IDENTIFICATION OF NOVEL GENETIC ELEMENTS CONTROLLING TRANSCRIPTIONAL REGULATION OF THE HUMAN Na^+/I^- SYMPORTER (NIS) GENE

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AUGUST 2006

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ABSTRACT

IDENTIFICATION OF NOVEL GENETIC ELEMENTS CONTROLLING TRANSCRIPTIONAL REGULATION OF THE HUMAN Na^+/I^- SYMPORTER (NIS) GENE

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The function of sodium iodide symporter (NIS) in mammary gland epithelial cells is essential for the accumulation of iodide in mother's milk, which is the first source of iodide for the synthesis of thyroid hormones in the newborn. In addition to the lactating mammary gland, NIS expression has been also detected in breast tumors. Several hormones and ligands have been implicated in the functional expression of NIS in the mammary gland and breast cancer cell line models but the molecular determinants governing this expression are not yet identified. In this study we aimed to identify *cis*- and *trans*-acting elements regulating NIS expression in the breast cancer cell line MCF-7 in response to all-*trans*-retinoic acid (tRA), and to assess the possible role of 17-β-estradiol (E2) in regulating the expression of NIS. Using comparative bioinformatics, we have identified several regions that were conserved in human, mouse and rat in the sequences flanking and including the NIS gene. By using luciferase reporter assays, we have established that conserved clusters 3 and 4 respond to tRA in MCF-7. We have also shown that putative retinoic acid response elements controlling tRA-induced NIS expression in MCF-7 are located in the first intron of this gene. This tRA-responsive NIS expression was also correlated with the estrogen receptor status of mammary gland cell lines and we investigated roles of ERα in the regulation of NIS expression. We showed that the suppression of endogenous ERα by RNA interference resulted in down-regulation of both basal and tRA-induced NIS expression in MCF-7, furthermore, we have also shown that (E2) is capable of up-regulating NIS expression in MCF-7. In the ERα negative cell line MDA-MB-231, re-introduction of ERα resulted in NIS expression in a ligand independent manner. The role of ERα in the regulation of NIS expression was supported by the identification of an estrogen response element (ERE) in the promoter of NIS, this ERE was conserved in human, mouse and rat. We have also showed that this ERE could respond to E2 stimulation, and that ERα occupies the NIS promoter by binding to this novel element *in vivo*. These results indicate that E2 and ERα contribute to the regulation of NIS in the breast cancer cell line MCF-7.

ÖZET

İNSAN Na^+/I^- SİMPORTIR GENİ TRANSKRİPSİYONUNUN DÜZENLENMESİNİ KONTROL EDEN YENİ GENETİK ELEMANLARIN BELİRLENMESİ

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Na+/I- taşıyıcı protein (Na+/I- Symporter protein, NIS) aktivitesi anne sütüne iyot taşınması ve dolayısı ile yeni doğan bebeğin tiroid hormonları üretebilmesi için şarttır. Süt üreten meme dokusuna ek olarak, artış gösteren NIS ifadesi meme tümörlerinde de teşhis edilmiştir. Birkaç hormon ve ligandın meme hücrelerinde NIS geni işlevsel ifadesinde etkili olduğu tesbit edilmiş olsa da, bu ifadeye yol açan moleküler faktörler, veya genetik belirleyiciler henüz tamamen belirlenmemiştir. Bu çalışmada, MCF-7 insan meme kanseri hücre hattında all-*trans*-retinoik asit (tRA) muamelesi ile NIS geni ifadesine yol açan *cis*- ve *trans*- etkili faktörleri belirledik, ve 17-β-estradiol (E2)'ün NIS geni regülasyonuna etki mekanizmalarını inceledik. Çalışma kapsamında, önce karşılaştırmalı biyoenformatik yöntemleri kullanarak insan sıçan ve farede NIS geninde korunmuş ekzon dışı bölgeleri belirledik. Daha sonra bu bölgelerden *cis*-etkili faktör potansiyeli olanların lüsiferaz genini aktive edeceği deney düzenekleri ile korunmuş bölge 3 ve 4'ün MCF-7 hücrelerinde tRA ligandına yanıt verdiğini gösterdik. Ayrıca, tRA'ya yanıtı kontrol eden elemanların NIS geninin ilk intronu içerisinde olabileceğine dair veriler elde ettik. Bunlardan başka, NIS gen ifadesi tRA yanıtının hücrelerdeki östrojen reseptör alfa (ERα) varlığı ile korelasyon gösterdiğini ortaya koyarak, bu faktörün NIS gen ifadesini kontrol ederken girdiği moleküler etkileşimleri gen dizisi düzeyinde belirledik. Çalışmalarımızda, RNA interferans metodu kullanarak MCF-7 hücrelerinde ERα gen düzeyini düşürdüğümüzde, hem bazal NIS ifadesinin hem de tRA ile indüklenen NIS geni ifadesinin düştüğünü gördük. Aynı zamanda, E2 ligandının NIS gen ifadesini artırıcı etkisini gösterdik. Ayrıca, ERα ifadesi olmayan MDA-MB-231 meme kanseri hücre hatlarına sonradan ERα geni verdiğimizde NIS ifadesinin E2'den bağımsız olarak arttığını görerek, bu artışa yol açan kontrol mekanizmasını detaylı olarak inceledik. Bu analizlerde, NIS geni kontrol bölgesinde fare ve sıçanda da korunmuş bir ERα yanıt elemanı (Estrogen Response Element) olduğunu, bu ERE'nin E2'ye yanıt verdiğini, ve ERα faktörünün buraya hem *in vitro* hem de *in vivo* şartlarda bağlandığını gösterdik. Bu sonuçlar, ERα ve E2'nin meme kanseri hücrelerinde NIS geni transkripsiyonunu kontrol ettiğini ortaya koymuştur.

To My Loving and Caring Wife

Neslihan

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1. INTRODUCTION

1.1. Biological Significance of Iodide Transport

Iodine is the heaviest element normally metabolized in biological material (Wolff, 1964), and iodide is a limiting element for the synthesis of hormones, which are essential for proper growth and development of many organs (Stubbe *et al.*, 1986). Because of the scarcity of this element (Wolff, 1964), several organisms evolved with a remarkable mechanism of collecting this rare element. One of the first organs to be known for its iodide concentrating mechanism is the thyroid gland (Carrasco, 1993). Moreover, indications to the presence of iodide concentrating mechanisms in other organs in human and animals date back to as early as 1856 when Claude Bernard described the presence of iodine in the salivary gland, and in 1859 as reports describing the presence of iodine in the milk of nursing mothers were available, as well as in hair, skin, ovaries, placenta, kidney, stomach and intestine [reviewed in (Brown-Grant, 1961)]. The availability of radio isotopes by 1945 improved the techniques by which lower amounts of iodide could be measured, and thus provided a better understanding regarding organs in which the iodide transporter was functional (Brown-Grant, 1961).

The expression of an iodide transporter in the thyroid has long been known, based on the fact that thyroid hormone synthesis requires the presence of an iodide concentrating mechanism in this organ (Wolff, 1964). The existence of mRNA capable of encoding a functional iodide transporter was illustrated by the expression of poly A RNA isolated from the thyroid cell line FRTL-5 in the oocytes of *X. laevis*, which led eventually to the cloning of the cDNA of the rat sodium iodide symporter (Vilijn and Carrasco, 1989; Dai *et al.*, 1996). The cloning of the sodium iodide symporter (see below) provided an invaluable tool for the analysis of NIS mRNA (and later, protein) expression in different tissues or organs. Since then, NIS mRNA

expression was demonstrated in several other organs of higher vertebrates. Using RT-PCR, hNIS mRNA was detected in the thyroid, salivary gland, parotid gland, submandibular gland, pituitary gland, pancreas, testis, mammary gland, gastric mucosa, prostate, ovaries, kidney, and placenta (Smanik *et al.*, 1997; Ajjan *et al.*, 1998a; Spitzweg *et al.*, 1998; Mitchell *et al.*, 2001; Spitzweg *et al.*, 2001). By Northern blot analysis, the expression of the human NIS mRNA was observed in thyroid, and in the parotid glands (Spitzweg *et al.*, 1998).

The sodium iodide (Na^+/I^-) symporter (NIS, the official nomenclature given by HUGO gene nomenclature committee is SLC5A5) is the fifth member of the sodium/solute carrier family 5 (SLC5A), a family of proteins that mediate the active transport of a variety of molecules including iodide. Twelve members of this family have been described so far, including 7 members involved in the transport of glucose (members 1, 2, 4 and 9-12). This family belongs to the solute carrier super-family which includes 45 solute carrier families and one solute carrier organic anion transporter family (Wain *et al.*, 2002; Wain *et al.*, 2004).

The rat iodide transporter was the first to be cloned as a result of a functional screening of a human cDNA library from FRTL-5, a rat thyroid cell line, in *Xenopus laevis* oocytes (Dai *et al.*, 1996). In the same year another report described the cloning of the human iodide transporter using cDNA prepared from human papillary carcinoma tissue; they amplified the hNIS cDNA fragment using primers derived from the nucleotide sequence of the rat mRNA of rNIS (Smanik *et al.*, 1996). In the year 2000, Tazebay *et al.*, showed that active transport of iodide in the lactating mammary gland in mice is mediated by the transporter encoded by the same gene (Tazebay *et al.*, 2000). Later, the mouse sodium iodide symporter was cloned from thyroid and lactating mammary gland tissues (Perron *et al.*, 2001; Pinke *et al.*, 2001). The hNIS cDNA encodes a 643-amino acid protein with 84% homology to the rat and the mouse genes. This gene encoding the human iodide transporter was mapped to chromosomal location 19p13.2-p12 using fluorescence *in situ* hybridization (Smanik *et al.*, 1997). The coding sequence of the hNIS gene is encoded by 15 exons, and the exon-intron junctions were also described (Smanik *et al.*, 1997).

1.1.1. Structure of the Na^+/I symporter

The first clues regarding the secondary structure of NIS came from the first report, in which the authors described the mRNA sequence of this gene (Dai *et al.*, 1996). In that report, analysis of the predicted protein sequence based on hydropathy analysis and secondary structure algorithms suggested that the cloned cDNA encodes a protein with 12 putative trans-membrane domains, and due to its hydrophilic composition, the carboxy-terminus was placed at the cytoplasmic side of the plasma membrane (Dai *et al.*, 1996).

The generation of an anti-NIS antibody allowed researchers to further analyze the structure and the post-translational modifications that lead to the mature functional protein. The cytoplasmic location of the carboxy terminus of NIS was confirmed using indirect immunofluorescence in permeabilized FRTL-5 cells (Levy *et al.*, 1997). Moreover, using this antibody, membrane fractions from FRTL-5 cells or COS cells transfected with NIS cDNA revealed a prominent immunoreactive polypeptide with a molecular weight of about 87 kDa, different from the predicted mass (65 kDa). This difference was attributed to post-translational modifications at 3 putative Asparagine (Asn) residues at positions 225, 485 and 497 by *N*-linked glycosylation (Levy *et al.*, 1997; Paire *et al.*, 1997), two of which (residues 485 and 497) were located in the predicted sixth extra-cellular loop. Site directed mutagenesis of these putative glycosylation sites demonstrated that NIS is processed at three Asn sites instead of two; thus placing the third glycosylation site (previously predicted in the third intracellular loop at position 225) in the cytoplasmic side of the membrane, because *N*-linked glycosylation occur at exposed extracellular facing sites in the endoplasmic reticulum during protein processing (Levy *et al.*, 1998).

Moreover, by using an amino-terminus FLAG-tagged NIS, engineered by site directed mutagenesis, Levy *et al.* (1998) showed that non-permeabilized as well as permeabilized cells were stained by anti-FLAG antibodies, an observation suggesting that the amino terminus of NIS is located at the extra-cellular side of the plasma membrane (Levy *et al.*, 1998). Based on that, the authors suggested a revised model for the secondary structure of NIS, in which the amino terminus faces extracellularly.

According to this current model (Figure 1.1), NIS is an intrinsic membrane protein, with 13 transmembrane helices (Levy *et al.*, 1998).

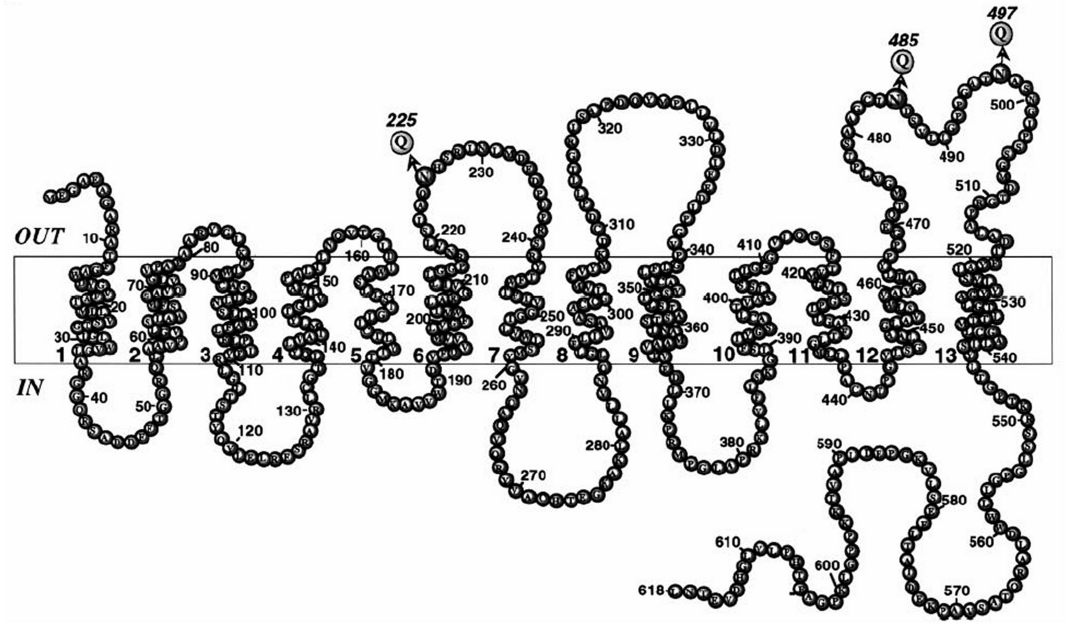

Figure 1.1. The current model for the secondary structure of NIS. In this revised model, NIS contains 13 transmembrane helices. According to this model, the hydrophilic loop containing Asn225 and the NH2 terminus faces extracellularly. All three *N*-linked glycosylation consensus sequences are indicated with Qs at positions 225, 485, and 497. Picture adapted from Levy *et al.* (1998).

1.1.2. Expression of the Na^+/I^- symporter

Ajjan *et al.* (1998) analyzed the expression of NIS in rat extra-thyroidal tissues by quantitative RT-PCR and they showed NIS transcripts in the stomach and in the lactating mammary gland and to a less extent in the small intestine (Ajjan *et al.*, 1998b). Expression of rNIS in the stomach was also demonstrated by another group (Kotani *et al.*, 1998). They compared the expression level of rNIS mRNA and protein in the rat stomach and the rat thyroid cell line FRTL-5, results of which revealed lower NIS protein in stomach in contrast to the level in FRTL-5, although

the mRNA expression level in the stomach was much higher than that of the thyroid cell line, this observation was reasoned to the degradation of the immature gastric NIS protein (Kotani *et al.*, 1998).

Tazebay *et al.* (2000) analyzed the expression of NIS protein in the mammary gland of mice in various physiological stages. A functional expression of mNIS protein starts at mid pregnancy (day 11 of a 19 day gestation period in mice), and reaches highest levels towards the end of pregnancy (day 18), which continues during lactation, such expression of mNIS protein was absent from the mammary gland of nubile mice (Tazebay *et al.*, 2000). They also provided evidence of an increased NIS expression in over 80% of human breast cancer samples compared to no expression in normal tissues from reductive mammoplasties, as well as in experimental mammary tumors induced by an activated *Ras* and *Neu* oncogenes under the control of the Murine Mammary Tumor Virus (MMTV) promoter in transgenic mice (Tazebay *et al.*, 2000).

In a more recent study (Wapnir *et al.*, 2003), NIS expression in a wide variety of normal and cancerous tissues was analyzed by immunohistochemical staining using an anti-NIS antibody. From 371 breast specimens (conventional whole tissue sections) analyzed, NIS expression was confirmed in 76% of invasive breast carcinoma and 88% of ductal carcinoma *in situ* samples, whereas 87% of normal counterparts were reported negative. NIS expression was also observed in a wide range of tumors of other origins, including prostate (74%), ovary (73%), lung (65%), colon (62.6%), and endometrium (56%). NIS protein was present in 75% of benign thyroid lesions and 73% of thyroid cancers (Wapnir *et al.*, 2003).

Expression of NIS mRNA in mouse tissues has been described by RT-PCR as well; high levels of mNIS expression were reported in the thyroid, stomach and in the lactating mammary gland, but with lower levels in small intestine, skin, brain, testis, mammary gland, ovary, spleen and in prostate gland (Perron *et al.*, 2001).

1.1.3. Function of the Na^+/I^- symporter

As the name implies, sodium/iodide symporter is the transporter responsible for the active transport of iodide from the blood stream into cells. This intra-cellular iodide

is then used in different physiological processes. This uptake process is sodium dependant (Bagchi and Fawcett, 1973), and NIS actively co-transports Na^+ and I^- with a stoichiometry of 2 Na^+:1 I^- (Eskandari *et al.*, 1997). The sodium-driven transport of iodide is maintained by an ouabain sensitive sodium-potassium ATP*ase*, which provides the energy required for this process [(Wolff and Halmi, 1963), and for a review see (Baker and Morris, 2004)]. NIS is also capable of transporting other ions with less affinity, including ClO_3^-, SCN^-, $SeCN^-$, NO_3^-, Br^-, BF_4^-, IO_4^-, BrO_3^-, but not perchlorate (ClO_4^-) (Eskandari *et al.*, 1997). In fact, ClO_4^- is a well known competitive inhibitor of iodide transport as well as NO_3^-, BF_4^-, SCN^-, 2,4-dinitrophenol, and cardiac glycosides (Wolff, 1964; Carrasco, 1993; Eskandari *et al.*, 1997).

1.1.3.1. Iodide requirement in hormone biosynthesis and development

It has been known for decades that thyroid hormone biosynthesis requires the presence of inorganic iodide, and the presence of an iodide trapping system is the first limiting step in this process (Wolff, 1964; Carrasco, 1993; Baker and Morris, 2004). Iodide is an essential constituent of the thyroid hormones triiodothyronine (T3) and thyroxin (T4). NIS located at the basolateral membrane of thyrocytes (Chambard *et al.*, 1983), transports iodide into the cells, which is then transported across the apical membrane into the follicular lumen (or colloid) by different anion transporters, such as pendrin and apical iodide transporter (Bidart *et al.*, 2000; Rodriguez *et al.*, 2002). In the colloid, thyroid peroxidase covalently incorporates transported iodide into tyrosine residues of the thyroid hormone precursor, thyroglobulin, in a process known as organification (Igo *et al.*, 1964; Carrasco, 1993). Iodinated thyroglobulin is then endocytosed, followed by phagolysosomal hydrolysis of the iodinated thyroglobulin releasing the thyroid hormones, which are then released into the blood stream, this process in mainly controlled by the thyroid stimulating hormone (Carrasco, 1993). A summary of these events is illustrated in Figure 1.2.

Thyroid hormones are essential for proper growth and maturation of skeletal muscles, nervous system and lungs of the fetus and the developing newborn (Stubbe *et al.*, 1986).

As mentioned before, NIS is also expressed in the lactating mammary gland, and functions to secrete iodide into mother's milk (Tazebay *et al.*, 2000), thus providing the first source of thyroid hormones to the newborn. To date, the biological relevance of NIS expression and iodide transport in organs other than the thyroid and the lactating mammary gland is not clear and further research is required to reveal the significance of iodide in the physiological processes in these organ systems.

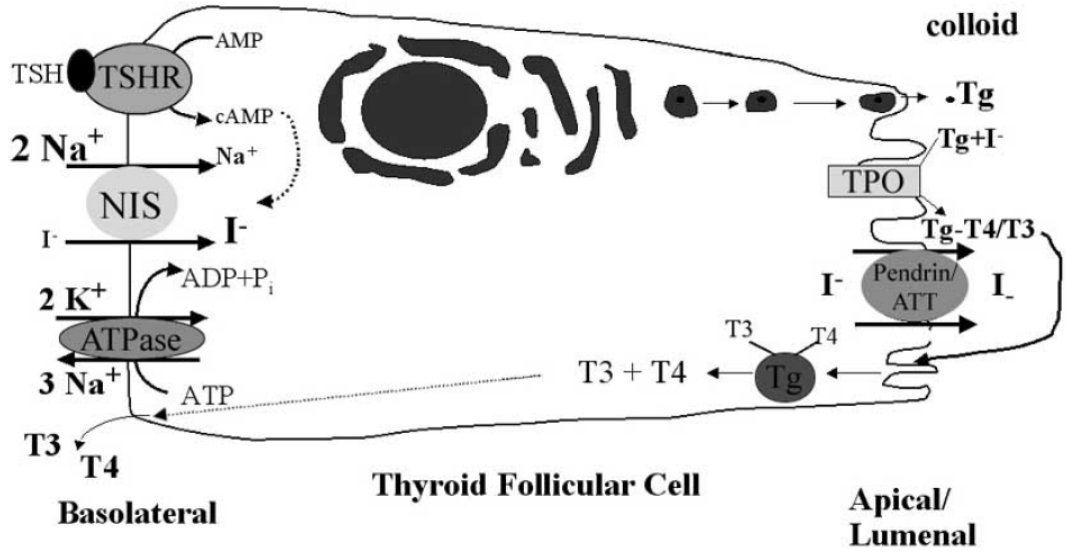

Figure 1.2. An illustration of thyroid hormone bio-synthesis in thyroid follicular cells. TSH stimulates the sodium dependent iodide transport into thyroid follicular cells, this process is maintained by the sodium-potassium ATP*ase*, providing the energy required for this process. Iodide uptaken into follicular cells is transported to colloid by pendrin. Placed at the basolateral membrane, thyroperoxidase (TPO) catalyzes the incorporation of iodide into tyrosine residues of thyroglobulin (Tg) to produce thyroid hormones T3 and T4. Iodinated Tg will be endophagocytosed into the cells and the hormones will be released into blood stream. Figure from Baker and Morris (2004).

1.1.4. Significance of iodide transport in the clinic

The ability of the thyroid gland to transport iodide is an absolute requirement for the synthesis of thyroid hormones. This characteristic, mediated by functional NIS

expression is also observed in abnormalities of the thyroid such as thyroid nodules and thyroid cancer (Wollman and Reed, 1960). This function of NIS – as the key transporter of iodide – has emerged as a valuable tool for the diagnosis and treatment of thyroid cancer. For decades, radioactive iodide played a major therapeutic role in the postoperative management of differentiated thyroid carcinoma (DTC) because of its effectiveness to ablate remnant thyroid tissue and metastases. Moreover, the degree and pattern of iodide accumulation in the thyroid, as revealed by scintigraphic imaging, is used as an aid in the differential diagnosis of thyroid nodules.

This characteristic of the thyroid gland, *i.e.* expression of NIS that is capable of iodide uptake, has been reported in the mammary gland during lactation and in breast cancer (Cho *et al.*, 2000; Tazebay *et al.*, 2000). NIS expression was also observed in a high percentage of human breast cancer specimens (with various pathologies) in contrast to no expression in normal tissues obtained from reductive mammoplasties (Tazebay *et al.*, 2000; Wapnir *et al.*, 2003). These results suggest that radioiodide administration may be effective as an adjuvant to surgical treatment of primary breast cancer, and/or as a tool in the diagnosis and treatment of metastatic disease. A major characteristic of the healthy thyroid gland is that it exhibits NIS activity for life, within boundaries set by thyroid regulatory factors such as thyroid stimulating hormone and iodide itself (Eng *et al.*, 1999; Riedel *et al.*, 2001). In contrast, the potential effectiveness of radioiodide therapy in breast cancer depends on whether NIS becomes functionally expressed in malignant mammary cells, given that it is not functionally expressed in healthy cells, except during pregnancy and lactation. It is notable that a single transport protein – NIS – catalyzes the same fundamental process – active Na^+-dependent I^- transport – in both tissues, but is regulated differently in each of them. These differences affect not only how NIS functions under normal conditions, but also how it can play a role in cancer management in both tissues.

Unlike the thyroid gland (being the only known organ to incorporate iodide into thyroid proteins), in mammary gland cells iodide is secreted into the milk, and this difference creates a challenge for the application of an effective dose of radioiodide for the treatment of malignant cells of the breast (Zuckier *et al.*, 2001).

Clearly, alternative strategies for detection of micrometastatic disease and for more effective and targeted systemic therapies are needed to improve survival in breast cancer, which remains the leading cause of cancer deaths in women (ages of 20-59) in developed countries (Greenlee *et al.*, 2000).

Recently, several researchers have reported the possible use of radioiodide for the treatment of cancers by forced expression of NIS in tumors of several origins, such as in prostate cancer (Spitzweg *et al.*, 2000; La Perle *et al.*, 2002), hepatoma (Haberkorn *et al.*, 2001), glioma (Cho *et al.*, 2002), neuroendocrine tumor cells (Schipper *et al.*, 2003), head and neck squamous cell carcinoma (Gaut *et al.*, 2004), colon cancer (Mitrofanova *et al.*, 2005; Scholz *et al.*, 2005), pancreatic tumors (Dwyer *et al.*, 2006a), and in ovarian tumor xenografts (Dwyer *et al.*, 2006b).

Insofar, as NIS is functionally expressed to a sufficient degree in cancerous cells, whether of thyroid, breast, or any other origin, radioiodide emerges as a potential diagnostic and therapeutic tool. A considerable amount of work has already been carried out concerning transcriptional regulation of NIS in thyroid gland [see (Pasca di Magliano *et al.*, 2000) and references within]. On the other hand, the molecular determinants of mammary gland NIS transcription are still relatively unknown. Therefore, an extensive study of *cis*- and *trans*-acting factors regulating the NIS gene in mammary gland might prove extremely valuable and informative for the efforts of establishing a novel diagnostic and/or therapeutic protocol against the breast disease.

1.2. Regulation of Na^+/I^- Symporter Gene Expression

1.2.1. Regulation in thyroid gland

In thyroid gland, thyroid stimulating hormone's (TSH) action elevates the intracellular level of cyclic AMP (cAMP), and this elevation is an important modulator of gene expression in thyrocytes (Ikuyama *et al.*, 1992; Armstrong *et al.*, 1995). Such effect of TSH on iodide uptake in the thyroid was first reported in the 1960s when researchers described increased iodide uptake in thyroid gland of rats treated with TSH in a cycloheximide dependent manner. This suggested that TSH actually is responsible for the synthesis of an enzyme that mediates iodide uptake

(Halmi *et al.*, 1960). This observation was later confirmed by Knopp and co-workers (1970) using bovine thyroid cells, and the inhibitory effect of actinomycin D when added together with TSH was also observed. In contrast, in cells treated with actinomycin D after 2 hours of TSH treatment iodide uptake was stimulated normally, suggesting that TSH treatment resulted in the synthesis of a specific RNA molecule. They also found that cyclohexamide blocked iodide uptake when added with TSH, however, if TSH and cyclohexamide were washed out after two hours, iodide uptake developed normally (Knopp *et al.*, 1970). In the same study, they also observed a similar effect of TSH when they incubated the cells with cAMP, and that the cellular levels of cAMP responded to increasing or decreasing concentrations of TSH, concluding that TSH in thyroid cells activates adenyl cyclase so that cAMP production is augmented, and then the production of a specific RNA molecule, which in turn induces the formation of specific stimulatory protein (Knopp *et al.*, 1970).

These early findings actually suggested a regulatory action of TSH on the expression of NIS in the thyroid gland. Regulation of NIS expression at the transcriptional level was more evident in research carried out after the cloning of the NIS gene (Dai *et al.*, 1996; Smanik *et al.*, 1996; Perron *et al.*, 2001; Pinke *et al.*, 2001), thus supporting results of earlier reports concerning regulation of NIS expression in thyrocytes. It has been shown that TSH activates the transcription of NIS via cAMP in a cyclohexamide-dependent manner (Kogai *et al.*, 1997). Later on, several reports characterized this TSH stimulated NIS transcription, clarifying this regulatory mechanism and confirming that, in thyroid gland, TSH regulates NIS dependent iodide transport both at post-translational and at transcriptional level (Levy *et al.*, 1997; Ohno *et al.*, 1999; Riedel *et al.*, 2001). Clues for TSH mediated post-translational regulation of NIS came from studies, in which membrane vesicles (prepared from FRTL-5 cells which lost iodide uptake as a result of prolonged deprivation of TSH) retained iodide uptake after stimulation by TSH, suggesting that NIS protein is present in the vesicles and a mechanism other than transcription might be required for proper NIS activity (Kaminsky *et al.*, 1994).

The regulatory effect of TSH on NIS protein was illustrated later on; researchers found out that the half life of NIS protein increases from 3 days to 5 days

in the presence of TSH, and that NIS is a phosphoprotein, whose phosphorylation is mediated by TSH (Riedel *et al.*, 2001). Moreover, TSH was found to modulate the intra-cellular distribution of NIS; in the presence of TSH, NIS is mainly located at the plasma membrane, whereas in TSH deprived cells NIS was translocated to intra-cellular compartments (Riedel *et al.*, 2001).

At transcriptional level, TSH dependent expression of NIS is mediated by an adenylate cyclase-cAMP pathway (Carrasco, 1993; Ohno *et al.*, 1999). Several groups isolated the 5' regulatory region of rat and human NIS genes in order to study *cis*- and *trans*-acting elements that regulate NIS transcription in thyrocytes (Endo *et al.*, 1997; Tong *et al.*, 1997; Behr *et al.*, 1998; Ryu *et al.*, 1998; Ohno *et al.*, 1999). It was reported that, a novel transcription factor, named "NIS TSH-responsive factor-1" or NTF-1, mediates the transcriptional regulatory effect of TSH, mediated by cAMP, on NIS promoter through a TSH responsive element (TRE) located between positions -420 and -385 of the rat NIS promoter in a thyroid specific manner (Ohmori *et al.*, 1998). Figure 1.3 illustrates the positions of *cis*-acting elements involved in the regulation of NIS transcription in the thyroid gland.

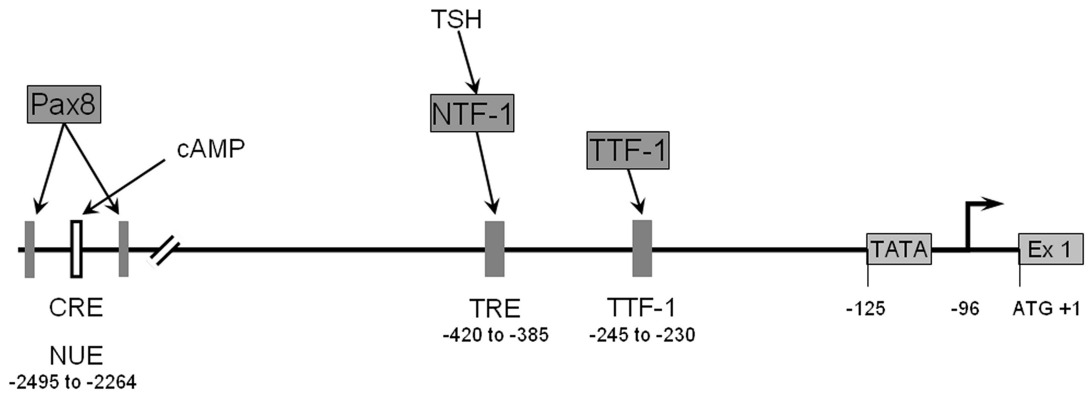

Figure 1.3. A representation of the factors involved in the regulation of NIS expression in the thyroid gland. Small boxes on the horizontal line represent the position of the known binding sites of the factors regulating the thyroid specific transcription of NIS. The position of TATA element and the first exon is indicated with horizontal boxes. The position of transcription start site is indicated by the right angle arrow, numbering is relative to the first base of the start codon.

Thyroid transcription factor-1 (TTF-1), which belongs to the Nk2 family of homeobox-containing genes in *Drosophila* (Guazzi *et al.*, 1990) was implicated in the regulation of several thyroid specific genes such as thyroid peroxidase (TPO), thyroglobulin (Tg) and thyroid stimulating hormone receptor (TSH-R) (Damante and Di Lauro, 1994). It was also found to activate the transcription of NIS in thyroid cells, and functional TTF-1 binding sites were found between nucleotide positions -245 to –230 bp of the rNIS promoter (Endo *et al.*, 1997). Mutations in the NTF-1 binding site (TRE) causing loss of the TSH response also resulted in a decrease in the TTF-1-induced promoter activity in transfection experiments. Suggesting that TTF-1-mediated thyroid specific expression of NIS is controlled by the TSH/cAMP-pathway (Endo *et al.*, 1997).

1.2.1.1. NIS upstream enhancer

The isolation and the cloning of the promoter and upstream regulatory region of the rat NIS gene facilitated the search for *cis*- and *trans*-acting genetic elements mediating thyroid-specific and TSH-regulated transcriptional activation (Endo *et al.*, 1997; Tong *et al.*, 1997; Ohno *et al.*, 1999). These studies resulted in the identification of a thyroid-specific transcriptional regulatory *cis*-acting element at the 5'-flanking region of the rat NIS gene (Ohno *et al.*, 1999). This enhancer region (NIS upstream enhancer, or NUE), is located between nucleotides -2495 to -2264 and contains binding sites for Pax8, a paired domain factor which is present both in thyroid and in kidney, and TTF-1, a homeodomain containing protein present in the developing thyroid, lung and diencephalon. In DN*ase* I footprinting studies carried by Ohno *et al.* (1999), it has been shown that Pax8 actually binds to two sites in this newly identified enhancer. Mutational analysis of these binding sites has shown that Pax8 binding as well as NIS transcription is reduced when these sequences are modified, suggesting a functional role for Pax8 in NUE transcriptional activity.

TTF-1 also binds at two different sequences in NUE element, and one of these two binding sites overlaps with Pax8 binding site, while the other is closely located (~20 nucleotides) but distinct from the second site. It was demonstrated that, when located at the 5' of the thymidine kinase (TK) promoter controlling a marker gene, the NUE element can activate the transcription of the marker in nonthyroid cell

lines in a cAMP-dependent manner, only if this construct is cotransfected with a Pax8 expression vector, and not with a TTF-1 expression vector. This suggests that Pax8 (and not TTF-1) is required for the transcriptional activation and cAMP stimulation of the NUE. Moreover, a mutation at another site containing a degenerate cAMP responsive element (CRE-like) sequence (5'-TGACGCA-3') does not interfere with Pax8 binding to NUE, but almost abolishes NUE activation by protein kinase A (PKA). This indicates that an unidentified element that binds to this degenerate CRE-like sequence acts synergistically with Pax8 in order to establish TSH-controlled transcription of NIS (Ohno *et al.*, 1999).

Further work revealed that this CRE-like element in the rNUE (named NUC) is a *bona fide* CRE and that it can be recognized by various members of the AP-1 and CREB family of transcription factors that modulate the transcriptional activity of NUE. Further more, using tethered dimers of b-Zip molecules, it has been shown that specific homo- or hetero-dimers of AP-1 can synergistically stimulate NUE activity in concert with Pax-8 (Chun *et al.*, 2004).

Thyroid specific transcription factor-1 (TTF-1), NIS TSH-responsive factor-1 (NTF-1) and Pax8 are the major transcription factors that have been implicated in thyroid specific transcription of rNIS (Endo *et al.*, 1997; Ohmori *et al.*, 1998; Ohno *et al.*, 1999). In accord with that, the Human NIS upstream enhancer (hNUE) was also identified; it was found to be localized at –9847 to –8968 bp relative to the hNIS gene start codon. It contains functional Pax8 and TTF-1 binding sites and a CRE-like sequence (Taki *et al.*, 2002). The enhancer was shown to be cell specific; it activates NIS transcription only in thyroid cell lines, and not in MCF-7 breast cancer or JEG-3 choriocarcinoma cells (Taki *et al.*, 2002).

1.2.1.2. Regulation of NIS by retinoids in the thyroid

As presented earlier, the ability of thyroid tumors to retain iodide uptake was used for decades for the treatment and diagnosis of thyroid cancer. However, in cases with advanced tumorigenesis, thyroid cells suspend this characteristic of iodide uptake due to progressive tumor associated de-differentiation of thyrocytes, thus leading to ineffective radioiodide therapy. It has been shown recently that in patients with

radioiodide resistant tumors, treatment with retinoic acid (RA: a well known agent with differentiation-inducing properties) reactivates the iodide uptake mechanism, and thus restoring the possibility of radioiodide based therapy (Simon *et al.*, 1996). Further characterization of this stimulatory effect of RA in thyroid cell models revealed that, RA treatment of normal non-transformed thyrocytes resulted in decreased iodide uptake and reduced NIS expression. On the other hand, both NIS mRNA and iodide uptake were elevated in human follicular thyroid carcinoma cell lines, suggesting that RA treatment could be used to up-regulate NIS expression and thus iodide uptake in tumor cells to be targeted differentially by radioiodide treatment (Schmutzler *et al.*, 1997).

Retinoic acids are derivatives of vitamin A, which play an important role in several physiological processes during embryonic development and in adult life (Pfahl and Chytil, 1996). They are also known for their potent proliferation-inhibiting and differentiation-inducing properties. Retinoic acid signals are mediated by nuclear receptors (Retinoic acid receptors, RAR and Retinoic X receptors, RXR), action of which can be seen as receptor-receptor interactions, or receptor-DNA interactions, as well as interactions with other regulatory proteins (Pfahl and Chytil, 1996). Transcription activation function of RARs is mediated by binding to DNA sequences called retinoic acid response elements (RARE) in the promoter of target genes (Giguere, 1994). The binding site of RARs may vary, depending on the target gene, and the consensus sequence is a hexamer (PuGG/TTCA). The classical RARE is composed of two direct repeats of this core motif, which are usually separated by 5 nucleotides, although functional direct repeats separated by 1, 2 or 10 nucleotides have been also reported (Giguere, 1994; Kato *et al.*, 1995).

The molecular determinants controlling this RA induced NIS expression in thyroid cells were investigated; it has been shown that RA exerts its up-regulatory effect on hNIS promoter through a RARE located at -1375 relative to the ATG codon (Schmutzler *et al.*, 2002). It has been shown that RAR binds to this element (DR10: 5'-AGGTCAn_{10}GGGTCC) and activates NIS transcription in response to RA stimulation, and that the RA stimulation and RAR binding were abolished due to mutations in either half site of this element (Schmutzler *et al.*, 2002). This evidence

of a direct stimulatory action of RA on NIS expression in thyroid cell lines, as well as the success in RA redifferentiation prior to radioiodide therapy, encouraged investigators to study the feasibility of radioiodide therapy after RA treatment in cancer patients with tumors of other origins (Spitzweg *et al.*, 2003; Abu *et al.*, 2005).

1.2.2. Regulation of NIS in mammary gland

In addition to the thyroid gland where iodide is used for thyroid hormone biosynthesis, iodide is also concentrated in lactating mammary gland, and sufficient supply of iodide in milk to the nursing newborn is essential for proper development of the nervous system, skeletal muscles, and lungs (Carrasco, 1993). Tazebay and colleagues previously carried out a study to assess the functionality as well as the regulation of NIS protein expression in various extrathyroidal tissues by western blots, and scintigraphic imaging in live animals after radioiodide or technetium-pertechnetate ($^{99m}TcO_4^-$, a substrate of NIS with shorter radioactive half-life as compared to radioiodide, $^{131}I^-$) injections (Tazebay *et al.*, 2000). They have provided immunological and biochemical evidence that iodide uptake in the lactating mammary gland is mediated by the same protein as in the thyroid, but with significant differences in the hormonal regulation of its expression in these two tissues.

in vivo experiments in mice demonstrated that in normal physiology, NIS expression is strictly linked to mammary development in gestation, and to lactation (Tazebay *et al.*, 2000). After delivery, NIS accumulation in mammary epithelial cells depends on suckling in a reversible manner. Non-lactating mammary gland tissue in female mice does not express NIS (and does not accumulate iodide) unless animals receive subcutaneous oxytocin treatments for three consecutive days. On the other hand, a similar treatment in ovariectomized mice is not sufficient for NIS up-regulation, and in such surgically treated animals administration of estradiol (E2) together with oxytocin is essential for functional expression of NIS. In these animals, a hormone combination including oxytocin and prolactin in addition to E2 leads to a robust NIS expression comparable to that in lactating mammary glands (Cho *et al.*, 2000; Tazebay *et al.*, 2000). The fact that E2 treatment was only essential in ovariectomized animals, whereas lactogenic hormones were sufficient for functional

NIS expression in normal (surgically untreated) virgin mice suggested that ovary functions and endogenous estrogens are essential in up-regulating NIS expression (Tazebay *et al.*, 2000).

The effect of prolactin was also described in studies performed using mouse mammary gland explants (Rillema and Rowady, 1997), it has been shown that physiological concentrations of prolactin were sufficient to increase the expression of NIS protein accompanied by an increased iodide uptake. This increase in NIS protein levels was inhibited by cyclohexamide and actinomycin D, suggestive of a prolactin role at both translational and transcriptional levels (Rillema *et al.*, 2000). In another study, the effect of prolactin was also manifested as it increased the iodide uptake when added to mouse mammary gland explants isolated from mid pregnancy mice and incubated with insulin and cortisol (Rillema *et al.*, 2002). In the same study, an effect of insulin on the iodide uptake was also observed, by itself or in combination with any of the other hormones applied. It was interesting to observe a significant increase in iodide uptake mediated by insulin in explants isolated from virgin mice too, in contrast to prolactin, which did not affect iodide uptake when applied alone. The increase in iodide uptake in response to the triple hormone treatment of explants from pregnant mice compared to no effect on virgin animal explants suggested that lactogenic hormone stimulation of iodide uptake required differentiated mammary gland cells (Rillema *et al.*, 2002). However, the molecular determinants contributing to this regulation by lactogenic and steroidal hormones affecting NIS expression or iodide uptake in the mammary gland during lactation were not described in those reports.

1.2.3. Regulation of NIS in human mammary carcinoma cell line MCF-7

In order to identify *cis*-acting elements responsible for hormone dependant regulation of NIS expression, an appropriate cell line model where functional analyses could be performed should be found. Despite a wide search by many groups including ourselves, a mammary gland cell line where NIS expression is regulated in response to estradiol, oxytocin or prolactin could not yet be identified. Nevertheless, RA which has a role in development, differentiation, and cell growth has been shown to up-regulate functional NIS expression in estrogen receptor (ER) positive, RA

receptor positive, MCF-7 human breast carcinoma cell line (Kogai *et al.*, 2000). In this cell line, NIS was shown to be inducible in response to 9-*cis*-retinoic acid (9cRA) and all-*trans*-retinoic acid (tRA), ligands that were previously known to induce iodide transport activity in dedifferentiated thyroid tumor metastatic tissues in humans (Kogai *et al.*, 2000; Schmutzler and Kohrle, 2000; Tanosaki *et al.*, 2003). Kogai *et al.* (2000) have shown that tRA have up-regulated both NIS expression and iodide transport in MCF-7 cells in a dose dependent manner. The absence of a similar increase in NIS mRNA levels in the ERα- MDA-MB-231 cell line after tRA treatment has led the authors to consider that ERα positivity of MCF-7 may have led to increased levels of RAR in the presence of E2, which may provide cellular conditions favorable for NIS expression (Kogai *et al.*, 2000). A correlation between the ERα status of mammary cell lines and 9-*cis*-retinoic acid (9cRA, a ligand for both RAR/RXR heterodimers and RXR/RXR homodimers) inducibility of NIS gene was also previously indicated (Tanosaki *et al.*, 2003).

In a separate study, Nkx-2.5, a homeobox transcription factor, was indicated as the mediator of tRA responsive NIS expression in MCF-7 cells (Dentice *et al.*, 2004). In MCF-7, RA was shown to first up-regulate the expression of Nkx-2.5 mRNA (reaching maximum levels after 6 hours), followed by NIS mRNA with maximum level at 12 hours, the iodide uptake increase was linear and followed the increase in NIS expression. Nkx-2.5 was also shown to interact with two sites in the rat NIS promoter; the site W was at -250 and the site N2 was at -542 relative to the ATG codon (Figure 1.4). They have also reported a correlation between Nkx-2.5 expression and lactation in mice (Dentice *et al.*, 2004).

Systemic tRA treatment in immunodeficient mice with MCF-7 xenograft tumors resulted in a significant increase in NIS expression and in iodide uptake. Similar treatment was also evident in transgenic mice carrying the oncogene polyoma virus middle T antigen (Kogai *et al.*, 2004) and the iodide accumulation in other organs was not affected by tRA treatment. The RA induced-NIS expression in MCF-7 was also found to be stimulated by dexamethasone. In the presence of tRA, dexamethasone increased NIS mRNA levels and iodide uptake significantly, in

addition to an inhibition of radioiodide efflux, resulting in a better drug combination to increase radioiodide toxicity in breast cancer cells (Unterholzner *et al.*, 2006).

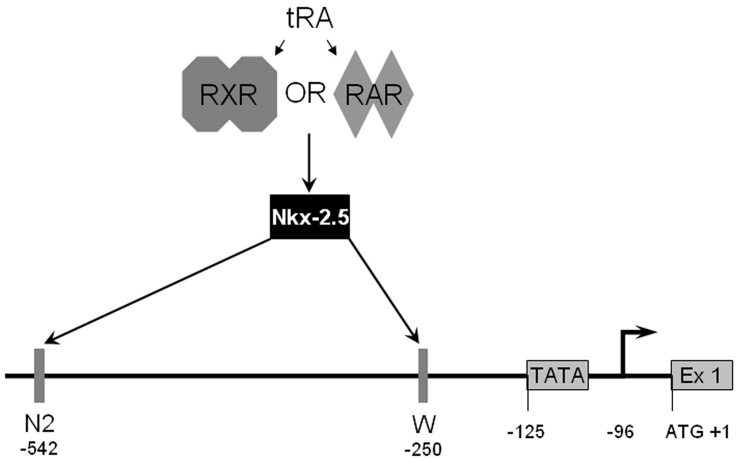

Figure 1.4. Regulatory elements involved in Nkx-2.5 dependant transcription of NIS in MCF-7. It was proposed that upon tRA stimulation, RA receptors will activate Nkx-2.5, which in turn will bind to two sites (N2 and W) in the promoter of rNIS leading to transcription. The position of these elements is indicated, and the numbering is relative to the start codon.

Recently, it was reported that in MCF-7 cells, treatment with prolactin, insulin, insulin-like growth factor-I (IGF-I), and IGF-II resulted in an increased expression of NIS both at transcriptional and at the protein levels. These ligands also resulted in an increased iodide uptake in MCF-7 cells in culture (Arturi *et al.*, 2005).

1.3. Aim of This Study

As presented above, the transcriptional regulation of the mammary gland NIS gene is not yet fully understood. Several hormones and ligands have been implicated in the increased functionality of this gene aiming to develop strategies for novel methods for the diagnosis and treatment of breast cancer. Since NIS is expressed in the normal mammary gland only during lactation, and in breast cancer, understanding the

molecular determinants regulating its expression and function will ultimately provide a broader vision on possible therapeutic applications.

Our aim is to identify *cis*- and *trans*-acting genetic elements involved in the transcriptional regulation of NIS in the mammary gland, and in breast cancer models.

1.3.1. Rationale

It has been reported recently that conserved patterns of gene expression could reflect conserved patterns of regulatory mechanisms and potentially conserved *cis*-acting elements (Negre *et al.*, 2005). Thus conserved regions in the sequences around and including NIS gene could harbor regulatory *cis*-acting elements involved in the mammary gland specific expression. We carried out a comparative bioinformatics analysis of the sequences flanking and including the NIS gene in human, mouse and rat searching for conserved regions, which possibly contain distal control regions regulating the expression of NIS in response to tRA in MCF-7 breast cancer cells. Such putative control elements were cloned in front of a luciferase reporter gene and they were used in functional assay experiments. This has led to the identification of several putative control regions.

It has been also shown that E2 is one of the hormones regulating functional NIS expression in the mammary gland, which when administered alone was sufficient to up-regulate its expression (Tazebay *et al.*, 2000). Moreover, the well known tRA-induced expression in MCF-7 was correlated with presence of estrogen receptor (ER), in contrast to MDA-MB-231, in which ER negativity and lack of RA response correlated. We studied the effect of individual or combined nuclear receptor ligands, such as 17-β-estradiol and tRA, on NIS transcription in the breast cancer cell line model MCF-7. We found out that tRA induces NIS expression in cell lines that were both ERα+ and RARα+. We also showed that ectopic expression of ERα in MDA-MB-231 restored basal NIS expression in a ligand independent manner. We show evidence to the direct involvement of ERα in NIS expression by the a functional ERE in NIS promoter.

2. MATERIALS AND METHODS

2.1. Sequence Information and Databases

Genomic DNA sequences to be analyzed for DNA conservation in non-coding sequences were obtained from the Genome Browser database at the University of California Santa Cruz (Kent *et al.*, 2002). The initial analysis was performed on a 310 kbp sequence from *Homo sapiens* (Release July 2003; Chr.19:17622612-17932611), *Rattus norvegicus* (Release June 2003; Chr. 16: 18813016-19123015), and *Mus musculus* (Release October 2003; Chr. 8: 71126739-71436738). Annotations for the human sequence were obtained from the Genome Vista database (Couronne *et al.*, 2003), and the sequences were aligned with the mVista tool (Frazer *et al.*, 2004) using a conservation level of 60% at a window length of 75 bp with the human sequence at the x axis.

The second analysis was performed on a shorter stretch of DNA of 90 kbp from *H. sapiens* (Release July 2003; Chr. 19: 17816282-17906905), *R. norvegicus* (release June 2003; Chr. 16: 19018698-19107000), and from *M. musculus* (Release October 2003; Chr. 8: 71152446-71242000). Annotations for the human sequence were obtained from the Genome Vista database, and sequences were aligned with the mVista tool using the human sequence at the x axis and at 50% conservation level and a window length of 75 bp.

2.1.1. PCR amplification of conserved regions

Common conserved regions among human, mouse and rat were selected for PCR amplification. Conserved regions clustered within 1 kbp were amplified as one larger fragment and subsequently cloned into the reporter vector (see Results section 3.1). PCR amplification was performed in 25 µl reaction volumes containing 0.8X PCR buffer, 3 mM $MgCl_2$, 200 µM dNTP, 10 pmoles of each primer, 5% DMSO, 1 U of

Taq DNA polymerase (Roche) and 100 ng of human genomic DNA. Thermal cycler conditions were an initial denaturation step at 94°C for 4 min; a loop cycle of 94°C, 30sec / 62°C, 30sec / 72°C, 40sec; and a final extension at 72°C for 7 minutes. Table 2.1 illustrates the position of each conserved region cluster and primers used for PCR amplification.

Table 2.1. Conserved region clusters used for PCR amplification. Genomic position of each cluster is indicated according to the human sequence of chromosome 19 Release July 2003.

Cluster Name	Genomic Position offset=17816282*	PCR Primers (5'→3')
Cluster A	15485-15502 15503-15707 16115-16200 16348-16420	hNISA-F: GGTACCACGCGTCGGAGAGCACGCCATGAAGG hNISA-R: AAGCTTCTCGAGGGCCTCCATGTGGATTCTTG
Cluster B	16695-16749 16821-17000 17622-17751	hNISB-F: GGTACCACGCGTAAGAATCCACATGGAGGCCC hNISB-R: AAGCTTCTCGAGTTGCCCTGCCTCAGTGTGAG
Cluster 1	18147-18210 18446-18555 18699-18791	hNIS1-F: GGTACCACGCGTCCTAAACCCAAGAATCACTG hNIS1-R: AAGCTTCTCGAGTTGCACTGAAGACCCCTCCC
Cluster 2*	21295-21350 21381-21452 21455-21554	hNIS2-F: GGTACCACGCGTAACACACAGGAGCAGTGAGG hNIS2-R: AAGCTTCTCGAGGGAGATGAGGGTATGCAGTG
Cluster 3	26374-26432 26673-26747 26861-27102 27311-27440	hNIS3-F: GGTACCACGCGTTCAGATTCCACCAGCTTATG hNIS3-R: AAGCTTCTCGAGGGGTTGCAGATTTATTGGGC
Cluster 4	28380-28458 28727-28822 28972-29037	H4N-F: GGTACCACGCGTCTCGGTGCTTTAACGGAAGG H4N-R: AAGCTTCTCGAGACTCCTGACCTCGTGATCCA
Cluster 5	34063-34108	hNIS5-F: GGTACCACGCGTTACACTCCACCTTGGATGAC hNIS5-R: AAGCTTCTCGAGGGATCTTTGGAACTCTCTGG
Cluster 8	47056-47245 47607-47619	hNIS8-F: GGTACCACGCGTAGCCCAGCCTCATCTTCAAG hNIS8-R: AAGCTTCTCGAGTGAACTCCTGGCCTCAAGCG
Cluster 9	65174-65232 65268-65369 65440-65528	hNIS9-F: GGTACCACGCGTTGACTCCACTTGCCTCCTAC hNIS9-R: AAGCTTCTCGAGTGGCGAAACCTTGTCTCTAC

* Offset value for cluster 2 regions was (17816282) based on Human-mouse comparison, each interval represents the individual conserved regions.

2.2. Plasmids

2.2.1. Reporter constructs

The luciferase reporter vectors, pGL3E1bLuc and phRL-TK were kindly provided by Roberto Di Lauro, Naples, Italy. The reporter pGL3E1bLuc is a modified version of the original plasmid pGL3-Basic (Promega), which contains the E1b TATA element (5'-TCG AGT CTA GAG GGT ATA TAA TGG ATC-3') between *Xho*I/*Bgl*II sites of pGL3-Basic; destroying the *Bgl*II site while keeping the *Xho*I site intact. PCR products containing conserved regions were cloned into *Mlu*I/*Xho*I sites of pGL3E1bLuc to produce the reporter plasmids named cluster A, cluster B and clusters 1, 2, 3, 4, 5, 8 and 9.

Cluster 3 derivatives containing individual or combined conserved regions were prepared by restriction endonuclease digestion of internal sequences, or by PCR. The plasmid Cl.3-ex containing the conserved region 3-1 was prepared by removing *Esp*I/*Xho*I fragment from cluster 3, likewise plasmids Cl.3 -pm, Cl.3 Δ3-4, Cl.3 -xp, Cl.3 -*Sac*I and Cl.3 -*Esp*I were prepared by removing the *Pvu*II/*Mlu*I, the *Pst*I/*Sma*I, the *Xho*I/*Pvu*II, the *Sac*I and the *Esp*I fragments respectively.

The plasmid Cl.3-2 was prepared by cloning a PCR product containing the conserved region 3-2 using primers *3-2F*: 5'-CTG CAG ACG CGT AGG CTG AGC TGA GAC TTG AA-3' and *3-2R*: 5'-AAG CTT CTC GAG GAG GAA TAA ATG GGA CGT GG-3' into *Mlu*I/*Xho*I sites of pGL3E1bLuc, likewise Cl.3-3 was prepared by inserting a PCR fragment containing the conserved region 3-3 (PCR primers were *3-3F*: 5'-GGT ACC ACG CGT CCA TTT ATT CCT CTG AGG CA-3' and *3-3R*: 5'-ACT AGT CTC GAG TGT CTG CTG TTG ACA GGT GG-3') into *Mlu*I/*Xho*I sites of pGL3E1bLuc.

The plasmid Cl.3/4 was prepared by removing the insert containing the conserved cluster 4 from Cl.4 (first, digesting with *Mlu*I followed by klenow filling of the cohesive ends and then digesting with *Xho*I) and inserting this fragment into the *Bam*HI (klenow filled to produce blunt ends) and *Sal*I sites of Cl.3 following the luciferase reporter gene.

Constructs containing the human NIS 5'UTR were prepared by inserting a PCR product (PCR primers were *hNIS5UTR-F* 5'-TGG CCT GTC TGT CCC AGT CCA GGG CTG A-3' and *hNIS5UTR-R* 5'-TCT CCA CGG CCT CCA TGG AGG GCG GGT GCG GA-3') amplified from human genomic DNA into the *Sma*I/*Nco*I sites of the reporter constructs.

The reporter pPS2XERE was prepared by ligating a synthetic double strand (ds) oligo-nucleotide containing two tandem copies of the pS2 ERE into *Mlu*I/*Xho*I sites of pGL3E1bLuc. The DNA sequence of this oligo-nucleotide was 5'-CGC GTA AGG TCA CGG TGG CCA CAC GCG TAA GGT CAC GGT GGC CAC CCC GTC-3'. Likewise, pNIS2XERE was created by inserting a synthetic ds oligo-nucleotide containing two tandem copies of the putative NIS ERE (5'-CGC GTA GGC GGA GTC GCG GTG ACC CGG CGG AGT CGC GGT GAC CCG GGA GC-3') into the *Mlu*I/*Xho*I sites of pGL3E1bLuc. The reporter plasmid pRARE-Luc was prepared by inserting a ds oligo-nucleotide containing 3 DR5 elements separated by 2 nucleotides in the *Mlu*I/*Xho*I sites of pGL3E1bLuc, the sequence of this oligo was (only the upper strand is shown and the direct repeat elements are underlined) 5'-CGC GTA GGT CAA ATG CAG GTC AAA AGG TCA AAT GCA GGT CAA AAG GTC AAA TGC AGG TCA C-3'. The reporter pC5-Luc was prepared by inserting a ds oligo nucleotide containing 5 tandem repeats of the C element harboring TTF-1/Nkx-2.5 binding site (Dentice *et al.*, 2004), the sequence of which was 5-CCC AGT CAA GTG TTC TT-3' in the *Mlu*I/*Xho*I sites of pGL3E1bLuc.

2.2.1.1. Site directed mutagenesis

Constructs harboring the retinoic acid response element (5'-AGG TCA AAG TCC TCC TGG GTC C-3'; bases 125-146 in cluster 3), were subjected to site directed mutagenesis based on data from Schmutzler *et al.* (2002). PCR based mutagenesis was performed in 50 µl reaction volumes containing 40 ng of target plasmid DNA, 1X *Pfu* buffer, 200 µM dNTP mix, 100 ng of each primer and 2.5 U of *Pfu*-Turbo DNA polymerase (Stratagene). The primer pair (the mutant nucleotides are underlined) *sense a125t/g127a*: 5'-GAC CAG AAC CTC CAG TGA TCA AAG TCC TCC TGG G-3' and *antisense a125t/g127a*: 5'-CCC AGG AGG ACT TTG

ATC ACT GGA GGT TCT GGT C-3'. Reaction conditions were an initial denaturation step at 95ºC for 30 seconds followed by 15 cycles of 95ºC for 30 seconds / 55ºC for 1 minute / 68ºC for 6 minutes and 20 seconds. Following the PCR reaction the tubes were cooled to 37ºC on ice and then 1 µl of *Dpn*I (10U/µl; Fermentas) was added and incubated at 37ºC for 1 hour. Following the *Dpn*I digestion of the parental (methylated) strand, 5 µl were used for transformation of super-competent *E. coli* (DH5α) cells.

Plasmids were rescued from single colonies and checked for the presence of the mutation by automated DNA sequencing. Similar protocol was used to create the ERE mutants in plasmids harboring the estrogen responsive element. The oligo nucleotide sequences of primers used for the mutagenesis are shown in table 2.3.

2.2.2. Expression vectors

The expression plasmid for Nkx-2.5 was kindly provided by Domenico Salvatore, Naples, Italy. The expression vector for ERα (pCMV-ERα) was prepared by inserting the *Eco*RI fragment (containing ERα coding sequence) form the plasmid pSG5ERpuro (kindly provided by Patrick Balaguer, Montpellier, France) into pcDNA3.1C (Invitrogen). Oligo-nucleotides for the knockdown of ERα were designed and supplied by Oligoengine, WA (N-19 targets 458 and 499 on NM_000125 were *sh-ER458*: 5'-TTC AGA TAA TCG ACG CCA G-3', and for *sh-ER499*: 5'-GTA CCA ATG ACA AGG GAA G-3'). These shRNA oligos were then cloned in the *Bgl*II/*Xho*I sites of pSuper-GFP/Neo (pSR, Oligoengine, WA) to generate the two knockdown constructs, pSR-ER-458 and pSR-ER-499.

2.3. Cell Culture

2.3.1. Cell lines

Human breast cancer cell lines BT-474, T-47D, BT-20, MDA-MB-453, MDA-MB-468, *hTERT*-HME1, MCF-7, MDA-MB-231 and MDA-66; Human cervical cancer cell line HeLa; rat thyroid cell line FRTL-5 and the monkey kidney cell line COS-7 were used in this study.

2.3.2. Growth media

Mammary gland, HeLa and COS-7 cell lines were maintained in high glucose Dulbecco's modified Eagle's medium (Gibco) [supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 1% L-glutamine (Biochrom)], abbreviated in the text as reg-DMEM, at 37°C in a 5% CO_2 incubator. MDA-66 was maintained in the above medium with the addition of 0.4 mg/ml Hygromycin (Roche). FRTL-5 cells were maintained in Coon's modified Ham's F12 medium supplemented with 2 mM glutamine, 5% FBS, 1% P/S and the 6 hormone mix (10 µg Insulin, 10 nM Hydrocortisone, 5 µg/ml Transferrin, 10 ng gly-his-lys acetate, 10 ng/ml somatostatin and 10 mU/ml Thyroid Stimulating Hormone (TSH)) at 37ºC in a 5% CO_2 incubator. These cells display a sensitivity to TSH-induced increase in cAMP levels, thus the presence of TSH is an absolute growth requirement.

2.3.3. Hormone induction

All-*trans*-retinoic acid (tRA) and 17-β-estradiol (E2) were purchased from Sigma. tRA was dissolved in DMSO, E2 was dissolved in ethanol as 10 mM stock solutions, and stored protected from light at -20°C.

2.3.3.1. tRA induction

In general tRA induction experiments were performed in reg-DMEM, unless otherwise indicated. tRA was applied to a final concentration of 1 µM for 12 hours when analyzing the expression of NIS by RT-PCR (24 hours induction was applied when performing luciferase reporter assay with tRA stimulation). After hormone induction, cells were rinsed with cold PBS, and harvested by trypsinization, cell pellets were divided into two tubes; RNA and protein extracts were prepared from the same sample.

2.3.3.2. E2 induction

Induction with E2 was performed in sf-DMEM (a phenol-red free DMEM (Sigma) supplemented with 10% dextran-coated-charcoal stripped FBS, 1% P/S and 1% L-glutamine). Two days before the addition of hormones, cells were fed with sf-DMEM in order to deplete the culture media from endogenous steroids and retinoids.

E2 was applied to a final concentration of 10 nM for 3 hours. After hormone induction cells were collected as mentioned above and divided into two tubes.

2.3.4. Transfection

Cell lines were transfected with plasmid DNAs using FuGENE-6 reagent (Roche). FuGENE:DNA ratios were determined experimentally to be 3:1 for MCF-7, HeLa, FRTL-5 and COS-7 and 6:1 for both MDA-MB-231 and for MDA-66. In general, cell lines were seeded in the appropriate culture container (depending on the assay to be performed) at an experimentally optimized density (depending on the cell line) so that they reach confluence at the time of the assay. For MCF-7 and FRTL-5, cells (90%-100% confluence in 100 mm dishes) are harvested by trypsinization and washed once with reg-DMEM, then resuspended in 10 ml complete medium, and diluted 1:7 in culture medium (3 ml cells in 20 ml medium), diluted cells are then seeded in 24-well plates for transfection prior to luciferase reporter assay; 400 µl cells per well. For MDA-MB-231 and MDA-66 dilution was 1:3 (6 ml cells in 20 ml medium), and for HeLa cells dilution ratio was 1:2.

2.3.4.1. Transient transfection with ERα

MDA-MB-231 cells were transfected with pCMV-ERα in 100 mm dishes, using FuGENE-6 (as described above) and 5 µg of the expression vector. Two days after transfection, media were replaced with fresh sf-DMEM containing 10 nM E2 or 1 µM tRA, or a combination of both hormones. After hormone induction cells were collected as mentioned above.

COS-7 cells grown in 150 mm dishes were transfected with the ERα expressing plasmid as described above. Two days after transfection, cells were treated with 10 nM E2 for 3 hours or with vehicle (EtOH) prior to harvesting. ERα transfected COS-7 cell were used for nuclear extract preparation.

2.4. Luciferase Reporter Assay

Cells were seeded in 24-well plates in reg-DMEM; so that they reach confluence at the time of the assay. Two days later, and 1 hour prior to transfection, cells were

washed twice with PBS, and the medium was replaced with culture medium (reg-DMEM or sf-DMEM depending on the hormone to be used) lacking antibiotics. Transfection was carried out with 200 ng of reporter vector plus 3 ng of phRL-TK to normalize for transfection efficiency. For E2 induction, two days post transfection, medium was changed with fresh sf-DMEM containing 10 nM E2 (or EtOH as vehicle control) and continued incubation for 6 hours, while in the case of tRA induction, 12 hours after transfection, culture medium was replaced with reg-DMEM containing 1 μM tRA (or DMSO as vehicle control) and continued incubation for 24 hours. Then the cells were harvested and luciferase reporter assay was performed using the Dual-Glo Luciferase Assay system (Promega). Luciferase values for all samples were normalized by first subtracting the background of no-transfection control, and then dividing firefly luciferase values over those of *Renilla* luciferase. Fold induction is relative to the value of the empty vector pGL3E1bLuc.

2.5. RNA Isolation

The expression level of NIS, pS2, RIP140 and GAPDH was monitored by semi-quantitative RT-PCR. RNA was prepared from cell pellets using the Nucleospin RNA II kit (Macherey-Nagel) as recommended by the manufacturer. The RNA preparation protocol included on-column DN*ase* treatment step, minimizing the presence of genomic DNA in the RNA samples. RNA concentrations were determined spectrophotometrically.

2.5.1. cDNA synthesis, RT-PCR

In general 2 μg of total RNA were used for cDNA synthesis using the Revert-Aid First Strand cDNA Synthesis Kit (Fermentas). Primers for semi-quantitative RT-PCR amplified corresponding transcripts from positions spanning two or more exonic sequences, except for the RIP140, for which, a -RT (where cDNA is prepared using the same amount of RNA but without adding reverse transcriptase to the tube) control was included to ensure the amplification from cDNA only. PCR primers were *RT-NIS-F*: 5'-CTC ATC CTG AAC CAA GTG AC-3', *RT-NIS-R2*: 5'-TAC ATG GAG AGC CAC ACC A-3', *RT-pS2-F*: 5'-CCA TGG AGA ACA AGG TGA TCT GC-3', *RT-pS2-R2*: 5'-GTC AAT CTG TGT TGT GAG CCG AG-3', *GAPDH-F*: 5'-

GGC TGA GAA CGG GAA GCT TGT CAT-3', *GAPDH-R*: 5'-CAG CCT TCT CCA TGG TGG TGA AGA-3', *RIP140-F*: 5'-GAC TCA TGG AGA AGA GCT TG-3', *RIP140-R*: 5'-ACA TGA TGA GGA GAC TCT GC-3'. PCR amplification was performed in 25 µl reaction volumes containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 10 pmoles of each primer, and 1 unit of *Taq* DNA polymerase (Fermentas). Thermal cycler conditions were an initial denaturation step at 95°C for 3 min; a loop cycle of 95°C, 30sec / 61°C, 30sec / 72°C, 30sec; and a final extension at 72°C for 10 minutes. The cycle number varied for each transcript amplified, for NIS it was 40 cycles, pS2 in MCF-7 was 15 cycles, and in MDA-MB-231/MDA-66 was 40 cycles. Cycle number for GAPDH was 19, and for RIP140 it was 22 cycles. PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized using the Gel Doc-2000 supported with the Multi-Analyst Ver.1.1 image analysis software (Bio-Rad).

2.6. Protein Isolation

The expression of ERα, RARα, and calnexin was examined by Western blot analysis. Cell pellets were incubated in lysis buffer for 30 minutes [50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40 and 1X protease inhibitor cocktail (Roche)], cell extracts were cleared by centrifugation at 13000 rpm for 30 minutes at 4ºC. Protein content was quantified using Bradford assay.

2.6.1. Western blot analysis

Twenty micrograms of whole cell extracts were denatured in gel loading buffer [50 mM Tris-HCl pH 6.8, 1% SDS, 0.02% bromophenol blue, 10% Glycerol and 5% 2-mercaptoethanol] at 95°C for 5 min, resolved by SDS-PAGE using a 10% gel, and electrotransferred onto PVDF membranes (Millipore). The membranes were blocked in Blotto (Tris-buffered saline containing 0.5% Tween 20 and 5% nonfat milk powder) for 1 hour at room temperature. The membranes were incubated with mouse monoclonal anti-hERα F-10 (1:500, Santa Cruz) for 16 hours at 4°C, washed 3 times with Blotto and incubated with peroxidase-conjugated goat anti-mouse (1:2000, Sigma) for 1 hour, immunocomplexes were then detected using ECL-plus (Amersham), and exposed to X-Ray films (AGFA) for 1 minute. The films were then

developed using a hyper-processor developer (Amersham). Membranes were then washed 3 times with Blotto, re-incubated with rabbit monoclonal anti-hRARα C-20 (1:1000, Santa Cruz) for 16 hours and then stained with goat anti-rabbit (1:2000, Sigma). The same protocol was repeated on the same membrane for the internal control calnexin using a rabbit anti-calnexin (1:5000, Sigma).

2.7. Suppression of ERα by shRNA

MCF-7 cells were transfected as described above, using pSR-ER-458, pSR-ER-499 and the empty vector control pSR. After transfection, cells were washed wish PBS, harvested by trypsinization, diluted and transferred to 24-well plates for selection with DMEM containing 0.5 mg/ml Geneticin (Sigma). Three weeks later, stably transfected colonies (expressing the EGFP marker) were transferred to new culture dishes and were allowed to grow for further analysis.

The presence of the knockdown construct was confirmed by PCR using genomic DNA isolated from each clone as a template, and the pSR insert screening primers from Oligoengine (*F*: 5'-GGA AGC CTT GGC TTT TG-3' and *R*: 5'-CGA ACG TGA CGT CAT C-3'). The level of ERα suppression was analyzed by western blot using ERα antibodies as described above. Clones with the lowest ERα expression as compared to the empty vector transfected cells were selected for NIS expression level analysis in response to tRA. The expression level of NIS was monitored by RT-PCR; band intensities were analyzed with the Multi-Analyst image analysis software (Ver.1.1; Bio-Rad), and normalized to the internal control GAPDH.

2.8. Electrophoretic Mobility Shift Assay (EMSA)

Electrophoretic mobility shift assay was performed using the Lightshift Chemiluminescent EMSA kit from Pierce Biotechnology according to the protocols provided. The overall protocol includes several steps; each was optimized to get a better shift and sharper bands. The first step was to prepare and pre-run the gel; we used a 6% native polyacrylamide gel in 0.5X TBE in a minigel electrophoresis apparatus (dimensions 8 x 8 x 0.1 cm). Pre-run was for 60 minutes at 100 V constant.

While the gel was in pre-electrophoresis, the binding reaction was prepared as described in table 2.2; 20 µl binding reactions were prepared and incubated at room temperature for 40 minutes. For the super-shift reaction 1µg of anti-ERα or anti-RARα antibodies were included to the reaction just before adding the Biotin labeled oligos. Prior to electrophoresis, 5 µl of 5X Loading Buffer were added to each sample, mixed by pipetting up and down (care was taken not to vigorously mix the samples) and then 20 µl of each sample was loaded onto the polyacrylamide gel. Total electrophoresis time was 50 minutes at 100 V constant (until the bromophenol blue dye has reached the bottom of the gel).

Table 2.2. EMSA Binding reaction preparation chart. Samples were prepared according to this chart, a set of 4 reactions was prepared for each target oligo, using ERE wt, ERE Mut1, ERE Mut2, ERE Mut3 and the RARE control.

		Biotin DNA	Biotin DNA + Nuclear Extract (N.E.)	Biotin DNA + N.E. + excess competitor	Biotin DNA + N.E. + Antibody
Binding Reaction Components	Ultra pure water	12*	8	6	3
	10X Binding Buffer	2	2	2	2
	1 µg/µl poly (dI•dC)**	-	-	-	-
	1 µg/µl Salmon sperm DNA***	1	1	1	1
	50% Glucerol	1	1	1	1
	100 mM $MgCl_2$	1	1	1	1
	1% NP-40	1	1	1	1
	2 pmol/µl Unlabeled Target DNA	-	-	2	-
	Nuclear Extract	-	4	4	4
	0.2 µg/ul Antibody	-	-	-	5
	10 fmol/µl Biotin labeled Oligo	2	2	2	2
	Total volume	20	20	20	20

*All volumes are expressed in µl; ** the non specific DNA competitor poly (dI•dC) was also included in binding reactions for the EBNA and RARE control experiments. *** Salmon sperm DNA is another non specific DNA competitor used for ERE containing oligos

Binding reactions were electro-transferred to Hybond-N+ nylon membranes (Amersham) using the EC140 Mini blot Module for wet transfer in pre-chilled 0.5X TBE. Transfer was performed at 380 mA constant for 45 minutes. Just after the transfer is complete (keeping the membranes damp) membranes were cross-linked in the GS Gene Linker (BioRad) applying 150 mJoule.

Chemiluminescent detection of Biotin-labeled DNA was performed according to the manufacturer's protocol and solutions. Briefly, UV cross-linked membranes were incubated in Blocking Buffer for 15 minutes, and then in conjugate/blocking buffer (1:300 dilution of the Stabilized Streptavidin-Horseradish Peroxidase Conjugate in the Blocking Buffer) solution for an additional 15 minutes. Membranes were washed 4 times (5 minutes each) with 1X washing buffer. Membranes were then incubated with Substrate Equilibration Buffer for 5 minutes and then membranes were incubated for 5 minutes with the Substrate Working Solution (a 1:1 mixture of Luminol Enhancer Solution and Stable Peroxide Solution). Membranes were exposed to X-ray films (AGFA) for 5-10 minutes and developed in the Hyper-Processor Developer (Amersham).

2.8.1. Nuclear extract preparation

Nuclear extracts used in EMSA experiments varied depending on the target DNA used, for the control experiments, Epstein-Barr Nuclear Antigen (EBNA) extract was used as well as the nuclear extract from MCF-7 cells (for the RARE control oligo). For experiments with ERE containing oligos nuclear extracts from COS-7 cells transfected with pCMV-ERα were used. In general nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology) according to the manufacturer procedure. Sub-confluent cells grown in 150 mm plates were harvested by trypsinization and collected by centrifugation at 1200 rpm, to the cell pellets (50 µl packed cell volume) 500 µl of ice-cold CERI (containing 1X protease inhibitor cocktail) was added and vortexed vigorously to completely resuspend the cell pellets and incubated on ice for 10 minutes. Then 27.5 µl of ice-cold CERII was added and mixed by vortexing for 5 seconds, tubes were incubated on ice for 1 minute then centrifuged at maximum speed for 5 minutes in a

pre-chilled centrifuge. Supernatants were collected and labeled as cytoplasmic extracts and stored at -80ºC. The insoluble pellets (containing nuclei) were resuspended in 150 µl ice-cold NER and vortexed at highest speed for 15 seconds every 5 minutes for a total of 45 minutes (tubes were kept on ice during intervals). Extracts were cleared by centrifugation at maximum speed for 10 minutes in a cold centrifuge, supernatants were collected and 40 µl aliquots were labeled nuclear extracts and stored at -80ºC.

2.8.2. Oligo-nucleotides

For control experiments, EBNA and RARE control oligo-nucleotides were used. The EBNA control oligo was included in the kit (5'-BIOTIN-…TAGCATATGCTA…-3'). For the retinoic acid receptor binding site and the estrogen receptor responsive element we used synthetic oligo-nucleotides (Metabion, Germany) containing the binding site as summarized in table 2.3.

Table 2.3. Oligo-nucleotide sequences used in EMSA reactions. The RARE oligo contains 2 DR5 elements separated by 2 nucleotides; underlined bases represent the direct repeat sequences. The ERE oligo-nucleotides contain the ERE binding site, the two half sites are in bold and the mutated bases are underlined.

Name	Sequence*
RARE	5'-AGGTCAAATGCAGGTCAAAAGGTCAAATGCAGGTCA-3'
ERE-wt	5'-CTAGGTCTGGAGGCGG**AGTCG**CGG**TGACC**CGGGAGCCC-3'
ERE-Mut1	5'-CTAGGTCTGGAGGCGG**G**GTC**T**CGG**A**GACCCGGGAGCCC-3'
ERE-Mut2	5'-CTAGGTCTGGAGGCGG**AGTCT**CGG**A**GACCCGGGAGCCC-3'
ERE-Mut3	5'-CTAGGTCTGGAGGCCG**G**GTCCGCT**G**GACCTTGGAGCCC-3'

* The upper strand is shown

2.8.2.1. Biotin labeling

Biotin 3' End Labeling Kit was used for labeling oligo-nucleotides used for EMSA binding reactions. The labeling reactions contained 1X TdT reaction buffer, 100 nM

unlabeled oligo, 0.5 µM Biotin-11-UTP and 10 U TdT in 50 µl reaction volumes. Tubes were incubated at 37ºC for 30 minutes and the reaction was terminated by adding 2.5 µl of 0.2 M EDTA. Then 50 µl chloroform:isoamyl alcohol were added to each tube to extract the TdT, vortexed and centrifuged. The aqueous phase was collected and stored at -20ºC.

For each target, complementary single strand oligos were separately labeled and then annealed by mixing equal amounts of each oligo followed by incubation at 90ºC for 2 minutes then slowly cooling to room temperature.

2.9. Chromatin Immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed essentially as described by the supplier of the reagents (Santa Cruz Biotechnology; protocol No. 12 on http://www.scbt.com) with the following modifications. MCF-7 cells (150 mm dish) grown in sf-DMEM were treated with 10 nM E2. Formaldehyde cross-linking [1% (v/v)] was done for 10 min at room temperature. Cross linking was terminated by the addition of glycine to a final concentration of 125 mM. Cells were scraped and the pellets were resuspended in 6 ml lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40 and 1X protease inhibitor cocktail) for 10 minutes on ice. The cell lysate was washed once with ice-cold PBS, and resuspended in 1.9 ml high salt lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS and 1X protease inhibitor cocktail) and sonicated in the ultrasonic processor UP50H (Hielscher Ultra-sonics, Germany) 3 times, 7 seconds each at 60% amplitude and a continuous cycle. At this point 100 µl of chromatin solution was removed and labeled “Input”.

Chromatin solution was pre-cleared by adding 100 µl of protein A-Sepharose 6MB (Sigma) as 50% slurry containing 0.5 mg/ml BSA, 200 µg/ml sonicated salmon sperm DNA in TE (pH 8.0) for 30 minutes at 4°C. Immunoprecipitation was performed at 4°C for 16 hours using anti-ERα antibodies and anti-FGFR-1 (C-15, Santa Cruz) antibodies (as negative control) as recommended by the supplier, then immunocomplexes were incubated with 100 µl protein A-Sepharose (50% slurry) for

2 hours at 4°C. Afterwards, beads were collected, washed and eluted as recommended in the Santa Cruz protocol. All samples, including the input, were reverse cross linked by incubating at 65°C with proteinase K for 16 hours, DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Isolated DNA was used for PCR amplification of ERα-precipitated fragments for NIS-ERE (*NIS-ChIP-F*: 5'-TGG CCT GTC TGT CCC AGT CCA GGG CTG A-3' and *NIS-ChIP-R*: 5'-GGG TTG CAG ATT TAT TGG GC-3'). *NF1-F*: 5'-TGC TAC TCT TTA GCT TCC TAC-3' and *NF1-R*: 5'-CCT TAA AAG AAG ACA ATC AGC C-3' primers were used as an ERE-unrelated control.

3. RESULTS

3.1. Comparative Genomics Identifies Potential *cis*-acting Elements Controlling NIS transcription

It has recently been shown that conserved gene expression patterns could reflect a conserved regulatory mechanism, and thus a possible existence of conserved regulatory *cis*-acting elements (Negre *et al.*, 2005). In order to identify putative distal control regions regulating the expression of NIS in response to tRA in mammary gland cells, we carried out a comparative genomic DNA analysis using the genome Vista tool (Bray *et al.*, 2003; Couronne *et al.*, 2003).

We analyzed a 90 kbp genomic DNA fragment including and flanking the NIS gene for sequences with at least 50% conservation over 75 bp stretches in human, mouse and rat (these criteria provided better resolution of the conserved regions). As a result of this analysis we were able to identify several non-coding regions with the above mentioned conservation criteria. As shown in figure 3.1 (a summary of these regions can be found in table 3.1), conserved regions clustered within 1 kbp were studied as single groups and were given the name cluster 1, cluster 2 and so forth, this grouping of conserved regions facilitated subsequent functional analyses.

The previously characterized NIS upstream enhancer (Ohno *et al.*, 1999; Taki *et al.*, 2002) is located in the coding sequence of the upstream gene RPL18A (cluster 1, regions 1-2 and 1-3), thus cluster 1 would serve as a positive control when studying the transcriptional activity of the selected clusters. We would expect this cluster to be transcriptionally active in the thyroid cell line FRTL-5.

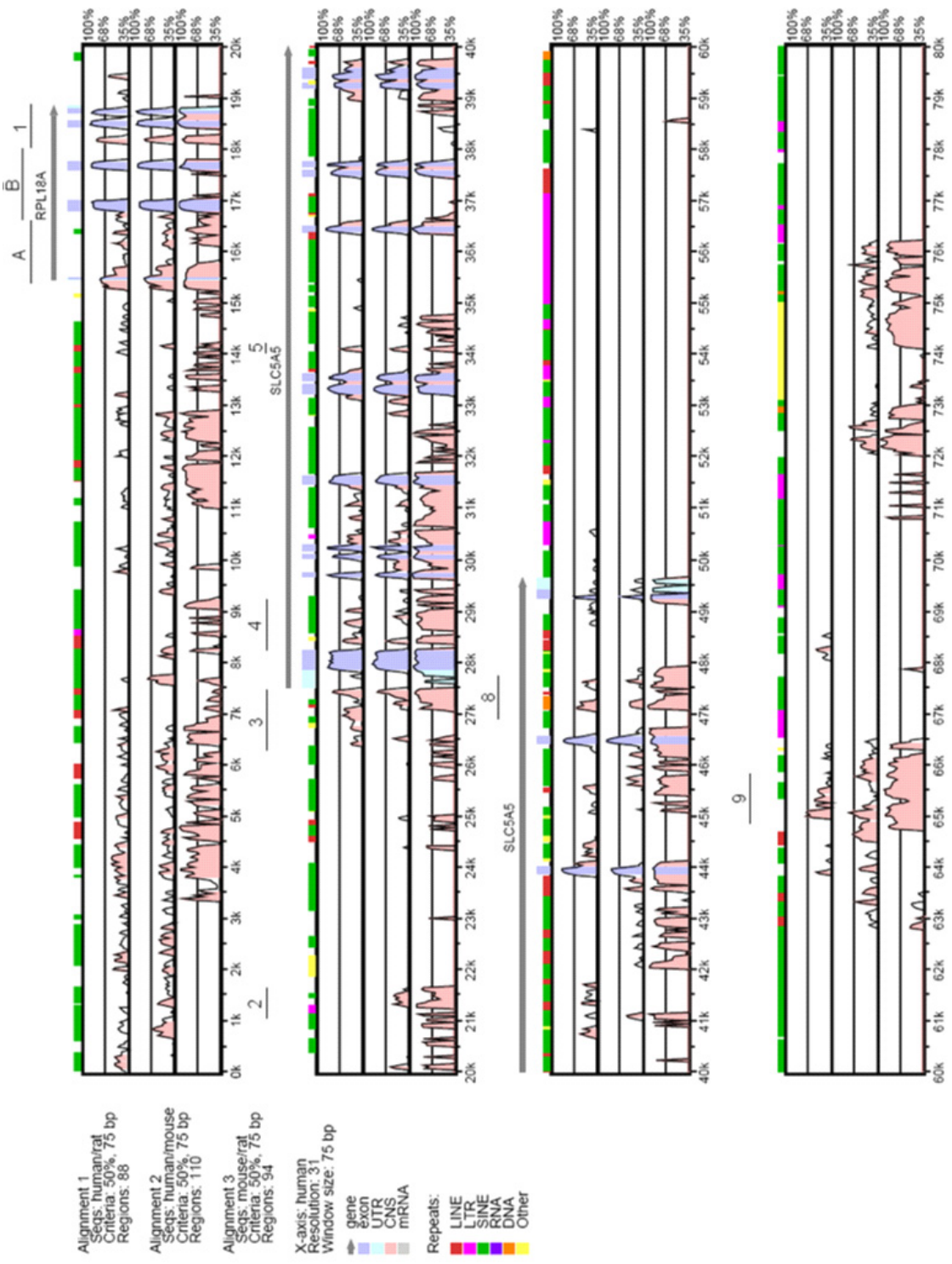

Figure 3.1. VISTA plot of conservation levels in a 90 kbp genomic DNA in human, mouse and rat. Percent nucleotide identities between human, mouse and rat DNA sequences are plotted as a function of position along the human sequence. Peaks of evolutionary conservation in overlapping exonic sequences are shaded blue. Aligned regions of more than 50% identity over 75 bases are shaded pink. Lines above each alignment indicate the position of the conserved cluster selected for PCR amplification.

Table 3.1. Summary of the conserved regions analyzed. Conserved regions were grouped into 9 clusters. The genomic position of each region is indicated according to the human sequence of chromosome 19 Release July 2003, as well as the percentage of conservation.

Cluster Name	Conserved region name	Genomic Position offset=17816282*	Conserved region length (bp)	Conservation Level (%)
Cluster A	A-1	15485-15502	18	94.4
	A-2	15503-15707	223	51.1
	A-3	16115-16200	92	53.3
	A-4	16348-16420	80	57.5
Cluster B	B-1	16695-16749	59	54.2
	B-2	16821-17000	180	86.1
	B-3	17622-17751	130	90.0
Cluster 1	1-1	18147-18210	64	84.4
	1-2	18446-18555	110	90.0
	1-3	18699-18791	93	90.3
Cluster 2*	2-1	21295-21350	59	57.6
	2-2	21381-21452	84	52.4
	2-3	21455-21554	100	55.0
Cluster 3	3-1	26374-26432	64	54.7
	3-2	26673-26747	79	51.9
	3-3	26861-27102	244	56.1
	3-4	27311-27440	130	63.1
Cluster 4	4-1	28380-28458	79	60.8
	4-2	28727-28822	97	61.9
	4-3	28972-29037	66	57.6
Cluster 5	5-1	34063-34108	46	76.1
Cluster 8	8-1	47056-47245	209	54.1
	8-2	47607-47619	13	100.0
Cluster 9	9-1	65174-65232	70	60.0
	9-2	65268-65369	104	54.8
	9-3	65440-65528	112	50.0

* Offset value for cluster 2 regions was (17816282) based on Human-mouse comparison, each interval represents the individual conserved regions.

3.1.1. Three conserved region clusters are transcriptionally active in MCF-7

To assess the functionality of the identified sequences we adopted the following strategy: putative regulatory region clusters were amplified by PCR using human genomic DNA as a template, and subsequently they were cloned in the luciferase reporter vector pGL3E1bLuc as described in Material and Methods section 2.2. Then these reporter constructs were used for transient transfection assays, after which the transcriptional potential of these conserved regions was assessed by reporter assays.

The following three cell lines were chosen for reporter assay experiments: MCF-7; a breast cancer cell line [of the luminal type (Charafe-Jauffret *et al.*, 2006)] in which, as shown in figure 3.2a, RA induction of NIS was previously observed (Kogai *et al.*, 2000), FRTL-5; a normal thyroid cell line in which the thyroid specific regulation of NIS has been characterized (Ohno *et al.*, 1999), and HeLa; a cervical cancer cell line with no NIS expression (Figure 3.2b).

Figure 3.2. tRA dependant expression of NIS in MCF-7. Cell lines grown in reg-DMEM were treated with tRA (1 μM) for the indicated periods. RNA was prepared and the expression of NIS was evaluated by RT-PCR. GAPDH was used as an internal control to verify the quality of the synthesized cDNA. A) MCF-7 cells respond to the tRA stimulation, with a maximum expression level at 12 hours, whereas, B) HeLa cells lack basal and tRA-induced NIS expression.

Cell lines were transiently transfected with the above indicated reporter constructs containing conserved region clusters, and the luciferase reporter assay was performed as indicated in Materials and Methods section 2.4. As anticipated, cluster 1 (harboring the NUE sequence) was transcriptionally active in the thyroid cell line FRTL-5 (Ohno *et al.*, 1999) (Figure 3.3a). Of the nine clusters studied, clusters 2, 3 and 4 activated the expression of the reporter gene significantly in MCF-7; cluster 3 being strongest, followed by clusters 2 and 4. The remaining clusters (clusters 5, 8, 9, A, and B) were not transcriptionally potent as compared to clusters 1, 2, 3, and 4.

3.1.2. Conserved clusters 3 and 4 respond to tRA in MCF-7

The main aim of this analysis was to determine if any of these conserved regions or clusters was responsive to tRA in the breast cancer cell line MCF-7, in which the

endogenous NIS was known to be overexpressed in response to RA (Kogai *et al.*, 2000). To address this aim, we carried out luciferase reporter assay experiments in which, MCF-7 cells were transiently transfected with the above indicated reporter constructs and were treated either with DMSO (as vehicle control) or with 1 μM tRA for 24 hours. Then by luciferase assays we monitored the stimulatory effect of tRA on the transcription of these clusters. As a result, we found out that only clusters 3 and 4 significantly responded to the tRA stimulus (Figure 3.3b). On the other hand, even being transcriptionally potent in all cell lines studied, clusters 1 and 2 did not respond to tRA induction in MCF-7. The remaining clusters were not responsive to tRA, and thus were not further analyzed.

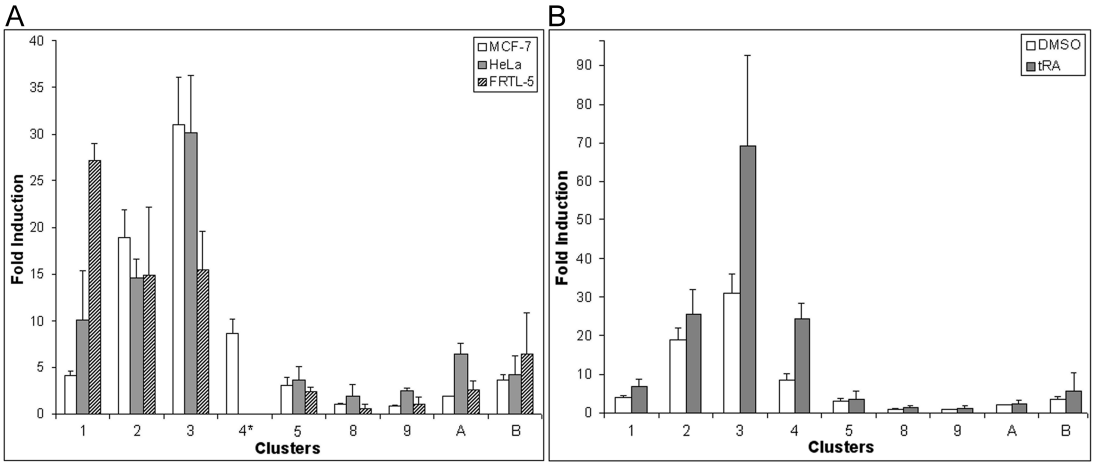

Figure 3.3. Conserved clusters 3 and 4 respond to tRA in MCF-7. Conserved clusters were amplified by PCR and cloned in the luciferase reporter plasmid pGL3E1bLuc. A) Three cell lines were first transfected with the reporter constructs containing conserved clusters to determine their transcriptional activity. Cluster 1 was transcriptionally active in FRTL-5 as expected; clusters 1, 2, 3 and 4 were transcriptionally active in all cell lines, while the others did not shown significant reporter gene expression. Transfection efficiency in all cells was more than 70% with no apparent cell death due to transfection with FuGENE. B) MCF-7 cells were transfected with all conserved clusters and treated with tRA or DMSO for 24 hours. Of the nine clusters analyzed, only clusters 3 and 4 were significantly responsive to tRA treatment. (*) The transcriptional activity of cluster 4 was not determined in HeLa or in FRTL-5.

3.1.3. tRA Response in conserved cluster 3 is not attributable to a single retinoic acid responsive element

Cluster 3 is a 1.1 kbp sequence upstream of the human NIS gene starting at -1138 bp relative to the transcription start site (Endo *et al.*, 1997; Behr *et al.*, 1998). We found that the sequence in cluster 3 is composed of 4 different conserved regions (Figure 3.4a), annotated in this text as regions 3-1 to 3-4, located immediately upstream of NIS transcription start site, including the minimal NIS promoter (Venkataraman *et al.*, 1998). In a previous study, the RA responsiveness of the human NIS promoter was attributed to a RA responsive site in the 5' region of our construct, located between bases 125-146 of the inserted sequence (Schmutzler *et al.*, 2002). Interestingly, this site was not in a conserved region (based on the bioinformatics data), yet it accounts for RA response in thyroid cell lines (Schmutzler *et al.*, 2002). In order to verify and determine the contribution of this site in tRA responsiveness in the context of cluster 3 in MCF-7, we created a construct carrying a mutation that has been previously shown to impair the RA response from this sequence by abolishing RAR binding to this element in thyroid cell lines (Schmutzler *et al.*, 2002). In our analysis, this mutation slightly decreased the transcriptional activation of the reporter gene; and could only reduce the tRA stimulated expression by about 27% (Figure 3.4b). This indicates that this site is not the major tRA responsive site, and the presence of another (other) tRA responsive element(s) in cluster 3 is highly possible.

In an effort to search for additional putative tRA responsive sites, we prepared a number of deletion constructs derived from the above mentioned reporter construct containing cluster 3 (Figure 3.5a) and analyzed the luciferase reporter expression upon tRA stimulation. As shown in figure 3.5b, constructs containing individual or combined conserved regions 3-1, 3-2 and 3-3 did not result in significant activation of the reporter and did not show tRA response, except for Cl3 -xp, which responded slightly to tRA but yet with great loss of the original signal from the parent clone, all the above mentioned constructs (Cl.3 -ex, Cl.3-2, Cl.3-3 and Cl.3 -xp) lack conserved region 3-4 and NIS TATA element, instead, they contained the E1b TATA sequence. On the other hand, constructs carrying the conserved region 3-4 retained most of the parental transcriptional activity; however, tRA responsiveness was lost. This reflects the importance of this immediate

upstream sequence (conserved region 3-4) in transcription of the NIS gene in MCF-7, and that a combined effect of this region with the upstream RARE may be required for maximal transcriptional activation in response to tRA.

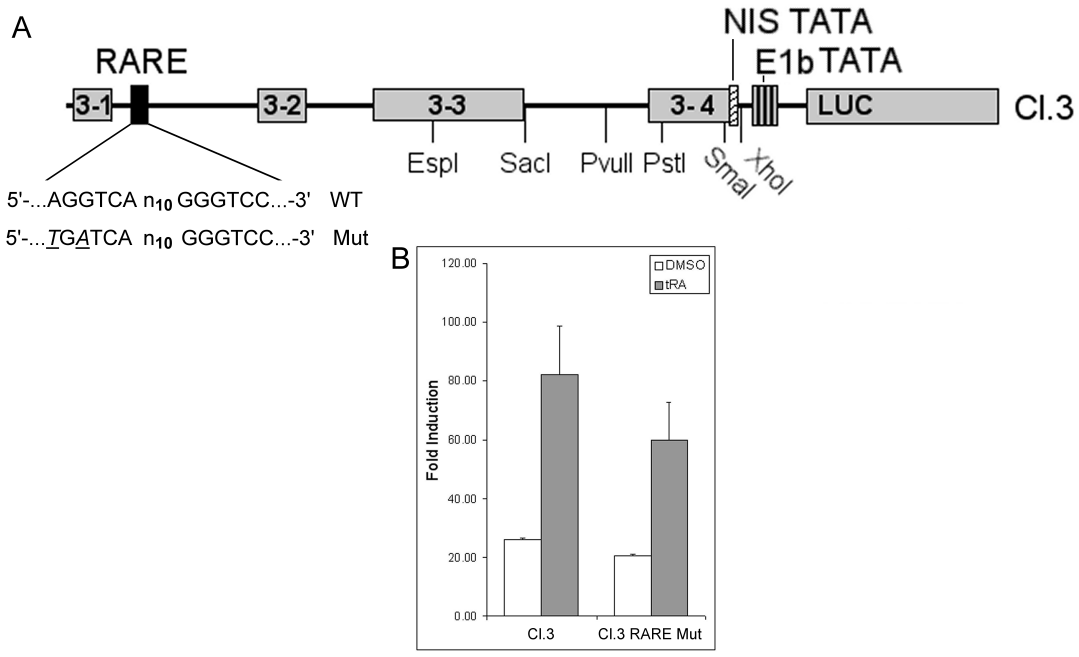

Figure 3.4. The retinoic acid responsive element in Cl.3 is not the major site for tRA response in MCF-7. A) Map of the reporter plasmid (Cl.3), the position of the 4 conserved regions is represented by the grey boxes labeled 3-1 to 3-4, the position of NIS TATA and E1b TATA elements is also indicated by striped boxes. The previously characterized RARE is indicated by the black box, the wild-type and the mutant sequences are expanded below the map. B) MCF-7 cells were transiently transfected with both reporters (Cl.3 and Cl.3 RARE Mut), cells were treated with tRA (or DMSO) for 24 hours and luciferase reporter assay was performed. Luciferase values were normalized to those of *Renilla* luciferase and the fold induction is represented relative to the empty vector.

3.1.3.1. Conserved region 3-4 in NIS promoter accounts for most of NIS transcription

To determine the importance of conserved region 3-4 for NIS transcription, we prepared a construct derived from Cl.3 with a deletion of a 101 bp in region 3-4

(Figure 3.5a). This deletion did not remove the NIS TATA element. MCF-7 cells were transfected with this reporter plasmid in the absence or presence of 1 μM tRA. The severe decrease in the transcriptional potential of cluster 3 is similar to Cl.3 -xp, in which the whole conserved region 3-4 was deleted. On the other hand, the overall tRA stimulation was maintained as in Cl.3 -xp. This is probably due to the presence of the upstream RA responsive element. There is the possibility that, in both cases (Cl.3 -xp and Cl.3 Δ 3-4) region 3-4 or at least the removed 101 bp stretch may contain elements that are required for increased expression of NIS and that this element may function in a cooperative manner with the upstream RA responsive site.

3.1.4. 5'UTR sequence has an up-regulatory effect on NIS transcription but does not increase the tRA responsiveness

As mentioned above, cluster 3 is a 1.1 kbp sequence containing 4 conserved regions; it starts at -1138 and ends at -45 bp relative to transcription start site of the human NIS gene. In order not to miss a possible effect of the 5'UTR on NIS transcription, we extended Cl.3 insert to include the sequence between -45 up to the ATG codon by inserting a PCR product amplified from human genomic DNA into the *Sma*I/*Nco*I sites of Cl.3 (figure 3.6a). This modification eliminated the E1b TATA element from the new construct (named Cl.3UTR). It is worth mentioning here that cluster 3 includes the NIS TATA element and for better comparison, we also reconstructed Cl.3 by inserting cluster 3 sequence from Cl.3 into pGL3 Basic (Figure 3.6a).

MCF-7 cells were transfected with the new constructs and the cells were treated with either DMSO or 1 μM tRA for 24 hours, and the luciferase reporter assay was performed as mentioned above. The first thing we noticed was the decrease in the magnitude of fold induction (4-fold) of Cl.3 without the E1b TATA when compared to the previous results obtained in the presence of the E1b TATA element (Figure 3.6b). This difference in magnitude is a reflection of the contribution of the E1b TATA element, and this change did not affect the overall tRA stimulation.

A significant increase in reporter activity was observed in the presence of 5'UTR sequences. The presence of the UTR in Cl.3 increased both basal and tRA-induced transcription by 98%. On the other hand, the overall stimulation by tRA was reduced by 4% (Figure 3.6b).

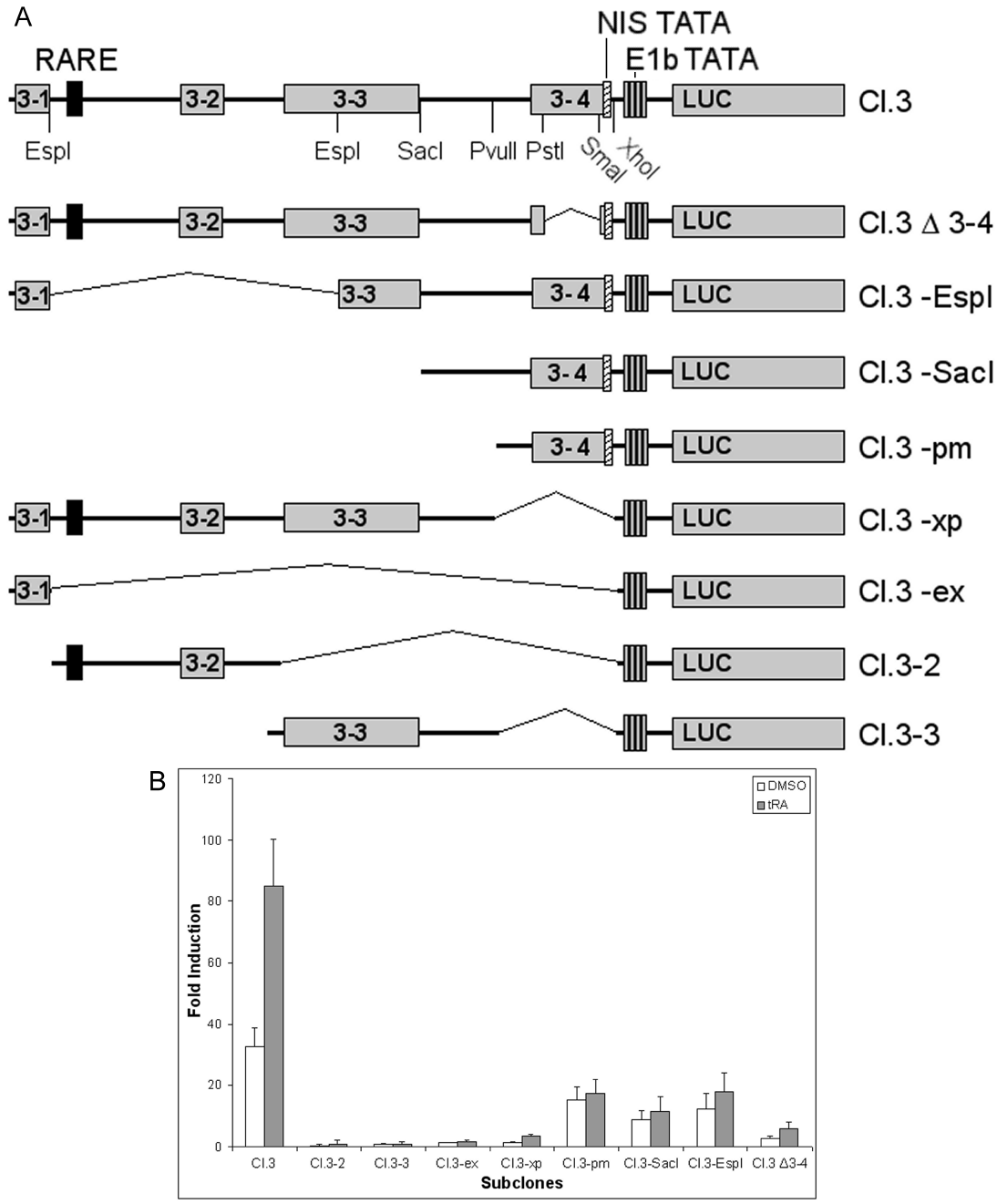

Figure 3.5. Conserved region 3-4 in cluster 3 is essential for proper transcriptional activation. A) Maps of Cl.3 derivatives, deletion mutants of Cl.3 were prepared to include individual or combined conserved regions. Positions of restriction enzymes used for the subcloning procedure are indicated. B) MCF-7 cells were transiently transfected with Cl.3 and the deletion mutants. Then the cells were treated with tRA (or DMSO) for 24 hours. Luciferase assay was performed and the values were normalized using the values of *Renilla* luciferase, fold induction was calculated relative to the empty vector.

3.1.5. Combining clusters 3 and 4 has a synergistic tRA response in the absence of UTR sequences

Cluster 4 is a 771 bp sequence located in the first intron of the human NIS gene. This cluster is composed of 3 conserved regions numbered from 4-1 to 4-3 (Figure 3.6a). A closer look at the sequence composition of this cluster revealed the presence of two putative RA responsive elements. The first is a perfect DR2 (Giguere, 1994) sequence (AGGTCA$_{(n_2)}$AGTTCA) located in the second conserved region 4-2 at 465 bp corresponding to the *Mlu*I site of the reporter vector and the second is a DR10 sequence (AGGTGG$_{(n_{10})}$AGGTCA) which is located just after the third conserved region (4-3) 744 bp relative to the *Mlu*I site in the reporter vector.

Although being located in a conserved region, it was surprising to find out that the sequence of the DR2 element was not conserved (in sequence or position) in human/mouse, or in human/rat alignments. Likewise, the DR10 element was found to be also not conserved in the similar alignments.

Our initial analysis revealed that this cluster responded to tRA stimulation (Figure 3.3b). To further analyze the functionality of this cluster in regulating the expression of the NIS gene in response to tRA, we wanted to study the combinatorial effect of clusters 3 and 4 in response to tRA compared to cluster 3 alone. To address this point, we prepared a reporter construct based on Cl.3 (without the E1b element) by adding cluster 4 sequence following the coding sequence of the luciferase reporter as shown in figure 3.6b, to produce Cl.3/4. Transient transfections were performed as described above, and the cells were incubated either with 1 μM tRA or with the vehicle DMSO for 24 hours.

It was interesting to see that both the basal and tRA stimulated transcriptional activation of the luciferase reporter were significantly increased in Cl.3/4 by 16% and 46%, respectively. Also the overall tRA stimulation was increased in Cl.3/4 by 26% when compared to Cl.3 (Figure 3.6b). To our surprise, the addition of the UTR sequence in Cl.3/4 reduced this enhanced tRA stimulation, such synergistic effect of these clusters could have been missed if we only used Cl.3/4UTR, because it reflects the arrangement of these clusters *in vivo*. On the other hand, such effect of the UTR

should not be considered suppressive, as these constructs may be missing additional sequences required for proper induction *in vivo*, and they lack chromatin structure.

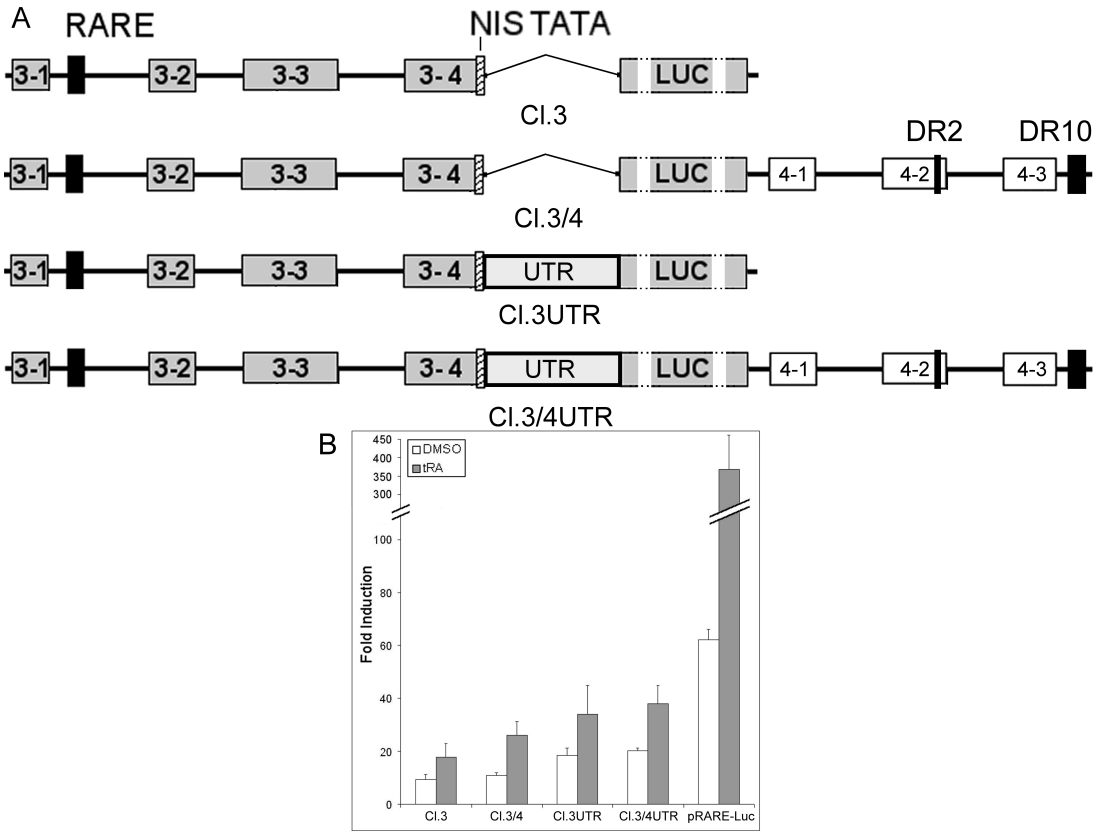

Figure 3.6. Combining clusters 3 and 4 has a synergistic tRA response in the absence of the UTR sequence. A) Maps representing the vectors used to analyze the combination of clusters 3 and 4, cluster 4 (right side of the map) was added to Cl.3 following the luciferase reporter. The UTR sequence was added to extend the sequence of cluster 3 up to the ATG of the reporter coding sequence. The putative RA responsive elements in cluster 4 sequence are represented with black boxes labeled DR2 and DR10. B) MCF-7 cells were transiently transfected with reporter constructs and the transcription of the luciferase reporter was measured in response to tRA stimulation (1 μM for 24 hours). Luciferase values for each construct were normalized to those of *Renilla* luciferase, fold induction is relative to the empty vector.

3.1.6. Nkx-2.5 reduces the transcriptional potential of Cl.3 in HeLa cells

In a recent study, the homeobox-containing transcription factor Nkx-2.5 was shown to be involved in the transcription of NIS in response to RA treatment (Dentice *et al.*, 2004). Two Nkx-2.5 binding sites were identified in the rat NIS promoter, and evidence indicated that these two sites (N2 and W) are required for maximal induction of NIS. Based on our bioinformatics analysis, the two identified sites are located in conserved regions of cluster 3: N2 and W are in regions 3-3 and 3-4, respectively, nevertheless, these elements were not found to be present in the human sequence. This may indicate the presence of Nkx-2.5 binding sites elsewhere in Cl.3.

In an effort to identify the binding sites of Nkx-2.5 in the human NIS promoter, we wanted to see the ability of Nkx-2.5 to up-regulate the transcriptional activity of Cl.3 (as it did for the rat NIS promoter). To this end, we co-transfected HeLa cells with the luciferase reporter containing cluster 3 and a construct expressing Nkx-2.5 or the empty vector pCMV-FLAG. After transfection we monitored the luciferase activity of the reporter gene and it was clear that Nkx-2.5 expression caused a severe decrease in the transcriptional activity of Cl.3 (Figure 3.7a). This result was very surprising when one considers that Dentice *et al.* (2004) had shown that Nkx-2.5 stimulated the expression of the reporter from a similar plasmid containing the rat NIS promoter in HeLa cells, and that forced Nkx-2.5 expression was sufficient to increase NIS expression and iodide uptake in the human cell line MCF-7 (Dentice *et al.*, 2004).

In parallel experiments we wanted to determine the intrinsic transcriptional activity of Nkx-2.5 in MCF-7 (Figure 3.7b). To do so, we transfected MCF-7 cell with a construct harboring 5 tandem repeats of the element C, which was shown previously to harbor the Nkx-2.5 binding sequence (pC5-Luc in figure 3.7c) and monitored its responsiveness to tRA stimulation (Dentice *et al.*, 2004). Interestingly, the pC5-Luc did not respond to tRA stimulation, and it did not show strong transcriptional activity either. On the other hand, the control vector pRARE-Luc responded very well to the stimulus (Figure 3.7b). These results indicate that, unlike what have been previously reported (Dentice *et al.*, 2004), in MCF-7 Nkx-2.5 expression is either very low or does not respond to tRA stimulation. Moreover, the

presence of ectopically expressed Nkx-2.5 had an unexpected negative effect on NIS promoter activity. Our results did not indicate an involvement of Nkx-2.5 in the transcription of NIS in MCF-7 in response to tRA. Based on these data, we declined pursuing further experiments on the involvement of Nkx-2.5 as a regulator of NIS expression in MCF-7 in response to tRA stimulation.

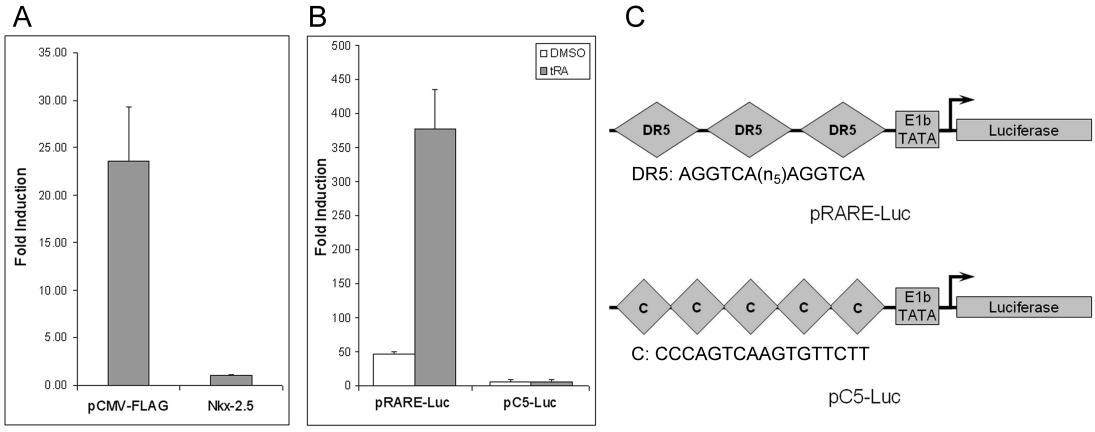

Figure 3.7. Nkx-2.5 has a negative effect on luciferase gene expression in Cl.3 in HeLa cells. A) HeLa cells grown in 24-well plates were co-transfected with Cl.3 and 20 ng of a vector expressing Nkx-2.5 (or the empty vector pCMV-FLAG), cells were collected and luciferase assay was performed, fold induction of Cl.3 is relative to the empty vector pGL3E1bLuc. B) MCF-7 cells were transfected with pC5-Luc, and pRARE-Luc to measure the tRA responsive activation of these elements, as shown in C) pC5-Luc contains 5 tandem repeats of the element "C", which contain the TTF-1/Nkx-2.5 binding site, while pRARE-Luc contains 3 tandem copies of DR5 elements in front of the E1b TATA.

3.2. Estrogen Receptor-α Activates Transcription of the Mammary Gland Sodium Iodide Symporter

3.2.1. tRA-responsive NIS expression is correlated with the presence of a functional ERα

By immunoblot experiments, we have screened 8 different human mammary gland cell lines that we have in our collection for the presence of ERα (Figure 3.8a). Cells were grown in regular DMEM containing 10% FBS (abbreviated as reg-DMEM in

this text, as opposed to phenol-red free DMEM supplemented with dextran-coated-charcoal treated steroid-free FBS, abbreviated as sf-DMEM). In parallel experiments, we either treated them with 1 μM tRA or with vehicle (DMSO) for 12 hours (h) before analyzing NIS expression by RT-PCR (Figures 3.8b and 3.8c). We have also monitored the expression of pS2, an estrogen-responsive gene widely used as a marker to monitor the functionality of ERα and/or E2 treatments in ERα+ cell lines (Jakowlew *et al.*, 1984; Demirpençe *et al.*, 2002).

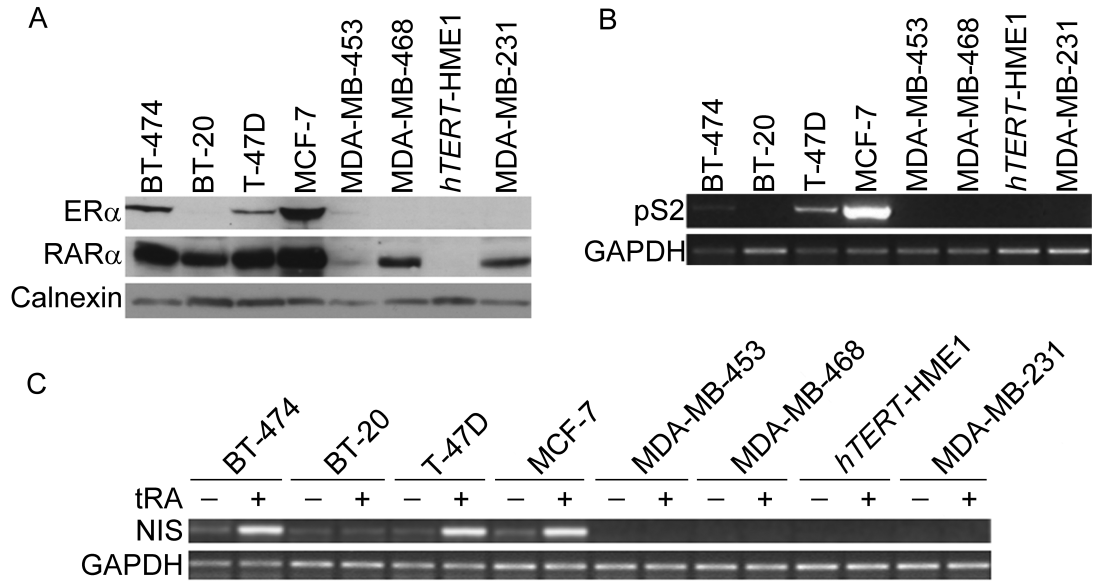

Figure 3.8. ERα positivity in mammary gland cell lines is correlated with tRA responsive NIS expression. Cells grown in reg-DMEM were used in these experiments. A) Immunoblot analysis of ERα and RARα expression in a variety of mammary gland cell lines. Total proteins were extracted, and electrophoresed samples were blotted using anti-human-ERα antibody and anti-human-RARα antibody, respectively. Calnexin expression was also monitored as an internal control. B) RT-PCR analysis of pS2 expression in the absence of E2 or tRA. cDNA was prepared using total RNA isolated from cell lines. The expression level of pS2 (an indicator of ERα activity) was monitored by PCR. C) Cell lines grown either in the presence (+) or absence (-) of 1 μM tRA were collected, and tRA responsive NIS gene expression was monitored by RT-PCR as described in B. Amplification of GAPDH gene cDNA was used as an internal control both in B and in C

Expression of pS2 in cells not treated with E2 but cultured in reg-DMEM (Figure 3.8b) was probably due to the well known estrogenic activity of phenol red, a pH indicator dye (Welshons *et al.*, 1988; Ortmann *et al.*, 1990).

Close correlation between ERα and pS2 expressions suggested that, as expected, pS2 gene expression was a reliable indicator of ERα activity (figure 3.8a and 3.8b). As a result of these analyses, we confirmed that BT-474, T-47D, and MCF-7 were both physically and functionally ERα+ (Figure 3.8a). Remaining cell lines such as BT-20, MDA-MB-453, MDA-MB-468, *hTERT*-HME1, and MDA-MB-231 were classified as ERα-. Basal expression of NIS (in the absence of tRA) was detected in all three cell lines with strong ERα positivity (BT-474, T-47D, MCF-7) and in one ERα- cell line (BT-20). On the other hand, tRA-induced NIS expression was strictly specific to cell lines that were expressing both ERα and RARα (Figure 3.8c).

3.2.2. E2 up-regulates tRA-induced NIS expression in MCF-7 cells

Retinoic acid is known to be a potent inducer of NIS in the cell line MCF-7, elevating the expression of NIS mRNA significantly, this tRA responsiveness of NIS expression was previously described and studied in detail by several other groups (Kogai *et al.*, 2000; Dentice *et al.*, 2004; Kogai *et al.*, 2004). In a different study, E2 was shown to up-regulate NIS expression in experimental animals (Tazebay *et al.*, 2000); based on that, we wanted to study the effect of E2 (when administered alone or in combination with tRA) on NIS expression in MCF-7. For that, we used cells cultured in sf-DMEM (to minimize basal estrogenic activity) and treated them with 1 μM tRA for 12 h or with 10 nM E2 for 3 h, or with both ligands for 12 h. Previous reports indicated that tRA applied as 1 μM gives a maximal expression of NIS after 12 hours [(Kogai *et al.*, 2000), see also figure 3.2a].

With respect to E2, it has also been shown that a concentration of 10 nM gives the highest level of pS2 expression after 3 hours (Métivier *et al.*, 2003; Merot *et al.*, 2004), and dose-response curves performed in our lab at the beginning of this study produced similar results (Elif Yaman, Uygar Tazebay, unpublished results),

thus whenever we applied these two ligands we used the above mentioned concentrations.

We then studied the expression of NIS, RARα, ERα, and pS2 genes in response to these ligands. It was difficult to detect an up-regulated NIS expression after E2 treatment; although the E2 regulated genes like RARα and pS2 were up-regulated (Figure 3.9a and 3.9b). In addition to the previously described tRA-responsive control of NIS transcription, we observed that the addition of E2 (together with tRA) increased NIS expression about 1.9±0.2 fold (average of four independent experiments, for a representative result see Figure 3.9b). More precisely, E2 administration had a cumulative effect on tRA up-regulation of NIS when these two ligands were administered together. This could be explained by an indirect role of E2, where ERα activates the transcription of RAR and thus NIS up-regulation is observed in the presence of tRA.

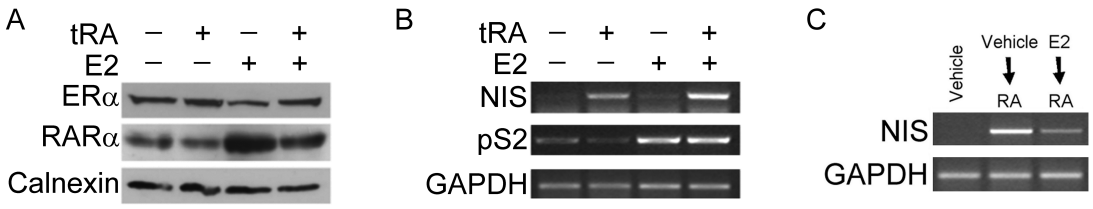

Figure 3.9. Estradiol has an up-regulatory effect on the tRA-induced NIS expression in MCF-7. MCF-7 cells grown in sf-DMEM were treated with 1 μM tRA for 12 hours, with 10 nM E2 for 3 hours, or with both ligands for 12 hours. Cells were then collected and divided into two, the first was used for protein analysis and the second was used for RNA analysis. A) Immunoblot analysis of the levels of ERα and RARα in ligand treated cells. After electrophoresis proteins were blotted using anti-human-ERα and anti-human-RARα antibodies, respectively. Calnexin expression was monitored as a loading control. B) Total RNA was extracted and used for cDNA synthesis. The expression level of NIS and pS2 was monitored by RT-PCR. C) MCF-7 cells were first treated with either EtOH (10 μl 95% EtOH in 10 ml sf-DMEM) or 10 nM E2 for 3 hours, then tRA was added to a final concentration of 1 μM. NIS expression was monitored by RT-PCR, and GAPDH expression was monitored as an internal control in B and C.

To test this, we treated MCF-7 cells cultured in sf-DMEM with tRA (1 μM for 12h) after priming with E2 (10 nM for 3h). Surprisingly, the level of tRA-induced NIS expression in cells that were first primed with E2 and then induced by tRA was lower than that of cells treated with tRA after priming with vehicle (Figure 3.9c). These results indicate that the role of E2 on NIS expression could be a direct involvement of ERα rather than indirectly by first inducing RAR.

3.2.3. Suppression of ERα by shRNA down-regulates NIS expression

To determine the functional relevance of ERα in NIS gene regulation, we suppressed endogenous ERα in MCF-7 by RNA interference (RNAi) method. For this, we used two alternative small hairpin RNA (shRNA) probes targeting different regions of ERα mRNA (sh-ER499 and sh-ER458; see Material and Methods). Cells were stably transfected either with empty vector (pSuper-GFP/Neo, OligoEngine, WA) carrying the GFP and the neomycin resistance (NeoR) marker genes, or with similar vectors carrying shRNA N-19 target sequences in addition to these two markers. Then, colonies originated from transfected cells that were both resistant to neomycin and that were green fluorescing were isolated and cultured separately. By western blots, we monitored the level of ERα suppression in a number of different cell colonies expressing sh-ER458 as compared to colonies transfected with empty vector (Figure 3.10a).

We have noticed that sh-ER458 was more potent than sh-ER499 in suppressing the endogenous ERα gene (Figure 3.10b), thus colonies originated from sh-ER458, and showed significant suppression of the endogenous ERα were selected for further studies (such as colonies 458-12 and 458-13, Fig. 3.10a). Subsequently, we treated these ERα suppressed MCF-7 colonies either with tRA or with vehicle (DMSO), and analyzed both tRA-induced and basal NIS expression (figure 3.10c). In these studies, for both 458-12 and 458-13, we observed about 60% decrease in basal and about 45% decrease in tRA induced expression of NIS as compared to empty vector transfected controls (Figure 3.10c); results indicating that ERα plays a role in both basal and tRA induced NIS gene expression.

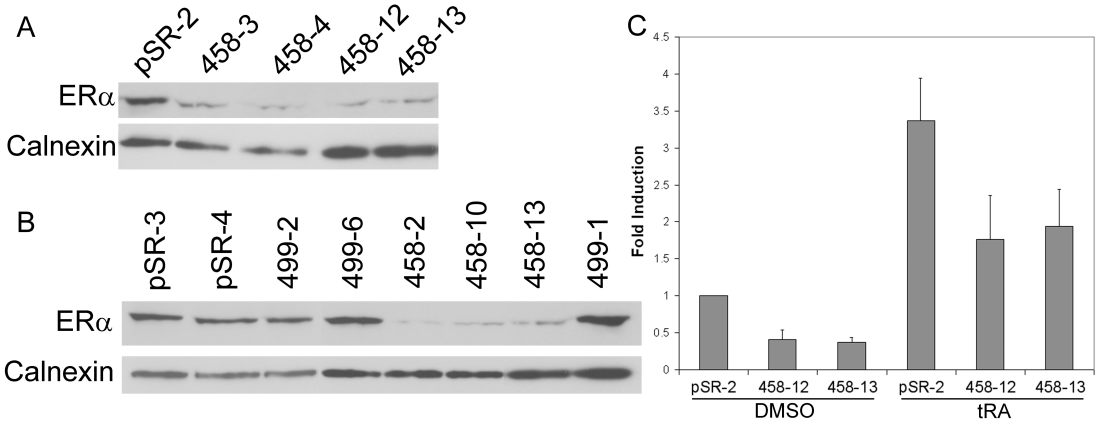

Figure 3.10. Suppression of ERα by shRNA down-regulates NIS expression in MCF-7 cells. MCF-7 cells were transfected either with empty vector pSR, with pSR-ER-458, or with pSR-ER499. Following a double selection procedure based on Geneticin (0.5 mg/ml) resistance and EGFP expression, clones were isolated and further analyzed. A and B) Representative western blot results showing the effect of sh-ER458 and sh-ER499 on the levels of ERα in several clones compared to the empty vector trasfected clones. Clones 458-12 and 458-13 (A) showed significant ERα suppression and were selected for tRA induction. C) Clones 458-12 and 458-13 were grown in reg-DMEM and treated with 1 μM tRA or with DMSO (5 μl in 10 ml culture medium) for 12h. After RNA isolation, cDNA was prepared using 2 μg total RNA, and subsequently used as a template for semi-quantitative RT-PCR analysis using NIS specific primers. Data represent the fold induction (average of four independent experiments) of NIS in 458-12 and 458-13 clones normalized to GAPDH control, and relative to the empty vector pSR.

3.2.4. Ectopic ERα expression in MDA-MB-231 up-regulates NIS expression

MDA-MB-231 cell line express RARα, a major component of tRA signaling mechanism as detected by immunoblots using anti-hRARα antibodies (Figure 3.8a above). Furthermore, these cells respond to tRA, as assessed by the RAR controlled RIP140 gene expression [Figure 3.11a; (Kerley *et al.*, 2001)]. RIP140 expression was also monitored in MCF-7 cells where, unlike MDA-MB-231, tRA-dependent NIS expression was observed (Figure 3.11a). This has shown that the level of tRA responsiveness is comparable in these two cell lines. However, although RA signaling pathway is intact, MDA-MB-231 cells do not express NIS in response to neither tRA nor any other ligand known to induce NIS in other cell systems, including the mammary tissue of experimental animals (Kogai *et al.*, 2000).

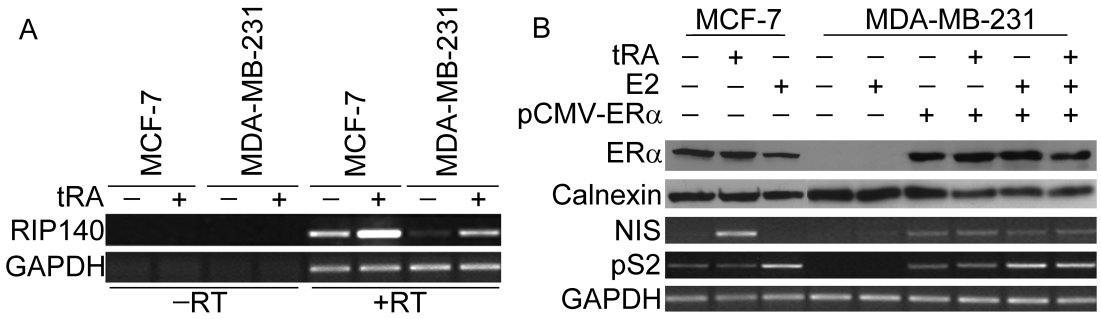

Figure 3.11. Transient expression of ERα in MDA-MB-231 cells leads to increased NIS expression in a ligand independent manner. A) Assessment of the tRA signaling pathways in MDA-MB-231 compared to MCF-7. Cells grown in reg-DMEM were either treated with tRA (+), or with DMSO (-) for 12 hours. Total RNA was extracted and the expression of RIP140, (a tRA responsive gene) was monitored by RT-PCR. As RIP140 is a mono-exonic gene, -RT controls where the reverse transcriptase enzyme was absent in reactions, were included. B) MDA-MB-231 cells growing in sf-DMEM were transiently transfected with a plasmid vector expressing ERα [lanes pCMV-ERα (+)]. Forty eight hours later, cells were treated with 10 nM E2 (3h), 1 μM tRA (12h), or with both ligands (12h). Then, cells were harvested, RNA and protein was extracted from the same sample. ERα expression status in transfected cells were checked and compared to the levels in MCF-7 cells by immunoblot. Calnexin expression was used as a loading control. NIS and pS2 expression levels in response to ligand treatments were assessed by RT-PCR. pS2 was used to verify the functionality of ERα and GAPDH expression was used as an internal control in A and B.

Because tRA-responsive NIS expression was only detected in both ERα+ and tRA-responsive cell lines (Figure 3.8c), we investigated whether introduction of ERα gene could restore tRA responsiveness of NIS expression in MDA-MB-231. For this purpose, we first transiently introduced an ERα expression vector (pCMV-ERα) to this ERα- mammary cell line, and studied ligand responsive NIS expression. Transfected cells expressed ERα at levels comparable to those in MCF-7 cells, and the receptor was functional as assessed by changes in pS2 expression levels (Figure 3.11b). By using RT-PCR we observed that in these cells, NIS basal expression increased to levels higher than that in MCF-7 cells grown in sf-DMEM media (Figure 3.11b). However, treatment of such transfected cells with E2, tRA, or E2 together with tRA did not lead to a further increase in this NIS basal expression (Figure 3.11b). The fact that, even the simultaneous addition of E2 and tRA did not

up-regulate NIS expression in these cells as it did in MCF-7 indicated that in this cellular context functional ERα expression was not sufficient to restore ligand-responsive NIS up-regulation.

We then used a genetically modified MDA-MB-231 cell line that was stably transfected with a vector expressing human ERα (Métivier *et al.*, 2003). First, we confirmed ERα and RARα status of this cell line named as MDA-66 (Figure 3.12b). Then, we analyzed functionality of these receptors by assessing modulations in expressions of tRA- and E2-responsive genes such as RIP140, RARα and pS2 (Figures 3.12a, 3.12b and 3.12c).

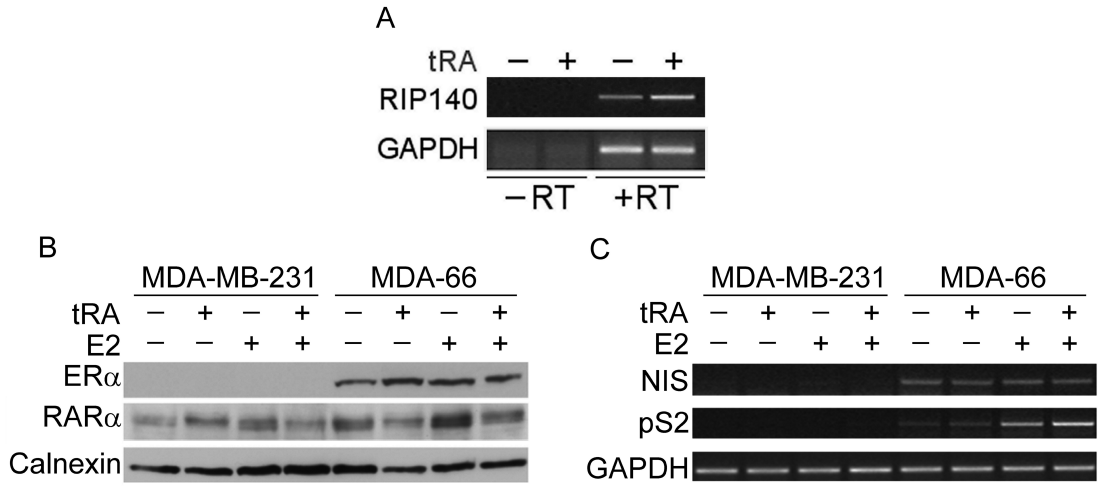

Figure 3.12. Stable expression of ERα in MDA-MB-231 cells leads to a higher basal expression of NIS in a ligand independent manner. A) Assessment of the tRA responsiveness in MDA-66. Cells grown in reg-DMEM were either treated with 1 μM tRA (+), or with DMSO (-) for 12 hours. Total RNA was extracted and the expression of RIP140 was monitored by RT-PCR. B) MDA-MB-231 cells stably transfected with hERα expressing vector (named MDA-66), as well as untransfected cells were cultured in sf-DMEM in the presence of 10 nM E2, 1 μM tRA, or both ligands. Cell pellets were collected for immunoblot and RT-PCR analyses (see panel C). Total proteins were extracted from cell pellets, then electrophoresed and blotted to immunoblot membrane, and changes in the expression of ERα and RARα were monitored by western blot. Calnexin expression was used as loading control. C, Total RNA was extracted from pellets collected as above, and total cDNA was prepared and submitted to RT-PCR analysis using NIS, pS2 and GAPDH gene specific primers. GAPDH expression was used as an internal control.

As expected, an E2-responsive up-regulation of RARα (Elgort *et al.*, 1996) was clearly observed in MDA-66. This result was additional evidence indicating that in these cells E2 signaling mechanism was intact (Figure 3.12b). Subsequently, we studied both the basal expression and E2/tRA responses of NIS gene in these cells (Figure 3.12c). Similar to what we observed in MDA-MB-231 cells that were transiently transfected with ERα, in MDA-66 cells the basal expression of NIS was remarkably increased from undetectable levels to levels higher than that in MCF-7 cells cultured in sf-DMEM (see below). Furthermore, in accordance with results obtained from transiently transfected MDA-MB-231 cells, the expression of NIS gene in MDA-66 cells was not responsive to treatments with E2, tRA, or E2 together with tRA (Figure 3.12c).

As anticipated, composition of growth media affected basal NIS expression, such that, it was reduced (to varying degrees) in cells cultured in sf-DMEM as compared to the ones in reg-DMEM (Figure 3.13). In MDA-66 cells, basal expression of NIS was affected comparably less by culture media composition, which could reflect differences in regulation of endogenous ERα in MCF-7 vs. vector-expressed ERα in MDA-66. On the other hand, when cultured in reg-DMEM, the basal expression of NIS was comparable in MDA-66 and in MCF-7 cells (Figure 3.13). Independent of culture media, NIS expression was not detected in the ERα-cell model; MDA-MB-231 cells (compare Figure 3.8c and 3.12c).

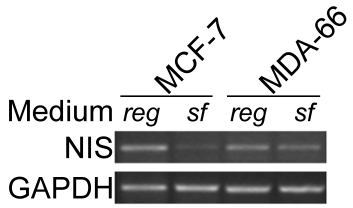

Figure 3.13. Growth media composition affects basal transcription of NIS both in MDA-66 and in MCF-7 cells. MCF-7 and MDA-66 cells were grown either in reg-DMEM (reg) or in dextran-coated charcoal-treated FBS containing media, sf-DMEM (sf). Then they were harvested, and total RNA that was extracted from cell pellets and was subjected to RT-PCR analysis using either NIS specific or the internal control GAPDH specific primers.

3.2.5. Identification of a novel non-canonical ERE in NIS promoter

To evaluate the possibility of a direct regulation of NIS gene by ERα, we first carried out an *in silico* analysis using the Dragon ERE finder program (Bajic *et al.*, 2003). We searched for possible EREs in a 3 kbp region upstream of the transcription start site in human NIS. As a result of this analysis we identified only one putative ERE sequence (Figure 3.14a), albeit it was a novel sequence which was not previously described as an ERE (Bajic *et al.*, 2003). This putative ERE, (5'-CG-GGTCA-CCG-CGACT-CC-3') was located 9 bp upstream of NIS TATA element (Figure 3.14a). This new element had the characteristic head-to-head inverted repeat sequences with high homology to the ERE consensus, and it was similar to previously established EREs (Figure 3.14b). Significantly, this putative NIS ERE sequence and its position vis-à-vis TATA element was also conserved in rat and mouse genomes (Figure 3.14c). In order to establish the transcriptional activation potential of this novel element in response to E2, we have constructed a reporter vector containing two tandem copies of putative NIS ERE sequence; followed by the E1b TATA element and the luciferase reporter gene, pNIS2XERE (see Materials and Methods). We also constructed a similar vector containing two copies of ERE sequence, which was previously shown to bind ERα and lead to E2 responsive up-regulation of the pS2 gene, and named this vector as pPS2XERE (Figure 3.15a).

We then transiently transfected MCF-7, MDA-MB-231, and MDA-66 mammary cell lines with these reporter vectors and studied E2 dependent luciferase activity. In these experiments, both pS2 ERE and putative NIS ERE showed significantly activated luciferase expression in response to E2 in both MCF-7 and in MDA-66 (figure 3.15b). About five fold stimulation by NIS ERE was obtained in MCF-7 cells in response to E2 treatment, whereas, under same conditions, pS2 ERE stimulated reporter gene expression about three fold. This indicated that NIS ERE is at least as sensitive to E2 as pS2 ERE, and clearly, it has the potential to mediate E2-dependent transcription. A similar result is obtained in MDA-66 cells, where NIS ERE-stimulated expression of the reporter 2.4 folds, whereas pS2 ERE-dependent stimulation was 3.4 fold. We also noticed that, in MCF-7 cells, in terms of potency, NIS ERE driven reporter gene expression was five times stronger than that of pS2.

The significant difference in magnitude of gene activation by NIS ERE in MCF-7 and MDA-66 cell lines is in accordance with our preceding results indicating the presence of a better ligand-dependent response in MCF-7 cells.

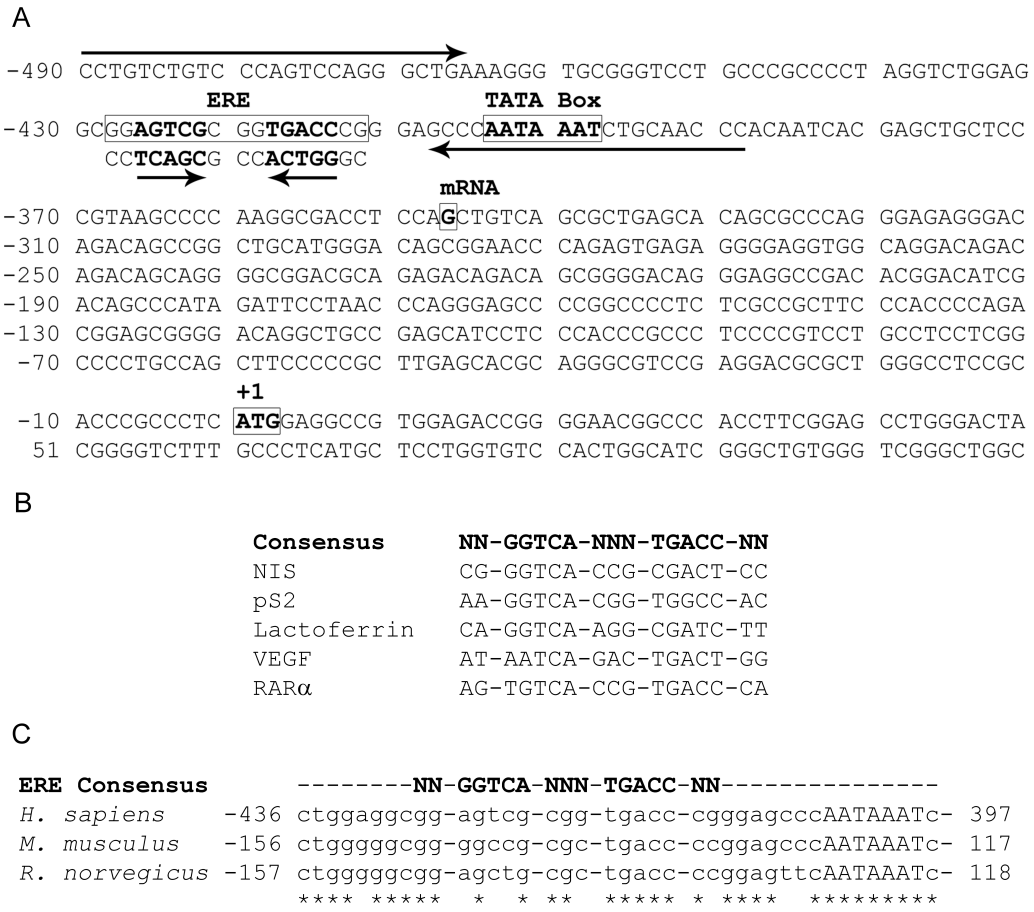

Figure 3.14. A putative novel ERE sequence is located in close proximity of NIS TATA box element in the promoter region. A) Human NIS gene proximal promoter region sequence is shown. The first codon (ATG) of NIS, transcription start site, TATA box element, and novel ERE sequences are indicated in boxes. Two inverted repeats of the ERE element located at the minus strand are shown by short inverted arrows. Position of primers used for the PCR amplification described in Figure 3.16 is also indicated by long arrows. B) Comparison of previously established ERE sequences that were found in several ER regulated genes, the putative NIS ERE sequence, and the consensus sequence. C) Comparison of NIS putative ERE sequence in human, mouse and rat genomes. Putative NIS EREs that were identified in all three genomes located in close proximity of TATA elements, and they fit to functional ERE consensus sequence. NIS TATA region is indicated by upper case letters. (*) signs indicate identical bases in human, mouse and rat sequences.

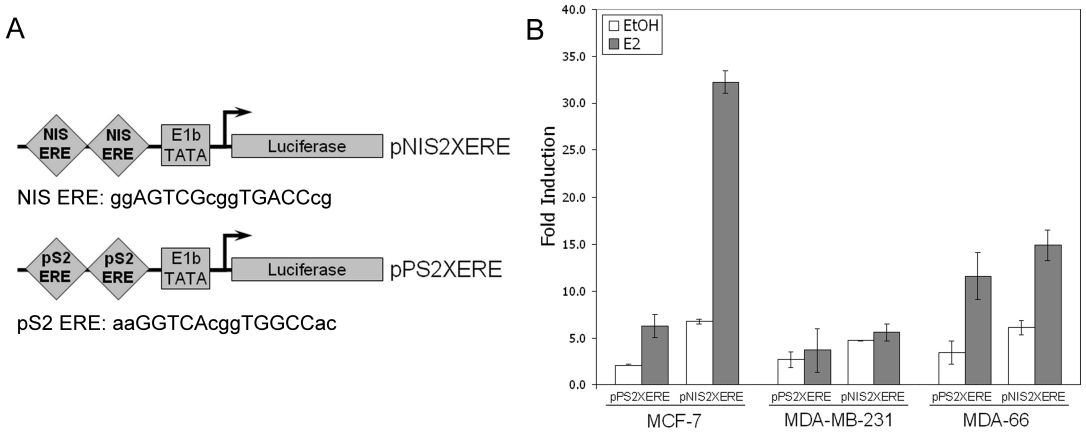

Figure 3.15. The novel ERE element in NIS promoter activates transcription in functional assays in response to E2. A) Map of reporter vectors (pGL3 based) containing the firefly luciferase under control of the E1b TATA element and two tandem repeats of either pS2 ERE (pPS2XERE) or NIS ERE (pNIS2XERE). B) MCF-7, MDA-MB-231, and MDA-66 cells were transiently transfected with reporter vectors presented in (A) and were then treated either with 10 nM E2 (3 h) or with vehicle (ethanol; 10 µl in 10 ml culture medium). Then, luciferase activities were measured, and normalized to the values of *Renilla* luciferase. Fold induction is relative to the empty vector. Data represent the average of four independent experiments.

Taken together, these results may reflect differences in terms of molecular components associated with ER activity. As expected, in our ERα- cell model, MDA-MB-231, neither NIS ERE, nor pS2 ERE has led to E2-dependent regulation of the luciferase reporter gene (Figure 3.15b). We concluded that this non-canonical ERE sequence located in NIS promoter can potentially act as a *cis*-acting element and respond to E2 in a proper cellular context.

3.2.6. Physical interaction of ERα with NIS promoter in vivo

To establish whether endogenous ERα can occupy the novel ERE in NIS promoter *in vivo*, we carried out ChIP experiments in E2 treated MCF-7 cells. In the presence of ERα antibodies, the NIS promoter was precipitated from formaldehyde cross-linked total cell lysates (Figure 3.16, lane 2). In contrast, neither control Fibroblast Growth Factor Receptor-1 (FGFR-1) antibodies, nor antibody uncoated protein-A Sepharose beads precipitated the NIS promoter above background levels (Figure 3.16, lanes 3

and 4, respectively). As expected, ERα antibody was unable to precipitate an unrelated DNA fragment corresponding to the NF1 gene exon 22 (Figure 3.16, lane 2). These data therefore demonstrate that endogenous ERα binds to the NIS gene promoter *in vivo*, thereby suggesting that at least part of the regulatory effects of ERα on NIS expression were due to a direct interaction between the receptor and NIS promoter (Figure 3.16).

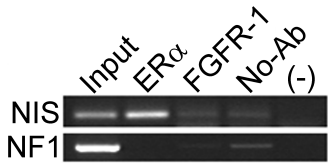

Figure 3.16. ERα occupies the promoter of NIS *in vivo*. MCF-7 cells grown in sf-DMEM were treated with 10 nM E2 and used for ChIP analysis using ERα specific antibody. DNA isolated from immunocomplexes was used as a template for PCR amplification using NIS promoter specific primers (indicated as long arrows in Fig. 3.14A), or unrelated intronic primers corresponding to the NF1 gene exon 22. Lanes: Input; the input DNA used for ChIP analysis, ERα; estrogen receptor-α precipitated DNA, FGFR-1; fibroblast growth factor receptor-1 precipitated DNA, No-Ab; DNA precipitated with protein A-Sepharose beads only (background control), and (-); negative PCR without template DNA.

3.2.7. Physical interaction of ERα with NIS ERE in vitro in response to E2

Results obtained by ChIP experiments indicated clearly that the NIS promoter is occupied by ERα; however, we wanted to test the specificity of this binding to the novel ERE. For this purpose we designed oligo-nucleotide probes harboring mutations in the ERE sequence to be used in electrophoretic mobility shift assay experiments. Three different mutants of ERE were examined (Table 2.3 in Materials and Methods), in the first mutant oligo (ERE-Mut1) 3 point mutations were introduced, the second oligo (ERE-Mut2) contained 2 point mutations; one in each half site, and the third, ERE-Mut3 contained a completely destroyed sequence that did not resemble an estrogen responsive element, where the wild type ERE (GG-

AGTCG-CGG-TGACC-CG) has been changed to CG-GGTCC-GCT-GGACC-TT; the underlined letters represent the changed nucleotides.

EMSA was performed using nuclear extracts obtained from COS-7 cells transiently transfected with pCMV-ERα in the absence or presence of 10 nM E2. All probes were shifted when incubated with COS-7:ERα nuclear extracts (Figure 3.17a). The binding of nuclear proteins to these oligos was specific since it was inhibited by incubation with 200-fold molar excess of the corresponding unlabelled oligo. In addition to that, the introduction of anti-ERα antibodies to the binding reaction resulted in another shifted band (a super shift) only in the wild type probe (similar to the super shifted band when the RARE control oligo was used together with anti-RARα antibody (Figure 3.17b)).

It was also clear that this binding of ERα to the wild type probe is triggered by E2, since nuclear extracts from E2 untreated COS-7:ERα cells did not produce a similar super shift as compared to the E2-treated cell extracts (Figure 3.17c). This super shift was not visible when the mutant probes were used; this is an indication that other nuclear proteins are responsible for the shift observed in these ERE mutant oligo-nucleotide probes (Figure 3.17a). The close localization of this ERE to the TATA element provides a clue to what could possibly be the binding protein; a possible explanation might be that the components of the basal transcriptional machinery could find binding sites in the used oligo-nucleotide probes (see Discussion).

3.2.8. ERE mutations in the context of cluster 3 did not affect tRA responsiveness

So far, we have shown that E2 enhances the tRA responsiveness of NIS in MCF-7 and we have shown evidence in favor of a direct interaction between ERα and the novel NIS ERE. This novel ERE is present in cluster 3 sequence, one of two clusters that showed tRA responsiveness in MCF-7. Based on that, we questioned the possibility of interplay between ERα and RARα through their responsive elements in the regulation of NIS in the context of cluster 3. For this purpose we created ERE mutants of cluster 3 by site directed mutagenesis using the oligo-nucleotides in table 2.3 (see Materials and Methods), and studied the expression of the reporter gene in

response to E2 or tRA stimulation (Figure 3.18). We have noticed that Cl.3 was not responsive to E2 treatment, suggestive of the existence of other elements that are essential for E2 regulated expression (Figure 3.18a).

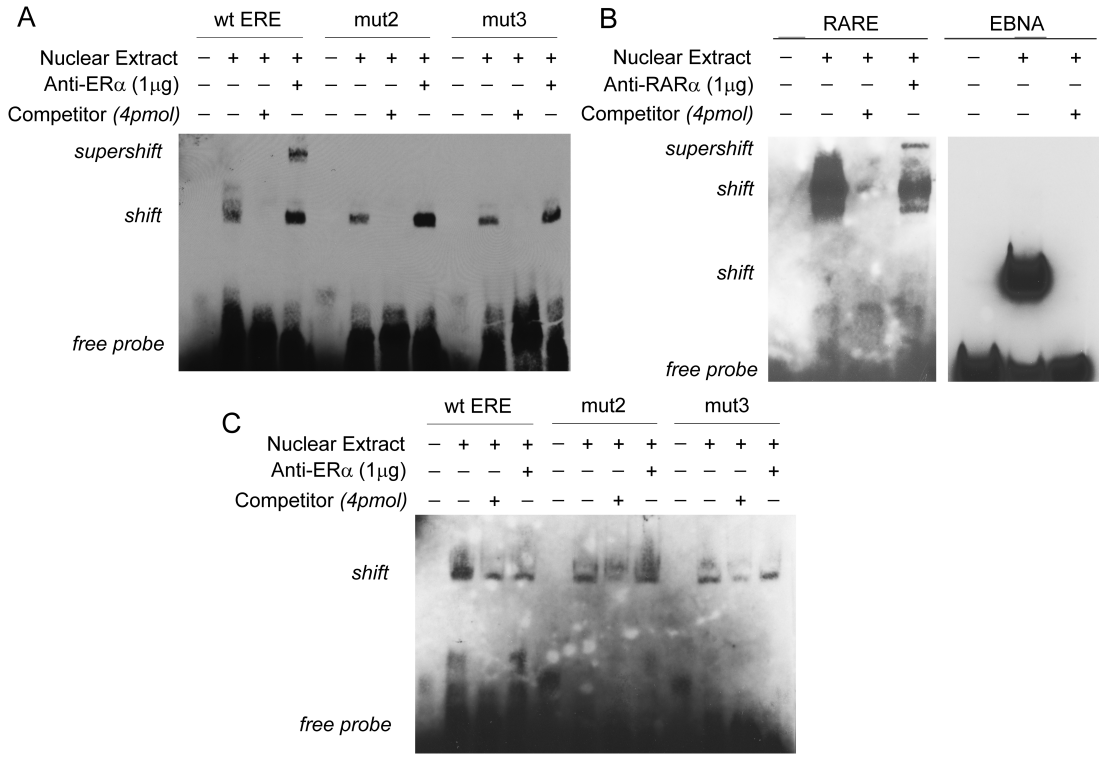

Figure 3.17. E2 stimulates the interaction of ERα with the novel NIS ERE in gel retardation assays. A) Nuclear extracts from COS-7 cells that were transfected with pCMV-ERα and treated with 10 nM E2, were incubated with biotin labeled oligo-nucleotide probes representing the wild type ERE or two different mutant variants of ERE (see table 2.3). For super shift analysis, 1 μg of anti-ERα antibody was also included in the binding reaction. Samples were resolved on a 6% non-denaturing acrylamide gel in TBE, transferred to Hybond N+ membranes and then incubated with streptavidin, and biotin labeled DNA probes were detected by chemiluminescence. The name of the probe used in each binding reaction is indicated on the top of each panel. B) Control EMSA experiments using oligo-nucleotides for the binding of RARα (RARE) and the Epstein-Barr Nuclear Antigen (EBNA). For RARE, nuclear extracts from MCF-7 were used. C) Nuclear extracts from COS-7 cells prepared as in (A) but not treated with E2 were used to test the binding of ERα to the biotin labeled probes in the absence of estradiol. All binding reactions were competed with 200-fold molar excess of corresponding unlabeled probes.

Unexpectedly, Cl.3 ERE mutant plasmids appears to be more potent (in driving the transcription of the reporter gene) than the wild type Cl.3. We were expecting that this mutation will reduce the activity of this construct as seen in previous experiments, in which NIS expression was down-regulated in response to ERα suppression (Figure 3.10). The reason for such result could be the absence of other elements in this plasmid, which are required for the regulation by ERα in the context of Cl.3. Very surprisingly, results of such experiments clearly showed an increase in the reporter gene activity as a result of the ERE mutations, with the exception of Cl.3 ERE Mut3, which was severely down-regulated. This increase in reporter activity was not confined to non-treated cells, but was also elevated in tRA treated cells; however, the overall tRA stimulation was unchanged when compared to the wild type sequence (Figure 3.18b).

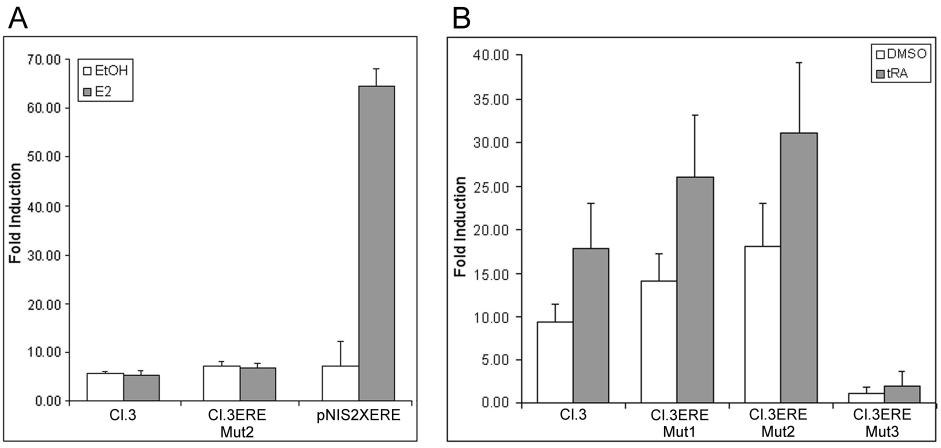

Figure 3.18. Mutants of the novel ERE sequence in the context of Cl.3 did not affect luciferase gene expression in response to tRA stimulation. A) MCF-7 grown in sf-DMEM were transfected with Cl.3 or with Cl.3 ERE Mut2, cells were then treated with 10 nM E2 and luciferase activity was measured, and normalized to the values of *Renilla* luciferase. E2 stimulation of MCF-7 was verified by the response obtained from the control plasmid pNIS2XERE. B) MCF-7 cells grown in reg-DMEM were transfected with Cl.3 and 3 different ERE mutant constructs, cells were then stimulated with 1 μM tRA for 24 hours. Cells were collected and luciferase assay was performed. Luciferase values from each construct were normalized to values of *Renilla* luciferase. Fold induction is relative to the empty vector.

3.2.9. E2 up-regulates NIS expression in MCF-7 in a dose dependant manner

Results obtained so far indicated the direct involvement of ERα in the expression of NIS through the novel ERE in the NIS promoter in MCF-7 cells. Indirect evidences were in favor of a direct role of E2 in NIS up-regulation. The first came from studies using animal models (Tazebay *et al.*, 2000), in which E2 administration was sufficient for increased NIS expression. Others include the decrease in NIS expression level in cells cultured in media depleted from steroids (sf-DMEM), and the E2 additive effect on tRA-induced NIS expression. More evidence was obtained from *in vitro* experiments, in which NIS ERE was shown to be responsive to E2, and this NIS ERE was bound to E2 activated ERα. Based on that, we decided to repeat the E2 response curves, this time using narrower intervals, since previous curves included E2 concentrations increased by 10 fold. For that, we cultured MCF-7 cells in sf-DMEM and treated them with increasing concentrations of E2 (0, 5, 10, 20, 50, and 100 nM) for 3 hours.

As indicated previously, we observed a significant decrease in the basal expression of NIS in cells cultured in sf-DMEM compared to those cultured in reg-DMEM. This change in the expression level makes it rather difficult to detect the presence of NIS transcript, yet a detectable increase in the expression of NIS was seen at 10 nM concentration (Figure 3.19a), which reached a maximum level at 20 nM E2 (2.7 fold increase as compared to EtOH treated control; this represents the average of 3 independent experiments), then starts to drop at higher concentrations (Figure 3.19b). This result shows for the first time a direct up-regulatory effect of E2 on the expression of NIS in MCF-7 cells.

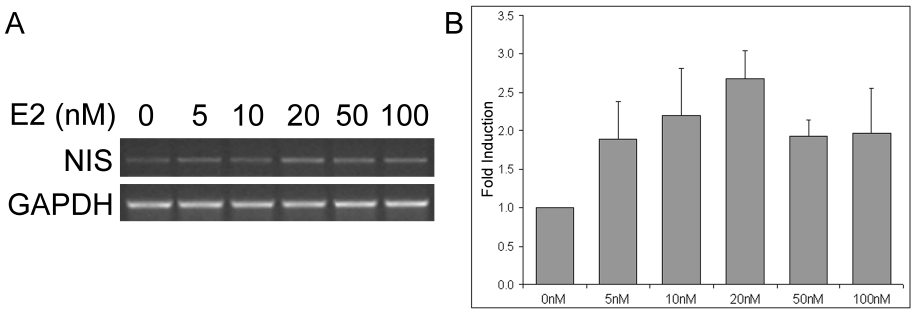

Figure 3.19. E2 up-regulates NIS expression in MCF-7 in a dose-dependant manner. A) MCF-7 cells grown in sf-DMEM were treated for 3 hours with increasing concentrations of E2 as indicated above each lane. Cells were collected and total RNA was extracted to be used for semi-quantitative RT-PCR. Changes in NIS expression in response to E2 treatment were monitored, as well as the internal control GAPDH. B) Quantification of NIS expression in response to E2. NIS band intensities were measured using the Multi-Analyst software (Bio-Rad) and values were normalized to those of GAPDH, fold induction is represented relative to the induction in non-treated cells, values of which were set to 1. Data represented is the average of 3 independent experiments.

4. DISCUSSION

The expression of NIS in the mammary gland and in breast cancer has led many groups to study the potential use of the function of this protein in the diagnosis and treatment of breast cancer. It was also clear that the regulation of NIS expression in the mammary gland is different than that of the thyroid gland, thus better understanding of the *cis*-acting elements and factors regulating this expression became a priority for many research groups including us. Unfortunately the lack of cell line models with NIS expression has limited the research to one cell line, MCF-7, in which NIS expression is controlled by RA stimulation. This study is composed of two main parts; both aimed to study the regulation of NIS expression by ligand activated nuclear receptors in the mammary gland cell line model MCF-7. The first involved the use of bioinformatics to search for *cis*-acting elements regulating NIS expression stimulated by tRA in MCF-7, and the second was to study the involvement of E2 and ERα in the control of NIS transcription. Our results showed that both ligands play an important role in regulation of NIS expression, and that the molecular pathways involved in this regulation functionally cooperate.

4.1. tRA Response Elements in Thyroid Cell Lines and in MCF-7 are Distinct

Using the genome Vista tools we analyzed the sequences flanking and including the NIS gene for conserved regions in the genomes of human, mouse and rat (Figure 3.1). This study was based on the recently shown correlation between conserved gene expression patterns and the conserved regulatory *cis*-acting elements (Negre *et al.*, 2005). The results of the initial analysis revealed several conserved regions which could be involved in the regulation of the transcription of this gene. Our first experimental data demonstrated the strength of such analysis; of the nine analyzed conserved clusters four were transcriptionally potent (Figure 3.3a). In particular conserved region cluster 1, which harbors the previously well studied NUE. Thyroid

specific transcription factors such as TTF-1 and Pax8 were shown to be involved in the regulation of NIS transcription by binding to sequences in the upstream enhancer in both rat and human cell line models (Ohno *et al.*, 1999; Taki *et al.*, 2002).

The second cluster to be of specific importance was cluster 3 for its strong response to tRA stimulation. This is the immediate upstream sequence in front of the NIS transcription start site including the previously described minimal promoter (Venkataraman *et al.*, 1998). The previously described RA responsive site controlling RA dependant NIS expression in thyroid cell lines (Schmutzler *et al.*, 2002) lies between conserved regions 3-1 and 3-2 in this cluster of four conserved regions (Figure 3.4a). And subsequent analyses revealed the importance of this cluster, specifically conserved region 3-4 in controlling NIS transcription.

Cluster 4 comes from the first intron of NIS; it encompasses 3 conserved regions (Figure 3.6a), and could be considered as important as cluster 3 for tRA dependant expression of NIS. tRA stimulation potential of this cluster was even higher than that of cluster 3 (Figure 3.3b). Several studies have previously illustrated the importance of intronic sequences in regulation of gene expression; one example is the ERE (estrogen responsive element) present in the first intron of the RA receptor alpha gene (Laganiere *et al.*, 2005). It was shown to be responsible for estrogen dependant transcription of RARα gene in breast cancer cell models. Another example comes from studies involved the understanding of *cis*-acting elements governing the expression of E-cadherin during the embryonic development of mice. Sequences in the second intron of E-cadherin were shown to be required for proper initiation and maintenance of E-cadherin expression in differentiated epithelia (Stemmler *et al.*, 2005). In a different study, the tissue-specific enhancer in the first intron of murine *Nfatc1* was shown to activate a heterogenic promoter and to direct gene expression in a subpopulation of endocardial cells of the developing heart (Zhou *et al.*, 2005).

Cluster 2 was transcriptionally potent in all cell lines studied, but it was not responsive to tRA stimulation and thus was not considered for further analysis, as with the remaining clusters which did not show relevant transcriptional activity in the

studied cell lines. Yet these conserved clusters could be of great value in the study of NIS expression in a different cellular context. For instance, if a cell model where NIS up-regulation by prolactin is found in the future, then these constructs could be used for the analysis of NIS expression in response to prolactin, and thus to identify *cis*-acting elements that regulate this physiological expression of NIS

RAs are potent molecules that have been used for redifferentiation therapy of many cancers because of their proliferation-inhibiting and differentiation-inducing abilities (Bastien and Rochette-Egly, 2004). They are ligands of a class of nuclear receptors called the retinoic acid receptors (RAR and retinoid x receptors; RXR) It has been shown that patients with poorly differentiated thyroid carcinomas lacking iodide transport responded to treatments with RA and showed an increased radioiodide transport (Simon *et al.*, 1996; Simon *et al.*, 1998; Schmutzler and Kohrle, 2000). It was reasonable to conclude that increased radioiodide transport was a reflection of an increased sodium iodide transporter activity and probably transcription (Schmutzler and Kohrle, 2000). Schmutzler *et al.* (2002) studied the involvement of RA and RAR in the transcription of NIS using human follicular thyroid carcinoma cell lines and found a dimeric retinoic acid responsive site (DR10) at -1375 relative to the ATG start codon of the human NIS gene. Their data showed that this site was responsive to RA stimulation (2.5 fold increase), and that blocking mutations in either half site abolished RAR binding to this element and thus the loss of RA response (Schmutzler *et al.*, 2002).

Based on the above summarized information, we carried out experiments to evaluate to contribution of this element (the DR10 at -1375) in the RA responsive NIS expression in the mammary gland cell model MCF-7. Conserved cluster 3 in our study is the upstream sequence of NIS gene (-1138 to -45 bp relative to transcription start site), it is composed of four conserved regions with high conservation levels (region 3-1: 54.7%; region 3-2: 51.9%; region 3-3: 56.1% and region 3-4: 63.1%). And the DR10 element lies between regions 3-1 and 3-2. We adapted the mutation previously shown to block RAR binding and RA responsiveness through this site (Schmutzler *et al.*, 2002), and constructed the plasmid Cl.3 RARE Mut (Figure 3.4a), to be used in reporter assays. In MCF-7, this mutation reduced reporter gene

expression mildly; the tRA uninduced expression was reduced by 22%, while the tRA stimulated expression was reduced by 27% and the overall tRA stimulation was only reduced by 7% (Figure 3.4b). These results clearly indicate that tRA responsive NIS transcription is carried out through either another RARE present elsewhere in this cluster, or through a cooperative regulation involving other *trans*-acting factors, action of which could be preceding the action of the tRA stimulus in the sequence of events leading to maximal NIS transcription.

We then analyzed cluster 3 for additional tRA responsive sites that could be present in this sequence. Results obtained in course of the experiments did not provide clues for the presence of another tRA responsive site (Figure 3.5b), instead it pointed out the possible importance of a single conserved region, namely 3-4. Subclones containing this conserved region restored most of the transcriptional activity of the parental cluster 3 (tRA response was lost though). In the absence of this conserved region a severe decrease in transcriptional activity was obvious; in particular a 101 bp deletion (sequence between the *Pst*I and *Sma*I sites) in region 3-4 was sufficient to reduce the transcriptional activity of this clone significantly. It should be mentioned here that tRA responsiveness of this construct was maintained, although with lower magnitude. This is probably due to the presence of the above mentioned upstream RARE, or yet an unidentified tRA responsive element. Interestingly, the importance of this small deletion was also shown in a different study, which involved the cloning and characterization of the human promoter for NIS (Behr *et al.*, 1998). In that study, a reporter construct of the human NIS promoter up to the *Bln*I site (-442 relative to the ATG codon) was shown to retain most of the transcriptional activity of the initial plasmid (up to -1644 bp relative to the ATG codon) in the rat thyroid cell line FRTL-5, whereas, a deletion of 30 bp (-412 at *Sma*I site) was sufficient to drastically reduce the activity of the reporter gene. The authors concluded that this sequence including the NIS TATA element (sequence between -443 to -395) is essential to confer promoter activity (Behr *et al.*, 1998).

As indicated previously, cluster 4 was the second sequence to be tRA responsive in MCF-7; this responsiveness could be attributed to two putative binding

sites that we found in the sequence of this cluster. The first was a perfect DR2 that lies in the second conserved region of this cluster. The second, not in a conserved region though, is a DR10 sequence with high similarity to the well known consensus (with two mismatches in the first half site). Apart from the sequence from cluster 4, we extended the analysis of the sequence around cluster 4, and interestingly, we found that the second half site of the DR10 is overlapping with another DR2 sequence identical to the DR2 in region 4-2, thus the first intron of NIS actually contains two DR2 elements and one overlapping DR10 element increasing the possibility of tRA mediated regulation of NIS in MCF-7 through the sequences in this intron. Retinoic acid responsive elements are composed typically of two direct repeats of the core motif PuG(G/T)TCA [reviewed in (Giguere, 1994)]. The two direct repeats in a classical RARE are separated by 5 nucleotides, yet direct repeats separated by 1 or 2 nucleotides have been shown to bind to RXR/RXR homodimers and RAR/RXR heterodimers, respectively (Giguere, 1994). Direct repeat half sites separated by 10 nucleotides (DR10) have been also shown to bind to nuclear receptors and respond to hormone stimulation (Kato *et al.*, 1995; Schmutzler *et al.*, 2002).

Cluster 4 was responsive to tRA stimulation, which was slightly higher than that of cluster 3 (Figure 3.3b), probably due to the presence of two putative responsive sites; yet the functionality and the contribution of these new elements has not been determined experimentally. We also studied the potential synergism between clusters 3 and 4 with regard to tRA responsiveness. Our data clearly showed an increase in the tRA response by 26% when compared to cluster 3 alone (Figure 3.6b).

The presence of the 5'UTR sequence in Cl.3 and in Cl.3/4 increased the transcriptional potential of these clusters (Figure 3.6b). This increase in the transcriptional activity was not accompanied by an increase in the overall tRA stimulation. On the other hand, unlike what was observed in Cl.3/4, the addition of the 5'UTR sequence to this plasmid decreased the enhanced tRA responsiveness. We were lucky in a sense; not having the UTR sequence in the first Cl.3/4 construct allowed us to identify the two RARE sequences in cluster 4, and subsequently

another RARE in the first intron of NIS, activity of which will be studied. In fact, Cl.3/4UTR is the closest resemblance to the arrangement of these conserved regions in the human genome, but the tRA response of the reporter gene was lower than that of the endogenous NIS expression level when stimulated by this ligand. Of course signals from reporter plasmid vectors will, to a certain extent, reflect the situation *in vivo*, but outcomes of these signals may also be different. One explanation could be the absence of essential sequences required for maximal response on the target DNA. Besides, ligand dependent nuclear receptors may exert differences in their transactivation mechanisms of target genes when studied in isolated experimental systems, in particular, the chromatin structure could play an important role in the transcriptional regulation of certain target genes (Métivier *et al.*, 2003; Kinyamu and Archer, 2004). Nuclear receptors are known for their ability to initiate transcription of target genes through chromatin remodeling followed by recruiting co-activators and other components of the transcriptional machinery (Métivier *et al.*, 2003). However, such molecular interaction may not be detected when studying their function using plasmid DNA based functional assays.

4.2. Estradiol and ERα are Involved in the Transcription of NIS

As mentioned earlier, MCF-7 was previously shown to have an up-regulated expression of NIS in response to RA stimulation. This RA-dependant expression of NIS was correlated to the presence of ERα, compared to neither expression nor tRA responsiveness in the ERα- cell line MDA-MB-231 (Kogai *et al.*, 2000). In order to determine the correlation between ERα and RA responsiveness we have carried out experiments using a selection of mammary gland cell line models and we treated them with tRA (a RAR-specific ligand) or with DMSO as vehicle control. We found that this ligand up-regulates NIS expression only in mammary gland cells that were both ERα+ and RARα+ (Figures 3.8a and 3.8c). A very similar result signaling a correlation between ERα status of mammary cell lines and 9cRA (a RAR/RXR-specific ligand) induction of NIS gene has also previously been shown (Tanosaki *et al.*, 2003). However, in our studies, we have also observed a basal expression of NIS in ERα+ mammary cell lines, as opposed to no detectable expression in ERα- cells (Figure 3.8). This suggested that besides playing its indirect role in modulating RAR

dependent regulation (Elgort *et al.*, 1996), ERα also plays a role in regulation or initiation of basal NIS expression (see below). The BT-20 cell line where ERα negativity and lack of basal NIS expression did not correlate was an exception (Figure 3.8). In previous studies where human breast tumor samples were analyzed, NIS up-regulation was also seen in some ERα- tumors (Tazebay *et al.*, 2000; Wapnir *et al.*, 2004), and BT-20 cells could provide a model for ERα-independent NIS regulation in human breast cancer.

In the absence of tRA, the expression of NIS gene was detected in four out of eight reg-DMEM-fed mammary gland cell lines tested including MCF-7 (Figure 3.8c). On the other hand, this basal expression of NIS diminished significantly in MCF-7 cells grown in sf-DMEM (Figures 3.9b and 3.13). This result indicated that charcoal-depleted factors (mainly steroids) contribute to the regulation of NIS gene expression. In fact, the effect of a steroid hormone, E2, on functional NIS expression was previously described in experimental animals (Tazebay *et al.*, 2000). In accordance with these observations, we demonstrated that in MCF-7 cells cultured in sf-DMEM, E2 treatment has an additive up-regulatory effect on tRA-stimulated NIS expression (Figure 3.9b). However, when administered alone (10 nM), such effect was rather difficult to detect, although other E2 stimulated genes such as RARα and pS2 were up-regulated (Figures 3.9a, 3.9b). Initial studies involved treatments with E2 were done with a concentration of 10 nM, based on a dose response curve established in our laboratory (E. Yaman and U. Tazebay, unpublished results). These results had indicated that a 10 nM concentration of E2 provides a maximum expression of the well known pS2 mRNA. However, in those past response curves E2 had been used with 10 fold increments; and seemingly this have hindered mild changes on NIS gene expression. But more recently, and after getting certain clues in favor of an E2 effect on NIS transcription (see below), we repeated this curve with a narrower intervals, and only then we were able to visualize for the first time the direct effect of E2 (20 nM) on NIS mRNA expression in MCF-7 (Figure 3.19).

The additive effect of E2 on tRA-stimulated NIS expression could well be explained by an indirect action of ERα on NIS gene expression according to a previously established interplay where ERα up-regulates RAR (Elgort *et al.*, 1996).

However, NIS expression in cells treated with tRA (12 h) after E2 priming (3 h) was not stronger than the expression of this gene in cells treated with tRA (12 h) alone (Figure 3.9c). This observation, although preliminary, did not provide a supportive evidence for the indirect role of E2-ERα regulatory pathway in NIS transcription. On the other hand, we demonstrated the direct action of ERα on NIS gene transcription (see below).

Previously, Kogai *et al.* (2005) reported that in MCF-7 cells E2 does not have any additive up-regulatory effect on tRA induced iodide transport. This contradicts with our results. One explanation of why E2 effect was not observed in iodide uptake tests (Kogai *et al.*, 2005) might be that the relatively moderate regulatory changes that could be detected by RT-PCR analysis may remain hidden in comparably less sensitive analytical methods such as enzyme activity assays. Another explanation could be the culture conditions. We have shown that media composition affected the level of NIS expression (figure 3.13). Therefore, given that the mild effects exerted by E2 treatment alone can only be detected when cells are grown in steroid free media, it would clearly be very difficult to detect in other culture conditions.

Analysis of NIS expression in MCF-7 cells where ERα was suppressed by RNA interference revealed the importance of this factor in both basal and tRA induced expression of NIS (Figure 3.10). The partial decrease in NIS mRNA levels in response to severe down-regulation of ERα may indicate redundant functions between ERα and other factors such as RARs and Nkx-2.5 regulating this gene (Kogai *et al.*, 2000; Dentice *et al.*, 2004). However, our analysis of Nkx-2.5 function has failed as presented in results part 3.2.6. The absence of a tRA dependant transcription of the reporter gene in the plasmid pC5-Luc (Figure 3.7b) may indicate that MCF-7 did not have a functional Nkx-2.5; another explanation is that Nkx-2.5 in MCF-7 did not respond to tRA stimulation. This was unexpected, because we are using the same MCF-7 clone (see Materials and Methods) that was used in the work of Dentice *et al.* (2004). On the other hand, expression of Nkx-2.5 in HeLa cells down-regulated the transcriptional activity of cluster 3 (Figure 3.7a), this effect could be explained by the observation of Dentice *et al.* (2004), where Nkx-2.5 up-regulated rat NIS promoter when up to 200 ng/dish of Nkx-2.5 expressing plasmid were used,

and then this effect was reversed with higher amounts. We have to mention here that we down scaled the amounts of plasmid DNA transfected to levels comparable with previously published protocols (Dentice *et al.*, 2004), however differences in transfection methods may have caused this negative effect; in that report, they have used the calcium phosphate precipitation method, a low efficiency method compared to FuGENE-6 mediated transfection, which may result in the introduction of more plasmid copies per cell.

Interestingly, previous studies have shown that in a rat thyroid cell line model, FRTL-5, activation of ER pathway by E2 down-regulates thyroid NIS gene expression (Furlanetto *et al.*, 1999; Kogai *et al.*, 2000). This suggests that common molecular elements may exert opposite regulatory effects on NIS gene expression in thyroid and in the mammary gland. As pregnancy is a physiological state, which is associated with increased needs for thyroid hormone (TH) synthesis (Glinoer, 2004), a potential reduction of the iodide available for TH synthesis of the mother could explain increased hypothyroidism cases in pregnancy and during lactation (Tazebay *et al.*, 2000; Glinoer, 2001). On the other hand, the above described regulatory actions of ERα on NIS gene expression might also provide an additional possible explanation to increased hypothyroidism cases in pregnancy and lactation when iodide uptake substantially increases in mammary glands (Tazebay *et al.*, 2000).

In an attempt to provide more supportive evidence to the relevance of ERα regulated NIS expression, we transiently transfected MDA-MB-231 (an ERα- cell line) with ERα expressing vector. Transfected cells expressed ERα at levels comparable to those in MCF-7 cells, and the receptor was functional as assessed by the increase in pS2 expression levels in response to E2 (Figure 3.11b). Interestingly, introduction of ERα significantly increased NIS gene expression, although it did not restore tRA responsiveness of this gene. We noticed that the ERα-activated NIS expression in MDA-MB-231 was even higher than the basal levels in the ERα+ cell model, MCF-7. On the other hand, when compared to tRA induced levels of NIS expression in MCF-7 cells, this ERα-activated expression of NIS was about three times lower (Figure 3.11b).

Although it seems correlated with tRA response, ERα activity together with tRA responsiveness was also not sufficient for ligand response either. In tRA-responsive MDA-MB-231 cells (as monitored by RIP140 regulation; Figure 3.11a), only the basal expression (and not E2 or tRA induced expression) of NIS was restored as a result of a both transient and stable ERα gene transfection (figures 3.11b and 3.12c). This result demonstrated a role of unliganded (or apo-) ERα in regulating basal expression of NIS gene. The absence of E2-induced NIS expression under these conditions may also be explained by the fact that we used 10 nM instead of 20 nM, however, this does not explain the lack of tRA responsiveness, or the lack of the additive effect of E2 on tRA-induced NIS expression when compared to MCF-7 treated with similar amounts.

Ususally, unliganded nuclear receptors are considered to be transcriptionally inactive. Upon ligand binding, receptor-associated co-repressors are exchanged for co-activators, resulting in the activation of transcription. However, recent studies indicate that this model is too simple and not adequate to explain the dynamic pattern of transcriptional regulation (Perissi and Rosenfeld, 2005). It has previously been shown that liganded ER is able to interact with a selective repressor protein illustrating an unpredictable mode of action for liganded receptors (Delage-Mourroux *et al.*, 2000). On the other hand, in a very recent report, the activator function of apo-ERα was also demonstrated (Métivier *et al.*, 2004). In this report, binding of apo-ERα to its cognate sequences initiates specific events sufficient for immediate response to E2, poising the pS2 gene for expression.

It has been shown that apo-ERα recruits several histone acetyl transferases as well as a histone methyl transferase, destabilizing nucleosomes positioned around apo-ERα binding site in pS2 gene promoter region. A very important suggestion of data gathered by Métivier *et al.* (2004) was that apo-ERα, by binding to its cognate sequences, induces a chromatin environment that is permissive for transcription to occur. Therefore, it is conceivable that similar mechanisms operate on NIS gene promoter, and apo-ERα is essential for holding the NIS gene at a transcriptionally competent state. This would indicate the absence of transcriptionally competent NIS gene loci in ERα- mammary cell lines, and thus explain the lack of tRA-responsive

NIS expression in these cells (Figure 3.8c). On the other hand, we could not detect ligand-dependent up-regulation of NIS gene in RARα+/ERα+ MDA-66 cells (Figure 3.12b. 3.12c). This result suggests that in addition to RARα and ERα, (an)other so far unidentified factor(s) are (is) essential for ligand-responsive expression of this gene. We must also point out that, when compared to MCF-7, MDA-66 cells are less differentiated, and as demonstrated by the contribution of different transactivation functions in ERα, the differentiation stage of breast cancer cell lines strongly affects the transcriptional activity of this receptor (Merot *et al.*, 2004). Moreover, the origin of these cell lines is also different; MCF-7 cells were derived from ductal carcinoma while MDA-MB-231 is mesenchymal-like. Based on data gathered by Charafe-Jauffret and colleagues (2006), these cell lines were classified into two different groups based on their genetic background, MCF-7 cells belong to the luminal type, while MDA-MB-231 cells belong to the mesenchymal type (Charafe-Jauffret *et al.*, 2006). Differences between these two types include the differential expression of gene sets, which include ERα and other transcription factors in addition to the expression of proteins with roles in the remodeling of the extracellular matrix (Charafe-Jauffret *et al.*, 2006). This could explain the differences in E2-responsive gene expression (or different responses according to growth media composition) that we observed between these two cell lines (Figures 3.8c, 3.12, 3.13).

Direct and indirect actions of ERs on NIS gene transcription are not mutually exclusive. Concerning possibilities of a direct action via ERα, it was interesting to detect a novel ERE sequence conserved in human, rat and mouse genomes in proximity (9 base pairs) of NIS TATA element (Figure 3.14). We have demonstrated that this ERE sequence has the capacity to activate gene expression in luciferase reporter assays in both MCF-7 and MDA-66 cells in response to E2 (Figure 3.15b). In fact, such a close localization of TATA and ERE elements is very unusual considering that all previously characterized ERE elements were shown to be localized at relatively distant positions to transcription start sites in corresponding genes [although varying remarkably between +23,088 and -2687 (Bourdeau *et al.*, 2004)]. However, it has also been known for long time that the response element preferences and DNA binding properties of nuclear receptors cannot be simply attributed to classical spacing, localization or orientation rules (Mader *et al.*, 1993).

The significant difference in magnitude of gene activation by NIS ERE in MCF-7 and MDA-66 cell lines may reflect differences in these two cell lines in terms of molecular components associated with ER activity. As expected, in our ERα- cell model, MDA-MB-231, neither NIS ERE, nor pS2 ERE has led to E2-dependent regulation of the luciferase reporter gene (Figure 3.15b). We concluded that this non-canonical ERE sequence located in NIS promoter can potentially act as a *cis*-acting element and respond to E2 in a proper cellular context.

By ChIP experiments, we have obtained evidences in support of *in vivo* physical interaction between ERα and the novel NIS ERE (Figure 3.16). Further evidences to the specific interaction of ERα with this novel ERE came from gel retardation assays (Figure 3.17). Using oligo-nucleotide probes representing this ERE element and 3 different mutants undoubtedly revealed that ERα interacts with the novel NIS ERE. The fact that this interaction was abolished with point mutations clearly shows the specificity of this ERα-ERE interaction. Interestingly, this binding of ERα was detected with the presence of E2 (Figure 3.17a), in support for the involvement of E2 in NIS regulation. When taken together with E2 responsive transcriptional activation of luciferase reporter gene via this ERE sequence in transfected MCF-7 cells, our EMSA and ChIP results provide an unequivocal evidence in support of the functionality of this site *in vivo*, and of a direct action of ERα on NIS promoter.

Cluster 3, as mentioned earlier, is the immediate upstream sequence of NIS gene including the minimal NIS promoter, interestingly, NIS ERE is part of this sequence and it was reasonable to assume the involvement of this element in the activity of this cluster in reporter assays. By site directed mutagenesis, we created a number of ERE mutants in the context of cluster 3 and examined the effect of such mutants on the overall activity of this plasmid and in response to E2 or tRA stimulation. Surprisingly, cluster 3 was not responsive to E2 treatment (Figure 3.18a), which indicated that E2 responsive NIS mRNA expression in MCF-7 requires other elements that are absent from this sequence and that ERα functions on NIS promoter in cooperation with other yet unidentified element(s). Knowing that ERα suppression resulted in down-regulation of both basal and tRA-induced NIS

expression (Figure 3.10c), it was interesting to find out that these ERE mutations did not affect the overall tRA response in cluster 3 when compared to the wild type plasmid (Figure 3.18b). Instead, a remarkable increase in the activity of cluster 3 was obvious for two of the mutants, and a severe decrease was clear only for the third mutant (Mut3 contains a completely destroyed ERE). It is unclear at the moment why these mutants would increase the transcriptional activity of cluster 3, knowing that these mutants do not interact with ERα according to results obtained by EMSA. One simple explanation is that ERα may exert negative regulation on NIS expression through this ERE, an argument that has been proven to be wrong according to the results obtained using RNAi method (and the overexpression of ERα in MDA-MB-231), which correlates the expression of NIS to the presence of a functional ERα.

On the other hand, the close localization of this ERE next to the TATA element may provide clues to explain this phenomenon and to how ERα might be involved in the regulation of NIS expression. The increased cluster 3 transcription in the presence of ERE mutations could be explained by a complex interplay between ERα and the other elements required for the general transcription machinery, sites of which are present in this narrow sequence. Analysis of this sequence for transcription factor binding sites revealed the presence of such sites, mainly for Sp1, Sp2, core promoter binding protein (CPBP) and GATA-1 binding. It is reasonable to assume that ERα acts as a key regulator of NIS expression; once bound to NIS ERE it is responsible for initiating expression in an estrogen dependent manner. This ERE sequence was among the deleted 101 bp sequence in Cl.3 Δ3-4, such deletion was sufficient to significantly reduce the reporter gene activity in MCF-7 (Figure 3.5b) in accordance with previously published data regarding the promoter of NIS (Behr *et al.*, 1998; Venkataraman *et al.*, 1998), providing another clue to the role played by this ERE and ERα in the expression of NIS. On the other hand, in thyroid cells for example, NIS expression is mainly controlled by the NIS upstream enhancer (Ohno *et al.*, 1999; Taki *et al.*, 2002), and the absence of bound ERα in these cells will free the promoter elements for general transcription. It has also been shown that NIS expression in thyrocytes was down-regulated in response to E2 treatment (Furlanetto *et al.*, 1999), according to the above argument, this could be accomplished by binding of ERα to NIS ERE leading to an impaired NUE driven transcription of NIS.

In mammary gland physiology, transport of iodide via NIS is observed after mid-gestation and during lactation (Tazebay *et al.*, 2000). Therefore, expression of NIS should be considered as one of the latest events in mammary gland development because it takes place in fully differentiated mammary epithelial lactocytes. So far, transcriptional molecular elements and ligands that were hitherto shown to regulate NIS expression were identified in studies that were either carried out in experimental animals or in a rather differentiated mammary cell line, MCF-7 (Cho *et al.*, 2000; Kogai *et al.*, 2000; Rillema *et al.*, 2000; Tazebay *et al.*, 2000; Rillema *et al.*, 2002; Schmutzler *et al.*, 2002; Tanosaki *et al.*, 2003; Dentice *et al.*, 2004; Kogai *et al.*, 2004; Kogai *et al.*, 2005; Dohan *et al.*, 2006). Related with this, a particularity of this study is that we also used a less differentiated tumor cell line such as MDA-MB-231 (Merot *et al.*, 2004) in establishing the role of apo-ERα as a factor that activates NIS expression. Therefore, to our opinion, a less obvious but interesting implication of our data is the very early role of apo-ERα in activation of NIS transcription. Close localization of ERE and the TATA element in NIS promoter (Figure 3.14) might provide an additional hint towards this possible early role of apo-ERα in initiating transcription in this promoter context. Future work will be needed to accurately establish the position of apo-ERα in the sequence of molecular interactions leading to NIS regulation in mammary gland lactocytes.

4.3. Conclusion

In this study, we have demonstrated that the expression of NIS in mammary gland cells involves the cooperation of two nuclear receptors, ERα and RAR. The mechanism by which the expression is regulated proved to be more complicated than one might presume. It has previously been shown that tRA up-regulates the expression of NIS in thyroid cell lines through the direct binding of RAR to the upstream RARE in NIS promoter (Schmutzler *et al.*, 2002), and more recent work illustrated that RAR is involved in the regulation of NIS indirectly through the activation of the cardiac homeobox transcription factor Nkx-2.5 (Dentice *et al.*, 2004). Nkx-2.5 expression coincided with the expression of NIS in both MCF-7 (a breast cancer cell line model) and murine lactating mammary gland; differences between the two systems may indicate differences in the molecular determinants

regulating this Nkx-2.5 dependant NIS expression. On the other hand, common signals regulating the expression of NIS in MCF-7 and during lactation may include E2 and ERα. Unfortunately, we have had difficulties in repeating the experiments of Dentice *et al.* (2004), which discouraged us to further study a possible –functional or mechanistic– interplay between Nkx-2.5 and ERα (see Results, Figure 3.7). On the other hand, we provided evidence in favor of the involvement of direct and indirect actions of E2 and ERα in the regulation of NIS.

Direct regulation of NIS by unliganded ERα (Apo-ERα) was demonstrated in the ER- cell line model MDA-MB-231, in which, the introduction of a functional ERα was sufficient to provide an expression level of NIS higher than that of the ERα+ cell line counter part MCF-7 when cultured in the same conditions. Other clues of a direct involvement of E2 and ERα in NIS regulation came from experiments in MCF-7, where NIS was up-regulated in response to E2 stimulation. In experiments where the endogenous ERα was suppressed by RNAi, we have seen that both basal and tRA-stimulated NIS expression was down-regulated as well. We provided evidence in support of a direct regulation of NIS by ERα by the identification of a functional ERE in the immediate NIS promoter, which was also shown to be occupied by ERα. Indirect effects of ERα on NIS expression could be also possible (Figure 4.1).

It is well known that ERα activates the transcription of RARα, which in turn will be responsible for NIS transcription in response to tRA. Experiments in MCF-7 where E2 and tRA were applied together provided evidence in favor of this point, but preliminary experiments in MCF-7 also showed that tRA stimulation in cells primed with E2 did not have the same additive effect seen when these two ligands were administered together. This observation could provide clues in favor of a cooperative mode of action, in which ERα and RARα may bind as heterodimers, a speculative hypothesis that must be tested.

The identified conserved regions could also be used for the analysis of NIS expression in cell line models to be identified in the future, in which NIS is regulated by other ligands such as prolactin. Moreover, such conserved regions might also be

used to screen cDNA libraries from lactating mammary gland by yeast one hybrid to search for novel transcription factors binding to these sequences and regulating NIS expression in a tissue specific manner.

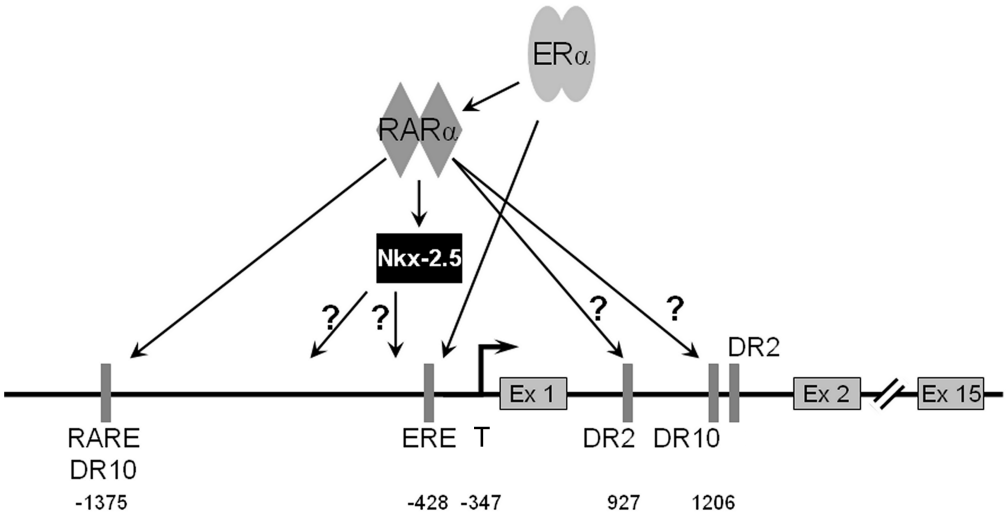

Figure 4.1. Proposed model of interactions of *cis*- and *trans*-acting factors involved in the stimulation of NIS expression in the mammary gland. Part of the NIS genomic fragment is represented by the horizontal line, where exons 1, 2 and 15 are represented by boxes with the label Ex 1, Ex 2 and Ex 15 respectively. The position of transcription start site is indicated by the right angle arrow. *cis*-acting elements are also shown with the location relative to the ATG codon is indicated by numbers beneath. Question marks indicated possibilities of binding of transcription factors to putative elements in human NIS gene region.

The DR2 and DR10 elements in cluster 4 are not yet fully characterized, and the involvement of these two elements, in NIS transcription might be assessed by studying mutants of these individual elements in reporter assays, and by studying the possible binding of RAR by techniques like EMSA and ChIP. In addition to the identified elements in this cluster, recently we found out that a third element (DR2) is also present in the sequence of the first intron of NIS just 4 bp away from the DR10 (the second half site of the DR10 overlaps with the first half site of this new DR2), which was not included in the sequence on cluster 4. Thus the sequence in cluster 4

should be extended to include this element and then one should study the effect of this new element on the transcription potential of cluster 4 and on the combined clusters 3 and 4 (Cl.3/4) in the near future.

Radioiodide ($^{131}I^-$ or $^{123}I^-$) and pertechnetate ($^{99m}TcO_4^-$) transport activity of NIS have successfully been used in detection, treatment, and follow-up of thyroid cancers (Dadachova and Carrasco, 2004). In addition, the up-regulatory effect of tRA on thyroid NIS expression was also previously established, and several clinical trials assessing RA redifferentiation therapy in dedifferentiated thyroid tumors and their metastases were also previously started (Grunwald *et al.*, 1998; Koerber *et al.*, 1999; Simon *et al.*, 2002). However, the potential of similar methods based on NIS activity in breast tumor cells still remains to be fully assessed. To our opinion, establishing molecular determinants, mechanisms, and ligands that have a role in NIS regulation is essential for successful implementation of possible NIS activity based novel methods for the management of malignant breast diseases.

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Unliganded estrogen receptor-α activates transcription of the mammary gland Na^+/I^- symporter gene

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Abstract

The function of sodium iodide symporter (Na^+/I^- symporter, or NIS) in mammary epithelial cells is essential for the accumulation of I^- in milk; the newborn's first source of I^- for thyroid hormone synthesis. Furthermore, increased mammary gland NIS expression has previously been shown in human breast cancer. Several hormones and factors including all-*trans*-retinoic acid (tRA) regulate the expression of NIS. In this study, using breast cancer cell lines, we established that tRA-responsive NIS expression is confined to estrogen receptor-α (ERα) positive cells and we investigated the role of ERα in the regulation of NIS expression. We showed that the suppression of endogenous ERα by RNA interference downregulates NIS expression in ERα positive mammary cells. Besides, in an ERα negative cell line, reintroduction of ERα resulted in the expression of NIS in a ligand-independent manner. We also identified a novel estrogen-responsive element in the promoter region of NIS that specifically binds ERα and mediates ERα-dependent activation of transcription. Our results indicate that unliganded ERα (apo-ERα) contributes to the regulation of NIS gene expression.
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In mammary gland lactocytes, sodium/iodide (Na^+/I^-) symport via NIS is required to secrete I^- in mother's milk [1]. I^- in milk is used by the newborn in thyroid hormone biosynthesis, and thus it plays an essential role in post-natal development of skeletal muscles, nervous system, and lungs [2]. In vivo experiments in mice have previously demonstrated that in normal physiology, NIS expression is strictly linked to mammary development in gestation, and to lactation [1]. Non-lactating mammary gland tissue in female mice does not express NIS unless animals receive subcutaneous oxytocin treatments for three consecutive days. On the other hand, a similar treatment in ovariectomized mice is not sufficient for NIS upregulation. In these surgically treated animals, administration of 17-β-estradiol (E2) together with oxytocin is essential for functional expression of NIS. The fact that E2 treatment was only essential in ovariectomized animals, whereas lactogenic hormones were sufficient for functional NIS expression in surgically untreated mice, suggested that ovary functions and endogenous estrogens are essential in upregulating NIS expression [1]. Unlike in non-lactating mammary gland tissue, in transgenic mice bearing experimental breast cancers triggered by *Erb-B2/neu* and *ras* oncogenes, functional expression of NIS significantly increases [1]. In the same study, human breast cancer specimens were also analyzed, and an increased NIS expression was detected in human invasive breast cancer and ductal carcinoma in situ, as compared to no expression of NIS in healthy breast samples obtained from reductive mammoplasty operations [1].

Recent studies with an ERα+ mammary cell line model, MCF-7, have led to the identification of additional hormones or ligands that control transcriptional regulation of NIS. In this cell line, the symporter gene was shown to be inducible in response to 9-*cis*-retinoic acid (9cRA) and all-*trans*-retinoic acid (tRA), ligands that were previously

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known to induce I^- transport activity in dedifferentiated thyroid tumor metastatic tissues in humans [3–5]. Kogai et al. [3] have shown that tRA have upregulated both NIS expression and iodide transport in MCF-7 cells in a dose-dependent manner. The absence of a similar increase in NIS mRNA levels in the ERα− MDA-MB-231 cell line after tRA treatment has led the authors to consider that ERα positivity of MCF-7 may lead to increased levels of retinoic acid receptor (RAR) in the presence of E2, which may provide cellular conditions favorable for NIS expression [3]. A correlation between the ERα status of mammary cell lines and 9cRA (a ligand for both RAR and retinoid x receptor (RXR) heterodimers and RXR/RXR homodimers) inducibility of NIS gene was also previously indicated [5]. In a separate study, Nkx2.5, a homeobox transcription factor, was indicated as the mediator of tRA-responsive NIS expression in MCF-7 cells [6].

In the present study, we investigated roles of E2 and ERα in transcriptional regulation of NIS in mammary cell lines. We first established a correlation between ERα status of mammary cell lines and tRA-responsive NIS expression. Then, we studied the roles of E2 and ERα in NIS regulation using two RA-responsive mammary cancer cell models such as the MCF-7 and MDA-MB-231 cell lines. We established that in a previously ERα− mammary cell line, MDA-MB-231, both transient and stable expression of ERα activates basal expression of NIS in an estrogen-independent manner (in the apo-ERα state; [7]). Furthermore, suppression of the endogenous ERα gene in MCF-7 cells by RNA interference method downregulated tRA induced NIS expression, indicating the role of ERα in regulation of the symporter gene. Subsequently, we have identified a novel ERE sequence located in close proximity (9 base pairs upstream) of the TATA element in NIS gene promoter. By chromatin immunoprecipitation (ChIP) experiments, we obtained strong evidences in support of a physical interaction between this novel *cis*-acting element and ERα in MCF-7 cells. Our results indicated a functional interaction between the unliganded ERα and RA-responsive pathways in NIS regulation in the mammary gland.

Materials and methods

Plasmids. The expression vector for ERα (pCMV-ERα) was prepared by inserting the *Eco*RI fragment (containing ERα coding sequence) form the plasmid pSG5ERpuro (kindly provided by Patrick Balaguer, Montpellier) into pcDNA3.1C (Invitrogen). The luciferase reporter vectors, pGL3E1bLuc and phRL-TK, were kindly provided by Roberto Di Lauro, Naples. The reporter pPS2XERE was prepared by ligating a synthetic double strand (ds) oligonucleotide containing two tandem copies of the pS2 ERE into *Mlu*I/*Xho*I sites of pGL3E1bLuc. The DNA sequence of this oligonucleotide was 5′-CGC GTA AGG TCA CGG TGG CCA CAC GCG TAA GGT CAC GGT GGC CAC CCC GTC-3′. Likewise, pNIS2XERE was created by inserting a synthetic ds oligonucleotide containing two tandem copies of the putative NIS ERE (5′-CGC GTA GGC GGA GTC GCG GTG ACC CGG CGG AGT CGC GGT GAC CCG GGA GC-3′) into the *Mlu*I/*Xho*I sites of pGL3E1bLuc. Oligonucleotides for the knockdown of ERα were designed and supplied by Oligoengine, WA (N-19 targets 458 and 499 on NM_000125 were sh-ER458: 5′-TTC AGA TAA TCG ACG CCA G-3′, and for sh-ER499: 5′-GTA CCA ATG ACA AGG GAA G-3′). These shRNA oligos were then cloned in the *Bgl*II/*Xho*I sites of pSuper-GFP/Neo (pSR, Oligoengine, WA) to generate the two knockdown constructs, pSR-ER-458 and pSR-ER-499.

Cell culture. Human mammary gland cell lines BT-474, T-47D, BT-20, MDA-MB-453, MDA-MB-468, *hTERT*-HME1, MCF-7, MDA-MB-231, and MDA-66 were used in this study. All-*trans*-retinoic acid (tRA) and 17-β-estradiol (E2) were purchased from Sigma. tRA was dissolved in DMSO, E2 was dissolved in ethanol as 10 mM stock solutions, and stored protected from light at −20 °C. All cell lines were maintained in high glucose Dulbecco's modified Eagle's medium [Gibco, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S) and 1% L-glutamine (Biochrom)], abbreviated in the text as reg-DMEM, at 37 °C in a 5% CO_2 incubator. MDA-66 was maintained in the above medium with the addition of 0.4 mg/ml Hygromycin (Roche). In general, hormone induction experiments were performed in sf-DMEM (a phenol red-free DMEM (Sigma) supplemented with 10% dextran-coated-charcoal stripped FBS, 1% P/S and 1% L-glutamine) unless otherwise mentioned. Two days before the addition of hormones, cells were fed with sf-DMEM in order to deplete the culture media from endogenous steroids and retinoids. tRA was applied to a final concentration of 1 μM for 12 h, while E2 was applied to a final concentration of 10 nM for 3 h. Cells were harvested by trypsinization and cell pellets were divided into two tubes to be used for both RNA and protein extract preparations.

Luciferase reporter assay. Cell lines were transfected with plasmid DNAs using FuGENE-6 reagent (Roche). FuGENE:DNA ratios were determined experimentally to be 3:1 for MCF-7 and 6:1 for both MDA-MB-231 and MDA-66. Cells were seeded in 24-well plates in reg-DMEM; so that they reach confluence at the time of the assay. Two days later, and 1 h prior to transfection, cells were washed twice with PBS, and the medium was replaced with sf-DMEM lacking antibiotics. Transfection was carried out with 200 ng of reporter vector plus 3 ng phRL-TK to normalize for transfection efficiency. Two days post transfection, medium was changed with fresh sf-DMEM containing 10 nM E2 (or EtOH as vehicle control) and continued incubation for 6 h. Then the cells were harvested and luciferase assay was performed using the Dual-Glo Luciferase Assay system (Promega). Luciferase values for all samples were normalized by first subtracting the background of no-transfection control and then dividing firefly luciferase values over those of *Renilla* luciferase. Fold induction is relative to the value of the empty vector pGL3E1bLuc.

Transient transfection with ERα. MDA-MB-231 cells were transfected with pCMV-ERα in 100 mm dishes, using FuGENE-6 (as described above) and 5 μg of the expression vector. Two days after transfection, media were replaced with fresh sf-DMEM containing 10 nM E2 or 1 μM tRA, or a combination of both hormones. After hormone induction, cells were rinsed with cold PBS, and harvested by trypsinization, cell pellets were divided into two tubes; RNA and protein extracts were prepared from the same sample.

RNA, cDNA, and semi-quantitative RT-PCR. The expression level of NIS, pS2, and GAPDH was monitored by semi-quantitative RT-PCR. RNA were prepared using the Nucleospin RNA II kit (Macherey–Nagel) as recommended by the manufacturer. In general 2 μg of total RNA were used for cDNA synthesis using the Revert-Aid First Strand cDNA Synthesis Kit (Fermentas). Primers for semi-quantitative RT-PCR amplified corresponding transcripts from positions spanning two or more exonic sequences. PCR primers were RT-NIS-F: 5′-CTC ATC CTG AAC CAA GTG AC-3′, RT-NIS-R2: 5′-TAC ATG GAG AGC CAC ACC A-3′, RT-pS2-F: 5′-CCA TGG AGA ACA AGG TGA TCT GC-3′, RT-pS2-R2: 5′-GTC AAT CTG TGT TGT GAG CCG AG-3′, GAPDH-F: 5′-GGC TGA GAA CGG GAA GCT TGT CAT-3′, GAPDH-R: 5′-CAG CCT TCT CCA TGG TGG TGA AGA-3′. PCR amplification was performed in 25 μl reaction volumes containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTP, 10 pmol of each primer, and 1 U *Taq* DNA polymerase (Fermentas). Thermal cycler conditions were an initial denaturation step at 95 °C for 3 min; a loop cycle of 95 °C, 30 s/61 °C, 30 s/72 °C, 30 s; and a final extension at 72 °C for 10 min. The cycle number varied for each transcript amplified, for NIS it was 40 cycles, pS2 in MCF-7 was 15 cycles,

and in MDA-MB-231/MDA-66 was 40 cycles. Cycle number for GAPDH was 19. PCR products were resolved on 2% agarose gels stained with ethidium bromide and visualized using the Gel Doc-2000 supported with the Multi-Analyst Ver.1.1 image analysis software (Bio-Rad).

Western blot analysis. The expression of ERα, RARα, and calnexin was examined by Western blot analysis. Cell pellets were incubated in lysis buffer for 30 min (50 mM Tris–HCl, pH 8.0, 250 mM NaCl, 0.1% Nonidet P-40, and 1× protease inhibitor cocktail (Roche)), cell extracts were cleared by centrifugation, and protein content was quantified using Bradford assay. 20 μg of whole cell extracts was denatured in gel loading buffer (50 mM Tris–HCl, pH 6.8, 1% SDS, 0.02% bromophenol blue, 5% 2-mercaptoethanol, and 10% glycerol) at 95 °C for 5 min, resolved by SDS–PAGE using a 10% gel, and electro-transferred onto PVDF membranes (Millipore). The membranes were blocked in Blotto (Tris-buffered saline containing 0.5% Tween 20 and 5% nonfat milk powder) for 1 h at room temperature. The membranes were incubated with mouse monoclonal anti-hERα F-10 (1:500, Santa Cruz) for 16 h at 4 °C, washed three times with Blotto, and incubated with peroxidase-conjugated goat anti-mouse (1:2000, Sigma) for 1 h, immunocomplexes were then detected using ECL-plus (Amersham), and exposed to X-ray films (AGFA) for 1 min. The films were then developed using a hyper-processor developer (Amersham). Membranes were then washed three times with Blotto, re-incubated with rabbit monoclonal anti-hRARα C-20 (1:1000, Santa Cruz) for 16 h, and then stained with goat anti-rabbit (1:2000, Sigma). The same protocol was repeated for the internal control calnexin using a rabbit anti-calnexin (1:5000, Sigma).

Suppression of ERα by shRNA. MCF-7 cells were transfected as described above, using pSR-ER458, pSR-ER499, and the empty vector control pSR. After transfection, cells were washed, diluted, and transferred to 24-well plates for selection with DMEM containing 0.5 mg/ml Geneticin (Sigma). Three weeks later, stably transfected colonies (expressing the EGFP marker) were transferred to new culture dishes and were allowed to grow for further analysis. The presence of the knockdown construct was confirmed by PCR using genomic DNA isolated from each clone as a template, and the pSR insert screening primers from Oligoengine (F: 5′-GGA AGC CTT GGC TTT TG-3′ and R: 5′-CGA ACG TGA CGT CAT C-3′). The level of ERα suppression was analyzed by Western blot using ERα antibodies as described above. Clones with lowest ERα expression as compared to the empty vector transfected clones were selected for tRA induction experiments.

Chromatin immunoprecipitation analysis. Chromatin immunoprecipitation (ChIP) was performed essentially as described by the supplier of the reagents (Santa Cruz Biotechnology; protocol No. 12 on http://www.scbt.com) with the following modifications. MCF-7 cells (150 mm dish) cultured in sf-DMEM were treated with 10 nM E2. Formaldehyde cross-linking (1% (v/v)) was done for 10 min at room temperature. Cross-linking was terminated by the addition of glycine to a final concentration of 125 mM. Cells were scraped and the pellets were resuspended in 6 ml lysis buffer (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, and 1× protease inhibitor cocktail) for 10 min on ice. The cell lysate was washed once with ice-cold PBS, resuspended in 1.9 ml high salt lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1× protease inhibitor cocktail), and sonicated in the ultrasonic processor UP50H (Hielscher Ultra-sonics, Germany) three times, 7 s each at 60% amplitude and a continuous cycle. At this point 100 μl of chromatin solution was removed and labeled "Input." Chromatin solution was precleared by adding 100 μl of protein A–Sepharose 6 MB (Sigma) as 50% slurry containing 0.5 mg/ml BSA, 200 μg/ml sonicated salmon sperm DNA in TE, pH 8.0, for 30 min at 4 °C. Immunoprecipitation was performed at 4 °C for 16 h using anti ERα antibodies and anti FGFR-1 C-15 (Santa Cruz) antibodies (as negative control) as recommended by the supplier, then immunocomplexes were incubated with 100 μl protein A–Sepharose (50% slurry) for 2 h at 4 °C. Afterwards, beads were collected, washed and eluted as recommended in the Santa Cruz protocol. All samples, including the input, were reverse cross-linked by incubating at 65 °C with proteinase K for 16 h, DNA was isolated by phenol/chloroform extraction and ethanol precipitation. Isolated DNA was used for PCR amplification of ERα-precipitated fragments for NIS-ERE (NIS-ChIP-F: 5′-TGG CCT GTC TGT CCC AGT CCA GGG CTG A-3′ and NIS-ChIP-R: 5′-GGG TTG CAG ATT TAT TGG GC-3′). NF1-F: 5′-TGC TAC TCT TTA GCT TCC TAC-3′ and NF1-R: 5′-CCT TAA AAG AAG ACA ATC AGC C-3′ were used as ERE-unrelated control.

Results and discussion

tRA-responsive NIS expression is correlated with the presence of a functional ERα

By immunoblot experiments, we have screened eight different human mammary gland cell lines that we have in our collection for the presence of ERα (Fig. 1A). Cells were cultured in regular DMEM containing 10% FBS (abbreviated as reg-DMEM in this text). In parallel experiments, we either treated them with 1 μM tRA or with vehicle (DMSO) for 12 h before analyzing NIS expression by RT-PCR (Fig. 1C). We have also monitored the expression of pS2, an estrogen-responsive gene widely used as a marker to monitor the functionality of ERα and/or E2 treatments in ERα+ cell lines [8,9]. Expression of pS2 in cells not treated with E2 but cultured in reg-DMEM (Fig. 1B) was probably due to the well-known estrogenic activity of phenol red, a pH indicator dye [10,11]. Close correlation between ERα and pS2 expressions suggested that, as expected, pS2 gene expression was a reliable indicator of ERα activity (Fig. 1A and B). As a result of these analyses, we confirmed that BT-474, T-47D, and MCF-7 were both physically and functionally ERα+ (Fig. 1A). Remaining cell lines such as BT-20, MDA-MB-453, MDA-MB-468, *hTERT*-HME1, and MDA-MB-231 were classified as ERα−. Basal expression (uninduced by tRA) of NIS was detected in all three cell lines with strong ERα positivity (BT-474, T-47D, and MCF-7) and in one ERα− cell line (BT-20). On the other hand, tRA-induced NIS expression was strictly specific to cell lines that were expressing both ERα and RARα, and not to cell lines that were only RARα+ (Fig. 1C). A similar result suggesting a correlation between ERα status of mammary cell lines and 9cRA (a RAR/RXR-specific ligand) induction of NIS gene has also previously been shown [5]. However, in our studies, we have also observed a basal expression of NIS in ERα+ mammary cell lines, as opposed to no detectable expression in ERα− cells (Fig. 1). This suggested that besides playing its indirect role in modulating RAR-dependent regulation [12], ERα also plays a role in regulation or initiation of basal NIS expression (see below). The BT-20 cell line could be considered as an exception, as ERα negativity and lack of basal NIS expression did not correlate (Fig. 1). In previous studies, where human breast tumor samples were analyzed, NIS expression was also seen in ERα− tumors [1,13]. Thus, BT-20 cells might provide a model for ERα− independent NIS gene activation in human breast cancer.

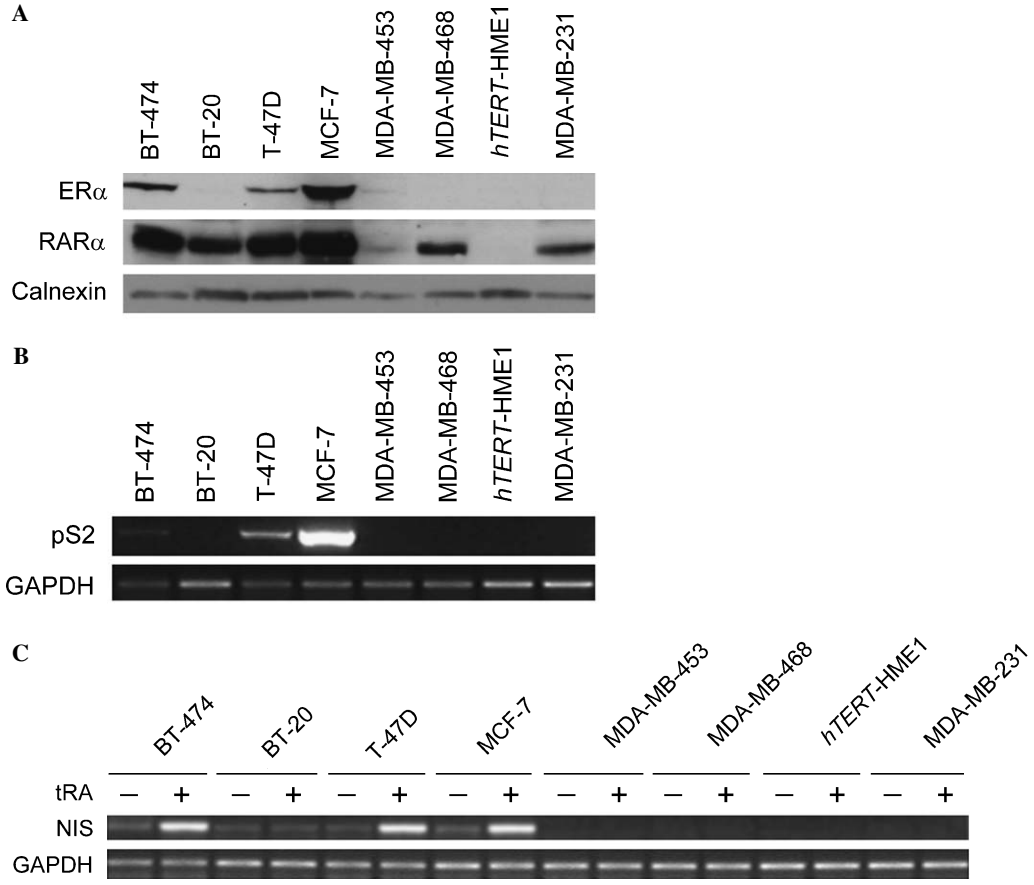

Fig. 1. ERα positivity in mammary gland cell lines is correlated with tRA-responsive NIS expression. (A) Immunoblot analysis of ERα and RARα expression in a variety of mammary gland cell lines, as indicated on top of each lane. Cells were grown in reg-DMEM, total proteins were extracted, and electrophoresed samples were blotted using anti-human-ERα antibody and anti-human-RARα antibody, respectively. Calnexin expression was also monitored with a similar method, and it was used as a gel loading control. (B) RT-PCR analysis of pS2 expression in cell lines grown in reg-DMEM in the absence of E2 or tRA. cDNA was prepared using total RNA isolated from cell lines. Then pS2 and GAPDH specific primers are used in PCR experiments, and accumulation of corresponding gene products was visualized. pS2 is a gene under control of ERα, and its expression was considered as an indicator of ERα activity. (C) Cell lines grown either in the presence (+) or absence (−) of 1μM tRA were collected, and tRA-responsive NIS gene expression was monitored by RT-PCR as described in (B). Amplification of GAPDH gene cDNA was used as an internal control both in (B) and in (C).

Suppression of ERα by shRNA downregulates NIS expression

To determine the functional relevance of ERα in NIS gene regulation, we suppressed endogenous ERα in MCF-7 by RNA interference (RNAi) method. For this, we used two alternative small hairpin RNA (shRNA) probes targeting different regions of ERα mRNA (sh-ER499 and sh-ER458; see Materials and methods). Cells were stably transfected either with empty vectors (pSuper-*GFP*/*Neo*, OligoEngine, WA) carrying the GFP and the neomycin resistance (Neo^R) marker genes, or with similar vectors carrying shRNA N-19 target sequences in addition to these two markers. Then, colonies originated from transfected cells that were both resistant to neomycin and that were green fluorescing were isolated and cultured separately. By Western blots, we monitored the level of ERα suppression in a number of different cell colonies expressing sh-ER458 as compared to colonies transfected with empty pSR vector (Fig. 2A). We then selected colonies with significant suppression for further studies (such as colonies 458-12 and 458-13, Fig. 2A). We also noticed that one of the two shRNAs was more potent (sh-ER458) in suppressing the endogenous ERα gene as compared to the other one (sh-ER499, results not shown). Subsequently, we treated these ERα suppressed MCF-7 colonies either with tRA or with vehicle (DMSO), and analyzed both tRA induced and basal NIS expression (Fig. 2B). In these studies, for both 458-12 and 458-13, we observed about 60% decrease in basal and about 45% decrease in tRA induced expression of NIS as compared to empty vector transfected controls (Fig. 2B); results indicating that ERα plays a role in both basal and tRA induced NIS gene expression. The partial decrease at NIS mRNA levels in response to severe downregulation of ERα may indicate redundant functions between ERα and other factors such as RARs and Nkx2.5 regulating this gene [3,6]. Interestingly, previous studies have shown that in a rat thyroid cell line model, FRTL-5, activation of ER pathway by E2 downregulates thyroid NIS gene expression [3,14]. This suggests that com-

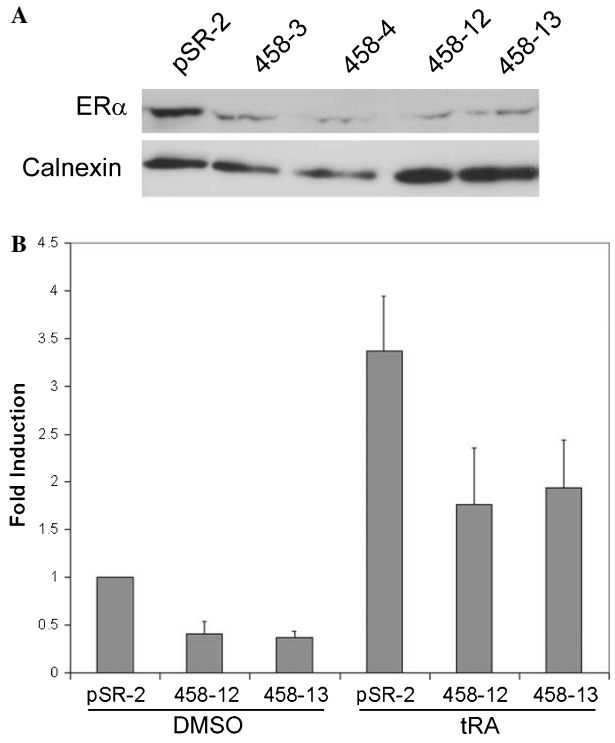

Fig. 2. Suppression of ERα by shRNA downregulates NIS expression in MCF-7 cells. MCF-7 cells were transfected either with empty vector pSR, with pSR-ER-458, or with pSR-ER499. Following a double selection procedure based on Geneticin (0.5 mg/ml) resistance and EGFP expression, clones were isolated and further analyzed. (A) A representative Western blot result showing the effect of sh-ER458 on the levels of ERα in four individual clones compared to an empty vector clone pSR-2. Clones 458-12 and 458-13 showed significant ERα suppression and were selected for tRA induction. (B) Clones 458-12 and 458-13 were grown in reg-DMEM and treated with 1μM tRA or with DMSO (5 μl in 10 ml culture medium) for 12 h. After RNA isolation, cDNA was prepared using 2 μg total RNA and subsequently used as a template for semi-quantitative RT-PCR analysis using NIS specific primers. Data represent the fold induction (average of four independent experiments) of NIS in 458-12 and 458-13 clones normalized to GAPDH control, and relative to the empty vector pSR.

mon molecular elements may exert opposite regulatory effects on NIS gene expression in thyroid and in the mammary gland. As pregnancy is a physiological state which is associated with increased needs for thyroid hormone (TH) synthesis [15], a potential reduction of the iodide available for TH synthesis of the mother could explain increased hypothyroidism cases in pregnancy and during lactation [16]. On the other hand, above-described regulatory actions of ERα on NIS gene expression might also provide an additional possible explanation to increased hypothyroidism cases in pregnancy and lactation when I^- uptake substantially increases in mammary glands [1].

Ectopic ERα expression in MDA-MB-231 upregulates NIS expression

MDA-MB-231 cell line expresses RARα, a major component of tRA signaling mechanism as detected by immunoblots using anti-human RARα antibodies (Fig. 1A). Furthermore, these cells respond to tRA, as assessed by the RAR controlled RIP140 gene expression ([17]; and results not shown). However, although RA signaling pathway is intact, MDA-MB-231 cells do not express NIS in response to either tRA or any other ligand known to induce NIS in other cell systems ([3]; and data not shown). Because tRA-responsive NIS expression was only detected in both ERα+ and RARα+ cell lines (Fig. 1C), we investigated whether introduction of human ERα gene could restore tRA-responsiveness of NIS expression in MDA-MB-231. For this purpose, we first transiently introduced an ERα expression vector (pCMV-ERα) to this ERα– mammary cell line and studied ligand-responsive NIS expression. Note that, in order to precisely control the concentration of supplemented steroids and other ligands, cells were cultured in phenol-red free DMEM supplemented with dextran-coated-charcoal treated steroid-free FBS (abbreviated as sf-DMEM). Transfected cells expressed ERα at levels comparable to those in MCF-7 cells, and the receptor was functional as assessed by the increase in pS2 expression levels in response to E2 (Fig. 3A). Interestingly, introduction of ERα significantly increased NIS gene expression, although it did not restore tRA-responsiveness of this gene. We noticed that the ERα-activated NIS expression in MDA-MB-231 was even higher than the basal levels in the ERα+ cell model, MCF-7. On the other hand, when compared to tRA induced levels of NIS expression in MCF-7 cells, this ERα-activated expression of NIS was about three times lower (Fig. 3A). A dose–response curve established using MCF-7 (and MDA-66, see below) cells has indicated that, as also reported by others, a concentration of 10 nM E2 was the optimal ligand concentration to be used for the highest level of pS2 gene induction in mammary cells cultured in vitro (results not shown, see [18,19]). Therefore, whenever we studied the effects of E2 we have added this ligand to cell culture medium at a 10 nM concentration. Treatment of ERα transfected cells with E2 (10 nM), tRA (1 μM), or E2 together with tRA did not lead to a further increase in this ERα activated NIS expression (Fig. 3A). In order to further confirm these results, we also used a genetically modified MDA-MB-231 cell line that was stably transfected with a vector expressing human ERα gene [19]. First, we confirmed ERα and RARα status of this cell line named as MDA-66 (Fig. 3B). Then, we analyzed functionality of these receptors by assessing modulations in expressions of tRA- and E2-responsive genes such as RARα, pS2, and RIP140 (Fig. 3B and C, and results not shown, respectively). As expected, an E2-responsive upregulation of RARα [12] was clearly observed in MDA-66. This result was an additional evidence indicating that in these cells E2 signaling mechanism was intact (Fig. 3B). Subsequently, we studied both the basal expression and E2/tRA responses of NIS gene in these cells (Fig. 3C). In accordance with the results obtained in MDA-MB-231 cells that were transiently transfected with ERα, in

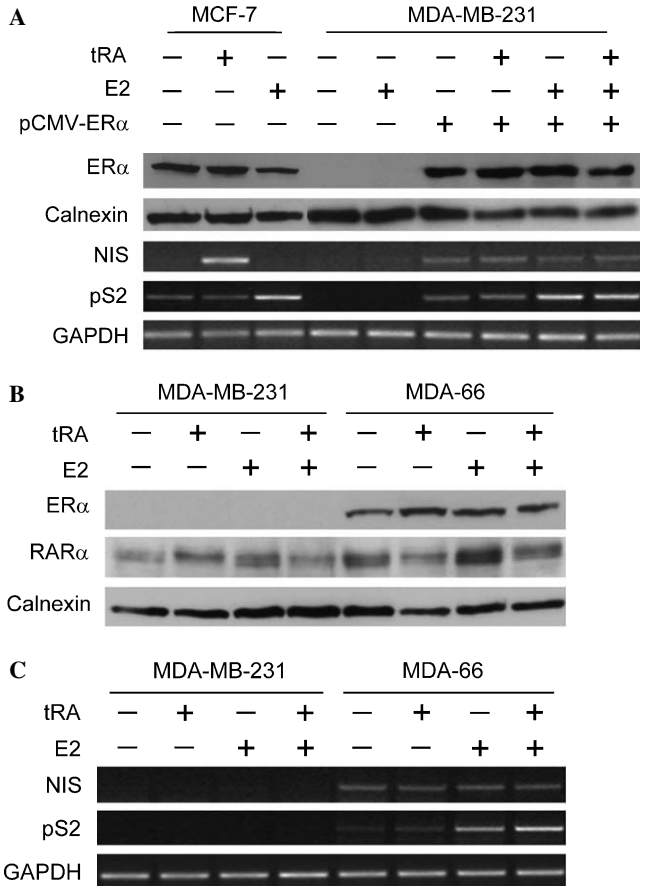

Fig. 3. Transient and stable expression of ERα in MDA-MB-231 cells leads to a higher basal expression of NIS in a ligand-independent manner. (A) MDA-MB-231 cells were transiently transfected with a plasmid vector expressing ERα under control of the CMV promoter [lanes pCMV-ERα(+)] in sf-DMEM. Forty-eight hours later cells were treated with 10 nM E2 (3 h), 1 μM tRA (12 h), or 10 nM E2 together with 1 μM tRA (12 h). Then, cells were harvested, divided to two, and one-half was used for extracting total proteins, and the other half was used for RNA extractions. ERα expression status in transfected cells was compared with those in MCF-7 cells by immunoblots using anti-human-ERα specific antibodies. Calnexin expression was used as a loading control. NIS and pS2 expressions in transfected cells and in MCF-7 cells in response to ligand treatments were assessed by RT-PCR. The ERα-responsive pS2 gene was used to monitor functionality of E2 and ERα. GAPDH expression was used to monitor the efficiency of the RT-PCR method, as an internal control. (B) MDA-MB-231 cells stably transfected with hERα expressing vector (named MDA-66), as well as untransfected cells were cultured in sf-DMEM in presence of 10 nM E2, 1 μM tRA, or both ligands. Then, they were harvested, and cell pellets were collected for immunoblot analysis and RT-PCR analysis (B). Total proteins were extracted from pellets obtained from ligand treated (as indicated with "+" signs on each lane) and untreated cells, then electrophoresed and blotted to immunoblot membrane. Subsequently, the membrane was treated with anti-human-ERα, anti-human-RARα, and anti-human-calnexin antibodies, respectively. Calnexin expression was used as loading control. (C) Total RNA was extracted from pellets collected as above, and total cDNA was prepared and submitted to the RT-PCR analysis using NIS, pS2, and GAPDH gene specific primers. GAPDH expression was used as an internal control.

MDA-66 cells the expression of NIS was remarkably increased, and it was not responsive to treatments with E2, tRA, or E2 together with tRA (Fig. 3C).

Taken together, these results indicated that ERα activity together with the intrinsic tRA-responsiveness was not sufficient for tRA responsive NIS expression in this ERα− mammary cell model (Fig. 3). Concerning the differences between cell lines, we must point out that, when compared to MCF-7, MDA-MB-231 (or MDA-66) cells are less differentiated, and it is known that the differentiation stage of breast cancer cell lines strongly affects the transcriptional activity of nuclear receptors [18]. In parallel to this, our results suggest that in addition to functionally expressed RARα and ERα, (an)other so far unidentified factor(s) are (is) essential for ligand-responsive expression of this gene in MDA-MB-231. On the other hand, in ovariectomized animals, even when administered alone, E2 was shown to significantly upregulate NIS expression [1]. This indicated that, additional factors that are present in hormonal and cellular microenvironment of mammary gland cells were needed for E2 responsive expression of NIS in isolated cells cultured in vitro.

Our results demonstrate a role of unliganded ERα (or apo-ERα) in regulating expression of NIS gene (Fig. 3). In general, unliganded nuclear receptors are considered to be transcriptionally unproductive or even repressive. Upon ligand binding, receptor-associated co-repressors are exchanged for co-activators, resulting in the activation of transcription. However, a growing body of evidence indicates that this model is too simple and not adequate to explain the dynamic pattern of transcriptional regulation [20]. It has previously been shown that liganded ERα is able to interact with a selective repressor protein illustrating an unpredictable mode of action for liganded receptors [21]. On the other hand, in a recent report, the activator function of apo-ERα was also demonstrated [7]. In this report, it was shown that apo-ERα recruits several histone acetyl transferases and a histone methyl transferase, destabilizing nucleosomes positioned around apo-ERα binding site in pS2 gene promoter region. An important suggestion of data gathered by Métivier et al. [7] was that apo-ERα, by binding to its cognate sequences, induces a chromatin environment that is permissive for transcription to occur. It is conceivable that similar mechanisms operate on NIS gene promoter, and apo-ERα is essential for holding the NIS gene at a transcriptionally competent state. This would suggest the absence of transcriptionally competent NIS gene loci in ERα− mammary cell lines, and thus explain the lack of both uninduced and tRA-responsive NIS expression in these cells (Fig. 1C).

Identification of a novel, non-canonical ERE in NIS promoter region

To evaluate the possibility of a direct regulation of NIS gene by ERα, we have first carried out an in silico analysis using the Dragon ERE finder program [22]. We searched for possible EREs in a 3 kb region upstream of the transcription start site in human NIS. As a result of this analysis we identified only one putative ERE sequence

(Fig. 4A), albeit it was a novel sequence which was not previously described as an ERE [22]. This putative ERE (5′-CG-GGTCA-CCG-CGACT-CC-3′) was located 9 bp upstream of NIS TATA element (Fig. 4A). This new element had the characteristic head-to-head inverted repeat sequences with high homology to the ERE consensus, and it was similar to previously established EREs (Fig. 4B). Significantly, this putative NIS ERE sequence and its position vis-à-vis TATA element was also conserved in rat and mouse genomes (Fig. 4C). In order to establish the transcriptional activation potential of this novel element in response to E2, we have constructed a reporter vector containing two tandem copies of putative NIS ERE sequence; followed by the E1b TATA element and the luciferase reporter gene, pNIS2XERE (see Materials and methods). We also constructed a similar vector containing two copies of ERE sequence which was previously shown to bind ERα and lead to E2-responsive upregulation of the pS2 gene, and named this vector as pPS2XERE. We then transiently transfected MCF-7, MDA-MB-231, and MDA-66 mammary cell lines with these reporter vectors and studied E2-dependent luciferase activity. In these experiments, both pS2 ERE and putative NIS ERE showed significantly activated luciferase expression in response to E2 in both MCF-7 and in MDA-66 (Fig. 5A). About 5-fold stimulation by NIS ERE was obtained in MCF-7 cells in response to E2 treatment, whereas, under same conditions, pS2 ERE stimulated reporter gene expression about 3-fold. A similar result is obtained in MDA-66 cells, where NIS ERE-stimulated expression of the reporter was 2.4-fold, whereas pS2 ERE-dependent stimulation was 3.4-fold. We also noticed that, in MCF-7 cells, in terms of potency, NIS ERE-driven reporter gene expression was five times stronger than that of pS2. These results indicated that NIS ERE has the potential to mediate E2-dependent transcription. In fact, such a close localization of TATA and ERE elements is very unusual considering that all previously characterized ERE elements were shown to be localized at relatively distant positions to transcription start sites in corresponding genes (although varying remarkably between +23,088 and −2687 [23]). However, it has also been known for long time that the response element preferences and DNA binding properties of nuclear receptors cannot be simply attributed to classical spacing,

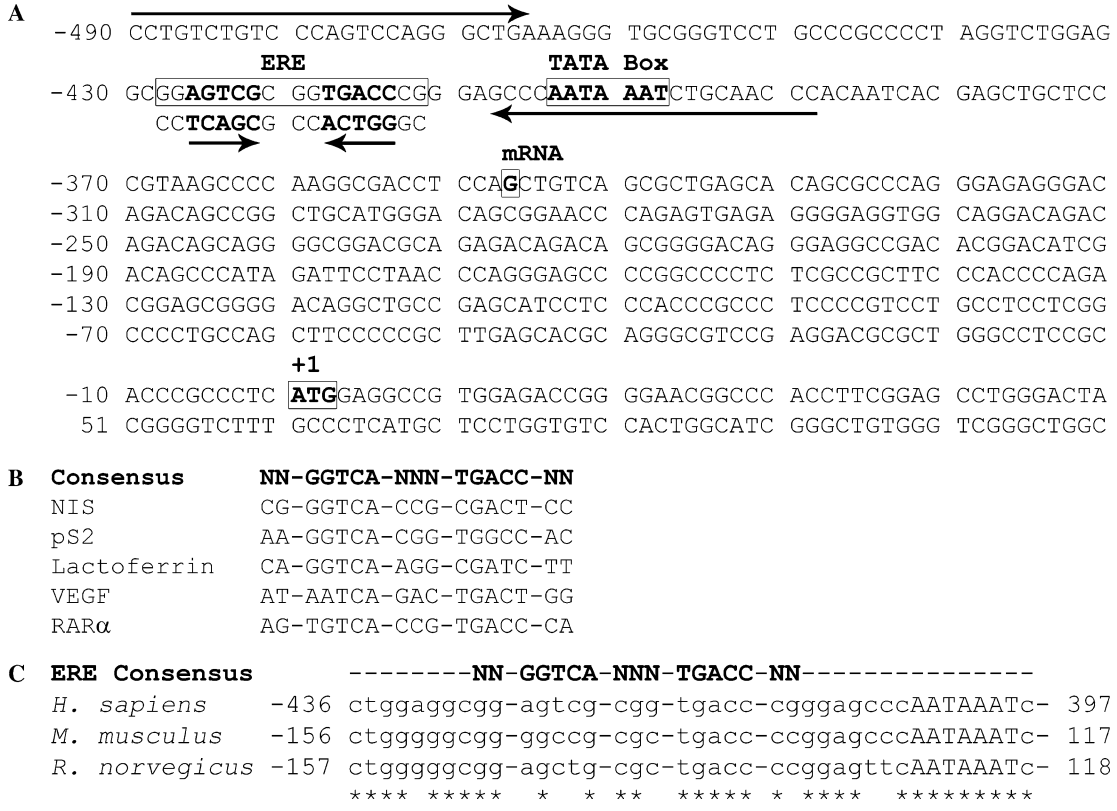

Fig. 4. A putative novel ERE sequence is located in close proximity of NIS TATA box element in the promoter region. (A) Human NIS gene proximal promoter region sequence is shown. The first codon (ATG) of NIS, transcription start site, TATA box element, and novel ERE sequences are indicated in boxes. Two inverted repeats of the ERE element located at the minus strand are shown by short inverted arrows. The position of primers used for the PCR amplification described in Fig. 5B is also indicated by long arrows. (B) Comparison of previously established ERE sequences that were found in several ER regulated genes, the putative NIS ERE sequence, and the consensus sequence. (C) Comparison of NIS putative ERE sequence in human, mouse, and rat genomes. Putative NIS EREs that were identified in all three genomes located in close proximity of TATA box elements, and they fit to functional ERE consensus sequence. NIS gene TATA element region is indicated by uppercase letters. (*) Signs indicate identical bases in human, mouse and rat sequences.

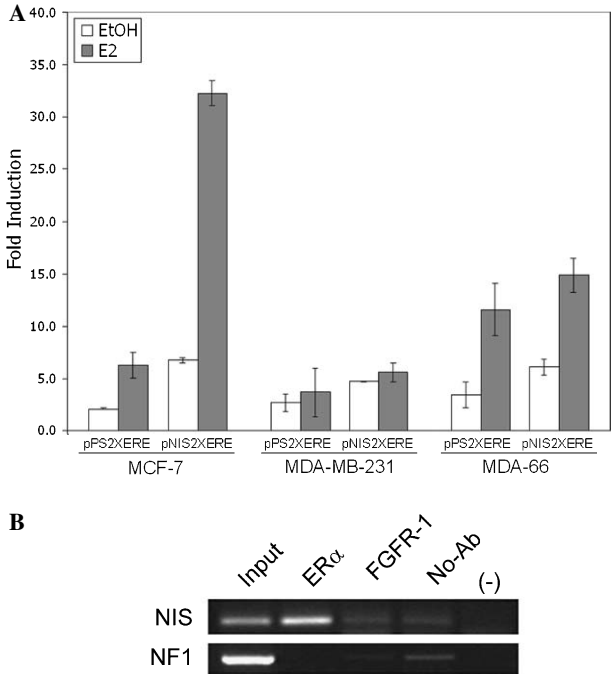

Fig. 5. Direct functional interaction between ERα and the NIS ERE sequence. (A) MCF-7, MDA-MB-231, and MDA-66 cells were transiently transfected with reporter vectors (pGL3 based) containing the luciferase gene under control of E1b TATA element and two tandem repeats of either pS2 gene ERE sequence (pPS2XERE) or NIS gene ERE sequence (pNIS2XERE). Transfected cells were treated either with 10 nM E2 (3 h) or with vehicle (ethanol; 10 μl in 10 ml culture medium). Then, luciferase activities were measured, and they were corrected using *Renilla* (phRL-TK) transfection efficiency control. Fold induction was calculated by normalizing luciferase values with those obtained from the empty vector. Data represent the average of four independent experiments. (B) MCF-7 cells grown in sf-DMEM and treated with 10 nM E2 were used for ChIP analysis using ERα specific antibody. DNA isolated from immunocomplexes was used as a template for PCR amplification using NIS promoter specific primers (indicated as long arrows in Fig. 4A), or unrelated intronic primers corresponding to *NF1* gene exon 22. Lanes: input, the input DNA used for ChIP analysis; ERα, estrogen receptor-α precipitated DNA; FGFR-1, fibroblast growth factor receptor-1 precipitated DNA; No-Ab, DNA precipitated with protein A–Sepharose beads only (background control); and (−), negative PCR without template DNA.

localization or orientation rules [24]. The significant difference in magnitude of gene activation by NIS ERE in MCF-7 and MDA-66 cell lines may reflect differences in these two cell lines in terms of molecular components associated with ER activity. As expected, in our ERα− cell model, MDA-MB-231, neither NIS ERE, nor pS2 ERE has led to E2-dependent regulation of the luciferase reporter gene (Fig. 5A). We concluded that this non-canonical ERE sequence located in NIS promoter can potentially act as a *cis*-acting element and respond to E2 in a proper cellular context.

To establish whether endogenous ERα can occupy the novel ERE in NIS promoter in vivo, we carried out ChIP experiments in MCF-7 cells. In the presence of ERα antibodies, the NIS promoter was precipitated from formaldehyde cross-linked total cell lysates (Fig. 5B, lane 2). In contrast, neither control Fibroblast Growth Factor receptor (FGFR-1) antibodies, nor antibody uncoated protein-A–Sepharose beads precipitated the NIS promoter above background levels (Fig 5B, lanes 3 and 4, respectively). As expected, ERα antibody was unable to precipitate an unrelated DNA fragment corresponding to NF1 gene exon 22 (Fig. 5B, lane 2). These data therefore demonstrate that in MCF-7 cells endogenous ERα binds to the NIS gene promoter in vivo, thereby suggesting that at least part of the regulatory effects of ERα on NIS expression were due to a direct interaction between the receptor and NIS promoter (Fig. 5B). Taken together with E2-responsive transcriptional activation of luciferase reporter via this ERE sequence in transfected MCF-7 cells, our ChIP results provide very strong evidence in support of the functionality of this site in vivo. These results indicate that apo-ERα and tRA-activated factors functionally interact in NIS regulation in breast cancer cell models such as MCF-7 and MDA-66.

In mammary gland physiology, transport of I^- via NIS is observed after mid-gestation and during lactation [1]. Therefore, expression of NIS should be considered as one of the latest events in mammary gland development because it takes place in fully differentiated mammary epithelial lactocytes. So far, transcriptional molecular elements and ligands that were hitherto shown to regulate NIS expression were identified in studies that were either carried out in experimental animals or in a rather differentiated mammary cell line, MCF-7 [1,3,5,6,25–31]. Related with this, a particularity of our study is that we used a dedifferentiated tumor cell line such as MDA-MB-231 [18] in establishing the role of apo-ERα as a factor that activates NIS expression. Therefore, to our opinion, a less obvious but interesting implication of our data is the very early role of apo-ERα in activation of NIS transcription. Close localization of ERE and the TATA element in NIS promoter (Fig. 4) might provide an additional hint towards this possible early role of apo-ERα in initiating transcription in this promoter context. Future work will be needed for accurately establishing the position of apo-ERα in the sequence of molecular interactions leading to NIS regulation in mammary gland lactocytes.

Radioiodide ($^{131}I^-$ or $^{123}I^-$) and pertechnetate ($^{99m}TcO_4^-$) transport activity of NIS has successfully been used in detection, treatment, and follow-up of thyroid cancers [32]. In addition, the upregulatory effect of tRA on thyroid NIS expression was also previously established, and several clinical trials assessing RA redifferentiation therapy in dedifferentiated thyroid tumors and their metastasis were also previously started [33–35]. However, the potential of similar methods based on NIS activity in breast tumor cells still remains to be fully assessed. To our opinion, establishing molecular determinants, mechanisms, and ligands that have a role in NIS regulation is essential for successful implementation of possible NIS activity based novel methods for the management of malignant breast diseases.

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