

Genome-wide analysis of SREBP-1 binding in mouse liver chromatin reveals a preference for promoter proximal binding to a new motif

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Lipid homeostasis in vertebrates is regulated by 3 sterol regulatory element binding protein (SREBP) isoforms. Here, we identify targets of SREBP-1 in mammalian liver using chromatin immunoprecipitation–high-throughput DNA sequencing. Antisera to SREBP-1 were used with liver chromatin from mice fed a high-carbohydrate diet after a fast, which leads to superinduction of hepatic SREBP-1c expression. SREBP-1–DNA complexes were subjected to massive parallel DNA sequencing using the Illumina Genome Analyzer II, resulting in 5.7 million sequence reads. Mapping these reads to the mouse reference genome identified 426 peaks of SREBP-1 binding vs. a control antibody. These binding peaks show a striking enrichment in proximal promoter regions, with 52% located within 1 kb upstream of a transcription start site. A previously undescribed sequence motif (5'-ACTACANNTCCC-3') was present in 76% of the total peaks, and we show that it is a functional SREBP-1 response element. Our analysis also reveals that an Sp1 consensus site is present as a “coregulatory” motif in 50% of the SREBP-1 binding peaks, consistent with previous functional studies. SREBP-1 bound not only to many well-characterized SREBP-1 target genes but to several other previously unknown targets in lipid and carbohydrate metabolism as well as many putative target genes in other diverse biological pathways.

ChIP-seq | fasting/refeeding | Sp1 | Kolmogorov–Smirnov test

All living organisms have evolved strategic regulatory systems to coordinate metabolic flux into potentially competing biochemical pathways to manage metabolite and end-product pools for optimal cellular fitness. This is particularly important in mammalian lipid homeostasis because cholesterol and fatty acids, the 2 major classes of mammalian lipids, are key to the structure and function of all cells, and unbalanced flux into these pathways can be both energy-inefficient and toxic. In vertebrates, lipid homeostasis is maintained in part by sterol regulatory element binding protein (SREBP) transcription factors, which are synthesized as \approx 125-kDa precursors containing 2 membrane-spanning domains that localize the immature proteins to the endoplasmic reticulum membrane (1).

Overlapping transcripts from 1 gene encode SREBP-1a and SREBP-1c isoforms, and the singular SREBP-2 is encoded from a distinct unlinked gene (2). There is an intricate system of nutrient sensing that relates cellular levels of cholesterol and other key lipid components to the expression and multistep membrane trafficking/ protease pathway that converts the membrane-bound SREBPs into the much smaller nuclear targeted transcription factors.

SREBPs activate genes encoding most of the key enzymes in cholesterol and fatty acid metabolism (3); thus, coupling SREBP processing to pathway flux provides a direct link between the activity of the regulatory factors and metabolic demand. Overexpression and targeted elimination studies in mice have produced crucial information on the roles of SREBPs in lipid regulation (3).

The available evidence indicates that SREBP-1 and SREBP-2 are mainly involved in fatty acid and cholesterol metabolism, respectively (3). However, there is still much to be learned about individual roles and physiological targets of the 3 mammalian SREBPs. Additionally, SREBPs activate genes not directly related to core reactions of lipid biosynthesis (4, 5); thus, additional studies are required to provide a more comprehensive understanding of SREBP function in physiology and metabolism.

We have performed a genome-wide analysis of SREBP-1 binding in liver using chromatin immunoprecipitation–high-throughput DNA sequencing (ChIP-seq) (6). We identified 426 SREBP-1 binding sites in the mouse liver genome, and, interestingly, 52% are located within 1 kb of the 5'-end of a mapped gene (the null expectation is 1%). We also performed an expression microarray analysis for genes regulated by the fasting/refeeding transition, in which SREBP-1c expression is dramatically increased. When we compared the fasting/refeeding differentially expressed genes with genes located close to SREBP-1 binding peaks, there was a high degree of correlation, indicating that our ChIP-seq data set contains a functional collection of SREBP-1c binding target genes.

The data also reveal a previously unrecognized DNA binding motif for SREBP-1, which matches an orphan motif that is highly conserved in promoters across several mammalian species, but a corresponding transcription factor was not known (7). We show that this previously undescribed motif is a functional SREBP-1 target using electrophoretic mobility-shift and transfection assays, further supporting the identity of SREBP-1 as the previously unidentified transcription factor.

Results

We prepared an antibody to a fragment of mouse SREBP-1 that shares no homology with SREBP-2 and used it to detect SREBP-1 in liver chromatin from a control group of mice, a fasted group, or a group that was fasted followed by refeeding a high-carbohydrate diet. The mature SREBP-1 was significantly decreased by fasting and increased to a level several-fold higher in the refeed sample (Fig. S1) than in the control group. Because this antibody is highly specific and robust, we used it to enrich SREBP-1 containing DNA fragments from the refeed chromatin using ChIP. In gene-specific ChIP analyses (Fig. S1), SREBP-1 association with 3 known SREBP target genes was confirmed; thus, we were encouraged to perform a genome-wide analysis of

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The authors declare no conflict of interest.

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A ChIP-seq reads & Peaks at min. ratio=5, min. reads =8.

Antibody	SREBP1	IgG
Seq Read (bp)	36	36
Total Reads	5,787,505	5,168,815
Peaks	426 (SREBP-1 vs IgG)	4 (IgG vs SREBP-1)
Nearest Genes	753	

B

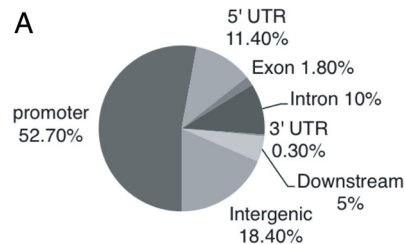
Symbol	SR1/IgG	RF/F
	F.C. (+/-)	F.C. (+/-)
SIRT6	2.88 (0.45)*	2.7 (0.46)**
RBL2	15.70 (1.09)*	2.45 (0.80)**
Rmnd1	5.51 (0.81)*	4.29 (0.86)**
LPIN1	1.59 (0.11)*	1.45 (0.198)
GSK3A	23.23 (2.67)*	3.33 (1.82)**
CDIPT	5.62 (0.49)*	3.17 (0.51)*
UBE2F	8.22 (0.47)*	3.79 (0.96)**
CDK5	5.47 (0.38)*	3.95 (1.16)**
SCARB1	5.47 (0.48)*	2.4 (1.24)
PDK1	4.37 (0.31)*	1.73 (0.35)**
LDLR	9.7 (1.2)*	4.14 (0.41)*
FASN	5.7 (0.64)*	6.9 (0.57)*

Fig. 1. ChIP-seq analysis for SREBP-1–DNA binding in hepatic chromatin. (A) Summary of peak analysis. (B) Peak validation. Ten randomly selected peaks were chosen for validation by gene-specific ChIP. The fold change (FC) is the fold increase for the signal from SREBP-1 antibody–enriched chromatin relative to a control IgG. SREBP-1 binding to these 10 peaks as well as to the LDL receptor and FAS promoters was also compared in hepatic chromatin prepared from fasting vs. re-fed mice as indicated. The negative control L32 showed no enrichment (31). * $P < 0.01$, ** $P < 0.05$

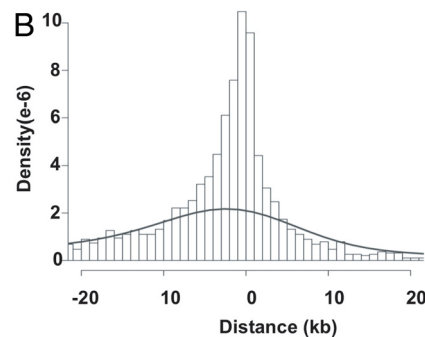
SREBP-1 binding. Chromatin from the SREBP-1 or IgG-enriched samples was used as a starting sample for high-throughput sequencing analysis (6).

Fig. 1A shows a summary of the sequencing analysis, and Fig. S2 shows the data for 1 peak with the sequence reads mapped onto a window in the UCSC Genome Browser Database. For defining SREBP-1 binding peaks, we set the minimum sequence reads to 8 and the minimum read ratio comparing SREBP-1 with IgG to 5. Using this stringent cutoff, we identified a total of 426 SREBP-1 binding sites. When the samples were reversed, there were only 4 peaks detected. Thus, the background is low and the specificity is high. We searched for the peak proximal genes (on both sides) and identified 753 total genes. Additional informa-

A



B



C

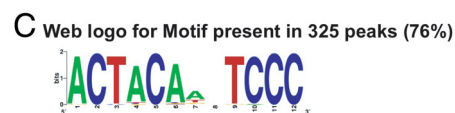


Fig. 2. Genome-wide distribution of ChIP-seq peaks. (A) Location of SREBP-1 binding peaks relative to known genes. The promoter and downstream regions are defined as 2 kb of 5' or 3' flanking DNA. (B) Peak distance relative to TSS of the closest gene. The dashed line shows the distribution of a random sequence of DNA the same size as the average peak. (C) The 426 peak regions were analyzed for overrepresented motifs using MEME (14). The top-scoring motif is shown.

tion on the peak locations is provided in Table S1, and the nearest gene list is provided in Table S2.

To validate the accuracy of the genome-wide binding data, we randomly picked 10 regions and performed ChIP analysis with peak specific primers. SREBP-1 binding to all 10 regions was enriched between 1.6- and 23-fold in the SREBP-1 chromatin relative to IgG (Fig. 1B). There was also a significant increase in SREBP-1 binding to most of the tested promoters in re-fed vs. fasted hepatic chromatin (Fig. 1B).

To find any commonalities among the SREBP-1 target genes, we searched the Database for Annotation, Visualization, and Integrated Discovery (DAVID) gene ontology (GO) database (8) for enrichment and found gene clusters in lipid ($P = 7e-4$) and carbohydrate metabolism ($P = 5.6e-3$) as expected (Table S3). Many of the genes in these categories are known SREBP targets, and others are previously unknown. Additionally, genes in other GO categories associated with intracellular protein movement and targeting ($P = 3.8e-7$), cell proliferation and differentiation ($P = 5.3e-4$), and apoptosis ($P = 3.6e-5$) showed significant enrichment. This is interesting and likely significant, because SREBPs undergo a multi-intracellular membrane itinerary during their life span (9) and are involved in cell proliferation (10) and apoptosis (11–13).

Interestingly, a peak distribution analysis revealed that 52% mapped to within 1 kb 5' of a gene transcription start site (TSS) and 11.4% were in 5' UTR regions. Altogether, this accounts for over 63% of the total SREBP-1 binding events, suggesting a strong preference for promoter proximal binding by SREBP-1. The remaining sites were distributed mostly within intergenic regions and introns, with very few located in 3' UTRs and exons (Fig. 2A). Overall, 95% of the peaks are located within 20 kb of

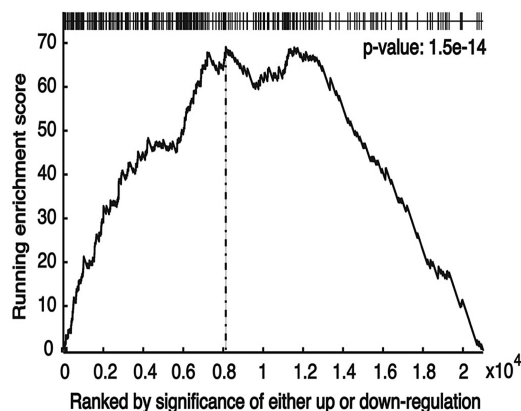


Fig. 4. Gene set enrichment analysis. The ChIP-seq peaks were analyzed for their representation within an expression array data set from refed vs. fasting liver as described in the text (18). Expression values for all genes in the microarray are ranked by fold regulation (from fasting to refed) on the x axis. The graph plots a running enrichment (KS) score on the ordinate across the complete differential expression data set on the abscissa. The location of each gene from the ChIP-seq data set within the ranked differential expression list is given as a thin vertical line on the horizontal axis displayed at the top of the graph. Here, the clustering of the ChIP-seq genes within the top portion of the differentially ranked genes is clearly visible ($P = 1.5 \times 10^{-14}$).

high degree of correlation indicates that the majority of the SREBP-1 binding sites identified by the ChIP-seq analysis are functionally important.

We also performed an unbiased search of the 5'-flanking regions of the genes that were differentially expressed by fasting/refeeding for a conserved motif that might emerge. Interestingly, the SREBP-1c motif described previously again emerged as the top motif using this unbiased data set, lending additional support for the pivotal role of SREBP-1c in mediating systematic gene expression changes during fasting/refeeding.

Discussion

We identified 426 SREBP-1 binding sites in chromatin from livers of mice treated to a fasting/high-carbohydrate feeding protocol that is known to increase hepatic levels of nuclear SREBP-1c, the major SREBP-1 isoform in liver, significantly. It is interesting that 52% of the binding sites are located in proximity to TSSs for mapped genes (Fig. 2). This is unusual because most genome-wide reports of transcription factor binding sites have shown no particular clustering or association relative to positions of known genes (19–22).

We also showed that there is a high correlation between the identity of genes located nearest to the SREBP-1 binding peaks and differentially expressed genes from RNA isolated from livers of fasted vs. fasted/refed mice. Because nuclear SREBP-1c levels are very low during fasting and dramatically induced by high-carbohydrate feeding, it is likely that the binding sites identified here are functionally important.

SREBPs are members of the basic helix-loop-helix (bHLHL) leucine zipper class of transcription factors, and most bHLH proteins dimerize and bind to an inverted repeat “E-box” element (23). The core E-box reads 5'-CANNTG-3', and individual family members exhibit unique preferences for specific bases in the center and flanking residues. All 3 SREBPs bind avidly to the E-box 5'-CACGTG-3' in vitro (24); however, in all the promoters carefully analyzed to date, none has been shown to be dependent on an E-box for activation by SREBP (2). The first well-characterized functional SRE was from the human LDL receptor promoter (25), which was used for affinity purification and subsequent cloning of SREBP cDNAs (26–28). This SRE element, 5'-ATCACCCCAC-3', is a direct repeat as opposed to an inverted repeat E-box. The

DNA binding flexibility that allows SREBPs to recognize E-boxes and direct repeat elements in vitro is attributable to a signature tyrosine residue that is unique to the SREBPs (24). All other E-box binding bHLH proteins contain an arginine at this position, which makes DNA contacts critical for specific recognition of E-box elements (29).

The crucial tyrosine allows the SREBP DNA binding domain to adopt an altered conformation to interact specifically with the direct repeat SRE element (29). We showed that the tyrosine residue was crucial to the nutritional regulatory response of SRE-containing promoters (30). Because this tyrosine was crucial for SRE element binding and sterol-dependent regulation, in vivo regulated SREs likely do not contain E-box motifs but, rather, some form of the direct repeat SRE similar to the LDL receptor promoter element. Consistent with this prediction, our ChIP-seq results identified a functional variant of the direct repeat SRE 5'-ACTACANNTCCC-3' as an in vivo recognition site in 76% of the SREBP-1 binding peaks.

In promoter activation studies, SREBPs are weak activators of transcription by themselves and require a neighboring coregulatory site for efficient activation and nutritional regulation (16). Transcription factors Sp1, NF-Y/CBF, and CREB have all been shown to function as coregulators in different SREBP-responsive promoters (2, 17). Consistent with these earlier studies, our genome-wide analysis identified an Sp1 site located close to 215 of the 426 peaks of SREBP-1 binding. However, predicted binding sites for NF-Y and CREB were not significantly enriched when analyzed on a genome-wide scale.

When the nearest genes to the SREBP-1 peaks were analyzed by GO software, clusters for lipid and carbohydrate metabolism were enriched as expected. Additionally, GO categories associated with intracellular membrane targeting and movement, apoptosis, cell proliferation, and proteolysis were significantly enriched (Table S3). These categories are interesting because SREBPs are known to undergo a regulated itinerary from endoplasmic reticulum to Golgi, followed by proteolytic maturation (9), and SREBPs are also associated with cell proliferation and apoptosis signaling (11–13).

Our analysis likely represents the binding pattern for SREBP-1c because it is the major hepatic isoform and it is specifically induced by the high-carbohydrate refeeding regimen used here (ref. 31; Fig. S1). However, because SREBPs function as dimers and 1c monomers can freely dimerize with both SREBP-1a and SREBP-2 (32), we cannot rule out the possibility that some of the binding sites identified here result from different monomeric combinations. It will be interesting to compare these results with future experiments under conditions in which only a single SREBP isoform is expressed.

The SREBP-1c binding motif identified by our studies corresponds to a motif identified in over 500 human promoters and is conserved in 317 promoters across diverse mammalian species (7). Although conservation of the motif was revealed by this earlier report (7), a corresponding transcription factor was not known. Our studies suggest that the unidentified transcription factor is SREBP-1c.

A genome-wide analysis of SREBP-1 binding in cultured human hepatoma HepG2 cells using ChIP on chip hybridization technology identified an E-box as a preferred site for SREBP-1 binding (33). The differences with our study may be that a cultured line of cells was used and SREBP-1a is more abundant than SREBP-1c in all cultured cell lines examined, including HepG2 (34). Additional studies are required to evaluate this further.

Materials and Methods

Animals. Six-week-old male C57BL/6 mice were obtained from Taconic and maintained on a chow diet (2020X; Harlan Teklad Global) for 1 week with a 12-h light/12-h dark cycle for acclimatization. The fasting/refeeding experi-

ments were performed as described elsewhere (31). Livers were removed, and $\approx 5\%$ slices were snap-frozen for later. The remaining tissue was immediately used for ChIP as described elsewhere in this article. Animal experiments were approved by the University of California, Irvine Institutional Animal Care and Use Committee (protocol 97–1545).

Preparation of Samples for ChIP Assays. Samples for ChIP assays using mouse tissues were prepared as described previously (31). Final DNA samples were analyzed for gene-specific ChIP (31) or ChIP-seq. The quantitative PCR oligonucleotide pairs for the mouse promoters are provided in Table S4. The library preparation protocol from Illumina was used to prepare samples for analysis with the Illumina/Solexa Genome Analyzer (6). Peak identification was performed using the software developed by the Wold laboratory at the California Institute of Technology (6). We used the default settings of a minimal number of 8 reads with a difference ratio of 5 between SREBP-1 antibody and control IgG to define peaks. At this cutoff, a false discovery rate of $<0.2\%$ was estimated by randomly generating a set of sequence reads corresponding to the size and number generated by the SREBP-1 antibody-enriched sample. Also, we did perform more stringent and less stringent analyses. These conditions resulted in fewer or more peaks as expected, but the most significantly enriched motif was the same as in Fig. 2.

EMSA. The sequences for the oligonucleotides used for the EMSA are provided in Table S4. The EMSA was performed with recombinant SREBP-1 protein (amino acids 1–490) as described elsewhere (16). Briefly, labeled probe and unlabeled competitor DNAs (as indicated in Fig. 3) were combined together on ice at the indicated molar equivalents before recombinant SREBP-1 protein was added. After a further 30-min incubation on ice, free DNA and DNA-protein complexes were resolved by native PAGE (16).

Transient Transfection. 293T cells were transfected with luciferase reporter constructs and a CMV- β -galactosidase internal control plasmid as described previously (32). Oligonucleotides used for plasmid cloning are provided in Table S4.

Motif Analysis. We applied the motif-finding program MEME (14) to SREBP-1 target peak regions to search for statistically overrepresented consensus SREBP-1 binding sites in the peak regions. Motifs were presented as position-dependent letter-probability matrices.

To find the binding sites for coregulators near the SREBP-1 binding sites, we masked all the SREBP-1 motif sites ($z > 3$) in the peak sequences with “N” and scanned the masked region ± 150 bp for coregulator binding sites using MEME. We also applied an enumeration-based method, k-mer analysis, to the sequences to search for coregulator motifs. For the k-mer analysis, we examined each motif length separately, from 6 to 15 bp. The following 11-mers were enriched in the extended peak regions ($z > 3$) and are matches to the Sp1 consensus site: CCCAGCCCCAG, CCCCAGCCCCA, CCAGCCCCAGC, GCCCAGCCCC, AGCCCCAGCCC, and CAGCCCCAGCC. The 6-mer CCAGCC is common to all these sites and is present in 215 of 426 extended peaks (± 150 bp; $z = 7.4$).

RNA Isolation, Quantitative PCR, and Gene Expression Profiling. Mouse liver total RNAs for refeeding and fasting were isolated using TRIzol (Invitrogen) and the RNeasy Mini Kit (Qiagen), followed by reverse transcription of 0.5 μ g of RNA with iScript Reverse Transcriptase (Bio-Rad) following the manufacturer's instructions. Analysis was performed using the standard curve method and normalization of all genes of interest to the housekeeping control L32. Gene expression profiling was carried out using the Mouse Gene 1.0 OST (Affymetrix) by hybridizing RNA from refeeding and fasting livers in triplicate. Differential expression was then assessed using the cyberT analysis program (35).

KS Analysis. The obtained ChIP-seq data were compared with expression microarray data by using a KS plot, a modified method of gene set enrichment analysis (18). The KS plot was obtained by calculating the running sum statistics for our SREBP-1 ChIP-seq gene set to observe enrichment in the ranked gene list from genes differentially expressed comparing the fast/refeeding expression microarray data.

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