Expression of the liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase mRNA in FAO-1 cells

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The hormonal regulation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression was studied in the rat hepatoma cell line FAO-1. Both 6-phosphofructo-2-kinase and fructose-2,6-bisphosphatase activities were detected in FAO-1 cells, at 68 % of the levels found in rat liver. Northern blot analysis showed that FAO-1 cells, like rat liver, contained a predominant species of bifunctional enzyme mRNA, which is 2.2 kb in size. A sensitive RNAase protection assay revealed the presence in FAO-1 cells of an additional mRNA species, which is generated when transcription is initiated from the skeletal muscle promoter of the rat liver/skeletal muscle gene. The liver/skeletal muscle mRNA ratio in FAO-1 cells was 10:1,

which is similar to that observed in rat liver. In contrast, in another rat hepatoma cell line, FTO-2B, only the skeletal muscle mRNA was detected. Insulin and dexamethasone induced the liver bifunctional enzyme mRNA in FAO-1 cells by 2–4-fold and 10–20-fold respectively in a concentration- and time-dependent manner, and their effects were antagonized by cyclic AMP. Transcription of the gene in FAO-1 cells, measured by nuclear run-on assays, was also enhanced by dexamethasone and insulin. It is concluded that the FAO-1 cell line is similar to liver with respect to both the preferential use of the liver promoter of the gene and its regulation by hormones, and is therefore an excellent model for the study of the hepatic expression of this gene.

INTRODUCTION

The functional specialization of the mammalian liver is dictated by specific regulation of gene transcription in the terminally differentiated hepatocyte [1]. Studies with cultured hepatoma cell lines have revealed that expression of many of the genes of hepatic carbohydrate metabolism is regulated in a complex manner by hormones such as glucagon, via its second messenger cyclic AMP (cAMP), glucocorticoids and insulin [2,3]. The effects of hormones on gene expression in hepatoma cells or primary hepatocytes are, in general, consonant with their established physiological actions: insulin increases the abundance of mRNAs that encode glycolytic enzymes [e.g. glucokinase, pyruvate kinase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6- P_2 ase)] and decreases mRNAs that encode gluconeogenic enzymes [e.g. phosphoenolpyruvate carboxykinase (PEPCK) and fructose-1,6-bisphosphatase (Fru-1,6- P_2 ase)]; cAMP has the opposite effects. Glucocorticoid hormones, on the other hand, have a more limited action, playing an important role in increasing PEPCK and 6PF-2-K/Fru-2,6- P_2 ase mRNA abundances, and they also have a permissive action in the regulation of pyruvate kinase mRNA by insulin [2,3]. However, while the *cis/trans* model of gene regulation has been successfully applied to an analysis of the PEPCK gene promoter in hepatoma cells, this approach has yielded only limited success with promoters for other genes, such as those for pyruvate kinase, glucokinase and 6PF-2-K/Fru-2,6- P_2 ase [2,3]. The fact that many of these genes are not expressed or regulated in standard cell culture lines has hampered this type of analysis. For example, hormonal and carbohydrate control of the liver-type pyruvate kinase and glucokinase genes, that is readily demonstrated *in vivo* (see [2,3] for a review), does not occur in hepatoma cells [4], and

can only be studied in primary hepatocytes [5]. However, with the exception of PEPCK, none of the liver-specific forms of glycolytic and gluconeogenic enzymes are expressed in primary hepatocyte cultures unless insulin and/or dexamethasone are added to the media. Furthermore, primary hepatocyte cultures present many technical problems, including reproducibility and survival time.

The bifunctional enzyme 6PF-2-K/Fru-2,6- P_2 ase catalyses the synthesis and hydrolysis of fructose 2,6-bisphosphate (Fru-2,6- P_2), a potent modulator of both glycolysis and gluconeogenesis, through its action on the activities of 6-phosphofructo-1-kinase (6PF-1-K) and Fru-1,6- P_2 ase [6,7]. 6PF-2-K/Fru-2,6- P_2 ase is expressed in all mammalian tissues [8,9]. The liver and skeletal muscle isoforms are different with respect to their N-terminal sequences, phosphorylation/dephosphorylation reactions and patterns of regulation by metabolites [7–9]. Both isoforms are products of a single 55 kb gene which contains 15 exons [10,11], with the muscle-specific transcript being initiated just distal to an upstream promoter containing exon 1_M and from which the liver exon 1_L is spliced out during processing. The liver-specific transcript is initiated just distal to a second promoter, upstream of exon 1_L, and generates exon 1_L. Either of these first exons (1_L or 1_M) is then spliced to the common exons 2–14. Cell-specific regulation of the liver and skeletal muscle forms of the enzyme is thus accomplished by the alternative first exons providing unique mRNAs in these tissues [2,3].

Both insulin and dexamethasone enhance bifunctional enzyme gene transcription in the rat hepatoma cell line FTO-2B [12], although the mRNA expressed was shown to be related to the skeletal muscle transcript. This finding raises doubt as to whether the same hormone response elements in the gene are involved in the regulation of the liver-specific mRNA that has been observed

Abbreviations used: cAMP, cyclic AMP; PEPCK, phosphoenolpyruvate carboxykinase; 6PF-1-K, 6-phosphofructo-1-kinase; 6PF-2-K/Fru-2,6- P_2 ase, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; Fru-1,6- P_2 ase, fructose-1,6-bisphosphatase; Fru-2,6- P_2 , fructose 2,6-bisphosphate; Fru-6- P , fructose 6-phosphate.

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in vivo [2,3]. In order to develop a cell culture model more suitable for studying regulation of the liver mRNA, we screened a number of rat hepatoma cell lines in an attempt to find a line that has a pattern of 6PF-2-K/Fru-2,6- P_2 ase gene expression and hormonal regulation similar to that found in rat liver *in vivo*. In this paper we report that FAO-1 cells meet these criteria.

MATERIALS AND METHODS

Materials

The random-primed DNA labelling kit, restriction endonucleases, nucleotides, bovine insulin and dibutyryl cAMP were purchased from Boehringer Mannheim. [α - 32 P]dATP, [α - 35 S]dATP, [α - 32 P]UTP and [3 H]uridine were from ICN Biomedicals. GeneScreen membrane was from Du Pont-New England Nuclear. Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical Corp. Fetal bovine serum and cell culture media were from Gibco. Other materials and chemicals were of the highest quality available.

Cell culture

Monolayer cultures of rat FAO-1 hepatoma-derived cells were grown in RPMI 1640 medium containing 10 mM glucose and supplemented with 10 % fetal bovine serum. FTO-2B cells were maintained in culture as described previously [12]. All experiments were carried out before the cell density reached confluency

and cells were incubated in serum-free medium during the hormonal treatment. Hormone effects were tested in the absence of serum to ensure that trace hormone concentrations in serum would not confound the results. The results are presented relative to untreated controls, incubated in the absence of serum, analysed at the same time as the treated cells. Since the cells were cultured in serum until confluency and then without serum for the duration of the experiment, it is possible that the basal level of expression changed during the experiment. In order to test whether the absence of serum in itself had any effect, cells were incubated with and without serum for up to 12 h. Relative to cells incubated with serum, the relative mRNA abundance (1.0 at zero time) was 0.90 ± 0.09 after 3 h, 0.95 ± 0.07 after 6 h, 1.30 ± 0.20 after 9 h and 1.20 ± 0.15 after 12 h (means $\pm$ S.E.M. from three experiments). The results indicate that removal of serum had little or no effect during the 12 h time course.

Measurement of 6PF-2-K/Fru-2,6- P_2 ase activity

FAO-1 cells ($\sim 4 \times 10^7$) were homogenized in medium composed of 100 mM KCl, 20 mM Tes, pH 7.8, 5 mM potassium phosphate, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride and 2.5 mg/l leupeptin. The enzyme was partially purified by a 6–21 % poly(ethylene glycol) 8000 precipitation [13]. Under these conditions the *in situ* phosphorylation state of the enzyme is preserved [13]. 6PF-2-K activity was assayed by measuring the rate of Fru-2,6- P_2 production from fructose 6-phosphate (Fru-6-P) and MgATP at pH 7.8 [13,14]. Fru-2,6- P_2 was assayed by its ability to activate pyrophosphate-dependent 6PF-1-K from potato tubers (Sigma) [15]. Fru-2,6- P_2 ase activity was assayed by measuring [32 P]P_i release from [2- 32 P]Fru-2,6- P_2 (10 μ M) [14] or by phosphoenzyme formation [14,16].

DNA hybridization probes

Four different hybridization probes were used to detect 6PF-2-K/Fru-2,6- P_2 ase mRNA (Figure 1a). A 1.4 kb *Eco*RI fragment was isolated from the cDNA for 6PF-2-K/Fru-2,6- P_2 ase that contained sequences transcribed from the third to the fourteenth exons, which are common to 6PF-2-K/Fru-2,6- P_2 ase from both liver and skeletal muscle [10,17,18]. A second probe was a 0.5 kb *Eco*RI fragment containing the 5' end of the cDNA for the bifunctional enzyme [13]. In order to detect tissue-specific mRNA expression, DNA fragments containing either the liver- or the muscle-specific first exon were isolated from a genomic clone (Figure 1a). The liver-specific probe is an *Eco*RI–*Ban*II fragment (299 bp) that includes most of exon 1_L, while the muscle-specific probe is a *Fok*I (220 bp) fragment that contains exon 1_M. The abundance of PEPCK mRNA was determined with a 1.5 kb *Pst*I fragment isolated from a cDNA clone (pPCK10rc) for the enzyme [19]. All DNA probes were random-primer labelled with [α - 32 P]dATP [20].

RNA isolation and analysis

RNA was isolated essentially according to Chirgwin et al. [21], with one extraction in 4 M guanidine thiocyanate followed by two successive extractions with 7.5 M guanidine hydrochloride. The abundance of the mRNA for 6PF-2-K/Fru-2,6- P_2 ase was determined either by Northern blot analysis [22] or by RNAase protection assay [23]. In both cases densitometric scans of the autoradiograms were carried out on an LKB-Pharmacia Ultrascan XL Laser Densitometer.

Figure 1 Skeletal muscle/liver bifunctional enzyme gene structure and hybridization probes for Northern blot analysis

(a) Structure of the rat 6PF-2-K/Fru-2,6- P_2 ase liver/skeletal muscle gene. Solid bars represent exons and connecting lines represent introns. The sequences used as hybridization probes are also indicated below the structure of the gene. B, *Ban*II; F, *Fok*I; R, *Eco*RI. (b) Northern blot of FAO-1 cell RNA (20 μ g) (lane 1), rat liver RNA (10 μ g) (lane 2) and rat skeletal muscle RNA (20 μ g) (lane 3). Hybridization was performed with the liver-specific DNA probe (probe B) shown in (a) as described in the Materials and methods section.

Ribonuclease protection assay

An RNA probe was constructed according to Maniatis et al. [23]. The 0.5 kb *EcoRI* fragment, previously described [13], was cloned into the *EcoRI* restriction site of the plasmid Bluescript SK(+). The plasmid was linearized by digestion with *AvaII*, and was then used to synthesize RNA using its T3 RNA polymerase promoter, with ribonucleotide triphosphates, including [α - 32 P]-CTP, and T3 RNA polymerase. The synthesized riboprobe was 390 bases long, complementary to 319 bases of the liver-specific mRNA, including exon 1_L, and to 192 bases of the skeletal muscle-specific mRNA. The probe was purified by electrophoresis in 6% polyacrylamide, in the presence of 8 M urea and Tris/borate/EDTA buffer. Samples of 10–50 μ g of total RNA from extracts of nearly confluent cells, and from rat liver and rat muscle, were incubated at 90 °C for 10 min with a molar excess (100 000 c.p.m.) of the purified riboprobe. The tubes were cooled slowly to 42 °C, and maintained at this temperature overnight. The assay was performed as described by Hod [24]. A mixture of RNAase T1 and RNAase A were used to degrade single-stranded unhybridized probe. After digestion, the protected fragments were collected by centrifugation (12 000 *g*; 15 min) at 4 °C in the presence of guanidine thiocyanate and propan-2-ol, and subjected to electrophoresis on DNA sequencing gels, as described previously [24]. The gels were then subjected to autoradiography and densitometric scanning.

Measurement of run-on gene transcription in isolated nuclei

The isolation of nuclei and the incorporation of [α - 32 P]UTP into nascent RNA transcripts of 6PF-2-K/Fru-2,6-*P*₂ase were performed as previously described [12].

cDNA library construction and screening

A cDNA library was constructed by using mRNA isolated from FAO-1 cell extracts, a cDNA synthesis kit from Pharmacia, and a λ ZAP II cloning kit from Stratagene. In order to obtain the 5' region of the 6PF-2-K/Fru-2,6-*P*₂ase gene, four different specific oligonucleotides, located in the common region of the gene, were used to prime the reverse transcriptase reaction. The library was screened with the 0.5 kb *EcoRI* cDNA probe [13]. Positive clones were excised *in vivo* with R408 helper phage.

DNA sequencing and analysis

All sequencing was done by the Sanger dideoxy method [25] using the Sequenase sequencing kit (U.S. Biochemicals, San Diego, CA, U.S.A.). Sequence analysis was carried out using the GCG software package [26].

Protein determination

Protein concentration was determined with the BCA Protein Assay Reagent from Pierce Chemical Co., using the enhanced protocol and with BSA as a standard.

RESULTS

Expression of 6PF-2-K/Fru-2,6-*P*₂ase in FAO-1 cells

Extracts from FAO-1 cells were assayed for 6PF-2-K activity at pH 7.8 with saturating concentrations of substrates (Fru-6-*P*,

5 mM; MgATP, 5 mM). Under these assay conditions, $V_{\max}$ values reflect the amount of enzyme protein [13]. FAO-1 cells contained about 27 μ units of 6PF-2-K activity per mg of cellular protein, while cells exposed for 12 h to insulin (0.1 μ M) or dexamethasone (1 μ M) had 6PF-2-K activities of about 61 and 81 μ units/mg respectively. The increase in 6PF-2-K activity was seen after a lag of 4–6 h. The extract from FAO-1 cells also exhibited Fru-2,6-*P*₂ase activity (20 μ units/mg of protein) (results not shown). In addition, we identified, by SDS/PAGE, a 32 P-labelled polypeptide in extracts incubated with [2- 32 P]Fru-2,6-*P*₂ that had a molecular mass of 55 kDa, which is identical to the size of the 6PF-2-K/Fru-2,6-*P*₂ase subunit from rat liver (results not shown). As with 6PF-2-K activity, a quantitatively similar enhancement in Fru-2,6-*P*₂ase activity was observed in cells treated with insulin or dexamethasone. The activity ratio of kinase to bisphosphatase was about 1.4, which is similar to that for the liver enzyme, but different from the activity ratios of 0.1–0.5 and 50 found in skeletal muscle and heart respectively [8]. The 6PF-2-K activity in livers from normal fed rats is approx. 40 μ units/mg of cellular protein [13]. The presence of 6PF-2-K/Fru-2,6-*P*₂ase in FAO-1 rat hepatoma cells was further corroborated by Western blot analysis of the cellular extract using an antibody against rat liver 6PF-2-K/Fru-2,6-*P*₂ase, which also revealed a 55 kDa subunit. These results indicate that the enzyme in FAO-1 cells is similar in amount and properties to 6PF-2-K/Fru-2,6-*P*₂ase from rat liver.

Identification of the mRNAs for 6PF-2-K/Fru-2,6-*P*₂ase in FAO-1 cells

The 5' region of the skeletal muscle mRNA contains sequences derived from an exon located about 5 kb upstream of the liver-specific exon (see Figure 1a for the structure of the 6PF-2-K/Fru-2,6-*P*₂ase gene). The remaining 13 exons are common to both forms of mRNA. A 1.4 kb cDNA probe [13,27] containing sequences common to liver and muscle mRNA (exons 3–14) hybridized with a 2.2 kb mRNA in FAO-1 cells (results not shown). A liver-specific probe hybridized with a 2.2 kb RNA from both FAO-1 cells and liver, but not with muscle RNA (Figure 1b). When the muscle-specific probe was used, a signal was observed only in the muscle RNA lane (results not shown), suggesting that the liver 6PF-2-K/Fru-2,6-*P*₂ase mRNA is the predominant species expressed in FAO-1 cells. It is not possible, however, to rule out the existence of other low-abundance 6PF-2-K/Fru-2,6-*P*₂ase mRNAs the concentrations of which may be below the detection limit of Northern blot analysis, or whose size and sequence are similar to those of the liver mRNA. Liver and skeletal muscle mRNAs differ in size by less than 200 bases, and cannot be easily distinguished by Northern blot analysis.

More conclusive results were obtained by an RNAase protection assay (Figure 2). Use of the 390-base RNA probe permitted a clear discrimination between the liver and muscle mRNAs (Figure 2). As predicted, the major protected fragment in liver RNA was 319 bases long, corresponding to the region complementary to the liver mRNA, while the major protected fragment in skeletal muscle RNA was 192 bases long. No protected bands were observed with yeast RNA. While liver and skeletal muscle contained both the liver and skeletal muscle transcripts, their abundances are clearly different in the two tissues: in liver the liver transcript is the predominant form, while in skeletal muscle the reverse is true. Quantification revealed that the liver mRNA constituted approx. 15% of the total bifunctional mRNA in skeletal muscle, while the skeletal muscle mRNA accounted for only about 10% of the total bifunctional mRNA in liver (Table 1). This result shows that both mRNAs

Figure 2 RNAase protection analysis

(a) The structure of the 500 bp fragment of the rat liver 6PF-2-K/Fru-2,6-P₂ase *EcoRI* cDNA after subcloning into Bluescript(+) and linearization by digestion with *Avall* is shown at the top. The regions of the probe predicted to be protected by the skeletal muscle and liver RNA after RNAase digestion are shown below the 390-base probe. (b) RNAase protection assay of RNA from FAO-1 cells, rat liver and rat skeletal muscle. Lane 1, undigested RNA probe (390 bases); the other lanes contain protected fragments after hybridization of the probe with RNA from different sources and digestion with RNAase: yeast RNA (20 µg) (lane 2), skeletal muscle (15 µg) (lane 3), rat liver (5 µg) (lane 4), FAO-1 cells (50 and 20 µg) (lanes 5 and 6 respectively). (c) RNAase protection assay of RNA from liver (10 µg) (lane 2), FTO-2B cells (50 µg) (lane 3) and skeletal muscle (20 µg) (lane 4). The undigested RNA probe is shown in lane 1.

Table 1 Abundance of the skeletal muscle and liver 6PF-2-K/Fru-2,6-P₂ase mRNAs in rat hepatoma cells relative to the abundance of the liver mRNA in liver

Skeletal muscle and liver mRNA abundances were assayed by RNAase protection as described in the Materials and methods section. The data are expressed relative to the abundance of the liver-specific mRNA (319 base protected fragment) in livers from fed rats, which was given a value of 1.0. The mRNAs were quantified by scanning laser densitometry of the 319 and 192 base protected fragments. The experiments represent the means $\pm$ S.E.M. of three experiments.

Tissue/cells	Relative mRNA abundance	
	Liver mRNA	Skeletal muscle mRNA
Liver from fed rats	1.00 $\pm$ 0.11	0.10 $\pm$ 0.01
Skeletal muscle from fed rats	0.15 $\pm$ 0.01	0.91 $\pm$ 0.08
FAO-1 cells	0.19 $\pm$ 0.02	0.02 $\pm$ 0.01
FTO-2B cells	0	0.14 $\pm$ 0.02

are expressed in these tissues and suggests that the use of alternate promoters of this gene is tissue-specific.

It was of interest to ascertain whether both bifunctional enzyme mRNAs are also expressed in hepatoma cell lines derived

Figure 3 Time courses of hormonal effects on 6PF-2-K/Fru-2,6-P₂ase mRNA induction

Cells were incubated in medium containing either 1 µM insulin (■) or 1 µM dexamethasone (□). Fetal serum was washed out from the incubation plate at time 0, just before the addition of the hormones. 6PF-2-K/Fru-2,6-P₂ase mRNA was determined by Northern blot analysis, and the changes in mRNA abundance are presented relative to untreated controls, incubated in the absence of serum, analysed at the same time as the treated cells, Dex, dexamethasone; Ins, insulin.

from rat liver. Application of the RNAase protection assay to RNA from FAO-1 cells showed a pattern of tissue-specific expression that is identical to that of rat liver (Figure 2b; Table 1), albeit at one-fifth the abundance. This is in contrast to enzyme activity levels, which are about two-thirds of those in liver. The explanation for this discrepancy is not clear, but may relate to different rates of translation and/or protein degradation in the FAO-1 cells versus the liver. Figure 2(c) shows that only the skeletal muscle mRNA is expressed in another rat hepatoma cell line, FTO-2B, a finding consistent with earlier studies of FTO-2B cell 6PF-2-K/Fru-2,6-P₂ase mRNA, which was recognized by a skeletal muscle-specific cDNA probe but not by a liver-specific probe [12]. Table 1 shows that the abundance of skeletal muscle mRNA in FTO-2B cells is slightly greater than that of the skeletal muscle mRNA in rat liver.

In order to rule out a possible dissimilarity in the sequences of the 5' ends of the liver and FAO-1 cell mRNAs, an FAO-1 cDNA library was screened as described in the Materials and methods section, and a clone containing the 5' end of the 6PF-2-K/Fru-2,6-P₂ase gene was isolated. Its sequence was identical to that of the first 328 bases of the liver 6PF-2-K/Fru-2,6-P₂ase cDNA (results not shown). This finding supports the hypothesis that the predominant FAO-1 cell bifunctional enzyme mRNA is identical to the liver-specific mRNA.

Hormonal control of 6PF-2-K/Fru-2,6-P₂ase gene expression in FAO cells

Figure 3 shows the time course of 6PF-2-K/Fru-2,6-P₂ase liver mRNA accumulation in FAO-1 cells incubated in the presence of 1 µM insulin or 1 µM dexamethasone. In both insulin- and dexamethasone-treated cells the abundance of the liver mRNA increased gradually, and attained maximum levels that were 2–4-fold and 20-fold higher respectively than the basal level. The increase in the level of 6PF-2-K/Fru-2,6-P₂ase mRNA in insulin-treated cells was noted only after a 4–6 h lag, while the onset

Figure 4 Hormonal regulation of 6PF-2-K/Fru-2,6- P_2 ase mRNA abundance in FAO-1 cells

Northern blot analysis of RNA (20 μ g) extracted from cells incubated for 12 h with no additions (lane 1), 0.1 μ M insulin (lane 2), 1 μ M insulin (lane 3), 0.1 μ M dexamethasone (lane 4), 1 μ M dexamethasone (lane 5), 10 μ M dexamethasone (lane 6), 0.5 mM dibutyl cAMP plus 1 mM theophylline (lane 7) or 0.5 mM monobutyl cAMP plus 1 mM theophylline (lane 8). In (a), a 1.4 kb *Eco*RI fragment (common probe; see Figure 1a) from the 6PF-2-K/Fru-2,6- P_2 ase rat liver cDNA was used as a probe. In (b), the probe was a 1.5 kb *Pst*I fragment from the cDNA for PEPCK.

Figure 5 Dose-response of insulin and dexamethasone action of 6PF-2-K/Fru-2,6- P_2 ase mRNA abundance in FAO-1 cells

FAO cells were incubated in media containing the indicated concentrations of insulin (Ins; ■) and dexamethasone (Dex; □) for 12 h. The abundance of 6PF-2-K/Fru-2,6- P_2 ase mRNA was determined by Northern blot analysis using the 1.4 kb common probe, and quantified by scanning densitometry. The results are expressed as described in the Materials and Methods section and in the legend to Figure 3.

of the response to dexamethasone was seen as early as 3 h, suggesting that the mechanism of induction of 6PF-2-K/Fru-2,6- P_2 ase mRNA may differ for the two hormones.

As can be seen in Figure 4(a), 0.1 μ M insulin was sufficient to increase the 6PF-2-K/Fru-2,6- P_2 ase mRNA abundance (lane 2), although a much larger increase was observed in the presence of dexamethasone (lanes 4, 5 and 6). As expected, the content of PEPCK mRNA in FAO-1 cells was increased by dexamethasone and cAMP (Figure 4b, lanes 4–6, and 7 and 8, respectively).

Figure 6 Effect of cAMP on the induction by insulin and dexamethasone of 6PF-2-K/Fru-2,6- P_2 ase mRNA in FAO-1 cells

The cells were incubated for 12 h under different conditions. Final concentrations were 1 μ M for insulin and dexamethasone (Dex), 0.5 mM for cAMP analogues (Mb-cAMP, monobutyl cAMP; Db-cAMP, dibutyl cAMP) and 1 mM for theophylline (Theo). The RNA was analysed as in Figure 4, and the results are expressed as described in the Materials and methods section and the legends to Figures 3 and 5. The experiment was repeated three times with essentially similar results.

Dibutyl cAMP plus theophylline, in the absence of insulin or dexamethasone, had no effect on 6PF-2-K/Fru-2,6- P_2 ase mRNA abundance.

Figure 5 shows dose-response curves of the induction of 6PF-2-K/Fru-2,6- P_2 ase liver mRNA in FAO-1 cells exposed to insulin or dexamethasone. Half-maximal effects of insulin and dexamethasone were obtained in the presence of 0.01 μ M of either hormone (Figure 5), concentrations that are within the physiological range for these hormones.

Dibutyl cAMP inhibits the induction of 6PF-2-K/Fru-2,6- P_2 ase mRNA by insulin and dexamethasone

Induction of 6PF-2-K/Fru-2,6- P_2 ase mRNA by either insulin or dexamethasone was counteracted by the simultaneous addition of cAMP analogues to the incubation medium (Figure 6). Monobutyl cAMP was sufficient to counteract the insulin induction of the gene, whereas the presence of both theophylline and dibutyl cAMP were required to antagonize the much more potent effect of dexamethasone. The cAMP analogues repressed the effect of saturating concentrations of dexamethasone and insulin, suggesting that they play a dominant role in regulating this gene's expression in FAO-1 cells. A similar dominant role of cAMP in affecting the induction of the skeletal muscle bi-functional enzyme mRNA has been reported for FTO-2B cells [12].

Induction of 6PF-2-K/Fru-2,6- P_2 ase mRNA is mediated by activation of gene transcription

The increase in mRNA may be explained by an increase in its rate of synthesis, by a decrease in its degradation rate, or by a combination of both mechanisms. Run-on transcription assays with isolated nuclei were performed to estimate the relative transcription rate of 6PF-2-K/Fru-2,6- P_2 ase gene in FAO-1 cells. As shown in Figure 7(a), there was an increase in the

Figure 7 Effect of insulin and dexamethasone on 6PF-2-K/Fru-2,6- P_2 ase gene transcription in isolated nuclei

Nuclei were isolated from cells incubated for 9 h in the presence of either 1 μ M insulin or 1 μ M dexamethasone. The nuclei were used in run-on transcription assays which were carried out as described in the Materials and methods section. Means $\pm$ S.E.M. from three independent experiments are shown.

relative transcription rate of the gene in cells exposed to either insulin or dexamethasone for 9 h. The time courses of induction of 6PF-2-K/Fru-2,6- P_2 ase gene transcription and mRNA abundance (Figure 3) showed that the rate of mRNA accumulation generally followed the changes in the relative synthesis rate of the mRNA (results not shown). These results strongly suggest that the mechanism for the increase in the 6PF-2-K/Fru-2,6- P_2 ase mRNA level in cells exposed to insulin or dexamethasone is mediated, in large part by stimulation of the transcription rate of the gene. For comparison, the transcriptional activity of the gene coding for PEPCK was also analysed in the same experiment (Figure 7b). As expected, the transcription of the PEPCK gene was enhanced in cells treated with dexamethasone and inhibited in cells incubated in the presence of insulin. The relatively small effects of insulin and dexamethasone on PEPCK transcription relate to the fact that measurements were done after 9 h. A similar extent of inhibition by insulin of PEPCK gene transcription has been documented previously [28]. The differential response in the relative transcription rates of the 6PF-2-K/Fru-2,6- P_2 ase and PEPCK genes in response to insulin and dexamethasone indicates that the enhancement in the transcription rate of 6PF-2-K/Fru-2,6- P_2 ase gene is specific.

DISCUSSION

Studies on the control of skeletal muscle/liver 6PF-2-K/Fru-2,6- P_2 ase gene expression have been done *in vivo* and in rat hepatoma FTO-2B cells. The amount of bifunctional enzyme protein decreases in livers of rats during starvation or diabetes, and is restored by refeeding a high-carbohydrate diet or by insulin administration respectively [13,29]. The amount of 6PF-2-K/Fru-2,6- P_2 ase mRNA is also reduced in liver and skeletal muscle of adrenalectomized rats, and is restored by administration of glucocorticoids which increase gene transcription [11,17,27]. This is a direct effect of glucocorticoids, since their addition to primary cultures of hepatocytes rapidly increases mRNA levels by nearly 100-fold [17]. Dexamethasone and insulin also induce bifunctional enzyme mRNA in FTO-2B cells, but these cells express a skeletal muscle 6PF-2-K/Fru-2,6- P_2 ase

mRNA, not a liver mRNA ([12]; the present paper). This paper clearly shows that, in contrast to FTO-2B cells, FAO-1 cells provide an excellent model system for studying the hormonal regulation of the liver-specific bifunctional enzyme mRNA.

The results of the present study also demonstrate that both the liver and skeletal muscle forms are expressed in muscle, liver and FAO-1 cells. The glucokinase gene provides another example of tissue-specific expression of different isoforms as a result of use of alternative first exons to generate pancreatic β -cell and liver isoforms [2]. However, the weight of evidence indicates that the β -cell glucokinase mRNAs are expressed only in β -cells and the liver glucokinase mRNAs are expressed only in liver, which suggests that, in contrast to the skeletal muscle/liver bifunctional enzyme gene, alternative promoter use in the glucokinase gene is strictly tissue-specific [30].

The use of both the muscle and liver promoters of the 6PF-2-K/Fru-2,6- P_2 ase gene in liver and skeletal muscle raises the question of whether the effects of hormones on bifunctional enzyme gene expression are differentially regulated, i.e. do hormones increase liver promoter activity in liver but not skeletal muscle promoter activity in muscle? Although data on this question are still limited, preliminary results with the RNAase protection assay (A. Vargas, unpublished work) have shown that both mRNAs were increased by insulin and glucocorticoids in FAO-1 cells, and previous work has demonstrated the same result in both skeletal muscle and liver of adrenalectomized rats after glucocorticoid administration [27]. Furthermore, although only the skeletal muscle transcript is expressed in FTO-2B cells, regulation of this transcript by insulin, dexamethasone and cAMP is similar to that observed for the liver-specific transcript *in vivo* and in primary cultures of hepatocytes [31], except that dexamethasone is a more potent inducer of 6PF-2-K/Fru-2,6- P_2 ase gene expression than insulin *in vivo* [13,29], in primary hepatocyte cultures [31] and in FAO-1 cells (present paper), but the reverse is true in FTO-2B cells [12]. The reason for this difference in hormone responsiveness is not known.

Analysis of RNA isolated from rat hepatoma H35 cells, from which both FTO-2B and FAO-1 cells were derived [32], also revealed a pattern of bifunctional enzyme gene expression that is similar to those of FAO-1 cells and rat liver (A. Vargas, unpublished work). Since FTO-2B cells were originally generated by treating a derivative cell line of H35 cells with mutagens and visible light in order to select for thymidine kinase-deficient cells [33], it is possible that this treatment and/or subsequent selective pressures on the cell line have resulted in an apparent loss of liver-specific factors that activate the liver promoter.

The finding that bifunctional enzyme gene expression is subject to regulation by the same set of hormones in FAO-1 and FTO-2B cells suggests that the same hormone response elements act on both promoters. Consistent with this idea, a unique glucocorticoid response unit, recently identified in the first intron of the gene, has been shown to enhance the activity of both promoters in transiently transfected cells [11]. This finding, and the results of LeMaigre et al. [34] and Darville et al. [35], argue strongly for the existence of two separate functional promoters of the gene. Based on the results presented here, it is reasonable to postulate the existence of tissue-specific regulatory systems which determine the relative activities of the liver and skeletal muscle promoters in their respective tissues. Previous work has demonstrated the presence of numerous binding sites for trans-acting factors in the 5'-flanking regions of the gene that influence the activities of both the liver and skeletal muscle promoters [34,35]. However, tissue-specific transcriptional factors responsible for the pattern of alternative promoter use in liver, FAO-1 cells and FTO-2B cells were not identified. Work is in progress,

using the rat hepatoma FAO-1 and FTO-2B cell lines as convenient model systems, to define the basis for first-exon selection of the skeletal muscle/liver gene.

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