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MOLECULAR MECHANISM OF THE ACTIVATION OF THE
RET/PTC1 ONCOGENE IN PAPILLARY THYROID CARCINOMAS
AND CHARACTERIZATION OF THE PROMOTER OF THE RAT
SODIUM IODIDE SYMPORTER GENE

DISSERTATION

Presented in Partial fulfillment of the Requirements for the Degree Doctor of Philosophy
in the Graduate School of The Ohio State University

By

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****

The Ohio State University

1997

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ABSTRACT

The PTC1 chimeric oncogene is generated by the rearrangement between the ret proto-oncogene and another gene named H4 (D10S170). The ret proto-oncogene encodes a receptor tyrosine kinase with GDNF (Glial cell line derived growth factor) as its ligand. The function of H4 is still unknown. Intrachromosomal rearrangement between the ret proto-oncogene and the H4 gene results in the fusion of the N-terminus of H4 to the tyrosine kinase domain of c-RET, forming the PTC1 oncogene.

The PTC1 oncogene has been detected only in human papillary thyroid carcinomas, but not in other type of tumors. Papillary thyroid carcinoma arises from thyroid follicular cells, where the ret proto-oncogene is not expressed. However, the expression of the PTC1 oncogene in papillary carcinoma is controlled by the H4 gene promoter. In order to study the transcriptional regulation of the PTC1 oncogene and to test the possibility that the specificity of the PTC1 oncogene to thyroid tumors is caused by the thyroid specific expression control of the H4 gene promoter, I first investigated the features of the H4 gene promoter. The results showed that H4 was expressed in various human tissues, including thyroid. The H4 gene promoter was localized to a region within 259 bp upstream of the ATG site by luciferase reporter assay. Furthermore, the H4 promoter can drive reporter gene expression in both thyroid and non-thyroid cells. Since the H4 gene is expressed in thyroid and non thyroid tissues, the specificity of the PTC1 oncogene to thyroid cancers cannot be explained by the regulatory control of expression of the H4 gene promoter.

The c-RET tyrosine kinase is activated by ligand-induced dimerization of the c-RET proteins. However, the RET tyrosine kinase in the PTC1 chimeric oncoprotein is constitutively activated. I hypothesized that the H4 portion of the PTC1 oncoprotein contains an
oligomerization domain which causes constitutive oligomerization and subsequent constitutive tyrosine kinase activation. Protein sequence analysis of the PTC1 oncprotein indicated that a potential leucine zipper domain, known to promote protein oligomerization, is present in the N-terminal region of H4. Indeed, oligomerization of PTC1 oncprotein was demonstrated by an in vitro assay using recombinant proteins containing the leucine zipper region. I further demonstrated that the PTC1 oncprotein forms dimers in vivo, and that the leucine zipper is responsible for this dimerization. I also demonstrated that the H4 leucine zipper mediated dimerization is essential for tyrosine hyper-phosphorylation and for the transforming activity of the PTC1 oncprotein. In addition, the transforming activity of PTC1 in NIH3T3 cells can be suppressed by introducing an inactive form of PTC1 oncogene into PTC1 transformed cells, presumably by forming inactive hetero-dimers between the two forms of PTC1.

The PTC1 oncogene causes not only transformation but also dedifferentiation of thyroid follicular cells. The iodide uptake activity, one of the differentiation markers of thyroid follicular cells, was lost in PTC1 oncogene transfected thyroid cells. The mRNA expression of Na⁺/I⁻ symporter (NIS), the molecule that mediates the active iodide uptake process in the thyroid gland, is down-regulated by PTC1 transformation. To determine the mechanisms involved in the transcriptional regulation of the NIS gene, a 16.4 kb genomic DNA fragment containing the 5' flanking region of the rat NIS gene was isolated and characterized. The transcription start sites of the rat NIS gene was localized to 98 bp upstream of the translation initiation site (ATG). A series of 5' flanking genomic DNA fragments, ranging from 234 bp to 8 kb, were tested for promoter activity by the luciferase reporter assay in both thyroid and non-thyroid cells. The DNA regulatory elements within the 8 kb of the 5’ flanking region were not sufficient to confer thyroid-specific transcription. Finally, consistent with the reduced radioiodide uptake activity observed in transformed thyroid cells, I demonstrated that the promoter activity of the rat NIS gene was suppressed in the PTC1 oncogene transformed NIH3T3 cells.

Taken together, in this dissertation, I studied the transcriptional regulation of the PTC1 oncogene and demonstrated that constitutive dimerization of the PTC1 oncprotein is essential for PTC1 transforming activity in the thyroid. In combination with other studies, I conclude
that constitutive oligomerization acquired by rearrangement or by point mutations may be a general mechanism for the activation of receptor tyrosine kinase oncogenes. In addition, I studied one of the downstream effects of the PTC1 oncogene and demonstrated that the NIS promoter activity is suppressed in PTC1 transformed cells.
ACKNOWLEDGMENTS

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PUBLICATIONS

1. Qiang Tong, Shunhua Xing and Sissy M. Jhiang "Leucine zipper mediated dimerization is essential for the hyperphosphorylation and oncogenic activity of PTC1 oncoprotein" The Journal of Biological Chemistry, 1997, 272, 9043-9047

2. John E. Sagartz, Sissy M. Jhiang, Qiang Tong, and Charles C. Capen "Thyroid stimulating hormone promotes growth of thyroid carcinomas in transgenic mice with targeted expression of the ret/PTC1 oncogene" Laboratory Investigation, 1997, 76, 307-318


5. Qiang Tong, Yishuan Li, Patricia A Sminik, Linda Fithian, Shunhua Xing, Ernest L Mazzaferri and Sissy M Jhiang "Characterization of promoter region and oligomerization domain of H4(D10S170), a gene frequently rearranged with the ret proto-oncogene" Oncogene, 1995, 10, 1781-1787

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FIELD OF STUDY

Major field: Biochemistry
Molecular Biology
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<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NIS</td>
<td>sodium iodide symporter</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>Tg</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>TPO</td>
<td>Thyroid peroxidase</td>
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<td>TSHr</td>
<td>Thyrotropin receptor</td>
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<td>GDNF</td>
<td>Glial cell line derived growth factor; serves as ligand for c-RET</td>
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<tr>
<td>ret proto-oncogene</td>
<td>Gene coding for the c-RET tyrosine kinase receptor, which binds to the ligand GDNF. The ret proto-oncogene is expressed in the central and peripheral nervous systems, as well as the excretory system</td>
</tr>
<tr>
<td>c-RET</td>
<td>The protein product of the ret proto-oncogene; Without ligand binding, its tyrosine kinase is inactive</td>
</tr>
<tr>
<td>PTC1 oncogene</td>
<td>The rearranged, activated form of the ret proto-oncogene, detected in human papillary thyroid carcinoma</td>
</tr>
<tr>
<td>PTC1 oncoprotein</td>
<td>The protein product of the PTC1 oncogene; Its tyrosine kinase is constitutively activated</td>
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CHAPTER 1

INTRODUCTION

Thyroid and Its Function

The thyroid gland, positioned just below the larynx, has two lobes that lie on either side of the trachea and are connected anteriorly. The basic structural unit of the mature thyroid is the thyroid follicle. These hollow spherical follicles are lined with a single layer of epithelial (follicular) cells, which synthesize thyroid hormones. The interior of the follicle is filled with a viscous colloid containing thyroglobulin which is secreted by follicular cells. Between the follicles, are the parafollicular cells with a neurocrest origin. The parafollicular cells are also called C-cells, because they produce calcitonin (Braverman and Utiger 1996).

The thyroid follicles actively accumulate iodide (I\textsuperscript{-}) from the blood by the sodium iodide symporter. On the apical membrane the iodide is oxidized to iodine (I\textsubscript{2}) by thyroid peroxidase on to the tyrosine residues of the thyroglobulin. The attachment of one or two iodines to the tyrosine produces moniodotyrosine (MIT) or diiodotyrosine (DIT) respectively. Two DIT can be coupled to form a tetraiodothyronine (T\textsubscript{4}). One MIT and one DIT can form a triiodothyronine (T\textsubscript{3}) molecule. Upon TSH stimulation, the follicular cells take up colloid and hydrolyze T\textsubscript{4}/T\textsubscript{3} from thyroglobulin and release the free T\textsubscript{4}/T\textsubscript{3} into the blood stream (Braverman and Utiger 1996).

The function of the thyroid is primarily regulated by thyroid-stimulating hormone (TSH, thyrotropin) released from the pituitary gland, which itself is regulated by the thyrotropin-releasing hormone (TRH) secreted from the hypothalamus (Braverman and Utiger 1996).
Thyroid Cancers

Thyroid cancer is a rare disease, accounting for less than 1% of cancer deaths. However, there are wide variations in the degree of malignancy, ranging from the relatively benign adenoma to the most malignant anaplastic carcinoma (Wynford-Thomas, 1993). Adenoma, follicular carcinoma, and papillary carcinoma are derived from the thyroid follicular cells, while medullary carcinoma is derived from the parafollicular cells. As for the highly dedifferentiated anaplastic carcinoma, its origin is unclear. Adenomas are solitary lesions composed of well-differentiated follicles. Follicular carcinomas, like adenomas, maintain the follicular formation. However, they are frequently associated with blood vessel invasion and metastasis to bone, brain, lung, and liver. In contrast, papillary thyroid carcinomas show papillae structure instead of follicles. However, the current diagnostic criteria of papillary carcinoma are its cytological features, the presence of large, light-stained nuclei with “grooved” appearance. Papillary thyroid carcinomas tend to have local lymphatic and lymph-node metastasis. Anaplastic carcinomas are quite malignant and occur predominantly in the elderly (Wynford-Thomas, 1993).

It is interesting that a single epithelial cell type (the thyroid follicular cell) can give rise to different tumor types. The study of thyroid carcinoma is important because it provides a special system to study the effects of different oncogenes and tumor suppressor genes on tumor initiation and progression.

Genetic Defects In Thyroid Cancers

The genetic abnormalities acquired by cancer cells lead to abnormal growth either by activation of growth-stimulating oncogenes or by inactivation of tumor-suppressor genes. Several oncogenes or tumor suppressor genes have been found to be involved in the development of thyroid carcinomas.

TSH stimulates the growth and function of thyroid follicular cells mainly through the cAMP pathway. TSH binds to TSH receptor (TSHR) and activates adenyl cyclase through G proteins. Point mutations in TSHR, the α subunit of Gs (gsp gene) and Gi2 (gip gene), which
lead to a constitutively elevated cAMP level, have been identified in thyroid adenomas (Russo, et al., 1995; Esapa, et al., 1997).

Mutations in the ras gene are the most common genetic defects in thyroid tumors. Point mutations in the ras genes that result in impairment of the GTPase activity of the RAS protein have been identified in about 50% of all types of thyroid tumors (Wynford-Thomas, 1993). It is believed that follicular carcinomas arise from preexisting adenomas, whereas most cases of papillary cancers do not (Wynford-Thomas, 1993). Furthermore, it was observed that ras was mutated more frequently in follicular tumors than in papillary tumors (Shi, et al., 1991). This pointed out a possible role for ras mutation in follicular type of thyroid tumors (Fig. 1.1).

Abnormalities of several receptor tyrosine kinases have been found in papillary thyroid carcinomas. Up to now, three forms of ret/PTC oncogenes have been identified in human papillary thyroid carcinomas. In addition, the trk gene which encodes the receptor for nerve growth factor is activated in about 10% of papillary carcinomas, where the tyrosine kinase domain of the TRK is fused with the TPR protein (Grieco, et al., 1992). In total, the rearrangement of ret or trk has been detected in 50% of human papillary thyroid carcinomas (Pierrotti, et al., 1995). Furthermore, overexpression of the met proto-oncogene, which encodes for the hepatocyte growth factor receptor, has been detected in more than 70% of papillary carcinomas (Di Renzo, et al., 1995). These findings indicate that activation of tyrosine kinase receptors is associated with the papillary type of thyroid tumors (Fig 1.1).

In addition to oncogenes, mutations of tumor suppressor genes have been detected in thyroid cancers. The p53 tumor suppressor gene is the most common genetic abnormality identified in human tumors. As mutations of the p53 gene have been detected in dedifferentiated anaplastic thyroid carcinomas, it was suggested that functional p53 may be essential for maintaining the differentiated phenotype of thyroid carcinoma (Fagin, 1995)(Fig. 1.1). The p16INK4a protein is an important negative regulator of mammalian cell proliferation. Loss of p16 activity has been found in a wide range of human cancers. Homozygous deletion of the entire p16 coding sequence was observed in two of three follicular and two of four papillary cancer cell lines. This high frequency of p16 mutation in differentiated thyroid tumors
suggests that p16 mutation might be a critical factor in the genesis of differentiated thyroid cancer (Jones, et al., 1996).

The ret Proto-oncogene

The ret proto-oncogene (Fig. 1.2) encodes a receptor-type tyrosine kinase (Takahashi, et al., 1988; Tahira, et al., 1990), whose ligand has been identified as glial-cell-line-derived neurotrophic factor (GDNF) (Trupp, et al., 1996; Treanor, et al., 1996; Jing, et al., 1996). The ret proto-oncogene was found to be expressed predominantly in certain cell lineages of the central and peripheral nervous systems, as well as the excretory system (Pachnis et al., 1993). Furthermore, the expression of the ret proto-oncogene was detected in some tumors of neural crest origin (Santoro, et al., 1990). A variety of mutations involving the ret proto-oncogene have been found to be associated with a number of human neuro-endocrine diseases, such as multiple endocrine neoplasia type 2 inherited cancer syndromes (Mulligan, et al., 1993; Donis-Keller, et al., 1993; Hofstra, et al., 1994), the congenital developmental defect Hirschsprung's disease (Romeo, et al., 1994), and some sporadic medullary thyroid carcinomas (Eng, et al., 1994).

The ret/PTC Oncogenes

The ret transforming gene (Fig. 1.2) was first identified in NIH3T3 transformants transfected with T cell lymphoma DNA. It was found that this oncogene was generated by rearrangement between an unknown gene rfp and the ret proto-oncogene (Takahashi and Cooper 1985). This rearrangement appears to happen during DNA transfection, because it was not detected in the original tumor. Additional in vitro rearranged forms of ret (retII and ret III, Fig. 1.2) have been isolated from NIH3T3 transformants transfected with human colon cancer DNA and human stomach carcinoma DNA (Ishizaka, et al., 1988; Koda 1988). In contrast, the ret/PTC oncogenes occurred in vivo, since their presence was confirmed in human papillary thyroid carcinomas. Up to now, three different forms (Fig. 1.2) of the ret/PTC oncogenes have been identified, in which the RET tyrosine kinase domain becomes fused with the N-terminal sequences of three different genes (Grieco, et al., 1990; Bongarzone, et al., 1993; Santoro, et
The PTC1 chimeric oncogene, formed by intra-chromosomal rearrangement between H4 (D10S170) and the ret proto-oncogene, has been detected in 2.5-30% of papillary thyroid carcinomas (Grieco, et al., 1990; Jhiang, et al., 1992; Jhiang and Mazzaferri, 1994). The PTC rearrangements occur only in papillary thyroid carcinomas and have not been detected in any non-thyroid tumors (Santoro, et al., 1993b). The product of the PTC1 oncogene is a fusion protein containing the N-terminus of H4 fused to the tyrosine kinase domain of the ret proto-oncogene (Grieco, et al., 1990). The H4 gene shows no significant homology to known genes and the function of H4 protein is currently unknown (Grieco, et al., 1994).

The PTC1 oncoprotein has been demonstrated to be hyperphosphorylated (Ishizaka, et al., 1992) and to exert transforming activity in NIH3T3 cells (Fusco, et al., 1987). The causative role of the PTC1 oncogene in human papillary thyroid carcinoma is further supported by the fact that our transgenic mouse model with targeted expression of the PTC1 oncogene in the thyroid gland develops papillary thyroid carcinomas (Jhiang, et al., 1996). As dimerization is considered to be a crucial step for receptor tyrosine kinase activation (Honegger, et al., 1990; Ullrich and Schlessinger, 1990), I hypothesized that both unscheduled expression of RET tyrosine kinase and constitutive oligomerization of PTC oncoproteins are responsible for their transforming activity in the thyroid. In chapter 2, I have demonstrated that the PTC1 chimeric oncogene shows unscheduled expression in the thyroid follicular cells and that recombinant proteins containing the putative leucine zipper domain of H4 form oligomeric complexes in vitro. In chapter 3, I further demonstrated that the leucine zipper of H4 is essential for the PTC1 dimerization in vivo, and for its tyrosine kinase activation and transforming activity.

The Sodium Iodide Symporter

The PTC1 oncogene not only causes transformation but also dedifferentiation of thyroid follicular cells. The iodide uptake activity, one of the differentiation markers of thyroid follicular cells, was lost in PTC1 oncogene transfected thyroid cells. The active iodide uptake by the normal thyroid gland, facilitate by the Na+/I symporter (NIS), is essential for the synthesis of the iodine-containing thyroid hormone (Taurog, et al., 1996). Transport of iodide
across the thyroid cell membrane is coupled to the transport of sodium, with the Na⁺ gradient generated by Na⁺-K⁺ ATPase as the driving force. The iodide transport system of the thyroid ensures that the radioiodide specifically reaches its target tissue. Thus, radioiodide has been used clinically to treat thyroid diseases including thyroid cancer. However, thyroid tumors take up radioiodide at much lower levels compared to normal thyroid tissues. 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 (Smanik, et al., 1997). Furthermore, reduced radioiodide uptake activity in the thyroid gland of our ret/PTC1 transgenic mouse is accompanied by down-regulation of the expression of mouse NIS (Cho, et al., manuscript in preparation). Indeed, many studies have shown that radioiodide uptake activity is readily lost when various oncogenes were expressed in immortalized rat thyroid cells (Trapasso, et al., 1996; Berlingieri, et al., 1993; Santoro, et al., 1993a). To ensure the effectiveness of radioiodide therapy, 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. In Chapter 4, I characterized the 5’ flanking genomic DNA fragments of the rat NIS gene to study the transcriptional regulation of NIS.
Thyroid follicular cells

\[ \text{ras} \quad \text{gsp} \quad \text{gip} \]

\[ \text{ret} \quad \text{trk} \quad \text{met} \]

Adenoma

Follicular carcinoma

Anaplastic carcinoma

Papillary carcinoma

\[ p53 \]

Fig. 1.1 Genetic defects in thyroid tumors (adapted from Wynford-Thomas)
Fig. 1.2 Schematic representation of the structure of \textit{ret} proto-oncogene and \textit{ret} rearrangements occurring during DNA transfection or in human papillary thyroid carcinomas.
CHARPTER 2

CHARACTERIZATION OF THE PROMOTER REGION AND OLIGOMERIZATION DOMAIN OF H4 (D10S170), A GENE FREQUENTLY REARRANGED WITH THE RET PROTO-ONCOGENE

Introduction

The H4 (D10S170) gene has been found to be rearranged with the ret proto-oncogene, to form the PTC1 oncogene, in 2.5 - 30 % of papillary thyroid carcinomas (Grieco et al., 1990; Jhiang et al., 1992; Santoro et al., 1992; Jhiang and Mazzaferri, 1994). The ret proto-oncogene encodes a tyrosine kinase receptor (Takahashi & Cooper, 1987; Takahashi et al., 1988; Tahira et al., 1990). Recently, germline mutations of the ret proto-oncogene have been found to be associated with three inherited cancer syndromes (Mulligan et al., 1993; Doniskeller et al., 1993; Hofstra et al., 1994; Eng et al., 1994; Carlson et al., 1994), and one congenital developmental defect (Romeo et al., 1994; Edery et al., 1994). The ret proto-oncogene was found to be expressed predominantly in certain cell lineages of the central and peripheral nervous systems, as well as the excretory system (Pachnis et al., 1993). Furthermore, the expression of the ret proto-oncogene was detected in some tumors of neural crest origin (Santoro, et al., 1990). Normally, the ret proto-oncogene is not expressed in thyroid follicular cells (Fabien et al., 1992), from which papillary thyroid carcinoma develops. However, since the PTC1 fusion protein contains the amino-terminus of H4 linked to the ret
tyrosine kinase domain, the expression of the chimeric PTC1 oncogene in papillary thyroid carcinoma is driven by the H4 gene promoter.

In the present study, the expression of the H4 gene in various human tissues was investigated, and the 5' flanking region of the H4 gene was cloned and initially characterized. We found that a short DNA segment from the 5' flanking region of the H4 gene was sufficient to promote the H4 gene expression in thyroid and non-thyroid cell lines.

In addition, protein sequence analysis indicated a potential leucine zipper (also called coiled-coil) domain in the N-terminal region of H4. Using an in vitro oligomerization assay, we showed that recombinant proteins containing the N-terminus of H4 can form oligomers. As dimerization is considered to be a crucial step for receptor tyrosine kinase activation (Honegger et al., 1990, Ullrich & Schlessinger, 1990), we hypothesize that both unscheduled expression of ret tyrosine kinase and constitutive oligomerization of PTC1 proteins are responsible for PTC1 transforming activity in thyroid.
Materials and methods

Cell lines

TPC-1 is a human papillary thyroid carcinoma cell line which expresses the PTC1 oncogene. PTC-UC1 is a human papillary thyroid carcinoma cell line. HeLa (ATCC CCL2) is a human cervical epithelioid carcinoma cell line. 8505C is a poorly differentiated human papillary adenocarcinoma cell line. Both TPC-1 and HeLa were maintained in DMEM (Dulbecco’s Modified Eagle Media) supplemented with 10% fetal bovine serum. PTC-UC1 was maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, 10 mU/ml TSH and 10 μg/ml insulin. 8505C cells were maintained in Minimum Essential Media supplemented with 10% non-essential amino acids, 4 mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, and 0.18% NaHCO3. All media contain 100 U/ml penicillin and 100 μg/ml streptomycin. All cells are incubated at 37 °C in a 5% CO2 enriched air atmosphere.

Cloning and characterization of the 5’ flanking region of the H4 gene

A leukocyte genomic library (van de Winkel et al., 1991) was screened with a cDNA probe corresponding to the 0.4 kb EcoRI fragment from the N-terminus of the ret/TPC-1 construct (Ishizaka et al., 1990). A positive clone containing a 13.5 kb insert was characterized by restriction enzyme mapping. Subsequently, a SacI - SalI 3 Kb fragment (the SacI site is located in the cloning site of the vector) was cloned into the pBluescript KS vector (Stratagene, La Jolla, CA 92037). Finally, the 0.5 Kb Sau3AI-SalI fragment was subcloned into the pBluescript KS vector and subjected to nucleotide sequencing, using Sequenase Kit from USB (Cleveland, OH 44122).

RT-PCR analysis of H4 expression

Total RNA was extracted from frozen human tissues and cell pellets using the method of Puissant and Houdebine (1990). RNA was analyzed by electrophoresis for its quality and quantity. One microgram of total RNA was reverse transcribed in a 20 μl reaction volume; 1 μl of product was used as a template for PCR. The primers used to amplify the H4 cDNA
fragments are derived from exon 1 and exon 2 of H4. The forward primer sequence is 5'-GTCGGGGGCATTTGCATCT-3', and the reverse primer sequence is 5'-TGAATTCTTCTTCGCTCAG-3' (Ito et al., 1993). As a control for cDNA integrity, a second pair of primers was used to amplify the retinoblastoma tumor suppressor (Rb) cDNA fragments (Lee et al., 1987). The forward primer sequence is 5'-AAGCAACCTCAGCCTCCAG-3', and the reverse primer sequence is 5'-CCTGGTGGAAGCATACTGCA-3'. The PCR reactions were carried out in 67 mM Tris-HCl, pH 8.3, 16.6 mM (NH₄)₂SO₄, 2.8 mM β-mercaptoethanol, 10% DMSO, 1 mM MgCl₂, 0.2 mM dNTP, 0.5-1.5 μM primers, and 0.05 U/μl Taq polymerase. The reaction profile consisted of one cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 2 min.

Engineering of luciferase reporter constructs

To characterize the H4 promoter region, the 3 Kb SacI-NcoI fragment and the 444 bp Sau3AI-NcoI fragment were separately cloned into the pGL2-basic vector (Promega, Madison, WI 53711). Subsequently, different lengths of the 5' flanking fragments were generated by unidirectional deletion from 5' end of the 444 bp Sau3AI-NcoI fragment.

Assay for promoter activity

The plasmids used for transfections were purified using the Maxi plasmid purification kit (QIAGEN, Santa Clarita, CA 91355), according to the manufacturer's protocol. The TPC-1 or PTC-UC1 cells were plated approximately 18 hr before transfection. To monitor the transfection efficiency, the pCH110 β-gal DNA construct (Pharmacia, Piscataway, NJ 08855) was co-transfected with the luciferase reporter constructs by lipofectin (GIBCO BRL, Gaithersburg, MD). The transfection medium was replaced after 8 hr incubation. The cells were harvested after an additional 40 hr incubation and tested for luciferase and β-galactosidase activity. For HeLa cells, the media was replaced 6 hr after transfection with lipofectAmine (GIBCO BRL, Gaithersburg, MD), and the cells were harvested after an additional 30 hr incubation. Luciferase activity and β-galactosidase activity were measured using the luciferase assay system (Promega, Madison, WI 53711), and the Galacto-Light
chemiluminescent β-galactosidase reporter assay system (Tropix, Bedford, MA 01730), respectively.

Expression of the recombinant proteins

The 0.35 kb NcoI-EcoRI fragment of PTCl, corresponding to the N-terminus of H4, was cloned in-frame into the pMALc vector (New England Biolab, Beverly, MA 01915) to generate MBP-H4N. MBP-PTCl was cloned by inserting the ret tyrosine kinase domain into the MBP-H4N construct. The 0.35 kb NcoI-EcoRI fragment was also cloned in-frame into the pGEX-2T vector (Pharmacia, Piscataway, NJ 08855) to generate GST-H4N. Furthermore, GST-H41-55 and GST-H460-101 were generated by specific deletion of the GST-H4N construct. GST-H41-55, encoding amino acid residues 1 to 55 of H4, does not contain the coiled-coil motif. GST-H460-101, encoding amino acid residues 60 to 101 of H4, contains the coiled-coil motif.

The recombinant proteins were expressed and purified essentially as described (Smith and Johnson, 1988). Briefly, protein expression was induced with 0.3 mM IPTG for 2 hr. The cell pellet was resuspended in MT-PBS (150 mM NaCl, 16 mM Na2HPO4, 4 mM NaH2PO4, pH 7.3) supplemented with protease inhibitors, and lysed by sonication. After centrifugation, the supernatant of the cell lysate was collected.

In vitro oligomerization assay

An aliquot of the GST or GST-H4N bacterial cell lysate was mixed with an aliquot of the MBP or MBP-PTCl lysate. MT-PBS was added to a final volume of 500 μl, and the mixtures were incubated overnight at 4 °C with rocking. The GST protein complexes were isolated by adding 20 μl of a 50% suspension of glutathione-agarose beads (sulphur linkage), followed by a 30 min incubation at 4 °C with rocking. After washing with MT-PBS, the bound proteins were dissociated from the beads by boiling in 20 μl SDS-PAGE loading buffer. The proteins were separated by SDS-PAGE and detected by staining with Coomassie Blue or by immunoblotting with an anti-MBP antibody (New England Biolab, Beverly, MA 01915).
Results

*H4 is expressed in various human tissues investigated by RT-PCR.*

The expression of the H4 gene in various human tissues was analyzed by RT-PCR, using primers specific to exon 1 and exon 2 of the H4 gene (Ito et al., 1993). Since the PTC1 rearrangement occurs in intron 1 of the H4 gene, only the unrearranged H4 transcripts can be amplified by these primers. As shown in Fig. 2.1a, H4 is expressed in 8505C cells and various human tissues examined, including thyroid. Interestingly, the TPC-1 cell line, which contains the PTC1 rearrangement, does not exhibit a detectable level of expression of H4. As a control, the retinoblastoma tumor suppressor gene cDNA fragments can be amplified successfully in all samples, including the TPC-1 sample (Fig. 2.1b). Therefore, the inability to amplify the wild type H4 cDNA fragments in TPC-1 cells is not due to compromise of the cDNA integrity, but rather the loss or silence of the wild type H4 allele. Indeed, the wild type H4 allele was demonstrated to be deleted in TPC-1 cells (Jossart, et al., 1996).

The nucleotide sequence of the immediate 5' flanking region of the H4 gene was determined.

A 13.5 Kb genomic DNA fragment containing the 5' region of H4 was isolated and characterized by restriction enzyme mapping (Fig. 2.2a). Subsequently, a DNA fragment of the immediate 5' end of the H4 gene was subcloned and sequenced (Fig. 2.2b). No TATA or CCAAT boxes were identified in this region. The nucleotide sequence is GC-rich and contains several potential Sp1 binding sites, a common feature of "house-keeping" genes which lack a TATA box (Renolds et al., 1984). Several potential AP-2 binding sites were also identified in this region.

The H4 gene promoter is localized within 259 bp upstream of the ATG site.

To investigate whether the 5'-flanking region of the H4 gene contains promoter activity, 3 kb and 0.4 kb genomic fragments of the H4 5' flanking region were separately inserted in front of the promoterless luciferase gene in the pGL2-basic vector. Transient transfection of these two constructs into three different cell lines did result in a significant
increase in luciferase activity (Fig. 2.3). To determine the major promoter region, serial deletions of the 0.4 kb fragment were constructed. As shown in Figure 2.3, deletion from the 5' end of this 0.4 kb fragment to -259 bp did not significantly affect its promoter activity. However, deletion of another 45 bp, to -214, reduced promoter activity to basal level. Therefore, the major H4 promoter region is located in the DNA fragment extending from -259 to +3 in the H4 gene. Interestingly, there are three potential AP2 binding sites located between -259 and -214, which may play a role in the transcriptional regulation of the H4 gene. Consistent with the ubiquitous expression pattern of the H4 gene, promoter activity was shown in three different cell lines investigated. However, in the thyroid cell lines TPC-1 and PTC-UC1, the optimal promoter activity was detected in the fragment from -259 to +3, whereas the optimal promoter activity in HeLa cells was observed in the -3 kb fragment (Fig. 2.3).

The N-terminus of the PTC-1 chimeric protein contains a potential coiled-coil motif, which may mediate constitutive oligomerization.

Analysis of the N-terminal sequence of H4 using the VAX Pascal program COILS 2.0, developed by Lupas et al (1991), revealed a region (Fig. 2.4) with a very high probability of forming a coiled-coil structure. Since coiled-coil domains are known to promote oligomerization of proteins, we designed experiments to test whether H4 forms oligomers in vitro (Fig. 2.5a). The N-terminus of H4 was fused with glutathione S-transferase (GST) to generate the fusion protein GST-H4N. In addition, PTC1 was fused with maltose binding protein (MBP) to generate MBP-PTC1. These fusion proteins, GST-H4N and MBP-PTC1, were separately expressed in E. coli and the crude lysates were incubated together. Glutathione-agarose beads were then applied to the mixtures to pull down the GST fusion proteins and their associated proteins. If the N-terminus of H4 can promote oligomerization, the MBP-PTC1 fusion proteins should associate with GST-H4N and will be precipitated along with GST-H4N by the glutathione-beads. The presence of MBP-PTC1 fusion proteins in the precipitate can be detected by immunoblot analysis using antibodies against MBP. As shown in Fig. 2.5b, the MBP-PTC1 was associated with GST-H4N but not with GST only. We further demonstrated that the presence of the coiled-coil domain is essential for the oligomerization of
the recombinant proteins. MBP-H4N can form oligomers with either GST-H4N or GST-H4Ni, which contains the putative coiled-coil domain, but not with GST or GST-H4N1-55, which lacks the coiled-coil domain (Fig. 2.5c). The results are summarized in Fig. 2.5d.

Discussion

RT-PCR results demonstrated that H4 is expressed ubiquitously in various human tissues investigated, including thyroid. Similar results were obtained by Northern blot analysis by others (Grieco et al., 1994). Therefore, when H4 is rearranged with the ret proto-oncogene, it promotes the unscheduled expression of the PTC1 chimeric protein in thyroid. We further localized the H4 promoter to a region within 259 bp upstream of the ATG site.

The oncogenic activation of the ret proto-oncogene by somatic rearrangement has been found to be restricted to papillary thyroid carcinoma in vivo (Santoro et al., 1993b). Since we demonstrated that the H4 gene is ubiquitously expressed, the specificity of the PTC1 activation cannot be due to thyroid-specific expression of the PTC oncogene. A second hypothesis is that somatic rearrangement of the ret proto-oncogene occurs only in thyroid. Ito et al. (1993) have demonstrated that PTC rearrangement could be induced by in vitro X-irradiation in both thyroid and non-thyroid cells. However, the in vitro X-irradiation of tumor cell lines may not accurately reflect the in vivo response of normal cells to external radiation exposure. Significantly, a high incidence of PTC activation was found in thyroid tumors of children from the area contaminated by the Chernobyl accident. (Ito et al., 1994). These results suggest that radiation exposure, a well recognized etiologic factor for papillary carcinoma, may play an important role in inducing PTC activation in thyroid.

Finally, it is possible that a thyroid-specific signal transduction pathway is required for PTC oncogenic function. However, PTC1 has shown transforming activity in immortalized mouse fibroblast NIH/3T3 cells (Fusco et al., 1987). Furthermore, the ret oncogene (in vitro activated ret) has been shown to induce a variety of tumors other than thyroid, depending on the promoter used to control the transgene expression in mice (Iwamoto et al., 1991a; 1991b).
Therefore, the mechanism underlying the specificity of the PTC oncogene in papillary thyroid carcinoma remains unclear. Transgenic mice expressing the PTC oncogene driven by the authentic H4 control elements would provide a valuable animal model for investigating the specificity of the PTC oncogene in papillary thyroid carcinoma. Our initial characterization of the H4 promoter region provides essential information for the establishment of this transgenic construct.

The PTC1 oncoprotein has been shown to be constitutively phosphorylated (Ishizaka et al., 1992). However, the mechanism underlying the constitutive phosphorylation is unknown. Our data demonstrate that the N-terminus of PTC-1 contains a coiled-coil domain and can form oligomers in vitro. As ligand-induced dimerization is considered to be a crucial step for receptor tyrosine kinase activation (Honegger et al., 1990, Ullrich & Schlessinger, 1990), our results support a model in which the N-terminus of H4 constitutively activates the tyrosine kinase of ret by inducing oligomerization and intermolecular cross-phosphorylation of the ret tyrosine kinase. Currently, three different forms of the PTC oncogene have been identified (Bongarzone et al., 1993; Santoro et al., 1994; Jhiang et al, 1994). The PTC2 oncoprotein has been shown to form dimers in vivo (Bongarzone et al., 1993). Furthermore, a potential coiled-coil domain has been identified in the N-terminus of the PTC3 protein (our unpublished data). In addition, the genes rearranged with the ret proto-oncogene in PTC2 and PTC3 have also been shown to be ubiquitously expressed (Bongarzone et al., 1993; Jhiang et al, 1994). Therefore, unscheduled expression of the ret tyrosine kinase in thyroid, and constitutive oligomerization of the PTC oncoproteins, appears to be a common mechanism of PTC oncogenic activation.

In addition to the PTC oncogene, constitutive oligomerization may serve as a common mechanism for oncogenic activation of other receptor tyrosine kinases. An amino acid substitution of the neu oncogene has been shown to induce oncoprotein aggregation, increased tyrosine kinase activity and transforming activity (Weiner et al., 1989). Furthermore, Rodrigues and Park (1993) have demonstrated that the leucine zipper domain in Tpr mediates dimerization of the tpr-met oncoprotein, and the constitutive dimerization is essential for its transforming activity. They further identified coiled-coil motifs in a number of rearranged
tyrosine kinase oncogenes, and proposed that constitutive dimerization may serve as a general mechanism for oncogenic activation of receptor tyrosine kinases. The common mechanism identified in a variety of tyrosine kinase oncogenes may provide an unique opportunity to design a common strategy to intervene with their oncogenic activity in a variety of tumors.
Fig. 2.1 Detection of the amplified human H4 cDNA fragments by agarose gel electrophoresis. (a) RT-PCR was performed with a pair of primers derived from exon 1 and exon 2 of H4. MW: 1 kb ladder marker (GIBCO BRL, Gaithersburg, MD). 8505C: a poorly differentiated human papillary adenocarcinoma cell line. TPC-1: a human papillary thyroid carcinoma cell line which expresses the PTC-1 oncogene. Control: no DNA template. (b) RT-PCR was performed with a pair of primers to amplify H4 cDNA fragments, along with a pair of primers to amplify the retinoblastoma tumor suppressor (Rb) gene cDNA fragments. PC4, PC9, PC27 and PC31 are human papillary thyroid tumors.
Fig. 2.2 Genomic structure of the 5' flanking region of H4 gene. (a) Restriction map of the 13.5 kb genomic DNA fragment of H4. The translation initiation site (ATG) is shown. B: BglII; E: EcoRI; H: HindIII; S: SalI. (b) Nucleotide sequence of the immediate 5' flanking region of H4. All numbering is relative to the ATG site. Putative binding sites for AP-2 and Sp-1 are indicated.
Luciferase Activity

<table>
<thead>
<tr>
<th>Luciferase Activity</th>
<th>TPC-1</th>
<th>PTC-UC1</th>
<th>Hela</th>
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<tbody>
<tr>
<td>- 3 kb</td>
<td>96</td>
<td>38</td>
<td>11</td>
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<tr>
<td>- 441 bp</td>
<td>82</td>
<td>32</td>
<td>4.5</td>
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<tr>
<td>- 353 bp</td>
<td>75</td>
<td>32</td>
<td>3.5</td>
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<tr>
<td>- 288 bp</td>
<td>87</td>
<td>32</td>
<td>5.9</td>
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<tr>
<td>- 259 bp</td>
<td>120</td>
<td>74</td>
<td>5.3</td>
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<tr>
<td>- 214 bp</td>
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<td>6.4</td>
<td>1.4</td>
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<tr>
<td>- 205 bp</td>
<td>5.2</td>
<td>5.4</td>
<td>1.2</td>
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<tr>
<td>- 145 bp</td>
<td>2.1</td>
<td>3.4</td>
<td>1.6</td>
</tr>
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Fig. 2.3  Transient expression analysis of the H4 gene promoter activity. Luciferase reporter constructs containing various lengths of the H4 5' flanking region were transfected into TPC-1, PTC-UC1 or HeLa cells. A β-gal construct was co-transfected with each construct, to normalize the transfection efficiency. The luciferase activity of each construct is reported relative to the luciferase activity of the pGL2-basic vector. The data represent at least two independent experiments. Duplicate transfections were performed in each experiment, and the variation between duplicates was within 17% of the mean. All numbering is relative to the ATG site.
Fig. 2.4 The N-terminus of the PTC1 chimeric protein, contributed by H4, contains a potential coiled-coil motif (LXXLXXX)n, which may mediate protein oligomerization.
Fig. 2.5 *In vitro* oligomerization of recombinant proteins containing the N-terminus of H4. (a) Procedure of the *in vitro* oligomerization assay. (b) MBP-PTC1 fusion protein was incubated with GST-H4N or GST. (c) MBP-H4N fusion protein was incubated with GST-H4N1-55, GST-H4N62-101, GST-H4N fusion proteins or GST protein. Glutathione-agarose beads were added to the mixtures to pull down GST or GST fusion proteins and other associated proteins. The precipitated complexes were separated by SDS-PAGE and stained with Coomassie blue (top panel) or immunoblotted with an anti-MBP antibody (bottom panel). The position of the MBP-PTC1 or MBP-H4 fusion protein in the corresponding immunoblot is indicated. The additional bands of lower molecular weight detected by the anti-MBP antibody were contributed by a degradation product of the MBP-PTC1 (middle band), and a weak cross-reaction with GST-H4N (lower band). (d) Schematic summary of the *in vitro* oligomerization assay results.
a.

Incubation of recombinant proteins.

Glutathione-agarose beads to pull down GST-protein complex.

SDS-PAGE

Western blot with anti-MBP antibody

(continued on next page)

Fig. 2.5
(Fig. 2.5, continued)

Fig. 2.5
d

(Fig. 2.5, continued)

Fig. 2.5

27
CHAPTER 3

LEUCINE ZIPPER MEDIATED DIMERIZATION IS ESSENTIAL FOR THE ACTIVATION OF THE PTC1 ONCOGENE

Introduction

The PTC1 chimeric oncogene, formed by intra-chromosomal rearrangement between H4 (D10S170) and the ret proto-oncogene, has been detected in 2.5-30% of papillary thyroid carcinomas (Grieco, et al., 1990; Jhiang, et al., 1992; Jhiang and Mazzaferr, 1994). The product of the PTC1 oncogene is a fusion protein containing the N-terminus of H4 fused to the tyrosine kinase domain of the ret proto-oncogene (Grieco, et al., 1990). The H4 gene shows no significant homology to known genes and the function of H4 protein is unknown (Grieco, et al., 1994). The ret proto-oncogene encodes a receptor-type tyrosine kinase (Takahashi, et al., 1988; Tahira, et al., 1990), whose ligand has been identified as glial-cell-line-derived neurotrophic factor (GDNF) (Trupp, et al., 1996; Treanor, et al., 1996; Jing, et al., 1996). A variety of mutations involving the ret proto-oncogene have been found to be associated with a number of human neuro-endocrine diseases, such as multiple endocrine neoplasia type 2 inherited cancer syndromes (Mulligan, et al., 1993; Donis-Keller, et al., 1993; Hofstra, et al., 1994), the congenital developmental defect Hirschsprung's disease (Romeo, et al., 1994), and some sporadic medullary thyroid carcinomas (Eng, et al., 1994).

The PTC1 oncoprotein has been demonstrated to be hyperphosphorylated (Ishizaka, et al., 1992) and to exert transforming activity in NIH3T3 cells (Fusco, et al., 1987). The
causative role of the PTCl oncogene in human papillary thyroid carcinoma is further supported by the fact that our transgenic mouse model with targeted expression of the PTCl oncogene in the thyroid gland develops papillary thyroid carcinomas (Jhiang, et al., 1996). However, the molecular mechanism of PTCl activation in the development of papillary thyroid tumors has yet to be elucidated. We have previously demonstrated that the PTCl chimeric oncogene shows unscheduled expression in the thyroid follicular cells and that recombinant proteins containing the putative leucine zipper domain of H4 form oligomeric complexes in vitro. As dimerization is considered to be a crucial step for receptor tyrosine kinase activation (Honegger, et al., 1990; Ullrich and Schlessinger, 1990), we hypothesized that both unscheduled expression of RET tyrosine kinase and constitutive oligomerization of PTCl proteins are responsible for PTCl transforming activity in the thyroid.

In this study, we further demonstrated that the leucine zipper region of H4 is responsible for the dimerization of the PTCl oncoprotein in vivo. Our data also indicate that the leucine zipper mediated dimerization is essential for tyrosine hyper-phosphorylation and the transforming activity of PTCl. Furthermore, the transforming activity of PTCl can be suppressed by introducing a loss-of-function mutant of PTCl into PTCl transformed NIH3T3 cells.

Materials and methods

Cell Lines and Antibodies

The African green monkey kidney cell line, COS-7 cells (ATCC 1651) was maintained in DMEM (Dulbecco’s Modified Eagle Media) supplemented with 10% fetal bovine serum. Both NIH3T3 and NIH3T3/PTCl were maintained in DMEM supplemented with 10% donor calf serum. All media contain 100 U/ml penicillin and 100 µg/ml streptomycin. All cells are incubated at 37 °C in a 5% CO₂ enriched air atmosphere. The monoclonal antibody MSJ was generated in our lab. Its epitope was tentatively mapped to amino acid residues 824 - 828 of RET (our unpublished data). The polyclonal antibody, C17, was raised against a synthetic
peptide (CKRRDYLDLAASTPSDSL) located at amino acid residues 1011 to 1027 of the C-terminus of the RET tyrosine kinase (Immuno-Dynamics, Inc, La Jolla, CA 92038). The C17 antibody was purified through an affinity column covalently bound with the synthetic peptide. The monoclonal antibody against phosphotyrosine (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY 12946).

**Plasmid Constructs**

The PTC1/CMV was constructed by excising the PTC1 cDNA from the plasmid TPC-1 (Ishizaka, et al., 1992), and inserting it into the XbaI and ApaI sites of pRc/CMV. The PTC1/LNCX was obtained by excising the PTC1 fragment from PTC1/CMV with HindIII and inserting it into the HindIII site of pLNCX. In order to clone PTC1Δzip/CMV and PTC1Δzip/LNCX, the T7 primer and another primer (5'-AAGGATCCCGGAACGGCGAGATGA-3'), which is located to a region just ahead of the leucine zipper, were used to perform PCR using PTC1/CMV as DNA template. The 250 bp PCR product was digested with BamHI and used to replace the 400 bp BamHI fragment of H4 in PTC1/CMV and PTC1/LNCX. In order to clone PTC1ΔN/CMV and PTC1ΔN/LNCX, a sense primer (5'-TATGGATCCGCCATGGCGCCGTCCGCTG-3'), which is located at the beginning of the leucine zipper region, was paired with an anti-sense primer (5'-AGTTCTTCCGAGGGAATTCC-3'), which is located at the beginning of the RET tyrosine kinase domain, to perform PCR on the PTC1/CMV template. A 200 bp BamHI fragment was excised from the PCR product, and used to replace the 400 bp H4 fragment of PTC1/CMV and PTC1/LNCX. The PTC1ΔC/CMV was generated by unidirectional deletion of the PTC1 insert, starting from the C-terminus of PTC1. A mutation (Arg^{897} → Glu), found in some patients with Hirschsprung's disease (Romeo, et al., 1994), was first introduced into c-ret cDNA by site-directed mutagenesis (Muta-gene M13 in vitro mutagenesis kit, Bio-Rad, Hercules, CA 94547) and later subcloned into PTC1/CMV and PTC1Δzip/CMV to obtain PTC1HS/CMV and PTC1ΔzipHS/CMV.
DNA Transfection

For transient transfection, $5 \times 10^5$ COS-7 cells were transfected with 10 - 20 $\mu$g of various DNA constructs using the calcium phosphate transfection kit (GIBCO BRL, Gaithersburg, MD). For the focus formation assay, $1.3 \times 10^5$ NIH3T3 cells were transfected with 0.3 $\mu$g of various DNA constructs. Three weeks later, the foci of transformed cells were stained with Giemsa and counted. For NIH3T3 stable transfectants, $4 \times 10^4$ NIH3T3 cells were transfected with 1 $\mu$g of various DNA constructs, and the G418 resistant colonies were screened for PTC1 expression by immunoprecipitating the cell lysates with the antibody C17, followed by immunoblot analysis with the antibody MSJ.

Immunoprecipitation and Immunoblot Analysis

Cells were lysed in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, and 0.5 mM Na$_3$VO$_4$. For immunoprecipitation, cell lysate were incubated at 4°C overnight with C17 polyclonal antibody which was covalently conjugated to protein A beads (Harlow and Lane, 1988). For the immunoblot assay, proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was blocked in TBST buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) containing 5% non-fat dry milk at 4°C overnight. After incubation with the primary antibody for one hour, and HRP-conjugated anti-mouse IgG for an additional hour, the membrane was treated with ECL reagent (Amersham, Arlington Heights, IL 60005) and exposed to X-ray film.

Cross-linking

The cross-linking experiments were carried out at room temperature with 0.01% glutaraldehyde in 50 mM triethanolamine (pH 8.2) and 100 mM NaCl, using 20-50 $\mu$l lysate of COS-7 cells transfected with various DNA constructs. Aliquots were removed at the times indicated and the reaction was terminated by boiling the mixture in SDS-PAGE sample buffer. The sample was resolved by SDS-PAGE and detected by immunoblot with the anti-RET antibody MSJ.
**Soft-agar Assay**

The soft agar assay (Xing et al., 1996) was carried out as follows. A bottom layer of agar was prepared in 60-mm Petri dishes using 3 ml of 0.5% noble agar (Difco, Detroit, MI 48232) in normal growth medium. Next, 3 ml of 0.35% noble agar in normal growth medium containing $9 \times 10^3$ cells were added on top of the bottom layer. Colonies greater than 0.5 mm were counted two weeks later.

**Results**

**PTC1 oncprotein forms dimers in vivo and its dimerization is mediated by the leucine zipper of H4**

To further investigate the oligomerization status of PTC1 oncprotein in eukaryotic cells, the PTC1 oncprotein was expressed in COS-7 cells by transient transfection with a DNA construct PTC1/CMV. The lysates of COS-7 transfectants expressing PTC1 were treated with glutaraldehyde to stabilize the oligomeric protein complexes (Fig.3.1b). Without cross-linking treatment, PTC1 oncprotein can be detected at the size of 53 kD, since the PTC1 used in our experiment is the alternative spliced isoform with shorter C-terminus (Tahira, et al., 1990). After 1 min treatment with glutaraldehyde, a 105 kD protein complexes can be readily detected. By 16 min, the PTC1 monomer can no longer be detected. We consistently observed that the PTC1 protein complexes had a stronger signal intensity than the PTC1 monomers in immunoblot detection. One possible explanation is that the formation of protein complexes may alter the conformation of the RET tyrosine kinase for better binding of the MSJ antibody to the epitope site.

To investigate the role of the leucine zipper of H4 in the formation of PTC1 protein complexes, two forms of PTC1 mutant, PTC1Δzip and PTC1ΔN, were constructed. PTC1Δzip had the leucine zipper region of H4 (amino acids residue 56-102) deleted. PTC1ΔN had the N-terminus of H4 (amino acids residue 3-52) deleted, but retained the leucine zipper (Fig.3.1a). As shown in Fig.3.1b, the PTC1Δzip (46 kD) fails to form protein complexes even
after 16 min treatment with glutaraldehyde. In contrast, PTClΔN (49 kD) retains the ability to form protein complexes. These data indicate that the formation of PTCl protein complex is mediated by the leucine zipper of H4.

A co-immunoprecipitation assay was performed to further investigate whether the PTCl forms homodimers. A mutated form of PTCl, PTClΔC, had the extreme C-terminus of PTCl (amino acids residue 345-461) deleted. Therefore, PTClΔC is no longer recognized by the polyclonal antibody C17 (Fig. 3.2a). When the PTClΔC was co-expressed in COS-7 cells with either PTCl, PTClΔzip, or PTClΔN, the PTClΔC encoded protein can not be immunoprecipitated by C17 unless it forms protein complexes with other forms of PTCl containing an intact C-terminus. The expression of PTCl and its mutants in various cell lysates is detected by the MSJ monoclonal antibody which can react with all forms of PTCl protein including PTClΔC (Fig. 3.2b). The PTClΔC product (38 KD), can not be immunoprecipitated by C17 when it was expressed alone or co-expressed with PTClΔzip (Fig. 3.2c, lane 2, lane 4), but can be co-immunoprecipitated when it was co-expressed with PTCl or PTClΔN (Fig. 2c, lane 3, lane 5). Taken together the results of the cross-linking experiment and the co-immunoprecipitation assay, we conclude that PTCl oncoproteins form homodimers in vivo, and the dimerization is mediated by leucine zipper domain of H4.

The H4 leucine zipper mediated dimerization is essential for tyrosine hyperphosphorylation of the PTCl oncoprotein

It has been shown that dimerization of receptor tyrosine kinases promotes the transphosphorylation of tyrosine residues on intracellular domains (Ullrich and Schlessinger, 1990). To investigate whether there is a correlation between dimerization and transphosphorylation, the tyrosine phosphorylation levels of PTCl, PTClΔzip and PTClΔN proteins expressed in COS-7 cells were examined (Fig. 3.3). The equivalent amount of PTCl, PTClΔzip and PTClΔN proteins used in the immunoblot analysis was also shown. The PTCl and PTClΔN oncoprotein are hyperphosphorylated on tyrosine residues. However, deletion of the leucine zipper region of PTCl dramatically reduced the tyrosine phosphorylation level of the PTClΔzip encoded protein.
The H4 leucine zipper mediated dimerization is required for the PTC1 transforming activity

To correlate the leucine zipper mediated dimerization and tyrosine hyper-phosphorylation with the transforming activity of PTC1, both focus formation assay and soft agar assay were performed to evaluate the transforming activity of PTC1, PTC1Δzip, or PTC1ΔN. The expression of PTC1, PTC1Δzip, or PTC1ΔN proteins encoded by the corresponding DNA constructs was initially confirmed by transient transfection in COS-7 cells, and the expression levels of these three proteins did not appear to be different in COS-7 cells. For focus formation assay, NIH3T3 fibroblast cells were transfected with either PTC1/LN CX, PTC1Δzip/LNCX, or PTC1ΔN/LNCX DNA constructs. The transfected NIH3T3 cells were either left in the plates for three weeks to score for the number of foci formed (loss of contact inhibition), or were under G418 selection to monitor the transfection efficiency. As shown in Table 3.1, although all DNA constructs showed equivalent transfection efficiency, NIH3T3 cells transfected with PTC1/LN CX or PTC1ΔN/LNCX, but not with PTC1Δzip/LNCX or vector DNA, lost contact inhibition and displayed ability to form foci.

For soft agar assay, NIH3T3 stable transfectants expressing PTC1, PTC1Δzip, or PTC1ΔN were established. We consistently observed that the protein expression levels of PTC1 or PTC1ΔN were higher than those of PTC1Δzip in corresponding NIH3T3 stable transfectants (Fig. 3.4). The clones expressing the lowest amount of PTC1 (PTC1-5 and PTC1-10) and a clone expressing the highest amount of PTC1Δzip (PTC1Δzip-3) and clones expressing both high (PTC1ΔN-2) and low amount (PTC1ΔN-1) of PTC1ΔN were selected for soft agar assay. The result of soft agar assay demonstrated that NIH3T3 cells expressing PTC1 or PTC1ΔN, but not PTC1Δzip, acquired anchorage-independent growth in soft agar (Table 3.2).

Although no difference in the ability to form focus was observed between cells expressing PTC1 and cells expressing PTC1ΔN (Table 3.1), fewer colonies were formed in soft agar for PTC1ΔN expressing cells, compared to PTC1 expressing cells (Table 3.2). Although two different clones of both PTC1 and PTC1ΔN were tested by soft agar assay, clonal effect can not be completely excluded. Alternatively, the N-terminal region of H4 may contain some elements contributing to the anchorage-independent growth of PTC1 but not to...
the loss of contact inhibition. Taken together, the results of focus formation assay and soft agar assay indicate that the leucine zipper mediated dimerization is required for the PTC1 transforming activity.

The PTC1 transforming activity can be suppressed by a loss-of-function PTC1 mutant through the formation of hetero-dimers

Since intermolecular phosphorylation of dimerized receptor tyrosine kinases is crucial for the PTC1 activation, it is possible to suppress the PTC1 transforming activity by introducing a loss-of-function PTC1 mutant to form inactive hetero-dimers with PTC1. A mutation at codon 897 of c-ret, changing arginine to glutamine, was identified in some patients with Hirschsprung’s disease (Romeo, et al., 1994). This mutant has been characterized as a loss-of-function mutation (Pasini, et al., 1995). PTC1HS, a PTC1 mutant containing this mutation, was transfected into the NIH3T3/PTC1 cells, which were established by transfecting NIH3T3 cells with human thyroid tumor genomic DNA that contains the naturally occurring PTC1 transforming gene (Grieco, et al., 1990). Since the NIH3T3/PTC1 cells do not have G418 resistance, NIH3T3/PTC1 cells transfected with various DNA constructs can be selected by G418, and the transformed phenotype of the G418 resistant colonies was evaluated by their ability to form foci. As shown in Fig. 3.5, approximately 68.6% of the mock transfected (pRc/CMV vector only) NIH3T3/PTC1 cells and 52.5% of the PTC1ΔzipHS/CMV transfected NIH3T3/PTC1 cells retained the ability to form foci. When analyzed by Student t-test, there is no significant difference between the effects of PTC1ΔzipHS and mock transfection. However, only 18.5% of PTC1HS/CMV transfected NIH3T3/PTC1 cells maintained transformed phenotype, which is significantly different from that of NIH3T3/PTC1 cells transfected with pRc/CMV vector (p < 0.001) or cells transfected with PTC1ΔzipHS/CMV (p < 0.05).
Discussion

In this study, we demonstrated that the PTC1 oncoprotein forms dimers in vivo, and the dimerization of PTC1 is mediated by the leucine zipper in the H4 portion of PTC1. Our data also indicated that the leucine zipper mediated dimerization is essential for the constitutive phosphorylation and the transforming activity of the PTC1 oncoprotein. Furthermore, a loss-of-function PTC1 mutant can reverse the transformed phenotype of NIH3T3/PTC1 cells, presumably by forming inactive heterodimers.

The PTC1 oncoprotein, with the extracellular and transmembrane domains of c-RET replaced by H4, is localized in the cytoplasm (Ishizaka, et al., 1992), instead of the plasma membrane of cells. It was proposed that the translocation of PTC1 from the cell membrane to the cytoplasm could have provided the structural basis for its escape from the modulatory effect of the membrane-associated protein kinase C (Lanzi, et al., 1992). However, our data indicate that loss of membrane localization is not enough for the activation of the RET tyrosine kinase, since PTC1Δzip, which is also localized in the cytoplasm, lost both tyrosine hyperphosphorylation and transforming activity. Our data indicated that the leucine zipper mediated dimerization is required for the activation of PTC1 tyrosine kinase.

In human papillary thyroid carcinomas, three different forms of the ret/PTC oncogenes have been identified, in which the RET tyrosine kinase domain becomes fused with the N-terminal sequences of three different genes (Grieco, et al., 1990; Bongarzone, et al., 1993; Santoro, et al., 1994; Jhiang, et al., 1994). In addition to PTC1, the PTC2 oncoprotein has also been shown to form dimers in vivo (Bongarzone, et al., 1993), and the dimerization is required for the mitogenic activity of PTC2 (Durick, et al., 1995). Although PTC3 has not been shown to form oligomers experimentally, a potential coiled-coil motif was identified in the ELE1 sequence of PTC3 (our unpublished data). In addition to ret/PTC oncogenes, RetI and RetII transforming genes, which were formed by rearrangements during in vitro transfection (Takahashi and Cooper, 1987; Ishizaka, et al., 1988), also form dimers in vivo (Cao and Etkin, 1995; and our unpublished data). Therefore, constitutive oligomerization of RET appears to be a common mechanism for the activation of rearranged RET oncoproteins. In fact,
constitutive oligomerization may serve as a common mechanism for oncogenic activation of other receptor tyrosine kinases in thyroid tumors. The Tpr protein, which is involved in a rearrangement with the tyrosine kinase domain of the TRK nerve growth factor receptor in human papillary thyroid carcinoma (Grieco, et al., 1992), contains a leucine zipper domain and forms dimers in vivo (Rodrigues and Park, 1993). All the tyrosine kinase oncogenes formed by rearrangement in human papillary carcinomas seem to be activated by constitutive oligomerization.

Rodrigues and Park have proposed that oligomerization may serve as a general mechanism for oncogenic activation of receptor tyrosine kinases in many types of tumors (Rodrigues and Park, 1993). They demonstrated that the leucine zipper domain in Tpr mediates dimerization of the tpr-met oncoprotein, and this constitutive dimerization is essential for its transforming activity. Not only rearrangement can cause oligomerization to activate receptor tyrosine kinases, point mutations have also been shown to promote constitutive dimerization of receptor tyrosine kinases. Point mutations of ret found in MEN2A patients, resulting in the substitution of one of five Cys residues in the extracellular domain, caused constitutive dimerization and activation of RET tyrosine kinase (Santoro, et al., 1995; Xing, et al., 1996). An amino acid substitution of the neu oncogene has also been shown to induce oncoprotein aggregation, increase tyrosine kinase activity and transforming activity (Weiner, et al., 1989). In addition, our experiment demonstrated that an inactive form of PTC1 can be used to suppress the PTC1 transforming activity. The suppression of the PTC1 transforming activity presumably is due to the formation of inactive heterodimers between the two forms of PTC1 oncoprotein. This strategy of suppressing the transforming activity by introducing an inactive form of tyrosine kinase oncogene may also apply to other receptor tyrosine kinase oncogenes, since oligomerization is proposed to be a general mechanism for oncogenic activation of receptor tyrosine kinases.
Fig. 3.1 The cross-linking of PTC1 protein complexes by glutaraldehyde depends on the presence of the leucine zipper. a) The schematic representation of PTC1 and its mutants used in this study. The localization and amino acid sequence of the leucine zipper of H4 is shown. b) Lysates of COS-7 cells expressing PTC1, PTC1Dzip, or PTC1DN were treated with 0.01% glutaraldehyde for the times indicated. The conjugated protein complexes were resolved on 7.5% SDS-PAGE gel and detected by immunoblot with MSJ antibody against RET. The potential dimer forms are indicated by arrows (→).
Fig. 3.1
Fig. 3.2 The co-immunoprecipitation of PTC1ΔC with PTC1 is mediated by the leucine zipper. a) The schematic representation of PTC1 and PTC1ΔC proteins and antibodies used in this experiment. b) The expression of PTC1 and its mutants in COS-7 cell lysates. Proteins in cell lysates were resolved on 9% SDS-PAGE and immunoblotted with MSJ antibody. c) Co-immunoprecipitation of PTC1ΔC with PTC1 or PTC1ΔN, but not with PTC1Δzip. COS-7 cell lysates were immunoprecipitated with the C17 antibody and then subjected to SDS-PAGE and immunoblot with MSJ antibody. Lysates of COS-7 cells transfected with various DNA constructs are indicated as follows: lane 1, PTC1; lane 2, PTC1ΔC; lane 3, PTC1 and PTC1ΔC; lane 4, PTC1Δzip and PTC1ΔC; lane 5, PTC1ΔN and PTC1ΔC.
Fig. 3.2
Fig. 3.3 The hyperphosphorylation of PTC1 depends on the leucine zipper mediated dimerization. Top panel, the tyrosine phosphorylation levels of PTC1 and PTC1Δzip were revealed by immunoblot with anti-phospho-tyrosine antibody 4G10. Bottom panel, equal amount of PTC1, PTC1Δzip and PTC1ΔN proteins used in the experiment is demonstrated by immunoblot with the MSJ antibody.
Fig. 3.4 The expression of PTC1, PTC1Δzip or PTC1ΔN proteins in the corresponding NIH3T3 stable transfectants. Lysates of various NIH3T3 stable transfectants (1x10^6 cells) were immunopreipitated with the C17 anti-RET polyclonal antibody, followed by immunoblotting with the MSJ anti-RET monoclonal antibody. Three stable transfectants of each DNA construct, PTC1 clone # 5, 7, 10; PTC1Δzip clone # 2, 3, 11; or PTC1ΔN clone # 1, 2, 3, were shown.
Fig. 3.5 The suppression of PTC1 transforming activity with a loss-of-function PTC1 mutant. NIH3T3/PTC1 cells (Grieco, et al., 1990) were transfected with pRc/CMV, PTC1HS/CMV, or PTC1ΔzipHS/CMV, using calcium phosphate method (Di Fiore, et al., 1987). The percentages shown on the chart represent the number of colonies which formed foci divided by the total number of G418 resistant colonies. Three independent experiments were performed, with each samples being tested in triplicate during each experiment. * indicates that the PTC1 transforming activity was significantly suppressed by PTC1HS/CMV compared to pRc/CMV vector (p < 0.001) or to PTC1ΔzipHS/CMV (p < 0.05).
<table>
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<tr>
<th>DNA Construct</th>
<th>Foci/pmole</th>
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<td>PTC1ΔN</td>
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<td>5.5X10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
</tbody>
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Table 3.1  Focus formation assay to compare the transforming activity of PTC1, PTC1Δzip, and PTC1ΔN in NIH3T3 cells
<table>
<thead>
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<th>Cell line</th>
<th>Number of colonies</th>
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<tr>
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<tr>
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<tr>
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<td>44 ± 6</td>
</tr>
<tr>
<td>PTC1ΔN-2</td>
<td>50 ± 16</td>
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</tbody>
</table>

Table 3.2  Soft-agar assay to compare the transforming activity of PTC1, PTC1Δzip, and PTC1ΔN in NIH3T3 stable transfectants
Chapter 4

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

Introduction

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 (Taurog, 1996). 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 (Mazzaferri 1996). 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 (Smanik, et al., 1997). Furthermore, reduced radioiodide uptake activity in the thyroid gland of our ret/PTC1 transgenic mouse is accompanied by down-regulation of the expression of mouse NIS (Cho, et al., manuscript in preparation). Indeed, many studies have shown that radioiodide uptake activity is readily lost when various oncogenes were expressed in immortalized rat thyroid cells (Trapasso, et al., 1996; Berlingieri, et al., 1993; Santoro, et al., 1993a).

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 (Carrasco, 1993). 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 (Kogai, et al., 1997). To study the transcriptional regulation of NIS, the promoter activity of the 5' flanking genomic DNA fragments of the rat NIS gene was characterized and compared to those of other known thyroid-specific genes.

Materials and methods

Cell lines

FRTL-5 rat thyroid cells (Ambesi-Impiombato, et al., 1980) 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) medium supplemented with 10% calf serum. The Rat-1 rat embryonic fibroblast cells were maintained in DMEM (Dulbecco's Modified Eagle Media) medium supplemented with 10% calf serum. The Rat-1 rat embryonic fibroblast cells were maintained in DMEM (Dulbecco's Modified Eagle Media) medium supplemented with 10% calf serum. The PC12N21 cell line (Burry 1993), 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' TGACTCGCGCTGCGACTCTC -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 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 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
The 2 kb nucleotide sequence 5' of the ATG translational initiation site was determined, using the Sequenase Kit (USB, Cleveland, OH 44122).

**Primer Extension Assay**

The rNIS33 primer (5'- CCATGGAGACAGGTGACTCG -3') corresponding to -16 to +4 bp relative to the ATG site was used for the primer extension assay (Sambrook, et al., 1989). The kinase labeled probe was co-precipitated with 10 µg RNA isolated from FRTL5, 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 RNase digestion, the remaining cDNA fragments were analyzed on a 6% sequencing gel, accompanied with a sequencing reaction using the same oligonucleotide as a ladder marker.

**Ribonuclease Protection Assay**

To prepare the riboprobe for rat NIS transcription start site mapping, a genomic fragment ranging from -352 to +4 relative to the ATG site was amplified by PCR, and subcloned into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA 92008). For evaluating the rat NIS transcript, a cDNA fragment ranging from +1752 to +1931 relative to the ATG site was amplified by PCR, and subcloned into the TA cloning vector pCR2.1. For evaluating the rat G3PDH transcript, a cDNA fragment was amplified by PCR with primer (5'- TGAAGGTCGGTGTGAACGGATTTGG -3') and primer (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 DNA with HindIII, the radioactive labeled anti-sense ribo-probes were synthesized with [α-32P]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 RNase T1 and RNase A mixture and 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)

The 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 amplified with an anchor primer provided with the kit and a specific nested primer rNIS31 (5’-AGTCGGGTCCCGGCACTGCGTTGG -3’). Upon confirming the amplified PCR products by Southern blot, a 250 bp fragment was re-amplified with the rNIS33 primer (5’- CCATGGAGACAGGTGACTCG -3’) and the UAP primer from the kit. The resulting PCR product was subcloned into TA cloning vector pCR2.1 and the nucleotide sequence was determined, using the Sequenase Kit (USB, Cleveland, OH 44122).

Plasmids

The ITPSS3/pGL2B plasmid was first constructed by inserting the 3 kb SstI genomic fragment of the immediate 5’ region of rat NIS gene 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 self-ligating the DNA construct after Mung-Bean nuclease treatment. The ITPSN8/pGL2B plasmid was constructed by inserting the most upstream 6 kb SstI fragment into the SstI site of ITPSN2/pGL2B. To generate ITP370/pGL2B and ITP230/pGL2B, either a 372 bp or a 234 bp genomic fragment of rat NIS gene was amplified with primer rNIS54 (5’- CAGTTTCTTCTCTCCAAAGCTGCGG AGAAAGG -3’) or rNIS55 (5’- GAATTCTTTAGACTGTGAGCGG -3’) paired with rNIS342 respectively, using rat genomic DNA as template. The resulting PCR products were first 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 bovine thyroglobulin gene promoter DNA fragment from the pSKTg plasmid, and inserting 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 x 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 x 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 the lysis buffer of 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. 4.1a). The 5' non-translated region of the rNIS gene was localized within a 2 kb SstI-Apal DNA fragment. This 2 kb DNA fragment was then subcloned and subjected to nucleotide sequence analysis (Fig. 4.1b). Comparing with the published rat cDNA nucleotide sequence (Dai, et al., 1996), the genomic DNA nucleotide sequence showed a discrepancy to the first 25 bp of the reported rat NIS cDNA nucleotide sequence (Fig. 4.1c). We performed 5' RACE (rapid amplification of the 5' cDNA end) 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 we cannot exclude the possibility of the presence of an intron, and the reported cDNA sequence was generated by differential splicing, we think it is unlikely. Because we found no EcoRI restriction site, which is located at the beginning of the published cDNA sequence, in the 5' flanking 8 kb genomic fragment of the rat NIS gene.

Based on the sequence analysis, the structure of rat NIS gene appears to be different from that of the Tg, TPO and 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 region (Damante and Di Lauro 1994). The Pax-8 binding site overlaps with the TTF-1 site proximal to the transcriptional start site. In contrast, the TSHr promoter shows a quite different structure. Two TTF-1 binding site has been found and no TTF-2 or Pax-8 binding sites are present in the 900 bp 5' flanking region of the TSHr gene (Ohmori, et al., 1995). In the 5' flanking region of the rat NIS gene, three potential TTF1 sites, at -480, -1736 and -1875
relative to the ATG translational initiation site, were identified (Fig 4.1d). The TTF-1 binding site has been shown to play an important role in mediating thyroid-specific expression of Tg, TPO and TSHr genes (Sinclair, et al., 1990; Fraccis-Lang, et al., 1992; Shimura, et al., 1994). Thus, the functional significance of these TTF-1 sites in rat NIS gene need to be investigated. No TTF-2 binding site can be identified in the 5’ flanking region of the rat NIS gene. 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 the 5’ flanking region of the rat NIS gene. 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 (Trapasso, et al., 1996). AP-2 was demonstrated to be able to mediate transcriptional activation of protein kinase C and cAMP-dependent protein kinase A (Imagawa et al., 1987). Since no CREB site can be identified, the TSH effect on the NIS 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 sites were determined with primer extension assay

Our 5’ RACE experiment resulted in three fragments started at -98, -93 and -92 bp relative to the ATG site. Consistent with this result, primer extension assay (Fig.4.2a) with a primer, located at -16 to +4 relative to the ATG site, showed an extended cDNA fragment specific to the FRTL-5 RNA, corresponding to -98 relative to the ATG site. We tentatively designate the -98 to be the start site for thyroid specific transcription. Ribonuclease protection assay with a probe ranging from -352 to +4 relative to the ATG translational initiation site revealed a protected fragment with a size consistent with other experiments (Fig 4.2b).

Both Tg and TPO promoters have a TATA box element located 20-30 nucleotides upstream of the transcriptional start site (Damante and Di Lauro 1994). However, TSHr promoter does not have a TATA box. In rat NIS 5’ flanking region, a potential TATA box
(AATAAAAT) was identified at -124 to -118 relative to the ATG translational initiation site. This putative TATA box is about 20 bp upstream of the putative transcriptional start site of the rat NIS gene. Several non-canonical TATA box sequences have been reported, such as the ATAAAAA in the gene for bovine elastin (Manohar and Anwar, 1994). It was also reported that the AATAAA retained 30% activity of the wild type TATAAA when assayed by a reconstituted in vitro transcription system (Wobbe and Struhl, 1990). Furthermore, by comparing the rat and human NIS 5' flanking sequence, we identified a stretch of 90 bp highly conserved sequence, ranging from -199 to -110 relative to the ATG translational initiation site for rat and -475 to -385 for human respectively (Kwon and Jhiang, unpublished data), sharing 75% identity between these two sequences.

The rat NIS promoter was mapped to -352 to -118 relative to the ATG site, and the 8 kb 5' flanking DNA fragment of the rat NIS gene is not sufficient to confer thyroid-specificity

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 rat NIS in three different rat cell lines, FRTL-5, Rat-1, and PC12 (Fig. 4.3). 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 significant reduction of promoter activity in FRTL-5 cells. However, significant decrease in promoter activity was also observed in two non-thyroid cell lines, the rat-1 and PC12 cells. The functional relevance of the TTF-1 site is yet to be determined.

None of the DNA fragments investigated was sufficient to confer thyroid-specific promoter activity, since all the DNA constructs showed promoter activity in both FRTL-5 thyroid cells and two non-thyroid cell lines, Rat-1 and PC12, which are embryonic fibroblast and pheochromocytoma cells respectively. Surprisingly, very high expression of the proximal rat NIS promoter was detected in Rat-1 and PC12 cells where the NIS mRNA is almost
undetectable (Fig. 4.2c). For comparison, a reporter DNA construct containing 2 kb bovine thyroglobulin promoter (Ledent, et al., 1990) had significant promoter activity in thyroid cells (FRTL-5) and undetectable promoter activity in non-thyroid cells, Rat-1 and PC12 cells. As shown in Fig. 4.3, the rNIS proximal promoter is weaker than the bovine Tg promoter.

Our results indicate that the rat NIS gene promoter is quite different from those of the Tg, TPO, and TSHr genes. For rat Tg and TPO genes, 170 bp and 420 bp DNA fragment upstream of the transcription start sites respectively can confer thyroid-specific expression (Damante and Di Lauro, 1994). As for rat TSHr, a 160 bp minimal promoter region containing one TTF-1 site can provide thyroid-specific expression (Ikuyama, et al., 1992). However, the 1.3 kb of the 5’ flanking region of the human thyroid peroxidase gene showed promoter activity in FRTL-5 cells, NIH3T3 cells and HepG2 cells (Foti, et al., 1990; Kikkawa, et al., 1990), but the 6.3 kb fragment demonstrated promoter activity only in FRTL-5 cells, but not in HepG2 cells (Kikkawa, et al., 1990). Interestingly, the expression of NIS gene has the most broad tissue spectrum. It has been shown that NIS is also expressed in non-thyroid tissues, such as salivary gland, mammary gland, gastric mucosa, and colon. However, both Tg and TPO are expressed exclusively in thyroid. The TSHr gene has been demonstrated to be expressed in retro-orbital fibroblasts and in adipose tissue, in addition to thyroid (Endo, et al., 1993). The difference in expression pattern must be a reflection of the difference of transcriptional regulation of these genes.

The differences of regulatory mechanisms between NIS and Tg, TPO, or TSHr 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 (Trapasso, et al., 1996). 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).
Proposed mechanisms underlying the thyroid preferential expression of rat NIS

Based on our current results, there are several possible mechanisms underlying the thyroid preferential expression of rat NIS gene. Since the discrepancy between the reported cDNA sequence and the genomic sequence might be due to the presence of an intron, 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 the rat NIS. It is also possible that additional elements located outside the studied 8 kb genomic fragment are required to inhibit the transcription of rat NIS gene in non-expressing tissues. In addition, DNA methylation has been shown to be an effective mechanism for transcriptional inhibition of tissue-specific genes in nonexpressing cells (Cedar 1988). The DNA at the 5' end of the human thyroglobulin gene is demethylated in thyroid and fully methylated in non-thyroid tissues (Libert et.al., 1986). It is possible that the endogenous NIS gene is only expressed in thyroid and other tissues where its DNA is demethylated, but not in tissues where its DNA is methylated. However, when we delivered unmethylated reporter DNA into cells, they can all be expressed, no matter the methylation state of their endogenous NIS gene. Finally, chromosomal structure might also be critical for the tissue-preferential expression of the NIS gene. 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.

The rat NIS promoter activity is reduced in PTC1 oncogene transformed cells

It has been shown that PTC1 causes dedifferentiation of thyroid cells, including the loss of iodide uptake activity, when transfected into thyroid cells (Santoro, et al., 1993a). In addition, both iodine concentration activity and NIS gene expression are significantly reduced in the thyroid of our PTC1 transgenic mouse in which the PTC1 oncogene is targeted-expressed in the mouse thyroid gland (Cho, et al., manuscript in preparation). To evaluate the effect of PTC1 oncogene on the transcriptional regulation of rat NIS, we introduced the rat NIS reporter DNA constructs into the NIH3T3 stable transfectant cells which either express PTC1 or a non-functional PTC1 mutant, PTC1ΔZip. The rat NIS promoter activity was found
to be reduced significantly in the PTC1 transformed cells (Fig. 4.4). However, no effect of PTC1 on NIS promoter activity can be demonstrated by transient co-transfection of the rat NIS promoter reporter DNA construct and the PTC1 expression DNA construct into FRTL-5 and Rat-1 cells. The suppression of rat NIS gene expression in PTC1 transformed NIH3T3 cells may be caused by secondary effects of transformed phenotype induced by PTC1. In addition, the NIS promoter activity was only reduced by less than 50%. This is well below over 90% reduction of NIS gene expression in tumors (Smanik et al., 1997). It is worth noting that factors such as the change of DNA methylation or chromosomal structure can not be revealed in our current assay system, since we used plasmid DNA constructs.

In summary, we demonstrated that the promoter structure of the rat NIS gene promoter is substantially different from that of other thyroid-specific genes. Further study of the transcriptional regulation of NIS gene expression will provide information which can be readily translated to improve the effectiveness of radioiodine therapy by increasing the expression of the endogenous NIS to a greater extent in patients with thyroid tumors prior to radioiodine therapy.
Fig. 4.1 The genomic structure 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 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.
rat NIS genomic fragment (16.4 kb)

Fig. 4.1

(continued on next page)
(Fig. 4.1, continued)

b

-1949  GAGCTCTTTATCTGCTGCTCTAAGCCT
-1920  CTGCTAGGTGGGAATGAGGCCAGCCAAACTCGGGGACCTTGAGACCACCACAGCTTTAGGT
       TTF-1
-1860  GAGGACTACACTGGATCATATGACAAACGGGTGTTTTTGTGTTGGTGTGGCGACGC
       T3R-6
-1800  ACCATCAGCGTTCGGTGTTTTTGAAGAGTATAGCCTGAAGCCTCCGGCTGTCTTGTCA
-1740  TTP-1
-1680  AGATATTTCATACGTATGACATGACATGCCCGGTTAGTTTTGAGATTTCTAACATCCAGT
       T3R-P
-1620  GCCAGCTAGAGTAAAGGCCTGCTCTATATCTATGAGAAGGGCATAAGGGGCACCGTGTCGA
-1560  AGAGAGTCTCTCTCCACTGAGTCATGGCCTGGCCACATCAAAGGAATGGGTGATCCCTCCTGA
-1500  CTATGCGATGTTCTCTCCTGTCATTTGGGCGGAGTGGCTGATCATTĂAGCTAGAGGA
-1440  ATCTGGAAGGTTGTTACCTCTCGAGACCTGGCTGGTTGATATTCTCCCTGATTAAAGATACCTCC
       GR
-1380  AGGGCATCGGATGCCACCTGGCCACCTGCTACCGCCTTCCCTTCGCCATTCTCCTCCACCC
-1320  ACATACTAAAATGTGTTTTTTTTGTGGAGCTGGGAAATGATGTGTTGACTAAAGCCAGCT
       AP-2
-1260  AGGAGTCAAAAGTGTAGGCCATTCTGGTCTACAAGGTGAGTTCCAGAACAGCCAGGGCTA
       AP-1
-1200  CACGGAGAGGCCCTATAGACATATTGCTGATGACATAGCAACACAGCTTCAGGCGCTCCTCCC
       GR
-1140  TGGTGCTGAGCAGCCGCTGATGCCCTGCTGCCAGCATGGAATCCATGCCCTACCTGCG
       GR
-1080  TCTGGAAGGCTCTCTCTCCCTCCCTGTTCTTGTGACCTCTTACCTGCTCCACCTAGGCTTA
-1020  CGAGCTGCCCCTATGCTAGGCTCTCGAAGCAGACTTCACCAGGGGCGGCGGAGTGCTGCGATATACA
       SP-1
-960   TCTAGACACAAAAACAGGGGCCGTGCGCTTAGATCTCATTACCTGCCGACCCCAAGAAACCGAGG
-900   ACTCCACAGCCAGCTCAACCCCTGAAACCCACGGCCAGTCCACACGAAAAGCGTCC
-840   CTTGAAGTCACATGCGGCAACTGCTCCTACCCAGATTATGCTGCGATAGAAGGGAGTG
       GR
-780   ATGGGCTCCACAAACACCCCTACTCTCCTACTCCAGCAGTAAAGGGAAGCCAAGGAGGA
-720   CTCACCCTGGCCGACCGGCTGAGCTGGGAAGGAAATGCGAGAACAGCAGGACGTGAGCA
       T3R-α
-660   ACGAGGCGTCGAGCTGCACTTGGCGGATGAGCTCATGTGACATGACACAT
       AP-1

Fig. 4.1

(continued on next page)
(Fig. 4.1, continued)

-600 CATAGGTCCAGAGTGCCCTTGTACAGGTGGGGAGTCCTAAAGCAGAAGAAAGAAATTCTC
-540 CAAGAGAACCTGAGTGCCTCCCACCGGGTTAGCTGAAGGGCAAGGTTGGACAGTTCCTTC
-480 TCCCCAGCTGGCGGAGAAAGGTAGATGCTCTCCTGGGGAAGGCGCTCCAGTGCTCGCTTTTT
TTF-1
-420 TCCTTATGGAGCCCCGAAGTCCAGGAAAGTGAACCCTAGTCCGGGTTTCCTCATAACTCC
-360 GCTTCCTAAATTCTTAGACTGTGAGCGGAGGTCTGAGCCGTCCATAGCCTTTCCCCATCT
-300 CTCCACAAACCTATACGGGAAACAGCCCTAGATGTGGGAGAAAGGGTACAGGAGACAGAGT
T3R-α
-240 GTTCCCCCAACCCGACTGCCCGCACCCCGAGGCTGCGGAGCTGCGCTGCCCGGAAGGGGT
-180 CGGGGTCCGCGCCGCCCTCCAGGGCCTGGGCGGAGCTGCAGCTCGACCCCGGAGTTTCAATTTA
SP-1  \rightarrow  SP-1
-120 ATCCCGAGGGCGAAAGCGAGTGAGCTGCAGGCGGACTGCGGACTCCT
-60 CCACTGACCGAGTGCCCCGGACGTCCTCCGCATCCTCTTCACCGAGTCACTGTCTCC

ATG GAG GGT GCC GAG GCC GGG
M E G A E A G

(Fig. 4.1, continued)
(Fig. 4.1, continued)

c

\begin{verbatim}
-109  -70'
rat NIS cDNA  GAATTCCGGGTCGACCACCGCGTCCGGCGGTGACTCGCGC
rat NIS gDNA  GAAAAGCGAGTGGATCGACGGCCCAGCGGTGACTCGCGC
5' RACE  GGATCGACGGCCCAGCGGTGACTCGCGC
\end{verbatim}

\begin{verbatim}
d
rat NIS 1  -480  TCCCAAGCTCG -469
rat NIS 2  -1740  TCTCAAGTCTCT -1729
rat NIS 3  -1876  CACCAAGCTTTA -1865
rat Tg A  ACTCAAGTATTC
rat Tg B  ACTCAAGTAGAG
rat Tg C  AGTCAAGTGTTT
rat TPO A  ACTCATAGAAAG
rat TPO B  TGCCAAGTGCTT
rat TPO C  ACTCAAGCTTTAG
Consensus  ACTCAAGTNNNN
\end{verbatim}

Fig. 4.1
Fig. 4.2 The determination of the rat NIS gene transcriptional start site. (a) Primer extension analysis of the rat NIS transcription start site. A DNA sequencing ladder, obtained using the same rNIS33 primer, was run in parallel. Arrows indicate the positions of the extended cDNA fragments relative to the ATG site. (b) Ribonuclease protection analysis of the rat NIS transcription start site, with a riboprobe corresponding to the 5' flanking region of the gene. Radioactive labeled 100 bp ladder (GIBCO BRL) was run in parallel as a size marker. (c) Ribonuclease protection analysis of the rat NIS gene expression in three cell lines as indicated, by using a riboprobe corresponding to the 3' of the gene. Ribonuclease protection analysis of rat G3PDH transcript was also performed on the same RNA samples to demonstrate RNA integrity.
### Fig. 4.2 (continued on next page)

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![Image of sequencing gel](image-url)
(Fig. 4.2, continued)

b

Fig. 4.2
(Fig. 4.2, continued)

c

Fig. 4.2
Fig. 4.3 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 pCL2Control 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 transcription start site (Ledent, et al., 1990).

<table>
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<th>Luciferase Activity</th>
<th>Luc</th>
<th>FRTL-5</th>
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<td>vector only</td>
<td></td>
<td>0</td>
<td>1.0</td>
<td>0.1</td>
</tr>
<tr>
<td>8kb (-8kb/+1)</td>
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<td>10</td>
<td>13</td>
<td>151</td>
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<td>372bp (-490/-118)</td>
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<td>234bp (-352/-118)</td>
<td></td>
<td>31</td>
<td>119</td>
<td>59</td>
</tr>
<tr>
<td>Tg promoter (-2036/+9)</td>
<td></td>
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<td>1.0</td>
<td>0.1</td>
</tr>
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</table>
Fig. 4.4 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 pCL2Control 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.
CHAPTER 5

FUTURE RESEARCH DIRECTIONS

The PTC1 oncogene has been detected only in papillary thyroid carcinoma. Three possible mechanisms were postulated which might account for the thyroid-specificity of the PTC1 oncogene. First, the PTC1 rearrangements might only occur in thyroid. Second, the PTC1 oncogenes might only be expressed in thyroid. Third, the components for PTC1 downstream signal transduction machinery might be only available in the thyroid. Our results in Chapter 2 demonstrated that the H4 gene is expressed in both thyroid and non-thyroid cells. Therefore, the PTC1 oncogene, which is controlled by the H4 gene promoter, also can be expressed in tissues other than thyroid. This excluded the second possibility. Furthermore, PTC1 oncogene was shown to be able to transform NIH3T3 cells, and transgenic mice with PTC1 oncogene as transgene under the control of H4 gene promoter developed tumors in the mammary gland instead of thyroid (Portella, et al., 1996). These results argued against the third possible mechanism. This leaves us with the first mechanism that PTC1 oncogene rearrangement preferentially occurs in thyroid. Further experiments need to be carried out to elucidate the actual mechanism for the thyroid-specific activation of the PTC1 oncogene.

Experimental results presented in Chapter 3 established links among dimerization of the PTC1 oncoprotein, the activation of its tyrosine kinase and its transforming activity. However, the intermediate signal transduction pathway which links the activation of tyrosine kinase of PTC1 and the final transformed phenotype of the cells is left uncharacterized. The study of the PTC1 signal transduction pathway is important because it will not only provide information for the process of PTC1 transformation, but also shed light on the ret proto-oncogene signal
transduction pathway. Two approaches can be taken: the first is to study protein components which can bind to and get phosphorylated by the PTC1 oncoprotein. The second approach starts with the genes which are regulated by the PTC1 oncogene at the transcriptional level. These genes can be identified by differential display method, and confirmed by Northern blot or in situ hybridization. By studying the transcription factors which regulate these PTC1 responsive genes, we can characterize the signal transduction pathway from downstream.

Furthermore, the effect of PTC1 oncogene in tumor progression needs to be characterized in a model system better than the NIH3T3 cells. A transgenic mouse model with targeted expression of the PTC1 oncogene in thyroid has been established (Jhiang, et al., 1996). All transgenic mice developed thyroid carcinomas. Further characterization of this transgenic mouse model may provide information of the PTC1 function in the tumor formation.

As for the transcriptional regulation of the sodium iodide symporter, more efforts need to be devoted to the identification of the DNA elements responsible for the thyroid preferential expression of the NIS gene. Furthermore, the effects of TSH, thyroid hormone and glucocorticoid on the expression of the NIS gene need to be characterized.
LIST OF REFERENCES


