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# Nucleoside transport-deficient mutants of PK-15 pig kidney cell line

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Previous studies indicated that PK-15 pig kidney cells express solely a nitrobenzylthioinosine-sensitive, equilibrative nucleoside transporter. In the present study, PK-15 cells were mutagenized by treatment with ICR-170 and nucleoside transport-deficient mutants selected in a single step in growth medium containing tubercidin and cytosine arabinoside at a frequency of about $2 \cdot 10^{-6}$. The mutants were simultaneously at least 100-times more resistant to tubercidin, cytosine arabinoside and 5-fluorodeoxyuridine than the wild-type parent cells. The mutants failed to transport thymidine and uridine and had lost all high affinity nitrobenzylthioinosine binding sites. Residual low level uptake of thymidine by the mutants was shown to be due to nonmediated permeation (passive diffusion), which explains the sensitivity of the mutants to growth inhibition by high concentrations of the nucleoside drugs. Passive diffusion of thymidine at a concentration of 16 $\mu$M was not rapid enough to support the growth of nucleoside transport-deficient mutant cells that had been made thymidine-dependent by treatment with methotrexate, whereas wild-type cells grew normally under these conditions. The nucleoside transport-deficient mutants exhibited about the same growth rate and plating efficiency (60–80%) as wild-type cells, but formed larger colonies than wild-type cells because of a more extensive spread of the cells on the surface of culture dishes. PK-15 cells adhere very strongly to the surface of culture dishes and have been transformed with high efficiency with plasmid DNA either via lipofection or electroporation.

## Introduction

Mammalian cells possess two basic types of nucleoside transport systems: equilibrative and $Na^+$-dependent, concentrative [1,2]. With few exceptions, all mammalian cells possess an equilibrative nucleoside transporter. Two forms of it have been distinguished on the basis of their sensitivity to inhibition by nitrobenzylthioinosine (NBTI) [1,2]. One form is strongly inhibited by nanomolar concentrations of NBTI (designated NBTI-sensitive) which results from the binding of NBTI to a nucleoside transporter-associated high affinity binding site on the plasma membrane ($K_d \leqq 1$ nM). The other form is not associated with such a binding site and is inhibited only by micromolar concentrations of NBTI (designated NBTI-resistant). Both forms, however, are inhibited by other inhibitors such as dipyridamole and dilazep. Some cells express only one or the other of these two equilibrative nucleoside transporters, but many cells express both in different proportions [2].

The distribution of $Na^+$-dependent, concentrative nucleoside transport is more limited. It has been demonstrated in epithelial cells of the kidney and intestine of various mammalian species and brush border membranes thereof where it seems to be involved in absorption of nucleosides into the blood [2–13]. This function is suggested by its localization in the brush border membrane and the presence of an equilibrative nucleoside transporter in the basolateral membranes [7,12]. Two forms of the concentrative transporter have been distinguished on the basis of substrate specificity. The more common form transports uridine and purine nucleosides. It is also present in mouse and rat macrophages, mouse lymphocytes and all mouse cell lines that have been investigated [14–21]. On the other hand, no $Na^+$-dependent, concentrative nucleoside transport has been detected in mouse and rat erythrocytes, rat and pig lymphocytes, Novikoff rat hepatoma cells, Chinese hamster ovary cells, and three lines of human T cells [16,17].

A second form of concentrative transporter that is specific for pyrimidine nucleosides, adenosine and adenosine analogs has been detected in mouse intesti-

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nal epithelial cells and rat and bovine renal brush border membrane vesicles [5,11,13] but not in other types of cells [16,17]. Both concentrative nucleoside transporters are highly resistant to inhibition by NBTI and other inhibitors of equilibrative nucleoside transport, such as dipyridamole and dilazep [6–17].

In a previous study we found that a pig kidney cell line, PK-15, does not express either of the two concentrative nucleoside transporters [22]. This finding is surprising since the PK-15 cells are believed to represent a single type of distal tubular cell which is normally thought to function in concentrative substrate transport. PK-15 cells express a cyclic AMP synthesis system that is responsive to calcitonin but not parathyroid hormone or vasopressin [23]. The cells also express very low alkaline phosphatase activity [23]. We found that the PK-15 cells only express NBTI-sensitive equilibrative nucleoside transport, but at a much lower level than observed in many other mammalian cell lines [22]. Furthermore, the transporter exhibits a much higher affinity for uridine and cytidine than those of other mammalian cells [22]. In the present study we have isolated by a single-step selection procedure variants of PK-15 cells that lack all nucleoside transport activity. These cells might be useful in various molecular studies because of their strong adherence to culture dish surfaces, their high plating efficiency and the finding that they can be readily transfected with plasmid DNA either by lipofection or electroporation. Only a few nucleoside transport-deficient mutants of cell lines are presently available and most are not well suited for transfection experiments. Nucleoside transport-deficient mutants have been isolated from mutagenized parent populations by a single-step selection procedure from S49 [24] and L1210 mouse leukemia cells [25] and human CEM lymphoma cells [26], all lines of nonadhering cells. A nucleoside transport deficient variant of adhering human colon carcinoma cells (HCT-8) has been isolated, but it was selected by long term culture in a medium containing increasing concentrations of 5-fluorouridine [27] and thus may harbor multiple mutations.

## Experimental procedures

*Cells.* PK-15 cells have been derived from a clone of a porcine kidney cell line (PK 2a) that was originally established in 1955 [23]. They were obtained from the American Type Culture Collection (ATCC; CCL-33) and propagated in monolayer culture in T-flasks in RPMI supplemented with 4% (v/v) fetal bovine serum and 4% (v/v) newborn bovine serum. Their model chromosome number is 37, that is one less the diploid number for pigs [28]. PK-15 cultures were routinely screened for mycoplasma contamination using a highly sensitive assay for adenosine phosphorylase activity [29,30]. All cultures used in the present study were mycoplasma-free. For enumeration, cells from culture dishes or plates were released by trypsinization and then counted in a Coulter counter. For determining the plating efficiency of PK-15 cells, 10-fold dilutions were prepared from suspensions of cells of known cell density and seeded into 6-cm petri plates. After 7–10 days of incubation, the medium was removed, the plates flooded with a solution of 0.1% (w/v) crystal violet in 50% (v/v) ethanol. After 1–2 min the stain was removed, the plates rinsed with water, and the colonies enumerated.

*Isolation of nucleoside transport-deficient mutants.* Slightly subconfluent cultures of PK-15 cells were incubated with growth medium containing 0.5 $\mu$g ICR-170 per ml at 37°C for 20 h. ICR-170 is a frame shift mutagen and has been previously used in the isolation of nucleoside transport-deficient mutants of mammalian cells [25,31]. No cytotoxic effects were apparent during the 20 h of incubation with the mutagen. After the 20 h of incubation, the cells were released from the culture flasks by trypsinization and a total of $2.6 \cdot 10^7$ cells were plated at a density of $7.2 \cdot 10^4$ cells/ml into ten 6-well tissue culture plates. Four days later, when the cells had adhered and divided once or twice, the medium was replaced with fresh growth medium containing 1 $\mu$M tubercidin (Tub) and 1 $\mu$M cytosine arabinoside (AraC). Incubation was continued and the medium replaced in 3–4 day intervals with fresh drug-containing medium. After 2 weeks of incubation the concentrations of both drugs were increased to 2.0 $\mu$M. At 4 weeks, a total of 38 colonies, each containing approximately 50 and 200 cells (see later Fig. 4) had developed in the 60 wells. The cells from four colonies (6-5, 10-6, 2-2 and 9-2) were cloned by limiting dilution. Clone 6-5 exhibited the most reliable growth pattern and has been analyzed in greater detail as a prototype of the isolated mutants (see later).

*Transport measurements.* All transport studies were conducted with monolayer cultures of PK-15 cells [22]. The cells were propagated in 24-well cell culture plates. When slightly subconfluent or confluent, the cultures were rinsed with RPMI and then overlaid with 0.5 ml of the same medium. [$^3$H]Thymidine, [$^3$H]uridine, [$^3$H]hypoxanthine, and [$^3$H]adenine uptake was measured by adding them, at timed intervals, to 4–8 wells of a plate. At the completion of the incubation, the medium was dumped out and the wells rapidly rinsed thrice (within 15 s) with ice-cold balanced salt solution (BSS) as described previously [16–18]. Where indicated the medium was supplemented with dipyridamole at least 2 min before nucleoside uptake was measured.

For assessing the metabolism of the radiolabeled nucleosides, RPMI-washed cell layers were extracted with 0.5 M trichloroacetic acid. The acid extracts were further processed and analyzed by ascending paper

chromatography using a solvent composed of 30 ml 1 M ammonium acetate (pH 5.0) and 70 ml of 95% ethanol (solvent 28) as described previously [14,32]. This procedure separates nucleoside tri-, di- and mono-phosphates and nucleosides from each other.

*Equilibrium binding of NBTI.* The equilibrium binding of [$^3$H]NBTI was measured with cells propagated in 24-well culture plates [17]. Cultures of cells in duplicate wells were overlaid with RPMI (0.5 ml/well) containing [$^3$H]NBTI to final concentrations of 0.1 to 10 nM. After 20 min of incubation at room temperature the culture fluid was analyzed for radioactivity. The wells were rinsed three times with ice cold BSS and the rinsed cells analyzed for radioactivity. Measured radioactivity was converted to concentrations of free ligand ($L_f$) and bound ligand ($L_b$), respectively, and the data were analyzed by the method of Scatchard [33].

*Fluorescent activated cell sorter (FACS) analyses of cells.* Cells released from culture dishes by trypsin treatment were washed in phosphate-buffered saline, pH 7.4 (PBS) and then fixed by incubation at a density of $3 \cdot 10^5$ cells/ml in a 0.5% (w/v) solution of paraformaldehyde at room temperature for 15 min. The cells were collected by centrifugation and suspended to the same density in 0.1% (v/v) Triton X-100 in PBS (PBS-Triton). After 5 min the cells were collected by centrifugation, washed once in PBS-Triton and then incubated with RNase (150 U/ml) at 37°C for 20 min. The cells were collected by centrifugation, washed once in PBS-Triton and suspended to $1.5 \cdot 10^6$ cells/ml in PBS containing 50 $\mu$g propidium iodide per ml. After 30 min at 4°C, the cells were collected by centrifugation, washed once in PBS, suspended in 0.5% paraformaldehyde and analyzed for volume and DNA content using a Becton Dickinson FACS IV.

*Transfection of PK-15 cells with pSV2-neo plasmid DNA.* A culture of *Escherichia coli* HB-101 containing the pSV2-neo plasmid was kindly provided by Dr. Peter Southern [34]. The 5.6-kb plasmid DNA was isolated by the alkaline lysis procedure [35]. Freshly plated subconfluent cultures of PK-15 cells were overlaid dropwise with a preincubated mixture of *Eco*RI-linearized pSV2-neo plasmid DNA and lipofectin™ reagent (Gibco BRL, Gaithersburg, MD) in RPMI following the direction of the manufacturer of lipofectin. After 12 h of incubation at 37°C, the mixture was replaced with RPMI supplemented with 10% (v/v) fetal bovine serum. After another 2 days of incubation, the cells were harvested by trypsinization and replated in the same medium at a density of $1 \cdot 10^4$ cells/ml as suggested by Southern and Berg [34]. One day later the medium was replaced with fresh growth medium containing 0.5 mg G418/ml, a concentration that completely inhibited the growth of wild-type PK-15 cells. The medium was replaced with fresh medium containing G418 every 3–4 days. After 2–3 weeks of culture, cells were harvested from selected colonies that had developed and replated in the presence of 1 mg G418/ml. Cells from the resulting cultures were cloned by limiting dilution and further propagated, and some clones were then analyzed for integrated plasmid DNA.

PK-15 cells were transfected by electroporation as described by Shigekawa and Dower [36] using an apparatus from Promega Biotech (X Cell 450). A suspension of $2.5 \cdot 10^6$ cells in 0.5 ml of RPMI containing 20 $\mu$g *Eco*RI-linearized pSV2-neo DNA was incubated at room temperature for 15 min and then electroporated at $E_0 = 625$ V/cm and $\tau = 7$ ms with a distance between electrodes of 0.4 cm. After 15 min of further incubation, the suspension was diluted to $1 \cdot 10^5$ cells/ml with RPMI containing fetal bovine serum to yield a final concentration of 10% (v/v). The cells were seeded into tissue culture plates, two days later the medium was supplemented with G418, and the cultures examined for the formation of colonies as described already.

Genomic DNA was extracted from transformed cells, restricted by incubation with *Eco*RI and *Xba*I and analyzed by Southern hybridization using procedures described previously [37]. The blots were hybridized with a 700 bp 'neo' probe. The probe was generated by digestion of pSV2-neo DNA with HindIII and BssHII and labeled by random priming with a kit (Prime-a-Gene) and [$\alpha$-$^{32}$P]dATP. The probe represents the one half of the 1.4 kb neo gene segment adjacent to the SV40 early promoter [34]. mRNA was isolated from transfected and untransfected (control) cells and analyzed by Northern blot hybridization as described previously [38] using the same 'neo' probe.

*Materials.* $^3$H-labeled nucleosides, nucleobases, and NBTI were purchased from Moravek Biochemicals (Brea, CA); unlabeled nucleosides, G418 (Geneticin), propidium iodide, and ICR-170 from Sigma (St. Louis, MO); and [$\alpha$-$^{32}$P]dATP from Amersham (Arlington Heights, IL).

## Results and Discussion

For the isolation of transport-deficient mutants, $2.6 \cdot 10^7$ PK-15 cells that had been mutagenized by treatment with ICR-170 were plated in the presence of 1 $\mu$M Tub and 1 $\mu$M AraC at concentrations (1 $\mu$M) that were found to almost completely inhibit the growth of the cells (see later Fig. 3). Exposure of the cells to two cytotoxic nucleosides that are phosphorylated by different kinases avoids the selection of mutants that are resistant because of a deficiency in kinase activity. Tub and AraC are both efficient substrates for the NBTI-sensitive nucleoside transporters of mammalian cells ($K_m \sim 100$ $\mu$M and 500 $\mu$M, respectively; Ref. 2), but Tub is phosphorylated by adenosine kinase [39]

whereas AraC is phosphorylated by deoxycytidine kinase [40]. After about 4 weeks of incubation of the cultures, 38 colonies with approx. 50 to 200 cells had developed. The cells from 16 colonies we examined were all found to be devoid of nucleoside transport activity as assessed by thymidine uptake measurements (see Fig. 1A). On the basis of a plating efficiency of the PK-15 cells of about 70% (see later) and assuming that the remaining colonies also contained cells lacking nucleoside transport activity, we calculated that these cells were present in the mutagenized population at a frequency of $2 \cdot 10^{-6}$. The cells from four colonies were cloned by limiting dilution and one of the clones (6-5), which was representative in its nucleoside uptake properties of all mutants we have analyzed, was studied in greater detail as a prototype.

Uptake of thymidine and uridine by the 6-5 PK-15 cells was extremely slow when compared to that by wild-type cells (Figs. 1A and B, respectively). After 120 min of incubation, a large proportion of the nucleosides taken up was recovered in phosphorylated products (mostly triphosphates). Thus, the 6-5 PK-15 cells did not lack uridine or thymidine kinase activities. Furthermore, since the intracellular concentration of thymidine equivalents after 120 min of incubation with thymidine was still only about 50% of that in the extracellular fluid (Fig. 1A), uptake was clearly not limited by the rate of phosphorylation. The slow uptake of thymidine by the 6-5 PK-15 cells was little affected by 10 $\mu$M dipyridamole (Fig. 1C), which completely inhibits nucleoside transport by wild-type PK-15 cells [22], and thymidine uptake by these cells was nonsaturable (Fig. 1D). The results indicate that the residual uptake of thymidine by the mutant reflects nonmediated permeation through the plasma membrane [1,2]. The velocities of thymidine and uridine uptake by the 6-5 cells (expressed as the first-order rate constant, $k$) in relation to their lipid solubilities (as measured by their octanol partition coefficients, $Z_{oct}$) is consistent with this conclusion. The $k/Z_{oct}$ ratios for the uptake of thymidine and uridine at 37°C by the 6-5 cells (Table I) were similar to those observed for the nonmediated permeation of these nucleosides into other types of mammalian cells [2]. The $k/Z_{oct}$ ratio for thymidine was lower than that for uridine. This difference is typical for the nonmediated permeation of deoxynucleoside and ribonucleosides [2]. The slight inhibition of thymidine uptake into 6-5 cells by

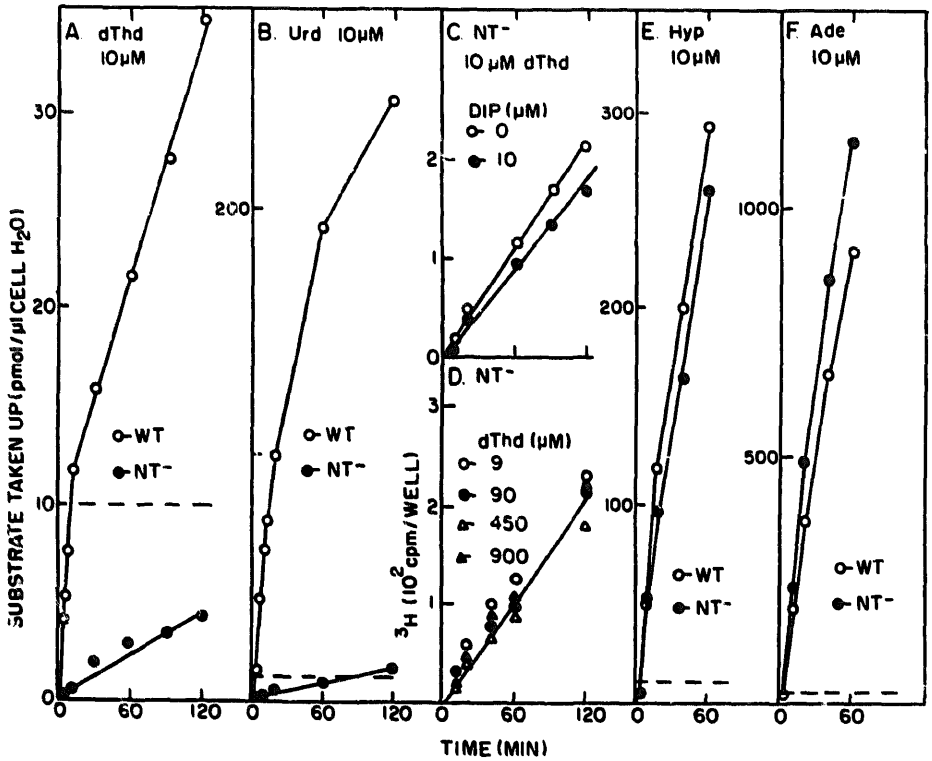


Fig. 1. Comparison of thymidine (A, C, D), uridine (B), hypoxanthine (E), and adenine (F) uptake by wild-type (WT) and nucleoside transport-deficient ($NT^-$) 6-5 PK-15 cells. Uptake of the substrates was measured in slightly subconfluent to confluent cultures in 24-well plates (2 to $5 \cdot 10^5$ cells/well) as described in Experimental procedures. The substrate concentrations were 10 $\mu$M (25 to 35 cpm/pmol), except in D where the medium was supplemented with [$^3$H]thymidine (390 cpm/$\mu$l) plus unlabeled thymidine to the indicated concentrations. In C, the medium was supplemented where indicated with 10 $\mu$M dipyridamole (DIP) before thymidine uptake was measured. The intracellular concentrations of substrate were calculated on the basis of an intracellular water space of 5 $\mu$l/$10^6$ PK-15 cells [22]. All points represent values from single wells. The broken lines indicate the intracellular concentration of substrate equivalent to that in the medium. Results comparable to those shown in frame A were obtained in two other experiments with 6-5 PK-15 cells and with other $NT^-$ PK-15 clones.

TABLE I

*First-order rate constants (k) for the uptake of thymidine and uridine by nucleosiae transport-deficient mutant of PK-15 cells, octanol partition coefficients ($Z_{oct}$) of the nucleosides and ratios of $k/Z_{oct}$*

The uptake velocity ($v$) was estimated from the data in Figs. 1A–D. The value for thymidine is the mean for the three experiments illustrated. $Z_{oct}$ is the ratio of substrate concentration in octanol/concentration in aqueous solution [2].

| Substrate | $v$ (pmol/$\mu$l cell $H_2O$ per min) | $k$ ($min^{-1}$) | $Z_{oct}$ | $k/Z_{oct}$ |
|---|---|---|---|---|
| Thymidine | 0.013 | 0.0013 | 0.0753 | 0.040 |
| Uridine | 0.7 | 0.011 | 0.0149 | 0.67 |

dipyridamole (Fig. 1C) was also not unexpected since dipyridamole is known to cause a slight inhibition of the nonmediated permeation of nucleosides into mammalian cells [41]. In contrast to the slow uptake of thymidine and uridine by the 6-5 cells, their uptake of hypoxanthine and adenine was as rapid as that by wild-type PK-15 cells (Figs. 1E and F, respectively). Combined the results indicate that the 6-5 cells have lost all nucleoside transport activity and that this loss of transport activity is the basis of their resistance to both Tub and AraC.

These conclusions are further supported by the finding that the mutant had lost practically all high-affinity NBTI-binding sites (Fig. 2). Wild-type PK-15 cells possess $4 \cdot 10^3$ to $2 \cdot 10^4$ high-affinity NBTI binding sites/cell (Ref. 22; see Fig. 2), whereas the nucleoside transport-deficient mutant was practically devoid of such sites. Furthermore, the mutant was simultaneously resistant to three different nucleoside analogs each of which is phosphorylated by a different kinase (Fig. 3). The mutant was at least 100-times more resistant than the wild-type cells. This difference in susceptibility is explained by the difference in rate of entry of the nucleosides via the nucleoside transport system

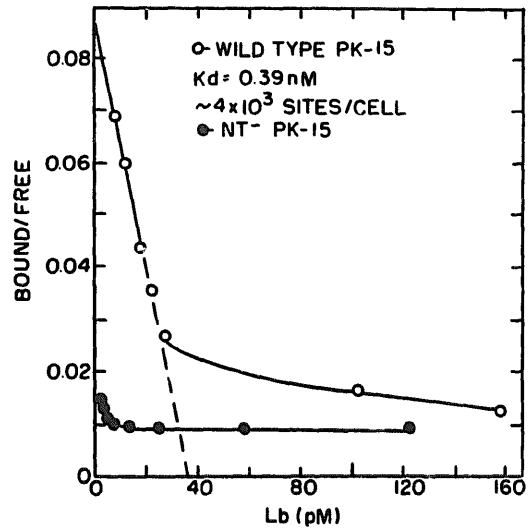


Fig. 2. Scatchard analyses of the equilibrium binding of [$^3$H]NBTI by wild-type and nucleoside transport-deficient ($NT^-$) 6-5 PK-15 cells. The equilibrium binding of [$^3$H]NBTI at concentrations ranging from 0.1 to 10 nM was measured in subconfluent cultures of wild-type and $NT^-$ PK-15 cells in 24-well plates ($2.5 \cdot 10^5$ cells/well) as described under Experimental procedures. The listed kinetic parameters for binding were calculated by the method of Scatchard [33].

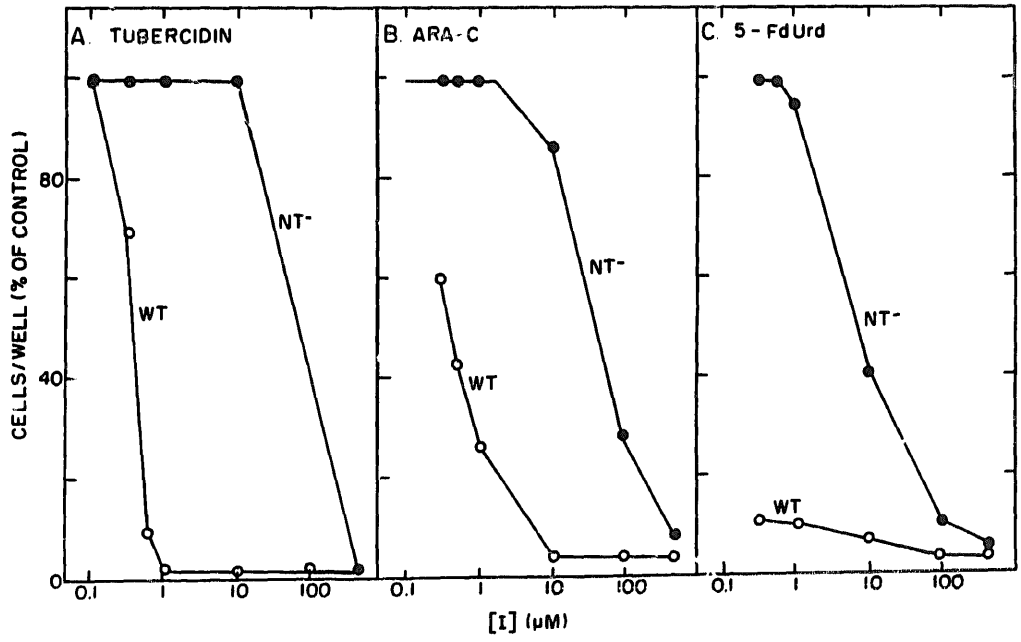


Fig. 3. Comparison of the inhibition of growth of wild-type (WT) and nucleoside transport-deficient ($NT^-$) 6-5 PK-15 cells by Tub (A), AraC (B), and 5-fluorodeoxyuridine (FdUrd; C). The cells were seeded into 24-well culture plates (at $5 \cdot 10^4$ cells/well) in the presence of the indicated concentrations of inhibitors. After 7 days of incubation at 37°C the cells were removed from each well by trypsinization and enumerated. The densities of the wild-type and $NT^-$ control cultures were $3.8 \cdot 10^5$ and $3.0 \cdot 10^5$ cells/well, respectively. All values are derived from results of duplicate wells.

into wild-type PK-15 cells and only via nonmediated permeation into the nucleoside transport mutant. Tub, AraC and 5-fluorodeoxyuridine are efficient substrates for the equilibrative nucleoside transporters of mammalian cells [2] and probably also enter the mutant cells via nonmediated permeation at similar rates as do natural nucleosides [2] since their octanol partition coefficients ($K_{oct} = 0.163 \pm 0.001$ for Tub; $0.0110 \pm 0.001$ for AraC, and $0.0527 \pm 0.0021$ for 5-fluorodeoxyuridine, determined as described in Ref. 41) were found to be comparable to those of natural purine and pyrimidine nucleosides [2].

The selection of PK-15 mutants that completely lack nucleoside transport activity at a frequency of $2 \cdot 10^{-6}$ supports our previous conclusion [22] that the only nucleoside transport system expressed in wild-type PK-15 cells is the NBTI-sensitive, equilibrative transporter. The growth rate of the nucleoside transport-deficient 6-5 PK-15 cells was about the same as that of wild-type PK-15 cells; their population doubling times ranged from 28 to 36 h (see later Fig. 5). The wild-type and 6-5 cells also exhibited about the same plating efficiencies (60–80%). However, we observed that the cell numbers/well of confluent cultures of 6-5 PK-15 cells were

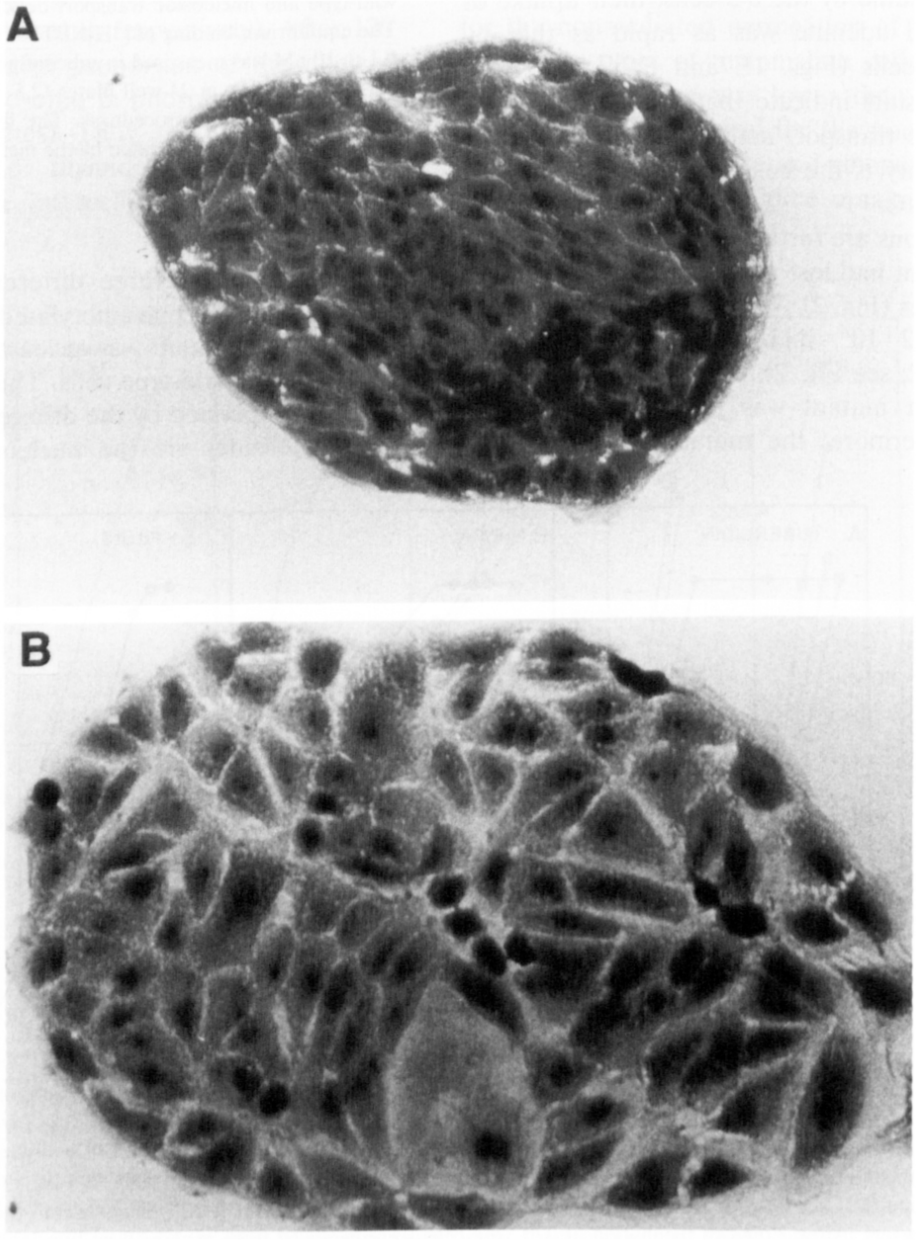


Fig. 4. Comparison of micrographs of colonies of wild-type (A) and nucleoside transport-deficient (B) PK-15 cells. The colonies of wild-type and 6-5 cells were photographed 8 days after plating single cell suspensions of the cells. Magnification of the micrographs = 160×.

generally 20–30% lower than those of confluent wild-type cells. This observation is probably explained by a more spread-out growth of the 6-5 cells as compared to those of the wild-type cells and a less tight packing of the mutant cells in colonies. These conclusions are indicated by a comparison of micrographs of colonies of wild-type PK-15 cells (Fig. 4A) and 6-5 PK-15 cells (Fig. 4B) taken at the same magnification. In colonies, the cells of the mutant appear to be much larger than the wild-type cells, but FACS analysis of the cells after their release from the culture dishes by trypsin treatment indicated that the volumes of the two types of cells were about the same, whether the cells were harvested from log phase or confluent, stationary-phase cultures (data not shown). The reasons for the more spread-out growth of the 6-5 cells is unknown, but it seems related to the nucleoside transport deficiency of the cells, since two other independently derived nucleoside transport-deficient mutants of PK-15 cells (5-4 and 7-2) exhibited the same growth pattern as the 6-5 cells (data not shown).

Although nucleosides enter the nucleoside transport-deficient PK-15 cells at significant rates by passive diffusion, the rate of thymidine permeation was not sufficient to significantly support the growth of these cells when made thymidine-dependent, at least not at the thymidine concentration that supported the growth of thymidine-dependent wild-type cells. As shown in Fig. 5, the growth of both wild-type and 6-5 cells was completely inhibited by 1 $\mu$M methotrexate. The inhibition of wild-type cells was completely reversed by the presence of 16 $\mu$M thymidine and 100 $\mu$M hypoxanthine in the medium, whereas the growth of the transport mutant remained inhibited. The death of the methotrexate-treated mutant cells was slightly delayed in the presence of thymidine and hypoxanthine, but then occurred more rapidly than in their absence. The results suggest that thymidine entered the mutant cells rapidly enough to transiently protect them until intracellular thymidine nucleotides became exhausted. Since the rate of nonmediated permeation varies in direct proportion to the substrate concentration, it seems likely that the methotrexate inhibition of the nucleoside transport-deficient mutant could be reversed by higher concentrations of thymidine in the medium. Nevertheless, no growth occurred under the conditions of the experiment depicted in Fig. 5, which thus might be suitable for the selection of nucleoside transport competent revertants that occur spontaneously or might be generated by transfection with DNA encoding the nucleoside transporter.

In a preliminary experiment we have demonstrated that PK-15 cells can be readily transfected with DNA either by lipofection or electroporation. The cells were transfected with linearized pSV2-neo plasmid DNA and transformed cells selected in medium containing

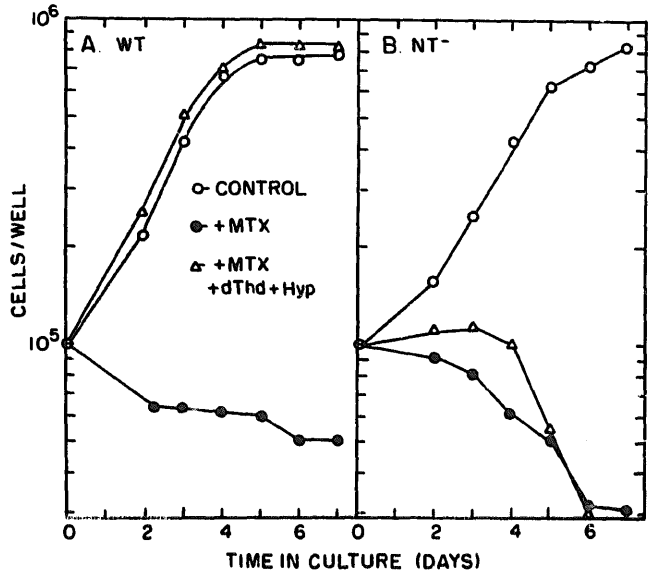


Fig. 5. Growth of wild-type (A) and nucleoside transport-deficient (B) PK-15 cells under thymidine-dependent and -independent growth conditions. Wild-type (WT) and nucleoside transport-deficient (NT⁻) 6-5 PK-15 cells were seeded into 6-well tissue culture plates at a density of about $1 \cdot 10^5$ cells/well. Where indicated the growth medium was supplemented with 1 $\mu$M methotrexate (MTX), 16 $\mu$M thymidine (dThd) and 100 $\mu$M hypoxanthine (Hyp). At the indicated times of incubation at 37°C, the cells of individual wells were released by trypsinization and enumerated in a Coulter counter.

the antibiotic G418 (see Experimental procedures). G418-resistant cells were isolated at a frequency of about $1.5 \cdot 10^{-4}$ after transfection by either lipofection or electroporation. In order to demonstrate the integration of the neo gene into host chromosomes, DNA was isolated from two clones (1 and 2) of one resistant colony, restricted by digestion with *Eco*RI or *Xba*I and analyzed by Southern hybridization using a 700-bp probe representing about one-half of the neo gene (Fig. 6A). Neo-specific fragments of about 5 kb and 7 kb were identified in *Xba*I- and *Eco*RI-digested DNA of transformed cells, respectively, but not in digests of DNA from nontransfected cells. Since the *Eco*RI-linearized pSV2-neo plasmid does not contain any internal *Eco*RI or *Xba*I restriction sites, these fragments must have been generated by cleavage of chromosomal sites adjacent to the integrated plasmid. The finding that the 5-kb *Xba*I fragment was smaller than the 5.6-kb pSV2-neo plasmid, therefore, indicates that only a portion of the plasmid containing the neo gene had become integrated. That the entire 1.4-kb neo gene had become integrated was verified by Northern blot hybridization of mRNA extracted from transformed cells using the neo-specific probe. A mRNA of about 2.5 kb was detected in transfected but not control cells (Fig. 6B). The size of the neo mRNA conformed to

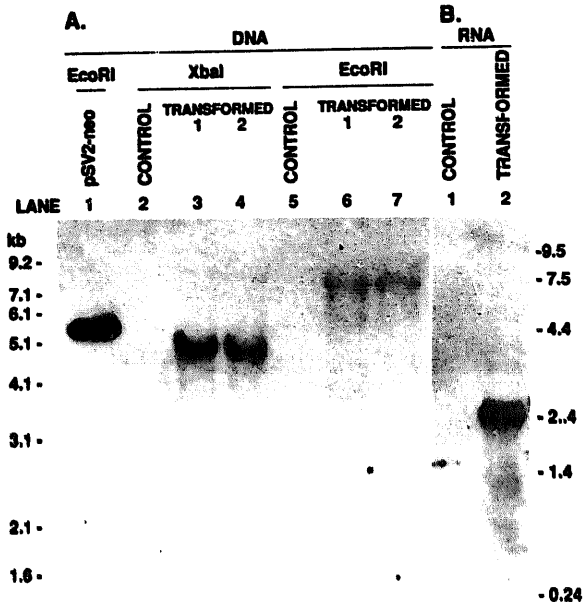


Fig. 6. Southern hybridization analyses of pSV2-neo DNA and DNA from transformed and untransformed PK-15 cells (A) and Northern hybridization analysis of RNA from these cells (B). (A) pSV2-neo DNA was linearized by incubation with *Eco*RI. DNA extracted from untransformed (control) and two clones of pSV2-neo transformed 6-5 PK-15 cells was restricted as indicated by incubation with *Eco*RI or with *Xba*I. The DNA fragments were separated by gel electrophoresis ina 0.8% agarose gel and transferred to a Nytran membrane. The blot was hybridized with a 700 bp, $^{32}$P-labeled 'neo'-specific DNA probe. (B) RNA extracted from untransformed and transformed 6-5 PK-15 cells was glyoxylated, electrophoresed in a 1% glyoxal-agarose gel and transferred to a Nytran membrane. The blot was hybridized with the same neo probe.

that expected for the neo mRNA transcribed from the pSV2-neo plasmid from the SV40 early promoter [34].

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