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Cloning and expression in *Escherichia coli* of a rat hepatoma cell cDNA coding for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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In liver, the 470-residue bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) catalyses the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis. In rat hepatoma (HTC) cells, this enzyme has kinetic, antigenic, and regulatory properties, such as insensitivity to cyclic AMP-dependent protein kinase and lack of associated FBPase-2 activity, that differ from those in liver. To compare the sequence of the HTC enzyme with that of the liver enzyme, we have cloned the corresponding fully-coding cDNA from HTC cells. This cDNA predicts a protein of 448 residues in which the first 32 residues of liver PFK-2/FBPase-2 including the cyclic AMP target sequence have been replaced by a unique *N*-terminal decapeptide. The rest of the protein is identical with the liver enzyme. An *N*-terminally truncated recombinant peptide of 380 residues containing the PFK-2 and FBPase-2 domains was expressed in *Escherichia coli* as a β-galactosidase fusion protein. It was recognized by anti-PFK-2 antibodies but its enzymic activities were barely detectable. In contrast, a cDNA fully-coding for the HTC enzyme could be expressed in *E. coli* as a β-galactosidase-free peptide that exhibited both PFK-2 and FBPase-2 activities. This peptide had those PFK-2 kinetic properties of the HTC enzyme that differ from the liver enzyme. These data, together with immunoblot experiments, suggest that the lack of associated FBPase-2 activity in HTC cells results from a post-translational modification of the enzyme rather than from the difference in amino acid sequence. As well as this peculiar type of PFK-2/FBPase-2 mRNA, HTC cells also contained low concentrations of the liver-type mRNA. Unlike in liver, neither mRNA was induced by dexamethasone in these cells.

INTRODUCTION

As shown by Warburg 60 years ago, a high aerobic glycolysis is a typical feature of the transformed cell phenotype. No fully satisfactory explanation for this phenomenon has been provided (Weinhouse, 1976). The most potent stimulator of glycolysis is fructose 2,6-bisphosphate, an ubiquitous allosteric activator of 6-phosphofructo-1-kinase (reviewed by Hue & Rider, 1987). The synthesis of fructose 2,6-bisphosphate is catalysed by 6-phosphofructo-2-kinase (PFK-2) and its degradation by fructose-2,6-bisphosphatase (FBPase-2). In liver, these two enzyme activities are borne by each subunit (54470 Da) of a homodimeric protein which is phosphorylated by cyclic AMP-dependent protein kinase. Cyclic AMP thereby inactivates PFK-2 and activates FBPase-2. In several tissues, fructose 2,6-bisphosphate concentration is controlled by growth factors, tumour promoters, and oncogene products (reviewed by Rousseau & Hue, 1989). Rat hepatoma cells of the dedifferentiated line HTC have a high glycolytic rate (Schamhart *et al.*, 1979) and their fructose 2,6-bisphosphate regulation differs from that in normal liver (Loiseau *et al.*, 1985). Thus, perturbations of fructose 2,6-bisphosphate control or expression of an abnormal PFK-2/FBPase-2 might contribute to the changes in glucose metabolism seen in transformed cells.

As one approach to this problem, we have compared the PFK-2/FBPase-2 purified from HTC cells with that from rat liver (Loiseau *et al.*, 1988). HTC cell PFK-2 has kinetic and antigenic properties different from those in liver and it is not a substrate of cyclic AMP-dependent protein kinase. Moreover, PFK-2 purified from HTC cells does not contain detectable FBPase-2 activity. Because no sequence information on the HTC enzyme was available, we have now cloned the cDNA corresponding to the HTC cell enzyme and have compared its sequence with that of the rat liver cDNA (Darville *et al.*, 1987) that encodes the full-length (470 amino acids) bifunctional PFK-2/FBPase-2 polypeptide. Besides low concentrations of a mRNA for the liver-type PFK-2/FBPase-2, HTC cells contained a mRNA encoding a PFK-2 devoid of the cyclic AMP-responsive phosphorylation site. When expressed in *E. coli*, this mRNA was translated into a protein that had several of the properties of HTC cell PFK-2.

Abbreviations used: bp, base pair(s); DTT, dithiothreitol; FBPase-2, fructose-2,6-bisphosphatase (EC 3.1.3.46); HTC, hepatoma tissue culture; IPTG, isopropyl-β-D-thiogalactopyranoside; LDH, lactate dehydrogenase (EC 1.1.2.3.); PEG, poly(ethylene glycol); PFK-2, 6-phosphofructo-2-kinase (EC 2.7.1.105); SSC, standard saline citrate; PAGE, polyacrylamide-gel electrophoresis.

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MATERIALS AND METHODS

Cells

HTC cells (clone 4.7 isolated by Dr. M. Marchand) were grown in suspension and the effect of dexamethasone was studied in serum-free medium as described (Loiseau *et al.*, 1985). Cells were centrifuged at 0 °C (2000 *g*, 2 min), washed twice in phosphate-buffered saline (0.15 M-NaCl, 2.5 mM-KCl, 8 mM-Na_2HPO_4 and 1.5 mM-KH_2PO_4 pH 7.45) and kept at −80 °C. To prepare cytosol, the cell pellets were resuspended (3 vol./g) in ice-cold buffer (50 mM-Hepes, 0.1 M-KCl and 15 mM-2-mercaptoethanol, pH 7.2) and disrupted with an Ultraturrax homogenizer (4 × 15 s). The homogenate was centrifuged at 4 °C in a Sorvall SS-34 rotor (10000 *g*, 10 min), then in a 50 Ti Beckman rotor (105000 *g*, 1 h). The supernatant (cytosol) was stored at −80 °C.

Purification and hybridization of nucleic acids

Total RNA was isolated from HTC cells by the LiCl/urea method (Auffray & Rougeon, 1980). Poly(A)-rich RNA (2–4% of total RNA) was purified by two cycles of oligo(dT)–cellulose (type 7 from Pharmacia) chromatography (Aviv & Leder, 1972). Dot blot hybridization of total RNA was performed as described using a chicken β-actin cDNA probe as a control (Crepin *et al.*, 1988). Phage DNA was prepared as described (Maniatis *et al.*, 1982) using the plate lysate method.

Construction and screening of the HTC cell cDNA library in λgt11

Poly(A)-rich RNA from HTC cells was tested for the mRNA of interest by dot blot hybridization to the rat liver PFK-2 cDNA probes RL2K-22c2 and RL2K-8. cDNA was synthesized with an Amersham kit that combines oligo(dT) priming and random priming of the first strand with 13-mer oligonucleotides (gift of Dr. J. N. Octave). After methylation and ligation of *Eco*RI linkers, the cDNA products were cut with *Eco*RI, purified by gel filtration (Bio-Gel A-50m; Bio-Rad) and ligated (Young & Davis, 1983*a*) in λgt11 (Protoclone λgt11 system; Promega). DNA was packaged *in vitro* (Gigapack; Genofit) and plated on *E. coli* Y1090 (Young & Davis, 1983*b*). Starting from 5 μg of poly(A)-rich RNA for each priming procedure, 1.2×10^7 recombinant clones were obtained. T4 DNA ligase, *E. coli* DNA polymerase I, *Eco*RI methylase and *Eco*RI restriction endonuclease were from Promega Biotech or Boehringer. Then, 5.4×10^6 recombinant clones were plated on *E. coli* Y1088 at high density (10^5 p.f.u. on 600 cm^2 Petri dishes). Replicas were made on nylon membranes (Amersham) according to the supplier's instructions and screened at low stringency. After prehybridization in 6 × SSC buffer (1 × SSC is 0.15 M-NaCl/15 mM-trisodium citrate), 10 × Denhardt's solution (Maniatis *et al.*, 1982) at 60 °C for 4 h, hybridization was carried out for at least 12 h at 60 °C in 25 mM-sodium phosphate buffer, pH 7.0, containing 3.5 × SSC, 1 × Denhardt's solution, 0.5% SDS, 2 mM-EDTA (pH 8) and 100 μg of heat-denatured herring sperm DNA/ml. The cDNA probes RL2K-8 and RL2K-22c2 were multiprime-labelled (Amersham) with [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) and added at 0.5×10^6 c.p.m./ml. Filters were washed twice in 2 × SSC/0.2% SDS for 30 min at 50 °C. Plaques which remained positive after three successive screenings were purified and analysed by restriction enzyme (Promega or Boehringer) mapping.

DNA sequencing

Following restriction with *Pst*I, *Eco*RI, *Rsa*I and *Bgl*II, cDNA fragments were purified on agarose gels (Lizardi *et al.*, 1984) and subcloned into the single-stranded M13 mp8 or/and mp9 bacteriophages (Messing *et al.*, 1977). The *E. coli* JM101 host strain (Yanisch-Perron *et al.*, 1985) was transformed with the phages by the calcium chloride method (Maniatis *et al.*, 1982). The nucleotide sequence of cDNA subclones was determined by the dideoxy chain termination method (Sanger *et al.*, 1977) in the presence of [^{35}S]dATP[αS] (Amersham, 600 Ci/mmol). The sequencing kits were the Sequenase (USB) or kilobase sequencing (Bethesda Research Laboratories) systems. Products were analysed on 4–6% polyacrylamide/urea gels.

Lysates from induced λgt11 recombinant lysogens

λgt11 recombinant lysogens in Y1089 were prepared as described (Huynh *et al.*, 1985). L-broth medium (200 ml) containing 50 μg of ampicillin/ml was inoculated with a 6-ml overnight culture of the lysogens of interest and grown at 32 °C until the A_{600} reached 0.6–0.8. The phages were heat-induced at 45 °C for 20 min. Production of the fusion protein was induced at 38 °C with 2 mM-IPTG. After shaking the cultures for 1 h, cells were harvested by centrifugation at 25 °C (8000 *g*, 10 min) and quickly suspended in a volume of cold lysis buffer (50 mM-Hepes-KOH, pH 7.5, 100 mM-KCl, 1 mM-potassium phosphate and 0.1 mM phenylmethanesulphonyl fluoride) equal to 1/50 of the culture volume. The suspension was frozen in 1 ml aliquots on solid CO_2 and allowed to thaw on ice for 3 h. Insoluble material was removed by centrifugation at 4 °C (10000 *g*, 10 min) and the supernatant was stored at −80 °C. PFK-2 was partially purified by poly(ethylene glycol) (PEG) precipitation followed by Blue-Sepharose chromatography as described (Loiseau *et al.*, 1988).

Peptide synthesis

A decapeptide GELTQTRLQK corresponding to the *N*-terminus of liver PFK-2 (to raise a L-antibody) and a nonapeptide EEKASKRTA corresponding to the putative *N*-terminus of HTC PFK-2 (to raise a M-antibody) were prepared by solid phase on a Beckman 990B automatic peptide synthesizer. Merrifield's solid phase method was applied. *t*-Butyloxycarbonyl-(N^{ϵ}-2-chlorobenzyl)lysine and *t*-butyloxycarbonylalanine resins (~ 0.3 mmol/g of resin) prepared from chloromethylated polystyrene resin (1% cross-linking) were submitted to repetitive cycles of deprotection [with 50% (v/v) trifluoroacetic acid in dichloromethane], neutralization [with 10% (v/v) triethylamine in dichloromethane] and coupling (with the use of a 4-fold molar excess of dicyclohexylcarbodi-imide). All amino acids were protected at the α-amino positions with *t*-butyloxycarbonyl groups and the following side-chain protectants were used: Arg, N^{ω}-tosyl; Glu, *O*-benzyl; Lys, N^{ϵ}-2-chlorobenzyl; Ser, *O*-benzyl; Thr, *O*-benzyl (Steward & Young, 1984). The peptides were cleaved from the resin with anhydrous HF in the presence of dimethyl sulphide, *p*-cresol and *p*-thiocresol as scavengers (low-HF/high-HF procedure). The crude peptides were

extracted from the resin with 10% (v/v) acetic acid. The product was chromatographed on a Sephadex G-25 (fine grade) column in 0.1 M-acetic acid and purified by reverse-phase h.p.l.c. on a Vydac C_{18} silica-gel column (system: trifluoroacetic acid/acetonitrile). Homogeneity of the synthesized peptides was determined by analytical h.p.l.c., t.l.c. and amino acid analysis.

Antibodies against synthetic peptides

The synthetic peptides (1 mg) were coupled with glutaraldehyde, at room temperature in the dark for at least 12 h, to 2 mg of keyhole limpet haemocyanin (Calbiochem) using the procedure of Avrameas & Ternyck (1969). Rabbits (New Zealand White) were immunized by multiple intradermal injections on day 0 with the peptide–haemocyanin in complete Freund's adjuvant (1:1) and on day 15 and every fortnight thereafter with 660 μg of peptide–haemocyanin in incomplete Freund's adjuvant. Rabbits were bled every other week beginning on day 28. γ-Globulins were precipitated from antisera with 45% $(NH_4)_2SO_4$, dissolved in one-third of the initial volume in phosphate-buffered saline and dialysed three times against the same buffer over a 16 h period at 4 °C. Their purity was checked by SDS/PAGE and they were stored at −20 °C. By e.l.i.s.a. (Coutelier *et al.*, 1986), the L- and M-antibodies recognized specifically their cognate synthetic antigen at dilutions up to $1:10^5$. These antibodies did not immunoprecipitate (Crepin *et al.*, 1988) PFK-2 activity from liver or HTC cells, as if they could not interact with the native enzyme. However, they were active in immunoblots (see the text).

Protein electrophoresis and immunoblotting

Proteins (up to 200 μg) were electrophoretically separated in a SDS/PAGE (8%) gel according to Laemmli (1970). After the run, the gel was equilibrated in transfer buffer (25 mM-Tris base, 192 mM-glycine, 20% methanol) and electroblotted onto an Immobilon PVDF membrane (Millipore) at 4 °C overnight at 50 V (150 mA) (Towbin *et al.*, 1979). Subsequent handling of the membrane followed the manufacturer's instructions. The antigens of interest (β-galactosidase and PFK-2) were detected by a specific primary antibody. These included anti-β-galactosidase (purified mouse monoclonal antibody from Promega, 1:5000), anti-PFK-2 rabbit polyclonal antibody IRL-6 (1:200), or antibodies (1:200) raised against the synthetic peptides. Pre-immune serum (1:200) was used as a control. Bound antibodies were detected by a peroxidase/anti-peroxidase complex (PAP from Prosan) revealed by oxidation of 4-chloro-1-naphthol (from BRL) to a violet product.

Labelling of PFK-2/FBPase-2 by fructose 2,6-[2-^{32}P]bisphosphate

PFK-2/FBPase-2 was phosphorylated by incubating the *E. coli* lysate or cytosol with 7 μM-fructose 2,6-[2-^{32}P]bisphosphate (5000 c.p.m./pmol), prepared as described by Van Schaftingen *et al.* (1982), in the presence of 20 mM-Tes (pH 7.5), 100 mM-KCl, 1 mM-dithioerythritol, 1 mM-potassium phosphate, 5 mM-$MgCl_2$, 1 mM-*sn*-glycerol 3-phosphate and 1 mM Mg-GTP at 30 °C for 30 s in 100 μl. The reaction was stopped by adding 4 μl of cold 100% trichloroacetic acid. After 10 min on ice, the incubation was neutralized by 20 μl of 2 M-Tris base and analysed by SDS/PAGE.

Assay of enzyme activities

PFK-2 activity was measured at pH 8.5 as described (Bartrons *et al.*, 1983) or at pH 7.1 in 50 mM-Hepes, 0.1 M-KCl, 20 mM-KF, 1 mM-DTT, 2 mM-fructose 6-phosphate, 5 mM-Mg-ATP and 5 mM-potassium phosphate. FBPase-2 activity was measured by the production of [^{32}P]P_i from fructose 2,6-[2-^{32}P]bisphosphate. The reaction was carried out at 30 °C in 500 μl containing 20 mM-Tes, pH 7.5, 100 mM-KCl, 5 mM-$MgCl_2$, 1 mM-DTT, 1 mM-*sn*-glycerol 3-phosphate, 1 mM-potassium phosphate, 1 mM-Mg-GTP and 10 μM-fructose 2,6-[2-^{32}P]-bisphosphate (50000 c.p.m./assay). The reaction was stopped by addition of 100 μl of 1 M-NaOH, and [^{32}P]P_i was separated from the substrate as described (Loiseau *et al.*, 1988).

Thermolysin digestion of PFK-2/FBPase-2

Thermolysin (Boehringer) solutions (0.01–1 mg/ml) were freshly prepared in buffer A (20 mM-Hepes, pH 7.5, 50 mM-KCl, 5 mM-$MgCl_2$, 2 mM-EDTA, 15 mM-2-mercaptoethanol and 1 mM-potassium phosphate) and clarified by centrifugation. PFK-2/FBPase-2 (5–20% PEG fraction in buffer A) was incubated for 15 min at 30 °C with thermolysin (0–100 μg/ml) and 2 mM-$CaCl_2$. The digestion was stopped with 10 mM-EGTA and 30 μl aliquots were assayed for PFK-2 activity at pH 7.1 and for FBPase-2 activity.

Other methods

Protein was determined (Bradford, 1976) with bovine serum albumin as a standard. cDNA probes were labelled with [α-^{32}P]dCTP (3000 Ci/mmol) using the Amersham Multiprime DNA labelling system. The BCL-2 (Crepin *et al.*, 1988) and IRL-6 (Darville *et al.*, 1987) antisera were prepared as described.

RESULTS

Cloning of 6-phosphofructo-2-kinase cDNA from HTC cells predicts a novel protein

A cDNA library in λgt11 (14×10^6 clones, 85% of which were recombinants) was prepared from HTC cell poly(A)-rich RNA. We did not try to identify positive clones with antibodies because none of our antisera raised against native chicken liver (BCL-2) or denatured rat liver (IRL-6) PFK-2/FBPase-2 gave positive signals with PFK-2, when immunoblotting cytosolic proteins from HTC cells. We therefore screened the library with two rat liver cDNA probes (Darville *et al.*, 1987), one corresponding to the 5′- and one to the 3′-moieties of PFK-2/FBPase-2 (Fig. 1). Probe 22c2 extends from the 5′-end to the *Eco*RI site of the fully-coding cDNA RL2K-22c; it corresponds to the first 446 bp which include the sequence for the first 90 amino acids of the liver bifunctional enzyme. Probe RL2K-8 is an incomplete cDNA of 1086 bp that includes the sequence for amino acids 167–470, i.e. the *C*-terminus of that same enzyme. In dot blot hybridization assays with total RNA from HTC cells, these probes gave signals that persisted under high stringency conditions (0.2 × SSC buffer, 65 °C). By screening 5.4×10^6 recombinants, 21 clones were detected with probe 22c2. Restriction with *Eco*RI showed that all these clones contained a fragment the length of which (1.4 kb) was equal to that of the 3′ *Eco*RI fragment of cDNA RL2K-22c. The clones contained a

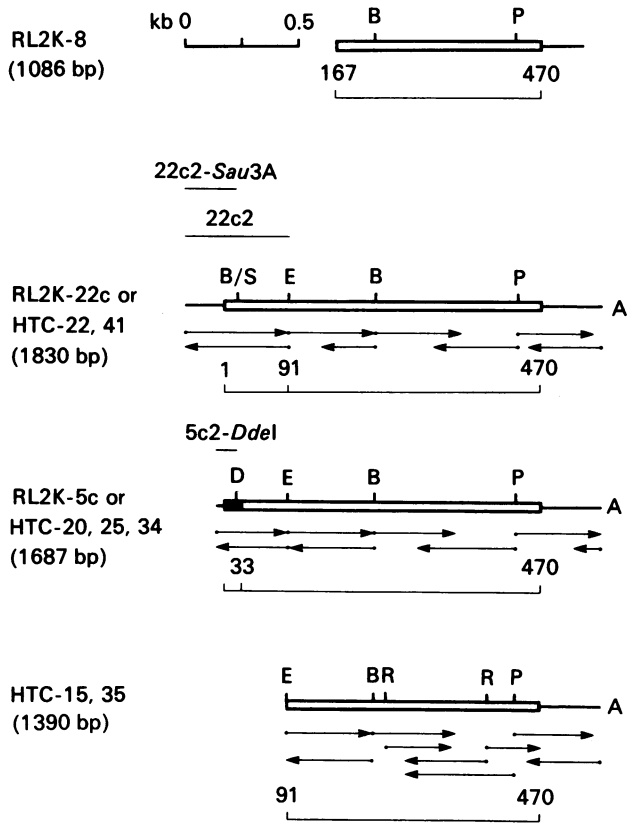

Fig. 1. Rat liver (RL) cDNA clones and probes, and restriction map with sequencing strategy of cDNA clones from HTC cells

Clone length is given in parentheses and does not include the length of the poly(A) tail (indicated by A when present) which varied between 9 and 77 nucleotides. The open bars correspond to translated regions with an amino acid scale that conforms to the numbering system used for the rat liver enzyme, as in Fig. 2. Note that the amino acid sequence is the same in all the clones, except for the filled area of clone RL2K-5c (identical to clones HTC-20, -25 and -34) which corresponds to amino acids that are specific to these clones. Sequences were not systematically determined on both strands when they were confirmatory of sequences obtained independently (Darville *et al.*, 1987; Crepin *et al.*, 1989) on identical clones. B, *Bgl*II; D, *Dde*I; E, *Eco*RI; P, *Pst*I; R, *Rsa*I; S, *Sau*3A.

second fragment which was about 300 bp long in nine of them (among which clones HTC-20, -25 and -34) and about 450 bp long in twelve of them (among which clones HTC-22 and -41). Interestingly, the 5′ *Eco*RI fragment of RL2K-22c is 446 bp long. Two more clones of 1.4 kb (HTC-15 and -35) were detected with probe RL2K-8. Restriction of clone HTC-15 with *Rsa*I, *Bgl*II, *Pst*I and *Hinf*I yielded a map identical to that of the 1.4 kb long 3′ *Eco*RI fragment of the rat liver cDNA RL2K-22c.

This survey therefore suggested that HTC cell poly(A)-rich RNA yielded two cDNA populations, one corresponding to a mRNA similar or identical to that of normal liver, and one corresponding to an incomplete copy of this mRNA or to a mRNA that differs from it at the 5′ end. The sequence of clones HTC-22 and -41 was identical with that of the rat liver RL2K-22c cDNA. Clones HTC-15 and -35 were identical; they are truncated versions of RL2K-22c, encoding residues 91–470 of PFK-2/FBPase 2. Sequencing of clones HTC-20, -25 and -34 showed that they were identical but corresponded to a different type of PFK-2/FBPase-2 mRNA. Indeed, their sequence differed from the liver-type at the 5′ end such that the first 32 amino acids of the liver enzyme (Darville *et al.*, 1987) are replaced by a unique sequence (a data bank search of similarity with known proteins was negative) of 10 amino acids (Fig. 2). Upstream from this sequence there is an open reading frame containing another AUG. However, this second AUG is within a nucleotide sequence incompatible with initiation of translation (Kozak, 1987). Our current work on a PFK-2/FBPase-2 gene encoding this mRNA, and localization of the transcription initiation site by S1 nuclease mapping (Darville *et al.*, 1989) and primer extension (Crepin *et al.*, 1989) suggest that translation does not start further upstream than indicated in Fig. 2. The predicted molecular mass of the HTC cell enzyme is therefore 52011 Da, as compared to 54470 Da for the liver enzyme. We have now found (Crepin *et al.*, 1989) that this HTC-type mRNA is present at low levels in normal liver and corresponds to the cDNA RL2K-5c cloned earlier (Darville *et al.*, 1987) from rat liver but not characterized at the time.

The putative protein encoded by the HTC-type mRNA does not possess the serine residue phosphorylated by cyclic AMP-dependent protein kinase in the liver enzyme (Ser-32). This provides an explanation for the lack of regulation of the HTC cell enzyme by cyclic AMP. The rest of the protein is identical to the liver-type enzyme including the PFK-2 and FBPase-2 catalytic domains and consensus sequences for nucleotide binding folds. The PFK-2 domain includes Cys-160, -183 and -198 and the FBPase-2 domain includes His-258 (Lively *et al.*, 1988) (Fig. 2). To investigate the actual properties of this enzyme, we expressed in *E. coli* the protein corresponding to the peculiar PFK-2/FBPase-2 mRNA cloned from HTC cells. The enzymic detection of this protein was facilitated by the fact that, unlike eukaryotes, prokaryotes have no detectable endogenous PFK-2/FBPase-2 activity (Hue & Rider, 1987).

Expression of the cloned protein in *E. coli*

Unlike for the liver-type full cDNA clones, the non-translated leader sequence is an open-reading frame in the fully-coding HTC-type cDNA clones (Fig. 2). Thus, we attempted to express in λgt11-containing *E. coli* the whole polypeptide encoded by the HTC-type cDNA, as a β-galactosidase fusion protein. We first determined which ones of our fully-coding HTC-type λgt11 clones found to be positive by cDNA hybridization did express immunodetectable PFK-2. The phages were plated on the *E. coli* Y1090 strain at 42 °C. A non-recombinant λ phage was used as a negative control. As positive control, we used clone RL2K-8, known to express immunodetectable PFK-2 under those conditions (Darville *et al.*, 1987). We also included the incomplete clones HTC-15 and -35 with the hope they were in-frame and in the right orientation. After induction with IPTG and transfer on nitrocellulose filters, the plaques were screened with the IRL-6 antibody known to recognize the RL2K-8 fusion protein. No antigen-antibody signal was seen with the non-recombinant λ clone. A positive signal was seen with the incomplete clone RL2K-8, as expected. A positive signal was also seen with clones HTC-15 and -35, and

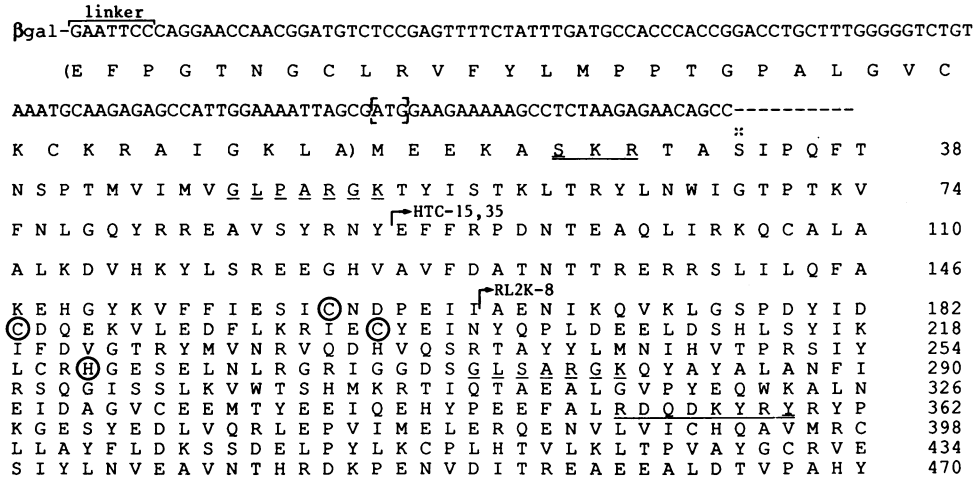

Fig. 2. Amino acid sequence of the protein encoded by cDNA HTC-34

This clone differs from normal rat liver PFK-2/FBPase-2 in terms of 5′ nucleotide sequence and corresponding *N*-terminal peptide sequence upstream from Ser-33 (asterisk). Numbers on the right refer to amino acids numbered as they occur in normal liver PFK-2/FBPase-2, starting here at Ser-33. The putative translation initiation site is in brackets. Between parentheses is the open reading frame which, in the expression vector described, links PFK-2/FBPase-2 to β-galactosidase. The critical cysteines of the PFK-2 domain and the critical histidine of the FBPase-2 domain are circled. The nucleotide binding motifs are indicated by dotted lines. Putative sites for phosphorylation by protein kinase C (Ser-28) and by tyrosine-specific protein kinase (Tyr-358) are underlined twice, within the consensus underlined once. Arrows show where the indicated clones begin.

Table 1. 6-Phosphofructo-2-kinase (PFK-2) and fructose-2,6-bisphosphatase (FBPase-2) activities in lysates from *E. coli* λgt11 recombinants and of purified enzymes

PFK-2 was assayed at pH 8.5. Data are means ± S.E.M. for the number of experiments given in parentheses. n.d., not detectable; n.s., *E. coli* phosphatase activity (34 ± 10 μunits/mg of protein, $n = 4$) considered as nonspecific because non-recombinant lysates could not be labelled with radioactive fructose 2,6-bisphosphate (see Fig. 3). This activity was subtracted from that measured with the recombinant clones.

Source	HTC cDNA coding portion	Activity (μunits/mg of protein) PFK-2	FBPase-2
E. coli lysates			
λ	Absent	n.d.	n.s.
HTC-35	Incomplete	0.2 ± 0.2 (4)	11 ± 10 (3)
HTC-25	Complete	9.9 ± 1.1 (6)	77 ± 5 (6)
HTC-34	Complete	10.9 ± 1.4 (7)	88 ± 9 (5)
Purified enzymes			
Rat liver	–	42400	13100
HTC cells	–	820	n.d.
Recombinant	–	162	2300

with the fully-coding clones, except HTC-20 which was presumably in the reverse orientation.

To produce preparative amounts of fusion proteins, strain Y1089 was lysogenized with clones HTC-25 and -34 which are identical and encode a full-length HTC-type enzyme, and with clone HTC-35 which encodes a *N*-terminally truncated protein sequence common to the HTC-type and liver-type enzymes. A non-recombinant λ phage was used as non-expressing control. Lysogenic cultures were induced with IPTG and the lysates were assayed for PFK-2 and FBPase-2 activities. No inclusion bodies were seen by phase contrast microscopy prior to lysis. As shown in Table 1, both activities were expressed by the fully-coding clones HTC-25 and -34. In contrast to the situation in HTC cells, when PFK-2 was purified from these lysates, FBPase-2 copurified with this enzyme. This is consistent with the integrity of the two catalytic domains of the bifunctional enzyme in the polypeptide sequence predicted from the nucleotide sequence of these clones (Fig. 2). The PFK-2 activity in lysates from clone HTC-25 was immunoprecipitable by BCL-2 antiserum (not shown), as is the case for the liver (Crepin *et al.*, 1988) and HTC cell (Loiseau *et al.*, 1988) enzyme. PFK-2 and FBPase-2 activities in lysates from bacteria containing the incompletely coding clone HTC-35 were respectively 50-fold and 7–8-fold less than in lysates from clones HTC-25 and -34.

A FBPase-2/PFK-2 activity ratio of 8 as seen in

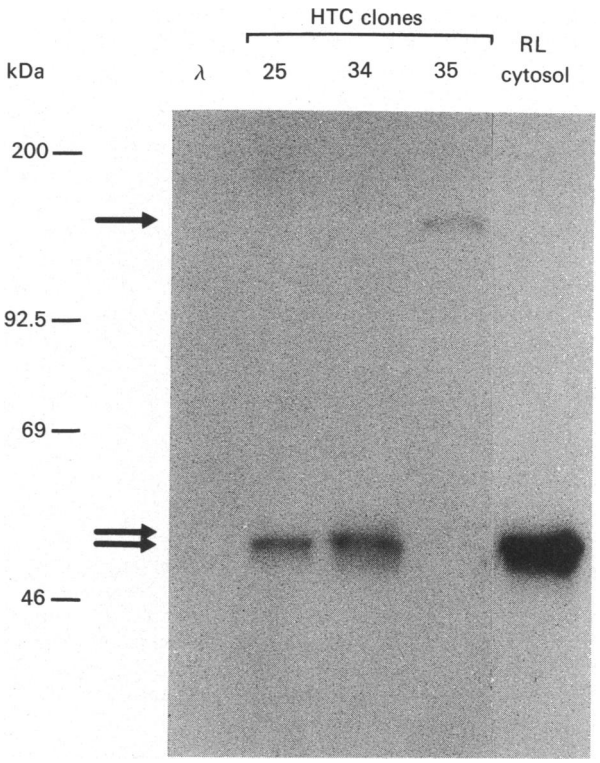

Fig. 3. Labelling by fructose 2,6-[2-^{32}P]bisphosphate of FBPase-2 in rat liver (RL) cytosol and in lysates from *E. coli* λgt11 recombinants

Autoradiogram after separation of proteins by SDS/PAGE is shown. Similar results were obtained in an independent experiment.

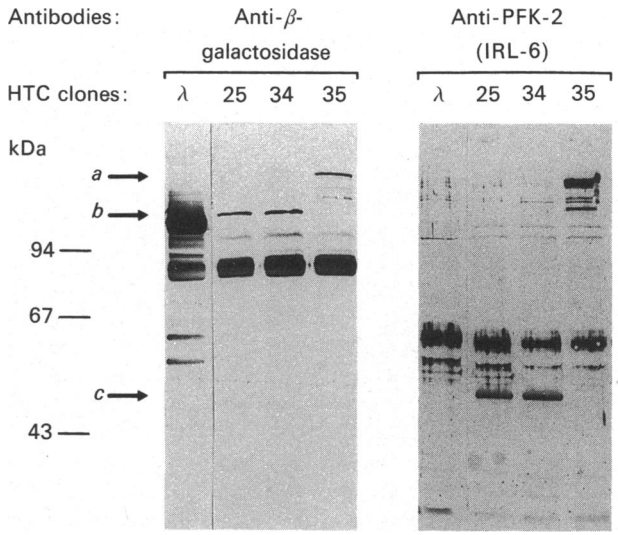

Fig. 4. Immunodetection of β-galactosidase and PFK-2 in lysates from *E. coli* λgt11 recombinants

Proteins were separated by SDS/PAGE and immunoblots of the gels were incubated with the antibodies indicated. Arrows point to β-galactosidase–PFK-2 fusion protein (*a*), β-galactosidase (*b*), and PFK-2 (*c*). The labelling of bands (*a*) and (*c*) with IRL-6 was specific since it was not seen with pre-immune serum.

Table 1 for the fully coding clones is reminiscent of that described for the muscle bifunctional enzyme, which is 5–10, as compared to 0.3 for liver (Table 1 and Hue & Rider, 1987). We therefore examined whether the FBPase-2 of the recombinant protein was, like muscle FBPase-2, 5-fold less sensitive to inhibition by fructose 6-phosphate than liver FBPase-2 (see Hue & Rider, 1987). This was indeed the case, with a K_i of 400 μM for the recombinant protein as compared with 75 μM for liver FBPase-2 (not shown). The mechanism of action of FBPase-2 involves a phosphoryl-enzyme intermediate as evidenced by the phosphorylation of His-258 by labelled fructose 2,6-bisphosphate (Pilkis *et al.*, 1984). The *E. coli* lysates from clones HTC-25, -34 and -35 were therefore incubated with fructose 2,6-[2-^{32}P]bisphosphate and analysed by SDS/PAGE. Autoradiography showed a single band with the HTC clones, thus revealing a *bona fide* FBPase-2, but no labelling of lysates from the non-recombinant λ clone. Liver cytosol treated similarly yielded the expected band at M_r 54500. The protein labelled in lysates from the incomplete clone HTC-35 had an M_r of about 155000 (Fig. 3), as expected from the predicted length of the β-galactosidase fusion protein. In contrast, the protein labelled in lysates from the fully-coding clones HTC-25 and -34 had an M_r of about 53000 and was much more radioactive than in clone HTC-35 (Fig. 3). Densitometry showed that the relative intensity (1:4.4:7.8) of labelling of the lysates from clones HTC-35, -25 and -34 reflected their relative FBPase-2 activity (Table 1). This experiment suggests that the bisphosphatase domain of the fully-coding clones is borne by a protein with a M_r similar to that expected from the sequence of the HTC-type PFK-2/FBPase-2 polypeptide (52011 Da), and that this protein has separated from β-galactosidase. This interpretation was confirmed by successive immunoblotting with a monoclonal anti-β-galactosidase antibody and with the anti-PFK-2 serum IRL-6. The incomplete clone HTC-35 expressed a protein of M_r about 155000 (Fig. 4, arrow *a*) that was detected with both antibodies. In contrast, two proteins were detected in the lysates from the fully-coding clones, one of M_r 116000 revealed by the anti-β-galactosidase antibody (Fig. 4*b*) and one of M_r 53000 revealed by the anti-PFK-2 antibody (Fig. 4*c*).

Thus, λgt11 recombined with a clone encoding amino acids 91–470, which are common to the liver-type and HTC-type enzyme, expressed a β-galactosidase fusion protein containing the PFK-2 and FBPase-2 domains but expressing much less of the corresponding activities (Table 1). On the other hand, λgt11 recombined with a fully-coding HTC-type clone expressed a protein cleaved from β-galactosidase and endowed with both the PFK-2 and FBPase-2 activities.

Comparison of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase expressed in *E. coli* with the HTC cell enzyme

HTC cell PFK-2 is not inhibited by *sn*-glycerol 3-phosphate, in contrast to the liver enzyme, and it is more sensitive than liver PFK-2 to inhibition by citrate, like the muscle enzyme (Loiseau *et al.*, 1988). We therefore determined whether these peculiar kinetic properties of HTC cell PFK-2 were retained following expression in *E. coli* and cleavage from β-galactosidase. This was the case (Fig. 5). Despite these similarities, one difference between

this recombinant PFK-2/FBPase-2 and the HTC cell enzyme was the absence from the latter of associated FBPase-2 activity and of specific labelling with fructose 2,6-bisphosphate (Loiseau *et al.*, 1988). We speculated that, in HTC cells, the enzyme has been cleaved such that its FBPase-2 domain was lost. This was ruled out by immunoblotting with the IRL-6 antibody, which indicated that the apparent M_r of PFK-2 partially purified from HTC cells was not shorter than that expected from the cloning data. Actually a minor (M_r 58000) and a major (M_r 56000) band were detected by this technique (Table 2). This is consistent with expression of the L-type and of the shorter HTC-type isoenzyme in these cells (see above). The discrepancy between these M_r values and those predicted from the cloning data (54470 and 52011 Da respectively) is discussed below. Thus, there is no evidence that the HTC-type enzyme has lost the FBPase-2 domain in HTC cells. However, interaction between distinct portions of this protein could mask latent FBPase-2 activity. El-Maghrabi *et al.* (1984) have indeed shown that the *N*-terminal portion of the liver-type enzyme inhibits FBPase-2 activity, since removal by limited thermolysin digestion of 200-odd *N*-terminal amino acids abolished PFK-2 activity but doubled FBPase-2 activity. The *N*-terminal amino acids of the HTC-type enzyme, which differ from those in the liver-type enzyme, might therefore inhibit FBPase-2 activity. The *E. coli*-expressed protein would exhibit FBPase-2 activity because the cleavage from β-galactosidase removes these inhibitory *N*-terminal amino acids of PFK-2. If these hypotheses were true, then limited thermolysin digestion of the enzyme in HTC cell extracts should reveal FBPase-2 activity. This treatment was therefore applied and the results compared with those obtained with the liver extracts. As expected, increasing concentrations of thermolysin abolished the PFK-2 activity and doubled the FBPase-2 activity of the bifunctional enzyme in liver extracts. However, at concentrations of thermolysin that abolished PFK-2 activity in HTC cell extracts, there was no stimulation of basal FBPase-2 activity (not shown). This basal thermolysin-insensitive FBPase-2 activity has kinetic properties quite different from those of liver FBPase-2 and it has been ascribed to a different enzyme (Loiseau *et al.*, 1988).

If, indeed, cleavage from β-galactosidase did not remove the predicted *N*-terminus of recombinant PFK-2, then antibodies raised against the *N*-terminus of the HTC-type PFK-2 should recognize the cleaved enzyme in recombinant *E. coli* lysates. A nonapeptide corresponding to those *N*-terminal amino acids (excluding the initial methionine) of the HTC-type enzyme which differ from the L-type enzyme (see Fig. 2) was therefore synthesized. A decapeptide corresponding to *N*-terminal amino acids that are unique to the L-type enzyme was also synthesized. These synthetic peptides were used to raise the specific M- and L-antibodies. When immunoblotting PFK-2 purified from rat liver, the M-antibody revealed a very faint band at M_r 53000 while the L-antibody revealed a major band at M_r 55000 (Table 2). This is consistent with our identification in rat liver of the mRNA for the M- and L-isoenzymes of PFK-2. When PFK-2 purified from HTC-25 *E. coli* lysates was immunoblotted with these antibodies, no signal was seen with the L-antibody, as expected, while a band of

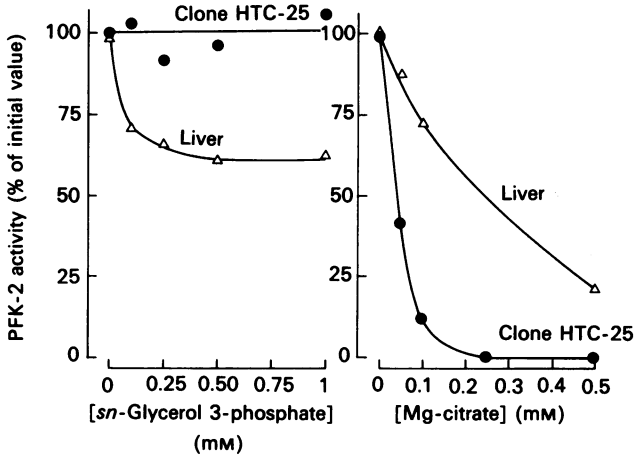

Fig. 5. Effect of *sn*-glycerol 3-phosphate and of citrate on PFK-2 activity

PFK-2 activity was measured at pH 7.1 in liver cytosol (25.1 μunits/mg of protein) and in a lysate (0.8 μunits/mg of protein) from *E. coli* lysogenized with λgt11 recombined with HTC-25 cDNA.

Table 2. Subunit M_r of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

The subunit molecular masses are those predicted from the amino acid sequences or the cloning data (*). The subunit M_r values (†, ‡) were obtained as follows: proteins from liver, HTC cells and recombinant *E. coli* lysates from clones HTC-25 and -34 were labelled with fructose 2,6-[2-^{32}P]bisphosphate before SDS/8%-polyacrylamide gel electrophoresis (†); the same samples were also immunoblotted with the three anti-PFK-2 antibodies (‡). The isoenzyme in parentheses occurs in much lower abundance than the other isoenzyme. n.d., not detectable.

Source	Isoenzyme	Predicted molecular mass (Da)*	Determined M_r			
			Covalent labelling†	IRL-6 antibody‡	M-antibody‡	L-antibody‡
Liver	L	54470	54500	55000	n.d.	55000
	(M)	52011	n.d.	53000	53000	n.d.
HTC cells	(L)	54470	n.d.	58000	n.d.	58000
	M	52011	n.d.	56000	56000	n.d.
Recombinant	M	–	53000	53000	53000	n.d.

M_r 53000 was seen with the M-antibody (Table 2). This experiment shows that the predicted *N*-terminus of the HTC-type enzyme is indeed retained in the recombinant protein following cleavage from β-galactosidase and it confirms the size of this protein (M_r 53000) already determined by labelling with fructose 2,6-bisphosphate (Fig. 3) and by immunoblot with the IRL-6 antibody (Fig. 4). As said above, the latter antibody identified in HTC cells a PFK-2 of apparent M_r greater than in *E. coli* lysates. This difference was now confirmed by immunoblotting PFK-2 purified from HTC cells, with the M- and L-antibodies. The M-antibody revealed a major band at M_r 56000 and the L-antibody revealed a minor band at M_r 58000 (Table 2).

We conclude from these experiments that both in HTC cells and in *E. coli* lysates the HTC-type enzyme contains the *N*-terminus as well as the FBPase-2 domain, as predicted from the cloning data. However, by SDS/PAGE, the migration of the protein extracted from HTC cells is slower than that expressed in *E. coli*. This might result from a covalent modification of the HTC protein which would also result in loss of FBPase-2 activity. This interpretation is supported by the fact that the L-isoenzyme, albeit expressed at low levels in HTC cells, does not contribute FBPase-2 activity either. Now, we observe that the apparent M_r of this L-isoenzyme in HTC cells is also about 3000 greater than in liver. Thus, the covalent modification postulated for the M-isoenzyme in HTC cells could also take place on the L-isoenzyme in these cells, as expected from their sharing an identical sequence of 438 amino acids.

Occurrence of two mRNAs for 6-phosphofructo-2-kinase in HTC cells and lack of induction by dexamethasone

Our cloning data suggest that HTC cells contain not only a peculiar PFK-2 mRNA but also the normal liver PFK-2/FBPase-2 mRNA. To evaluate their relative concentration, total RNA from HTC cells was dotted on nitrocellulose filters and hybridized with three mutually exclusive cDNA probes (Fig. 1). These probes were 5c2-*Dde*I which is specific for the HTC-type enzyme, 22c2-*Sau*3A which is specific for the liver enzyme, and HTC-15 or RL2K-8 which are common to both forms of the enzyme.

Hybridization signals were obtained with all these probes. The intensity of hybridization was stronger with the 5c2-*Dde*I than with the 22c2-*Sau*3A probe. Densitometric analysis indicated that the concentration of the HTC-type mRNA was at least 3-fold higher than that of the liver-type mRNA in HTC cells. Northern blot analysis (not shown) of poly(A)-rich RNA from HTC cells indicated that two populations of RNA, one of 1.9 kb and one of 2.1 kb, hybridized with the HTC-15 probe, as expected from expression of both the HTC-type and the liver-type PFK-2/FBPase-2 mRNA by these cells.

We had shown that glucocorticoid treatment of HTC cells produces a stable increase in the $V_{max.}$ of PFK-2 after a 2 h lag period and that this is inhibited by actinomycin D (Loiseau *et al.*, 1985). The availability of HTC-type cDNA probes now allowed us to determine whether glucocorticoids increased the concentration of the corresponding mRNA. Three independent cultures of HTC cells were exposed for 5 h to 0.5 μM-dexamethasone or to the vehicle. As expected, PFK-2 activity increased from 1.5 ± 0.4 μunit/unit of LDH in control cultures to 2.7 ± 0.8 μunit/unit of LDH in glucocorticoid-treated cultures (mean ± S.E.M., $P = 0.05$, paired *t* test). Total RNA from these cells was dotted on filters and assayed with the three cDNA probes described above. A chicken β-actin cDNA probe was used as a control. Treatment of the cells with dexamethasone did not influence the relative intensities of hybridization despite the increase in PFK-2 activity. Based on densitometric analysis and correction for β-actin mRNA, the ratios for mRNA from dexamethasone-treated cells to mRNA from control cells were 0.9 with the HTC-15 probe, 1.1 with the 22c2-*Sau*3A probe, and 0.9 with the 5c2-*Dde*I probe. Thus, the effect of dexamethasone does not appear to result from an increased amount of mRNA. Instead, it may result from the induction of a protein that modifies HTC cell PFK-2 covalently. Consistent with this interpretation, the enzyme partially purified from dexamethasone-treated cells was less thermolabile *in vitro* than that from untreated cells and was not recognized by antibodies that did immunoprecipitate PFK-2 from untreated cells. For this reason, we could not determine by immunotitration whether or not dexamethasone influenced the PFK-2 content of HTC cells.

DISCUSSION

We have shown here that HTC cells contain two mRNAs for PFK-2/FBPase-2, one present at low concentration which codes for the bifunctional enzyme found in normal liver, and one present at higher concentration which codes for a distinct protein. This protein lacks the cyclic AMP-dependent phosphorylation site at the *N*-terminus, as expected from the properties of the HTC-type enzyme, and it contains PFK-2 and FBPase-2 domains identical to those of the liver enzyme. Hybridization experiments with cDNA probes (Crepin *et al.*, 1989) now indicate that this novel protein could correspond to the muscle isoenzyme of PFK-2/FBPase-2. Consistent with this hypothesis, we have shown here that, when this putative isoenzyme is expressed in *E. coli*, it displays kinetic properties of PFK-2 and of FBPase-2 that resemble those of the muscle rather than the liver enzyme. In that respect, the unique *N*-terminus of the cloned HTC-type enzyme exerts on these two activities a control similar to that observed for the fully phosphorylated liver enzyme, namely low PFK-2 and high FBPase-2 activities. This *N*-terminus contains a sequence Ser-Xaa-Arg (Fig. 2) that could serve as a target for phosphorylation by protein kinase C (Woodget *et al.*, 1986). Phorbol esters stimulate PFK-2 activity in fibroblasts (Bosca *et al.*, 1985). However, HTC cell PFK-2 activity remained unchanged following incubation of the cells with phorbol myristate acetate under the same conditions (not shown). Another feature that distinguishes the behaviour of this enzyme in HTC cells and liver is the mechanism by which it is induced by glucocorticoids. While Colosia *et al.* (1988) have reported that the concentration of mRNA for PFK-2/FBPase-2 increases in the liver of adrenalectomized rats treated with dexamethasone, we found no change in PFK-2 mRNA concentration, despite increased PFK-2 activity, following incubation of HTC cells with dexamethasone.

A recombinant clone containing a truncated cDNA (HTC-35) expressed a hybrid polypeptide consisting of β-galactosidase fused to a sequence common to the HTC

and liver enzyme (residues 91–470) and known to contain the PFK-2 and FBPase-2 domains. These domains were identified by anti-PFK-2 antibodies and by labelling of the FBPase-2 catalytic site. Still, the PFK-2 activity was barely detectable. One reason might be the absence from this clone of the nucleotide binding motif, i.e. residues 48–54 (Fig. 2). The FBPase-2 domain was poorly labelled, consistent with the low activity detected. Since the liver enzyme is a homodimer, the low FBPase-2 activity of the fusion protein could result from failure of the enzyme to dimerize in HTC-35 lysates. This interpretation seems unlikely because Tauler *et al.* (1988) have shown that the FBPase-2 domain of the liver enzyme can be labelled with fructose 2,6-bisphosphate and can be catalytically active in monomeric form when it is separated from the first 218 amino acids. Whether or not dimerization occurs in *E. coli* expressing the HTC-35 truncated clone, it is probable that β-galactosidase hinders the PFK-2/FBPase-2 activities within the fusion protein. This hypothesis is consistent with the behaviour of the recombinant protein expressed by the fully-coding clones HTC-25 and -34. This protein became active as a PFK-2/FBPase-2 bifunctional enzyme following cleavage from β-galactosidase. The mechanism of this cleavage is unknown. In any case, our data show that proper folding of this complex protein can occur in *E. coli* lysates. This opens interesting perspectives for studying structure–function relationships by site-directed mutagenesis.

One property by which the recombinant protein differed from the HTC cell enzyme was FBPase-2 activity. This activity was displayed by the recombinant protein as expected from the cDNA and derived amino acid sequence and it correlated with specific labelling of the protein by fructose 2,6-bisphosphate, a unique property of FBPase-2. By the latter criterion, as well as by immunoblot, the recombinant protein had a subunit M_r similar to that predicted from the deduced amino acid sequence. We failed in our attempts at performing direct *N*-terminal sequencing of the recombinant protein and of the HTC enzyme. However, antibodies against synthetic peptides corresponding to the putative *N*-terminal amino acids reacted with the enzyme from the two sources, showing that they contained these amino acids. We therefore speculate that, in HTC cells, the FBPase-2 domain of the protein remains silent because of subunit interactions that do not occur in *E. coli* or of post-translational modification(s). Note in this context that PFK-2/FBPase-2 contains a motif Arg-Xaa-Xaa-Asp-Xaa-Xaa-Xaa-Tyr (Fig. 2) for phosphorylation by tyrosine-specific protein kinase (Woodget *et al.*, 1986). This is a potential regulatory site. A covalent modification of His-258 such that it can no longer bind fructose 2,6-bisphosphate is another possibility. Such modification(s) would explain the up-shift displayed by the HTC enzyme in SDS gels when compared to the recombinant enzyme. Loss of PFK-2-associated FBPase-2 activity in HTC cells might result in a higher steady-state concentration of fructose 2,6-bisphosphate and higher glycolytic rate.

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