

## Thyroid hormone transport by 4F2hc-IU12 heterodimers expressed in *Xenopus* oocytes

J W A Ritchie, G J Peter, Y-B Shi<sup>1</sup> and P M Taylor

Department of Anatomy and Physiology, University of Dundee, Dundee, DD1 4HN, Scotland UK

<sup>1</sup>Laboratory of Molecular Embryology NICHD, National Institutes of Health, Bethesda MD 20892-543, USA

(Requests for offprints should be addressed to P M Taylor; Email: p.m.taylor@dundee.ac.uk.)

### Abstract

Thyroid hormone (TH) action and metabolism require hormone transport across cell membranes. We have investigated the possibility that TH are substrates of amino acid transport (System L) mediated by heterodimers of 4F2 heavy-chain (hc) and the light-chain (lc) permease IU12. Co-expression of 4F2hc and IU12 cDNAs injected into *Xenopus* oocytes induces saturable, Na<sup>+</sup>-independent transport of triiodothyronine (T<sub>3</sub>), thyroxine (T<sub>4</sub>) (K<sub>m</sub> of 1.8 and 6.3 μM respectively), tryptophan and phenylalanine. Induced TH and

tryptophan uptakes are inhibited by excess BCH (synthetic System L substrate). Induced TH uptake is also inhibited by excess reverse tri-iodothyronine (rT<sub>3</sub>), but not by triiodothyroacetic acid (TRIAc) (TH analogue lacking an amino acid moiety). T<sub>3</sub> and tryptophan exhibit reciprocal inhibition of their 4F2hc-IU12 induced uptake. Transport pathways produced by 4F2hc-lc permease complexes may therefore be important routes for movement and exchange of TH (as well as amino acids) across vertebrate cell membranes, with a potential role in modulating TH action.

### Introduction

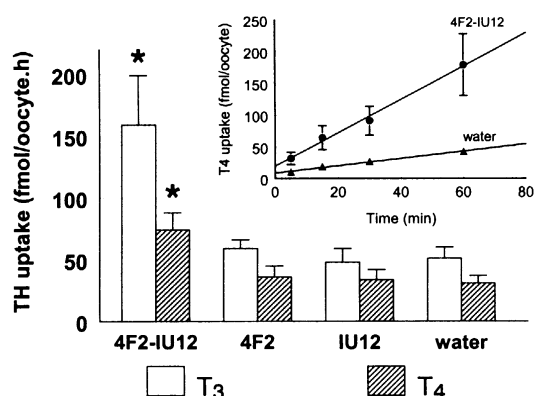
The thyroid gland produces both thyroxine (T<sub>4</sub>) and triiodothyronine (T<sub>3</sub>) and releases them into the blood circulation, although much circulating T<sub>3</sub> (the more active hormone) is generated by monodeiodination of T<sub>4</sub> in liver and kidney (Oppenheimer *et al.* 1996, Hennemann & Visser 1997). The major source of nuclear receptor-bound T<sub>3</sub> in many tissues (eg liver) is the blood T<sub>3</sub> pool, although some tissues (eg brain) generate T<sub>3</sub> endogenously from T<sub>4</sub> (Oppenheimer *et al.* 1996, Hennemann & Visser 1997). Movement of thyroid hormones between intra- and extra-cellular fluid compartments across the cell membrane is therefore an important step for modulation of hormone action and metabolism. Surprisingly, the specific mechanisms by which thyroid hormones cross the cell membrane are not fully understood, although movement by simple diffusion is likely to be a minor component of their total blood-tissue exchange (Hennemann *et al.* 1986, Blondeau *et al.* 1988, Chantoux *et al.* 1995, Blondeau *et al.* 1993, Zhou *et al.* 1992). Thyroid hormone (TH) transport into cells is inhibited by a wide variety of substances, including certain amino acids (notably tryptophan) (Blondeau *et al.* 1993, Zhou *et al.* 1990, Samson *et al.* 1992, Kemp & Taylor 1997), bilirubin (Chantoux *et al.* 1993), bilirubin conjugates (Chantoux *et al.* 1993) and various structurally unrelated drugs (Chantoux *et al.* 1993, Abe *et al.* 1998). Recent reports (Abe *et al.* 1998, Friesema *et al.* 1999) show that thyroid hormones and sulfated derivatives are transported by organic anion transporters such as Ntcp and oatp1-3, but the molecular mechanism by which thyroid hormones and amino acids interact has not been elucidated.

There is evidence for a close functional link between transport of aromatic amino acids and thyroid hormones in erythrocytes (Zhou *et al.* 1992, Samson *et al.* 1992), hepatocytes (Blondeau *et al.* 1988, Kemp & Taylor 1997) (by System T in both cases), placental choriocarcinoma cells (Prasad *et al.* 1994) and astrocytes (Blondeau *et al.* 1993) (by System L).

Recent studies have identified several members of a new family of amino acid permeases (eg LAT1, IU12/ASUR4 (Prasad *et al.* 1999, Mastroberardino *et al.* 1998, Torrents *et al.* 1998)) which exhibit activation of amino acid transport, having functional characteristics of System L, only when co-expressed with 4F2 heavy-chain (hc) glycoprotein. The highly-hydrophobic permeases light-chain (lc) interact covalently with 4F2hc to produce a functional, heteromeric 'transporter unit' in the cell membrane (Prasad *et al.* 1999, Mastroberardino *et al.* 1998, Torrents *et al.* 1998). The *Xenopus* lc permease IU12 (Torrents *et al.* 1998) is an early T<sub>3</sub>-response gene up-regulated during intestinal development (Liang *et al.* 1997) and it has been suggested that IU12 is involved in the signal transduction pathway of T<sub>3</sub>-induced metamorphosis (Liang *et al.* 1997). Here we demonstrate that the System L-like amino acid transport activity induced by co-expression of IU12 and 4F2hc in *Xenopus* oocytes accepts thyroid hormones as substrates, identifying an additional route for trans-membrane hormone transport which is also consistent with the proposed role of IU12 in transduction of T<sub>3</sub> signals.

### Materials and Methods

*Xenopus laevis* toads were purchased from the South African

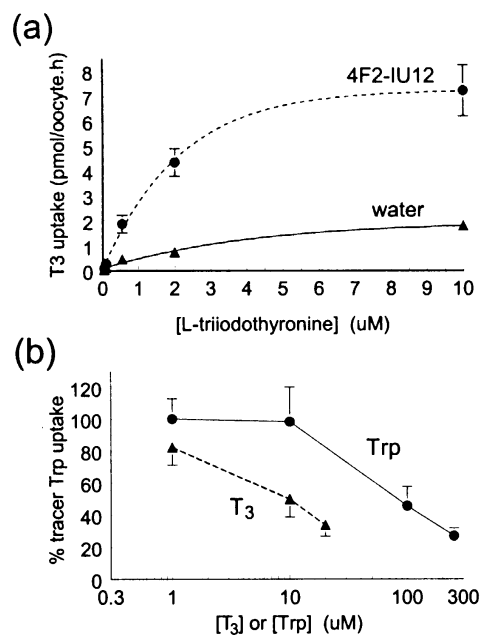


**Figure 1** Uptake of thyroid hormones ( $T_3$ ,  $T_4$  at  $0.1 \mu\text{M}$ ) by *Xenopus* oocytes 4 days after nuclear injection of 4F2hc and IU12 cDNA alone or in combination. Control oocytes were injected with water. Each bar represents mean uptake  $\pm$  S.E.M. measured in 5 separate batches of oocytes (using 8-11 individual oocytes per batch). \*, Uptake value significantly different from corresponding value in water-injected oocytes with  $P < 0.01$ . Inset: - time courses of  $0.1 \mu\text{M}$  [ $^{125}\text{I}$ ] $T_4$  uptake into oocytes injected with 4F2-IU12 DNAs or water (each point represents mean uptake  $\pm$  S.E.M. for 8-10 oocytes).

*Xenopus* Facility. Chemicals were obtained from Sigma (UK) with the exception of Collagenase A (Boehringer, UK) and Ultraspec water (Ambion, UK). Radiotracers were purchased from NEN (UK). cDNAs encoding IU12 (2.3 Kb EcoRI/ApaI fragment from pBluescriptSK-IU12 (Liang *et al.* 1997)) and human 4F2hc (1.85 Kb EcoRI/BamHI fragment from pSP65-4F2 (Teixeira *et al.* 1987)) were subcloned into the multiple-cloning region of pSG5 (an SV-40 driven expression plasmid).

Oocytes were isolated by collagenase treatment (Peter *et al.* 1996) of ovarian tissue obtained from mature female *Xenopus laevis* toads. Defolliculated, stage V-VI (prophase-arrested) oocytes were selected and maintained at  $18^\circ\text{C}$  in Modified Barths Medium (MBM) containing (in mM): 88 NaCl, 1 KCl, 2.4  $\text{NaHCO}_3$ , 0.82  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.66  $\text{NaNO}_3$ , 0.75  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 5.0 HEPES, pH 7.6 with Tris base, 10 mg/liter gentamycin sulphate. For DNA injection, oocytes were transferred into individual wells of Tetraski plates pre-filled with MBM and centrifuged at 500 g for 10 minutes at  $18^\circ\text{C}$ , which causes migration of the nucleus to the cell surface and facilitates nuclear injection (Mertz & Gurdon 1977). The visible nucleus of each oocyte was injected with 2 ng DNA in 15 nl Ultraspec water using a pneumatic delivery system (Peter *et al.* 1996). For co-injection studies, 2 ng of both DNAs (ie 4F2hc/IU12) were injected. Nuclei of control oocytes were injected with Ultraspec water. Oocytes were incubated in MBM at  $18^\circ\text{C}$  for 4 days to allow expression of injected DNA before experimentation.

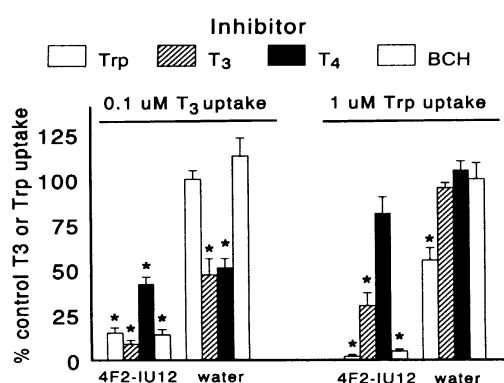
Thyroid hormone transport in oocytes was measured as influx of [ $^{125}\text{I}$ ]-labelled  $T_3$  and  $T_4$  tracers using a procedure



**Figure 2** (a) Uptake of  $T_3$  by oocytes injected with 4F2hc-IU12 DNAs or water as a function of external  $T_3$  concentration. Data are mean uptake  $\pm$  S.E.M. for 9-11 oocytes at each point, 4 days post-injection. Smallest error bars are masked by symbols. 4F2hc-IU12 induced  $T_3$  transport had an apparent  $K_m$  of  $1.8 \mu\text{M}$  and  $V_{max}$  of  $6.4 \pm 0.3 \text{ pmol/oocyte.h}$ . Tryptophan uptake in the same batch of oocytes had a  $K_m$  of  $70 \mu\text{M}$  and  $V_{max}$  of  $180 \pm 54 \text{ pmol/oocyte.h}$ . (b) Concentration-dependent inhibition of 4F2hc-IU12 induced [ $^3\text{H}$ ]tryptophan uptake by unlabelled  $T_3$  or tryptophan. Data show uptake of tryptophan ( $1 \mu\text{M}$  tracer) in presence of increasing concentrations of unlabelled inhibitor, as a percentage of control uptake in absence of inhibitor. Each point represents mean value  $\pm$  S.E.M. for 8-11 4F2hc-IU12-injected oocytes, after appropriate correction for uptake in water-injected oocytes.

described previously for amino acid uptake (Peter *et al.* 1996). All experiments were carried out at  $22^\circ\text{C}$  using  $\text{Na}^+$ -free transport buffer (unless otherwise stated) containing 100 mM tetramethylammonium chloride (TMACl), 2 mM KCl; 1 mM  $\text{CaCl}_2$ ; 1 mM  $\text{MgCl}_2$ ; 10 mM HEPES, pH 7.5 with Tris). Radiolabelled amino acid uptake ([ $^3\text{H}$ ]-phenylalanine or [ $^3\text{H}$ ]tryptophan over 30 min) was also measured (Peter *et al.* 1996). BCH (5 mM) was used as a specific inhibitor of amino acid transport System L.

Data are expressed as mean values  $\pm$  standard error of the mean (S.E.M.;  $n$  = number of observations). Experimental measurements in each batch of oocytes were made on 7 - 11 individual oocytes. Differences between mean values were assessed using Students unpaired t-test, with significance assigned at  $P < 0.05$ .



**Figure 3** Inhibition of T<sub>3</sub> (0.1 μM) and tryptophan (1 μM) uptake by iodothyronines, tryptophan and 2-endoamino-bicycloheptane-2-carboxylic acid (BCH) in *Xenopus* oocytes injected with 4F2hc-IU12 cDNAs or water. Inhibitor concentrations were 10 μM for T<sub>3</sub> and T<sub>4</sub>, 5 mM for BCH and 10 mM for tryptophan. Each bar represents uptake in the presence of inhibitor as a percentage of control uptake measured in absence of inhibitor (mean value ± S.E.M. for 7-11 oocytes). Control T<sub>3</sub> uptakes were 71 ± 7 and 22 ± 3 fmol/oocyte.h for 4F2hc-IU12- and water-injected oocytes respectively. Control tryptophan uptakes were 10.2 ± 0.4 and 2.1 ± 0.1 pmol/oocyte.h for 4F2hc-IU12- and water-injected oocytes respectively. Similar results were obtained using a different batch of oocytes. \* Significant reduction of uptake in presence of inhibitor ( $P < 0.005$ ).

## Results

Functional expression of 4F2hc-IU12 heterodimers in oocytes after nuclear injection of cDNAs was established in preliminary experiments (data not shown) as a marked, 2-endoamino-bicycloheptane-2-carboxylic acid (BCH)-inhibitable induction of uptake of [<sup>3</sup>H]phenylalanine and [<sup>3</sup>H]tryptophan (averaging 120 pmol/oocyte.h for both amino acids at 50 μM) relative to uptake in water-injected cells (10 pmol/oocyte.h). Induced phenylalanine uptake was stable between 2-4 days post-injection. Uptake of both T<sub>3</sub> and T<sub>4</sub> (0.1 μM) was also significantly increased in 4F2hc-IU12 injected oocytes compared to oocytes injected with a single DNA or water (Fig. 1). The time course of [<sup>125</sup>I]-labelled T<sub>3</sub> and T<sub>4</sub> uptake into oocytes was linear for least 2 hours (eg Fig. 1); all TH studies reported below involved a 60 min uptake period using oocytes at 4 days post-injection. The average increase in uptake over control (water- or IU12-injected oocytes) was 2.1 ± 0.5 and 2.7 ± 0.35 times for T<sub>4</sub> and T<sub>3</sub> respectively ( $n = 5$  batches of oocytes). Induced TH uptakes were saturable and showed mutual cross-inhibition (Figs 2a, 3; data shown for T<sub>3</sub> only). Induced T<sub>3</sub> and T<sub>4</sub> uptakes measured over a concentration range of 0.05-10 μM (the latter close to the limit of iodothyronine solubility) had for T<sub>3</sub>, an apparent  $K_m$  value of 1.8 μM and  $V_{max}$  of 6.4 ± 0.3 pmol/oocyte.h (Fig.

2a) and for T<sub>4</sub>, a  $K_m$  of 6.3 μM and  $V_{max}$  of 2.0 ± 0.6 pmol/oocyte.h. Induced 0.1 μM TH uptake differed markedly from basal TH uptake measured in control (water-injected) oocytes in that it was significantly inhibited by excess BCH and tryptophan (Fig. 3, data shown for T<sub>3</sub> only). The 4F2hc-IU12 induced uptake of [<sup>3</sup>H]-tryptophan is also inhibited by BCH (Fig. 3) and shows concentration-dependent inhibition by unlabelled T<sub>3</sub> and tryptophan (Fig. 2b), although inhibition by T<sub>4</sub> (10 μM) has not achieved statistical significance (Fig. 3). The natural iodothyronine reverse tri-iodothyronine (rT<sub>3</sub>) (10 μM) inhibited induced 0.1 μM T<sub>3</sub> uptake by 41 ± 4% ( $n = 3$  preparations), but the synthetic iodothyronine analogue triiodoacetic acid (TRIAc) did not inhibit T<sub>3</sub> (or tryptophan) uptake in either 4F2hc-IU12- or water-injected oocytes (data not shown).

Substitution of TMA<sup>+</sup> with 100 mM Na<sup>+</sup> did not have a significant effect on TH or tryptophan uptake, nor did preincubation of oocytes with 1 nM T<sub>3</sub> and T<sub>4</sub> for 48 h prior to transport measurement at 4-days post-injection.

## Discussion

In the present study, we provide evidence which indicates that amino acid transport activity produced by 4F2hc-IU12 heterodimers accepts thyroid hormones (T<sub>4</sub> and T<sub>3</sub>) as substrates. The 4F2hc-IU12 induced uptakes of T<sub>3</sub> and tryptophan in oocytes show mutual inhibition and are both Na<sup>+</sup>-independent and inhibited by excess BCH, confirming that the expressed transport activity is System L. To our knowledge, this is the first report of thyroid hormone transport by a cloned amino acid transporter. Other recent studies (Abe *et al.* 1998, Friesama *et al.* 1999) demonstrate that organic anion transporters also accept TH and other iodothyronines as substrates, providing both Na<sup>+</sup>-dependent (Ntcp (Friesama *et al.* 1999)) and Na<sup>+</sup>-independent (oatp 1-3 (Abe *et al.* 1998, Friesama *et al.* 1999)) transport pathways. The present results indicate that amino acid transporters producing System L-like activity may provide an important route for physiologically-relevant movements of TH across cell membranes. Furthermore, in combination with the knowledge that TH are also substrates for organic anion transporters, the results provide a rational basis for explaining the wide variety of reported inhibitors of cellular TH transport (Zhou *et al.* 1990, Samson *et al.* 1992, Kemp & Taylor 1997, Chantoux *et al.* 1993, Abe *et al.* 1998.).

4F2hc-IU12-induced TH transport is inhibited by rT<sub>3</sub>, indicating that System L may transport a variety of iodothyronines. T<sub>4</sub>, T<sub>3</sub> and rT<sub>3</sub> are large, iodinated tyrosine derivatives retaining an amino acid functional motif which presumably enables recognition by the System L binding site; this view is supported by the observation that TRIAC (a T<sub>3</sub> analogue lacking the amino acid moiety) does not inhibit induced TH or tryptophan uptake. 4F2hc-IU12 induced TH transport appears to be of high affinity relative to tryptophan transport but, in contrast, there is a marked sequential reduction in apparent  $V_{max}$  for induced transport of tryptophan,

T<sub>3</sub> and T<sub>4</sub> (180, 6.4 and 2 pmol/oocyte.h respectively in the same oocyte batch). This reduction in transport capacity correlates with increasing size of the substrate molecule and may therefore reflect increasing steric hinderence of the transport mechanism, especially by the large iodides of T<sub>3</sub> and T<sub>4</sub>. Bulky phenylglucosides show analogous reductions in apparent V<sub>max</sub>, for the glucose transporter SGLT1 (Lostao *et al.* 1994), due to impeded translocation across the cell membrane.

System L transport and 4F2hc protein are ubiquitously expressed in mammalian tissues ((Palacin *et al.* 1998, Taylor *et al.* 1999) for review), although the lc permeases identified thus far have distinct, relatively restricted tissue distributions (Prasad *et al.* 1999, Mastroberardino *et al.* 1998, Torrents *et al.* 1998, Liang *et al.* 1997, Gaugitsch *et al.* 1992). We have not examined TH transport properties of lc permeases other than IU12 but, given the broad substrate scope of amino acid transport related to expression of 4F2hc (and its analogue NBAT) (Palacin *et al.* 1998), we might predict that TH are carried by mammalian homologues of IU12 such as LAT1 (which also expresses System L-like activity) (Prasad *et al.* 1999, Mastroberardino *et al.* 1998), providing important routes for TH transport and exchange in many tissues. A counter-transport mechanism for cellular TH accumulation driven by hetero-exchange of T<sub>3</sub> with intracellular aromatic amino acids has been proposed (Zhou *et al.* 1992, Zhou *et al.* 1990), which is consistent with the known mechanism of 4F2hc-lc induced amino acid transport (Prasad *et al.* 1999, Mastroberardino *et al.* 1998, Torrents *et al.* 1998). This type of exchange might include physiologically-relevant T<sub>4</sub>/T<sub>3</sub> exchanges across cell membranes in tissues such as liver and kidney (Hennemann & Visser 1997, Zhou *et al.* 1992, Taylor *et al.* 1998). The K<sub>m</sub> for T<sub>3</sub> uptake reported here for 'cloned' System L (1.8 µM) is of similar order to those reported for TH uptake by cloned organic anion transporters (Abe *et al.* 1998, Friesema *et al.* 1999), and also has a value similar to the K<sub>m</sub> for T<sub>3</sub> uptake (reportedly by System L<sub>1</sub>) in cultured astrocytes (Blondeau *et al.* 1993). However this value is at least an order of magnitude higher than those reported in other cell types (eg hepatocytes, erythrocytes) where TH are transported by a mechanism termed System T (Blondeau *et al.* 1988, Chantoux *et al.* 1995, Zhou *et al.* 1992, Zhou *et al.* 1990, Kemp & Taylor 1997). It is possible that System T transport is mediated by a novel, as yet unidentified, lc permease with higher affinity fo TH. An alternative possibility is that TH transport in certain tissues is facilitated by binding of hormone to surface receptors as a pre-requisite to transport across the cell membrane (Zhou *et al.* 1990, Kemp & Taylor 1997, Samson *et al.* 1996, Gharbi-Chihi & Torresani 1981). We (Kemp & Taylor 1997, Taylor *et al.* 1998) and others (Samson *et al.* 1992, Gharbi-Chihi & Torresani 1981) have hypothesized that interactions between receptor and transporter proteins enable thyroid hormones to be 'channelled' to amino acid transport mechanisms in the cell membrane under certain circumstances. It is conceivable that TH receptor proteins are brought into functional contact with lc permeases by mutual interactions within a 4F2hc-related

protein complex. 4F2hc-IU12, Ntcp and oatp transporters expressed in oocytes all accept both T<sub>3</sub> and T<sub>4</sub> as substrates, therefore responsibility for apparent differences between the transport mechanism for the two hormones in certain mammalian cell types (eg Hennemann *et al.* 1986, Chantoux *et al.* 1995, Kemp & Taylor 1997)) must reside elsewhere, possibly at the level of surface TH receptors (Kemp & Taylor 1997, Taylor *et al.* 1998).

Regulation of TH action and inter-organ TH metabolism involves combined action of transporters for free TH and conjugates (notably TH sulfates) at the cell membrane in series with intracellular deiodinases and sulfotransferases/sulfatases (Oppenheimer *et al.* 1996 Hennemann *et al.* 1997, Taylor *et al.* 1999). The K<sub>m</sub> values for T<sub>3</sub> transport by 4F2hc-IU12 and organic anion transporters are markedly higher than plasma free TH concentrations, but this would ensure a linear change of uptake with free hormone concentration as required of a component in TH signalling (Oppenheimer *et al.* 1996, Hennemann *et al.* 1997, Taylor *et al.* 1999). On the other hand, the T<sub>3</sub> K<sub>m</sub> value for 4F2hc-IU12 is lower than those reported for non-iodinated amino acid substrates (eg Trp, Leu, Phe, Gln, His) of 4F2hc-lc permease heterodimers (K<sub>m</sub> >20 µM (Prasad *et al.* 1999, Mastroberardino *et al.* 1998, Torrents *et al.* 1998)), which should help T<sub>3</sub> compete effectively for transport under physiological conditions. Thyroid hormones play an important role in vertebrate development (Oppenheimer *et al.* 1996, Hennemann *et al.* 1997, Liang *et al.* 1997, Shi *et al.* 1996). IU12 is a TH-regulated gene, at least during amphibian development (Liang *et al.* 1997), providing a possible mechanism by which the hormone regulates its own action and metabolism by modulating cellular entry. Tissue-specific, temporal regulation of expression of IU12 (Liang *et al.* 1997) (or other lc permeases) may also help determine cellular competence to respond to extracellular TH signals; this may have particular importance during development (Shi *et al.* 1996). Expression of mammalian homologues of IU12 is associated with cell activation and carcinogenesis as well as tissue development (Prasad *et al.* 1999, Gaugitsch *et al.* 1992, Sang *et al.* 1995). Our results indicate that the functions of these proteins may include modulation of TH signalling as well as amino acid supply for protein synthesis and other processes of cell metabolism.

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