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Sodium butyrate increases glucose transporter expression in LLC-PK$_1$ cells

(kidney epithelial cell/mRNA expression/short-chain fatty acid/starvation)

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ABSTRACT **The effect of sodium butyrate on the expression of the facilitated glucose transporter (GT) was investigated in the pig kidney cell line LLC-PK$_1$. When cells were treated with butyrate, GT mRNA expression was remarkably enhanced with a maximal effect at 5 mM. Levels of GT mRNA were increased at 1 day after butyrate treatment and continued to increase for at least 4 days; however, acetate and propionate did not affect GT mRNA levels significantly. The induction of GT mRNA by butyrate was accompanied by an increase in GT function. The expression of GT mRNA decreased in HepG2, HT-29, and COS cells by treatment with butyrate for 1 day. Interestingly, glucose deprivation of LLC-PK$_1$ cells reduced the induction of GT mRNA by butyrate, although starvation itself slightly enhanced steady-state GT mRNA levels. Therefore, expression of GT in LLC-PK$_1$ cells is strongly induced by butyrate by a pathway that apparently depends on the presence of glucose in culture medium.**

Facilitated transport of D-glucose in nonpolarized cells is regulated by various conditions. In cultured fibroblasts, glucose transport and the number of facilitated glucose transporters (GTs) in plasma membranes are increased when cells are deprived of glucose (starvation) (1, 2). Other factors such as transformation (3, 4), phorbol esters (5, 6), hypertonicity (7), and insulin (8) also increase GT activity in nonpolarized cells. In the epithelia of small intestine and renal proximal tubules, there are two types of glucose transport systems: (*i*) a Na^+-independent system localized to the basolateral membranes and (*ii*) a Na^+-dependent glucose transporter (NaGT) present in brush-border membranes. These transporters differ in energy requirements and in biochemical and immunological characteristics (9). In contrast to the extensive studies on GT regulation in nonpolarized cells, there are few reports on GT regulation in epithelial cells possessing both GT and NaGT.

Recent advances in molecular biology have generated important information on GT and NaGT. Mueckler *et al.* (10) published the sequence of cDNA encoding GT in the human hepatoblastoma line HepG2. Birnbaum *et al.* (11) reported the sequence of GT in rat brain, showing it to be 97% identical with that of HepG2. These cDNAs have been used to study the distribution and regulation of GT mRNA expression in tissues and cells (12–14). In addition, Hediger *et al.* (15) reported the nucleotide sequence of the gene encoding NaGT in rabbit small intestine.

These developments prompted us to study the regulation of GT in LLC-PK$_1$ cells, an established cell line derived from pig kidney (16) and a good model system for studying glucose transport in differentiated epithelial cells (17–19). In the course of the studies on GT regulation, we found that sodium butyrate affected GT mRNA expression in cultured hepatocytes (unpublished data). Butyrate, a four-carbon short-chain fatty acid, has many biological effects on mammalian cells in culture. Thus, it can induce changes in cell morphology and growth rate, hyperacetylation of histones, and enhanced production of some proteins and their mRNAs (20–25). In the present report, we show that GT mRNA in LLC-PK$_1$ cells is significantly enhanced by butyrate and that the mechanism of induction is different from that produced by starvation.

MATERIALS AND METHODS

Cell Cultures. LLC-PK$_1$ cells were generously provided by D. Ausiello (Massachusetts General Hospital, Boston). Culture cells were fed with Dulbecco's modified Eagle's medium (DMEM; GIBCO) supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml), and cells were used for experiments at confluence. For starvation experiments, glucose was omitted from the medium and dialyzed fetal bovine serum was substituted. All cultures were maintained in an atmosphere of 5% CO_2/95% air at 37°C.

RNA Extraction and Hybridization. Medium was aspirated from 100-mm Corning culture dishes and cells were washed once with isotonic phosphate-buffered saline. Cells were lysed with 1.5 ml of RNA lysis solution (5 M guanidine thiocyanate/10 mM Tris·HCl/10 mM EDTA, pH 7.5), and total RNA was isolated as described (26). After measuring RNA concentration spectrophotometrically, RNA was fractionated in 1% formaldehyde/agarose gels and blotted onto nitrocellulose filters. GT mRNA was detected with a riboprobe prepared from prGT-1, the cloning vector containing a 2655-nucleotide GT cDNA (11). GT riboprobe was transcribed from pSP65 (27) containing a *Bam*HI fragment of GT cDNA (nucleotides 264–708) in the antisense orientation. Blots were hybridized with a ^{32}P-labeled GT riboprobe at 55°C and then washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS). Filters were exposed to Kodak XAR-5 film at −70°C with an intensifying screen.

Transport Studies. LLC-PK$_1$ cells on a permeable support were prepared by seeding cells on polycarbonate membranes (pore size, 5 μm; 25 mm in diameter; Nuclepore) coated with rat tail collagen (10 μg/cm^2) (type I; Collaborative Research, Waltham, MA). Cells grown on polycarbonate membranes were washed by immersing in solution A (1.8 mM $CaCl_2$/5.4 mM KCl/0.8 mM $MgSO_4$/116.4 mM choline chloride/1.0 mM KH_2PO_4/10.0 mM Hepes, pH 7.4) and then incubated for 30 min in solution A containing 5 mM 3-*O*-methyl-D-[^3H]glucose at 25°C. Efflux was initiated by placing the polycarbonate membranes in solution A without substrate and stopped at the indicated times by washing in ice-cold solution A. After solubilizing cells with 0.1 M NaOH/0.1%

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Abbreviations: GT, facilitated glucose transporter; NaGT, Na^+-dependent glucose transporter.

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SDS, the remaining radioactivity was measured by liquid scintillation counting.

RESULTS

Fig. 1 shows an RNA blot analysis of GT mRNA expression after sodium butyrate treatment of LLC-PK_1 cells. The riboprobe prepared from the cDNA encoding rat brain GT detected GT mRNA as a single band (2.5 kilobases) in LLC-PK_1 cells (lanes 1 and 2). A transcript of identical size was also detected when a nick-translated probe prepared from the full-length cDNA was used (data not shown). In neither case was there a significant difference between the pattern obtained after low- or high-stringency washing. Thus, expression of a single mRNA homologous with that of rat brain GT was confirmed in LLC-PK_1 cells. As shown in Fig. 1, lanes 3–6, butyrate markedly enhanced the expression of GT mRNA. The increase was observed 1 day after butyrate treatment and was continuous for up to 4 days. In a separate experiment, the GT mRNA level was found to be unaltered after treatment with butyrate for 12 hr (data not shown). We tested other short-chain fatty acids and found that the enhanced expression of GT was specifically induced by butyrate since neither acetate nor propionate affected GT expression (lanes 7 and 8, respectively). The maximum effect of butyrate on GT expression was achieved at a concentration of 5 mM (Fig. 2).

Increased GT mRNA levels were accompanied by increases in glucose transport activity as determined by 3-*O*-methyl-D-[^{3}H]glucose efflux from butyrate-treated LLC-PK_1 cells. 3-*O*-Methyl-D-glucose is a nonmetabolizable sugar that is preferentially transported by GT in the kidney (28). Furthermore, sodium-free medium was used to minimize any 3-*O*-methyl-D-[^{3}H]glucose transport by NaGT. Under these experimental conditions, 3-*O*-methyl-D-[^{3}H]glucose efflux from LLC-PK_1 cells should reflect the GT activity in basolateral membranes. As shown in Fig. 3, 3-*O*-methyl-D-[^{3}H]-glucose efflux was higher in butyrate-treated cells than in control cells. Thus, increased GT activity accompanied increased mRNA levels in butyrate-treated LLC-PK_1 cells, presumably due to increased protein synthesis.

To determine whether butyrate affects GT expression in other cell types, we also tested HepG2, HT-29, and COS cells. HepG2, a cell line derived from a human hepatoblastoma (29), constitutively expresses GT mRNA at a high level (10). HT-29 and COS cells are cell lines derived from a human colon carcinoma (30) and monkey fibroblasts (31), respectively. As shown in Fig. 4, the expression of GT mRNA was confirmed in these cells, and the size of GT

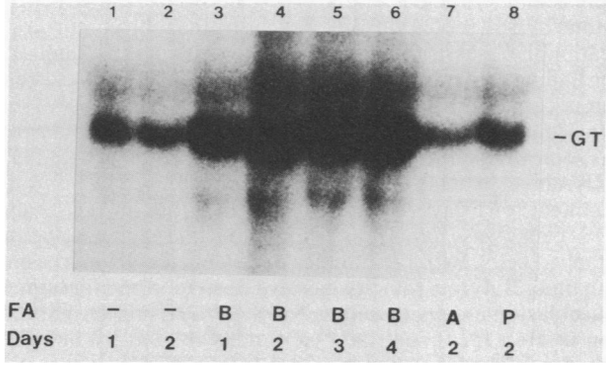

FIG. 1. RNA blot analysis of GT mRNAs in LLC-PK_1 cells. Samples (25 μg of total RNA per lane) were obtained from LLC-PK_1 cells cultured in the absence or presence of 5 mM short-chain fatty acids (FA) for indicated periods. −, Control; B, butyrate; A, acetate; and P, propionate.

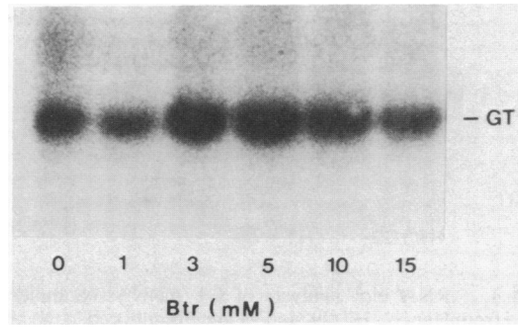

FIG. 2. RNA blot analysis of GT mRNAs in LLC-PK_1 cells. Samples (25 μg of total RNA per lane) were obtained from LLC-PK_1 cells cultured in the absence or presence of indicated concentrations of butyrate (Btr) for 1 day.

mRNAs in these cells was essentially the same as in LLC-PK_1 cells. However, in contrast to LLC-PK_1 cells, the expression of GT mRNA was not enhanced by butyrate in any of these three cell lines; in fact, the level of GT mRNA was significantly decreased. Thus, in the four cell lines tested, the butyrate effect on GT mRNA induction was not a general phenomenon but was observed only in LLC-PK_1 cells.

Since glucose deprivation increases GT activity in nonpolarized cells (1, 2), we considered the possibility that inhibition of glucose-phosphorylating enzymes (20) might be a mechanism by which butyrate could induce GT. Although the precise mechanism by which glucose starvation affects GT activity remains unclear, the number of GT molecules in plasma membranes was found to be increased by measuring D-glucose-specific cytochalasin B binding (2, 32, 33). As shown in Fig. 5, when we measured the expression of GT mRNA, we found that it was slightly enhanced by starvation but that the extent of induction was small compared to that induced by butyrate. To further explore the relationship between the effect of butyrate and starvation, when we combined these two conditions, we found surprisingly that the induction of GT mRNA by butyrate during starvation was decreased when compared with the effect of butyrate in the presence of glucose. GT mRNA levels under these conditions

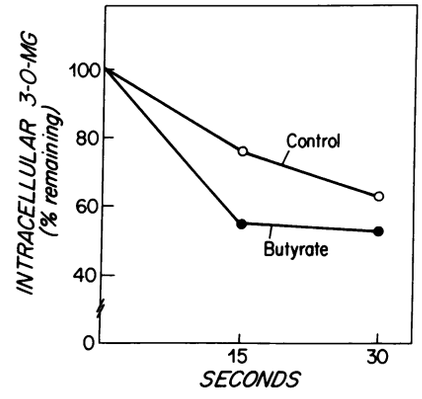

FIG. 3. Effect of butyrate treatment on 3-*O*-methyl-D-glucose (3-O-MG) efflux from LLC-PK_1 cells. LLC-PK_1 cells grown on a permeable support were treated with (●) or without (○) 5 mM butyrate for 2 days. Cells were preloaded with 5 mM 3-*O*-methyl-D-[^{3}H]glucose (5 μCi/ml; 1 Ci = 37 GBq), and efflux was initiated by placing cells in solution A without substrate. At the indicated times, the efflux was stopped by washing, and the remaining radioactivity was measured. For the estimation of 0-time values (100% remaining), cells were washed immediately after 3-*O*-methyl-D-[^{3}H]glucose was preloaded. Each point represents the mean value of five experiments performed in two determinations.

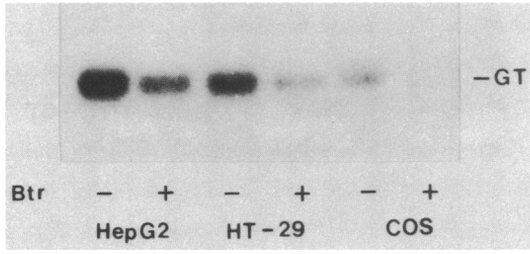

FIG. 4. RNA blot analysis of GT mRNAs. Samples were obtained from HepG2, HT-29, and COS cells cultured in the absence (−) or presence (+) of 5 mM butyrate (Btr) for 1 day. For HepG2 and HT-29 cells, 12.5 μg of total RNA per lane was used, and for COS cells, 25 μg of total RNA per lane was used.

were quantitated densitometrically (Biomed Instruments, Fullerton, CA) as follows: for butyrate, a 4.7-fold increase; for starvation, a 2.5-fold increase; for butyrate under starvation, a 1.8-fold increase against control (mean value of three experiments). In addition, when the same nitrocellulose blots were rehybridized with a ^{32}P-labeled actin probe, there was no significant difference in actin mRNA levels under these experimental conditions. These results in LLC-PK$_1$ cells indicate that (*i*) starvation is a weak inducer of GT mRNA compared with butyrate, (*ii*) the mechanism of GT mRNA induction by butyrate is different from that of starvation, and (*iii*) the induction of GT mRNA by butyrate depends, at least in part, on the presence of glucose in the culture medium.

DISCUSSION

The pig kidney cell line LLC-PK$_1$ was chosen to study GT regulation in epithelial cells. LLC-PK$_1$ cells have a transepithelial glucose transport system similar to that of kidney proximal tubules and small intestinal epithelia, since they possess both GT and NaGT (17–19). Consistent with the presence of GT mRNA in kidney (10, 11), we observed GT mRNA expression in cultured LLC-PK$_1$ cells. Our results show that GT mRNA expression is increased about 5-fold by butyrate in LLC-PK$_1$ cells and that this enhancement is fully achieved with glucose in the culture medium.

Butyrate has been reported to modulate the expression of various proteins and genes such as metallothionein I (22), transglutaminase (23), ferritin (24), and the α subunit of gonadotropin (25) in mammalian cells; however, the precise mechanism of butyrate induction remains unclear. In general, butyrate induces hyperacetylation of histones by inhibiting histone deacetylation (21), and alteration of histone acetylation may affect transcription of some genes (34, 35). However, this is probably not the case with GT mRNA induction in LLC-PK$_1$ cells since propionate, which is nearly as effective as butyrate in producing hyperacetylation of histones (21, 36), failed to induce GT mRNA (Fig. 1, lane 8). Anderson and Bridges (37) studied the effect of short-chain fatty acids on the rate of glucose production and glycolysis in isolated rat hepatocytes and found that both acetate and butyrate, but not propionate, increased glucose production and decreased glycolysis. However, we found that acetate did not affect GT mRNA level in LLC-PK$_1$ cells (Fig. 1, lane 7). Thus, the induction of GT mRNA by butyrate cannot be explained simply by its effect on glucose metabolism; however, we cannot exclude the possibility that histone hyperacetylation and alteration of glucose metabolism by butyrate act synergistically to induce GT mRNA.

Although the expression of GT mRNA in LLC-PK$_1$ cells was enhanced by starvation, the induction was weak compared to that produced by butyrate. In addition, the effects of these two stimuli on GT mRNA expression were not additive.

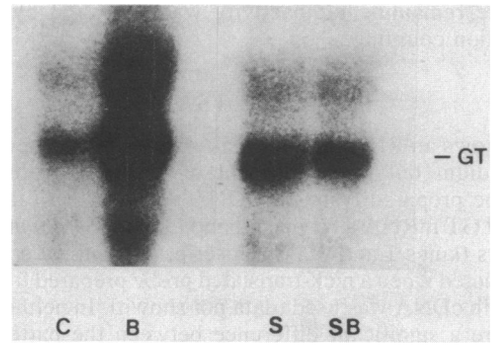

FIG. 5. RNA blot analysis of GT mRNAs in LLC-PK$_1$ cells. Samples (25 μg of total RNA per lane) were obtained from LLC-PK$_1$ cells cultured in the absence or presence of butyrate and D-glucose for 2 days. C, control; B, 5 mM butyrate; S, starvation (no glucose); and SB, 5 mM butyrate under starvation.

On the contrary, the induction of GT mRNA by butyrate was actually reduced by starvation. Thus, it is unlikely that butyrate, reportedly an inhibitor of glucose phosphorylation (20), mimics starvation by interfering with glucose utilization. However, further studies on the biological effects of butyrate on LLC-PK$_1$ cells are needed to confirm the speculations described above.

McClure and Cox (38) reported that the expression of the α subunit of the glycoprotein hormone was induced by millimolar concentrations of butyrate in HeLa cells and that this induction was strikingly dependent on the presence of glucose (or mannose) in the culture medium. They suggested that the induction of the α subunit was closely correlated with protein glycosylation in general and α-subunit glycosylation in particular. In the presence of glucose, the induction of gonadotropin α-subunit synthesis by butyrate was shown to be the result of an increased rate of transcription of α subunit gene (25). In addition, a subsequent study by Cox *et al.* (39) demonstrated that the induction of the α subunit and its mRNA by butyrate was inhibited by 2-deoxy-D-glucose, a sugar known to inhibit protein glycosylation. Based on their findings, Cox *et al.* (39) concluded that 2-deoxy-D-glucose affects the production of the glycoprotein hormone α subunit in HeLa cells not only by posttranslational (or cotranslational) modification but also by an alteration of mRNA synthesis (or degradation). Those observations and the present results suggest that glucose and/or its normal metabolites may have an important role in the transcriptional regulation (or the stability of mRNA) of certain genes. Although the mechanism of sugar dependence on butyrate-induced gene expression is not clear, 2-deoxy-D-glucose had no effect on histone hyperacetylation (39), indicating that chromatin modification, if necessary, is insufficient to explain the induction. Our finding that propionate did not affect GT mRNA expression is consistent with this interpretation.

In HepG2, HT-29, and COS cells, the effect of butyrate was somewhat different from that in LLC-PK$_1$ cells—i.e., GT mRNA level in these cell lines actually decreased by butyrate treatment for 1 day. A toxic effect of butyrate on HT-29 and COS cells was found, as shown by the appearance of detached cells, with the number of detached cells increasing with time. Butyrate toxicity has also been reported for human neuroblastoma cells in culture (20). So, GT mRNA expression in HepG2, HT-29, and COS cells treated by butyrate only for 1 day was shown as data (Fig. 4). However, it is important to note that GT expression returned to control levels or higher when HepG2 and HT-29 cells were treated with butyrate for 2 days, indicating butyrate had dual effects in these cells (data not shown). In any case, it is not surprising that different cell lines respond differently to the same

stimulus. For example, transformation by the *src* oncogene enhanced GT mRNA levels in rat cells such as Rat-1 and Fisher rat 3T3 but not in chicken embryo fibroblasts (13, 40). Also, starvation enhances GT mRNA levels in LLC-PK_1, chicken embryo fibroblasts, and COS cells (present study and unpublished observations) but not in 3T3-C2 murine fibroblasts (2). Thus, regulation of GT mRNA expression appears to be cell-type-specific and species-specific. However, further data are necessary to elucidate the mechanism(s) underlying GT regulation in mammalian cells.

In conclusion, butyrate, a short-chain fatty acid, increases GT mRNA expression in LLC-PK_1 cells in a time- and concentration-dependent manner. These cells serve as a useful model of functional epithelia to study GT regulation. Extension of this study to NaGT should provide important information on the regulation of net transepithelial glucose transport in the kidney and the small intestine.

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1. Kletzien, R. F. & Perdue, J. F. (1975) *J. Biol. Chem.* **250,** 593–600.
2. Haspel, H. C., Wilk, E. W., Birnbaum, M. J., Cushman, S. W. & Rosen, O. M. (1986) *J. Biol. Chem.* **261,** 6778–6789.
3. Kletzien, R. F. & Perdue, J. F. (1975) *Cell* **6,** 513–520.
4. Inui, K., Tillotson, L. G. & Isselbacher, K. J. (1980) *Biochim. Biophys. Acta* **598,** 616–627.
5. O'Brien, T. G. (1982) *J. Cell. Physiol.* **110,** 63–71.
6. Yamada, K., Tillotson, L. G. & Isselbacher, K. J. (1986) *J. Cell. Physiol.* **127,** 211–215.
7. Toyoda, N., Robinson, F. W., Smith, M. M., Flanagan, J. E. & Kono, T. (1986) *J. Biol. Chem.* **261,** 2117–2122.
8. Simpson, I. A. & Cushman, S. W. (1986) *Annu. Rev. Biochem.* **55,** 1059–1089.
9. Kasahara, M., Inui, K., Takano, M. & Hori, R. (1985) *Biochem. Biophys. Res. Commun.* **132,** 490–496.
10. Mueckler, M., Caruso, C., Baldwin, S. A., Panico, M., Blench, I., Morris, H. R., Allard, W. J., Lienhard, G. E. & Lodish, H. F. (1985) *Science* **229,** 941–945.
11. Birnbaum, M. J., Haspel, H. C. & Rosen, O. M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5784–5788.
12. Flier, J. S., Mueckler, M., McCall, A. L. & Lodish, H. F. (1987) *J. Clin. Invest.* **79,** 657–661.
13. Flier, J. S., Mueckler, M. M., Usher, P. & Lodish, H. F. (1987) *Science* **235,** 1492–1495.
14. Birnbaum, M. J., Haspel, H. C. & Rosen, O. M. (1987) *Science* **235,** 1495–1498.
15. Hediger, M. A., Coady, M. J., Ikeda, T. S. & Wright, E. M. (1987) *Nature (London)* **330,** 379–381.
16. Hull, R. N., Cherry, W. R. & Weaver, G. W. (1976) *In Vitro* **12,** 670–677.
17. Rabito, C. A. & Ausiello, D. A. (1980) *J. Membr. Biol.* **54,** 31–38.
18. Misfeldt, D. S. & Sanders, M. J. (1981) *J. Membr. Biol.* **59,** 13–18.
19. Handler, J. S. (1983) *J. Exp. Biol.* **106,** 55–69.
20. Prasad, K. N. & Sinha, P. K. (1976) *In Vitro* **12,** 125–132.
21. Sealy, L. & Chalkley, R. (1978) *Cell* **14,** 115–121.
22. Birren, B. W. & Herschman, H. R. (1986) *Nucleic Acids Res.* **14,** 853–867.
23. Byrd, J. C. & Lichti, U. (1987) *J. Biol. Chem.* **262,** 11699–11705.
24. Rouault, T. A., Hentze, M. W., Dancis, A., Caughman, W., Harford, J. B. & Klausner, R. D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6335–6339.
25. Darnell, R. B. (1984) *Mol. Cell. Biol.* **4,** 829–839.
26. Gattoni-Celli, S., Kirsch, K., Kalled, S. & Isselbacher, K. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 6127–6131.
27. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K. & Green, M. R. (1984) *Nucleic Acids Res.* **12,** 7035–7056.
28. Silverman, M. (1976) *Biochim. Biophys. Acta* **457,** 303–351.
29. Knowles, B. B., Howe, C. C. & Aden, D. P. (1980) *Science* **209,** 497–499.
30. Pinto, M., Appay, M.-D., Simon-Assmann, P., Chevalier, G., Dracopoli, N., Fogh, J. & Zweibaum, A. (1982) *Biol. Cell* **44,** 193–196.
31. Gluzman, Y. (1981) *Cell* **23,** 175–182.
32. Yamada, K., Tillotson, L. G. & Isselbacher, K. J. (1983) *J. Biol. Chem.* **258,** 9786–9792.
33. Pessin, J. E., Tillotson, L. G., Yamada, K., Gitomer, W., Carter-Su, C., Mora, R., Isselbacher, K. J. & Czech, M. P. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 2286–2290.
34. Dobson, M. E. & Ingram, V. M. (1980) *Nucleic Acids Res.* **8,** 4201–4219.
35. Reeves, R. (1984) *Biochim. Biophys. Acta* **782,** 343–393.
36. McKnight, G. S., Hager, L. & Palmiter, R. D. (1980) *Cell* **22,** 469–477.
37. Anderson, J. W. & Bridges, S. R. (1984) *Proc. Soc. Exp. Biol. Med.* **177,** 372–376.
38. McClure, D. S. & Cox, G. S. (1984) *Arch. Biochem. Biophys.* **233,** 93–105.
39. Cox, G. S., McClure, D. S. & Cosgrove, D. M. (1987) *Mol. Cell. Biol.* **7,** 1592–1601.
40. White, M. K. & Weber, M. J. (1988) *Mol. Cell. Biol.* **8,** 138–144.