Effects of transformation by *v-fps* on nucleoside transport in Rat-2 fibroblasts

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Important cellular nutrients, including nucleosides and hexose sugars, are rapidly taken up by cells, largely through mediated carrier systems. The present study examined nucleoside and hexose transport activity in normal Rat-2 fibroblasts and clonal derivatives that expressed either the wild-type (C10) or a temperature-sensitive mutant (NA9) form of *v-fps*, a transforming protein-tyrosine kinase. Initial uptake rates (transport) of adenosine, thymidine, 3-*O*-methylglucose and 2-deoxyglucose were greater in *v-fps*-transformed cells than in normal cells. Elevated transport rates were seen in cells that expressed the temperature-sensitive mutant *v-fps* only after growth at a temperature that was permissive for protein-tyrosine kinase activity. Nucleoside transport rates declined with increasing cell density in both normal and *v-fps* transformed cells. Analysis of the sensitivity of adenosine transport to inhibition by nitrobenzylthioinosine (NBMPR) indicated that Rat-2 fibroblasts, like many other rat cell types, possess at least two nucleoside transport systems, which can be distinguished by differences in sensitivity to NBMPR. Although both transport activities were elevated in *v-fps*-transformed cells, a greater increase was seen in the NBMPR-sensitive component than in the NBMPR-insensitive component. Mass law analysis of the binding of [³H]NBMPR indicated that transformed cells had either the same number (NA9) or a smaller number (C10) of NBMPR-binding sites than normal cells, and photolabelling of membrane proteins with [³H]NBMPR identified polypeptides with similar electrophoretic mobilities (55–75 kDa) in both normal and transformed cells. Thus transformation by *v-fps* resulted in an increase in NBMPR-sensitive transport activity which was not related to either the number of NBMPR-binding sites or the apparent molecular mass of NBMPR-binding polypeptides.

INTRODUCTION

At least four different types of nucleoside transport systems exist in various cells and tissues (Gati & Paterson, 1989). These include a non-concentrative system that is inhibited by nanomolar concentrations of nitrobenzylthioinosine (NBMPR) and is termed equilibrative-sensitive (es), a non-concentrative system that is unaffected by micromolar concentrations of NBMPR and is termed equilibrative-insensitive (ei), and two sodium-dependent concentrative systems that have distinct substrate specificities and lack sensitivity to NBMPR (Jakobs & Paterson, 1986; Vijayalakshmi & Belt, 1988). Whereas a few cell types express only a single nucleotide transport system, many others co-express multiple systems.

Transformation, which alters the growth properties and metabolism of cells, is often accompanied by increases in the transport of exogenous nutrients (for review, see Bettger & McKeahan, 1986). Increases in the activity of glucose transport systems during neoplastic transformation are well documented (Inui *et al.*, 1980; Klip *et al.*, 1984; Weber *et al.*, 1984; Hiraki *et al.*, 1988; Kitigawa *et al.*, 1989). Regulation of the GLUT1 (erythrocyte-type, HepG2) transport system has been extensively studied (Gould & Bell, 1990), and the predominant mechanism responsible for activation following oncogenic transformation is increased expression of the structural gene encoding the GLUT1 protein (Flier *et al.*, 1987; Birnbaum *et al.*, 1987; Hiraki *et al.*, 1988; Kitigawa *et al.*, 1989; White & Weber, 1990). In contrast, transformation-dependent increases in nucleoside uptake have been associated with changes in intracellular metabolism rather than transport across the plasma membrane (Quinlan & Hochstadt, 1974; Koren *et al.*, 1978; Rozengurt *et al.*, 1978). In fact, reviews

in the nucleoside transport literature have concluded that there is little, if any, experimental evidence to suggest that nucleoside transport is regulated in any fashion (Plagemann & Wohlhueter 1980; Plagemann *et al.*, 1988).

Nucleosides are important cellular nutrients and are used as anticancer and antiviral drugs. Changes in nucleoside transport activity during neoplastic transformation could greatly alter utilization of physiological nucleosides and susceptibility to pharmacologically active nucleoside analogues. In the light of recent advances in our understanding of nucleotide transport processes we have re-examined the question of regulation of this transport activity during oncogenic transformation, using a well-defined transformation model.

Fujinami sarcoma virus (FSV) contains a transforming gene (*v-fps*) with codes for a cytoplasmic transforming protein (P130^{gag-fps}) which intrinsic tyrosine-specific protein kinase (PTK) activity. In this work, we have examined nucleoside transport processes in (1) normal Rat-2 (R2) fibroblasts, (2) a stable transfectant of R2 fibroblasts that expresses wild-type P130^{gag-fps} (C10), and (3) a transfectant of R2 fibroblasts with a temperature-sensitive mutant p130^{gag-fps} which causes transformation at the permissive temperature (NA9; DeClue *et al.*, 1987). The temperature-sensitive mutant allowed comparison of nucleoside transport processes in cells that were genetically identical but expressed different levels of *v-fps* (PTK) activity when grown at permissive and non-permissive temperatures. Since elevation of the membrane transport of glucose is well documented in transformed cells, we also examined glucose transport activity in parallel studies with *v-fps*-transformed cells. For both nucleosides and hexoses, cellular uptake was measured using rapid assay techniques that provided measurements of

Abbreviations used: NBMPR, nitrobenzylthioinosine {6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine}; PTK, protein-tyrosine kinase; PBS, phosphate-buffered saline; R2 Rat-2 fibroblast; es, equilibrative-sensitive; ei, equilibrative-insensitive.

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uptake as early as 3 s after permeant addition, thus providing initial rates of uptake (transport). We describe here changes in nucleoside transport activity with cell density and changes in both nucleoside and glucose transport associated with transformation of R2 fibroblasts, apparently as a result of the PTK activity of the *v-fps* oncoprotein. To the best of our knowledge, this is the first demonstration of changes in nucleoside transport activity associated with expression of a particular oncoprotein.

MATERIALS AND METHODS

Cells and media

The cells used in this study were fibroblasts (lacking thymidine kinase; Topp, 1981) and clonal derivatives obtained by transfection of R2 fibroblasts with DNA encoding wild-type P130^{*gag-fps*} (C10 fibroblasts) or a temperature-sensitive form of P130^{*gag-fps*} (NA9 fibroblasts; a gift from Dr. I. Sadowski, University of British Columbia, Vancouver, Canada). The origins of these cell lines have been described (Weinmaster *et al.*, 1984; Sadowski *et al.*, 1986; DeClue *et al.*, 1987). R2 and C10 fibroblasts exhibit normal and transformed phenotypes respectively under standard culture conditions. NA9 fibroblasts exhibit a normal phenotype when grown at the non-permissive temperature (39 °C) for P130^{*gag-fps*}, and a partially transformed phenotype when grown at the permissive temperature (33 °C).

Cells were maintained in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal bovine serum, and were incubated at 37 °C (or, where indicated, at 39 °C or 33 °C) in a humidified atmosphere of 5% CO₂ in air. Cells were harvested by trypsin treatment and cell numbers were determined by electronic particle counting.

For transport and NBMPR-binding experiments, cells from actively proliferating cultures were plated at low (unless otherwise noted) cell densities [(1–5) × 10⁵ cells/dish] in 60 mm-diam. tissue culture dishes. Assays were conducted with 2- or 3-day cultures that had been incubated at 37 °C or, in experiments which assessed effects of the temperature-sensitive mutant, that had been incubated for 2 days at 39 °C [designated NA9(39 °C) or R2(39 °C)] or at 33 °C [designated NA9(33 °C) or R2(33 °C)].

Colony formation in soft agar was measured as previously described (DeClue *et al.*, 1987). Briefly, trypsin-treated cells (1 × 10⁶) were suspended in warmed culture medium (42 °C) containing 0.36% agar and poured into 60 mm dishes containing a layer of hardened 0.6% agar in Dulbecco's modified Eagle's medium. Suspensions were allowed to solidify at room temperature and were then incubated at either 39 °C or 33 °C. On day 7, clusters containing eight or more cells were counted as colonies. Triplicates for each cell type and culture condition were analysed and the average colony number was determined.

The PTK activity of P130^{*gag-fps*} was measured as described (Meckling-Hansen *et al.*, 1987). Briefly, R2, C10 or NA9 cells cultured in 60 mm dishes at 39 °C or 33 °C were lysed in non-ionic detergent, and soluble P130^{*gag-fps*} was precipitated with a monoclonal antibody directed against the *gag* portion of the molecule. Washed immunoprecipitates were incubated with 20 mM-Hepes containing 10 mM-MnCl₂ and 2 μCi of [γ-³²P]ATP at 22 °C for 5 min, and labelled proteins were analysed by SDS/PAGE followed by autoradiography.

Transport assay

Assays (three or four cultures/condition) were performed individually at 22 °C in transport buffer (3 mM-K₂HPO₄, 1.8 mM-CaCl₂, 1 mM-MgCl₂, 144 mM-NaCl and 20 mM-Tris). Cultures were washed twice with transport buffer and assayed either immediately or after incubation with various inhibitor solutions.

Uptake intervals, which were timed by metronome signals, were initiated by adding 1.5 ml of transport buffer containing ³H-labelled permeant and were ended by aspirating the permeant solution (2 s before the end point) and immersing the dish (at the end point) in 1 litre of ice-cold stopper solution [phosphate-buffered saline (PBS) containing the appropriate transport inhibitor]. PBS consisted of 137 mM-NaCl, 5.4 mM-KCl, 1.1 mM-KH₂PO₄ and 1.1 mM-Na₂HPO₄, pH 7.4. The dishes were washed at least twice more by immersion in 1–2 litres of cold PBS and then drained. Cellular material was solubilized in 1.25 ml of 0.5 M-KOH at 37 °C for 1 h, and 1 ml of the resulting hydrolysate was combined with 10 ml of Tritonol (Pande, 1976) for determination of radioactivity by liquid scintillation counting. Protein content was determined using the Bio-Rad (Mississauga, Ontario, Canada) kit and BSA as a standard. Cell numbers were determined by the Coulter method with cultures that had been treated identically, except that non-radioactive permeant was used and cells were collected by trypsin treatment. Initial transport rates were estimated by drawing tangents to the computer-generated best-fit second-order polynomial rate equations.

For nucleoside transport assays, the stopper solution consisted of PBS containing 100 μM-dilazep, a potent inhibitor of nucleoside transport (Gati & Paterson, 1989). Uptake at time zero was determined by first incubating cells with transport buffer containing 100 μM-dilazep at 4 or 22 °C for 5 min and then assaying uptake in the presence of 100 μM-dilazep. When [³H]adenosine was the permeant, an inhibitor of adenosine deaminase (deoxycytosine; final concentration 1 μM) was added to the growth medium 60 min before transport assays. For NBMPR inhibition studies, cells were incubated for 15–30 min with NBMPR in transport buffer and then assayed for nucleoside uptake.

For glucose transport assays, the stop solution consisted of PBS and 100 μM-phloretin. Uptake at time zero was determined by first incubating cells with transport buffer containing 100 μM-phloretin at 4 or 22 °C for 5 min, and then assaying uptake in the presence of 100 μM-phloretin.

Cell volume determination

Cell volume was determined by two independent methods, both of which utilized trypsin-treated cells. In the first (Harley *et al.*, 1982), cells that had been incubated for 10 min at 22 °C in transport buffer containing ³H₂O, [¹⁴C]sucrose or [³H]poly(ethylene glycol) were spun through oil, and the intracellular volume of the cell pellet was calculated from the difference between the total water volume and the extracellular volume of the pellet. In the second method (Burres & Cass, 1986), modal cell volume was estimated from cell volume distributions determined with an electronic cell counter equipped with a 100-channel particle analyser (model zF Coulter counter with channelizer II; Coulter Electronics, Hialeah, FL, U.S.A.).

Because parental and transformed R2 fibroblasts lack thymidine kinase activity (Topp, 1981), thymidine represents a non-metabolized or poorly metabolized permeant in these cells. Intracellular water space was estimated from uptake (2–15 min) of [³H]thymidine on attached cells. These estimates differed from the estimates utilizing trypsin-treated cells by less than 15%.

Binding assay

Cultures, prepared as described for transport experiments, were washed twice with binding medium (RPMI 1640 medium without bicarbonate and glucose, and supplemented with 0.56 g of NaCl/litre) and then incubated (22 °C, 10 min) with 1 ml of binding medium with or without 10 μM-NBMPR (to determine non-specific binding). Binding reactions were initiated by adding

binding medium (1 ml per dish) containing graded concentrations of [^3H]NBMPR (0.1–40 nM). After incubation for 30 min at 22 °C (with occasional gentle mixing), 1 ml of binding medium was taken from each dish for determination of free NBMPR, and the remainder was aspirated and discarded. The dishes were washed twice by immersion in 1–2 litres of cold PBS (little if any specific binding was lost in this step) and drained well, and cellular material was hydrolysed by incubation (1 h, 37 °C) with 1.25 ml of 0.5 M-KOH. Portions of 1 ml of the hydrolysate and medium samples were analysed for ^3H content by liquid scintillation counting as in the transport assay. Specific binding was calculated by subtracting values of non-specific binding (from dishes that were incubated with excess non-isotopic NBMPR) from values for total binding (determined from dishes that were incubated with [^3H]NBMPR only). The affinity (K_d) and number (B_{max}) of NBMPR-binding sites for each cell line was estimated by Scatchard analysis (Rosenthal, 1967).

Photoaffinity labelling and electrophoretic analysis of membrane preparations

Cells from subconfluent cultures (about 5×10^6 cells/100 mm dish) were scraped into PBS with a rubber policeman, harvested and washed twice with PBS by centrifugation (500 g), resuspended (3×10^6 cells/ml) in Hepes-buffered saline (150 mM-NaCl, 20 mM-Hepes, 2 mM-MgCl₂, 1 mM-EDTA, pH 7.1), chilled on ice, swollen by addition of 0.25 vol. of ice-cold water, and disrupted by sonication (Braunsonic 1510; 4×20 s pulses). Plasma-membrane-enriched fractions were prepared and stored as previously described (Hogue *et al.*, 1990).

Membranes were photolabelled under equilibrium binding conditions with [^3H]NBMPR using a modification of the procedure described by Hogue *et al.* (1990). Membrane suspensions (about 300 μg in 100 μl ice-cold 50 mM-Tris, pH 7.4) were combined with equal volumes of labelling buffer (50 mM-Tris, pH 7.4, and 20 mM-dithiothreitol) containing 100 nM-[^3H]NBMPR alone or together with 10 μM -NBMPR (for determination of non-specific labelling) and incubated for 30 min on ice. The suspensions were placed in the centre of 35 mm tissue culture dishes and irradiated for 5 min with u.v. light on a chilled surface 7 cm from the cooling jacket of a 450 W mercury arc lamp (Canrad-Hanovia, Newark, NJ, U.S.A.). The irradiated membranes were pelleted in an Airfuge, resuspended in ice-cold chase buffer (50 mM-Tris, pH 7.4, and 10 μM -NBMPR), incubated for 15 min on ice, washed twice with chase buffer and resuspended in 50 μl of chase buffer. The resulting mixtures were combined with 50 μl of 2 $\times$ SDS sample buffer for SDS/PAGE by the method of Laemmli (1970). The electrophoretic mobilities of molecular mass standards were determined following slicing of Coomassie Blue-stained lanes. The mobility of photolabelled material was assessed by analysis (liquid scintillation counting) of the ^3H content of 2 mm gel slices, which were solubilized in 3 % Protosol in Econofluor (Dupont, Boston, MA, U.S.A.) and incubated overnight at 37 °C.

Chemicals

Cell culture medium and sera were from Gibco (Burlington, Ontario, Canada), and tissue culture dishes were from Nunc (Roskilde, Denmark) or Falcon (Becton Dickinson, Lincoln Park, NJ, U.S.A.). L-Adenosine and NBMPR, which were synthesized as described (Acton *et al.*, 1964; Paul *et al.*, 1975), were provided by Dr. A. R. P. Paterson (Edmonton, Alberta, Canada), and unlabelled nucleosides were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[^3H]Adenosine (33 Ci/mmol) and [^3H]NBMPR (30 Ci/mmol) were from Moravek Biochemicals (Brea, CA, U.S.A.). Other [^3H]nucleosides ([*methyl*- ^3H]thymidine, [2,8- ^3H]adenosine) were either from Moravek Biochemicals or

from ICN Biomedicals (Montreal, Quebec, Canada). [^3H]Nucleosides were purified by h.p.l.c. using a Whatman Partisil 10-0DS-3 M9 (25 cm) column eluted with 50 % methanol ([^3H]NBMPR) or with a methanol/water gradient (other [^3H]nucleosides). 3-*O*-Methyl[^3H]glucose (60 Ci/mmol) and 2-deoxy[^3H]glucose (30 Ci/mmol) were from ICN Biochemicals or Dupont of Canada (Mississauga, Ontario, Canada). Dilazep was a gift from F. Hoffman–La Roche and Co. (Basel, Switzerland).

RESULTS

Characteristics of a clonal derivative of R2 fibroblasts expressing temperature-sensitive P130^{gag-fps}

The cellular morphology of NA9 cells, which express temperature-sensitive P130^{gag-fps}, varied depending on whether cells were grown at the permissive (33 °C) or non-permissive (39 °C) temperature for PTK activity. Morphologically, NA9 cells grown at 39 °C were indistinguishable from parental (normal) R2 cells grown at 33, 37, or 39 °C. In contrast, after 2 days in culture at 33 °C, NA9 cells exhibited a partially transformed morphology and became progressively more fusiform with time. P130^{gag-fps} isolated by immunoprecipitation of cell lysates from NA9(39 °C) cells had only a trace of PTK activity, whereas that from NA9(33 °C) cells had the same level of immunoprecipitable kinase activity as wild-type P130^{gag-fps} from C10 cells (results not shown).

In addition to having fusiform morphology in monolayer culture, R2 cells that have been transformed with wild-type *v-fps* (e.g. C10 cells) display anchorage-independent growth. We examined the possibility that NA9 cells grown at the permissive temperature might also grow in an anchorage-independent fashion. Colony formation in soft agar was assessed after 7 days of culture at 39 °C or 33 °C. NA9(33 °C) cells produced colonies in soft agar (0.015 colonies/cell plated), although only half as many as did C10(33 °C) cells (0.034 colonies/cell plated). R2(39 °C), R2(33 °C) and NA9(39 °C) cells all failed to grow in soft agar (< 0.00004 colonies/cell plated). Thus NA9 cells, when grown at the permissive temperature for PTK activity, exhibited phenotypic characteristics (fusiform morphology, anchorage-independent growth) that are commonly associated with oncogenic transformation.

Transport properties of normal and temperature-sensitive P130^{gag-fps}-transformed R2 fibroblasts

Nucleoside and glucose transport rates were determined in NA9 cells grown at permissive and non-permissive temperatures (33 °C and 39 °C respectively) for temperature-sensitive P130^{gag-fps} PTK activity to determine if acquisition of the transformed phenotype was accompanied by changes in transport properties. In the experiments shown in Table 1, cultures of NA9 cells were grown at 39 °C or 33 °C for 48 h and then assayed at room temperature (about 22 °C) for transport activity using a metabolized permeant (adenosine) and two non-metabolized permeants (thymidine and 3-*O*-methylglucose). Parallel studies were conducted with R2 cells to assess the effects of growth temperature on the transport activity of cells that do not express P130^{gag-fps} PTK activity.

For each of the three permeants (adenosine, thymidine and 3-*O*-methylglucose), initial rates of uptake (transport) were elevated more than 2-fold in NA9(33 °C) cells relative to R2(39 °C), R2(33 °C) and NA9(39 °C) cells. There were also small changes in thymidine transport (40 % increase) and 3-*O*-methylglucose transport (30 % decrease) in R2(33 °C) cells relative to R2(39 °C) cells. Since the transport activity of normal R2 cells was affected only slightly by culture temperature, the increases in transport

Table 1. Transport rates in R2 and NA9 cells at permissive and non-permissive temperatures for transformation

NA9 and R2 cells $[(3-6) \times 10^5$ cells/dish] were cultured at 39 °C or 33 °C for 2 days and then assayed for uptake of 4 μM -[^3H]adenosine, 50 μM -[^3H]thymidine or 200 μM -3-*O*-methyl[^3H] glucose. Initial rates of uptake (pmol/s per μl of cell water) were calculated from time courses from 3 to 15 s after addition of ^3H -labelled permeant. Results are means $\pm$ S.E.M.

Cells	Uptake (pmol/s per μl)		
	Adenosine	Thymidine	3- <i>O</i> -Methylglucose
NA9(39 °C)	0.14 ± 0.02	3.7 ± 0.8	2.7 ± 0.4
NA9(33 °C)	0.35 ± 0.02	8.7 ± 1.2	5.0 ± 0.3
R2(39 °C)	0.17 ± 0.02	3.6 ± 0.4	2.8 ± 0.4
R2(33 °C)	0.17 ± 0.01	5.2 ± 0.5	2.0 ± 0.3

Table 2. Binding of NBMPR to R2 and NA9 cells grown at permissive and non-permissive temperatures for transformation

NA9 and R2 cultures were incubated for 48 h at the indicated temperatures, and for assay of binding (conducted at 22 °C) the cultures (5×10^5 cells/dish) were incubated with graded concentrations of [^3H]NBMPR (0.05–20 nM) as described in the Materials and methods section. The amount of specifically bound NBMPR was calculated, and K_d and B_{max} values were determined from Scatchard plots (as in Fig. 3).

Cell line	Growth temperature (°C)	K_d (nM)	B_{max} (sites/cell)
R2	39	0.4	1.3×10^5
	33	0.6	5.9×10^5
NA9	39	1.7	1.9×10^5
	33	0.9	6.6×10^5

activity observed when NA9 cells were grown at the permissive temperature must have been induced, directly or indirectly, by activation of P130^{gag-fps} PTK activity.

In human erythrocytes, which express only NBMPR-sensitive transport, the number of binding sites with high affinity for NBMPR is directly proportional to transport activity (Cass *et al.*, 1974), and nucleoside transport and NBMPR-binding activity are associated with a single 55 kDa polypeptide (Kwong *et al.*, 1988). In the experiments of Table 2, the effect of growth temperature on expression of NBMPR-binding sites was measured to determine if the elevation in nucleoside transport activity in NA9 cells at the permissive temperature could have resulted from an increase in the number of NBMPR-sensitive transport elements. However, growth at the lower temperature resulted in a > 3-fold increase in NBMPR-binding sites in both R2 and NA9 cells, and the total number of binding sites was the same in both cell types, which indicated that growth temperature affected NBMPR-binding activity in a similar fashion in both cell types. Therefore the elevation in nucleoside transport activity observed in NA9(33 °C) cells was not accompanied by an increase in the number of NBMPR-binding sites, suggesting a qualitative change in the nucleoside transport systems of the transformed cells.

Transport properties of normal and wild-type P130^{gag-fps}-transformed R2 fibroblasts

Studies with NA9 cells established that activation of *v-fps* PTK activity resulted in increases in both nucleoside and glucose

Fig. 1. Density-dependence of adenosine transport by R2 and C10 cells

R2 (○) and C10 (■) cells were plated at graded cell densities and assayed for uptake of 4 μM -adenosine 2 days later. Transport rates were determined as described in Table 1 from time courses of 3 to 15 s. Transport is plotted against the density of cultures at the time of the assay. The cell density at which R2 cells stopped proliferating is indicated by the arrow.

Fig. 2. Adenosine uptake by R2 and C10 cells

Uptake of 1 μM - (a) and 5 μM - (b) [^3H]adenosine by R2 (○, ●) and C10 (□, ■) cells $[(4-5) \times 10^5$ cells/dish] was determined in the absence (○, □) or presence (●, ■) of 500 nM-NBMPR as described in the Materials and methods section. Initial rates were estimated from tangents drawn to the initial portion of computer-generated best-fit curves, and were expressed as pmol/s per μl of cell water in the absence (–) or presence (+) of NBMPR. For 1 μM -adenosine, the rates were: R2(–), 0.2; R2(+), 0.1; C10(–), 0.3; C10(+), 0.1. For 5 μM -adenosine, the rates were: R2(–), 0.4; R2(+), 0.2; C10(–), 1.2; C10(+), 0.3.

Fig. 3. Concentration-effect relationships for NBMPR inhibition of adenosine uptake in R2 and C10 cells

Uptake of $4 \mu\text{M}$ -adenosine in a 5 s interval was measured at each of 10 concentrations of NBMPR in low-density cultures $[(4-5) \times 10^5 \text{ cells/dish}]$ of R2 ($\circ$) and C10 ($\bullet$) cells. Uptake is expressed as a percentage of that in control cells, which were incubated in the absence of NBMPR. Control transport rates were 3.4 ± 0.3 and $1.9 \pm 0.2 \text{ pmol/s per } \mu\text{l}$ of cell water for C10 and R2 cells respectively. Each point is the average calculated from five dishes. The Figure is representative of four similar experiments.

transport activity. Because of the effects of growth temperature on expression of NBMPR-binding sites in both R2 and NA9 cells, further studies were undertaken with the C10 line, a permanently established clonal derivative of R2 cells which expresses wild-type $\text{P130}^{\text{agg-fps}}$. Since R2 cells are normal and C10 cells are fully transformed at 37°C (Weinmaster *et al.*, 1984), nucleoside transport and NBMPR-binding activities could be examined in normal and *v-fps*-transformed cells without the additional complication introduced by culture temperature.

R2 cells, like other normal cultured fibroblasts, display contact inhibition of growth at cell densities at which the transformed C10 cells continue to proliferate. Since proliferative status might have effects on membrane transport processes that are unrelated to the activation of *v-fps* PTK activity, experiments were undertaken to determine densities for plating of R2 and C10 cells that yielded cultures with similar proliferative activities, and to determine if nucleoside transport activity was affected by culture density. R2 and C10 cells were plated at several different cell densities and initial rates of uptake of $4 \mu\text{M}$ -adenosine were determined 2 days later (Fig. 1). Both cell lines displayed similar marked decreases in adenosine influx at higher cell densities, suggesting that density is a major determinant of transport activity in both the transformed and normal cells. Subsequent analyses were carried out with cultures of low cell density (about $5 \times 10^5 \text{ cells/dish}$), at which all cell lines treated were proliferating.

Results from a typical set of experiments, which measured uptake of $1 \mu\text{M}$ - and $5 \mu\text{M}$ -adenosine by R2 and C10 fibroblasts, are illustrated in Fig. 2. At both concentrations, transport activity was greater (53 % and 200 % at 1 and $5 \mu\text{M}$ respectively) in the transformed cells than in the normal cells. These differences were not due to changes in membrane permeability, since the uptake of L-adenosine, which enters cells primarily by passive diffusion (Gati *et al.*, 1989), was similar in both R2 and C10 cells. (L-Adenosine, the enantiomer of D-adenosine, was used to

Fig. 4. Thymidine transport in R2 and C10 cells

Uptake of $50 \mu\text{M}$ - $[\text{^3H}]$ thymidine was measured in low-density cultures $[(3-4) \times 10^5 \text{ cells/60 mm dish}]$ of R2 ($\circ$) and C10 ($\square$) cells as described in the Materials and methods section. Transport rates were 9.4 and $3.6 \text{ pmol/s per } \mu\text{l}$ of cell water for C10 and R2 cells respectively.

measure membrane permeability in experiments that were similar to those of Fig. 2. Rates of uptake of $4 \mu\text{M}$ -L- $[\text{^3H}]$ adenosine by R2 and C10 cells were negligible and ranged from 0.01 to $0.03 \text{ pmol/per } \mu\text{l}$ of cell water.) Similarly, D-adenosine transport was nearly completely eliminated by addition of 1 mM non-radioactive thymidine to assays (results not shown). Both cell types exhibited at least two components of adenosine transport, since $1 \mu\text{M}$ -NBMPR did not completely inhibit uptake of adenosine.

Results from a large series of experiments with adenosine suggested that the relative levels of expression of NBMPR-sensitive transport differed between R2 and C10 cells and between NA9(39°C) and NA9(33°C) cells. For example, when transport rates were determined with $5 \mu\text{M}$ -adenosine (see Fig. 2), NBMPR-sensitive transport represented 48 % of total transport in R2 cells and 74 % in C10 cells. To better define these differences in the NBMPR-sensitive and NBMPR-insensitive components of transport in R2 and C10 cells, concentration-effect relationships were determined for NBMPR inhibition of uptake of $4 \mu\text{M}$ -adenosine during 5 s intervals (Fig. 3). Adenosine uptake was inhibited in both R2 and C10 cells at low NBMPR concentrations ($< 10 \text{ nM}$), with decreases in uptake of about 20–40 %, furthermore, as the NBMPR concentration was increased above 10 nM, the inhibition profiles for the two cell types became markedly different. In C10 cells, adenosine uptake was decreased substantially between 10 and 500 nM, with less than 20 % of activity remaining at 500 nM-NBMPR, whereas in R2 cells there was little, if any, change in adenosine uptake at NBMPR concentrations between 10 nM and $1 \mu\text{M}$. These results suggested that, although a small component of adenosine uptake in both R2 and C10 cells is highly sensitive to NBMPR, the major component in C10 cells is moderately NBMPR-sensitive and that in R2 cells is NBMPR-resistant. Since the literature describes equilibrative transport systems as either equilibrative-sensitive (es) or equilibrative-insensitive (ei), we refer to both the highly NBMPR-sensitive and the moderately NBMPR-sensitive components in C10 cells as es, and do not hereafter distinguish between the two components. Utilization of 500 nM– $1 \mu\text{M}$ -NBMPR in our experiments ensured that only NBMPR-insensitive transport would remain in both cell types.

To further evaluate transformation-related differences in

nucleoside transport activity, transport of thymidine was measured in R2 and C10 cells. Since R2 fibroblasts (and hence their derivatives) lack thymidine kinase activity (Topp, 1981), thymidine is a non-metabolized permeant. As was seen with adenosine, the transformed cells exhibited greater absolute rates of thymidine transport and a larger component of NBMPR-sensitive thymidine transport than normal cells (Fig. 4). A decrease in thymidine transport rates at higher culture densities was also observed (compare rates obtained for the low-density cultures of Fig. 4 with those for the high-density cultures of Table 3).

Table 3. Thymidine transport in high-density cultures of R2 and C10 cells

Transport rates for 50 μM -[^3H]thymidine were estimated from time courses of uptake from 3 to 15 s in high-density cultures of R2 cells (1.5×10^6 cells/dish) and C10 cells (6×10^6 cells/dish) in the absence or presence of 1 μM -NBMPR. Transport rates (pmol/s per μl of cell water) were calculated from time courses in the absence (total) or presence (ei) of NBMPR; the NBMPR-sensitive component (es) is the difference between total and ei.

Cells	Transport (pmol/s per μl)		
	Total	ei	es
R2	3.1 ± 0.4	0.8 ± 0.07	2.3 ± 0.5
C10	4.4 ± 0.5	0.5 ± 0.03	3.9 ± 0.5

Fig. 5. Scatchard analysis of binding of NBMPR to R2 and C10 cells

A representative experiment in which site-specific binding of NBMPR to cultures (5×10^5 cells/60 mm dish, in duplicate) of R2 (a) and C10 (b) cells is shown. Cultures were incubated for 30 min with graded concentrations of [^3H]NBMPR in the presence and absence of 10 μM non-radioactive NBMPR, and specific binding was calculated as described in the Materials and methods section. Kinetic parameters: (a) K_d 0.1 nM, B_{max} 110000 sites/cell; (b) K_d 0.1 nM.

Fig. 6. Photoaffinity labelling of R2 and C10 membranes with [^3H]NBMPR

Membrane proteins were separated on SDS/PAGE (12% gels) and gel slices were analysed as described in the Materials and methods section. ^3H counts incorporated into membrane proteins are plotted as a function of migration distance (slice number) for R2 ($\circ$, $\bullet$) and C10 ($\square$, $\blacksquare$) membranes that were photolabelled in the absence ($\circ$, $\square$) or presence ($\bullet$, $\blacksquare$) of excess (5 μM) unlabelled NBMPR. The migration positions of standard molecular mass markers are shown at the top of the diagram, and were used to estimate the apparent molecular masses of photolabelled species.

Interaction of NBMPR with normal and wild-type P130^{cas-fps}-transformed R2 fibroblasts

If the difference in NBMPR-sensitive nucleoside transport activity between C10 and R2 cells was due to a difference in the number of NBMPR-sensitive transport elements, C10 cells should exhibit greater numbers of high-affinity NBMPR-binding sites than R2 cells, assuming that NBMPR-binding sites represent functional transporters. High-affinity binding of [^3H]NBMPR to C10 and R2 cells is shown in Fig. 5. Although C10 cells exhibited fewer total binding sites (0.79×10^5 sites/cell) than R2 cells (1.1×10^5 sites/cell), since C10 cells were considerably smaller than R2 cells, the overall binding-site densities per unit cell volume of the two cell types were almost identical (At 5×10^5 cells per dish, R2 and C10 cells respectively had volumes of 2.3 μl and 1.3 μl /10⁶ cells, yielding binding site densities of 5.5×10^{10} and 6.1×10^{10} sites/ μl). Thus, although the binding-site densities of the two cell types were the same, C10 cells had higher rates of NBMPR-sensitive transport than R2 cells, indicating that the relationship between numbers of NBMPR-binding sites and functional NBMPR-sensitive nucleoside transport elements is complex.

Although the NBMPR-binding properties of R2 and C10 cells were similar, it was possible that the NBMPR-sensitive transport elements differed structurally in normal and transformed cells. NBMPR-binding polypeptides were identified in plasma membrane fractions of R2 and C10 cells by site-specific photolabelling with [^3H]NBMPR (Fig. 6). The electrophoretic profiles of the photolabelled material from R2 and C10 membranes were similar, with a relatively broad major peak (consisting of polypeptides of 55–75 kDa) being specifically labelled in both cell types. Lower-molecular-mass species, probably proteolytic

fragments of the major NBMPR-binding protein(s), were also labelled with [^3H]NBMPR. NBMPR-binding polypeptides of similar apparent molecular mass (62–80 kDa) have been identified in rat erythrocytes and various rat cell lines (Jarvis & Young, 1986; Gati & Paterson, 1989).

Hexose transport in R2 fibroblasts expressing wild-type P130^{gag-fps}

Results of numerous earlier studies (Hatanaka *et al.*, 1969; Hatanaka & Hanafusa, 1970; Kawai & Hanafusa, 1971; Kletzien & Perdue, 1974) have led to the general conclusion that glucose transport is elevated in transformed cells. In our own experiments hexose uptake was measured over short (3–30 s) and extended (1–10 min) time periods. For 2-deoxyglucose, C10 cells exhibited higher initial rates of uptake than R2 cells (13 and 4.2 pmol/s per μl of cell for C10 and R2 cells respectively). These studies also demonstrated that short time courses (3–30 s) provided better estimates of transport rates than uptake curves generated over 1–15 min. Transport of the non-metabolized hexose 3-*O*-methylglucose was 2–3-fold higher in C10 cells than in R2 cells. The results with 3-*O*-methylglucose confirmed the hypothesis that R2 fibroblasts transformed by wt P130^{gag-fps} have increased hexose transport rates relative to their normal counterparts.

DISCUSSION

This study evaluated the effects of transformation by *v-fps* on membrane transport processes in Rat-2 fibroblasts. We have shown that nucleotide transport processes can indeed be regulated in response to changes in culture density or as a result of oncogenic transformation. The temperature-sensitive *v-fps* mutant, NA9, expresses P130^{gag-fps} with thermolabile PTK activity because of a mutation in the non-catalytic SH2 domain, which regulates interaction of the kinase with host cell proteins (Sadowski *et al.*, 1986). Regardless of the culture temperature (39, 37 or 33 °C), wild-type levels of P130^{gag-fps} are expressed in NA9 cells, but only at the permissive temperature (33 °C) does temperature-sensitive P130^{gag-fps} possess significant PTK activity. Activation of PTK activity in NA9 cells by growth at 33 °C was sufficient to induce phenotypic changes characteristic of transformation and was accompanied by substantial increases in transport of both nucleosides and hexoses.

R2 fibroblasts, like other rat cell types (Plagemann & Wohlhueter, 1985; Jarvis & Young, 1986; Gati & Paterson, 1989), possess at least two types of nucleoside transport: the NBMPR-sensitive route(s) are inhibited by nanomolar (1–500 nM) concentrations of NBMPR, and the NBMPR-insensitive route functions in the presence of micromolar concentrations of NBMPR. Entry of adenosine by both routes appeared to be largely mediated, since uptake of the enantiomer L-adenosine was negligible and D-adenosine transport was almost completely inhibited by thymidine. R2 fibroblasts had approx. 10^5 NBMPR-binding sites per cell, with high affinity ($K_d = 0.1$ nM) for NBMPR, and polypeptides of molecular mass 55–75 kDa were specifically photolabelled with [^3H]NBMPR. NBMPR-binding polypeptides mediate nucleoside transport in human erythrocytes (Kwong *et al.*, 1988), and it is likely that the 55–75 kDa polypeptides identified by photolabelling with NBMPR are involved in transport via the high-affinity NBMPR-sensitive route in R2 fibroblasts.

Transport of adenosine and thymidine was decreased at higher cell densities in both normal R2 cells and transformed C10 cells. These density-dependent changes may be responses regulated by physical contact between cells or through signalling molecules that are altered as cultures become more crowded. Since cell size decreased at higher densities, it is also possible that volume

changes regulated transport rates. The effects of density on transport rates necessitated careful control of culture conditions, especially in experiments that compared transport processes of normal and transformed cells.

The NBMPR-sensitive component of adenosine transport was elevated to a greater extent than the NBMPR-insensitive component in both C10 and NA9(33 °C) cells, relative to R2 and NA9(39 °C) cells, suggesting that the mechanism up-regulating nucleoside transport activity during transformation preferentially stimulated the NBMPR-sensitive system. Concentration–effect profiles for inhibition of adenosine transport by NBMPR revealed a highly NBMPR-sensitive component in both cell types and an additional moderately NBMPR-sensitive component in C10 cells that was not seen in R2 cells. The physical relationship between these components, which are defined in terms of their sensitivity to NBMPR, is unknown, but is likely to be complex. It was not possible to define these components in terms of kinetic parameters. There was no evidence of Na⁺-dependent nucleoside transport systems in Rat-2 fibroblasts, since adenosine transport rates were unaffected by omission of Na⁺ from the assay medium for both the normal and the transformed cells (results not shown).

The binding-site densities for NBMPR were the same in R2 and C10 cells and were affected equally by culture temperature in R2 and NA9 cells. NBMPR is a small hydrophobic molecule that partitions into membranes, and some of the binding sites detected in the binding assay with intact cells may have been internal. NBMPR has proven to be an excellent probe for the study of the nucleoside transporter of human erythrocytes, as these cells possess a single transporter type and lack internal membrane-bound organelles. In the present study we found that differences in NBMPR-sensitive transport activity between normal and transformed cells were not accompanied by differences in NBMPR-binding site densities, indicating that NBMPR-binding sites are not equivalent to functional NBMPR-sensitive transport elements. C10 and NA9(33 °C) cells may differ from R2 and NA9(39 °C) cells in the relative proportions of intracellular and plasma-membrane binding sites, resulting in differences in the number of functional NBMPR-sensitive transporter elements in the plasma membranes of the normal and transformed cells. It is also possible that the increased nucleoside transport activity following transformation was due to increased capacity of existing transport elements. Definition of the transport components present in R2 fibroblasts at the molecular level will probably be necessary before we can satisfactorily explain the differences in sensitivity to NBMPR of adenosine and thymidine transport seen with transformation.

In Rat-2 fibroblasts, regulation of nucleoside transport processes appears to occur, both in response to changes in cell density and as a result of oncogenic transformation. Thus nucleoside transport elements may be more than constitutive carrier molecules, but may indeed play important roles in developmental and differentiation processes. As immunological and molecular probes become available, we will be able to examine the mechanism(s) by which transformation and culture density regulate nucleoside transport. Also, it will be of interest to examine whether other cytoplasmic PTKs besides *v-fps* or other oncoproteins have similar effects on nucleoside transport activity and whether such changes are associated with altered sensitivity of transformed cells to nucleoside drugs.

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