Identification of a novel rat hepatic gene induced early by insulin, independently of glucose

Sandrine COFFY*, Jean-François DECAUX*, Jean GIRARD*, Yves DE KEYZER*¹ and Maryam ASFARI†^{1,2}

*Institut Cochin, INSERM U567, CNRS UMR8104, Département d'Endocrinologie, Université Paris 5, 24 rue du Faubourg Saint-Jacques, 75014 Paris, France, and †Merck-Santé, Centre de Recherche, 4 Avenue du Président François Mitterrand, 91384 Chilly-Mazarin, Cedex, France

We used mRNA differential display to identify new genes induced early after exposure to insulin. Our screening strategy was based on the comparison of gene expression during the time course of insulin induction in the liver of 12-day-old suckling rats both *in vivo* and *in vitro*. A novel, early induced transcript, EIIH, was identified that encodes a 353-amino-acid protein with several features suggesting that it may be secreted or bound to membranes. EIIH is also distantly related to a variety of LRR (leucine-rich repeat) proteins. Insulin treatment increased EIIH mRNA levels in the hepatocytes of suckling, fasted adult and STZ (streptozotocin)-treated diabetic rats, where insulin was required to maintain the basal level of EIIH expression. EIIH expression

was induced during the suckling/weaning transition, and remained detectable thereafter. Tissue distribution analysis in adult rats revealed a pattern of expression mainly in the liver, intestine and islets of Langerhans, closely following that of the Glut2 (glucose transporter 2), suggesting that it may play a role in carbohydrate metabolism. EIIH may be a primary target of the transcriptional regulation by insulin, and may therefore constitute a new model to study the mechanisms by which insulin acts on gene transcription.

Key words: differential display, gene expression, hepatocyte, insulin, rat, streptozotocin.

INTRODUCTION

Insulin plays a major role in glucose homeostasis through regulation of carbohydrate and lipid metabolism. Activation of insulin receptors in the main insulin-target tissues, such as liver, adipose tissue and skeletal muscle, leads to modulations of different metabolic pathways at multiple levels, including transcriptional control of gene expression (for a review, see [1]). This transcriptional effect is well documented in the liver, where insulin has been shown to modulate expression of specific target genes involved in a series of cellular pathways and metabolic functions, including glycolysis, gluconeogenesis, fatty acid oxidation and lipogenesis. Whereas a group of these genes, such as L-PK (L-type pyruvate kinase), ACC (acetyl-CoA carboxylase), S14 (spot 14), FAS (fatty acid synthase), ACL (ATP-citrate lyase) and SDC1 (stearoyl-CoA desaturase 1) require both insulin and glucose for full transcriptional activation [2,3], other genes, such as GK (glucokinase) [4] LXR α (liver X receptor α) [5], SREBP-1c (sterol-regulatory-element-binding protein-1c) [2] and, as shown recently, SHARP-2 (split- and hairy-related protein-2) [6], are regulated by insulin, independently of glucose. While the roles of SREBP-1c and LXR α as early intermediates of insulin action on the expression of genes such as GK, S14, ACC and FAS are well demonstrated [2,4], more detailed studies on transcription/activity of liver SREBP-1c [2,7], LXR α [5] and GK [4,8] have demonstrated that insulin-dependent expression of these genes required the expression/activation of other proteins yet to be identified. The characterization of new, purely insulin-responsive genes in the liver should provide insights into the mechanisms of insulin action on metabolic pathways.

Numerous studies have attempted to identify insulin-regulated genes using different screening approaches [9,10] or analysing

global differences of gene expression in the liver of diabetics rodents [11,12]. An obstacle to the identification of the transcripts regulated by insulin in intact animals has been the interference associated with physiological changes in blood glucose concentration or contra-regulatory hormones such as glucagon, and often the *in vitro* models suffered from the lack of physiological relevance.

We used a double screening based on the mRNA DD/RT-PCR (differential display/reverse transcriptase-PCR) method to identify genuine transcriptional targets of insulin. We compared gene expression in the liver of rats before and after insulin secretion induced physiologically by glucose administration. Simultaneously, we compared the transcription levels in hepatocyte primary cultures during the time course of insulin induction. Although differential approaches have been used previously to identify insulin target genes, the present study is the first one based on DD/RT-PCR under physiological conditions in the liver. In addition, our experiments were designed to selectively identify genes induced by insulin from those that also require glucose or other hormones. In the present paper, we report the identification, cloning and expression pattern of a novel early insulin-induced hepatic gene, EIIH, in rats and show that its expression is controlled by insulin, independently of glucose.

EXPERIMENTAL

Animals

Wistar adult male (6- or 7-week-old) or pregnant female rats were purchased from Janvier (Le Genest-St-Ilse, France) and were maintained on a 12 h light/dark cycle in our animal facility for 1 week before experiments, and had free access to water and chow

Abbreviations used: ACC, acetyl-CoA carboxylase; DD, differential display; FAS, fatty acid synthase; GK, glucokinase; Glut2, glucose transporter-2; IRS-1, insulin receptor substrate 1; LRR, leucine-rich repeats; LXR α , liver X receptor α ; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; S14, spot 14; SREBP-1c, sterol-regulatory-element-binding protein-1c; STZ, streptozotocin.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (email maryam.asfari@merck.fr).

The nucleotide sequence data reported for the full-length EIIH cDNA will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY533242.

pellets (65 % carbohydrate, 11 % fat and 24 % protein, by energy content). All experiments conformed to the relevant guidelines of the French Ministry of Agriculture for scientific experimentation on animals.

Diabetes mellitus was induced by one intravenous injection of STZ (streptozotocin; Sigma) at 80 mg/kg of body mass 5 days before the experiment. Blood glucose concentration was monitored daily with a glucometer (Roche Diagnostics). Only animals with a blood glucose concentration higher than 300 mg/dl on two separate days were included in the study. Human insulin (Novo Nordisk) was administered by intraperitoneal injection at 20 units/kg of body mass. Rats were killed either before or 30 min after insulin injection, and livers were kept at -80°C until processed.

Rats (12-day-old) were separated from their mother 3 h before the experiments. For *in vivo* studies, they were force-fed with a solution of glucose (5 g/kg) and were killed at different time intervals. Liver was rapidly excised and frozen at -80°C until RNA preparation.

For ontogenesis studies, pregnant females on day 19 and 20 of gestation were anaesthetized with pentobarbital (120 mg/kg of body mass) and foetal livers were collected after Caesarean section. Newborns were obtained after normal delivery, and their livers were used at 0, 5, 10, 15, 20, 25, 30 and 35 days after birth.

Primary hepatocyte culture, insulin and 17β -oestradiol treatment

Suckling (12-day-old) or 24 h fasted adult rats were anaesthetized with pentobarbital (50 mg/kg of body mass). Hepatocytes were isolated by liver perfusion as described previously [13]. After measurement of cell viability by Trypan Blue exclusion, cells were suspended in M199 medium without glucose (Invitrogen) supplemented with 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 0.1 % (w/v) BSA, lactate/pyruvate (10 and 1 mM) and 2 % (v/v) Ultrosor G (Invitrogen). Hepatocytes were seeded at a density of 8×10^6 cells/100-mm-diameter Petri dish, and cultures were maintained at 37°C and 5 % CO_2 . After 4 h of plating, the supernatant containing unattached cells was removed and replaced with 10 ml of the same medium without BSA and Ultrosor G for 16–18 h. Finally, 100 nM insulin was added to the cultured medium.

For 17β -oestradiol (Sigma) treatment, adult rat hepatocytes were cultured in Iscove's medium (Invitrogen) supplemented with 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 10 % charcoal-treated serum (Biowest) on collagen-coated six-well dishes (Becton Dickinson) overnight. The cells were then pre-incubated in Iscove's medium with 0.1 % BSA for 4 h, and incubated in hormone-containing medium at the indicated concentrations for 3 h.

RNA isolation

Total RNAs used in the DD/RT-PCR steps were isolated by the CsCl step-gradient-centrifugation method [14]. Total RNAs used for other experiments were isolated with the RNAwiz™ kit (Ambion) or the RNeasy kit (Qiagen) with minor modifications for whole livers and cultured hepatocytes: we doubled the suggested lysis buffer volume and a centrifugation step (10 min at 5000 g) was added to clear the supernatant used in subsequent steps.

RNA blot analysis

RNA samples (10 μg) were separated on a 1 % (w/v) agarose gel containing 2.2 M formaldehyde and transferred on to nylon membranes (Hybond; Amersham Biosciences). The membranes were hybridized in formamide-containing solutions according to standard procedures [15]. Probes were labelled by random priming

with [α - ^{32}P]dATP (Amersham Biosciences), using the Strip-EZ kit (Ambion). The GK cDNA (1.8 kb) was prepared as described previously [16], and the 600 bp EIIH fragment cloned by DD/RT-PCR was used as a probe. Hybridized membranes were exposed to autoradiographic films (BioMax, Kodak) with intensifying screens (Dupont Cronex) overnight at -80°C . Dehybridization was performed according to the strip-EZ DNA kit instructions.

DD/RT-PCR

RNAs (2 μg) were reverse-transcribed for 1 h at 42°C with 4 units of Omniscript RT (Qiagen) and modified oligo(dT) primers consisting of a mixture of three one-base-anchored primers: T10: 5'-CATTATGCTGAGTGATATC(T)₁₁A-3', T11: 5'-CATTATGCTGAGTGATATC(T)₁₁G-3' and T12: 5'-CATTATGCTGAGTGATATC(T)₁₁C-3' at 300 nM. cDNAs were diluted 10-fold before PCR.

DD/RT-PCR was performed with the Delta Differential Display kit (Clontech) according to the manufacturer's recommendations with few modifications. Briefly, 2.5 units of Expand Long Template DNA polymerase (Roche) were used in 20 μl of PCR mixtures containing 2 μl of diluted cDNA, 350 μM dNTP, 2.5 μCi of [α - ^{32}P]dATP (2000 Ci/mmol; Amersham Biosciences) and 0.3 μM arbitrary primer provided in the kit and one-base-anchored modified oligo(dT) primer (T10, T11 or T12) to prime the reaction. As a control for genomic DNA contamination, a sample of each RNA solution was subjected to PCR without prior reverse transcription. Amplification was carried out for three cycles at 94°C for 2 min, 40°C for 5 min and 68°C for 5 min, then 35 cycles at 94°C for 45 s, 60°C for 1 min and 68°C for 2 min, followed by a 68°C step for 7 min. A 10 % portion of the radiolabelled PCR product was separated by electrophoresis on a 4.5 % (v/v) polyacrylamide/7 M urea denaturing sequencing gels (HR-1000, Genomix). The dried gels were exposed to autoradiographic films (BioMax, Kodak) for 1 day. Differentially expressed bands were excised, eluted at 100°C for 5 min in Tris/EDTA buffer and re-amplified with the same primer set. The cDNAs were purified with QIAquick gel extraction kit (Qiagen) after agarose gel electrophoresis. A total of 90 PCR primers combinations were used. The primer set that gave the EIIH differentially expressed were P6: 5'-ATTACCCTCACTAAATGCTGGGTG-3' and T11.

5'-RACE (rapid amplification of cDNA ends) PCR and EIIH cDNA cloning

The 5'-end of EIIH cDNA was cloned by 5'-RACE PCR using the SMART™ RACE cDNA amplification kit (Clontech) and RNA from insulin-induced adult rat hepatocytes, together with EIIH gene-specific primers (5'-GGGCTGGGCTAGTGTGAGAAGACAGAGC-3' and 5'-GGAGAGCTGAGATGTGGCCCTGGCG-3'). The full-length cDNA sequence (2489 bp) was determined with fluorescent automated sequencing. The full-length EIIH cDNA, was obtained by end-to-end PCR with the primers 5'-ACGCGGGGGGGTTCGCCTAGGTG-3' and 5'-GATGGAAA-GAGCTCTTACATGTGTTTATT-3'. This fragment was sub-cloned into the pTarget vector (Promega), named pTarget-EIIH and sequenced.

In vitro transcription and translation of EIIH cDNA

pTarget-EIIH DNA was used for *in vitro* transcription and translation in the TNT-coupled reticulocyte lysate system (Promega) with [^{35}S]methionine (15 mCi/ml) (Amersham Biosciences). The translated products were analysed on a 10 % (v/v) polyacrylamide gel. The gel was dried and then exposed overnight to autoradiographic film (BioMax, Kodak).

Analysis of mRNA expression by real-time quantitative RT-PCR

Real-time PCR was performed with the LightCycler apparatus (Roche Molecular Biochemicals) using SYBR Green I. Total RNA (0.5 µg) was reverse-transcribed for 1 h at 42 °C in a 20 µl final reaction containing 1 × RT buffer, 10 mM DTT (dithiothreitol), 250 nM random hexamers (Promega), 250 ng of Oligo(dT)₁₅ (Promega), 2 mM each dNTP and 100 units of Superscript II RT (Invitrogen). A volume of 2.5 µl of a 1/60 dilution of first-strand cDNAs was amplified in a 10 µl PCR reaction containing 0.5 µM each primer, 2 mM MgCl₂ and 1 × LightCycler DNA Master SYBR Green I mix (Roche Molecular Biochemicals). Samples were incubated in the LightCycler apparatus for an initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles. Each cycle was 95 °C for 15 s, 58 °C for 7 s, and 72 °C for 15 s. The following oligonucleotide primers (Invitrogen) were used: cyclophilin gene (GenBank® accession number M19533), 5'-ATGGCACTGGTGGCAAGTCC-3' and 5'-TTGCCATTCTGGACC-CAAA-3'; GK gene (GenBank® accession number NM_012565), 5'-GTGTACAAGCTGCACCCGA-3' and 5'-CAGCATGCAAG-CCTTCTTG-3'; EIIH gene, 5'-CCACATCTCAGCTCTCCAC-3' and 5'-CACACCCAGAACAGAGTCTTAC-3'. SYBR Green I fluorescence emission was determined after each cycle. The relative amounts of all mRNAs were quantified by the second derivative maximum method of the LightCycler software. Amplification of specific transcripts was confirmed by melting-curve profiles generated at the end of each PCR. Product length and PCR specificity were checked further by 2% (w/v) agarose gel electrophoresis and ethidium bromide staining.

Tissue-specific expression of EIIH mRNA by RT-PCR

Tissues were removed from 7-week-old adult rats fed *ad libitum* and were used immediately for RNA preparation. Islets of Langerhans were isolated from two pancreases by ductal injection of collagenase (type XI, 1 mg/ml; Sigma), followed by centrifugation on Ficoll gradients as described previously [17]. cDNA synthesis was performed at 42 °C for 1 h in a total reaction volume of 20 µl with 2 µg of total RNA, 500 ng of oligo(dT) primers (Invitrogen) and 200 units of MMLV (Moloney murine leukaemia virus) RT (Superscript II, Invitrogen). cDNAs (2 µl) were used for subsequent PCR amplification with the following specific primers: cyclophilin gene, 5'-GGGCTCCGTTGTCTTCCTTTT-3' and 5'-CTCCACTTCGATCTTGCCACAG-3'; EIIH gene, 5'-ACGCGGGGGGTCGCCTAGGTG-3' and 5'-GATGGAAA-GAGCTCTTACATGTGTTTATT-3'; GK gene, 5'-GGACCAA-GGGCTTCAAGGCCTCT-3' and 5'-GTACACGGAGCCATCC-ACGCC-3'; Glut2 (glucose transporter 2) gene (GenBank® NM_012879), 5'-GACAAGATCACCGGAACCTTGCC-3' and 5'-TCTGGCTTTCTGGACAGAAGAGC-3'. PCRs were performed in a final volume of 40 µl containing 15 pmol of each primer using the following cycling protocol: 3 min at 94 °C, followed by a variable number of cycles (22 cycles for cyclophilin, 28 cycles for Glut2, and 32 cycles for GK and EIIH) consisting of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, followed by a final step at 72 °C for 7 min. PCR products (10 µl) were analysed on a 1.5% (w/v) agarose gel stained with ethidium bromide.

RESULTS

Isolation of differentially expressed genes by DD/RT-PCR and confirmation of differential expression

In order to identify physiologically relevant hepatic genes regulated by insulin, we simultaneously compared the changes in gene expression in response to insulin in two different models

Figure 1 Identification of EIIH by DD/RT-PCR and analysis of its induction pattern by insulin

(A) RNA blot analysis of GK mRNA level of 12-day-old rat hepatocytes in primary cultures stimulated by 100 nM insulin (left-hand panel) and in the liver of pups of the same age after glucose force-feeding (5 g/kg of body mass; right-hand panel) for the indicated times. Total RNA (10 µg) was hybridized with a 1.8 kb GK probe and autoradiographed for 20 h. Lower panels show ethidium-bromide-stained 18 S RNAs used as a control for RNA loading. (B) DD/RT-PCR cDNA pattern obtained with primers P6 and T11. The arrow points to EIIH cDNA. (C) RNA blot analysis of the induction pattern of EIIH mRNA by insulin. Hybridization of the same membrane as in (A) with the 600 bp EIIH probe. The duration of insulin treatment is indicated above each panel.

using mRNA DD/RT-PCR: (i) hepatocytes from suckling rats cultured in the absence of insulin and glucose for 16 h, and then treated with 100 nM insulin for a period of 24 h, during which they were processed for RNA isolation at selected time intervals (Figure 1, left-hand panels); (ii) suckling rats force-fed with a glucose solution, leading to an increase in circulating insulin *in vivo*. The livers were removed every 1 h after force-feeding over a time period of 5 h (Figure 1, right-hand panels). In order to be considered as a candidate for further analysis, the same differentially expressed genes had to be identified both in the liver of intact rats and in cultured hepatocytes during the time course of insulin treatment.

As a control for the insulin effect in both models, we examined the induction of GK, a well-known hepatic gene induced by insulin. RNA blot analysis showed the expected accumulation of GK mRNA in response to insulin treatment, following a kinetics of induction similar to that previously described both for the *in vivo* [13] and *in vitro* [8] conditions (Figure 1A).

We then proceeded to mRNA DD/RT-PCR screening in these samples using 90 sets of primers as described in the Experimental section. Improved electrophoresis conditions enabled us to visualize fragments ranging in size from 300 to 2500 bp. Figure 1(B) shows part of a DD/RT-PCR gel obtained with the primer pair P6 and T11.

The whole mRNA DD/RT-PCR procedure was repeated with RNA samples isolated from a second independent, but similar, experiment (different animals and cell cultures), with a more

limited series of time points (results not shown). The expression profiles observed in this second set of experiments were very similar to the first one, showing a very good reproducibility of the method.

The global analysis of the profile of cDNA bands did not reveal significant changes in the levels of the majority of the bands during the insulin treatment period, suggesting that insulin has no general action on gene transcription. However, some bands presented a different pattern in one or the other situation, confirming differences in gene transcription between whole liver and isolated hepatocytes as reported previously [18]. Only a few bands exhibited a common pattern of induction or repression in both situations, and most of them were induced in response to insulin. These bands were eluted from DD/RT-PCR gels, subjected to a second amplification of PCR using the same primers as before and then gel-purified. Direct nucleotide sequencing of the DD/RT-PCR products identified the GK gene as one of the up-regulated genes and the PEPCK (phosphoenol pyruvate carboxy-kinase) gene as one of the down-regulated (results not shown), as expected from our experimental design.

Most of the candidate cDNAs identified initially did not yield a detectable specific band during our RNA blot confirmation step, or when hybridized to multiple mRNA species gave rise to more or less intense smears, and therefore their differential expression could not be confirmed. Nevertheless, we identified a 600 bp cDNA corresponding to a new gene up-regulated by insulin (Figure 1B). We called this gene 'early induced hepatic gene' (EIIH), since its pattern of expression exhibited an early up-regulation under insulin stimulation for both the *in vivo* and the *in vitro* models. To confirm the DD results, we analysed the same total RNA samples used for DD/RT-PCR screening by RNA blot using the purified EIIH band as a probe (Figure 1C). The radiolabelled EIIH cDNA probe hybridized to a single mRNA of approx. 2.5 kb. Although RNA blot analysis did not follow the pattern of expression observed in DD/RT-PCR experiments exactly, insulin repeatedly induced an early increase in EIIH gene expression in both insulin-treated hepatocytes and in the liver of glucose force-fed rats (Figures 1A and 1C, and results not shown). Basal EIIH expression was low in both models before insulin action. In the primary cultures of hepatocytes, EIIH expression reached a maximum level 6 h after the addition of insulin, and then progressively returned to basal level in less than 24 h. A rapid induction by insulin was also observed *in vivo* with an obvious increase 3 h after glucose force-feeding. These results showed that EIIH mRNA was indeed inducible by insulin under both experimental conditions, and was thus a good candidate for a physiologically relevant gene, transcriptionally induced by insulin in the liver.

Effect of insulin on EIIH mRNA level in hepatocytes from suckling and fasted adult rats

To determine more precisely the impact of insulin on EIIH gene expression, we measured the hormone effect in several conditions. We studied the early phase of specific EIIH mRNA accumulation in primary cultures of 12-day-old suckling rat hepatocytes. RNA blot analysis confirmed that this induction is rapid and transient, being detectable as soon as 2 h after insulin addition reaching a maximum level 4 h later, before decreasing sharply (Figure 2A). A similar profile was observed in adult hepatocyte primary cultures, where EIIH mRNA expression was induced within the first 1 h of insulin treatment, peaked at 4 h and then progressively decreased (Figure 2B). EIIH mRNA was present as a faint band in the basal state in adult hepatocytes, whereas it was undetectable in the 12-day-old suckling rat hepatocytes (Figure 2A). Finally, in both

Figure 2 Time-course analysis of GK and EIIH mRNAs accumulation by RNA blot analysis

Cells were cultured in the absence of glucose and insulin for 16 h. Insulin (100 nM) was added, and total RNA was prepared at the times indicated above the panels. (A) 12-day-old suckling and (B) fasted adult rats after insulin treatment. The bottom panels of (A) and (B) show ethidium-bromide-stained 18 S RNA. It should be noted that the increase of EIIH mRNA at 24 h was not observed in other experiments. Blots are representative of three independent experiments.

cases, GK expression, as a control of the insulin effect, showed the classical induction pattern with the expected longer lag in 12-day-old suckling rat hepatocytes. [4, 19]. Taken together, these data indicate a primordial role of insulin in the accumulation of EIIH mRNA, independently of glucose.

Structure analysis of the full-length EIIH cDNA

We cloned the missing 5' part of the rat EIIH cDNA by RACE from insulin-induced adult liver RNA. The full-length mRNA contains a 1062 bp open reading frame located in the 5' half encoding a putative protein of 353 amino acids (see the supplementary data at <http://www.BiochemJ.org/bj/385/bj3850165add.htm>). The sequence surrounding the initiator codon is in good agreement with the Kozak consensus sequence for initiator codons [20].

A database search with the full-length EIIH cDNA sequence (GenBank® accession number AY533242) revealed high identity, 51.2%, with the coding region of a human cDNA named E2IG4 (GenBank® accession number AF191019), cloned as an oestrogen-induced gene in the mammary tumour cell line MCF-7 [21]. Further analysis with genome sequences revealed orthologous genes in mouse, with 79.3% identity, and zebrafish (GenBank® accession numbers XM_145954.2 and BC047843.1 respectively). However, these orthologues do not belong to a known gene family, and, together with EIIH, may constitute the first identified members of a new gene family. The chromosomal localization was determined by genome sequence analysis with human, rat and mouse databases. The human gene maps to chromosome locus 11q13.3, the rat gene to chromosome locus 1q32 and the mouse gene to chromosome 7.

Analysis of EIIH putative protein

In vitro transcription/translation in the presence of [³⁵S]methionine was used to confirm the coding potential of the 2.5 kb EIIH cDNA. The predicted EIIH protein had a calculated molecular mass of 38 kDa, similar to the estimated size of the largest and

Figure 3 EIIH mRNA expression in liver during development

Total RNA was extracted from the liver of rats at the days indicated above the blot, and was hybridized with EIIH and GK probes. Birth and weaning are indicated by vertical arrows. The rats were weaned at day 20 on to a high-carbohydrate, low-fat diet. Bottom panels, 18 S RNA stained with ethidium bromide. The blot is representative of two independent experiments.

most major band seen on SDS/PAGE analysis confirming the potential functionality of this open reading frame (results not shown). The deduced rat EIIH protein displayed 85 % identity with the human E2IG4 protein (GenBank® accession number NP_056331) [21], indicating further that E2IG4 is the human equivalent of EIIH protein.

Further comparisons were performed with protein motif databases in an attempt to predict a function for EIIH protein. A large number of proteins were indeed identified that could be categorized as a LRR (leucine-rich repeat) family of proteins. These proteins contain LRR motifs, consisting of 20–29 residues, with a conserved 11-residue consensus sequence: LXXLXXN/CXL where X represents any amino acid [22,23]. Most of these proteins are found in eukaryotes, their resemblance is almost entirely restricted to the LRR motif and possess very diverse functions. The only common characteristic among these proteins is their ability to interact with other proteins at their LRR [23]. PROSITE analysis revealed nine LRR motifs in EIIH, and four of these nine motifs are perfect matches with the consensus LRR motifs that are conserved in human, rat and mouse EIIH proteins, suggesting that they may be important for EIIH function. Other known motifs, such as phosphorylation and N-glycosylation sites, were also identified, and were conserved in rat, human and mouse EIIH sequences (see the supplementary data at <http://www.BiochemJ.org/bj/385/bj3850165add.htm>). It also includes a leucine zipper-like pattern (285–306), the significance of which is questionable, given the high proportion of leucine within EIIH sequence (70/353, 19.8 %) and the presence of several conserved proline residues within the pattern. Similar to E2IG4 and its mouse equivalent, the rat EIIH protein contains a N-terminal hydrophobic region that may represent a signal peptide as determined using the SignalP program, thus suggesting that EIIH may be the precursor to a secreted or membrane-associated protein. However, none of these observations allowed predicting a function for EIIH protein.

Induction of the EIIH mRNA during development in rat liver

In order to better approach the role of the EIIH protein, we established the profile of its mRNA accumulation during development by RNA blot analysis (Figure 3). EIIH mRNA became detectable after birth and during the suckling period as a weak signal. It increased sharply during weaning and remained detectable thereafter. GK mRNA levels became detectable during weaning, reaching a maximum 25 days after birth and were decreased at day 35. However, the apparent decrease of EIIH mRNA may be, like the one observed for GK, related to the nutritional conditions

Figure 4 Effect of insulin on EIIH mRNA levels in the liver of diabetic STZ-treated rats

Diabetes was induced by injection of STZ 5 days before the experiment. Intraperitoneal injection of insulin was performed 30 min before rats were killed. The bars represent the relative EIIH (A) and GK (B) mRNA levels as determined by real-time RT-PCR after normalization with cyclophilin expression levels. Results are means $\pm$ S.E.M. of four (STZ rats treated by insulin) and six (controls and STZ rats) animals. Statistical significance was calculated using ANOVA between STZ-treated and control rats ($*P < 0.05$), STZ-treated and STZ-treated insulin-injected rats ($**P < 0.05$), and control and STZ-treated insulin-injected rats ($\#P < 0.05$) for EIIH mRNA. The real-time RT-PCR measurement was repeated twice with similar results.

at the moment of sacrifice, and the associated variations of insulin secretion.

EIIH mRNA levels in the liver of STZ-diabetic rats

Adult diabetic rats obtained by STZ treatment, which selectively destroys β -cells of the pancreas, were used to examine the effects of insulin deprivation on EIIH expression in the liver.

We used quantitative real-time RT-PCR analysis to assess EIIH and GK mRNA levels in control and STZ-diabetic rats 30 min after one injection of insulin (20 units/kg of body mass). After normalization for cyclophilin mRNA levels, we observed a reduction of approx. 50 % in EIIH mRNA levels in the liver of STZ rats compared with the controls (36.2 ± 4.7 % compared with 100 ± 17.1 %; Figure 4A). Insulin injection increased its level approx. 1.5- and 4-fold compared with control and STZ-treated animals respectively (151.4 ± 33.5 %). As reported previously [24], a dramatic reduction (approx. 10-fold) in GK mRNA in the liver of STZ-treated rats compared with the controls (6.5 ± 2.8 % compared with 100 ± 17.2 %) was detected, and insulin treatment restored its level to the control value (Figure 4B). Taken together, these data demonstrate that insulin plays a determinant role in regulating EIIH expression *in vivo*.

Tissue distribution of EIIH, Glut2 and GK mRNAs in rat

EIIH mRNA tissue distribution was analysed by RT-PCR in adult rats. It was expressed at high levels in the liver, small intestine (duodenum and ileum) and placenta (Figure 5A). It was not or barely detectable in other tissues, including whole pancreas, adipose tissue, skeletal muscle, kidney, spleen, brain, lung and testis. Since several EIIH-expressing tissues are also known to express Glut2, Glut2 mRNA distribution was studied in parallel in the same samples. As expected, the Glut2 RT-PCR signal essentially followed EIIH mRNA expression, with the exception of testis, where EIIH was not detected. Although Glut2 is known

Figure 5 Tissue distribution analysis of EIIH and Glut2 mRNAs by RT-PCR

RT-PCR products (10 μ l) were analysed by electrophoresis on a 1.5% (w/v) agarose gel. **(A)** Whole-organ tissue distribution. Li, liver; Pa, pancreas; AT, adipose tissue; SM, skeletal muscle; K, kidney; S, spleen; D, duodenum; I, ileum; B, brain; Pl, placenta; Lu, lung; Te, testis; -, no cDNA. **(B)** Expression in whole pancreas (Pa) and isolated pancreatic islets of Langerhans (IsL). **(C)** Expression in whole brain (B), dissected hypothalamus (H) and pituitary gland (Pi). The sizes of the RT-PCR products were 2489 bp for EIIH, 717 bp for Glut2, 738 bp for GK and 568 bp for cyclophilin. Cyclophilin was used as an indication of cDNA input in the reactions.

Figure 6 Northern blot analysis of the oestradiol-treated adult rat hepatocytes *in vitro*

Adult rat hepatocytes were treated with 17 β -oestradiol and insulin at the indicated concentrations, and RNAs were analysed by Northern blotting as described in the Experimental section. Blots are representative of three separate experiments. Dex, dexamethasone; E2, 17 β -oestradiol.

to be expressed in the endocrine pancreas and some brain areas [25], we could not detect its expression using total RNA prepared from whole tissue. Given that these tissues contain multiple cell populations, we analysed mRNA from purified pancreatic islets of Langerhans, hypothalamus and pituitary tissues by the same RT-PCR systems. As expected, Glut2 expression could be detected in pancreatic islets and hypothalamus mRNAs. Again, in these selectively enriched samples, EIIH mRNA showed a distribution pattern similar to that of Glut2, except for the pituitary, where EIIH expression, but not Glut2, was detectable (Figures 5B and 5C). These data suggest a possible relationship between these two genes and demonstrate EIIH expression in the endocrine pancreas. Finally, in the same series of tissues, GK mRNA analysis showed the expected expression profile, essentially restricted to the liver, endocrine pancreas, some brain areas, and, to a lesser extent, the placenta and testis.

Effects of 17 β -oestradiol on EIIH gene expression

Since the human orthologue of EIIH, E2IG4, has been shown to be expressed in response to 17 β -estradiol [21], it was of interest to see if EIIH also responds to 17 β -oestradiol. Indeed, EIIH mRNA was increased in the adult rat hepatocytes within 3 h of exposure to increasing concentrations of 17 β -oestradiol (Figure 6).

DISCUSSION

The aims of the present study was to identify novel early insulin-induced genes for further characterization of insulin action at cellular/molecular levels and to disclose new pharmacological targets for the treatment of insulin-related pathologies and, principally, diabetes.

Liver was chosen for it is one of the major targets of insulin playing a pivotal role in glucose homeostasis. Our experimental scheme is original as it allows the dissociation the glucose effects from the insulin action. We focused on genes that are regulated rapidly and markedly in order to select those that are likely to represent authentic primary targets of insulin.

Induction of the EIIH gene preceded that of the GK gene which is thought to require prior *de novo* synthesis, and thus is probably the result of an indirect mechanism [8,19]. In contrast, the early response of the EIIH gene suggests that it is dependent on a different mechanism, and can be considered as a new model to identify promoter elements involved in the direct regulation of transcription by insulin. Nevertheless, our results do not exclude a possible role of insulin on the stability of pre-existing EIIH mRNA, especially in the adult rat hepatocytes. A search for putative transcription-factor-binding sites in the genomic region preceding the 5' end of EIIH cDNA showed a cluster of potential binding sites approx. 2 kb upstream of the initiator codon, including sites for GATA-1 and 2, SRY (sex-determining region Y), HNF (hepatocyte nuclear factor) 2 and 3, and SREBP-1 approx. 200 bp upstream of several CREs (cAMP-responsive elements), which altogether may represent a promoter or a transcriptional regulatory region. Although we have demonstrated that EIIH responds to 17 β -oestradiol in rat hepatocytes, our search did not identify EREs (oestrogen-responsive elements) in this region. This could be explained by the facts that, first, it has already been reported that oestradiol/oestrogen receptors ER α and ER β can bind to the SRE (sterol regulatory element) region and induce gene transcription [26,27], and, secondly, the upstream 2 kb region is just a theoretical promoter region and the real EIIH promoter region remains to be determined.

Despite cloning of the full-length EIIH cDNA, the nature and activity of the EIIH protein remains obscure. It is noteworthy that the EIIH gene appears to be unique in most species, suggesting that the protein plays a specific role. EIIH protein only shows identities with LRR motifs of proteins belonging to different families. Indeed, the LRR is quite frequently found in proteins, but its function is poorly characterized in terms of biological activity, and these proteins are involved in many diverse biological processes, such as pathogen recognition, brain development apoptosis or plant disease resistance [22], but not in insulin action, so far.

The EIIH gene appears to be the rat orthologue of E2IG4, encoding a protein identified in human mammary gland tumour cells through its transient induction by oestradiol [21]. Interestingly enough, the E2IG4 gene has been mapped to chromosome locus 11q13.3 in the human genome, within the region that was designated as IDDM4 (insulin-dependent diabetes mellitus 4) for it shows linkage to Type I diabetes susceptibility [28,29].

For elucidation of possible EIIH function, we can hypothesize that, since EIIH is inducible by both insulin and oestradiol, at least in certain cell types, its function could be at the conjunction of the two hormones' actions (cross-talk) in those cell types. Previous reports on the induction of insulin receptor and IRS-1 (insulin receptor substrate 1) gene by oestradiol support this hypothesis [30,31]. This effect of oestradiol on the early components of insulin activity can be considered as the major interdependent action, or cross-talk, between the two hormones, and is emphasized further by the finding that the long-term high blood levels of

insulin and oestrogen reduces the IRS-1 levels in the hepatocytes and leads to insulin resistance [32,33].

On the other hand, EIIH mRNA tissue distribution is remarkably similar to that of Glut2. In contrast with other glucose transporters, Glut2 is expressed in a limited subset of tissues with high K_m glucose transport activities, such as intestine, kidney, liver, pancreatic β -cells and mammary gland cells [34,35]. Although Glut2 is neither under insulin nor oestradiol regulation, a common protein linking the insulin and oestradiol activities to Glut2 function could be envisaged. In this view, EIIH would be a good candidate, since its gene is expressed soon after insulin stimulation in tissues that had had no previous exposure to high glucose concentrations and so should adapt rapidly to glucose absorption.

In conclusion, we have identified a new gene, termed EIIH, rapidly inducible by insulin independently of glucose at the transcriptional level *in vitro* as well as *in vivo*, and mainly expressed in the liver. The hypothetical protein represents a new class of insulin-responsive molecules, and might constitute an important component of the physiological and/or cellular response to this hormone.

This work was supported by a grant from Merck-Santé to S. C. A patent, FR0304835, for the human orthologue of EIIH, E2IG4, as a target for drug development and treatment of diabetes using EIIH as a model, was filed on April 13 2003. We thank Jean-Georges Fisher and Ismaila Ba for their excellent technical assistance, Nathalie Marchand for help with animal care, Franck Letourneur for his advice on quantitative RT-PCR, and Franck Mauvais-Jarvis and Catherine Postic for stimulating discussions.

REFERENCES

- O'Brien, R. M., Streeper, R. S., Ayala, J. E., Stadelmaier, B. T. and Hornbuckle, L. A. (2001) Insulin-regulated gene expression. *Biochem. Soc. Trans.* **29**, 552–558
- Foufelle, F. and Ferré, P. (2002) New perspectives in the regulation of hepatic glycolytic and lipogenic genes by insulin and glucose: a role for the transcription factor sterol regulatory element binding protein-1c. *Biochem. J.* **366**, 377–391
- Fukuda, H., Noguchi, T. and Iritani, N. (1999) Transcriptional regulation of fatty acid synthase gene and ATP citrate-lyase gene by Sp1 and Sp3 in rat hepatocytes (1). *FEBS Lett.* **464**, 113–117
- Nouspikel, T. and Iynedjian, P. B. (1992) Insulin signalling and regulation of glucokinase gene expression in cultured hepatocytes. *Eur. J. Biochem.* **210**, 365–373
- Tobin, K. A., Ulven, S. M., Schuster, G. U., Steiner, H. H., Andresen, S. M., Gustafsson, J.-Å. and Nebb, H. I. (2002) Liver X receptors as insulin-mediating factors in fatty acid and cholesterol biosynthesis. *J. Biol. Chem.* **277**, 10691–10697
- Yamada, K., Kawata, H., Shou, Z., Mizutani, T., Noguchi, T. and Miyamoto, K. (2003) Insulin induces the expression of the SHARP-2/Stra13/DEC1 gene via a phosphoinositide 3-kinase pathway. *J. Biol. Chem.* **278**, 30719–30724
- Osborne, T. F. (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J. Biol. Chem.* **275**, 32379–32382
- Bossard, P., Parsa, R., Decaux, J. F., Iynedjian, P. and Girard, J. (1993) Glucose administration induces the premature expression of liver glucokinase gene in newborn rats: relation with DNase-I-hypersensitive sites. *Eur. J. Biochem.* **215**, 883–892
- Diamond, R. H., Du, K., Lee, V. M., Mohn, K. L., Haber, B. A., Tewari, D. S. and Taub, R. (1993) Novel delayed-early and highly insulin-induced growth response genes: identification of HRS, a potential regulator of alternative pre-mRNA splicing. *J. Biol. Chem.* **268**, 15185–15192
- Rome, S., Clement, K., Rabasa-Lhoret, R., Loizon, E., Poitou, C., Barsh, G. S., Riou, J. P., Laville, M. and Vidal, H. (2003) Microarray profiling of human skeletal muscle reveals that insulin regulates approximately 800 genes during a hyperinsulinemic clamp. *J. Biol. Chem.* **278**, 18063–18068
- Dhabbi, J. M., Mote, P. L., Cao, S. X. and Spindler, S. R. (2003) Hepatic gene expression profiling of streptozotocin-induced diabetes. *Diabetes Technol. Ther.* **5**, 411–420
- Lan, H., Rabaglia, M. E., Stoehr, J. P., Nadler, S. T., Schuele, K. L., Zou, F., Yandell, B. S. and Attie, A. D. (2003) Gene expression profiles of nondiabetic and diabetic obese mice suggest a role of hepatic lipogenic capacity in diabetes susceptibility. *Diabetes* **52**, 688–700
- Narkewicz, M. R., Iynedjian, P. B., Ferré, P. and Girard, J. (1990) Insulin and tri-iodothyronine induce glucokinase mRNA in primary cultures of neonatal rat hepatocytes. *Biochem. J.* **271**, 585–589
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299
- Kingston, R. E. (2002) Preparation and analysis of RNA. In *Current Protocols in Molecular Biology*, vol. 1 (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., eds.), John Wiley & Sons, New York
- Iynedjian, P. B., Ucla, C. and Mach, B. (1987) Molecular cloning of glucokinase cDNA: developmental and dietary regulation of glucokinase mRNA in rat liver. *J. Biol. Chem.* **262**, 6032–6038
- Sutton, R., Peters, M., McShane, P., Gray, D. W. and Morris, P. J. (1986) Isolation of rat pancreatic islets by ductal injection of collagenase. *Transplantation* **42**, 689–691
- Baker, T. K., Carfagna, M. A., Gao, H., Dow, E. R., Li, Q., Searfoss, G. H. and Ryan, T. P. (2001) Temporal gene expression analysis of monolayer cultured rat hepatocytes. *Chem. Res. Toxicol.* **14**, 1218–1231
- Bossard, P., Decaux, J. F., Juanes, M. and Girard, J. (1994) Initial expression of glucokinase gene in cultured hepatocytes from suckling rats is linked to the synthesis of an insulin-dependent protein. *Eur. J. Biochem.* **223**, 371–380
- Kozak, M. (1987) An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res.* **15**, 8125–8148
- Charpentier, A. H., Bednarek, A. K., Daniel, R. L., Hawkins, K. A., Lafilin, K. J., Gaddis, S., MacLeod, M. C. and Aldaz, C. M. (2000) Effects of estrogen on global gene expression: identification of novel targets of estrogen action. *Cancer Res.* **60**, 5977–5983
- Andrade, M. A., Perez-Iratxeta, C. and Ponting, C. P. (2001) Protein repeats: structures, functions, and evolution. *J. Struct. Biol.* **134**, 117–131
- Kobe, B. and Kajava, A. V. (2001) The leucine-rich repeat as a protein recognition motif. *Curr. Opin. Struct. Biol.* **11**, 725–732
- Iynedjian, P. B., Gjinovci, A. and Renold, A. E. (1988) Stimulation by insulin of glucokinase gene transcription in liver of diabetic rats. *J. Biol. Chem.* **263**, 740–744
- Li, B., Xi, X., Roane, D. S., Ryan, D. H. and Martin, R. J. (2003) Distribution of glucokinase, glucose transporter GLUT2, sulfonylurea receptor-1, glucagon-like peptide-1 receptor and neurotrophin-1 messenger RNAs in rat brain by quantitative real time RT-PCR. *Mol. Brain Res.* **113**, 139–142
- Streicher, R., Kotzka, J., Muller-Wieland, D., Siemeister, G., Munck, M., Avci, H. and Krone, W. (1996) SREBP-1 mediates activation of the low density lipoprotein receptor promoter by insulin and insulin-like growth factor-I. *J. Biol. Chem.* **271**, 7128–7133
- Lopez, D., Sanchez, M. D., Shea-Eaton, W. and McLean, M. P. (2002) Estrogen activates the high-density lipoprotein receptor gene via binding to estrogen response elements and interaction with sterol regulatory element binding protein-1A. *Endocrinology* **143**, 2155–2168
- Davies, J. L., Kawaguchi, Y., Bennett, S. T., Copeman, J. B., Cordell, H. J., Pritchard, L. E., Reed, P. W., Gough, S. C., Jenkins, S. C., Palmer, S. M. et al. (1994) A genome-wide search for human type 1 diabetes susceptibility genes. *Nature (London)* **371**, 130–136
- Hashimoto, L., Habita, C., Beressi, J. P., Delepine, M., Besse, C., Cambon-Thomsen, A., Deschamps, I., Rotter, J. I., Djoulah, S., James, M. R. et al. (1994) Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q. *Nature (London)* **371**, 161–164
- Gonzalez, C., Alonso, A., Gruesso, N. A., Diaz, F., Esteban, M. M., Fernandez, S. and Patterson, A. M. (2001) Effect of treatment with different doses of 17- β -estradiol on insulin receptor substrate-1. *J. Pancreas (Online)* **2**, 140–149
- Molloy, C. A., May, F. E. and Westley, B. R. (2000) Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. *J. Biol. Chem.* **275**, 12565–12571
- Gonzalez, C., Alonso, A., Diaz, F. and Patterson, A. M. (2003) Dose- and time-dependent effects of 17 β -oestradiol on insulin sensitivity in insulin-dependent tissues of rat: implications of IRS-1. *J. Endocrinol.* **176**, 367–379
- Yuan, L., Ziegler, R. and Hamann, A. (2002) Chronic hyperinsulinism induced down-regulation of insulin post-receptor signaling transduction in Hep G2 cells. *J. Huazhong Univ. Sci. Technol. Med. Sci.* **22**, 313–316
- Grover-McKay, M., Walsh, S. A., Seftor, E. A., Thomas, P. A. and Hendrix, M. J. (1998) Role for glucose transporter 1 protein in human breast cancer. *Pathol. Oncol. Res.* **4**, 115–120
- Thorens, B. (1996) Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. *Am. J. Physiol.* **270**, G541–G553