Biochem. J. (1990) 271, 585–589 (Printed in Great Britain)

Insulin and tri-iodothyronine induce glucokinase mRNA in primary cultures of neonatal rat hepatocytes

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Glucokinase (EC 2.7.1.2) first appears in the liver of the rat 2 weeks after birth and increases after weaning on to a high-carbohydrate diet. We investigated the hormonal regulation of glucokinase (GK) mRNA in primary cultures of hepatocytes from 10–12-day-old suckling rats. GK mRNA was undetectable in such cells after 48 h of culture in serum-free medium devoid of hormones. Addition of insulin or tri-iodothyronine (T_3) to the medium resulted in induction of GK mRNA. The effects of insulin and T_3 were dose-dependent and additive. Dexamethasone alone did not induce GK mRNA, but enhanced the response to insulin and decreased the response to T_3. Induction of GK mRNA by insulin was not affected when the medium glucose concentration was varied between 5 and 15 mM, nor when culture was conducted in glucose-free medium supplemented with lactate and pyruvate or galactose. The time course of initial accumulation of GK mRNA in response to insulin was characterized by a lag of 12 h and an induction plateau reached after 36 h. If hepatocytes were then withdrawn from insulin for 24 h and subsequently subjected to a secondary stimulation by insulin, GK mRNA re-accumulated with much faster kinetics and reached the fully induced level within 8 h. Both primary and secondary responses to insulin were abolished by actinomycin D. These results provide insight into the role of hormonal stimuli in the ontogenic development of hepatic glucokinase.

INTRODUCTION

Glucokinase (GK; ATP:D-glucose 6-phosphotransferase; EC 2.7.1.2), a regulatory enzyme of glucose metabolism in liver, does not appear during ontogenic development before the third week of extra-uterine life in rodents (Walker & Holland, 1965; Jamdar & Greengard, 1970). Enzyme activity and mRNA can first be detected in rat liver around day 14 after birth, and reach adult levels by day 30 (Walker & Holland, 1965; Jamdar & Greengard, 1970; Spence, 1983; Iynedjian *et al.*, 1987). This time period coincides with weaning and is characterized by a major nutritional shift, from the high-fat intake provided by maternal milk to a carbohydrate-rich diet supplied as laboratory food pellets. Concomitantly the plasma insulin/glucagon ratio, which is low during suckling, undergoes a sustained increase (Girard *et al.*, 1977). That these reciprocal changes in circulating insulin and glucagon could be instrumental in the emergence of GK is suggested by the fact that, in the adult animal, transcription of the GK gene in liver is turned on by insulin and off by glucagon (Iynedjian *et al.*, 1988, 1989).

The importance of carbohydrate intake for the development of hepatic GK has been underscored by the experiments of Haney *et al.* (1986), who were able to induce the enzyme prematurely by rearing rat pups, from 1 day of age, on an artificial formula rich in glucose oligomers. Artificially fed animals exhibited 30% of adult hepatic GK activity after 3 days and 70% after 9 days. Conversely, the normal development of the enzyme can be delayed by manoeuvres that prevent the post-weaning increase of the plasma insulin/glucagon ratio, such as weaning to a carbohydrate-free diet, starvation or induction of alloxan diabetes (Walker & Holland, 1965; Walker & Eaton, 1967). Thus several lines of evidence point to hyperinsulinaemia (and hypoglucagonaemia) as a triggering factor for the developmental induction of hepatic GK. In addition, both thyroid hormones and glucocorticoids appear to be necessary for the normal development of liver GK (Jamdar & Greengard, 1970; Partridge *et al.*, 1975).

Given the difficulty of defining individual hormone effects in the intact animal, we were interested in using hepatocytes from newborn rats to study the induction *de novo* of GK mRNA by hormones. Primary induction of GK activity in such cells by insulin and other factors has already been documented (Nakamura *et al.*, 1979; Wakelam & Walker, 1980). In the present paper we report on the effects *in vitro* of insulin, thyroid hormones, glucocorticoids and glucose on GK mRNA in hepatocytes isolated from 10–12-day-old rats, i.e. at a stage preceding the spontaneous development of the enzyme *in vivo*.

MATERIALS AND METHODS

Animals

Litters from the first pregnancies of Wistar rats from our breeding colony were used. Litters were culled to 10 pups on the day after birth. Animals were housed at 22 °C with light from 7:00 to 19:00 h. Pups between 10 and 12 days of age were separated from their mother just before the isolation of hepatocytes.

Hepatocyte culture

Hepatocytes were isolated essentially according to the method of Seglen (1976). The animals (6–8) were anaesthetized (50 mg of pentobarbital/kg body weight, subcutaneous) and the liver was perfused for 10 min at a flow rate of 3.5 ml/min per liver with

Abbreviations used: GK, glucokinase; MEM, minimal essential medium with Earle's salts; PBS, phosphate-buffered saline; T_3, tri-iodothyronine; PEPCK, phosphoenolpyruvate carboxykinase.

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Ca^{2+}-free Hepes buffer (10 mM-Hepes, 138 mM-NaCl, 3 mM-KCl and 0.7 mM-Na_2HPO_4, pH 7.6) and then digested with Hepes buffer supplemented with 0.025% (w/v) collagenase and 2.2 mM-$CaCl_2$ for 10 min. At the end of the collagenase perfusion the hepatocytes were suspended in minimal essential medium with Earle's salts (MEM) supplemented with 0.1% BSA, filtered through a nylon mesh and washed several times in the above medium. The cells were then resuspended in the seeding medium consisting of MEM supplemented with 10 mM-glucose, 1 mM-lactate, 0.1 mM-pyruvate, 0.1% BSA, 2% Ultroser G, penicillin (10 units/ml), streptomycin (100 μg/ml) and kanamycin (50 μg/ml), and plated at a density of 10^7 cells/100 mm dish (Nunclon, Roskilde, Denmark). After 4 h for adherence, the medium was changed to a serum-free medium similar to the seeding medium but devoid of Ultroser G. Other additives or changes in the medium are noted in the legends of Figures. Cells were incubated in air/CO_2 (19:1) at 37 °C and the medium was changed every 24 h.

In some experiments, the hepatocytes from suckling rats were cultured for 48 h in MEM containing 100 nM-insulin and 100 nM-dexamethasone, after which the monolayers were rinsed three times with PBS (phosphate-buffered saline: 138 mM-NaCl, 3 mM-KCl, 22 mM-Na_2HPO_4 and 1.5 mM-NaH_2PO_4, pH 6) containing 1% BSA, and incubated for a further 10 min in this buffer to remove insulin from its receptors (Sasaki *et al.*, 1984). The cells were then cultured for 24 h with dexamethasone alone. At this time, the medium was changed to one containing 100 nM-insulin in addition to dexamethasone, and cells were processed for RNA isolation at selected intervals.

Isolation of cell RNA

Cells were washed twice with ice-cold PBS, pH 7.6, and lysed with 5 M-guanidine thiocyanate, 25 mM-sodium citrate, 0.5% sodium sarcosyl and 100 mM-β-mercaptoethanol. The RNA was isolated from cell lysates by centrifugation (175000 *g*, 12) through 5.7 M-CsCl containing 0.1 M-EDTA. Extraction of RNA from whole livers was performed according to Chirgwin *et al.* (1979), with purification of RNA by pelleting through CsCl. For electrophoresis, samples of 10 μg of total RNA per gel lane were denatured in 6.6 mM-formamide, 0.85 mM-formaldehyde, 20 mM-Mops, 5 mM-sodium acetate and 1 mM-EDTA. Electrophoresis was done overnight at 2 V/cm in gels containing 1% agarose, 2.2 M-formaldehyde, 100 mM-Mops, 1 mM-EDTA and 5 mM-sodium acetate. Resolved RNA was then transferred on to a nylon membrane by capillary transfer. Before hybridization, the blots were stained with 0.05% Methylene Blue to verify the positions of 18 S and 28 S RNA and the absence of degradation (Maniatis *et al.*, 1982).

Hybridization probes were the 1800 bp *Eco*R1–*Eco*R1 insert of pUC-GK1 (Iynedjian *et al.*, 1987), the 2600 bp *Pst*1–*Pst*1 insert from PCK-10 (Yoo-Warren *et al.*, 1983), or the 1100 bp *Pst*1–*Pst*1 insert from RSA-13 (Sargent *et al.*, 1979). They were labelled with [^{32}P]dCTP by the multiprime labelling system (Feinberg & Vogelstein, 1983) to a specific radioactivity of $(1–2) \times 10^9$ d.p.m./μg.

Filters were prehybridized at 42 °C in 45% formamide, 4 × SSC (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate), 100 mM-NaH_2PO_4, pH 6.5, 0.1% sodium pyrophosphate, 0.1% SDS, 5 × Denhardt's (1 × Denhardt's is 0.02% BSA/0.02% polyvinylpyrrolidone/0.02% Ficoll 400) and 75 μg of denatured single-stranded salmon sperm DNA/ml with shaking for a minimum of 6 h. Hybridization was performed overnight at 42 °C in the same solution containing 10% dextran sulphate and 0.5 ng of the radiolabelled probe/ml. Filters were washed in 2 × SSC/0.1% SDS at 42 °C for 1 h, 1 × SSC/0.1% SDS at 65 °C for 1 h and then 0.1 × SSC/0.1% SDS for 15 min at 65 °C. Filters were exposed to Kodak X-Omat film at −70 °C with intensifying screens. Autoradiographic signals were quantified with a GS 300 scanning densitometer and software package (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.).

Materials

Culture medium was from Eurobio (Paris, France). Fatty acid-free BSA, fraction V, was from Armour Pharmaceutical Co. (Kankakee, IL, U.S.A.). Ultroser G was purchased from IBF (Villeneuve la Garenne, France). Human insulin was from Novo Industrie (Paris, France). Dexamethasone was from Merck Sharp

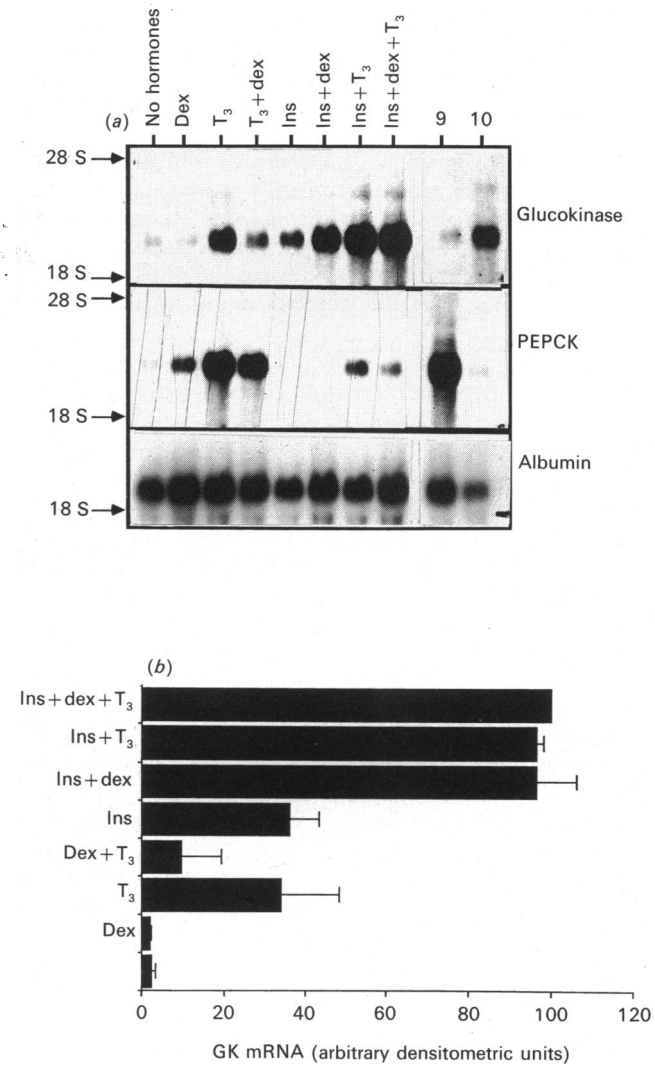

Fig. 1. Effect of dexamethasone (dex), T_3 and insulin (ins) on GK mRNA, PEPCK mRNA and albumin mRNA in primary cultures of hepatocytes from suckling rats

After adherence, hepatocytes were cultured for 48 h in MEM supplemented with 0.1% albumin, 10 mM-glucose, 1 mM-lactate, 0.1 mM-pyruvate, antibiotics and 100 nM of the indicated hormones. Total RNA was isolated and subjected to Northern blot analysis with the GK1, PCK10 or RSA-13 probes as described in the Materials and methods section. (*a*) Autoradiograph from a representative experiment. Lane 9, RNA from the liver of an adult rat fasted for 48 h; lane 10, RNA from the liver of an adult rat fasted for 48 h and re-fed on a high-carbohydrate diet for 6 h. (*b*) Accumulation of GK mRNA in response to the hormonal treatments. Quantification by scanning densitometry. Data are means ± S.E.M. of three separate experiments.

& Dohme (Paris, France) and tri-iodothyronine (T_3) was from Sigma (St. Louis, MO, U.S.A.). CsCl was from Gibco (Cergy Pontoise, France). Guanidine thiocyanate was from Fluka (Buchs, Switzerland). Collagenase was purchased from Boehringer-Mannheim (Meylan, France). Restriction enzymes were from Promega (Madison, WI, U.S.A.). All chemicals were of the highest available grade. Nylon membranes (Hybond-N filters) were bought from Amersham.

RESULTS

Multihormonal regulation of GK mRNA in neonatal hepatocytes

At the time of cell isolation, GK mRNA was not detectable in hepatocytes from 10–12-day-old rat pups (results not shown). After 48 h in hormone-free medium, GK mRNA remained undetectable. The effects of insulin, dexamethasone and T_3 on the level of GK mRNA after 48 h of culture are illustrated in Fig. 1. A representative autoradiograph is presented in Fig. 1(*a*) and the compilation of data from three separate experiments is shown in Fig. 1(*b*). As may be seen, a clear-cut induction of GK mRNA occurred in response to either T_3 or insulin. Both hormones together exerted additive effects. Dexamethasone by itself did not induce GK mRNA. However, culture in the presence of dexamethasone plus insulin resulted in markedly greater induction than with insulin alone. This level of induction was not further enhanced by supplementation of the culture medium with T_3 in addition to dexamethasone and insulin. Surprisingly, the build-up of GK mRNA in response to T_3 plus dexamethasone was smaller than with T_3 alone. We can at present provide no explanation for this observation. Note in Fig. 1(*a*) that maximal induction in the present cell culture system resulted in GK mRNA levels comparable with the highest levels seen in intact animals, that is in adult rats refed on carbohydrates following a 48 h period of fasting.

Hormonal effects on the mRNA coding for cytosolic phosphoenolpyruvate carboxykinase (EC 4.1.1.32; PEPCK) were monitored in the same cells (Fig. 1*a*, middle panel). The amount of PEPCK mRNA was high in freshly isolated hepatocytes (results not shown) and declined to very low levels after 48 h of culture without hormones. T_3 and to a lesser extent dexamethasone prevented the loss of PEPCK mRNA. When insulin was present together with T_3 and/or dexamethasone, PEPCK mRNA was decreased below the levels seen with T_3 and/or dexamethasone alone.

Albumin mRNA did not significantly change over the course of the culture or in response to hormones (Fig. 1*a*, lower panel).

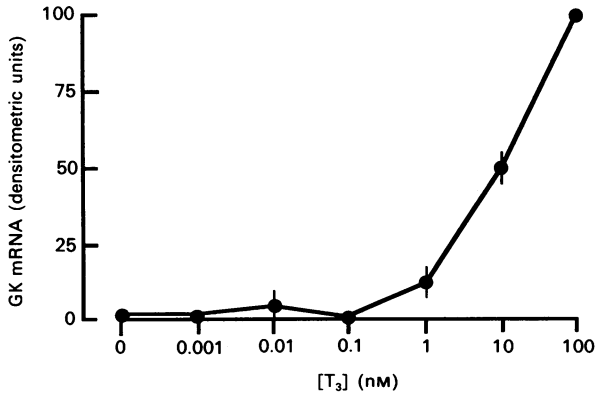

Fig. 2. Dose–response relationship for T_3-induced accumulation of GK mRNA in cultured hepatocytes from suckling rats

After adherence, hepatocytes were cultured for 48 h in medium with T_3 at the indicated concentrations. Total RNA was extracted and subjected to Northern blot analysis with the GK1 probe. GK mRNA was quantified by scanning autoradiographs. Data are means and ranges of values for two distinct experiments.

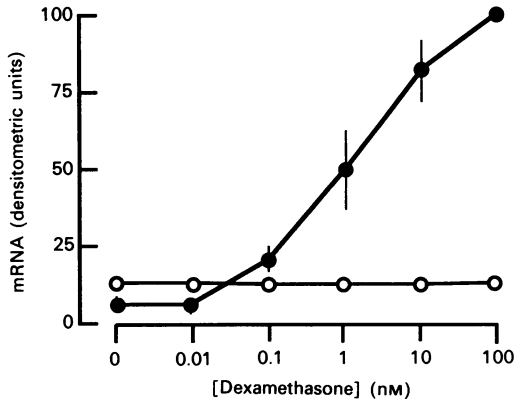

Fig. 3. Dose–response relationship for permissive effect of dexamethasone on insulin-induced accumulation of GK mRNA

After adherence, hepatocytes were cultured for 48 h in MEM supplemented with 5 nM-insulin and increasing concentrations of dexamethasone. GK mRNA (●) was quantified as in Fig. 2. Albumin mRNA (○) was quantified after hybridization of the blots with the RSA-13 probe. Data are means and ranges of values for two distinct experiments.

Concentration-dependence of effects of T_3 and dexamethasone on GK mRNA

The dose–response relationship for the induction of GK mRNA by T_3 is shown in Fig. 2. Induction of GK mRNA became detectable at a T_3 concentration of 1 nM and was maximal at 100 nM.

As shown above, dexamethasone acting alone does not induce GK mRNA. In the experiments summarized in Fig. 3, we have investigated the enhancement, by increasing concentrations of dexamethasone, of the response to a low concentration of insulin (5 nM). The permissive effect of dexamethasone was manifest between 1 and 100 nM. The albumin mRNA concentration was unaffected, in agreement with the data in Fig. 1(*a*).

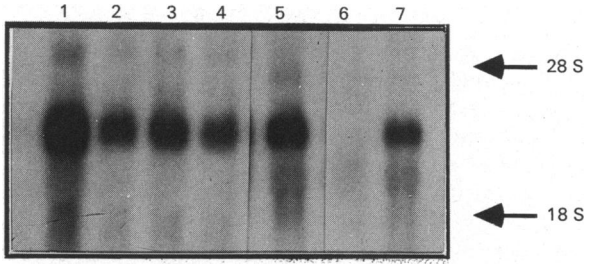

Fig. 4. Insulin induction of GK mRNA in neonatal hepatocytes cultured at different glucose concentrations

After adherence, hepatocytes were cultured for 48 h with 100 nM-dexamethasone, 100 nM-insulin and the indicated substrates. Glucose concentrations in the medium were measured when hepatocytes were cultured with lactate plus pyruvate or galactose. The glucose concentration remained below 1.5 mM under these conditions. Total cellular RNA was extracted and subjected to Northern blot analysis with the GK1 probe. A representative autoradiograph is shown. The experiment was repeated three times with similar results. Lane 1, 5 mM-lactate plus 0.5 mM-pyruvate; lanes 2–4, 5 mM-, 10 mM- and 15 mM-glucose respectively; lane 5, 10 mM-galactose; lane 6, RNA from the liver of an adult rat fasted for 48 h and re-fed for 6 h with a high-carbohydrate diet.

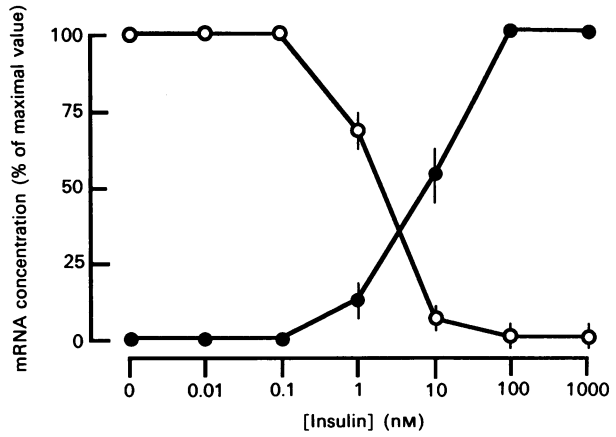

Fig. 5. Dose–response relationship for insulin-induced accumulation of GK (●) and PEPCK (○) mRNA in primary cultures of hepatocytes from suckling rats

After adherence, hepatocytes were cultured for 48 h in medium supplemented with 100 nM-dexamethasone and increasing concentrations of insulin. RNA was extracted and subjected to Northern blot analysis with the GK1 or PCK10 probes as described in the Materials and methods section. Data are means and ranges of values from two distinct experiments.

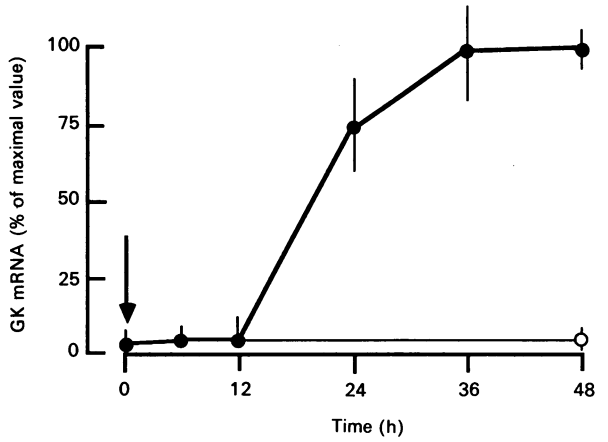

Fig. 6. Time course of the accumulation of GK mRNA upon primary stimulation by insulin

After adherence, hepatocytes were cultured in medium containing 100 nM-dexamethasone in the absence (○) or presence (●) of 100 nM-insulin. The cells were processed for RNA isolation at the indicated times after hormone addition. Total cellular RNA was extracted and subjected to Northern blot analysis with the GK1 probe. Quantification of GK mRNA was obtained by scanning autoradiographs. Data are means ± S.E.M. of five separate experiments.

Insulin-dependent induction of GK mRNA in glucose-free medium

In earlier work using hepatocytes from newborn rats, high concentrations (> 5 mM) of glucose or other sugars in culture medium were found to be necessary for insulin-dependent induction of GK activity (Wakelam & Walker, 1980, 1981). In the present experiments, however, changing the medium glucose concentration between 5 and 15 mM did not affect the amplitude of GK mRNA induction by 100 nM-insulin in presence of 100 nM-dexamethasone (Fig. 4). Exogenous glucose was indeed dispensable, since a full-scale increase in GK mRNA occurred in glucose-free medium containing 5 mM-lactate plus 0.5 mM-pyruvate, or 10 mM-galactose (Fig. 4). No induction of GK mRNA occurred in the absence of insulin when the medium glucose concentration was increased from 5 to 15 mM (results not shown).

Concentration-dependence of insulin effect on GK mRNA

The log dose–response relationship shown in Fig. 5 was established with neonatal hepatocytes cultured with a fully permissive concentration of dexamethasone (100 nM). Under these conditions, 1 nM-insulin was marginally effective in inducing GK mRNA, and 100 nM elicited a maximal response. The physiological concentrations of insulin in the portal blood lie within this range (Smadja *et al.*, 1988). A reciprocal de-induction of PEPCK mRNA was noted in the same cells at similar insulin concentrations (Fig. 5).

Time course of GK mRNA induction during primary and secondary stimulation by insulin

All of the time-course experiments presented here were performed in the presence of dexamethasone to maximize the effect of insulin. The kinetics for the initial accumulation of GK mRNA in neonatal hepatocytes in response to insulin are depicted in Fig. 6. Exposure to insulin for 12–24 h was necessary for the rise in GK mRNA to become evident. The induction plateau was attained 36 h after the initial challenge by insulin. Actinomycin D (0.4 μg/ml) completely prevented the insulin-dependent induction of GK mRNA (results not shown), supporting the view that active transcription is necessary for GK mRNA accumulation.

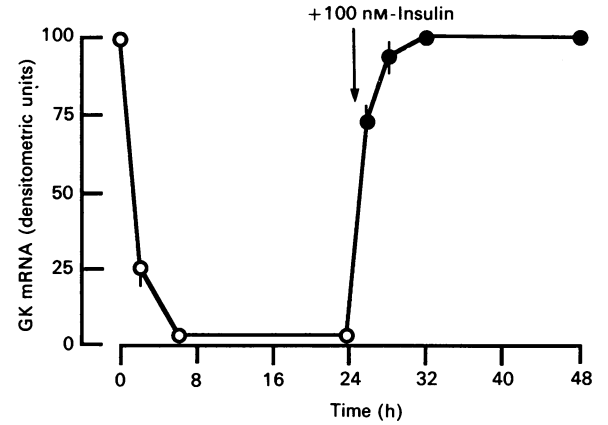

Fig. 7. Time course of accumulation of GK mRNA upon secondary stimulation by insulin

After adherence, hepatocytes were cultured for 48 h in medium containing 100 nM-insulin plus 100 nM-dexamethasone. At this time (time zero on the abscissa), the cells were washed in acid medium (see details in the Materials and methods section) to remove insulin from its receptors and the culture was continued for 24 h in medium with 100 nM-dexamethasone alone. The medium was then changed for one containing 100 nM-insulin in addition to dexamethasone. Total cellular RNA was extracted and subjected to Northern blot analysis with the GK1 probe. Quantification of GK mRNA was obtained by scanning autoradiographs. Data are means and ranges of values from three distinct experiments.

The kinetics of GK mRNA induction in hepatocytes subjected to a secondary challenge with insulin are presented in Fig. 7. In this protocol, the cells were first allowed to induce GK mRNA to the plateau by 48 h of culture with insulin, as in previous experiments. At this time (time zero in Fig. 7), insulin was withdrawn from the cells by an acid wash and culture was continued in medium devoid of insulin for a 24 h period.

Withdrawal of insulin resulted in the rapid decay of GK mRNA, which fell below detectable levels within 8 h. Re-addition of insulin at the 24 h time point elicited a prompt increase in the amount of GK mRNA, such that the fully induced level was reached within 8 h of secondary stimulation. The rapid re-appearance of GK mRNA was prevented by actinomycin D (results not shown). It should be emphasized that control hepatocytes cultured with dexamethasone alone for 72 h, and then challenged for the first time with insulin, displayed the same slow response as cells stimulated early in culture (results not shown). Thus prior exposure to insulin, rather than simple aging of the cells in culture, is instrumental for the rapid response to insulin shown in Fig. 7.

DISCUSSION

The present data provide insight into the role of hormonal stimuli in the ontogenic development of GK. During the suckling–weaning transition, plasma insulin, thyroid hormone and glucocorticoid concentrations rise (Girard *et al.*, 1977; Henning, 1978; Walker *et al.*, 1980). Both insulin and T_3 are shown here to induce the appearance of GK mRNA *de novo* when added to primary cultures of hepatocytes from rat pups aged 10–12 days. It is then probable that both of these hormones act in concert in the intact animal to trigger the rise of hepatic GK. We have also shown that the synthetic glucocorticoid dexamethasone, although devoid of effect by itself, enhances the inductive response to insulin. The direct action of insulin on the hepatocyte does not depend on the extracellular glucose concentration. Furthermore, the simple addition of glucose at high concentrations to the culture medium does not result in induction of GK mRNA. We therefore conclude that the role of carbohydrates *in vivo* is an indirect one, mediated by their insulinotropic action and the concomitant fall in plasma glucagon concentration (Girard *et al.*, 1977). The latter event is probably critical, because glucagon is known to turn off transcription of the GK gene, even in the presence of high concentrations of insulin (Iynedjian *et al.*, 1989). The administration of insulin to adult diabetic rats results in rapid stimulation of the transcription of the GK gene, as shown by run-on assay with isolated nuclei (Iynedjian *et al.*, 1988; Magnusson *et al.*, 1989). The specific stimulation of GK gene transcription by insulin and the ensuing increase in GK mRNA concentration has also been shown in cultured hepatocytes from adult rats. In adult cells, a strong insulin-dependent activation of gene transcription occurs within 1 h, and the mRNA is induced to maximal levels within 4–8 h after insulin addition (Iynedjian *et al.*, 1989). In contrast, the initial induction of GK mRNA in cultured hepatocytes from suckling rats exhibited a lag-time of 12 h and reached the plateau only after 36 h of insulin exposure. Subsequent to this slow initial response, hepatocytes maintained in culture and subjected to a second insulin challenge displayed much faster kinetics of induction, comparable with the time course seen in adult cells. The acceleration of the insulin effect during secondary stimulation is reminiscent of similar observations in other models of hormone action on gene expression, such as the oestrogen-dependent induction of ovalbumin in the immature chick oviduct or of vitellogenin in rooster liver (Palmiter, 1972; Burch & Weintraub, 1983). In the vitellogenin system, it has been shown that primary activation of the gene by oestradiol results in durable changes at the chromatin level which persist after withdrawal of the hormone. Such changes are thought to be instrumental for the rapid responsiveness upon secondary stimulation (Burch & Weintraub, 1983). It is tempting to speculate that effects of this type might take place at the level of the GK transcriptional unit upon stimulation by insulin.

The mechanism underlying the effect of T_3 on GK mRNA in neonatal hepatocytes has not been defined. Since induction occurs *de novo*, it probably results from a transcriptional effect. The thyroid hormones are known to be involved in the transcriptional regulation of a number of other genes encoding key enzymes of carbohydrate and lipid metabolism (Goodridge, 1987).

This work was supported in part by a grant from the Fondation pour la Recherche Médicale (Paris) (to J. G.) and by a grant (32-25664-88) from the Swiss National Science Foundation (to P. B. I.). M. R. N. was a Fellow of the Association of Medical School Pediatric Department Chairmen and was supported by the Pediatric Scientist Training Program (grant HD-00850 from NIH).

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Received 17 April 1990/18 June 1990; accepted 29 June 1990