Regulation of hepatic metabolic pathways by the orphan nuclear receptor SHP

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SHP (small heterodimer partner) is an important component of the feedback regulatory cascade, which controls the conversion of cholesterol to bile acids. In order to identify the bona fide molecular targets of SHP, we performed global gene expression profiling combined with chromatin immunoprecipitation assays in transgenic mice constitutively expressing SHP in the liver. We demonstrate that SHP affects genes involved in diverse biological pathways, and in particular, several key genes involved in consecutive steps of cholesterol degradation, bile acid conjugation, transport and lipogenic pathways. Sustained expression of SHP leads to the depletion of hepatic bile acid pool and a concomitant accumulation of triglycerides in the liver. The mechanism responsible for this phenotype includes SHP-mediated direct repression of downstream target genes and the bile acid sensor *FXRα*, and an indirect activation of *PPARγ* and *SREBP-1c* genes. We present evidence for the role of altered chromatin configurations in defining distinct gene-specific mechanisms by which SHP mediates differential transcriptional repression. The multiplicity of genes under its control suggests that SHP is a pleiotropic regulator of diverse metabolic pathways.

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Introduction

Bile acid synthesis represents the major pathway for the elimination of excess cholesterol from the body (Chiang, 2002, 2004; Russell, 2003). Bile acids, the end products of the cholesterol catabolic pathways, serve several important physiological functions, including solubilization of cholesterol, vitamins and other lipids in the intestine (Chiang, 2002, 2004; Russell, 2003). Because of their intrinsic toxicity,

intracellular bile acid levels are tightly controlled by a complex regulatory cascade, which modulates their own synthesis. The molecular target of this feedback regulatory loop has been shown to be the *CYP7A1* gene, encoding the rate-limiting enzyme of the ‘classical’ cholesterol catabolic pathway (Goodwin *et al*, 2000; Lu *et al*, 2000). Previous studies identified the nuclear receptor *FXRα* as the major hepatic bile acid sensor that governs bile acid synthesis and transport (Makishima *et al*, 1999; Parks *et al*, 1999; Wang *et al*, 1999). Bile acids are potent ligands of *FXRα*, which induces the expression of *SHP* (small heterodimer partner). Elevated levels of *SHP* in turn lead to transcriptional repression of the *CYP7A1* gene, by inhibiting the activity of the nuclear receptor *LRH-1* on the *CYP7A1* promoter (Goodwin *et al*, 2000; Lu *et al*, 2000). In line with this, mice deficient in *SHP* exhibit impaired feedback regulation of bile acid production, although compensatory, *SHP*-independent repression pathways can also operate (Kerr *et al*, 2002; Wang *et al*, 2002; Holt *et al*, 2003). *SHP* is an atypical orphan nuclear receptor, which lacks a DNA-binding domain (Seol *et al*, 1996). It contains an N-terminal receptor dimerization domain, which mediates its recruitment to promoters via interaction with various nuclear receptors. Previous *in vitro* studies have identified a number of potential interaction partners for *SHP*, including *LRH-1*, *HNF-4*, *ER*, *PPARs*, *PXR*, *CAR* and *NF-κB* (Seol *et al*, 1996, 1997; Johansson *et al*, 2000; Lee *et al*, 2000; Kim *et al*, 2001; Ourlin *et al*, 2003; Bae *et al*, 2004). These observations indicate that *SHP* may regulate a broad array of genes in various biological pathways. *SHP* is expressed at low levels in the liver and is transiently induced by bile acid treatment (Goodwin *et al*, 2000; Lu *et al*, 2000). Because bile acids can affect the expression of a variety of genes independently of *SHP* (Kerr *et al*, 2002; Wang *et al*, 2002; Holt *et al*, 2003), the identity of the bona fide targets of this nuclear receptor and its *in vivo* contribution to the feedback repression of genes involved in cholesterol catabolic cascade remained elusive.

To address this issue, we generated transgenic mice, which express *SHP* in the liver independently of bile acid signaling. Global gene expression profiling combined with chromatin immunoprecipitation (ChIP) assays identified a large number of target genes regulated by *SHP* in a direct or indirect manner. Detailed analysis of the genes involved in cholesterol and triglyceride metabolism revealed a central role of *SHP* in the regulatory network controlling their expression. Furthermore, we present *in vivo* evidence for distinct promoter-specific repression mechanisms, which may provide the molecular basis for the differential inhibition of individual genes by *SHP*.

Results

Transgenic mice expressing SHP in the liver

In order to obtain a comprehensive view of the genes affected by *SHP*, we generated transgenic mice constitutively expres-

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sing SHP in hepatocytes. The construct used for transgenesis contained a Flag-tagged human *SHP* cDNA in front of the transthyretin (*TTR*) promoter, which drives transgene expression, specifically in the liver (Yan *et al*, 1990). Of the several

transgenic lines obtained, we chose for further analysis those expressing SHP about seven-fold above the endogenous levels (Figure 1A and B), which correspond to the induction levels detected by bile acid treatment of primary hepatocytes (Goodwin *et al*, 2000). Transgene expression in the liver was evident after birth and reached maximum levels after weaning, when the animals were placed on a normal chow diet. The livers of transgenic animals were visibly enlarged and pale in color. As judged by immunostaining with α Flag antibody, transgene expression was uniform in all hepatocytes (Figure 1C). Hematoxylin–eosin (H&E) staining of liver sections revealed no gross morphological alterations in transgenic livers, although some eosinophilic intracytoplasmic vacuolization could be observed. On the other hand, the majority of hepatocytes in SHP-expressing liver sections stained positive with oil red O, pointing to an extensive lipid accumulation (Figure 1C). Consistent with the observed fatty-liver phenotype, hepatic triglyceride levels were markedly increased and the concentration of hepatic bile acids was significantly decreased (Table I). Interestingly, serum bile acid and triglyceride levels were similar in wild-type and SHP-Tg animals, while serum total cholesterol and HDL cholesterol levels were significantly reduced. In the sera of SHP-Tg mice, the concentrations of C4 (7 α -hydroxy-4-cholesten-3-one), the product of CYP7A1-catalyzed reaction (Russell, 2003), and β -sitosterol, a plant sterol supplied by ingestion (Russell, 2003), dropped to about 26 and 36% of the levels detected in wild-type animals, respectively. The serum levels of lanosterol, an intermediate of the *de novo* cholesterol synthesis pathway (Russell, 2003; Chiang, 2004), increased about two-fold (Table I). These results suggest that in SHP-Tg mice, major defects occur in bile acid synthesis, lipogenesis, *de novo* cholesterol biosynthesis and absorption pathways.

Genes affected by SHP expression in the liver

To investigate the changes in hepatic gene expression patterns caused by constitutive expression of SHP, we performed global transcriptional profiling using pools of RNA samples

Figure 1 SHP expression and histological analysis of SHP-Tg mice. (A) Quantitative RT-PCR analysis of RNAs from the livers of 60-day-old SHP-Tg mouse lines and nontransgenic wild-type littermates (control) was performed using primer sets amplifying both mouse and human SHP cDNA. Numbers at the bottom represent fold difference between GAPDH-normalized values, compared to the first control sample. (B) Western blot analysis of hepatic nuclear extracts from 60-day-old SHP-Tg and wild-type (control) mice was performed by using α SHP antibody, to detect total SHP protein, and α Flag antibody, to detect transgene-derived protein expression. (C) Liver sections from 60-day-old SHP-Tg and wild-type (control) mice were stained with H&E, oil red O and α Flag as indicated.

Table I Physiological parameters in SHP-Tg mice^a

	Wild type	SHP-Tg	SHP-Tg/wild type
Liver weight (g)	1.29 $\pm$ 0.17	1.55 $\pm$ 0.12*	1.20
% body weight	4.25 $\pm$ 0.28	6.28 $\pm$ 0.34**	1.47
<i>Serum</i>			
Triglycerides (mM)	0.32 $\pm$ 0.07	0.36 $\pm$ 0.14	
Total cholesterol (mM)	2.90 $\pm$ 0.24	1.90 $\pm$ 0.75*	0.65
Free cholesterol (mM)	0.41 $\pm$ 0.07	0.53 $\pm$ 0.20	
VLDL cholesterol (mM)	0.014 $\pm$ 0.01	0.031 $\pm$ 0.025	
LDL cholesterol (mM)	0.10 $\pm$ 0.02	0.13 $\pm$ 0.08	
HDL cholesterol (mM)	2.80 $\pm$ 0.2	1.70 $\pm$ 0.70*	0.60
C4 (nM)	142 $\pm$ 27	38 $\pm$ 10**	0.26
Lanosterol (nM)	320 $\pm$ 72	803 $\pm$ 298*	2.50
β -Sitosterol (nM)	3711 $\pm$ 485	1356 $\pm$ 329**	0.36
Bile acids (μ M)	12.3 $\pm$ 2.3	13.2 $\pm$ 2.2	
<i>Liver extract</i>			
Bile acids (μ mol/g liver)	2.49 $\pm$ 0.36	0.31 $\pm$ 0.06**	0.12
Triglycerides (μ g/g liver)	7.9 $\pm$ 1.2	19.4 $\pm$ 1.7**	2.45
Cholesterol (μ g/g liver)	2.86 $\pm$ 0.40	2.61 $\pm$ 0.33	

^aAll values are means $\pm$ standard deviations from five male animals, fasted for 8 h before specimen collection. Statistical analysis was performed by unpaired Student's *t*-test. **P* < 0.05; ***P* < 0.005.

Figure 2 Analysis of the mRNA levels of genes involved in bile acid (A) and cholesterol (B) homeostasis. RT-PCR assays were performed with liver RNA prepared from 60-days-old SHP-Tg and wild-type (control) mice. The values obtained were normalized to GAPDH values and are expressed as fold change compared to the wild-type data. Bars represent mean values and standard deviations from RNA samples of at least five individual mice. * $P < 0.05$; ** $P < 0.01$. Black bars, control animals. Gray bars, SHP-Tg animals.

prepared from wild-type and SHP-Tg livers. As shown in Supplementary Table 1, a large number of genes involved in diverse biological pathways were affected. These include genes playing roles in various metabolic, stress and inflammatory response, transport, detoxification, cell cycle, signaling, cell adhesion and transcriptional control processes. Using a filter of two-fold change as the cutoff, we found that 67 genes were downregulated and 48 genes were upregulated in SHP-Tg livers.

Several of these genes participate in cholesterol–bile acid and lipid homeostasis, indicating that the observed phenotype of SHP-Tg animals is associated with defects in the expression of multiple genes involved in the regulation of these pathways. Therefore, our subsequent analyses were focused on these particular pathways. In order to validate the microarray results, we performed quantitative RT-PCR assays using liver RNA samples from individual animals. As expected, *CYP7A1* was downregulated in SHP-Tg mice (Figure 2A). Interestingly however, *CYP8B1* and *CYP7B1*, genes encoding two downstream enzymes of the classical and alternative bile acid biosynthetic pathway, were more sensitive to SHP-dependent repression than *CYP7A1*. Similar changes were observed in their protein levels (Supplementary Figure 2). The mRNA levels of bile acid-CoA:amino-acid *N*-acyltransferase (*BAT*), involved in the more downstream event of bile acid conjugation, were also significantly decreased in SHP-Tg livers (Figure 2A). These results show that SHP inhibits several key steps of the bile acid production cascade, pointing to its central role in the negative feedback regulatory pathway that prevents accumulation of bile acids beyond physiological levels. On the other hand, we detected

decreased expression of genes coding for bile salt export pumps (*BSEP* and *MDR2*), and also for the *NTCP* gene, whose product is the major mediator of hepatic basolateral bile acid uptake (Trauner and Boyer, 2003) (Figure 2A). Collectively, the above changes explain the decreased hepatic bile acid pool and the essentially unaffected serum bile acid levels in SHP-Tg mice (Table I). In this respect, we note that, although the hepatic bile acid pool size was greatly reduced, it was not entirely diminished. The residual concentrations of hepatic bile acids may arise from the downregulation of the major bile acid export pump, *BSEP*, and the unaltered expression of the gene encoding the second bile acid import protein *OATP* (Trauner and Boyer, 2003) (Figure 2A).

Analysis of other genes involved in cholesterol and lipid homeostasis revealed an interesting pattern. While the expression levels of apolipoproteins, microsomal triglyceride transfer protein (*MTP*), liver fatty acid-binding protein (*L-FABP*) and the LDL receptor were not affected, reciprocal changes in the expression of the HDL receptor (Trigatti *et al*, 2003) *SR-BI* and the *ABCA-1* transporter (Knight, 2004) were detected in SHP-Tg livers (Figure 2B). These differences are expected to result in decreased reverse cholesterol uptake and increased cholesterol efflux from hepatocytes, which should be manifested in hypercholesterolemia. However, serum cholesterol levels in SHP-Tg animals were decreased, suggesting that the changes in *SR-BI* and *ABCA-1* expression may correspond to compensatory mechanisms aimed at the acquisition of balanced concentrations of cholesterol. The main mechanism by which serum cholesterol levels decrease in SHP-Tg mice most likely involves impaired absorption, as suggested by the decreased serum sitosterol levels, a plant sterol, exclusively supplied by food ingestion (Table I). Impaired absorption is also an expected outcome of decreased bile acid production and secretion. In addition, we measured lanosterol levels in the sera to estimate the effects of SHP on *de novo* cholesterol synthesis. Lanosterol levels were increased in SHP-Tg mice (Table I), which could be interpreted as an increased *de novo* synthesis activity consequent to reduced absorption. However, our gene expression analysis suggests that the observed lanosterol accumulation is a consequence of the decreased expression of the lanosterol demethylase (*CYP51b*), rather than stimulation of *de novo* cholesterol synthesis, as judged by the unaltered expression of *HMG-CoA* reductase, the rate-limiting enzyme of the pathway (Figure 2B). In contrast to serum, the levels of cholesterol in the liver were not significantly altered in SHP-Tg mice (Table I). This could be due to the opposing effects of reduced degradation, which is expected to increase levels, and the reduced uptake, or *de novo* synthesis, which are expected to decrease the concentration of hepatic cholesterol.

In order to understand the molecular basis of the fatty liver phenotype in SHP-Tg mice, we studied the expression of several lipogenic genes. As shown in Figure 3A, the fatty acid translocase (*CD36*), which facilitates the uptake of long-chain fatty acids, was highly upregulated in SHP-Tg livers. Furthermore, the mRNA levels of other genes involved in fatty acid and triglyceride biosynthesis, such as the fatty acid synthase (*FAS*), ATP citrate lyase (*ACL*), acetyl-CoA carboxylase (*ACC-1*) and stearoyl-CoA reductase (*SCD1*), were also increased significantly (Figure 3A). These findings suggest that the elevated levels of hepatic triglycerides (Table I) and the positive oil red O staining of SHP-Tg livers (Figure 1) are

Figure 3 Analysis of the mRNA levels of genes involved in lipogenesis (A) and transcription factors (B). The results are presented as in the legend of Figure 2. Black bars, control animals. Gray bars, SHP-Tg animals.

the result of an increased expression of several lipogenic genes.

Next, we tested the expression of the major transcription factors, which have been implicated in the regulation of the studied genes. In agreement with the previously proposed autoregulatory loop (Goodwin *et al*, 2000; Lu *et al*, 2000), we found that the expression of endogenous *SHP* was severely downregulated in SHP-Tg mice. Interestingly however, the mRNA levels of *FXR α* , the main inducer of *SHP* and other genes in response to bile acids, were also significantly reduced (Figure 3B). This finding defines a novel component of the crossregulatory network controlling bile acid homeostasis. We did not observe significant differences between wild-type and SHP-Tg mice in the expression of *LRH-1*, *HNF-4 α* , *LXR α* , *HNF-1 α* , *CAR*, *PXR*, *RXR α* and *PPAR α* . On the other hand, the expression of *SREBP-1c* and *PPAR γ 1*, which encode the major regulators of lipogenic genes (Shimano *et al*, 1996; Osborne, 2000; Kersten, 2001; Herzig *et al*, 2003; Yu *et al*, 2003), was upregulated (Figure 3B). A schematic summary of the above regulatory effects is shown in Figure 5.

Molecular mechanisms involved in SHP-dependent repression and activation of target genes

In order to determine whether SHP is involved directly or indirectly in the repression or activation of the above genes, we analyzed the recruitment of SHP to the corresponding regulatory regions by ChIP assays. In parallel, we studied the occupancy of the respective promoters by HNF-4 α and LRH-1, two SHP-interacting factors, which have been implicated in the regulation of the studied genes (Repa and Mangelsdorf, 2000; Hayhurst *et al*, 2001; Schoonjans *et al*, 2002; del

Castillo-Olivares *et al*, 2004; Fayard *et al*, 2004; Inoue *et al*, 2004; Pare *et al*, 2004). In wild-type animals, HNF-4 α could be detected in all genes that were downregulated by SHP. LRH-1 occupancy was also evident in most genes, except those of *NTCP* and *SR-BI* (Figure 4A). In SHP-Tg mice, both factors dissociated from the *CYP8B1* and *CYP7B1* genes, but remained associated with the other promoters. Importantly, with the exception of the *SR-BI* gene, we could detect SHP recruitment into the regulatory regions of all downregulated genes only in samples from SHP-Tg livers, suggesting that SHP is directly involved in their repression.

Previous *in vitro* studies suggested that a multistep mechanism is involved in SHP-dependent transcriptional repression (Boulias and Talianidis, 2004). This includes coactivator competition, histone deacetylation, followed by histone 3 (H3)-K9 methylation and stable association of SHP itself with chromatin. In principle, any of the above individual events should result in efficient transcriptional repression, which raised the theoretical possibility that depending on the promoter context of different genes, SHP-mediated repression may involve one or more of the above steps. *In vivo* evidence for such gene-specific, differential mode of action was obtained by the more detailed analysis of the *CYP7A1*, *SHP* and *CYP8B1* promoters (Figure 4B). As shown above, the three regulatory regions were occupied by SHP in SHP-Tg mice, but HNF-4 and LRH-1 occupancy was preserved only on the *CYP7A1* and *SHP* genes. CBP and RNA pol-II dissociated from all promoters in SHP-Tg animals; however, HDAC-1 recruitment, H3 deacetylation and H3-K9 methylation were observed only at the regulatory regions of the *SHP* and *CYP8B1* genes (Figure 4B). These findings suggest that the mechanism of *CYP7A1* repression involves coactivator (e.g. CBP) competition but not subsequent deacetylation and H3-K9 methylation as observed for the *SHP* and *CYP8B1* genes. A further distinction can be made between the mechanisms operating in the repression of *SHP* gene, where both interacting partners (HNF-4 and LRH-1) are present on the promoter under repressive conditions, and that functioning on the *CYP8B1* gene where HNF-4 and LRH-1 are absent. For this latter case, the results suggest that the alteration in chromatin structure leads to complete dissociation of transcription complex components, with the exception of SHP and HDAC-1. SHP remains associated with the promoter, probably via its intrinsic ability to interact with underacetylated or K9-methylated H3 (Boulias and Talianidis, 2004). In light of the functioning of distinct repression mechanisms on different genes, we note that in SHP-Tg mice the expression of the *CYP7A1* gene was repressed to a lesser extent than that of *SHP* and *CYP8B1*. This points to a possible correlation between the degree of inhibition and the actual repression mechanism employed, depending on the contribution of histone deacetylation and H3-K9 methylation in the process. In line with this, we observed an occupancy pattern similar to that found in *CYP7A1* gene at the promoters of *BAT*, *BSEP*, *NTCP* and *FXR* genes, which were downregulated to a lesser extent, and an occupancy pattern similar to that found in *CYP8B1* gene at the promoter of *CYP7B1*, which was more dramatically downregulated in SHP-Tg mice (data not shown).

We also examined SHP occupancy on selected lipogenic genes, whose expression was increased in SHP-Tg mice. The lack of recruitment of SHP to the *SREBP-1c*, *CD36* and *FAS* promoters points to an indirect effect (Figure 4C). In SHP-Tg

Figure 4 Analysis of transcription factor recruitment (A, C), RNA pol-II occupancy, and chromatin modifications (B). Soluble, formaldehyde-crosslinked chromatin was prepared from the livers of 60-days-old SHP-Tg and wild-type (control) mice and subjected to immunoprecipitations with the indicated antibodies. The DNAs in the precipitates were analyzed by PCR using primer sets amplifying the indicated promoter regions of different genes. Left: Autoradiogram of a representative ChIP experiment from one control and one SHP-Tg mouse. Right: Quantitative analyses of experiments performed on three different control and three different SHP-Tg samples. All data, obtained with liver extracts from individual animals, were first normalized to the corresponding input values (% input) and expressed as fold enrichment over the values obtained with control antibody. Bars represent average fold enrichments calculated from the three experiments. The error bars indicate variations between the signals obtained in samples from three individual mice. White bars, control animals. Black bars, SHP-Tg animals.

Discussion

(Watanabe *et al*, 2004). The bile acid sensor FXR α activates the expression of the orphan nuclear receptor SHP, which is a repressor of *CYP7A1* gene coding for the rate-limiting enzyme of bile acid synthesis (Goodwin *et al*, 2000; Lu *et al*, 2000).

The aim of this study was to obtain a comprehensive view of bona fide target genes regulated by SHP. The results suggest that SHP is a pleiotropic regulator, influencing the expression of several genes involved in diverse biological processes, including regulation of metabolic pathways, stress and inflammatory response, detoxification and cell cycle control. Detailed analysis of the genes involved in cholesterol and lipid homeostasis revealed a central role of SHP in a complex regulatory circuit, schematically presented in Figure 5. The results demonstrate that SHP represses genes of the bile acid synthesis, conjugation and transport pathway, as well as genes involved in cholesterol uptake and synthesis. Several genes coding for consecutive steps of the bile acid production cascade are repressed by SHP, suggesting that SHP action is important for the complete blocking of the entire pathway.

The identification of the *FXR α* gene as a direct target of SHP extends previous models aimed at comprehending the regulatory cascades involved in bile acid homeostasis, by indicating a role for SHP in the recently discovered alternative bile acid-induced feedback regulatory mechanism (Holt *et al.*, 2003). *FXR α* is the main activator of the *SHP* gene in response to bile acids and other genes like *BSEP* and *FGF19* (Sinal *et al.*, 2000; Holt *et al.*, 2003). Accordingly, bile acid-mediated activation of *FXR α* leads to the induction of two signaling cascades. The first operates through activation of *FGF19*, which would signal JNK activation in neighboring cells (Holt *et al.*, 2003), while the other involves induction of *SHP* expression in the same cell (Goodwin *et al.*, 2000; Lu *et al.*, 2000). Once SHP levels increase to a critical point, it will also repress *FXR α* expression and consequently the *FXR α* -*FGF19* pathway, thus limiting its function to a short time

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period. Under physiological conditions, SHP-dependent regulation is also expected to be transient, as suggested by its strong repressive effect on its own gene. Downregulation of *FXR α* may also contribute to the decline of *SHP* expression. Taken together, we propose that SHP may play an auto-feedback regulatory role in both inhibitory pathways, which are central for the acquisition of balanced intracellular levels of cholesterol and bile acids.

In mice, *FXR α* mRNAs are produced by alternative splicing of exon 5 and the use of two alternative promoters that drive transcription from either exon 1 or 3 (Zhang *et al*, 2003). Previous studies have shown that upon fasting, the *FXR α 3 α 4* variant is selectively induced by the action of PGC-1 on the internal promoter, which plays a role in the fasting-dependent decrease of triglyceride synthesis (Zhang *et al*, 2004). Interestingly however, fasting and PGC-1 overexpression also increased SHP expression independently of FXR (Zhang *et al*, 2004). Our ChIP experiments suggest that SHP can be recruited to both *FXR α 1 α 2* and *FXR α 3 α 4* promoters, which is consistent with the downregulation of all FXR isoforms in SHP-Tg mice. Therefore, we speculate that upon fasting conditions, SHP may play a modulatory role in controlling PGC-1-mediated induction of *FXR α* .

Consistent with the molecular characteristic of SHP as a transcriptional repressor, we could detect SHP on the promoters of most downregulated genes. On the contrary, it was not recruited to any of the genes that were upregulated in SHP-Tg mice, suggesting that activation of these genes is due to an indirect effect. Among the genes induced in SHP-Tg mice, those involved in the lipogenesis pathway are of special interest. The role of SHP and the LXR α -SREBP1 regulatory axis in the reciprocal relationship between bile acid synthesis and triglyceride production has recently been put forward (Watanabe *et al*, 2004). In mice fed cholic acid, the hepatic expression of *SREBP-1c* and several other lipogenic genes was downregulated, an effect that was compromised in mice lacking SHP (Watanabe *et al*, 2004). In agreement with these findings, we observed that reduction of hepatic bile acid pool size in SHP-Tg mice correlates with the upregulation of *SREBP-1c* and lipogenic gene expression, with a concomitant accumulation of triglycerides in the liver. However, in our animal model, SHP is expressed at levels that mimic bile acid-induced conditions. Therefore, our results suggest that increased expression of SHP can also induce the activation of *SREBP-1c* and lipogenic genes by an indirect mechanism, which does not involve its recruitment to the corresponding gene regulatory regions. As outlined above, *SREBP-1c* activation is likely to be mediated by the action of SHP on cholesterol catabolic enzymes, thus influencing the intracellular concentration of metabolic intermediates that activate LXR α . This, points to a dual role for SHP in the regulation of *SREBP-1c* and lipogenic genes. Depending on the actual intracellular metabolic milieu, SHP may repress *SREBP-1c* expression, as indicated by studies in cholic acid-fed mice (Watanabe *et al*, 2004), or it may induce the lipogenic pathway through the activation of LXR α when it is expressed for a long period of time, as is the case in our SHP-Tg animal model.

The genes responsible for the fatty liver phenotype of SHP-Tg mice (lipogenic genes, CD36) are similar to those found in SREBP-1-Tg mice (Horton *et al*, 2003) and in mice where PPAR γ 1 was overexpressed by adenovirus-mediated gene

transfer (Yu *et al*, 2003). In the latter animal model however, induction of several adipocyte-specific genes (aP2, adipsin, adiponectin) was also implicated in the fatty liver phenotype. In SHP-Tg mice, we did not observe significant changes in the levels of adipocyte-specific genes (data not shown), suggesting that the fatty liver phenotype is solely mediated by the upregulation of fatty acid translocase CD36 and lipogenesis-related genes. We speculate that the approximate 3.5-fold increase of endogenous PPAR γ 1, observed in SHP-Tg mice, is sufficient for the upregulation of certain already expressed genes, like CD36, but not for the expression of adipocyte-specific genes in the liver, which may require very high levels of PPAR γ , such as those achieved by Ad-PPAR γ 1 infection (Horton *et al*, 2003).

Another important conclusion of this study concerns the gene-specific molecular repression mechanisms, schematically presented in Supplementary Figure 1. Selective associations of SHP with promoters provide the first level of specificity. Since SHP does not bind to DNA, it is recruited to promoters via interactions with other nuclear receptors. HNF-4 α and LRH-1 are the best-studied factors that provide a molecular platform for targeting SHP into regulatory regions (Goodwin *et al*, 2000; Lee *et al*, 2000; Lu *et al*, 2000). We consistently found occupancy by one or both of these factors in promoters where SHP was recruited. However, SHP did not associate with all promoters occupied by HNF-4, like *SR-BI* and others (data not shown), suggesting that the transcription initiation complex formed on HNF-4-containing regions can influence the recruitment of SHP. With respect to genes where SHP is recruited, the context of the particular promoter may provide an additional specificity by influencing different steps of the repression mechanism. SHP has the ability to displace coactivator proteins from nuclear receptors (Seol *et al*, 1997; Johansson *et al*, 2000; Lee *et al*, 2000; Kemper *et al*, 2004), functionally interact with the histone deacetylase HDAC-1-containing complexes (Kemper *et al*, 2004), the H3-K9 methylase G9a and with underacetylated or K9-methylated H3 (Boulias and Talianidis, 2004). As shown in this study, one or more of the above steps are utilized in distinct regulatory regions. In certain genes, coactivator displacement is not followed by histone deacetylation and methylation, while in others, like in the case of the *CYP8B1* gene, H3 deacetylation and H3-K9 methylation correlate with the dissociation of HNF-4 and LRH-1 from the promoter. In support of the above, it has been demonstrated that *in vitro* binding of LRH-1 to the *CYP8B1* promoter is inhibited by SHP, whereas binding to the *CYP7A1* promoter was not affected (del Castillo-Olivares and Gil, 2001). The selective recruitment of SHP and the molecular decisions between the actual repression mechanisms employed are likely to be governed by the availability of specific interaction surfaces for coregulator recruitment on different genes. They may also provide the molecular basis of differential repression of individual genes. For example, the participation of H3-K9 methylation in the repression mechanism in some genes may influence the potential of its reactivation when SHP expression declines, as it is considered a stable repressive mark whose elimination requires histone exchange (Kouzarides, 2002; Vermaak *et al*, 2003). Thus, we propose that distinct repressive promoter complexes and chromatin configurations may play a determining role in regulating the extent and possibly the duration of transcriptional inhibition.

Materials and methods

Transgenic mice and histological analysis

The Flag epitope containing human SHP cDNA (Boulias and Talianidis, 2004) was inserted into the *StuI* site of the pTTR1-ExV3 plasmid (Yan *et al*, 1990). The 6.2 kb *HindIII* fragment containing the mouse transthyretin enhancer/promoter, intron 1, Flag-SHP cDNA and SV40 poly-A site was used to microinject CBA-CaX57Bl/10 fertilized oocytes. Founder animals were identified by Southern blotting and crossed with F1 CBA-CaX57Bl/10 mice to generate lines. Transgenic lines expressing SHP about seven-fold above endogenous levels were maintained in grouped cages in temperature-controlled (23°C) virus-free facility on a 12 h light/dark cycle and were fed a standard rodent chow diet (4RF21, Mucedola) and water *ad libitum*. About 1/4 of the animals died between 2 and 4 weeks of age, while the rest survived at least 6 months. All of the experiments were performed with mice at 2 months of age.

For histological analysis, livers were fixed in 4% paraformaldehyde and embedded in paraffin. Liver sections (8–10 mm thick) were used for staining with H&E. Frozen sections of formalin-fixed livers were stained with oil red O (0.3% in isopropanol) for 10 min and washed with excess water. For immunostaining with α Flag antibody, frozen liver sections were fixed in methanol, blocked with 1% BSA containing PBS, followed by incubation with a polyclonal Flag antibody (Sigma) and AlexaFluor568 (Molecular Probes) secondary antibody as described (Kistaki and Talianidis, 1997). Light and fluorescence images were observed using Zeiss Axioscope 2 Plus microscope.

Serum and tissue chemistry

Serum samples were prepared from whole blood, collected from the hearts of anesthetized animals. Hepatic extracts were prepared from whole livers containing the gall bladder by homogenization either in 75% ethanol or in chloroform/methanol (2:1). The ethanol extracts were incubated for 2 h at 50°C and after centrifugation, the supernatants were further diluted with 75% ethanol and then with 25% PBS and used for measurement of bile acids using the colorimetric assay kit from Trinitron. Chloroform/methanol extracts were centrifuged at 3000 r.p.m. and after the addition of 1/5 volume of 0.9% NaCl, the supernatants were recentrifuged at 2000 r.p.m. The lower organic phase containing lipids was lyophilized. After reconstitution in 1% Triton X containing PBS, the samples were used for triglyceride measurements.

The assays for plasma triglycerides and total cholesterol were performed using a Cobas Mira analyzer and reagent systems (Roche). Free cholesterol levels were determined using an enzymatic colorimetric method from Waco Chemicals. The cholesterol distribution profiles were measured by using a size-exclusion high-performance liquid chromatography system, SMART, with Superose® 6 PC 3.2/30 column (Amersham Pharmacia Biotechn.). Data were integrated using a Chromeleon chromatography data system (Gynkotek HPLC, Germering). The distribution of lipoproteins was continuously measured as total cholesterol using enzymatic colorimetric reagents. The integrated area of the fractions was expressed in molar concentration. The various peaks in the profiles were designated VLDL, LDL and HDL for simplicity, even though it was clear that the separation was determined primarily by the size of the lipoproteins.

RNA preparation and expression profiling

Total RNA was prepared by the guanidinium isothiocyanate extraction method and, after digestion with DNase I, was further purified by using the RNeasy kit from Quiagen. Reverse transcription and quantitative RT-PCR assays were performed as described previously (Kouskouti *et al*, 2004). The nucleotide sequence of primers sets is shown in Supplementary Table 2.

For microarray analysis, two wild-type and two SHP-Tg RNA pools were prepared. Each pool consisted of equal amounts of five

individual RNA samples from male animals. cDNA was generated by reverse transcription and subsequently a Cy-dye label incorporated using the Klenow fragment (Smith *et al*, 2003). Each cDNA sample was split into two, one half labeled with Cy3-dCTP and the other half with Cy5-dCTP. Reciprocally labeled wild-type and SHP-Tg cDNA samples were then mixed to generate four different target combinations. These targets were used for hybridization of Mouse Known Gene SGC Oligonucleotide Arrays microarrays, comprising 7500 known mouse genes (http://www.hgmp.mrc.ac.uk/Research/Microarray/HGMP-RC_Microarrays/description_of_arrays.jsp#3). Hybridization and washing were performed as described (Smith *et al*, 2003). After washing, the slides were scanned using the Affymetrix 428 scanner and data extracted with ImaGene 4.2 (BioDiscovery). Normalization was performed using a two-dimensional loess function to eliminate spatial variation, as implemented in the YASMA (yet another statistical microarray analysis) package for R. Data were then imported into GeneSpring (Silicon Genetics) for further analysis. Potential differentially expressed genes were selected using a filter of above 1.5-fold change in three of the four hybridizations. These data have been deposited in the ArrayExpress microarray database of the European Bioinformatics Institute (<http://www.ebi.ac.uk/arrayexpress/Experiment code: E-MEXP-294>).

Chromatin immunoprecipitation

In order to crosslink the transcription complexes in their native tissue environment, anesthetized mice were perfused by inserting a catheter into portal vein and cutting the vena cava inferior. The livers were perfused successively with PBS, 1% formaldehyde and 0.125 M glycine, for 10 min each. Crosslinked nuclei were purified by centrifugation through a sucrose gradient as described (Soutoglou *et al*, 2001). Immunoprecipitations, washings and PCR analysis were performed as described (Hatzis and Talianidis, 2002; Soutoglou and Talianidis, 2002). Crosslinked liver extracts from three individual wild-type and three individual SHP-Tg mice were processed and quantified separately. The antibodies used in this study were as follows: mouse polyclonal SHP antibody was prepared by immunization of Balb-C mice with recombinant His-tagged, full-length SHP protein purified from *Escherichia coli* under denaturing conditions. After three boosts in 1 month intervals, sera were collected and tested in different applications. A rabbit polyclonal antibody against LRH-1 was raised by immunization of New Zealand White female rabbits with recombinant full-length human LRH-1 protein, purified under native conditions. The antibody against HNF-4 α has been described (Hatzis and Talianidis, 2001). Antigen specificities were confirmed by IP-Western blot assays with crosslinked chromatin or native nuclear extracts and by competition experiments. Antibodies against CBP, RNA pol-II, SREBP1, PPAR γ 1, LXR α , CYP7A1, CYP7B1 and CYP8B1 were from Santa Cruz Biotechnology. Anti-HDAC1, anti-dimethyl H3-K9, anti-acetyl-H3 and anti-acetyl-H4 were from Upstate Biotechnology.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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References

Bae Y, Kemper JK, Kemper B (2004) Repression of CAR-mediated transactivation of CYP2B genes by the orphan nuclear receptor, short heterodimer partner (SHP). *DNA Cell Biol* **23**: 81–91

Boulias K, Talianidis I (2004) Functional role of G9a-induced histone methylation in small heterodimer partner-mediated transcriptional repression. *Nucleic Acids Res* **32**: 6096–6103

- Chiang JY (2002) Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* **23**: 443–463
- Chiang JY (2004) Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms. *J Hepatol* **40**: 539–551
- del Castillo-Olivares A, Campos JA, Pandak WM, Gil G (2004) The role of alpha1-fetoprotein transcription factor/LRH-1 in bile acid biosynthesis: a known nuclear receptor activator that can act as a suppressor of bile acid biosynthesis. *J Biol Chem* **279**: 16813–16821
- del Castillo-Olivares A, Gil G (2001) Suppression of sterol 12alpha-hydroxylase transcription by the short heterodimer partner: insights into the repression mechanism. *Nucleic Acids Res* **29**: 4035–4042
- Fayard E, Auwerx J, Schoonjans K (2004) LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. *Trends Cell Biol* **14**: 250–260
- Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Wilson TM, Kliewer SA (2000) A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol Cell* **6**: 517–526
- Hatzis P, Talianidis I (2001) Regulatory mechanisms controlling human hepatocyte nuclear factor 4alpha gene expression. *Mol Cell Biol* **21**: 7320–7330
- Hatzis P, Talianidis I (2002) Dynamics of enhancer–promoter communication during differentiation-induced gene activation. *Mol Cell* **10**: 1467–1477
- Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ (2001) Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol Cell Biol* **21**: 1393–1403
- Herzig S, Hedrick S, Morante I, Koo SH, Galimi F, Montminy M (2003) CREB controls hepatic lipid metabolism through nuclear hormone receptor PPAR-gamma. *Nature* **426**: 190–193
- Holt JA, Luo G, Billin AN, Bisi J, McNeill YY, Kozarsky KF, Donahee M, Wang da Y, Mansfield TA, Kliewer SA, Goodwin B, Jones SA (2003) Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev* **17**: 1581–1591
- Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL (2003) Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc Natl Acad Sci USA* **100**: 12027–12032
- Inoue Y, Yu AM, Inoue J, Gonzalez FJ (2004) Hepatocyte nuclear factor 4alpha is a central regulator of bile acid conjugation. *J Biol Chem* **279**: 2480–2489
- Johansson L, Bavner A, Thomsen JS, Farnegardh M, Gustafsson JA, Treuter E (2000) The orphan nuclear receptor SHP utilizes conserved LXXLL-related motifs for interactions with ligand-activated estrogen receptors. *Mol Cell Biol* **20**: 1124–1133
- Kemper JK, Kim H, Miao J, Bhalla S, Bae Y (2004) Role of an mSin3A-Swi/Snf chromatin remodeling complex in the feedback repression of bile acid biosynthesis by SHP. *Mol Cell Biol* **24**: 7707–7719
- Kerr TA, Saeki S, Schneider M, Schaefer K, Berdy S, Redder T, Shan B, Russell DW, Schwarz M (2002) Loss of nuclear receptor SHP impairs but does not eliminate negative feedback regulation of bile acid synthesis. *Dev Cell* **2**: 713–720
- Kersten S (2001) Mechanisms of nutritional and hormonal regulation of lipogenesis. *EMBO Rep* **2**: 282–286
- Kim YS, Han CY, Kim SW, Kim JH, Lee SK, Jung DJ, Park SY, Kang H, Choi HS, Lee JW, Pak YK (2001) The orphan nuclear receptor small heterodimer partner as a novel coregulator of nuclear factor-kappa b in oxidized low density lipoprotein-treated macrophage cell line RAW 264.7. *J Biol Chem* **276**: 33736–33740
- Knight BL (2004) ATP-binding cassette transporter A1: regulation of cholesterol efflux. *Biochem Soc Trans* **32**: 124–127
- Kouskouti A, Scheer E, Staub A, Tora L, Talianidis I (2004) Gene-specific modulation of TAF10 function by SET9-mediated methylation. *Mol Cell* **14**: 175–182
- Kouzarides T (2002) Histone methylation in transcriptional control. *Curr Opin Genet Dev* **12**: 198–209
- Ktistaki E, Talianidis I (1997) Modulation of hepatic gene expression by hepatocyte nuclear factor 1. *Science* **277**: 109–112
- Latasa MJ, Griffin MJ, Moon YS, Kang C, Sul HS (2003) Occupancy and function of the –150 sterol regulatory element and –65 E-box in nutritional regulation of the fatty acid synthase gene in living animals. *Mol Cell Biol* **23**: 5896–5907
- Lee YK, Dell H, Dowhan DH, Hadzopoulou-Cladaras M, Moore DD (2000) The orphan nuclear receptor SHP inhibits hepatocyte nuclear factor 4 and retinoid X receptor transactivation: two mechanisms for repression. *Mol Cell Biol* **20**: 187–195
- Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J, Mangelsdorf DJ (2000) Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol Cell* **6**: 507–515
- Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ, Shan B (1999) Identification of a nuclear receptor for bile acids. *Science* **284**: 1362–1365
- Osborne TF (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. *J Biol Chem* **275**: 32379–32382
- Ourlin JC, Lasserre F, Pineau T, Fabre JM, Sa-Cunha A, Maurel P, Vilarem MJ, Pascussi JM (2003) The small heterodimer partner interacts with the pregnane X receptor and represses its transcriptional activity. *Mol Endocrinol* **17**: 1693–1703
- Pare JF, Malenfant D, Courtemanche C, Jacob-Wagner M, Roy S, Allard D, Belanger L (2004) The fetoprotein transcription factor (FTF) gene is essential to embryogenesis and cholesterol homeostasis and is regulated by a DR4 element. *J Biol Chem* **279**: 21206–21216
- Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD, Lehmann JM (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* **284**: 1365–1368
- Repa JJ, Mangelsdorf DJ (2000) The role of orphan nuclear receptors in the regulation of cholesterol homeostasis. *Annu Rev Cell Dev Biol* **16**: 459–481
- Russell DW (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* **72**: 137–174
- Schoonjans K, Annicotte JS, Huby T, Botrugno OA, Fayard E, Ueda Y, Chapman J, Auwerx J (2002) Liver receptor homolog 1 controls the expression of the scavenger receptor class B type I. *EMBO Rep* **3**: 1181–1187
- Seol W, Choi HS, Moore DD (1996) An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. *Science* **272**: 1336–1339
- Seol W, Chung M, Moore DD (1997) Novel receptor interaction and repression domains in the orphan receptor SHP. *Mol Cell Biol* **17**: 7126–7131
- Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL (1996) Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. *J Clin Invest* **98**: 1575–1584
- Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G, Gonzalez FJ (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**: 731–744
- Smith L, Underhill P, Pritchard C, Tymowska-Lalanne Z, Abdul-Hussein S, Hilton H, Winchester L, Williams D, Freeman T, Webb S, Greenfield A (2003) Single primer amplification (SPA) of cDNA for microarray expression analysis. *Nucleic Acids Res* **31**: e9
- Soutoglou E, Talianidis I (2002) Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. *Science* **295**: 1901–1904
- Soutoglou E, Viollet B, Vaxillaire M, Yaniv M, Pontoglio M, Talianidis I (2001) Transcription factor-dependent regulation of CBP and P/CAF histone acetyltransferase activity. *EMBO J* **20**: 1984–1992
- Trauner M, Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* **83**: 633–671
- Trigatti BL, Krieger M, Rigotti A (2003) Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol* **23**: 1732–1738
- Vermaak D, Ahmad K, Henikoff S (2003) Maintenance of chromatin states: an open-and-shut case. *Curr Opin Cell Biol* **15**: 266–274
- Wang H, Chen J, Hollister K, Sowers LC, Forman BM (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* **3**: 543–553
- Wang L, Lee YK, Bundman D, Han Y, Thevananthar S, Kim CS, Chua SS, Wei P, Heyman RA, Karin M, Moore DD (2002) Redundant pathways for negative feedback regulation of bile acid production. *Dev Cell* **2**: 721–731
- Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD, Auwerx J (2004) Bile acids lower

- triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *J Clin Invest* **113**: 1408–1418
- Yan C, Costa RH, Darnell Jr JE, Chen JD, Van Dyke TA (1990) Distinct positive and negative elements control the limited hepatocyte and choroid plexus expression of transthyretin in transgenic mice. *EMBO J* **9**: 869–878
- Yu S, Matsusue K, Kashireddy P, Cao WQ, Yeldandi V, Yeldandi AV, Rao MS, Gonzalez FJ, Reddy JK (2003) Adipocyte-specific gene expression and adipogenic steatosis in the mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression. *J Biol Chem* **278**: 498–505
- Zhang Y, Castellani LW, Sinal CJ, Gonzalez FJ, Edwards PA (2004) Peroxisome proliferator-activated receptor- γ coactivator 1 α (PGC-1 α) regulates triglyceride metabolism by activation of the nuclear receptor FXR. *Genes Dev* **18**: 157–169
- Zhang Y, Kast-Woelbern HR, Edwards PA (2003) Natural structural variants of the nuclear receptor farnesoid X receptor affect transcriptional activation. *J Biol Chem* **278**: 104–110