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Transformation and pH Homeostasis of Fibroblasts Expressing Yeast H^+-ATPase Containing Site-Directed Mutations

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Mouse fibroblasts expressing a yeast proton-pumping ATPase show tumorigenic transformation (R. Perona, and R. Serrano, Nature (London) 334:438–440, 1988). By expressing site-directed mutations of the yeast ATPase with different levels of activity, a close correlation has been found between enzyme activity, tumorigenic transformation, and intracellular pH measured by weak-acid distribution. Fibroblasts expressing the yeast proton-pumping ATPase showed increased capability to grow at acidic pH and to resist lethal acidification mediated by reversal of the Na^+-H^+ antiporter. Measurements with microelectrodes in individual cells demonstrated electrical hyperpolarization and confirmed the increased pH of cells expressing yeast ATPase. These results indicate that the yeast enzyme expressed in mouse fibroblasts has electrogenic proton-pumping activity and that this activity deregulates fibroblast growth. This suggests a connection between the biophysical phenomena of proton transport, intracellular pH, and membrane potential and the biochemical regulatory circuits based on protein kinases and transcription factors.

Several lines of evidence indicate that intracellular alkalinization above a threshold pH value of 7.1 to 7.2 is necessary for proliferation of animal cells and is mediated in part by a Na^+-H^+ exchanger activated by growth factors and oncogenes (2–5, 9, 18, 27). A more controversial issue is whether intracellular alkalinization is sufficient to induce proliferation. This problem has been approached in the past by using alkaline media and ammonia to manipulate intracellular pH. In some cell types, such as invertebrate eggs, artificial alkalinization induces proliferation in the absence of specific mitogens (2, 4, 5). On the other hand, in mammalian fibroblasts these manipulations fail to induce growth (9). Some reports describing induced proliferation of fibroblasts by increased extracellular pH (30) have been criticized on the basis that precipitation of calcium phosphate occurs under this condition. Such precipitates, rather than the elevation of cell pH, may be responsible for the observed mitogenic effect (5). Therefore, it has been concluded that increased proton transport and intracellular alkalinization are necessary (permissive) but not sufficient for the proliferation of mammalian cells (5, 9, 27).

On the other hand, investigation of human tumors in vivo with ^{31}P nuclear magnetic resonance indicates that a common feature of all tumors examined is an elevation of intracellular pH (12), and there are indications that chronic abnormalities in local pH may have both a direct and an indirect role in the etiology of epithelial human cancer (6). In addition, intracellular pH is increased after transformation of fibroblasts by mutagens (11).

Since alkaline media and ammonia both have toxic side effects on animal cells (2, 4, 18), the different results obtained with invertebrate and mammalian cells may reflect differences in sensitivity to these side effects. Therefore, we have introduced a more specific approach to manipulation of intracellular pH which involves expressing the gene of a yeast proton-pumping ATPase. Mouse fibroblasts expressing the yeast proton pump are tumorigenic, but the activity of the yeast enzyme in the mouse cells was not demonstrated (14). This point is addressed here. We utilized site-directed mutations of yeast ATPase to establish a correlation between ATPase activity, fibroblast transformation, and intracellular pH. In addition, we demonstrate an alteration of pH homeostasis in cells expressing the yeast proton pump.

MATERIALS AND METHODS

Cells and growth conditions. NIH 3T3 cells and derived cell lines were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% newborn calf serum (GIBCO Laboratories).

Plasmids and oligonucleotides. The basic expression plasmid (pSV_hAT_5) has already been described (14). It contains the coding region of wild-type yeast ATPase under the control of the simian virus 40 (SV40) promoter. The 3.8-kilobase *Cla*I fragments (25) of mutant ATPase genes *pma1-213* (Glu-233→Gln [16]), *pma1-219* (Lys-474→Gln [16]), *pma1-217* (Asp-378→Glu [16]), *pma1-236* (Lys-379→Gln [17]), and *pma1-245* (deletion of last 18 amino acids [15]) were subcloned into pSV_hAT_5 by substitution of the wild-type *Cla*I fragment. Plasmid pDMT, containing the polyomavirusmiddle-T antigen gene under control of the SV40 promoter, was kindly provided by Lorraine Chalifour (National Research Council, Montreal, Canada). Antisense oligonucleotides against the first six codons of the yeast ATPase (GGATGATGTATCAGTCAT) and against the 3′ nontranscribed region of an actin gene (GGCCGTTAAT CATCTTTCAAC, 413-Act [Leandro Sastre, unpublished data]) were synthesized with an Applied Biosystems DNA synthesizer and purified by high-pressure liquid chromatography.

Transfection and transformation assays. For the generation of cell lines expressing different ATPase genes, NIH 3T3 cells (10^6) were suspended in 0.4 ml of phosphate-buffered saline (0.14 M NaCl, 3 mM KCl, 10 mM NaP_i [pH 7.4]) containing 10 μg of the desired expression plasmid and 0.1 μg of the pSV_2neo plasmid (nonlinearized) and subjected to a single pulse from an electroporation apparatus (Bio-Rad

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Laboratories). The capacitor unit was charged with 500 μF and 300 V. Cells were then diluted with DMEM supplemented with serum and plated into dishes (100 mm in diameter). Two days later, G-418 (1.5 mg/ml) was added into the medium and individual resistant colonies were isolated with cylinders. The presence of the ATPase gene was verified by Southern analysis (data not shown).

In order to quantitate transforming capacity, we performed transfection experiments as described above but with 6 μg of the ATPase expression plasmids and 3 μg of the pSV_2neo plasmid. Transformation was scored by counting G-418-resistant colonies with transformed phenotypes (highly refractile cells showing dense and disordered growth). After the percentage of transformation was determined, mass culture of each plate was performed and 10^6 cells suspended in phosphate-buffered saline were injected subcutaneously into several sites of 7-week-old male BALB/c *nu/nu* mice. Tumor appearance was scored weekly for 3 months.

Measurement of intracellular pH and membrane potential. Measurement of intracellular pH by the distribution of 3-*O-methyl*-D-[1-^{3}H]glucose and [7-^{14}C]benzoic acid (Du Pont, NEN Research Products) was as described previously (3). The medium contained 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-Tris (pH 7.4). Measurements with double-barreled H^+-selective microelectrodes (31) were carried out under the same conditions. The H^+-selective barrel contained an ETH1907 (Fluka)-based liquid proton sensor. The reference barrel was filled with 3 M KCl and used to record membrane potential. Electrical coupling between ion-selective and reference microelectrodes was measured by passing 1-nA square pulses through the reference barrel. Electrodes with capacitive coupling above 5% (excluding the capacitance transient) were rejected. The bath reference electrode was a low-resistance 3 M KCl microelectrode, and it was used for calibrating ion-sensitive microelectrodes. The potential of the H^+-selective electrode was monitored with one of the probes of a WPI F223A electrometer. The potential of the reference barrel was recorded with a WPI M-707 amplifier and electronically substracted from the H^+-selective potential, giving a differential signal from which pH could be read directly. Proton-selective microelectrodes were calibrated in solutions buffered to different pHs between 6.8 and 8.0, giving slopes between 52 and 57 mV per pH unit.

Acid suicide technique and thymidine incorporation. Cells were seeded in 96-well dishes (2×10^4 cells per well). After 24 h, culture medium was replaced by LiCl saline solution (130 mM LiCl, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES-Tris [pH 7.4] [19]) and the cells were incubated for 2 h. The medium was aspirated and replaced with choline chloride acid-saline solution [130 mM choline chloride, 5 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM 2-(*N*-morpholino)ethanesulfonic acid–Tris (pH 5.5) (19)]. At various times, the medium was aspirated and replaced by DMEM supplemented with 10% newborn calf serum. After 2 h, [6-^{3}H]thymidine (Amersham Corp.) (10 μCi/ml) was added, and 24 h later the cells were washed twice with phosphate-buffered saline and isolated with a cell harvester, and their radioactivity was determined with a scintillation counter.

For the determination of growth at different pHs, cells(2×10^4 per well) were grown in 96-well dishes and24 h after seeding, the medium was replaced with bicarbonate-free DMEM containing 10% serum, 10 μCi of [6-^{3}H]thy-midine per ml, and 20 mM buffer adjusted to the desiredpH with HCl {PIPES [piperazine-*N*-*N'*-bis(2-ethanesulfonic acid)] for pH values of 4.0 to 7.0, HEPES for pH values of 7.1 to 7.5, and HEPPS (*N*-2-hydroxyethylpiperazine-*N'*-3-propane sulfonic acid) for pH values of 7.6 to 8.2}. After 24 h, the radioactivity incorporated was determined as described above.

Measurement of ATP hydrolysis in isolated membranes. All operations during membrane preparation were carried out at 2 to 4°C. Lyophilized cells (10 to 30 mg of total protein) were suspended in 5 ml of medium with 20% glycerol, 10 mM Tris hydrochloride (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, and 0.25 mM phenylmethylsulfonyl fluoride. After homogenization with a glass homogenizer, they were sonicated for 20 s with a B-12 Branson Sonifier with microtip (setting 5, 60 W). After debris was removed by centrifugation for 5 min at 3,000 rpm (Sorvall SS-34 rotor), a total membrane fraction was obtained by centrifugation for 1 h at 45,000 rpm (Beckman 65 rotor). The pellet was suspended with 0.5 ml of the homogenization buffer and stored at −70°C.

ATP hydrolysis was measured as described previously (23), at 37°C and pH 5.7, in the presence of 5 mM azide to inhibit mitochondrial ATPase, 50 mM nitrate to inhibit vacuolar ATPases, and 0.5 mM ouabain to inhibit Na,K-ATPase.

RESULTS

Correlation between yeast H^+-ATPase activity, tumorigenic transformation, and intracellular pH. We wanted to investigate whether the increased pH and tumorigenicity of fibroblasts expressing the yeast proton-pumping ATPase (14) were caused by the catalytic activity of the enzyme or by some unexpected effect of the yeast protein in the mammalian cell.

Site-directed mutants of the yeast ATPase with different levels of activity have recently been constructed (15–17). Expression plasmids with either the mutants or the wild-type ATPase were introduced into NIH 3T3 cells by cotransfection with the neomycin-resistance plasmid (pSV_2neo) (100:1, ATPase-neomycin resistance plasmid). Cell lines transfected with active ATPase genes (PMA1 and pma1-245) showed disordered growth patterns. This morphological transformation was more evident in cells transfected with the hyperactive mutant gene *pma1-245*. This is a deletion of the last 18 amino acids of the ATPase, which constitute an inhibitory domain mediating the physiological regulation of the enzyme (15). Some patches of disordered growth were present in cell lines transfected with the *pma1-217* and *pma1-236* alleles, which have lower activities than the wild type (16, 17). The two inactive mutant genes (*pma1-213* and *pma1-219* [16]) produced cell lines with normal morphologies.

In order to quantitate the transforming capacities of the different ATPase genes, we performed cotransfection experiments into NIH 3T3 cells using a 2:1 ratio of ATPase-expressing plasmid and pSV_2neo. After selection with G-418, we determined the percentage of clones with transformed morphology, and the results are summarized in Table 1. The percentage of densely growing clones increased with the ATPase activity of the expressed gene. This correlation was also true for the tumorigenic capacity of the cell lines once injected into nude mice. Although one of the ATPase alleles with low activity (*pma1-217*) showed some capacity of inducing disordered growth of the cells, the potential to induce tumors was present only in clones which express an ATPase with relatively high activity (70% of wild-type

TABLE 1. Correlation between ATPase activity, tumorigenic transformation, and intracellular pH of fibroblasts expressing different mutants of yeast ATPase

Gene expressing ATPase	Activity (%)[a]	Transformation (%)[b]	Tumorigenicity (latency period [wks])[c]	Cell pH[d]
None		<5	0/6	7.09
PMA1	100	50	6/6 (4)	7.32
pma1-213	<10	<5	0/4	7.09
pma1-219	<10	<5	0/4	7.10
pma1-217	20	10	0/4	
pma1-236	70	31	4/6 (8)	7.19
pma1-245	300	69	6/6 (2)	7.41

[a] Percentage of ATPase activity with respect to that of wild-type (PMA1) ATPase when expressed in yeast. In the case of *pma1-217*, the percentage of proton-pumping activity is given because this mutation causes partial uncoupling (data are from references 15 through 17).

[b] Percentage of G-418-resistant fibroblast clones (n, 20 to 25) which have transformed morphology. Each value is the average of two experiments differing by less than 5%.

[c] Number of nude mice developing tumors/total number of animals and time required for the appearance of the tumors.

[d] Cells were washed and incubated in buffer without bicarbonate. Each value is the average of two determinations differing by less than 0.05 pH units.

activity expressed from allele *pma1-236*). Both the incidence (from four of six animals for allele *pma1-236* to all animals for more active ATPases) and the latency period before appearance of the tumors (from 8 weeks for allele *pma1-236* to 2 weeks for the hyperactive mutant) correlated with the activity of the ATPase allele transfected into the cells. By all criteria, the most active ATPase gene in transformation was the hyperactive mutant gene *pma1-245*. When expressed in yeast cells and in the absence of a specific mechanism of activation triggered by glucose fermentation, this mutant enzyme has about three times more activity than wild-type ATPase (15). It is likely that the activating system operating in yeast cells is not present in animal cells and therefore that the mutant is hyperactive in those cells.

We have investigated whether the capacity of inducing tumors correlates with the proton-pumping activity the ATPase allele expressed by the fibroblasts. Intracellular pH was measured by the distribution of benzoic acid, and as the ATPase activity of the gene was increased, a more alkaline intracellular pH was obtained in the corresponding cell line (Table 1). These data agree with previous preliminary results comparing the wild type and an inactive allele (14).

Therefore, a close correlation has been found between H^+-ATPase activity, morphological transformation, tumorigenicity, and intracellular pH (Table 1). These results strongly suggest that the effects of yeast ATPase on fibroblasts are due to the proton-pumping activity of the enzyme.

Altered pH homeostasis of fibroblasts expressing yeast ATPase. A direct demonstration of the activity of yeast ATPase in fibroblasts was attempted by measuring ATP hydrolysis in isolated membranes. Under the conditions described in Materials and Methods, membranes from control fibroblasts and from fibroblasts expressing yeast ATPase exhibited rates of ATP hydrolysis (resistant to azide, nitrate, and ouabain) of 2.5 and 3.5 nmol/min per mg of protein, respectively (averages of three determinations [standard deviation, 0.3]). The inhibition by the ATPase inhibitors vanadate and erythrosine B (both at 0.1 mM) was 60% in control cells and 75% in cells transfected with yeast ATPase. This inhibition allows a correction for ATP hydrolysis mediated by nonspecific phosphatases (23, 24). It can be calculated that ATPases sensitive to the above inhibitors have activities of 1.5 and 2.6

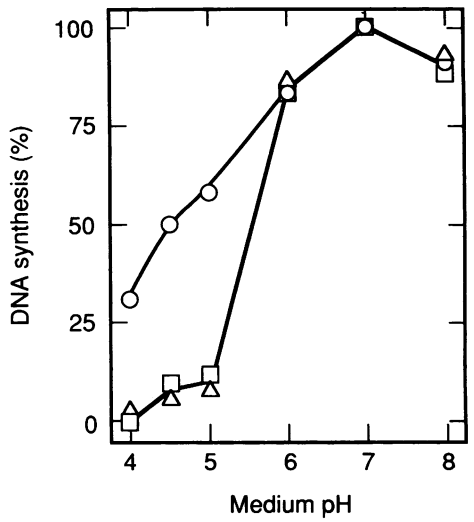

FIG. 1. pH dependence of DNA synthesis in cells expressing wild-type yeast ATPase (○), inactive ATPase mutant pma1-213 (□), or middle-T antigen (△). Cells were grown in multiwell dishes, shifted to bicarbonate-free DMEM supplemented with 10% calf serum, and adjusted to the indicated pH. [^{3}H]thymidine incorporation was determined after 24 h. DNA synthesis of 100% corresponds to 44,700 to 44,900 cpm for the different cell lines, and each point is the average of two determinations differing by less than 5%.

nmol/min per mg of protein in control cells and in cells transfected with yeast ATPase, respectively. This significant difference may reflect the activity of the yeast enzyme. However, the lack of fully specific inhibitors for yeast ATPase (24) prevents definitive conclusions until the enzyme is purified from the animal cells.

An indirect demonstration of the activity of the yeast proton-pumping ATPase was made by analyzing pH homeostasis of the cells. Fibroblasts expressing the yeast ATPase tolerate acidic media much better than both nontransformed cells and cells transformed by the polyomavirus middle-T antigen (Fig. 1). The latter control indicates that transformation by itself, although it produces serum-independent activation of proton efflux mediated by the H^+-Na^+ antiporter (3–5), does not confer tolerance to very acidic media. This is in agreement with the report that fibroblasts with chemically induced mutations which result in the hyperactive H^+-Na^+ antiporter have very low rates of DNA synthesis at pH values below 6 (11). Cells expressing yeast H^+-ATPase exhibit substantial DNA synthesis at pH values below 5. This could be explained by the presence in these cells of a very active proton-extruding activity that is clearly of a different nature than the endogenous H^+-Na^+ antiporter.

The same conclusion was reached by submitting the cells to the acid suicide test of Pouyssegur et al. (19). In this experiment, the cells are first loaded with Li and then incubated in acid medium with choline as monovalent cation. Under these conditions, the Na^+-H^+ exchanger catalizes the efflux of Li^+ in exchange for H^+ influx and the cells become acidified and die. Fibroblasts expressing the yeast ATPase were less acidified and maintained higher rates of DNA synthesis than nontransformed cells or cells transformed by the polyomavirus middle-T antigen (Fig. 2). An important control is presented in Fig. 3, where it is shown that the difference in acid tolerance between ATPase-expressing cells and control cells was abolished by an antisense oligonucleotide against the first six codons of the yeast

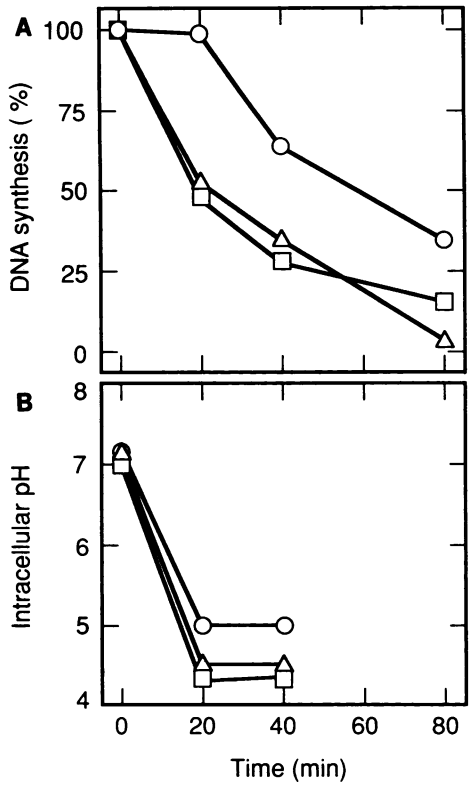

FIG. 2. Cell viability (A) and intracellular pH (B) after intracellular acidification mediated by reversal of the Na^+-H^+ antiport. Symbols are as defined in the legend to Fig. 1. Cells were loaded with LiCl, washed, and incubated for the indicated times in choline chloride solution at pH 5.5. (A) Cell viability was estimated by replacing the acid solution with culture medium containing 10% calf serum and determining the incorporation of [^{3}H]thymidine after 24 h. DNA synthesis of 100% corresponds to 44,900 to 45,100 cpm for the different cell lines, and each point is the average of four determinations (standard deviations, 2 to 5%). (B) Intracellular pH was determined by the distribution of benzoic acid. Each values is the average of two determinations differing by less than 0.05 pH units.

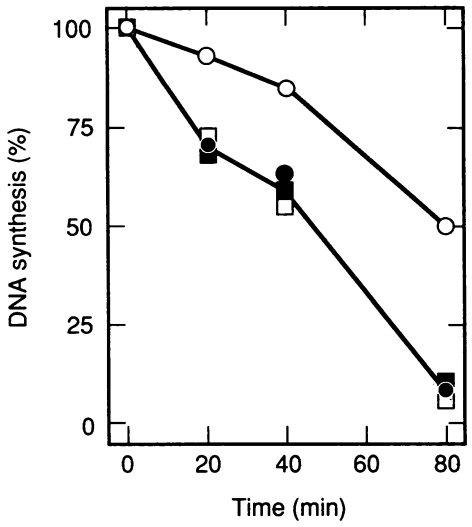

FIG. 3. Effect of ATPase antisense oligonucleotides on the sensitivity of fibroblasts to intracellular acidification. Symbols are as defined in the legend to Fig. 1, and experimental conditions were the same as those for Fig. 2A, except that in the case of the closed symbols, ATPase antisense oligonucleotide (20 μM) was present during the last 16 h of growth and during the incubations for acid loading. DNA synthesis of 100% corresponds to 41,900 to 42,100 cpm for the different cell lines, and each point is the average of four determinations (standard deviation, 3 to 7%).

ATPase. Antisense oligonucleotides are very effective in reducing expression of genes in animal cells, because they are transported into the cells by a specific uptake system (8). An antisense oligonucleotide against actin was without effect on the same cells (data not shown). It must be pointed out that since the experiments were carried out in the absence of bicarbonate, known proton transport systems of animal cells could not extrude protons under these conditions (5). Therefore, the capacity to maintain a higher rate of DNA synthesis under acidifying conditions is probably due to the activity of the yeast proton-pumping ATPase.

Measurements with microelectrodes. In order to confirm the increased pH of ATPase-expressing cells by alternative methods, we have utilized double-barreled microelectrodes inserted into single cells (31). This also allowed the determination of the electrical membrane potential. The increased pH of ATPase-expressing cells was confirmed by the microelectrode measurements (Fig. 4). In a typical experiment, with a medium pH of 7.4, entrance of the microelectrode into the ATPase-expressing cells resulted in a deflection to pH 7.5, while entrance into control cells resulted in deflection to the opposite direction (to pH 7.2). Average values for 5 to 6 control cells and cells expressing the wild-type ATPase were 7.2 and 7.5, respectively (standard deviation, 0.05 pH units). Cells expressing the yeast ATPase had a greater average membrane potential than control cells (40 versus 20 mV) (Fig. 5). This is in accordance with the electrogenic character of yeast ATPase (24).

DISCUSSION

The first conclusion of these experiments is that the degree of transformation, tumorigenicity, and increased pH of fibroblasts expressing site-directed mutations of yeast H^+-ATPase correlates with the activity of the different enzymes. Therefore, the proton-pumping activity of the yeast enzyme is responsible for the physiological alterations of the cell

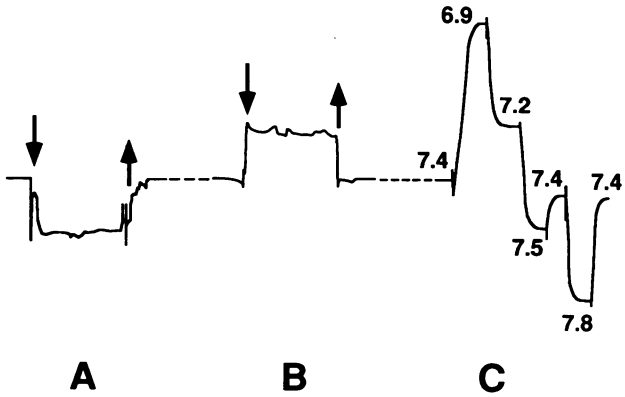

FIG. 4. Measurement of intracellular pH in single cells with microelectrodes. (A) Cell expressing wild-type yeast; (B) control cell not expressing yeast ATPase; (C) calibration of the electrode. Arrows indicate entry (↓) and exit (↑) of the electrode from the cells.

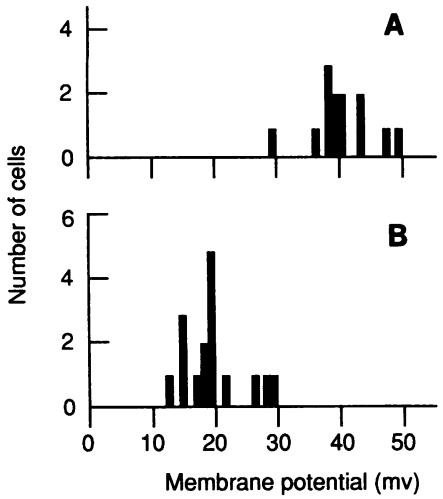

FIG. 5. Measurement of electrical membrane potential in individual cells with microelectrodes. (A) Cells expressing wild-type yeast ATPase; (B) cells expressing the inactive mutant pma1-213.

lines and not any unexpected interactions of the yeast protein with the animal cells. In addition, the increased intracellular pH, membrane potential, and tolerance to different acidification protocols of cells expressing yeast ATPase are expected from the electrogenic proton-pumping activity of the enzyme.

The second conclusion is that increased proton transport and intracellular alkalinization due to the expression of the yeast proton pump can increase the growth of mammalian fibroblasts in the absence of specific mitogens (Fig. 6). This agrees with previous results obtained by utilizing high external pH and ammonia to alkalinize invertebrate eggs (2, 4, 5), chicken embryo cells (21), and mouse NIH 3T3 fibroblasts (30). However, before a definitive conclusion can be reached about the mechanism of this phenomenon, we need to investigate whether cells expressing yeast ATPase exhibit

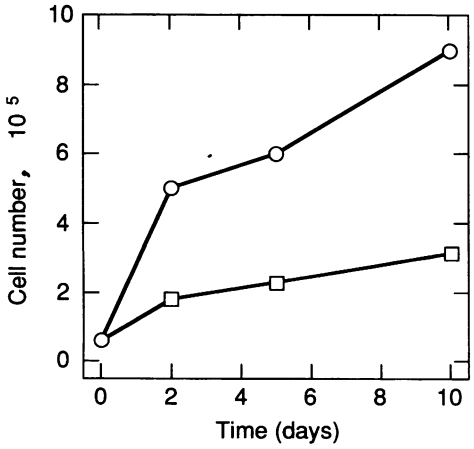

FIG. 6. Growth of ATPase-expressing cell lines in low-serum medium. Symbols are as defined in the legend to Fig. 1. Cells were plated in 35-mm wells in DMEM supplemented with 10% calf serum. After 6 to 12 h, cells were counted to confirm accurate plating, medium was removed, and the cells were fed with DMEM containing 0.5% calf serum. Cells were trypsinized and counted at the days indicated. Each point is the mean of two experiments differing by less than 10%.

autocrine growth factor production or increased levels of growth factor receptors.

A different question, however, is whether the proliferation induced by either growth factors (22) or oncogenes (3) in quiescent mammalian cells can be explained by the small increase in cellular pH usually observed (2, 4, 5). Two lines of evidence suggest that this is not the case. In the first place, raising the external pH may increase the intracellular pH to the same level as that induced by growth factors without triggering proliferation (9). It must be pointed out, however, that in these experiments, artificial alkalinization induced some increase of DNA synthesis in the absence of serum (10% of the serum-induced value) and that toxic side effects of the manipulation may complicate the interpretation of the results (see the introduction). The second line of evidence has been accumulated more recently and refers to the effect of bicarbonate. In the presence of physiological levels of bicarbonate, the operation of a Na^+-dependent chloride-bicarbonate exchanger maintains intracellular pH within the permissive range for growth (about 7.2) in the absence of growth factors. However, no proliferation is observed under these conditions (1, 7, 28).

We must indicate, however, that expression of yeast H^+-ATPase results in a higher capability for proton transport and intracellular alkalinization than is provided by either the activation of the Na^+-H^+ exchanger by growth factors and oncogenes or the operation of the bicarbonate-dependent system. This is apparent from the results in Fig. 1, which show that ATPase-expressing cells tolerate acidic media much better than serum-stimulated cells or oncogene-transformed cells. In addition, Gillies and co-workers (University of Arizona, Tucson) have recently demonstrated that fibroblasts expressing yeast ATPase have higher intracellular pH levels (0.2 to 0.3 pH units) than control cells, even in the presence of serum and bicarbonate (R. J. Gillies, R. Martinez-Zaguilan, G. Martinez, R. Serrano, and R. Perona, submitted for publication). Therefore, it seems that the large increase in proton transport and cell pH produced by expressing yeast ATPase is mitogenic, while the smaller increase produced by growth factors, oncogenes, and bicarbonate is only permissive for the growth response, which requires some additional effects by specific mitogens.

These results suggest an interaction between the biochemical regulatory circuit of the cell cycle based on receptors, second messengers, protein kinases, and transcription factors (10, 13) and biophysical factors such as proton transport, cell pH, and membrane potential (4, 5). It has been suggested that regulation by intracellular pH may be a primitive method of control which does not require special receptor molecules. Metabolic enzymes involved in energy metabolism, in protein, RNA, and DNA synthesis (2), and in polymerization reactions involving H^+ release or uptake (29) are very sensitive to changes in pH. It has been speculated that proton transport evolved very early in primitive cells to control intracellular pH (20). Since components of the biochemical regulatory circuit are also pH sensitive, intracellular pH may function as a synergistic messenger, which provides a metabolic context within and through which the actions of other effectors are integrated (2). In addition, the transition of mammalian cells from the quiescent to the proliferative state is probabilistic (26), and biophysical parameters such as pH may affect the probability of steps of this regulatory pathway.

It was important to show by genetic engineering of proton transport that drastic perturbations in pH homeostasis can trigger the growth of mammalian cells. Perhaps this response

is a vestige of the primitive biophysical control circuit, replaced in cells of higher animals by a more sophisticated, but still pH-sensitive, biochemical control circuit.

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LITERATURE CITED

1. **Bierman, A. J., E. J. Cragoe, S. W. de Laat, and W. H. Moolenaar.** 1988. Bicarbonate determines cytoplasmic pH and suppresses mitogen-induced alkalinization in fibroblastic cells. J. Biol. Chem. **263**:15253–15256.
2. **Busa, W. B., and R. Nuccitelli.** 1984. Metabolic regulation via intracellular pH. Am. J. Physiol. **246**:R409–R438.
3. **Doppler, W., R. Jaggi, and B. Groner.** 1987. Induction of v-mos and activated Ha-ras oncogene expression in quiescent NIH 3T3 cells causes intracellular alkalinisation and cell-cycle progression. Gene **54**:147–153.
4. **Epel, D., and F. Dube.** 1987. Intracellular pH and cell proliferation, p. 363–393. *In* A. L. Boynton and H. L. Leffert (ed.), Control of animal cell proliferation, vol. II. Academic Press, Inc., New York.
5. **Grinstein, S., D. Rotin, and M. J. Mason.** 1989. Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. Biochim. Biophys. Acta **988**: 73–97.
6. **Harguindey, S., L. M. A. Aparicio, and S. M. Algarra.** 1989. Integrated etipathogenesis of cancer of mucosal surfaces with emphasis on the digestive track: an appraisal. J. Biol. Response Modif. **8**:1–10.
7. **L'Allemain, G., S. Paris, and J. Pouyssegur.** 1985. Role of a Na^+-dependent Cl^-/HCO_3^- exchange in regulation of intracellular pH in fibroblasts. J. Biol. Chem. **260**:4877–4883.
8. **Loke, S. L., C. A. Stein, X. H. Zhang, K. Mori, M. Nakanishi, C. Subasinghe, J. S. Cohen, and L. M. Neckers.** 1989. Characterization of oligonucleotide transport into living cells. Proc. Natl. Acad. Sci. USA **86**:3474–3478.
9. **Moolenaar, W. H., L. H. K. Defize, and S. W. de Laat.** 1986. Ionic signalling by growth factor receptors. J. Exp. Biol. **124**: 359–373.
10. **Murray, A. W., and M. W. Kirschner.** 1989. Dominoes and clocks: the union of two views of the cell cycle. Science **246**:614–621.
11. **Ober, S. S., and A. B. Pardee.** 1987. Intracellular pH is increased after transformation of chinese hamster embryo fibroblasts. Proc. Natl. Acad. Sci. USA **84**:2766–2770.
12. **Oberhaensli, R. D., P. J. Bore, R. P. Rampling, D. Hilton-Jones, L. J. Hands, and G. K. Radda.** 1986. Biochemical investigation of human tumours in vivo with phosphorus-31 magnetic resonance spectroscopy. Lancet **ii**:8–11.
13. **Pardee, A. B.** 1989. G1 events and regulation of cell proliferation. Science **246**:603–608.
14. **Perona, R., and R. Serrano.** 1988. Increased pH and tumorigenicity of fibroblasts expressing a yeast proton pump. Nature (London) **334**:438–440.
15. **Portillo, F., I. F. Larrinoa, and R. Serrano.** 1989. Deletion analysis of yeast plasma membrane H^+-ATPase and identification of a regulatory domain at the carboxyl-terminus. FEBS Lett. **247**:381–385.
16. **Portillo, F., and R. Serrano.** 1988. Dissection of functional domains of the yeast proton-pumping ATPase by directed mutagenesis. EMBO J. **7**:1793–1798.
17. **Portillo, F., and R. Serrano.** 1990. Growth control strength and active site of yeast plasma membrane ATPase studied by site-directed mutagenesis. Eur. J. Biochem. **186**:501–507.
18. **Pouyssegur, J., A. Franchi, G. L'Allemain, and S. Paris.** 1985. Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. FEBS Lett. **190**:115–119.
19. **Pouyssegur, J., C. Sardet, C. Franchi, G. L'Allemain, and S. Paris.** 1984. A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. Proc. Natl. Acad. Sci. USA **81**:4833–4837.
20. **Raven, J. A., and F. A. Smith.** 1976. The evolution of chemiosmotic energy coupling. J. Theor. Biol. **57**:301–312.
21. **Rubin, H.** 1973. pH, serum and Zn^{2+} in the regulation of DNA synthesis in cultures of chick embryo cells. J. Cell. Physiol. **82**:231–238.
22. **Schuldiner, S., and E. Rozengurt.** 1982. Na^+/H^+ antiport in Swiss 3T3 cells: mitogenic stimulation leads to cytoplasmic alkalinization. Proc. Natl. Acad. Sci. USA **79**:7778–7782.
23. **Serrano, R.** 1988. H^+-ATPase from plasma membranes of *Saccharomyces cerevisiae* and *Avena sativa* roots: purification and reconstitution. Methods Enzymol. **157**:533–544.
24. **Serrano, R.** 1989. Structure and function of plasma membrane ATPase. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**:61–94.
25. **Serrano, R., M. C. Kielland-Brandt, and G. R. Fink.** 1986. Yeast plasma membrane ATPase is essential for growth and has homology with $(Na^+ + K^+)$, K^+ and Ca^{2+}-ATPases. Nature (London) **319**:689–693.
26. **Shields, R.** 1977. Transition probability and the origin of variation in the cell cycle. Nature (London) **267**:704–707.
27. **Soltoff, S. P., and L. C. Cantley.** 1988. Mitogens and ion fluxes. Annu. Rev. Physiol. **50**:207–223.
28. **Szwergold, B. S., T. R. Brown, and J. J. Freed.** 1989. Bicarbonate abolishes intracellular alkalinization in mitogen stimulated 3T3 cells. J. Cell. Physiol. **138**:227–235.
29. **Williams, R. J. P.** 1988. Ion pumps and cell shapes. Trends Biochem. Sci. **13**:249.
30. **Zetterberg, A., and W. Engstrom.** 1981. Mitogenic effect of alkaline pH on quiescent, serum starved cells. Proc. Natl. Acad. Sci. USA **78**:4334–4338.
31. **Zuethen, T.** 1980. How to make and use double-barreled ion-selective microelectrodes. Curr. Top. Membr. Transp. **13**:31–47.