

REPORT

Core transcriptional regulatory circuitry in human hepatocytes

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Received 7.11.05; accepted 24.2.06

We mapped the transcriptional regulatory circuitry for six master regulators in human hepatocytes using chromatin immunoprecipitation and high-resolution promoter microarrays. The results show that these regulators form a highly interconnected core circuitry, and reveal the local regulatory network motifs created by regulator–gene interactions. Autoregulation was a prominent theme among these regulators. We found that hepatocyte master regulators tend to bind promoter regions combinatorially and that the number of transcription factors bound to a promoter corresponds with observed gene expression. Our studies reveal portions of the core circuitry of human hepatocytes.

Molecular Systems Biology 2 May 2006; doi:10.1038/msb4100059

Subject Categories: functional genomics; metabolic and regulatory networks

Keywords: autoregulation; hepatocyte; transcriptional regulation; regulatory hierarchy; chromatin immunoprecipitation

Introduction

The liver performs a number of complex functions essential for life including the uptake and storage of glucose, synthesis of bile acids, production of plasma proteins, and drug detoxification. These functions are carried out by hepatocytes, which comprise the bulk of the liver tissue. We and others have begun to use genome-scale approaches to determine the transcriptional regulatory circuitry of hepatocytes (Friedman *et al.*, 2004; Odom *et al.*, 2004; Phuc Le *et al.*, 2005; Rubins *et al.*, 2005; Zhang *et al.*, 2005). These studies have been limited because they focused on a small number of regulators and used low-resolution technology that explored only a subset of proximal promoters in the mammalian genome. We mapped the promoter occupancy of six master regulators in primary human hepatocytes using chromatin immunoprecipitation (ChIPs) combined with DNA microarrays representing large (10 kb) portions of promoter regions for most annotated human genes. Our results provide a high-resolution, genome-wide overview of the core transcriptional circuitry of human hepatocytes.

Results and discussion

Identification of transcription factor binding sites

To initiate mapping of the transcriptional regulatory circuitry of primary human hepatocytes, we selected regulators known

to be critical to hepatocyte biology based on genetic experiments in mouse or human (HNF1 α , HNF4 α , FOXA2/HNF3 β , HNF6/ONECUT1, CREB1, and USF1) (Table I, Supplementary Table S1) (Kuo *et al.*, 1992; Pani *et al.*, 1992; Cereghini 1996; Duncan *et al.*, 1998; Zaret 2002; Costa *et al.*, 2003; Montminy *et al.*, 2004; Pajukanta *et al.*, 2004; Lee *et al.*, 2005). These liver-enriched transcription factors can also play important roles in other tissues (e.g. kidney and pancreatic islets) (Bell and Polonsky, 2001). We then determined promoter occupancy for these six regulators by combining ChIPs with microarrays that have high resolution and extensive promoter coverage (Materials and methods). We employed DNA microarrays that contain 60-mer oligonucleotide probes covering the region from -8 to $+2$ kb relative to the transcript start sites for almost 18 000 annotated human genes, which were compiled from five independent databases (Boyer *et al.*, 2005). Most known transcription factor binding sites occur within $-8/+2$ kb of the transcription start site (Boyer *et al.*, 2005); this is also true for the transcription factors studied here, although some binding sites have been identified in other regions (Tronche *et al.*, 1997; Rada-Iglesias *et al.*, 2005). The sites occupied by transcription factors were represented by peaks of ChIP-enriched DNA that spanned neighboring probes (examples in Supplementary Figure S1). The coverage of promoter regions averaged one 60 mer for each 250 bases of sequence (Boyer *et al.*, 2005).

Table 1 Transcriptional master regulators profiled in primary human hepatocytes

Regulator	Function	PFAM category	Genes bound
HNF1 α	Metabolic control	POU homeodomain	1016
HNF4 α	Development, metabolism	Nuclear receptor	4519
HNF6 (ONECUT1)	Development	CUT homeodomain	1306
HNF3 β (FOXA2)	Development	Forkhead	890
CREB1	cAMP response	bZIP	2197
USF1	Glucose, lipid metabolism	Basic helix-loop-helix	1632

Identification of transcriptional regulatory motifs

We analyzed this high-resolution data using previously reported methods (Lee *et al*, 2002) to identify transcriptional network regulatory motifs (the simplest units of network structure), and thus to determine how these six key hepatocyte regulators contribute to autoregulatory loops, multicomponent loops, feed-forward loops, and multi-input motifs (Figure 1, Supplementary Table S2) (Lee *et al*, 2002; Milo *et al*, 2002; Shen-Orr *et al*, 2002; Odom *et al*, 2004).

Several aspects of the regulatory circuitry present among these master regulators have been noted or suggested previously. The network is highly interconnected (Figure 1A), and combinatorial control plays an important role in directing gene expression (Krivan and Wasserman, 2001). HNF4 α and HNF1 α bound each others promoters; the FOXA2 promoter was bound by both HNF6 and FOXA2; and the HNF4 α promoter is occupied by multiple HNF factors (Figure 1A, Supplementary Figure S1) (Pani *et al*, 1992; Duncan *et al*, 1998; Bailly *et al*, 2001; Costa *et al*, 2003; Briancon *et al*, 2004; Odom *et al*, 2004). Our data further showed the promoter of transthyretin, an archetypic hepatocyte gene, is occupied by HNF1 α , HNF4 α , FOXA2, and HNF6 as predicted from extensive site-specific and sequence-based analysis (Supplementary Figure S1) (reviewed in Costa *et al*, 2003).

Feed-forward loops were observed for seven combinations of transcriptional regulators (Figure 1). FOXA2 is involved with three separate feed-forward motifs, and potentially acts as a master regulator via the feed-forward motif for over 180 genes. This is consistent with previous suggestions that FOXA2 is at the top of regulatory hierarchies within the liver as well as other tissues (Lee *et al*, 2005). Single input motifs are present for all six regulators, although it is likely that many of the genes presently classed as single input are probably coordinately regulated and cobound by other as-yet-uncharacterized factors. Multi-input motifs were present for most combinations of transcriptional regulators (Figure 1B, Supplementary Table S2).

Prevalence of autoregulation among hepatocyte regulators

A remarkable feature of the portions of hepatocyte regulatory circuitry we studied here is the frequency of autoregulatory loops: five of the six regulators (83%) occupied their own promoters (Figure 1A, Supplementary Figure S2). Consistent with this, we use hypothesis-driven binding sequence analysis

(MacIsaac *et al*, 2006) to determine that optimized binding sequences for each transcription factor, and their presence close to each autoregulatory binding event (Supplementary information). Most bacterial transcription factors form autoregulatory loops (Thieffry *et al*, 1998; Shen-Orr *et al*, 2002). However, an analysis of nearly all yeast transcription factors has shown that only 10% have autoregulatory loops, suggesting that this form of regulation occurs much more rarely in eukaryotes (Lee *et al*, 2002; Harbison *et al*, 2004). These observations prompted us to consider the possibility that autoregulatory motifs are general features of eukaryotic transcription factors at the top of regulatory hierarchies or that play key roles in major cellular processes. When we inspected data for all transcription factors in yeast (Harbison *et al*, 2004; Supplementary information), we found that master regulators of key cellular processes were significantly more likely to autoregulate than other regulators (Supplementary Table S3 and S4, Supplementary Material). For instance, STE12 and TEC1 form autoregulatory loops: STE12 is the master regulator of mating and TEC1 is a key regulator of pseudohyphal growth (Zeitlinger *et al*, 2003).

In mammalian cells, transcription factors that have autoregulatory loops are frequently considered master regulators of tissues or key processes. These include, for example, OCT4 in embryonic stem cells (Boyer *et al*, 2005; Okumura-Nakanishi *et al*, 2005), MyoD and MyoG in muscle (Tapscott and Weintraub, 1991; Blais *et al*, 2005), FOXA2 in hepatocytes (Pani *et al*, 1992; Lee *et al*, 2005), and PU.1 in myeloid cells (Chen *et al*, 1995) (Supplementary Table S5). Importantly, most characterized mammalian transcription factors have been investigated because they play key roles in a particular tissue or cellular process. However, there are transcription factors (such as USF1) that do not occupy their own promoters (Supplementary Table S6), indicating that autoregulation is not simply a universal feature of mammalian transcription factors (Bateman, 1998).

Autoregulatory motifs may be a general feature of transcription factors that play key roles in major cellular processes because they impart stability to regulatory networks (Becskei and Serrano, 2000; Brandman *et al*, 2005). Master regulators of hepatocytes, like those of other tissues, are known to play both positive and negative regulatory roles (Briancon *et al*, 2004); both positive and negative feedback loops allow systems to be resistant to noise (Becskei and Serrano, 2000; Rosenfeld *et al*, 2002; Brandman *et al*, 2005). These characteristics may be crucial for regulators that are responsible for tissue-specific programs in higher eukaryotes.

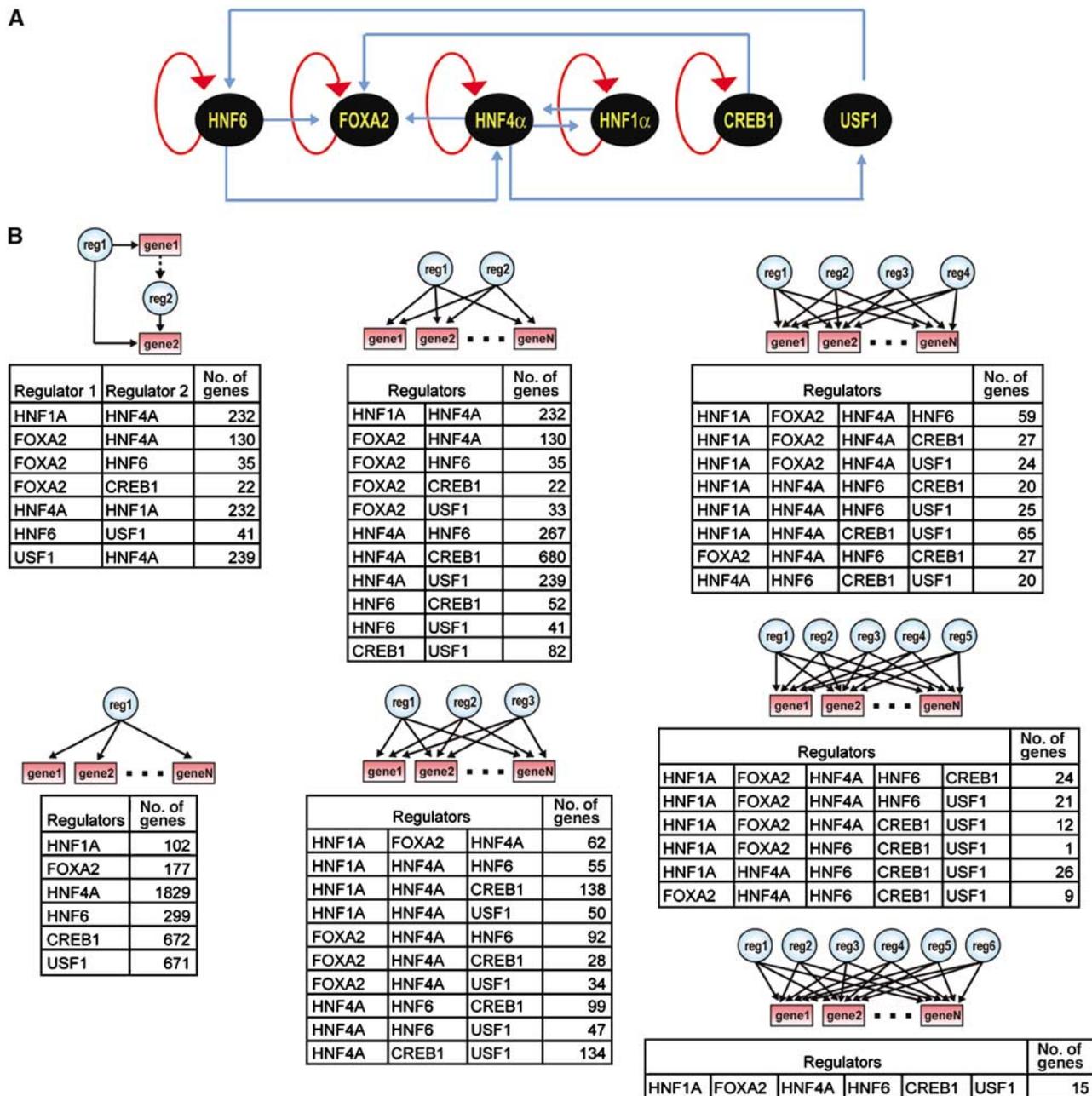


Figure 1 (A) Transcription factor crosstalk and autoregulation in the core regulatory circuitry of human hepatocytes. Regulators are shown as black ovals, with genomic occupancy of promoter regions indicated by blue or red (for autoregulatory loops) lines. (B) Frequency of network motifs in core hepatocyte regulatory network. For clarity, only combinations potentially controlling 20 or more genes are shown for the two-, three-, and four-factor multi-input motifs. (See also Supplementary Table S2).

Statistical enrichment of multiple binding events and correlation of promoter occupancy with gene expression

Well-characterized liver promoters are often controlled combinatorially by multiple liver-enriched transcription factors (Cereghini, 1996; Krivan and Wasserman, 2001; Costa *et al*, 2003; Friedman *et al*, 2004; Phuc Le *et al*, 2005; Rada-Iglesias *et al*, 2005). This prompted us to inspect our genome-wide binding data for evidence of enrichment in combinatorial

promoter occupancy. Comparison of the experimental data against randomized binding data (randomized by assuming all factors bind independently) revealed a statistically significant enrichment in the number of promoters bound by two or more transcriptional regulators. The enrichment generally increases with the number of transcriptional regulators bound (Figure 2A, Supplementary Figure S3). For instance, 1188 genes are bound by three or more regulators, whereas we would expect by random chance to see 345 genes cobound by three or more factors (z -score 45.8). Similar analyses of four,

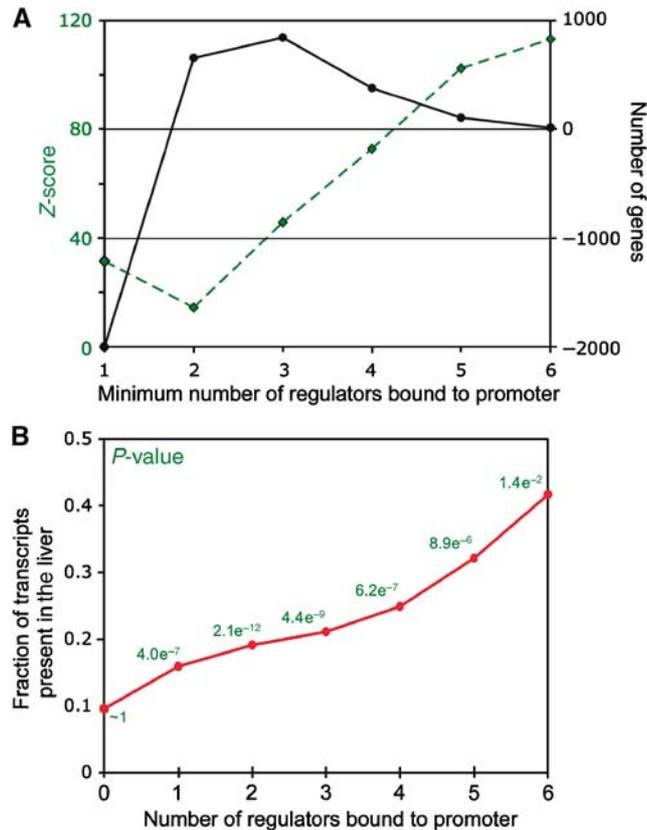


Figure 2 (A) Combinatorial occupancy of promoter regions is significantly enriched, using expectations calculated assuming a binomial distribution of binding events. The difference in numbers between expected and actual binding events is shown (black) with the z-score of the difference (green) (see also Supplementary Figure S3). (B) Comparison of the number of bound transcription factors to gene expression. *P*-values were determined using a hypergeometric calculation, and transcripts present were established by analysis of publicly available compendiums of tissue-specific gene expression (see also Supplementary Figure S4).

five, and six bound regulator combinations yield z-scores that increase with increasing number of bound regulators (Figure 2B). These results are consistent with previous suggestions that liver transcriptional regulation is controlled by multiple transcription factors acting in concert.

It might be expected that the presence of larger numbers of liver-specific transcription factors at a promoter region would increase the probability that the associated gene was expressed. We tested this hypothesis by classifying genes by the presence or absence of transcripts in the liver (Su *et al*, 2004), and comparing these categories with the number of regulators bound to corresponding promoters. We found a strong correspondence between these two values (Figure 2B), although the statistical significance drops at higher multi-input motifs due to small numbers of genes in the higher multi-input motifs. This correspondence is independent of the stringency used to call a transcript present (Supplementary Figure S4). Nevertheless, there are transcripts expressed in the absence of binding by these six master regulators, and there are genes bound by multiple factors, which are not appreciably expressed in human hepatocytes. These observations highlight the complexity of the hepatocyte transcriptional program.

Conclusions

We have used a systematic approach to identify the set of human promoters bound by six master regulators that are essential for proper liver development and function. The results show that these regulators form a highly interconnected core circuitry in human hepatocytes, identify the local regulatory network motifs created by regulator–gene interactions, and reveal that autoregulation is a predominant theme among liver-enriched transcription factors. The data support previous predictions that these factors co-occupy many genes to control hepatocyte gene expression, and there exists a direct correspondence between the number of regulators bound to a promoter region and the probability that a gene is expressed. This initial analysis of a portion of the regulatory circuitry in human liver should lay the foundation for future efforts to more fully elucidate the hepatic transcriptional program.

Supplementary information

Supplementary information including Materials and Methods is available at *Molecular Systems Biology* website (www.nature.com/msb). Also supporting website at <http://web.wi.mit.edu/young/autoregulation/>.

Data Accession Number

Data accession number at ArrayExpress is E-WMIT-9.

Acknowledgements

We thank the Whitehead Center for Microarray Technology and Whitehead Bioinformatics and Research Computing; E Herbolsheimer, S Strom, and W Gordon for experimental and computational support; FC Wardle, G Gerber, and C Harbison for helpful discussions. This work was supported by NIH Grants DK68766 and DK20595 (GIB), DK070813 (DTO), DK68655, and HG002668 (RAY). RAY and DKG are consultants for Agilent Technologies.

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