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TR Phosphorylation & Nuclear Import

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TR PHOSPHORYLATION & NUCLEAR IMPORT

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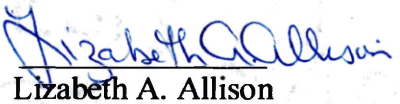

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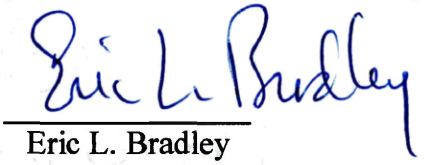

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Abstract

The thyroid hormone receptor α (TRα) is a transcription factor, which can activate or repress gene expression in response to thyroid hormone. In addition, some of its actions, including DNA binding and transcriptional activation, are thought to be regulated by phosphorylation. Results presented here, using *Xenopus* oocyte microinjection assays, demonstrate that a phosphorylated form of TRα is present in the oocyte nucleus, whereas unphosphorylated TRα remains cytoplasmic. Changes in the phosphorylation state of TRα occur rapidly in *Xenopus* oocytes and point to the possibility that phosphorylation occurs in the nucleus. Furthermore, increasing the overall phosphorylation state of the oocyte leads to enhanced nuclear retention of TRα, suggesting that phosphorylation may have an important role in regulating nuclear localization of TRα. However, serine 12, a well-characterized casein kinase II phosphorylation site, is not necessary for this enhanced nuclear retention, nor is this site necessary for nuclear import. Taken together, these data provide evidence that phosphorylation of one or more sites other than serine 12, while not directly involved in nuclear import, play an important role in regulating nuclear retention of TRα. These findings provide a foundation for future studies on the role of phosphorylation in the regulation of TRα.

TR PHOSPHORYLATION & NUCLEAR IMPORT

Introduction

The thyroid hormone receptor (TR), like other members of the nuclear receptor superfamily, is a transcription factor that activates or inhibits the expression of target genes in response to ligand. These transcription factors play a vital role in the normal development and maintenance of homeostasis in many organisms. Mutant alleles of members of this family have also been linked to a variety of diseases including various forms of cancer and resistance to thyroid hormone. In recent years, phosphorylation of transcription factors such as TR has been shown to be an important regulatory factor in many systems. Along with tremendous advances in our understanding of TR phosphorylation, there has also been a great deal of confusion in the literature, as well as a host of new questions to be answered. This thesis will address the importance of phosphorylation in the nuclear localization of TR. The following introduction will review what is currently known about the thyroid hormone and how TR mediates its functions. What is known about the structure of TR, its subcellular trafficking, and how it acts on target genes will be addressed.

Thyroid hormone production and regulation

Two of the most important hormones in the body are triiodothyronine (T_3) and tetraiodothyronine (T_4), collectively known as the thyroid hormones. T_3 is the physiologically active form; T_4 is converted to T_3 at the target tissue. A tight endocrine pathway regulates thyroid hormone release (Fig. 1). Regulation begins in the brain,

specifically the hypothalamus. The hypothalamus releases thyrotropin releasing hormone (TRH) which travels through the blood of the hypothalamo-hypophysial portal system. This hormone then acts on the pituitary gland, specifically the adenohypophysis. The adenohypophysis secretes thyroid stimulating hormone (TSH) into the bloodstream, which travels through the blood and acts on the thyroid gland. The binding of TSH to its cell surface receptor on the thyroid gland begins a cellular cascade which ultimately leads to the production and release of the thyroid hormones (Wilson, 1998). The thyroid hormones then travel through the blood as a complex with specific binding proteins to their target organs or tissues. These specific binding proteins are thyroxine-binding globulin (TBG) and transthyretin (TTR) (Wilson, 1998). Once T_3 reaches its target cell, it is believed to enter the cell by a carrier-mediated, energy-dependent process, though some hormone may diffuse passively through the plasma membrane due to its lipophilic constitution (Ichikawa et al., 2000; Wilson, 1998). It then enters the nucleus, possibly in association with cytoplasmic thyroid hormone-binding protein (CTBP). Once in the nucleus, T_3 is released from CTBP and binds TR (Ichikawa et al., 2000; Nagasawa et al., 1995).

There are multiple isoforms of TR, which may compete for the limited numbers of thyroid hormone response elements available (Leitman et al., 1996). Two genes code for TRα and TRβ respectively. The TRα locus is located on chromosome 17 in humans. Alternative splicing creates two isoforms, TRα1 and TRα2. TRα2 is incapable of binding T_3 and it is believed that it may act as a repressor of T_3-dependent gene transcription (Tsai and O'Malley, 1994; Wilson, 1998). The TRβ locus is found on human chromosome 3.

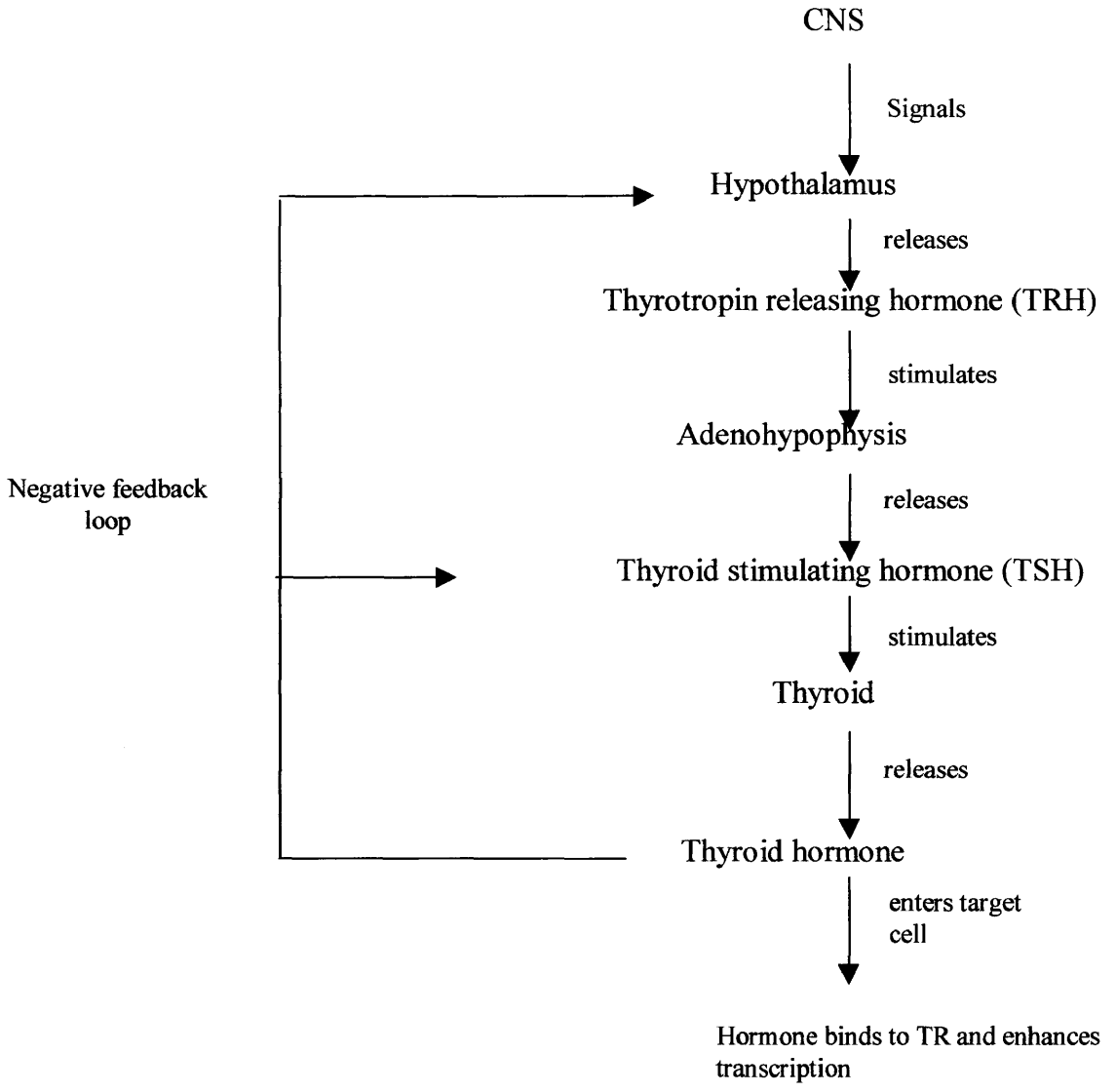


Figure 1. Secretory pathway of the thyroid hormones. Regulation begins in the hypothalamus with the release of TRH. TRH stimulates the adenohypophysis to secrete TSH. TSH then binds to cell surface receptors on the thyroid gland, which leads to the release of the thyroid hormones. The thyroid hormone then enters the cell and enters the nucleus, binds to TR and, in most cases, enhances transcription.

Alternative splicing and separate promoters create the isoforms TRβ1 and TRβ2 (Gauthier et al., 1999; www.thyroidmanager.org/Chapter16/16d-text.htm). In the absence of ligand, TR is usually loosely bound to target gene sequences. However, upon hormone binding, a conformational change is induced in TR, which strengthens the receptor's association with DNA. Furthermore, hormone binding promotes receptor dimerization (Tsai and O'Malley, 1994), which will be discussed in a later section.

Role of thyroid hormone in the body

T_3 has been shown to have an important role in reproduction, differentiation of certain cell types, development of various tissues, and maintenance of homeostasis in adults. These functions of T_3 are mediated by the TRs (Lin et al., 1992; Wilson, 1998). Some specific functions of T_3 include control of amphibian metamorphosis, and, in mammals, cardiac output, ventilation, mobilization of food stores, and body temperature control via thermogenesis and sweating mechanisms (Eliceiri and Brown, 1994; Sachs et al., 2000; Wilson, 1998). Many of these studies concerning the effects of T_3 involve the use of knockout mice, who lack either the TRα gene, the TRβ gene, or both. For example Rüsch et al. (1998) demonstrated that TRβ deficient mice have abnormal hearing development. They further pointed out that these mice have problems with potassium conductance, which occurs at the inner ear hair cells. Studies of TRβ deficient mice have also shown that this isoform is the main regulator for TSH release and, in its absence, extremely high levels of T_3 and T_4 can be found in the blood. TRα can stimulate TSH release, but at a much less efficient level than TRβ (Forrest and Vennström, 2000; Gauthier et al., 1999).

TRα deficient mice have also been produced. These mice die within five weeks due to growth arrest, and have major defects in bone and small intestine development (Fraichard et al., 1997; Gauthier et al., 1999). Further studies suggest that TRα1 may be involved in both control of heart rate and body temperature. TRα knockout-mice had an average heart rate 20% lower than the wild type and also showed a 0.5°C lower body temperature (Wikström et al., 1998). Mice lacking TRα1 and TRα2 also have a deficiency in T and B lymphocyte development. B lymphocytes were most profoundly affected, showing a considerable lack of B cell progenitor proliferation. However, what remains unclear is how TRα is controlling the B cell population, whether by direct interaction or by creating an environment that promotes B cell differentiation (Arpin et al., 2000).

As stated earlier, T_3 is very important for development and adult homeostasis. Deficiency or overproduction of T_3 or T_4, or genetic defects in TR can lead to a disease state. In humans, both hyper- and hypothyroidism have been documented (Wilson, 1998). There are two main forms of hyperthyroidism. One form is caused by mutant TSH receptors that are activated in the absence of hormone and stimulate the thyroid gland to produce an excess of T_3. The second form is Graves' disease which is caused by autoantibodies produced against an individual's own TSH receptors. These autoantibodies activate the same pathway as a normal TSH receptor bound by hormone. Individuals with hyperthyroidism have dramatic weight loss due to a large increase in metabolic rate, excessive sweating due to increased heat production, weakness associated with muscle loss, and presence of goiter (an enlarged thyroid gland) (Wilson, 1998). A major cause of hypothyroidism is a deficiency of iodine, a component of both T_3 and T_4.

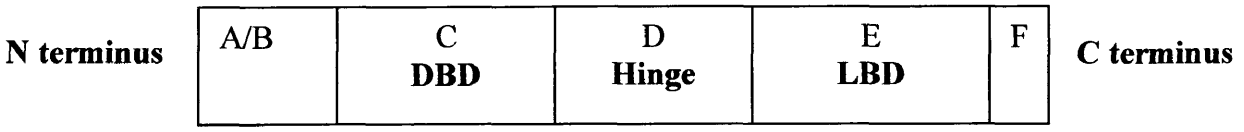
Hypothyroidism leads to cretinism which is diagnosed by especially short stature, obesity, malformed legs, and mental retardation (Wilson, 1998). Resistance to thyroid hormone is the major heritable disease associated with mutations in TRβ. Mutations are primarily located in the T_3-binding domain and the adjacent hinge region. Inheritance of this disease is autosomal dominant but resistance is never complete. Individuals with this disease experience a wide range of symptoms including goiter, abnormally high T_3 levels, and varying degrees of slowed development (www.thyroidmanager.org/Chapter16/16d-text.htm).

Characteristics of TR and other members of the nuclear receptor superfamily

TR is a member of the large steroid/thyroid superfamily of nuclear receptors, which includes the receptors for steroid hormones, vitamin D receptor, retinoic acid receptor (RAR), and the retinoid X receptor (RXR), as well as orphan receptors whose ligand and/or function are currently unknown. These receptors act as transcription factors which can either repress or enhance transcription of target genes (Weigel, 1996). TRs bind to DNA at specific sites called thyroid hormone response elements (TREs) as monomers, homodimers, and as heterodimers with RXR (Leitman et al., 1996; Tsai and O'Malley, 1994). These TREs can be inverted, direct, or even everted repeats, with each half site having a similar homology to the estrogen response element (Tsai and O'Malley, 1994).

All members of this family share functional amino acid domains named A through F (Fig. 2). The A/B regions of the N-terminus are weakly conserved, are variable in length and contain an autonomous transcriptional activation function

A



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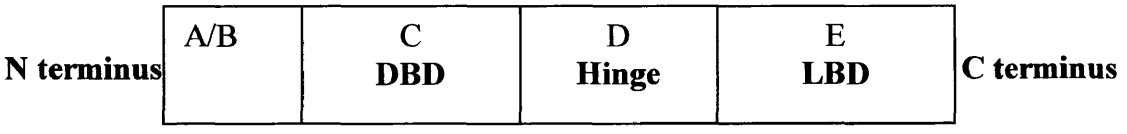

Figure 2. A. Domains of the nuclear receptor superfamily. The A/B domains are weakly conserved. The C domain contains the DNA binding domain (DBD). The D domain contains the variable hinge region. The E domain contains the highly conserved ligand binding domain (LBD). The F domain is weakly conserved, is absent in some members such as the thyroid hormone receptor and has no known function. **B. Domains of the thyroid hormone receptor.** The A/B region contains the phosphorylation site at serine 12. The D domain contains the NLS.

(Robyr et al., 2000). The C region includes the highly conserved DNA binding region, which is comprised of two Zn^{2+} containing motifs called "zinc fingers" (Robyr et al., 2000; Weigel, 1996). The D domain is a variable hinge region. Domain E is also highly conserved and contains the ligand binding domain, a second transactivation function, a dimerization domain, and a region involved in nuclear localization. This second transactivation function is an amphipathic α-helix that is highly conserved and is critical for transcriptional activation (Robyr et al., 2000). The final region F has no known function and is highly variable across the superfamily, including complete absence of the region in TR, the progesterone receptor (PR), RAR, and RXR (Robyr et al., 2000).

TR binding to DNA

TRs, like their counterparts in the superfamily, can bind DNA in multiple forms. First, as mentioned above, TR can bind to TREs as a monomer, homodimer, or heterodimer. These various protein-protein interactions regulate transcriptional activity (Weigel, 1996). For example, TR preferentially forms heterodimers with RXR. There are three RXR isoforms, α, β, and γ. Thus, the regulatory outcome of binding of TR to its target sequence will depend upon to which RXR variant it is bound (Weigel, 1996). Also, as stated earlier, there are multiple isoforms of TR which may compete for the limited numbers of TREs available. Furthermore, the distribution of these isoforms is different. TRα is found mainly in the brain, gut, and lung (Macchia et al., 2000). TRβ shows a wider distribution and appears to be more important at the adult stage (Forrest and Vennström, 2000). Thus, several mechanisms exist which can alter the effect of T_3.

The effect not only depends on how TR binds to DNA, but also which isoform has bound the hormone.

Nuclear import

Since all proteins are synthesized in the cytoplasm, nuclear proteins must have a mechanism to enter the nucleus in order to perform their activity. As transcription factors, members of the steroid/thyroid superfamily fall into this category. Traffic from the cytoplasm to the nucleus and vice versa is regulated through the nuclear pore complex. The nuclear pore is a protein complex embedded in the nuclear membrane. It is composed of 50-100 subunits which form a three-dimensional structure that has an eightfold rotational symmetry. Both faces have rings that are connected via central spokes creating a central channel for proteins to pass through. The cytoplasmic face has eight filaments, which project away from the nucleus into the cytoplasm. The nuclear face of this structure has a basket-like structure, which points inward (Corbett and Silver, 1997; Newmeyer, 1993).

Proteins entering, exiting, or even shuttling between the cytoplasm and the nucleus generally have a signal, which allows them to be properly targeted. Shuttling is the phenomenon where a protein seems to be localized to a specific subcellular compartment; however, in reality, individual proteins are being both imported and exported, with one of the rates substantially slower than the other leading to a more localized state. Both nuclear localization signals (NLS) and nuclear export signals (NES) have been identified. Two signals which have been well characterized are the simian virus 40 large T-antigen (also known as the classical NLS) and the nucleoplasmin bipartite NLS. The classical NLS is a stretch of basic amino acids, while the bipartite

NLS is made up of two basic amino acid stretches separated by a 10 residue spacer region.

For larger proteins, both import and export are usually energy-dependent processes, although export may not specifically require GTP hydrolysis. Passive diffusion does occur in some cases with proteins that are less than 40-60 kD, though many of these smaller proteins are actively imported since this pathway is more efficient (Corbett and Silver, 1997; Guichon-Mantel et al., 1996; Kaffman and O'Shea, 1999; Okamoto et al., 1999). Results of a series of experiments by Bunn et al. (2001) strongly suggest that, in *Xenopus* oocytes, TR undergoes passive diffusion for its entry into the nucleus. The authors showed that TR import in *Xenopus* oocytes is temperature and energy-independent. Furthermore, they demonstrated that inhibitors of signal-mediated import, wheat germ agglutinin and Ran Q69L, do not inhibit the nuclear import of TR. Interestingly, TR can also enter the oocyte nucleus by a signal-mediated pathway. This latter pathway will be discussed in more detail later.

The mechanism for the import of many proteins with a classical NLS begins with the cargo protein binding directly to the importin α subunit of the importin α- importin β heterodimer complex (Fig. 3). Following binding, this complex is targeted to the cytoplasmic filaments of the nuclear pore complex by the importin β subunit. At the nuclear pore, in the presence of Ran-GDP, the importin-cargo protein complex is translocated into the nucleus through the central channel of the nuclear pore complex. In a separate pathway, Ran-GDP also enters the nucleus and is converted to Ran-GTP by the Prp20p and RCC1 exchange factors. Within the nucleus Ran-GTP binds to the importin-cargo complex, which causes the dissociation of the importin complex from the

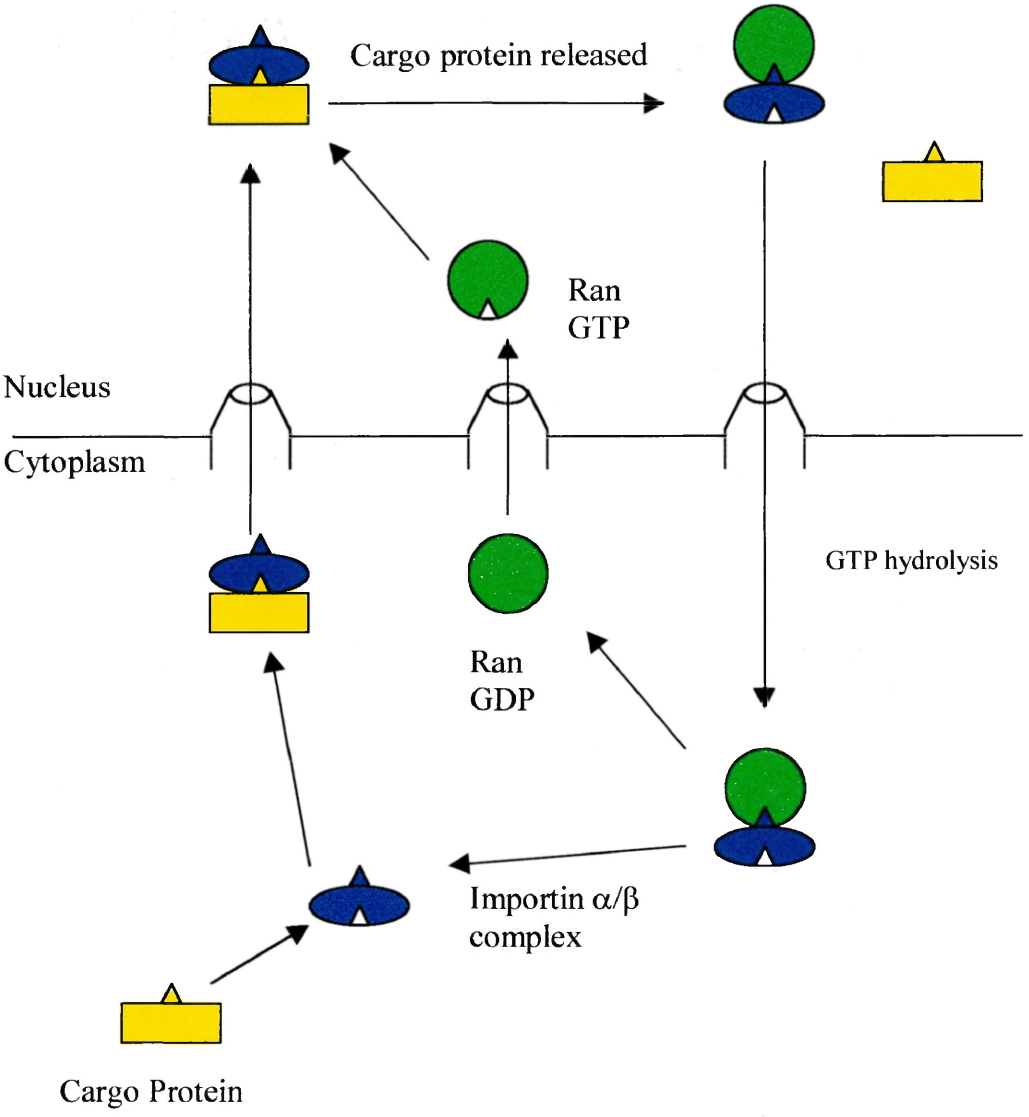

Figure 3. Nuclear import of proteins with a classical NLS. The cargo protein bearing a classical NLS binds the importin α/β complex and is translocated to the nucleus. Ran GTP binds to this complex causing the release of the cargo protein. The Ran/importin complex is exported to the cytoplasm for another round of import.

cargo protein. After dissociation, the importin subunits are recycled back to the cytoplasm in a process that requires GTP hydrolysis by Ran. The cargo protein is then able to perform its biological function within the nucleus (Corbett and Silver, 1997; Kaffman and O'Shea, 1999; Nigg, 1997).

Nucleocytoplasmic Shuttling

Nuclear receptors, including the glucocorticoid receptor (GR), estrogen receptor (ER), PR, and recently TR have been shown to undergo rapid nucleocytoplasmic shuttling (Bunn et al., 2001; Newmeyer, 1993). These receptors localize to different parts of the cell at steady state. In the absence of hormone, GR, the mineralocorticoid receptor (MR) and possibly the androgen receptor (AR) are localized to the cytoplasm of the cell. In the presence of hormone, these receptors enter the nucleus and enhance transcription. On the other hand, the progesterone, estrogen, and thyroid hormone receptors are primarily localized to the nucleus in the absence of ligand and in the presence of ligand activate transcription. Interestingly, ER and PR are not associated with DNA in the absence of ligand while TR is bound to DNA. ER and PR only bind their target sequences in the presence of ligand (Haché et al., 1999; Tsai and O'Malley, 1994). These localizations are not absolute, however, and depend on the cell type. For example, in *Xenopus* oocytes, TR can be found in both the cytoplasm and the nucleus at steady state (Bunn et al., 2001).

All these receptors apparently contain NLSs allowing them to enter the nucleus and, once there, they interact with the DNA to turn on the appropriate genes based on the hormonal signal (Haché et al., 1999; Tsai and O'Malley, 1994). Interestingly, GR and PR

contain multiple NLSs (Guichon-Mantel et al., 1996; Haché et al., 1999). In the presence of ligand, GR acts as a shuttling protein that continuously travels between the cytoplasm and the nucleus. The first NLS, NL1 is located in the C-terminal end of the DNA binding domain of GR and is the main signaling motif. However, a second ligand-dependent NLS, NL2, has been found in the ligand-binding domain of GR (Haché et al., 1999). As mentioned earlier, PR also has multiple NLS sequences. The first, a hormone-independent sequence, is found in the hinge region and is very similar to the classic NLS. A second NLS is located in the second zinc finger of the DNA binding domain. This signal is regulated by the mechanisms that control the activity of DNA binding. A third weaker ligand-dependent signal is present in the steroid-binding domain. In contrast, human AR has had only one NLS identified, a region of twenty amino acids. It is located in the hinge region of the receptor and has the same properties as the bipartite signal previously mentioned. Similarly, human ER contains a forty-eight amino acid sequence located in the hinge region between the DNA and hormone-binding domains (Guichon-Mantel et al., 1996).

Research has suggested that TR can follow a receptor-mediated pathway for import into the nucleus, although in *Xenopus* oocytes a co-existing passive pathway has been demonstrated (Bunn et al., 2001). Despite indirect evidence for a NLS in TR, it has proved difficult to define a NLS as both necessary and sufficient for nuclear localization. A basic sequence in the D domain, which is conserved in all TR isoforms, has been shown to target cytoplasmic proteins to the nucleus, albeit weakly, in that nuclear localization and retention were not complete (Dang and Lee, 1989). Other experiments, using various methods, have shown that this region is indeed important for import;

however, by itself, it is not efficient at promoting nuclear import (Boucher et al., 1988; Lee and Mahdavi, 1993; Zhu et al., 1998). Therefore, other factors not yet discovered must be involved in regulating TR subcellular movement (Bunn et al., 2001).

Phosphorylation of transcription factors and viral proteins

The phosphorylation of transcription factors and viral proteins has been shown to be an important regulatory factor in many systems. For example, Kann et al. (1999) showed, using digitonin permeabilized cell *in vitro* nuclear import assays, that only phosphorylated hepatitis B virus core particles were targeted to the nucleus. Nuclear uptake of the virus core particle was shown to involve a NLS-mediated pathway. They also demonstrated that the phosphorylated core particle interacts directly with nuclear pore complex proteins via a nuclear pore binding signal, and can inhibit nuclear import of other nuclear proteins by competing for these binding sites. In conclusion, the authors suggest that phosphorylation of the core subunit causes a conformational change that exposes the COOH-terminal NLS so that the phosphorylated residues are no longer accessible to phosphatases, allowing entry of the viral particle into the nucleus (Kann et al., 1999).

Similarly, it has been shown that nuclear import of Dorsal, a member of the Rel family of proteins in *Drosophila*, is regulated by phosphorylation (Drier et al., 1999). Dorsal is of prime importance in *Drosophila* development. This protein is involved in the formation of the ventral-to-dorsal nuclear gradient. Dorsal is normally found in the cytoplasm bound to the I-κB-related protein Cactus. In response to a ventral signal, signal-dependent phosphorylation of both Cactus and Dorsal leads to the degradation of

Cactus and nuclear targeting of Dorsal. This phosphorylation event is necessary for nuclear import and thus is vital to the development of the adult fly (Drier et al., 1999).

Phosphorylation has also been implicated as playing a role in regulating the nuclear import of NF-κB, another member of the Rel family of proteins. NF-κB is a transcription factor found in B cells, which are involved in the human inflammatory immune response. In this system, phosphorylation has been shown to be important as a method of masking and unmasking nuclear localization sequences (NLSs) (Moroianu, 1999). In the inactive state NF-κB is found in the cytoplasm, bound to the inhibitor I-κB, thus masking the NLS of NF-κB. Activation begins by binding of a bacterial lipopolysaccharide to a cell surface receptor. This binding initiates a signaling cascade, which eventually leads to the activation of IKKα, a serine-specific kinase. This kinase then phosphorylates two serine residues on I-κB, causing I-κB to dissociate from NF-κB. This dissociation unmasks the NLS, exposing it to the nuclear import machinery and thus allowing import of NF-κB into the nucleus (Moroianu, 1999).

Kaffman et al. (1998) demonstrated that the phosphorylated form of the transcription factor Pho4 could not bind to Pse1, which is a member of the importin β family, and thus could not be imported into the nucleus. Pho4 is a yeast transcription factor required for phosphate starvation-specific gene expression. This protein is regulated by changes in the extracellular concentration of inorganic phosphate. Under conditions of high phosphate, Pho4 resides in the cytoplasm and is phosphorylated, thus resulting in repression of genes active under low phosphate conditions. However, under phosphate-starved conditions, Pho4 becomes dephosphorylated by inhibition of the

kinase responsible for the normal levels of phosphorylation. This unphosphorylated form is then able to bind to Pse1 and this complex is then transported into the nucleus.

Another study has shown that dephosphorylated NLS tagged-albumin conjugates were unable to undergo nuclear transport in digitonin-permeabilized cells (Mishra and Parnaik, 1995). This model is very effective because albumin does not have a NLS, allowing direct study of phosphorylation of specific sequences of the various NLSs. Dephosphorylation also prevented the conjugates from binding to the nuclear pore complex, an essential step in signal-mediated nuclear transport. Interestingly when the cells were treated with protein kinase C or A (PKC and PKA), the conjugates were phosphorylated and nuclear transport was restored.

Steroid receptor phosphorylation

Members of the steroid/thyroid hormone receptor superfamily are phosphoproteins. Many of their phosphorylation sites are serine and threonine residues; however, some members of the family can be phosphorylated at tyrosine residues (Weigel, 1996). Evidence for phosphorylation has been provided by experiments using several techniques, including altered mobility on a SDS-polyacrylamide gel, detection of phosphotyrosine using a phosphotyrosine specific antibody, and [^{32}P] labeling studies (Tsai and O'Malley, 1994). These studies have shown that phosphorylation may have a role in hormone binding, DNA binding, transcriptional activation, and nuclear import (specifically nucleocytoplasmic shuttling of GR) (Tsai and O'Malley, 1994), as described below.

Role of phosphorylation in hormone binding

Studies have shown that phosphorylation may play a role in hormone binding by nuclear receptors. For example, using *in vitro* analysis, Auricchio's research group has shown that phosphorylation is involved in hormone binding of ER (Tsai and O'Malley, 1994). They showed that treatment with nuclear phosphatase results in the loss of hormone binding. Furthermore, they demonstrated that treatment of ER with Ca^{2+} calmodulin kinase phosphorylates tyrosine residues, and restores the hormone binding activity of ER (Castoria et al., 1993). While these earlier studies suggested a role for phosphorylation in hormone binding, more recent work has suggested an alternatative interpretation. The current belief is that members of the superfamily are typically basally phosphorylated. This phosphorylation level is then increased with ligand binding (Weigel, 1996). However, this new idea does not completely invalidate earlier work and a great deal of research continues on the subject.

Role of phosphorylation in DNA binding

Phosphorylation has also been shown to have a role in DNA binding of nuclear receptors. Many of these studies have focused on ER and PR. For example, it has been shown that, upon treatment with estradiol, ER becomes phosphorylated in MCF-7 breast adenocarcinoma cells (Denton et al., 1992). They then treated these cells with a potato acid phosphatase. The result was a decrease in phosphorylation of ER as measured by decreasing ^{32}P levels. They further demonstrated that the dephosphorylated receptor had a lower affinity for specific DNA binding sites. Other groups have reported similar results. Phosphorylating human ER on tyrosine 537 led to its dimerization with other

ERs in human MCF-7 cells. Further, in agreement with Denton et al. (1992), dephosphorylation caused a decrease in binding of ER to its specific response element as shown by electrophoretic gel mobility shift assays (EMSA) (Arnold et al., 1995).

Studies with PR have yielded conflicting results. Using zinc finger mutants, Takimoto et al., (1992) demonstrated that hormone-induced phosphorylation of human PR includes DNA-independent and DNA-dependent stages. They proposed a three-stage cascade of phosphorylation. First, there is basal phosphorylation where low levels of phosphates are incorporated into the receptors. The second stage begins when hormone binding causes a conformational change exposing additional residues to phosphorylation. The third stage occurs when the receptor binds to DNA. Once again, this binding is proposed to cause a conformational change that exposes new residues for phosphorylation.

In support of DNA-dependent phosphorylation, Bagchi et al. (1992) reported that binding of PR to its specific response element occurs prior to phosphorylation. However, the authors reported that they saw no ligand-induced phosphorylation, suggesting that liganded receptor is not a prerequisite for DNA binding. They propose a model in which the receptor is synthesized and then complexed with heat shock protein 90 (hsp90) in the cytoplasm. Hormone binding causes a release of this complex, and PR forms a dimer and binds to its response element in the nucleus. Binding to DNA then induces phosphorylation of the receptor (Bagchi et al., 1992). Beck et al. (1992) provides additional evidence suggesting that phosphorylation is not necessary for DNA binding. In this study, T47D breast cancer cells were treated with 8-bromo-cAMP, an activator of cAMP-dependent protein kinases and okadaic acid which is a known inhibitor of proteins

phosphatases 1 and 2A. Neither compound modified the DNA binding activity of the receptor, as assayed by EMSA of whole cell extracts from treated and untreated cells.

Role of phosphorylation in transcriptional activation

While the role of phosphorylation in hormone and DNA binding remains undecided, its role in nuclear receptor activation is becoming more firmly established. A number of steroid receptors have been utilized in these studies. While Beck et al. (1992) showed that phosphorylation did not alter the DNA binding affinity of PR, their results suggested that indeed phosphorylation enhanced transcription of target genes. In their study, both 8-bromo-cAMP and okadaic acid enhanced PR-mediated transcription 3 to 4 fold in the presence of progestin. Furthermore, H8, a cyclic nucleotide-dependent protein kinase inhibitor, blocked gene expression in the presence of the hormone. Taken together, these results suggest a role for phosphorylation in activation of progestin-dependent gene expression by PR. Researchers working with ER have reported similar results. Mutagen studies have shown that serine 118 is important in transcriptional activation of human ER. Mutation of this residue to an alanine residue led to a reduction in ER-mediated gene transcription (Joel et al., 1998). In their study, the authors demonstrate that $pp90^{rsk1}$, a ribosomal kinase, phosphorylates human ER at serine 167. This phosphorylation was also shown to enhance transcription of target genes (Joel et al., 1998). Similarly, the human vitamin D receptor (VDR) has been shown to have enhanced transcriptional activity in the presence of casein kinase II (CK-II) (Jurutka et al., 1996). Thus, phosphorylation of members of the nuclear receptor superfamily seems to play a role in their ability to activate transcription.

Role of phosphorylation in nucleocytoplasmic shuttling

As previously mentioned phosphorylation seems to play an important role in the nuclear transport of many proteins. Since the steroid receptors are phosphoproteins and undergo nucleocytoplasmic shuttling, research on the role of phosphorylation in this phenomenon is receiving a great deal of attention. For example, numerous studies have suggested that phosphorylation regulates GR shuttling. GR distribution seems to be regulated by protein phosphatases 1 and 2A and tyrosine kinases in rat fibroblasts (DeFranco et al., 1991). The authors showed that a high dose of okadaic acid leads to poor nuclear retention. Since okadaic acid is known to inhibit these two phosphatases, these findings suggest that phosphorylated GRs are not capable of recycling and remain cytoplasmic (DeFranco et al., 1991). Another paper demonstrating the importance of GR phosphorylation state on subcellular localization used digitonin-permeabilized cell nuclear export assays. In this study, tyrosine kinase inhibitors prevented GRs from being phosphorylated, leading to an inhibition of nuclear export (DeFranco, 1997).

TR Phosphorylation

TR, like its counterparts in the steroid receptor superfamily, is a phosphoprotein. Both rat and chicken TRα have a well-characterized casein kinase II phosphorylation site at serine 12 in the N-terminal region (Glineur et al., 1989; Goldberg et al., 1988). In addition, v-ErbA, a viral oncogenic homolog of chicken TRα, has a cAMP-dependent protein kinase A site at serines 28/29 (Glineur et al., 1990). Researchers have demonstrated that the oncoprotein must be phosphorylated for full biological activity as a transcriptional repressor, as kinase inhibitors reduce the function of v- ErbA (Glineur et

al., 1990). In contrast, Katz et al. (1995) reported that phosphorylation of the TRα2 variant in rats reduces its ability to bind to DNA. When TRα2 was dephosphorylated an increase in repressor activity was observed. Another report suggests that phosphorylation may be involved in regulating the levels of TRβ1 in different cell types. Okadaic acid was used to enhance phosphorylation by inhibiting phosphatase activity. This increase in phosphorylation led to increased levels of TRβ1, which the authors attributed to greater stability of phosphorylated TRβ1 in the various cell types (Ting et al., 1997).

Lin et al. (1992) showed that in addition to promoting receptor stability, phosphorylation led to an increase in the binding of TR to the TREs. The authors proposed a model in which phosphorylation allows TR to bind with nuclear accessory proteins by inducing a conformational change. Several other groups have suggested that phosphorylation may be involved in DNA binding of TR. However, Sugawara et al. (1994) and Bhat et al. (1994) reported conflicting results. Sugawara et al. (1994) reported no change in DNA binding of RXR/TRβ1 heterodimers after the phosphorylation of human TRβ1(hTRβ1). In contrast, Bhat et al. (1994) reported an increase in DNA-binding by heterodimers after phosphorylation of hTRβ1. Sugawara et al. (1994) did report an increase in homodimer binding of the phosphorylated receptor, while unphosphorylated TRβ1 bound DNA as a monomer. Adding confusion to the DNA binding issue, is a report that suggests phosphorylation does not alter the affinity of receptor dimers for DNA. However, this report also suggested that phosphorylation greatly inhibited the ability of TR monomers to bind to DNA (Tzagarakis-Foster and Privalsky, 1998).

Jones et al. (1994) used okadaic acid to increase phosphorylation of TR, and isoquinoline sulfonamide (H7), which inhibits phosphorylation by acting against a wide range of kinases, to test the effect of phosphorylation of TR on transcriptional activation. Okadaic acid, by inhibiting phosphatase activity augmented the transcriptional activity of a TRE-mediated reporter luciferase gene in the presence of T_3, in monkey kidney CV-1 cells. Conversely, H7 lowered transcriptional activity as it inhibited kinase activity. Another report showed that T_3 binding leads to a three-fold increase in phosphorylation of TRβ1. Based on protease studies the authors suggested that phosphorylated TR undergoes a conformational change, which may play a role in transcriptional activation (Ting and Cheng, 1997). Thus, phosphorylation of TR is involved in regulating transcription. However, currently the mechanism is not yet understood. It is not clear whether phosphorylation is directly or indirectly affecting regulation through the receptor or the other proteins involved.

Use of Xenopus oocytes as a model system

Xenopus oocytes were first used for microinjection experiments in the 1970s. Since that time, this system has been used to study a number of mechanisms and important pathways, including characterization of protein kinases and their inhibitors as well as studies of the meiotic machinery (Lacal, 1999). Oocytes have been used to determine gene function, which has greatly enhanced our understanding of various secretory products, as well as aiding in the identification of many membrane receptors. Examples include characterization of the Na^+ and K^+ channels and the GABA transporters. In addition, the Ras and Mos oncogenic pathways were first studied in

Xenopus oocytes (Lacal, 1999). These studies were possible because the oocytes demonstrate a unique ability to transcribe and translate most injected foreign DNA and RNA. Their large size also makes them ideal for the recovery of proteins of interest (Lacal, 1999).

Oocyte microinjection assays are especially suited for TR research. Researchers have already used these types of assays to study the regulation of transcription by TR (Lacal, 1999; Nagl et al., 1995), and the ability to inject into both the cytoplasm and the nucleus makes this system ideal for studying nuclear import and export (Bunn et al., 2001). Numerous examples can be found in the literature citing the use of oocyte microinjection to study nuclear import, including studies of RNA transport, as well as the transport of heat shock proteins (Lacal, 1999; Murdoch and Allison, 1996). Furthermore, since in oocytes TR is not entirely localized to the nucleus, factors that enhance nuclear retention can be studied.

Questions addressed in this thesis

As the preceding review has shown, phosphorylation has been implicated in many regulatory roles, not only in the function of nuclear receptors but for other proteins as well. Although there is a large body of literature available on the nuclear receptors, only a small amount is dedicated to TR. Much of the TR literature has focused on the effects of gene knockouts on murine development. This research has provided great insight into the many functions of TR; however, this type of research does not shed light on the various molecular interactions of TR, at the level of protein-protein and DNA-protein interactions. The eukaryotic cell has developed into a complex structure of many

compartments. The trafficking of regulatory factors that occurs between compartments is vital to the development of an organism; thus understanding the nuclear import mechanisms of these factors is essential. Ultimately, to answer the fundamental question of how TRs regulate such a range of diverse functions in so many tissue types, additional molecular and cellular biology techniques will have to be applied. With these combined approaches many of the developmental questions will then be able to be answered. In this thesis research, three main questions were addressed.

1) Is nuclear TR phosphorylated in *Xenopus* oocytes?
2) Does phosphorylation of TR occur in the cytoplasm or the nucleus?
3) Is phosphorylation of TR an important mechanism for regulating its nuclear import and retention?

Materials and Methods

Gene constructs

pT3-rTRα, an expression vector containing rat TRα1 under the control of the T3 RNA polymerase promoter, was a gift from M. Lazar (University of Pennsylvania School of Medicine, Philadelphia, PA). This plasmid was purified from host DH5α cells using Qiagen MidiPreps (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions.

In vitro transcription/translation of ^{35}S-TRα

^{35}S-labeled TRα was synthesized using the TNT Rabbit Reticulocyte Lysate System (Promega, Madison, WI). The following reaction mixture was prepared: 25 µl TNT Rabbit Reticulocyte Lysate; 2 µl TNT Reaction Buffer; 1 µl TNT T3 RNA Polymerase; 1 mM Amino Acid Mixture, Minus Methionine; 40 U RNasin Ribonuclease Inhibitor; 1 µg of pT3-rTRα DNA template; and 20 µCi L-[^{35}S]methionine (1000 Ci/mmol, *in vivo* cell labeling grade; Amersham Life Science, Arlington Heights, IL), for a final reaction volume of 50 µl.

The reaction was incubated at 30°C for 90 minutes. 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography confirmed the presence of ^{35}S-TRα.

Xenopus oocyte microinjection

Microinjection needles were made from 1 mm glass capillaries using a Narshige PC-10 needle puller. After making the needle, the tip was broken to 20 μm diameter, confirmed using a stage micrometer. Microinjections were performed using a Pico-Pump (Medical Systems Corp., Greenvale, NY) with a pressure of 20 psi and a pulse time of 60 msec, thus injecting a volume of 20 nl (~100 pg of ^{35}S-TRα).

Surgeries were performed to remove ovarian lobes from adult *Xenopus laevis*. Procedures were approved by the Institutional Research on Animal Subjects Committee. Oocytes were processed as previously described (Allison et al., 1991; Allison et al., 1993). The oocytes were transferred to small culture dishes with mesh along the bottom containing O-R2 medium (82.5 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.0 mM Na_2HPO_4, 5.0 mM Hepes, 3.8 mM NaOH). Healthy stage V-VI oocytes (approximately 1 mm in diameter) were selected for microinjection. Oocytes have a pigmented animal pole and a lightly colored vegetal pole (Lacal, 1999). For nuclear injections the animal pole was turned upwards and the 20 nl sample was injected into the pole (Figure 4A). Placing the animal pole upwards provides an approximate location for the nucleus; this type of injection is a "blind" injection. A successful injection yields a red nucleus after dissection due to the presence of hemoglobin in the rabbit reticulocyte lysate. For cytoplasmic injections the vegetal pole was turned upward and the sample was injected into the pole (Figure 4B).

After injection, oocytes were incubated in O-R2 solution containing 100 μg/ml cycloheximide (Sigma Chemical Co., St. Louis, MO), which prevents *de novo* protein synthesis and thus prevents incorporation of excess ^{35}S-methionine into endogenous

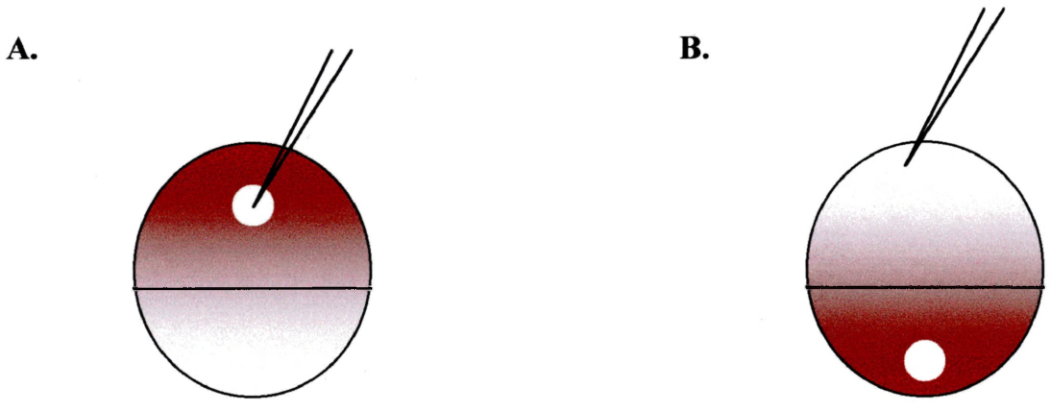

Figure 4. Microinjection of *Xenopus oocytes*. **A**. Nuclear microinjection. **B**. Cytoplasmic microinjection.

proteins. Cycloheximide does not interfere with nuclear import of proteins in *Xenopus* oocytes (Murdoch and Allison, 1996). The oocytes were left on ice for 15-20 minutes to promote wound healing, and were subsequently incubated at 20°C for various durations.

Dephosphorylation assay

^{35}S-TRα was microinjected into the cytoplasm of *Xenopus* oocytes, followed by incubation for 6 hours at 20°C. Subsequently, the oocytes were manually dissected in Nuclear Isolation Medium (NIM) (83 mM KCl; 17 mM NaCl; 10 mM Tris, pH 7.2; 1 mM phenylmethylsulfonyl fluoride [PMSF]). Nuclei were separated from the cytoplasm by making a small tear in the animal pole. Nuclei were then gently removed from the cytoplasm. Six nuclei and six cytoplasms were pooled per sample. The cytoplasms were homogenized in Homogenization Buffer (1% Triton X-100; 100 mM NaCl; 20 mM Tris-HCl, pH 7.6; 1 mM PMSF). The cytoplasms were centrifuged at 9,000 x g for 5 minutes to pellet the yolk and pigment. The supernatant was added to 5 vol acetone and allowed to precipitate overnight at -80 °C.

The nuclei were brought to a final volume of 12 µl with NIM. The dephosphorylation reaction was prepared as follows for a final volume of 40 µl: 12 µl nuclear extract; 20 U Calf Intestinal Alkaline Phosphatase (Promega); 1X Dephosphorylation Buffer (Promega). A mock reaction was prepared with nuclear extract and 1X Dephosphorylation Buffer. Both the dephosphorylation and mock reactions were incubated at 37°C for 1 hour. Samples were then precipitated overnight in 5 vol acetone. Both fractions were centrifuged and the acetone was removed. The sample pellets were allowed to air dry, and then resuspended in 20 µl of 1X SDS-PAGE Sample Buffer (125 mM Tris, pH 6.8; 1% SDS; 5% glycerol; 0.005% bromophenol blue;

10 mM DTT), and boiled for 3 minutes. Samples were separated by 12% SDS-PAGE. The gel was then fixed for at least 30 minutes in isopropanol: H_2O: acetic acid (25: 65:10). After fixing, the gel was transferred to Amplify (Amersham) and was agitated for 30 minutes. Amplify increases the detection efficiency of ^{35}S by converting the beta emissions to light when exposed to X-ray film. The gel was then dried for one hour at 80°C under vacuum using a BioRad Gel Dryer (Hercules, CA). The film was exposed to Kodak X-OMAT film and stored at -80°C for 2 weeks. The film was then developed using a Konica X-ray film processor.

Treatment of oocytes with Okadaic Acid (OA), and Isobutyl-methyl-xanthine (IBMX)

^{35}S-TRα was microinjected into the cytoplasm of *Xenopus* oocytes. After injection the oocytes were incubated in O-R2, cycloheximide, and dimethylsulfoxide (DMSO) as the control. DMSO is the solvent vehicle for the various compounds and as such the same percentage of DMSO was added to the control as was added to the various treatments. Other oocytes were incubated for 3 hours at 20°C in either OA (100 μM) or IBMX (125 μM). H7, an inhibitor of various kinases, and forskolin, a PKA activator were used; however, both compounds proved to be toxic to the oocytes and the experiments were discontinued. After incubation, oocytes were dissected in ice cold 1% trichloroacetic acid (TCA) or, in later experiments, ice cold Nucleus Isolation Buffer (NIB) (25 mM Tris, pH 8.0; 10% glycerol; 5 mM $MgCl_2$; 2 mM DTT). Six nuclei and six cytoplasms were pooled separately and homogenized in Homogenization Buffer. The cytoplasms were centrifuged at 9000 x g and the supernatant was added to 5 vol acetone. The nuclei and cytoplasms were processed in the manner previously described. The X-

ray films were scanned, and the bands quantified using Scion Image Analysis software (Scion Corporation, Frederick, MD). Replicate experiments were performed and treatments were then compared using the Student's t test to determine significance of the differences between the control and experimental data sets (with *n* representing the number of samples of six pooled cytoplasms or nuclei).

Compartment studies

^{35}S-TRα was microinjected into the cytoplasm or the nucleus of *Xenopus* oocytes. The oocytes were then either immediately dissected in NIB or were allowed to incubate at 20°C for 3 hours prior to dissection. A successful cytoplasmic injection was indicated by the absence of a red nucleus, while a successful nuclear injection presented a red nucleus. All samples were processed as described above.

SDS-PAGE

Before preparing the separating gel, the gel plates, spacers, and combs of the BioRad Mini Protean ii apparatus (Hercules, CA) were cleaned with 95% ethanol. The gel box was then set up according to the manufacturer's instructions. The gel solution (10-12% acrylamide mix [29:1 acrylamide: bisacrylamide; BioRad Laboratories], 375 mM Tris, pH 8.8, 0.1% SDS, 0.1% ammonium persulfate [APS], and 0.04% TEMED) was poured between the gel plates. Water-saturated butanol was added to the top of the gel to prevent oxygen from interfering with polymerization. The separating gel was allowed to polymerize for 30 minutes.

After the separating gel polymerized, the butanol was removed by rinsing with water. The stacking gel was then prepared by mixing 5.1% acrylamide mix, 0.13 M Tris, pH 6.8, 0.1% SDS, 0.1% APS, and 0.1% TEMED. This solution was poured on top of the separating gel and the comb was inserted. The stacking gel was allowed to polymerize for 30 minutes. Following polymerization, the comb was removed and the gels were assembled in the gel apparatus. SDS-PAGE Running Buffer (192 mM glycine; 25 mM Tris, pH 8.2; 3 mM SDS) was poured into inner and outer sections of the gel apparatus.

Protein pellets were resuspended in 20 µl of 1X SDS-Page Sample Buffer. All gels contained one lane of BioRad Kaleidoscope Pre-stained Standards, so that the size of the proteins could be compared with the known sizes of the proteins in the standard. All samples were incubated in a boiling water bath for 3 minutes, and then loaded into the lanes of the gel using BioRad Gel Loading Tips. Samples were electrophoresed at 150 V until the bromophenol blue dye reached the end of the gel plates.

In vitro phosphorylation of TRα

TRα was phosphorylated under the following reaction conditions in a total volume of 25 µl: 2 µl Casein Kinase II Buffer (New England BioLabs, Beverly, MA) (20 mM Tris-HCl; 50 mM KCl; 10 mM $MgCl_2$); 1.25 µl of 1 µM ATP; 500 U Casein Kinase II (New England BioLabs); 10 µCi Easytides γ ^{32}P-ATP (Perkin Elmer Life Sciences (NEN); 3000 Ci/mmol) or 10 µCi Redivue γ ^{32}P-ATP (Amersham; 3000 Ci/mmol); and 5 µl ^{35}S-labeled TRα in TNT rabbit reticulocyte lysate. The appropriate mock reaction was also prepared using 5 µl of rabbit reticulocyte lysate in place of the ^{35}S-labeled TRα.

The reaction mixture was incubated for 30 minutes at 30°C, followed by the addition of 25 µl of 2X SDS-PAGE Sample Buffer to the reaction. Samples were analyzed by 12% SDS-PAGE and fluorography. Two X-ray films were placed over the dried gel for about 3 hours. The use of two films provides a method to visualize not only phosphorylated TRα but also the ^{35}S-labeled TRα. The ^{32}P that is incorporated into the phosphorylated TR exposes both films, however, the ^{35}S-labeled TR only exposes the film closest to the dried gel. This method takes advantage of the fact that ^{32}P is a stronger beta emitter and thus penetrates both films.

Microinjection of phosphorylated TRα

Phosphorylated ^{35}S-labeled TRα was injected into the cytoplasm of *Xenopus* oocytes. The oocytes were then either immediately dissected or allowed to incubate at 20°C for 3 hours and were then manually dissected and analyzed for nucleocytoplasmic distribution as previously described.

Mutagenesis

To introduce mutations into TRα, the Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Inc.) was used. The goal of this procedure was to introduce three separate mutations into TRα at position 12, which is normally a serine residue that is phosphorylated by casein kinase II. Unfortunately, despite numerous attempts this procedure was unsuccessful at introducing the mutations, and, consequently, this strategy was abandoned.

A PCR based strategy was then used to introduce the three mutations at position 12 as well as a deletion of positions 1-12. PCR was a viable option since the position of interest was close to the N terminus. Five primers were designed in order to introduce the mutations and to create restriction sites for subcloning. The right primer introduced a BamHI restriction site while the four different left primers introduced a SacI restriction site; bold letters indicate the introduced mutations. The right primer was as follows: 5'- GGT GGA TCC TTA GAC TTC CTG ATC C. The serine to alanine primer used was as follows: 5'- C CGA GCT CGA ATG GAA CAG AAG CCA AGC AAG GTG GAG TGT GGG **GCC** GAC CCA. The serine to threonine primer used was as follows: 5'- C CGA GCT CGA ATG GAA CAG AAG CCA AGC AAG GTG GAG TGT GGG **ACC** GAC CCA. The serine to glutamic acid primer used was as follows: 5'- C CGA GCT CGA ATG GAA CAG AAG CCA AGC AAG GTG GAG TGT GGG **GAG** GAC CCA. The deletion mutant primer used was as follows: 5'- C CGA GCT CGA ATG GAC CCA GAG GAG AAC.

The following PCR reaction was set up in a total volume 50 µl: 1.25 µl of 20 µM left primer (Gene Link Inc., Foster City, CA); 1.25 µl of 20 µM right primer (Gene Link Inc.); 5.0 µl 10X Pfu Buffer (Stratagene, LaJolla, CA); 1.0 µl of 10 mM PCR nucleotide mix (Stratagene); 2.5 U of Turbo Pfu DNA polymerase (Stratagene); 10 ng pT3-rTRα. PCR was then performed in the Gene Amp PCR system 2400 (Perkin Elmer, Foster City, CA) under the following parameters, beginning with 95°C for 5 minutes. The next cycle was performed 30 times: 95 °C for 30 seconds, 65 °C for 1 minute (55 °C for the glutamic acid mutant), and 72 °C for 1.5 minutes. The following cycle was performed one time: 95

°C for 30 seconds, 65 °C for 1 minute (55 °C for the glutamic acid mutant), and 72 °C for 7 minutes.

Following PCR, agarose gel electrophoresis was used to confirm the presence of PCR products. The PCR products were then purified using the Qiagen PCR Purification Kit according to the manufacturer's instructions. Sequential restriction digests were then performed on the purified PCR product and the pGEM 4Z vector (Promega). The first restriction digests were conducted in a final volume of 40 µl with either 4 µg pGEM 4Z vector or 30 µl purified PCR product, and 40 U SacI (New England BioLabs), 4 µl 10X NeBuffer I, and 4 µl 10X BSA (New England BioLabs). The reactions were then incubated for 3 h at 37°C. These reactions were then purified using the Qiagen PCR Purification Kit according to the manufacturer's instructions. Following the purification a second restriction digest was performed: 30 µl purified DNA from first digest, 40 U BamHI (New England BioLabs), 4 µl 10X BamHI buffer, 4 µl 10X BSA (New England BioLabs). This reaction was then incubated overnight at 37°C.

After incubation overnight, 4 µl of 10X-glycerol dye (0.2 M EDTA, 50% glycerol, 0.25% Bromophenol Blue, 0.25% Xylene cyanol) were added to each digest and samples were electrophoresed on a 1% agarose gel. The gel was stained with 0.1 µg/ml EtBr and the bands were visualized on a UV transilluminator and excised. The excised bands containing the DNA were purified using the Qiagen Gel Purification Kit according to the manufacturer's instructions. The digested pGEM 4Z vector and the digested PCR product were ligated (3:1 vector: insert ratio) together in the following reaction in a total of 10 µl: 100 ng digested pGEM 4Z, 133 ng digested PCR product, 3 U

T4 DNA ligase (Promega), and 1 μl 10X ligase buffer. The reaction was then incubated overnight at 16°C.

Following the overnight ligation, XL10-Gold ultracompetent cells (Stratagene) were transformed with 5 μl of the ligation reaction according to the manufacturer's instructions. The transformed cells were then plated and grown overnight at 37 °C on LB plates containing ampicillin and X-gal. White colonies, containing the insert, were selected and cultured overnight. DNA was extracted and purified from these cultures using the Qiagen Mini Prep kit according to the manufacturer's instruction. A 0.7% agarose gel was used to screen for the presence of the insert by comparing the size of nonrecombinant pGEM4Z with recombinant pGEM4Z containing the mutant TRα insert. The successfully ligated products ran at a slower rate on the gel than the faster band representing the nonrecombinant vector. SacI/BamHI digests were then performed to confirm the correct orientation of the insert in the recombinant plasmids.

DNA Sequencing

To confirm the success of the PCR-based mutagenesis of TRα, manual dideoxy sequencing was performed using the Sequenase Version 2.0 DNA sequencing kit (USB, Cleveland, OH). Figure 5 shows the results of the sequencing reactions for the threonine and alanine mutants, which clearly contain the appropriately altered sequence at the codon for amino acid 12. Due to the guanosine-rich-sequence in the region of interest of the glutamic acid mutant, the sequence was ambiguous; however, the mutation was presumed correct due to the absence of the wild type sequence (data not shown). Before preparing the gel and reactions, the plates were cleaned with Alconox, 70% ethanol, and

acetone. After cleaning the plates, one side of each plate was coated with Sigmacote (Sigma). The base and side spacers were then cleaned with 70% ethanol. The spacers were arranged between the gel plates to prevent leaks and then the plates and spacers were clamped together.

When the plates were ready, the gel solution was prepared. The gel was made as followed: 42 g urea, 10 ml 10X TBE, 15 ml of 40% Acrylamide Stock (19:1 acrylamide : bisacrylamide, BioRad), and brought to a final volume of 100 ml with ddH_2O. The solution was then heated in a 37°C water bath for 10 minutes, filtered with a 0.22 micron filter, and brought to a final volume of 100 ml. The solution was then left on ice for 5 minutes. Immediately prior to pouring the gel, 800 µl 10% APS and 50 µl TEMED were added to the solution.

While the gel polymerized the pGEM4Z-TRα mutant constructs were prepared for sequencing. The DNA was denatured in the following reaction: 1 µg of DNA and 1 µl of 2N NaOH. The reaction was briefly centrifuged and allowed to incubate at room temperature for 5 minutes. To the above reaction, 5 µl of 4 M NH_4Ac, pH 7.0, and 50 µl of 100 % ethanol were added. After mixing, the DNA was allowed to precipitate for 1 h at –20°C. The reaction was then centrifuged at 12,000 rpm for 25 minutes at 4°C in a 5417R centrifuge (Eppendorf, Westbury, NY). The supernatant was discarded and the pellet washed with 25 µl of 70% ethanol. The DNA was then centrifuged for 5 minutes at 4°C , the supernatant removed, and the pellet air dried briefly.

The annealing reaction was then prepared as follows: 5 µl TE, pH 8.0, 2 µl 5X Sequenase buffer, and 30 ng of the SP6 promoter primer (Promega). The reaction mixture was resuspended 40X on ice, and placed in water heated to 65°C. The samples

were then allowed to cool to room temperature in the water, usually for about 20-30 minutes. The sequencing reaction was prepared as follows while the annealing reaction mixture cooled: 1 μl DTT, 0.4 μl 5X labeling mixture, 1.6 μl H_2O, 0.5 μl ^{35}S-αdATP (10 μCi/μl, 1000 Ci/mmol, Amersham), and 0.25 μl Sequenase. To each tube of annealed DNA, 3.75 μl of the sequencing reaction was added and allowed to incubate for 7 minutes at room temperature. During this time 2.75 μl of dideoxynucleotides were added to the wells of microtiter plates (U-shaped wells, Nunc). Following the incubation, 3.25 μl of the sequencing reaction were added to each well containing the dideoxynucleotides. The samples were then incubated in a 37°C water bath for 5 minutes. Subsequently, the microtiter plates were placed on ice and 4 μl of stop buffer were added to each well.

Prior to loading the samples, the sequencing apparatus (BioRad) was run for 1 h at 62W to pre-warm the gel. Samples were then heated for 1.5 minutes at 80°C, and 4 μl of each sample were electrophoresed for 90 minutes at 62W. After completion of the run, the plates were removed from the sequencing apparatus and separated. Whatman paper was placed over the gel to remove it from the plate. The gel was then covered with Saran Wrap and dried under vacuum at 80°C for 2 h on a BioRad Gel Dryer. The dried gel was allowed to cool under vacuum, and was then exposed to X-ray film overnight at room temperature. The next day the film was developed using a Konica X-ray film processor and the results were analyzed. Having confirmed the mutant sequence, the mutant TRα expression vectors that were generated by PCR-based mutagenesis were named as follows: pGEM4Z-rTRα (ala), pGEM4Z-rTRα (thr), pGEM4Z-rTRα (glu), for the substitution of alanine, threonine, or glutamic acid at position 12, respectively; and pGEM4Z-rTRα Δ12, for the mutant with a deletion of the first 12 amino acids.

Microinjection of TRα mutants

^{35}S-labeled mutant TRs were generated by *in vitro* transcription/translation as described earlier, using pGEM4Z-rTRα (ala), pGEM4Z-rTRα (thr), pGEM4Z-rTRα (glu), and pGEM4Z-rTRα Δ12 expression vectors as templates. The ^{35}S-labeled mutant TRs were injected into the cytoplasm of *Xenopus* oocytes. The oocytes were allowed to incubate at 20°C for 3 h and were then manually dissected and analyzed for nucleocytoplasmic distribution as previously described.

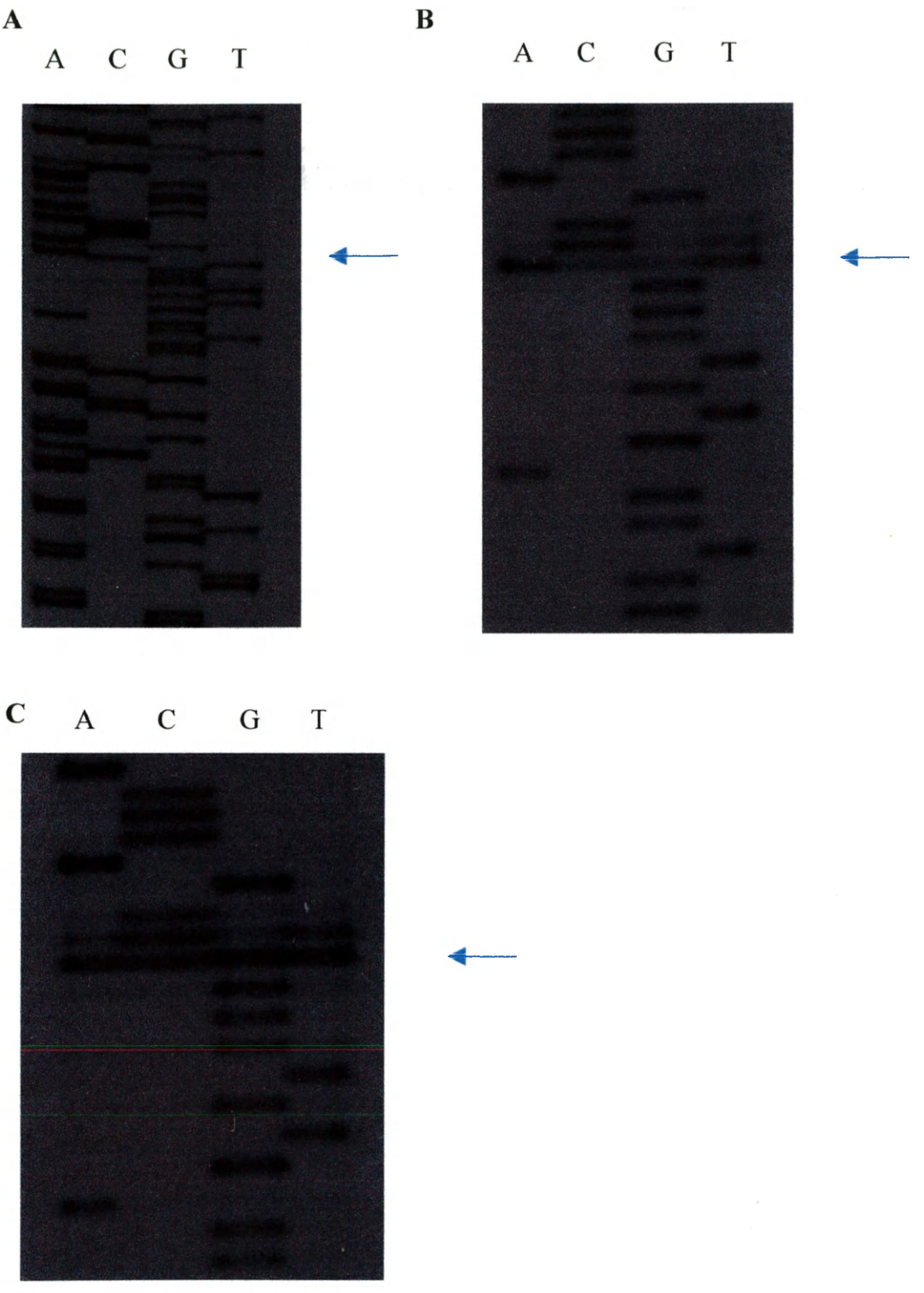

Figure 5. Manual sequencing of TRα and mutants. A. Wild type serine (TCA) **B.** Threonine mutant (ACC) **C.** Alanine mutant (GCC). Blue arrows indicate the codon for the amino acid at position 12.

Results

A phosphorylated form of TRα is present in the nucleus of Xenopus oocytes

Prior *Xenopus* oocyte microinjection studies on nuclear import characteristics of TRα showed the very interesting finding that TRα recovered from nuclear fractions has a slightly altered mobility on denaturing SDS polyacrylamide gels when compared to TRα recovered from the cytoplasm (Fig 6A). The nuclear TR band has an upward shift indicating slower movement through the gel matrix. Such an upward mobility shift often represents the phosphorylated form of a protein (Ronchini and Capobianco, 2000; Savouret et al., 1994; Tsai and O'Malley, 1994). Thus, it was of interest to determine whether the altered mobility of TRα was due to phosphorylation of nuclear TRα.
In order to assess the phosphorylation state of nuclear TR, ^{35}S-TRα was microinjected into the cytoplasm of *Xenopus* oocytes, and the oocytes were incubated 3 h to allow sufficient time for nuclear import of TRα. A time of 3 h was chosen based on other injection studies, which varied the incubation time. These experiments showed that nuclear import reached steady-state after 3 h (data not shown). The ability to dissect oocytes and separate the nucleus from the cytoplasm makes it possible to manipulate both fractions. Thus, nuclear samples were treated with alkaline phosphatase or with a mock treatment consisting of buffer alone, while cytoplasmic fractions remained untreated. Alkaline phosphatase dephosphorylates proteins (DeFranco et al., 1991; Sugawara et al., 1994). Figure 6B shows that TRα recovered from nuclear samples treated with alkaline

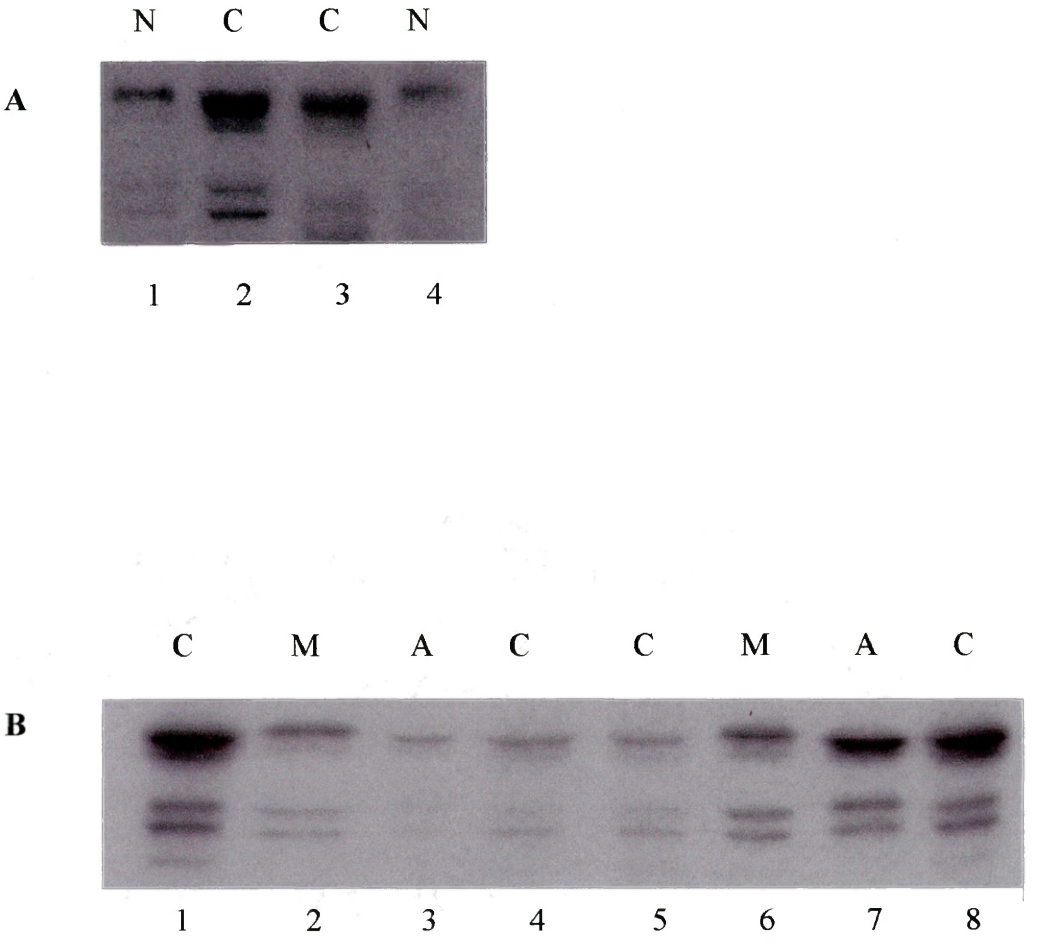

Figure 6(A). Nucleocytoplasmic distribution of TRα in *Xenopus* oocytes. *Xenopus* oocytes were cytoplasmically injected with ^{35}S-TRα. After 3 h incubation at 20°C, the oocytes were manually dissected into cytoplasmic (C) and nuclear (N) fractions. Proteins were separated by 12% SDS-PAGE, followed by fluorography. **(B) A phosphorylated form of TRα is present in the nucleus of *Xenopus* oocytes.** *Xenopus* oocytes were cytoplasmically microinjected with ^{35}S-TRα. After 3 h incubation at 20°C, oocytes were manually dissected and proteins extracted from 6 cytoplasmic (C) or nuclear fractions. Nuclear fractions were treated with alkaline phosphatase (A) (lanes 3 and 7) and incubated for 1 h at 37°C or were mock-treated with buffer alone (M) (lanes 2 and 6). Cytoplasmic fractions (lanes 1,4,5 and 8) were left untreated. Proteins were separated by 12% SDS-PAGE, followed by fluorography.

phosphatase had the same mobility as TRα recovered from untreated cytoplasmic samples (compare lanes 3 and 7 with lanes 1, 4, 5 and 8). The mock-treated samples (lanes 2 and 6) maintained the same mobility as untreated nuclear samples (Fig 6A, lanes 1 and 4), and show altered mobility of nuclear TRα compared with cytoplasmic TRα. These data suggest that a phosphorylated form of TRα is present in the nucleus of *Xenopus* oocytes.

The phosphorylation state of Xenopus oocytes modulates the nuclear distribution of TRα

The above results suggest that TRα recovered from the nucleus of *Xenopus* oocytes is phosphorylated. In order to assess the importance of phosphorylation in the nuclear localization of TRα, the phosphorylation state of the oocyte was manipulated. ^{35}S-TRα was microinjected into the oocyte cytoplasm and three treatments were administered during the 3 h incubation period. One group was treated with IBMX, while another group was treated with okadaic acid. The third group was the control, which was incubated in an equivalent amount of the solvent DMSO. Both IBMX and okadaic acid are known to enhance the general phosphorylation state of cells (Bhat et al., 1994; Jones et al., 1994); IBMX acts as a phosphodiesterase inhibitor and protein kinase A activator, while okadaic acid prevents the activity of both phosphatase 1 and 2A (Ting et al., 1997).

Figure 7A shows the results of these experiments. In the presence of IBMX, nuclear localization of TRα was enhanced when compared with the nucleocytoplasmic distribution of TRα in untreated oocytes (compare lanes 2 and 4). In the absence of IBMX, on average, 33% of cytoplasmically injected ^{35}S-TRα was localized to the oocyte

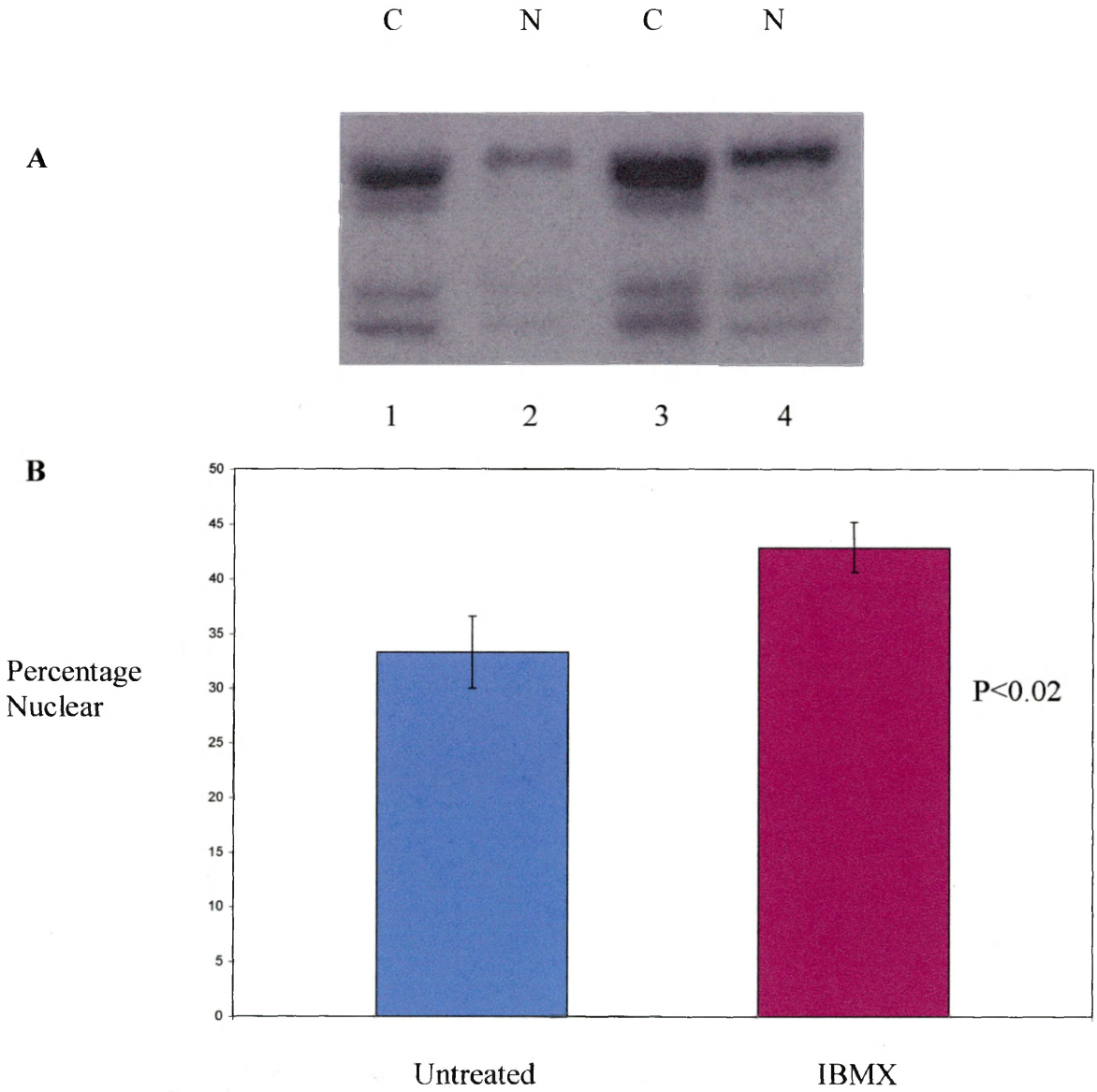

Figure 7(A). The phosphorylation state of *Xenopus* oocytes modulates the nuclear distribution of TRα. Oocytes were cytoplasmically injected with ^{35}S-TR and incubated for 3 h at 20°C without (lanes 1 and 2) or with IBMX (125μM) (lanes 3 and 4). The oocytes were manually dissected into cytoplasmic (C) and nuclear (N) fractions, and analyzed as described in Fig 6. Representative results are shown. **(B) Summary of nuclear distribution of TRα in IBMX-treated *Xenopus* oocytes.** Films were scanned and the percent of nuclear TR was quantified by densitometry, using Scion imaging software. A student t-test was performed. Untreated, n=13; IBMX-treated, n=17 (where "n" represents the number of six pooled cytoplasms or nuclei).

nucleus. After treatment with IBMX, on average, the amount of ^{35}S-TRα localized to the nucleus after cytoplasmic injection increased significantly to 43% ($P < 0.02$) (Fig 7B). A similar trend was observed when nuclear import assays were carried out in the presence of okadaic acid (data not shown). However, okadaic acid increased oocyte mortality. Thus, insufficient replicates were obtained for statistical analysis. In summary, taken together, these data suggest that phosphorylation plays a role in the nuclear import and/or nuclear retention of TRα.

Phosphorylation/dephosphorylation of TRα in Xenopus oocytes is a rapid process

Having shown that the nucleocytoplasmic distribution of TRα can be manipulated by altering the phosphorylation state of the oocyte, it was of interest to determine the length of time necessary not only for phosphorylation but for transport into the nucleus. Once again, the *Xenopus* oocyte system was utilized. One group of oocytes was cytoplasmically microinjected with ^{35}S-TRα, a second was microinjected into the nucleus. A successful nuclear injection was determined by the presence of a red nucleus after manual dissection. The presence of a red nucleus indicated that the needle penetrated the nucleus and successfully injected the red lysate containing the ^{35}S-TRα. Within each of these treatments two incubation times were employed. Some oocytes were manually dissected immediately after microinjection. The remainder were incubated for 3 h at 20°C prior to dissection. The oocytes were then processed as previously described.

Figure 8 presents the results of this experiment. After immediate dissection, cytoplasmically microinjected TRα remained primarily in the cytoplasm, and showed the

faster mobility by SDS-PAGE indicative of unphosphorylated TRα (Fig 8A, lanes 1 and 2). In a few samples a very small fraction of TRα localized to the nucleus and showed the slower mobility by SDS-PAGE indicative of phosphorylated TRα (data not shown). This result suggests that the changes in phosphorylation state of TRα can occur quite rapidly. After 3 h, 36% of TRα localized to the nucleus, and, as expected, showed an altered mobility compared with cytoplasmic TRα (lanes 3 and 4). Nuclear microinjections yielded varying results. After immediate dissection, TRα remained largely nuclear in all samples and showed the slower mobility indicative of phosphorylated TRα (lanes 5 and 6). However, in some samples a small fraction of TRα either leaked into the cytoplasm from the injection site or was rapidly exported from the nucleus. This cytoplasmic TRα showed the faster mobility indicative of unphosphorylated TRα.

Interestingly, in one set of nuclear samples, TRα did not show an altered mobility; however, this result was not seen in any of the replicate experiments. This observation could be the result of variability in metabolic activity seen between batches of oocytes from different frogs, as well as the variability in the length of time taken for the "immediate" dissection. While the dissections occurred within an average of ten minutes, they certainly did not all occur at the same instant since the dissections were done manually. Since the changes in phosphorylation state appear to occur very rapidly, the window to observe the shift in mobility could certainly be missed in individual oocytes. This point seems to be supported by observations of oocytes that were allowed to incubate for 3 h after microinjection of TRα into the nucleus. Microinjected TRα remained primarily nuclear, with a small percentage of TRα localized in the cytoplasm

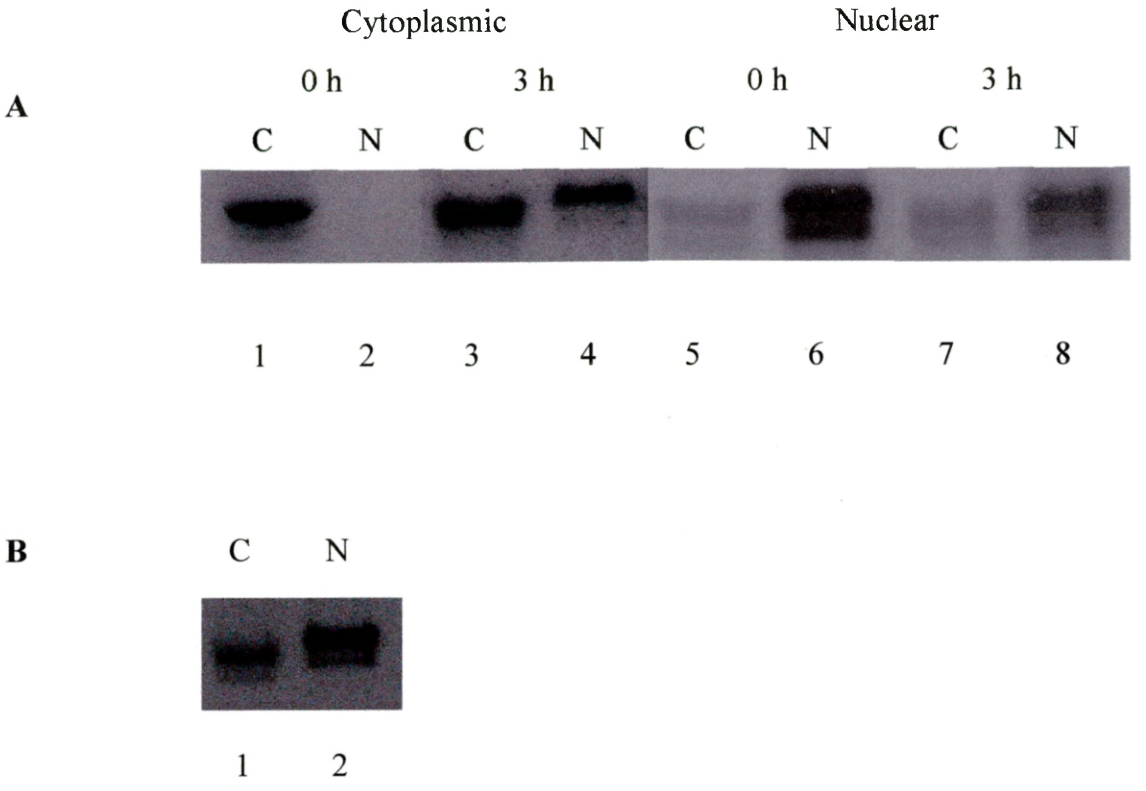

Figure 8. (A) Phosphorylation/dephosphorylation of TRα in *Xenopus* oocytes occurs rapidly. Oocytes were cytoplasmically injected with ^{35}S-TRα and were dissected immediately (lanes 1 and 2), or were incubated for 3 h at 20°C and then manually dissected (lanes 3 and 4). Alternatively, oocytes were injected into the nucleus and were dissected immediately (lanes 5 and 6), or were incubated for 3 h at 20°C and then manually dissected (lanes 7 and 8) into cytoplasmic (C) and nuclear (N) fractions, and analyzed as described in Fig 6. Representative results are shown (n=34). **(B) ^{35}S-TRα added to oocyte extracts changes phosphorylation state.** Uninjected oocytes were manually dissected into cytoplasmic (C) and nuclear fractions (N). Following dissection, ^{35}S-TRα was added to both cytoplasmic and nuclear extracts. Proteins were acetone-precipitated and analyzed by SDS-PAGE and fluorography.

(lanes 7 and 8). All the samples of nuclear TRα showed an altered mobility by SDS-PAGE, compared with the samples of TRα that had been exported to the cytoplasm. In summary, taken together, these studies suggest that phosphorylation/dephosphorylation of TRα is a rapid process and is complete after 3 h.

Further support for rapid changes in the phosphorylation state of TRα can be seen in Fig. 8B. Cytoplasmic and nuclear extracts were prepared from uninjected *Xenopus* oocytes (lanes 1 and 2). Subsequently, ^{35}S-TRα was added to both extracts. Proteins were then precipitated overnight in acetone. The extracted proteins were separated by SDS-PAGE and analyzed by fluorography. Interestingly, ^{35}S-TRα added to the nuclear extract showed an altered mobility when compared to the ^{35}S-TRα added to the cytoplasmic extract. There are at least two possible interpretations of this data. First, the rabbit reticulocyte lysate used for *in vitro* translation of ^{35}S-TRα, is known to have some kinase activity and could be phosphorylating TRα (Ohno et al., 2000; Promega, 2000). In this scenario, the cytoplasmic extract must contain phosphatases, which rapidly dephosphorylate TRα and alter the mobility of TRα in the cytoplasmic extract when compared to TRα in the nuclear extract where phosphatases are absent or in low amounts. A second possibility is that the rabbit reticulocyte lysate does not phosphorylate TRα. This scenario would suggest the presence of kinases in the nuclear extract, which rapidly phosphorylate TRα and thus cause the mobility shift. Distinguishing between these two possibilities will require further experimentation. It should also be noted that these scenarios may not be mutually exclusive. Eukaryotic cells are compartmentalized and contain different sets of proteins in each compartment to perform different functions. Thus, the cytoplasm may contain an overall phosphatase

activity while the nucleus may contain an overall kinase activity. The data presented here do not rule out this possibility. Regardless of which scenario is correct, these findings demonstrate the important point that changes in the phosphorylation state of TRα in *Xenopus* oocytes occur rapidly, within minutes.

Microinjection of in vitro-phosphorylated TRα

The results of the nuclear import assays suggested that the general phosphorylation state of the oocyte is important for the nuclear localization of TRα, and that changes in the phosphorylation state of TRα in oocytes occur rapidly. Thus, it was of interest to attempt to manipulate the phosphorylation state of TRα itself *in vitro*, and then to determine the effect of microinjecting *in vitro*-phosphorylated TRα on nuclear import and nuclear retention. As stated in the Introduction, the nuclear receptors are phosphoproteins. These receptors have been extensively studied and many of the phosphorylation sites have been identified. Rat TRα has a well-characterized phosphorylation site found at serine 12, which is phosphorylated by casein kinase II (Glineur et al., 1989; Goldberg et al., 1988). Interestingly, TRs isolated from chicken have more than one well-characterized phosphorylation site and these additional residues (serine 28,29) are phosphorylated by protein kinase A (Glineur et al., 1989; Tzagarakis-Foster and Privalsky, 1998). To ensure that serine 12 was the residue phosphorylated, two *in vitro* reactions were performed using both casein kinase II and protein kinase A to test the ability of each kinase to phosphorylate rat TRα *in vitro*. The results from these control experiments showed that only casein kinase II phosphorylated TRα (data not shown), suggesting that indeed serine 12 was being correctly targeted since this residue

has been shown to be phosphorylated by casein kinase II. Thus, for subsequent experiments, ^{35}S-TRα was incubated with casein kinase II and γ^{32}P ATP for 30 minutes. This phosphorylation reaction allowed for the incorporation of the second label to the protein at serine 12. The $^{35}S^{32}$P-TRα was microinjected into the cytoplasm of *Xenopus* oocytes. After a 1 h or 3 h incubation, the oocytes (n=36) were manually dissected and proteins extracted from the nuclear and cytoplasmic fractions. The proteins were then separated by SDS-PAGE, and dried gels were exposed to two X-ray films. Using two films provides a method to visualize both the phosphorylated and unphosphorylated forms, as the ^{32}P will expose both films while the ^{35}S will only expose the film closest to the dried gel (Murdoch and Allison, 1996).

Unfortunately, this method proved to be ineffective in that ^{32}P-TRα was undetectable in the proteins recovered from the oocytes, although the ^{35}S-TRα was detectable. This result was surprising since ^{32}P-TRα bands could be detected after the phosphorylation reaction (data not shown). However, the rabbit reticulocyte lysate used in the transcription/translation reaction to produce ^{35}S-TRα contains a number of abundant proteins that can be phosphorylated (Promega, 2000). Multiple bands were seen on both films after the phosphorylation reaction, suggesting that a large percentage of the ^{32}P label was incorporated into these other proteins. Furthermore, since only a small amount of TRα is translated using this *in vitro* system, and only a small amount of protein was injected into the oocytes, it is possible that, in combination with inefficient labeling of TRα, the amount of ^{32}P-TRα recovered from each fraction was below the sensitivity limits of the X-ray film. The possibility also remains that after injection TRα was dephosphorylated and then re-phosphorylated with non-radioactive phosphorus

within the oocytes, making it impossible to visualize phosphorylated TR by this method. This method of *in vitro* phosphorylation may prove useful once a substantial amount of purified TRα can be produced by over-expression in bacteria, after construction of an appropriate expression vector.

Phosphorylation of TRα at serine 12 is not required for nuclear import or nuclear retention

Having shown that nuclear TRα is phosphorylated and that IBMX significantly enhances the nuclear localization of TRα by altering the phosphorylation state of the oocyte, it was of interest to determine which phosphorylation site in TRα is important for nuclear import and nuclear retention of TRα. As stated earlier, serine 12 is a well-characterized phosphorylation site and thus was chosen as the site to analyze (Glineur et al., 1989; Goldberg et al., 1988). Four mutant expression vectors for TRα were constructed; a deletion mutant which had the sequence coding for the first 12 amino acids deleted and three mutants with nucleotide substitutions converting the codon for serine to codons for alanine, threonine, or glutamic acid, respectively. These latter three mutations were chosen for the following reasons: 1) alanine residues cannot be phosphorylated and thus test the importance of phosphorylation at this site, 2) threonine residues can be phosphorylated and thus test whether it is a requirement for a serine at this position, and, 3) glutamic acid residues cannot be phosphorylated but mimic the negative charge associated with phosphorylation. *In vitro*-translated TRα mutants or wild type TRα were then microinjected into the cytoplasm of *Xenopus* oocytes and incubated for 3 h at 20°C to allow the nucleocytoplasmic distribution of TRα to reach a steady-state.

All the mutants showed a similar nucleocytoplasmic distribution when compared to the wild type (Fig. 9A). In this set of experiments, 39% (n=18) of wild type TRα localized to the nucleus. Similarly, 40% (n=10) of the glutamic acid mutant, 40% (n=14) of the alanine mutant, 42% (n=12) of the threonine mutant, and 39% (n=8) of the Δ12 deletion mutant localized to the nucleus after cytoplasmic injection. Statistical analysis confirmed that the distribution patterns of mutant and wild type TRαs were not significantly different (student's t-test, $P>0.15$). It should be noted that the percentage of wild type TRα localized to the nucleus is higher than seen in the experiments summarized in Fig. 7B. This result could simply be due to the differences seen between different batches of oocytes. However, in the experiments shown in Fig. 7, control oocytes were incubated in medium containing DMSO and it is possible that DMSO slightly inhibits nuclear import in an unknown fashion.

Surprisingly, the alanine, glutamic acid, and Δ12 deletion mutants, which cannot be phosphorylated at position 12, all showed an altered mobility on SDS-polyacrylamide gels similar to the altered mobility of wild type TRα previously described (Fig. 6A). To determine if the altered mobility of these mutants was due to phosphorylation, alkaline phosphatase assays were performed on the alanine and deletion mutants as well as the wild type TRα. Figure 9B shows the results for the wild type and the Δ12 deletion mutant. Alkaline phosphatase treated nuclear samples had the same mobility as untreated cytoplasmic samples (compare lanes 1 and 3 and lanes 4 and 6). The mock-treated nuclear samples maintained their altered shift when compared to the untreated cytoplasmic samples or the alkaline phosphatase-treated samples (compare lane 2 with lanes 1 and 3, and lane 5 with lanes 4 and 6). Likewise, alanine mutant TRα recovered

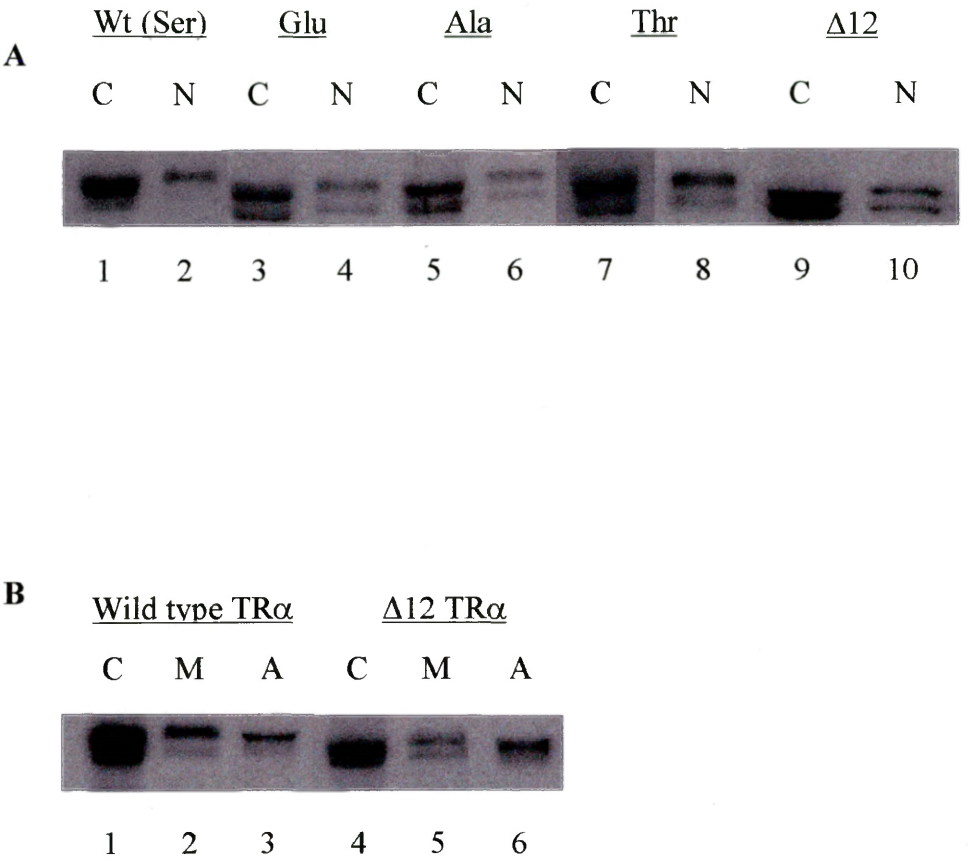

Figure 9(A). Phosphorylation of TRα at serine 12 is not required for nuclear import or nuclear retention. ^{35}S-labeled position 12 mutants were injected into the cytoplasm of *Xenopus* oocytes. The oocytes were incubated for 3 h and divided into cytoplasmic (C) and nuclear (N) fractions and analyzed as described in Fig. 6. Lanes 1 and 2 represent wild type TRα (serine at position 12); 3 and 4, the glutamic acid mutant; 5 and 6, the alanine mutant; 7 and 8, the threonine mutant; and 9 and 10, the Δ12 deletion mutant. **(B) TRα has other phosphorylation sites.** *Xenopus* oocytes were cytoplasmically microinjected with ^{35}S-TRα (lanes 1-3) and the Δ12 deletion mutant (lanes 4-6). After 3 h incubation at 20°C, oocytes were manually dissected and proteins extracted from 6 cytoplasmic (C) or nuclear fractions. Nuclear fractions were treated with alkaline phosphatase (A) and incubated for 1 h at 37°C or were mock-treated with buffer alone (M). Cytoplasmic fractions were left untreated. Proteins were separated by 12% SDS-PAGE, followed by fluorography.

from alkaline phosphatase treated nuclear fractions did not show an altered mobility after SDS-PAGE; instead the mutants had the same mobility as mutant TRα recovered from untreated cytoplasmic samples (data not shown). Thus, the mobility shift demonstrated by the mutants appears to be due to phosphorylation, suggesting that there are other important, but cryptic phosphorylation sites within TRα.

Discussion

Phosphorylation has been shown to be important in regulating a number of cellular and molecular events, including nuclear import and retention of transcription factors. In the present study, *Xenopus* oocyte microinjection assays have shown that phosphorylation of a well-characterized casein kinase II site at serine 12 is not necessary for the nuclear import and retention of TRα. However, a phosphorylated form of TRα is present in the oocyte nucleus and increasing the phosphorylation state of the oocyte enhances nuclear retention of TRα. Furthermore, changes in the phosphorylation state of TRα occur rapidly and are compartment-specific. Taken together, these data provide evidence that, phosphorylation of a site(s) other than serine 12 has an important role in regulating the nuclear activity of TRα.

Phosphorylation of serine 12 is not necessary for the nuclear import and retention of TRα

Since TRα is a phosphoprotein, there is the potential that some of its functions are regulated by phosphorylation. These functions could include DNA binding, hormone binding, transcriptional activation, dimer formation, and nuclear import and retention. TRα has recently been shown to be a shuttling protein and, thus, phosphorylation could play a role in regulating its intracellular movement (Bunn et al., 2001). Serine 12 is the most well-characterized phosphorylation site, making this residue a good candidate for

mutagenesis (Glineur et al., 1989; Goldberg et al., 1988). Furthermore, this residue is conserved in all members of the TR family suggesting an important biological role. Results from this research suggest, however, that serine 12 is not necessary for the nuclear import and retention of rat TRα. The individual mutants generated in this study not only removed the ability of this residue to be phosphorylated (alanine mutant and Δ12 deletion mutant), but also mimicked the negative charge found on a phosphorylated residue (glutamic acid mutant) (Glineur et al., 1990). All the mutant proteins were imported into the nucleus and showed a similar nucleocytoplasmic distribution when compared to the wild type. Most strikingly, none of the first twelve amino acids, which include another predicted phosphorylation site, a serine at position 6 (Fig. 10), were shown to be necessary for nuclear import. The Δ12 deletion mutant also entered the nucleus and showed the same nucleocytoplasmic distribution as full length TR. This mutant also showed an altered mobility on denaturing SDS polyacrylamide gels, providing further evidence that there are other phosphorylation site(s). These observations are in agreement with an earlier report showing that the phosphorylation site at position 12 in chicken TRα was not essential for nuclear localization in cultured mammalian cells (Andersson and Vennström, 1997). Interestingly, chicken TRα has a well-characterized protein kinase A phosphorylation site at serines 28 and 29; however, this site is not conserved in rat TRα (Jones et al., 1994; Sugawara et al., 1994). This is of interest since this site has been shown to be important for the biological activity of the oncogenic homolog of TRα v-*erb*A (Glineur et al., 1989).

The results reported here showing that phosphorylation of serine 12 is not necessary for nuclear import of TRα do not rule out the possibility that phosphorylation

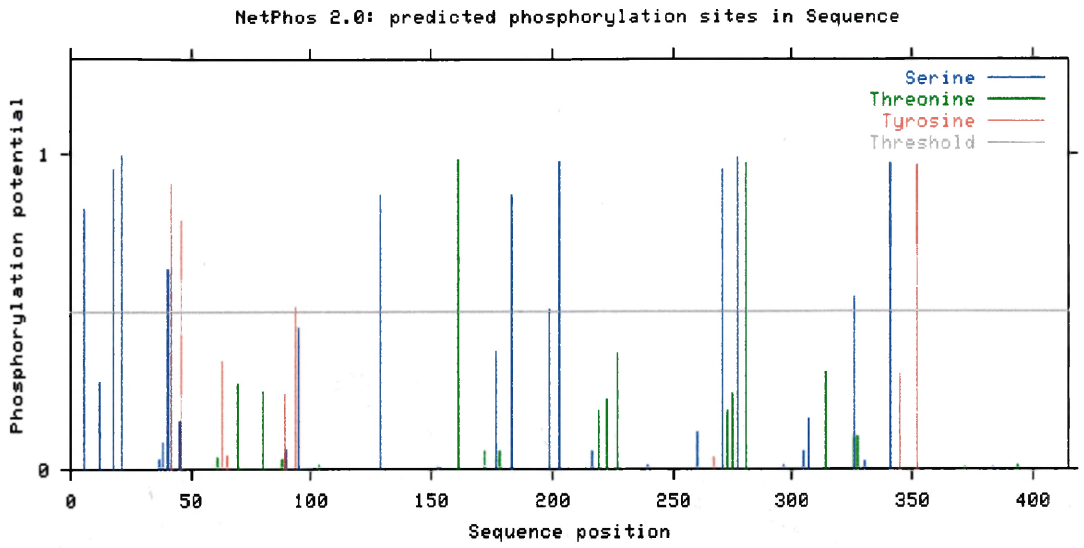

Figure 10. Predicted phosphorylation sites in TRα. Results showing putative phosphorylation sites using the NetPhos 2.0 search engine. Threshold is the likelihood that a particular site will be phosphorylated. This value is set at 50, any site below this number or close to it is not likely to be a phosphorylation site, while higher values are more likely to be a true phosphorylation site.

of one or more other sites is necessary for nuclear import. All the mutants retained their ability to be phosphorylated, suggesting that there is indeed at least one other phosphorylation site that has yet to be characterized. Phosphorylation of these additional individual site(s) may be necessary for nuclear import, or these sites may work in consort to contribute to the phosphorylation state of TRα, allowing nuclear import. Thus, these sites would be able to maintain the necessary phosphorylation state for import, even in the event of the loss of one of the other sites.

Phosphorylation has been shown to have a role in the nuclear import of a number of proteins including Dorsal, Pho4, and the hepatitis B core particle (Drier et al., 1999; Kaffman et al., 1998; Kann et al., 1999). Of particular interest is the regulation of the transcription factor Pho4, since it has been shown that its nuclear activity is regulated by its phosphorylation state. When Pho4 is unphosphorylated by the actions of a kinase inhibitor, transcription occurs; however, when Pho4 is phosphorylated, it is exported to the cytoplasm. Furthermore, this transcription factor has multiple phosphorylation sites with different functions including import, export, and binding to other transcription factors (Komeili and O'Shea, 1999). The phosphorylation of these different sites provides tight regulation of the activity of Pho4. These results could provide a model for TRα, since it is clear from the results of the present study that there are multiple phosphorylation sites present within TRα. The phosphorylation or dephosphorylation of these sites could regulate the specific activities of TRα.

TRα has been shown to shuttle rapidly between the nucleus and cytoplasm in mammalian cells (Bunn et al., 2001). Phosphorylation could play a role in this shuttling, since it has been shown that the glucocorticoid receptor (GR) shuttling is regulated by

phosphorylation (Dean et al., 2001; DeFranco et al., 1991). In this case, researchers reported that protein phosphatase types 1 and 2A regulated the shuttling of GR. Inhibiting the phosphatases led to a loss of nuclear retention, which caused GR to remain in the cytoplasm (DeFranco et al., 1991). Further evidence for the role of phosphorylation in the shuttling of GR is provided by Dean et al. (2001). They report that protein phosphatase 5 (PP5) is involved in the regulation of GR shuttling. Suppression of PP5 results in the nuclear accumulation of phosphorylated GR and leads to an increase in transcriptional activity (Dean et al., 2001). The mechanisms suggested for GR shuttling may provide a model for TR shuttling.

It is clear that phosphorylation plays a role in the activity of a host of transcription factors, thus finding and characterizing the cryptic sites in TRα provides an important area of research. These uncharacterized sites may explain some of the conflicting results found in the literature regarding the importance of phosphorylation for hormone binding and DNA binding, as well as the importance of various phosphorylation sites for other activities of TRα. It is certainly possible that differing experimental conditions could trigger these cryptic sites in different ways, thus creating the confusion seen in the literature. For example, many of the DNA binding studies (see Introduction, "*TR phosphorylation*") used different cell lines, which have varying phosphatase and kinase concentrations. It is conceivable that in these different backgrounds some phosphorylation sites of TRα would be more active than others, thus yielding different results. This possibility is not without precedence; for example, the relative importance of phosphorylation of Dorsal in *Drosophila* (see Introduction, "*Phosphorylation of transcription factors and viral proteins*") varies in different backgrounds. In a wild-type

signaling background there was only a weak loss of function in phosphorylation mutants of Dorsal (Drier et al., 1999). However, when these same mutants were placed in a Cactus mutant background the Dorsal phosphorylation mutants were completely destabilized. These results demonstrate that the design of the experiment is critical and must be considered when analyzing the data.

Phosphorylation enhances nuclear retention of TRα

There are many examples in the literature demonstrating the importance of phosphorylation in the activity of transcription factors (DeFranco et al., 1991; Komeili and O'Shea, 1999; Moroianu, 1999). Of particular interest, several reports have shown that phosphorylation of nuclear receptors can alter DNA binding affinity. In some cases, DNA binding of ER and PR, as well as TR, has been shown to be enhanced in the presence of compounds known to increase phosphorylation (Arnold et al., 1995; Bagchi et al., 1992; Bhat et al., 1994; Denton et al., 1992; Lin et al., 1992). However, there are other reports which demonstrate that phosphorylation is not necessary for DNA binding. Despite these conflicting reports, increased nuclear retention of phosphorylated TRα because of enhanced DNA binding remains an intriguing possibility, since it has also been shown that phosphorylation of TRα can increase transcriptional activation of T_3-responsive genes (Jones et al., 1994; Ting et al., 1997). Despite serine 12 not being necessary for nuclear import of TRα, it is clear from the results of this thesis and previous studies that phosphorylation plays a role in the nuclear events of TRα. In *Xenopus* oocytes, TRα is not localized exclusively to the nucleus. This lack of localization allowed the manipulation of the phosphorylation state of the oocytes and in

turn changed the localization pattern of TRα. Increasing the overall phosphorylation state of *Xenopus* oocytes by treatment with IBMX, a known inhibitor of phosphodiesterase and a protein kinase A activator, led to a significant increase in the nuclear localization of TRα. This result is consistent with studies mentioned above which showed that phosphorylation can enhance DNA binding *in vitro* and increase dimerization with RXR, both of which would promote nuclear localization (Bhat et al., 1994; Tzagarakis-Foster and Privalsky, 1998). Further evidence that phosphorylation enhances nuclear localization of TRα is provided by the current finding that phosphorylated TRα injected into the nucleus of *Xenopus* oocytes remains predominantly nuclear even after 3 hours. Taken together, these results lead to several interesting questions. Does the phosphorylated form of TRα bind DNA with greater affinity *in vivo*, thus promoting nuclear retention? Does phosphorylation increase the rate of nuclear import or slow down the rate of nuclear export of TRα?

Phosphorylation, by enhancing DNA binding could allow more TRα to remain in the nucleus, which would in turn lead to an increase in transcription in the presence of T_3, and to an increase in gene repression in the absence of T_3. Mammalian cells show a different distribution of TRα compared with *Xenopus* oocytes, as TRα is primarily nuclear but individual receptors undergo rapid nucleocytoplasmic shuttling (Bunn et al., 2001). As discussed earlier, GR also undergoes shuttling and this shuttling is regulated by phosphorylation/dephosphorylation signals (Dean et al., 2001; DeFranco et al., 1991). A similar model could be applied to TRα shuttling in mammalian cells, in which phosphorylation could effect the rate of import or export. Nuclear TRα could be largely phosphorylated. However, at any given time, an individual receptor could be

dephosphorylated and exported, and then quickly reimported. Nuclear retention of TRα could thus be the result of the export machinery being unable to bind a phosphorylated receptor. There is evidence that phosphorylation/dephosphorylation can directly alter the binding affinity of a protein for its import/export machinery. One example is in yeast where Gln3p, a transcription factor for the nitrogen catabolite repressible gene, has its nucleocytoplasmic distribution determined by its phosphorylation state. When phosphorylated this protein is unable to bind to its import receptor and thus remains cytoplasmic (Carvalho et al., 2001). This inability to bind TR could also be the result of the phosphorylated receptor's increased association with DNA, as previously discussed, or of the receptor binding to the nuclear matrix. The nuclear matrix is an insoluble array of filaments which provides the structure and compartmentalization of the nucleus (DeFranco, 1997). There is evidence that unliganded GR can either bind to the nuclear matrix after release from chromatin or undergo nuclear export (DeFranco, 1997; Yang et al., 1997). TR has also been shown to associate with the nuclear matrix (C. Bunn, N. Hollingshead, P. Garcia, L. Allison, unpublished results). Thus, a model could be proposed in which this binding would prevent access to the export machinery and slow down the rate of export. Clearly, more work needs to be done in both mammalian cells and *Xenopus* oocytes to test this model and complete our understanding.

Changes in phosphorylation state occur rapidly in Xenopus oocytes

To be effective as a molecular and signaling mechanism, a signal must be rapid. This present study demonstrates that dephosphorylation/phosphorylation of TRα in

Xenopus oocytes occurs, within minutes or less. These results are in agreement with work done with PHAX, a nuclear export protein (Ohno et al., 2000). In this study, PHAX was *in vitro* translated in rabbit reticulocyte lysate and microinjected into either the nucleus and cytoplasm of *Xenopus* oocytes. The authors report that PHAX is subject to rapid dephosphorylation in the cytoplasm, while PHAX injected into the nucleus remains phosphorylated (Ohno et al., 2000). Furthermore, unphosphorylated PHAX, when injected into the nucleus underwent phosphorylation (Ohno et al., 2000). A similar distribution is reported here: TRα injected into the nucleus remained primarily nuclear after 3 h and showed the slower mobility on SDS-PAGE associated with phosphorylation, while TRα found in the cytoplasm was unphosphorylated and had a slower mobility. Furthermore, TRα injected into the cytoplasm remained primarily in the cytoplasm after immediate dissection and showed the same mobility as cytoplasmic fractions after 3 h demonstrating the dephosphorylated form of TRα.

Ohno et al. (2000) showed that rabbit reticulocyte lysate has kinase activity and is capable of phosphorylating PHAX. Interestingly the authors also demonstrated that PHAX can be phosphorylated by casein kinase II *in vitro*. This activity was confirmed by adding rabbit reticulocyte lysate to PHAX translated in bacteria, which is unphosphorylated. In the presence of the lysate PHAX was phosphorylated and showed an altered mobility on SDS-PAGE. This kinase activity is of particular interest since it has been shown that TRα can also be phosphorylated by casein kinase II at serine 12 and several of the putative sites, specifically serine 45 and serine 203, show casein kinase II consensus sites (Fig.10). Unfortunately, a bacterial expression vector, that would generate an unphosphorylated TRα, since eukaryotic proteins do not undergo post-translational

modification in bacteria, is not currently available. Thus, direct confirmation of phosphorylation occurring in the nucleus cannot be performed yet. However, taken together the results presented here suggest that, similarly to PHAX, phosphorylation occurs rapidly and probably occurs in the nucleus. More experiments will need to be performed in order to demonstrate that phosphorylation occurs in the nucleus of *Xenopus* oocytes. If this is indeed the case, this would suggest that phosphorylation of TRα does not directly effect nuclear import but, instead, enhances nuclear retention and inhibits export.

Future directions

Research on TR function and its regulation has expanded greatly over the last decade; however, more research is needed. First, as Fig. 10 shows, there are a number of potential phosphorylation sites. One or several of these sites could be important in the cellular and molecular events described in this study, such as nuclear retention and nuclear import and export. Clearly, more work needs to be done to confirm the presence of these sites, including carrying out site-directed mutagenesis to learn how these regions effect receptor behavior. In addition, more research needs to be done on manipulating the phosphorylation state of the cell system being studied. There are a host of inhibitors and activators of kinases and phosphatases available that could be used, including protein phosphatase 1 and 2A, PKA, H7, or forskolin, as well as phosphate depletion assays. Antibodies against specific kinases found in the cell system being used could provide a great deal of information. To complete the story of phosphorylation, this work needs to be complemented with mammalian cell line studies, including phosphate depletion

assays, and nucleocytoplasmic shuttling assays in heterokaryons, as well as *in vitro* nuclear import assays. These assays will help complete the picture and allow for the development of a model for nuclear retention and localization. Finally, the development of a bacterial expression vector for TRα would greatly enhance the study of phosphorylation. The ability to purify mg quantities of protein would make it easier to study the localization and movement of this receptor. A greater quantity of protein would make it possible to phosphorylate TRα *in vitro*, and, as mentioned earlier, TRα generated from bacteria would not be phosphorylated and thus could be used to provide direct evidence for the role of phosphorylation in nuclear retention and for the specific sites of phosphorylation.

Conclusion

With regard to TRα the role of phosphorylation in cellular and molecular events remains enigmatic. Results presented here show that phosphorylation increases nuclear retention of TRα in *Xenopus* oocytes. The mechanism remains unclear, however, and this uncertainty generates exciting new directions for research. Furthermore, this research shows that the first 12 amino acids are not necessary for nuclear import. Similarly, the phosphorylation state of serine 12 is not necessary for either nuclear import or retention. However, given that nuclear TRα is phosphorylated, it remains to be determined whether the phosphorylation state of TRα is directly involved in its nuclear import or whether phosphorylation occurs in the nucleus and plays a direct role in promoting nuclear retention.

Phosphorylation plays a major role in human health. Over 700 protein kinases and phosphatases are encoded by the human genome (Cohen, 2001). A host of human diseases are the result of mutations or malfunctions in the phosphorylation machinery, including some forms of leukemia, Non-Hodgkins lymphoma, and diabetes (Cohen, 2001). Furthermore, many pathogens produce virulence factors that target the phosphorylation machinery, most notably the bacteria *Yersinia* which causes bubonic plague (Cohen, 2001). Malfunctions in TR function such as mutations in TRβ lead to the disease resistance to thyroid hormone. The resulting disease has various symptoms including goiter and slow development. Thus, understanding the mechanisms regulated by phosphorylation, and the proteins which carry out these functions, is of great importance.

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