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# Cloning of a rat kidney cDNA that stimulates dibasic and neutral amino acid transport and has sequence similarity to glucosidases

(cystine transport/cystinuria/4F2 antigen)

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ABSTRACT **The transport of amino acids across cell membranes is believed to be mediated by integral membrane proteins with distinct substrate specificities. Using expression cloning in *Xenopus* oocytes and assaying for the uptake of $^{14}$C-labeled cystine, we isolated a 2.3-kilobase cDNA (D2) from a rat kidney library. D2 is expressed specifically in kidney and intestine and induces the transport of both neutral and cationic amino acids. The deduced amino acid sequence predicts a 78-kDa protein with a single transmembrane domain, a structure not typical of the known membrane transport proteins, which generally have multiple membrane-spanning regions. The putative extracellular region is highly similar to the 4F2 heavy-chain cell surface antigen and to a family of α-glucosidases, which raises the possibility that D2 encodes a transport activator or regulatory subunit.**

Cystine and the dibasic amino acids lysine, arginine, and ornithine are malabsorbed in the kidney and jejunum of patients with cystinuria, an observation that has led to the suggestion that these amino acids are transported via a single high-affinity transporter (1–3). There is disagreement as to the existence of a second low-affinity renal transporter for cystine alone (4, 5). The murine ecotropic retroviral receptor has recently been shown to be a dibasic amino acid transporter, most likely the ubiquitous $y^+$ system (6, 7). It does not transport cystine (J. Cunningham, personal communication) and is therefore unlikely to play a role in cystinuria. As a preliminary experiment to expression cloning of amino acid transporters in general, several groups have recently demonstrated the uptake of cystine (8, 9) and dibasic and neutral amino acids (10, 11) into oocytes injected with kidney and intestine mRNA. The uptake of arginine and alanine is stimulated specifically by the injection of rabbit kidney mRNA in the size range of 1.8–2.4 kilobases (kb) and may represent activity similar to the broad spectrum system $b^{0,+}$, a transporter previously observed only in the mouse blastocyst (12, 13).

We report here the use of expression cloning methods to isolate a rat kidney cDNA clone that induces the uptake of cystine and dibasic and neutral amino acids. The sequence of this clone* suggests a previously undescribed mechanism of amino acid transport. A cDNA clone from rabbit kidney with similar uptake characteristics and a high degree of sequence similarity was cloned simultaneously and independently as reported in the following paper (14).

## METHODS

**Preparation and Microinjection of Poly(A)$^+$ RNA.** RNA was harvested from tissues of adult female Sprague–Dawley rats and from LLC-PK$_1$ and OK cell lines by the guanidinium isothiocyanate (BRL) method using cesium/trifluoroacetic acid (Pharmacia). mRNA was isolated by oligo(dT)-cellulose chromatography (Pharmacia). Collagenase-treated and manually-defolliculated *Xenopus laevis* oocytes were microinjected as described (15), generally 40–50 ng of mRNA in 50 nl. Rat kidney cortex mRNA was size-fractionated by using a preparative agarose gel electrophoresis apparatus (15, 16).

**Transport Measurements.** Uptake of $^{14}$C-labeled substrates into oocytes was measured 3 days after injection (15). Oocytes were incubated for 1 hr in 0.75 ml of uptake solution containing 0.5–1.5 μCi (1 Ci = 37 GBq) of the $^{14}$C-labeled substrate and either 100 mM $Na^+$ or 100 mM *N*-methyl-D-glucosamine. Unlabeled amino acids were added to bring the total amino acid concentration to 15 μM (50 μM for glucose) for all determinations except the inhibition and kinetics experiments, where additional unlabeled amino acids were added to the uptake mixtures. Each data point represents the mean of the uptake into five to eight oocytes. All amino acids were the L form, except where noted. For the kinetics of cystine uptake, $K_m$ and $V_{max}$ values were calculated from Lineweaver–Burk plots. Uptake into water-injected oocytes was subtracted for each concentration point.

**Library Construction and Clone Isolation.** The mRNA size fraction that caused peak stimulation of cystine uptake was used to construct a directional cDNA library (17) in the expression vector pBluescript (Stratagene), which was then electroporated into ElectroMAX DH10B cells (BRL). Complementary RNA (cRNA) was *in vitro* transcribed [modification of the method of Krieg and Melton (18)] from pools of 600–700 clones and injected into oocytes. A pool that induced the uptake of [$^{14}$C]cystine 10-fold over background was identified and sequentially subdivided and *in vitro* transcribed until a single clone (D2) was identified. All D2-injected oocytes were injected with 40–50 ng of cRNA in 50 nl as described above. For the hybrid depletion experiment, RNA was denatured, hybridized to sense or antisense oligonucleotides (0.25 μg/μl; sense, nucleotides 76–94; antisense, nucleotides 285–302; Midland Certified Reagent, Midland, TX) in 50 mM $Na^+$ for 1 hr at 42°C, and injected into oocytes (total of 40–50 ng of RNA per oocyte) (19).

**Northern Analysis and *in Vitro* Translation.** Ten micrograms of each mRNA was electrophoresed on a 1% formaldehyde/agarose gel and blotted onto nitrocellulose filters (Schleicher & Schuell). The D2 insert (positions 1–1696) was labeled by using the $^{T7}$QuickPrime kit (Pharmacia). Hybridization was performed for 18 hr at 37°C in a 50% formamide hybridization mixture. Washing was done in 0.1× standard saline citrate/0.1% SDS at 50°C for low stringency and at 65°C for high stringency. Exposure shown was 16 hr for rat tissue lanes and, from a different blot, 72 hr for the OK and LLC-PK$_1$ lanes. *In vitro* translation with D2 cRNA was performed by using a rabbit reticulocyte lysate system plus or

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Abbreviation: cRNA, complementary RNA.

*The nucleotide sequence of clone D2 has been deposited in the GenBank data base (accession no. M80804).

minus canine pancreatic membranes (Promega) and endoglycosidase H (Boehringer Mannheim) (20).

**Sequencing and Sequence Analysis.** D2 cDNA was subcloned by using fragments produced by internal restriction sites, and both strands were completely sequenced by using a $^{T7}$Sequencing kit (Pharmacia); synthetic oligonucleotide primers were used to complete the sequencing. Identification of similar sequences resulted from a search of the GenBank, European Molecular Biology Laboratory, Swiss-Prot, and Protein Identification Resource data bases; sequence analysis and alignment was performed using software described (21).

**Materials.** Radiochemicals were from New England Nuclear, enzymes were from Pharmacia, and other chemicals were from Sigma, except as noted.

## RESULTS

Using an expression cloning technique similar to that previously used to clone the $Na^+$/glucose cotransporter (22), we screened for uptake of [$^{14}$C]cystine into *Xenopus* oocytes. Cystine uptake by oocytes was 5 times higher than control values after the injection of rat jejunum mRNA and 134 times higher after the injection of rat kidney mRNA (Fig. 1*A*). Size fractionation of kidney mRNA resulted in a single size range (1.8–2.5 kb) that induced cystine uptake and that was used to construct a unidirectional cDNA library (Fig. 1*A Inset*). Progressively smaller pools of cDNA clones were *in vitro* transcribed and injected into oocytes until the cRNA from a single clone (D2) able to induce cystine uptake 200- to 400-fold over control values was isolated. Pools of clones that did not contain D2 demonstrated uptake at levels identical to those of water-injected controls (data not shown). Hybrid depletion of D2 from kidney mRNA by incubation with an antisense oligonucleotide to a region near the 5′ end of D2 resulted in a decrease in cystine uptake to 3.9% of maximum, suggesting that D2 is responsible for most if not all of the cystine uptake observed after injection of kidney mRNA (Fig. 1*B*). In addition to inducing uptake of cystine, D2 also induced uptake of lysine, arginine, and ornithine and, quite surprisingly, histidine, alanine, serine, leucine, citrulline, glutamine, asparagine, methionine, and phenylalanine (Fig. 1*C*). Dibasic amino acids in excess inhibited the uptake of neutral amino acids and, likewise, neutral amino acids in excess inhibited the uptake of dibasic amino acids, suggesting that uptake is occurring via the same transporter (Table 1). Uptake is stereospecific; it is inhibited more by the addition of unlabeled L as opposed to D amino acids. Partial inhibition did occur with the D isomers, especially for lysine. Uptake of dibasic amino acids and leucine is mostly $Na^+$ independent (Table 2), although alanine demonstrated significant sodium dependence (30–40% of the total), which raises the question of the role of D2 in sodium-dependent alanine uptake. The $K_m$ value for cystine is 67.3 $\mu$M, with a $V_{max}$ of 0.96 nmol per oocyte per hr. This value is in accord with previously reported cystine $K_m$ values for the proposed high-affinity cystine and dibasic amino acid transporter (1, 4).

In Northern blots screened at low stringency, clone D2 cRNA hybridized intensely to a species of 2.2 kb and weakly to another of 4.4 kb in mRNA from rat kidney and intestine (Fig. 2). A similar species was also seen in mRNA from the kidney-derived cell line LLC-$PK_1$ and, very weakly, in mRNA from OK cells. These bands remain after high-stringency screening. At low stringency after a long exposure (data not shown), weak bands (50- to 100-fold less intense) were seen at 2.2 kb in rat pancreas and liver, at 2.4 kb in heart and brain, and at >10 kb in spleen. These weak bands were not seen after high-stringency washing and probably represent related sequences (see below). No hybridization was seen to mRNA from lung or skeletal muscle.

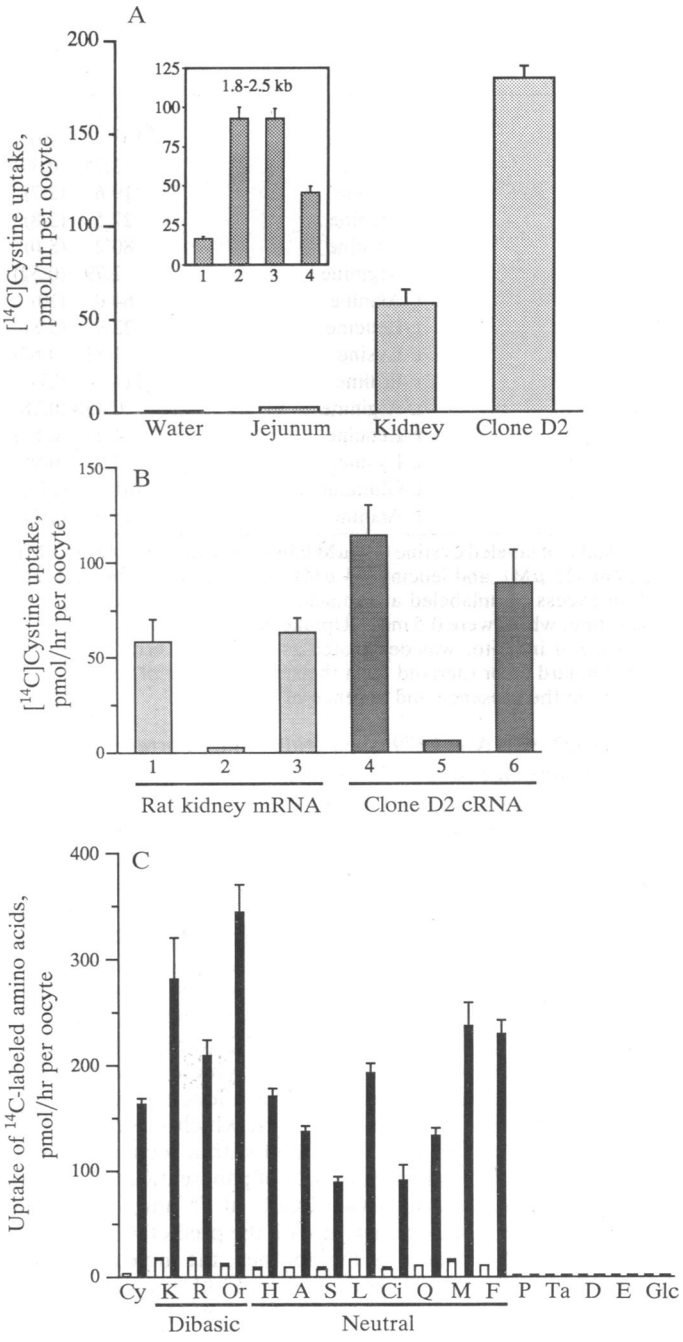


FIG. 1. (*A*) Uptake of $^{14}$C-labeled L-cystine into *Xenopus* oocytes. Oocytes were injected with water, rat kidney cortex and jejunum mRNA, or clone D2 cRNA. (*Inset*) Peak uptake induced by injection of size fractions of mRNA. Fractions 1–4 represent average mRNA sizes of 1.8, 2.1, 2.3, and 2.5 kb, respectively. Vertical bars give the standard error. (*B*) Hybrid depletion of cystine uptake. Rat kidney mRNA and clone D2 cRNA were each incubated with an antisense oligonucleotide to a region near the 5′ end of clone D2 and then injected into oocytes. After a 3-day incubation, [$^{14}$C]cystine measurements were performed as described. The mean of the uptake into water-injected oocytes has been subtracted. Bars 1 and 4 show uptake into oocytes injected with rat kidney mRNA and with clone D2 cRNA, respectively. Bars 2 and 5 show that uptake is decreased to 3.9% and 5.0%, respectively, after incubation with an antisense oligonucleotide; incubation with a sense oligonucleotide (bars 3 and 6) had a minimal effect. We see qualitatively identical results with mRNA from cortex or medulla (data not shown). (*C*) Uptake of amino acids into D2 cRNA-injected oocytes. Oocytes injected with D2 cRNA (solid bars) take up dibasic and neutral amino acids, but not proline, taurine, glucose, or acidic amino acids. Open bars represent water-injected oocytes. Cy, L-cystine; Or, L-ornithine; Ci, L-citrulline; Ta, taurine; Glc, glucose. Other amino acids are indicated by the single-letter code.

Table 1. Inhibition and stereospecificity of uptake

| $^{14}$C-labeled amino acid | Inhibitor | % uptake (SE) |
|---|---|---|
| L-Cystine | L-Cystine | 37.1 (5.4) |
| | D-Cystine | 103 (10) |
| | L-Lysine | 2.75 (0.37) |
| | D-Lysine | 19.6 (2.7) |
| | L-Alanine | 27.5 (2.8) |
| | D-Alanine | 80.2 (8.0) |
| L-Lysine | L-Arginine | 2.79 (0.52) |
| | L-Alanine | 64.0 (10) |
| | L-Leucine | 22.9 (5.8) |
| L-Alanine | L-Lysine | 1.81 (0.67) |
| | L-Proline | 114 (27) |
| | L-Arginine | 0.450 (0.28) |
| | L-Leucine | 4.27 (0.87) |
| L-Leucine | L-Lysine | 2.95 (0.95) |
| | L-Glutamic acid | 101 (17) |
| | L-Alanine | 13.5 (1.7) |

Uptake of labeled cystine (2.2 $\mu$M labeled species), lysine (15 $\mu$M), alanine (15 $\mu$M), and leucine (15 $\mu$M) was assessed in the presence of an excess of unlabeled amino acids (2 mM for all except D- and L-cystine, which were 0.5 mM). Uptake of labeled amino acids in the absence of inhibitor was designated as 100%. The error represents the standard error (derived from the standard error of the means of uptake in the presence and absence of inhibitor).

The D2 cDNA is 2279 nucleotides long, corresponding to the message size seen on Northern analysis. We identified an open reading frame that codes for a 683-amino-acid protein with a molecular mass of 78 kDa. The deduced amino acid sequence is shown in Fig. 3. It has seven potential N-glycosylation sites, all toward the C-terminal end. An internal hydrophobic region (residues 87–107), which is predicted to be membrane spanning (31), makes it likely that D2 codes for a type II membrane glycoprotein with a hydrophilic cytoplasmic N terminus, a single membrane-spanning domain, and an extracellular C terminus. This structure is unlike that of previously cloned transporters and channels (refs. 6, 22, 32, and 33, for example), most of which have multiple membrane-spanning regions. There is a leucine zipper motif at the C terminus (residues 548–569), which raises the possibility that the protein is associated with a second subunit.

*In vitro* translation in the absence of pancreatic microsomal membranes demonstrates two bands at 75 and 78 kDa on SDS/PAGE (Fig. 4), consistent with the predicted size of the protein. In the presence of membranes, the molecular mass increases to 87 and 90 kDa. This 12-kDa increase was partially reversed (to 77 and 80 kDa) by treatment with endoglycosidase H, indicating that the protein is glycosylated and suggesting that the C terminus is extracellular. It also indicates that cleavage of the internal hydrophobic region does not occur. The presence of two products suggests the use of alternate translation initiation sites (Met-13, -15, or -65), at least *in vitro*.

Search of protein sequence data bases (December, 1991) demonstrates significant similarity between the proposed extracellular C-terminal domain of the D2 protein and a family of carbohydrate-metabolizing enzymes, including $\alpha$-amylases and $\alpha$-glucosidases from both prokaryotic and eukaryotic organisms. An alignment of the D2 amino acid sequence and those of several members of this protein family is shown in Fig. 3. Similarity is greatest for the *Drosophila* hypothetical protein L (41% identity; alignment not shown) and the *A. aegypti* (yellow fever mosquito) salivary gland maltase-like gene (38% identity) (26, 27). D2 shares a number of highly conserved residues with the members of this protein family, including one and possibly two aspartic acid residues (Asp-311 and Asp-356) postulated to be part of the catalytic site (34). Porcine pancreatic amylase, a calcium-binding enzyme, has been crystallized and the structure resolved to 2.9 Å (25). Two of the proposed calcium ligands in porcine pancreatic amylase are conserved in D2. There is also significant sequence identity (29%) between D2 and the human and mouse 4F2 heavy-chain cell surface antigens, type II membrane glycoproteins involved in cell growth (30, 35, 36). D2 and the proposed $y^+$ transporter do not show any sequence similarity.

Table 2. $Na^+$ dependence of transport

| Substrate | % $Na^+$ independence (SE) |
|---|---|
| L-Cystine | 87.2 (7.6) |
| L-Lysine | 99.9 (10) |
| L-Alanine | 63.2 (8.0) |
| L-Leucine | 97.7 (10) |

Uptake of labeled amino acids into D2-injected oocytes in the presence of 100 mM $Na^+$ was compared to uptake when $Na^+$ was replaced with 100 mM *N*-methyl-D-glucosamine. Uptake in the presence of $Na^+$ is designated as 100%. The error is as in Table 1.

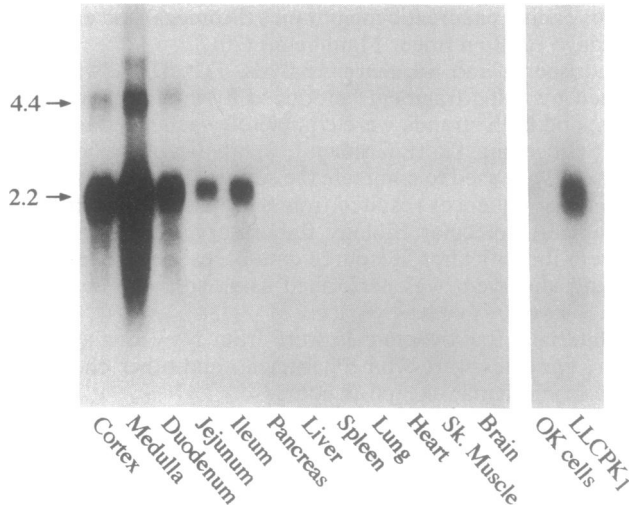


FIG. 2. Tissue distribution of the D2 message. The low-stringency Northern blot of RNA from rat tissues and kidney cell lines probed with $^{32}$P-labeled D2 shows a strong 2.2-kb and a weaker 4.4-kb signal in kidney and intestine (especially medulla and duodenum). In some blots prepared from different lots of mRNA, jejunum gave a stronger signal than duodenum. LLC-$PK_1$ and OK cells have the smaller species. Sk., Skeletal.

## DISCUSSION

We have cloned a cDNA (D2) that, when *in vitro* transcribed, induces the uptake of cystine and dibasic and neutral amino acids in cRNA-injected *Xenopus* oocytes. The deduced amino acid sequence of D2 predicts a 78-kDa protein with a single transmembrane region, making it a protein unlike most previously cloned transporters. A potassium channel with a single transmembrane domain, however, has recently been cloned (37, 38). D2 might oligomerize to form a functional transporter with multiple transmembrane regions. The similarity between D2 and glucosidases, including conservation of several important sites, suggests, however, that D2 is a carbohydrate-metabolizing enzyme, although we have been unable to demonstrate $\alpha$-amylase or maltase activity in cellular fractions from D2-injected oocytes (data not shown). The specific expression of D2 in kidney and jejunum is consistent with its involvement in transport. The D2 protein might function as the regulator for a native oocyte transporter (perhaps by altering glycosylation or up-regulating transporter production), acting either intracellularly or at the cell surface, or as the regulatory subunit of a heterooligomeric

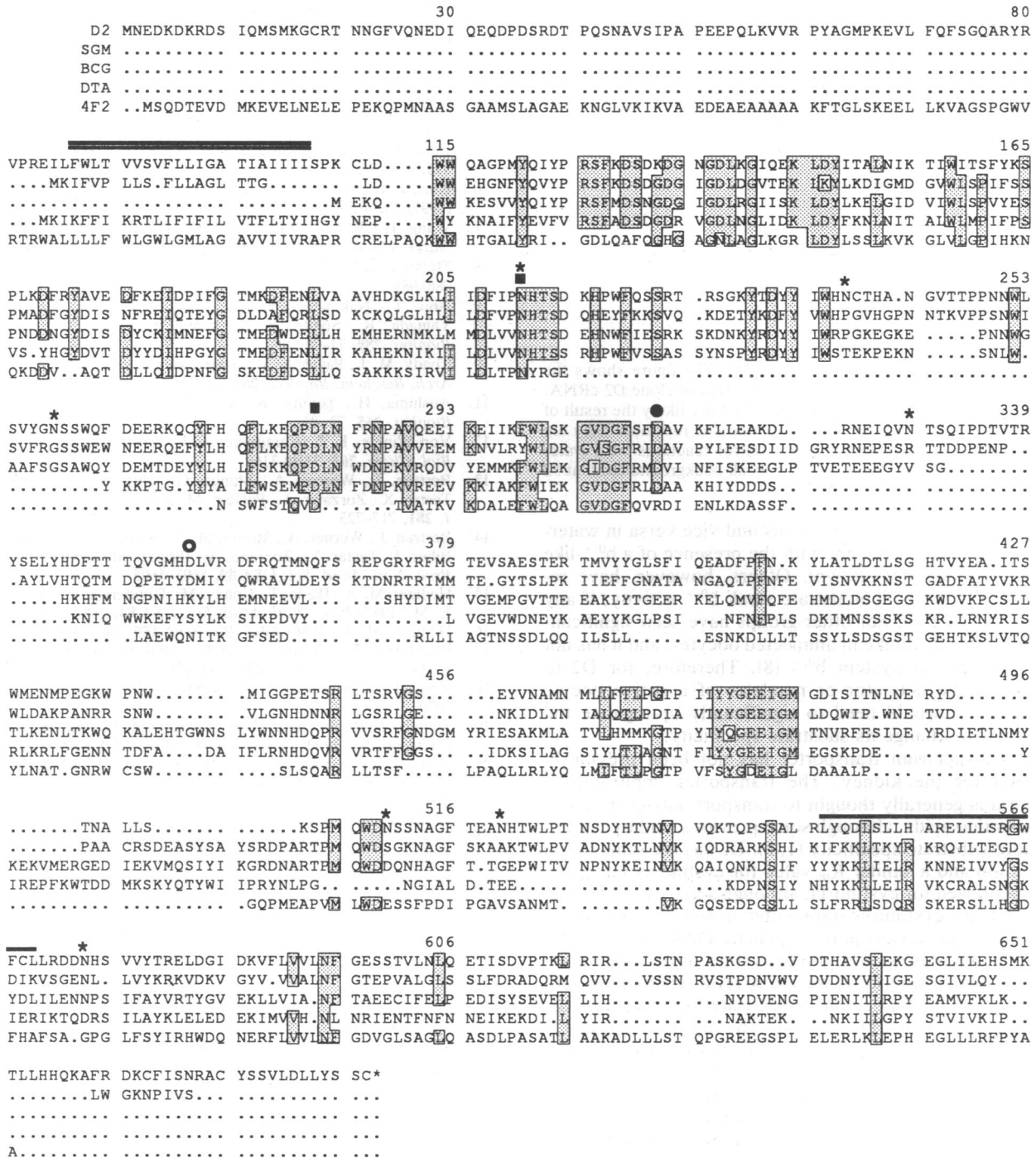

FIG. 3. Deduced amino acid sequence of D2 and its alignment with similar sequences. The deduced amino acid sequence of D2 is the top sequence. There are four potential methionine start codons (at residues 1, 13, 15, and 65). The Met-1 AUG codon shows the highest match with the Kozak consensus initiation sequence [CC(A/G)CC (23)] and is the most likely initiator codon given that it is the first (24). Stars represent potential N-linked glycosylation sites. The putative transmembrane domain and the leucine zipper motif are marked by double and single lines, respectively. Aspartic acid residues believed to be catalytic in porcine pancreatic amylase and conserved in D2 are indicated by circles (see text). Asp-356 (○) does not line up in this figure with the proposed catalytic asparatic acid in the known glucosidases, but lies within a similar sequence. Asn-211 and Asp-281 (■) correspond to the proposed calcium-binding ligands Asn-100 and Asp-167 in porcine pancreatic amylase (25). The amino acid sequence of D2 is aligned with those of glucosidases and the human 4F2 heavy-chain antigen. Sequences with the highest degree of similarity [except the *Drosophila* hypothetical proteins D, H, and L, which are of unknown function (26)] are shown. SGM, *Aedes aegypti* salivary gland maltase-like protein (27); BCG, *Bacillus cereus* oligo-1,6-glucosidase (28); DTA, *Dictyoglomus thermophilum* amylase (29); 4F2, 4F2 heavy chain antigen (30). The *A. aegypti* salivary gland maltase-like protein and *D. thermophilum* amylase have 5′ signal sequences; *B. cereus* oligo-1,6-glucosidase, which is cytoplasmic, lacks a 5′ hydrophobic region. Residues that are conserved in four or five of the sequences are boxed.

transporter. 4F2, which is involved in cell growth in an as yet undefined way, exists as a heterodimer attached to a smaller, nonglycosylated light chain. In analogy to this protein, the D2 protein might attach to a second subunit. The leucine zipper motif at the C terminus of D2 might mediate dimerization.

If D2 is not itself a transporter, the uptake we observe represents the activity of a native oocyte transporter. The mouse blastocyst transporter $b^{0,+}$ (12) is a $Na^+$-independent transporter with broad specificity for neutral and dibasic amino acids. We see significant inhibition of dibasic amino

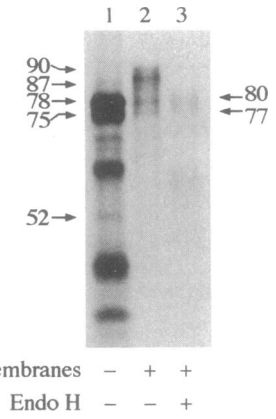


FIG. 4. *In vitro* translation of clone D2. The figure shows the result of SDS/10% PAGE of *in vitro* translation of clone D2 cRNA. Bands at molecular masses <75 kDa in lane 1 are likely the result of initiation of translation from internal methionine residues. They are not seen in lanes with membranes. Control reactions with water instead of mRNA (not shown) show a weak background band at 52 kDa. Endo H, endoglycosidase H.

acid uptake by neutral amino acids and vice versa in water-injected oocytes, consistent with the presence of a $b^{0,+}$-like transporter (data not shown). Others, however, have reported that oocytes demonstrate weak $b^{0,+}$ activity, if any (13, 39). Neither we nor other groups have seen significant saturable cystine uptake in uninjected oocytes, and it has not been reported for system $b^{0,+}$ (8). Therefore, for D2 to function as the activator or regulator of a native oocyte transporter, it would need to significantly up-regulate the transporter or change its substrate specificity.

A broad-spectrum transporter has not been previously described for the kidney. The transporter implicated in cystinuria is generally thought to transport only cystine and dibasic amino acids, although some previous studies suggest a broader substrate specificity (1, 5). D2 has the same tissue distribution and a similar $K_m$ value for cystine as the transporter proposed for cystinuria, although some evidence suggests that the cystinuria transporter is sodium-dependent, unlike D2-induced transport. A genetic defect in D2 could explain the increased excretion of cystine and dibasic amino acids seen in cystinuria; there are several renal amino acid transport systems that could compensate for abnormal neutral amino acid reabsorbtion (5, 40). The results of our hybrid depletion experiment suggest that D2 plays a major role in renal cystine transport; D2 is likely the species responsible for the cystine uptake into oocytes that was reported by McNamara *et al.* (8). Examination of the D2 gene in patients with cystinuria may prove useful in understanding the disease.

Our data raise the interesting possibility that amino acid transport in the kidney proceeds via a transporter with a single transmembrane region. Alternatively, transport may require activation by a glucosidase-like protein, a concept not previously considered in models of renal transporters. The role of D2 in transport—and its potential role in determination of transporter substrate specificity—remain to be addressed.

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