Journal of Cellular Biochemistry 36:249–260 (1988)
Growth Regulation of Cancer 97–108

Effect of Mutations Affecting Na^+:H^+ Antiport Activity on Tumorigenic Potential of Hamster Lung Fibroblasts

Alain E. Lagarde, Arlette J. Franchi, Sonia Paris, and Jacques M. Pouysségur

Mount Sinai Hospital Research Institute, Division of Cancer and Cell Biology, and Department of Medical Genetics, University of Toronto, 600 University Avenue, Toronto, Ontario, M5G 1X5, Canada (A.E.L.); Centre de Biochimie, CNRS, Université de Nice, Parc Valrose, 06034 Nice, France (A.J.F., S.P., J.M.P.)

Mutants unable to regulate intracellular pH through the Na^+:H^+ antiport system were found to evolve tumors less frequently than wild-type CCL39 hamster lung fibroblasts, after transplantation in athymic nude mice. When rare tumors arose, they comprised cells which were transformed in vitro, but which upon retransplantation grew at a lower rate than tumor cells originating from CCL39 cells. Both parental and mutant cells became transformed after transfection of the activated Harvey *ras* oncogene, but transfectants derived from the mutants had a weaker tumorigenic potential. These results suggest that transformed characteristics can be acquired independently from the Na^+:H^+ antiporter. However, the presence of this system provides a selective growth advantage when cells are confronted with natural environments, as it occurs during the expansion of tumors in a host.

Key words: Na^+:H^+ antiport, pH regulation, cell proliferation, neoplastic transformation

It is well established that an amiloride-sensitive Na^+:H^+ antiport system catalyzes the reversible and electroneutral exchange of Na^+ and H^+ ions across the plasma membrane of mammalian cells [1]. This system allows intracellular pH (pHin) to be maintained within narrow values compatible with cell viability and optimal proliferation [2]. It also becomes activated shortly after quiescent cells are stimulated to divide in response to growth factors or tumor-promoting phorbol esters [3–7]. The

Abbreviations used: pHin, intracellular pH; pHo, extracellular pH; THR, α-thrombin; EGF, Epidermal growth factor; INS, insulin; DME/F12, Dulbecco's modified minimal essential medium/Ham's F12 medium; FCS, fetal calf serum; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid; Mes, 2-N-morpholino ethane sulfonic acid; Mops, 3-N-morpholino propane sulfonic acid; DMA, 5-N,N′-dimethylamiloride; DIDS, 4,4′-diisothiocyanostilbene-2,2′disulfonic acid.

Received February 19, 1987; revised and accepted August 18, 1987.

modest but persistent pHin alkalinization which results from this activation is believed to trigger rate-limiting reactions implicated in the conversion of growth factor signals into a proliferative response [8]. However, in bicarbonate-buffered medium, pHin may also be adjusted through the coordinate action of carbonic anhydrase and of a Na^+-dependent HCO_3^-:Cl^- exchange system [9,10]. It has therefore been argued that a functional Na^+:H^+ antiport is not an essential component of the signal transducing pathways conducting the mitosis [11–13].

Methods were recently developed to isolate Na^+:H^+ antiport-defective mutants from Chinese hamster lung fibroblasts (CCL39) [14], mouse L cells [15], and pig kidney epithelial cells [16]. The division of mutants of the CCL39 cell line was found to obey the same dependence on extracellular pH (pHo) as their parent, in regular HCO_3^-/CO_2-buffered medium [2]. However, in the absence of bicarbonate, these mutants needed to be exposed to pHo values above 7.2, instead of 6.8, before they could be recruited to proliferate in response to factors such as α-thrombin (THR) and insulin (INS) [2,14]. In addition, they could not compensate for acute intracellular acidifying conditions with enough efficiency to prevent the process from becoming lethal [12].

In this study we examined the consequences that the loss of a functional Na^+:H^+ antiport activity might have on the acquisition of malignant attributes and on the development of tumors. We found that mutants unable to catalyze Na^+:H^+ exchange implanted less successfully in nude mice or grew at a slower rate than cells whose transport activity was intact. This trend was observed regardless of whether or not the cells had inherited an elevated anchorage-independent growth potential, a reduced requirement for growth factors, or were transformed with an activated *ras* oncogene. We conclude that if the Na^+:H^+ antiporter plays no crucial role in the transformation process, it does confer a selective advantage to malignant tumor cells proliferating in situ.

MATERIALS AND METHODS

Cell Lines

The growth factor dependence and malignant evolution of the Chinese hamster lung fibroblast CCL39 cell line (ATCC) were described in previous reports [17–19]. 39T10 is a malignant cell line which was isolated from a tumor growing at the site of subcutaneous (sc) inoculation, after transplanting CCL39 cells into athymic nude mice [17]. PS33, PS200, and PS120 are three mutants which were obtained after mutagenic treatment of CCL39 fibroblasts, followed by selection under conditions which specificially eliminate cells mediating Na^+:H^+ exchange [14]. The pHO6T1 plasmid was transfected into CCL39 and PS120 cells by Ca^{++}-phosphate precipitation, followed by selection in G418 (400 μg/ml)-containing medium, and 39ras5, 120ras1, and 120ras5 colonies were isolated. This vector contains the 6.6-kilobase (kb) Harvey *ras* oncogene of the human EJ bladder carcinoma, flanked by two potent viral enhancers, as well as the *neo*-resistance marker [20]. Expression of the human Ha-*ras* gene in the transfectants was verified by immunoprecipitation of the p21 protein with a specific antibody (Triton Biosciences Inc., Alameda, CA). Karyotype analysis indicated that all cell lines retained the same modal chromosomal number (2n = 22) as diploid CCL39 cells.

Tissue Culture Conditions

Cells were routinely propagated in DME/F12 medium (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine, 2 g/liter sodium bicarbonate, 5 μg/ml human transferrin, and 10% FCS, without antibiotics. In experiments designed to test individual growth factors, FCS was either omitted (serum-free medium, SFM) or substituted with bovine insulin (INS 10 μg/ml), mouse EGF (10 ng/ml), or human α-thrombin (THR, 1 U/ml) from Sigma Chemical Co. (St. Louis, MO). Cells were plated in FCS-containing medium to allow attachment, and were subsequently rinsed twice with SFM before being exposed to purified factors on day 0. Cell counts were determined after a 6-day incubation period at 37°C in a humidified 5% CO_2–95% air atmosphere. The mean number of cell population doublings occurring between day 0 and 6 was calculated from duplicate cultures.

Cell growth in HCO_3^-/CO_2-free medium was followed at daily intervals by substituting sodium bicarbonate with 30 mM Hepes buffer adjusted to pH 7.0, 7.4, and 7.8. These media also included hypoxanthine (50 μM), uridine (50 μM), and dialyzed FCS (10%) and were replaced every 2 days, as described previously (14).

Colony Formation in Agarose

Eight thousand cells resuspended in SFM were mixed with 0.3% agarose (Type I, Sigma) and the appropriate concentrations of FCS (0 to 10%) and layered on a 0.5% agarose basal layer in 35-mm Petri dishes. Duplicate dishes were incubated for 8 days at 37°C in a humidified atmosphere of 5% CO_2–95% air. Colonies were scored under a microscope using a calibrated grid. Results were expressed as a percentage of colonies formed per seeded cell.

Measurement of DNA Synthesis

Cells inoculated in 24-well tissue culture plates were starved for 24 hr before being exposed to bicarbonate-free medium supplemented with dialyzed FCS (10%) and [methyl-^{3}H] thymidine (Amersham Corp., Oakville, ON, Canada; 1 μCi/ml, 2 μM). The amount of radioactivity incorporated into trichloroacetic acid-precipitable material during the next 24 hr in a CO_2-free incubator was determined by liquid scintillation counting. In this assay, which measures the re-initiation of DNA synthesis following G_0-arrest, the pH of the medium was initially adjusted with 30 mM of either Mes (pH = 6.25 to 6.7) or Mops (pH = 7.0 to 8.2), as described earlier [2,14].

Tumorigenicity Assays

Inocula comprising 1, 5, or 10 × 10^5 viable cells suspended in 0.2 ml phosphate-buffered saline were injected subcutaneously (sc) into the flank of 1-month-old BALB/c (*nu/nu*) mice. The presence of a progressively growing and localized tumor was assessed twice a week by palpation. Its size was measured with a ruler at weekly intervals thereafter. When tumors reached a size equivalent to 10 cm^3, the animals were sacrificed and inspected for the presence of macroscopic metastases in the lungs.

Measurement of Na^+ Uptake and Intracellular pH

The rate of $^{22}Na^+$ uptake driven by an outwardly directed proton efflux, was estimated exactly as described previously [12,14]. Conditions for measuring pHin

variations in response to NH_4^+-induced cytoplasmic acidification were also reported earlier [10].

RESULTS

Tumorigenic Potential of CCL39 Variants Exhibiting Altered Na^+:H^+ Antiport Activity

Earlier observations demonstrated that CCL39 fibroblasts were not tumorigenic at the onset since their injection in the peritoneal cavity or the tail vein of athymic nude mice resulted in no tumor formation [18]. However, inoculation of CCL39 cells at a sc site allows the outgrowth of rare variants which have inherited a complete transformed phenotype [17–19]. Accordingly, a relatively long latency period (~1 month) precedes the emergence of tumors in CCL39-injected animals, as shown in Figure 1. To address the question of whether the loss of a functional Na^+:H^+ antiport activity would affect this clonal evolution process, three independent mutants were characterized.

PS33, PS200, and PS120 mutants were isolated using a procedure which counterselects cells undergoing an acute and persistent intracellular acidification through the Na^+:H^+ antiport system [14]. Unlike PS200 and PS120 cells, PS33 cells retained some 30–40% normal antiport activity presumably because only one instead of two alleles of the responsible gene had been inactivated in this mutant [14]. As illustrated in Figure 1, sc injection of these mutants into nude mice led to the formation of tumors in 80% (PS33), 25% (PS120), and 0% (PS200) of the animals, respectively. Increasing the size of the inocula by a factor of ten did not enhance the incidence of tumors, and did not shorten their time of appearance. These findings suggest that unlike cells which are fully (CCL39) or partially (PS33) competent for mediating Na^+:H^+ exchange, mutants completely devoid of activity are not (PS200) or less frequently (PS120) converted into malignant variants once they are confronted with in vivo environments prevailing in nude mice.

As summarized in Table I, the growth characteristics of mutant and parental cells in tissue culture did not differ appreciably. In defined medium containing bicarbonate (pHo = 7.4) they arrested easily in G_0 upon removal of growth factors, and the same relative numbers of cell population doublings were induced in response to either serum, THR or THR and INS. EGF, with or without INS, appeared to be

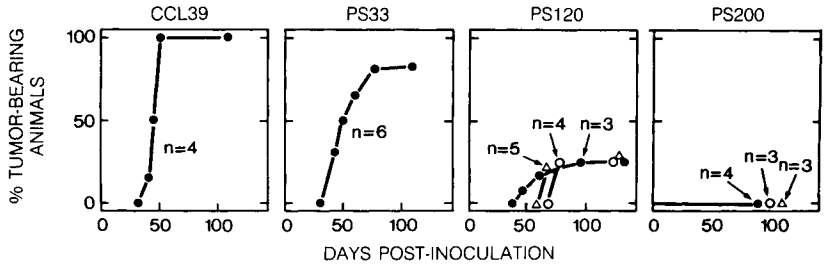

Fig. 1. Tumorigenicity of antiport-defective CCL39 mutants. Single cells suspensions containing 1× (●), 5× (○), and 10 × 10^5 cells (△) were injected subcutaneously into the flank of 4–6-week-old Balb/c athymic nude mice. The animals were followed on a weekly basis for the emergence of tumors growing progressively at the site of inoculation. n = number of animals inoculated per group.

TABLE I. Growth Properties of Wild-Type and Mutant Cell Lines*

Medium composition	CCL39	PS33	PS120	PS200	39T10	PS120-T1
No. of cell population doublings						
SFM	−0.5	−0.9	0.5	−0.8	2.9	1.8
INS (10 μg/ml)	1.1	−0.3	1.3	0.2	3.0	3.8
EGF (10 ng/ml)	2.2	0.2	0.6	−0.2	5.4	3.0
THR (1 U/ml)	3.9	3.4	3.2	2.3	5.5	4.3
INS + EGF	4.8	1.4	2.0	1.6	5.8	5.2
INS + THR	4.5	4.9	3.5	3.7	5.8	5.1
Serum: (0.1%)	0.9	0.2	2.1	0.1	5.2	3.2
(5%)	5.2	4.3	4.5	5.3	5.9	5.7
% colonies in agarose						
Serum: (0%)	0	0	0	0	0.5	0.1
(1%)	0	0	0	0	8.0	3.5
(10%)	2.0	1.5	0.5	1.4	27.0	32.0

*In normally bicarbonate buffered medium (pHo = 7.4) as described in Materials and Methods.

less efficient in promoting the division of PS120 and PS200 cells, but this was also observed for PS33 mutants. The mutants had the same limited capacity to grow as spheroids in semisolid agarose as parental cells. The slightly lower plating efficiencies manifested by the mutants are within the range of clonal variability observed in CCL39 cell populations. The only discernible difference between PS200, PS120, and PS33 and CCL39 cells resides in the inability of the former two mutants to re-initiate DNA synthesis in response to growth factor stimulation when the extracellular pH is below 7.2 [2,14]. As illustrated in Figure 2, at this pHo value CCL39 cells replicated DNA at 30% of its optimal level, whereas PS120 cells had to be exposed to pHo = 7.6 in order to reach a comparable rate of division. Thus, we presume that the nature of the environments prevailing in nude animals is such that antiport-defective mutants do not survive or proliferate as well as competent cells. Consequently, the spontaneous emergence of transformed variants from these mutants might be handicapped, thus explaining the rarity with which tumors arose and progressed. When tumors arise occasionally, as is the case in some PS120-injected animals (Fig. 1) they should comprise cells having selectively overcome conditions that restrict the process of malignant transformation. To examine this question we establish cell lines from PS120 and CCL39 tumors and we compared their characteristics.

Malignant Properties of Tumor Cell Lines Derived From Na^+:H^+ Antiport-Competent Versus -Deficient Cells

As shown in Figure 3, when PS120 tumor-derived cells (PS120-T1) were re-implanted into nude mice, tumors formed in 100% of the animals. These tumors arose not only systematically but also earlier than those induced in PS120- or CCL39-injected mice (Fig. 1). They progressed steadily to reach a median size of ~3 cm^3 after 50 days. Likewise, CCL39 tumor-derived cells (39T10) produced tumors which expanded rapidly but which eventually attained a tenfold larger size (Fig. 3). This significant difference in the rate at which 39T10 and PS120-T1 tumors progressed might reflect the intrinsically weaker growth potential of PS120-T1 tumor cells which cannot regulate pHin through the Na^+:H^+ antiport system.

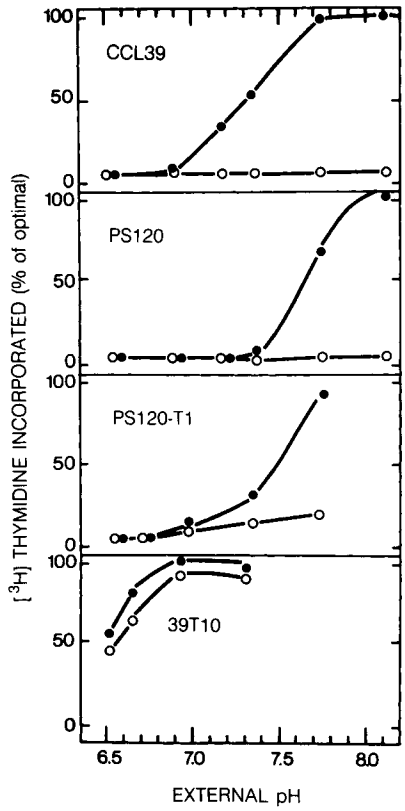

Fig. 2. pH dependence of the rate of DNA synthesis in CCL39, PS120, PS120-T1, and 39T10 cell lines. Cells were exposed to SFM for 24 hr in HCO_3^-/CO_2^- buffered medium, pHo = 7.4. They were then exposed to bicarbonate-free medium buffered with Mes or Mops, containing [^{3}H]thymidine, with (●) or without (○) 10% dialyzed serum. The amount of radioactivity incorporated 24 hr later was determined.

Results listed in Table I indicate that both 39T10 and PS120-T1 cells inherited features commonly associated with the transformed phenotype. They escaped G_0-arrest controls and therefore divided at a substantial rate in serum-free medium; they responded better to INS, EGF, or THR than CCL39 and PS120 parental cells; and they formed colonies in agarose at a ~20-fold higher frequency. However, PS120-T1 differed from 39T10 cells in that their rate of DNA replication (Fig. 2) and proliferation (Fig. 4A) were submitted to the same pHo controls as their PS120 progenitors. Under the same conditions (i.e. in bicarbonate-free medium), 39T10 tumor cells replicated DNA between pHo 6.5 and 7.5, regardless of whether or not they were stimulated by growth factors (Fig. 2). Therefore external pH had a much greater restrictive influence on the division of PS120-T1 cells than on that of 39T10 cells.

Additional experiments depicted in Figure 4B and 4C confirm the absence of a functional $Na^+:H^+$ antiport system in PS120-T1 cells. Firstly these cells were unable to rapidly re-establish an initial pHin value following NH_4^+-induced acid load (Fig. 4B). CCL39 cells were shown previously to readjust acidification [12]. In both PS120 and PS120-T1 cells, protection against a persistent cytoplasmic acidification was

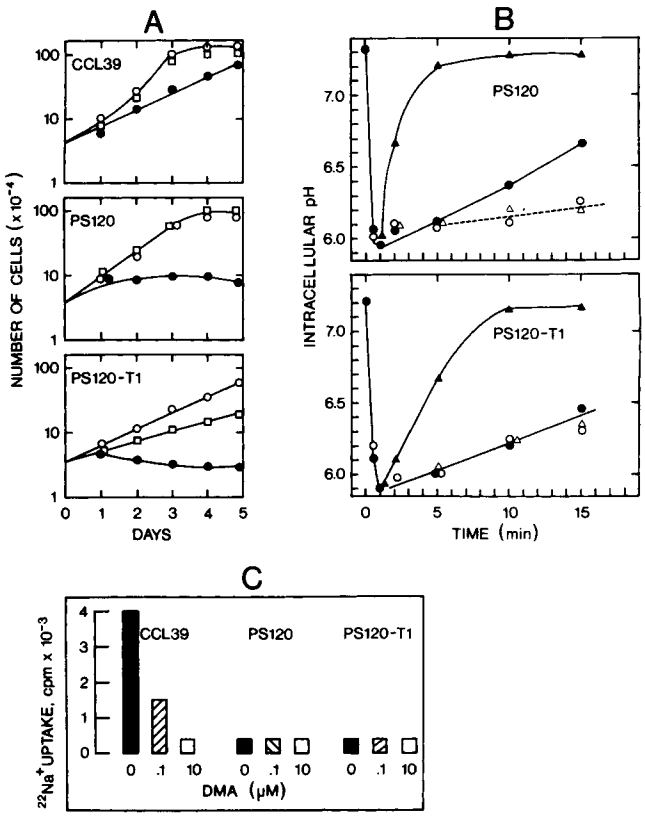

Fig. 3. Properties of PS120 and PS120-T1 cell lines. **A:** Cell proliferation in bicarbonate/CO_2-free medium buffered at pHo 7.0 (●), 7.4 (□), and 7.8 (○). Medium supplemented with 10% dialyzed fetal calf serum, hypoxanthine, uridine, and appropriate buffer solutions was changed on day 0, 2, and 4, as described previously [14]. **B:** Kinetics of pHi recovery following NH_4^+-induced intracellular acidification in the absence (●) and presence of 1 mM DIDS (○), 5 mM $NaHCO_3$ (▲), 5 mM $NaHCO_3$ and 1 mM DIDS (△). Cells were pre-incubated with 20 mM NH_4Cl for 45 min. At time 0, extracellular NH_4^+ was removed and radiolabelled benzoic acid intracellular content was measured at time intervals to determine pHi as described previously [10]. **C:** Rate of $^{22}Na^+$ influx in CCL39, PS120, and PS120-T1 cells in the absence and presence of 5-N, N′-dimethylamiloride (DMA). Conditions for measuring $^{22}Na^+$ uptake driven by H^+ efflux were described previously [14].

afforded through the Na^+-dependent HCO_3^- exchange system [10], as indicated by experiments involving addition of bicarbonate ions (Fig. 4B). This protection was abolished when the specific inhibitor of the anionic exchange system, DIDS, was added in conjunction with bicarbonate (Fig. 4B). Secondly, no amiloride-sensitive $^{22}Na^+$ uptake could be detected in PS120 or PS120-T1 cells (Fig. 4C). These findings indicate that the oncogenic events which were at the origin of the PS120-T1 tumor did not involve the partial recovery of a functional Na^+:H^+ antiport activity, or the expression of another transport system possessing an equivalent pH-regulating function. Instead, in the course of their first passage into the animal, PS120 cells acquired an enhanced proliferative potential whose expression remains restricted by adverse environmental conditions requesting appropriate pHin regulation.

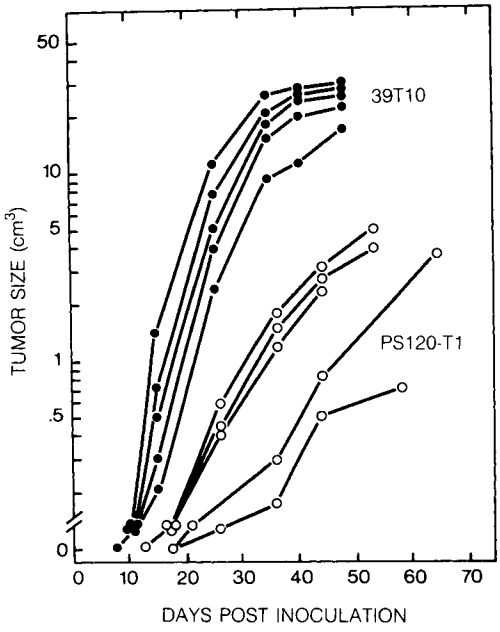

Fig. 4. Tumorigenicity of 39T10 and PS120-T1 tumor-derived cells. 1×10^5 cells were injected sc into groups of six Balb/c nude mice, and tumor size was measured at regular intervals.

Properties of CCL39 and PS120 Harvey ras Transfectants

The possibility that the differential tumorigenic potential of 39T10 and PS120-T1 cells resulted from the deregulation of unrelated oncogenes rather than from their distinct ability to respond to pH variations could not be excluded. We therefore investigated whether the deliberate introduction of a potent oncogene into CCL39 and PS120 cells would confer upon them similar transformed characteristics. A plasmiol vector encoding the activated form (VAL^{12} mutation) of the human Ha-*ras* oncogene was transfected into CCL39 and PS120 fibroblasts, and three neomycin-resistant colonies—39ras5, 120ras1, and 120ras5—were characterized. Immunoprecipitation experiments of p21 proteins indicated that 120ras5 cells express ~5-fold less *ras* products than 39ras5 and 120ras5 (not shown). This level was nevertheless tenfold higher than in CCL39 or PS120 cells because of the strong viral promoters driving the *ras* gene [20]. All three transfectants were transformed according to conventional criteria (Fig. 5): they were highly refractile and displayed altered morphological features; they cloned in semisolid agarose with an elevated frequency; and they divided in the absence of growth factor stimulation. However, whereas sc inoculation of 39ras5 cells into nude mice led to the systematic formation of tumors growing at a rate of ~5 cm^3 per month, tumors formed in only 75% and 50% of the cases in 120ras5 and 120ras1-injected animals, respectively (Fig. 5). Furthermore, some of the latter tumors grew for approximately a month and subsequently regressed so that the overall incidence of persisting tumors was respectively 100% (39ras5), 50% (120ras1), and 25% (120ras5). Another noticeable difference between the two classes of *ras*-transfectants was that 39ras5 tumors were invasive and disseminated to the lungs, whereas only one animal among those injected with 120ras1 and 120ras5 cells showed pulmonary metastases, after 60 days. Histological sections of 120ras1 and

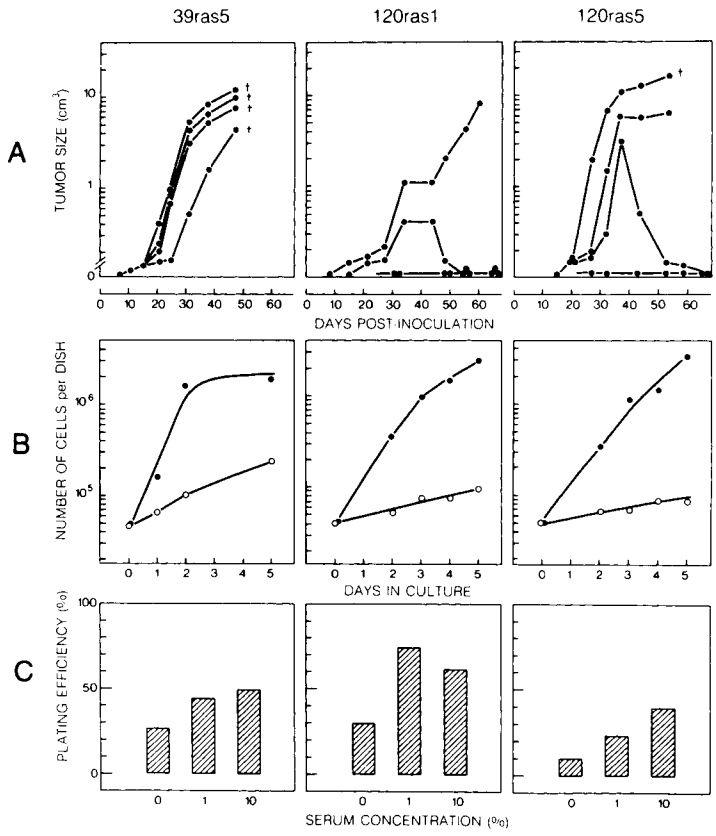

Fig. 5. Properties of 39ras5, 120ras1, and 120ras5 transfected cell lines. **A:** Tumorigenic potential. 1×10^5 cells were injected sc into groups of four nude mice, and tumor size was measured at regular intervals. Pulmonary metastases were detected in all 39ras5-injected animals at day 50, but in only one 120ras5-injected mouse (†). **B:** Proliferation in serum-free (○) and serum-containing medium (●) buffered at pHo = 7.4 with bicarbonate-CO_2. Serum was removed at day 1, and on day 0 monolayers were fed with either SFM or 5% serum. Cultures were trypsinized at daily intervals and cell numbers were determined. Mean of duplicated determinations is shown. **C:** Percentage colonies forming in semisolid agarose supplemented with 0%, 1% and 10% serum.

120ras5 tumors at day 50 revealed extensive zones of necrosis extending from the center to the periphery. Therefore, despite the fact that transfection of the *ras* oncogene induced similar transformed characteristics in parental and mutant cells the tumor-forming ability of cells unable to mediate Na^+:H^+ exchange was comparatively lower.

DISCUSSION

The mechanisms by which the Na^+:H^+ antiporter regulates the intracellular pH of mammalian cells are receiving considerable attention [1]. In this study we attempted to confirm the role previously attributed to this system in the pH control of cell proliferation [2,21]. To this end we took advantage of the recent isolation of

fibroblast mutants unable to catalyze Na^+:H^+ exchange [14] to examine whether such a defect would affect the extent of cell division, as determined in an in vivo context. We compared preneoplastic, malignant, and *ras*-transformed CCL39-cell lines, lacking or not Na^+:H^+ antiport activity, for their ability to evolve tumors after implantation into nude mice.

We first showed that two mutants (PS200, PS120) induced tumors less frequently than their CCL39 parent. We believe the significantly lower tumor incidence is directly accountable for the complete lack of transport activity since another mutant (PS33) which had retained residual activity was nearly as tumorigenic as CCL39 cells (Fig. 1). However, we ignore the reasons that PS200 did not form tumors at all, whereas PS120 cells did so occasionally. We found no discernible difference between these two mutants concerning their rate of proliferation in response to growth factors or in semisolid media (Table I). They similarly failed to take up $^{22}Na^+$ ions in an amiloride-sensitive manner, and did not replicate at external pHs below 7.2, two properties associated with the loss of a functional Na^+:H^+ antiport activity [2,14]. We noticed that PS200 and PS120 mutants were characteristically less responsive to EGF at concentrations which are weakly mitogenic to normal CCL39 cells. This observation is unlikely to explain the modest tumorigenic potential of these mutants, since PS33 cells were also less responsive to EGF, but were nevertheless able to form tumors. Fewer EGF binding sites (50% less than normal) were detected on the membrane of PS120 cells, but it was demonstrated that the process of ligand internalization was not impaired in such a mutant [22]. The tumorigenic potential of CCL39 fibroblasts was previously shown to reside in the propensity of these cells to generate authentic malignant variants at low frequency when implanted at a sc site in nude mice [17,18]. We speculate that conditions prevailing in this particular environment are poorly permissive to mutant cells which require either high concentrations of Na^+ and bicarbonate ions, or a slightly alkaline medium (pHo $>$, 7.2) [2,14] in order to undergo a minimum number of divisions before malignant variants arise.

The lack of antiport activity did not preclude cells from acquiring a completely transformed phenotype. Not only did rare PS120-derived tumors such as PS120-T1 comprise cells which manifested transformed features in vitro (Table I), but mutant cells could also become transformed after transfection of the Ha-*ras* oncogene (Fig. 5). However, despite the fact that PS120-T1, 120 ras1, and 120ras5 cells inherited an increased capacity to grow without anchorage and partially escaped growth factor requirements, their tumorigenic potential was comparatively weaker than that of 39T10 and 39ras 5 cells. If PS120-T1 cells initiated tumors much earlier and more frequently than PS120 cells, these tumors expanded less rapidly than 39T10 cells which were not impaired in the ability to exchange Na^+ and H^+ ions (Fig. 2). Likewise, tumors arose less frequently and did not always persist in animals challenged with *ras*-transformed antiport-less mutants, whereas they were extremely aggressive in mice receiving implants of *ras*-transformed competent cells (Fig. 5). We believe these differences reflect the distinct fate of cell populations that can or cannot overcome exposure to low pH values. It is well documented that hypoxia and acidosis develop in rapidly growing tumors, and that these factors are in part responsible for necrosis [23,24]. Direct measurements using microelectrodes [25] and telemetry capsules [26] have revealed that the intratumoral pH of some tumors may be as low as 6.3 ± 0.1. Such values are clearly below the threshold (pHo = 6.8) at which fibroblasts can divide [2,27]. Antiport-deficient cells whose threshold pH is

even more alkaline (pHo = 7.2) would conceivably be vulnerable even under less severe conditions. The excessive production of lactic acid by tumor cells is recognized to be responsible in part for the marked acidification of their immediate environment [28]. This process has clearly been shown to be one of the metabolic consequences of activation of the *ras* oncogene [29]. Levels of lactate production would have to be compared among the cell lines that we characterized to determine whether there is any correlation between the stage of transformation, the extent of acidification due to lactic acid release, and the kinetics of tumor expansion in vivo.

The present report strongly suggests that the $Na^+:H^+$ antiporter is a limiting factor in cell proliferation by virtue of its ability to maintain a steady pHin under conditions when other pHin-regulating systems operate less efficiently. Its controls apply to normal as well as to neoplastically transformed cells. The recent suggestion that this exchange system contributes to the development of the malignant phenotype [27] is contradicted by our observation showing that CCL39 fibroblasts can acquire this phenotype even when this function is deleted by mutations. We also obtained evidence that CCL39 mutants which overexpress the $Na^+:H^+$ antiport activity [30] do not inherit a selective advantage to grow as tumors in comparison to CCL39 parental cells (unpublished results).

The conclusions drawn from this study regarding the role of the $Na^+:H^+$ antiporter in the development of tumors assume that mutations abrogating the exchange activity are indeed affecting the gene encoding the transporter molecule. The possibility that they affect a distinct protein which would regulate the antiporter as well as other cellular processes cannot be formally excluded. However, recent transfection experiments point to the contrary [15], and the molecular characterization of the human gene which can restore a wild-type phenotype in Hamster mutants should resolve this ambiguity.

ACKNOWLEDGMENTS

This work was supported by grants from the National Cancer Institute of Canada (N.C.I.C.), the Centre National de la Recherche Scientifique, and the Institut National de la Recherche Médicale. A.E.L. is the recipient of an N.C.I.C. Research Scholarship award, and of a short-term I.C.R.E.T.T. fellowship. We gratefully acknowledge the technical assistance of J. Dixon and the secretarial help of Astrid Eberhart.

REFERENCES

1. Moolenar WH: Annu Rev Physiol 48:363, 1986.
2. Pouysségur JA, Franchi A, L'Allemain G, Paris S: FEBS Lett 190:115, 1985.
3. Besterman JM, Cuatrecasas P: J Cell Biol 99:340, 1984.
4. Burns CP, Rozengurt E: Biochem Biophys Res Commun 116:931, 1983.
5. Cassel D, Rothenberg P, Zhyang Y, Deuel TF, Glaser L: Proc Natl Acad Sci USA 80:6224, 1983.
6. Grinstein S, Cohen S, Goetz D, Rothstein A, Gelfand EW: Proc Natl Acad Sci USA 82:1429, 1985.
7. Moolenar WH, Tertoolen LGJ, de Laat SW: J Biol Chem 259:7563, 1984.
8. Rozengurt E: Science 234:161, 1986.
9. Jentsch TJ, Stahlknecht TR, Hollwede H, Fisher DG, Keller SK, Wiederholt: J Biol Chem 260:795, 1985.
10. L'Allemain G, Paris S, Pouysségur J: J Biol Chem 260:4877, 1985.
11. L'Allemain G, Franchi A, Cragoe E, Pouysségur J: J Biol Chem 259:4313, 1984.
12. L'Allemain G, Paris S, Pouysségur J: J Biol Chem 259:5809, 1984.

13. Besterman JM, Tyrey SJ, Cragoe EJ, Cuatrecasas P: Proc Natl Acad Sci USA 81:6762, 1984.
14. Pouysségur J, Sardet C, Franchi A, L'Allemain G, Paris S: Proc Natl Acad Sci USA 81:4833, 1984.
15. Franchi A, Perucca-Lostanlen D, Pouyssegur J: Proc Natl Acad Sci USA 83:9388, 1986.
16. Agarwal NH, Haggerty E, Adelberg E, Slayman C: Am J Physiol 251:C825, 1986.
17. Perez-Rodriquez R, Chambard JC, Van Obberghen-Schilling E, Franchi A, Pouysségur J: J Cell Physiol 109:387, 1981.
18. Renwick D, Franchi A, Pouysségur J, Lagarde A: J Natl Cancer Inst 77:105, 1986.
19. Van Obberghen-Schilling E, Perez-Rodriquez R, Franchi A, Chambard JC, Pouysségur J: J Cell Physiol 115:123, 1983.
20. Spandidos DA, Wilkie NM: Nature 310:469, 1984.
21. Busa WB, Nuccitelli R: Am J Physiol 246:R409, 1984.
22. Hwang J, Pouysségur J, Willingham MC, Pastan I: J Cell Physiol 128:18, 1986.
23. Rotin D, Robinson B, Tannock IF: Cancer Res 46:2821, 1986.
24. Rotin D, Wan P, Grinstein S, Tannock IF: Cancer Res 47:1497, 1987.
25. Jain RK, Shah SA, Finney PL: J Natl Cancer Inst 73:429, 1984.
26. Hinsull SM, Colson RH, Franklin A, Watson BW, Bellamy D: J Natl Cancer Inst 73:463, 1984.
27. Ober SS, Pardee AB: Proc Natl Acad Sci USA 84:2766, 1987.
28. Sauer LA, Douchy RT: J Biol Chem 260:7496, 1985.
29. Racker E, Resnick RJ, Feldman R: Proc Natl Acad Sci USA 82:3535, 1985.
30. Franchi A, Cragoe E, Pouysségur J: J Biol Chem 261:14614, 1986.