# Expression of a Cloned γ-Aminobutyric Acid Transporter in Mammalian Cells†

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ABSTRACT: The cDNA clone GAT-1, which encodes a $Na^+$- and $Cl^-$-coupled GABA transporter from rat brain, has been expressed in mammalian cells using three different systems: (1) transient expression upon transfection of mouse $Ltk^-$ cells with a eukaryotic expression vector containing GAT-1; (2) stable expression in L-cells transfected with the same vector; (3) transfection of HeLa cells infected with a recombinant vaccinia virus expressing T7 RNA polymerase. Similar results both qualitatively and quantitatively were obtained with all systems. The GABA transporter expressed in HeLa and L-cells retains all the properties described previously for GABA transport into synaptosomes and synaptic plasma membrane vesicles. It was fully inhibited by *cis*-3-aminocyclohexanecarboxylic acid (ACHC) and not by β-alanine. The $K_M$ for GABA transport and the $IC_{50}$ for ACHC inhibition were similar to the presynaptic transporter. Accumulated [$^3$H]GABA was released from transfected cells by dissipating the transmembrane $Na^+$ gradient with nigericin or by exchange with unlabeled external GABA. Accumulation was stimulated by both $Na^+$ and $Cl^-$ in the external medium. However, in the absence of external $Cl^-$, a small amount of GABA transport remained which was dependent on GAT-1 transfection. Functional expression of the GABA transporter was abolished by tunicamycin. An antitransporter antibody specifically immunoprecipitates a polypeptide with an apparent molecular mass of about 70 kDa from GAT-1-transfected cells. When cells were grown in the presence of tunicamycin, only a faint band of apparent mass of about 60 kDa was observed. We conclude that GAT-1 encodes the ACHC-sensitive GABA transporter subtype which, upon expression in mammalian cells, exhibits properties very similar to those of the native system. Sensitivity to tunicamycin suggests that N-linked glycosylation is important for the functional expression of this membrane protein.

High-affinity, sodium-dependent neurotransmitter transport, measured in a variety of brain preparations, is thought to terminate the overall process of synaptic transmission (Iversen, 1971, 1973; Kuhar, 1973; Bennett et al., 1974). The rat brain γ-aminobutyric acid (GABA)[1] transporter is one of the most abundant and well studied [reviewed in Kanner (1983)] of these proteins.

The transporter catalyzes cotransport of sodium, chloride, and GABA (Radian & Kanner, 1983; Kanner, 1978, 1983; Keynan & Kanner, 1988). The stoichiometry of the system was estimated to be 2–3 $Na^+$:$Cl^-$:GABA (Keynan & Kanner, 1988). The protein was purified from rat brain and reconstituted into liposomes in an active form (Radian & Kanner, 1985; Radian et al., 1986). This glycoprotein migrates as an 80-kDa polypeptide on SDS–PAGE. Polyclonal antibodies prepared against this 80-kDa band immunoprecipitate GABA transport activity (Radian et al., 1986).

Kinetic and pharmacological studies suggest the presence of a variety of GABA transporter subtypes. There are at least two types of high-affinity GABA transporters (Kanner & Bendahan, 1990). One is inhibited by ACHC and seems to be neuronal (Bowery et al., 1976; Neal & Bowery, 1977). The other is more sensitive to β-alanine and is suspected to be of glial origin (Schon & Kelly, 1975). Low-affinity GABA transport has been also observed and appears to consist of two components. One is due to high-affinity transport in inverted vesicles. Transport from the cytosolic side to the exterior has a 100-fold higher $K_M$ than transport from the cell exterior to the cytoplasm (Mabjeesh & Kanner, 1989). Moreover, in intact synaptosomes which are right-side-out, there is an additional low-affinity GABA transporter (Wood & Sidhu, 1986; Mabjeesh & Kanner, 1989).

Recently, a high-affinity GABA transporter has been cloned, and its functional properties have been examined in *Xenopus* oocytes (Guastella et al., 1990). That expression system is not very convenient and suffers from great variability, at least with regard to GABA transport. Therefore, we decided to find conditions for the transient and stable expression of the cloned transporter in mammalian cells. Using these systems, we have been able to define unambiguously the subtype of the cloned transporter, to study the effect of N-glycosylation and to provide evidence that low-affinity GABA transport is not a property of the cloned high-affinity transporter.

## Experimental Procedures

### *Materials*

Protein A–Sepharose CL-4B, tunicamycin, the antibiotic G418, and trypsin inhibitor were purchased from Sigma. [$^3$H]GABA (47.6 Ci/mmol) was from the Nuclear Research Center, Negev, Israel, and from NEN; [$^{35}$S]methionine (1000 Ci/mmol) was from Amersham. ACHC was synthesized for us by Erwin Gross in the pilot plant at the School for Applied Sciences (The Hebrew University). The tissue culture medium, serum, penicillin/streptomycin, and L-glutamine were

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[1] Abbreviations: ACHC, *cis*-3-aminocyclohexanecarboxylic acid; AOAA, (aminooxy)acetic acid; CMV, cytomegalo virus; DMEM, Dulbecco's modified Eagle's medium; DOTMA, *N*-[1-[2,3-bis(oleoyloxy)-propyl]]-*N*,*N*,*N*-trimethylammonium chloride; GABA, γ-aminobutyric acid; L-cells, mouse $Ltk^-$ cells; PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 8 mM $Na_2HPO_4$, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2 \cdot 6H_2O$, pH 7.4; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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purchased from Gibco/BRL and from Biological Industries, Kibbutz Bet-Ha'Emek, Israel. All the restriction enzymes and the transfecting reagent (DOTMA) were obtained from Boehringer Mannheim. TransfectACE (Rose et al., 1990) was a generous gift from Dr. John K. Rose, Department of Pathology, Yale University School of Medicine. Bluescript II SK$^-$ was from Stratagene. The vaccinia/T7 recombinant virus vTF7-3 was a gift from Dr. Bernard Moss (NIH). HeLa cells were obtained from the American Type Culture Collection and from Dr. Aharon Razin (The Hebrew University). Mouse Ltk$^-$ cells (L-cells) and pL32-neomycin were obtained from Dr. Oded Meyuhas (The Hebrew University). pCMV$\beta$ (MacGregor & Caskey, 1989) was a gift from these authors and was passed on to us by Dr. Abraham Feinsod (The Hebrew University).

*Methods*

HeLa cells and L-cells were cultured in DMEM supplemented with 10% fetal calf serum (heat-inactivated), 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 2 mM L-glutamine at 37 °C in 5% $CO_2$.

*Expression Vectors.* A Bluescript II SK$^-$ plasmid, pT7-GAT-1, encoding the cloned $Na^+$- and $Cl^-$-dependent GABA transporter from rat brain was excised in vivo from the GAT-1 $\lambda$-ZAPII clone described by Guastella et al. (1990). pCMV$\beta^-$-GAT-1 was prepared using standard cloning procedures (Sambrook et al., 1989). The plasmid pCMV$\beta$ was digested with *Not*I to delete the $\beta$-galactosidase gene, and the GAT-1 cDNA was inserted in its place. The orientation of the insert relative to the CMV promoter was verified by *Sac*I and *Hin*dIII digestions. The resulting plasmid was used for transfection of L-cells.

*Infection/Transfection in HeLa Cells.* Growth and titration of the vTF7-3 virus and the infection/transfection procedure were the same as described (Blakely et al., 1991). In some experiments, DOTMA was used instead of lipofectin (BRL) or dimethyldioctadecylammonium bromide (transfectACE, Gibco-BRL) as the cationic lipid, at exactly the same concentrations.

*Transient Expression of pCMV$\beta^-$-GAT-1 in L-Cells.* L-Cells were plated in 24-well plates at 70% confluency. After 24 h of growth, the cells were transfected (1.2 $\mu$g of DNA/well) using the calcium phosphate coprecipitation method (Graham & Van Der Eb, 1973) and were incubated for another 24 h. Subsequently, the medium was replaced with fresh medium, and transient expression was assayed 48 h after transfection.

*Stable Expression of GABA Transport in L-Cells.* A total of $6 \times 10^5$ cells were stably cotransfected with pCMV$\beta^-$-GAT-1 (20 $\mu$g) and a plasmid, pL32-neomycin, (1.3 $\mu$g) encoding the bacterial neomycin resistance gene driven by the mouse ribosomal protein L32 gene promoter (positions −159 to +77) (Dr. Oded Meyuhas, unpublished results). After 48 h, cells were transferred to selective medium containing 400 $\mu$g of active G418 per milliliter, and stably transformed clones expressing the GABA transporter were isolated after 3–5 weeks of selection.

*GABA Transport in HeLa and L-Cells.* At 6–12-h post-transfection, HeLa cells were washed with PBS, and each well was incubated in 0.2 mL of PBS containing 0.2 $\mu$Ci of [$^3$H]GABA (47.6 Ci/mmol). Transport reactions were carried out at room temperature for 5 min unless indicated otherwise in the figure legends. Transport was terminated by aspirating the medium and washing twice with 1 mL of cold PBS. Cells were lysed with 1% SDS (0.5 mL/well), and the radioactivity was measured by liquid scintillation counting. Transport into L-cells was carried out 48 h after transfection as above, except that 2 $\mu$Ci/mL [$^3$H]GABA was present. Efflux was measured as described in the legend to Figure 4. Unless otherwise indicated, the results represent averages of triplicate points with standard deviations of 2–5% of the mean value. In each case, two or more replicate experiments gave essentially identical results.

*Separation of L-Cell Extracts after GABA Transport by Thin-Layer Chromatography (TLC).* Analysis of accumulated radiolabel was performed according to the method of Martin and Smith (1972). After transfection of L-cells growing in 24-well plates with pCMV$\beta^-$-GAT-1, the cells were allowed to accumulate [$^3$H]GABA by transport for 1 h with or without 100 $\mu$M AOAA. Cells were extracted with 70% ethanol (30 $\mu$L/well), unlabeled GABA (12.5 $\mu$g/30 $\mu$L) was added, and samples were applied to 250-$\mu$m silica gel G thin-layer plates. Two control samples were also applied, each containing 12.5 $\mu$g of unlabeled GABA + 0.5 $\mu$Ci of [$^3$H]GABA. One of the controls also contained an extract of cells which had not taken up [$^3$H]GABA. The plate was placed in a chamber equilibrated for 15 min in the solvent mixture of butanol/acetic acid/water (60:20:20, by volume) and developed for 150 min. The developed plate was dried in an oven at 110 °C for 1 h. Spots were visualized by iodine vapor. All the lanes were cut out, and the radioactivity was determined using a liquid scintillation counter.

*Immunoprecipitation of the GABA Transporter from HeLa Cells Transfected with pT7-GAT-1.* HeLa cells were plated on 24-well plates, infected, and transfected as indicated above. Twelve hours after transfection, the cells were washed with methionine-free DMEM containing 100 units/mL penicillin, 100 $\mu$g/mL streptomycin, and 2 mM glutamine and incubated for 30 min in this medium (1 mL/well). Subsequently, cells were washed once with 190 mM NaCl and incubated for 1 h at 37 °C with 0.5 mL of 190 mM NaCl containing [$^{35}$S]-methionine (25 $\mu$Ci/mL). The cells were washed 3 times in cold PBS and lysed by incubation for 30 min at 4 °C with 0.5 mL of RIPA buffer (0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, and 0.25 mM PMSF) containing trypsin inhibitor (1 mg/mL). The lysates were centrifuged for 15 min at 10000 rpm at 4 °C. To the clear lysate was added 10 $\mu$L of the appropriate antibody, and the mixture was shaken end over end in the cold room for 16 h. Then 10 mg of protein A–Sepharose Cl-4B was added, and the shaking was continued for another 4 h. The samples were centrifuged, and the pellets were washed 4 times with 1 mL of RIPA buffer and then one wash with a solution containing 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 100 mM NaCl. The beads were sedimented and resuspended in 100 $\mu$L of 2-fold-concentrated SDS–PAGE sample buffer. After SDS–PAGE, the gel was stained with Coomassie Blue and destained, incubated for 30 min at room temperature with Amplify (Amersham), and dried. Radioactive bands were visualized by autoradiography.

*SDS Gel Electrophoresis.* Samples were prepared and analyzed on discontinuous SDS–polyacrylamide gels (4% stacking gel, 10% separating gel) as described by Laemmli (1970). Size standards (Pharmacia) were run in parallel and visualized by Coomassie Blue staining.

*Protein Determination.* Cells were lysed by addition of 2% $Na_2CO_3$ in 0.1 N NaOH (200 $\mu$L/well). Aliquots were taken for protein determination (Lowry et al., 1951).

## Results

*Kinetics of [$^3$H]GABA Uptake.* The GABA transporter cDNA insert in the Bluescript-derived plasmid pT7-GAT-1

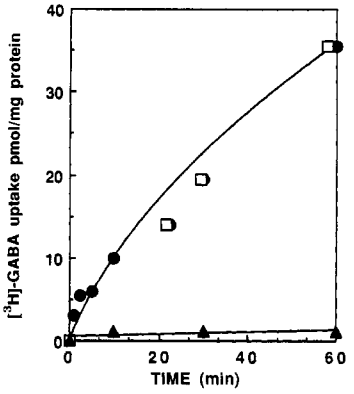


FIGURE 1: Time course of [$^3$H]GABA uptake in L-cells transfected with pCMV$\beta^-$-GAT-1. L-Cells were transfected with pCMV$\beta^-$-GAT-1 (filled circles, squares) or with the same vector without insert (pCMV$\beta^-$, triangles), and transport was carried out for the times indicated on the abscissa as described under Experimental Procedures. The transport medium was the standard NaCl-containing medium with (squares) or without (circles) 100 $\mu$M AOAA; 0.16 mg of protein was present in each well. Points represent mean values from triplicates.

is transcribed under control of the T7 promoter. The GAT-1 cDNA was further subcloned into the mammalian expression vector pCMV$\beta^-$. This places the coding sequence under the control of the CMV promoter. Three systems were evaluated for expression of the GABA transporter from these plasmids. The CMV construct (pCMV$\beta^-$-GAT-1) was tested in transiently and stably transfected L-cells. The pT7-GAT-1 plasmid was transfected into HeLa cells infected with vTF7-3, a recombinant vaccinia virus which directs expression of bacteriophage T7 RNA polymerase (Fuerst et al., 1986; Blakely et al., 1991).

The uptake of [$^3$H]GABA into L-cells transiently transfected with pCMV$\beta^-$-GAT-1 is illustrated in Figure 1. Uptake continued for at least 60 min, and did not reach plateau even after 90 min (data not shown). The uptake is dependent on GAT-1 cDNA, since no uptake was observed when L-cells were transfected with the vector pCMV$\beta^-$ alone (Figure 1). In the presence of 100 $\mu$M AOAA, which specifically inhibits GABA transaminase (the first enzyme in GABA catabolism), the time course of GABA accumulation was unchanged (Figure 1). After 60 min of incubation, we extracted the radioactivity accumulated by these cells and analyzed it by TLC. The radiolabel accumulated in cells treated with or without AOAA migrated with a mobility identical to that of authentic GABA. Thus, the accumulated GABA is apparently not metabolized.

Similar results were obtained using L-cells stably transfected with pCMV$\beta^-$-GAT-1 and with vTF7-3-infected HeLa cells transfected with pT7-GAT-1. Analysis of the initial rate of [$^3$H]GABA influx at various [$^3$H]GABA concentrations yielded values for the apparent $K_M$ and $V_{max}$ for the three expression systems which are given in Table I. In all cases, a $K_M$ of about 4 $\mu$M was observed. This value is in excellent agreement with that observed in synaptic plasma membranes (Kanner, 1978), reconstituted systems (Radian et al., 1986), and the expressed GAT-1 in oocytes (Guastella et al., 1990). The kinetics of GABA transport were also examined using an extended range of GABA concentrations (up to 500 $\mu$M) to test for the presence of a low-affinity component. No significant increase in rate was observed above 20 $\mu$M GABA, indicating that low-affinity GABA transport was not expressed in these cells.

*Ion Dependence of GABA Transport.* The GABA transporter of synaptic plasma membrane vesicles typically exhibits

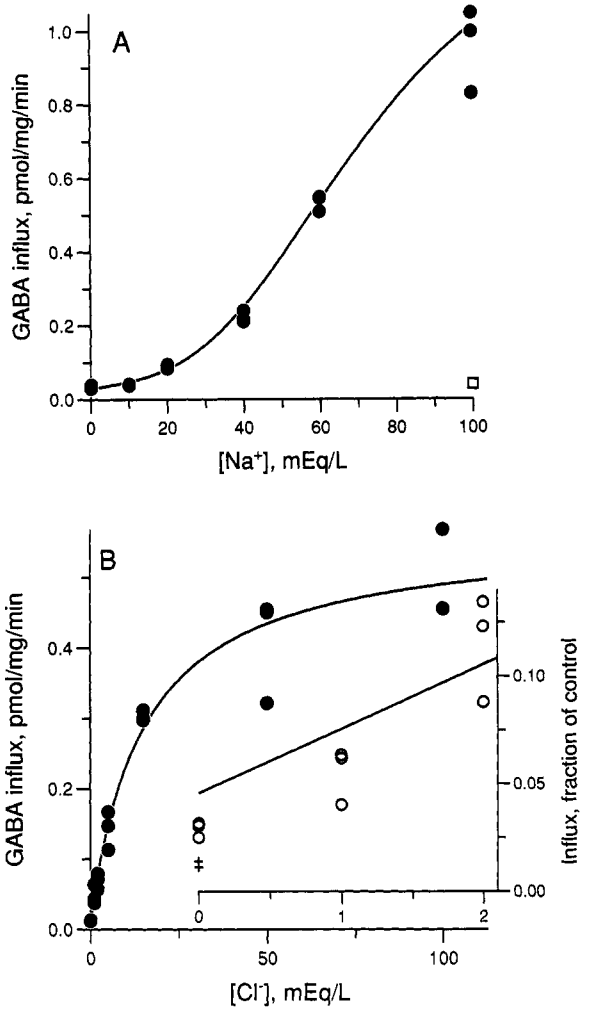


FIGURE 2: Sodium and chloride concentration dependence of GABA transport in vaccinia/T7-infected HeLa cells. HeLa cells were infected with vTF7-3 and transfected with pT7-GAT-1 as described under Experimental Procedures. Transport was measured after 5-min incubation in a medium consisting of 5 mM $KP_i$, pH 7.5, 0.5 mM $MgSO_4$, 0.3 mM $CaCl_2$, and 100 mM NaCl. LiCl (A) and sodium isethionate (B) were used as equimolar replacements for NaCl to achieve the indicated $Na^+$ and $Cl^-$ concentrations. Transport in the absence of pT7-GAT-1 is indicated in (A) (square). In the inset to panel B, influx at low $Cl^-$ concentrations is shown as the fraction of control (100 mM NaCl). The line is the linear least-squares fit to all influx points between 0 and 5 mequiv/L $Cl^-$. The "+" symbols represent influx into cells transfected with a control plasmid (pBluscript II SK$^-$). A Buchler–Cotlove chloridimeter was used to measure free [$Cl^-$] in the medium of the nominally 0 $Cl^-$ samples after incubation. Using this assay, free [$Cl^-$] was estimated at approximately 75 $\mu$equiv/L. The [$Na^+$] dependence was repeated at least twice, and the [$Cl^-$] dependence at least 3 times, with identical results. The average standard deviation of triplicate points was 5–10% of the mean except for the measurements at low [$Cl^-$] which were 10–20% of the mean.

a characteristic dependence on $Na^+$ and $Cl^-$ concentrations. The same ion dependence was shown by GAT-1-directed GABA transport into transfected HeLa and L-cells. As an example, Figure 2 illustrates the ion dependence of GABA transport expressed using the vaccinia/T7 system. Transport was dramatically stimulated by both $Na^+$ (Figure 2A) and $Cl^-$ (Figure 2B), similar to GABA transport in rat brain derived preparations. The sodium concentration dependence (Figure 2A) exhibited the sigmoidal behavior originally described by Martin and Smith (1972), while the dependence of transport on $Cl^-$ concentration was hyperbolic (Figure 2B). The weak inhibition of GABA transport by $Li^+$ and isethionate reported by Shank et al. (1987) may influence the shapes of

Table I: Kinetic Parameters of GABA Uptake in Transfected Cells[a]

| transfected cell | $K_M$ ($\mu$M) | $V_{max}$ [pmol min$^{-1}$ (mg of protein)$^{-1}$] |
|---|---|---|
| vTF7-3 HeLa | 5.1 ± 1.5 | 167.3 ± 23.4 |
| L-cells (transient) | 5.0 ± 0.58 | 220.0 ± 84 |
| L-cells (stable) | 4.7 ± 1.3 | 349.4 ± 28.6 |

[a] Initial rates of [$^3$H]GABA influx were measured in L-cells transiently and stably transfected with pCMV$\beta^-$-GAT-1 and in vTF7-3-infected HeLa cells transfected with pT7-GAT-1. Transport was measured after 5 min in PBS containing 0.8 $\mu$M [$^3$H]GABA with 0–10 $\mu$M unlabeled GABA. Net transport rates were determined by subtracting transport rates into untransfected cells (HeLa) or rates in the presence of 50 mM unlabeled GABA (L-cells). $K_M$ and $V_{max}$ were determined using nonlinear regression analysis of the corrected $v$ vs $S$ data. Similar $K_M$ and $V_{max}$ values were measured in replicate experiments.

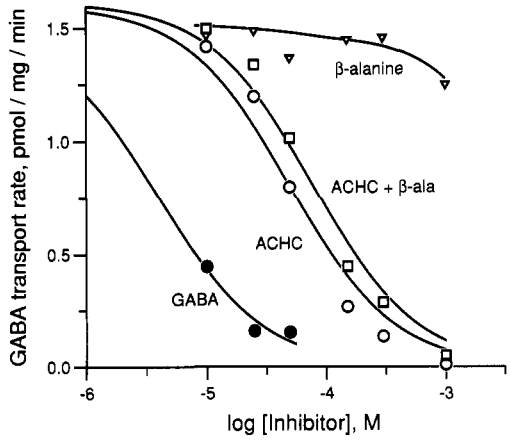


FIGURE 3: Inhibition of GABA uptake by substrate analogues in L-cells transfected with pCMV$\beta^-$-GAT-1. Ten-minute transport rates were measured in L-cells 48 h after transfection. Substrate analogues were present in the influx medium at the final concentrations indicated on the abscissa. In the case where $\beta$-alanine and ACHC were present together, each was present at half of the indicated concentrations. Unlabeled GABA (open circles); ACHC (filled circles); $\beta$-alanine (squares); $\beta$-alanine and ACHC (triangles).

these curves somewhat. However, the shape of the $Na^+$ dependence in particular is more likely to result from the known requirement for more than one $Na^+$ ion in GABA transport (Keynan & Kanner, 1988). In this system, it was possible to measure the rate of GAT-1-directed GABA transport in medium substantially free of $Cl^-$. The inset to Figure 2B shows GABA influx at low $Cl^-$ concentration depicted as the fraction of control influx measured in 100 mM NaCl. The "+" symbols at zero $Cl^-$ represent influx into cells transfected with a control plasmid lacking the GAT-1 insert. The increased flux in pT7-GAT-1-transfected cells was invariably observed in at least three separate experiments. We conclude that $Cl^-$ stimulates GABA influx approximately 100-fold but is not absolutely required for GABA transport. Similar experiments at low $Na^+$ concentration failed to demonstrate $Na^+$-independent influx (not shown).

*Inhibition of GABA Transport by Substrate Analogues.* The use of the selective inhibitors ACHC and $\beta$-alanine to determine the subtype of the expressed transporter is illustrated in Figure 3. ACHC fully inhibited the process with a half-maximal effect at 100 $\mu$M. In contrast, $\beta$-alanine did not significantly inhibit. Furthermore, $\beta$-alanine did not augment the inhibitory action of ACHC. For a comparison, the high affinity of unlabeled GABA toward the transporter—as compared to ACHC—is illustrated by inhibition of [$^3$H]GABA influx (Figure 3). Although the experiment shown is from L-cells transiently transfected with pCMV$\beta^-$-GAT-1, similar

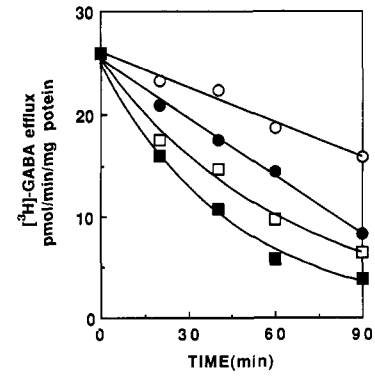


FIGURE 4: Effect of external GABA and nigericin on efflux of previously accumulated [$^3$H]GABA. L-cells were transfected with pCMV$\beta^-$-GAT-1 for 48 h. The cells were loaded with [$^3$H]GABA by transport for 1 h as described under Experimental Procedures. Subsequently, the medium was removed by aspiration, and the cells were washed once with 1 mL of the efflux medium (see below). The cells were then incubated with 1 mL of the efflux medium for the indicated times, and reactions were stopped as described under Experimental Procedures. The efflux medium contained PBS (open circles) with 5 $\mu$M nigericin (filled circles), 50 $\mu$M GABA (open squares), or 5 $\mu$M nigericin + 50 $\mu$M GABA (filled squares).

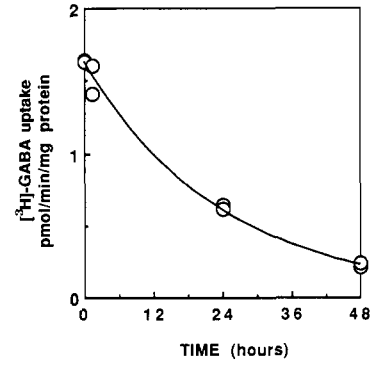


FIGURE 5: Effect of tunicamycin on GABA transport. Cells from a stable clone expressing the GABA transporter were plated and incubated in a medium containing DMEM, fetal calf serum, and antibiotics as detailed under Experimental Procedures. At the indicated times, tunicamycin (10 $\mu$g/mL final concentration) was added to individual wells. After 48 h, GABA transport was measured for 5 min as described under Experimental Procedures. The times indicated on the abscissa reflect the incubation time with tunicamycin (all the cells were grown for a total of 48 h).

results were observed with the vaccinia/T7 system (data not shown).

*Efflux of GABA.* The reversibility of the uptake process is illustrated in Figure 4. In this experiment, L-cells, transfected with pCMV$\beta^-$-GAT-1, were allowed to take up [$^3$H]GABA. Subsequently, the radioactivity was removed and fresh unlabeled medium added to the cells. A slow efflux of [$^3$H]GABA was observed, which is markedly enhanced by unlabeled GABA. This results from exchange of internal for external substrate. Exchange reflects a partial reaction catalyzed by the transporter, as opposed to efflux which represents the full translocation cycle (Nelson & Rudnick, 1979; Kanner et al., 1983). Nigericin is expected under these experimental conditions to increase the internal $Na^+$ concentration (by exchanging internal $K^+$ for external $Na^+$). Figure 4 demonstrates that this increase in internal $Na^+$ concentration also increased efflux. Moreover, efflux was increased further when GABA and nigericin were added together to the efflux medium.

*Glycosylation.* The GABA transporter is glycosylated, like most biochemically characterized eukaryotic membrane proteins (Radian et al., 1986). We have examined the effect of

glycosylation in L-cells stably expressing the GABA transporter. Incubation with 10 μg/mL tunicamycin [a specific inhibitor of N-linked glycosylation) (Elbein, 1984)] caused a time-dependent inhibition of GABA transport (Figure 5). Similar results were obtained using transiently transfected HeLa and L-cells (see also Figure 6).

*Immunoprecipitation.* The product of the GAT-1 cDNA is recognized by the polyclonal antibody directed against the purified $Na^+$- and $Cl^-$-coupled GABA transporter (Radian et al., 1986). In the experiment shown in Figure 6, vTF7-3-infected HeLa cells transfected with pT7-GAT-1 were incubated in the presence of [$^{35}$S]methionine for 1 h immediately prior to solubilization, immunoprecipitation, and separation by SDS gel electrophoresis. Figure 6A is an autoradiogram showing that the polyclonal antibody specifically immunoprecipitated $^{35}$S-labeled components that migrated (lane 6) as a 70-kDa band and a 140-kDa band, similar to the migration of purified rat brain GABA transporter, which also aggregates under these conditions (Kanner et al., 1989). These two bands were not observed under control conditions (control plasmid, lanes 1–4; control serum, lane 5; or both, lane 1). The other minor bands observed are not specific; i.e., they were observed also under the control conditions (see also Figure 6B) and reflect other components cross-reacting with antibodies present in the crude antitransporter antiserum. This was further substantiated by the use of an affinity-purified antibody (Figure 6B, lane 1). In the presence of tunicamycin, the antiserum immunoprecipitated smaller amounts of a 65-kDa band (Figure 6A, lane 8). This probably represents nonglycosylated transporter.

## Discussion

In this study, we have examined the properties of the cloned $Na^+$- and $Cl^-$-coupled GABA transporter from rat brain in several mammalian expression systems. Two of these give rise to transient expression and, with one, stable expression is obtained. All systems exhibited GABA transport with similar characteristics, which closely resembled the process in native rat brain membrane vesicles. This includes substrate and inhibitor affinity (Table I and Figure 3), [$Na^+$] and [$Cl^-$]-dependence (Figure 2A,B, respectively), and reversibility (Figure 4). This reversibility provides additional evidence that the radioactive GABA accumulated is not metabolized. Stable expression was achieved by cotransfecting L-cells with two plasmids, one (pL32-neomycin) containing the selectable neomycin gene and the other (pCMV$\beta^-$-GAT-1) containing the GAT-1 insert driven by the CMV promoter. pCMV$\beta^-$-GAT-1 by itself led to transient expression of GABA transport into L-cells at rates and to extents higher than those obtained in the vaccinia/T7 system. The vaccinia/T7 system, however, is very convenient since it leads to high expression within 6–20 h. At later times, however, expression is impaired because of the cell damage caused by the vaccinia virus. This system is particularly useful for screening the ability of new clones constructed in vectors with a T7 promotor (such as pBS and pGEM) to express a functional protein. The only requirement for such new constructs is that the cDNA is in the correct orientation for transcription from the T7 promoter.

The GAT-1 cDNA has also been expressed in *Xenopus* oocytes (Guastella et al., 1990). One issue not clearly resolved in the oocyte system is the subtype classification of the cloned GABA transporter, presumably due to relatively high variability of this expression system. The experiments in this study clearly indicate that the cloned transporter is of the ACHC-sensitive subtype (Figure 3). This is not unexpected, since the transporter preparation from which microsequence information was used to obtain the clone is also of this subtype (Radian et al., 1986; Kanner & Bendahan, 1990). On the other hand, it can be anticipated that the two transporter subtypes might be highly homologous, especially in light of the extensive homology between GABA and norepinephrine transporters

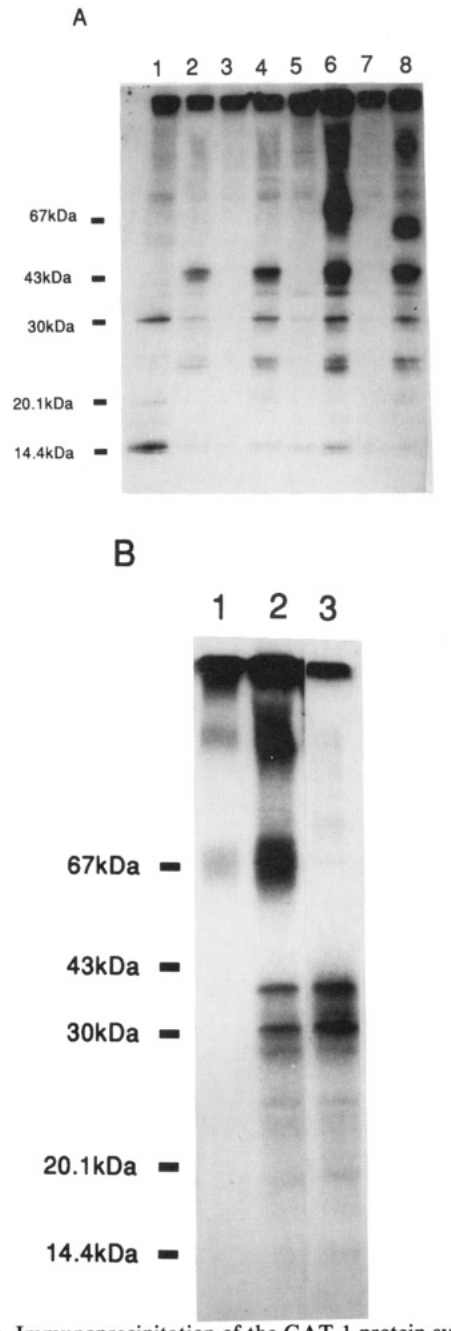


FIGURE 6: Immunoprecipitation of the GAT-1 protein synthesized in vivo in HeLa cells. GAT-1 expression was directed by pT7-GAT-1 in the vaccinia/T7 expression system in HeLa cells. The cells were labeled with [$^{35}$S]methionine, lysed, and immunoprecipitated with the indicated antibody. The immunoprecipitates were analyzed on SDS–PAGE as described under Experimental Procedures. All HeLa cells were infected with vTF7-3 and transfected either with control plasmid (Bluescript SKII$^-$) or with pT7-GAT-1. (A) Preimmune serum was used in odd-numbered lanes and anti-GABA transporter antiserum in the even-numbered lanes. The transfection conditions were as follows: lanes 1 and 2, Bluescript II SK$^-$; lanes 3 and 4, the same with tunicamycin (10 μg/mL); lanes 5 and 6, pT7-GAT-1; lanes 7 and 8, pT7-GAT-1 with tunicamycin (10 μg/mL). Tunicamycin was added together with the DNA. (B) Lane 1, immunoprecipitation with the affinity-purified antibody against the GABA transporter (Radian et al., 1990); lane 2, immunoprecipitation with anti-GABA transporter antiserum; lane 3, HeLa cells transfected with control plasmid (Bluescript II SK$^-$) and immunoprecipitated with anti-GABA transporter antiserum.

(Guastella et al., 1990; Nelson et al., 1990; Pacholczyk et al., 1991). Thus, an oligonucleotide probe based on one of the subtypes might have hybridized with a cDNA coding for the other. However, it is still not clear whether two separate genes exist for the two subtypes. Other possibilities, including posttranslational modification, also could result in an altered substrate binding site.

There is a considerable complexity in GABA transporter subtypes. In addition to high-affinity subtypes (Kanner & Bendahan, 1990), low-affinity GABA transport ($K_M$ 100–200 $\mu$M) has also been observed (Mabjeesh & Kanner, 1989). One type of low-affinity transporter observed in synaptic plasma membrane vesicle preparations represents inside-out vesicles into which GABA is transported from the cytoplasmic to the external face of the membrane. The remaining low-affinity transport is apparently due to a separate system. Since the plasma membranes of the transfected cells used here are all "right-side-out", the expression systems described here allow us to determine if this separate low-affinity system is related to the high-affinity system. Since only high-affinity transport was observed (Table I), it seems that the low-affinity system is not encoded by the GAT-1 cDNA.

As with the $Na^+$-coupled glucose transporter (Birnir et al., 1990), tunicamycin appears to block functional expression of the GABA transporter (Figure 5). Tunicamycin inhibition of N-linked asparagine glycosylation could affect GABA uptake in various ways. The N-linked oligosaccharides may be required for proper processing, correct membrane targeting, protein stability, or function. At this time, we cannot differentiate between the various possibilities, including other effects of tunicamycin, except to note that in cells incubated in the presence of tunicamycin, the major polypeptide reacting with antibodies to the GABA transporter has a higher electrophoretic mobility than the native GABA transporter (Figure 6).

Appropriate posttranslational processing in mammalian expression systems represents a distinct advantage for expressing cloned mammalian transport proteins. Another advantage that these systems offer is the ability to study and quantify the various aspects of the synthesis, function, and regulation of the transporter that have been difficult so far to address in synaptosomes, or with mRNA-injected oocytes. We conclude that the expression systems described here can serve as a model system for studying the biochemistry, cell biology, pharmacology, physiology, and molecular biology of the cloned $Na^+$- and $Cl^-$-coupled GABA transporter.

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