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Cell Biology

Intracellular pH is increased after transformation of Chinese hamster embryo fibroblasts

(Na^+/H^+ antiporter/growth control)

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ABSTRACT **These studies reveal that a series of tumorigenic Chinese hamster embryo fibroblast (CHEF) cell lines maintain an internal pH (pH_i) that is 0.12 ± 0.04 pH unit above that of the nontumorigenic CHEF/18 parental line. This increase of pH_i in the tumorigenic CHEF cells is not due to autocrine growth factor production or to the persistent activation of pathways previously shown to modulate Na^+/H^+-antiporter activity present in the CHEF/18 line. These findings suggest that the defect in pH_i regulation in the tumorigenic CHEF/18 derivatives lies in the Na^+/H^+ antiporter itself. Further studies to determine the biological significance of an increased pH_i show that the external pH (pH_o)-dependence curve for initiation of DNA synthesis in the tumorigenic CHEF lines is shifted by approximately 0.2 pH unit toward acidic values relative to that of the nontumorigenic CHEF/18 parent. These data show a critical role for pH_i in the regulation of DNA synthesis in Chinese hamster embryo fibroblasts and demonstrate that aberrations in pH_i can contribute to the acquisition of altered growth properties.**

A rapid increase in intracellular pH (pH_i) due to the stimulation of an amiloride-sensitive Na^+/H^+ antiporter is a nearly universal response of quiescent cells to growth factor addition (1–3); the apparent ubiquity of this response makes pH_i an attractive candidate for a mitogenic signal pathway. Evidence that pH_i is indeed involved in the control of cell proliferation comes from several sources. Specifically, the mitogenic response of cells to growth factors occurs only over a narrow range of extracellular pH (pH_o) values (3, 4), the increase of pH_o can raise pH_i and lead to cell proliferation in the absence of added growth factors in some systems (5–7), and, finally, inhibition of the ability of cells to increase pH_i in response to growth factors can prevent cell proliferation after growth factor addition (8, 9). These findings support a critical role for pH_i in the entry of cells into the DNA-synthesis phase of the cell cycle and suggest that alterations in pH_i may contribute to abnormal proliferative responses such as those seen in tumorigenic cells.

We are reporting elsewhere (10) the characterization of a growth factor-stimulatable Na^+/H^+ antiporter that modulates pH_i in the nontransformed, nontumorigenic Chinese hamster embryo fibroblast (CHEF) line CHEF/18. In the present study, we investigate three tumorigenic lines sequentially derived from the CHEF/18 cells. Our findings show that maintainance of an increased pH_i is an early event associated with the acquisition of the tumorigenic phenotype in this cell series. We further demonstrate that this pH_i increase is a result of Na^+/H^+-antiporter activity, occurs in the absence of autocrine growth-factor production or persistent second-messenger-system activation, and correlates with an altered proliferative response. These findings suggest a role for pH_i itself as a contributory agent in the development of tumorigenicity in CHEF cells.

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MATERIALS AND METHODS

Cell Culture. The four CHEF lines used in this paper were developed as follows: the CHEF/18 clonal line was isolated from a culture of a Chinese hamster embryo; these cells are nontransformed and nontumorigenic (11). After nine doublings of a CHEF/18 population mutagenized by addition of ethyl methanesulfonate at 200 μg/ml, the clonal line 21-2 was derived after a single round of selection in methylcellulose; this line is weakly tumorigenic (two tumors in 12 nude mice). 21-2-M3 is a clone derived from 21-2 after three additional rounds of selection in methylcellulose and is strongly tumorigenic (two tumors in four nude mice). T30-4 is a subclone from a tumor arising after subcutaneous injection of 10^7 21-2-M3 cells into a nude mouse; its tumorigenic potential is equivalent to that of 21-2-M3. The 21-2, 21-2-M3, and T30-4 lines form colonies in low serum concentrations (12).

All of these CHEF lines were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO H21) supplemented with 10% fetal calf serum in humidified 10% CO_2/90% air at 37°C. Cells were arrested in G_0/G_1 by growing to confluency in 35-mm dishes, followed by two washes in serum-free medium and a 24-hr incubation in serum-free DMEM (13).

Measurement of Intracellular pH. pH_i measurements were made with [^{14}C]benzoic acid essentially as described (8). Cultures were first equilibrated for 60 min at the indicated pH in HCO_3^--free glucose–saline medium containing 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 20 mM of one of the following buffers: Pipes for pH values 6.0–7.0, Hepes for pH values 7.1–7.5, or *N*-(2-hydroxyethyl)-piperazine-*N*′-3-propanesulfonic acid (EPPS) for pH values 7.6–8.2. Buffer pH was adjusted with HCl. After the equilibration period, [7-^{14}C]benzoic acid was added at 1 μCi/ml (1 Ci = 37 GBq). After incubation at 22°C for various times in the presence or absence of 10% fetal calf serum as indicated, the dishes were washed rapidly four times with ice-cold phosphate-buffered saline (pH 7.4). Cells were then solubilized in 0.1 M NaOH, and the radioactivity was assayed by liquid scintillation spectrometry.

The radioactivity measured was corrected for radioactivity trapped in the external space and the loss of intracellular radioactivity during the wash procedure; pH_i was calculated as described (8). Total cell protein was determined as described by Lowry *et al.* (14). Intracellular water space was

Abbreviations: pH_i, intracellular pH; CHEF, Chinese hamster embryo fibroblast; pH_o extracellular pH; EGF, epidermal growth factor; TFP, trifluoperizine; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride.

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calculated from the equilibrium uptake of 3-*O*-methyl-D-[1-^{3}H]glucose (8). The value obtained (5 μl/mg of protein) was found to be identical in all four CHEF lines.

Thymidine Incorporation. Confluent, serum-starved cells were stimulated to enter S phase by the addition of HCO_3^--free DMEM containing 10% fetal calf serum, 2 μCi of [^{3}H]dThd per ml, and buffer as described above (Pipes, Hepes, or EPPS). Unstimulated controls had no fetal calf serum addition. At appropriate intervals, samples were washed rapidly four times with ice-cold phosphate-buffered saline. Cells were then precipitated with 5% trichloroacetic acid, the acid-insoluble material was dissolved in 0.1 M NaOH, and the radioactivity was assayed by liquid scintillation spectrometry (15).

Preparation of Conditioned Medium. DMEM was conditioned as described by Cherington and Pardee (16). Briefly, cells were grown to approximately 90% confluence in 60-mm dishes by using DMEM with 10% fetal calf serum. This medium was replaced with serum-free DMEM, changed again after 60 min, and changed a third time after 12 hr. Fresh serum-free DMEM was then added to each dish (3 ml per dish), and the dishes were incubated for 48 hr. The medium was then collected, centrifuged at 100,000 × *g* for 30 min, diluted 1:1 with serum-free DMEM, and stored at −20°C.

Protein Kinase C Measurements. After treatment of cells with the indicated agents, protein kinase C was isolated, and its activity was determined as described by Melloni *et al.* (17). The final assay mixture (200 μl) contained 5 μM [γ-^{32}P]ATP, 100 μg of histone (type III-S), 10 μg of phosphatidylserine, 0.2 μg of dioleoylglycerol, 1 mM $CaCl_2$, and 30 μl of protein kinase C preparation in 50 mM sodium borate (pH 7.5). For certain experiments, $CaCl_2$ and phospholipids were omitted and the reaction was done in the presence of 5 mM EDTA. In all cases incubations were carried out for 10 min at 30°C and were terminated by the addition of 1 ml of 10% trichloroacetic acid containing 2 mg of bovine serum albumin. Samples were collected on Whatman GF/C glass filters, and radioactivity was determined by using liquid scintillation spectrometry.

Calcium Uptake Determinations. Confluent G_0/G_1-arrested cells were preequilibrated for 30 min at 22°C in serum-free DMEM, $^{45}CaCl_2$ was then added at 0.75 μCi/ml, and the incubations were continued for the indicated times. Experiments were terminated by washing the dishes rapidly four times in ice-cold phosphate-buffered saline (pH 7.4). After solubilization in 0.1 M NaOH, cell-associated radioactivity was determined by liquid scintillation spectrometry.

Materials. [7-^{14}C]Benzoic acid, 3-*O*-methyl-D-[1-^{3}H]glucose, [γ-^{32}P]ATP, $^{45}CaCl_2$, and [^{3}H]dThd were from New England Nuclear. Fetal calf serum was from HyClone (Logan, UT). Amiloride, histone type III-S, phosphatidylserine, dioleoylglycerol, trifluoperizine (TFP), 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8), and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were purchased from Sigma. The protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was obtained from Seikagaku America (St. Petersburg, FL). All other chemicals were of the highest grade commercially available.

RESULTS

Tumorigenic CHEF Lines Maintain High pH_i. The CHEF line CHEF/18 contains an amiloride-sensitive Na^+/H^+ antiporter that is activated by growth factors (10). A series of tumorigenic CHEF/18 derivatives (21-2, 21-2-M3, and T30-4) maintained a pH_i that was 0.12 ± 0.04 pH unit above that of CHEF/18 regardless of pH_o values (Fig. 1). As all three of the tumorigenic cell lines had an altered pH_i, the lesion responsible for this phenomenon must have occurred during the mutagenesis of CHEF/18 to produce derivative line 21-2 and, hence, is coincident with the initial observations of the tumorigenic phenotype.

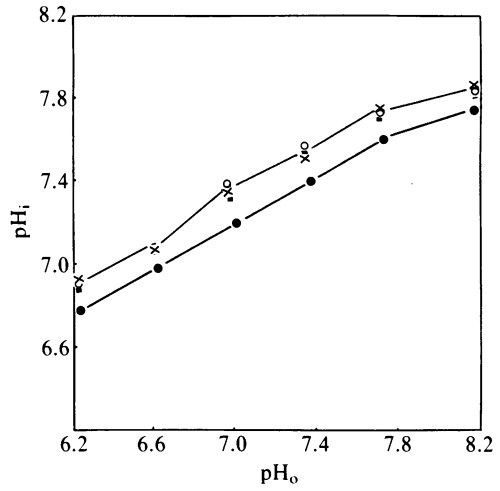

FIG. 1. pH_i as a function of pH_o in quiescent CHEF cells. G_0/G_1-arrested cultures of CHEF/18 (●), 21-2 (○), 21-2-M3 (×), or T30-4 (■) were equilibrated for 60 min in glucose–saline medium at the indicated pH. This medium was then replaced by the same one containing [^{14}C]benzoic acid. pH_i was determined after 15 min. Each point is the mean of triplicate determinations.

Altered pH_i Is a Result of Na^+/H^+ Antiporter Activation. Since these studies were carried out in HCO_3^--free medium, the observed differences in pH_i between the tumorigenic CHEF cells and CHEF/18 could not have been due to alterations in HCO_3^-/Cl^- exchange or Na^+/HCO_3^- cotransport. These findings implicate a Na^+/H^+-exchange system in the control of pH_i in these four CHEF lines. To verify the involvement of a Na^+/H^+ antiporter in the maintainance of the altered pH_i observed in the 21-2, 21-2-M3, and T30-4 tumorigenic CHEF lines, cells were assayed for their ability to recover from an intracellular acid load induced by incubating the cells with NH_4^+ (18). Upon removal of the NH_4^+, a transient decrease in pH_i was seen, followed by a rapid return to normal levels (Fig. 2*A*). It is apparent that the maximal decrease in pH_i induced by NH_4^+ was the same in all four cell

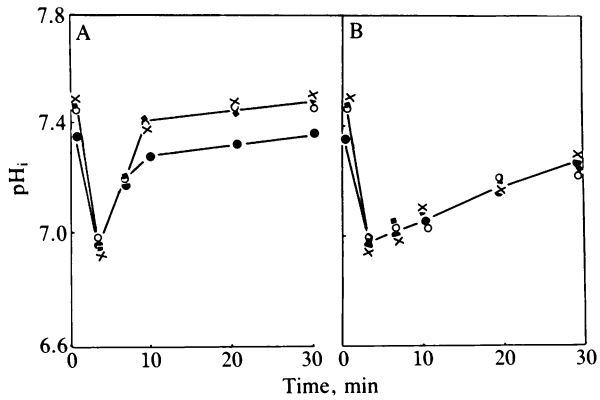

FIG. 2. Recovery of pH_i after exposure of cells to NH_4^+. Confluent cultures of CHEF/18 (●), 21-2 (○), 21-2-M3 (×), or T30-4 (■) were preequilibrated for 30 min in glucose–saline medium (pH 7.2) containing 20 mM NH_4Cl. Cells were then rinsed rapidly twice in standard glucose–saline medium. Cells were next incubated in standard glucose–saline medium containing [^{14}C]benzoic acid in the absence (*A*) or presence (*B*) of 100 μM amiloride for the indicated times. pH_i was then determined as described. Each point is the average of triplicate determinations. pH_i at time 0 was determined by adding [^{14}C]benzoic acid to the preequilibration medium for 5 min.

lines. The rate of recovery was also identical in the tumorigenic and nontumorigenic cells.

To ascertain that the recovery from the acid load was indeed due to a Na^+/H^+ antiporter, acid-loaded cells were treated with 100 μM amiloride, a concentration found to inhibit >80% of growth factor-stimulated increases in pH_i in CHEF/18 cells (10). This drug hindered the ability of all cell lines to recover from the acid load (Fig. 2*B*). These data show that a Na^+/H^+-exchange system does play a crucial role in the return of pH_i to control levels in all four CHEF lines after cytoplasmic acidification and, thus, implicate this antiporter in the maintainance of increased pH_i levels in the 21-2, 21-2-M3, and T30-4 cells.

Increase of pH_i in Tumorigenic CHEF Cells Is Not Due to Autocrine Growth Factor Production. One possible explanation for the increased pH_i values in the tumorigenic CHEF cells is the synthesis of autocrine growth factors resulting in continuous stimulation of the Na^+/H^+ antiporter. Previous work by Cherington and Pardee has shown no evidence for such growth factor production in the CHEF/18 and T30-4 lines (16). We extended these studies to the 21-2 and 21-2-M3 cells to show that the conditioned medium from 21-2 or 21-2-M3 cultures was unable either to stimulate the growth or to increase the pH_i of the nontumorigenic CHEF/18 line (Table 1). These results indicate that the 21-2 and 21-2-M3 lines do not secrete autocrine growth factors or other agents capable of influencing pH_i.

Tumorigenic CHEF Cells Do Not Have Altered Protein Kinase C Activity. CHEF/18 cells contain a protein kinase C-mediated pathway for modulation of the Na^+/H^+ antiporter (10). Alterations in protein kinase C activity or distribution could thus lead to abnormal control of the Na^+/H^+ antiporter and consequently to aberrant pH_i values (2). Therefore, we examined protein kinase C activity in our four CHEF cell lines. No significant differences in protein kinase C activity were found among the CHEF/18, 21-2, 21-2-M3, and T30-4 cells (Table 2). In addition H-7, a specific protein kinase C inhibitor (19), did not detectably reduce pH_i in the tumorigenic lines (Table 3), though it did result in a >80% inhibition of protein kinase C activity (Table 2). These two pieces of evidence argue against alterations in the protein kinase C-mediated antiport activation pathway as an explanation for the increased pH_i in the tumorigenic CHEF cells.

Manipulations of Ca^{2+} or Calmodulin Do Not Affect pH_i in the Tumorigenic CHEF Cells. The Na^+/H^+ antiporter of CHEF/18 cells can be modulated by a Ca^{2+}/calmodulin-mediated mechanism distinct from the aforementioned protein kinase C-dependent process (10); abnormalities in this pathway could also result in aberrant pH_i in the tumorigenic cell lines. Since epidermal growth factor (EGF), an agent that uses this Ca^{2+}/calmodulin mechanism to affect pH_i, acts by causing a Ca^{2+} influx rather than by mobilizing intracellular Ca^{2+} stores (10), we first examined the effects of Ca^{2+}-free medium on the pH_i of the 21-2, 21-2-M3, and T30-4 cells. Our results show that the pH_i of the tumorigenic CHEF lines was no more sensitive to depletion of external Ca^{2+} than was that of CHEF/18 (Table 3). Furthermore, studies using $^{45}CaCl_2$ did not reveal any significant differences in Ca^{2+} influx among the four cell lines (Fig. 3). These experiments show that the tumorigenic CHEF cells are not raising their internal pH by altering Ca^{2+} uptake.

As it is conceivable that the tumorigenic cells may have obviated their need for external Ca^{2+} by using intracellular Ca^{2+} stores, we conducted studies using TMB-8, an antagonist of intracellular Ca^{2+} release (20). Our results demonstrate that TMB-8 had no effect on the pH_i of any of the CHEF lines (Table 3). The calmodulin antagonists W-7 and TFP (21) were likewise without effect on the pH_i of the tumorigenic CHEF lines, even when used at concentrations previously shown to block >85% of the EGF-induced rise in CHEF/18 pH_i (10). These findings show that a constitutive activation of the Ca^{2+}/calmodulin pathway for modulation of the Na^+/H^+ antiporter is not responsible for the increased pH_i found in the tumorigenic CHEF lines.

Changes in pH_i Affect S Phase Entry. Recent evidence has suggested a role for pH_i in the regulation of cellular proliferation, protein synthesis, and S6 ribosomal protein phosphorylation (7). To determine whether the observed aberrations in the pH_i regulation of the tumorigenic CHEF/18 derivatives relate to their altered growth properties, we examined reinitiation of DNA synthesis in density-arrested G_0/G_1 cultures of CHEF/18, 21-2, 21-2-M3, and T30-4 cells (Fig. 4*A*). In all cell lines, stimulation of [^{3}H]dThd incorporation by serum was dependent on pH_o values. The curves for entry of the tumorigenic cells into S phase were shifted by approximately 0.2 pH unit toward acidic values relative to that of CHEF/18. The lower limits for S-phase entry were found to be 6.4 for 21-2, 21-2-M3, and T30-4 and about 6.6 for CHEF/18. At pH_o values more acidic than these, the incorporation of [^{3}H]dThd remained at the levels found in quiescent cells. The relative tolerance of the tumorigenic CHEF lines to acidic pH_o values is consistent with the increased pH_i found in these cells in the quiescent state. Changes in [^{3}H]dThd incorporation were not a result of cell death or damage due to pH_o variation, as plating efficiency was not altered at pH_o values from 6.4 to 8.0 (unpublished observations).

The importance of internal pH in the regulation of cell proliferation is emphasized by a plot of [^{3}H]dThd incorporation versus pH_i values, which shows that the pH_i curves for [^{3}H]dThd incorporation are identical in all four lines (Fig. 4*B*) and, thus, indicates a controlling role for pH_i in the entry of cells into S phase. Similar results have been reported by Pouyssegur *et al.* (22) using Chinese hamster lung fibroblast mutants that have altered Na^+/H^+-antiporter activity.

Table 1. Effect of conditioned medium (CM) from tumorigenic cells on CHEF/18 growth and pH_i

Conditions	Cells × 10^{-5}	pH_i
DMEM/0.2% FCS	0.56	7.35 ± 0.04
21-2 CM	0.55	7.37 ± 0.05
21-2-M3 CM	0.55	7.34 ± 0.04
DMEM/10% FCS	2.14	7.58 ± 0.07

CHEF/18 cells were plated at 0.3 × 10^5 cells per 35-mm dish in DMEM containing 10% fetal calf serum (FCS). After 24 hr, cells were shifted to the conditions shown above. Cultures were assayed for pH_i after 15 min or for cell number per 35-mm dish after 48 hr.

Table 2. Protein kinase C activity in CHEF cells

Addition	Protein kinase C activity, pmol of ^{32}P per min/mg of protein			
	CHEF/18	21-2	21-2-M3	T30-4
None	443 ± 38	427 ± 36	453 ± 41	431 ± 33
H-7	69 ± 40	56 ± 35	47 ± 43	62 ± 31

Confluent quiescent cells were incubated in serum-free DMEM with or without 100 μM H-7 for 30 min prior to determination of protein kinase C activity. Results are the means ± SEM of duplicates.

DISCUSSION

Our examination of several tumorigenic lines sequentially derived from the nontransformed, nontumorigenic CHEF line CHEF/18 has shown that these derivatives maintain a resting pH_i that is increased by 0.12 pH unit above that of the parental cells. This change in pH_i is due to the action of a

Table 3. Effect of inhibitors of protein kinase C and calmodulin on pH_i

Addition or replacement	pH_i			
	CHEF/18	21-2	21-2-M3	T30-4
None	7.35 ± 0.04	7.46 ± 0.05	7.46 ± 0.03	7.46 ± 0.04
H-7	7.34 ± 0.05	7.45 ± 0.03	7.43 ± 0.05	7.45 ± 0.05
Mn^{2+} for Ca^{2+}	7.30 ± 0.03	7.39 ± 0.06	7.41 ± 0.04	7.41 ± 0.04
TMB-8	7.37 ± 0.05	7.46 ± 0.03	7.45 ± 0.03	7.46 ± 0.05
W-7	7.36 ± 0.04	7.45 ± 0.04	7.48 ± 0.07	7.46 ± 0.04
TFP	7.33 ± 0.06	7.43 ± 0.05	7.44 ± 0.06	7.43 ± 0.06

Studies were conducted as described in *Materials and Methods* with the following modifications. For investigations using the inhibitors H-7 (100 μM), TMB-8 (50 μg/ml), and TFP (20 μM), the appropriate agent was present during the last 30 min of the preequilibration and thereafter. In experiments using W-7 (100 μM), the inhibitor was present throughout the entire preequilibration and subsequent assay. Studies in nominally Ca^{2+}-free medium were conducted in glucose–saline medium in which the normal 2 mM $CaCl_2$ content was replaced with an equivalent concentration of $MnCl_2$. Assay time was 15 min. Results are the means ± SEM of three determinations.

Na^+/H^+ antiporter since the increased pH_i values in the tumorigenic CHEF cells were observed in the absence of HCO_3^- but were affected by amiloride (Fig. 2). We have demonstrated that these cells do not produce autocrine growth factors, nor do they exhibit persistent activation of either the protein kinase C or Ca^{2+}/calmodulin-mediated pathways previously shown to modulate the Na^+/H^+ antiporter in CHEF/18 cells. These findings suggest an alteration in the functioning of the antiporter itself may be responsible for the aberrant pH_i observed in the tumorigenic CHEF cells.

Further support for an altered Na^+/H^+ antiporter in the 21-2, 21-2-M3, and T30-4 lines comes from studies using acid-loaded cells (Fig. 2). The rate of recovery from the acid load was identical in all four cell lines; only the final resting value differed. The simplest interpretation of these results is that the CHEF/18 derivatives have the same number of Na^+/H^+ antiporters as in the parental line, but that the antiporters in the tumorigenic lines are altered such that they have a higher "set point" (1) and, hence, maintain a higher pH_i.

Our work demonstrates that aberrations in pH_i control can confer altered growth properties, specifically relative responsiveness in an acidic environment. Thus, activation of the Na^+/H^+ antiporter may facilitate abnormal growth and tumor formation *in vivo*. Other evidence for the involvement of a Na^+/H^+-exchange system in the development of tumorigenicity comes from several sources. Known tumor promoters such as the phorbol esters stimulate Na^+/H^+ exchange in various systems (1). Moreover, some primary tumor tissues and tumor-derived cell lines secrete growth factor-like proteins (e.g., v-sis protein, transforming growth factor α) that are expected to mimic normal growth factors (platelet-derived growth factor and EGF, respectively) in their ability to cause antiporter activation. Finally, abnormal Na^+/H^+ antiporter activity could explain the high rates of lactic acid production often observed in tumorigenic tissues, since phosphofructokinase, the rate-limiting enzyme in glycolysis, is quite responsive to increases in pH (1).

In spite of the evidence supporting the involvement of pH_i in the development of the tumorigenic phenotype, such a change in pH_i is not obligatory for the acquisition of tumorigenicity in CHEF cells since we have found two weakly tumorigenic CHEF/18 derivatives whose pH_i values are indistinguishable from that of the parental line (unpublished observations). Moreover, the extent of the change in pH_i does not correlate with the degree of tumorigenicity in the CHEF lines examined here: 21-2-M3 and T30-4, both of which cause tumors in nude mice with high frequency (11), have the same pH_i as 21-2, a weakly tumorigenic line from which they were serially derived. These data suggest that a change in pH_i, while relatively benign in itself, may enhance the tumorigenic potential of other cellular lesions. Such may be the case with T30-4, a line that has lost its requirement for EGF (12) in addition to maintaining an increased pH_i. This hypothesis is consistent with the current understanding of the acquisition of the tumorigenic state as being a complex,

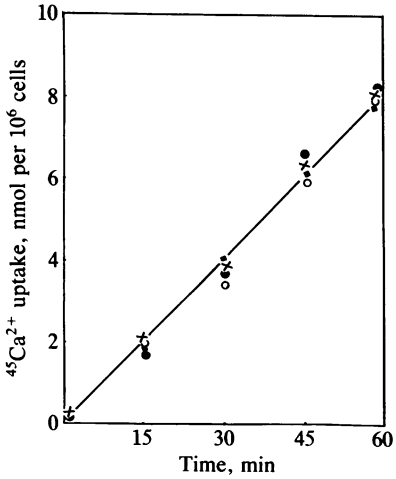

FIG. 3. $^{45}Ca^{2+}$ uptake by CHEF cells. Confluent, quiescent CHEF/18 (●), 21-2 (○), 21-2-M3 (×), or T30-4 (■) cells were preequilibrated for 30 min in serum-free DMEM. $^{45}CaCl_2$ was then added at 0.75 μCi/ml. Incubations were continued for the indicated times, and cell-associated radioactivity was determined.

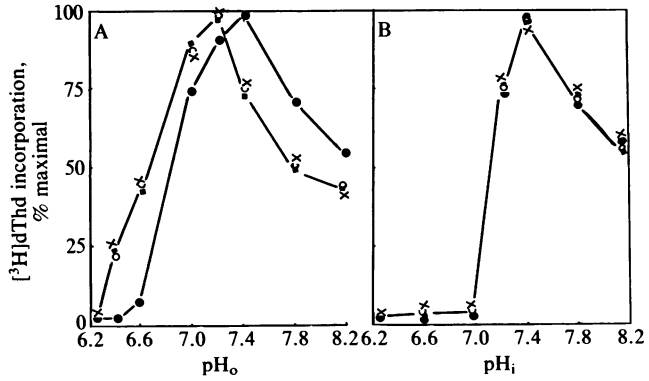

FIG. 4. pH dependence of DNA synthesis in CHEF cells. Confluent quiescent cells were prepared as described. Cultures were then shifted to HCO_3^--free DMEM supplemented with 10% fetal calf serum and buffered to the indicated pH. [^{3}H]dThd uptake was determined at 24 hr and is plotted against the measured pH_o (*A*) or pH_i (*B*) at that time. Cells: ●, CHEF/18; ○, 21-2; ×, 21-2-M3; ■, T30-4. The 100% incorporation value was 2.46 × 10^6 cpm per 35-mm dish; no significant difference in the maximal incorporation value was detected among the four cell lines.

multistep process capable of being caused by combinations of many different factors (2).

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