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Formation of a large, complex domain of histone hyperacetylation at human 14q32.1 requires the serpin locus control region

Euan W. Baxter, W. Jason Cummings and R. E. K. Fournier*

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N., Seattle WA, 98109-1024, USA

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ABSTRACT

The human serine protease inhibitor (serpin) gene cluster at 14q32.1 is a useful model system to study cell-type-specific gene expression and chromatin structure. Activation of the serpin locus can be induced *in vitro* by transferring human chromosome 14 from non-expressing to expressing cells. Serpin gene activation in expressing cells is correlated with locus-wide alterations in chromatin structure, including the *de novo* formation of 17 expression-associated DNase I-hypersensitive sites (DHSs). In this study, we investigated histone acetylation throughout the proximal serpin subcluster. We report that gene activation is correlated with high levels of histone H3 and H4 acetylation at serpin gene promoters and other regulatory regions. However, the locus is not uniformly hyperacetylated, as there are regions of hypoacetylation between genes. Furthermore, genetic tests indicate that locus-wide controls regulate both gene expression and chromatin structure. For example, deletion of a previously identified serpin locus control region (LCR) upstream of the proximal subcluster reduces both gene expression and histone acetylation throughout the ~130 kb region. A similar down regulation phenotype is displayed by transactivator-deficient cell variants, but this phenotype can be rescued by transfecting the cells with expression cassettes encoding hepatocyte nuclear factor-1α (HNF-1α) or HNF-4. Taken together, these results suggest that histone acetylation depends on interactions between the HNF-1α/HNF-4 signaling cascade and the serpin LCR.

INTRODUCTION

A fundamental aspect of cell specialization is the organization of the genome into a chromatin structure that is permissive for the pattern of gene activity of that particular cell type. Early studies demonstrated that expressed genes often reside in nuclease-accessible chromatin (1); more recently, it has been shown that gene-rich domains are enriched in open chromatin fibers (2). These expression-associated chromatin states are formed not only by interactions between cell-specific factors and their DNA binding sites, but also by covalent modifications of histones and other chromosomal proteins in active genomic regions [reviewed in (3)].

The cluster of human serine protease inhibitor (serpin) genes at 14q32.1 has been a useful model system for studying the regulation of cell-specific gene expression and chromatin structure. The 14q32.1 serpin locus occupies ~370 kb of genomic DNA, and the eleven serpin genes in the region are organized into three subclusters of four, three and four serpin genes each (4). The proximal serpin subcluster contains four serpin gene sequences—α1-antitrypsin (α1AT, SERPINA1), an antitrypsin-related pseudogene (ATR, SERPINA2), corticosteroid binding globulin (CBG, SERPINA6) and protein Z-dependent protease inhibitor (ZPI, SERPINA10). These genes are highly expressed in the liver, but they are repressed in most other cell types. The cell-specific changes in gene expression and chromatin structure that occur during hepatic differentiation can be recapitulated *in vitro* by transferring human chromosome 14 from non-expressing to expressing cell types (5). This forms the basis of a system for the genetic analysis of the locus by targeted chromosomal modification (6).

Activation of the proximal serpin subcluster by chromosome transfer results in the expression of the α1AT, CBG and ZPI genes at hepatic cell-typical levels, as well as the *de novo* formation of 17 expression-associated DNase I-hypersensitive sites (DHSs) (5). DNase I hypersensitivity

*To whom correspondence should be addressed. Tel: +1 206 667 5217; Fax: +1 206 667 6522; Email: kfournie@fhcrc.org
Present address:
Euan W. Baxter, Molecular Medicine Unit, Clinical Research Building, St. James University Hospital, Leeds, LS9 7TF, UK

generally reflects an altered structure of the nucleosomal array such that DNA in the 200–400 bp linker region is more accessible to nuclease digestion (7). This altered chromatin structure can be induced by the binding of specific transcription factors (8). Clusters of DHSs often comprise regulatory regions that are important for high-level, cell-specific gene expression (9). In the proximal serpin subcluster, expression-associated DHSs appear throughout an ~130 kb region that extends from a cluster of three sites at ~−24 kb to a single site at ~+100 kb. Within this interval, there are four expression-associated DHSs in the ~8.0 kb region upstream of α1AT that contains the serpin locus control region (LCR). In contrast, there are only seven constitutive DHSs in the entire proximal subcluster in non-expressing cells (5).

Cell-specific expression of many liver genes is mediated by a group of liver-enriched transactivators that include hepatocyte nuclear factor-1α (HNF-1α), a homeodomain protein (10), the HNF-3 family of forkhead-related proteins (11), the CCAAT/enhancer binding protein (C/EBP) (12) and HNF-4, a steroid hormone receptor-like protein (13). HNF-4 transactivates a number of liver genes, including transthyretin, apolipoprotein CIII and HNF-1α (14). HNF-1α activates many liver genes, including α1AT (15). HNF-3 can bind to silent chromatin and alter its structure (16). HNF-6 also plays an important role in liver-specific gene expression, and it binds to the promoters of HNF-3β and a number of other liver genes (17).

Transactivator-deficient hepatoma variants have been useful tools for defining the roles of specific transactivators in regulating gene expression and chromatin structure. For example, by selecting cells that were unable to activate the α1AT promoter/enhancer in *trans*, hepatoma cell variants deficient in HNF-1α and HNF-4 expression were obtained (18). These HNF-1α- and HNF-4-deficient cells failed to express any serpin genes in the proximal subcluster. Furthermore, the variants did not display the 17 expression-associated DHSs normally found in this region. However, transfecting the cells with expression plasmids encoding either HNF-1α or HNF-4 partially rescued the variant phenotypes both in terms of gene expression and DHS formation (19). Thus, HNF-1α and HNF-4 are required for both cell-specific gene expression and chromatin structure in the proximal serpin subcluster.

Another genetic approach to investigate mechanisms that regulate the serpin locus has been targeted mutagenesis. Mutant serpin alleles were generated by homologous recombination in DT40 microcell hybrids (6) containing human chromosome 14, and the specifically modified chromosomes were transferred to rat hepatoma cells for functional tests (20). A mutant chromosome that lacked the ~8.0 kb region immediately upstream of the α1AT hepatic promoter failed to activate serpin gene expression after chromosome transfer, and expression-associated DHSs did not form on the mutant allele. These and other data define a serpin LCR that regulates both gene activity and chromatin structure (20).

The nucleosomal histones of active genes are marked by many post-translational modifications that are thought to affect the accessibility of chromatin to regulatory proteins (3). Acetylation of histone N-termini is associated with actively transcribed chromatin [reviewed in (21)]. Furthermore, hyperacetylation of histones H3 and H4 is correlated with transcriptional activity on a genome-wide scale (22). The acetylation of histones is catalyzed by several specific histone acetyl transferases (HATs) (23). Many transcription factors seem to exert their effects, at least in part, by acting both as DNA-binding factors and as docking surfaces for HATs [reviewed in (23)]. In the proximal serpin subcluster, the HAT CBP (CREB-binding protein) seems to play a role in activating the α1AT expression in differentiating CaCo-2 cells (24).

To further investigate the chromatin structure of the proximal serpin subcluster in various genetic contexts, we used histone acetylation as a marker in wild-type, variant and specifically modified cells. We report that gene promoters and other regulatory regions in the proximal serpin subcluster showed strong cell-type-specific histone hyperacetylation in expressing cells, and this cell-specific chromatin structure required both the HNF-1α/HNF-4 signaling cascade and the serpin LCR.

MATERIALS AND METHODS

Cell lines and culture conditions

F(14n)2 and F(14n)14 are rat hepatoma cells that contain a single, wild-type copy of human chromosome 14, and R(14n)6 cells are rat fibroblasts that contain a wild-type copy of human chromosome 14 (5). F(Δ8.0) hybrids are rat hepatoma cells that contain a mutant copy of human chromosome 14, in which an ~8.0 kb segment of genomic DNA just upstream of the hepatic α1AT promoter had been deleted specifically (20). H11 is a hepatoma variant cell line selected for its inability to activate the α1AT promoter/enhancer in *trans*; these cells are deficient in the expression of HNF-1α and HNF-4 (19). H11(14n)D and H11(14n)E are H11 derivatives that contain a wild-type copy of human chromosome 14 (19). EH1 and EH4 are stable transfectants prepared by transfecting H11(14n)E cells with expression plasmids encoding HNF-1α or HNF-4, respectively (19). The cells were grown in 1:1 Ham's F12:DMEM with 10% fetal bovine serum and various selective agents, as described previously (5,19). Fluorescence *in situ* hybridization was performed periodically on each cell line to ensure uniform retention of human chromosome 14, as described previously (25).

Chromatin immunoprecipitation (ChIP) assays

A total of 1×10^8 cells were washed in phosphate-buffered saline and fixed in 50 ml of 1 × Paro-fixation buffer containing fresh formaldehyde for 5 min (26). The fixation was stopped by the addition of one-tenth volume of 2.5 M glycine, and the fixed chromatin was washed once in Paro rinse 1 and twice in Paro rinse 2 containing 10 mM sodium butyrate before being resuspended in 1.8 ml of dilution buffer [1%Triton X-100, 4 mM EDTA, 40 mM Tris (pH 8.1), 300 mM NaCl, 10 mM sodium butyrate and protease inhibitors (Roche 'complete mini' tablets, Catalog no. 1-836-153)]. The chromatin was sonicated in five 30 s pulses using a microtip-equipped Fisher Scientific 550 Sonic dismembrator at setting 5. The chromatin was cooled for 30 s in an ice-ethanol bath between sonication pulses. Cell debris was pelleted by centrifugation at 13000 r.p.m., and the supernatant was separated into 200 µl aliquots that were stored at −70°C. Input DNA was prepared

as described previously (26), and an aliquot was run on a 1% agarose gel to ensure that the DNA was properly fragmented. The fragment size ranged from ~600 bp to ~2 kb, with even ethidium bromide staining, suggesting that most molecules were <2 kb.

Chromatin was pre-cleared using 60 µl of Protein A-Agarose beads (Upstate Biotechnologies Catalog no. 16-156) that had been washed three times with Protein A wash buffer [10 mM Tris (pH 8), 1 mM EDTA and 10 mg/ml BSA]. The pre-cleared chromatin was incubated overnight with 5 µg of polyclonal antibody to modified histones. Rabbit polyclonal IgG antibodies against histone H3 acetylated at lysine residues 9 and 14 (Catalog no. 06-599) and all forms of acetyl-histone H4 (Catalog no. 06-866) were obtained from Upstate Biotechnologies. Precipitations were also carried out without antibody or with equivalent amounts of purified IgG from non-immunized rabbits (Upstate Biotechnologies Catalog no. 12-370) as controls for non-specific interactions.

The antibody–chromatin complexes were precipitated by incubation with 60 µl of Protein A-Agarose beads (Upstate Biotechnologies, Catalog no. 16-156) for 30 min at 4°C, then washed and eluted from the beads as described previously (26). Reversal of crosslinks and proteinase K digestion of the remaining proteins were also as described, but the DNA was purified from the digestions using a PCR purification system (Qiagen Catalog no. 28104).

Specific DNA sequences in the immunoprecipitates were amplified by PCR. Fifty microliter reactions contained 1 µCi [α-^{32}P]dCTP (3000 Ci/mmol, PerkinElmer Life Sciences) with each cold dNTP at a final concentration of 100 µM. Two pairs of primers were used—a test pair, and a control pair which amplified a 218 bp sequence from the non-expressed Goosecoid gene (27), which is ~380 kb upstream of α1AT on human chromosome 14 (4). All reactions were carried out in a PerkinElmer 9700 using 30 cycles and an annealing temperature of 58°C. The intensities of the bands were measured using a Typhoon PhosphorImager and Image Quant software. All amplicons on human chromosome 14 were in the range of 200–500 bp, and each primer pair was tested using rat genomic DNA to verify that no rat sequences were amplified. Each primer pair was also tested to ensure that they amplified a single copy DNA sequence. The positive control was the promoter of the rat prolyl-4-hydroxylase (rP4H) gene (28), which is expressed in all the cell lines used in this study. Sequences of the primers used in the ChIP assays are presented in Appendix 1, which is available online.

Increasing amounts of template DNAs (1, 2, 4, 8, 25, 50 and 100 ng) were amplified in separate experiments to ensure that amplification and detection of the test PCR products were within the linear range. The ratios of intensities of test and control amplicons remained constant across the range of template concentrations.

RESULTS

Genomic organization of the proximal serpin subcluster and surrounding regions

The genomic organization of an ~1 Mb segment of human 14q32.1 that includes the serpin locus and its proximal and distal neighbors has been described previously (4). Figure 1 shows an ~320 kb segment of this region that contains the proximal serpin subcluster- α1AT, ATR, CBG and ZPI and its most immediate neighbors. Note that on this map, and throughout this report, we define position +1 bp as the hepatic transcription start site of the α1AT gene. This corresponds to the G residue at position 93924694 of the human chromosome 14 sequence fixed in May 2004. As assessed by the formation of expression-associated DHSs (5), the proximal serpin subcluster extends from ~45 kb upstream of α1AT

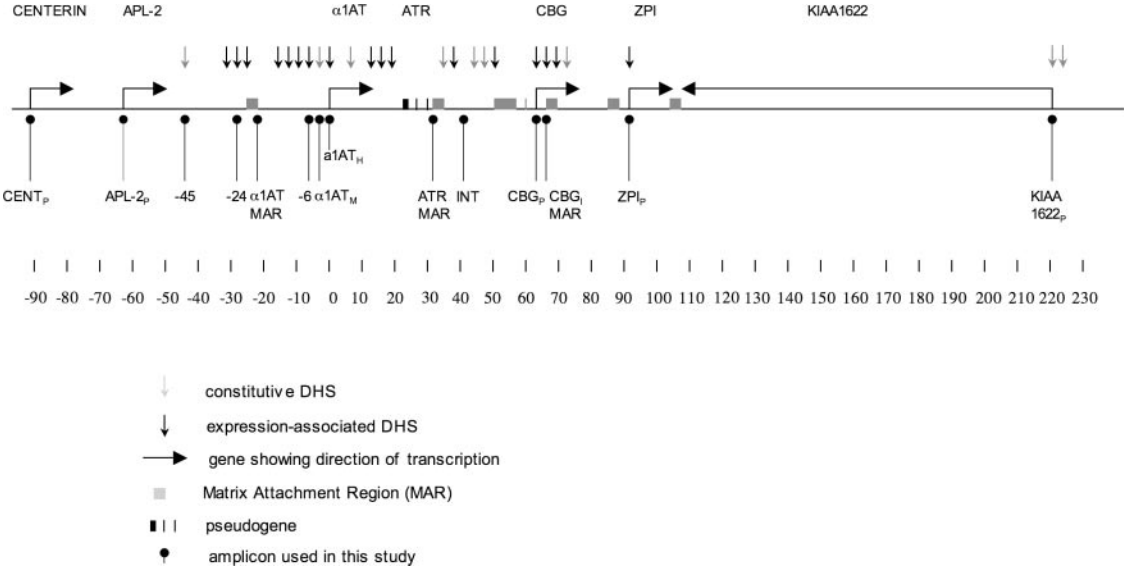

Figure 1. Genomic structure of the proximal serpin subcluster and surrounding regions. The proximal serpin subcluster (α1AT, ATR, CBG and ZPI) extends from ~−45 to ~+110 kb. This region contains 17 expression-associated DHSs, 9 constitutive DHSs, and 6 MARs. The positions of the PCR amplicons used in ChIP assays described in this report are indicated.

(i.e. position ~−45 kb in Figure 1) to just downstream of ZPI at position ~+110 kb. As shown in the figure, the centerin and APL-2 genes, members of the central serpin subcluster (4), are just distal (telomeric) to ~−45 kb. The large KIAA 1622 transcription unit, a non-serpin sequence that occupies the interval from ~+110 to ~+210 kb, is just centromeric to the proximal serpin subcluster.

Histones H3 and H4 are hyperacetylated in hepatoma hybrids but not fibroblast hybrids

Previous work has shown that active promoters and other expression-associated DHSs are commonly sites of histone hyperacetylation, and domains of hyperacetylation can be broadly correlated with gene activity [reviewed in (21)]. Furthermore, constitutively hyperacetylated matrix attachment regions (MARs) may act as boundaries between expressed and non-expressed chromatin (29). To determine whether a domain of accessible/modified chromatin at the proximal serpin subcluster could be defined in this way, ChIP experiments were performed. We also investigated whether MARs in the region (30) marked boundaries between modified and unmodified chromatin.

ChIPs were performed and specific DNA sequences were amplified by PCR as described in Materials and Methods. The PCR amplicons (Figure 1) included sequences from the promoters of the human genes encoding centerin ($CENT_P$) and anti-proteinase-like 2 ($APL\text{-}2_P$), the macrophage ($\alpha1AT_M$) and hepatic ($\alpha1AT_H$) promoters of α1AT, and the promoters of CBG (CBG_P), ZPI (ZPI_P) and KIAA 1622 ($KIAA\ 1622_P$). Amplicons near a constitutive DHS at ~−45 kb, a cluster of three expression-associated DHSs at ~−24 kb and an expression-associated DHS within the serpin LCR at ~−6 kb were also analyzed. As histone acetylation within MARs has also been described, we also tested single-copy sequences within the α1AT MAR at ~−18 kb, the ATR MAR at ~+28 kb and the CBG intronic MAR at ~+68 kb (Figure 1). An intergenic DNA segment (INT) between ATR and CBG (~+35 kb) was also tested. All the PCRs in this study included an internal control, consisting of primers from the human Goosecoid gene, which is ~380 kb upstream of α1AT (4). Goosecoid is expressed during embryogenesis (27), but not in any of the cell lines used in this study. The relative amounts of H3 and H4 acetylation are expressed as ratios of test over control band intensities for the specific antibody fractions over those of pre-immune antibody controls (marked IgG in the figures). The results are averages of independent ChIP assays, with standard deviations indicated.

Representative examples of ChIP assays from expressing cells [F(14n)14, a rat hepatoma hybrid containing human chromosome 14] and non-expressing cells [R(14n)6, a rat fibroblast hybrid containing human chromosome 14] are shown in Figure 2. High levels of histone H3 and H4 acetylation were seen at the hepatic α1AT promoter (~20-fold enrichment) and the ZPI promoter (~13-fold) in F(14n)14 hybrid cells. Interestingly, histone acetylation at the CBG promoter was more modest in this cell line (~3-fold), although CBG and ZPI expression levels were similar (data not shown). In contrast, there was no apparent enrichment for acetylated histones at the α1AT, CBG or ZPI promoters in the non-expressing fibroblast hybrid, R(14n)6. The promoter of the KIAA 1622 gene, which is expressed in both hybrid cell types, was enriched for acetylated histones (3- to 6-fold) in both cell types. This was similar to the pattern of histone acetylation at the promoter of the rP4H gene, a constitutively

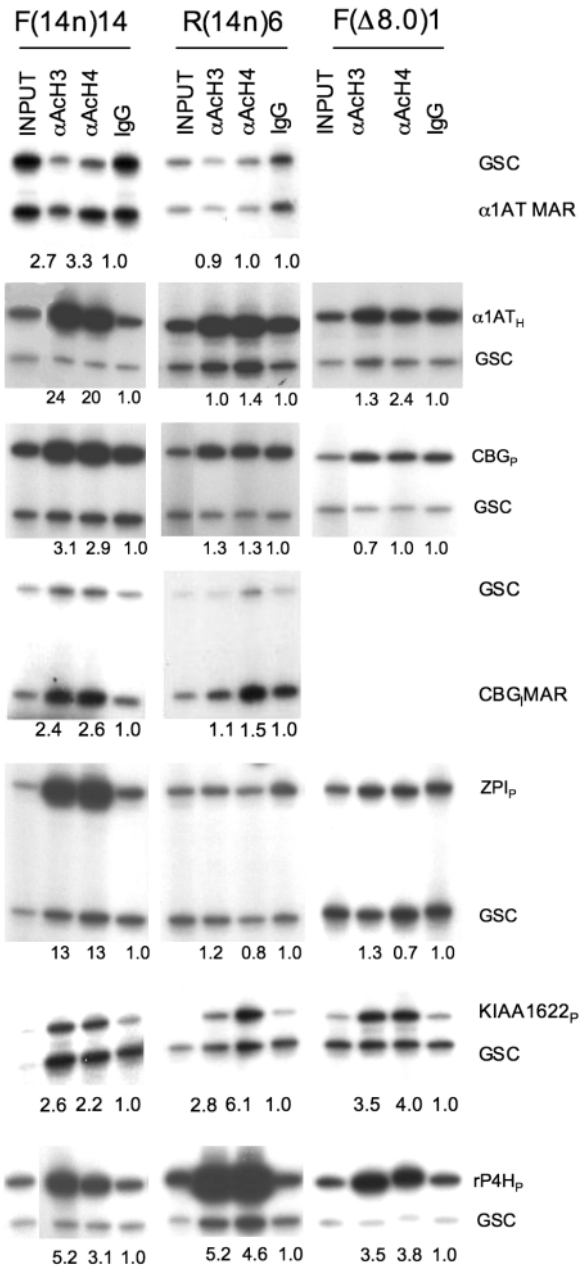

Figure 2. Histone acetylation in the proximal serpin subcluster in expressing, non-expressing and Δ8.0 mutant hybrid cells. Representative examples of duplex PCRs used to quantitate levels of histone H3 (αAcH3) and H4 (αAcH4) AcH4) acetylation in expressing [F(14n)14], non-expressing [R(14n)6] and Δ8.0 mutant hybrid cells. Results for amplicons from the α1AT MAR, the α1AT hepatic promoter ($\alpha1AT_H$), the CBG promoter (CBG_P), the CBG intron 1 MAR (CBG_IMAR), the ZPI promoter (ZPI_P), the KIAA1622 promoter ($KIAA_P$) and the ubiquitously expressed rP4H promoter ($rP4H_P$) are shown. All experimental determinations were normalized to signals from the non-expressed Goosecoid (GSC) control, which is ~380 kb distal to α1AT on human chromosome 14. Input–input DNA. IgG-pre-immune serum control. The numbers below each lane are the levels of the test amplicon relative to the Goosecoid control amplicon in the same sample, normalized to the IgG control, as quantitated using a Typhoon PhosphorImager and Image Quant software.

expressed gene that served as an internal control. Finally, modest cell-specific enrichment (2- to 3-fold) of acetylated H3 and H4 was observed in MARs upstream of α1AT and within CBG intron 1 in F(14n)14 cells (Figure 2).

The pattern of histone acetylation across the ∼320 kb genomic region that extends from the central serpin subcluster, across the proximal serpin subcluster, and through the large KIAA1622 transcription unit is summarized in Figure 3. There was no apparent enrichment for acetylated histones H3 or H4 in the centerin or APL-2 promoters or at a constitutive DHS at ∼−45kb in either expressing F(14n)14 cells (Figure 3; H3, black bars; H4, dark gray bars) or non-expressing R(14n)6 cells (Figure 3; H3, light gray bars; H4, white bars). However, expression-associated enrichments for acetylated histones H3 (∼6-fold) and H4 (∼8-fold) were seen in the region around −24 kb, a region that contains three expression-associated DHSs. Thus, expression-associated DHS formation and histone hyperacetylation extend to a region at least 24 kb but <45 kb upstream of the α1AT gene. These observations suggest that the distal boundary of the chromatin domain that contains the proximal serpin subcluster lies between ∼−45 and −24 kb.

Moving further from left (distal) to right (proximal) through the proximal serpin subcluster (Figure 3), modest (2- to 3-fold) levels of cell-specific histone acetylation were seen within the MAR upstream of α1AT (∼−18 kb). In contrast, high levels of H3 (∼15-fold) and H4 (>20-fold) acetylation were observed in serpin LCR sequences (20) near −6.0 kb. These levels of histone hyperacetylation were comparable with those at the very active α1AT hepatic promoter, which were the highest enrichments observed in the entire subcluster. Thus, histone hyperacetylation marks an important regulatory region within this chromatin domain. High levels of cell-specific histone H3 and H4 acetylation (∼10-fold) were observed at the macrophage promoter of α1AT, which has low activity in hepatic cells (31), and at the hepatic promoter (15–25-fold), which is ∼2 kb further downstream.

Downstream of α1AT, modest levels of histone acetylation (2- to 3-fold) were observed in the MAR downstream of ATR, but no hyperacetylation was seen in an intergenic DNA segment in the same region. As noted above, histone acetylation in the CBG promoter was much less than that of the α1AT or ZPI promoters (3- to 4-fold versus 10- to 20-fold), and only slight enrichment (∼3-fold) was detected in the MAR within

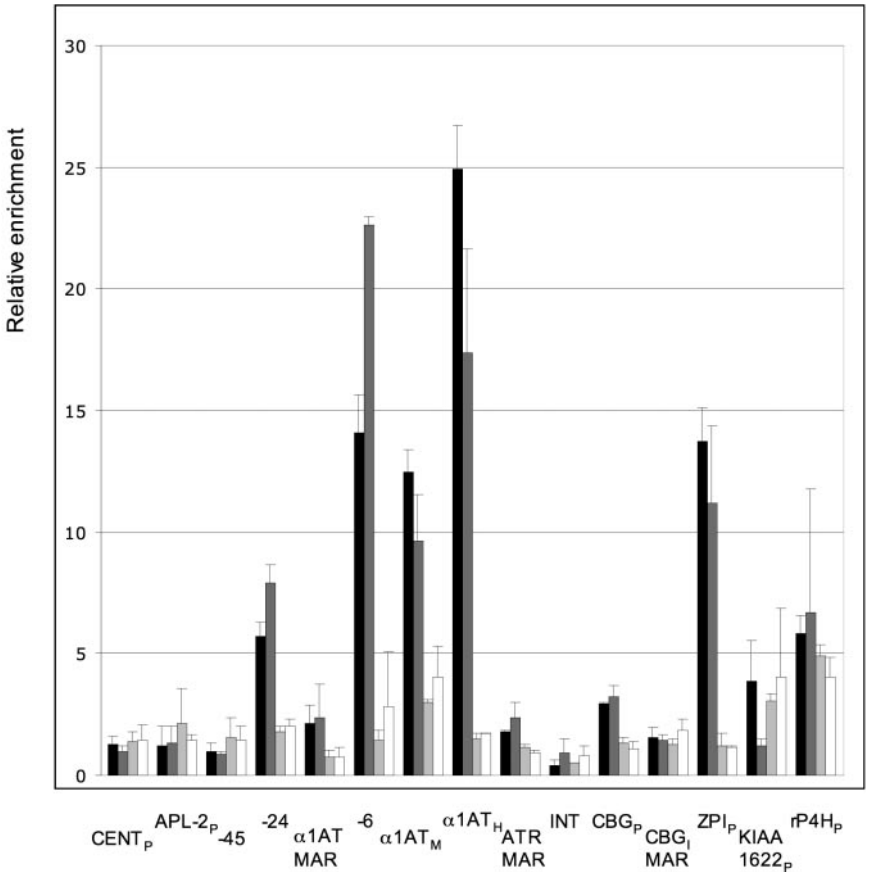

Figure 3. Patterns of histone acetylation in the proximal serpin subcluster in expressing and non-expressing cells. The graph shows patterns of histone acetylation across the proximal serpin subcluster in expressing and non-expressing cells from distal (left) to proximal (right). The black and dark gray bars represent histone H3 and H4 acetylation, respectively, in expressing F(14n)14 cells, whereas the light gray and white bars represent histone H3 and H4 acetylation, respectively, in non-expressing R(14n)6 cells. Amplicons that were assayed in these experiments included promoter regions of the Centerin (CENT$_P$), APL-2 (APL-2$_P$), α1AT (A1AT$_M$ and α1AT$_H$), CBG (CBG$_P$), ZPI (ZPI$_P$), KIAA 1622 (KIAA 1622$_P$), and rat prolyl-4-hydroxylase (rP4H$_P$) genes. Results from amplicons in the regions of expression-associated DNase I-hypersensitive sites at −45, −24 and −6 kb are also shown, as are those from the α1AT MAR, the ATR MAR and the CBG Intron 1 MAR, as well as an intergenic (INT) sequence between ATR and CBG. The positions of these amplicons on the genomic map of the region are shown in Figure 1. Each column represents the average of at least two independent determinations, with the standard deviations shown.

CBG intron 1. The last highly acetylated region in the subcluster was at the ZPI promoter; cell-specific enrichment of acetylated histones in this region was ~10-fold. The KIAA1622 promoter, which is ~115 kb further downstream and active in both cell types, was slightly hyperacetylated relative to the Goosecoid control in both hepatoma and fibroblast hybrids.

These data indicate that the proximal serpin subcluster resides within a complex chromatin domain in which histone acetylation varies considerably. The most highly acetylated regions in the domain are upstream of α1AT, and these include both the hepatic and macrophage α1AT promoters as well as the serpin LCR. A cluster of DHSs at ~−24 kb is also hyperacetylated in expressing cells. Finally, histones at the ZPI promoter were highly acetylated, but cell-specific enrichment of acetylated histones in other regions of the proximal subcluster tended to be only 2- to 3-fold.

Deletion of the serpin locus control region reduces histone acetylation throughout the proximal subcluster

Deletion of chromosomal DNA sequences in the ~8.0 kb region upstream of α1AT results in a mutant serpin allele that is refractory to cell-specific gene activation and chromatin remodeling (20). Thus, when the Δ8.0 mutant allele is transferred to rat hepatoma recipient cells, activation of α1AT, CBG and ZPI gene expression fails to occur, and none of the expression-associated DHSs in the proximal serpin subcluster is formed (20). These genetic data define a serpin LCR that regulates α1AT, CBG and ZPI transcription, and the DHS mapping data indicate that the functional domain so defined extends from at least −24 to +100 kb (20). To determine whether histone acetylation was also controlled by the serpin LCR in this functionally defined domain, ChIP experiments were performed using wild-type and Δ8.0 mutant serpin alleles.

Representative ChIP assays for the hepatic α1AT promoter ($\alpha1AT_H$), the CBG promoter (CBG_P), the ZPI promoter (ZPI_P), the KIAA 1622 promoter ($KIAA\ 1622_P$), and the rP4H promoter ($rP4H_P$) in wild-type [F(14n)14] and mutant [F(Δ8.0)1] hybrid cells are shown in Figure 2. As described above, the hepatic promoter of α1AT was highly enriched for acetylated histones H3 (~25-fold) and H4 (~20-fold) in hepatoma hybrid cells containing wild-type human chromosome 14 relative to fibroblast hybrids containing the same chromosome (Figure 2). However, there was no enrichment for acetylated histones at the hepatic promoter of α1AT in hepatoma cells that contained the Δ8.0 mutant allele (Figure 2). Similarly, the cell-specific enrichments for acetylated histones that were seen at the CBG and ZPI promoters in the wild-type serpin allele were completely abolished in the Δ8.0 mutant allele (Figure 2). In contrast, histone acetylation at the KIAA 1622 and rP4H promoters was largely unaffected affected by the Δ8.0 mutation.

Levels of histone acetylation across the wild-type and Δ8.0 mutant serpin alleles are summarized in Figure 4. The results shown for the Δ8.0 mutant allele represent averages from analyses of two independent hybrid cell clones, F(Δ8.0)1 and F(Δ8.0)6, which each contained the Δ8.0 mutant chromosome. Expression-associated histone acetylation at the ~−24 kb DHSs, the α1AT hepatic promoter, the CBG promoter and the ZPI promoter were all abolished in the Δ8.0 mutant allele, although histone acetylation at the KIAA1622 and rP4H promoters was comparable in mutant and wild-type cells. Thus, the serpin LCR is required for histone hyperacetylation across the entire proximal subcluster, and the functional domain defined by the Δ8.0 mutation encompasses a structural domain that is defined by both DHS formation (20) and cell-specific histone acetylation.

HNF-1α and HNF-4 are required for histone hyperacetylation in the proximal serpin subcluster

Variant cell lines that are deficient in the expression of specific transactivators are useful genetic tools. We previously isolated and characterized hepatoma cell lines that were unable to activate the α1AT hepatic promoter/enhancer in *trans* (18,19,32). These cells lines failed to express the liver-enriched transactivators HNF-1α and HNF-4; consequently, they were deficient in the expression of an array of genes whose expression requires HNF-1α and/or HNF-4, although other liver-specific genes continued to be expressed (18). Furthermore, when wild-type human chromosome 14 was transferred into these transactivator-deficient variants, α1AT, CBG and ZPI gene activation failed to occur, and none of the expression-associated DHSs in the proximal serpin subcluster was formed (19). However, stable transfection of the variant cells with expression plasmids encoding HNF-1α or HNF-4 partially rescued their variant phenotypes (19). To determine whether these changes in gene activity and chromatin structure were accompanied by alterations in histone acetylation across the locus, ChIP experiments were performed.

H11(14n)D and H11(14n)E are independent hybrid lines generated by transferring wild-type human chromosome 14 into the HNF-1α-deficient, HNF-4-deficient hepatoma variant line, H11 (19). Representative ChIP assays for the macrophage ($\alpha1AT_M$) and hepatic ($\alpha1AT_H$) promoters of α1AT, the CBG promoter (CBG_P), the ZPI promoter (ZPI_P), the KIAA 1622 promoter ($KIAA\ 1622_P$), and the rP4H promoter ($rP4H_P$) in H11(14n)D and H11(14n)E hybrid cells are shown in Figure 5A and B, respectively. As shown in the figure, none of the cell-specific enrichments for acetylated histones that were observed at serpin promoters in wild-type hepatoma hybrids [Figure 2, F(14n)14] was seen in the H11(14n)D (Figure 5A) or H11(14n)E (Figure 5B) variant cells.

Stable transfection of HNF-1α-deficient, HNF-4-deficient hepatoma variants with expression plasmids encoding HNF-1α or HNF-4 partially rescued their variant phenotypes, both in terms of serpin gene activation and DHS formation (19). In such 'rescued' variants, cell-specific histone hyperacetylation was partially restored. For example, transfecting H11(14n)E cells with expression plasmids encoding HNF-4 and HNF-1α generated the EH4 and EH1 transfectants, respectively. Histone hyperacetylation at the α1AT macrophage promoter, the α1AT hepatic promoter, the CBG promoter and the ZPI promoter was partially (50–70% of wild-type) restored in the EH4 transfectant line (Figure 5A). Similar effects were seen in the EH1 transfectant, although the magnitude of some of these effects was smaller (Figure 5B).

The patterns of histone acetylation across the proximal serpin subcluster in wild-type, variant and 'rescued' variant cells are summarized in Figure 5C and D. As summarized

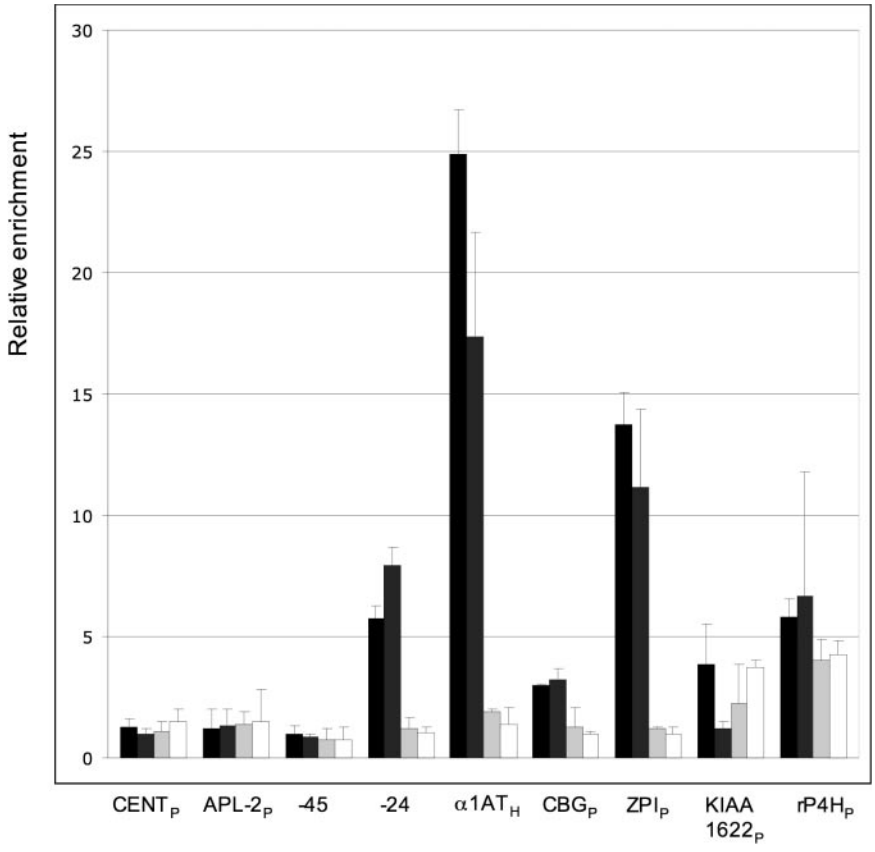

Figure 4. Cell-specific histone acetylation in the proximal subcluster requires the serpin locus control region. The graph shows patterns of histone acetylation across the proximal serpin subcluster in wild-type and Δ8.0 mutant cells from distal (left) to proximal (right). The black and dark gray bars represent histone H3 and H4 acetylation, respectively, in expressing F(14n)14 cells, whereas the light gray and white bars represent histone H3 and H4 acetylation, respectively, in mutant F(Δ8.0)1 cells. The amplicon designations are as described in the legend to Figure 3, and their genomic positions are shown in Figure 1. Each column represents the average of at least two independent determinations, with the standard deviations shown.

above, transfection of the H11(14n)E variant cells with HNF-4 largely rescued histone hyperacetylation at serpin promoters (Figure 5A and C). However, ectopic HNF-4 expression in these cells had little effect on histone acetylation at DHSs upstream (−24 kb and −6 kb) of α1AT (Figure 5C), including ∼−6 kb region, which contains important components of the serpin LCR (20). Ectopic HNF-1α expression also rescued histone hyperacetylation in the proximal serpin subcluster (Figure 5D). These data demonstrate that the functional domain defined by a *cis* acting mutation, the Δ8.0 mutant allele, was recapitulated by variant cells whose deficiency phenotypes functioned in *trans*. This provides further support for the view that the proximal serpin subcluster is an independent functional and structural genomic domain.

DISCUSSION

The human serpin locus at 14q32.1 contains 11 serpin genes (4). These genes are arranged in three subclusters—the well-studied proximal serpin subcluster, which includes α1AT, ATR, CBG and ZPI; a central subcluster, which includes vaspin, centerin and APL-2 (BX248259); and a distal subcluster, which contains the KAL, PCI, AACT and KAL-like genes. Genes in the proximal and distal subclusters are expressed in the liver, but genes in the central subcluster are not. This suggests that the locus might be organized into distinct functional domains that are differentially regulated in different cell types.

The first evidence for long-range regulation within the serpin locus was the identification of a locus control region upstream of α1AT that is required for cell-specific activation of the entire proximal subcluster (20). Originally defined by an 8.0 kb deletion extending from −8.4 to −.32 kb, the serpin LCR is required for cell-specific activation of the α1AT, CBG and ZPI genes in hepatic cells. The serpin LCR is also necessary for the formation of expression-associated DHSs throughout this ∼130 kb region (20). Thus, locus-wide controls affect both gene activity and chromatin structure in the proximal serpin subcluster. The experiments described in this report provide insight into the nature of those controls, and they further define the functional domain that encompasses the proximal serpin subcluster.

Expression-associated DHSs in the proximal serpin subcluster are distributed throughout the region that extends from ∼−25 to ∼+100 kb (5). Formation of DHSs in this interval requires the serpin locus control region (20). Moreover, none of these DHSs is formed in transactivator-deficient hepatoma variants that lack HNF-1α and HNF-4 (19). In contrast, DHSs at −45 kb (just proximal to the central serpin

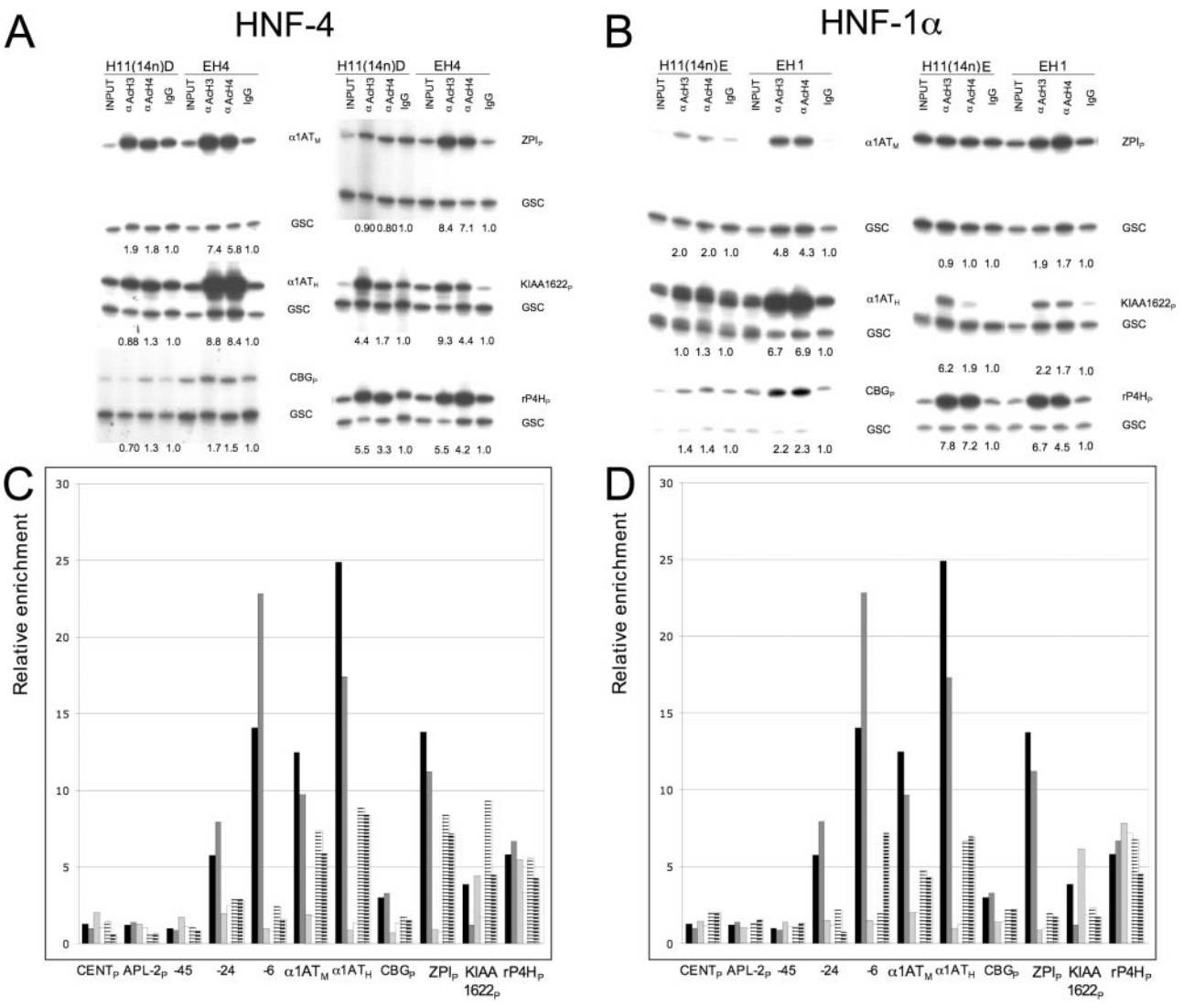

Figure 5. Histone acetylation in transactivator-deficient variant cells. (**A** and **B**) Representative examples of duplex PCRs used to quantitate levels of histone H3 (αAcH3) and H4 (αAcH4) acetylation in variant H11(14n)D cells (A) and H11(14n)E cells (B). Levels of histone H3 and H4 acetylation in H11(14n)E cells stably transfected with expression plasmids encoding HNF-4 [EH4, (A)] or HNF-1α [EH1, (B) are also shown. Results for amplicons from the α1AT macrophage promoter ($\alpha1AT_M$), the α1AT hepatic promoter ($\alpha1AT_H$), the CBG promoter (CBG_P), the ZPI promoter (ZPI_P), the KIAA1622 promoter ($KIAA_P$) and the ubiquitously expressed rP4H promoter ($rP4H_P$) are shown. All experimental determinations were normalized to signals from the non-expressed Goosecoid (GSC) control, which is ∼380 kb distal to α1AT on human chromosome 14. Input–input DNA. IgG- pre-immune serum control. The numbers below each lane are the levels of the test amplicon relative to the Goosecoid control amplicon in the same sample, normalized to the IgG control, as quantitated using a Typhoon PhosphorImager and Image Quant software. (**C** and **D**) Patterns of histone acetylation across the proximal serpin subcluster in wild-type, variant and rescued variant cells. The amplicon designations are as described in the legend to Figure 3, and their genomic positions are shown in Figure 1. The black and dark gray bars represent histone H3 and H4 acetylation, respectively, in wild-type F(14n)14 cells (C and D). The light gray and white bars represent histone H3 and H4 acetylation, respectively, in H11(14n)D (C) or H11(14n)E (D) variant cells. The small stripe bars and large stripe bars in (C) represent histone H3 and H4 acetylation, respectively, in the rescued variant EH4, an H11(14n)E derivative stably transfected with an expression plasmid encoding HNF-4. Note that ectopic expression of HNF-4 in these cells largely restored cell-specific histone acetylation at serpin gene promoters (50–70% of wild-type) but had little effect on histone acetylation at DHSs further upstream of α1AT (∼−24 kb and ∼−6 kb). The small stripe bars and large stripe bars in (D) represent histone H3 and H4 acetylation, respectively, in the rescued variant EH1, an H11(14n)E derivative stably transfected with an expression plasmid encoding HNF-1α. Ectopic expression of HNF-1α in these cells partially restored cell-specific histone acetylation at serpin promoters.

subcluster) and +209 kb (in the KIAA 1622 promoter) are not affected either in hepatoma variants or in cells carrying LCR deletions. Thus, genetic lesions that affect the expression of the proximal serpin subcluster both in *trans* and in *cis* provide evidence for a functional domain of ∼130 kb.

Hyperacetylation of histones H3 and H4 within the proximal serpin subcluster is correlated with gene activity. Furthermore, the active chromatin domain marked by acetylated histones is largely coincident with that defined by expression-associated DHSs. For example, acetylated histones are neither enriched at the centerin or APL-2 promoters in hepatic cells, nor at the −45 kb DHS. However, both H3 and H4 are hyperacetylated in the region at ∼−24 kb, which contains three expression-associated DHSs. At the other (proximal) end of the domain, there is strong, cell-specific enrichment for acetylated histones at the ZPI promoter, but not at the promoter of the

next neighboring gene, KIAA1622. These data suggest that the distal end of the domain is between ~−45 and −24 kb, and the proximal end is at ~+110 kb, where transcripts from ZPI and KIAA1622 terminate. Interestingly, a MAR has been mapped to +109 kb (4), which may represent a boundary between adjacent chromatin domains [reviewed in (33)].

Levels of histone hyperacetylation within the proximal serpin subcluster varied from region to region. For example, the highest levels of H3 and H4 acetylation were seen at the −24 kb DHSs (5- to 8-fold), the serpin locus control region (15- to 20-fold), the macrophage promoter of α1AT (~10-fold), the hepatic α1AT promoter (15–25-fold) and the ZPI promoter (10- to 15-fold). Modest levels of histone acetylation were seen in the ATR MAR and the CBG promoter (3- to 4-fold), and no enrichment at all was detected in intergenic DNA between ATR and CBG or in the CBG intron 1 MAR. Thus, a complex pattern of histone acetylation marks the active form of the proximal serpin domain. This suggests that the activated state of the subcluster does not come about via spreading of hyperacetylation from regions of highest acetylation, e.g. the serpin LCR, to neighboring chromatin. The complex pattern of histone modification within the serpin subcluster is similar to that of the mouse β-globin locus, where regions of histone hypoacetylation define subdomains within larger regions of nuclease sensitivity (34).

Histone hyperacetylation in the proximal serpin subcluster was affected by genetic lesions that affect serpin gene expression both in *cis* and in *trans*. The entire pattern of histone hyperacetylation was disrupted in hepatoma variant cells that fail to express HNF-1α and HNF-4, but this variant phenotype could be rescued, at least in part, by transfection of the cells with cDNAs encoding the transactivators. However, it appeared that histone acetylation at serpin promoters was more responsive to transactivator rescue than acetylation at upstream regulatory regions, such as the serpin LCR. These partial phenotypes are reminiscent of those observed in cells containing sub-deletions of the serpin LCR (20). Thus, there may exist a hierarchy of controls that affect subdomains of the proximal serpin subcluster, each in a discrete way.

The serpin LCR (20) is one of the most highly hyperacetylated regions in the proximal serpin subcluster. High levels of histone acetylation in this region are consistent with its identification as a locus-wide regulatory element, as other important control regions, including the β-globin LCR and the human growth hormone (hGH) LCR, are hyperacetylated in expressing cell types (35). However, serpin LCR deletions behave very differently from β-globin LCR deletions, because histone acetylation at the β-globin promoter is not affected by globin LCR deletions, whereas serpin LCR deletions reduce histone acetylation at serpin promoters and other expression-associated DHSs. In this regard, serpin LCR deletions are more like those of hGH LCR deletions, which reduce histone acetylation in a large genomic domain (36).

Constitutively hyperacetylated MARs seem to act as boundaries between regions of expressed and non-expressed chromatin in the chicken β-globin locus (29). However, in the serpin subcluster, MAR hyperacetylation is expression-associated. Furthermore, MARs do not act as chromatin boundaries within the proximal subcluster, because serpin LCR deletions affect histone acetylation both upstream and downstream, irrespective of the presence of intervening MAR sequences. These observations suggest that mammalian MARs may have a variety of different functions *in vivo*.

The identification of the proximal serpin subcluster as a discrete functional entity is an important step in understanding the ways in which gene activity and chromatin structure are regulated in this complex chromatin domain.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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