Expression of rat liver Na⁺/L-alanine co-transport in *Xenopus laevis* oocytes

Effect of glucagon *in vivo*

Manuel PALACIN,* Andreas WERNER,† Jürgen DITTMER,† Heini MURER† and Jürg BIBER†‡

*Department of Biochemistry and Physiology, University of Barcelona, 08028 Barcelona, Spain, and †Institute of Physiology, University of Zürich-Irchel, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland

Poly(A)⁺ RNA (mRNA) isolated from rat liver was injected into *Xenopus laevis* oocytes, and expression of Na⁺/L-alanine transport was assayed by measuring Na⁺-dependent uptake of L-[³H]alanine. Expression of Na⁺/L-alanine transport was detected 3–7 days after mRNA injection, and was due to an increment of the Na⁺-dependent component. After injection of 40 ng of total mRNA, Na⁺-dependent uptake of L-alanine was 2.5-fold higher than in water-injected oocytes. In contrast with Na⁺/L-alanine transport by water-injected oocytes, expressed Na⁺/L-alanine transport was inhibited by *N*-methylaminoisobutyric acid, was inhibited by an extracellular pH of 6.5 and was saturated at approx. 1 mM-L-alanine. After sucrose-density-gradient fractionation, highest expression of Na⁺/L-alanine uptake was observed with mRNA of 1.9–2.5 kb in length. Compared with mRNA isolated from control rats, mRNA isolated from glucagon-treated rats showed a approx. 2-fold higher expression of Na⁺/L-alanine transport. The results demonstrate that both liver Na⁺/L-alanine transport systems (A and ASC) can be expressed in *X. laevis* oocytes. Furthermore, the data obtained with mRNA isolated from glucagon-treated rats suggest that glucagon regulates liver Na⁺/L-alanine transport (at least in part) via the availability of the corresponding mRNA.

INTRODUCTION

Amino acids are accumulated in mammalian cells by Na⁺-independent and Na⁺-dependent transport processes, which have been well characterized in terms of their substrate specificities and kinetic properties (Kilberg, 1982; Collarini & Oxender, 1987; Saier *et al.*, 1988). In hepatocytes the uptake of L-alanine is catalysed by two Na⁺-dependent transport systems (type A and ASC), which contribute to about a similar degree to overall transport. Although overlapping substrate specificity has been demonstrated, these two transport systems can be distinguished by the interaction with *N*-methylaminoisobutyric acid (MeAIB), which is exclusively transported by system A (Le Cam & Freychet, 1977; Kilberg *et al.*, 1979).

Although no direct evidence exists that systems A and ASC represent different molecular entities, this seems very likely on the basis of their different physiological regulation. Whereas the ASC system is barely regulated, the activity of the hepatic system A is under the control of the hormones glucagon and insulin and is also controlled by the supply of L-alanine (adaptation) (Shotwell *et al.*, 1983). Based on studies with various inhibitors, up-regulation of the activity of system A (as induced, e.g., by glucagon) is dependent on protein synthesis *de novo*, most probably owing to an enhanced transcription rate (Shotwell *et al.*, 1983; Saier *et al.*, 1988).

Improvement in our understanding of amino acid transport and its regulation in mammalian cells is mainly compromised by our scarce knowledge about the structural identity of the different carriers. Since no specific and high-affinity inhibitors are available, two main strategies have been used to identify mammalian amino acid carriers. Several methods for the reconstitution of neutral-amino-acid transport systems (such as systems A and ASC) have been described (McCormick *et al.*, 1985; Bracy *et al.*, 1987; Quesada & McGivan, 1988; Fafournoux *et al.*, 1989). In a more direct approach, McCormick & Johnstone (1988) have

described a 120–130 kDa protein of Ehrlich ascites cells, which upon reconstitution showed L-alanine transport with characteristics of the system A. Further, by using thiol-specific reagents in combination with substrate protection, a 20 kDa polypeptide has been postulated as a essential component of liver alanine transport mediated by either system A or ASC (Hayes & McGivan, 1983).

An alternative strategy to identify L-alanine transport systems would be their functional expression in *Xenopus* oocytes, as has been demonstrated for the intestinal Na⁺/D-glucose transport (Hediger *et al.*, 1987). In the present paper we demonstrate expression of Na⁺/L-alanine uptake by *Xenopus laevis* oocytes which have been injected with poly(A)⁺ RNA isolated from rat liver. The results indicate that both systems A and ASC are expressed in *X. laevis* oocytes and that both transport systems are encoded by mRNA of 1.9–2.5 kb in length. In addition, we demonstrate that liver mRNA isolated from glucagon-injected rats leads to increased expression of Na⁺/L-alanine transport activity in the oocytes. Thus these results suggest that liver Na⁺/L-alanine transport is regulated (at least in part) by glucagon via an increased availability of mRNA.

EXPERIMENTAL

Oocytes and injections

Xenopus laevis frogs were obtained from H. Kähler, Institute für Entwicklungsbiologie, Hamburg (Germany). Small clumps of oocytes were treated with collagenase (Boehringer, Mannheim, Germany) at 2 mg/ml in a Ca²⁺-free solution (ORII: 82.5 mM-NaCl, 2 mM-KCl, 1 mM-MgCl₂, 10 mM-Hepes/Tris, pH 7.5) for 2 × 90 min to remove the follicular layer. After thorough washing with ORII and modified Barth's solution [mBS: 88 mM-NaCl, 1 mM-KCl, 0.82 mM-MgSO₄, 0.41 mM-CaCl₂, 0.33 mM-Ca(NO₃)₂, 2.4 mM-NaHCO₃, 10 mg of gentamycin/ml, 10 mM-Hepes/Tris, pH 7.6] the oocytes were kept in mBS overnight at 18 °C.

Abbreviations used: MeAIB, *N*-methylaminoisobutyric acid.

‡ To whom correspondence and reprint requests should be addressed.

Healthy-looking stage V–VI oocytes were injected with 50 nl of mRNA (0.2–1 mg/ml of water) or water with a semi-automatic injector (Inject + Matic-system; J. A. Gabay, Geneva, Switzerland). If not otherwise stated, oocytes were incubated at 18 °C for 5 days in mBS before the uptake measurements.

Isolation and fractionation of poly(A)⁺ RNA (mRNA)

Liver mRNA from rats (Sprague–Dawley, 200–250 g body wt.) was extracted either by the caesium trifluoroacetate/guanidinium thiocyanate method according to the protocol given by Pharmacia AB (Molecular Biology Division, Uppsala, Sweden) or by the phenol-extraction method as described by Chomczynski & Sacchi (1987). Both extraction methods resulted in mRNA that gave similar expression of L-alanine transport. Rats were injected intraperitoneally with either 1 mg of glucagon (Sigma)/100 g body wt. in a total volume of 1.5 ml of 0.9 % NaCl or with saline alone (paired control). Then, 5 h after glucagon injection, the animals were killed and 3–6 g of liver was used for mRNA isolation. Oligo(dT)–cellulose for poly(A)⁺ RNA purification was purchased from Boehringer and used in accordance with the manufacturer's protocol. After denaturation (3 min at 65 °C), 100–200 µg of mRNA was fractionated on a linear sucrose gradient (6–20 %, w/w) in 15 mM-Pipes/0.25 % sarcosyl/5 mM-EDTA (pH 6.5) (Goldin *et al.*, 1986). After centrifugation for 18 h at 24000 rev./min and 4 °C (Kontron TST-41 rotor; r_{av} = 11.4 cm), 0.5 ml fractions were collected, precipitated with ethanol and extensively washed with 70 % ethanol. The final samples were stored in water at –70 °C.

Electrophoretic analysis

The mean size of the fractionated mRNA was determined by electrophoresis on a denaturing 1%-agarose submarine gel stained with ethidium bromide. For calibration, size markers were purchased from BRL (Gaithersburg, MD, U.S.A.). Northern-blot analysis for α -tubulin was performed as described by Eckardt *et al.* (1990). Briefly, after electrophoresis the RNA was subjected to limited hydrolysis by NaOH treatment before RNA transfer to a nylon filter in SSC buffer (0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0). The filter was first pre-hybridized in 50 % (w/v) formamide/4 × SSC/25 mM-sodium phosphate (pH 6.8)/5 × Denhardt's solution [0.1 % poly(vinylpyrrolidone) 360, 0.1 % Ficoll 400, 0.1 % BSA]/1 % SDS and 200 µg of sheared salmon sperm DNA/ml. Hybridization with 25 ng of α -tubulin cDNA that had been labelled with [³²P]dCTP to a specific radioactivity of 1.5×10^8 c.p.m./µg was performed at 42 °C overnight in 1 × Denhardt's solution/5 × SSC/0.1 % SDS/10 % dextran sulphate. Mismatched hybrids were removed by washing the filter twice in 0.1 × SSC at 54 °C. After drying, the filter was exposed to X-ray films (Dupont, Cronex 4). Quantitative analysis of the autoradiographs was performed by densitometry.

Flux measurements

To measure uptake of L-[³H]alanine or methylamino[1-¹⁴C]-isobutyric acid ([¹⁴C]MeAIB) (both purchased from NEN Radiochemicals), 14–20 oocytes per individual time point or condition were first washed in Na⁺-free uptake solution (100 mM-choline chloride, 2 mM-KCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 10 mM-Hepes/Tris, pH 7.5). Half of the oocytes were then placed in 100 µl of the Na⁺-containing uptake solution (same as before, but with 100 mM-NaCl replacing choline chloride) containing the desired concentration (see legends) of L-[³H]alanine (3–10 µCi/100 µl) or [¹⁴C]MeAIB (1 µCi/100 µl). The other half was used for measurement of the Na⁺-independent uptake in Na⁺-free choline chloride-containing uptake solution with the same radioisotopic composition. If not otherwise stated, the measurements were performed for 30 min at 25 °C. After incubation, the oocytes

were washed 3–4 times with 4 ml of cold Na⁺-free uptake solution containing in addition 1 mM of the tested amino acid. Single oocytes were transferred to a scintillation vial, dissolved in 0.2 ml of 10 % SDS and counted for radioactivity in 3 ml of scintillation fluid.

RESULTS

Characterization of endogenous L-alanine uptake by water-injected oocytes

As reported previously, stage V and IV oocytes exhibit endogenous Na⁺-dependent L-alanine transport activity (Van Winkle, 1988; Campa & Kilberg, 1989). Thus it was important first to characterize Na⁺/L-alanine uptake in water-injected oocytes, which served as a control throughout this study. As illustrated in Fig. 1(a), net Na⁺-dependent uptake of L-alanine was linear during the first 30 min of the incubation assay. Whereas total L-alanine uptake (in the presence of Na⁺) increased constantly, L-alanine uptake in the presence of choline chloride was already saturated after 15 min. Linearity for net Na⁺-dependent L-alanine uptake over the 30 min time period was also observed at concentrations of 0.2 mM- and 1 mM-L-alanine in the uptake solution (results not shown).

To test for the possible presence of the Na⁺/L-alanine transport system type A, which is characterized by its tolerance for some *N*-monomethylated substrates (Christensen, 1984), Na⁺/L-alanine transport by water-injected oocytes was determined in the presence of 10 mM- or 20 mM-MeAIB. In contrast with the data reported by Jung *et al.* (1984), who could not demonstrate any effect of MeAIB in non-injected oocytes, Na⁺/L-alanine uptake by water-injected oocytes was significantly stimulated by 22 % (see Table 1). A similar stimulation of Na⁺/L-alanine transport oocytes in the presence of MeAIB has been reported by Campa

Fig. 1. Time course of the uptakes of L-[³H]alanine and [¹⁴C]MeAIB by water-injected oocytes

At 3 days after injection, the uptake of 50 µM-L-alanine (a) and of 0.2 mM-MeAIB (b) was measured in the absence (■) or in the presence of 100 mM-Na⁺ (□). Net Na⁺-dependent L-alanine uptake (△) was calculated as the difference between the uptakes in the presence of Na⁺ and choline. The data represent means ± s.e. of five determinations. If not included, errors bars were smaller than the symbol.

Table 1. Expression (and inhibition by MeAIB) of Na⁺/L-alanine uptake in oocytes injected with water or liver mRNA

The data represent means $\pm$ S.E. from 12 independent experiments (10 oocytes per experiment) using three different liver mRNA preparations. At 5 days after injection, net Na⁺/L-alanine uptake was determined at 0.2 mM-L-alanine. * $P < 0.05$ by Student's paired t test compared with the absence of MeAIB.

Oocytes injected with	L-Alanine uptake (pmol/30 min per oocyte)		Effect of MeAIB
	MeAIB absent	MeAIB present	
Water	6.9 $\pm$ 1.0	8.5 $\pm$ 1.6*	+22 $\pm$ 7%
mRNA	16.1 $\pm$ 2.2	14.4 $\pm$ 3.2*	-12 $\pm$ 5%
Ratio of uptake (mRNA/water)	2.4 $\pm$ 0.2	1.6 $\pm$ 0.1	

& Kilberg (1989). As a more direct test for the presence of system A, the uptake of 0.2 mM-[¹⁴C]MeAIB was studied in the absence and presence of Na⁺ (Fig. 1b). In agreement with the lack of the inhibition of L-alanine transport by MeAIB, no Na⁺-dependent transport of MeAIB was observed. These results therefore suggest that in water-injected oocytes the L-alanine transport system A is absent, or for some reason is not detectable. It must be noted that a small rate of Na⁺/MeAIB transport in non-injected oocytes has been demonstrated by Campa & Kilberg (1989). Since in that study oocytes had been defolliculated manually, it might, however, be possible that defolliculation by collagenase (as used in the present study) could have inactivated such a small system-A-like transport activity.

Na⁺/L-alanine co-transport by mRNA-injected oocytes

Oocytes were injected with various amounts of liver mRNA and routinely analysed 5 days later. Significant expression was observed already after 3 days, and was maximal after 5 days, since after incubation of the oocytes for 7 days expression was not increased any further (results not shown). As shown in Fig. 2, dose-dependent expression of Na⁺/L-alanine transport was observed after injection of 9 and 18 ng of mRNA. At saturating amounts of mRNA (36 ng) net Na⁺/L-alanine uptake was stimulated by approx. 2-fold compared with the water-injected oocytes. It is concluded that the increased Na⁺/L-alanine transport is entirely due to an expression of the Na⁺-dependent component of L-alanine uptake, since the Na⁺-independent component was not changed (12 $\pm$ 1 and 11 $\pm$ 1 pmol of L-alanine/oocyte per 30 min in mRNA and water-injected oocytes respectively; for conditions see Table 1). Table 1 summarizes the results obtained with 12 independent batches of oocytes and

three different mRNA preparations. As illustrated, injection of saturating amounts of mRNA led to a mean of a 2.4-fold higher rate of Na⁺/L-alanine transport compared with that for water-injected oocytes.

Na⁺-dependent transport of L-alanine in hepatocytes is due to the activity of the system type A (inhibitable by MeAIB) and the system type ASC; these two transport systems contribute approximately equally to liver L-alanine transport (Edmondson *et al.*, 1979; Kilberg *et al.*, 1979). Therefore, to decide if the transport system type A or type ASC or both are expressed in the oocytes, and to distinguish expressed transport activity from an eventually activated endogenous transport, L-alanine transport by injected oocytes was measured in the presence of 10 mM-MeAIB. As illustrated in Table 1, Na⁺/L-alanine transport in mRNA-injected oocytes was inhibited significantly by 12% by MeAIB, whereas in water-injected oocytes the presence of MeAIB stimulated Na⁺/L-alanine transport by 22%. As a result, an apparent inhibitory effect by MeAIB on Na⁺/L-alanine transport in mRNA-injected oocytes can be calculated as being 34%. Therefore it is suggested that injection of liver mRNA into the oocytes led to an expression of both type A and ASC systems in approximately equal amounts. Interestingly, however, uptake of 0.2 mM-MeAIB by oocytes injected with total mRNA could not be demonstrated with certainty. Even after prolonged incubations (up to 2 h) the uptake of MeAIB in the presence of Na⁺ was not significantly different from that in the absence of Na⁺ (results not shown).

A further way to distinguish the two L-alanine transport systems is by their pH-dependency (Le Cam & Freychet, 1977; Kilberg *et al.*, 1980). Those authors reported pH-sensitivity of the transport system A, but not of the system type ASC. As shown in Fig. 3, Na⁺/L-alanine transport by injected oocytes was inhibited by 40% when the external pH was lowered from pH 7.0 to 6.5. In contrast, transport of L-alanine by water-injected oocytes was not affected by this change in external pH.

In a third set of experiments, uptake of L-alanine into water- and mRNA-injected oocytes was measured at two different concentrations of L-alanine, to distinguish further between expression of rat liver L-alanine transport and an eventually activated endogenous L-alanine transport. The apparent K_m of Na⁺-dependent transport of L-alanine in isolated rat liver plasma-membrane vesicles is above 1 mM (Sips *et al.*, 1980; Rosenthal *et al.*, 1985). On the other hand, the apparent K_m for Na⁺/L-alanine transport in oocytes was reported to be around 20 μ M (Jung *et al.*, 1984). The data presented in Fig. 4 demonstrate that Na⁺-dependent transport of L-alanine by water-injected oocytes is saturated at 0.2 mM, whereas in mRNA-injected oocytes Na⁺/L-alanine transport was saturated above 1 mM. Together with the data presented above, it is therefore concluded that injection of the liver mRNA most likely leads to an expression of

Fig. 2. Dose-response of Na⁺/L-alanine uptake expression

Water or 9, 18 or 38 ng of liver mRNA dissolved in 50 nl of water was injected into oocytes. After 5 days Na⁺/L-alanine transport was measured with 1 mM-L-alanine. The data represent net Na⁺/L-alanine transport (means $\pm$ S.E., $n = 7$).

Fig. 3. Dependence on external pH of expressed Na^+/L -alanine uptake

Oocytes were injected with water ($\square$) or 36 ng of liver mRNA ($\blacksquare$). After 5 days Na^+/L -alanine transport (1 mM) was measured at the indicated external pH values. The percentages of uptake were calculated on the basis of the respective transport rates at pH 7.5 (100%), which were 10.6 pmol for water-injected and 22.2 pmol for mRNA-injected oocytes. The data represent two experiments performed with two different mRNA preparations.

Fig. 4. Concentration-dependence of net Na^+/L -alanine uptake in oocytes

The oocytes were injected with 50 nl of water ($\square$) or 36–41 ng of liver mRNA from two different preparations ($\blacksquare$). After 5–6 days of incubation, Na^+/L -alanine uptake was determined at 0.2 mM- and 1 mM-L-alanine, for 7–10 oocytes, in both Na^+ -containing and Na^+ -free media. The data represent means $\pm$ S.E. from 4 different batches of oocytes.

the liver L-alanine transport systems A and ASC rather than to an activation of the endogenous L-alanine transport activity.

Fractionation of mRNA

Purified rat liver mRNA was fractionated on a sucrose density gradient. After injection of pooled fractions (see Fig. 5a), two pools (fractions 10–13 and fractions 14–16) could be identified, which led to an expression of Na^+/L -alanine transport similar to that observed with total mRNA. A more detailed analysis of the two positive pools revealed that expression of Na^+/L -alanine transport by both systems (A and ASC) is encoded by mRNA present in fractions 13–16, corresponding to mRNA species of 1.9–2.5 kb in length (Fig. 5b; see also Fig. 6).

Effect of treatment with glucagon *in vivo*

Glucagon has been demonstrated to increase liver L-alanine transport by stimulating the type A transport system, possibly via an increased transcription rate (Shotwell *et al.*, 1983; Saier *et al.*, 1988). Therefore mRNA was isolated from rats injected with glucagon (1 mg/100 g body wt.) for 5 h and tested in the oocyte expression system. As demonstrated in Table 2, mRNA

Fig. 5. Expression of Na^+/L -alanine transport with fractionated mRNA

(a) Oocytes were injected with water, total mRNA and pooled mRNA fractions; 5 days later net Na^+/L -alanine uptake was determined at 0.2 mM-alanine. The bars represent means $\pm$ S.E. obtained from 7–10 oocytes. (b) Analysis of single fractions of mRNA isolated from control rats ($\square$) and glucagon-treated rats ($\blacksquare$). The following amounts of mRNA from pooled fractions 4–12, separate fractions 13, 14, 15, 16 and 17, and pooled fractions 18–21, were injected, respectively: control, 11, 20, 33, 25, 20, 11 and 2 ng; glucagon, 12, 17, 44, 37, 13, 4 and 2 ng.

Table 2. Effect of mRNA isolated from glucagon-treated rats on expression of Na^+/L -alanine transport

The data represent means $\pm$ S.E.M. of five independent experiments using two different mRNA preparations. In each experiment 10 oocytes were injected with 40 ng of mRNA. Uptake of Na^+/L -alanine was determined at day 5 after injection at 0.2 mM-L-alanine. *Value significantly different ($P < 0.05$) from injections with control mRNA by Student's *t* test.

Oocytes injected with	L-Alanine uptake (pmol/30 min per oocyte)
Water	5.5 $\pm$ 0.3
mRNA (control)	12.0 $\pm$ 1.4
mRNA (glucagon)	17.2 $\pm$ 1.6*

isolated from glucagon-treated rats resulted in a significantly higher expression of L-alanine transport (3.2-fold over background) compared with an injection of the same amount (40 ng) of mRNA isolated from paired (saline-injected) control rats (2.2-fold over background).

To test for the specificity of this effect, a Northern-blot analysis using α -tubulin cDNA as a probe was performed. As Fig. 6 shows, no significant difference in the patterns of the total mRNAs was observed, nor was the amount of α -tubulin mRNA

changed in the two mRNA preparations. Densitometric analysis of the α -tubulin signal revealed less than 10% difference between the two mRNA samples (results not shown).

Fractionation on sucrose density gradients of mRNA isolated from glucagon-treated rats and expression of the fractionated mRNA is shown in Fig. 5(b). Similarly to the control mRNA, the fractions 13–16 were found to be positive with respect to expression of L-alanine uptake. In agreement with the observation made after injection of total mRNA, injection of fractionated mRNA from glucagon-treated rats resulted in higher expression of L-alanine uptake, especially with the fractions 14–16. It should be noted that the small differences in the injected amounts of mRNA (see legend to Fig. 5b) are most likely due to the contamination by 18 S rRNA (see Fig. 6), and therefore could not account for the observed increased expression of Na⁺/L-alanine transport by the mRNA fractions obtained from glucagon-treated rats.

In contrast with the fractions obtained with control mRNA, a

Fig. 6. Agarose-gel electrophoresis and Northern-blot analysis with α -tubulin cDNA of liver mRNA isolated from either control rats or glucagon-treated rats

(a) Electrophoresis of total control mRNA (lane 1; 0.91 μ g), control-mRNA fractions 12 (0.22 μ g), 13 (0.403 μ g), 14 (0.66 μ g), 15 (0.50 μ g) and 16 (0.40 μ g) (lanes 3–7), total mRNA from glucagon-treated rats (lane 2; 0.93 μ g) and the corresponding fractions 12 (0.2 μ g), 13 (0.33 μ g), 14 (0.88 μ g), 15 (0.75 μ g) and 16 (0.26 μ g) (lanes 9–13). Lane 8 contained mRNA size standards. (b) Hybridization of total mRNA isolated from control rats (lane 1) and glucagon-treated rats (lane 2) with α -tubulin cDNA. Each lane contained 3.8 μ g of total mRNA.

Table 3. Net Na⁺-dependent uptake of [¹⁴C]MeAIB

Oocytes were injected either with total mRNA (40 ng) or fractionated mRNA (37 ng of fraction 15) isolated from glucagon-treated rats. The data represent the means $\pm$ S.E. of two experiments (10 oocytes each) using two different mRNA samples. After 6 days, the uptake of 0.2 mM-MeAIB was determined. *Significantly different from zero ($P < 0.05$) by Student's t test.

Oocytes injected with	MeAIB uptake (pmol/30 min per oocyte)
Water	0.4 $\pm$ 0.6
Total mRNA	0.2 $\pm$ 0.8
Fraction 15	1.0 $\pm$ 0.4*

slight expression of Na⁺-dependent MeAIB transport was observed with mRNA isolated from glucagon-injected rats. Significant Na⁺-dependent transport of MeAIB could be demonstrated with fraction 15 of mRNA isolated from glucagon-treated animals (Table 3). In oocytes injected with the other fractions or with total mRNA isolated from glucagon-treated animals, significant Na⁺-dependent transport of MeAIB was, however, never observed.

DISCUSSION

As demonstrated in this work, injection of mRNA isolated from rat liver into *X. laevis* oocytes leads to higher Na⁺-dependent L-alanine transport compared with water-injected oocytes, owing to expression of mRNA species of 1.9–2.9 kb in length. To our knowledge, this is the first report demonstrating expression of liver Na⁺/L-alanine transport in the oocytes. Aoshima *et al.* (1988) reported expression of kidney and small-intestinal Na⁺-dependent amino acid uptake, but no size fractionation was performed in those studies.

Since *X. laevis* oocytes exhibit endogenous Na⁺/L-alanine transport (Jung *et al.*, 1984; Van Winkle, 1988; Campa & Kilberg, 1989), it was important to show whether the increased transport of L-alanine was due to expression of liver mRNA or to an activation of the endogenous L-alanine transport owing to the injection of mRNA. The latter possibility has been ruled out for two reasons: first, Na⁺-dependent transport of L-alanine by water-injected oocytes is clearly saturated at 0.2 mM-L-alanine, whereas it is not in oocytes injected with rat liver mRNA. These data are in agreement with the apparent K_m for Na⁺-dependent transport of L-alanine reported for *X. laevis* oocytes, which is 0.02 mM (Jung *et al.*, 1984) and with the apparent K_m of rat liver Na⁺/L-alanine transport, which has been reported to be above 1 mM when determined with isolated liver plasma-membrane vesicles (Sips *et al.*, 1980; Rosenthal *et al.*, 1985). Second, by lowering the external pH value from 7.0 to 6.5, Na⁺/L-alanine transport by mRNA-injected oocytes was clearly inhibited by 40%, whereas Na⁺/L-alanine transport by water-injected oocytes was not inhibited, or was even slightly stimulated. Similar inhibition of Na⁺/L-alanine transport by low pH values has been reported for L-alanine transport in hepatocytes (Le Cam & Freychet, 1977; Kilberg *et al.*, 1980).

Having established that rat liver Na⁺/L-alanine transport is expressed in the oocyte system, the question of the nature of the expressed system was addressed. In hepatocytes, Na⁺/L-alanine transport is due to two transport systems (type A and ASC) which contribute to about the same degree to the overall L-alanine transport (Edmondson *et al.*, 1979; Kilberg *et al.*, 1979). These two transport systems can best be distinguished by the *N*-monomethylated substrate MeAIB, which inhibits specifically system A (Shotwell *et al.*, 1983; Christensen, 1984; Saier *et al.*, 1988). Another way to distinguish between the two systems is by their pH-dependency. At pH values lower than 7, system ASC is barely affected, whereas system A is almost completely inhibited at pH 6 (Le Cam & Freychet, 1977; Kilberg *et al.*, 1980). The following lines of evidence suggest that injection of rat liver mRNA results in the expression of both Na⁺/L-alanine transport systems (A and ASC).

(i) Expression of the type A system was analysed in two ways: by the inhibitory effect of MeAIB on L-alanine transport and by uptake studies with radiolabelled MeAIB. Inclusion of MeAIB in the uptake medium significantly inhibited Na⁺/L-alanine transport by mRNA-injected oocytes by 12%, but also stimulated Na⁺/L-alanine transport in water-injected oocytes by 22% (see Table 1). Thus MeAIB apparently inhibited L-alanine uptake by 34%. However, the nature of the stimulatory effect of

MeAIB in water-injected oocytes is unknown, and therefore the actual contribution of system A to the expressed Na⁺/L-alanine transport therefore cannot be answered. When [¹⁴C]MeAIB was used as the substrate, significant Na⁺-dependent uptake of MeAIB by oocytes injected with total mRNA (isolated from either control or glucagon-injected rats) could never be observed, although the inhibitory effect of MeAIB is suggestive of the presence of system A. This discrepancy could be explained by the low concentration (0.2 mM) of MeAIB used (low specific radioactivity of [¹⁴C]MeAIB) and by the lower turnover rate of system A for MeAIB compared with L-alanine, as has been demonstrated in uptake studies with hepatocytes (Edmondson *et al.*, 1977; Kilberg *et al.*, 1979; Hayes & McGivan, 1982). In support of this would be the observation that in oocytes injected with the mRNA fraction exhibiting highest expression of L-alanine transport (fraction 15 of mRNA isolated from glucagon-injected rats) transport of MeAIB was significantly stimulated in the presence of Na⁺ (Table 3).

(ii) Further evidence for the expression of system A was obtained by transport experiments at different pH values. In hepatocytes, system A is almost completely inhibited at pH 6.0, whereas system ASC shows only a very low sensitivity to pH values lower than 7 (Le Cam & Freychet, 1977; Kilberg *et al.*, 1980, 1981). The results obtained with the uptake studies with mRNA-injected oocytes clearly demonstrate a 40% inhibition of net Na⁺-dependent transport of L-alanine, again pointing to the conclusion that in mRNA-injected oocytes the systems A and ASC are probably expressed to about an equal amount.

The results obtained from the size-fractionation experiments indicate that the liver Na⁺-dependent L-alanine transport systems are encoded by mRNA of length 1.9–2.5 kb. Since, as discussed above, overall Na⁺/L-alanine transport by mRNA-injected oocytes has to be regarded as mediated by both systems A and ASC, it is concluded that each transport system (A and ASC) is encoded by mRNA of length 1.9–2.5 kb. The predicted molecular mass of such transport systems would therefore range between 65 and 90 kDa. There exists one report demonstrating a putative size of a protein associated with system A activity; by immunoprecipitation and reconstitution experiments a 120–130 kDa protein from Ehrlich ascites-tumour cells has been suggested to be involved in L-alanine transport by system A (McCormick & Johnstone, 1988).

Glucagon administered *in vivo* strongly increases the activity of transport system type A and also, but to a much lesser extent, the system type ASC in hepatocytes (Handlogten & Kilberg, 1984). As demonstrated with intact hepatocytes and purified liver plasma membranes, this effect of glucagon on system A is manifested by an increase in the V_{max} (Fehlmann *et al.*, 1979; Schenerman & Kilberg, 1986). Two mechanisms for the regulation of the liver system A by glucagon have been described. Besides an acute protein-synthesis-independent effect of glucagon (Edmondson & Lumeng, 1980; McGivan *et al.*, 1981), a long-term effect of glucagon has been described, which is dependent on mRNA and protein synthesis (Pariza *et al.*, 1976; Barber *et al.*, 1983). The results obtained in the present study with liver mRNA isolated from glucagon-treated rats suggest that the glucagon stimulates (at least in part) liver Na⁺/L-alanine transport via an increased availability of mRNA resulting either from increased transcription or from altered stability. In agreement with the effect of glucagon *in vivo* on system A is the observation that significant Na⁺-dependent transport of MeAIB could be observed with mRNA (fraction 15) obtained from glucagon-treated rats. However, it cannot be concluded that glucagon increases solely the transcription of the A system, since similar inhibition of Na⁺/L-alanine transport by MeAIB and by pH 6.5 was observed for both mRNAs obtained from control and

glucagon-treated animals (results not shown). That the observed effect of glucagon with respect to the Na⁺/L-alanine transport must be regarded as rather specific is indicated by the Northern-blot analysis, demonstrating that the abundance of the message for α -tubulin was not altered by the glucagon treatment.

We conclude that the liver Na⁺-dependent transport systems for L-alanine (systems A and ASC) are functionally expressed and detected in *X. laevis* oocytes after injection of liver mRNA. Further, mRNA-fractionation experiments suggest that both systems (A and ASC) are encoded by mRNA of length 1.9–2.5 kb. These observations should now allow isolation and characterization of the cDNAs encoding the liver Na⁺-dependent transport systems for L-alanine.

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