

# A Role for Thyroid Hormone Transporters in Transcriptional Regulation by Thyroid Hormone Receptors

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Thyroid hormones (THs) must be taken up by target cells to act at the genomic level through binding to nuclear thyroid hormone receptors (TRs). Extensive study has been made of mechanisms by which TH-bound TRs regulate transcription, yet little is known about the critical upstream step, *i.e.* how THs enter the cell. Growing evidence suggests that saturable transport mechanisms mediate the greater part of TH movement across the plasma membrane and have important roles in the regulation of TH bioavailability. For example, System L is a multifunctional transport system serving as a plasma membrane transporter of THs and amino acids in mammalian cells. We have used two complementary systems, the *Xenopus* oocyte (which

has negligible basal System L activity) and the mammalian BeWo cell line (which has System L activity for TH transport), to investigate the role of this representative TH transporter in nuclear action of THs. We demonstrate that overexpression of System L in *Xenopus* oocytes increases both cytoplasmic and nuclear delivery of THs from external medium and also enhances transcriptional activation by TRs. Conversely, blocking endogenous System L activity in BeWo cells with specific inhibitors reduces both TH uptake and TR function. These results indicate that plasma membrane TH transporters such as System L may have important roles in gene regulation by TRs. (*Molecular Endocrinology* 17: 653-661, 2003)

THYROID HORMONES (THs; T<sub>4</sub> and T<sub>3</sub>) regulate growth, development, and critical metabolic functions in vertebrates. Both hormones are secreted by the thyroid gland, although most plasma T<sub>3</sub> is produced by extrathyroidal deiodination of T<sub>4</sub>. Major effects of THs are exerted at the genomic level through binding of hormone (predominantly T<sub>3</sub>) to nuclear thyroid hormone receptors (TRs; *e.g.* Ref. 1 for review). The plasma free hormone concentration appears to be the key determinant of biological activity (2). The proportion of nuclear receptor-bound T<sub>3</sub> originating from plasma T<sub>3</sub> or local deiodination of T<sub>4</sub> varies between tissues, but it is clear that both hormones must first cross the plasma membrane of target cells to exert genomic effects (2, 3). Although THs have a lipophilic nature, the polar amino acid side chain retards their passage across cell membranes such that they are likely to partition into, but not diffuse across, them (4). There is growing evidence (*e.g.* Refs. 2, 5-7) that saturable transport mechanisms (rather than diffusion) mediate the greater part of TH movement across the plasma membrane and have important roles in the

regulation of TH metabolism and bioavailability. A variety of TH transport mechanisms have recently been identified, including transporters shared with certain amino acids [*e.g.* System L (8-10)] or organic anions [*e.g.* members of the oatp family (6, 11)]. The recognition that these transporters are of major importance for delivery of TH to the cell interior raises the strong possibility that TH transport across the cell membrane is an important but overlooked step for control of cellular TH signaling and action (2, 12). Nevertheless, beyond some evidence to suggest that active processes help extracellular THs reach cell nuclei (13-15), there is surprisingly little information regarding the overall importance of membrane transport in control of TH delivery to nuclear TRs and thus of cellular TH action.

In the present study we have attempted to clarify this issue using two different but complementary experimental systems, namely 1) the *Xenopus* oocyte and 2) mammalian (BeWo) cell culture. Our earlier studies have shown that System L [a transport activity produced by members of the SLC7 gene family (16)] is a multifunctional transport system that serves as a plasma membrane transporter of TH as well as amino acids in a variety of cell types (*e.g.* Refs. 5, 17, and 18). The System L TH transporter was first identified as an amino acid transporter and, for simplicity, we retain

Abbreviations: BCH, 2-Amino[2,2,1] heptane-2-carboxylic acid; MBM, modified Barth's medium; RXR, retinoic acid receptor; TH, thyroid hormone; TR, thyroid hormone receptor.

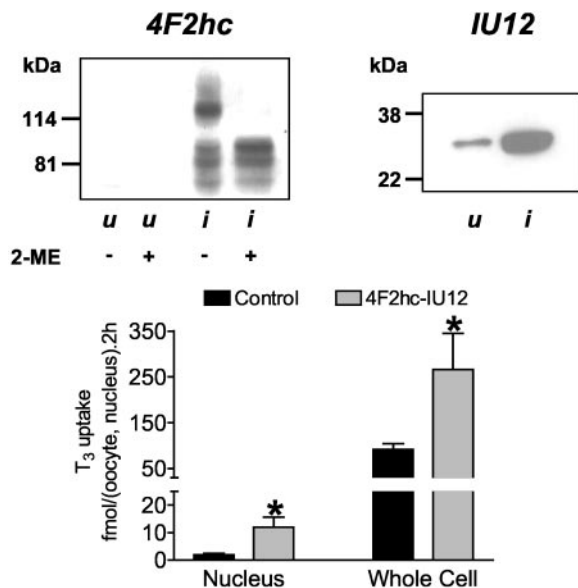
here the conventional nomenclature for amino acid transporters (16). We focus here on System L as a representative TH transporter and demonstrate that it has an important role not only in the cellular uptake of TH but more importantly in overall delivery of extracellular TH to the nucleus, where the hormone exerts its biological function by regulating transcription by TRs. We show that in the *Xenopus* oocyte, which has negligible basal System L activity, overexpression of a System L transporter leads to increased cellular uptake and nuclear appearance of TH and also enhances transcriptional activation by TRs. Similarly, System L blockade in the mammalian BeWo cell line is shown to inhibit cellular uptake and nuclear delivery of TH and reduce transcriptional activation by TRs. These results indicate that plasma TH transporters such as System L may have important roles in mediating and/or regulating the biological functions of THs.

## RESULTS

### Overexpression of System L TH Transporter in *Xenopus* Oocytes Enhances Cellular Uptake and Nuclear Appearance of TH

We first investigated whether overexpression of System L transporter could render a cell more sensitive to effects of external TH. For this series of experiments we used the *Xenopus* oocyte. The System L transporter is formed as a heteromeric complex consisting of a heavy-chain glycoprotein (4F2hc) and a light-chain permease of the glycoprotein-associated amino acid transporter family (16): here we use IU12, the *Xenopus* LAT1 permease (19). The *Xenopus* oocyte has low levels of IU12, which can be enhanced by injecting IU12 mRNA (Fig. 1, upper panel). This low level of endogenous IU12 is insufficient for maximal function of overexpressed 4F2hc (8). 4F2hc and IU12 proteins overexpressed together in *Xenopus* oocytes (Fig. 1, upper panel) form heteromeric complexes in oocyte membranes, as visualized on Western blots under nonreducing conditions (shown here for 4F2hc only).

To investigate the function of these 4F2hc-IU12 heteromeric complexes, we studied the uptake of  $T_3$  into oocytes from the external medium. As shown in Fig. 1 (lower panel), the overexpression of 4F2hc-IU12 heteromeric complexes led to the formation of functional System L (LAT1-like) transporter activity (8, 19), resulting in a 3-fold increase in whole-cell  $T_3$  uptake, which is blocked by the competing System L substrate 2-amino [2,2,1] heptane-2-carboxylic acid (BCH) (8). This increased  $T_3$  uptake resulted in a marked increase in overall delivery of extracellular  $T_3$  to the oocyte nucleus (6.25-fold increase in nuclear appearance over the same period; Fig. 1, lower panel). Increased nuclear  $T_3$  delivery appeared unlikely to reflect any increase in TH transport capacity of the nucleus under these circumstances, because System L transporters overexpressed in oocytes do not localize to the nu-



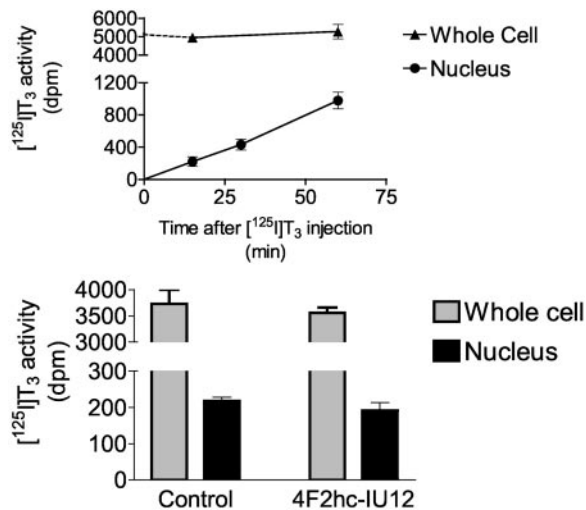
**Fig. 1.** Overexpression of System L Transporter Increases Cellular Uptake and Nuclear Appearance of  $^{125}I$ - $T_3$  in *Xenopus* Oocytes

Oocytes were coinjected with cRNA of System L subunits (4F2hc and IU12) 3 d before the experiment. *Upper panel*, Detection of overexpressed 4F2hc and IU12 proteins in oocyte membranes. Western blots probed with antibodies to detect 4F2hc (50  $\mu$ g membrane protein/lane) or IU12 (15  $\mu$ g membrane protein/lane). No 4F2hc protein is detected in membranes from native uninjected (u) oocytes, although IU12 is expressed at relatively low levels in these cells. In 4F2hc-IU12 cRNA injected cells (i), 4F2hc appears as a broad band with two distinct peaks (at 82 and 90 kDa; probably reflecting different glycosylation states) under reducing conditions (+2-mercaptoethanol; 2-ME), whereas under nonreducing conditions it appears predominantly as higher molecular weight species consistent with formation of a heteromeric 4F2hc-IU12 holotransporter. *Lower panel*, Functional expression of System L as a TH transporter. Oocytes were incubated in uptake buffer containing 100 nM  $^{125}I$ - $T_3$  for 2 h and processed into nuclear and extranuclear fractions for radioactivity assay. Whole-cell uptake is the sum of  $^{125}I$ - $T_3$  associated with nuclear and extranuclear fractions. Representative result showing mean uptake/appearance  $\pm$  SEM for single batch of oocytes (three to six measurements per data point). \*, Value significantly different from respective control value ( $P < 0.05$  by Student's *t* test).

clear membrane in detectable quantities (19, 20). Nevertheless, we confirmed this directly by showing that the initial rate of nuclear appearance of  $^{125}I$ - $T_3$  injected into the cytoplasm was equal in control and System L-overexpressing oocytes (Fig. 2). These results indicate that the surface permeability of the oocyte to  $T_3$  is a key factor limiting movement of the hormone from extracellular medium to the cell nucleus.

### System L TH Transporter Facilitates Transcriptional Activation by TR

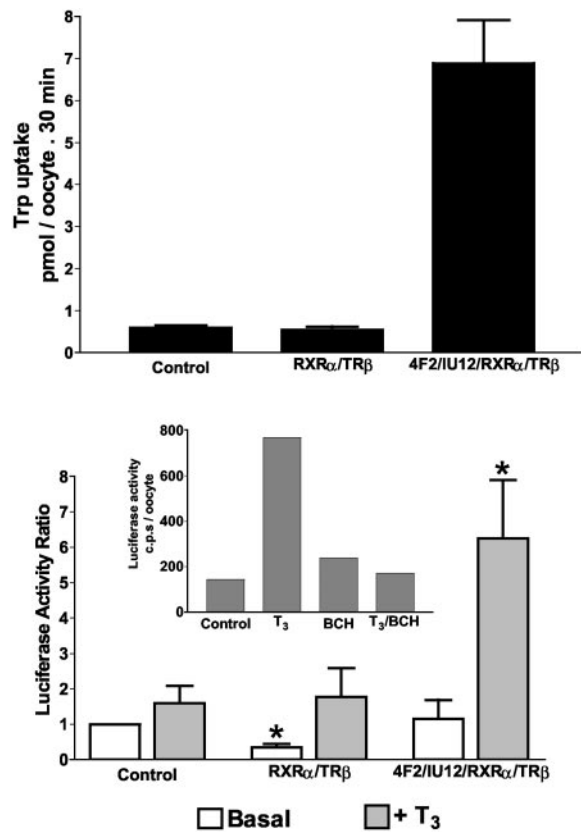
As the major biological effects of THs are mediated through transcriptional regulation by TRs, we re-



**Fig. 2.** Overexpression of System L Transporter in *Xenopus* Oocytes Does Not Increase Nuclear Uptake of  $^{125}\text{I}$ - $\text{T}_3$  Injected into the Cytoplasm

Oocytes were coinjected with cRNA of System L subunits (4F2hc and IU12) 3 d before the experiment as indicated. *Upper panel*, Time course of nuclear appearance of cytoplasmic  $^{125}\text{I}$ - $\text{T}_3$ . The injected  $^{125}\text{I}$ - $\text{T}_3$  tracer bolus distributes in the cytoplasm and subsequently appears in the oocyte nucleus with a linear time course over a postinjection period of at least 1 h. There is no significant leakage of  $^{125}\text{I}$ - $\text{T}_3$  from oocytes over this period [whole-cell disintegrations/min (dpm) data]. Whole-cell radioactivity is the sum of  $^{125}\text{I}$ - $\text{T}_3$  disintegrations/min associated with nuclear and extranuclear fractions. Representative result showing mean disintegrations/min  $\pm$  SEM for three measurements per data point (symbols mask small error bars in some cases). *Lower panel*, Whole-cell and nuclear  $^{125}\text{I}$ - $\text{T}_3$  activities 30 min after injection of  $^{125}\text{I}$ - $\text{T}_3$  into cytoplasm of intact oocytes. Here, nuclear  $^{125}\text{I}$ - $\text{T}_3$  activity reflects initial-rate appearance of tracer in oocyte nuclei over a 30-min period (see *top panel* data). Results shown are mean disintegrations/min  $\pm$  SEM for six experiments. There was no significant difference between either whole-cell or nuclear  $^{125}\text{I}$ - $\text{T}_3$  activities from control and System L (4F2hc-IU12)-overexpressing oocytes.

soned that changes in TH delivery to the cell nucleus should be important for TH action. To investigate whether the System L TH transporter can thus participate in gene regulation by TRs, we took advantage of the ability to establish a TH-dependent transcriptional system in the *Xenopus* oocyte (21). The *Xenopus* oocyte has little endogenous TR to allow TH-dependent transcription (Fig. 3 and Ref. 21). On the other hand, exogenous TRs can be easily introduced into the oocyte by microinjection of the cRNA encoding TRs. As retinoic acid receptor (RXR) is important for TR function in the oocyte (21), we coinjected TR and RXR cRNAs to allow the accumulation of TR/RXR heterodimers in the oocyte nucleus, thus establishing a TH-responsive system. TR/RXR expression had little effect on basal TH uptake; neither did it prevent the increase in System L transport activity produced by coexpression of System L transporter subunits (Fig. 3, *upper panel*).



**Fig. 3.** Overexpression of System L Transporter Stimulates  $\text{T}_3$ -Dependent Gene Transcription in *Xenopus* Oocytes

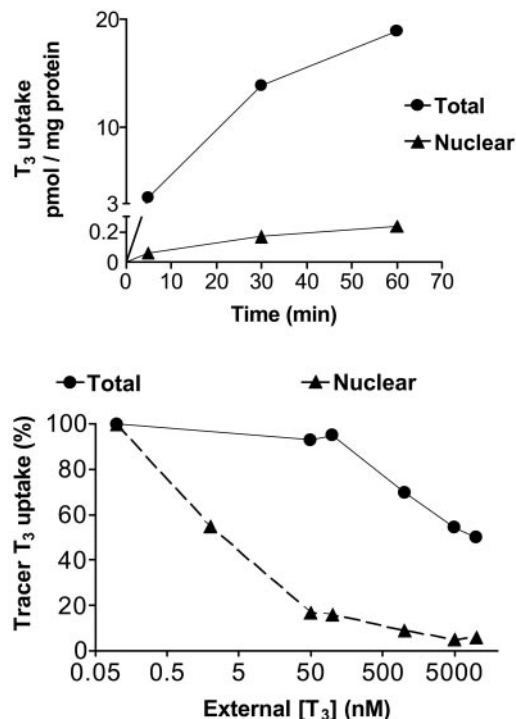
Oocytes were coinjected with cRNA of System L subunits (4F2hc and IU12) and/or thyroid receptors ( $\text{TR}\beta$  and  $\text{RXR}\alpha$ ) 3 d before commencement of experiments to allow expression of the encoded proteins. *Upper panel*, System L transport activity (measured as uptake of  $10\ \mu\text{M}$  L- $[\text{3H}]$ tryptophan in NaCl transport buffer) is strongly expressed in oocytes injected with cRNA for transporter subunits but not TRs. *Lower panel*, The  $\text{T}_3$ -dependent luciferase reporter TREpGLB was injected directly into the nuclei of intact oocytes expressing transporter subunits and/or TRs. These oocytes were then incubated in MBM  $\pm$   $100\ \text{nM}$   $\text{T}_3$  for 24 h and lysed for assay of luciferase activity as an index of reporter gene expression. The *main figure* shows the ratio of luminescent counts for different experimental oocyte groups compared with oocytes injected with TREpGLB only (basal control). Data are presented as mean value  $\pm$  SEM from  $n = 4$  oocyte batches (each experiment involving seven to nine individual oocytes per batch). \*, Value significantly different from basal control value ( $P < 0.05$  by Student's  $t$  test). The *inset* illustrates the inhibitory effect of  $5\ \text{mM}$  BCH on stimulation of TREpGLB reporter expression by  $10\ \text{nM}$   $\text{T}_3$  over 24 h in oocytes overexpressing 4F2hc/IU12/RXR $\alpha$ /TR $\beta$ . Raw luciferase activity data [luminescent counts per second (c.p.s.) per oocyte] are shown from experiments using a single batch of oocytes.

To investigate whether the transporter influences TR function, we used a reporter consisting of the TH-dependent element of the *Xenopus* TR $\beta$ A promoter driving the expression of the luciferase gene (TREpGLB; Ref. 22). When the  $\text{T}_3$ -responsive lucif-

erase reporter was injected into oocyte nuclei, it was expressed at a low basal level, and the addition of TH to the culture medium had little effect on its expression (Fig. 3, *lower panel*). Overexpression of System L transporter alone did not produce any significant activation of the reporter by TH (data not shown). Overexpression of TR/RXR in oocytes to produce a TH-responsive system caused a repression of the basal reporter expression, as reported earlier (21, 23) and the addition of TH reversed this repression but with little additional activation (Fig. 3, *lower panel*). In contrast, when System L transporter was coexpressed with TR/RXR the addition of TH led to strong activation of the reporter (Fig. 3), demonstrating a potentially key permissive role for plasma membrane TH transporters in nuclear function of TH. The importance of System L in the present case was confirmed by the ability of BCH to block the TH activation but not the basal expression of the reporter (Fig. 3, *inset, lower panel*).

#### System L Transporter Participates in Cellular TH Uptake in BeWo Cells

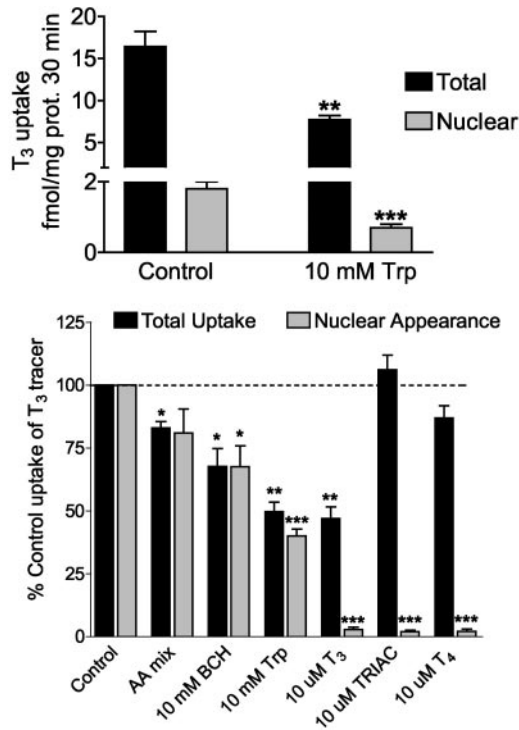
Having established that overexpressed System L transporter could contribute substantially to gene regulation by TRs in *Xenopus* oocytes, we proceeded to examine whether this representative TH transporter might be important for TH function in somatic cells. For this purpose we studied the BeWo human chorionic carcinoma cell line, which exhibits a significant component of saturable TH uptake mediated by System L and blocked by BCH (5). In preliminary experiments we established that nuclear appearance as well as total uptake of  $^{125}\text{I}$ - $\text{T}_3$  tracer in BeWo cells increased progressively for at least 60 min (Fig. 4). In subsequent experiments with BeWo cells we used an experimental duration of 30 min (to maximize tracer appearance in nuclei under at least pseudoinitial rate-uptake conditions) and a physiologically relevant  $^{125}\text{I}$ - $\text{T}_3$  tracer concentration of 100 pM [a value below the level at which nuclear TRs would exhibit full saturation (1, 2)]. Under these conditions, nuclear appearance of  $^{125}\text{I}$ - $\text{T}_3$  tracer was significantly reduced at much lower external concentrations of unlabeled  $\text{T}_3$  than was total cellular uptake (Fig. 4, *lower panel*). These kinetic observations on nuclear  $^{125}\text{I}$ - $\text{T}_3$  appearance most likely reflect competition between the radiotracer and excess unlabeled  $\text{T}_3$  for binding to TRs and other nuclear TRs, rather than competition for uptake at the nuclear membrane. The data for total cellular  $^{125}\text{I}$ - $\text{T}_3$  uptake confirm our previous studies with BeWo cells (5) showing that greater than 50% of  $\text{T}_3$  uptake is through saturable transport pathways in this cell type. The amino acid tryptophan, a known competitor of  $\text{T}_3$  transport in BeWo cells (8) and a System L substrate, reduced (by  $\leq 55\%$ ) total uptake and nuclear appearance of  $^{125}\text{I}$ - $\text{T}_3$  when added in excess (10 mM) to the uptake buffer (Fig. 5). Specific blockade of amino acid transport System L using excess BCH (10 mM) also significantly reduced both total uptake and nuclear appearance of



**Fig. 4.** Nuclear Appearance of  $^{125}\text{I}$ - $\text{T}_3$  in BeWo Cells

*Upper panel*, Time course of whole-cell (total) and nuclear uptake of 50 nM  $^{125}\text{I}$ - $\text{T}_3$  (each point represents mean value of three separate wells). *Lower panel*, Concentration dependence of  $^{125}\text{I}$ - $\text{T}_3$  uptake. BeWo cells were incubated for 30 min in NaCl uptake buffer containing 100 pM  $^{125}\text{I}$ - $\text{T}_3$  tracer with excess unlabeled  $\text{T}_3$  as indicated ( $\leq 10 \mu\text{M}$ , a value close to the solubility limit for iodothyronines at physiological pH). Representative values show percent control uptake (total and nuclear) for a single passage of BeWo cells (each point represents mean value of three separate wells).

$^{125}\text{I}$ - $\text{T}_3$  in BeWo cells (Fig. 5, *lower panel*). A mixture of 20 natural amino acids at physiological plasma concentrations (24) inhibited  $^{125}\text{I}$ - $\text{T}_3$  tracer uptake into BeWo cells by only 18% (representing  $\sim 38\%$  inhibition of saturable uptake; Fig. 5, *lower panel*), confirming that TH can compete reasonably effectively with other natural substrates for cellular uptake at the plasma membrane. Amino acid inhibitors of  $\text{T}_3$  transport at the cell surface also reduced overall nuclear appearance of  $^{125}\text{I}$ - $\text{T}_3$  but with no apparent disturbance of nuclear uptake/binding kinetics (the nuclear to cytosol  $^{125}\text{I}$ - $\text{T}_3$  ratio was  $\sim 0.12$  in all cases), thus demonstrating that amino acids did not significantly affect behavior of  $\text{T}_3$  within the cell. In contrast, the iodothyronine analog 3,3',5-triiodothyroacetic acid does not interact with  $^{125}\text{I}$ - $\text{T}_3$  uptake mechanisms at the plasma membrane, but markedly inhibits nuclear appearance of  $^{125}\text{I}$ - $\text{T}_3$  (presumably by competing with  $\text{T}_3$  for nuclear receptor sites). A broadly similar result is also seen with  $\text{T}_4$ , which is a relatively poor substrate for System L (8) and at 10  $\mu\text{M}$  has only a minor inhibitory effect on total  $^{125}\text{I}$ - $\text{T}_3$  uptake into BeWo cells under the experimental conditions used. The substrate



**Fig. 5.** Effects of Putative Inhibitors of Cellular T<sub>3</sub> Uptake on Whole-Cell (Total) and Nuclear Uptake of <sup>125</sup>I-T<sub>3</sub> in BeWo Cells

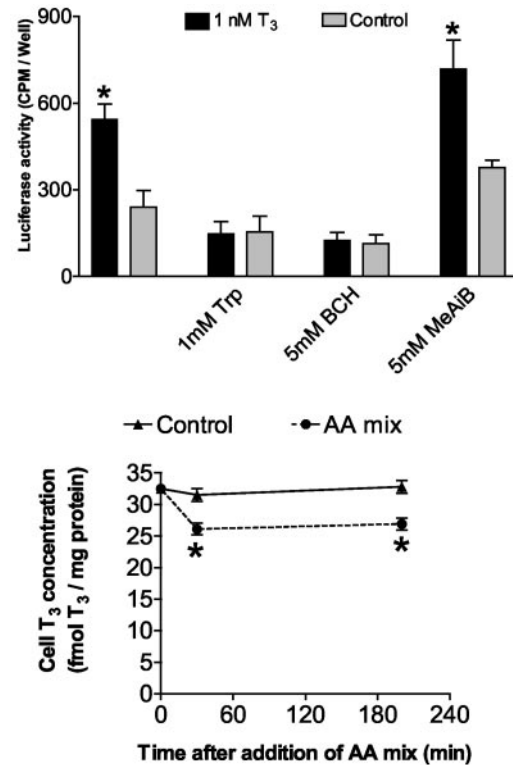
Cells were incubated in NaCl uptake buffer containing 100 pM <sup>125</sup>I-T<sub>3</sub> for 30 min with inhibitor as indicated. Data are presented as mean value ± SEM for n = 5–9 individual plates of BeWo cells. *Upper panel* shows the inhibitory effect of tryptophan on rate of <sup>125</sup>I-T<sub>3</sub> uptake. *Lower panel* summarizes inhibitory effects of various substances, expressed as percent control uptake (total and nuclear) remaining in presence of inhibitor. AA mix, A mixture of 20 natural amino acids at physiological plasma concentrations (see Ref. 24 for exact composition). Statistical significance of differences from control values are indicated as \*, P < 0.05; \*\*, P < 0.005; \*\*\*, P < 0.0001 (by Student's *t* test). TRIAC, 3,3',5-Triiodothyroacetic acid.

selectivities of plasma membrane transporters and nuclear receptors for iodothyronines are therefore clearly distinct, at least in relative terms.

**Blockade of System L Inhibits Transcriptional Regulation by TR in BeWo Cells**

The data described above indicated to us that plasma membrane transporters play critical roles in nuclear accumulation of THs in BeWo cells, at least under our experimental conditions. We next investigated whether our observed acute effects of the TH uptake inhibitors BCH and tryptophan on nuclear binding of T<sub>3</sub> were mirrored by a reduced activation of TH-dependent gene transcription over a longer time period. We used a T<sub>3</sub>-responsive luciferase reporter (25) introduced into BeWo cells via a replication-deficient adenovirus vector. This luciferase reporter showed ef-

fectively linear increases in expression over 24 h under all experimental conditions (data not shown). The reporter exhibited T<sub>3</sub>-dependent activation of transcription, which was suppressed over a 24-h period in the presence of System L substrates (Fig. 6, *upper panel*) at concentrations that would be expected to compete out T<sub>3</sub> uptake into cells through this route (5, 8, 10). In contrast, *N*-methylaminoisobutyric acid (a specific competing substrate of amino acid transport System A) does not interact with TH uptake in BeWo cells (5) and was without effect on T<sub>3</sub>-dependent activation of transcription.



**Fig. 6.** Effects of Amino Acids on T<sub>3</sub>-Dependent Transcription and T<sub>3</sub> Concentration in BeWo Cells

*Upper panel*, Tryptophan (Trp) and BCH suppress T<sub>3</sub>-dependent induction of luciferase reporter gene expression in BeWo cells. Representative result showing luciferase activity in lysates of cells containing T<sub>3</sub>-dependent luciferase reporter (introduced by adenoviral vector) after 24-h incubation period in NaCl uptake buffer + 25 mM glucose +/- 1 nM T<sub>3</sub> and/or inhibitors. Values are mean luminescent counts per min/well ± SEM for n = 3 experiments. \*, Value significantly different from respective control (P < 0.05 by Student's *t* test). Similar results were obtained using a different BeWo cell passage. *Lower panel*, Amino acids (AA) alter steady-state distribution of T<sub>3</sub> across BeWo cell membrane. BeWo cells were preequilibrated with 100 pM <sup>125</sup>I-T<sub>3</sub> tracer for 2 h, and then exposed to fresh medium containing <sup>125</sup>I-T<sub>3</sub> at the same concentration and specific activity +/- a mixture of physiological amino acids at normal plasma concentrations [AA mix (24)]. Data are presented as mean cell T<sub>3</sub> concentration ± SEM for n = 6 separate measurements. \*, Value significantly lower than respective control (P < 0.05 by Student's *t* test). MeAIB, *N*-methylaminoisobutyric acid.

### Extracellular Amino Acids Influence Intracellular TH Concentration in BeWo Cells

Our results indicate that the activity of TH transporters such as System L at the plasma membrane is likely to enhance cellular responsiveness to a change in extracellular TH concentration. Equally, changes in TH flux at the cell surface due to other factors (*e.g.* increased competition for transport from other substrates or altered transporter gene expression) might also be predicted to influence the intracellular concentration of  $T_3$  and thus affect TH actions. We therefore progressed to investigate the possibility that amino acids, given that they interact with TH transporters at the cell surface, might influence steady-state distribution of TH across the plasma membrane. BeWo cells were pre-equilibrated with  $^{125}\text{I}$ - $T_3$  and then exposed to fresh medium containing  $^{125}\text{I}$ - $T_3$  at the same concentration and specific activity  $+/-$  a mixture of physiological amino acids at normal plasma concentrations (24). The addition of the amino acid mixture produced a small but significant decrease in the total quantity of  $T_3$  in cells within 30 min (indicative of a net shift in transmembrane TH concentration gradient), which persisted for at least 3 h (Fig. 6, *lower panel*).

### DISCUSSION

TH action is mediated largely through TR-dependent gene regulation in the cell nucleus. It is clear that TH in the plasma must first cross the surface membrane of target cells to reach the nucleus. Several different types of TH transporter have now been identified (*e.g.* Refs. 2, 6, and 12 for review), although the extent to which they each contribute to delivery of plasma TH to the cytosol and thus ultimately to transcription through TR is largely unknown. Our experiments using *Xenopus* oocytes presensitized to TH by coexpression of TR and RXR demonstrate that increasing surface permeability to TH by overexpressing a representative TH transporter (here we use System L) enhances the cellular response to TH in terms of transcriptional activation. Conversely, blockade of  $T_3$  uptake into BeWo cells using competing System L substrates markedly blunts cellular  $T_3$  sensitivity in terms of the activation of transcription through nuclear TRs. The fact that competitive blockade of a different amino acid transporter (System A) fails to inhibit TH-induced transcription in such experiments (Fig. 6) confirms the specificity of the effect to System L and  $T_3$ ; this also argues against the possibility that our observations result from indirect suppression of transcription and/or translation (*e.g.* in response to osmotic effects of exogenous amino acid competitors). An immediate and important conclusion to be drawn from our studies is that  $T_3$  is a transportable substrate of System L (reaching the nucleus and activating TH-dependent gene transcription through this route) rather than simply a high-affinity

inhibitor of amino acid transport as proposed elsewhere (10, 26). On a broader front, the results provide direct evidence that plasma membrane transport of  $T_3$  at physiologically relevant concentrations (100 pM to 1 nM) through System L may be an important determinant of nuclear  $T_3$  availability and TH action. Furthermore, the observation that a mixture of 20 amino acids at physiological plasma concentrations has a relatively small effect on  $^{125}\text{I}$ - $T_3$  tracer uptake into BeWo cells both confirms that THs can compete effectively with other natural substrates for cellular uptake and supports the idea that saturable transport mechanisms may contribute significantly to cellular TH delivery *in vivo*. Plasma TH levels exhibit relatively slow adjustment *in vivo* and factors other than membrane transport (notably TH synthesis, release, and metabolism) also have vital roles in TH signaling and action. Nevertheless, our data show clearly that TH transporters such as System L participate in regulating cellular  $T_3$  actions over physiologically relevant periods of at least 24 h. System L may have a widespread role in cellular TH signaling, given that it is expressed ubiquitously in mammalian cells (16) and accepts both  $T_3$  and  $T_4$  as substrates with relatively high affinity [albeit the less potent  $T_4$  is transported poorly in comparison to  $T_3$  (8)]. Other TH transporters including various members of the oatp family are expressed in a highly tissue-specific manner (6) and may therefore also be anticipated to contribute to TH signaling and action to a greater or lesser degree in particular cell types. Such cell-specific complements of TH transporters may not only facilitate (and also possibly regulate) delivery and bioavailability of TH to the cell interior, but also provide a therapeutic target for TH disorders (6, 12).

Membrane transport, unlike diffusion, offers the possibility of regulating TH actions at the point of cellular entry (or exit). Specific functional properties of a TH transporter may enable it to contribute at more subtle and gradual levels of control of hormone action, appropriate to the whole organism. For example, we have found that external amino acids are capable of altering steady-state intracellular  $T_3$  concentration, at least in BeWo cells. This may be related, at least in part, to specific transport properties of System L (probably the major TH transporter in this cell type), which operates as an obligate exchanger of substrates [with different Michaelis-Menten ( $K_m$ ) values for influx and efflux (27)] thus enhancing the potential influence of *trans*-substrates on TH distribution. Such influences may conceivably have a role in fine tuning of TH-regulatory mechanisms *in vivo*.

For oocytes overexpressing System L, the increased rate of appearance of TH tracer in the nucleus is proportionally greater than that in the whole cell (6.25-fold as opposed to 3-fold control value, respectively; see Fig. 1, *lower panel*). These results are consistent with the suggestion (3) that plasma THs may be selectively channeled to nuclear TRs in certain cell types (thus further enhancing the importance of membrane transport for TH signaling) rather than merely

entering a general metabolic TH pool within the cytoplasm. In contrast to these results, we found no evidence that  $T_3$  was targeted preferentially to the nucleus of oocytes overexpressing System L if the  $^{125}\text{I}$ - $T_3$  tracer was injected directly into the cell, bypassing the transport step (See Fig. 2). It seems evident, therefore, that any channeling that occurs is likely to be initiated close to the inner cell surface, immediately after the TH molecule is released from the TH transporter. Unfortunately, the mechanisms by which transported THs move from the inner face of the plasma membrane to the cell nucleus remain largely unknown. At least some cytoplasmic THs may simply diffuse to the nucleus, although discrete cytosolic TH-binding proteins that could conceivably help channel TH to the nucleus have been identified (e.g. Ref. 28). Cytoskeletal disruptors such as colchicine have been reported to significantly retard nuclear TH appearance in certain cell types (13), and there is also some evidence (29) that a proportion of unbound TRs may reside in the cytosol and translocate to the nucleus after cellular TH stimulation, possibly as a TR + TH + chaperone complex. Our observation that blockade of System L in BeWo cells suppresses TH-dependent transcription (Fig. 6, upper panel) to a greater extent than might be predicted by the reduction in nuclear TH appearance (Fig. 5, lower panel) provides additional evidence that plasma TH may be selectively channeled to active nuclear TR. It is noteworthy in this regard that  $T_3$  receptor sites on the nuclear envelope distinct from nuclear TR have been reported (30), in which case a proportion of TH binding sites associated with the nuclear fraction may not be directly involved with TH-dependent transcriptional activation. Partial inhibition of nuclear TH appearance through blockade of the System L TH transporter would be sufficient to almost totally suppress TH-dependent transcription in BeWo cells if the inhibited part reflected blockade of a pathway associated specifically with active nuclear TRs.

In summary, we have presented evidence that plasma membrane uptake of  $T_3$  through System L (a representative TH transporter) may be an important determinant of nuclear  $T_3$  delivery from plasma and hence cellular TH action. Nevertheless, the reported presence of multiple TH transport systems in many cell types (2, 5–7) makes it likely that individual TH transporters contribute to shared control of intracellular TH availability and action, in conjunction with processes such as TH metabolism. Differences in tissue sensitivity to TH may arise, at least in part, from developmental and/or tissue-specific regulation of expression of TH transporters (5, 6). For example, the effective TH level in individual tissues during amphibian metamorphosis, which is believed (31) to be critical for temporal regulation of tissue-specific transformations, appears to correlate with expression of the System L transporter subunit IU12 (32). Interactions between transport of TH and other substrates of TH transporters (e.g. amino acids for System L, organic anions for oap

family members) may also have pathological implications (2, 12). With regard to amino acids, there are pathophysiological circumstances revealing a relationship between tryptophan and TH distributions and abundance that might reflect competition between these substrates for the L-type transporter *in vivo*. For example, tryptophan-deficient chickens show signs of relative hyperthyroidism compared with pair-fed controls (33). Equally, in newly diagnosed hypothyroid patients the cerebrospinal fluid concentrations of tryptophan and tyrosine [both important neurotransmitter precursors delivered across the blood-brain barrier by System L (10)] are elevated and correlate positively with extent of hypothyroid state; cerebrospinal fluid concentrations of these amino acids also decrease significantly during treatment to normalize the thyroid state (34). High dosages of the dopamine precursor L-dopa [another substrate for System L (35) that should compete with TH for transport by this route] are also reported to alter plasma thyroid status (36) and free TH concentrations (37), although interpretation of such data *in vivo* is complicated by possible interactions between dopaminergic and TH systems.

## MATERIALS AND METHODS

### Chemicals

Unless otherwise specified, chemicals were obtained from Sigma (Poole, UK). [ $^{125}\text{I}$ ] $T_3$  and [ $^3\text{H}$ ]-L-tryptophan radiotracers were purchased from Perkin-Elmer Corp. (Norwalk, CT).

### Studies Using *X. laevis* Oocytes

Oocytes were isolated by collagenase treatment of ovarian tissue obtained from mature female *Xenopus laevis* toads (South African *Xenopus* facility, Noordhoek, S.A.) using methods described previously (38). Defolliculated, stage V–VI (prophase-arrested) oocytes were selected and maintained at 18 C in modified Barth's medium (MBM) containing (in millimolar concentration): 88 NaCl, 1 KCl, 2.4 NaHCO<sub>3</sub>, 0.82 MgSO<sub>4</sub>, 0.66 NaNO<sub>3</sub>, 0.75 CaCl<sub>2</sub>, and 5.0 HEPES, pH 7.6 (adjusted with Tris base), and 10 mg/liter gentamycin sulfate.

A System L amino acid transporter was overexpressed in oocytes using the following procedure. Plasmids containing cDNA for the System L transporter subunits 4F2hc [kindly provided by Dr. L. C. Kuhn, Institute of Physiology, University of Zurich, Zurich, Switzerland (39)] and IU12 (32) were linearized before cRNA synthesis (mMessage mMachine, Ambion, Inc., Austin, TX). Individual oocytes were coinjected with 4F2hc and IU12 cRNA (40 ng of each cRNA in 50 nl cytoplasmic injectate) on the day of isolation. Oocytes were incubated in MBM for 2–4 d to allow expression of injected cRNA before experimentation. In certain studies the nuclear hormone receptors RXR $\alpha$  and TR $\beta$  were also overexpressed in oocytes using similar methods (21).

Initial-rate uptake of radiotracers into oocytes was measured over periods of up to 2 h using methods described previously (8). At the end of certain uptake experiments, oocyte nuclei were isolated using the following procedure: groups of three oocytes were rinsed and incubated in 1 ml ice-cold 10% trichloroacetic acid for 10 min, after which the visible nuclei were excised using needles under a microscope. All three nuclei were solubilized together in 0.5 ml of

0.2 M NaOH and processed for radioactive scintillation counting. Radioactivity in the residual oocyte debris (extranuclear fraction) was measured similarly. Nuclear uptake of tracer  $^{125}\text{I-T}_3$  (~0.1 kBq in 20 nl water) injected into cytoplasm of intact oocytes was monitored using similar procedures; groups of three  $^{125}\text{I-T}_3$  injected oocytes were processed together into nuclear and extranuclear fractions for radioactivity assay at timed periods post injection, as described above.

A  $\text{T}_3$ -responsive luciferase reporter construct was also used to investigate  $\text{T}_3$ -dependent activation of transcription in the oocyte expression system. This DNA construct, TREpGLB (22), was made by inserting the 1.6-kb *SacI-BglII* fragment of the *Xenopus laevis* TR $\beta$ A promoter (23) upstream of the luciferase reporter gene in pGL2B plasmid (Promega Corp., Madison, WI). The construct was injected directly into the nucleus of oocytes (13 ng in 18 nl water) 3 d after isolation/cRNA injection. Oocytes were allowed to recover from injection for 3 h in MBM and then transferred to MBM  $\pm$   $\text{T}_3$  and/or the synthetic System L substrate BCH at specified concentrations for 24 h. At the end of this incubation period, oocytes were rinsed and lysed individually in Steady-Glo luciferase assay reagent (Promega Corp.) within single wells of a 96-well plate before luminescence counting.

Plasma membranes were isolated from uninjected (native) *X. laevis* oocytes and those overexpressing 4F2hc and IU12 (3 d post injection) using methods described previously (40). Plasma membrane samples were subjected to SDS-PAGE under both reducing and nonreducing conditions (presence or absence of 5% 2-mercaptoethanol, respectively) and electrophoretically transferred to a nitrocellulose membrane using a wet transfer apparatus. The membrane was then probed by Western blotting for 4F2hc (as described in Ref. 5) or IU12. IU12 was detected using 1:500 dilution of serum from a rabbit coimmunized with two synthetic peptides based on the IU12 primary amino acid sequence [MAADS-VKRRQSGASKTEEDRQ (residues 1–22) and RYKKPELER-PIKVN (residues 419–432)] and visualized using a horseradish peroxidase-conjugated antirabbit IgG secondary antibody (1:5000 dilution) and enhanced chemiluminescence.

### Studies Using BeWo Cell Culture

The BeWo human choriocarcinoma cell line (gift from Dr. H. McArdle, Rowett Research Institute, Aberdeen, UK) was maintained in continuous culture in 90% Hams F-12 nutrient mixture, 10% fetal calf serum, at 37 C in an atmosphere of 95% air-5%  $\text{CO}_2$ . BeWo cells were cultured on 175-cm<sup>2</sup> flasks and subcultured onto dishes or multiwell plates as required for experimentation; culture media were changed every second day and on the day before experimentation.

Radiotracer uptake experiments were performed at 37 C on BeWo cells that had just reached confluence. Basic NaCl uptake buffer contained, in millimolar concentration: 121 NaCl, 4.9 KCl, 2.5  $\text{MgSO}_4$ , 20 Tris hydrochloride, and 1  $\text{CaCl}_2$  at pH 7.4. Initial-rate radiotracer uptakes were measured using cells grown on 24-well plates as described previously (5). To measure nuclear uptake of radiotracer, BeWo cells were grown on 10-cm culture dishes and then incubated in NaCl uptake buffer containing 100 pM  $^{125}\text{I-T}_3$  for 30 min followed by rapid subcellular fractionation [Nuclei EZ-Prep kit (Sigma, St. Louis, MO) used according to the manufacturer's instructions] to produce highly purified nuclear and cytosolic fractions. These fractions were assayed for radioactivity and protein as described previously (5). In certain experiments, BeWo cells were preequilibrated with 100 pM  $^{125}\text{I-T}_3$  for 2 h, and then exposed to fresh medium containing  $^{125}\text{I-T}_3$  at the same concentration and specific activity  $\pm$  a mixture of physiological amino acids at normal plasma concentrations (24). After incubation periods of 30 or 200 min, cells were rinsed thoroughly and then processed and assayed as described above.

In separate experiments, a  $\text{T}_3$ -responsive luciferase reporter in a replication-deficient adenovirus vector [Ad5-tk-

palx3-Luc (25, 41)] was introduced into BeWo cells to investigate  $\text{T}_3$ -dependent activation of transcription. Cells were cultured in QBSF-51 medium containing 10% charcoal-treated FBS for 24 h before addition of adenovirus (at a multiplicity of infection of 5) for 3 h. Virus was then removed and cells incubated in fresh QBSF-51 medium for at least 24 h before experiments were begun. Cells were then washed in warm PBS and incubated in NaCl uptake buffer + 25 mM glucose  $\pm$   $\text{T}_3$  and/or inhibitors for timed periods of up to 24 h (41). Reporter expression was assessed by luciferase assay using the Promega Corp. Bright-Glo system (according to the manufacturer's instructions) and measuring sample luminescence on a MicroBeta counter (Wallac, Inc., Gaithersburg, MD).

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### REFERENCES

1. Yen PM 2001 Physiological and molecular basis of thyroid hormone action. *Physiol Rev* 81:1097–1142
2. Hennemann G, Docter R, Friesema EC, de Jong M, Krenning EP, Visser TJ 2001 Plasma membrane transport of thyroid hormones and its role in thyroid hormone metabolism and bioavailability. *Endocr Rev* 22:451–476
3. Larsen PR, Silva JE, Kaplan MM 1981 Relationships between circulating and intracellular thyroid hormones: physiological and clinical implications. *Endocr Rev* 2:87–102
4. Lai CS, Korytowski W, Niu CH, Cheng SY 1985 Transverse motion of spin-labeled 3,3',5-triiodo-L-thyronine in phospholipid bilayers. *Biochem Biophys Res Commun* 131:408–412
5. Ritchie JW, Taylor PM 2001 Role of the system L permease LAT1 in amino acid and iodothyronine transport in placenta. *Biochem J* 356:719–725
6. Fujiwara K, Adachi H, Nishio T, Unno M, Tokui T, Okabe M, Onogawa T, Suzuki T, Asano N, Tanemoto M, Seki M, Shiiba K, Suzuki M, Kondo Y, Nunoki K, Shimosegawa T, Iinuma K, Ito S, Matsuno S, Abe T 2001 Identification of thyroid hormone transporters in humans: different molecules are involved in a tissue-specific manner. *Endocrinology* 142:2005–2012
7. Neves FA, Cavalieri RR, Simeoni LA, Gardner DG, Baxter JD, Scharschmidt BF, Lomri N, Ribeiro RC 2002 Thyroid hormone export varies among primary cells and appears to differ from hormone uptake. *Endocrinology* 143:476–483
8. Ritchie JW, Peter GJ, Shi YB, Taylor PM 1999 Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in *Xenopus* oocytes. *J Endocrinol* 163:R5–R9
9. Friesema EC, Docter R, Moerings EP, Verrey F, Krenning EP, Hennemann G, Visser TJ 2001 Thyroid hormone transport by the heterodimeric human system L amino acid transporter. *Endocrinology* 142:4339–4348
10. Uchino H, Kanai Y, Kim do K, Wempe MF, Chairoungdua A, Morimoto E, Anders MW, Endou H 2002 Transport of

- amino acid-related compounds mediated by L-type amino acid transporter 1 (LAT1): insights into the mechanisms of substrate recognition. *Mol Pharmacol* 61: 729–737
11. Friesema EC, Docter R, Moerings EP, Stieger B, Hag-enbuch B, Meier PJ, Krenning EP, Hennemann G, Visser TJ 1999 Identification of thyroid hormone transporters. *Biochem Biophys Res Commun* 254:497–501
  12. Shi YB, Ritchie JW, Taylor PM 2002 Complex regulation of thyroid hormone action: multiple opportunities for pharmacological intervention. *Pharmacol Ther* 94: 235–51
  13. Halpern J, Hinkle PM 1982 Evidence for an active step in thyroid hormone transport to nuclei: drug inhibition of L-<sup>125</sup>I-triiodothyronine binding to nuclear receptors in rat pituitary tumor cells. *Endocrinology* 110:1070–1072
  14. Horiuchi R, Cheng SY, Willingham M, Pastan I 1982 Inhibition of the nuclear entry of 3,3',5'-triiodo-L-thy-ro-nine by monodansylcadaverine in GH3 cells. *J Biol Chem* 257:3139–3144
  15. Pontecorvi A, Lakshmanan M, Robbins J 1988 Different intracellular and intranuclear transport of triiodothyronine enantiomers in rat skeletal myoblasts. *Endocrinology* 123:2922–2929
  16. Verrey F, Meier C, Rossier G, Kuhn LC 2000 Glycopro-tein-associated amino acid exchangers: broadening the range of transport specificity. *Pflugers Arch* 440:503–512
  17. Blondeau JP, Beslin A, Chantoux F, Francon J 1993 Triiodothyronine is a high-affinity inhibitor of amino acid transport system L1 in cultured astrocytes. *J Neurochem* 60:1407–1413
  18. Ritchie JW, Collingwood CJF, Taylor PM 2001 Effect of hypothyroidism on pathways for iodothyronine and tryptophan uptake into rat adipocytes. *Am J Physiol* 280: E254–E259
  19. Torrents D, Estevez R, Pineda M, Fernandez E, Lloberas J, Shi YB, Zorzano A, Palacin M 1998 Identification and characterization of a membrane protein ( $\gamma$ +L amino acid transporter-1) that associates with 4F2hc to encode the amino acid transport activity  $\gamma$ +L. A candidate gene for lysinuric protein intolerance. *J Biol Chem* 273: 32437–32445
  20. Mastroberardino L, Spindler B, Pfeiffer R, Skelly PJ, Loff-ing J, Shoemaker CB, Verrey F 1998 Amino-acid trans- port by heterodimers of 4F2hc/CD98 and members of a permease family. *Nature* 395:288–291
  21. Wong J, Shi YB 1995 Coordinated regulation of and transcriptional activation by *Xenopus* thyroid hormone and retinoid X receptors. *J Biol Chem* 270:18479–18483
  22. Amano T, Leu K, Yoshizato K, Shi YB 2002 Thyroid hormone regulation of a transcriptional coactivator in *Xenopus laevis*: implication for a role in postembryonic tissue remodeling. *Dev Dyn* 223:526–35
  23. Wong J, Shi YB, Wolffe AP 1995 A role for nucleosome assembly in both silencing and activation of the *Xenopus* TR  $\beta$  A gene by the thyroid hormone receptor. *Genes Dev* 9:2696–2711
  24. Christie GR, Hajduch E, Hundal HS, Proud CG, Taylor PM 2002 Intracellular sensing of amino acids in *Xenopus laevis* oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. *J Biol Chem* 277: 9952–9957
  25. Menjo M, Yamaguchi S, Murata Y, Hayashi Y, Nagaya T, Ohmori S, Refetoff S, Seo H 1999 Responsiveness to thyroid hormone is enhanced in rat hepatocytes cultured as spheroids compared with that in monolayers: altered responsiveness to thyroid hormone possibly involves complex formed on thyroid hormone response elements. *Thyroid* 9:959–967
  26. Prasad PD, Leibach FH, Mahesh VB, Ganapathy V 1994 Relationship between thyroid hormone transport and neutral amino acid transport in JAR human choriocarci-noma cells. *Endocrinology* 134:574–581
  27. Meier C, Ristic Z, Klauser S, Verrey F 2002 Activation of system L heterodimeric amino acid exchangers by intra-cellular substrates. *EMBO J* 21:580–589
  28. Vie MP, Blanchet P, Samson M, Francon J, Blondeau JP 1996 High affinity thyroid hormone-binding protein in human kidney: kinetic characterization and identification by photoaffinity labeling. *Endocrinology* 137:4563–4570
  29. Zhu XG, Hanover JA, Hager GL, Cheng SY 1998 Hor-mone-induced translocation of thyroid hormone recep-tors in living cells visualized using a receptor green flu-orescent protein chimera. *J Biol Chem* 273:27058–27063
  30. Sidransky H, Verney E 1999 Hormonal influences on tryptophan binding to rat hepatic nuclei. *Metabolism* 48: 144–152
  31. Shi YB, Wong J, Puzianowska-Kuznicka M, Stalow MA 1996 Tadpole competence and tissue-specific temporal regulation of amphibian metamorphosis: roles of thyroid hormone and its receptors. *Bioessays* 18:391–399
  32. Liang VC, Sedgwick T, Shi YB 1997 Characterization of the *Xenopus* homolog of an immediate early gene asso-ciated with cell activation: sequence analysis and regu-lation of its expression by thyroid hormone during am-phibian metamorphosis. *Cell Res* 7:179–193
  33. Carew Jr LB, Alster FA, Foss DC, Scanes CG 1983 Effect of a tryptophan deficiency on thyroid gland, growth hor-mone and testicular functions in chickens. *J Nutr* 113: 1756–1765
  34. Sjoberg S, Eriksson M, Nordin C 1998 L-thyroxine treat-ment and neurotransmitter levels in the cerebrospinal fluid of hypothyroid patients: a pilot study. *Eur J Endo-crinol* 139:493–497
  35. Kageyama T, Nakamura M, Matsuo A, Yamasaki Y, Takakura Y, Hashida M, Kanai Y, Naito M, Tsuruo T, Minato N, Shimohama S 2000 The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. *Brain Res* 879:115–121
  36. Minozzi M, Faggiano M, Lombardi G, Carella C, Cris-cuolo T, Scapagnini U 1975 Effect of L-dopa on plasma TSH levels in primary hypothyroidism. *Neuroendocrinol-ogy* 17:147–153
  37. Baruch AL, Davis C, Hodkinson HM 1976 Causes of high free-thyroxine index values in sick euthyroid elderly pa-tients. *Age Ageing* 5:224–227
  38. Peter GJ, Davidson IG, Ahmed A, McIlroy L, Forrester AR, Taylor PM 1996 Multiple components of arginine and phenylalanine transport induced in neutral and basic amino acid transporter-cRNA-injected *Xenopus* oocytes. *Biochem J* 318:915–922
  39. Teixeira S, Di Grandi S, Kuhn LC 1987 Primary structure of the human 4F2 antigen heavy chain predicts a trans-membrane protein with a cytoplasmic NH2 terminus. *J Biol Chem* 262:9574–9580
  40. Wang Y, Tate SS 1995 Oligomeric structure of a renal cystine transporter: implications in cystinuria. *FEBS Lett* 368:389–392
  41. Hayashi Y, DePaoli AM, Burant CF, Refetoff S 1994 Expression of a thyroid hormone-responsive recombi-nant gene introduced into adult mice livers by replica-tion-defective adenovirus can be regulated by endoge-nous thyroid hormone receptor. *J Biol Chem* 269: 23872–23875