Conservation from rat to human of cytosolic phospho*enol*pyruvate carboxykinase and the control of its gene expression

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Structural conservation of cytosolic phospho*enol*pyruvate carboxykinase protein and mRNA sequence was found in all species examined from rodents to human. The mitochondrial isoenzyme, in all species tested, represents a distinct protein. Moreover, irrespective of the ratio of cytosolic to mitochondrial isoenzyme, cytosolic phospho*enol*pyruvate carboxykinase activity in the human as in the rat is controlled at the level of gene expression and through the same multiple hormonal stimulation. This evolutionary conservation of the cytosolic phospho*enol*pyruvate carboxykinase structure and mode of regulation supports the enzymes' physiological importance in mammals.

*Phospho*enol*pyruvate carboxykinase* *Gene expression*

1. INTRODUCTION

The coexistence in tissue of isoenzymes catalyzing the same enzymatic reaction is a frequent phenomenon in biology, although the presence of one form alone may be functionally sufficient. In this case, isoenzymes undergo evolutionary changes in structure as well as in the control of their cellular level. Families of genes representing several aspects of such a divergence have been reported [1–5].

We have studied phospho*enol*pyruvate carboxykinase (EC 4.1.1.32 PEPCK) which catalyzes the first committed gluconeogenic reaction. There are two PEPCK forms, one mitochondrial and the other cytosolic. Both share the same catalytic activity but represent distinct protein entities with no immune cross-reactivity between them [6–8]. The coexistence of the two isoenzymes does not seem necessary since the ratio of the cytoplasmic to mitochondrial forms differs in various species [6–10].

Despite the diverse representations of the two PEPCK forms, the crucial role of the cytosolic PEPCK in glucose metabolism in mammals is demonstrated by the fact that in most mammals studied it is absent in the fetus and appears in the liver at birth along with gluconeogenesis (review Benvenisty et al. [11]). Additionally, detailed studies in the rat have shown that cytosolic PEPCK is an adaptive enzyme and the expression of its gene is under multiple hormonal control in a tissue-specific manner (review [11,12]). Thus, cytosolic PEPCK provides a unique opportunity to examine whether its physical structure as well as the complex hormonal control of the expression of its gene are conserved, irrespective of the ratio of

Abbreviations: dBcAMP, *N*,6-O,2-dibutyryladenosine-3′5′-monophosphate; IgG, immunoglobulin; PEPCK, phospho*enol*pyruvate carboxykinase (GTP) (EC 4.1.1.32); SSC, standard saline citrate; poly(A)-rich RNA, RNA containing poly(A) at its 3′-end

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cytosolic to mitochondrial forms in various mammalian species.

2. EXPERIMENTAL

2.1. *Animals and tissues*

Six-week-old male, sabra rats and mice (H.U. strains), Syrian hamsters, *Psamomys obesus*, guinea pigs and a cat (unknown age) were all from the Hebrew University breeding center. Chinese hamsters (same age) were from the Weizman Institute breeding center. Human kidney was obtained after nephrectomy. The human hepatoma cell line PLC/PRF/5 was adapted to culture and maintained in RPM I medium as in [13,14]. For PEPCK activity cells were washed 3 times with cold 0.15 M NaCl, collected with a rubber policeman, and frozen and thawed 3 times in PEPCK buffer [15]. The 100000 × *g* supernatant was taken for enzyme assay [15–17] and protein determination [18].

2.2. *Immunoassay of PEPCK*

Catalytic activity, immunotitration and immunoprecipitation of PEPCK were performed as in [15–17] and slab gel electrophoresis was according to Maizel [19].

2.3. *RNA extraction and its analysis by Northern and dot-blot hybridization*

Poly(A)-rich RNA was prepared as in [21,22] from total RNA extracted with guanidine thiocyanate from livers of various mammals or from human kidney [20]. Total RNA from human hepatoma cells was similarly extracted, using a CsCl gradient according to Chirgwin et al. [20]. The RNA yield was consistently 200 $\mu g/10^6$ cells, irrespective of treatment. Total RNA (20–50 μg) or poly(A)-rich RNA (1–10 μg) were denatured and size-fractionated by electrophoresis on an agarose gel containing 2.2 M formaldehyde [23]. The size of PEPCK RNA was determined from the migration of the markers (bacterial 16 S and 23 S and mammalian 28 S and 18 S ribosomal RNA). Total RNA from human hepatoma cells of each treatment was applied to nitrocellulose filters in a series of dots containing 3–12 μg RNA. Northern and dot blotting and hybridization were performed as in [21,22] using an *Eco*RI 5.4-kb fragment of genomic PEPCK DNA [24] as a probe. For measurements of thermal stability of hybrids, poly(A)-rich RNA from rat liver and human kidney was dot-blotted on nitrocellulose filters and hybridized with ^{32}P-nicktranslated PEPCK cDNA clone (pPCK-10 [24]). After washing at room temperature the filters were incubated for 30 min in 0.1 × SSC, 0.1% SDS at various temperatures ranging from 50 to 80°C. The filters were then washed in 0.1 × SSC at room temperature. The radioactivity retained on the filters was quantified by densitometry of the autoradiographic images. Densitometry, using Helena Quick Scan R and D densitometer, of the autoradiographic images, was performed and the area under the peaks weighed yielding proportionately linearly increasing density with increasing amounts of RNA per dot up to 12 μg.

3. RESULTS AND DISCUSSION

3.1. *Structural conservation of mammalian cytosolic PEPCK and its mRNA*

The degree of antigenic homology of cytosolic PEPCK from various mammals and the size of the PEPCK protein were determined using rat liver cytosolic PEPCK antibody [17]. Immunotitration of PEPCK activity in liver cytosols consistently resulted in a linear inactivation of PEPCK with increasing amounts of antibody, although the slopes obtained with several species varied considerably (fig.1b). Thus, identical slopes were obtained with PEPCK from rats, mice and hamsters, that of the human PEPCK deviated about 3-fold, and those of the fasted cat (24 h) and guinea pigs (48 h) deviated 4–10-fold, respectively. In contrast, human mitochondrial PEPCK activity as well as that of the guinea pig and cat, was not neutralized by the antibody confirming previous results from rats [6], baboons [7] and chickens [8] on the discrete antigenic entities of the two PEPCK isoenzymes. The discrete immunochemical reaction and the size of the cytosolic PEPCK protein were determined from the analysis of the dissociated immunoprecipitate by electrophoresis on SDS–polyacrylamide slab gels (fig.1a). Three protein bands were revealed in all precipitates after staining, two of which correspond to the antibody heavy and light chains and one band equal in size to rat cytosolic PEPCK (74000). Thus in all species (rats, Chinese and Syrian hamsters, guinea pigs,

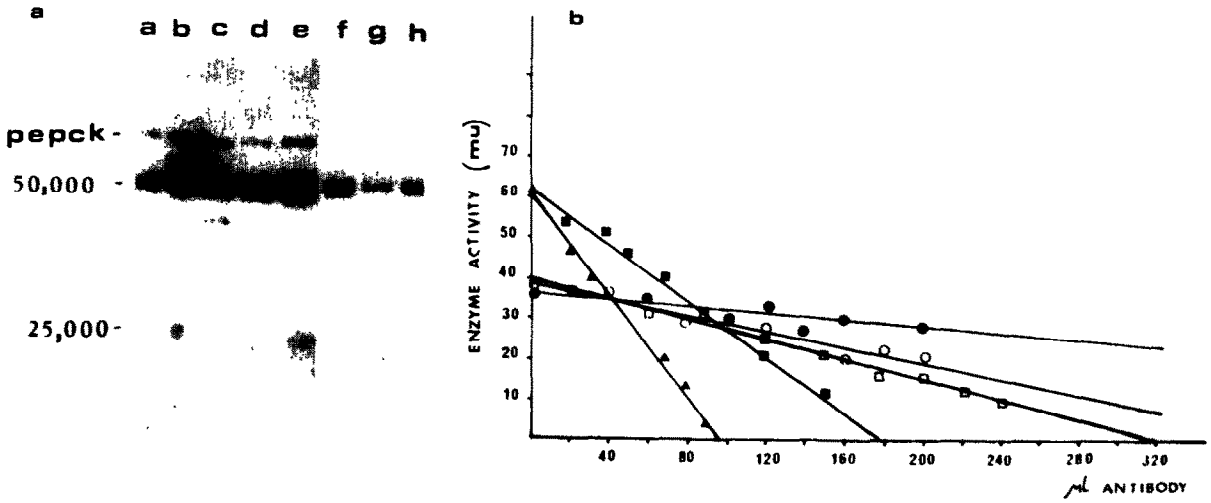

Fig.1. (a) SDS–polyacrylamide gel electrophoresis of immune precipitated cytosolic PEPCK from various mammals. 50000 and 25000 are heavy and light chains of IgG. (a) Syrian hamster; (b) Chinese hamster; (c) fasted guinea pig; (d) mouse; (e) rat; (f) *Psamomys obesus*; (g) human; (h) rat. (b) Immunotitration of PEPCK activity from various mammalian species. Tissue extracts containing 40–80 munits of PEPCK catalytic activity were treated with increasing amounts of rat PEPCK antibody and the residual activity remaining in the supernatant determined. (▲) A representative line of immunotitrations of cytosolic PEPCK from fasted rats, mice and Syrian and Chinese hamsters; (■) human; (□) fasted cat; (○) fasted guinea pig; (●) a representative line of immunotitrations of mitochondrial PEPCK (human, guinea pig, and a cat).

Psamomys obesus and the human) the immunoreactive PEPCK is of the same molecular size (fig.1a).

The homology and size of cytosolic PEPCK mRNA from various species was determined by Northern blot hybridization analysis of poly(A)-rich RNA extracted from livers (various species) and human kidney or cultured hepatoma cells. A ^{32}P-nicktranslated PEPCK genomic fragment (5.4 kb [24]) was used as a probe. One major hybridized band of a similar size (2800 bases) as previously determined for rat cytosolic PEPCK mRNA [25] was visualized in RNA from rats, mice, human (kidney or cultured hepatoma cells) and fasted guinea pig and cat (fig.2b). The hybridized sequences remained stable under relatively stringent wash conditions (0.1 × SSC at 52°C), indicating homology of the PEPCK sequences. This, together with the similarity of PEPCK antigenicity suggest a considerable degree of conservation of the primary structure of cytosolic PEPCK proteins in all examined species. Thermal stability analysis was employed to estimate the degree of sequence homology between the human and rat PEPCK mRNAs (fig.2a). Poly(A)-rich RNA from rat liver and human kidney were immobilized on nitrocellulose filters and hybridized with ^{32}P-nicktranslated rat PEPCK cDNA, containing almost the entire coding sequence [24]. The resulting melting profiles reveal 14°C difference between the melting temperatures (T_m) of hybrids involving rat PEPCK mRNA (T_m = 74°C) and those involving the human mRNA (T_m = 60°C). Assuming that 1°C of T_m results from about 1.5% mismatched base-pairs [27], the decreased T_m of hybrids involving human mRNA, reflects about 21% of sequence diversity with respect to the rat, and probably accounts for the deviated immunotitration.

3.2. *Control of cytosolic PEPCK gene expression*

The multiplicity of hormones affecting the activity of liver cytosolic PEPCK in the rat is a feature inherent to this enzyme and of great physiological significance to its role in gluconeogenesis (review [11,12]). Detailed studies in the rat have shown that hormones modulate the activity of cytosolic PEPCK by controlling the expression of its gene [21,22,25,26]. The availability of a human hepatoma cell line in culture [13],

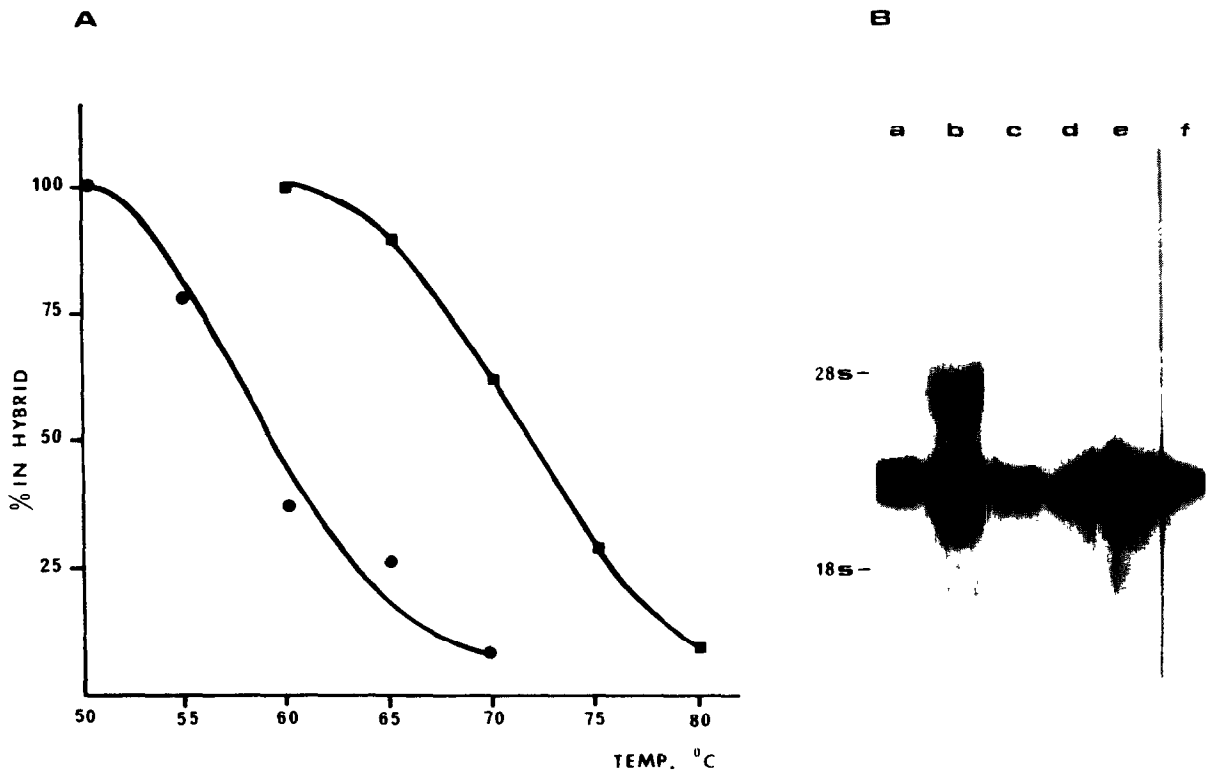

Fig.2. (A) Thermal stability of hybrids of rat cytosolic PEPCK cDNA with PEPCK mRNA from rat and human. 4 μg poly(A)-rich RNA from rat liver (■), and human kidney (●) were immobilized on nitrocellulose squares (1 cm) and hybridized with ^{32}P-nicktranslated PEPCK cDNA (pPCK-10); the filters were washed as described in section 2. The autoradiographic images of the radioactivity retained on the filters were quantified by weighing the area under the densitometric peaks and plotted. Four μg of wheat germ RNA similarly immobilized served as a control for non-specific binding and retention of radioactivity throughout the experiment. (B) Northern blot hybridization of RNA from different mammalian species. Quantities, as specified, of poly(A)-rich RNA from different mammalian species were assayed by Northern blot hybridization as described in section 2. (a) Rat liver, 4 μg; (b) mouse kidney, 2.4 μg; (c) AKR/J mouse liver, 5.2 μg; (d) 48 h-fasted guinea pig liver, 8 μg; (e) 24 h-fasted cat liver, 25 μg; (f) human kidney, 10 μg.

which retains many of the synthetic properties of the normal liver [14], provided a suitable system in which to examine the hormonal control of human PEPCK activity. The physical similarity of the cytosolic PEPCK and its mRNA with that of the rat (figs 1,2) enabled examination of the control of the human cytosolic PEPCK gene expression. Thus, in cells maintained in the absence of serum for 12–20 h, the addition of dBcAMP and dexamethasone together, increases the activity of the enzyme and this effect is abolished by the concomitant addition of insulin (table 1). The abundance of PEPCK mRNA sequences in human cells was assayed by dot-blot hybridization with ^{32}P-nicktranslated rat genomic DNA as a probe and quantified by densitometry of the autoradiographic images. It is evident that the relative abundance of PEPCK RNA sequence is increased considerably by the inducers and that this effect is abolished by insulin (table 1).

Similar results were previously found in Reuber H35 rat hepatoma cells from measurements of the rate of synthesis of PEPCK [28] and recently from measurements of the abundance of PEPCK mRNA and its synthesis [29]. Since evidence from the rat suggests that the cytosolic PEPCK gene is a unique sequence [24] it is conceivable that control elements in the gene or in its flanking regions are responsible for the multiple hormonal modulations of its transcription. The direct and similar ef-

Table 1

Induction of cytosolic PEPCK activity and its mRNA in human hepatoma cells in culture and its abolishment by insulin

Addition to medium	Cytosolic PEPCK activity (munit/mg protein)	Abundance of human PEPCK mRNA	
		Exp.A (densitometric units)	Exp.B
Serum	9.3	42	n.d.
None	7.2	8	n.d.
Inducers	15.3	105	251
Inducers + insulin	9.1	20	17

Cells were incubated for 20 h (exp.A) and 12 h (exp.B) in the absence of serum with the specified additions. dBcAMP, 5×10^{-4} M, dexamethasone, 2×10^{-7} M (inducers) and insulin, 6.7×10^{-6} M (exp.A) and 6.7×10^{-7} M (exp.B) were added where specified. Abundance of mRNA in human hepatoma cells was determined by dot-blot hybridization and expressed in arbitrary densitometric units. n.d., not determined

fects of hormones on the abundance of PEPCK mRNA in both the rat and human cultured hepatoma cells, makes it very likely that in the human as well, hormones modulate the activity of cytosolic PEPCK at the transcription level. It is noteworthy that this similarity in hormonal regulation is apparent despite the different proportions of cytosolic PEPCK in the rat (95%) and human cells (50%). These findings provide a suitable basis for identifying and studying structural elements of the human PEPCK gene responsive to these hormones. Conservation of the hormonal control of cytosolic PEPCK from rat to human is inherent to its physiological function. This together with the evolutionary conservation of its structural characteristics support the enzyme's importance to mammalian independent life.

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