0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 5
Printed in U.S.A.

Expression of Rat Liver Cell Membrane Transporters for Thyroid Hormone in *Xenopus laevis* Oocytes*

ROELOF DOCTER, EDITH C. H. FRIESEMA, PAUL G. J. VAN STRALEN, ERIC P. KRENNING, MARIA E. EVERTS†, THEO J. VISSER, AND GEORG HENNEMANN

Departments of Internal Medicine III and Clinical Endocrinology (R.D., E.C.H.F., P.G.J.v.S., E.P.K., M.E.E., T.J.V., G.H.), and Nuclear Medicine (E.P.K.), Erasmus University Medical School, Rotterdam, The Netherlands

ABSTRACT

The present study was conducted to explore the possible use of *Xenopus laevis* oocytes for the expression cloning of cell membrane transporters for iodothyronines. Injection of stage V–VI *X. laevis* oocytes with 23 ng Wistar rat liver polyadenylated RNA (mRNA) resulted after 3–4 days in a highly significant increase in [^{125}I]T_3 (5 nM) uptake from 6.4 ± 0.8 fmol/oocyte·h in water-injected oocytes to 9.2 ± 0.65 fmol/oocyte·h (mean ± SEM; n = 19). In contrast, [^{125}I]T_4 (4 nM) uptake was not significantly stimulated by injection of total liver mRNA. T_3 uptake induced by liver mRNA was significantly inhibited by replacement of Na^+ in the incubation medium by choline$^+$ or by simultaneous incubation with 1 μM unlabeled T_3. In contrast, T_3 uptake by water-injected oocytes was not Na^+ dependent. Fractionation of liver mRNA on a 6–20% sucrose gradient showed that maximal stimulation of T_3 uptake was obtained with mRNA of 0.8–2.1 kilobases (kb). In contrast to unfractionated mRNA, the 0.7- to 2.1-kb fraction also significantly stimulated transport of T_4, and it was found to induce uptake of T_3 sulfate (T_3S). Because T_3S is a good substrate for type I deiodinase (D1), 2.3 ng rat D1 complementary RNA (cRNA) were injected either alone or together with 23 ng of the 0.8- to 2.1-kb fraction of rat liver mRNA. Compared with water-injected oocytes, injection of D1 cRNA alone did not stimulate uptake of [^{125}I]T_3S (1.25 nM). T_3S uptake in liver mRNA and D1 cRNA-injected oocytes was similar to that in oocytes injected with mRNA alone, showing that transport of T_3S is independent of the metabolic capacity of the oocyte. Furthermore, coinjection of liver mRNA and D1 cRNA strongly increased the production of $^{125}I^-$, showing that the T_3S taken up by the oocyte is indeed transported to the cell interior.

In conclusion, injection of rat liver mRNA into *X. laevis* oocytes resulted in a stimulation of saturable, Na^+-dependent T_4, T_3 and T_3S transport, indicating that rat liver contains mRNA(s) coding for plasma membrane transporters for these iodothyronine derivatives. (*Endocrinology* **138:** 1841–1846, 1997)

T_4 IS THE main secretory product of the thyroid gland, which is enzymatically converted in peripheral tissues to the biologically active hormone T_3. About 80% of the plasma T_3 production in man results from this extrathyroidal pathway, in which the liver plays a dominant role (1). The conversion of T_4 to T_3 as well as the further deiodination of iodothyronines is effected by different types of deiodinases in tissues (2). Transport across the plasma membrane is required for intracellular deiodination. During the last 15 yr evidence has accumulated that the plasma membranes of different tissues contain one or more specific transport proteins for T_3 and T_4. The transport process appears to be temperature and energy dependent, and is inhibited by hormone analogs and compounds that disturb the Na^+ gradient across the plasma membrane (see Refs. 3 and 4 for comprehensive reviews). Although much is known about the physiology of transmembrane T_4 and T_3 transport, little is known about the molecular mechanisms of these processes. Studies with a monoclonal antibody that inhibits T_4 and T_3 transport into rat hepatocytes showed immunoprecipitation of a plasma membrane protein from rat liver with a M_r of about 55 kDa (5). In another study, photoaffinity labeling of rat erythrocyte membranes with [^{125}I]T_3 resulted in the identification of a 45-kDa protein (6). Additional studies using the above monoclonal antibody and other inhibitors of plasma membrane transport also indicated that this transport process is rate limiting for subsequent metabolism of thyroid hormone (7). Other workers have shown that this transport process is a determinant for the nuclear occupancy of thyroid hormone (8, 9). Thus, plasma membrane transport may play an important role in the overall regulation of thyroid hormone bioactivity.

No information is as yet available about the structure of this transport protein(s). During the last years a large number of plasma membrane transporters (for instance, for amino acids, organic anions, bile acid, sulfate, and water) have been cloned following their expression in *Xenopus laevis* oocytes after microinjection of messenger RNA (mRNA) coding for these proteins (10–16). Therefore, we have adopted this technique to express the thyroid hormone transport protein(s) using total rat liver polyadenylated [poly(A)$^+$] RNA (mRNA) and fractions thereof as a first step in the cloning process.

Materials and Methods

Materials

T_3 was purchased from Sigma Chemical Co. (St. Louis, MO), [3′,5′-^{125}I]T_4 (>19 MBq/nmol), [3′-^{125}I]T_3 (>29 MBq/nmol), and L-[^{3}H]arginine (>2.22 MBq/nmol) were obtained from RCC Amersham (Aylesbury, UK). [3′-^{125}I]T_3 sulfate ([^{125}I]T_3S) was prepared by reaction of [^{125}I]T_3 with chlorosulfonic acid in dimethylformamide (both from

Received October 30, 1996.

Address all correspondence and requests for reprints to: R. Docter, Ph.D., Laboratory for Endocrinology, University Hospital Dijkzigt, Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands.

* A preliminary report of this work was presented at the 11th International Thyroid Congress, September 1995, Toronto, Ontario, Canada.

† Recipient of a fellowship from the Royal Netherlands Academy of Arts and Sciences.

Merck, Darmstadt, Germany) and purified by Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden) chromatography as previously described (17). [^{125}I]T_4 was purified before use with the same method. All other chemicals were of reagent grade.

Animals

Two- to 3-yr-old adult *Xenopus laevis* females were obtained from the Hubrecht Laboratory (Utrecht, The Netherlands). They were maintained in a water-filled tank with three dark sides at a temperature of 18–22 C. A 12-h light, 12-h dark cycle was maintained to reduce seasonal variations in oocyte quality. Frogs were fed twice a week. The water was changed about 2 h after feeding.

Livers of male Wistar rats, female Sprague-Dawley rats, and female Fisher rats were used to prepare mRNA. Animals had free access to food and water and were kept in a controlled environment (21 C) with constant day length (12 h).

Oocyte isolation and RNA injections

Oocytes were prepared as described previously (18), with some modifications. Ovarian fragments were removed from *X. laevis* females under MS-222 anesthesia (Sigma; 1 g/liter 3-aminobenzoic acid ethyl ester, in tap water) and hypothermia. Small lumps containing 20–50 oocytes were washed in calcium-free ORII (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). To remove follicular layers, the lumps were incubated twice for 90 min each time at room temperature in ORII with 2 mg/ml collagenase A (Boehringer Mannheim, Mannheim, Germany) on a rotator. The oocytes were washed thoroughly five times with ORII and subsequently five times with MBS [modified Barth's solution, 88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.4 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.4), containing 10 IU/ml penicillin, and 10 μg/ml streptomycin]. The oocytes were sorted manually on morphological criteria, such as size, polarization, pigmentation, and absence of follicular layer debris. Healthy-looking stage V–VI oocytes (19) were transferred to six-well tissue culture plates and incubated in MBS at 18 C in the dark.

The next day, oocytes were injected with 0.23–23 ng RNA in 23 nl water or with water alone (control) using a Nanoject system (Drummond Scientific Co., Broomall, PA). Injected oocytes were maintained in MBS at 18 C for 3–4 days, with a daily change of medium.

mRNA isolation

A commercial kit (Stratagene, La Jolla, CA) was used for the isolation of mRNA from rat liver tissue according to the manufacturer's protocol. Tissue was homogenized in guanidinium isothiocyanate buffer with β-mercaptoethanol. After dilution, precipitated proteins were removed by centrifugation, and the mRNA was bound to oligo(deoxythymidine)cellulose. After several wash steps, mRNA was eluted with elution buffer at 65 C. For size-fractionation, rat liver mRNA (150 μg) in water was heated to 65 C for 5 min and then loaded on a linear 6–20% (wt/vol) sucrose gradient containing 15 mM piperazine-*N*,*N*′-[2-ethanesulfonic acid] (PIPES) (pH 6.4), 5 mM Na_2-EDTA, and 0.25% (wt/vol) Sarkosyl. The gradient was centrifuged for 19 h at 4 C at 25,000 rpm (80,000 $\times$ g_{av}) in a Beckman SW 41 rotor (Beckman, Palo Alto, CA). Subsequently, 0.7-ml fractions were collected from the bottom of the tubes. Total and size-fractionated mRNA were precipitated with 0.3 M sodium acetate in ethanol (20), resuspended in water at a concentration of 1 μg/μl, and stored at −80 C. mRNA concentrations were estimated by measuring the absorption at 260 nm (20). The size ranges of mRNAs in each fraction were estimated by electrophoresis of the fractions on 1% agarose gel and staining with ethidium bromide. Each fraction contains a maximum concentration of one size of mRNAs ($mRNA_{max}$) with gradually lower concentrations of smaller and larger species of mRNA, extending about 0.6 kilobase (kb) on each side of the $mRNA_{max}$. To combine data from different gradient experiments, results were grouped according to the $mRNA_{max}$ in each fraction, *i.e.* less than 0.5, 0.5–1.5, 1.5–2.5, and more than 2.5 kb, respectively.

In vitro transcription

Complementary RNA (cRNA) coding for the arginine (Arg) transporter rBAT (16) or for rat type I deiodinase (D1) (21) was prepared by *in vitro* transcription using the AmpliScribe T3 transcription kit (Epicentre Technologies, Madison, WI) according to the protocol for synthesis of capped cRNA. For capping, the m7G[5′]ppp[5′]G cap analog was used (Epicentre Technologies). pBluescript DNAs containing the respective complementary DNAs (cDNAs) as insert were used as template after linearization with *Xho*I (Boehringer Mannheim). After transcription, the DNA template was digested using ribonuclease-free deoxyribonuclease I, and the incubation mixture was extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (Life Technologies, Breda, The Netherlands) and once with chloroform. The cRNA in the final water phase was precipitated with an equal volume of 5 M ammonium acetate, incubated on ice for 30 min, and centrifuged for 10 min at 4 C. cRNA pellets were dissolved in water and stored at −80 C.

Uptake assays

Groups of 10 oocytes were washed for 1 min at 18 C in choline$^+$-containing incubation buffer (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 10 mM Tris, pH 7.5). Subsequently, the oocytes were incubated for 1 h at 25 C in 0.1 ml of the same buffer containing 50 μM Arg and 370 kBq/ml [^{3}H]Arg or with 4 nM [^{125}I]T_4 (60 kBq/ml), 5 nM [^{125}I]T_3 (60 kBq/ml), or 1.25 nM [^{125}I]T_3 sulfate ([^{125}I]T_3S; 90 kBq/ml). Uptake of these labeled compounds was also tested in Na^+ buffer (same buffer with 100 mM NaCl instead of choline chloride) to assess Na^+-dependent uptake. After 1 h, incubation buffer was removed, and the oocytes were washed four times with 2.5 ml ice-cold Na^+ buffer containing 0.1% BSA. Oocytes were transferred to new tubes or scintillation vials and counted individually.

Metabolism assays

Groups of 10 oocytes were transferred to a 96-well tissue culture plate. Subsequently, the oocytes were incubated at 18 C in the dark in 0.1 ml MBS containing 1.25 nM [^{125}I]T_3S (90 kBq/ml). After 18 h, the incubation medium was removed, and the oocytes were transferred to tubes and washed 4 times with 2.5 ml ice-cold Na^+ buffer containing 0.1% BSA. Each group of 10 oocytes was divided into 2 groups of 5 oocytes, transferred to new tubes, counted, homogenized in 0.1 ml 0.1 M NaOH, and centrifuged. The supernatants were analyzed for $^{125}I^-$, [^{125}I]T_3S, and [^{125}I]T_3 by Sephadex LH-20 chromatography as previously described (22).

Statistics

Data are presented as the mean ± SEM. Statistical significance was evaluated by Student's *t* test for unpaired observations. Data from the gradient experiments were analyzed by ANOVA, using the Studentized range for comparison of group means.

Results

Injection of 0.23 ng rBAT cRNA, which expresses Na^+-independent transport of L-arginine, L-cysteine, and L-leucine (16), resulted in a rise in [^{3}H]Arg uptake from 10 (water-injected) to 205 pmol/oocyte·h in choline$^+$-containing medium (Table 1) without a change in T_3 uptake in both the presence and absence of Na^+. Injection of 23 ng total liver mRNA resulted in a moderate, but significant ($P < 0.001$), increase in T_3 uptake from 6.4 ± 0.8 (water-injected) to 9.2 ± 0.65 fmol/oocyte·h (Table 1). This increase was completely blocked by replacing Na^+ in the uptake medium by choline$^+$, indicating that the increase in T_3 uptake is Na^+ dependent. From the data in Table 1, it is also clear that water-injected oocytes exhibited an endogenous T_3 uptake that was not significantly inhibited by replacement of Na^+ by choline$^+$. Furthermore, it appeared that this endogenous uptake was highly dependent on the batch of oocytes used (range, 1.7–13.9 fmol T_3/oocyte·h). To eliminate this variable endogenous uptake, we have taken the difference in uptake between

TABLE 1. Uptake of T_3 and arginine by *X. laevis* oocytes

Injected material	Uptake[a]		
	T_3 (Na^+) (fmol/oocyte)	T_3 (choline$^+$) (fmol/oocyte)	Arg (choline$^+$) (pmol/oocyte)
Water	6.4 ± 0.8	5.0 ± 0.5	10 ± 1.8
0.23 ng rBAT cRNA	6.5 ± 0.7	5.1 ± 0.4	205 ± 18.7[b]
23 ng liver mRNA	9.2 ± 0.65[c]	5.5 ± 0.5	

Results represent the mean ± SEM of 19 (T_3) or 14 (Arg) experiments.
[a] Ten oocytes injected 4 days previously with water, rBAT cRNA, or Wistar rat liver mRNA were incubated for 1 h with 5 nM [^{125}I]T_3 or 50 μM [^{3}H]Arg in 0.1 ml medium with Na^+ or choline$^+$.
[b] $P < 0.001$ *vs.* water-injected oocytes.
[c] $P < 0.01$ *vs.* water-injected oocytes.

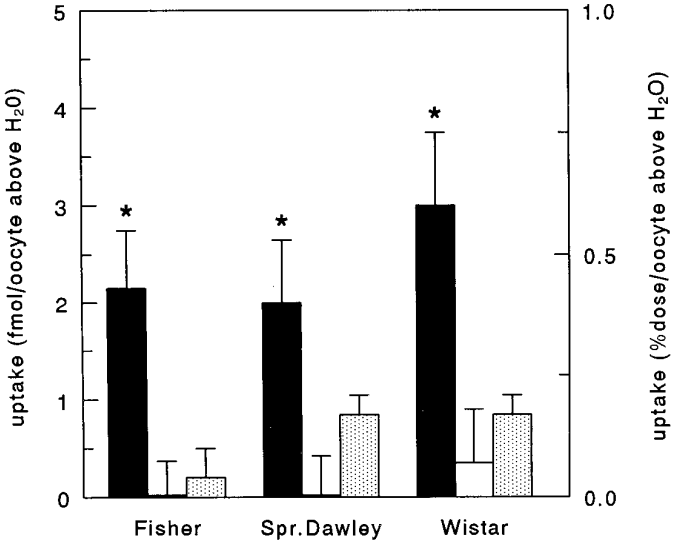

FIG. 1. Induction of T_3 uptake by oocytes after injection of total liver mRNA from different rat strains. Groups of 10 oocytes were injected with 23 ng/oocyte liver mRNA or with water, and after 4 days they were incubated for 1 h at 25 C with 5 nM [^{125}I]T_3 in 0.1 medium containing Na^+ (■), choline$^+$ (□), or Na^+ and 1 μM unlabeled T_3 (▨). The results show the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes, and are presented as the mean ± SEM of 10 oocytes. *Left y*-axis, Uptake of [^{125}I]T_3 in femtomoles per oocyte/h; *right y*-axis, uptake as a percentage of the added [^{125}I]T_3. *, $P < 0.001$ *vs.* zero.

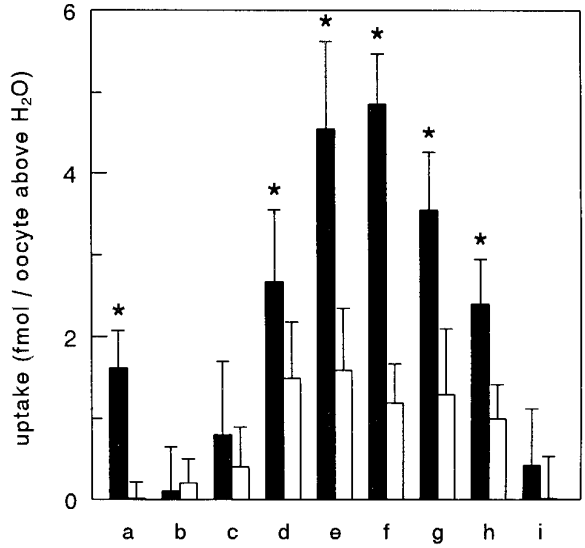

FIG. 2. Induction of T_3 uptake by oocytes after injection of various size fractions of rat liver mRNA. Groups of 10 oocytes were injected with 23 ng/oocyte liver mRNA or with water, and after 4 days they were incubated for 1 h at 25 C with 5 nM [^{125}I]T_3 in 0.1 ml medium containing Na^+ (■) or choline$^+$ (□). The results show the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes, and are presented as the mean ± SEM of 10 oocytes. Size ranges of RNA fractions: a, unfractionated mRNA; b, 3.0–4.5; c, 2.2–3.5; d, 1.5–3 kb; e, 1.25–2.5 kb; f, 0.8–2.1 kb; g, 0.5–1.4 kb; h, 0.3–1.25 kb; and i, 0.2–0.9 kb. *, $P < 0.001$ *vs.* zero.

mRNA-injected and water-injected oocytes in each further experiment as a measure of mRNA-induced T_3 uptake.

Figure 1 shows the induction of T_3 uptake by oocytes injected with 23 ng mRNA prepared from livers of three different rat strains and sexes. No significant difference was found among the preparations. mRNA-induced T_3 uptake in choline$^+$-containing medium was not significantly different from zero, indicating that the expressed transport system is completely Na^+ dependent. Furthermore, addition of 1 μM unlabeled T_3 inhibited the uptake of [^{125}I]T_3 by more than 50%.

Total Sprague-Dawley rat liver mRNA was size-fractionated on a 6–20% sucrose gradient, and the mRNA size ranges were determined by agarose gel electrophoresis. Of each fraction, 23 ng mRNA were injected into oocytes. The fractions containing mRNA of 0.8–2.1 kb showed the largest stimulation of Na^+-dependent T_3 uptake. A typical experiment is depicted in Fig. 2.

In 9 fractionation experiments, water-injected oocytes showed a T_4 uptake of 3.7 ± 0.7 fmol/oocyte·h and a T_3 uptake of 7.0 ± 1.3 fmol/oocyte·h. Injection of 23 ng total liver mRNA did not stimulate the uptake of T_4 significantly. mRNA-induced uptake of T_4 amounted to only 0.12 ± 0.08 fmol/oocyte·h, whereas mRNA-induced T_3 uptake in the same experiments was 0.95 ± 0.16 fmol/oocyte·h ($P < 0.001$; Fig. 3a). However, after fractionation of rat liver mRNA on a 6–20% sucrose gradient, uptake of T_4 was also significantly induced, in particular with mRNA of 0.5–2.5 kb, similar to T_3 uptake (Fig. 3, c and d).

Figure 4 shows that uptake of T_3S during 1 h was stimulated by injection of oocytes with a 0.8- to 2.1-kb fraction of rat liver mRNA, and that this uptake was Na^+ dependent. mRNA-induced T_3S uptake was inhibited by 50% in the presence of 1 μM unlabeled T_3S (data not shown). As basal uptake of T_3S by water-injected oocytes was low, injection of rat liver mRNA produced a relatively much larger signal compared with the effect on T_3 uptake. Injection of 2.3 ng D1 cRNA did not stimulate T_3S uptake, whereas injection of a

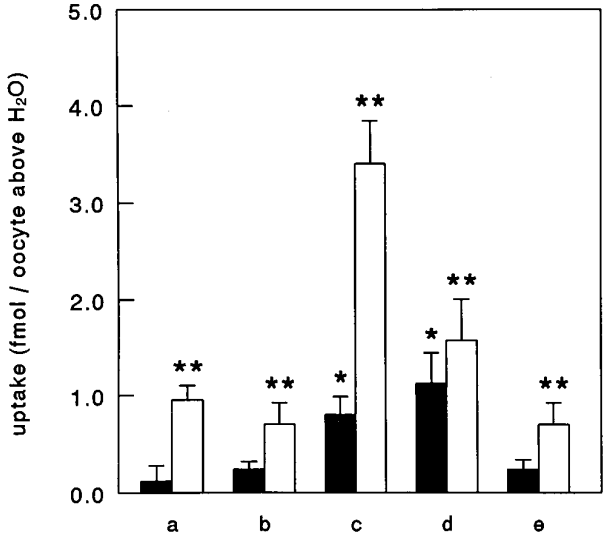

FIG. 3. Induction of T_4 (■) and T_3 (□) uptake by oocytes after injection of various size fractions of rat liver mRNA. Groups of 10 oocytes were injected with 23 ng/oocyte liver mRNA or with water, and after 4 days they were incubated for 1 h at 25 C with 5 nM [^{125}I]T_3 or 4 nM [^{125}I]T_4 in 0.1 ml Na^+ medium. The results show the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes and are presented as the mean ± SEM of nine experiments. Size ranges of RNA fractions: a, unfractionated mRNA; b, $mRNA_{max}$ less than 0.5 kb; c, $mRNA_{max}$ 0.5–1.5 kb; d, $mRNA_{max}$ 1.5–2.5 kb; e, $mRNA_{max}$ more than 2.5 kb. c *vs.* b: T_4, $P < 0.05$; T_3, $P < 0.001$; c *vs.* d: T_4, P = NS; T_3, $P < 0.01$; d *vs.* e: T_4, $P < 0.01$; T_3, P = NS. **, $P < 0.001$; *, $P < 0.01$ (*vs.* zero).

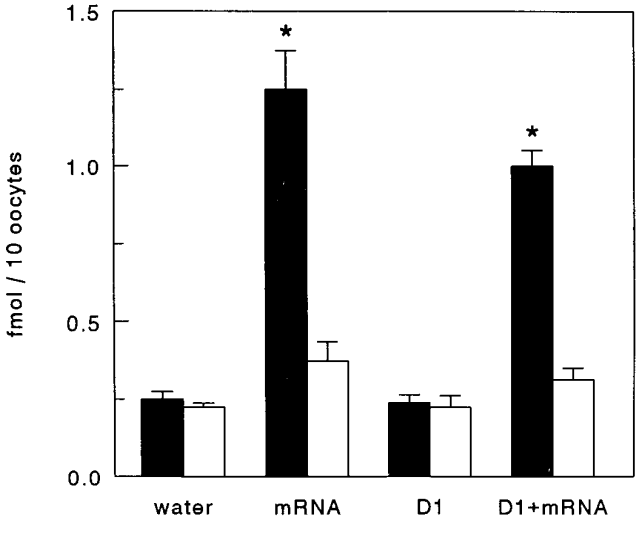

FIG. 4. T_3S uptake by oocytes injected with water, 0.8–2.1 kb rat liver mRNA, D1 cRNA, or the combination of liver mRNA and D1 cRNA. Groups of 10 oocytes were injected with 23 ng/oocyte mRNA, 2.3 ng/oocyte D1 cRNA, or both, and after 4 days they were incubated for 1 h at 25 C with 1.25 nM [^{125}I]T_3S in 0.1 ml medium containing Na^+ (■) or choline$^+$ (□). Data are presented as the mean ± SEM of 10 oocytes. *, $P < 0.001$ *vs.* water-injected oocytes.

mixture of 0.8–2.1 kb liver mRNA and D1 cRNA stimulated T_3S uptake to a similar extent as liver mRNA alone. Figure 5 shows the results of prolonged incubations (18 h) of similarly injected oocytes with [^{125}I]T_3S. Total T_3S uptake after 18 h was obviously higher than with 1-h incubations (Fig. 4).

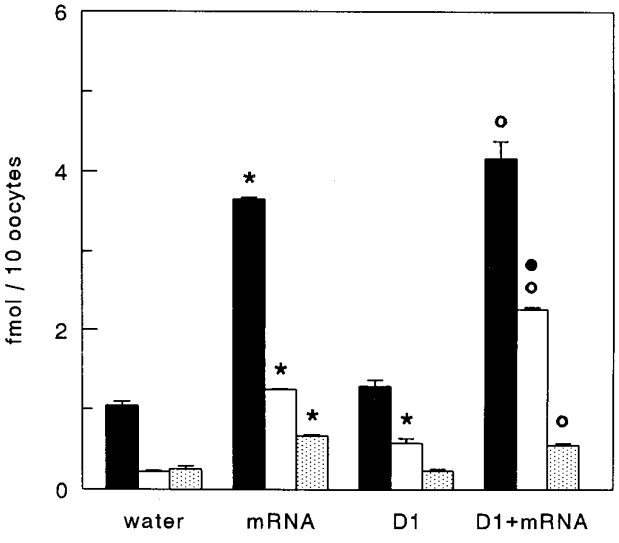

FIG. 5. Metabolism of T_3S in oocytes injected with water, 0.8–2.1 kb rat liver mRNA, D1 cRNA, or the combination of liver mRNA and D1 cRNA. Groups of 10 oocytes were injected with water, 23 ng/oocyte mRNA, 2.3 ng/oocyte D1 cRNA, or both, and after 4 days they were incubated for 18 h at 18 C with 1.25 nM [^{125}I]T_3S in 0.1 ml Na^+ medium. Results show the total amount of radioactivity (■), I^- (□), and T_3 (▨) in the oocytes at the end of the incubation and are presented as the mean ± SEM of four pools of five oocytes. *, $P < 0.001$ *vs.* water-injected oocytes; o, $P < 0.001$ *vs.* D1 cRNA- or water-injected oocytes; ●, $P < 0.001$ *vs.* liver mRNA-injected oocytes.

Total T_3S uptake was low in water- or D1 cRNA-injected oocytes, but was strongly stimulated in oocytes injected with 0.8–2.1 kb liver mRNA. Total T_3S uptake by oocytes injected with liver mRNA together with D1 cRNA was similar to uptake of oocytes injected with rat liver mRNA alone. In water-injected oocytes, deiodination of T_3S and deconjugation of T_3S to T_3 were very low. Deconjugation was similarly low in D1 cRNA-injected oocytes, but deiodination of T_3S was stimulated by expression of D1 ($P < 0.01$). In oocytes injected with liver mRNA, more T_3S was hydrolyzed than in water- or D1 cRNA-injected oocytes ($P < 0.001$), and T_3S was also deiodinated to some extent ($P < 0.001$). Finally, injection of oocytes with the combination of liver mRNA and D1 cRNA led to a much larger production of iodide from T_3S than injection of either RNA alone ($P < 0.001$).

Discussion

Our present findings that Na^+-dependent uptake of T_3, T_4, and T_3S is induced in *X. laevis* oocytes by the injection of rat liver mRNA are in agreement with our previous suggestion that transport of iodothyronines into rat hepatocytes proceeds via a plasma membrane transporter, as it is a saturable process dependent on temperature, the intracellular ATP concentration, and the Na^+ gradient over the cell membrane (3, 23). Because the oocytes exhibit an endogenous uptake system for T_3 which is independent of the Na^+ gradient, we have defined mRNA-induced T_3 uptake as the difference between T_3 uptake by mRNA-injected oocytes and that by water-injected oocytes in the same medium. Our finding that mRNA-induced T_3 uptake is completely abolished by replacement of Na^+ in the uptake medium by choline$^+$ strongly suggests that this uptake is tightly coupled to the

Na^+ gradient. This may also explain the ATP dependence of uptake of T_4 and T_3 by hepatocytes, because ATP is necessary to maintain the Na^+ gradient over the plasma membrane (24, 25). Livers from different rat strains contain similar amounts of mRNA coding for the T_3 transporter, showing the same Na^+ dependence and similar saturability with unlabeled T_3.

Uptake of T_4, T_3, and T_3S by oocytes is induced by the injection of certain mRNA species, but not by others. Thus, T_3 uptake by oocytes injected with rBAT cRNA is not different from that by water-injected oocytes. Similarly, injection of D1 cRNA does not induce T_3S uptake. Furthermore, not all rat liver mRNA fractions tested stimulate T_3 uptake; mRNAs of 0.2–0.9 and 2.2–4.5 kb have no effect, but mRNA of 0.8–2.1 kb induces a 2.5-fold greater stimulation of T_3 uptake than crude rat liver mRNA. Taken together, our results suggest that the size of the mRNA coding for the T_3 transporter is 1.5 ± 0.5 kb, a size large enough to code for a protein of 45–55 kDa, previously estimated by immunoprecipitation and photoaffinity labeling of putative cellular T_3 transport proteins (5, 6).

Induction of T_4 uptake in oocytes is undetectable after the injection of total rat liver mRNA. However, when liver mRNA is fractionated on a 6–20% sucrose gradient, a highly significant stimulation is found in fractions with $mRNA_{max}$ ranging between 0.5–2.5 kb. This stimulation is significantly higher than that by fractions with $mRNA_{max}$ smaller than 0.5 kb or larger than 2.5 kb. Although the stimulation of T_4 uptake seems somewhat larger in fractions in which $mRNA_{max}$ ranges between 1.5–2.5 kb, there is no significant difference from that in the fractions in the 0.5–1.5 kb size range. The largest stimulation of T_3 uptake was found in fractions containing $mRNA_{max}$ of 0.5–1.5, significantly higher than the smaller and larger mRNA size fractions. Therefore, stimulation of T_4 and T_3 uptake seems to peak in different mRNA fractions, suggesting that T_4 and T_3 transporters are translated from different mRNAs. This is in agreement with previous indirect evidence, suggesting different mechanisms for T_3 and T_4 uptake in liver cell membranes. Uptake of T_4 is lower than that of T_3, which is also in accordance with previous findings using rat hepatocytes (26), indicating that the V_{max} for the specific uptake of T_4 was 3.5-fold lower than that for T_3 uptake.

Uptake of T_3 and T_3S by oocytes is linear during the first hour (data not shown), suggesting that the 1-h uptake data represent binding and/or transport at the level of the cell membrane. Although the Na^+ dependence of the initial T_3 uptake process strongly suggests that this represents transmembrane transport, subsequent metabolism of T_3 would be an unequivocal indication that the hormone indeed enters the oocytes. Unfortunately, T_3 is not metabolized by oocytes. Induction of D1 by injection of its cRNA (21) does not change the situation, because T_3 is a poor substrate for this enzyme. On the other hand, [3′-^{125}I]T_3S is rapidly deiodinated by D1, initially in the inner ring and subsequently in the outer ring with liberation of $^{125}I^-$ (27). Our results indicate that the relative increase of T_3S uptake by *X. laevis* oocytes after the injection of rat liver mRNA is much greater than that in T_3, although the absolute rate of T_3S uptake is less than that of T_3 even in mRNA-injected oocytes. A similar difference was found between T_3 and T_3S uptake in rat hepatocytes (28). Like T_3 uptake, liver mRNA-induced T_3S uptake by oocytes is Na^+ dependent, whereas endogenous T_3S uptake by water-injected oocytes is Na^+ independent. The finding that deiodination of T_3S is highest in oocytes coinjected with D1 cRNA and rat liver mRNA indicates that T_3S is indeed transported to the cell interior as D1 is an intracellular membrane protein (29). Furthermore, it is clear that uptake of T_3S in mRNA plus D1 cRNA-injected oocytes is similar to that in oocytes injected with mRNA alone. This indicates that plasma membrane transport is independent of the metabolic capacity of the oocyte, underlining the rate-limiting potential of the transport process for entry and subsequent metabolism of thyroid hormone (7).

In conclusion, we present a system for expression cloning of cDNA coding for rat liver T_4, T_3, and T_3S transporter(s) based on the 0.8- to 2.1-kb mRNA fraction. This technique may lead to the molecular characterization of thyroid hormone plasma membrane transport proteins and to a better understanding of the molecular mechanism of translocation of thyroid hormone across the plasma membrane of target cells.

Acknowledgments

We thank Prof. Heini Murer and Dr. Daniel Markovich, Department of Physiology, University of Zurich (Zurich, Switzerland), for their generous gift of rBAT cDNA and for the opportunity offered to R.D. and P.G.J.v.S. to work with *X. laevis* oocytes in their laboratory. Furthermore, we wish to thank Prof. P. Reed Larsen, Peter Bent Brigham and Womans' Hospital, Harvard Medical School (Boston, MA), for his generous gift of D1 cDNA.

References

1. **Hennemann G** 1986 Thyroid hormone deiodination in healthy man. In: Hennemann G (ed) Thyroid Hormone Metabolism. Marcel Dekker, New York and Basel, pp 277–295
2. **Visser TJ** 1990 Importance of deiodination and conjugation in the hepatic metabolism of thyroid hormone. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, pp 255–283
3. **Docter R, Krenning EP** 1990 Role of cellular transport systems in the regulation of thyroid hormone bioactivity. In: Greer MA (ed) The Thyroid Gland. Raven Press, New York, pp 233–254
4. **Kragie L** 1994 Membrane iodothyronine transporters. I. Review of physiology. Endocr Res 20:319–341
5. **Mol JA, Krenning EP, Docter R, Rozing J, Hennemann G** 1986 Inhibition of iodothyronine transport into rat liver cells by a monoclonal antibody. J Biol Chem 261:7640–7643
6. **Samson M, Osty J, Blondeau J P** 1993 Identification by photoaffinity labeling of a membrane thyroid hormone-binding protein associated with the triiodothyronine transport system in rat erythrocytes. Endocrinology 132:2470–2476
7. **Hennemann G, Krenning EP, Polhuys M, Mol JA, Bernard BF, Visser TJ, Docter R** 1986 Carrier-mediated transport of thyroid hormone into rat hepatocytes is rate-limiting in total cellular uptake and metabolism. Endocrinology 119:1870–1872
8. **Halpern J, Hinkle PM** 1982 Evidence for an active step in thyroid hormone transport to nuclei: drug inhibition of L-^{125}I-triiodothyronine binding to nuclear receptors in rat pituitary tumor cells. Endocrinology 110:1070–1072
9. **Gonçalves E, Laksmanan M, Robbins J** 1989 Triiodothyronine transport into differentiated and undifferentiated mouse neuroblastoma cells (NB41A3) Endocrinology 124:293–300
10. **Hagenbuch B, Lübbert H, Stieger B, Meier PJ** 1990 Expression of the hepatocyte Na^+/bile acid cotransporter in *Xenopus laevis* oocytes. J Biol Chem 265:5357–5360
11. **Markovich D, Bissig M, Sorribas V, Hagenbuch B, Meier PJ, Murer H** 1994 Expression of rat renal sulfate transport systems in *Xenopus laevis* oocytes. J Biol Chem 269:3022–3026
12. **Bertran J, Werner A, Stange G, Markovich D, Biber J, Testar X, Zorzano A, Palacin M, Murer H** 1992 Expression of Na^+-independent amino acid transport in *Xenopus laevis* oocytes by injection of rabbit kidney cortex mRNA. Biochem J 281:717–723
13. **Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ** 1994 Expres-

sion cloning of a rat liver Na^+-independent organic anion transporter. Proc Natl Acad Sci USA 91:133–137
14. **Bissig M, Hagenbuch B, Stieger B, Koller T, Meier PJ** 1994 Functional expression cloning of the canicular sulfate transport system of rat hepatocytes. J Biol Chem 269:3017–3021
15. **Deen PMT, Verdijk MAJ, Knoers NVAM, Wieringa B, Monnens LAH, van Os CH, van Oost BA** 1994 Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. Science 264:92–95
16. **Bertran J, Werner A, Moore ML, Stange G, Markovich D, Biber J, Testar X, Zorzani A, Palacin M, Murer H** 1992 Expression cloning of cDNA from rabbit kidney cortex that induces a single transport system for cystine and dibasic and neutral amino acids. Proc Natl Acad Sci USA 89:5601–5605
17. **Mol JA, Visser TJ** 1985 Synthesis and some properties of sulfate esters and sulfamates of iodothyronines. Endocrinology 117:1–7
18. **Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW, Meier PJ** 1991 Expression of the hepatocellular chloride-dependent sulfobromophtalein uptake system in *Xenopus laevis* oocytes. J Clin Invest 88:2146–2149
19. **Dumont JA** 1972 Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. J Morphol 136:153–180
20. **Maniatis T, Fritsch EF, Sambrook J** 1982 Molecular Cloning–A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, pp 461–468
21. **Berry MJ, Banu L, Larsen PR** 1991 Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. Nature 349:438–440
22. **Docter R, Krenning EP, Bernard HF, Visser TJ, Hennemann G** 1988 Inhibition of uptake of thyroid hormone by preincubation with *N*-bromoacetyl-3,3′,5-triiodothyronine. Endocrinology 123:1520–1525
23. **Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G** 1982 Decreased transport of thyroxine (T_4), 3,3′,5-triiodothyronine (T_3) and 3,3′,5′-triiodothyronine (rT_3) into rat hepatocytes in primary culture due to a decrease of cellular ATP content and various drugs. FEBS Lett 140:229–233
24. **Pedersen PL, Carafoli E** 1987 Ion motive ATPases. Ubiquity, properties, and significance to cell function. Trends Biochem Sci 12:146–150
25. **Lechene C** 1988 Physiological role of the Na-K pump. In: Skou JC, Nørby JG, Maunsbach AB, Esmann M, eds. The Na^+,K^+-pump. B. Cellular Aspects, Progress in Clinical and Biological Research. Liss, New York, vol 268:171–194
26. **Krenning EP, Docter R, Bernard HF, Visser TJ, Hennemann G** 1981 Characteristics of active transport of thyroid hormone into rat hepatocytes. Biochim Biophys Acta 676:314–320
27. **Visser TJ, Mol JA, Otten MH** 1983 Rapid deiodination of triiodothyronine sulfate by rat liver microsomal fraction. Endocrinology 112:1547–1549
28. **Everts ME, Visser TJ, van Buuren JCJ, Docter R, de Jong M, Krenning EP, Hennemann G** 1994 Uptake of triiodothyronine sulfate and suppression of thyrotropin secretion in cultured anterior pituitary cells. Metabolism 43:1282–1286
29. **Fekkes D, van Overmeeren E, Hennemann G, Visser TJ** 1980 Solubilization and partial characterization of rat liver iodothyronine deiodinases. Biochim Biophys Acta 613:41–51