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Stimulation of system y^+-like amino acid transport by the heavy chain of human 4F2 surface antigen in *Xenopus laevis* oocytes

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ABSTRACT **A kidney cortex cDNA clone (rBAT) has recently been isolated, which upon *in vitro* transcription and capping complementary RNA (cRNA) and injection into *Xenopus laevis* oocytes induces a system $b^{0,+}$-like amino acid transport activity. This cDNA encodes a type II membrane glycoprotein that shows significant homology to another type II membrane glycoprotein, the heavy chain of the human and mouse 4F2 surface antigen (4F2hc). Here we demonstrate that injection of human 4F2hc cRNA into oocytes results in the activation of a cation-preferring amino acid transport system that appears to be identical to the y^+-like transport already present in the oocyte. This is based on the following results: (*i*) Injection of *in vitro* transcripts from 4F2hc cDNA (4F2hc cRNA) into oocytes stimulates up to 10-fold the sodium-independent uptake of L-arginine and up to 4.1-fold the sodium-dependent uptake of L-leucine. In contrast, 4F2hc cRNA does not increase the basal sodium-independent uptake of L-leucine. (*ii*) Basal and 4F2hc cRNA-stimulated sodium-independent uptake of L-arginine is completely inhibited by L-leucine in the presence of sodium. Similarly, the basal and 4F2hc cRNA-stimulated sodium-dependent uptake of L-leucine is entirely inhibited by L-arginine. (*iii*) The stimulation of sodium-independent uptake of L-arginine and the stimulation of sodium-dependent uptake of L-leucine induced by injection of 4F2hc cRNA are both completely inhibited by dibasic L amino acids and to a lesser extent by D-ornithine. (*iv*) Both basal and 4F2hc cRNA-stimulated sodium-independent uptake of L-arginine show two additional characteristics of the system y^+ transport activity: inhibition of L-arginine uptake by L-homoserine only in the presence of sodium and an increase in the inhibition exerted by L-histidine as the extracellular pH decreased. Our results allow us to propose that an additional family of type II membrane glycoproteins (composed by rBAT and 4F2hc) is involved in amino acid transport, either as specific activators or as components of amino acid transport systems.**

Amino acids cross the plasma membrane of cells via sodium-independent and sodium-dependent carriers. In eukaryotic cells, several carriers have been defined based on ion dependency, substrate specificity, and kinetic properties (1). Our knowledge of the structural identity of these carriers is limited. Recently, two cDNAs related to amino acid transport activity in mammalian cells have been isolated: (*i*) The murine receptor for the ecotropic murine leukemia virus has been identified as being responsible for system y^+, a sodium-independent cation-preferring amino acid carrier (2, 3). (*ii*) As described in the two preceding papers, kidney cortex cDNAs from rabbit and rat [named rBAT ($b^{0,+}$ amino acid transporter-related protein) (4) and D2 (5), respectively] have been cloned, which upon injection into oocytes induce system $b^{0,+}$ (6), a sodium-independent carrier for cationic and neutral amino acids (including L-cystine). The predicted protein for the ecotropic murine leukemia virus receptor contains several putative membrane-spanning domains (7), as do other known membrane carriers (8–11). To the contrary, the predicted proteins for rBAT and D2, corresponding to type II membrane glycoproteins, contain a cytoplasmic N terminus, only one putative membrane-spanning domain, and a large putative extracellular domain that is N-glycosylated (4, 5). This suggests that rBAT (and D2) may not be the complete carrier, but may be alternatively (*i*) a monomer of the active homooligomer carrier, (*ii*) a specific activator of the carrier, or (*iii*) a subunit of the carrier.

The protein rBAT (and also D2) (4, 5) shows amino acid sequence homology with the human and mouse 4F2 cell surface antigen heavy chain (4F2hc) (12–14). As illustrated in Fig. 1, the predicted rBAT protein is believed to be a type II membrane glycoprotein (4), as has been shown for 4F2hc (16–18). Both proteins show a very similar localization of the single putative transmembrane domain within their sequences. The overall maximal amino acid sequence identity between the two proteins is 30%, with 52% sequence similarity (26% identity in the alignment shown in Fig. 1). Furthermore, there is significant amino acid sequence homology in the putative extracellular domains of both rBAT and 4F2hc with a family of α-amylases and α-glucosidases, which lack transmembrane domains (15, 19–21). Interestingly, neither rBAT nor 4F2hc proteins have the conserved aspartic acid (or glutamic acid) residues, which are a crucial feature of the proposed catalytic site for amylases, α-glucosidases, and transglucanosylases (22). Consistent with this sequence difference is the fact that α-amylase or maltase activity could not be demonstrated in oocytes injected with D2 complementary RNA (cRNA) (5).

The 4F2 antigen is a heterodimer containing an 85-kDa glycosylated heavy chain covalently linked to a 40-kDa highly hydrophobic light chain (16–18). Although its function remains unknown, this protein has been proposed to be involved in the modulation of intracellular Ca^{2+} levels (23). The expression of 4F2 antigen is known to be highly regulated at the onset of cell proliferation (16, 24, 25). In this study we demonstrate that human 4F2hc (12), in analogy to rBAT, induces amino acid transport activity when injected in *Xenopus* oocytes.

METHODS

Oocytes and Injections. *Xenopus laevis* were obtained from H. Kähler (Institut für Entwicklungsbiologie, Hamburg, F.R.G.). Healthy looking stage V–VI oocytes (for preparation see ref. 30) were injected with water or cRNA made *in vitro* from clones 4F2hc or rBAT (0.05 mg/ml in water) using a

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Abbreviations: 4F2hc, 4F2 surface antigen heavy chain; MeAIB, 2-(methylamino)isobutyric acid; cRNA, complementary RNA.

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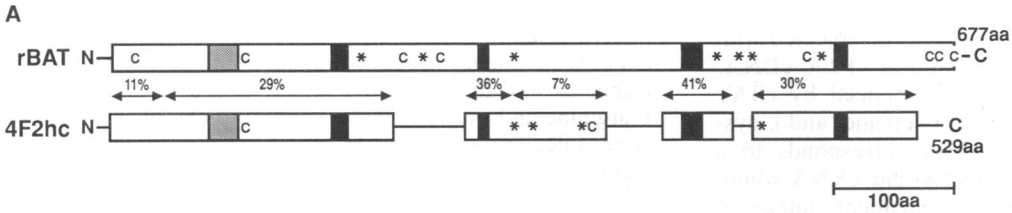

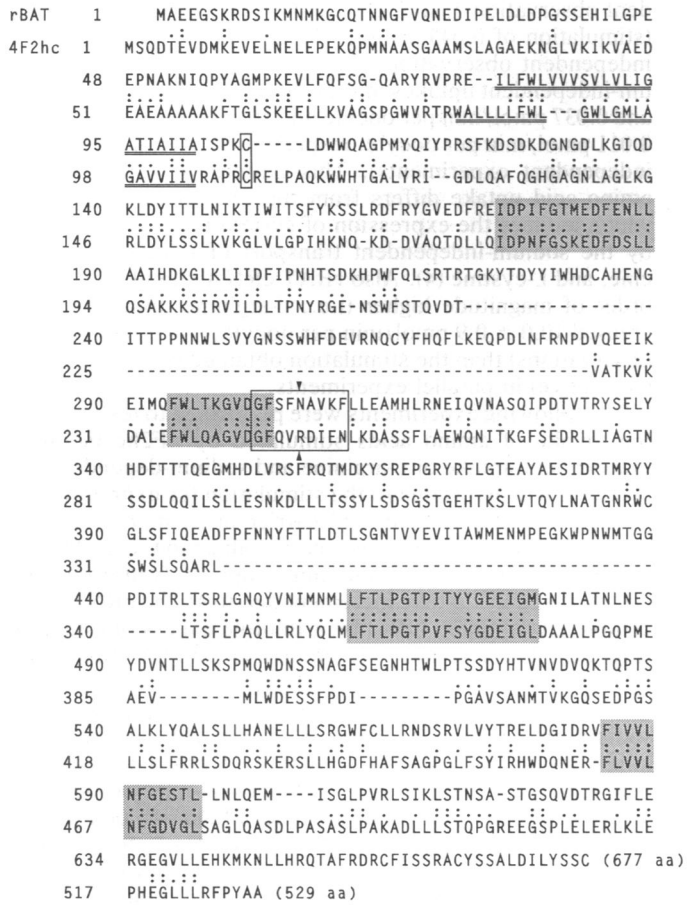

FIG. 1. Schematic representation of the structural analogy and the amino acid sequence homology between 4F2hc and rBAT. (*A*) Schematic representation of rBAT and 4F2hc proteins showing that rBAT, in analogy to 4F2hc, contains a cytoplasmic N terminus, a putative transmembrane domain (indicated by stippled bars at amino acid residues 80–102 and 82–104 for rBAT and 4F2hc, respectively), and a large putative extracellular domain with several potential N-glycosylation sites (indicated by asterisks). The proteins in this alignment show 26% identity (45% similarity) in their overall amino acid sequence. Comparison of the predicted amino acid sequences (percent identity) within the indicated segments of both proteins is shown between the schematic drawings. Four amino acid sequence fragments (i.e., 10–18 amino acid residues long) are highly conserved (67–80% identity) in both proteins and are shaded in black. The positions of cysteine residues (C) within the sequences are indicated in the scheme. Interestingly, one of the two cysteine residues in 4F2hc (amino acid residue 109) is conserved in rBAT in a very similar localization, next to the transmembrane domain (amino acid residue 106). Gaps for the alignment smaller than 10 amino acid residues are not represented or combined. aa, amino acids. (*B*) Comparison of the predicted amino acid sequences of rBAT (upper line) and 4F2hc (lower line). Identical and similar amino acids are indicated by colons and single dots, respectively. Highly conserved amino acid sequences, of at least 10 amino acid residues, between both proteins are shaded. The conserved cysteine residue in both sequences is indicated (small box). The putative transmembrane domain in both proteins is double underlined. Residues that resemble the amino acid sequence of one of the two postulated catalytic regions of amylases, α-glucosidases, and transglucanosylases (15) are boxed. In this region, the proposed catalytic residue (aspartate or glutamate) for these carbohydrate-metabolizing enzymes is substituted by asparagine and arginine in rBAT and 4F2hc proteins, respectively (indicated by the arrowheads).

semiautomatic injector (Inject + Matic; J. A. Gabay, Geneva). For the entire study, the injected volume was 50 nl. The amount of cRNA injected (2.5 ng for both clones) caused saturated responses in the oocytes. Subsequently, the oocytes were incubated at 18°C for 3–4 days in modified Barth's solution.

Synthesis of Transcripts from cDNA (cRNA). rBAT- (4) and human 4F2hc- (12) containing plasmids were isolated by using a miniprep kit (Qiagen). The rBAT cDNA, cloned in pBluescript SK^+, was linearized by *Kpn* I digestion and *in vitro* transcribed by using T3 RNA polymerase (Promega) in the presence of m^7GpppG (New England Biolabs). After 1 hr, all DNA was digested using DNase I (Pharmacia), and the cRNA was precipitated with ammonium acetate and ethanol. The cRNA was finally resuspended in 10 μl of water, an aliquot was quantitated by absorbance at 260 nm, and transcript integrity was checked on a 1% agarose/formaldehyde gel. For 4F2hc, cloned in pSP65, the plasmid was linearized with *Hin*dIII, and SP6 RNA polymerase was used for *in vitro* transcription. The sample was then treated as for rBAT.

Uptake Measurements. To measure the uptake of different amino acids, seven or eight oocytes per condition were washed once in amino acid-free uptake solution (100 mM choline chloride or 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes/Tris at pH 7.5). The oocytes were placed in 90 μl of uptake solution containing the appropriate amino acid (50 μM) at 10–20 μCi/ml (1 Ci = 37 GBq). Amino acid inhibition studies were performed by adding 5 mM inhibitor to this uptake solution. Uptakes were measured after a 30-min incubation for 4F2hc-injected oocytes and their controls (water-injected oocytes) and after 10 min for rBAT-injected oocytes and their controls (water-injected oocytes). Under these conditions, we found linear uptake rates in cRNA-injected and water-injected oocytes (data not shown). After incubation, the oocytes were washed three times with 4 ml of ice-cold stop solution (20 mM L-leucine, 20 mM L-arginine, 80 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes/Tris at pH 7.5). Each oocyte was then transferred to a scintillation vial, dissolved in 0.2 ml of 10% SDS, and then counted in 3 ml of scintillation fluid.

Data of uptake are expressed as pmol/min per oocyte in the basal condition (water-injected oocytes) and in the stimulated condition (cRNA-injected oocytes) or as the increment of uptake due to the injected cRNA (uptake in cRNA-injected oocytes minus uptake in water-injected oocytes). Student's *t* test was used for statistical analysis.

RESULTS AND DISCUSSION

To test the hypothesis that 4F2hc, which is structurally related to rBAT, is also a specific activator or a component

of an amino acid transport system, we measured the uptake of isotopically labeled amino acids by oocytes injected with cRNA prepared by *in vitro* transcription of 4F2hc cDNA. Three amino acids whose transport is induced by rBAT cRNA in oocytes—that is, L-arginine, L-leucine, and L-cystine (4)—were tested. Fig. 2*A*, which corresponds to a representative experiment, shows that 4F2hc cRNA stimulates, in the oocytes, the sodium-independent uptake of L-arginine and the sodium-dependent uptake of L-leucine. The stimulation of the sodium-independent uptake of L-arginine induced by 4F2hc cRNA was 0.87 ± 0.15 pmol/min per oocyte in six independent observations (i.e., six different batches of oocytes). The stimulation of the sodium-dependent uptake of L-leucine was 0.79 ± 0.22 pmol/min per oocyte in four independent observations. That represents an average increment of 320% (range of 70–900%) and 180% (range of 70–310%) over the sodium-independent uptake of L-arginine and the sodium-dependent uptake of L-leucine of water-injected oocytes, respectively. The variability in the stimulation is due to variability in the basal (i.e., water-injected oocyte) amino acid uptake. The cRNA of 4F2hc did not stimulate sodium-dependent uptake of L-arginine (stimulation of 0.03 ± 0.20 pmol/min per oocyte; $n = 3$ independent observations), sodium-independent uptake of L-leucine (stimulation of 0.043 ± 0.030 pmol/min per oocyte; $n = 3$ independent observations), or sodium-dependent and sodium-independent uptakes of L-cystine (stimulations of −0.014 and 0.037 pmol/min per oocyte and stimulations of 0.010 and 0.011 pmol/min per oocyte, respectively; averages from two independent experiments). This pattern of stimulation of amino acid uptake differs from the one elicited by rBAT, which induces the expression of system $b^{0,+}$, characterized by the sodium-independent transport of L-arginine, L-leucine, and L-cystine (4). Also rBAT cRNA stimulation is one order of magnitude higher (i.e., L-arginine uptake was increased 10.0 ± 0.9 pmol/min per oocyte; $n = 3$ independent observations) than the stimulation obtained by 4F2hc cRNA (see above) in parallel experiments.

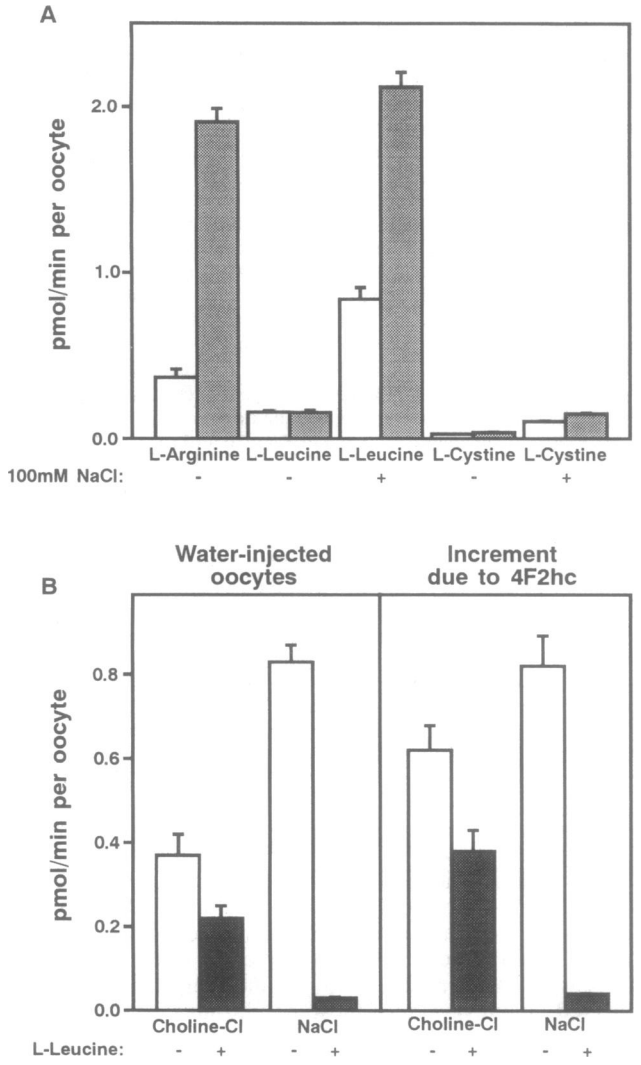

FIG. 2. (*A*) Activation of oocyte amino acid uptake by 4F2hc cRNA. Oocytes injected with 50 nl of water (open bars) or 50 nl of water containing 2.5 ng of 4F2hc cRNA (stippled bars) were assayed after 3 days for uptake of the indicated amino acids. The uptakes of these amino acids, at a concentration of 50 μM, were determined during 30-min incubations in the presence of 100 mM NaCl or 100 mM choline chloride. The bars represent the mean ± SEM obtained from seven or eight oocytes per group of a representative experiment. (*B*) Inhibition of L-arginine uptake by L-leucine in the presence and absence of sodium. Oocytes were injected and assayed for 50 μM amino acid transport, and the uptake values are presented as indicated below. Uptake assay was performed in the absence (open bars) and presence (shaded bars) of 5 mM L-leucine. The increment of amino acid transport due to 4F2hc was calculated as the difference between the uptake in 4F2hc cRNA-injected oocytes minus that in water-injected oocytes. Inhibition of L-arginine uptake by L-leucine was statistically significant ($P \leq 0.05$) in all groups.

To confirm that the amino acid uptake stimulated by 4F2hc corresponds to the activation of a cation-preferring amino acid carrier, we tested inhibition by some cationic and neutral amino acids (Fig. 3). The inhibition profile of sodium-independent L-arginine uptake by the tested amino acids, at 100-fold excess concentration, was identical for the basal activity and for the activity stimulated by 4F2hc cRNA (i.e., increment due to 4F2hc) (Fig. 3*A*). Furthermore it corresponds to the inhibition pattern expected for a stereospecific cation-preferring amino acid transport (26–28): dibasic L amino acids > L-leucine and D-ornithine >> L-alanine and 2-(methylamino)isobutyric acid (MeAIB). The amino acid inhibition profile of L-leucine uptake in the presence of sodium (Fig. 3*B*) was also identical for the basal activity and the component stimulated by 4F2hc cRNA: dibasic L amino acids and L-leucine inhibited almost completely those uptake activities, whereas inhibition by D-ornithine and also by L-alanine was only partial. MeAIB was ineffective as an inhibitor. These results suggest that (*i*) 4F2hc cRNA stimulates a transport activity for the sodium-independent uptake of L-arginine and sodium-dependent uptake of L-leucine, which is a transport activity already present in the water-injected oocytes; (*ii*) neutral amino acids (i.e., L-leucine and

The following experiments were performed to test whether the uptake of amino acids stimulated by 4F2hc (sodium-independent uptake of L-arginine and sodium-dependent uptake of L-leucine) is due to the stimulation (or expression) of a single transport amino acid carrier or of more than one transport system. In the presence of sodium in the incubation medium, both the basal L-arginine uptake (water-injected oocytes) and the uptake increment due to 4F2hc cRNA were completely inhibited (95%) by a 100-fold excess of L-leucine (Fig. 2*B*). These results are compatible with the stimulation by 4F2hc of a component of transport shared by L-arginine and L-leucine, the latter being transported in the presence of sodium. In the absence of sodium (choline chloride medium), inhibition of this transport activity by 5 mM L-leucine was ≈40% in water-injected and 4F2hc cRNA-injected oocytes. These results are consistent with the stimulation of a y^+-like system activity by 4F2hc cRNA. System y^+, for cationic amino acids, is inhibited by neutral amino acids with a higher affinity in the presence than in the absence of sodium (26–28). This system has been reported to be a major component of sodium-independent uptake of cationic amino acids in *Xenopus* oocytes (29, 30).

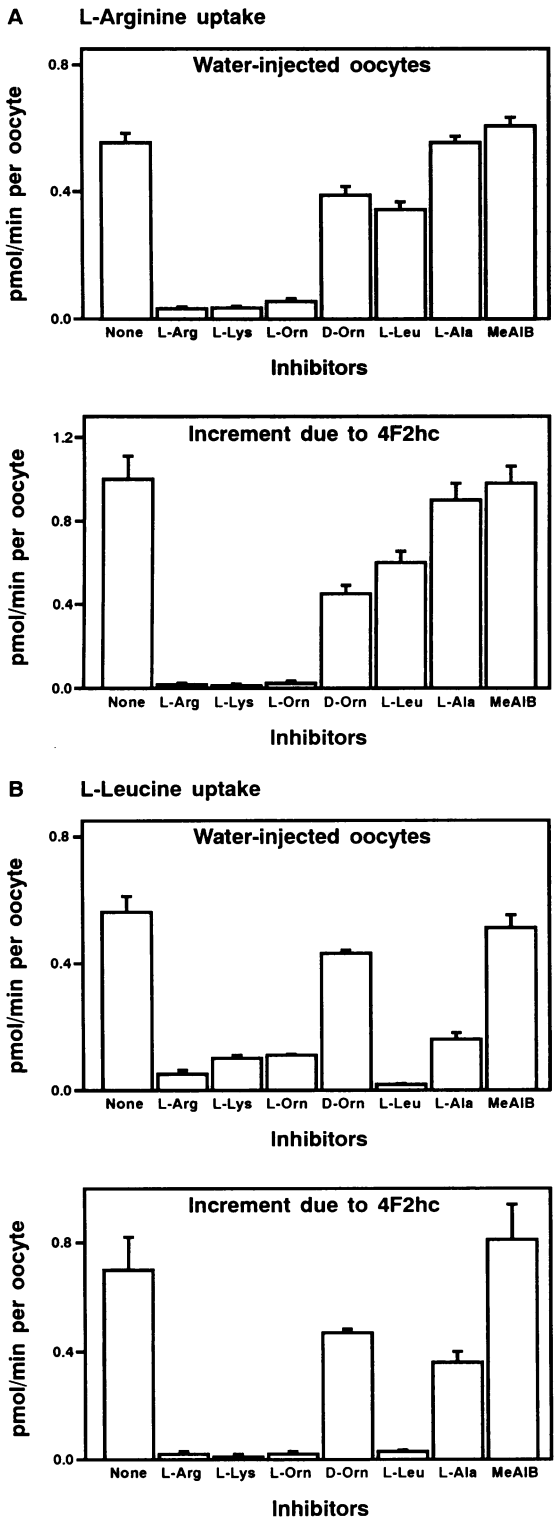

FIG. 3. Inhibition by different amino acids of the basal and 4F2hc-stimulated amino acid uptake. Oocytes injected with 50 nl of water or 50 nl of water containing 2.5 ng of 4F2hc cRNA were assayed 3 days later for uptake of 50 μM L-arginine in 100 mM choline chloride medium (*A*) and uptake of 50 μM L-leucine in 100 mM NaCl medium (*B*). Amino acids tested as inhibitors were added to the incubation medium to a final concentration of 5 mM. The bars represent the mean ± SEM obtained from seven or eight oocytes per group of a representative experiment. Inhibition of L-arginine uptake by all the tested amino acids, with the exception of L-alanine and MeAIB, was significant ($P \leq 0.01$), both when basal uptake or the increment due to 4F2hc was studied. Inhibition of L-leucine uptake by all the tested amino acids, with the exception of MeAIB, was significant ($P \leq 0.01$) when basal uptake or the increment due to 4F2hc was studied. Orn, ornithine.

L-alanine) interact better with this transport activity stimulated by 4F2hc cRNA in the presence of sodium in the medium. In summary, our data show that 4F2hc cRNA injection in oocytes stimulates a cation-preferring sodium-independent transport activity of amino acids (system y^+-like) that is already present in oocytes.

To further examine whether 4F2hc stimulates a system y^+-like activity, we studied the sodium dependency of L-homoserine inhibition. It has been demonstrated that the activity of system y^+ is not inhibited by L-homoserine (5 mM; 100-fold excess concentration) in the absence of sodium, whereas it is largely inhibited by this amino acid in the presence of 100 mM sodium (27), and that this transport system carries L-homoserine only in the presence of sodium (3). Fig. 4 shows that the basal L-arginine uptake activity of *Xenopus* oocytes and the component stimulated by 4F2hc cRNA are both inhibited by L-homoserine exclusively in the presence of sodium.

Since system y^+ requires a cationic side chain in its substrates, we measured L-histidine inhibition as a function of extracellular pH. Because nitrogen on the imidazole side chain of L-histidine has a pK_a of 6.1, the majority of these molecules are cationic below this pH and are transported by system y^+ (2). Fig. 5 shows that L-histidine (5 mM) inhibits the basal activity and the component stimulated by 4F2hc cRNA of (50 μM) L-arginine uptake, as a function of pH in the uptake medium: the L-histidine inhibition is greater as the pH decreases.

We conclude that 4F2hc cRNA injection into oocytes results in the stimulation of a transport activity, already present in stage V–VI oocytes, which shows several characteristics of a cation-preferring amino acid carrier (system y^+-like). System y^+ has recently been identified as the ecotropic murine leukemia virus receptor (2, 3), a protein that shares no sequence homology with 4F2hc (7, 12). This supports the possibility that 4F2hc acts, in *Xenopus* oocytes, as a specific activator or as a component of the active y^+ carrier. The structural homology of the 4F2hc surface antigen and the rBAT protein, and their functional similarity as inducers of two different amino acid transport processes, suggests the existence of an additional family of type II

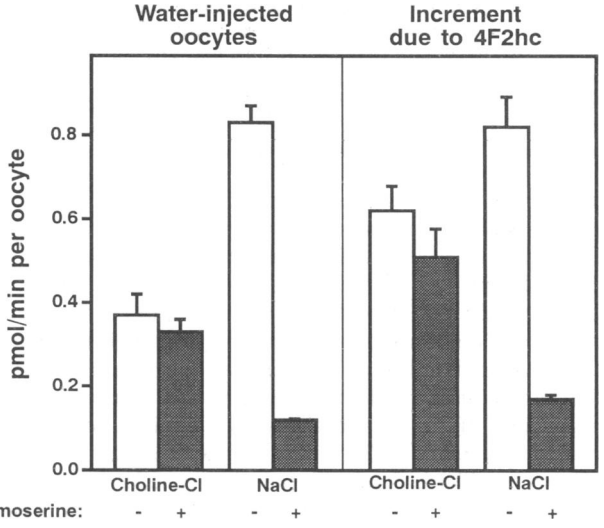

FIG. 4. Inhibition of L-arginine uptake by L-homoserine in the presence and absence of sodium. Oocytes injected with water (50 nl) or 2.5 ng of 4F2hc cRNA were assayed for uptake of 50 μM L-arginine as indicated in Fig. 2. The uptake assay was performed in the absence (open bars) and presence (shaded bars) of 5 mM L-homoserine. The bars represent the mean ± SEM obtained from seven or eight oocytes per group of a representative experiment. Inhibition by L-homoserine was only significant ($P \leq 0.001$) in the presence of sodium. A second independent experiment showed identical results.

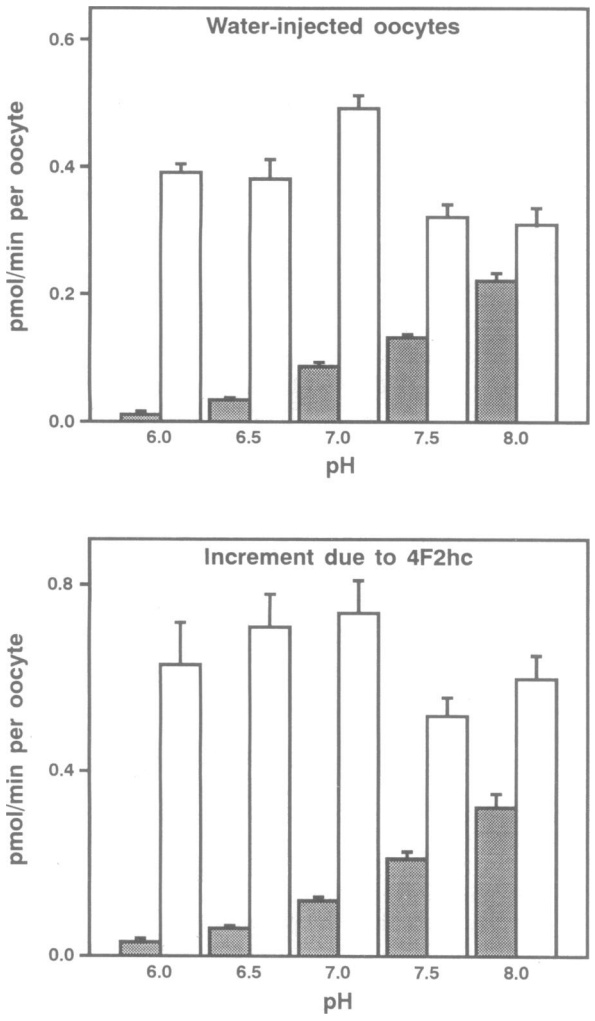

FIG. 5. Inhibition of L-arginine uptake by L-histidine as a function of extracellular pH. Oocytes injected with 50 nl of water or 50 nl of water containing 2.5 ng of 4F2hc cRNA were assayed 3 days later for uptake of 50 μM L-arginine in different media at the pH values indicated. The choline chloride uptake media differ in their content of Tris·HCl necessary to reach the final pH. The bars (open for basal uptake; shaded for the uptake in the presence of 5 mM L-histidine) represent the mean ± SEM obtained from seven or eight oocytes per group of a representative experiment. Inhibition by L-histidine was significant ($P \leq 0.01$) in all the groups.

membrane glycoproteins, which are regulators or structural elements of amino acid transport systems in eukaryotic cells. Human and murine 4F2hc are known to be associated by disulfide linkage to a different subunit (light chain; refs. 16–18), and rBAT protein conserves one of the two cysteine residues of human and murine 4F2hc potentially implicated in this covalent bond (Fig. 1). Therefore, it appears unlikely that the active amino acid transporters are homooligomers of rBAT and 4F2hc, respectively. Rather it is proposed that rBAT protein and 4F2hc could play an analogous role as the Na^+/K^+-ATPase β subunit (another type II membrane glycoprotein) that, upon injection of its RNA in *Xenopus* oocytes, supports the maturation of active pumps containing the endogenous catalytic α subunit (31). In fact, foreign β subunits of the Na^+/K^+-ATPase also support, in the oocytes, the functional expression of foreign α subunits (32). However, further investigations are needed to test whether 4F2hc and rBAT are, in analogy to the Na^+/K^+-ATPase β subunit, elements of the transport systems y^+ and $b^{0,+}$ or, alternatively, specific regulators of their activity.

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