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# Differentiation-dependent expression of the $Na^+$/glucose cotransporter (SGLT1) in LLC-$PK_1$ cells: Role of protein kinase C activation and ongoing transcription

(renal epithelial cells/mRNA degradation)

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**ABSTRACT** **We examined changes in the mRNA level of SGLT1, a $Na^+$/glucose cotransporter, by the differentiation status of LLC-$PK_1$ renal epithelial cells. Proliferating (undifferentiated) cells revealed no detectable SGLT1 mRNA by Northern blot analysis. However, when cells became confluent and differentiated into polarized monolayers, there was an abrupt appearance of the SGLT1 mRNA. When confluent (differentiated) cells were dedifferentiated by reseeding at a subconfluent density, SGLT1 mRNA levels decreased quickly to nondetectable levels ($t_{1/2}$ = 1.5 h), while the mRNA levels of γ-glutamyltranspeptidase, another differentiation marker, decreased only slowly ($t_{1/2}$ > 40 h). This decrease in SGLT1 mRNA was completely blocked by H-7, a protein kinase inhibitor. Since protein kinase C was highly activated in the undifferentiated cells and treatment of differentiated cells with a phorbol ester also induced quick and complete loss of SGLT1 mRNA ($t_{1/2}$ = 1.5 h) but not of γ-glutamyltranspeptidase mRNA, protein kinase C activation appears to be involved in the dedifferentiation-induced decrease in SGLT1 mRNA. Although the phorbol ester-induced decrease in the SGLT1 mRNA level was blocked completely by inhibition of transcription, inhibitors of translation blocked the decrease in mRNA levels only partially.**

The renal epithelial cell line LLC-$PK_1$ expresses a $Na^+$/glucose cotransport activity in a differentiation-specific manner (1). This cotransport activity is similar to that found in the renal outer medulla and the small intestine (2) and has properties characteristic of the high-affinity $Na^+$/glucose cotransporter SGLT1 (3). By cDNA cloning (4), we demonstrated SGLT1 expression in this cell line.

Various mechanistic proposals have been offered to explain the dependence of $Na^+$/glucose cotransport expression in LLC-$PK_1$ cells on differentiation. $Na^+$/glucose cotransporter expression is correlated with the development of differentiation-specific structures such as microvilli, which form only when cultures become confluent (1). Disruption of differentiation-specific structures (e.g., tight junctions) decreases the expression of $Na^+$/glucose cotransport activity (5). Increased cAMP levels accelerate $Na^+$/glucose cotransporter induction (6) and LLC-$PK_1$ mutant lines defective in protein kinase A activity have impaired expression of both $Na^+$/glucose cotransporter activity and differentiation-specific structures (7). On the other hand, phorbol ester [phorbol 12-*O*-tetradecanoate 13-acetate (TPA)] inhibits the expression of $Na^+$/glucose cotransport activity (6). These studies suggest the possible role of both protein kinase A and protein kinase C in the regulation of $Na^+$/glucose cotransport activity.

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In an earlier study (4), we provided evidence that the modulation of $Na^+$/glucose cotransport activity in LLC-$PK_1$ cells induced by changes in medium glucose concentrations occurred at the level of mRNA abundance. We therefore hypothesized that changes in $Na^+$/glucose cotransport activity during differentiation of LLC-$PK_1$ cells are also regulated by the amount of SGLT1 mRNA. In support of that hypothesis, we now demonstrate that SGLT1 mRNA is not detectable in undifferentiated LLC-$PK_1$ cells and appears only after cells differentiate. Furthermore, we present evidence that the loss of SGLT1 mRNA during LLC-$PK_1$ cell proliferation is dependent on protein kinase C.

## MATERIALS AND METHODS

**Cell Culture.** LLC-$PK_1$ cells were maintained as described (4) and used at passages 202–217. For experiments using differentiated cells, one million cells were plated on a 6-cm dish and used 7–10 days later when cell monolayers showed abundant domes. The cell density of differentiated monolayers was $6 \times 10^5$ cells per $cm^2$.

**Northern Blot Analysis.** Total cellular RNA was extracted (8) and subjected to Northern blot analysis as described (4). Autoradiographic signals were quantitated by scanning (Hewlett–Packard ScanJet IIP) and analyzed (NIH Image Software) using a Macintosh computer. Filters could be stripped by boiling in 0.1% SDS and rehybridized at least three times without loss of sensitivity.

**Nucleic Acid Probes.** Porcine SGLT1 cDNA plasmid pPSGT-B1 was as described (4). Porcine γ-glutamyltranspeptidase (γ-GT) cDNA plasmid psGT18/2 (9) was provided by H. G. Gassen (Technische Hochschule, Darmstadt, Germany) and rat α-tubulin cDNA was from A. Rustgi (Massachusetts General Hospital). cDNA probes were labeled with [$\alpha$-$^{32}$P]dCTP by random priming.

**Protein Kinase C Assay.** For rapidly proliferating cells, one million cells were inoculated into a 15-cm dish and cultured for 48 h (50% confluent). Preparation of cell homogenate, separation of soluble and particulate fractions, and partial purification of protein kinase C by DEAE-cellulose column chromatography were performed as described (10). Kinase assay was performed using the Protein Kinase C Assay System (BRL).

## RESULTS

**SGLT1 mRNA Levels During LLC-$PK_1$ Cell Differentiation and Dedifferentiation.** We have found (4) that differentiated

Abbreviations: DRB, dichloro-β-D-ribofuranosylbenzimidazole; TPA, phorbol 12-*O*-tetradecanoate 13-acetate; γ-GT, γ-glutamyltranspeptidase.

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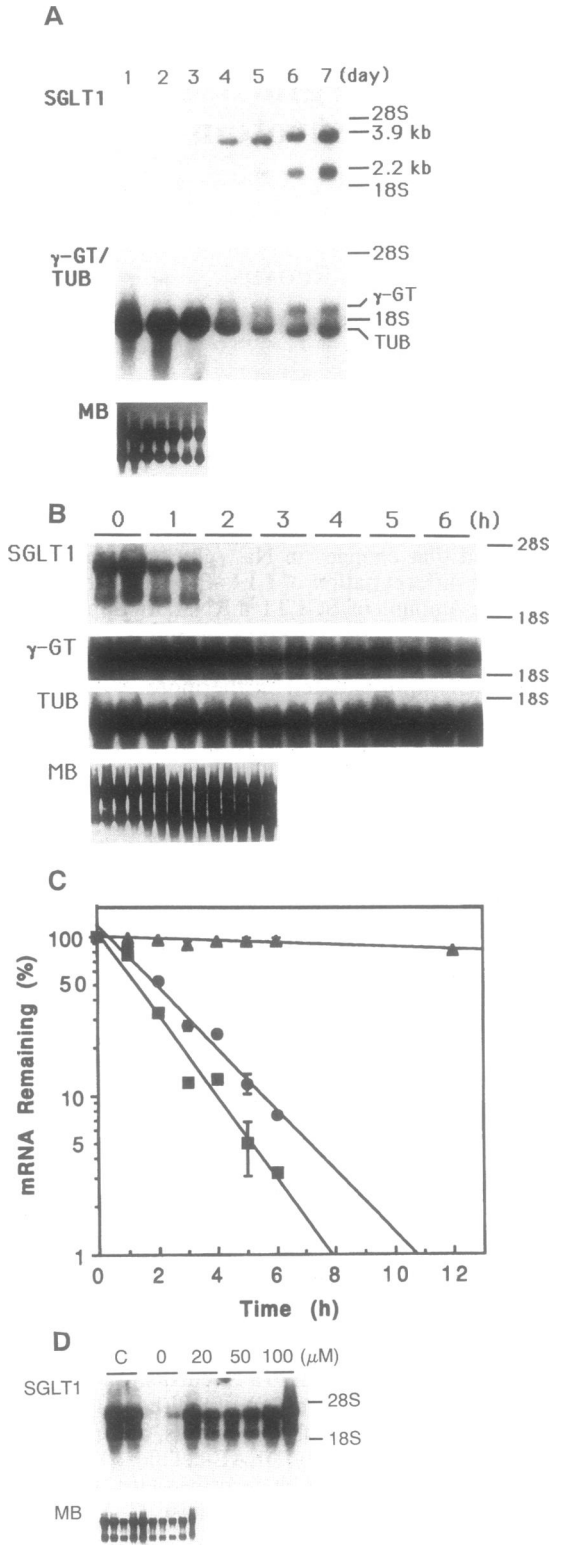


FIG. 1. Time course of SGLT1 and γ-GT mRNA levels during culture of LLC-PK$_1$ cells. Differentiated LLC-PK$_1$ cells were harvested by trypsinization and reseeded at $6 \times 10^3$ cells per cm$^2$ in 6-cm dishes. Total RNA was extracted at the indicated times after reseeding and subjected to Northern blot analysis. Note that there are two molecular species of SGLT1 mRNA (3.9 kb and 2.2 kb). (*A*) Induction of SGLT1 and γ-GT mRNAs by cell differentiation. Cells became confluent and differentiated at day 4. TUB, α-tubulin; MB, methylene blue staining of RNA on the filter. (*B*) Rapid decrease in SGLT1 mRNA level after reseeding. (*C*) Densitometric quantitation of data in *B* (■, 3.9-kb SGLT1; ●, 2.2-kb SGLT1; ▲, γ-GT). (*D*) Block of dedifferentiation-induced decrease in SGLT1 mRNA by H-7. Cells were reseeded as in *B* in the presence of H-7 at the indicated concentrations. Total RNA was extracted at 6 h from duplicated samples. Lanes C represent positive controls of mRNA from differentiated cells.

LLC-PK$_1$ cells express two molecular species of SGLT1 mRNA (3.9 kb and 2.2 kb) that are transcribed from the same gene and differ only in the length of the 3′ untranslated sequences (4). By Northern blot analysis, these SGLT1 mRNAs were absent in subconfluent cultures but appeared abruptly on day 4 when the cells reached confluence and began to form domes, a morphological criterion of differentiation (Fig. 1*A*). The mRNA level of γ-GT, another differentiation marker of this cell line (7), also increased during culture differentiation. On the other hand, the mRNA level of α-tubulin dropped significantly (3.5-fold) when cells became confluent (day 4), in accordance with previous reports (11).

When LLC-PK$_1$ cells were trypsinized and reseeded at subconfluent density to induce dedifferentiation, SGLT1 mRNA disappeared quickly within 6 h ($t_{1/2}$ = 1.5 h; Fig. 1 *B* and *C*), while γ-GT mRNA level decreased very slowly ($t_{1/2}$ > 40 h) and α-tubulin mRNA level remained unchanged.

Since several recent studies have demonstrated a role of protein kinase C in regulating specific mRNA levels (12–14), we examined the effects of H-7 [1-(5-isoquinolinylsulfonyl)-2-methylpiperazine], a potent inhibitor of protein kinases A and C (15), on the differentiation-induced loss of SGLT1 mRNA. When cells were reseeded at subconfluent density in the presence of H-7, the decrease in SGLT1 mRNA levels was effectively blocked (Fig. 1*D*), indicating the importance of protein kinase activation in this phenomenon. No cytotoxicity was observed during the 6 h of treatment at the H-7 concentrations used.

**TPA-Induced Decrease in SGLT1 mRNA.** To distinguish whether protein kinase A or protein kinase C is involved in the dedifferentiation-induced loss of SGLT1 mRNA, differentiated LLC-PK$_1$ cells were exposed to 8-bromo-cAMP or vasopressin to activate protein kinase A or to TPA to activate protein kinase C. Activation of protein kinase A did not result in a rapid decrease in SGLT1 mRNA levels, although prolonged treatment (48 h) resulted in a partial decrease (Y. Watanabe, T.O., and D.B.R., unpublished data). It had previously been shown that activation of protein kinase A in proliferating LLC-PK$_1$ cells accelerated the appearance of $Na^+$/glucose cotransport activity during differentiation, but the activation of protein kinase C suppressed it (6, 7).

In contrast, activation of protein kinase C by TPA induced a rapid decrease of SGLT1 mRNA levels comparable to that induced by reseeding (Fig. 2). The concentration of TPA used (0.1 μM) has been shown to activate protein kinase C in this cell line (16). After a 2-h lag period, SGLT1 mRNAs decreased quickly ($t_{1/2}$ of the rapid decrease phase = 1.5 h); at 6 h, these levels dropped to about 15% of the control (Fig. 2*B*). When cells were pretreated with 0.5 μM TPA for 24 h to down-regulate protein kinase C, the SGLT1 mRNA levels returned to the control values and these were not affected by further stimulation with TPA (data not shown). Like the induction of dedifferentiation by reseeding, TPA treatment resulted in a very slow decrease in γ-GT mRNA ($t_{1/2}$ > 10 h; Fig. 2*C*); α-tubulin mRNA level increased by 1.5-fold at 6 h (Fig. 2*A*). These results suggest the importance of protein kinase C in the dedifferentiation-induced decrease in SGLT1 mRNA. Interestingly, the mRNA for porcine $Na^+$/amino acid cotransporter SAAT1, which has 74% nucleotide sequence homology to SGLT1 (17), was stable in the presence of TPA (T.S. and D.B.R., unpublished data). This result supports the concept that decreased SGLT1 mRNA levels after protein kinase C activation are specific for SGLT1.

The estimated half-lives of SGLT1 and γ-GT mRNAs in differentiated LLC-PK$_1$ cells were 11.6 h and 25 h, respec-

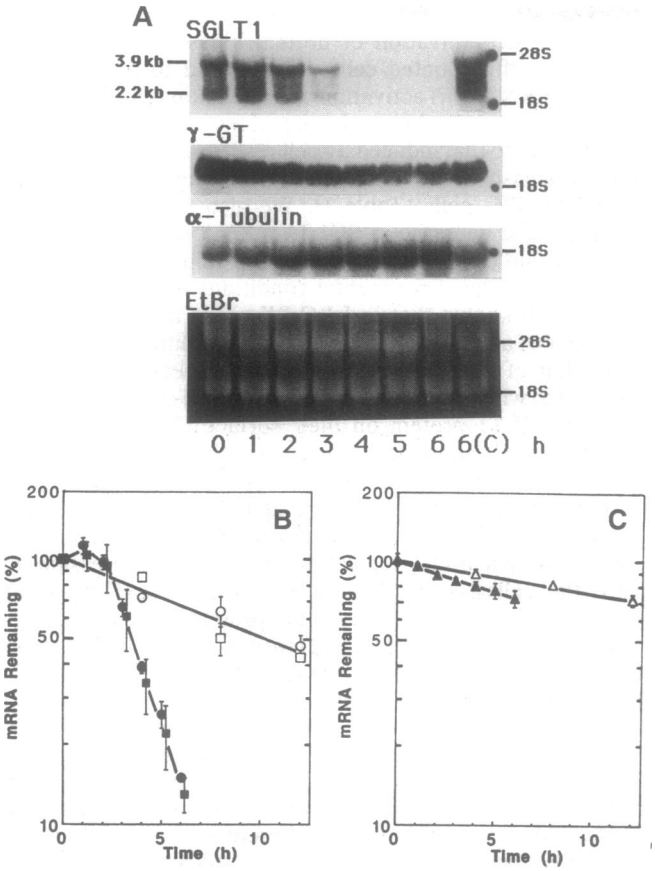


FIG. 2. Time course of SGLT1 and γ-GT mRNA levels after TPA or dichloro-β-D-ribofuranosylbenzimidazole (DRB) treatment. (*A*) Differentiated LLC-PK$_1$ cells were treated with 0.1 μM TPA for the indicated periods and total RNA was extracted for Northern blot analysis. Lane 6(C) is positive control of 6 h without TPA. EtBr, ethidium bromide staining of the RNA gel. (*B*) Decrease in SGLT1 mRNA level after TPA (0.1 μM) or DRB (25 μg/ml) treatment was determined by Northern blot analysis and quantitated by densitometry. Squares, 3.9-kb SGLT1; circles, 2.2-kb SGLT1; solid symbols, TPA treated; open symbols, DRB treated (mean ± SEM of three cultures). (*C*) Decrease in γ-GT mRNA level. The same blots used for *B* were reprobed for γ-GT mRNA. Solid triangle, TPA treated; open triangle, DRB treated.

tively, as determined by blocking transcription with DRB (Fig. 2 *B* and *C*), an inhibitor of RNA polymerase II (18). Similar half-lives were observed when transcription was blocked by actinomycin D (data not shown). The inhibitor concentrations used were similar to those used by other investigators with this cell line (19, 20). The discrepancy between the mRNA half-life after DRB treatment (11.6 h) and half-life value at the rapid decrease phase after TPA treatment described above (1.5 h) may suggest active degradation of SGLT1 mRNA (see *Discussion*).

**Protein Kinase C Activity.** If the activation of protein kinase C is important in the dedifferentiation-induced decrease in SGLT1 mRNA, it should be found to be activated in the dedifferentiated LLC-PK$_1$ cells. Dawson and Cook (10) reported that both TPA treatment and reseeding activated protein kinase C in this cell line by using histone as the substrate for the kinase assay. We confirmed this finding by using a synthetic peptide substrate specific for protein kinase C (Table 1). In confluent (differentiated) cells, most (84%) of the protein kinase C was found in the soluble fraction, indicating it was not activated. However, TPA treatment of differentiated cells resulted in the redistribution of protein kinase C from the soluble to the particulate fraction (44%), consistent with activation of this enzyme. On the other hand, in the growing (undifferentiated) cells, most (97%) of the protein kinase C was recovered in the particulate fraction, indicating strong activation of this kinase. Note that the total protein kinase C activity was reduced when activated, consistent with the proteolytic degradation of the activated enzyme as reported (21).

**Complete Prevention of TPA-Induced Decrease in SGLT1 mRNA by Transcription Blockers.** During examination of the effects of transcription blockers and TPA on SGLT1 mRNA levels, we found that pretreating cells with transcription blockers (actinomycin D and DRB) for 30 min before TPA stimulation precluded the effect of TPA (Fig. 3 *A* and *B*). Moreover, transcription blockers were able to abrogate the TPA effect even when they were added after the loss of SGLT1 mRNA had begun (Fig. 3 *C* and *D*). Since actinomycin D and DRB block transcription by different mechanisms (22), these results indicate the importance of on-going transcription in the decrease in SGLT1 mRNA level, although the mechanism of this phenomenon remains to be elucidated.

We also examined the possible effects of translation blockers on the TPA-induced decrease in SGLT1 mRNA levels. We found that neither cycloheximide nor anisomycin altered the decrease in SGLT1 mRNA levels when added 3 h after TPA (Fig. 3 *C* and *D*). However, pretreatment of the cells with translation blockers for 30 min before TPA addition partially prevented the TPA-induced SGLT1 mRNA loss as estimated by densitometry: the level of the 3.9-kb SGLT1 mRNA decreased to 22 ± 1% of its initial level (mean ± SEM, $n = 9$) with TPA treatment for 6 h, but decreased only to 49 ± 3% ($n = 3$) when cells were pretreated with anisomycin and to 65 ± 8% ($n = 3$) when cells were pretreated with cycloheximide. No evidence of cytotoxicity was observed throughout the experiments.

## DISCUSSION

We have shown in LLC-PK$_1$ cells that SGLT1 mRNA is detectable only when the cells are differentiated (Fig. 1*A*) and is quickly lost when cells were reseeded to induce dedifferentiation (Fig. 1 *B* and *C*). The involvement of protein kinase C in the rapid decline of SGLT1 mRNA by cell dedifferentiation seems likely, because (*i*) H-7, a protein kinase

Table 1. Intracellular localization of protein kinase C in confluent and subconfluent LLC-PK$_1$ cells

| Cells | *n* | Protein kinase C, pmol/per min per mg | |
|---|---|---|---|
| | | Soluble fraction | Particulate fraction |
| Confluent | 6 | 23.71 ± 0.69 (84.1 ± 0.8%) | 4.47 ± 0.25 (15.9 ± 0.8%) |
| Confluent TPA-treated | 6 | 9.17 ± 0.29 (55.7 ± 3.8%) | 7.66 ± 1.03 (44.3 ± 3.8%) |
| Subconfluent | 3 | 0.06 ± 0.03 (3.0 ± 1.7%) | 1.70 ± 0.08 (97.0 ± 1.7%) |

Confluent (differentiated) LLC-PK$_1$ cell monolayers (7 days after seeding), confluent monolayers treated with 0.1 μM TPA for 1 h, or subconfluent (undifferentiated) cells (2 days after seeding, 50% confluent) were lysed and separated into soluble and particulate fractions. Protein kinase C in each fraction was partially purified by DEAE-cellulose column chromatography and assayed. Data are expressed as means ± SEM with the cellular distributions (percent of total) shown in parentheses.

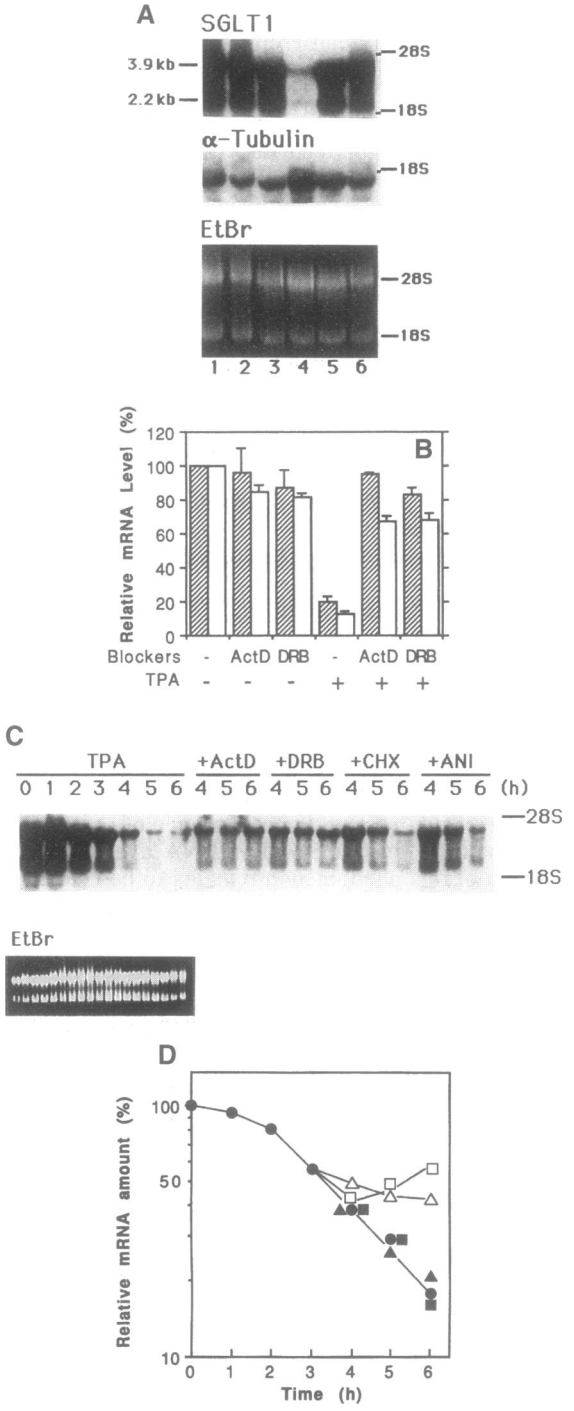


FIG. 3. Effects of transcription or translation blockers on TPA-induced decrease in SGLT1 mRNA. (*A*) Differentiated LLC-PK$_1$ cells were treated for 30 min with either actinomycin D (5 μg/ml) or DRB (25 μg/ml) followed by addition of TPA (0.1 μM). Total RNA was extracted at 6 h and subjected to Northern blot analysis. Lanes: 1, untreated cells; 2, actinomycin D (ActD) alone; 3, DRB alone; 4, TPA alone; 5, actinomycin D/TPA; 6, DRB/TPA. (*B*) Densitometric representation. Bars: hatched, 3.9-kb SGLT1; open, 2.2-kb SGLT1. Each bar represents the mean ± SEM of three cultures. (*C*) Differentiated LLC-PK$_1$ cells were first treated with TPA (0.1 μM) and, after 3 h, transcription or translation blockers were added [actinomycin D (ActD), 5 μg/ml; DRB, 25 μg/ml; cycloheximide (CHX), 10 μg/ml; anisomycin (ANI), 25 μM]. At indicated times after TPA treatment, total RNA was extracted for Northern blot analysis of SGLT1 mRNA. EtBr, ethidium bromide staining of the RNA gel. (*D*) Densitometric representation of *C*. ●, TPA alone; □, TPA/actinomycin D; △, TPA/DRB; ■, TPA/cycloheximide; ▲, TPA/anisomycin. Data shown in *C* and *D* were reproduced three times.

blocker, stabilized SGLT1 mRNA in undifferentiated cells (Fig. 1*D*), (*ii*) activation of protein kinase A by 8-bromo-cAMP in differentiated cells did not lead to rapid loss of SGLT1 mRNA, (*iii*) activation of protein kinase C by TPA in differentiated cells induced quick loss of SGLT1 mRNA comparable to that induced by cell reseeding (Fig. 2), and (*iv*) protein kinase C was activated in proliferating undifferentiated LLC-PK$_1$ cells (Table 1). We also observed that the TPA-induced decrease in SGLT1 mRNA required on-going transcription (Fig. 3), but the effect of inhibiting translation depended on the experimental conditions.

It is well known that in LLC-PK$_1$ cells the $Na^+$/glucose cotransport activity serves as a marker of differentiation (1). Induction of cotransporter activity has been found to be associated with an increase of LLC-PK$_1$ cells that express the 75-kDa SGLT1 protein on their surface (23). Our present results strongly suggest that changes in SGLT1 mRNA levels contribute to the regulation of $Na^+$/glucose cotransport activity during differentiation and dedifferentiation. It should be noted that our current conclusion does not rule out a role for post-translational regulation of SGLT1 expression in LLC-PK$_1$ cells as evidenced by the studies of LasHeras *et al.* (24) since we did not measure either protein levels or activity.

We currently interpret the decrease in SGLT1 mRNA upon dedifferentiation or TPA treatment to be due to specific mRNA degradation on the basis of two lines of evidence. (*i*) The rate of SGLT1 mRNA loss after dedifferentiation or TPA treatment was much greater than that observed after treating cells with transcription blockers (Figs. 1 and 2). (*ii*) Since *SGLT1* gene transcription was below the detection limit by nuclear run-on assay in either differentiated or undifferentiated cells, the turnover rate of SGLT1 mRNA appears to be low (T.S. and D.B.R., unpublished data). Takahama and Singer (14) provided an example of destabilization of a specific mRNA by protein kinase C activation by showing that CD4 mRNA degradation is enhanced by TPA treatment of precursor thymocytes. While our data support the role of post-transcriptional regulation of SGLT1 expression in LLC-PK$_1$ cells, we cannot definitively exclude the contribution of transcriptional regulation. Further studies will be required to assess the overall contribution of transcriptional and post-transcriptional components in the regulation of the amounts of SGLT1 mRNA.

The rapid decrease of mRNA level with dedifferentiation or TPA treatment appeared specific to SGLT1 mRNA because (*i*) mRNA of γ-GT, another differentiation marker, did not decrease rapidly under either condition (Figs. 1 and 2) and (*ii*) the mRNA of SAAT1, a $Na^+$-coupled solute cotransporter homologous to SGLT1, was stable in the presence of TPA.

The strict dependency of TPA-induced SGLT1 mRNA decrease on on-going transcription (Fig. 3) suggests a requirement for the regulated expression of TPA-inducible genes that inhibit *SGLT1* gene transcription and/or enhance SGLT1 mRNA degradation. Although our current findings do not provide a detailed mechanism involved in the regulation of SGLT1 mRNA, they strongly suggest the existence of an indirect pathway by which protein kinase C regulates the specific mRNA amount, not by direct phosphorylation of effector molecules (e.g., ribonucleases) but by the induction of genes of as yet unknown function.

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