

# Chemical Inducers of Differentiation in a Long-Term Renal Cell Line

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The long-term renal epithelial cell line LLC-PK<sub>1</sub> expresses at confluence several differentiated characteristics of renal proximal tubule including Na<sup>+</sup>/glucose cotransport and several brush border membrane hydrolases. The differentiation-inducing chemical hexamethylene bisacetamide (HMBA) triggers a dramatic induction of Na<sup>+</sup>/glucose symport, trehalase and maltase, expressed as an increase in the number of cells in the culture that express the differentiated phenotype. Characteristics of the induction response are reviewed in terms of proposed mechanisms of inducer action. New evidence suggests that in addition to elevation of intracellular Na levels mediated by partial inhibition of the sodium pump, HMBA treatment also alters polyamine levels via effects on ornithine decarboxylase. These responses may be mediated by HMBA effects on protein kinase C activity. The possible role of polyamine fluctuations and DNA demethylation in mediating HMBA effects on differentiated gene expression is currently being investigated.

## Introduction

In recent years, considerable attention has focused on the expression of renal functions in cell culture. These experimental systems offer the advantages associated with the growth of a single cell type *in vitro*, including the opportunity to examine effects of variables such as cell growth and metabolism, hormonal environment, substratum, cell-cell interactions, cell polarity, and specific gene expression.

This review will focus on factors that influence the expression of the differentiated phenotype of the long-term cell line LLC-PK<sub>1</sub> derived from kidney proximal tubule. This polarized epithelial cell line expresses in culture a number of differentiated functions characteristic of proximal tubule including Na<sup>+</sup>/glucose cotransport activity and several brush border membrane disaccharidase activities such as trehalase and maltase (1). These activities are expressed after the development of a high cell density and confluent monolayer and are localized in the apical membrane.

## Enhancement of a Differentiated Phenotype by Exogenous Inducers

### Categories of Inducers

Certain differentiation-inducing chemicals trigger a dramatic progressive increase in expression of several differentiated functions after addition to confluent monolayers. The biological activity of these inducers was first detected by the visible increase in dome formation that followed their addition to MDCK cells, another long-

term renal cell line, of distal tubule or collecting duct origin (2). Domes result from transepithelial fluid transport in cell culture and reflect both differentiated and undifferentiated functions, key contributors being Na,K-ATPase activity, cell adhesiveness to the substratum, development of occluding junctions, and membrane polarization. Subsequently, it was demonstrated that certain inducers triggered enhanced levels of other differentiated functions in LLC-PK<sub>1</sub> cells, including Na<sup>+</sup>/glucose symport activity (3,4) and several microvillar hydrolase activities (1).

Inducers fell into three major categories: a) polar compounds such as hexamethylene bisacetamide (HMBA), dimethylformamide (DMF), and dimethyl sulfoxide (DMSO) (2,5); b) physiological compounds such as butyrate and adenosine (2,5); and c) inhibitors such as 5-azacytidine, an inhibitor of DNA methylation and ouabain (6), an inhibitor of Na,K-ATPase. These agents are known to stimulate cell differentiation in a wide range of cell types, suggesting that similar intracellular mechanisms operate regardless of the final pathway of differentiation. However, the mechanism by which they stimulate the development of a differentiated phenotype is not understood.

## Inducible Markers in Renal Cell Cultures

### Microvillar Hydrolases

Trehalase, maltase,  $\gamma$ -glutamyltranspeptidase, leucine aminopeptidase, and alkaline phosphatase activities were identified in the apical membrane of LLC-PK<sub>1</sub> cultures (1). Inhibitor and kinetic characteristics of these activities closely resembled those of renal proximal tubule. Microvillar hydrolase activities were barely detectable in sparse,

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subconfluent cultures, but upon development of a confluent cell density and functional membrane polarization, these activities were significantly and coordinately increased.

Treatment of confluent cultures with the inducer HMBA produced a dramatic increase in both trehalase and maltase activities (1). Activities of other hydrolases such as leucine aminopeptidase and alkaline phosphatase were not affected. Induction was a progressive response, reaching a plateau after 20 to 30 days of exposure to HMBA (Fig. 1). The development of a confluent monolayer was a prerequisite for increased expression after inducer addition; if inducers were added to subconfluent cultures, increased trehalase activity did not appear until after the development of a confluent cell density. Similar results were obtained whether inducer was added at 1 day after plating cells at an initial confluent cell density or after addition to cells grown to confluence from an initial sparse density. Induced trehalase was localized in the apical membrane and activity exhibited an unchanged  $K_m$  for trehalase but increased  $V_{max}$ . Induction of trehalase was blocked by cycloheximide after a 24-hr lag period, suggesting the existence of an intracellular pool of trehalase with a long half-life. When extracts from control and HMBA-induced cells were mixed, activities were additive, suggesting that increased levels of trehalase activity were not due to synthesis of an activator or removal of an inhibitor.

*N,N*-Dimethylformamide (DMF) was also effective in inducing both trehalase and maltase activities. Activities increased progressively over a 30-day period. In this case,

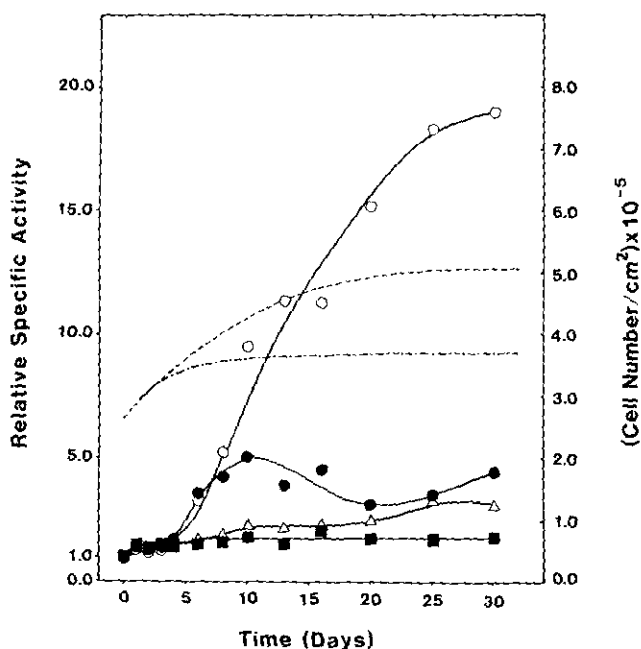


FIGURE 1. Induction of trehalase and maltase by HMBA. Trehalase activity in control (●) and HMBA-treated cultures (○). Maltase activity in control (■) and HMBA-treated cultures (△). Cell density in control (- - -) and HMBA-treated cultures (- · - ·). From Yoneyama and Lever (1).

induction of maltase exceeded that of trehalase. DMF was far more cytotoxic than HMBA; if activities are normalized to cell number or DNA content, the magnitude of induction by DMF is far greater than if normalized to total cell protein.

DMF and HMBA differed in reversibility characteristics. While effects of DMF were freely reversible after removal of inducer, effects of HMBA persisted over 4 days after removal.

## Commitment Period

A 48-hr exposure to HMBA was sufficient to commit the cells to commence a program of increased trehalase expression. The subsequent degree of induction increased as a function of time in culture after the initial exposure and the inducer concentration. Cells appeared to retain a memory of inducer treatment for at least 1 week, but induction was reversed after trypsinization and replating in the absence of inducer.

## Cell Patterning Response to HMBA

In order to further investigate the mechanism of increased trehalase expression after inducer treatment of LLC-PK<sub>1</sub> cultures, fluorescence immunolocalization studies were carried out using a polyclonal antibody to trehalase purified from pig kidney cortex (7).

The antibody recognized a 100 to 110 kD protein by Western blot analysis of HMBA-induced LLC-PK<sub>1</sub> cell extracts but this band was not visible in uninduced cell extracts. The antibody inhibited trehalase activity in cell extracts, whereas no inhibition was observed after incubation with a control antibody; also, trehalase activity was immunoprecipitated after overnight incubation with antitrehalase IgG.

Figure 2 shows that HMBA-induced cultures contain a larger number of trehalase positive cells than uninduced confluent cultures. Trehalase staining was localized in the apical membrane and exhibited the typical punctate staining pattern characteristic of apical membrane proteins. Interestingly, trehalase-positive cells were not randomly distributed over the monolayer but were organized into clusters and strands of cells (Fig. 2). These clusters and strands were also distinguishable from the flattened areas of the monolayer by phase contrast optics (Fig. 2). The relationship of this phenomenon to inducer action was established by the observation that the number of trehalase positive cells increased as a function of HMBA concentration up to an optimum of 10 mM and were also increased as a function of duration of exposure to inducer. The kinetics of the appearance of trehalase-positive cells correlated well with the kinetics of the appearance of increased trehalase activity.

It is unlikely that the trehalase-positive cells in strands may represent a preexisting cell population rather than an induced phenotype. Six clonal lines derived from a parental LLC-PK<sub>1</sub> culture exhibited induction of trehalase and development of strand formation, indicating that a single cell has the potential to give rise to

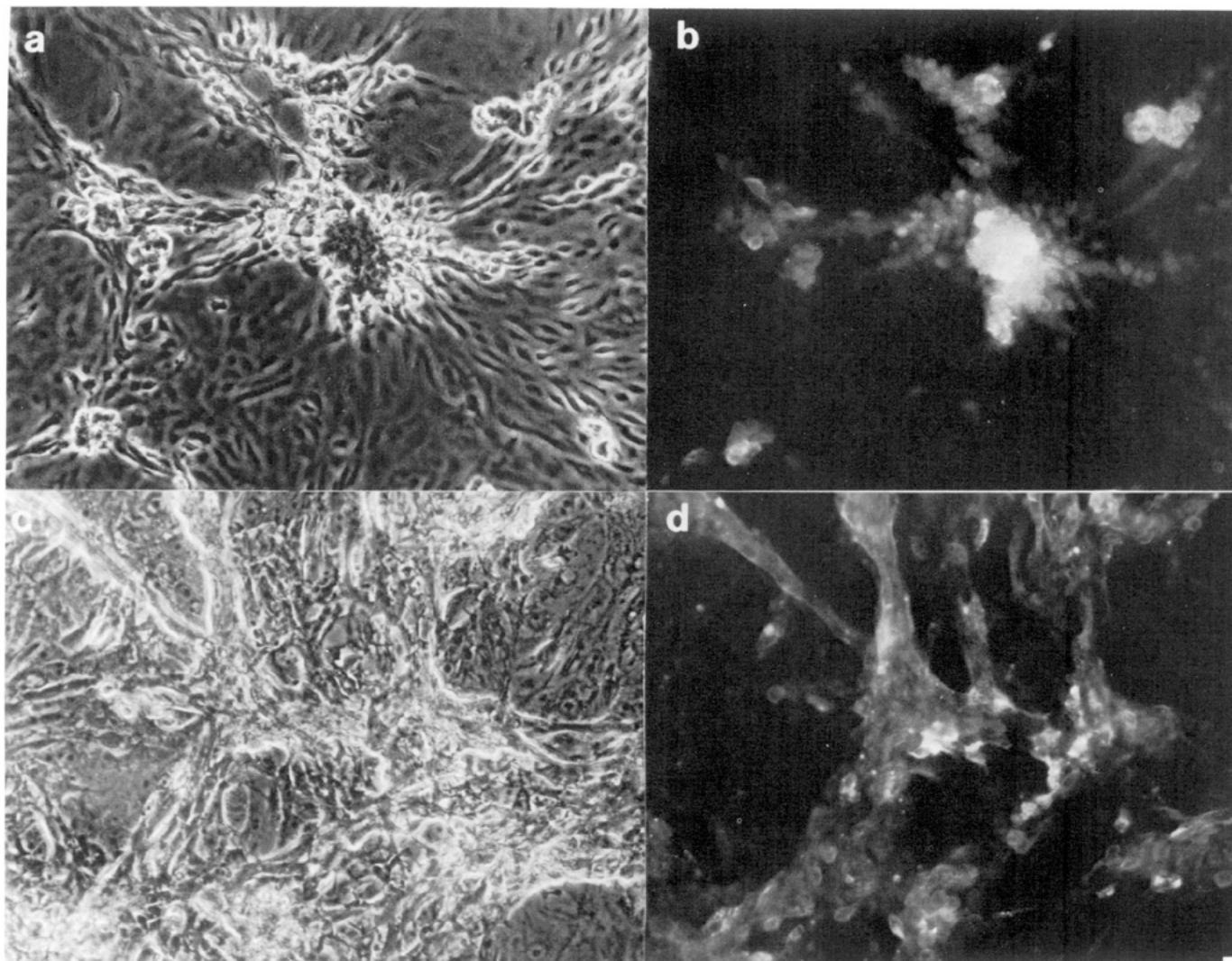


FIGURE 2. Immunofluorescence localization of trehalase in HMBA-induced cultures. (a) Phase contrast and (b) fluorescence optics, control cultures. (c) Phase contrast and (d) fluorescence optics, cultures treated for 2 weeks with 10 mM HMBA. Cultures were stained with rabbit polyclonal antitrehalase antibody and FITC-conjugated goat antirabbit second antibody, as described previously (?). Bar =  $\mu\text{m}$ .

Figure 3 illustrates a scanning electron micrograph of a LLC-PK<sub>1</sub> monolayer. Both flattened and strand-forming regions of the monolayer exhibit characteristic microvilli on the apical membrane surface, and this morphology is unaltered after HMBA treatment apart from an increased incidence of cells in strands. Interestingly, well-developed cilia are evident on most cells in the monolayer, as shown in more detail in Figure 3B. Cilia formation in LLC-PK<sub>1</sub> monolayers has also been observed by Mullin and Klein-zeller (8). These cilia presumably function in the proximal tubule *in vivo* to facilitate fluid flow.

Induction of trehalase expression could be dissociated from effects on cell patterning. DMF induces the same increase in trehalase activity as HMBA but does not elicit the cell patterning and cell strand-forming response. In this case, trehalase-positive cells were scattered at random over the monolayer and all cells exhibited a flattened cuboidal morphology (?). Thus, clustering of trehalase-positive cells is not a prerequisite for increased expression of trehalase.

One hypothesis to explain the different actions of HMBA and DMF on the spatial distribution of trehalase-positive cells over the monolayer proposes passage of intracellular mediators of differentiation through cell-cell junctions. Expression of the differentiated phenotype, monitored by the number of trehalase-positive cells, can be viewed as a stochastic event, the probability of which is increased after inducer treatment. The result is an increased number of positive cells in the population. HMBA would regulate the levels of intracellular mediators (e.g., ions, polyamines) that could diffuse to adjacent cells via cell-cell junctions, accounting for the clustering of trehalase-positive cells. By contrast, DMF would not promote cell-cell communication, resulting in isolated, randomly distributed trehalase-positive cells.

Another possible explanation is based on division of committed cells. A diminished rate of cell division persists in a confluent monolayer. HMBA treatment does not appreciably inhibit cell proliferation, whereas DMF is strongly inhibitory.

## Induction by Growth on a Permeable Support

Growth of LLC-PK<sub>1</sub> monolayers on polycarbonate filters (Nuclepore, 5  $\mu$ m pore size) led to increased trehalase expression and extensive development of strand formation, approaching the levels observed after HMBA treatment of monolayers grown on plastic. Trehalase expression was almost exclusively confined to cell strands. These observations indicate that growth of monolayers on a permeable substratum mimics the effects of HMBA on monolayers grown on plastic.

Enhancement of differentiated gene expression by growth on collagenous, basal lamina or permeable substrata have been noted in other epithelial cell types (9), but the mechanism of these effects is not understood. It has been proposed that enhanced access of nutrients to basolateral transport sites is responsible. Extensive changes in epithelial morphology have been described under these growth conditions, including a more columnar cell shape and deposition of a basal lamina (10).

## Role of Cell-Cell Contact and Cell Density

Trehalase expression was greatly decreased in sparse, subconfluent cultures, although a few positively staining cells were occasionally visible in the center of cell islands, a region of high local cell density. This result could not be explained by removal of preexisting trehalase by trypsinization before replating at low cell density. When a confluent monolayer was wounded with a pipette, the local region of rapid cell proliferation and low cell density thus created was devoid of trehalase-positive cells. These results lend support to the interpretation that the cells do not terminally differentiate.

One may conclude from these findings that induction of increased trehalase expression requires cell-cell contact and a high local cell density. However, these conditions are not sufficient, since only a fraction of cells in an induced confluent monolayer express trehalase. Since alterations in cell proliferation, cell shape and volume, cytoskeleton, and cell-cell junction formation are intimately coupled to changes in cell density, it is difficult to pinpoint the contribution of each variable. Densely confluent monolayers are capable of a diminished rate of cell division and tight junctions have been observed to persist even in mitotic cells (11).

## Induction of Trehalase by Glucose Deprivation: Dissociation from Induction by HMBA

When LLC-PK<sub>1</sub> cultures were switched to medium in which glucose was replaced by galactose, induction of trehalase was observed (Fig. 4). As noted in the case of induction by HMBA, trehalase induction accompanying glucose deprivation was a slow, adaptive process occurring over 20 to 30 days. An alternate carbohydrate source was required to observe induction; supplementation with

galactose was optimal with less induction observed in the presence of mannose and fructose. Concentrations of glucose above 5 mM were sufficient to block induction. The nonmetabolizable glucose analog  $\alpha$ -methyl glucose permitted slightly increased levels of trehalase compared with glucose-containing medium, suggesting that sugars exert their effects after interaction with a cellular receptor rather than by generation of an intracellular metabolite.

Clonal sublines were derived nonselectively from the parental population and fell into two categories: Cells that exhibited trehalase induction after glucose replacement by galactose, and cells that did not exhibit trehalase induction after glucose replacement. Both categories exhibited inducibility of trehalase by HMBA. These results suggest that trehalase induction after glucose deprivation occurs by a different mechanism than induction observed after HMBA treatment.

In clones maintained for 2 weeks in glucose-free, galactose-supplemented media, glycogen levels and lactate production were diminished, but ATP levels were unchanged. Activities of glucose-6-phosphatase, a rate-limiting enzyme of gluconeogenesis, were dramatically elevated. Isolation of gluconeogenic LLC-PK<sub>1</sub> clones was recently described (12). In this case, clones were selected for ability to proliferate well under glucose-deprived conditions. These results indicate that the LLC-PK<sub>1</sub> line contains cells that retain the capability for gluconeogenesis associated with renal proximal tubule.

## Induction of Na<sup>+</sup>/Glucose Symport Activity

Amsler and Cook (3) have demonstrated that addition of HMBA to confluent LLC-PK<sub>1</sub> monolayers accelerated the development of Na<sup>+</sup>/glucose symport activity. This response was blocked by addition of the tumor promoter TPA, suggesting that the protein kinase C system may be involved. By contrast, the facilitative type of glucose transporter is induced by TPA (13). Using phlorizin, a specific high-affinity ligand that serves to titrate the number of active symporter units, Amsler and Cook (4) demonstrated that the induced transport activity represented an increased number of symporter sites.

Using monoclonal antibodies that specifically bind the Na<sup>+</sup>/hexose symporter of LLC-PK<sub>1</sub> cells (14,15), we carried out fluorescence immunolocalization of symporter expression in LLC-PK<sub>1</sub> monolayers. Antibodies recognized a 75 kD protein in cell extracts as determined by Western blot analysis; this is the same apparent molecular weight observed in renal brush border membranes from pig kidney. The symporter was purified to homogeneity from pig kidney brush border membranes and active fractions could be identified by reconstitution of Na<sup>+</sup>-dependent phlorizin binding activity (16). The purified preparation was a glycoprotein.

Immunolocalization studies revealed that HMBA-induced monolayers contained a dramatic increase in the number of symporter-positive cells. As noted for trehalase, symporter-positive cells were organized in clusters

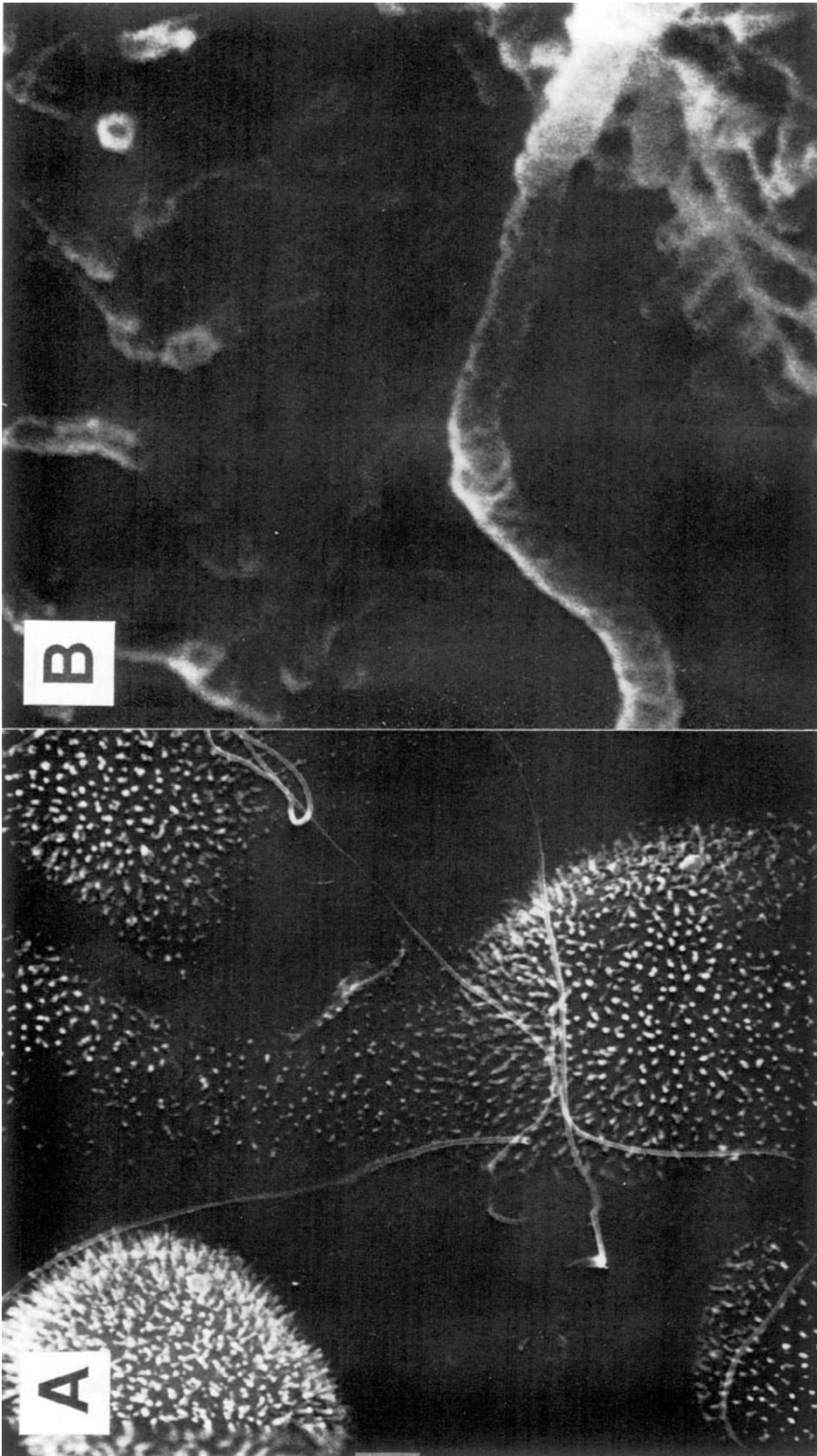


FIGURE 3. Cilia formation in LLC-PK<sub>1</sub> cell monolayers. (A) HMBA-induced culture; (B) close-up of cilium.

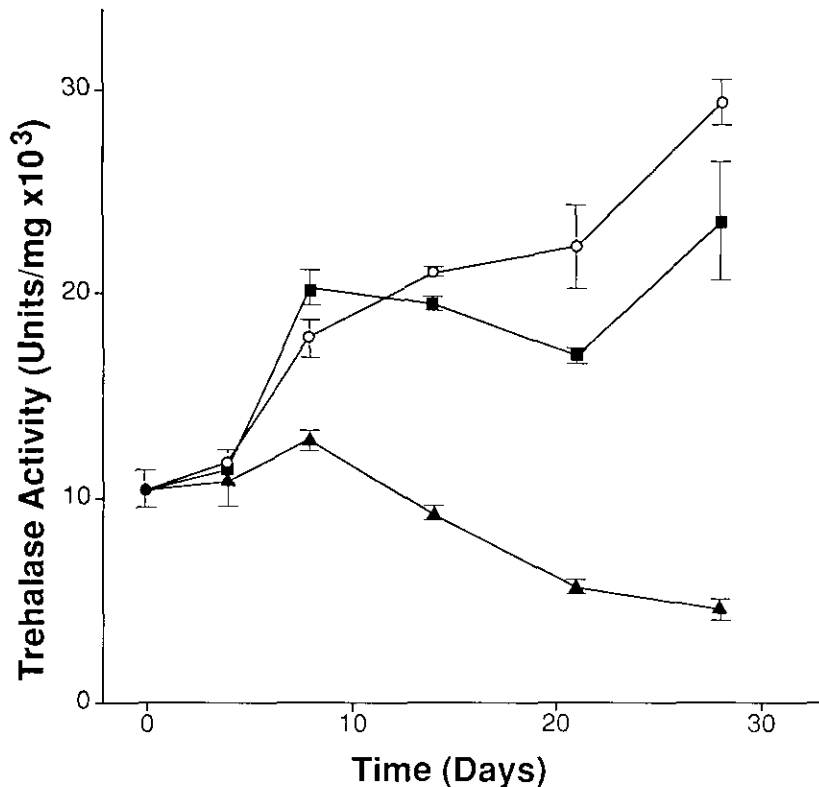


FIGURE 4. Induction of trehalase after glucose deprivation. Glucose-grown cultures were switched on day 0 to medium supplemented with either (○) 10% undialyzed serum but no hexoses; (▲) 10% dialyzed serum plus 25 mM glucose; or (■) 10% dialyzed serum plus 25 mM galactose.

or strands rather than randomly distributed in the monolayer.

Taken together, immunolocalization studies have demonstrated in the case of two differentiated markers, trehalase and the  $\text{Na}^+$ /glucose symporter, that HMBA enhances the differentiated phenotype by increasing the proportion of cells in the culture that express differentiation markers, rather than by increasing the expression of these markers uniformly over the cell population. HMBA appears to facilitate a commitment step that turns on a program of differentiated expression.

## Mechanism of Action of Inducers

Although the precise mechanisms that regulate differentiated gene expression in renal epithelial monolayers are not understood, studies using chemical inducers and cell variants have provided some information. Although HMBA and DMF are equally potent as inducers, they clearly operate by different mechanisms. Effects of DMF are readily reversible after removal of inducer (1), whereas after a 48-hr commitment period, cells retain a memory of HMBA exposure (7). HMBA stimulates increased intracellular cyclic AMP levels after 24 hr, whereas DMF does not affect cyclic AMP levels (17). HMBA induces the cell patterning response; DMF does not (7). Variant MDCK cells were isolated that were resistant to induction by HMBA and by DMF (18).

An early event common to both inducers is elevation of intracellular  $\text{Na}^+$  levels. This is achieved after a 24-hr in-

cubation with inducers, requires *de novo* protein synthesis, and involves a 30 to 40% reduction in the transport efficiency of the  $\text{Na},\text{K}\text{-ATPase}$  (6,19). The significance of this finding is underscored by the observation that inducer-resistant variants were refractory to pump inhibition by HMBA (18). Inhibition was not due to a reduction in the number of pump units, as shown by titration with tritiated ouabain, could not be explained by decreased accessibility to basolateral pump sites, and did not result from changes in ATP levels. Since  $\text{Na}^+/\text{Ca}^{2+}$  antiport has been demonstrated in LLC-PK<sub>1</sub> cells (20), it is predicted that increased  $\text{Na}^+$  levels would result in increased intracellular  $\text{Ca}^{2+}$  levels. This may in turn activate  $\text{Ca}^{2+}$ -dependent protein kinases, phospholipases, or other potential components of a regulatory cascade. We were unable to demonstrate increased  $\text{Ca}^{2+}$  levels using quin-2 within the first 24 hr after inducer treatment. It is possible that these effects are too transient to be measured under our conditions.

Increased intracellular  $\text{Na}^+$  levels have been observed to trigger differentiation in a wide variety of cell types, suggesting a fundamental role. Ouabain promoted differentiation in mouse embryos (21) and murine erythroleukemia (MEL) cell cultures (22).

## Insights into the Mechanism of HMBA Action Obtained with MEL Cells

**Intracellular Metabolites.** HMBA is now entering phase 1 clinical trials as an anticancer agent used in

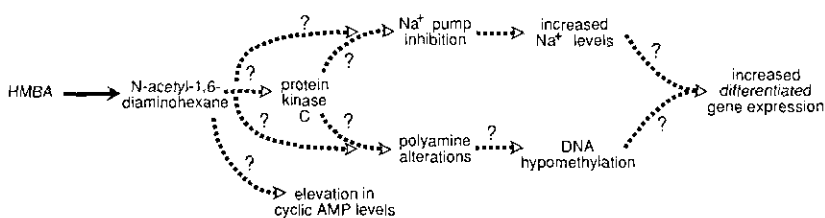


FIGURE 5. A working hypothesis to explain induction of cell differentiation by HMBA.

differentiation therapy (23). As a result, its intracellular metabolites have been identified in both human and animal studies (24,25). Metabolites included 6-acetamidohexanoic acid; *N*-acetyl-1,6-diaminohexane; 1,6-diaminohexane; 6-aminohexanoic acid and its lactam, caprolactam. The acetyl moieties of HMBA can be incorporated into lipids and protein (26). *N*-Acetyl-1,6-diaminohexane also accumulates after HMBA addition to MEL cells and exogenous addition of this metabolite is able to trigger the complete differentiation process in MEL cells (27). This implies that HMBA is not directly responsible for induction but acts via its catabolites.

**Polyamines.** HMBA addition to MEL cells caused a decrease in ornithine decarboxylase activity (ODCase), as well as changes in polyamine levels (28). The polyamine biosynthetic enzyme ODCase is negatively modulated by a variety of diamines, including the HMBA metabolite *N*-acetyl-1,6-diaminohexane (27). Strong evidence that changes in cellular polyamines may mediate HMBA-induced differentiation was provided by observations that difluoromethylornithine (DFMO), an irreversible ODCase inhibitor, stimulated differentiation of MEL cells (29). Methyl glyoxal bis(guanyl hydrazone), a competitive inhibitor of *S*-adenosylmethionine decarboxylase, produced opposing changes in polyamines and blocked induction by HMBA. Findings from this study suggested that polyamine-dependent events early in the commitment period were necessary for induction of differentiation.

**DNA Hypomethylation.** In addition to its effects on polyamine biosynthesis, DFMO dramatically increases intracellular levels of decarboxylated *S*-adenosylmethionine (SAM) (30). Since *S*-adenosylmethionine is the methyl donor in most intracellular methylation reactions, accumulation of its intracellular derivative might be expected to influence cellular methylation.

HMBA-induced differentiation in MEL cells is accompanied by a genome-wide DNA hypomethylation (31). Furthermore, addition of 5-azacytidine, an inhibitor of DNA methylation, could induce MEL cell differentiation (31). Hypomethylation during MEL cell differentiation was transient, resulting from rapid demethylation followed by *de novo* methylation (32). Razin et al. (32) have provided elegant evidence that DNA demethylation during HMBA induction of MEL cell differentiation does not require DNA replication but represents an active enzymatic replacement of 5-methylcytosine by cytosine in nonreplicating DNA.

**Protein Kinase C.** Tumor promoters such as phorbol 12-myristate 13-acetate inhibit differentiation in both

MEL cells (33) and in LLC-PK<sub>1</sub> cultures (3). Protein kinase C is a specific receptor for the tumor promoter (34) and is believed to be intimately involved with signal transduction in cell proliferation and differentiation in a variety of cell types (35). Melloni et al. (36) have presented several lines of evidence that HMBA induction of MEL cell differentiation involves a proteolytically activated form of protein kinase C that does not require Ca<sup>2+</sup> or phospholipid for its catalytic activity. Polyamines have recently been shown to inhibit protein kinase C association with the membrane (37). Other categories of inducers, such as dimethyl sulfoxide, cause a decrease in phosphatidylinositol metabolites inositol triphosphate and diacylglycerol (38). Diacylglycerol activates protein kinase C and inhibits differentiation.

### Possible Model to Explain HMBA Effects on Cell Differentiation

Figure 5 presents a hypothetical scheme to interpret HMBA-mediated induction of differentiated gene expression, based on findings outlined previously. It appears likely that HMBA does not act directly but via one of its intracellular metabolites, *N*-acetyl-1,6-diaminohexane. Among the earliest intracellular events are a transient activation of protein kinase C (within 2 hr) followed by its depletion, a reduction in intracellular spermidine and putrescine and a rapid transient DNA demethylation, possibly influenced by changes in polyamine levels. Also within the first 24 hr, increased intracellular Na<sup>+</sup> levels are detected, mediated by inhibition of the Na<sup>+</sup>,K<sup>+</sup>-ATPase. Protein kinase C has been proposed to regulate Na<sup>+</sup>,K<sup>+</sup>-ATPase activity (39) and also mediate induction of ornithine decarboxylase activity (40). The temporal and causal relationship between these proposed mediators of differentiation and intermediate steps in the cascade of events have not yet been elucidated.

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