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Identification of holocarboxylase synthetase chromatin binding sites in human mammary cell lines using the DNA adenine methyltransferase identification technology

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ABSTRACT

Holocarboxylase synthetase (HCS) is a chromatin protein that is essential for mediating the covalent binding of biotin to histones. Biotinylation of histones plays crucial roles in the repression of genes and repeats in the human genome. We tested the feasibility of DNA adenine methyltransferase identification (DamID) technology to map HCS binding sites in human mammary cell lines. Full-length HCS was fused to DNA adenine methyltransferase (Dam) for subsequent transfection into breast cancer (MCF-7) and normal breast (MCF-10A) cells. HCS docking sites in chromatin were identified by using the unique adenine methylation sites established by Dam in the fusion construct; docking sites were unambiguously identified using methylation-sensitive digestion, cloning, and sequencing. In total, 15 novel HCS binding sites were identified in the two cell lines, and the following 4 of the 15 overlapped between MCF-7 and MCF-10A cells: inositol polyphosphate-5-phosphatase A, corticotropin hormone precursor, ribosome biogenesis regulatory protein, and leptin precursor. We conclude that DamID is a useful technology to map HCS binding sites in human chromatin and propose that the entire set of HCS binding sites could be mapped by combining DamID with microarray technology.

Holocarboxylase synthetase (HCS)[2] is a chromatin protein [1] that plays a pivotal role in catalyzing histone biotinylation [1,2]. Histone biotinylation is a recently discovered epigenetic mark [3]. Biotin is unique because biotin serves as an essential covalently bound cofactor for several mammalian carboxylases and also functions as a covalent histone modification. Initially, biotinylation of histones was thought to be catalyzed by biotinidase [4]. However, subsequent studies revealed that HCS is much more important than biotinidase for biotinylation of histones in vivo [1,5–7] despite biotinidase demonstrating histone biotinyl transferase activity in vitro [8–10]. Several histone biotinylation sites have been identified using recombinant histone biotin ligases, including HCS, and synthetic histone tail peptides. The identified biotinylation sites are K9, K13, K125, K127, and K129 in histone H2A [9]; K4, K9, K18, and perhaps K23 in histone H3 [7,10]; and K8, K12, and perhaps K5 and K16 in histone H4 [7,8,11] (where K represents lysine). In vitro biotinylation of K8, K12, and K16 in histone H4 has been confirmed by mass spectrometric analysis of mammalian histones [11]. The existence of biotinylated histones was recently questioned [12], but findings in three independent laboratories [13–15], in addition to ours [2,3,7,8,10,16], suggest that biotinylation is a natural histone modification. One might consider the evidence in two of these publications [13,14] to be circumstantial. However, the third study [15] used liquid chromatography–tandem mass spectrometry (LC–MS/MS) to unambiguously demonstrate that approximately 50% of the histones in *Candida albicans* are biotinylated [15].

Histone biotinylation is a relatively rare event; less than 0.1% of histones are biotinylated in humans [3,13]. However, the abundance of an epigenetic mark is not the sole determinant of its biological importance. For example, only approximately 3% of cytosines are methylated, but the role of DNA methylation in gene regulation is substantial and undisputed [17]. Likewise, serine-14 phosphorylation in histone H2B and histone poly(ADP-ribosylation) are detectable only after induction of apoptosis and major DNA damage, respectively, yet the role of these epigenetic marks in cell death is unambiguous [18–20]. Despite the low global abundance of biotinylated histones, the epigenetic mark appears to be highly enriched in specific loci, consistent with its regulatory function. For example, evidence suggests that roughly one of three histone H4 molecules is biotinylated at K12 in telomeric repeats [21]. Moreover, K12-biotinylated histone H4 (H4K12bio), K9-biotinylated H3 (H3K9bio), and K18-biotinylated H3 (H3K18bio) are

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[2] *Abbreviations used:* HCS, holocarboxylase synthetase; LC–MS/MS, liquid chromatography–tandem mass spectrometry; DamID, DNA adenine methyl transferase identification; Dam, DNA adenine methyltransferase; PCR, polymerase chain reaction; LF 2000, lipofectamine 2000; cDNA, complementary DNA; qRT–PCR, quantitative real-time PCR; BLAST, basic local alignment search tool.

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enriched at transcriptionally repressed loci in mammalian genomes, suggesting a role for histone biotinylation in gene regulation [5,6,16,22]. The important role of HCS and subsequent biotinylation of histones has been confirmed in various studies. For example, phenotypes of HCS knockdown include decreased life span and heat survival in *Drosophila melanogaster* [1] and increased transcriptional activity of retrotransposons in metazoans, predisposing test organisms to chromosomal instability [6].

HCS has a dispersed and distinct distribution in chromatin; however, its chromatin binding sites are poorly defined [1]. Therefore, the objective of this study was to employ DNA adenine methyl transferase identification (DamID) technology [23] to generate the first insight into HCS chromatin binding sites and to pave the way for more comprehensive mapping efforts in future studies. Briefly, DamID technology is based on fusing DNA adenine methyltransferase (Dam) from *Escherichia coli* to a chromatin protein or transcription factor of interest (e.g., HCS) [23]. On expression of the fusion protein in cultured cells or in an intact organism such as *Drosophila,* HCS guides Dam to HCS binding sites, where Dam creates a unique adenine methylation tag that is absent in most eukaryotes [24]. Adenine methylation sites can be identified by methylation-sensitive sequencing procedures.

In this proof-of-concept study, DamID technology was used in mammary epithelial cell lines to map genomic HCS binding sites in human chromatin for the first time, thereby identifying potential aberrant epigenetic marks in breast cancer and setting the ground for future breast cancer studies.

Materials and methods

Cloning HCS–Dam fusion plasmid

The pIND (V5) EcoDam [23,25] vector was obtained from Bas van Steensel's laboratory at the Netherlands Cancer Institute. pIND (V5) EcoDam codes for *E. coli* Dam and contains a multiple cloning site upstream of the Dam open reading frame. An HCS–Dam fusion construct was generated as follows. Full-length human HCS was polymerase chain reaction (PCR)-amplified using HCS–pET41a as a template [2], AccuPrime Pfx DNA polymerase SuperMix (Invitrogen, Carlsbad, CA, USA), and the following primers: 5′-GTTCGAATTCATGGAAGATAGACTCCACATGG-3′ (forward, *Eco*RI site underlined) and 5′-GTTTCTCGAGCCGCCGTTTGGGGAGGATGAGG-3′ (reverse, *Xho*I site underlined). Following amplification, HCS and pIND (V5) EcoDam vector were digested with *Eco*RI and *Xho*I (Fermentas, Glen Burnie, MD, USA), ligated using a Fast-Link DNA Ligation Kit (Epicenter Biotechnologies, Madison, WI, USA), and transformed into MAX Efficiency DH5α Competent Cells (Invitrogen). The HCS–Dam fusion plasmid was sequenced (Eurofin MWG Operon, Huntsville, AL, USA) to confirm its identity and was denoted as "HCS–Dam". Two control plasmids were used in HCS mapping studies: (i) plasmid pIND V5 EcoDam [11] codes for Dam only and was used to identify artifactual binding of Dam to chromatin that was not mediated by HCS; (ii) plasmid HCS codes for full-length human HCS and was used to identify artifactual adenine methylation in the absence of Dam. Plasmid HCS was generated as described above for HCS–Dam, but the following reverse primer was substituted for the original reverse primer: 5′-GTTTTCTAGATTACCGCCGTTTGGGGAGGATGAGG-3′ (*Xba*I site underlined). The stop codon in this reverse primer terminates translation after the HCS open reading frame.

Cell culture

MCF-7 and MCF-10A cells (American Type Culture Collection [ATCC], Manassas, VA, USA) were used for all studies. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (ATCC) with the following supplements: 1% (v/v) L-glutamine (Invitrogen), 10% (v/v) fetal bovine serum (Invitrogen), 1% (v/v) antibiotic/antimycotic (Invitrogen), 1% (v/v) nonessential amino acids (Invitrogen), and 0.06% (v/v) bovine insulin (Sigma–Aldrich, St. Louis, MO, USA). MCF-10A cells were cultured in mammary epithelial growth media (MEGM) + bullet kit (Lonza, Basel, Switzerland), 1% (v/v) fetal bovine serum, and 0.05% (v/v) cholera toxin (Sigma–Aldrich).

Transfection of cells with HCS–Dam and control plasmids

For transfection with HCS–Dam, EcoDam, and HCS plasmids, 3.5×10^6 cells were seeded in T-75 flasks and allowed to adhere for 18 h, after which time cells were transfected with one of the three plasmids as follows. DNA was complexed with Lipofectamine 2000 (LF 2000, Invitrogen) or FugeneHD (Roche, Madison, WI, USA) in serum-free opti-MEM medium (Invitrogen). For MCF-7 cells, 6.75 μg of each plasmid was complexed with 10.2 μl of LF 2000 in a 1:1.5 DNA/lipid ratio (μg of DNA/μl of LF 2000). These complexes were allowed to incubate for 20 min and were then added to each designated flask. For MCF-10A cells, 8.25 μg of each plasmid was complexed with 20.625 μl of FugeneHD in a 1:2.5 DNA/FugeneHD ratio (μg of DNA/μl of FugeneHD). These complexes were incubated for 15 min and were then delivered to each designated flask. Cells were collected and lysed 48 h after transfection using protocols specific for isolation of RNA or genomic DNA.

Quantitative real-time PCR

Total RNA was collected using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and reverse-transcribed using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The complementary DNA (cDNA) was used to confirm successful transfection of cells with plasmids HCS–Dam, EcoDam, and HCS using the following primers and SYBR Green (Qiagen) in quantitative real-time PCR (qRT–PCR) expression analysis: (i) HCS–Dam, 5′-GCAACTCCTTCGACATGCTGAGAAA-3′ (forward, positioned in HCS region) and 5′-TCTTCATGCCGGTACGCGTAGAAT-3′ (reverse, positioned in Dam region); (ii) Dam, 5′-TCTGGTTGAGCGCCATATTCCAGT-3′ (forward) and 5′-TGTACAAAGCCAGCAGTTCGTCCA-3′ (reverse); and (iii) HCS, 5′-ATGGAAGATAGACTCCACAT-3′ (forward) and 5′-TGAGACCTGATCCTTAACTTCC-3′ (reverse). Glyceraldehyde 3-phosphate dehydrogenase was used as the reference gene for qRT–PCR normalization using primers 5′-TCCACTGGCGTCTTCACC-3′ (forward) and 5′-GGCAGAGATGATGACCCTTT-3′ (reverse) [6]. The cycle threshold values were used to calculate amplicon abundance [26].

Mapping HCS binding sites of adenine-methylated sequences

Genomic DNA was purified by using the DNeasy Mini Kit (Qiagen) 48 h posttransfection. DNA was digested with *Dpn*I (Fermentas) and purified using a PCR Purification Kit (Qiagen). The digested DNA was ligated into the pBlueScript II sk(+) vector after vector linearization with *Bam*HI (Fermentas); the Fast-Link DNA Ligation Kit was used for ligation. Note that digestion with *Dpn*I and *Bam*HI produces compatible overlaps. The ligation mixture was transformed into MAX Efficiency DH5α Competent Cells, and the cells were plated on Luria–Bertani (LB)-ampicillin plates. White colonies were randomly selected for sequencing by using the T7 promoter primer (Eurofins MWG Operon, Huntsville, AL, USA). HCS binding loci were identified by matching sequences against human genome using the National Center for Biotechnology Information (NCBI's) basic local alignment search tool (BLAST) and were defined as sequences within the genes and sequences up to 100,000 bp upstream or downstream of the genes.

Statistics

The GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA) was used to plot qRT–PCR graphs. Data are presented as means ± standard errors. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA), followed by post hoc Tukey's test. Values were considered as statistically significant if $P < 0.05$.

Results and discussion

Plasmid expression

Transfection of mammary cells with plasmids Dam–HCS, EcoDam, and HCS produced the expected expression patterns compared with nontransfected control cells. When cDNA was analyzed using PCR primers for HCS, the transcript abundance was 170- and 62-fold greater in MCF-7 cells transfected with HCS and HCS–Dam, respectively, than in nontransfected control cells (Fig. 1A); the abundance of HCS transcript was not altered by transfection with EcoDam (negative control) because it did not contain HCS. When cDNA was analyzed using PCR primers for the HCS–Dam fusion protein, the transcript abundance was 2000-fold greater in MCF-7 cells transfected with HCS–Dam than in nontransfected control cells (Fig. 1B); the abundance of HCS–Dam fusion transcript was not altered by transfection with EcoDam and HCS (negative controls) because these controls lacked HCS–Dam fusion. When cDNA was analyzed using PCR primers for Dam, the transcript abundance was 660- and 800-fold greater in MCF-7 cells transfected with EcoDam and HCS–Dam, respectively, than in nontransfected control cells (Fig. 1C); the abundance of Dam transcript was not altered by transfection with HCS (negative control) because it did not contain Dam. Results were similar in MCF-10A cells (Fig. 2).

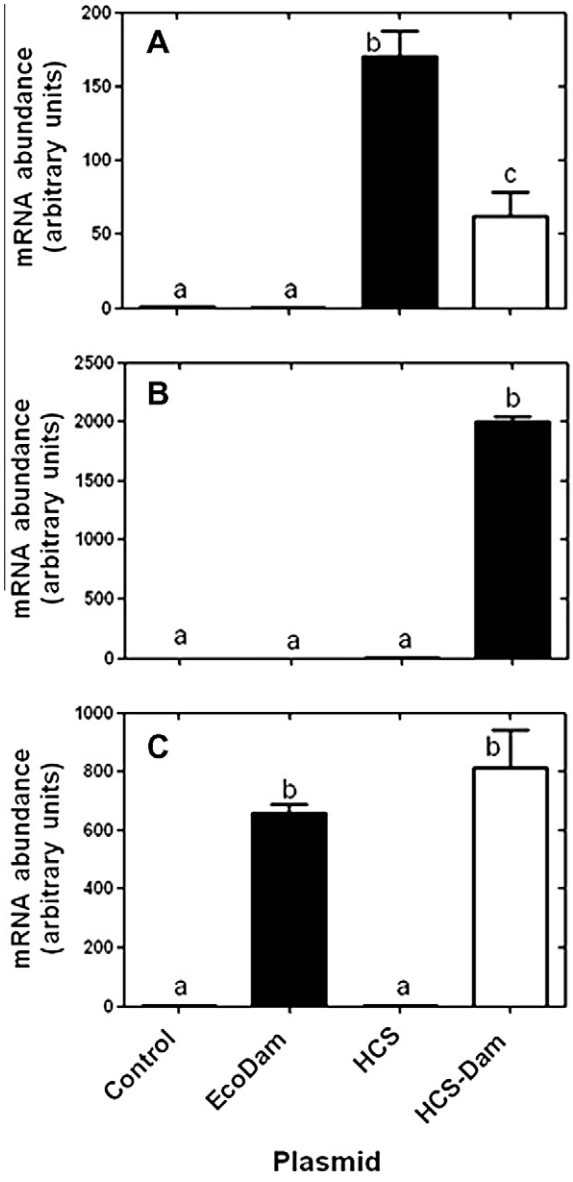

Fig.1. Transcript abundance in MCF-7 cells transfected with plasmids HCS–Dam, EcoDam, and HCS. Transcripts were quantified by qRT–PCR using gene-specific primers for HCS (A), HCS–Dam fusion (B), and EcoDam (C). Each bar represents the mean ± standard error ($n = 3$). Bars with different letters indicate significant differences ($P < 0.05$) between treatments. mRNA, messenger RNA.

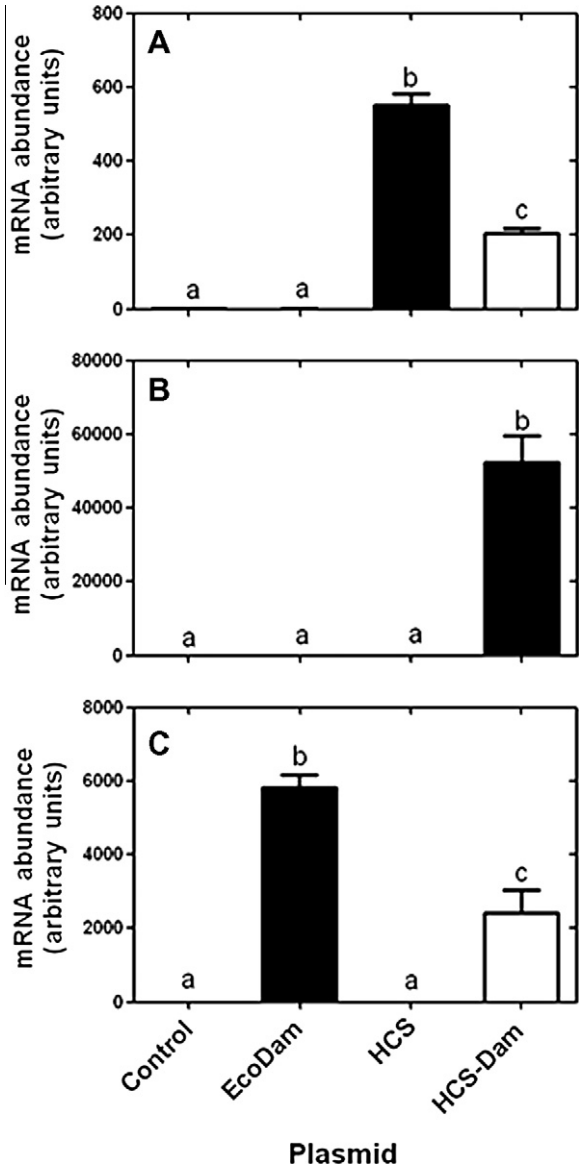

Fig.2. Transcript abundance in MCF-10A cells transfected with plasmids HCS–Dam, EcoDam, and HCS. Transcripts were quantified by qRT–PCR using gene-specific primers for HCS (A), HCS–Dam fusion (B), and EcoDam (C). Each bar represents the mean ± standard error ($n = 3$). Bars with different letters indicate significant differences ($P < 0.05$) between treatments. mRNA, messenger RNA.

Table 1
Genomic HCS binding sites in MCF-7 cells.

MCF-7 HCS binding locus	Gene(s)	Chromosome	GenBank accession number(s)
5′-CCACAGAATCAGGGGATAACGCAGGAAAGAACAT-3′	Inositol polyphosphate-5-phosphatase A	10	NM_005539.3
5′-CAGGAAAGAACATGTGAGCAAAAG-3′	Corticotropin-releasing hormone precursor (CRH) and homolog of yeast ribosome biogenesis regulatory protein (RRS1)	8	NM_000756.2 NM_015169.3
5′-ATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTA-3′	Cadherin 8 and cadherin 11 type 2 preproproteins	16	NM_001796.2 NM_001797.2
5′-ACCATGCTCTTTCCCCCTTGCAACCTCACTCATTCTCTCTCCTATTC-3′	Schwannomin interacting protein 1 (SCHIP1)	3	NM_014575.2
5′-GGTTTTTATCTTTGCAAGCAGCAGAT-3′	Neuron navigator 3 (NAV3)	12	NM_014903.4
5′-ATTCCACACAACATACGAGC-3′	Leptin precursor	7	NM_000230.2
5′-AAAGGGATTTTGGGGCATGAGATCATC-3′	Apoptosis inhibitor 5 isoform b	11	NM_006595.3
5′-AAAGGGATTTTGGGGCATGAGATCATC-3′	Netrin-G1 ligand	11	NM_020929.1

Table 2
Genomic HCS binding sites in MCF-10A cells.

MCF-10A HCS binding locus	Gene(s)	Chromosome	GenBank accession number(s)
5′-CCACAGAATCAGGGGATAACGCAGGAAAGAACAT-3′	Inositol polyphosphate-5-phosphatase A	10	NM_005539.3
5′-TCGCCCTATAGTGAGTCGTAT-3′	*Homo sapiens* myosin, heavy chain 9, nonmuscle (MYH9)	22	NM_002473.4
5′-CAGGAAAGAACATGTGAGCAAAAG-3′	Corticotropin-releasing hormone precursor (CRH) and homolog of yeast ribosome biogenesis regulatory protein (RRS1)	8	NM_000756.2 NM_015169.3
5′-TGAGCTAACTCACATTAATTGCGTTG-3′	SRY (sex-determining region Y)–box 4 and prolactin	6	NM_003107.2 NM_001163558.1
5′-CCTGTTTTGCAGGGTGCTTT-3′	Purinergic receptor P2X5 isoforms A and B	17	NM_002561.2
5′-GAGCTCCAGCTTTTTTTCTCTTTAG-3′	Tetratricopeptide repeat domain 7B	14	XM_510118.2
5′-CCTGGGGTGCCTAATGAGTG-3′	CUB and sushi multiple domains 1	8	Q96PZ7-1
5′-ATTCCACACAACATACGAGC-3′	Leptin precursor	7	NM_000230.2

HCS-docking sites

Following the validation of expression of fusion and control plasmids, these plasmids were expressed in MCF-7 and MCF-10A cell lines, genomic sequences methylated by Dam were cloned into the pBlueScript II sk(+) cloning vector, and then positive clones were obtained and sequenced. When five clones from HCS–Dam positive MCF-7 cells were annotated by BLAST, eight unique HCS binding sequences, within specific genes, were detected (Table 1). Each clone returned more than one sequence because of the complementary ends created during digestion with *Dpn*I and the random ligation of these fragments during ligation and cloning. Transfection with plasmid HCS (negative control) did not produce any clones in MCF-7 cells. We conclude that HCS greatly enhances the binding of Dam at distinct genomic loci. Results in MCF-10A cells were similar to those in MCF-7 cells, including five clones identified from HCS–Dam positive MCF-10A cells that contained eight unique sequences when annoted by BLAST (Table 2).

Within these data, some important observations were noted. First, when MCF-10A cells were transfected with plasmid HCS, one clone with one single sequence was identified compared with the seven clones in cells transfected with HCS–Dam, indicating that the false positive rate due to these artifacts might be up to 14% in Dam-based assays. In addition, for the MCF-10A cell line, a locus within X-linked neuroligin 4 gene was disregarded as a potential HCS docking site because it appeared to be due to artifactual methylation by pIND (V5) EcoDam control. No such artifactual methylation by Dam was observed for MCF-7 cells. Finally, three HCS docking sites were found to be common between MCF-7 and MCF-10A cells, indicating a 26% overlap in the HCS binding regions identified in the two epithelial cell lines and increasing confidence that the results produced by HCS–Dam technology are real. Note that clones were selected randomly in this proof-of-concept study; therefore, one should not expect a 100% overlap in clones. Comprehensive maps can be generated only by using microarray-based technologies or high-throughput sequencing and may show an expected overlap of 100% between the identified genes because both cell lines used in this study are of epithelial origin or, alternatively, could allow determination of nonoverlapping loci specific for either cell type. In addition, note that the following gene sets share a common HCS docking locus: *CRH* and *RRS1* (Tables 1 and 2), cadherin 8 and cadherin 11 (Table 1), and SRY (sex-determining region Y)-box 4 and prolactin (Table 2).

This study proves the feasibility of the DamID technology to identify, for the first time, potential HCS binding sites in human mammary epithelial cell lines. DamID technology eliminates the need for large cell numbers and antibodies; thus, it represents a viable alternative method to the chromatin immunoprecipitation (ChIP) assay, a frequently used tool to investigate DNA–chromatin interactions. With the establishment of the ability of DamID technology to identify chromatin protein binding sites, the future goal of this project is to use the technology along with cDNA microarray sequencing to monitor chromatin proteins in small cell samples such as oocytes. Although the combination of techniques employed in this study provided only a snapshot of events occurring at the genomic level, it proves the DamID technology's utility and, when combined with cDNA microarray sequencing, has the potential for full genome analysis.

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