcctgtcagctct-3′, KpnI and BamHI sites are underlined) and Luc-587F (5′-aggatctggcaagcgtcctgtcagctct-3′, BamHI site is underlined and Xhol site is given in italics)/Luc-R1 as the primer pairs separately. The products were gel-purified and digested with KpnI/BamHI and BamHI/HindIII, followed by ligation together into pGL3-basic, pre-digested with KpnI/HindIII, producing luciferase reporter plasmid F4Δ-pGL3-basic, containing hNIS promoter region (−1252 to −348 bp) upstream of luciferase-coding sequence with the region −667 to −588 bp deleted.

LucF4/Ddel-2R (5′-aggatctggcaagcgtcctgtcagctct-3′) and Ddel-2F (5′-aggatctggcaagcgtcctgtcagctct-3′)/Luc-R1 were used as PCR primer pairs, and F1-pGL3-basic was used as a template for PCR to amplify fragment-Δ5′ and fragment-Δ3′. These two fragments were gel-purified and digested separately with BamHI and BgII, followed by ligation together. LucF4/Luc-R1 were used with this ligation product as templates to amplify fragment-Δ. Ddel-3F (5′-ggatccctggcaagcgtcctgtcagctc-3′) and Luc-R1 were used as the primer pair with the fragment-Δ as a template for PCR to amplify DNA fragment-Δ5′. Luc-F4 and Ddel-3R (5′-aggatctggcaagcgtcctgtcagctc-3′) were used as the primer pair with the fragment-Δ as a template to amplify DNA fragment-ΔΔ5′. The fragment-Δ3′ and the fragment-ΔΔ5′ were gel-purified and digested separately with BgII/HindIII and KpnI/BamHI, followed by ligation together into pGL3-basic pre-digested with KpnI/HindIII to get the NRBS-D and NRBS-P double-deletion luciferase reporter plasmid (F4ΔΔ-pGL3-basic) that contains hNIS promoter region from −1252 to −348 bp, with double deletions at −999 to −938 bp covering NRBS-D and at −653 to −615 bp covering NRBS-P. The BamHI and BgII sites are underlined.

Transient assay with luciferase reporter constructs

Transient transfection assays tested luciferase activities from mutant hNIS promoter reporter constructs in response to 10 μg/ml cycloheximide (CHX; added 1 h before transfection). Luciferase reporter plasmids/pUC18/phRG-B (Promega) were transfected into KAK-1 cells using Lipofectamine 2000 (Invitrogen), and 24 h later, mixtures were replaced with media containing CHX. Luciferase and Renilla luciferase activities were determined 24 h later (Dual-Luciferase Assay Kit; Promega). Transient transfection assays monitored the effects of 30 μM PJ34 (applied 1 h before transfection) in KAK-1 cultures. After 24 h, transfection mixtures were replaced with media containing PJ34. Luciferase and Renilla activities were determined 24 h later. Luciferase activity was normalized to Renilla to account for variations in transfection efficiency (triplicate transfections as mean ± S.D.). The effects of different treatments or different mutations are calculated by the ratio of the normalized luciferase activity to the normalized luciferase activity of the control samples.

Electrophoretic mobility shift assay

Nuclear extracts were prepared using NucBuster Protein Extraction kit (Novagen/EMD Biosciences, Inc). Antibodies against respective antigens were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA (TTF-1 (F-12), TTF-2 (S-18, F-17, and V-20), Pax8 (A-15), Sp1 (E-3), c-Fos (H-125), AP-2α (C-18), and c-Jun (H-79)), and rabbit polyclonal antibody against PARP-1 was obtained from Roche Diagnostics. Electrophoretic mobility shift assay (EMSA) probes were end-labeled using T4 poly nucleotide kinase with γ-P32-ATP. Reactions were performed according to the instruction given in the EMSA Accessory Kit (Novagen). Antibodies or cold competitive probes were added as indicated. Mixtures were incubated on ice for 30 min, and 2 μl of loading dye were added and the samples were resolved on 7.5% PAGE/0.5×TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH8) and run overnight, and then the gels were dried and autoradiographed. Competitive cold EMSA probes were prepared by annealing sense and antisense single-strand synthetic oligos (Supplementary Table 2, see section on supplementary data given at the end of this article). EMSA Probe A (126 bp, −684 to −565 bp) was amplified using Muta-F4 (5′-aaaaaagagaggtcttgagctgtc-3′) and Muta-R20 (5′-tttacaccacattcgccttcag-3′). EMSA Probe B (196 bp, −531 to −348 bp) was amplified with F6.1 (5′-aggagatecgcacagctcctgtcagctc-3′, KpnI site is underlined) and Luc-R1 (5′-aggagcttgaggtgtctgtcagctc-3′, HindIII site is underlined). Plasmid F1-pGL3-basic was used as the PCR template.
Multiple probes were prepared, namely EMSA probe SHIFT-1 (200 bp, −1667 to −1468), EMSA probe SHIFT-2 (200 bp, −1467 to −1268), EMSA probe SHIFT-3 (200 bp, −1267 to −1068), EMSA probe SHIFT-4 (200 bp, −1067 to −868), and EMSA probe SHIFT-5 (166 bp, −873 to −708) using PCR with LucF1 (5'-gaggtaccatgctgcaagaaggggctgtga-3') and Shift-R1 (5'-ctgtggaatgaatcatctg-3') for the SHIFT-1 probe; Shift-F2 (5'-cattgggaaggggaacag-3') and Shift-R2 (5'-caagttggtgggaga-3') for the SHIFT-2 probe; Shift-F3 (5'-gttctctcatatatgtag-3') and Shift-R3 (5'-ttctttattcatgctgca-3') for the SHIFT-3 probe; Shift-F4 (5'-taataggaagcactcata-3') and Shift-R4 (5'-taaaccgccgcttggaacct-3') for the SHIFT-4 probe; and Shift-F5 (5'-ggtcagtgctctcagc-3') and Shift-R5 (5'-tgaataccttgagctatgg-3') for the SHIFT-5 probe. The plasmid F1-pGL3-basic was used as the PCR template. The Comp-1 probe, containing NRBS-P, was prepared by annealing Comp-1F (5'-gcctttttgacgacctttccaccctgtcaacagca-3') and Comp-1R (5'-gttctctcatatatgtag-3') for the SHIFT-1 probe; Shift-F3 (5'-taataggaagcactcata-3') and Shift-R4 (5'-taaaccgccgcttggaacct-3') for the SHIFT-4 probe; and Shift-F5 (5'-ggtcagtgctctcagc-3') and Shift-R5 (5'-tgaataccttgagctatgg-3') for the SHIFT-5 probe. The 'nicked' Comp-1 probe was prepared by annealing the following three oligos: nick-Comp-1-F01 (5'-gcctttttgacgacctttccaccctgtcaacagca-3'), 5'-phosphorylated nick-Comp-1-F02 (5'-tctgctgttgacaggtgggga-aattgaggtctaaaaa-3'), and Comp-1R. The nicked/1 bp deletion Comp-1 probe was prepared by annealing nick-Comp-1-F01, 5'-phosphorylated nick-Comp-1-F03 (5'-tctgctgttgacaggtgggga-aattgaggtctaaaaa-3'), and Comp-1R. The SHIFT-414 probe was prepared by annealing SHIFT-414F (5'-ctgtggaatgaatcatctg-3') and SHIFT-414R (5'-gcctttttgacgacctttccaccctgtcaacagca-3').

For u.v.-cross-linking analysis, EMSA was performed, followed by u.v.-cross-linking on ice using HB-2000 Hybridizer (UVP Inc., Upland, CA, USA) at 400 mJ/cm² for 20 times at 254 nm. Samples were added with the SDS-PAGE loading dye, heated at 95 °C for 5 min, and resolved on 7% SDS-PAGE, and the gel was dried and autoradiographed.

**LC/MS/MS analysis**

A biotinylated affinity probe was prepared using F1-pGL3-basic plasmid as a template and wtF4 (5'-tgaggtctggaagggctgtg-3')/Biotin-R22 (5'-BioTEG/tgcctctcatctccatgc-3') as the primer pair. A control probe was prepared similarly, but using the wtF20 (5'-catggggaaggggggacct-3')/Luc-R1 primer pair. The probes were gel-purified and quantitated. Dynabeads M-280 streptavidin (Invitrogen) were washed thrice (2 M NaCl, 1 mM EDTA, and 10 mM Tris–HCl; pH 7.5), and were then again washed twice (1×EMSA buffer; 100 mM KCl, 20 mM HEPES, 0.2 mM EDTA, and 20% glycerol; pH 8.0). Beads were blocked in bovine insulin solution (5 mg/ml; 1×EMSA buffer, 20 °C) for 15 min, and then washed twice and resuspended in 1×EMSA buffer. Five hundred microliters of basal KAK-1 cell nuclear extract (8 mg/ml) mixed with 220 μl 1×EMSA buffer, 40 μl salmon testes DNA (500 ng/μl; Sigma), 40 μl poly dI:dC (0.01 U/l, Sigma), 40 μl dithiothreitol (DTT; 10 mM), 8 pmol biotinylated affinity probe, and 32 pmol control probe were incubated (4 °C, 30 min, agitated), and then 100 μl of pre-blocked M-280 beads were added and incubated (4 °C, 1 h). Magnetically collected beads were washed five times (500 μl 1×EMSA buffer with 0.5 mM DTT, 3 min, 4 °C), and were finally washed (500 μl cold HBSS), resuspended in cold HBSS, and sent for LC/MS/MS analysis.

Beads were eluted with NH₄HCO₃ at increasing concentrations. Eluates (acidified with formic acid and evaporated to dryness) were digested with trypsin. Digests were acidified, dried, and reconstituted with 10 μl of 5% acetonitrile. Five-microliter samples, injected in a C18 capillary column, were eluted (acetonitrile–H₂O gradient) and analyzed by LC/MS/MS (Finnigan LTQ, Thermo Fisher Scientific, Waltham, MA, USA). The MS–MS spectra were analyzed using Mascot (Matrix Science www.matrixscience.com) protein database search engine against human proteins in SwissProt database.

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP-IT Express kit (Active Motif, Carlsbad, CA, USA) and basal cultured Cal-62 cells. Chromatin DNA, isolated from formaldehyde-fixed cells, was sheared by sonication. Human PARP-1 (hPARP-1)/chromatin DNA complex was immunoprecipitated using rabbit anti-hPARP-1 polyclonal antibodies (Roche Diagnostics), and collected using protein-G-coupled magnetic beads. The immunoprecipitated chromatin DNA was eluted from the beads, heated to reverse cross-linking, and treated with Proteinase K before using as a template for PCR amplification. Interactions between PARP-1 and different genomic DNA regions were examined using the following respective PCR primer pairs: the hNIS promoter region containing NRBS-P (−764 to −604 bp) using NRBS-P-F (5'-tgaggtctggaagggctgtg-3') and NRBS-P-R (5'-ctgtggaatgaatcatctg-3') as the primer pair, and the hNIS promoter region containing NRBS-D (−1043 to −892 bp) using NRBS-D-F (5'-tacggtctggtggtggaacct-3') and NRBS-D-R (5'-ggggagagccattccagag-3').
as the primer pair. The PCR amplifications were performed using TaKaRa PrimeStar DNA polymerase, and the reaction parameters are specified in the respective figures.

Results

NRBS-P is refined by site-directed mutagenesis coupled with luciferase reporter assay

Previous work (Li et al. 2007), utilizing luciferase reporter constructs containing serial deletions of hNIS promoter regions with CHX treatment, provided evidence that a region between −774 and −478 bp (NRBS-P) is a binding site for a trans-active transcriptional repressor, NIS-repressor. To further refine NRBS-P, site-directed mutations were introduced into the region spanning −724 to −534 bp (Fig. 1A). Twenty-three 6 bp mutations, in which all G, C, or T were mutated to A, were designed to evaluate the importance of the G, C bases. The sites of mutations were selected to cover the sequences systematically, resulting in 22 mutant hNIS promoter constructs. Effects of CHX treatment on these constructs are shown (Fig. 1B) with data normalized to F-4.5-pGL3-basic control vector. Results demonstrate that mutation 10 resulted in a remarkable reduction in normalized luciferase activity. In consideration of the activity of flanking mutations (mutations 8–11), it appears to define NRBS-P to a region from −653 to −621 bp from the hNIS translation start site.

Factors in nuclear extracts, prepared from KAK-1, KAT-18, SW1736, and a primary culture of anaplastic thyroid cancer, bind to NRBS-P as analyzed by EMSA

NIS promoter fragment (−684 to −565 bp; Probe A), encompassing NRBS-P region, was used to probe KAK-1 nuclear extract in EMSA. The result (Fig. 1C) reveals three Probe A-specific bands (one major band and two minor bands; lanes 1–8). Band signals were increased with increased amounts of nuclear extract (lanes 2 and 3), diminished by competition with excess cold Probe A (lane 4), and unaltered by increasing sonicated salmon sperm DNA (nonspecific DNA competitor; lanes 5–6) or poly dI:dC (artificial nonspecific DNA competitor; lanes 7–8), demonstrating factor(s) in KAK-1 nuclear extract binding specifically to Probe A. Probe B (−533 to −348 bp; downstream from Probe A), when incubated with the same KAK-1 nuclear extract, failed to produce these bands. Also, EMSA signals seen with Probe B were unaltered by increased amounts of nuclear extract or by adding cold Probe B (lanes 9–12), demonstrating that these signals resulted from nonspecific binding from KAK-1 nuclear extract.

When the nuclear extracts prepared from KAT-18, SW1736, and histologically verified anaplastic thyroid cancer tissue were tested with the radioactive Probe A in EMSA experiments, the same EMSA signals were found, as shown in Fig. 2, further confirming that the Probe A-binding factor(s), demonstrated in KAK-1 cells, also exist in STR-unique thyroid cancer cell lines and anaplastic thyroid carcinoma tissue.

NRBS-P is further refined and mapped to −648 to −620 bp using KAK-1 nuclear extract

Further refinement of the promoter region responsible for EMSA bands seen with KAK-1 nuclear extract is shown in Fig. 3A (lanes 1–4), with different cold PCR fragments being used to compete against radiolabeled Probe A (−684 to −565 bp). Unlabeled Probe C (−774 to −645 bp) and Probe D (−605 to −348 bp) did not competitively diminish EMSA bands, denoting that sequences responsible for gel-shift bands resided in hNIS promoter region spanning −645 to −605 bp. In Fig. 3B, excess cold double-stranded oligonucleotides Comp-2 (−633 to −595 bp, lane 5; also lane 12 in Fig. 3C), Comp-0.9 (−660 to −627 bp, lane 6), Comp-1.1 (−653 to −630 bp, lane 7), and Comp-1.4 (−638 to −615 bp, lane 10) all failed to compete against radiolabeled Probe A in EMSA. In contrast, excess cold Comp-1 (−653 to −615 bp) completely erased these bands (lane 4, Fig. 3B, and lane 11,

Figure 2 Electrophoretic mobility shift assay (EMSA) using nuclear extracts prepared from KAK-1 (lanes 2–3), SW1736 (lanes 4–5), KAT-18 (lanes 6–7), and primary culture of an anaplastic thyroid carcinoma tissue sample (lanes 8–9). Lane 1 contains radiolabeled Probe A only. Comparison of EMSA signals in lanes 2, 4, 6, and 8 with those in lanes 3, 5, 7, and 9 (containing 30× excess unlabeled Probe A) respectively reveals Probe A-specific signals. The arrows point to Probe A-specific bands.
Fig. 3C), while two other oligonucleotides Comp-1.2 (−648 to −625 bp, lane 8, Fig. 3B) and Comp-1.3 (−643 to −620 bp, lane 9, Fig. 3B) effectively competed against radiolabeled Probe A. This further mapped the sequence responsible for the characteristic EMSA bands to −648 to −620 bp of hNIS promoter, which is consistent with NRBS-P region identified in functional studies using luciferase reporter assays with 22 mutant hNIS promoter reporter constructs.

**KAK-1 nuclear extract loses binding to Probe A at increasing salt concentrations**

Figure 3D demonstrates that major EMSA bands, seen with KAK-1 nuclear extract and radiolabeled Probe A, gradually diminished in the presence of higher concentrations of KCl (lanes 3–12). Addition of 0.2 M KCl to the EMSA reaction mixture reduced the bands by ~50% (lane 4) compared with the controls without extra KCl (lane 2). This is consistent with typical salt elution conditions for protein transcription factors from their DNA-binding sites (Gadgil et al. 2001).

**LC/MS/MS analysis identifies hPARP-1 as a likely candidate for NIS-repressor component**

KAK-1 nuclear extract was incubated with biotinylated Probe A and Dynabeads M-280 to isolate the bound protein factors. Dynabead eluates, analyzed by LC/MS/MS, revealed the predominance of hPARP-1, with a Mascot score of 852 (probability-based Mowse Score >33 indicates identity, P < 0.05) with 50 peptides and 459 amino acids matched, and 42% of the sequence covered with 39 unique peptides (shown in bold in Supplementary Table 3, see section on supplementary data given at the end of this article).

**PARP-1 is associated with endogenous hNIS promoter in Cal-62 cells under basal culture conditions**

ChIP assays, utilizing a polyclonal anti-PARP-1 antibody, demonstrated that PARP-1 binds to a −764 to −604 bp region (containing NRBS-P; lane 4, Fig. 4A) and a −1043 to −892 bp region (containing NRBS-D, discussed later; lane 4, Fig. 4B) in the endogenous hNIS promoter in Cal-62 cells under basal culture conditions without endogenous hNIS transcription. Antibodies, recognizing TTF-1 (lanes 5 in Fig. 4A and B) and TTF-2 (lanes 6 in Fig. 4A and B), did not precipitate these two DNA regions.
To evaluate whether purified PARP-1 associates with NRBS-P, we performed EMSA using Cal-62 nuclear extract, commercial PARP-1 (Trevigen, Gaithersburg, MD, USA), and radiolabeled Comp-1 and Comp-1 variants (Fig. 5). Compared with Cal-62 nuclear extract (Fig. 5A and B, lanes 2 and 3 in each), commercial PARP-1 failed to produce any gel-shift band (Fig. 5A and B, lanes 4 and 5 in each) using Comp-1 probe (containing NRBS-P). Considering that PARP-1, when functioning in a DNA-repair role, binds to nicked DNA substrate, we introduced a ‘nick’ in the Comp-1 probe, and demonstrated that this does not cause commercial PARP-1 to produce any gel-shift band (Fig. 5A, lanes 9 and 10). Figure 5B (lanes 9 and 10) shows results utilizing a ‘nicked’ Comp-1 probe that also has a 1 bp deletion (nicked Comp-1/1 bp deletion) and also fails to produce any gel-shift band with commercial PARP-1. This suggests that hPARP-1 does not, by itself, directly bind NRBS-P.

Oligonucleotides containing consensus DNA-binding sites of multiple thyroidal transcription factors (AP-1, AP-2, CREB, Pax8, Sp1, TTF-1, and TTF-2) failed to compete against NRBS-P probe suggesting that these factors do not directly bind to NRBS-P.

Since hNIS expression is tissue specific, thyroidal transcription factors need to be considered as candidates for binding to NRBS, possibly as components of NIS-repressor. We investigated whether cold oligonucleotides, containing consensus binding sites for known effective thyroidal transcription factors, could compete against Probe A (containing NRBS-P). Figure 3C shows that cold double-stranded oligonucleotides containing consensus binding sites for AP-1 (lane 4), AP-2 (lane 5), CREB (lane 6), Pax8 (lane 7),
Sp1 (lane 8), TTF-1/Pax8 (lane 9), and TTF-2 (lane 10) failed to alter EMSA patterns seen using radiolabeled Probe A, suggesting that these factors did not bind directly to Probe A.

Pharmacological inhibition of hPARP-1 with PJ34 increased hNIS promoter activity in KAK-1 cells with luciferase reporter assay

PJ34 is a potent PARP-1 inhibitor (Abdelkarim et al., 2001). Its effects on hNIS transcription were evaluated using pGL3-basic, F4-pGL3-basic (containing hNIS promoter; −1252 to −348 bp), and F4Δ-pGL3-basic luciferase reporter constructs (same as F4-pGL3-basic with sequence −667 to −588 bp, which encompasses NRBS-P, deleted). Results (Fig. 6) revealed that 1) the normalized luciferase activities from both F4Δ-pGL3-basic and F4-pGL3-basic increased significantly ($P<0.05$, one-way ANOVA) compared with those from pGL3-basic control vector under basal culture conditions, indicating that the hNIS promoter sequences were functional; 2) the normalized luciferase activities from F4Δ-pGL3-basic increased significantly ($P<0.05$, one-way ANOVA) compared with those from F4-pGL3-basic under basal cell culture conditions, consequent to a negative regulatory site (NRBS-P) being removed; 3) PJ34 treatment (30 μM for 2 days) stimulated the normalized luciferase activity from all constructs compared with the respective counterparts without PJ34 ($P<0.05$, Student’s $t$-test); 4) the stimulation ratio, defined as the ratio of the normalized luciferase activity of samples with PJ34 treatment to that of those without PJ34 treatment, increased significantly from pGL3-basic to F4-pGL3-basic ($P<0.001$, one-way ANOVA) and from pGL3-basic to F4Δ-pGL3-basic ($P<0.01$, one-way ANOVA), and decreased significantly from F4-pGL3-basic to F4A-pGL3-basic ($P<0.01$, one-way ANOVA). Thus, pharmacological inhibition of hPARP-1 using PJ34 increases hNIS promoter activity, and the sequence from −667 to −588 bp (that encompasses NRBS-P) is important for this effect; however, when the normalized luciferase activity from F4Δ-pGL3-basic construct with PJ34 treatment was compared to that without PJ34 treatment, a strong stimulation was still apparent. This suggested that one or more additional DNA sequences (presumably additional NRBS) were responsible for NIS-repressor activity and the effect of PJ34.

The second NRBS (NRBS-D) in the hNIS promoter

Five additional EMSA probes, SHIFT-1, -2, -3, -4, and -5, were prepared using PCR, radiolabeled, and used to probe KAK-1 nuclear extract in EMSA. The EMSA results shown in Fig. 7 indicate that 1) there is no specific signal for SHIFT-1 probe (Fig. 7A, lane 5), covering −1667 to −1468 bp; 2) there are multiple faint specific signals for SHIFT-2 probe (Fig. 7A, lane 8), covering −1467 to −1268 bp; 3) there is no specific signal for SHIFT-3 probe (Fig. 7A, lane 11), covering −1267 to −1068 bp; 4) there is one strong specific signal for SHIFT-4 probe (Fig. 7B, lane 5), covering −1067 to −868 bp; and 5) there is no specific signal for SHIFT-5 probe (Fig. 7B, lane 8), covering −873 to −708 bp. This shows that KAK-1 nuclear extract contains one or more factors that can bind to the sequence from −1067 to −868 bp in the hNIS promoter, further upstream from NRBS-P. We designated this region as a distal NRBS (NRBS-D).

The core sequence of NRBS-D is homologous to that of NRBS-P and demonstrates cross-competition between both sites

Seven PCR fragments and three annealed double-stranded oligonucleotides were used as unlabeled competitors against the radiolabeled SHIFT-4 probe in EMSA to determine the core sequence for NRBS-D. The seven PCR fragments are as follows: SHIFT-4.1 (150 bp; −1017 to −868), SHIFT-4.2 (100 bp; −967 to −868), SHIFT-4.3 (150 bp; −1067 to −918), SHIFT-4.4 (100 bp; −1017 to −918), SHIFT-4.5 (150 bp; −1017 to −868), SHIFT-4.6 (140 bp;
The three annealed double-stranded oligonucleotides are as follows: ds-411 (5'-tttattcctctgaggcagggtctattttat-3', 30 bp; K1017 to K988), ds-412 (5'-tgaggcagggtctattttatccttgttaca-3', 30 bp; K1007 to K978), and ds-413 (5'-tctattttatccttgttacagatggggaaa-3', 30 bp; K997 to K968). Only the sequences of the sense strands are listed. Probe A and annealed double-stranded Comp-1 (both containing NRBS-P) were also included as cold competitors, as we considered NRBS-D to be an additional binding site for NIS-repressor, which had already been demonstrated to bind to NRBS-P.

Further analysis used an unlabeled annealed double-stranded oligonucleotide (double-stranded SHIFT-414; 5'-ccttgttacagatggggaaaactaaggccca-3', 30 bp; K987 to K958). Results exhibited a strong competition against the radiolabeled Probe A in EMSA (Fig. 8A, lane 5). This suggests that the NIS-repressor, binding to NRBS-D, also binds to NRBS-P. Thus, NRBS-D and NRBS-P can cross-compete efficiently against each other in EMSA, indicating that NIS-repressor, in KAK-1 nuclear extract, can bind to either NRBS-P or NRBS-D in the hNIS promoter region. These results were reproduced using Cal-62 nuclear extract and both NRBS-D and NRBS-P probes in EMSA (data not shown).
AzaC, sodium butyrate, and PJ34 treatments do not alter EMSA signals in KAK-1 and Cal-62 cells

Since AzaC and sodium butyrate increase hNIS transcription in KAK-1 cells (Li et al. 2007), it was intriguing to determine whether the mechanism of their activity was independent of protein binding to NRBS-P using EMSA. Results (Fig. 3A) revealed no change in the gel-shift signals with nuclear extract from KAK-1 cells treated with AzaC (0.5 mM for 2 days). Gel-shift signal intensity increased with greater amounts of nuclear extract (lanes 5, 6, and 8), and diminished with competition by cold Probe A (lane 7). Likewise, nuclear extract prepared from cells treated with sodium butyrate (1 mM for 2 days; Fig. 3A) had a similar lack of effect upon EMSA band patterns, with signal intensity being increased by greater amounts of nuclear extract (lanes 9, 10, and 12) and diminished with competition by cold Probe A (lane 11).

Figure 9 EMSA studies that depict the effects of 5-azacytidine (AzaC), sodium butyrate (NaB), and PJ34 treatments (for 3 days each) of KAK-1 and Cal-62 cells using their respective nuclear extracts and the radiolabeled Comp-1 probe (containing NRBS-P) in (A) or the radiolabeled SHIFT-414 probe (containing NRBS-D) in (B). In both A and B, all lanes have radiolabeled probes and additional reactants as follows: lane 1, EMSA buffer mixture without nuclear extract; lane 2, basal Cal-62 nuclear extract; lane 3, same as lane 2 with 50× cold probe; lane 4, AzaC-treated Cal-62 nuclear extract; lane 5, NaB-treated Cal-62 nuclear extract; lane 6, PJ34-treated Cal-62 nuclear extract; lane 7, basal KAK-1 nuclear extract; lane 8, same as lane 7 with 50× cold probe; lane 9, AzaC-treated KAK-1 nuclear extract; lane 10, NaB-treated KAK-1 nuclear extract; and lane 11, PJ34-treated KAK-1 nuclear extract. The arrows point to probe-specific bands.

Supershift assays suggest that TTF-2 associates with NRBS-P and NRBS-D

In supershift assays, the antibodies against human Sp1 (E-3), c-Jun (H-79), c-Fos (H-125), AP-2α (C-18), TTF-1 (F-12), Pax8 (A-15), and PARP-1 failed to alter the EMSA signal mobilities, suggesting that their respective antigens are not associated with the NRBS-P site.

Figure 10 Supershift experiments evaluating antibodies against thyroid-related transcription factors using Cal-62 nuclear extract with radiolabeled Comp-1 probe (containing NRBS-P) or radiolabeled SHIFT-414 probe (containing NRBS-D). Experiments depicted in A and B were performed using Comp-1 probe, while experiments depicted in C used SHIFT-414 probe. In all three sections, lane 1 contains probe only, with all other lanes containing basal Cal-62 nuclear extract, and lane 3 contains 50× cold respective probes. In (A), specific antibodies were added to respective lanes as follows: lane 4, anti-TTF-1; lane 5, anti-TTF-2 (S-18); lane 6, anti-Pax8; lane 7, anti-Sp1; lane 8, anti-c-Jun; lane 9, anti-c-Fos; lane 10, anti-AP-2α; and lane 11, anti-PARP-1. In both (B and C), specific antibodies were added as follows: lane 4, anti-TTF-2 (S-18); lane 5, anti-TTF-2 (F-17); and lane 6, anti-TTF-2 (V-20).
This is consistent with the results showing that their respective consensus DNA target sequences are unable to compete against NRBS-P. The anti-TTF-2 antibody (S-18) shifted the EMSA signals, changing the mobility of one of the bands, showing faster migration, and simultaneously changing the single Comp-1-specific signal into multiple constituent bands with faster migration on the gel, as shown in Fig. 10A, lane 5. We attempted to further verify this phenomenon with two additional anti-TTF-2 commercial antibodies, recognizing different TTF-2 epitopes. Both these antibodies (F-17 and V-20) failed to alter the EMSA signals as achieved with the S-18 antibody. This indicates that TTF-2 is a constituent of the protein factors responsible for the EMSA signals with NRBS-P (Fig. 10B) and NRBS-D probes (Fig. 10C), suggesting that human TTF-2 is likely to be part of the NIS-repressor complex.

Electrophoretic analysis of factors in KAK-1 nuclear extract u.v.-cross-linked with NRBS-P suggests that NIS-repressor is a protein complex

To evaluate the composition of NIS-repressor, KAK-1 nuclear extract was mixed with radiolabeled Comp-1 probe (−653 to −615 bp, containing NRBS-P) as in the EMSA experiments. This was followed by u.v.-cross-linking, and it was resolved on SDS-PAGE. Figure 11 (lane 5) demonstrates three major regions that are diminished in intensity by the addition of cold Comp-1 probe (A–C). This is likely to result from multiple protein factors bound to the Comp-1 probe, and suggests that NIS-repressor binds to NRBS-P as a protein complex.

The hNIS promoter construct, containing a NRBS-D/NRBS-P double deletion, is still responsive to PJ34 treatment

When transiently transfected into KAK-1 cells, the normalized luciferase activities from F4ΔΔ-pGL3-basic (the NRBS-D/NRBS-P-double deletion reporter construct) were still stimulated by PJ34 treatment (30 μM for 2 days), although at only half of the magnitude of the F4-pGL3-basic plasmid (containing wild-type hNIS promoter), as shown in Fig. 12.

Pharmacological inhibition of hPARP-1 with PJ34 stimulates endogenous hNIS mRNA transcription in both KAK-1 and Cal-62 cells

PJ34 was used to treat KAK-1 cells (30 μM) and Cal-62 cells (24 μM) for 3 days. Cells were collected into TRIzol, and qRT-PCR was used to quantitate...
endogenous human NIS mRNA levels under basal culture conditions and following PJ34 treatment. After normalization to the 18s rRNA level, endogenous human NIS levels were increased by 65-fold in KAK-1 cells, and by 18-fold in Cal-62 cells consequent to PJ34 treatment (shown in Table 1). Analysis of Pax8 mRNA levels in both KAK-1 and Cal-62 cells treated with PJ34 revealed a tenfold stimulation of Pax8 in KAK-1 cells, but no stimulation in Cal-62 cells. This suggests that PJ34 stimulation of hNIS transcription in these cells is not strictly consequent to the expression of Pax8.

Despite increased hNIS mRNA levels consequent to PJ34 treatment, both KAK-1 cells and Cal-62 cells did not express NIS protein as measured by western blot. In addition, evaluation of radioiodide uptake in these cells after this treatment failed to reveal any significant uptake (data not shown).

Table 1 Relative human sodium–iodide symporter (hNIS) mRNA levels in monolayer cultures following treatment with PJ34 (30 µM for 3 days; n = 3)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Treatment</th>
<th>Relative hNIS mRNA level per 10^9 18s RNA</th>
<th>Fold stimulation (relative to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAK-1</td>
<td>Control</td>
<td>≤ 8 ± 2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PJ34</td>
<td>524 ± 370</td>
<td>65</td>
</tr>
<tr>
<td>Cal-62</td>
<td>Control</td>
<td>4 ± 2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PJ34</td>
<td>71 ± 8</td>
<td>18</td>
</tr>
</tbody>
</table>

Discussion

Radioiodine therapy remains the only known effective systemic tumoridical treatment for thyroid carcinomas. Unfortunately, around 10% of such cancers and most dedifferentiated thyroid cancers fail to concentrate radioiodine consequent to the loss of NIS expression (Robbins et al. 1991, Ain 2000). For this reason, efforts to understand the mechanisms of this loss may lead to new treatments to restore NIS expression, permitting effective therapy with radioiodine. Our previous study provided evidence of a trans- active protein factor (complex) suppressing NIS transcription under basal conditions, possibly accounting for the loss of human NIS expression in some thyroid cancers, in addition to or in combination with other postulated mechanisms that also cause loss of hNIS expression in dedifferentiated thyroid cancer cells. We named this trans-active protein factor, NIS-repressor, and mapped its binding site in the proximal NIS promoter (NRBS; NRBS-P) (Li et al. 2007). This repressor may function in concert with or independent of epigenetic effects on NIS expression via NIS promoter methylation and histone deacetylation (Venkataraman et al. 1999).

In an effort to characterize and identify the composition of NIS-repressor, we further refined NRBS-P to sequences from −648 to −620 bp, and found an additional NRBS at −987 to −958 bp (NRBS-D; relative to the NIS translation start site). The homology between NRBS-D and NRBS-P core sequences is 83% in a 23 bp region, with two A/G and two T/C transitions. This 23 bp consensus sequence (5’T-G(G/A)GCT(T/C)A(G/A)TTTCCCCA(T/C)CTGT-3′) is in opposite orientation between NRBS-P and NRBS-D in the hNIS promoter that are 310 bp apart from each other. A human genome homology search (NCBI/BLAT/blastn suite) shows this consensus sequence to occur (at >90% homology) within two kilobases of the 5′-end of more than 20 different genes in the human genome. Among them are genes for kinases, receptors, and transporters. The significance of any functional effects of NIS-repressor upon these genes is intriguing, but unknown at this point.

EMSA evidenced that proteins in KAK-1 nuclear extract bind to NRBS-P, and these are considered to constitute NIS-repressor. Electrophoretic analysis of these nuclear extract proteins, which were u.v.-cross-linked to the radiolabeled NRBS-P probe, revealed multiple bands, suggesting that NIS-repressor is a protein complex. Several thyroidal transcription factors (Sp1, AP-1, AP-2, TTF-1, and Pax8), previously shown to affect NIS transcription, were excluded as candidates for NIS-repressor components. This is because double-stranded oligonucleotides (containing their respective consensus DNA-binding sites) failed to compete against a radiolabeled NRBS-P probe in EMSA, and the supershift experiment using antibodies against these transcription factors did not alter the EMSA signals. Unexpectedly, one antibody against hTTF-2 (S-18), but not two other antibodies (F-17 and V-20), altered the migration of the probe–protein complex in the supershift assays, suggesting that human TTF-2 is associated with, or part of, the NIS-repressor complex.

In consideration of the effects of AzaC and NaB on restoring NIS transcription (Venkataraman et al. 1999), it was interesting to note that these agents did not alter the EMSA pattern using either KAK-1 or Cal-62 nuclear extracts. This suggests that NIS-repressor represents a mechanism of hNIS gene regulation that is likely independent from postulated epigenetic mechanisms.

The hPARP-1 (EC 2.4.2.30) was identified, by proteomic analysis of the nuclear extract from KAK-1
cells, as the principal candidate for a component of the NIS-repressor complex. PARP-1 was initially known for its role as a DNA-damage sensor, repair, and signaling protein. Later studies have shown that PARP-1 also participates in additional critical cellular activities, such as apoptosis, genetic stability, and gene transcription (Schreiber et al. 2006). PARP-1 was reported to be able to bind to regulatory sequences by itself (Zhang et al. 2002, Chiba-Falek et al. 2005), modify some transcription factors or signal proteins by poly(ADP-ribosyl)ation (Miyamoto et al. 1999), and influence other protein factors by heterocomplex formation (Simbulan-Rosenthal et al. 2003). A recent study has revealed that PARP-1 has widespread effects upon transcription of diverse genes, either as a positive or negative transcription factor (Krishnakumar et al. 2008).

The ChIP analysis of Cal-62 cells with anti-PARP-1 antibody shows that PARP-1 is associated with NRBS-P and NRBS-D in Cal-62 cells under basal culture conditions without NIS transcription. Furthermore, PJ34, an agent that has been demonstrated as an inhibitor of PARP-1 enzymatic activity (Abdelkarim et al. 2001), effectively stimulated luciferase activity from NIS promoter constructs and also stimulated endogenous hNIS transcription in both KAK-1 and Cal-62 cells, confirming that PARP-1 is part of a negative regulatory factor for hNIS gene transcription. Despite the ChIP data indicating that PARP-1 is associated with the hNIS promoter region containing NRBS-P and NRBS-D, a commercial anti-hPARP-1 polyclonal antibody (that had been effective in the ChIP assay) failed to alter the EMSA pattern in supershift analysis, possibly consequent to inaccessibility of the epitopes within the protein complex.

EMSA studies with commercial preparations of hPARP-1 protein failed to produce the same signals as did the nuclear extract from KAK-1 cells or Cal-62 cells. This purified commercial PARP-1 protein also failed to reproduce the same EMSA signals as the nuclear extract from Cal-62 cells when using nicked or nicked/1 bp deletion (gapped) NRBS-P probes, suggesting that PARP-1 affinity for nicked DNA could not account for NRBS binding. Our working hypothesis is that PARP-1 does not directly bind to the NRBS sequence; rather, it is associated with other proteins that contain the critical NRBS-binding domain. In addition, PARP-1 may participate in NIS-repressor activity through its poly(ADP-ribosyl)ation activity. When this is inhibited by PJ34, it may compromise the assembly, stability, or activity of the NIS-repressor protein complex.

Overexpression of Pax8 has been reported to restore hNIS expression in ARO cells (now re-identified as a colon cancer-derived cell line, HT-29 (Schweppe et al. 2008); just as with KAK-1; Presta et al. 2005); however, when we treated KAK-1 with PJ34, Pax8 mRNA increased tenfold above control cells, suggesting that Pax8 may be involved in the effect of PJ34 on hNIS transcription in KAK-1 cells. Despite this, PJ34 treatment did not affect the Pax8 mRNA level in Cal-62 cells despite increasing hNIS mRNA levels. This indicates that Pax8 stimulation is not necessary for the effect of PJ34 on hNIS transcription in the genotypically verified human thyroid anaplastic cancer cell line, Cal-62.

The response of the NRBS-D/NRBS-P-double deletion reporter construct to PJ34 is puzzling. There are a number of possible explanations. For one, it is possible that the 300 bp region between NRBS-P and NRBS-D contains binding site(s) for stimulatory transcription factors, and these sites are made sterically inaccessible by the NIS-repressor complexes bound to NRBS regions in the intact promoter, becoming accessible to stimulate transcription in the double deletion promoter construct. Alternatively, since NRBS has been found to be associated with a wide range of genes, it is possible that the levels of additional trans-active regulatory factors, independent of the NRBS sequences on our reporter constructs, are altered by PJ34 treatment. This could attenuate the expected loss of response to PJ34 through removal of the two NRBS sequences. These different, but not mutually exclusive, possibilities warrant further investigation.

Since our data suggest that PARP-1 represses hNIS expression, while in a protein complex that we term NIS-repressor, and that PARP-1 also functions independently in multiple intracellular roles, it is not possible to gauge the activity of NIS-repressor by measurement of PARP-1 expression. When the NIS-repressor constituents are fully characterized, it will be interesting to see if NIS-repressor is overexpressed, compared with normal thyroid tissue or differentiated thyroid cancers, in dedifferentiated thyroid cancers, or in thyroid cancers that are unable to concentrate radiiodine. These NIS-repressor constituents could provide additional therapeutic targets for restoration of iodide transport.

Although we found that PJ34 treatment of KAK-1 and Cal-62 cells increased the endogenous hNIS mRNA level significantly, we could not find hNIS protein expression after this treatment with western blotting nor functional NIS activity using radiiodide uptake assays. This appears to be related to low hNIS mRNA levels, even after PJ34 treatment, as
radioiodide uptake activity was evident in both KAK-1 and Cal-62 cells after they were transfected with an hNIS-coding sequence driven by a cytomegalovirus promoter in the pCR3.1 vector. This suggests that the translation of mature hNIS mRNA and the posttranslational processing of hNIS protein are intact in these cell lines.

KAK-1 has been used as a model system in thyroid cancer research for more than 16 years (Ain et al. 1992). Unfortunately, in mid-2007, after two laboratories introduced STR genotyping into their analysis of thyroid cancer cell lines, it became apparent that many established thyroid cancer cell lines, including KAK-1, were cross-contaminated by other cell lines. The STR genotype of KAK-1 has been demonstrated to match that of the HT-29 colon cancer cell line (Schweppe et al. 2008), suggesting it to be a poor model system to study thyroid cancer. Serendipitously, colon carcinoma cells have been reported to express intracellular hNIS (Wapnir et al. 2003), providing a rationale for use of our KAK-1 data regarding hNIS transcriptional regulation. Additionally, we provide confirmation of key elements of this study using STR-verified thyroid carcinoma cell lines and histologically confirmed thyroid cancer tissues. Nonetheless, all future works on hNIS regulation in thyroid cancer must be performed using bona fide cell lines.

In summary, we have defined two core sequences in the hNIS promoter, NRBS-D and NRBS-P, which are both binding sites for a trans-active transcriptional repressor, NIS-repressor. Proteomic analysis revealed PARP-1 as an important constituent of the NIS-repressor protein complex. A known inhibitor of PARP-1 enzymatic activity, PJ34, causes increased endogenous transcription of hNIS in genotypically verified thyroid cancer cells. Supershift data also suggest that TTF-2 is likely to be a component of the NIS-repressor complex. The NRBS consensus sequence may have regulatory importance for multiple diverse human genes. For clinical thyroid oncology, NIS-repressor may ultimately prove a useful target in efforts to restore the effectiveness of radiiodine therapy to dedifferentiated thyroid cancers.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-09-0156.

Declaration of interest
W Li has nothing to declare. K B Ain is an inventor on US Patent no. 6,015,376 (issued on 1/18/00) and US Patent no. 7,029,879 (issued on 4/18/06).

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