Identification and Characterization of a Prostaglandin Transporter

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Carrier-mediated prostaglandin transport has been postulated to occur in many tissues. On the basis of sequence homology, the protein of unknown function encoded by the rat matrin F/G complementary DNA was predicted to be an organic anion transporter. Expression of the matrin F/G complementary DNA in HeLa cells or *Xenopus* oocytes conferred the property of specific transport of prostaglandins. The tissue distribution of matrin F/G messenger RNA and the sensitivity of matrin F/G–induced prostaglandin transport to inhibitors were similar to those of endogenous prostaglandin transport. The protein encoded by the matrin F/G complementary DNA is thus preferably called PGT because it is likely to function as a prostaglandin transporter.

Prostaglandins (PGs) and thromboxanes (Txs) play widespread physiological, pathophysiological, and therapeutic roles in health and disease (*1*). Although PGs are often assumed to traverse membranes by simple diffusion, they are charged anions at physiological pH and diffuse poorly across model biological membranes such as that of the rabbit erythrocyte (*2–4*). This limited simple diffusion appears to be augmented, at least in some circumstances, by carrier-mediated transport. For example, PGE_1, PGE_2, and $PGF_{2\alpha}$ are cleared rapidly on passage through the pulmonary circulation, whereas prostacyclin (PGI_2) is not (*5–8*). Because intracellular enzymes (PG dehydrogenases) metabolize PGI_2 as well as PGE_1, PGE_2, and $PGF_{2\alpha}$ (*9*), selective PG clearance by the pulmonary vascular bed must be attributable to differential transport across the plasma membrane of pulmonary cells. Additional evidence supports the presence of a specific PG transporter (or transporters) in the lung (*10–12*). Many epithelia, including those in the liver, kidney, choroid plexus, anterior uvea, and uterus, also engage in active vectorial PG transport (*13–17*). Studies with perfused kidney tubules indicate that such transport is a two-step process, consisting of active, energy-dependent basolateral uptake followed by passive apical secretion (*13*).

The rat liver bromosulfophthalein (BSP)–bile salt transporter, "oatp" (*18*), shows 37% amino acid identity to the predicted protein encoded by the rat matrin F/G complementary DNA (cDNA) (*19*). Translation of the matrin F/G protein has been thought to begin at the ATG codon at nucleotides 385 to 387, and the protein was originally proposed to be a DNA binding protein (*19*). Further analysis shows that the ATG codon at nucleotides 88 to 90, within the same open reading frame (ORF), lies in a more favorable consensus for translation initiation (*20*), in which case the translated protein would be extended by 99 amino acids at the NH_2-terminus as compared with that originally proposed (Fig. 1A). Kyte-Doolittle hydropathy analysis of the predicted NH_2-terminally extended protein revealed strong similarities to oatp, suggesting that the two are closely related members of the transporter superfamily characterized by 12 membrane-spanning domains (Fig. 1B).

To test directly whether the matrin F/G cDNA encodes an organic anion transporter, we transiently expressed oatp and matrin F/G cDNAs in HeLa cell monolayers or in *Xenopus* oocytes (*21*). Expression of oatp resulted in the expected 5- to 20-fold increase in [^{35}S]BSP uptake relative to background in both expression systems (*22*). Although PGs were not transported by oatp, they markedly inhibited BSP transport [inhibition constant (K_i) ~14 μM (*22*)], suggesting that prostanoids might interact with the binding site of oatp or related transporters (matrin F/G). Indeed, expression of the matrin F/G cDNA in either HeLa cells or *Xenopus* oocytes was associated with a rapid and marked increase in the transport of PGE_1, PGE_2, and $PGF_{2\alpha}$ (Fig. 2). In contrast, the stable prostacyclin analog iloprost and the prostacyclin metabolite 6-keto

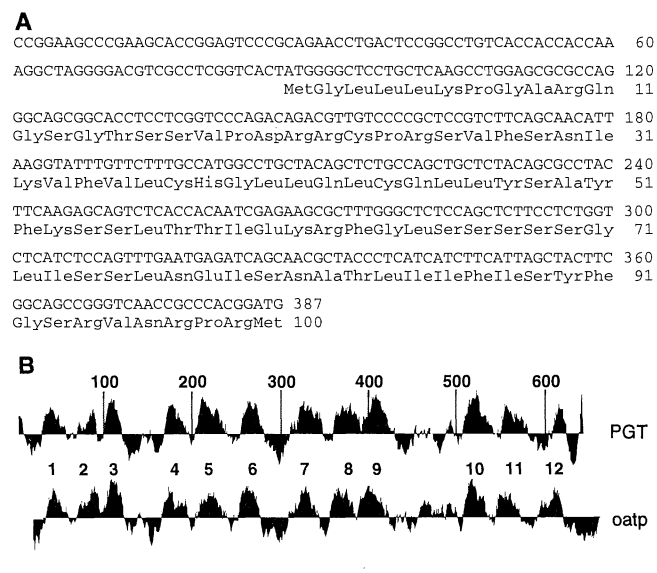

Fig. 1. (**A**) Complementary DNA and deduced amino acid sequences corresponding to the NH_2-terminal region of matrin F/G with the ATG codon beginning at nucleotide 88 as the translation start site. The ATG codon at nucleotide 385 is the translation start site originally proposed (*19*). (**B**) Hydropathy analysis of the NH_2-terminally extended matrin F/G protein (PGT) and oatp (*18, 19*) based on the Kyte-Doolittle algorithm with a window of 13 residues (*33*). Numbers at top refer to amino acids of PGT, with the ATG codon at nucleotide 88 encoding residue 1; numbers between plots indicate putative membrane-spanning regions.

Table 1. Prostanoid K_m and inhibitor K_i values for PGT-mediated uptake into HeLa cell monolayers determined by measurement of [^{3}H]$PGF_{2\alpha}$ transport during 1 min at 27°C.

Prostanoid	K_m (nM)	Inhibitor	K_i (μM)
PGE_1	70 ± 26	Bromcresol green	3.6 ± 0.2
PGE_2	94 ± 16	Bromosulfophthalein	5.8 ± 0.3
$PGE_{2\alpha}$	104 ± 19	Indocyanine green	35 ± 1.1
TxB_2	423 ± 69	Furosemide	47 ± 4.6
6-Keto $PGF_{1\alpha}$	7569 ± 1200	*p*-Aminohippurate	>400
Iloprost	86900 ± 36000	Indomethacin	>400*
Arachidonate	96000 ± 11700	Probenecid	>400*

*Mean of two monolayers from a single transfection. Other values are means ± SEM from three to five separate transfections.

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$PGF_{1\alpha}$ were not transported to any significant degree (Fig. 2C). Transport of prostanoids appeared to be specific because several other organic anions (glutathione, *p*-aminohippurate, taurocholate, urate, unconjugated and conjugated bilirubin, and estradiol glucuronide) were not transported (*22*).

To test the hypothesis that the ATG codon at nucleotide 88 is required for functional expression of prostanoid transport, we engineered a truncated cDNA that lacks ATG 88 but contains ATG 385 and the remaining shared ORF (*21*). Expression of the truncated construct was associated with only background PGE_2 transport (Fig. 2A).

Because PG transport was blocked by anion transport inhibitors (*22*) and because the predicted protein has 12, rather than 7, hydrophobic domains, matrin F/G cDNA encodes a PG transporter and not a PG receptor (*23*). Thus, we will hereafter refer to the transporter as PGT (prostaglandin transporter).

The background uptake of PGE_1, PGE_2, $PGF_{2\alpha}$, TxB_2, and 6-keto $PGF_{1\alpha}$ was extremely low in both expression systems. In contrast, the background uptake of arachidonate (1 nM) was high (≥40 fmol/min per milligram of protein in HeLa cells, ≥0.5 fmol/30 min per oocyte). After PGT expression, arachidonate transport was slightly above background in only one of three groups of oocytes and in neither of two separate HeLa cell transfections (*22*). Thus, arachidonate is probably not transported to a significant extent by PGT.

At 37°C, PGE_1, PGE_2, and $PGF_{2\alpha}$ accumulation by HeLa cell monolayers transfected with PGT cDNA saturated at a level 150- to 500-fold that observed with monolayers transfected with the control plasmid (*22*). At 27°C, initial uptake could be resolved and was approximately linear over 5 to 10 min (Fig. 2B). The rank order of PGT-mediated uptake rates over 1 min for HeLa cells at prostanoid concentrations (1 nM) well below the Michaelis constant (K_m) was $PGE_1 \approx PGE_2 \approx PGF_{2\alpha} > TxB_2 \gg$ 6-keto $PGF_{1\alpha} \approx$ iloprost (Fig. 2C). Qualitatively similar results were obtained with oocytes (*22*). We determined the affinity of PGT for prostanoid substrates by assessing the ability of unlabeled prostanoids to inhibit $PGF_{2\alpha}$ uptake (*24*) (Fig. 2D). The affinity of PGT was high for PGE_1, PGE_2, and $PGF_{2\alpha}$ ($K_m \leq 10^{-7}$ M), intermediate for TxB_2, and low for 6-keto $PGF_{1\alpha}$, iloprost, and arachidonate (Table 1). The rank order of affinities was thus similar to that for uptake rates (Fig. 2C).

Northern (RNA) blot analysis of rat total RNA (*25*) revealed transcripts of ~4 kb in several tissues (Fig. 3A) and transcripts of

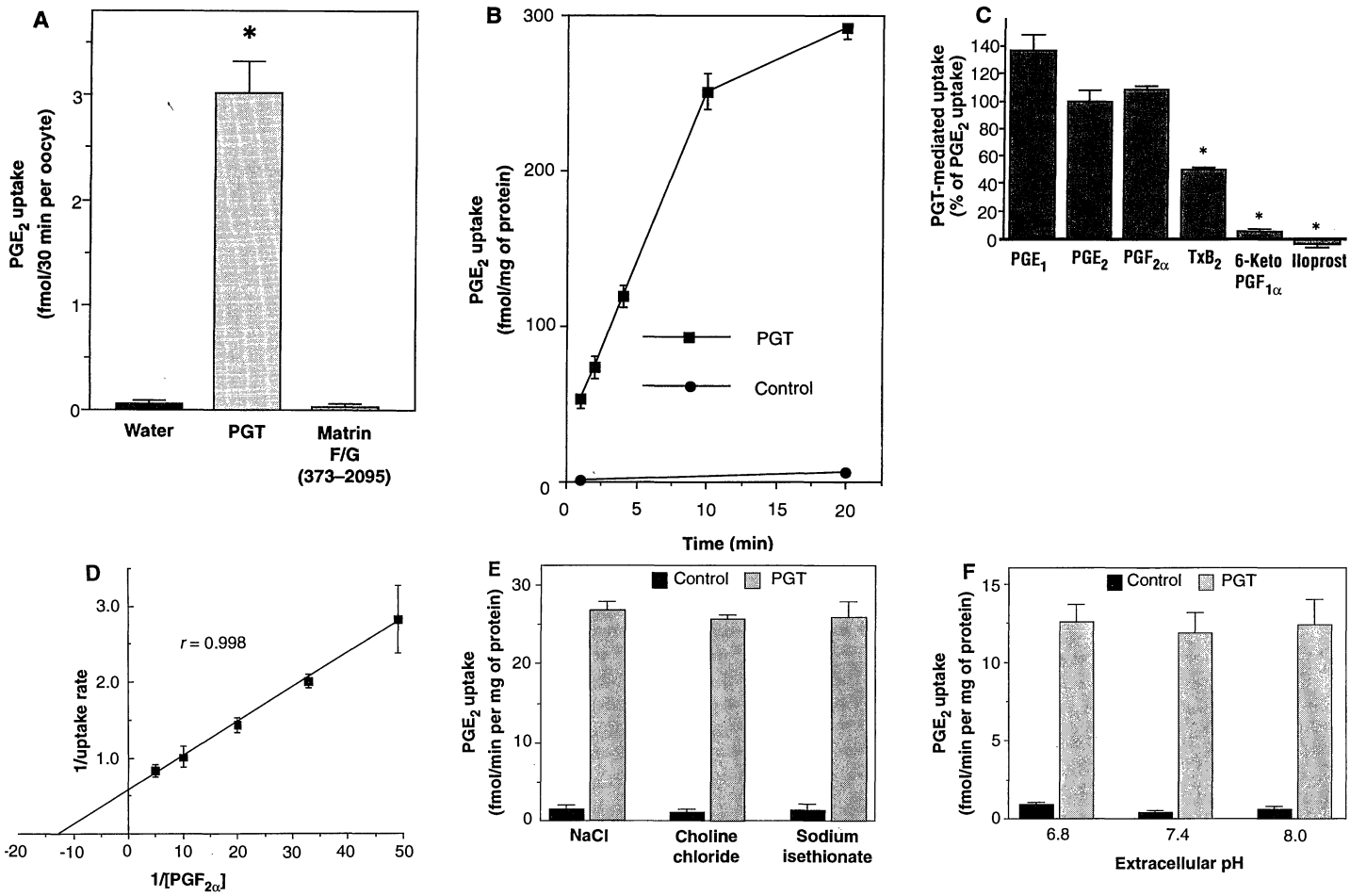

Fig. 2. (**A**) Uptake of [^{3}H]PGE_2 into *Xenopus* oocytes injected with water, cRNA transcribed from matrin F/G cDNA containing the ATG codon at nucleotide 88 (PGT), or cRNA transcribed from a 5′ truncated matrin F/G cDNA lacking ATG 88 but containing ATG 385 [matrin F/G(373–2095)]. Data are means±SEM of 8 to 10 oocytes. *$P < 0.05$ versus water-injected oocytes (analysis of variance). (**B**) Time course of [^{3}H]PGE_2 uptake into HeLa cells transfected with matrin F/G cDNA (PGT) or control plasmid (pBluescript KS, Stratagene). (**C**) One-minute PGT-mediated prostanoid uptake into transfected HeLa cell monolayers. Data are means ± SD of three or four monolayers. *$P < 0.05$ versus PGE and PGF uptake (analysis of variance). (**D**) Double-reciprocal plot for PGT-mediated $PGF_{2\alpha}$ transport into transfected HeLa cells. The units of $PGF_{2\alpha}$ concentration ([$PGF_{2\alpha}$]) and uptake are micromolar and picomoles per minute per milligram of protein, respectively. In this particular experiment, the Michaelis constant was 80 nM. (**E**) Effect of substitution of Na^+ and Cl^- with choline and isethionate, respectively, on PGE_2 uptake into transfected HeLa cells. The measured Na^+ concentration of the choline chloride solution was 1.2 mM. Data are means ± SD of two monolayers. (**F**) Effect of extracellular pH on PGE_2 uptake into transfected HeLa cells. Data are means ± SD for two to four monolayers for (B), (D), and (F).

~2.4 and ~5 kb in the brain. Analysis of polyadenylated [poly(A)$^+$] RNA revealed smaller transcripts in the brain (~2.4 kb) and stomach (~0.8, ~1.0, and ~1.4 kb), as well as ileum, jejunum, and kidney (~1.5 kb) (Fig. 3B). The matrin F/G cDNA is 2.75 kb in length and lacks a 3′ polyadenylation signal (*19*), so it may not represent a full-length clone. It is not clear whether the apparent enrichment for smaller transcripts after selection of poly(A)$^+$ RNA represents higher degrees of polyadenylation.

The presence of PGT mRNA in tissues containing epithelia, but not in heart or skeletal muscle (Fig. 3A), suggests that PGT may mediate one of the steps in epithelial carrier-mediated PG transport (*13–17*). Replacement of extracellular Na^+ with choline had no effect on PGE_2 transport into HeLa cells transfected with PGT cDNA (Fig. 2E), suggesting that PGT mediates passive transport rather than the active, Na^+-dependent step (*13*). Similarly, changing the inwardly directed proton gradient by rapidly altering the extracellular pH had no effect on transport (Fig. 2F), indicating that PGT does not function as a PG/H^+ cotransporter.

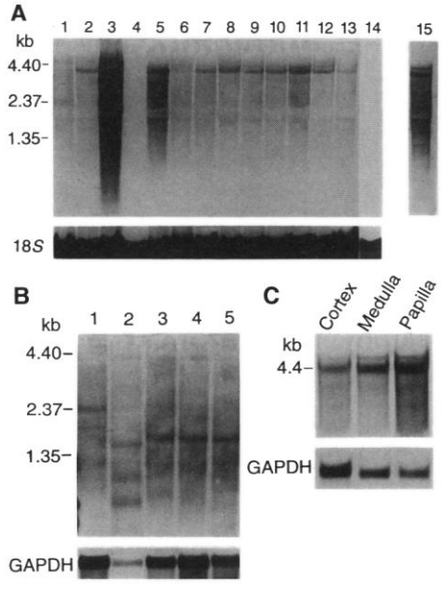

Fig. 3. Northern blot analysis of PGT mRNA abundance in rat tissues. (**A**) Total RNA. Lanes: 1, brain; 2, stomach; 3, lung; 4, skeletal muscle; 5, liver; 6, testis; 7, colon; 8, ileum; 9, jejunum; 10, duodenum; 11, kidney; 12, eye; 13, uterus; 14, heart. Each lane contained 55 μg of total RNA with the exception of lane 15, which contained 30 μg of lung total RNA. The positions of molecular size standards are shown in kilobases. 18*S*, 18*S* RNA. (**B**) Poly(A)$^+$ RNA (2 μg per lane). Lanes: 1, brain; 2, stomach; 3, ileum; 4, jejunum; 5, kidney. GAPDH, glyceraldehyde-3-phosphate dehydrogenase mRNA. (**C**) Total RNA (55 μg per lane) from renal cortex, outer medulla, and papilla.

Replacement of extracellular Cl^- with isethionate had no effect on transport (Fig. 2E), which suggests that PGT is not a PG/Cl exchanger.

We explored further a role for PGT in epithelial transport by examining the effects of inhibitors. Indocyanine green, bromcresol green, indomethacin, furosemide, *p*-aminohippurate, and probenecid inhibit PG transport when applied to intact epithelia (*11–16, 26–28*). Of these agents, *p*-aminohippurate, indomethacin, and probenecid had no effect on PGT-mediated $PGF_{2\alpha}$ uptake into HeLa cells (Table 1), which suggests that these three drugs act at a different step in overall transepithelial PG transport. For those agents that did inhibit PGT, the K_i values were similar to those obtained for the lung, kidney, anterior uvea, and choroid plexus [bromcresol green, 2 to 4 μM (*11, 15, 16, 26, 27*); indocyanine green, 16 μM (*12*); and furosemide, 25 to 80 μM (*15, 27*)]. Thus, the tissue distribution of its mRNA, its sensitivity to known blockers of prostanoid transport, and its substrate selectivity indicate that PGT likely mediates one of the steps in the transepithelial transport of prostanoids.

Although transepithelial PG transport has not been described in the renal papilla, renal papillary collecting duct cells are a site of abundant PGE_2 and $PGF_{2\alpha}$ synthesis (*29*). Newly synthesized PGE_2 and $PGF_{2\alpha}$ are preferentially released across the basolateral membrane of papillary (but not cortical) collecting duct cells in primary culture (*30*). Similar sidedness has been described for the isolated rat colon (*31*). Such polarized prostanoid release suggests a carrier-mediated process. A passive transporter such as PGT could facilitate PG efflux if PG concentrations in the cytoplasm were higher than those in the plasma. PGT mRNA is readily detected in the rat colon (Fig. 3A), and, in the kidney, it is most abundant in the papilla (Fig. 3C). Although it is not known in which membranes PGT is expressed in the renal papilla and colon, these results raise the possibility that PGT or similar transporters mediate the translocation of newly synthesized polar prostanoids across plasma membranes in these organs.

After their release from peripheral tissues, the gastrointestinal tract, and the renal papilla, PGE_1, PGE_2, and $PGF_{2\alpha}$ are removed from the circulation by carrier-mediated transport in pulmonary, hepatic, and renal vascular beds, respectively (*5–8*). Because PGT mRNA is most abundant in the lung, liver, and kidney (Fig. 3), and because the rank order for prostanoid clearance by the lung ($PGE_1 \approx PGE_2 \approx PGF_{2\alpha} > TxB_2 > PGI_2$ (*5, 7, 10–12, 32*) is the same as that of transport by PGT (Fig. 2C and Table 1), PGT may mediate the vascular clearance of prostanoids.

In summary, the substrate specificity, inhibitor sensitivity, and distribution of PGT mRNA suggest that PGT may mediate the release of newly synthesized PGs from cells, the transepithelial transport of PGs, and the clearance of PGs from the circulation. The latter two functions would confer on PGT a primary role in defining PGE_1, PGE_2, and $PGF_{2\alpha}$ as autocoids. Because these PGs are functionally stable in blood for at least 2 min (*5*), they could potentially circulate and activate remote receptors if their actions were not terminated locally by carrier-mediated transport and enzymatic degradation. Indeed, when pulmonary PG clearance in intact animals is blocked by the sustained infusion of indocyanine green at concentrations equivalent to the K_i for PGT, vasodilatory PGE passes through the pulmonary vascular bed to the arterial circulation and substantially reduces systemic blood pressure (*12*). Thus, PGT may be important in the maintenance of normal blood pressure.

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21. For oocyte expression, water or complementary RNA (cRNA) that had been transcribed in vitro from matrin F/G cDNA and capped was injected into *Xenopus* oocytes (50 ng of cRNA per oocyte). Uptake studies were performed 2 to 3 days after injection by washing of oocytes three times in Waymouth's solution, incubating for various periods at 27°C with radioactive substrate (~0.25 μCi/ml; total concentration, ~1 nM), washing three times with ice-cold Waymouth's solution, and lysing in 0.5 ml of 10% SDS. Oocyte-associated radioactivity was determined by liquid scintillation spectroscopy. PG uptake in oocytes was linear over 60 min. For some experiments, the 1723–base pair Hinc II–Bam HI fragment of matrin F/G cDNA, which lacks ATG 88 but contains ATG 385 and the ORF to the stop codon at nucleotide 2017, was subcloned into pGEM-4Z, thus placing the coding sequence under the control of the T7 promoter. For HeLa cell expression, cells were grown to 80% confluence on 35-mm dishes and then infected with recombinant vaccinia virus vTF7-3 (10 plaque-forming units per cell) [T. R. Fuerst *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8122 (1986)]. Thirty minutes after infection, cells were transfected with matrin F/G cDNA (10 μg/ml) plus lipofectin (20 μg/ml) [R. D. Blakely *et al.*, *Anal. Biochem.* **194**, 302 (1991)]. After 3 hours of incubation, vaccinia virus and the DNA-lipofectin complex were removed, and the cells were maintained overnight in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Uptake studies were performed 19 hours after transfection. Monolayers were washed three times with culture medium without serum and incubated for various times at 27°C with radioactive substrate (0.05 μCi/ml per dish; total concentration, ~0.2 nM). Uptake was stopped by washing cells once with ice-cold Waymouth's solution containing 5% bovine serum albumin and then four times with Waymouth's solution alone. Cells were scraped and the associated radioactivity was determined by liquid scintillation spectroscopy.
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24. For substrate K_m determinations (Table 1), [^{3}H]$PGF_{2\alpha}$ uptake was determined in duplicate HeLa cell monolayers in the presence of various concentrations of unlabeled $PGF_{2\alpha}$, PGE_1, PGE_2, TxB_2, 6-keto $PGF_{1\alpha}$, arachidonate, or iloprost (0 and 20 nM to 10 μM). A K_m or K_i value for a given prostanoid and a single transfection was calculated by a nonparametric method [R. Eisenthal and A. Cornish-Bowden, *Biochem. J.* **139**, 715 (1974)]. For inhibitor K_i determinations, [^{3}H]$PGF_{2\alpha}$ uptake was determined with duplicate HeLa cell monolayers in the presence of 0, 10, or 100 μM inhibitor. The concentration of [^{3}H]$PGF_{2\alpha}$ (0.2 nM) was 500-fold less than the K_m for $PGF_{2\alpha}$ (104 nM). Therefore, K_i can be approximated as $[i]v_i/(v-v_i)$, where v is the 1-min uptake value without inhibitor, v_i is the 1-min uptake value with inhibitor, and $[i]$ is the inhibitor concentration [K. D. Neame and T. G. Richards, *Elementary Kinetics of Membrane Carrier Transport* (Wiley, New York, 1972)].
25. The 3′ untranslated region of the matrin F/G cDNA was removed at the Bam HI site (nucleotide 2055). The remaining cDNA was subcloned into pGEM3Z and linearized with Nco I, and an antisense digoxigenin-labeled cRNA probe was generated with SP6 RNA polymerase. Total RNA was isolated from Sprague-Dawley rat tissues by grinding in liquid nitrogen with a mortar and pestle followed by guanidinium-acid-phenol extraction [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. Polyadenylated RNA was prepared with oligo(dT) magnetic beads (Poly-A Track, Promega). RNA was separated by glyoxal agarose gel electrophoresis and transferred to a Hybond N (Amersham) membrane, which was then incubated with the digoxigenin-labeled probe. After washing the membrane twice with 0.1× standard saline citrate at 65°C, labeled complexes were visualized by chemiluminescence autoradiography (2-hour exposure) (Amersham). The extent of loading of lanes was established by methylene blue staining and by probing for 18*S* RNA or glyceraldehyde-3-phosphate dehydrogenase mRNA.
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A Neuropeptide Gene Defined by the *Drosophila* Memory Mutant *amnesiac*

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Mutations in genes required for associative learning and memory in *Drosophila* exist, but isolation of the genes has been difficult because most are defined by a single, chemically induced allele. Here, a simplified genetic screen was used to identify candidate genes involved in learning and memory. Second site suppressors of the *dunce* (*dnc*) female sterility phenotype were isolated with the use of transposon mutagenesis. One suppressor mutation that was recovered mapped in the *amnesiac* (*amn*) gene. Cloning of the locus revealed that *amn* encodes a previously uncharacterized neuropeptide gene. Thus, with the cloning of *amn*, specific neuropeptides are implicated in the memory process.

Two genes essential for learning and memory in *Drosophila* have been cloned and sequenced. The *dnc* gene encodes a adenosine 3′,5′-monophosphate (cAMP)–specific phosphodiesterase (*1*). A second mutation, *rutabaga* (*rut*), encodes a Ca^{2+}- and calmodulin-sensitive adenylate cyclase (*2*, *3*). Both genes are components of the adenylate cyclase second messenger pathway, and the cloning of these two loci relied on knowledge of the biochemical activities of the gene products. However, full exploitation of the genetic potential to dissect learning and memory mechanisms requires a direct progression from the genetic mutation to the isolation of the gene. Such an approach has been key in other areas of *Drosophila* research, but application of the same techniques to learning mutants is hampered by the labor intensive nature of behavioral testing. For example, mutagenesis by the mobilization of transposable elements or by x-rays greatly facilitates cloning of the disrupted genes. Unfortunately, the lower mutagenesis efficiencies when compared with chemical mutagens, combined with the difficulty of direct behavioral screening, make these traditional approaches problematic.

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Table 1. Fertility of wild-type and mutant flies. Single female flies of the indicated genotypes were placed individually in culture vials, and the percentage of females laying eggs and the average number of progeny produced after 18 days were recorded (*n* refers to the number of female flies assayed). The dnc^{m11} allele was used.

Genotype	*n*	Females laying eggs (%)	Avg. no. of progeny ± SEM
Wild type	50	98	>40
dnc/dnc	84	14	0
dnc P(19A)/dnc P(19A)	70	57	1.7 ± 0.5
dnc P(19A)/dnc	50	74	2.3 ± 0.6
dnc amn/dnc amn	290	66	3.9 ± 1.0
dnc amn/dnc	37	73	1.9 ± 0.7