Spot 14 protein interacts and co-operates with chicken ovalbumin upstream promoter-transcription factor 1 in the transcription of the L-type pyruvate kinase gene through a specificity protein 1 (Sp1) binding site

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In hepatocytes, the amount of the Spot 14 (S14) protein is closely related to the full expression of enzymes involved in the glycolytic and lipogenic pathways. In the present study we address the role played by this protein in the control of transcription of the L-type pyruvate kinase (L-PK) gene in primary hepatocytes. We show that human S14, which by itself does not bind to the L-PK promoter, physically interacts with the human chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1) and induces the switch of this factor from a repressor to an activator. However, the enhancing activity of S14 and COUP-TF1 depends on the presence of a proximal GC-rich box (the L0 element) that specifically binds nuclear proteins from the livers of rats fed a

glucose-rich diet. Moreover, the L0 element, which strongly binds dephosphorylated specificity protein 1 (Sp1), loses all affinity when this factor is phosphorylated by cAMP-dependent protein kinase. Mutations that affect binding of Sp1 and nuclear proteins to the L0 box also decrease basal transcription and impair glucose responsiveness of the promoter. These results therefore shed light on the mechanism by which the S14 protein, whose concentration rapidly rises after glucose intake, contributes to the full activity of the L-PK promoter.

Key words: glucose, glycolysis, hepatocytes, lipogenesis.

INTRODUCTION

In the mammalian liver, glycolysis and fatty acid synthesis, together with gluconeogenesis, contribute to glucose homeostasis. For instance, a carbohydrate-rich diet induces glycolysis and fatty acid synthesis so that glucose, initially converted into pyruvate, is transformed into triacylglycerols, which represent the storage form of energy. These integrated processes involve the co-ordinated activity, as well as the co-regulated synthesis, of several enzymes and are strictly controlled by the nutritional status of animals via both hormonal and nutrient-induced adaptation [1,2]. It is now well established that, besides short-term regulation of some key enzyme activities, another consequence of a carbohydrate-rich diet is the increased transcription of genes encoding enzymes of glycolytic and lipogenic pathways, such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC) and L-type pyruvate kinase (L-PK) [3–5]. Conversely, fasting or dietary unsaturated fats induce the repression of these genes [6].

Interestingly, in humans and rodents, the Spot 14 (S14) gene, which encodes a small acidic protein, shares a similar pattern of regulation with genes involved in the lipogenic pathway. Indeed, in the rat, the expression of this gene, first described as a thyroid-responsive gene [7], has been shown to be restrained to meta-

bolically responsive tissues that synthesize lipids [8]. In addition, its transcription was increased in liver by a high-carbohydrate diet or injection of insulin [9]. In contrast, a diet rich in polyunsaturated fatty acids [10] or high plasma glucagon levels [11] greatly decreased its expression. Despite this close correlation with lipogenic enzymes in terms of gene regulation, the specific function of the S14 protein remains unclear, although reported data have suggested that the expression of S14 precedes that of enzymes involved in the lipogenic pathway. For instance, S14 gene expression was controlled by circadian factors so that the peak of its expression slightly preceded the feeding period [12]. In addition, during the re-feeding of fasted animals, the synthesis of the S14 mRNA was first observed in the perivenous zone of the hepatic lobule where glucose is mainly consumed and coincided with the expression of the ACC gene [13]. More recently, it has been shown that transfection of hepatocytes with an S14 antisense oligonucleotide led to reduced triacylglycerol formation and an attenuated expression of several genes specifically involved in the lipogenic pathway, such as the ATP citrate lyase, malic enzyme and FAS genes [14]. In addition, when primary hepatocytes were transfected with this antisense oligonucleotide and a reporter gene directed by the promoter of the L-PK gene, which codes for one of the key regulatory enzymes of

Abbreviations used: ACC, acetyl-CoA carboxylase; COUP-TF1, chicken ovalbumin upstream promoter-transcription factor 1; hCOUP-TF1, human COUP-TF1; DR4-ME, direct repeat present in the rat malic enzyme; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FAS, fatty acid synthase; GST, glutathione S-transferase; HNF, hepatocyte nuclear factor; IP6, inverted palindrome of the same hexameric site spaced by six residues; L-PK, L-type pyruvate kinase; Oct1, octamer-binding protein 1; PKA, cAMP-dependent protein kinase; PP1, protein phosphatase type 1; RXR, retinoid X receptor; S14, Spot 14; hS14, human S14; Sp1, specificity protein 1; T3, 3,3',5-tri-iodo-L-thyronine; T3R, thyroid hormone receptor; URL, unprogrammed reticulocyte lysate.

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the glycolytic pathway, a clear decrease in the promoter activity was observed [14].

These results opened up the possibility that the function of the S14 protein could be investigated by studying its implication in L-PK gene regulation. L-PK expression has been widely investigated and, although some molecular mechanisms still remain unclear, it has been ascertained that the proximal promoter conferred cell-type specificity and was largely responsible for the regulation by hormones and nutrients. In this proximal promoter, several *cis*-acting elements have previously been identified and function as a unit, since their association shows transcriptional synergistic activation [15,16]. Among them, the L4 (also called LIII, which extends from nt -170 to -150) and L3 (also called LII, which extends from nt -149 to -126) elements have been considered to support full carbohydrate responsiveness and tissue-specific expression [17,18]. More proximal to the transcription start point, the L2 (nt -116 to -99) and L1 (also named LI, nt -94 to -76) boxes have been shown to bind nuclear factor 1 and hepatic nuclear factor (HNF) 1 respectively [19].

In the present study we report experimental data that may shed light on the role played by S14 in L-PK gene expression. The S14 protein, which cannot by itself bind to the proximal promoter, modifies the behaviour of the chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1), a transcription factor whose role in the regulation of the L-PK gene has never been clearly elucidated. We show that human S14 (hS14) interacts with human COUP-TF1 (hCOUP-TF1) and up-regulates L-PK gene expression through a specificity protein 1 (Sp1) binding site localized in the proximal promoter between nt -57 and -47. This element, which we propose to name L0, is shown to be a keystone of the promoter's regulation, since mutations in L0 diminish basal transcriptional activity and strongly attenuate glucose responsiveness. In addition, inasmuch as we demonstrate that the propensity of Sp1 to recognize the L0 element is dependent on its phosphorylation state, this transcription factor might be involved in the down-regulation of L-PK expression by cAMP [20,21].

EXPERIMENTAL

Materials

Taq DNA polymerase, restriction enzymes and the Klenow fragment of *Escherichia coli* DNA polymerase I were obtained from Gibco BRL (Cergy-Pontoise, France). Okadaic acid and cAMP-dependent protein kinase (PKA) inhibitor were purchased from Calbiochem (Meudon, France), and antiproteases were obtained from Roche (Meylan, France). GSH-Sepharose® 4B, anti-[glutathione S-transferase (GST)] antibodies and radiochemicals were purchased from Amersham Pharmacia Biotech (Les Ulis, France). Exgen 500 was from Euromedex (Souffelweyheim, France), all oligonucleotides were synthesized by Genset (Paris, France) and all other chemicals were from Merck (Fontenay-sous-bois, France) or Sigma (L'isle d'Abeau, France). Recombinant Sp1 was obtained from Promega (Lyon, France). Goat anti-(hCOUP-TF1) IgG and goat immunoglobulin solutions were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Plasmids

The 640 bp fragment including the entire coding sequence for the hS14 protein [22] was obtained by specific amplification from nucleate blood cell DNA. The PCR product was inserted into the *Sma*I cloning site of the pBK-CMV plasmid (Stratagene, La

Jolla, CA, U.S.A.), then subcloned into the pSG5 vector (Stratagene), downstream of the T7 promoter, thus producing pSG5-hS14. pSG5-human HNF4 α 2, pGEM3Z-rat RXR α (where RXR corresponds to retinoid X receptor), pCMV-human Sp1 and pCDNA-hCOUP-TF1 were gifts from Dr B. Laine (Unité INSERM459, Faculté de Médecine, Lille, France), Dr J. A. Gustaffson (Department of Biosciences, Karolinska Institute, Huddinge, Sweden), Dr R. Tjian (Howard Hughes Medical Institute, University of California, Berkeley, CA, U.S.A.) and Dr G. Salbert (UPRES-A 6026, Université de Rennes, Rennes, France) respectively. The construction of the recombinant plasmid pSG5-human T3R (where T3R corresponds to thyroid hormone receptor), which expresses human T3R β has been described previously [23]. The different L-PKluc gene reporters, named -183 L-PKluc, -150 L-PKluc, -96 L-PKluc and -54 L-PKluc, contained the -183/+11, -150/+11, -96/+11 and -54/+11 sequences of the rat L-PK gene respectively, fused to the firefly luciferase gene. Mutant -183 L-PKluc was generated by replacing both the -50 and -51 cytosine nucleotides in the -183 L-PK promoter with thymine nucleotides using the Quick Change site-directed mutagenesis kit (Stratagene). All plasmids were checked by sequencing.

In vitro translation and electrophoretic mobility shift assay (EMSA)

Labelling of the neosynthesized double-stranded oligonucleotides was performed by *E. coli* DNA polymerase I (Klenow fragment) in the presence of [α -³²P]dCTP. The oligonucleotide sequence referred to as L3 Box L-PK corresponded to the -146/-122 sequence of the rat L-PK gene. The -66/-37 sequence, which is strongly similar to an inverted GC box, was thus referred to as Sp1L-PK. Mutant Sp1L-PK corresponded to this sequence, except that both the -51 and -50 cytosines were replaced by thymines, and has been shown to prevent Sp1 binding [24]. Therefore sequences were as follows: L3 Box-L-PK, 5'-gatcCTGGA-CTCTGGCCCCCAGTGTACg-3'; Sp1L-PK, 5'-gateccCTCAT-CTGAGCCAGGCCCCATCCCACTGACg-3'; and mutant Sp1L-PK, 5'-gateccCTCATCTGAGCCAGGTTCCATCCCACTGACg-3' (lower-case nucleotides represent nucleotides that do not belong to the genomic sequence. They were used as templates for the radiolabelling with *E. coli* DNA polymerase I).

The sequences of the inverted palindrome of the same hexameric site spaced by six residues (IP6) that preferentially binds a homodimeric form of T3R β [25], as well as that of the direct repeat present in the rat malic enzyme (DR4-ME), which is thought to bind the T3R β -RXR α heterodimer [26], were: 5'-gateccTTATTGACCCCAGCTGAGGTCAAGTTACGg-3' and 5'-gateccAGGACGTTGGGGTTAGGGGAGGACAGTGG-ACg-3' respectively.

The sequence of the consensus Sp1 oligonucleotide was 5'-GATCCCATTCGATCGGGGCGGGGCGAGCG-3'. In all experiments, the amount of labelled oligonucleotide used was 1 ng (approx. 20×10^3 c.p.m.) and, when used, the amount of added unlabelled oligonucleotide was 100-fold greater than that of the labelled oligonucleotide. Expression plasmids encoding the sequences for hS14, HNF4 α 2, COUP-TF1 and T3R β as well as the rat RXR α cDNAs were *in vitro* transcribed and translated using the TNT T7-coupled reticulocyte lysate system, according to the manufacturer's instructions (Promega). EMSAs were performed as described previously [23]. Briefly, neotranslated proteins (in 4 μ l of the incubation medium) and 5 footprint units of human recombinant Sp1 or an equivalent volume of unprogrammed reticulocyte lysate (URL) were preincubated at 25 °C for 30 min, in 20 mM Tris/HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 10 %

(v/v) glycerol, 1 mM MgCl₂ and 2.5 mM dithiothreitol (DTT). When nuclear extracts were present, 5 µg of protein was used per well in 20 mM Hepes/KOH (pH 7.9) containing 50 mM KCl, 5 mM DTT, 0.2 mM EDTA, 10 % (v/v) glycerol, 50 nM okadaic acid and 200 nM PKA inhibitor. When necessary, 2 µl of anti-(COUP-TF1) IgG, 4 µl of anti-S14 IgG [27] or 2 µl of goat Ig solution was added to the incubation medium. The addition of ³²P-labelled oligonucleotides was followed by an additional 30 min incubation at 25 °C in a total volume of 50 µl. The oligonucleotide–protein complexes were then resolved using a non-denaturing 5 % (w/v) polyacrylamide gel. Electrophoresis was carried out at 100 V for 4–6 h in 0.09 M Tris, 0.009 M borate and 2 mM EDTA, pH 8.3. Gels were then dried and autoradiographed.

Liver nuclear extracts

Male Sprague–Dawley rats (180–200 g) were fasted for 48 h and re-fed for 24 h with either a standard diet (A-03; Usine d'Alimentation Rationnelle, Villemaison-sur-Orge, France) or a glucose-supplemented diet [standard diet with free access to drinking water supplemented with 1.7 % (w/v) glucose]. Nuclear extracts were then prepared according to the procedure described by Hasegawa et al. [28], and were used immediately. Okadaic acid (50 nM) and the PKA inhibitor 5-24 (200 nM) were present throughout the preparation of the nuclear extracts.

Transient transfection assays

Primary hepatocytes were isolated from male Sprague–Dawley rat livers by a collagenase perfusion method [28a]. Hepatic cells (6 × 10⁵ cells/well) were then plated on to collagen-coated 6-well plates, and transfections were carried out 4 h later using a polyethyleneimine transfection method (Exgen 500; Euromedex). In all experiments, 2 µg of reporter plasmid (–183L-PKluc, mutant –183 L-PKluc, –150 L-PKluc, –96 L-Pkluc or –54 L-PKluc) and 100 ng of each expression vector (pSG5-hS14, pCDNA-hCOUP-TF1 and pCMV-human Sp1) were used per well in the absence of serum. Control transfections were undertaken with corresponding void plasmids. A cotransfection with 1 µg of pSEAP control (ClonTech Laboratories, Saint-Quentin, France; this vector expresses SEcreted Alkaline Phosphatase under the control of the early simian virus 40 promoter) was added in order to normalize transfection efficiencies. Following transfection for 3 h, the media were supplemented with 10 % (v/v) fetal calf serum, 50 units/ml penicillin, 50 µg/ml streptomycin and 0.1 unit/ml insulin. Cultures were continued for 48 h in the presence of either 5.5 or 25.5 mM glucose. Hepatocytes were then lysed and analysed for luciferase and alkaline phosphatase activities. Relative luciferase activities are presented as means ± S.D. for three independent experiments performed in triplicate.

Expression of GST–hS14 fusion protein and protein–protein interactions *in vitro*

The coding sequence of hS14 was subcloned into the *Bam*H1 site of pGEX-6P1 (Amersham Pharmacia Biotech) after directed mutagenesis. The reading frame was then checked by direct sequencing. The chimaeric GST–hS14 protein was produced in *E. coli* BL21, whereas the GST protein alone was similarly generated from the void plasmid. Synthesis was induced by the addition to the cultures of 100 µM isopropyl β-D-thiogalactoside followed by incubation for 2 h. Bacteria were then lysed by sonication in PBS supplemented with 0.25 % Triton X-100, 2 mM EDTA, 1 mM MgCl₂, 1 mM DTT and an antiprotease

cocktail (1 µg/ml pepstatin, 2 µg/ml leupeptin and 2 µg/ml aprotinin). Lysates were then checked for protein production by Western-blot analysis, using an anti-GST antibody. Each protein was purified using GSH–Sepharose® 4B-affinity resin according to the manufacturer's instructions. Identical amounts of different ³⁵S-labelled proteins were incubated with either GST–agarose or GST–hS14–agarose in TKN buffer [50 mM Tris/HCl (pH 8.0), 100 mM KCl, 140 mM NaCl, 0.5 % Nonidet P40, 10 % (v/v) glycerol and an antiprotease cocktail (1 µg/ml pepstatin, 2 µg/ml leupeptin and 2 µg/ml aprotinin)] for 2 h at 4 °C with gentle rocking. Sepharose matrix washings were performed in centrifugal filter units (Millipore) by two rinses with TKN buffer, followed by two rinses with 20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 0.5 % Nonidet P40 and an antiprotease cocktail (1 µg/ml pepstatin, 2 µg/ml leupeptin and 2 µg/ml aprotinin). Bound proteins were finally released from Sepharose with 10 mM GSH in 50 mM Tris/HCl (pH 8.0). Released proteins were then resolved by SDS/PAGE, and the gel was subsequently dried and then analysed in a PhosphorImager (FLA-2000; Fujifilm, Tokyo, Japan).

Co-immunoprecipitation of COUP-TF1 and hS14

³⁵S-labelled hS14 protein and unlabelled COUP-TF1 were *in vitro* synthesized and preincubated at 4 °C for 30 min in TKN buffer. Goat anti-(hCOUP-TF1 antibodies) (4 µg) were then added for 2 h at 4 °C with gentle rocking. Protein G–Sepharose (100 µl) was added and used to gather protein complexes that were pelleted by gentle centrifugation (500 g for 2 min). Following four washes with TKN buffer, complexes were disrupted by boiling for 4 min in 50 µl of 2 × Laemmli buffer. Proteins were then separated by SDS/PAGE. Dried gels were subsequently analysed as described above.

RESULTS

S14 counteracts COUP-TF1 inhibition of L-PK promoter activity

Since previous observations had suggested that, at least in hepatocytes cultured in high glucose concentrations, the S14 protein could play an important role in the full activity of the L-PK promoter [14], we first searched for possible binding of the S14 protein to sequences of this promoter. To this end, several overlapping oligonucleotides, whose sequences together corresponded to the whole –183 promoter region, were tested, but none of them could bind the hS14 protein. In addition, as seen in Figure 1(A), cotransfection of primary hepatocytes with plasmid coding for S14 did not produce any modification of the luciferase activity irrespective of the glucose concentration. These observations did not exclude, however, the possibility that S14 counteracts effects of factors known to inhibit the transcription directed by this promoter. As members of the COUP-TF family had been shown to diminish both the basal transcription [29], and the glucose induction of the L-PK promoter [30], we sought functional interactions between S14 and COUP-TF1. In fact, whereas cotransfection of primary hepatocytes with pCDNA-hCOUP-TF1 alone strongly diminished the basal and glucose-induced transcription directed by the –183 promoter, overexpression of S14 counteracted COUP-TF1 effects and fully restored both basal transcription and glucose responsiveness (Figure 1A).

S14 protein prevents COUP-TF1 from binding to the L3 element of the L-PK promoter

The L3 element of the L-PK promoter has been shown to bind both HNF4 and COUP-TF1 [17,29], although the binding of

Figure 1 S14 protein interferes with COUP-TF1 binding to the L3 box

(A) Primary hepatocytes prepared from rat livers using a collagenase perfusion method were cultured in the presence of 5.5 or 25.5 mM glucose (grey and black bars respectively). Cells were transfected with 2 μ g of -183 L-PKluc reporter plasmid and, when indicated (+) 100 ng of pSG5-hS14 or pCDNA-hCOUP-TF1 plasmids. The amount of the transfected expression plasmids was determined from dose-effect experiments and did not result in squelching. Luciferase activity was normalized to alkaline phosphatase activity. Results are presented as the fold induction compared with the activity of cells transfected with the -183 L-PKluc plasmid plus pSG5 and pCDNA void plasmids (first two columns). Values are presented as means $\pm$ S.D. for three independent experiments performed in triplicate. (B) EMSA of a labelled oligonucleotide corresponding to -146/-122 of the rat L3 box L-PK promoter sequence, with, when indicated by +, URL, neosynthesized hS14, hCOUP-TF1, human HNF4 α 2 or 2 μ l of anti-(hCOUP-TF1) immunoglobulins solution (AbCOUP-TF1). (C) EMSA of labelled oligonucleotides corresponding to the IP6 or DR4-ME sequences, in the presence (+) of URL, neosynthesized hS14, human T3R β or rat RXR α . In (B and C) arrows indicate the position of specific shifted bands, and the asterisks indicate non-specific binding.

COUP-TF1 has been questioned [31]. As shown in Figure 1(B), the L3 box clearly bound COUP-TF1 and HNF4, although the former with weaker affinity. In the presence of S14, however, the binding of COUP-TF1 was significantly inhibited, whereas that of HNF4 remained unmodified. The faint residual band that we observed in the presence of the S14 protein was totally removed when COUP-TF1 and S14 were incubated in the presence of specific antibodies known to prevent COUP-TF1 from binding to its cognate element [32]. This experiment was repeated three times and in the presence of S14 the binding of COUP-TF1 to the L3 element diminished by $75 \pm 10\%$. The S14/COUP-TF1 molar ratio was 2:1 and had been previously optimized by experiments performed with increasing amounts of S14 protein. The possibility that such a displacement could be due to non-specific artefacts was checked by similar experiments performed with related nuclear receptors, such as T3R β and RXR α . As shown in Figure 1(C), no displacement of the binding of T3R homodimers or heterodimers to IP6 and DR4-ME elements respectively, was observed.

hS14 protein and hCOUP-TF1 physically interact *in vitro*

The effect of S14 could be due to physical interaction between this protein and COUP-TF1, and this point was checked by two different methods. First, a plasmid coding for an GST-hS14 fusion protein was constructed and the chimaeric protein was used in a protein-protein interaction assay. When bound to a GSH-Sepharose matrix, the hS14-GST fusion protein was shown to specifically retain [35 S]methionine-labelled hCOUP-TF1 (Figure 2A). Whereas no interaction between hS14 and human HNF4 α 2, human T3R β or rat RXR α could be observed (Figure 2B), hS14-GST protein was able to interact with labelled hS14 (Figure 2A), which confirmed a previous observation [33].

A second experiment based on co-immunoprecipitation was undertaken to test the physical interaction between hS14 and hCOUP-TF1 proteins. As seen in Figure 2(C), goat anti-

(hCOUP-TF1) antibodies specifically coprecipitated unlabelled hCOUP-TF1 and labelled hS14. Interestingly, in this case a molecular ratio of 2:1 for hS14/hCOUP-TF1 was also found to be optimal.

COUP-TF1 and S14 protein increase the L-PK promoter activity in the absence of the L4 and L3 elements

Since co-operation between L3 and L4 elements had been shown to be necessary for the full inhibitory effect of COUP-TF1 [29], it was interesting to determine whether the counteracting effect of S14 was maintained in the absence of the L4 element. To this aim, different constructions of the L-PK promoter (-150 L-PKluc that excluded the L4 element and -96 L-PKluc that was devoid of both the L4 and L3 elements) were tested by co-transfection in primary hepatocytes overexpressing S14, COUP-TF1 or both. Interestingly, the inhibitory effect of COUP-TF1 that we observed when this factor was cotransfected with the -183 L-PK promoter was converted into a clear activatory effect when the L-PK promoter was devoid of the L4 box. More importantly, overexpression of COUP-TF1 alone also increased the activity of -96 L-PKluc, which did not contain the L3 element. It is noteworthy that in both cases transactivation of COUP-TF1 was largely potentiated by cotransfection with pSG5-hS14 (Figure 3). This unexpected result led us to wonder whether the activatory effect of COUP-TF1 that was enhanced by S14 might be due to the binding of COUP-TF1 to a cryptic site or mediated through another factor able to bind to the proximal promoter.

Dephosphorylated Sp1 is able to bind to the proximal promoter

The presence of a cryptic site was rapidly eliminated, since we could not find any evidence for binding of COUP-TF1 to the -96/+11 sequence of the rat L-PK promoter (results not

Figure 2 hS14 protein and hCOUP-TF1 physically interact *in vitro*

(A) Autoradiography of dried electrophoretic gels run with proteins eluted from the GSH–Sepharose 4B matrix. Matrices were loaded with equal amounts of GST protein (lanes 2 and 5) or GST–hS14 fusion protein (lanes 3 and 6). Radiolabelled hS14 (lanes 2 and 3) or hCOUP-TF1 (lanes 5 and 6) were then incubated with loaded matrices. A 1/20 quantity of the hS14 (lane 1) or hCOUP-TF1 (lane 4) labelled protein input was run in parallel as a reference. (B) Autoradiography of dried electrophoretic gels run with proteins eluted from the GSH–Sepharose 4B matrix. Matrices were loaded with equal amounts of GST protein (lanes 2, 5 and 8) or GST–hS14 fusion protein (lanes 3, 6 and 9). Radiolabelled human HNF4α2 (lanes 2 and 3), rat RXRα (lanes 5 and 6) or human T3Rβ (lanes 8 and 9) were then incubated with loaded matrices. A 1/20 quantity of the human HNF4 α2 (lane 1), rat RXRα (lane 4) or human T3Rβ (lane 7) protein input was run in parallel as a reference. (C) Autoradiography of gels loaded with incubations of ³⁵S-labelled hCOUP-TF1 (lanes 2 and 7) or unlabelled hCOUP-TF1 plus ³⁵S-labelled hS14 (lanes 3–5 and 8–10) in the presence of Protein G–Sepharose (lanes 2–5) or goat anti-(hCOUP-TF1) antibodies (Ab COUP-TF1) coupled to Protein G–Sepharose (lanes 7–10). In lanes 1 and 6, ³⁵S-labelled hS14 was incubated in the presence of Protein G–Sepharose or anti-(hCOUP-TF1) antibodies coupled to Protein G–Sepharose respectively. The hS14:hCOUP-TF1 molar ratio was 1:1, 2:1 or 3:1 as indicated.

shown). However, between nt –56 and –47 we discerned a sequence that could be considered to be an inverted GC box. The most proximal L-PK promoter (–66/–37) was thus tested (using an EMSA) for its ability to bind purified human recombinant Sp1 factor. As shown in Figure 4, the migration of this labelled oligonucleotide in acrylamide gels was delayed in the presence of the Sp1 factor, but was unmodified when tested with COUP-TF1 or S14 protein only. The shifted band was totally

Figure 3 COUP-TF1 and S14 co-operatively increase L-PK promoter activity

Primary hepatocytes prepared from rat livers by a collagenase perfusion method were cultured in the presence of 5.5 mM glucose. Cells were transfected with 2 μg of –150 L-PKluc or –96 L-PKluc reporter plasmids and, when indicated (+), 100 ng of pCDNA-hCOUP-TF1 and/or pSG5-hS14 plasmids. Luciferase activity was normalized to alkaline phosphatase activity. Results are presented as the fold induction compared with the activity of cells transfected with corresponding reporter plasmids plus pSG5 and pCDNA void plasmids (first column of each graph). Values are presented as means ± S.D. for three independent experiments performed in triplicate.

Figure 4 Sp1 factor binds to the proximal L-PK promoter

EMSA of labelled oligonucleotides corresponding to the Sp1 consensus sequence (cons Sp1) or the Sp1-L-PK sequence in the presence of URL or human recombinant Sp1, hS14, hCOUP-TF1, PKA-treated Sp1 (PKA trt) or PP1-treated Sp1 (PP1 trt). When indicated (+ cons Sp1), 100 ng of unlabelled Sp1 consensus oligonucleotide was added as a competitor.

displaced by a 100-fold excess of unlabelled oligonucleotide corresponding to a consensus Sp1 response element. In addition, we observed that the affinity of Sp1 for the oligonucleotide was

Figure 5 Specific nuclear proteins are able to bind to the inverted GC box

EMSA of oligonucleotides corresponding to Sp1L-PK (lanes 1–11) and mutant Sp1L-PK (Mut Sp1; lanes 12 and 13) incubated (+) with URL, neosynthesized hS14, hCOUP-TF1, 2 μ l of goat immunoglobulins, 4 μ l of anti-S14 IgG (AbS14) or 2 μ l of COUP-TF1 immunoglobulins solution (AbCOUP-TF1). Labelled oligonucleotides were incubated with an equal amount of nuclear extracts from livers of rats fed a glucose-rich diet (Glucose +) or a standard diet (Glucose -). As competitor unlabelled consensus Sp1 oligonucleotide (consSp1) was added at 100 $\times$ concentration compared with labelled Sp1L-PK oligonucleotide (lane 3).

greatly enhanced by its dephosphorylation, since the preincubation of this factor with protein phosphatase type 1 (PP1) led to an increase in the intensity of the shifted band. In contrast, when phosphorylated by the catalytic unit of mouse PKA, Sp1 completely lost its affinity for the oligonucleotide.

Specific nuclear proteins are able to bind to the proximal inverted GC box

To address the physiological significance of these observations, we decided to explore the possibility for nuclear proteins to bind to the Sp1L-PK element, containing the inverted GC box. To this aim, nuclear extracts were prepared from livers of rats fed a glucose-enriched diet for 12 h before sacrifice. As seen in Figure 5, the migration of the labelled Sp1L-PK oligonucleotide was delayed by these nuclear extracts and two specific bands of high molecular mass could be observed (lane 2). Conversely, when an identical amount of nuclear extracts prepared from livers of rats fed a standard diet were tested, delayed bands were hardly discernible (lane 1). Nuclear extracts could be displaced from binding to Sp1L-PK by a 100-fold excess of consensus Sp1 oligonucleotide. Moreover an oligonucleotide mutated so as to render it incapable of binding Sp1 was unable to bind nuclear extracts prepared from livers of rats fed a glucose-enriched diet (lane 13). Interestingly, when nuclear extracts were supplemented with S14 protein, either alone or together with COUP-TF1, we observed an intensification of the top band and the complete disappearance of the bottom one. Conversely no binding could

Figure 6 Sp1, COUP-TF1 and hS14 co-operate to activate L-PK promoter activity

Primary hepatocytes were prepared from rat livers and cultured in the presence of 5.5 or 25.5 mM glucose (grey or black bars respectively). Cells were transfected with -183 L-PKluc, mutated -183 L-PKluc (Mut -183 L-PKluc), -96 L-PKluc or -54 L-PKluc reporter plasmids as indicated. Cells were cotransfected with 100 ng of pCMV-Sp1, pCDNA-hCOUP-TF1 or pSG5-hS14 when indicated (+) or with corresponding void vectors when indicated (-). (A) Luciferase activity was normalized to alkaline phosphatase activity. Values are presented as means $\pm$ S.D. for three independent experiments performed in triplicates. (B) Results are presented as the fold induction compared with the activity of cells transfected with corresponding reporter plasmids plus void plasmids. Values are presented as means $\pm$ S.D. for three independent experiments performed in triplicate.

be observed when liver nuclear extracts from rats fed a standard diet were supplemented with COUP-TF1, S14 or both (results not shown). The nature of the proteins involved in these complexes was then addressed by pretreatment of nuclear extracts with specific antibodies. As seen in Figure 5, antibodies directed against either S14 or COUP-TF1 led to a clear fading of the top

band irrespective of the presence or absence of S14 or COUP-TF1. These observations support the hypothesis that hS14 and COUP-TF1 are necessary components of the larger complex.

Sp1, COUP-TF1 and S14 co-operate to fully activate the L-PK promoter

To confirm the involvement of Sp1, COUP-TF1 and S14 in the activation of the L-PK promoter, hepatocytes in primary cultures were cotransfected with plasmids expressing the luciferase gene reporter under the control of -54 , -96 and -183 L-PK promoter sequences. Each of these plasmids was then cotransfected with Sp1 alone or in association with COUP-TF1 and S14. As seen in Figure 6, the cotransfection of Sp1 alone induced a slight increase in the luciferase activity when the reporter gene was under the control of each promoter fragment. When associated with the overexpression of Sp1, the transfection of primary hepatocytes with COUP-TF1 led to a 4–5-fold increase, which in the presence of hS14 became 10-fold greater than the corresponding basal value. Moreover when the -183 L-PK promoter was mutated in such a way that the -50 and -51 cytosines were replaced by two thymines, we observed a strong decrease in the activity of the -183 L-PK promoter. This mutation has been shown to weaken the affinity of Sp1 for the Sp1L-PK sequence [24].

It was interesting to consider the role that glucose might play when cells overexpressing Sp1 were transfected with S14 protein or COUP-TF1. All experiments were performed in the presence of 0.1 unit/ml insulin to ensure the complete metabolism of glucose. When cells, in which the reporter gene was under the control of the -183 promoter, were cotransfected with plasmids expressing COUP-TF1 and S14, the basal level of L-PK was greatly enhanced, but the induction by 25.5 mM glucose was preserved so that combining transfection and high glucose-concentration led to a 10-fold increase in the basal promoter activity. In contrast, when shortened promoters directed the luciferase gene, incubation of primary hepatocytes with high glucose concentrations did not produce any further increase in the promoter activity. Lastly, when the reporter gene was under the control of the mutant -183 L-PK promoter, transcription in the presence of glucose was greatly attenuated, which supported the hypothesis that the inverted GC box was necessary for the full response to glucose.

DISCUSSION

In the present study, our aim was to address the mechanism by which the S14 protein could play a role in the transcription process of the L-PK promoter. This role had previously been suggested from experiments performed in hepatocytes treated with anti-S14 oligonucleotide in the presence of a high glucose concentration [14]. However, when we overexpressed S14 in primary hepatocytes cultured at a low glucose concentration we did not notice any increased activity in the L-PK promoter (Figure 1). We therefore decided to address the possibility that S14 played a role in the transcription process by interfering with an inhibitory factor. Inasmuch as it had been demonstrated that COUP-TFs could inhibit the promoter activity of the L-PK gene [29,30], we sought for a possible interaction of S14 with these nuclear receptors.

Evidence for a functional interaction between COUP-TF1 and S14 is provided by experiments depicted in Figure 1: the co-expression of S14 in hepatocytes abrogated the inhibitory activity of COUP-TF1. In addition, physical interaction between these proteins is directly demonstrated by the co-immunoprecipitation

of hS14 by anti-(COUP-TF1) antibodies and by the trapping of COUP-TF1 by immobilized S14 protein. It is noteworthy that this interaction was stronger when experiments were performed in incubation buffers of high ionic strength (results not shown), which suggests the involvement of non-ionic bonds. Under similar conditions, we did not observe any physical interaction between hS14 and human HNF4 α 2, rat RXR α or human TR β , other receptors related to COUP-TF1 within the nuclear receptor family [34]. Interestingly, we observed a strong interaction between GST-hS14 and radiolabelled hS14, which confirms that S14 may behave as a homodimer, as previously suggested by another group [35]. This would also explain why physical interaction was stronger when a molar ratio of 2:1 between hS14 and hCOUP-TF1 was used.

This first set of data led us to address the role played by COUP-TFs, orphan receptors belonging to the steroid/thyroid/retinoid family. These nuclear receptors are generally considered as negative regulators of transcription [36,37], but some reports have underlined the possibility that they behave as transcription activators [38,39]. To date, in the context of the L-PK gene, these nuclear receptors have been considered as inhibitors of promoter activity [29,30], although a recent report has shown that COUP-TF1 can act as an activator [40]. In the present study we show that COUP-TF1 has dual activities that depend on the presence of the L4 box or S14 protein. Indeed in the absence of S14, the L4 element was necessary for COUP-TF1 to behave like an inhibitory agent, whereas COUP-TF1 enhanced the promoter activity in the absence of this element.

Unexpectedly, our observations also demonstrated that the activatory effect of COUP-TF1 was obviously not mediated through interaction with the L3 element, since it persisted in hepatocytes expressing the luciferase under the control of the -96 L-PK promoter, thus in the absence of this element. This observation supports previous reports showing that, even without binding to DNA, COUP-TF1 can act as an activator through protein–protein interaction with DNA-bound factors, such as HNF4 [41], octamer-binding protein 1 (Oct1) [42], or the ubiquitous Sp1 factor. This latter factor has been shown to co-operate through physical interaction with COUP-TF1 in the activation of the nerve growth factor inducible-A ('NGFI-A') promoter [24], and in the transcriptional activity of the HIV-1 long terminal repeat [43]. Interestingly, a few reports have provided evidence for the involvement of Sp1 in the transcriptional regulation of several genes that encode metabolic enzymes belonging to the glycolytic and lipogenic pathways, for example FAS [44], ATP citrate lyase [45], ACC [46], aldolase A and pyruvate kinase M2 [47].

In the present study we show that interaction between COUP-TF1 and Sp1 is actually involved in the activation of the L-PK promoter, since cotransfection of Sp1 in cells that already expressed COUP-TF1 reinforced the activatory effect of the nuclear receptor. This effect was linked to the presence of an inverted GC box that is located in the most proximal promoter (nt -57 to -47). Indeed, this region, which specifically bound Sp1 (Figure 4), played an important role in the L-PK transcription, since mutation of its core sequence resulted in a dramatic decrease in both the basal activity and the Sp1-mediated activatory effect of COUP-TF1 (Figure 6). This GC box also specifically bound nuclear complexes only present in the livers of rats fed a glucose-enriched diet. Treatment of these nuclear extracts with antibodies directed against COUP-TF1 prevented them from binding to the GC box, which means that COUP-TF1 is involved in their binding capacity. Taken together these observations suggest that, in addition to four previously identified elements, the L-PK promoter contains a functional site which we

propose to name L0. This L0 site would recognize Sp1 or a related Sp1-like protein, and functional interaction between Sp1 and COUP-TF1 would support the activatory behaviour of COUP-TF1.

In hepatocytes, the L-PK gene, like other genes coding for regulatory enzymes of the glycolytic and lipogenic pathways, has been shown to be negatively regulated by glucagon. In addition, the glucagon effect seems to be dominant over the induction of the L-PK expression by glucose or insulin [20]. It has been proposed that the responsiveness to cAMP was supported by the -183 promoter, more specifically by the L3 and L4 boxes [48,49]. Our results provide an alternative explanation for the rapid and dominant effect of glucagon on the expression of the L-PK gene. Indeed, we show that the binding of Sp1 to the L0 element was strictly dependent on its dephosphorylation by PP1, which is known to counteract PKA-induced phosphorylation. It can thus be suggested that the phosphorylation of Sp1 via PKA, which prevents the binding of Sp1 to the L0 element (Figure 4), would contribute to the rapid and dominant decrease of the L-PK promoter activity that is observed in the presence of glucagon. In addition, several reports have underlined the fact that the phosphorylation state of Sp1 could be linked to glucose regulation. For example, Daniel et al. [50] demonstrated a phosphorylation-dependent binding of Sp1 to the proximal promoter of the ACC gene. They linked this binding to glucose regulation, since they showed that, at least in adipocytes, the binding of Sp1 to its cognate element was enhanced by treatment of cells with high glucose concentrations. This enhanced binding was correlated with an increase in PP1 activity [50]. Pyruvate kinase M2 and aldolase A promoter activities have also been shown to be regulated by glucose-induced dephosphorylation of Sp1 [47]. In the current study, the L0 element was shown to bind nuclear extracts from the livers of rats fed on a glucose-enriched, but not a standard, diet. The fact that the L0 element was able to specifically bind the dephosphorylated, but not phosphorylated, form of Sp1 provides an indirect argument for considering that nuclear extracts may contain a dephosphorylated form of Sp1 or an Sp1-like related protein.

S14, whose expression is restrained to lipogenic tissues, has been proposed to act as an integrator of hormonal and nutritional stimuli, since its expression was rapidly induced after injection of 3,3',5-tri-iodo-L-thyronine (T3) and/or a glucose-enriched diet. Our data demonstrate that in cells cultured at low glucose concentrations the overexpression of S14 abrogates the clear inhibitory effect of COUP-TF1. Since S14 can physically interact with COUP-TF1 and prevent it from binding to the L3 element, a first hypothesis would be that S14 could act by rendering the L3 element able to bind another factor instead of COUP-TF1, for example HNF4, which could in turn interact with L4 bound activatory factors as previously suggested [29]. This hypothesis would explain why, in the context of the -183 promoter, overexpression of S14 together with that of COUP-TF1 totally restores the glucose responsiveness of this promoter. Nevertheless this hypothesis does not account for the role played by Sp1. Indeed, when Sp1 was coexpressed in these cells, COUP-TF1 and S14 strongly enhanced the -183 promoter activity at low, as well as high, glucose concentrations. It must be noted that the somewhat higher activity that the -183 promoter elicited when cells were maintained at 25.5 mM glucose is certainly due to the presence of L3 and L4 boxes. Indeed this effect of glucose was not observed when the -183 promoter was replaced by -96 and -54 promoters, which did not include these elements. Interestingly, in cells transfected with these constructs, neither the transfection of S14 (see Figure 3) nor that of Sp1 (see Figure 6) could induce a large increase in the promoter activity in the

absence of COUP-TF1. Moreover, since COUP-TF1 or S14 cannot substitute for Sp1 in binding to the L0 element, we hypothesize that S14 could also act like a coactivator or a stabilizer of the Sp1-COUP-TF1 complex bound to L0 through the dephosphorylated form of Sp1. This hypothesis is indirectly supported by the observation that L0-bound nuclear complexes prepared from the livers of rats fed a glucose-rich diet contained both S14 and COUP-TF1, although neither COUP-TF1 nor S14 could induce the binding of endogenous proteins when they supplemented nuclear extracts from livers of rats fed a standard diet.

Our data thus provide an example of protein-protein interactions conferring to COUP-TF1 a dual activity since, in the context of the -183 LPK promoter, the presence of Sp1 and S14 proteins induces the switch of COUP-TF1 from a repressor to an activator. Interestingly, a comparable mechanism has been revealed by studies on the variant HNF1 promoter [42]. Indeed in the proximal promoter of this gene, binding of COUP-TF1 to a direct repeat 1 ('DR1') sequence did not result in transactivation, but COUP-TF1 transactivated the promoter through recruitment of a neighbouring octameric site by physical interaction with Oct1. Therefore in this example too, COUP-TF1 exhibited transactivating properties when removed from its own DNA element and recruited into a proximal complex with a DNA-bound protein.

In the context of the L-PK promoter, whose expression has been shown to be T3-permissive [20], a large amount of S14 protein due to a high glucose intake and/or a high level of T3, could thus contribute to L-PK gene expression, by forming a multimeric complex including both COUP-TF1 and dephosphorylated Sp1. Given that the expression of S14 has been shown to be necessary for the full expression of lipogenic genes, it would be interesting to determine whether a similar mechanism is involved in their regulation. This might explain the co-ordinated synthesis of several genes, as well as the pleiotropic effects of T3 in hepatocytes.

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