

# Functional production and reconstitution of the human equilibrative nucleoside transporter (hENT1) in *Saccharomyces cerevisiae*

## Interaction of inhibitors of nucleoside transport with recombinant hENT1 and a glycosylation-defective derivative (hENT1/N48Q)

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We have produced recombinant human equilibrative nucleoside transporter (hENT1) in the yeast *Saccharomyces cerevisiae* and have compared the binding of inhibitors of equilibrative nucleoside transport with the wild-type transporter and a N-glycosylation-defective mutant transporter. Equilibrium binding of <sup>3</sup>H-labelled nitrobenzylmercaptapurine ribonucleoside {6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine; NBMPR} to hENT1-producing yeast revealed a single class of high-affinity sites that were shown to be in membrane fractions by (1) equilibrium binding (means ± S.D.) of [<sup>3</sup>H]NBMPR to intact yeast ( $K_d$  1.2 ± 0.2 nM;  $B_{max}$  5.0 ± 0.5 pmol/mg of protein) and membranes ( $K_d$  0.7 ± 0.2 nM;  $B_{max}$  6.5 ± 1 pmol/mg of protein), and (2) reconstitution of hENT1-mediated [<sup>3</sup>H]thymidine transport into proteoliposomes that was potently inhibited by NBMPR. Dilazep and dipyrindamole inhibited NBMPR binding to hENT1 with IC<sub>50</sub> values of 130 ± 10 and 380 ± 20 nM respectively. The role of N-linked glycosylation in the interaction of NBMPR with hENT1 was examined by the quantification of binding of [<sup>3</sup>H]NBMPR to yeast producing either wild-type

hENT1 or a glycosylation-defective mutant (hENT1/N48Q) in which Asn-48 was converted into Gln. The  $K_d$  for binding of NBMPR to hENT1/N48Q was 10.5 ± 1.6 nM, indicating that the replacement of an Asn residue with Gln decreased the affinity of hENT1 for NBMPR. The decreased affinity of hENT1/N48Q for NBMPR was due to an increased rate of dissociation ( $k_{off}$ ) and a decreased rate of association ( $k_{on}$ ) of specifically bound [<sup>3</sup>H]NBMPR because the values for hENT1-producing and hENT1/N48Q-producing yeast were respectively 0.14 ± 0.02 and 0.36 ± 0.05 min<sup>-1</sup> for  $k_{off}$ , and (1.2 ± 0.1) × 10<sup>8</sup> and (0.40 ± 0.04) × 10<sup>8</sup> M<sup>-1</sup> · min<sup>-1</sup> for  $k_{on}$ . These results indicated that the conservative conversion of an Asn residue into Gln at position 48 of hENT1 and/or the loss of N-linked glycosylation capability altered the binding characteristics of the transporter for NBMPR, dilazep and dipyrindamole.

**Key words:** nitrobenzylmercaptapurine ribonucleoside, nucleoside transport.

## INTRODUCTION

Nucleoside transporters are integral membrane proteins that mediate the uptake and release of physiological nucleosides and nucleoside drugs used in anti-cancer and anti-viral therapies (reviewed in [1–4]). The transport of nucleosides and nucleoside analogues must occur in a mediated fashion because these highly hydrophilic compounds do not readily diffuse across lipid bilayers. Seven distinct nucleoside transport processes have been demonstrated in mammalian cells on the basis of substrate specificity, inhibition by diagnostic agents, and mechanism of transport [1–3]. Four of these have now been identified by molecular cloning and functional expression of cDNA species in oocytes of *Xenopus laevis* ([5–13], and M. W. Ritzel, S. Y. M. Yao, A. Ng, J. R. Mackey, C. E. Cass and J. D. Young, unpublished work). These transporters comprise two functionally distinct and structurally unrelated protein families. The concentrative nucleoside transporters (CNTs) of mammalian cells are Na<sup>+</sup>-dependent symporters that move nucleosides into cells

against their concentration gradients; cDNA species encoding representatives of the two major subfamilies (CNT1, pyrimidine-nucleoside selective; CNT2, purine-nucleoside selective) have been cloned from human and rat tissues ([5,6,8,9,13], and M. W. Ritzel, S. Y. M. Yao, A. Ng, J. R. Mackey, C. E. Cass and J. D. Young, unpublished work). The equilibrative nucleoside transporters (ENTs) are bidirectional, broadly selective transporters that catalyse the facilitated diffusion of nucleosides down their concentration gradients. The ENTs have been subdivided on the basis of their sensitivity to inhibition by nanomolar concentrations of nitrobenzylmercaptapurine ribonucleoside {NBMPR; 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine} into *es* (equilibrative sensitive) and *ei* (equilibrative insensitive) transporters. cDNA species encoding the *es* and *ei* transporters (ENT1 and ENT2 respectively) have been cloned from human placenta, cultured HeLa cells and rat intestine [9,11,12,14].

In humans, the *es* and *ei* cDNA species are predicted to encode two related proteins (hENT1, hENT2) of 50 % identity and 57 % similarity composed of 456 amino acid residues with a molecular

Abbreviations used: CMM, complete minimal medium; ENT, equilibrative nucleoside transporter; *es*, equilibrative and sensitive to inhibition by NBMPR; MTX, methotrexate; NBMPR, nitrobenzylmercaptapurine ribonucleoside (also known as nitrobenzylthioinosine), 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine; NBTGR, nitrobenzylthioguanosine; SAA, sulphanilamide.

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mass of 50.2 kDa, three potential N-linked glycosylation sites and 11 transmembrane domains [11,12,14]. The predicted topology of hENT1 assumes a single N-linked glycosylation site, which was thought to be in the large extracellular loop between the first two transmembrane domains from the N terminus [12] on the basis of an earlier demonstration that the *es* transporter of human erythrocytes is glycosylated very close to one end of the protein [15]. The assignment of Asn-48 as the site of the N-linked glycosylation of hENT1 was confirmed by an analysis of the electrophoretic mobility of recombinant hENT1 in which the Asn was replaced with Gln (M. Sundaram, S. Y. M. Yao, E. Chomey, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work). The role of N-linked glycosylation in the biosynthetic processing and function of hENT1 is not known. The perturbation of N-linked glycosylation of membrane proteins in a murine cell line that possesses three nucleoside transport processes produced complex changes in uridine transport kinetics but had little, if any, effect on the abundance or apparent affinity of the *es* transporter for NBMPR [16]. Similarly, the partial removal of carbohydrate from the ENT1 transporter by digestion with endo- $\beta$ -galactosidase had no effect on the inhibition by dipyrindamole of NBMPR binding [15]. hENT2 has a similar N-linked glycosylation site at Asn-48 plus a second, downstream site, also in the large extracellular loop, at Asn-57 [11,14]. It is not yet known whether the hENT2 sites are glycosylated.

We have previously shown that cDNA species encoding nucleoside transporters can be identified by phenotypic complementation in the yeast *Saccharomyces cerevisiae* by assessing the growth of a yeast strain that is deficient in thymidine transport under selection conditions that prevent the production of dTMP *de novo* [17]. Here we demonstrate the functional expression of hENT1 cDNA in *S. cerevisiae* by the following criteria: survival of yeast under the selection conditions, the presence of hENT1 mRNA and protein in yeast, the mediated uptake of thymidine by intact yeast and proteoliposomes reconstituted from detergent-solubilized membranes, the inhibition of thymidine uptake by NBMPR and the high-affinity binding of NBMPR to intact yeast and yeast membrane fractions. We have examined the role of N-linked glycosylation in hENT1 function by comparing the equilibrium binding of [ $^3$ H]NBMPR to yeast producing either wild-type hENT1 or a mutant in which Asn-48 was converted into Gln (hENT1/N48Q). hENT1/N48Q exhibited a decreased affinity for NBMPR relative to that of hENT1, which was due to an increase in the dissociation rate and a decrease in the association rate. The apparent affinities of hENT1/N48Q for dipyrindamole and dilazep, which were examined by assessing their relative abilities to inhibit the binding of [ $^3$ H]NBMPR under equilibrium binding conditions, increased relative to that of hENT1. Our results, which demonstrated the production of recombinant hENT1 in yeast in quantities sufficient for analysis of the equilibrium binding and dissociation kinetics of the prototypic inhibitors of *es*-mediated transport in mammalian cells, support earlier conclusions that the transport inhibitors (NBMPR, dipyrindamole and dilazep) bind to the *es* transporter protein at or near the nucleoside recognition site.

## EXPERIMENTAL

### Plasmid construction

The hENT1 cDNA was inserted into a yeast expression vector (pYES2) containing the inducible Gal1 promoter. The resulting plasmid (pYhENT1) was produced by using the cDNA encoding hENT1 [12] as the template for amplification by PCR and the following 5' *Kpn*I-containing and 3' *Sph*I-containing primers

(restriction sites underlined): 5'-GCT GGTACC ATG ACA ACC AGT CAC CCT C-3' and 5'-GCA GCATGC CAC AAT TGC CCG GAA-3'. pYhENT1 incorporated several features important in the heterologous expression of recombinant proteins in yeast, including the addition of an optimized Kozak consensus sequence [18,19] that was engineered to encompass the ATG start codon and was inserted into pYES2 immediately downstream of, and thus under the control of, the Gal1 promoter. pYN48Q [a plasmid containing a mutant construct in which Asn-48 of hENT1 had been changed to Gln by site-directed mutagenesis (M. Sundaram, S. Y. M. Yao, E. Chomey, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work)] was obtained by PCR amplification of the N48Q coding region using 5' *Kpn*I and 3' *Sph*I. The fidelity of both the wild-type and mutant constructs was confirmed by sequencing the cDNA species and the regions of the plasmids that included the cloning sites.

### Strains and media

KY114 (MAT $\alpha$ , gal, ura3-52, trp1, lys2, ade2, his d2000) was the parental yeast strain used to generate KTK, a strain that expresses herpes simplex thymidine kinase [17]. Other strains were generated by transformation of the yeast/*Escherichia coli* shuttle vector pYES2 (Invitrogen, Carlsbad, CA, U.S.A.) into strain KTK by using a standard lithium acetate method [20]. The cDNA inserts were under transcriptional control of the inducible GAL1 promoter, in which the GAL1 promoter was induced or repressed by using galactose or glucose respectively as the carbon source. Yeast strains were maintained in complete minimal medium (CMM) containing 0.67% yeast nitrogen base (Difco, Detroit, MI, U.S.A.), lysine, adenine, histidine and either 2% (w/v) glucose (CMM/GLU) or 2% (w/v) galactose (CMM/GAL). Strain KTK was maintained in CMM that was also supplemented with uracil. Agar plates contained CMM with various supplements and 2% (w/v) agar (Difco). Plasmids were propagated in the *E. coli* strain TOP10F' (Invitrogen) and maintained in Luria broth [21] with ampicillin (50  $\mu$ g/ml).

### Phenotypic complementation assay

The complementation assay used to detect the presence of functional recombinant hENT1 was based on the ability of an introduced cDNA to rescue yeast defective in thymidine transport from dTMP starvation when cultured in the presence of exogenously supplied thymidine [17]. In brief, yeast cells grown in CMM/GLU were harvested and transferred to CMM/GAL; after an additional 8–10 h they were transferred to plates with either CMM/GLU or CMM/GAL that also contained sulphanimide (SAA) at 6 mg/ml and methotrexate (MTX) at 50  $\mu$ g/ml (CMM/GLU/MTX/SAA and CMM/GAL/MTX/SAA respectively) in the absence or presence of different concentrations of thymidine. Plates were incubated at 30 °C for 3.5 days and then assessed for colony formation. In some cases the plating media were supplemented with test compounds for an assessment of their ability to prevent complementation.

### RNA isolation and Northern analysis

Total RNA was isolated by the hot acid/phenol method [22] from proliferating yeast grown in (1) CMM/GLU, (2) CMM/GAL or (3) CMM/GAL/MTX/SAA that also contained various concentrations of thymidine. Total RNA (10  $\mu$ g) was resolved electrophoretically on a denaturing 1.0% (w/v) agarose/

formaldehyde gel and transferred to Hybond-N membranes (Amersham, Oakville, ON, Canada).  $^{32}\text{P}$ -probes were prepared by labelling a cDNA fragment (nt 1–648 of the hENT1-coding region) with the use of a T7 Quick Prime Kit (Pharmacia, Dorval, PQ, Canada). Membranes were incubated with  $^{32}\text{P}$ -probes and then washed twice under high-stringency conditions. Hybridization complexes were detected by autoradiography or phosphorimaging with a FUJIX Bio-Imaging Analyzer Bas 1000 (DNA Sequencing Laboratory, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada).

### Production of hENT1 polyclonal antibodies

Polyclonal antibodies against hENT1 were produced in rabbits against a synthetic peptide, corresponding to residues 254–272 of the predicted hENT1 protein sequence, and conjugated via an additional C-terminal Cys residue to ovalbumin. Peptide-specific antibodies were affinity-purified from the resultant serum by chromatography with peptide immobilized by its Cys residue to iodoacetyl agarose (SulfoLink gel; Pierce, Rockford, IL, U.S.A.).

### Preparation of yeast membranes

Yeast cells with either pYhENT1 or pYES2 were grown in CMM/GAL/MTX/SAA containing 40  $\mu\text{M}$  or 1 mM thymidine respectively to a  $D_{600}$  of 0.8–1.5. Cells were collected by centrifugation at 500  $g$  for 5 min, washed three times with breaking buffer [10 mM Tris/HCl (pH 7.5)/0.2 mM EDTA/0.2 mM dithiothreitol] containing protease inhibitors (Complete Protease Inhibitors; Boehringer Mannheim, Laval, PQ, Canada), and lysed by vortex-mixing in the presence of glass beads (425–600  $\mu\text{m}$ ; Sigma, Mississauga, ON, Canada) for 15 min. Unbroken cells and the glass beads were removed from the lysate by centrifugation at 500  $g$  for 5 min, and the membrane fractions were obtained by centrifugation of lysates at 120000  $g$  for 60 min. The resulting membrane pellets were resuspended in breaking buffer that contained protease inhibitors. The samples were either used immediately or frozen at  $-80^\circ\text{C}$  in breaking buffer containing 15% (v/v) DMSO.

### Electrophoresis and immunoblotting

SDS/PAGE was performed [23] with membranes, prepared as described above, that had been solubilized in 4 M urea, after which proteins were transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA, U.S.A.). The resulting membranes were subjected to the following treatments at  $4^\circ\text{C}$ : (1) incubation in TTBS (Tris-buffered saline/0.2% Tween 20) containing 5% (w/v) skimmed milk powder, (2) incubation in TTBS with primary antibodies (see above) and 1% (w/v) skim milk powder, (3) washing with TTBS, (4) incubation with TTBS containing horseradish peroxidase-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) and 1% (w/v) skimmed milk powder, (5) washing with TTBS, and (6) detection by enhanced chemiluminescence (ECL<sup>®</sup>; Amersham) and autoradiography.

### Thymidine uptake in *S. cerevisiae*

The uptake of [ $^3\text{H}$ ]thymidine by exponentially proliferating yeast was measured by using the 'oil stop' method as described for cultured cells [16,24], with the following modifications. Yeast cells were grown in CMM/GAL/MTX/SAA containing thymidine to a  $D_{600}$  of 0.8–1.5, washed three times with CMM/GAL, then resuspended in CMM/GAL at a concentration of  $6 \times 10^7$  cells/ml. Uptake was initiated by the addition of [ $^3\text{H}$ ]thymidine to yeast cultures. Triplicate 250  $\mu\text{l}$  portions of the

cultures were removed at regular intervals and uptake was terminated immediately by centrifugation of the yeast cells at 12000  $g$  for 2 min through oil. The oil was removed by aspiration and the pellets were solubilized with 5% (v/v) Triton X-100 for determination of the radioactive content by scintillation counting.

### [ $^3\text{H}$ ]NBMPR binding

Intact yeast cells or total membrane fractions prepared from yeast as described above were assessed for their ability to bind [ $^3\text{H}$ ]NBMPR (Moravek Biochemicals, Brea, CA, U.S.A.) by using a filtration assay described previously [25], with the following modifications. Yeast cells with pYhENT1, pYN48Q or pYES2 were grown in CMM/GAL/MTX/SAA containing 40  $\mu\text{M}$  thymidine (pYhENT1, pYN48Q) or 1 mM thymidine (pYES2) to a  $D_{600}$  of 0.8–1.5. Cells were washed three times in binding buffer [100 mM KCl/10 mM Tris/HCl (pH 7.4)/0.1 mM  $\text{MgCl}_2$ /1 mM  $\text{CaCl}_2$ ] and resuspended in binding buffer at  $3 \times 10^7$  cells/ml. Cell concentrations were determined with an electronic particle counter. Duplicate samples ( $2.25 \times 10^6$  cells per assay; 15  $\mu\text{g}$  of protein per assay) were incubated at  $22^\circ\text{C}$  for 40 min with graded concentrations of [ $^3\text{H}$ ]NBMPR (0.12–24 nM) in binding buffer in the absence or presence of 10  $\mu\text{M}$  dilazep [a gift from F. Hoffman La Roche (Basel, Switzerland)]. Yeast cells were collected on Whatman GF/B filters (Whatman, Springfield Mill, KY, U.S.A.) under vacuum and the filters were washed four times with binding buffer. The radioactive content was measured by scintillation counting. The amount of [ $^3\text{H}$ ]NBMPR that bound specifically was calculated as the difference between the amount of [ $^3\text{H}$ ]NBMPR that bound in the absence of 10  $\mu\text{M}$  dilazep and the amount that bound in its presence. Binding of [ $^3\text{H}$ ]NBMPR to yeast membrane fractions was determined by the same procedure (with 20  $\mu\text{g}$  of protein per assay). The inhibition of [ $^3\text{H}$ ]NBMPR binding by dilazep or dipyrindamole was measured by preincubating the assay mixtures for 10 min with graded concentrations of dilazep or dipyrindamole before the addition of [ $^3\text{H}$ ]NBMPR. The apparent inhibition constants ( $K_i$  values) were calculated by the method of Cheng and Prusoff [26] from the observed  $\text{IC}_{50}$  values.

### Kinetics of dissociation and association of [ $^3\text{H}$ ]NBMPR

Intact yeast cells with pYhENT1 or pYN48Q (grown in CMM/GAL/MTX/SAA with 40  $\mu\text{M}$  thymidine) were equilibrated with 2.5 nM [ $^3\text{H}$ ]NBMPR for 40 min at  $22^\circ\text{C}$ . The dissociation process was initiated by introducing a  $10^4$ -fold excess of unlabelled NBMPR to yeast cells such that the dissociated [ $^3\text{H}$ ]NBMPR molecules were diluted by unlabelled NBMPR, thereby preventing any reassociation of [ $^3\text{H}$ ]NBMPR. Portions of the assay mixtures were removed at regular intervals and the yeast cells were collected on Whatman GF/B filters under vacuum. Filters were washed twice with binding buffer and the radioactive content was measured by scintillation counting. The dissociation rate constants were calculated by using a non-linear one-phase exponential decay equation. The association process was assessed by the addition of 2.5 nM [ $^3\text{H}$ ]NBMPR to yeast cells. Portions of the assay mixtures were removed at regular intervals and the yeast cells were collected on Whatman GF/B filters under vacuum and washed twice with binding buffer; the radioactive content was measured by scintillation counting. The association rate constants were calculated by using a non-linear one-phase exponential association equation.

### Reconstitution of hENT1-mediated thymidine transport into proteoliposomes

Reconstitution of functional recombinant hENT1 into proteoliposomes was performed by an adaptation of a procedure used previously for the reconstitution of *es* transporters from mammalian cells [27–29]. Membranes prepared from yeast cells with pYhENT1 (grown in CMM/GAL/MTX/SAA with 40  $\mu$ M thymidine) were solubilized in binding buffer containing 1.0% (w/v) octylglucoside (Sigma) and 0.15% (w/v) aolectin for 1 h at 4 °C with mixing. Solubilized membranes (200  $\mu$ g/ml protein) were supplemented with sonicated lipids (Avanti Polar Lipids, Alabaster, AL, U.S.A.) consisting of phosphatidylcholine (bovine brain), cholesterol, phosphatidylethanolamine (egg) and phosphatidylserine (bovine brain) at a molar ratio of 33:33:26:8 and a trace amount ( $10^5$  d.p.m./ml) of [ $^{14}$ C]cholesteryl oleate (Amersham). The removal of octylglucoside and formation of proteoliposomes was performed by gel filtration (Sephadex G-50 medium, 1.5 cm  $\times$  38 cm column) of the lipid/protein/detergent mixtures at a flow rate of 1 ml/min. Proteoliposomes were collected in the void volume and were either used immediately or frozen in ethanol/solid CO<sub>2</sub> and stored at –80 °C. The uptake of [ $^3$ H]thymidine into proteoliposomes was initiated by the addition of 100  $\mu$ l portions of proteoliposome suspension (approx. 5  $\mu$ g of protein, with or without inhibitors) to 25  $\mu$ l of [ $^3$ H]thymidine prepared in binding buffer. Uptake was terminated by centrifuging the proteoliposome mixture through Sephadex G-50 fine minicolumns equilibrated with a mixture of transport inhibitors [10 mM adenosine/10  $\mu$ M dipyridamole/10  $\mu$ M nitrobenzylthioguanosine (NBTGR) prepared in binding buffer]. The eluate was collected and the protein,  $^{14}$ C and  $^3$ H contents were determined. Results were normalized by using [ $^{14}$ C]cholesteryl oleate as an internal standard to quantify the lipid content of proteoliposomes. The mediated uptake of [ $^3$ H]thymidine was calculated from the difference between total uptake and non-mediated uptake, which was measured in the presence of 10 mM adenosine, 10  $\mu$ M dipyridamole and 10  $\mu$ M NBTGR. The effect of NBMPR on [ $^3$ H]thymidine uptake was determined by first preincubating the samples for 10 min with graded concentrations

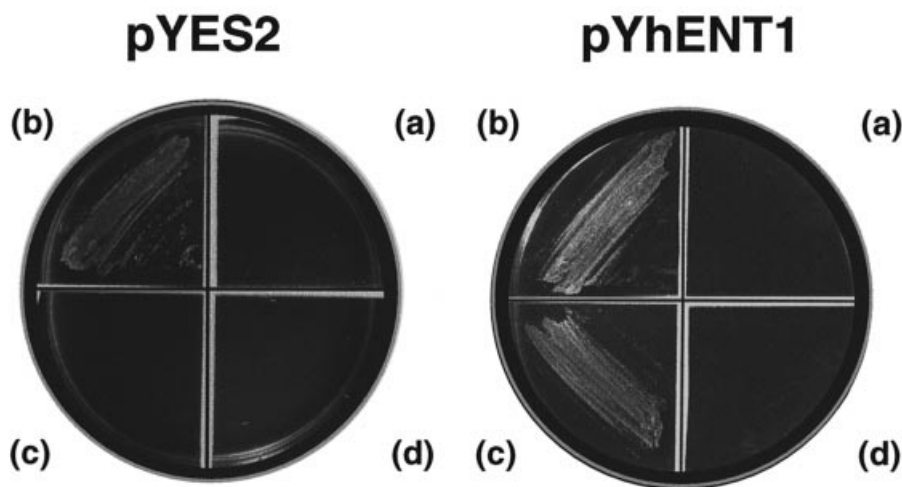
of NBMPR prepared in binding buffer, after which [ $^3$ H]thymidine was added to assay mixtures as described above.

### RESULTS

#### pYhENT1 complementation of a thymidine transport deficiency in yeast

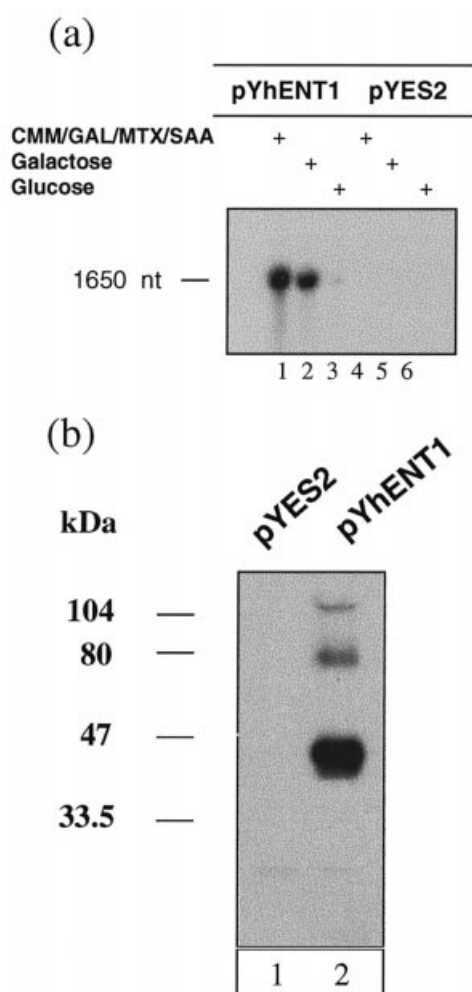
In *S. cerevisiae*, the inhibition of thymidylate synthase by SAA and MTX results in the depletion of dTMP pools and the inhibition of growth [17,30]. This MXT/SAA-imposed growth arrest can be overcome by supplying low concentrations of thymidine to the growth medium if the yeast cells acquire the capacity to both transport and phosphorylate thymidine. Although the strain (KTK) used in this study was engineered to contain herpes simplex thymidine kinase, it cannot transport thymidine and thus will not grow in MTX/SAA-containing media that lack thymidine or have low concentrations (less than 200  $\mu$ M) of thymidine, but will grow in media with thymidine at high concentrations (at least 1 mM) because of entry by passive diffusion.

The experiments shown in Figure 1 established that expression of the hENT1 cDNA rescued KTK cells from drug-imposed depletion of dTMP. Yeast cells with pYhENT1 or pYES2 were grown under conditions of thymidylate starvation in the presence of either galactose (inducer) or glucose (repressor) with various concentrations of thymidine. pYES2-containing and pYhENT1-containing yeast cells were unable to form colonies in thymidine-free medium (Figure 1a, negative control for growth) but did so in the presence of 1 mM thymidine (Figure 1b, positive control for growth). pYhENT1-containing yeast cells also formed colonies in the presence of 10  $\mu$ M thymidine but only when plated on medium with MTX/SAA that was supplemented with galactose, the inducer required for efficient transcription of the cDNA insert in the pYES2 vector (Figure 1c). There was no growth when pYhENT1-containing yeast cells were plated on medium with MTX/SAA that was supplemented with glucose, the repressor used to block transcription of the hENT1 insert (Figure 1d). These results indicated that expression of the cDNA encoding



**Figure 1** Functional complementation of a thymidine transport deficiency in yeast by recombinant hENT1

Yeast cells containing either pYES2 or pYhENT1 were streaked on CMM/GAL/MTX/SAA medium (inducing conditions, thymidylate starvation) that contained 0  $\mu$ M thymidine (a), 1 mM thymidine (b) or 10  $\mu$ M thymidine (c). Yeast cells were also streaked on CMM/GLU/MXT/SAA medium (repressive conditions, thymidylate starvation) that contained 10  $\mu$ M thymidine (d). Growth on plates was assessed after 3.5 days.



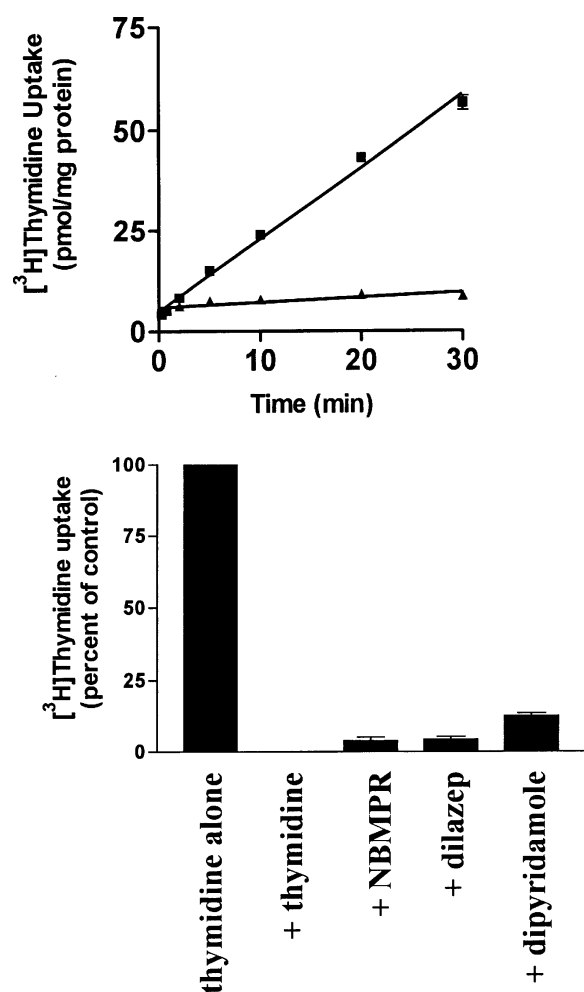
**Figure 2** Production of hENT1 mRNA and recombinant protein in yeast

(a) Total RNA from yeast with pYhENT1 (lanes 1–3) or pYES2 (lanes 4–6) was subjected to Northern analysis with a  $^{32}\text{P}$ -labelled hENT1 cDNA fragment and then detected by autoradiography. Growth conditions were as follows: CMM/GAL/MTX/SAA medium that contained either 40  $\mu\text{M}$  thymidine (lane 1) or 1 mM thymidine (lane 4); CMM/GAL medium (lanes 2 and 5); CMM/GLU medium (lanes 3 and 6). (b) Membranes, prepared as described in the Experimental section, from yeast with pYES2 (lane 1) or pYhENT1 (lane 2) grown in CMM/GAL/MTX/SAA medium that contained 1 mM or 40  $\mu\text{M}$  thymidine respectively, were subjected to immunoblotting with hENT1-specific polyclonal antibodies. The positions of molecular mass markers are indicated (in kDa) at the left.

hENT1 was required to complement the growth arrest imposed by thymidylate starvation and were consistent with the acquisition of thymidine-transport capability resulting from growth in the presence of the inducer (galactose).

#### Production of hENT1 mRNA and protein

Northern analysis was performed to determine whether mRNA corresponding to hENT1 cDNA was present in pYhENT1-containing yeast (Figure 2a). RNA samples were prepared from yeast cells with either pYhENT1 or pYES2 that were cultured (1) in the presence of drugs in conditions under which extracellular thymidine could be salvaged for growth (CMM/GAL/MTX/SAA plus 40  $\mu\text{M}$  or 1 mM thymidine respectively) or (2) in the absence of drugs under conditions that either induced or repressed transcription of the cDNA insert. A single band of 1650 nt was

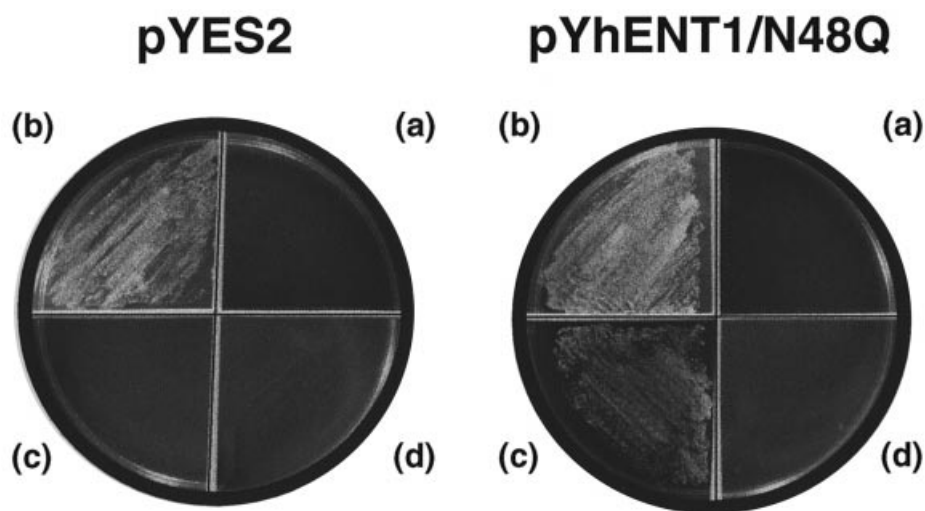


**Figure 3** Cellular uptake of  $[^3\text{H}]$ thymidine by yeast producing recombinant hENT1

Upper panel: uptake of 1.0  $\mu\text{M}$   $[^3\text{H}]$ thymidine was measured, as a function of time, into yeast with pYhENT1 (■) or pYES2 (▲) grown in CMM/GAL/MTX/SAA medium (inducing conditions) that contained respectively 40  $\mu\text{M}$  or 1 mM thymidine. Lower panel: yeast cells with pYhENT1 or pYES2 were grown as described for the upper panel. Cells were incubated for 20 min with 1.0  $\mu\text{M}$   $[^3\text{H}]$ thymidine alone (100% of uptake) or in the presence of either 10 mM thymidine, 1  $\mu\text{M}$  NBMPR, 10  $\mu\text{M}$  dilazep or 10  $\mu\text{M}$  dipyrindamole. The 'background' quantity of  $[^3\text{H}]$ thymidine adsorption on pYES2-containing yeast was subtracted from the 'stimulated' quantity observed for pYhENT1-containing yeast. Results are means  $\pm$  S.D. for triplicate determinations of the cellular content of thymidine equivalents (i.e. thymidine and its metabolites). Error bars are not shown where values were smaller than that represented by the symbols. Two separate experiments gave similar results.

detected in pYhENT1-containing yeast grown in medium with galactose and to a smaller extent in yeast grown in medium with glucose. No signal was present in the pYES2-containing cells under any of the experimental conditions. These results, which demonstrated the presence of hENT1 mRNA in cells with pYhENT1, indicated enhanced transcription of hENT1 cDNA by activation of the Gal1 promoter.

Western analysis was performed to confirm the presence of recombinant hENT1 protein in pYhENT1-containing yeast (Figure 2b). Yeast cells with either pYhENT1 or pYES2 were subjected to thymidylate starvation in the presence of galactose (inducing conditions) and thymidine (40  $\mu\text{M}$  or 1 mM respectively) that permitted the yeast (pYhENT1-containing or pYES2-containing) to grow at approximately the same rates. Im-



**Figure 4** Functional complementation of a thymidine transport deficiency in yeast by recombinant hENT1/N48Q

Yeast cells containing either pYES2 or pYN48Q were streaked on CMM/GAL/MTX/SAA medium (inducing conditions, thymidylate starvation) that contained 0  $\mu$ M thymidine (a), 1 mM thymidine (b) or 10  $\mu$ M thymidine (c). Yeast cells were also streaked on CMM/GLU/MXT/SAA medium (repressive conditions, thymidylate starvation) that contained 10  $\mu$ M thymidine (d). Growth on plates was assessed after 3.5 days.

munoblotting with an anti-hENT1 polyclonal antibody directed against a synthetic peptide epitope (hENT1 residues 254–272) demonstrated the presence of immunoreactive material in membranes of pYhENT1-containing yeast that was not present in membranes of pYES2-containing yeast. The predominant immunoreactive species was observed as a broad band of 40–45 kDa and two minor reactive species of 80 and 120 kDa. The predicted molecular mass of non-glycosylated hENT1 is 50.2 kDa [12].

#### Effects of inhibitors of nucleoside transport on hENT1-dependent thymidine rescue

NBMPR, dilazep and dipyridamole have been used as pharmacological probes in the identification of *es*-mediated processes [3,4]. hENT1-dependent complementation in yeast was assessed in the presence of these classic *es* inhibitors (results not shown). Yeast cells with either pYhENT1 or pYES2 were tested under inducing conditions in medium with MTX/SAA alone or medium that also contained one of the three *es* inhibitors. Each of the inhibitors blocked hENT1-dependent colony formation in the presence of a low concentration (10  $\mu$ M) of thymidine but had no effect on colony formation in the presence of a high concentration (1 mM) of thymidine. The ability of the *es* inhibitors to block the thymidine rescue of pYhENT1-containing yeast at low thymidine concentrations indicated the production of recombinant hENT1 that had retained its ability to recognize, and be inhibited by, NBMPR, dilazep and dipyridamole.

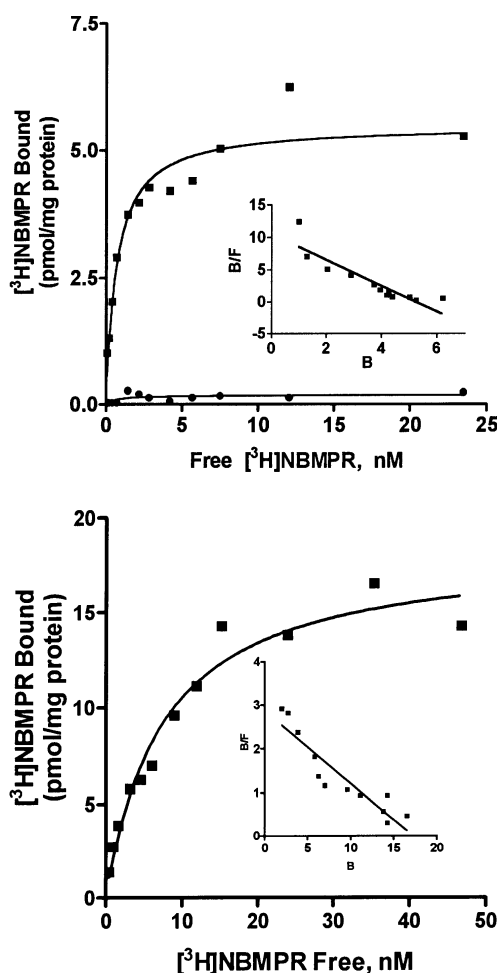
#### Yeast cells with hENT1 exhibit increased cellular uptake of [<sup>3</sup>H]thymidine

The uptake of 1.0  $\mu$ M [<sup>3</sup>H]thymidine was measured in yeast with either pYhENT1 or pYES2 that had been cultured in MTX/SAA-containing medium with the inducer and a thymidine supplement (40  $\mu$ M or 1 mM respectively) to allow growth (Figure 3). Thymidine uptake by pYhENT1-containing yeast was greater than by pYES2-containing yeast (Figure 3, upper panel) and was inhibited by the addition of an excess of unlabelled thymidine (Figure 3, lower panel). The addition of any one of the

*es* transport inhibitors (NBMPR, dilazep or dipyridamole) decreased thymidine uptake by hYhENT1-containing yeast to levels only slightly above those observed with pYES2-containing yeast (Figure 3, lower panel). These results indicated that the expression of pYhENT1 was accompanied by the acquisition of the capacity for cellular uptake of thymidine by a process that was mediated and inhibitor-sensitive, a further indication that pYhENT1-containing yeast, when grown in the presence of galactose, produced functional recombinant hENT1.

#### pYhENT1/N48Q complementation of a deficiency in thymidine transport in yeast

hENT1 is predicted to contain a single N-linked glycosylation site in the large extracellular loop between transmembrane domains 1 and 2 at Asn-48 [12]. The role of N-linked glycosylation in the biosynthetic processing and function of hENT1 is not known. A cDNA in which the N-linked glycosylation site of hENT1 (Asn-48) was converted to Gln by site-directed mutagenesis (M. Sundaram, S. Y. M. Yao, E. Chomey, C. E. Cass, S. A. Baldwin and J. D. Young, unpublished work) was cloned into the pYES2 expression vector to generate pYN48Q, which was then functionally expressed in yeast. When yeast cells with either pYN48Q or pYES2 were exposed to MTX/SAA in the presence of galactose (inducer) or glucose (repressor), pYN48Q-containing and pYES2-containing yeast formed colonies in the presence of 1 mM thymidine (Figure 4b) but not in thymidine-free medium (Figure 4a). Colonies were also formed by pYN48Q-containing yeast in the presence of galactose and 10  $\mu$ M thymidine (Figure 4c), whereas none were observed in the presence of glucose and 10  $\mu$ M thymidine (Figure 4d). Yeast cells with pYN48Q or pYES2 were also tested under inducing conditions in medium containing MTX/SAA and 10  $\mu$ M thymidine alone or with 1  $\mu$ M NBMPR, 10  $\mu$ M dilazep or dipyridamole. Although colonies were observed when pYN48Q-containing yeast cells were plated in medium containing thymidine, none were observed in the presence of the inhibitors (results not shown). Thus the expression of pYN48Q cDNA in yeast complemented the growth



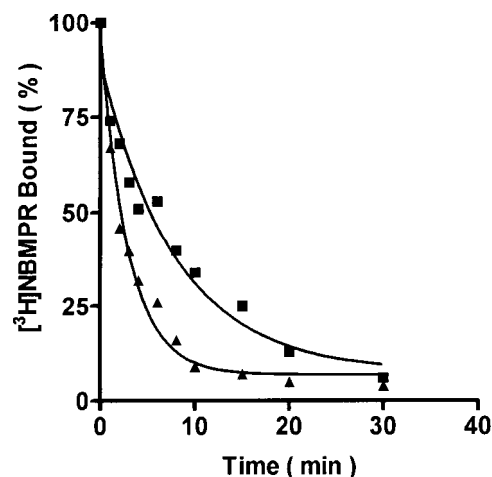
**Figure 5** Equilibrium binding of [ $^3\text{H}$ ]NBMPR to yeast producing either recombinant hENT1 or hENT1/N48Q

Upper panel: equilibrium binding of [ $^3\text{H}$ ]NBMPR (0.12 nM–0.24 nM) to yeast harbouring pYhENT1 (■) or pYES2 (●) grown in CMM/GAL/MTX/SAA medium (inducing conditions) that contained 40  $\mu\text{M}$  or 1 mM thymidine respectively. Results are presented as the amounts of specifically bound [ $^3\text{H}$ ]NBMPR as a function of the free [ $^3\text{H}$ ]NBMPR. Each point is the average of duplicate determinations (individual values differed from the value shown by 11% at most). Three experiments gave similar results, yielding a  $B_{\text{max}}$  of  $5.0 \pm 0.5$  pmol/mg of protein and a  $K_d$  of  $1.2 \pm 0.2$  nM (means  $\pm$  S.D.). Inset to upper panel: Scatchard analysis of specific binding of [ $^3\text{H}$ ]NBMPR to pYhENT1-containing yeast. Lower panel: equilibrium binding of [ $^3\text{H}$ ]NBMPR to pYN48Q-containing yeast was performed as described for the upper panel. Each point is the average of duplicate determinations (individual values differed from the value shown by 13% at most). Six experiments gave similar results, yielding a  $B_{\text{max}}$  of  $15 \pm 1.1$  pmol/mg of protein and a  $K_d$  of  $10.5 \pm 1.6$  nM (means). Inset to lower panel: Scatchard analysis of specific binding of [ $^3\text{H}$ ]NBMPR to pYN48Q-producing yeast.

arrest imposed by thymidylate starvation, by enabling thymidine rescue by a process that was blocked by the *es* inhibitors. These results indicated that the N-glycosylation mutant had retained its capacity for inhibitor-sensitive thymidine transport. They also demonstrated that glycosylation at Asn-48 was not required for the production of functional hENT1 in yeast.

#### Binding of [ $^3\text{H}$ ]NBMPR to hENT1-producing and hENT1/N48Q-producing yeast

Equilibrium binding studies with human erythrocytes and membrane vesicles have yielded values for binding-site numbers ( $B_{\text{max}}$ ) and affinities ( $K_d$ ) that represent the interaction of

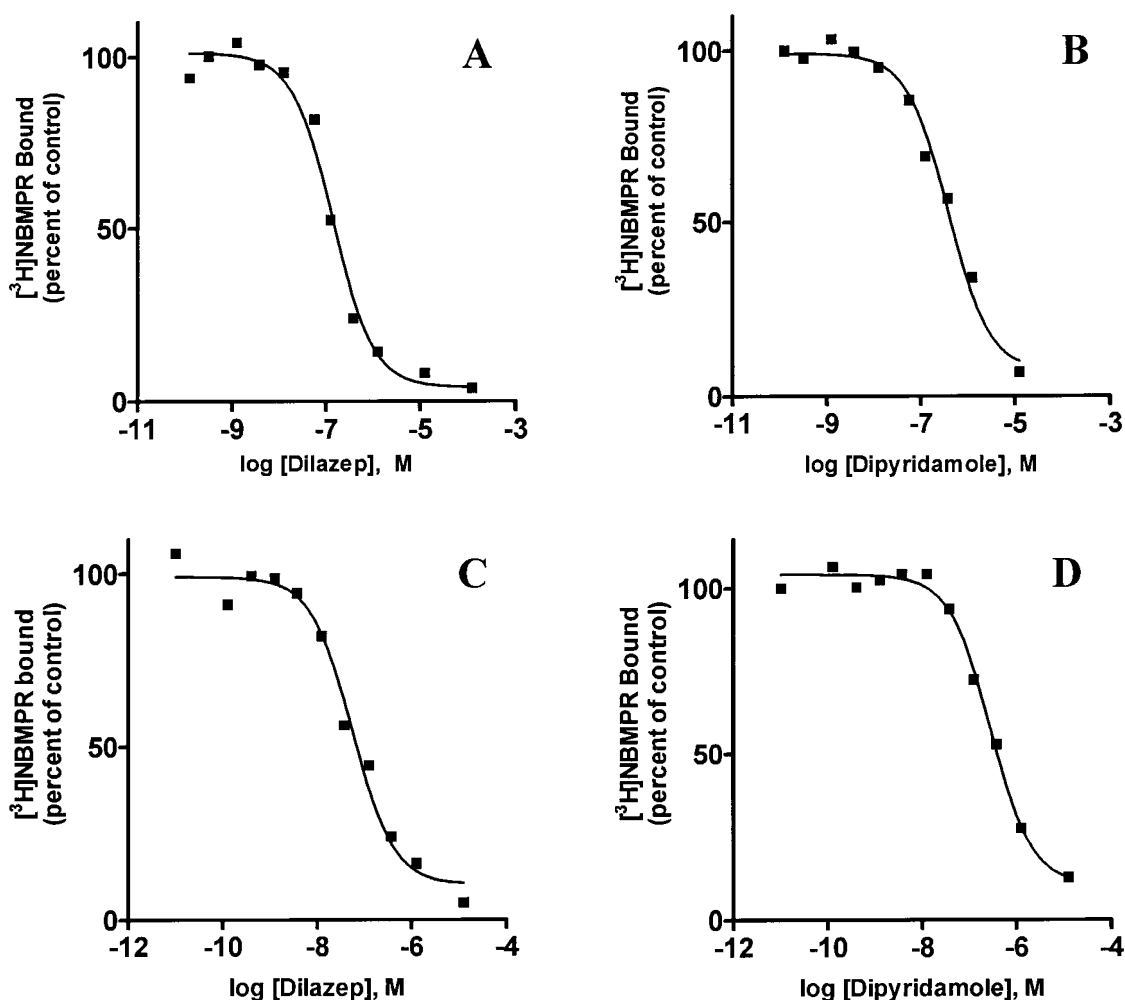


**Figure 6** Dissociation of [ $^3\text{H}$ ]NBMPR from yeast producing either recombinant hENT1 or hENT1/N48Q

Yeast cells containing pYhENT1 (■) or pYN48Q (▲), that had been grown in CMM/GALMTX/SAA medium containing 40  $\mu\text{M}$  thymidine, were incubated in the presence of 2.5 nM [ $^3\text{H}$ ]NBMPR and allowed to reach equilibrium. Excess (5  $\mu\text{M}$ ) unlabelled NBMPR was then added to the cells and the amount of [ $^3\text{H}$ ]NBMPR that remained bound was measured. Specific binding of [ $^3\text{H}$ ]NBMPR is shown as a function of time. Values for dissociation rate constants were estimated with a non-linear one-phase exponential decay equation. Each point is the average of duplicate determinations; individual values differed from the value shown by 6% at most (pYhENT1) or less than 5% (pYN48Q). Three experiments gave similar results for the apparent rate of dissociation for hENT1 and N48Q, yielding means  $\pm$  S. D. of  $0.14 \pm 0.02$  and  $0.36 \pm 0.05$   $\text{min}^{-1}$  respectively.

[ $^3\text{H}$ ]NBMPR with the *es* transporter protein [3], now known to be hENT1 [1,12]. Equilibrium binding of [ $^3\text{H}$ ]NBMPR to pYhENT1-containing yeast was examined under conditions similar to those used previously in studies with mammalian cells [16]. Yeast cells were incubated with 8.0 nM [ $^3\text{H}$ ]NBMPR alone or in the presence of 10  $\mu\text{M}$  dilazep, a tight-binding competitive inhibitor of *es*-mediated transport that is commonly used to distinguish between specific and non-specific binding of NBMPR to the nucleoside recognition site of the transporter. Equilibrium was reached within 20–30 min of exposure of pYhENT1-containing yeast to [ $^3\text{H}$ ]NBMPR (results not shown). In subsequent binding experiments, cells (or membrane vesicles) were incubated with [ $^3\text{H}$ ]NBMPR for 40 min to ensure equilibrium between free and bound ligand.

The presence of high levels of dilazep-sensitive binding of [ $^3\text{H}$ ]NBMPR to pYhENT1-containing yeast, when grown under inducing conditions, led to the use of reversible binding of NBMPR under equilibrium conditions to quantify the functional recombinant transporter in yeast. Studies with the purified *es* transporter of human erythrocytes [31] suggested that the transporter (a heterogeneously glycosylated polypeptide of 45–55 kDa) binds NBMPR with high affinity ( $K_d \leq 1.0$  nM) and a 1:1 stoichiometry. In the experiments shown in Figure 5 (upper panel), equilibrium binding was determined at graded concentrations (0.12–24 nM) of [ $^3\text{H}$ ]NBMPR with intact pYhENT1-containing or pYES2-containing yeast cells that were grown in liquid cultures in medium with MTX/SAA, galactose and either 40  $\mu\text{M}$  (pYhENT1) or 1 mM (pYES2) thymidine. The amount of [ $^3\text{H}$ ]NBMPR specifically bound was determined by conducting binding assays in the presence and in the absence of excess dilazep (results not shown). Dilazep-sensitive binding of [ $^3\text{H}$ ]NBMPR to pYhENT1-containing yeast was saturable with a  $B_{\text{max}}$  of  $5.0 \pm 0.5$  pmol/mg of protein and a  $K_d$  of  $1.2 \pm 0.2$  nM



**Figure 7** Inhibition by dilazep and by dipyridamole of [ $^3\text{H}$ ]NBMPR binding to yeast producing either recombinant hENT1 or hENT1/N48Q

Yeast cells containing pYhENT1 or pYN48Q were grown in CMM/GAL/MTX/SAA medium that contained 40  $\mu\text{M}$  thymidine. The pYhENT1-containing yeast cells were incubated with 0.5 nM [ $^3\text{H}$ ]NBMPR alone or together with graded concentrations (0.125 nM to 12  $\mu\text{M}$ ) of either dilazep (**A**) or dipyridamole (**B**); the pYN48Q-containing yeast cells were incubated with 6 nM [ $^3\text{H}$ ]NBMPR alone or with graded concentrations of dilazep (**C**) or dipyridamole (**D**). The concentrations of [ $^3\text{H}$ ]NBMPR differed between pYhENT1-containing and pYN48Q-containing yeast because of the differences in  $K_d$  values for NBMPR binding. Results are presented as the percentages of [ $^3\text{H}$ ]NBMPR bound as a function of the logarithm of the concentration of dilazep or dipyridamole. The amount of [ $^3\text{H}$ ]NBMPR that bound in the absence of additives was taken as 100% binding. Each point is the average of duplicate determinations; individual values differed from the value shown by 12% at most. Three experiments gave similar results, yielding  $\text{IC}_{50}$  values for dilazep and dipyridamole inhibition of NBMPR binding to hENT1 of  $130 \pm 10$  and  $380 \pm 20$  nM respectively, and to hENT1/N48Q of  $50 \pm 5$  and  $230 \pm 3$  nM respectively (means  $\pm$  S.D.).

(means  $\pm$  S.D.). There was no [ $^3\text{H}$ ]NBMPR binding to yeast with pYES2. These results demonstrated that the expression of pYhENT1 in yeast was accompanied by the acquisition of NBMPR-binding activity, evidently the consequence of functionally active, recombinant hENT1 with an affinity for NBMPR that was similar to those of the native *es* transporters of erythrocytes and cultured cells [3,4]. Thus, if we assume a 1:1 stoichiometry between NBMPR binding and recombinant hENT1, there were approx.  $10^4$  functionally active molecules per cell.

The ability of pYN48Q, which encodes a hENT1 variant lacking the capacity for N-glycosylation, to complement the thymidine-transport defect of yeast phenotypically under selection conditions (see Figure 4) suggested that functionally active hENT1/N48Q was produced and processed to the plasma membrane. Equilibrium binding of [ $^3\text{H}$ ]NBMPR to pYN48Q-containing yeast was examined (Figure 5, lower panel) under conditions identical with those used with pYhENT1-containing

yeast (Figure 5, upper panel). NBMPR binding was saturable, with a  $K_d$  of  $10.5 \pm 1.6$  nM and a  $B_{\text{max}}$  of  $15.0 \pm 1.1$  pmol/mg of protein. The 10-fold increase in  $K_d$  for yeast with the mutant protein, relative to that for yeast with the wild-type protein, suggested that the conversion of Asn into Gln induced a structural change in the vicinity of the NBMPR-binding site that lowered the affinity for NBMPR. The 3-fold increase in  $B_{\text{max}}$  in hENT1/N48Q-producing yeast, relative to that of hENT1-producing yeast, suggested that the quantity of functionally active (with respect to NBMPR binding) mutant hENT1 was greater than that observed with wild-type hENT1.

#### Dissociation and association of [ $^3\text{H}$ ]NBMPR from hENT1-producing and hENT1/N48Q-producing yeast

The rates of dissociation of [ $^3\text{H}$ ]NBMPR from intact pYhENT1-containing and pYN48Q-containing yeast (grown under selection conditions) were determined (Figure 6) to investigate the basis of



the decreased affinity of [ $^3$ H]NBMPR for hENT1/N48Q. The apparent dissociation rate constants for yeast producing either recombinant hENT1 or recombinant hENT1/N48Q were  $0.14 \pm 0.02$  and  $0.36 \pm 0.05 \text{ min}^{-1}$  respectively. It therefore seems that the conformational changes resulting from the conversion of Asn-48 into Gln and/or the absence of the N-linked oligosaccharide led to an accelerated dissociation of [ $^3$ H]NBMPR from its binding site, resulting in a weaker binding of NBMPR by recombinant hENT1/N48Q than by recombinant hENT1. This conformational change is also reflected in changes in the apparent association rate constants (results not shown).  $K_{\text{on}}$  values for hENT1 and hENT1/N48Q were determined as  $(1.20 \pm 0.1) \times 10^8$  and  $(0.40 \pm 0.04) \times 10^8 \text{ M}^{-1} \cdot \text{min}^{-1}$  respectively.

#### Inhibition of binding of [ $^3$ H]NBMPR to hENT1-producing and hENT1/N48Q-producing yeast by dilazep and dipyridamole

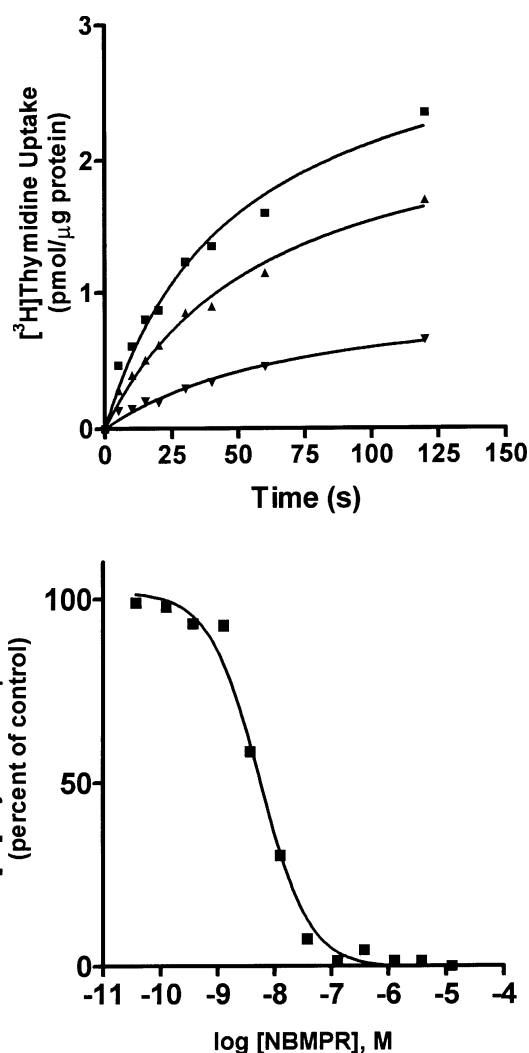
Dilazep and dipyridamole are potent inhibitors of NBMPR binding to mammalian cells, and their inhibition of *es*-mediated transport activity has been attributed to their interaction with sites that are the same as, or overlap with, the NBMPR-binding sites [32–34]. The apparent relative affinities of dilazep and dipyridamole for recombinant hENT1 and hENT1/N48Q were estimated by assessing their abilities to inhibit the binding of [ $^3$ H]NBMPR to pYhENT1-containing and pYN48Q-containing yeast (Figure 7). Dilazep and dipyridamole decreased [ $^3$ H]NBMPR binding to pYhENT1-containing yeast with  $\text{IC}_{50}$  values of  $130 \pm 10$  and  $380 \pm 20 \text{ nM}$  respectively, and to pYN48Q-containing yeast with  $\text{IC}_{50}$  values of  $50 \pm 5$  and  $230 \pm 3 \text{ nM}$  respectively. The apparent  $K_i$  values, estimated by the method of Cheng and Prusoff [26], for the inhibition of NBMPR binding to hENT1-producing and hENT1/N48Q-producing yeast were respectively 90 and 30 nM for dilazep, and 260 and 150 nM for dipyridamole. These results indicated that the conversion of Asn-48 into Gln in hENT1 resulted in conformational changes that increased the apparent affinity of the *es* transporter for dilazep and dipyridamole.

#### Binding of NBMPR to hENT1 in yeast membranes

Experiments were undertaken to confirm that the binding of NBMPR to pYhENT1-containing yeast represented the interaction with membrane-associated recombinant hENT1 (results not shown). Crude membranes, isolated from cells grown under inducing conditions, were incubated with graded concentrations of [ $^3$ H]NBMPR in the presence or absence of excess dilazep (for the detection of non-specific binding). A Scatchard analysis of the dilazep-sensitive (i.e. specific) component of binding indicated the interaction of [ $^3$ H]NBMPR with a single class of binding sites with a  $B_{\text{max}}$  of  $6.5 \pm 1.0 \text{ pmol/mg}$  of protein and a  $K_d$  of  $0.7 \pm 0.2 \text{ nM}$ . These results demonstrated that membranes prepared from pYhENT1-containing yeast possessed high-affinity NBMPR-binding sites in larger quantities than those observed in intact pYhENT1-containing yeast, suggesting that most of the NBMPR-binding sites were membrane-associated.

#### Reconstitution of thymidine transport activity into hENT1-containing proteoliposomes

The experiments shown in Figure 8 were undertaken to determine whether membrane-associated recombinant hENT1 could be solubilized and functionally reconstituted into proteoliposomes.



**Figure 8** Demonstration of recombinant hENT1 in yeast membranes by reconstitution of thymidine transport activity

Upper panel: crude membranes prepared from yeast with pYhENT1, as described for Figure 2, were solubilized with octylglucoside and reconstituted into liposomes. The uptake of  $20 \mu\text{M}$  [ $^3$ H]thymidine into proteoliposomes was measured for the indicated durations in the absence (■, total uptake) or presence (▲, non-mediated uptake) of a mixture of transport inhibitors ( $10 \text{ mM}$  adenosine,  $10 \mu\text{M}$  dipyridamole and  $10 \mu\text{M}$  NBTGR). hENT1-mediated uptake of [ $^3$ H]thymidine (▼) was determined by subtracting the total uptake from the non-mediated uptake. Each point is the average of duplicate determinations (individual values differed from the values shown by 8% at most). Two experiments gave similar results, yielding an average initial rate of thymidine uptake of  $7 \pm 1 \text{ pmol/s}$  per mg of protein. Lower panel: hENT1-containing liposomes were prepared as described for the upper panel and then incubated with  $20 \mu\text{M}$  [ $^3$ H]thymidine for 20 s in the presence or absence of graded concentrations of NBMPR ( $0.0375 \text{ nM}$  to  $12.5 \mu\text{M}$ ). Results are presented as the relative amounts of hENT1-mediated [ $^3$ H]thymidine uptake as a function of the concentration of NBMPR. hENT1-mediated [ $^3$ H]thymidine uptake in the absence of NBMPR was taken as 100% uptake. Each point is the average of duplicate determinations (individual values differed from the values shown by 9% at most). Two experiments gave similar results, yielding an average  $\text{IC}_{50}$  of  $5.5 \text{ nM}$  for the inhibition of thymidine uptake by NBMPR.

Membranes prepared from pYhENT1-containing yeast cells that were grown under inducing conditions were solubilized with octylglucoside. Exogenously supplied lipids were used to prepare proteoliposomes by a procedure used previously to reconstitute native *es* transporter from human and mouse cells [27–29]. The time course of uptake of  $20 \mu\text{M}$  [ $^3$ H]thymidine (Figure 8, upper

panel) was determined into proteoliposomes alone (total uptake) or in the presence of a mixture of nucleoside transport inhibitors (non-mediated uptake). The differences between the calculated initial rates of uptake were used to calculate the initial rate of transport ( $7 \pm 1$  pmol/s per mg of reconstituted protein). The concentration–effect relationship of the inhibition of [ $^3$ H]thymidine uptake into proteoliposomes by NBMPR (Figure 8, lower panel) yielded an  $IC_{50}$  of  $5.5 \pm 0.5$  nM. These results demonstrated that the functional reconstitution of thymidine transport activity from membranes of pYhENT1-expressing yeast could be achieved, and that the sensitivity of nucleoside transport by reconstituted recombinant hENT1 to inhibition by NBMPR was similar to that previously observed with erythrocytes and cultured cell lines [29,35,36].

## DISCUSSION

The availability of cDNA species encoding the ENT proteins of rat and human cells has allowed detailed kinetic studies of the recombinant transporters in oocytes of *Xenopus laevis* to be performed [9,12]. Cultured cells have also been used for the functional expression of hENT2 [14]. However, these heterologous expression systems have not produced sufficient quantities of recombinant protein to permit a detailed analysis of the physical characteristics of the recombinant transporter proteins. Several integral membrane proteins have been introduced into the yeast *S. cerevisiae* with the production of substantial quantities of functional recombinant protein [37–40]. We report here the first demonstration of functional expression of wild-type and mutant hENT1 cDNA in yeast. Although equilibrium binding analysis with [ $^3$ H]NBMPR has been used extensively for quantification of the *es* transporter in cells and membrane preparations, multiple *es* transporter isoforms with similar affinities for NBMPR would appear as a single transporter type in equilibrium binding studies. The functional expression of the hENT1 cDNA species in yeast permitted a detailed analysis of the binding of NBMPR to the recombinant transporter proteins in an *es*-null background, and identified differences in binding affinities between the wild-type and N-glycosylation-defective mutant transporter proteins.

Yeast cells lack the capacity for mediated uptake of extracellular thymidine [41], a feature that permits the expression of nucleoside transporter cDNA species by imposing a drug selection (MTX, SAA) that results in the depletion of thymidylate and in cell death, unless a mechanism for salvage of extracellular thymidine is present and functional in the recipient yeast [17,30]. We demonstrated that the expression of hENT1 cDNA in yeast subjected to thymidylate starvation was responsible for the observed growth of yeast when exogenous thymidine was supplied to the growth medium. Northern analysis confirmed that (1) hENT1 mRNA was present in pYhENT1-containing yeast but not pYES2-containing yeast, and (2) greater quantities of hENT1 mRNA were present in pYhENT1-containing yeast grown in the presence of galactose (inducing conditions) than in yeast grown in the presence of glucose (repressive conditions). Inhibition of transport by nanomolar concentrations of NBMPR, dilazep or dipyrindamole is diagnostic of the *es* transport process of mammalian cells [3,4], and thymidine rescue of pYhENT1-expressing yeast from thymidylate starvation was blocked by addition of excess (1 or 10  $\mu$ M) NBMPR, dilazep or dipyrindamole to the growth medium. pYhENT1-containing yeast, but not pYES2-containing yeast, accumulated radioactivity during 20 min incubations with [ $^3$ H]thymidine; this uptake was shown to occur in a mediated fashion and to be inhibited by NBMPR, by dilazep and by dipyrindamole.

hENT1 is an integral membrane protein and thus was expected to be present in membrane fractions of pYhENT1-expressing yeast. Immunoblotting of yeast membranes with hENT1-specific polyclonal antibodies (directed against a synthetic peptide corresponding to residues 254–272) demonstrated the presence of immunoreactive protein in pYhENT1-expressing yeast but not in pYES2-expressing yeast. The predominant immunoreactive species exhibited an apparent molecular mass of 40–45 kDa and thus corresponded to the hENT1 monomer because the predicted molecular mass of non-glycosylated hENT1 is 50.2 kDa. The native *es* transporter (recognized by photoaffinity labelling with [ $^3$ H]NBMPR) migrates during SDS/PAGE with a wide range of mobilities (42–60 kDa) among different species and cell types [3,4]. These differences in molecular mass have been attributed to various degrees of N-linked glycosylation [15,16,35,36,42]. The larger minor immunoreactive species (80 and 120 kDa) were probably hENT1 dimers and trimers respectively. Immunoblotting of detergent-solubilized yeast membranes under reducing and non-reducing conditions yielded identical patterns of staining (results not shown), suggesting that disulphide bonds were not involved in the oligomerization of hENT1.

A further demonstration that recombinant hENT1 was located primarily in yeast membranes came from results of studies of equilibrium binding of [ $^3$ H]NBMPR with membrane preparations from pYhENT1-containing yeast. Crude membranes from pYhENT1-containing yeast that had been grown under inducing conditions exhibited high-affinity binding of NBMPR ( $K_d$   $0.7 \pm 0.2$  nM;  $B_{max}$   $6.5 \pm 1.0$  pmol/mg of protein). NBMPR bound to pYhENT1-expressing yeast and yeast membranes with  $K_d$  values similar to those found for the *es* transporters of a variety of mammalian cell types [3]. The number of NBMPR-binding sites observed in pYhENT1-expressing yeast was similar to the values reported [43] for human erythrocytes (approx.  $10^4$  sites per cell), suggesting, because NBMPR binds to the *es* transporter protein with an apparent stoichiometry of 1:1 [31], that there were approx.  $10^4$  functionally active hENT1 molecules per cell. Although the *es* transporter protein of human erythrocytes has been purified to homogeneity by immunoaffinity chromatography, the quantities obtained were small [31]. The level of hENT1 production in yeast opens the way for use of the yeast expression system for the production of recombinant hENT1 protein for purification.

NBMPR binding to the *es* transporter occurs at the exofacial surface [25,44]; its binding site is believed to be the same as, or to overlap with, the nucleoside recognition site for permeation [44,45]. Examination of NBMPR binding to the N-glycosylation-defective mutant hENT1/N48Q revealed a decrease to one-tenth, relative to hENT1, in the affinity of the recombinant protein for NBMPR. The decreased affinities were shown to be due in part to increased rates of dissociation because  $k_{off}$  values of specifically bound [ $^3$ H]NBMPR from hENT1-producing and hENT1/N48Q-producing yeast were  $0.14 \pm 0.02$  and  $0.36 \pm 0.05$  min $^{-1}$  respectively. The decreased affinities were also due to changes in the rates of NBMPR association:  $k_{on}$  values were determined as  $(1.20 \pm 0.1) \times 10^8$  and  $(0.40 \pm 0.04) \times 10^8$  M $^{-1}$ ·min $^{-1}$  for hENT1-producing and hENT1/N48Q-producing yeasts respectively.

The decreased affinity of hENT1 for NBMPR after the alteration of Asn-48 implicates this residue, and/or the N-glycosylated oligosaccharide, in the binding of NBMPR to hENT1. The region of hENT1 involved in the binding of inhibitor has recently been defined by experiments in which portions of the human and rat *es* transporters (hENT1, rENT1) were swapped with each other, thereby exploiting the intrinsic differences in dipyrindamole sensitivity between the two transporters [46]. The

characteristics of the resulting hENT1/rENT1 chimaeras indicated that the region responsible for sensitivity to dipyrindamole (and therefore to NBMPR) is contained within the N-terminal half of hENT1, primarily in transmembrane domains 3–6 (residues 100–261). Our present results suggest that the region N-terminal to the third transmembrane domain, most probably the large extracellular loop connecting the first and second transmembrane domains, also contributes to the formation of the nucleoside recognition site of hENT1. We have previously suggested that NBMPR binds to hENT1 in the N-terminal half of the protein within 15 kDa of the site of the N-glycosylation site [15].

When examined under equilibrium conditions, dilazep and dipyrindamole exhibit competitive inhibition of NBMPR's binding to the *es* transporter of human erythrocytes and other cell types and are therefore thought to interact, like NBMPR, at or near the nucleoside recognition site at the exofacial surface of the transporter [32–34,44,47]. The ability of dilazep and dipyrindamole to interact with recombinant hENT1 was demonstrated by assessing the binding of NBMPR to hENT1-producing yeast in the presence of graded concentrations of either of the two *es* inhibitors. Dilazep and dipyrindamole inhibited the binding of NBMPR with  $IC_{50}$  values of  $130 \pm 10$  and  $380 \pm 20$  nM, which yielded apparent inhibitory constants ( $K_i$ ) for dilazep and dipyrindamole of 90 and 260 nM respectively. These values are similar to those observed elsewhere in studies with the native *es* transporter [3,4,27]. The  $IC_{50}$  values ( $50 \pm 5$  and  $230 \pm 3.2$  nM) for inhibition of the binding of NBMPR to hENT1/N48Q-producing yeast by dilazep and dipyrindamole yielded apparent  $K_i$  values of 30 and 150 nM respectively. These results suggest that the conversion of Asn-48 into Gln resulted in conformational differences between hENT1 and hENT1/N48Q that increased the affinity of the latter for dilazep and for dipyrindamole.

The abundance of recombinant hENT1 in pYhENT1-expressing membranes provided a source of recombinant hENT1 for reconstitution into proteoliposomes, thereby permitting a direct demonstration of hENT1-dependent transport activity. Reconstituted proteoliposomes, prepared from hENT1-producing yeast, exhibited a mediated uptake of thymidine that was inhibited by NBMPR ( $IC_{50}$   $5.5 \pm 0.5$  nM) at concentrations similar to those seen previously [36] with intact BeWo cells ( $IC_{50}$  1.6 nM), which have recently been shown by reverse-transcriptase-mediated PCR to express the hENT1 gene [48]. Native *es* transporters have previously been solubilized from mammalian cells and reconstituted into proteoliposomes [27–29,49], although in several of these studies the cells used also contained other nucleoside transporters. The reconstitution of recombinant hENT1 from yeast membranes into proteoliposomes has advantages because of the absence of endogenous thymidine-transporting and NBMPR-binding activities and because of the opportunities presented through recombinant DNA techniques for modification of the hENT1 amino acid sequence.

In conclusion, *S. cerevisiae* has proved to be a useful model system in which to study recombinant hENT1. Phenotypic complementation of thymidylate starvation indicated that hENT1 cDNA was functionally expressed in yeast and exhibited the expected sensitivity to inhibition by NBMPR, by dilazep and by dipyrindamole. Northern and Western analyses confirmed the production of hENT1 mRNA and recombinant protein respectively in pYhENT1-containing yeast grown in medium that contained an inducer (galactose) for expression of the cDNA insert. Recombinant hENT1 was produced at moderately high levels in intact yeast and was present mostly (perhaps entirely) in yeast membranes. Equilibrium binding analysis demonstrated

site-specific interaction of NBMPR with the nucleoside recognition site on recombinant hENT1 and provided a means of evaluating the effects of dilazep and dipyrindamole on NBMPR binding. The conservative conversion of Asn-48 into Gln, with the consequent loss of N-linked glycosylation capability, decreased the affinity of hENT1 for NBMPR and increased its affinity for dilazep and dipyrindamole, suggesting that Asn-48, and/or the N-linked oligosaccharide, influences the structure(s) of the nucleoside and inhibitor recognition site(s) of hENT1. Finally, recombinant hENT1 retained its ability to transport thymidine after solubilization with octylglucoside and reconstitution into proteoliposomes, opening the door to use of the yeast expression system for the production of transport-competent recombinant hENT1 for protein purification.

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