Glucose-6-phosphate dehydrogenase mRNA sequence abundance in primary cultures of rat hepatocytes

Effect of insulin and dexamethasone

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Hepatic glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) is subject to nutritional regulation. To assess the possible role of hormones in this regulation, the amounts of G6PDH mRNA were studied in primary cultures of rat hepatocytes treated with insulin and dexamethasone, alone or in combination. Relative concentrations of G6PDH mRNA were directly assessed by a dot-blot hybridization procedure with nick-translated cDNA probes. G6PDH sequence abundance increased when the cultures were treated with insulin or dexamethasone, but the G6PDH mRNA induced by dexamethasone was not expressed at the protein level as active enzyme. In cultures treated with insulin and dexamethasone in combination, enzyme activity and G6PDH sequence abundance were greater than those induced by insulin alone. Our results directly demonstrate that G6PDH mRNA amounts are modulated in liver by these two classes of hormones and can partially account for the dietary induction of the enzyme observed *in vivo*.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PDH) is the key rate-limiting enzyme in the pentose phosphate pathway. The activity of the enzyme in rat liver is regulated by the nutritional and hormonal state of the animal. Enzyme activity increases when an animal is starved and re-fed with a high-carbohydrate diet (Tepperman & Tepperman, 1958; Volpe & Vagelos, 1976) or after administration of insulin (Weber & Convery, 1966; Rudack *et al.*, 1971; Nepokroeff *et al.*, 1974) or glucocorticoids (Berdanier *et al.*, 1976; Wurdeman *et al.*, 1978; Berdanier & Shubeck, 1979).

Previous studies from our laboratory have demonstrated that insulin and glucocorticoids, alone or in combination, affect enzyme activity in primary cultures of rat hepatocytes (Kelley & Kletzien, 1984) and that the increase in enzyme activity can usually be accounted for by an increase in the relative rate of synthesis of G6PDH (Stumpo & Kletzien, 1985). Translation studies *in vitro* demonstrated that the observed increase in the relative rate of synthesis is correlated with an increase in functional mRNA (Stumpo & Kletzien, 1984). Although these studies and others (Miksicek & Towle, 1982) have indicated that G6PDH activity is regulated at a pretranslational level, other investigators (Sun & Holton, 1978) have suggested that an increase in translational efficiency may be largely responsible. These previous studies have used translation *in vitro* in rabbit reticulocyte lysates to assay functional mRNA indirectly. In the study reported here, we have used cDNA probes developed in our laboratory (Kletzien *et al.*, 1985) to assay G6PDH mRNA directly in primary cultures of rat hepatocytes treated with various hormones. The results of this study establish that the hormone insulin and the synthetic

glucocorticoid dexamethasone increase G6PDH mRNA sequence abundance.

MATERIALS AND METHODS

Animal preparation and cell culture

Male Sprague–Dawley rats (200–300 g) were obtained from Hilltop Farms (Scottsdale, PA, U.S.A.). Rats were maintained in individual cages with free access to laboratory chow and water. Animals were starved for 48 h before isolation of hepatocytes. Hepatocytes were isolated and maintained in culture as previously described (Kelley & Kletzien, 1984). Insulin (46.7 munits/ml) and dexamethasone (0.5 μ M) were added 2 h after plating, and re-added with each change of medium. Poly(A)⁺ RNA was isolated from these cultures after 48 h of treatment.

Isolation of RNA

Poly(A)⁺ RNA was isolated from the primary cultures of hepatocytes by using a guanidinium thiocyanate extraction procedure and oligo(dT)–cellulose chromatography as previously described (Stumpo & Kletzien, 1984).

Dot-blot hybridization

Poly(A)⁺ RNA quantification. Details of this procedure have been described elsewhere (Kletzien *et al.*, 1985). Briefly, 0.25 μ g of each sample of poly(A)⁺ RNA was fixed to a nylon membrane and incubated in the presence of a [³²P]oligo(dT) probe (E. A. Jones & R. F. Kletzien, unpublished work). The oligo(dT) probe was prepared by tailing (dT)_{12–18} with [³²P]dTTP to a total length of 30–150 bases. After washing, the membrane was

Abbreviations used: G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); poly(A)⁺ RNA, polyadenylated RNA.

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subjected to autoradiography and analysis with a soft-laser scanning densitometer (Zeineh; Biomed Instruments, Fullerton, CA, U.S.A.). After autoradiography the dots containing the RNA were removed and the amount of radioactivity bound to the RNA was determined by scintillation counting. These values (expressed relative to the normal control) were used as correction factors for the data obtained from the G6PDH-specific probe for relative poly(A)⁺ RNA content. This conversion enables the comparison of the G6PDH cDNA probe data to be made on the same relative concentration of poly(A)⁺ RNA.

G6PDH mRNA quantification. In this assay the RNA samples (0.25 µg/dot) were fixed to GeneScreen (New England Nuclear, Boston, MA, U.S.A.) by using a u.v.-light cross-linking technique described by Church & Gilbert (1984). The membrane was then hybridized in the presence of ³²P-labelled cDNA clones (pGDF-34 and pGDC-4) specific for G6PDH mRNA (Kletzien *et al.*, 1985). After washing and autoradiography, analysis was carried out as described above.

RESULTS AND DISCUSSION

Verification of techniques

G6PDH mRNA in hepatocytes is present in low abundance (Stumpo & Kletzien, 1984; Kletzien *et al.*, 1985) and represents approx. 0.02% of the total mRNA even under conditions of enzyme induction. The low abundance of this mRNA necessitated that the poly(A)⁺ RNA fraction be isolated by oligo(dT)-cellulose chromatography before analysis for G6PDH-specific sequences. Each RNA sample was subjected to electrophoresis in the presence of formaldehyde, and we found that variable amounts of rRNA contaminated the poly(A)⁺ preparations even after two cycles of oligo(dT)-cellulose chromatography. Since different amounts of rRNA per µg of input RNA would ultimately affect the quantification of G6PDH mRNA sequences as analysed by G6PDH cDNA probes, we needed to assess the poly(A)⁺ RNA concentration in each sample. To do this, each RNA sample was analysed by dot-blot hybridization in the presence of an oligo(dT) probe (see the Materials and methods section). The results of this assay are shown in

Table 1, and permitted us to assess relative poly(A)⁺ RNA concentrations. Therefore the subsequent quantification of the G6PDH mRNA sequences was related back to the relative poly(A)⁺ concentrations.

Previous work from this laboratory reported the molecular cloning of G6PDH mRNA sequences (Kletzien *et al.*, 1985) and the use of cDNA probes in assessing the nutritional modulation of mRNA amounts in intact animals. In that study we were uncertain as to the location of the cDNA sequences with respect to the protein-coding portion of the mRNA. We have now constructed nearly full-length cDNA probes and have carried out analysis of the nucleotide sequence (R. S. Fritz, D. J. Stumpo & R. F. Kletzien, unpublished work). For the study reported here we have used two cDNA probes (280-base-pair fragment of pGDF-34 and 340-base-pair fragment of pGDC-4) that contain an open reading frame for G6PDH, indicating that the cDNA probes are specific for G6PDH mRNA.

Analysis of G6PDH mRNA amounts

Dexamethasone and insulin maximally stimulate G6PDH activity and relative rate of synthesis in hepatocytes by 48 h of treatment (Stumpo & Kletzien, 1984, 1985). Therefore we examined the effect of treating primary cultures of hepatocytes for 48 h with dexamethasone and insulin, individually or in combination, on G6PDH mRNA sequences. Primary cultures of hepatocytes were established from three rats, and the poly(A)⁺ RNA was isolated from each set of cultures. The preparations were analysed by 'Northern' blotting for G6PDH mRNA sequences (results not shown). Although the 'Northern' analysis was not used for quantification of G6PDH sequences, it did establish that the poly(A)⁺ RNA preparations all contained a single undegraded band of G6PDH mRNA activity of about 2300 bases.

The G6PDH mRNA sequences were quantitatively assessed by dot-blotting the samples, followed by cross-linking the RNA to the membrane with u.v. light. These results (Table 2) established that dexamethasone alone caused about a 1.44–1.81-fold increase in G6PDH mRNA sequences without increasing enzyme activity or relative rate of enzyme synthesis. Surprisingly, insulin alone caused a more modest increase in G6PDH mRNA sequences (1.34–1.60-fold) while substantially increasing both the relative rate of enzyme synthesis and G6PDH activity. When both hormones were used in combination, there was a synergistic increase in G6PDH mRNA sequences relative to the control cultures. A synergistic response was also noted in the previously measured enzyme parameters.

The results of this study establish that dexamethasone increases the amounts of G6PDH mRNA, but this RNA is not expressed at the protein level as active enzyme. We detected only a 1.8-fold increase in G6PDH sequence abundance, compared with the 4-fold increase in functional mRNA reported by Stumpo & Kletzien (1984). In the present study the samples compared were of equal relative poly(A)⁺ RNA concentrations, and the G6PDH sequences were directly assayed by hybridization. We consider that the standardization of the poly(A)⁺ RNA concentrations enabled us to assess accurately the G6PDH sequence abundance in these samples.

Treatment of cultures with dexamethasone and insulin in combination elevated enzyme activity and G6PDH mRNA amounts more than those observed with insulin

Table 1. Quantification of poly(A)⁺ RNA

Hepatocytes (nine or ten 150 mm-diam. culture plates from the livers of three rats) were treated with dexamethasone (0.5 µM) and/or insulin (46.7 munits/ml) for 48 h and the poly(A)⁺ RNA fraction was isolated. The poly(A)⁺ RNA concentration of these samples was determined as described in the Materials and methods section. The results are expressed as means ± s.d. for three determinations.

Condition	Oligo(dT)	
	(c.p.m.)	(× control)
Control	839.3 ± 85.6	1.00
Dexamethasone	1201.3 ± 31.8	1.43
Insulin	1007.7 ± 39.7	1.20
Dexamethasone + insulin	911.5 ± 22.5	1.08

Table 2. Comparison of G6PDH mRNA amounts, relative rate of synthesis and enzyme activity in cultured hepatocytes treated with dexamethasone and insulin

Poly(A)⁺ RNA from hepatocyte cultures described in Table 1 was analysed for G6PDH sequence abundance with a 280-base-pair fragment of pGDF-34 (similar results were observed when pGDC-4 was used as the probe). The results are means $\pm$ S.D. for three determinations and are expressed as percentages of the control. G6PDH activity was determined in cytosol samples from the hepatocyte cultures, and values are means $\pm$ S.E.M. for six determinations. Differences were analysed by Student's *t* test: a versus b, *P* 0.001; a versus c, *P* 0.002; a versus d, *P* 0.001.

Condition	G6PDH mRNA (%)		Relative rate of synthesis ($\times 10^3$)*	Enzyme activity (munits/mg of protein)
	(c.p.m.)	(densitometer)		
Control	1.00 $\pm$ 0.07 ^a	1.00 $\pm$ 0.03	3.48 $\pm$ 1.02	8.41 $\pm$ 0.70
Dexamethasone	1.81 $\pm$ 0.10 ^b	1.44 $\pm$ 0.14	3.66 $\pm$ 0.83	7.80 $\pm$ 0.30
Insulin	1.59 $\pm$ 0.07 ^c	1.34 $\pm$ 0.05	4.89 $\pm$ 0.83	15.05 $\pm$ 0.90
Dexamethasone + insulin	3.04 $\pm$ 0.16 ^d	3.17 $\pm$ 0.28	10.10 $\pm$ 3.30	21.80 $\pm$ 1.40

* From Stumpo & Kletzien (1985).

alone. Insulin appears not only to increase G6PDH mRNA but also to affect a translational event, since the dexamethasone-induced G6PDH mRNA is expressed as active protein only when both hormones are present. It is also possible that the dexamethasone-induced mRNA may represent a G6PDH mRNA species that is translated less efficiently. However, analysis of the poly(A)⁺ RNA by 'Northern' blots from each culture condition revealed that only a single mRNA species of 2300 bases hybridized to the G6PDH probes. A demonstration of the pretranslational event(s) involved in the control exerted by these hormones will require the isolation of the 5' flanking region of the gene with its control elements.

In this study, dexamethasone and insulin accounted for an approximate 3-fold increase in G6PDH sequence abundance and enzyme activity. Thus results from these tissue-culture studies do not completely account for the 13-fold increase in enzyme activity observed in the starved/re-fed animal (Kletzien *et al.*, 1985). This would indicate the involvement of some other hormone(s) or factor(s) in the dietary regulation of G6PDH activity.

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