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PRINCIPAL INVESTIGATOR: Lori Wallrath, Ph.D.

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, Iowa 52242

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13. ABSTRACT (Maximum 200 Words) The five-year survival rate for individuals with a primary breast tumor is 97%. In contrast, the five-year survival rate of individuals with breast cancer metastasis is 23%. Clearly, understanding the molecular mechanisms of metastasis are essential for the prediction and prevention of metastatic disease. Studies of the chromatin protein Heterochromatin Protein 1-alpha (HP1 ^{Hsα}) will provide insights on the molecular changes that accompany breast cancer metastasis. HP1 ^{Hsα} is down-regulated in highly invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells. This down-regulation is also observed in metastatic patient tissues. We have determined that transcriptional down-regulation of HP1 ^{Hsα} in highly invasive/metastatic breast cancer cells occurs through an E-box element in the promoter region. To determine how levels of HP1 ^{Hsα} regulate invasive properties we have expressed HP1 ^{Hsα} in highly invasive/metastatic breast cancer cells and knocked down levels of HP1 ^{Hsα} in poorly invasive/metastatic breast cancer cell lines. Our results demonstrate that addition of HP1 ^{Hsα} into highly invasive cells reduces their invasive potential. Furthermore, HP1 ^{Hsα} dimerization is required for this reduction. Subsequent studies will be aimed at changes in gene expression that correlate with altered invasion.			
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INTRODUCTION

Breast cancer accounts for approximately 30% of all diagnosed cancer and is the most common malignancy in women. The lethality of breast cancer is mainly attributed to its ability to metastasize throughout the body. The five-year survival rate for individuals with metastatic breast cancer is 23%, in contrast to 95% for those with non-metastatic breast cancer (American Cancer Society). Although there has been significant progress made in the identification and understanding of breast cancer over recent years, there remains a need for the identification of molecular markers that definitively distinguish poorly invasive/non-metastatic tumors from highly invasive/metastatic tumors. To identify such prognostic/predictive markers, a clearer understanding of the progression of the metastatic disease state is required.

We have identified a molecular marker that is a candidate breast cancer metastasis suppressor. This marker, termed Heterochromatin Protein 1 ($HP1^{Hsc\alpha}$), is an evolutionarily conserved non-histone chromosomal protein (1, 3, 7). HP1 primarily localizes to centric heterochromatin where it plays a role in chromosome segregation and silencing of genes brought into juxtaposition with heterochromatin (4, 6). HP1 also localizes within the gene-rich euchromatic regions of the genome where it is proposed to play a role in gene regulation (1, 17). We discovered that $HP1^{Hsc\alpha}$, one of three HP1 proteins in humans, was significantly down-regulated in highly invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells (9). This observation was specific for $HP1^{Hsc\alpha}$, and not the other two HP1 family members. We discovered a similar correlation with $HP1^{Hsc\alpha}$ levels in breast cancer patients: $HP1^{Hsc\alpha}$ was abundant in the nuclei of cells from primary breast tumors, but dramatically reduced in cells of metastatic tissue (9). Given the role of HP1 proteins in gene regulation (1, 13, 14), we hypothesize that down-regulation of $HP1^{Hsc\alpha}$ alters the expression of genes involved in invasion and/or metastasis.

BODY

Each task and progress on that task is discussed below. All tasks are identical to those stated in the approved STATEMENT OF WORK in the original proposal.

Task 1: Determine the consequences of $HP1^{Hsc\alpha}$ expression on tumor metastasis markers and global transcriptional expression in human breast cancer cells.

This task is based on the observation that poorly invasive/non-metastatic breast cancer cells (MCF-7) have levels of $HP1^{Hsc\alpha}$ similar to most cell types, whereas, highly invasive/metastatic cells (MDA-MB-231) have low levels of $HP1^{Hsc\alpha}$ (9). Given the role of $HP1^{Hsc\alpha}$ in gene regulation, we hypothesize that altering levels of $HP1^{Hsc\alpha}$ results in changes in gene expression. To experimentally address this hypothesis we proposed to modulate the levels of $HP1^{Hsc\alpha}$ in both MCF-7 and MDA-MB-231 cells and assay for changes in gene expression. An adenovirus delivery system was utilized to increase levels of $HP1^{Hsc\alpha}$ in MDA-MB-231 cells. This system was used due to our inability to isolate MDA-MB-231 cells that stably expressed a tagged $HP1^{Hsc\alpha}$ transgene. Transient

transfection with *HP1^{Hsa}*-containing plasmids was not an option due to the low transfection efficiencies (2-5%) obtained with this cell line. In contrast, adenovirus infection results in up to 100% of the cells receiving the transgene (based on EGFP scoring). We constructed adenoviruses containing wild type and mutant *HP1^{Hsa}* transgenes tagged at the amino terminus with EGFP. From our work on *Drosophila* HP1 (12) and the work of others on mammalian and *S. pombe* HP1 proteins (11, 19), amino terminal tags do not appear to interfere with HP1 function. Western analysis demonstrated that MDA-MB-231 cells infected with *HP1^{Hsa}* adenoviruses express levels of *HP1^{Hsa}* protein similar to that of MCF-7 cells at 48 hours post infection (Figure 1A). We have demonstrated that expression of *HP1^{Hsa}* in MDA-MB-231 reduced *in vitro* invasion by 30% relative to controls (see Task 3 below). We are currently performing RT-PCR analysis with primers for 20 genes known to be involved in invasion and metastasis. We anticipate that genes such as *E-cadherin*, which promote an invasive phenotype, will decrease in expression, while genes such as *MMP-1*, which inhibit an invasive phenotype, will increase in expression upon expression of the *EGFP-HP1^{Hsa}* transgene.

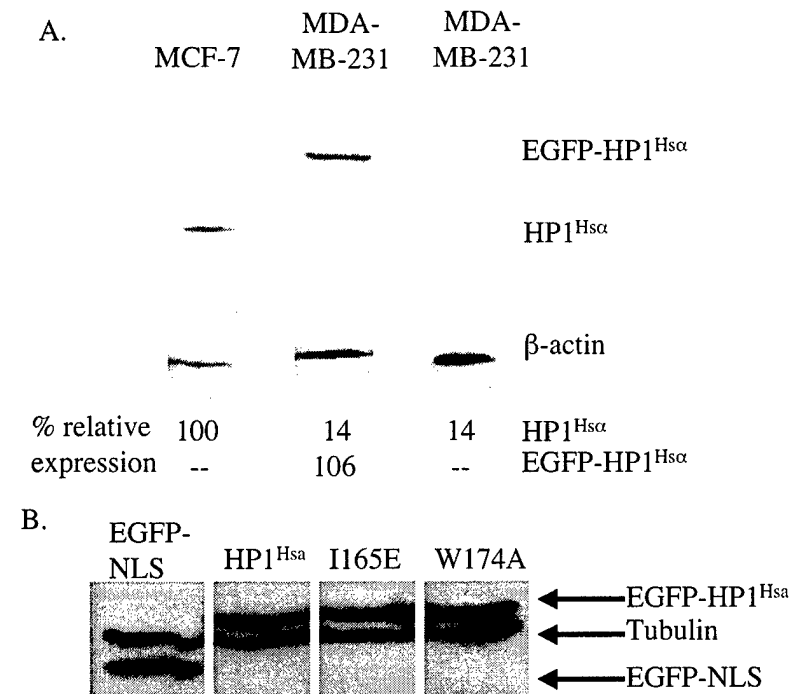


Figure 1. Protein expression of adenoviral constructs in MDA-MB-231 cells. (A) Western analysis of EGFP- *HP1^{Hsa}* protein levels 24 hour post infection compared to endogenous *HP1^{Hsa}* protein levels in uninfected MDA-MB-231 cells and MCF-7 cells (anti-*HP1^{Hsa}* antibody from Upstate Biotechnology). Anti-β-actin antibodies (U. of Iowa Hybridoma Core) are used to detect β-actin as a loading control. (B) Western analysis showing similar expression of EGFP-NLS, *HP1^{Hsa}*, I165E, and W174A 24 hours post infection.

As a counter experiment to the *EGFP-HP1^{Hsa}* expression studies described above, we are also performing RNAi experiments to knock-down the levels of HP1^{Hsa} in MCF-7 cells. We generated four RNAi plasmid constructs directed to different regions within the *HP1^{Hsa}* mRNA. These constructs were tested for their ability to knock-down HP1^{Hsa} in kidney 293T cells. These cells were used because they have a high transfection efficiency compared with MCF-7 cells. Western analysis demonstrated that two of the four constructs (618 and 272, Figure 2A) caused a 90% knock-down of HP1^{Hsa} compared to untransfected and GFP transfected control samples 48 hours post transfection (data not shown). The *HP1^{Hsa}* sequences within the two successful constructs were subsequently cloned into adenoviral vectors. Infection of MCF-7 cells with the resulting adenoviruses demonstrated a 95% knock-down at 48 post infection relative to uninfected MCF-7 cells (Figure 2C and D). We are currently in the process of comparing levels of knock-down with an adenoviral siGFP construct directed against GFP as a non-specific control. We are also performing *in vitro* invasion assays to determine whether MCF-7 cells gain invasive ability upon knock-down of HP1^{Hsa}. To examine changes in gene expression, RNA from MCF-7 cells infected with a control RNAi construct and the *HP1^{Hsa}* RNAi construct will be isolated for microarray analysis (HG-U133, Affymetrix). The University of Iowa DNA Core Facility has experience processing Affymetrix microarray chips. Furthermore, our laboratory has performed a similar microarray analysis on wild type and HP1 mutant *Drosophila*. The microarray analysis on HP1^{Hsa} knock-down cells will allow us to determine genes that might be involved in the metastatic process in breast cancer cells.

Simultaneous with the generation of the plasmid/viral constructs used for HP1^{Hsa} knock-down, we also purchased an siRNA Pool (Dharmacon) containing short dsRNAs to four regions of *HP1^{Hsa}* mRNA. These short dsRNA molecules were initially used for transfection in HeLa and 293T cells that have a high efficiency of transfection. Such experiments gave rise to 99% knock-down of HP1^{Hsa} compared with cells transfected with a missense dsRNAi as a control (data not shown). Attempts to achieve HP1^{Hsa} knock-down in MCF-7 cells by transfection of the HP1^{Hsa} dsRNA pool was initially met with difficulties, most likely due to the low transfection efficiencies of MCF-7 cells. However, upon trying the seventh transfection reagent (Lipofectamine 2000), success was observed. We obtain 95% HP1^{Hsa} knock-down relative to untransfected and missense controls (Figure 2B and data not shown). Thus, we currently have two independent methods for achieving knock-down of HP1^{Hsa} in MCF-7 cells. We are moving forward with the viral constructs rather than the Dharmacon siRNAs since the adenoviral constructs are more cost efficient.

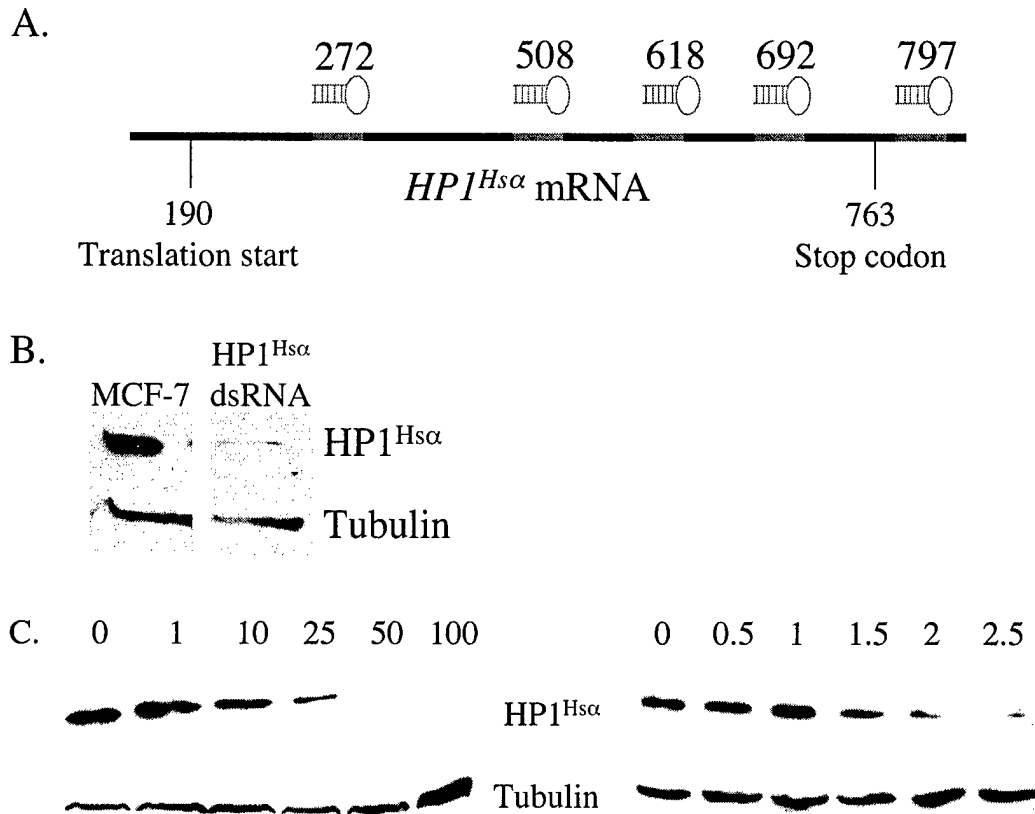


Figure 2. Knock-down of HP1^{Hsa} in MCF-7 cells. (A) Diagram of *HP1^{Hsa}* mRNA with regions corresponding to siRNA constructs labeled. (B) Western analysis of HP1^{Hsa} levels 48 hours after transfection of MCF-7 cells with Dharmacon dsRNAi. HP1^{Hsa} was detected with monoclonal antibodies specific for HP1^{Hsa} (Upstate Biotechnology); tubulin was detected with anti-tubulin antibodies (U. of Iowa Hybridoma Core) as a loading control. (C) Western analysis of HP1^{Hsa} levels 48 hours after infection with adenovirus containing construct 618. Antibodies are as described for (B). Numbers above the lanes correspond to the microliters of virus applied to the cell culture (D) Western analysis of HP1^{Hsa} levels 48 hours after infection with adenovirus containing construct 272. Antibodies are as described for (B). Numbers above the lanes correspond to microliters of virus applied to the cell culture.

Task 2: Determine the molecular mechanisms of *HP1^{Hsa}* down-regulation in human breast cancer invasion/metastasis.

The rationale for this task is that *HP1^{Hsa}* mRNA and HP1^{Hsa} protein levels in MDA-MB-231 cells are reduced 40% and 80%, respectively, compared to that of MCF-7 cells (9). We concluded that one component of the down-regulation acted at the transcriptional

level. Initially, we sequenced fragments of the *HP1^{Hscα}* gene from MCF-7 and MDA-MB-231 genomic DNA to examine the sequences for differences between the two cell types. No differences were found within the coding region or 150 bp upstream of the transcription start site. In the absence of genetic sequence changes, we examined the promoter region of *HP1^{Hscα}* for epigenetic alterations between the two cell lines. DNA methylation is frequently associated with down-regulation of gene expression in cancer cells (5, 16). Bisulfite sequence analysis revealed that the promoter region of *HP1^{Hscα}* is hypomethylated in both cell types (performed in collaboration with M. Klädde, Texas A&M). Last, we reasoned that differential regulation between the two cell lines could be due to altered regulation of transcription factors acting within the promoter region. Constructs with varying amounts of promoter sequences fused to a *luciferase* reporter gene were generated. These constructs were transfected into MCF-7 and MDA-MB-231 cells and luciferase expression was measured. The results demonstrated that differential regulation occurs through an E-box consensus element within the promoter region. Details of these experiments have been published (15) (see Appendix).

Task 3: Determine the domains of *HP1^{Hscα}* required for invasion and metastasis.

HP1^{Hscα} has a two-domain structure consisting of an amino chromo domain (CD) and a carboxy chromo shadow domain (CSD). The CD associates with methylated lysine 9 of histone H3 (8) and is thought to be the primary mechanism of *HP1* localization within centric heterochromatin. The CSD homodimerizes (2); this dimerization generates a surface that interacts with a variety of proteins possessing a pentapeptide motif (18). In order to determine the domains of *HP1^{Hscα}* involved in metastasis, we generated four mutant constructs: 1) a truncation that leaves just the CD, 2) a truncation that leaves just the CSD, 3) a point mutation, I165E, that disrupts the dimerization of *HP1* (10) and 4) a point mutation, W174A (10), that disrupts interaction with the pentapeptide (18). Due to the high costs associated with viral packaging, only the wild type and two point mutations have been packaged into adenoviruses. Infection with the control nuclear-tagged EGFP construct did not significantly alter invasion when compared with the uninfected MDA-MB-231 cells (data not shown). Viral titers were adjusted to achieve similar amounts of protein production for all constructs by western analysis (Figure 1B). Infection with the wild type *HP1^{Hscα}* construct correlated with a 30% reduction in invasion compared with the nuclear-tagged EGFP (Figure 3). A 30% reduction was also observed for the W174A mutation, suggesting that interactions with pentapeptide motif containing proteins do not play a role in regulating invasion. In contrast, the point mutation that disrupts *HP1^{Hscα}* homodimerization, I165E, exhibit invasion levels similar to the nuclear-tagged EGFP control. Thus, *HP1^{Hscα}* dimerization appears to be essential for the regulation of *in vitro* invasion.

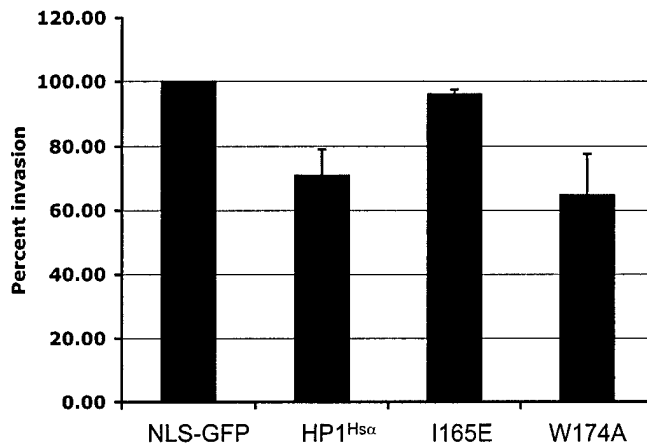


Figure 3. HP1^{Hsc} alters *in vitro* invasion of MDA-MB-231 cells. MDA-MB-231 cells were infected with adenovirus constructs expressing wild type and mutant HP1^{Hsc} and nuclear-tagged EGFP as a control. The I165E mutation disrupts HP1 homodimerization. The W174A mutation disrupts interaction with pentapeptide containing proteins, while allowing homodimerization.

KEY RESEARCH ACCOMPLISHMENTS

- Generated adenoviral vectors that express nuclear-tagged EGFP and mutant HP1^{Hsc} amino terminally tagged with EGFP.
- Demonstrated that adenoviral vectors express stable protein products at 168 hours post infection.
- Demonstrated that two of the four RNAi plasmid constructs effectively knocked-down expression of HP1^{Hsc} in HeLa and 293T cells.
- Demonstrated that two adenoviral RNAi constructs effectively knock-down expression of HP1^{Hsc} in MCF-7 breast cancer cells.
- Demonstrated that short dsRNA oligos effectively knock-down levels of HP1^{Hsc} in MCF-7 breast cancer cells.
- Identified an E-box consensus element as a major contributor to the differential regulation of HP1^{Hsc} observed between MCF-7 and MDA-MB-231 cells.
- Addition of exogenous HP1^{Hsc} to MDA-MB-231 cells (to levels comparable to MCF-7 HP1^{Hsc} expression) correlates with a reduction in *in vitro* invasion.
- A single point mutation that disrupts dimerization, eliminates the ability of HP1^{Hsc} to cause a reduction in *in vitro* invasion.

REPORTABLE OUTCOMES

Publication

Norwood, L.E., Grade, S.K., Cryderman, D.E., Hines, K.A., Furiasse, N., Toro, R. Li, Y., Dhasarathy, A., Kladde, M.P., Hendrix, M.J.C. and Kirschmann, D.A. and L.L. Wallrath (2004). Conserved properties of HP1^{Hsc α} . Gene (In Press).

Poster Abstracts

Norwood, L.E., Wright, L., Margaryan, N.M, Cryderman, D.E., Hendrix, M.J.C., Kirschmann, D.A. and L.L. Wallrath (2004). Heterochromatin Protein 1: Development of a novel breast cancer metastasis marker. University of Iowa Review of the 2003 26th San Antonio Breast Cancer Symposium.

Moss, T.J., Norwood, L., Ferraro, D., Sloat, S., Hitchler, M.J. Hendrix, M.J.C., Kirschmann, D.A. and L.L. Wallrath. Structural and functional analysis of Heterochromatin Protein 1 in breast cancer cells. University of Iowa Review of the 2003 26th San Antonio Breast Cancer Symposium.

CONCLUSIONS

This year we have made significant progress on all three tasks of the original proposal. We have determined that the differential regulation of *HP1^{Hsc α}* between MCF-7 cells and MDA-MB-231 cells occurs through an E-box consensus element in the promoter region. Several transcription factor families have members that bind to this consensus sequence. Subsequent research to identify the factor involved at the *HP1^{Hsc α}* promoter is likely to be complex and beyond the scope of this proposal. Therefore, we have finished Task 2, which has resulted in one publication.

We are now successfully able to modulate levels of HP1^{Hsc α} through adenoviral delivery of wild type and mutant HP1^{Hsc α} transgenes and siRNA constructs. This success sets the stage for exciting experimentation to examine *in vitro* invasion, gene expression and a domain structure/functional analysis of HP1^{Hsc α} . We have already demonstrated that wild type HP1^{Hsc α} when introduced into MDA-MB-231 cells reduces *in vitro* invasion by 30% compared to nuclear EGFP controls. This reduction depends on the ability of HP1^{Hsc α} to dimerize. We anticipate that a minimum of two additional publications will result within the next year. One publication will focus on the effects of expressing HP1^{Hsc α} in MDA-MB-231 cells and the other will focus on the knock-down of HP1^{Hsc α} in MCF-7 cells, both publications targeted to journals such as Cancer Research.

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APPENDICES

1. One Publication
2. Two Poster Abstracts



Conserved properties of HP1^{Hsa}

Laura E. Norwood^a, Stephanie K. Grade^a, Diane E. Cryderman^a, Karrie A. Hines^a,
Nicholas Furiasse^a, Rafael Toro^a, Yuhong Li^a, Archana Dhasarathy^b, Michael P. Kladde^b,
Mary J.C. Hendrix^c, Dawn A. Kirschmann^c, Lori L. Wallrath^{a,*}

^aThe Department of Biochemistry, The University of Iowa, 3136 MRF, Iowa City, IA 52242, USA

^bThe Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX, USA

^cThe Department of Anatomy and Cell Biology, The University of Iowa, Iowa City, IA, USA

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Abstract

Heterochromatin protein 1 Hsa (*HP1^{Hsa}*) is one of three human proteins that share sequence similarity with *Drosophila* HP1. HP1 proteins are enriched at centric heterochromatin and play a role in chromatin packaging and gene regulation. In humans, *HP1^{Hsa}* is down-regulated in highly invasive/metastatic breast cancer cells, compared to poorly invasive/non-metastatic breast cancer cells. To gain insight into this differential regulation, we have cloned the *HP1^{Hsa}* gene and characterized its genomic region. *HP1^{Hsa}* is located on human chromosome 12q13.13, 589 bp upstream of the divergently transcribed *hnRNPA1* gene. Analysis of the promoter region revealed that differential regulation of *HP1^{Hsa}* between the two types of breast cancer cells is lost upon mutation of an USF/c-myc transcription factor binding site located 172 bp upstream of the predicted *HP1^{Hsa}* transcription start site. These findings provide insights into the down-regulation of *HP1^{Hsa}* in highly invasive/metastatic breast cancer cells. To examine the functional properties of *HP1^{Hsa}*, experiments were performed using *Drosophila melanogaster* as a genetic system. When human *HP1^{Hsa}* was expressed in transgenic *Drosophila*, silencing of reporter genes inserted at centric and telomeric locations was enhanced. Furthermore, expression of *HP1^{Hsa}* rescued the lethality of homozygous *Su(var)2-5* mutants lacking HP1. Taken together, these results demonstrate the participation of *HP1^{Hsa}* in silent chromatin formation and that *HP1^{Hsa}* is a functional homologue of *Drosophila* HP1.

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Keywords: Breast cancer metastasis; *Drosophila*; Gene silencing; Heterochromatin

1. Introduction

Heterochromatin protein 1 (HP1) was first discovered in *Drosophila melanogaster* and has since been found in a variety of eukaryotes from *Schizosaccharomyces pombe* to humans (Eissenberg and Elgin, 2000). *Drosophila*, mice and humans have three HP1 family members. All HP1 proteins

have a conserved amino domain termed the chromo domain (CD) and a carboxy domain termed the chromo shadow domain (CSD), separated by a less conserved hinge region (Eissenberg and Elgin, 2000). The HP1 CD binds to methylated lysine nine of histone H3; this interaction is important for HP1 localization at centric regions of chromosomes (Brehm et al., 2004).

The HP1 CSD homodimerizes, forming a site of interaction for several nuclear proteins possessing a pentapeptide motif (PxVxL), including the HP1 CSD itself (Cowieson et al., 2000; Smothers and Henikoff, 2000). The hinge region of some HP1 family members interacts with RNA and histone H1 (Nielsen et al., 2001; Muchardt et al., 2002). Thus, HP1 can be thought of as a bridging molecule that links various nuclear proteins to the chromosome.

The known functions of HP1 proteins are largely based on genetic data. HP1 was identified in *Drosophila* muta-

Abbreviations: bp, base pair(s); kb, kilobase(s); hp1, heterochromatin protein 1; CD, chromo domain; CSD, chromo shadow domain; CAF1, chromatin assembly factor 1; µg, microgram; EGFP, enhanced green fluorescent protein; PCR, polymerase chain reaction; SRACE, 5' rapid amplification of cDNA ends; CMV, cytomegalovirus; USF, upstream stimulatory factor.

* Corresponding author. Tel.: +1-319-335-7920; fax: +1-319-384-4770.

E-mail address: lori-wallrath@uiowa.edu (L.L. Wallrath).

53 genesis screens for modifiers of heterochromatic gene
54 silencing (Weiler and Wakimoto, 1995). Mutations in
55 the *Drosophila* gene encoding HP1, *Su(var)2-5*, are ho-
56 mozygous lethal; heterozygotes show suppression of si-
57 lencing of genes placed near heterochromatin, implying a
58 role for HP1 in chromatin packaging. In *S. pombe*,
59 mutations in the HP1-like protein Swi6 lead to chromo-
60 some segregation defects (Ekwall et al., 1995). Informa-
61 tion from studies of HP1 in model organisms in
62 mammals allows one to infer the function of HP1 homologs in
63 mammalian cells, where genetic assays are not currently
64 available.

65 In humans, *HP1^{Hsa}* is specifically down-regulated in
66 highly invasive/metastatic breast cancer cells compared
67 with poorly invasive/non-metastatic breast cancer cells,
68 both at mRNA and protein levels (Kirschmann et al.,
69 2000). Consistent with these cell culture phenotypes, stain-
70 ing of breast cancer tissue samples with antibodies to
71 *HP1^{Hsa}* showed that *HP1^{Hsa}* was decreased in distant
72 metastases compared to primary tumor tissues (Kirschmann
73 et al., 2000). In this study, we identify sequences within the
74 *HP1^{Hsa}* promoter region that are responsible for differential
75 expression in metastatic vs. nonmetastatic breast cancer cell
76 lines. In addition, we show here that *HP1^{Hsa}* is a functional
77 homolog of *Drosophila* HP1.

78 2. Materials and methods

79 80 2.1. Isolation of genomic clones

81 A lambda genomic library made from the whole placenta
82 of a 27-year-old healthy Caucasian female (Clontech) was
83 screened to recover *HP1^{Hsa}* genomic clones. 1.5×10^6
84 independent clones were screened using standard techni-
85 ques. *HP1^{Hsa}* cDNA was random prime labeled with 32 P-
86 dATP (Amersham Multiprime labeling kit) and used for
87 hybridization. Southern analysis was used to identify frag-
88 ments within the clones containing *HP1^{Hsa}*. These frag-
89 ments were isolated and sequenced to determine intron/exon
90 boundaries of the *HP1^{Hsa}* gene.

91 92 2.2. 5'RACE

93 RNA was isolated from MCF-7 cells using TRIzol
94 reagent (Life Technologies). RNA was amplified according
95 to the 5'RACE System (Life Technologies) using a primer
96 specific to a region 34–59 bp downstream of the stop
97 codon. cDNA was amplified at 50 °C instead of the typical
98 42 °C to minimize secondary structure. The cDNA was
99 PCR amplified using the abridged anchor primer (Life
100 Technologies) specific to the C-tailed cDNA and a primer
101 specific to the fifth exon. An additional extension cycle at
102 72 °C for 3 min was added at the end of the PCR cycles.
103 The PCR products were cloned (TA cloning system, Invi-
104 trogen) and sequenced.

105 106 2.3. Cells and culture conditions

MCF-7 cells were kindly supplied by Dr. F. Miller
(Karmanos Cancer Institute, Detroit, MI). MDA-MB-231
cells were obtained from the American Type Culture Col-
lection (ATCC, Manassas, VA). Cell lines were maintained
as previously described (Kirschmann et al., 1999).

107 108 109 110 111 112 2.4. Bisulfite genomic sequencing

113 Genomic DNA was isolated and analyzed by bisulfite
114 genomic sequencing as previously described (Kladde et al.,
115 1996). PCR products, amplified from bisulfite-deaminated
116 DNA using JumpStart *Taq* DNA polymerase (Sigma), were
117 purified and subjected to primer extension as described
118 (Kladde et al., 1996), except that the final concentrations
119 of dNTPs (A, C, T) and ddGTP were 50 and 150 μ M,
120 respectively. Exclusion of dGTP from the PCR product
121 primer extension reactions generated high termination effi-
122 ciencies (>96%) (Kladde et al., 1996) at template cytidines
123 (nucleotides that were $^{5\text{m}}\text{C}$ residues in vivo).
124

125 126 2.5. Plasmid constructs

127 Fragments of the *HP1^{Hsa}* promoter region (positions
128 –600, –466, –418, –286, –166, and –110 bp
129 relative to transcription start at +1) fused to exon one
130 (+143) were cloned into the pGL3-Basic vector (Promega).
131 Forward primers used for generating the deletion constructs
132 were as follows: –600 bp primer 5'-GCAGAAAGGAGC-
133 GAGCTCACGAACGTATG-3', –466 bp primer 5'-
134 CCTGCTATTGAGCTCTGGTGCCACATTGC-3', –418
135 bp primer 5'-GGTCGTTCTACGAGCTCTCCACC-3',
136 –286 bp primer 5'-CTTCCACGAGCTCATATTACAGT-
137 CAAG-3', –166 bp primer 5'-GTAAAATGCGCA-
138 GCTCTGCGCA-3', –110 bp primer 5'-CGTGAAATG-
139 GAGCTCAGGAGTAGG-3'. The reverse primer used for
140 all of the *HP1^{Hsa}* promoter deletion constructs recognizes
141 *HP1^{Hsa}* exon one and the pGL3-Basic vector: 5'-
142 AGATCTCGAGCCCGGGATTGAGAGTGATCA-3'. The
143 mutant transcription factor binding sites were generated
144 using the QuikChange site-directed mutagenesis kit (Stra-
145 tagene) and the following primers: δ EF1 (m δ EF1):
146 5'-CGCATTAAGAAGTTCCCCCCCCCTCTGAGAA-
147 CACG-3' and 5'-CGTGTCTCAGAGGGGGGGG-
148 GAAGTCTTAATGCG-3', c-myc (mmyb): 5'-
149 CGTTTTGGCGGGCCCCCCCCCTTGCAGAAAGG-3'
150 and 5'-CCTTCTGCGCAAGGGGGGGGGCCCCGCC-
151 AAAACG-3', USF'c-myc site at position –109 (mUSFp):
152 5'-CCTCTGAGACCCCCCAATGGCGGGCAGGAG-
153 TAG-3' and 5'-CTACTCCTGCCGCCATTGGGG-
154 GGGTCTCAGAGG-3', USF/c-myc site at position
155 –172 (mUSFd): 5'-CTCTTGTGACCGGGGG-
156 GGGAGTAAAATGGCG-3' and 5'-CGCCATTT-
157 TACTCCCCCCCCGGTCAACAAGAG-3'. The double
158 mutants of mmyb and mUSF1 (mmybUSFp) and mUSF1

159 and mUSF2 (mUSFpUSFd) were also made using the
160 above primers.

161

162 2.6. Transient transfection assays

163 MCF-7 and MDA-MB-231 cells were grown to 80%
164 confluency. A total of 1 µg of DNA, including 0.5 µg of
165 promoter construct and 0.5 µg of *CMV-lacZ* (kind gift of
166 Dr. Andrew Russo), was transfected into the cell lines
167 using Effectene Transfection Reagent (Qiagen). The cells
168 were grown for 48 h, collected with Cell Culture Lysis
169 Reagent (Promega), and assayed for luciferase and β-
170 galactosidase expression. Luciferase expression was mea-
171 sured using Luciferase Assay Substrate (Promega) to
172 monitor expression from *HP1^{Hsα}* promoter constructs. β-
173 Galactosidase expression was measured using Galacto-
174 Light Plus System (Applied Biosystems) to normalize
175 for transfection efficiency. Light units were measured on
176 a 96-well plate luminometer (Dynex). Normalized lucifer-
177 ase light unit measurements were set relative to light unit
178 measurements obtained for a promoter construct contain-
179 ing 4 kb of *HP1^{Hsα}* upstream sequences, including exon
180 one. This 4-kb construct gives uniform low levels of
181 expression in both cell lines. These data were analyzed
182 using the Microsoft Excel two samples unequal variance
183 Student's *t*-test.

184

185 2.7. P-element construct and *Drosophila* germ line 186 transformation

187 *HP1^{Hsα}* was fused in frame with *EGFP* and inserted
188 into the P-element vector pCaSpeRhs-act ([http://thummel.
189 genetics.utah.edu/vector%20map%20htmls/pcasper-hs-act.
190 html](http://thummel.genetics.utah.edu/vector%20map%20htmls/pcasper-hs-act.html)) containing an *hsp70* promoter to drive expression of
191 the fusion gene and a mini-*white⁺* gene for selection of
192 transformants. To generate an untagged *HP1^{Hsα}* construct,
193 *HP1^{Hsα}* cDNA was inserted into the P-element vector
194 pCaSpeR-hs-act. Both resulting P-element constructs were
195 independently injected into *y, w^{67c23}* *Drosophila* embryos,
196 along with P-turbo helper plasmid encoding transposase,
197 according to standard germ-line transformation procedures.
198 Daily heat-shock treatments lead to an estimated threefold
199 higher expression of *HP1^{Hsα}*-EGFP than the endogenous
200 HP1 protein as judged by Western analysis (data not
201 shown).

202

203 2.8. *Drosophila* genetics

204 All *Drosophila* stocks were raised on standard corn meal
205 sucrose media (Shaffer et al., 1994) at 25 °C. Females with
206 the genotype *Pf^{w+}, hsp70-HP1^{Hsα}*; *Su(var)2-5⁰⁴/CyO*,
207 *GFP* were crossed to males of the genotype *Su(var)2-5⁰²/
208 CyO, GFP*. Crosses were heat shocked at 37 °C for 45 min
209 daily. Rescue of lethality was indicated by the presence of
210 straight winged adults, representing the genotype *Pf^{w+},
211 hsp70-HP1^{Hsα}*; *Su(var)2-5⁰⁴/Su(var)2-5⁰²*.

2.9. Immunostaining of polytene chromosomes

Third instar larvae were heat shocked at 37 °C for 1 h and
allowed to recover at room temperature for 2 h. Salivary
glands were dissected, fixed, squashed and stained with a
monoclonal antibody to HP1 (C1A9) and a polyclonal
antibody to GFP (Molecular Probes) according to published
procedures (Platero et al., 1995).

2.10. Northern analysis

RNA for Northern analysis was isolated from third instar
larvae after heat shock at 37 °C for 1 h according to
published procedures (Wallrath et al., 1990). Levels of
mRNA produced by the heterochromatic transgenes were
measured by hybridization with *barley* cDNA sequences
fused to the *hsp26* transgene and labeled with ³²P-dATP
(Amersham) using random prime labeling (Amersham). The
rp49 cDNA was used as a control for RNA loading.

3. Results

3.1. Structure of the *HP1^{Hsα}* genomic region

HP1^{Hsα} is down-regulated in highly invasive/metastatic
breast cancer cells in comparison to poorly invasive/non-
metastatic breast cancer cells (Kirschmann et al., 2000). To
better understand the mechanism of *HP1^{Hsα}* down-regula-
tion, we have determined the structure of the *HP1^{Hsα}*
genomic region, including the promoter region (Fig. 1).
Clone F2-10 contains exons two and three surrounded by
repetitive sequences typically found in introns. This clone
spans a region approximately 9 kb upstream of exon two to
300 bp downstream of exon three. Exon two contains the
methionine translation start codon. Clone F2-11 contains
exons three, four, and five. A second screen, using sequen-
ces corresponding to exon one and 150 bp upstream as a
probe, identified four clones containing the *HP1^{Hsα}* prom-
oter region. Clone 3-4 contains 11 kb of the *HP1^{Hsα}* promoter
region in addition to exon one that is 5' untranslated
sequence. Taken together, *HP1^{Hsα}* is encoded by five exons
spanning 38 kb.

5'RACE was performed using a primer to *HP1^{Hsα}*
(positions +799 to +824) to identify the potential
transcription start site. Three products, having their 5'
ends within 22 bp of each other, were identified. We
designated +1 as the 5' end of the longest 5'RACE
product, extending exon one of *HP1^{Hsα}* an additional 54
bp upstream as compared to the NCBI CBX5 cDNA
sequence NM_012117 (Fig. 1).

A bioinformatics analysis was performed on *HP1^{Hsα}*
promoter region sequences. Using MatInspector V2.2
<http://transfac.gbf.de/TRANSFAC/>) at stringent conditions
(core sim 1.0, matrix sim 0.95), sequences from -601 to
+143 were analyzed for known transcription factor binding

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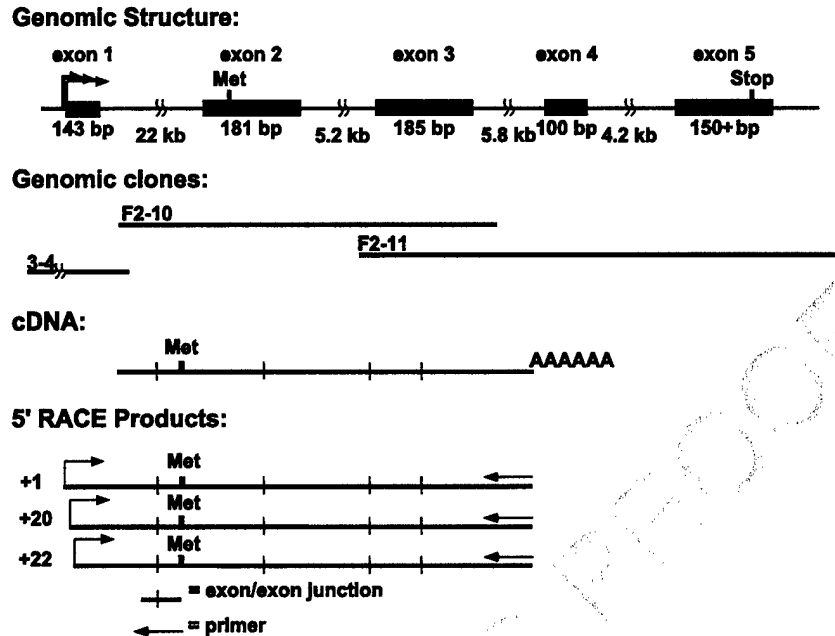


Fig. 1. The genomic structure of the human $HP1^{Hsa}$ gene with the lengths of each exon and intron in bp or kb are shown. Transcription start sites are assigned on the basis of 5RACE products. The translation methionine start (ATG) and stop are shown. The genomic clones are represented by lines under the corresponding genomic regions. Three clones obtained using 5RACE start within 54 bp upstream of the cDNA sequences previously published in Genbank. The clone containing the most 5' sequence was designated +1; additional clones start at +20 and +22.

264 sites. Using these criteria, 37 binding sites, some over-
265 lapping with each other, were identified within this region,
266 but no TATA box was identified. The lack of a TATA box is
267 consistent with having multiple transcription start sites
268 (Gum et al., 2003).

269 The *hnRNPA1* gene is divergently transcribed, starting at
270 position - 589 bp upstream of the $HP1^{Hsa}$ transcription start
271 (Fig. 3A). Although the promoter regions of *hnRNPA1*
272 and $HP1^{Hsa}$ are in close proximity, they appear to be
273 independently regulated in breast cancer cells. Unlike the
274 different levels of $HP1^{Hsa}$ observed in the two breast
275 cancer cell lines, *hnRNPA1* levels are unchanged be-
276 tween MDA-MB-231 and MCF-7 cells (data not shown).
277 DNase I footprinting of the region between the *hnRNPA1*
278 and $HP1^{Hsa}$ genes was performed using HeLa cells
279 (Biamonti et al., 1993). Six potential transcription factor
280 binding sites found in our bioinformatics analysis of the
281 $HP1^{Hsa}$ promoter region correspond to the DNase I
282 footprints previously identified, including two SP1 sites,
283 two CAAT boxes, a CREB/c-jun site, two USF/c-myc
284 sites, and a c-myc site (Biamonti et al., 1993) (Fig. 3A).
285 A δ EF1 site within the promoter region is also of
286 interest. The human AREB6 repressor protein that binds
287 to the δ EF1 site is up-regulated in highly invasive/
288 metastatic cell lines compared to poorly invasive/non-
289 metastatic cell lines (Kirschmann et al., 1999). Therefore,
290 the δ EF1 site was analyzed as a candidate regulatory
291 element (Fig. 3A).

3.2. Mechanism of $HP1^{Hsa}$ differential regulation

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293
294 The well-characterized breast cancer cell lines MDA-
295 MB-231, which is highly invasive/metastatic, and MCF-7,
296 which is poorly invasive/non-metastatic, were used to de-
297 termine the mechanism of differential regulation of $HP1^{Hsa}$.
298 One possible explanation for differential expression of
299 $HP1^{Hsa}$ is that a mutation in the $HP1^{Hsa}$ gene within
300 MDAMB-231 cells results in reduced expression. The
301 coding region, splice junctions, portions of the introns,
302 and 150 bp of the promoter region of $HP1^{Hsa}$ from MDA-
303 MB-231 and MCF-7 cells were sequenced and compared.
304 No differences between the $HP1^{Hsa}$ genomic sequences of
305 the two cell lines were found. Therefore, differential regu-
306 lation of $HP1^{Hsa}$ is not likely due to mutations within the
307 sequenced regions of $HP1^{Hsa}$.

308 As a second possibility to explain the differential expres-
309 sion, we investigated the DNA methylation status of $HP1^{Hsa}$
310 in both cell types. In many cases transcriptional regulation
311 in cancer cells is under control of DNA methylation,
312 particularly for genes near CpG islands (Dalloi et al.,
313 2003). A CpG island within exon one of the *hnRNPA1* gene
314 (-482 to -899 from $HP1^{Hsa}$ transcription start) was
315 identified using CpGReport (<http://www.ebi.ac.uk/>). Both
316 strands of the $HP1^{Hsa}$ promoter region (bases -900 to
317 +168), including the CpG island, were subjected to bisulfite
318 sequencing to determine DNA methylation of $HP1^{Hsa}$ in the
319 MDA-MB-231 and MCF-7 cell lines (Fig. 2 shows the

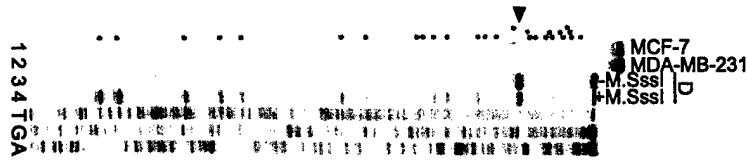


Fig. 2. Absence of detectable 5-methylcytosine (5mC) within the $HP1^{Hsa}$ promoter region in MCF-7 and MDA-MB-231 cells. DNA from both cell lines (lanes 1 and 2) was subjected to a sensitive variation of genomic bisulfite sequencing that is able to detect low levels of 5mC . Analysis of sequences from -220 to $+168$ of the $HP1^{Hsa}$ promoter is shown. Plasmid DNA containing the $HP1^{Hsa}$ promoter methylated in vitro by *M. SssI* (lane 4) provides a marker for modified CpG sites (filled circles). Since the plasmid was isolated from a *dcm+* strain of *E. coli*, methylation at a *dcm* site (arrow head) was also detected (lanes 3 and 4). Methylation at the *dcm*- and *M. SssI*-modified sites demonstrates the signal intensity that is commensurate with high and moderate levels of DNA methylation, respectively. Thus, MCF-7 and MDA-MB-231 cells do not have detectable levels of 5mC . Reactions carried out on purified plasmid DNA are labeled D.

320 methylation status of bases -220 to $+168$; bases -900 to
 321 -220 are not shown). Limited DNA methylation, if any,
 322 was observed in either cell type, and the methylation status
 323 was unchanged between the two cell lines throughout the
 324 $HP1^{Hsa}$ promoter region and exon one, including the CpG
 325 island within *hnRNPA1*. Thus, methylation does not appear
 326 to be involved in the differential regulation of $HP1^{Hsa}$.
 327 As a third explanation for the differential regulation, we
 328 hypothesized that differential expression might arise
 329 through different interactions between transcription factors
 330 and *cis*-acting DNA elements of the $HP1^{Hsa}$ promoter in
 331 the two cell types. Fragments of the $HP1^{Hsa}$ promoter,
 332 including untranslated exon one, were cloned upstream of
 333 a luciferase reporter gene. These constructs were co-trans-
 334 fected into MDAMB-231 and MCF-7 cells along with a
 335 plasmid containing the *CMV* promoter driving a *lacZ*
 336 reporter gene. Expression of luciferase and β -galactosidase
 337 was measured in light units. Luciferase expression was
 338 normalized to β -galactosidase expression to account for
 339 differences in transfection efficiency. Levels of luciferase
 340 above background were observed from all constructs,
 341 indicating that the cloned promoter region possessed
 342 transcriptional activity. A 5.6-fold difference in luciferase
 343 activity was observed between the MDA-MB-231 and
 344 MCF-7 cell lines for the largest construct, $-600/+143$,
 345 containing 13 sequences between the $HP1^{Hsa}$ and the
 346 *hnRNPA1* predicted transcription start sites (p -value=
 347 0.0021) (Fig. 3B and C). This difference in expression
 348 is similar to that observed for endogenous $HP1^{Hsa}$ gene
 349 between the two cell types (Kirschmann et al., 2000).
 350 Deletions that removed successive amounts of 5' sequences
 351 showed a reduction in the fold change in expression
 352 between the two cell types, suggesting the loss of regu-
 353 latory sequences (Fig. 3B). In particular, the differences in
 354 expression between the $-166/+143$ construct in the two
 355 cell types were barely statistically significant (p -val-
 356 ue=0.0608). One caveat of these deletion constructs is

that vector DNA sequences are brought into close prox-
 imity of the $HP1^{Hsa}$ promoter, possibly contributing to
 regulation.

To better identify elements involved in differential regu-
 lation of $HP1^{Hsa}$ between the two cell types, constructs
 containing mutations within candidate transcription factor
 binding sites were analyzed. These constructs allowed for
 the retention of 600 bp of upstream sequences. Of particular
 interest was a δ EF1 binding site at position -125 (Fig. 3A).
 This site can be bound by the human homolog of the
 chicken δ EF1 protein, AREB6, a transcriptional repressor
 (Ikeda et al., 1998) that is up-regulated in highly invasive/
 metastatic cell lines, including MDA-MB-231, compared to
 poorly invasive/non-metastatic cell lines, such as MCF-7
 (Kirschmann et al., 1999). Thus, the δ EF1 binding site was a
 promising candidate for regulating differential expression of
 $HP1^{Hsa}$. A mutation in the conserved δ EF1 site ($m\delta$ EF1)
 was constructed by replacing the 4 bp core binding site and
 5 bp of surrounding sequence with nine cytosines in the
 context of the $-600/+143$ construct. Differential expres-
 sion between the two cell lines was still observed (2.87-fold
 difference, p -value=0.0004) (Fig. 3C). Therefore, the δ EF1
 site does not appear to be involved in differential regulation
 of $HP1^{Hsa}$.

Several additional candidate transcription factor-binding
 sites were also investigated for their effects on differential
 regulation. These include a c-myc site within exon one of
 $HP1^{Hsa}$ (Fig. 3A). Mutation of this site ($mmyb$), replacing
 the core binding region and surrounding bases with nine
 cytosines, retained differential expression (ninefold differ-
 ence, p -value=0.0124) (Fig. 3C). Furthermore, mutation of
 two USF/cmyc sites located at positions -109 and -172 ,
 designated USFd (distal) and USFp (proximal), respectively,
 was tested for effects on expression (Fig. 3A). Mutations in
 the individual sites ($mUSFp$ and $mUSFd$), double mutations
 of both USF/c-myc sites ($mUSFpUSFd$), and mutation of
 USFp in combination with mutation of the c-myc site

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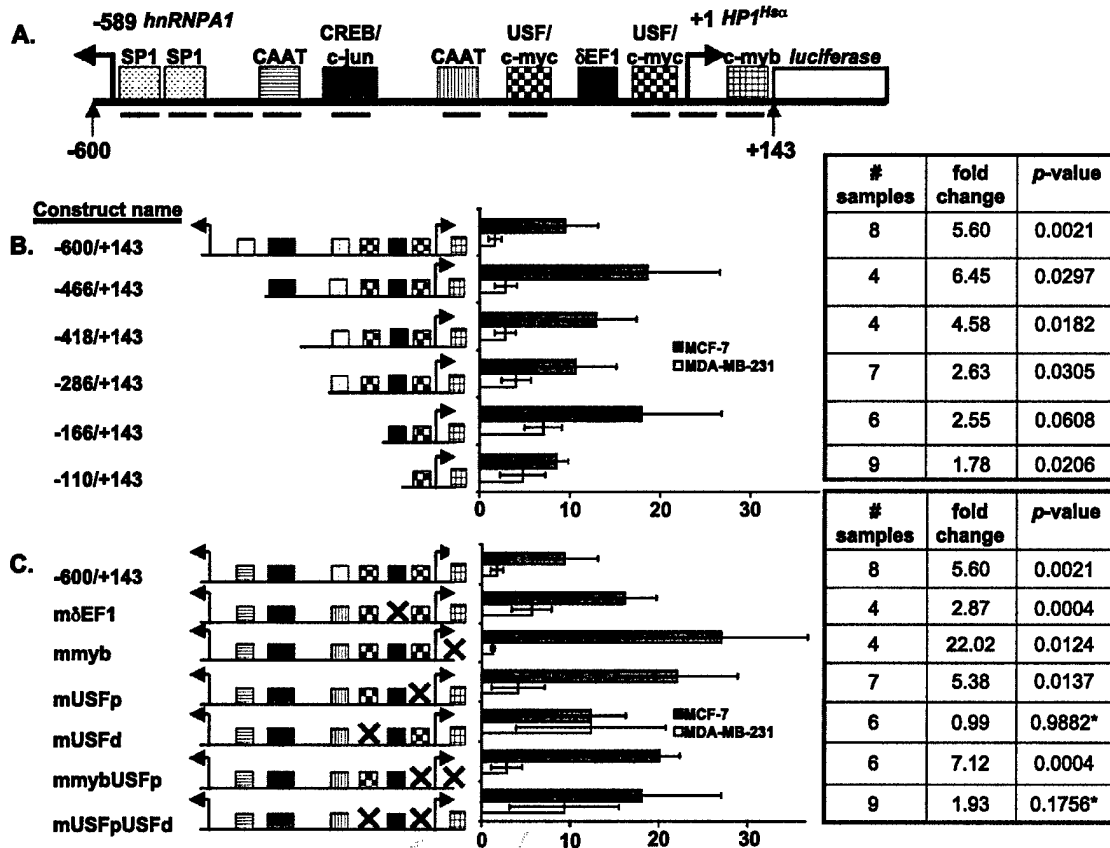


Fig. 3. (A) Diagram of the promoter region between the divergently transcribed *hnRNPA1* and *HP1^{Hsx}* genes. Consensus transcription factor binding sites that were previously shown to be footprinted by protein in HeLa cells (Biamonti et al., 1993) are indicated by black lines. Transcription factor binding sites identified by our bioinformatics searches are indicated by the name above the boxes. (B) Results of luciferase assays from 5' deletion constructs containing *HP1^{Hsx}* promoter fragments fused to a *luciferase* reporter gene. The constructs indicate the 5' and 3' sequence boundaries. The constructs were transfected into a metastatic/highly invasive cell line, MDA-MB-231 and a nonmetastatic/poorly invasive cell line, MCF-7. The number of samples, fold change between expression in MCF-7 and MDA-MB-231 cells, and *p*-values are indicated for each construct. (C) Results of mutational analysis of the *HP1^{Hsx}* promoter constructs. Construct names reflect the site(s) mutated. Asterisks mark *p*-values that show no statistical difference in expression between the two cell lines, indicating a loss of differential regulation.

394 (mmybUSFp) were examined. All of the mutations replaced
 395 the core 4 bp of the transcription factor binding sites and
 396 surrounding sequence with eight cytosines. The mUSFp
 397 mutation retained differential expression (5.38-fold differ-
 398 ence, *p*-value=0.0137) (Fig. 3C). The mmybUSFp double
 399 mutant also retained differential expression (7.12-fold dif-
 400 ference, *p*-value=0.0004). In contrast, mUSFd eliminated
 401 differential expression (0.99-fold difference, *p*-value=
 402 0.9882). Consistent with this finding, mUSFpUSFd disrup-
 403 ted differential expression (*p*-value=0.1756). Thus, the
 404 USFd site at position -172 is required for differential
 405 expression of *HP1^{Hsx}* between MCF-7 and MDA-MB-231
 406 cell lines.

407

408 3.3. Human *HP1^{Hsx}* functions similar to *Drosophila HP1*

409 In addition to understanding *HP1^{Hsx}* regulation, we also
 410 aimed to understand the functional properties of this protein.

411 *HP1* proteins are highly conserved between species, sug-
 412 gesting related functions. *HP1* was first identified in *Dro-*
 413 *sophila* where functional studies have been performed,
 414 demonstrating a role in gene silencing (Weiler and Waki-
 415 moto, 1995). Three human *HP1* family members, *HP1^{Hsx}*,
 416 *HP1^{Hsβ}* and *HP1^{Hsy}*, show a high degree of amino acid
 417 sequence identity with *Drosophila HP1*; however, it is
 418 unclear which family member is the functional homolog
 419 of *Drosophila HP1*. *HP1^{Hsy}* shows 44% overall amino acid
 420 sequence identity with *Drosophila HP1*, 60% in the CD, and
 421 38% in the CSD. These values are similar to those of
 422 *HP1^{Hsx}* showing 44% overall, 65% in the CD, and 43%
 423 in the CSD. *HP1^{Hsβ}* has slightly greater overall amino acid
 424 identity to *Drosophila HP1* than *HP1^{Hsx}* (46% vs. 44%).
 425 The *HP1^{Hsβ}* CD shows slightly more identity with the
 426 *Drosophila HP1* CD than the *HP1^{Hsy}* and *HP1^{Hsx}* CDs
 427 (68% vs. 60% and 65%, respectively). In contrast, the
 428 *HP1^{Hsβ}* CSD shows less identity to the *Drosophila HP1*

429 CSD than the $HP1^{Hs\alpha}$ CSD (39% vs. 43%). In sum,
430 comparisons of the amino acid sequences of human and
431 Drosophila HP1 identified only minor differences in the
432 percent identity without immediately suggesting a function-
433 al homologue.

434 Another protein feature that might suggest similar function
435 between Drosophila HP1 and a human HP1 is the
436 chromosome localization pattern. Drosophila HP1 shows
437 enrichment at heterochromatic regions and localizes to
438 approximately 200 euchromatic sites on larval polytene
439 chromosomes (Fanti et al., 2003). $HP1^{Hs\alpha}$ and $HP1^{Hs\beta}$
440 predominantly localize to centric heterochromatin, showing
441 partial overlap with anticentromere antibodies (Minc et al.,
442 1999). In contrast, $HP1^{Hs\gamma}$ localizes to centric heterochro-
443 matin and euchromatic regions (Minc et al., 2000). Based on
444 this localization data, $HP1^{Hs\gamma}$ appears to have a more similar
445 pattern to that of Drosophila HP1.

446 To investigate the functional properties of the $HP1^{Hs\alpha}$
447 protein and determine whether it is a functional homologue
448 of Drosophila HP1, we generated transgenic *Drosophila* that
449 expressed an $HP1^{Hs\alpha}$ -EGFP fusion gene under the control
450 of an *hsp70* heat shock promoter. Homozygous $HP1^{Hs\alpha}$ -
451 EGFP larvae were heat shocked 1 h at 37 °C and allowed to
452 recover for 2 h at room temperature. Salivary glands were
453 dissected from the larvae, squashed and stained with anti-
454 bodies that recognize EGFP and Drosophila HP1. The
455 results indicated that $HP1^{Hs\alpha}$ -EGFP localized to the chro-
456 mosome center (the site of fusion of all the centromeres), the

heterochromatic fourth chromosome, and euchromatic sites 457
in a pattern that completely overlapped with endogenous 458
Drosophila HP1 (Fig. 4A). It was possible that co-localiza- 459
tion was due to interactions between the $HP1^{Hs\alpha}$ CSD and 460
the Drosophila HP1 CSD, since CSDs have been shown to 461
dimerize (Cowieson et al., 2000). Therefore, we assayed the 462
localization of $HP1^{Hs\alpha}$ -EGFP in larvae lacking endogenous 463
Drosophila HP1. $HP1^{Hs\alpha}$ -EGFP showed the same pattern of 464
localization on larval polytenes with or without endogenous 465
HP1, indicating that $HP1^{Hs\alpha}$ associates with chromosomes 466
by similar mechanisms as Drosophila HP1 (Fig. 4B). 467

468 Overexpression of Drosophila HP1 enhances silencing of
469 genes repressed by heterochromatin (Weiler and Wakimoto,
470 1995). To test whether $HP1^{Hs\alpha}$ -EGFP has a similar function,
471 $HP1^{Hs\alpha}$ -EGFP was overexpressed (twofold over endoge-
472 nous HP1) in stocks carrying a tagged *hsp26* heat shock
473 gene inserted at different heterochromatic locations. Expres-
474 sion of $HP1^{Hs\alpha}$ -EGFP by daily heat shock resulted in a 40%
475 reduction in *hsp26* expression from a centric transgene (Fig.
476 5). Similarly, expression of $HP1^{Hs\alpha}$ -EGFP resulted in a 50%
477 reduction in *hsp26* expression from a telomeric transgene
478 (Fig. 5). These data demonstrate that the human $HP1^{Hs\alpha}$
479 protein participates in gene silencing and has similar func-
480 tions as Drosophila HP1.

481 Several homozygous lethal mutations exist in *Su(var)2-5*,
482 the Drosophila gene encoding HP1 (Eissenberg and Hartnett,
483 1993). These mutations were used to determine whether
484 $HP1^{Hs\alpha}$ -EGFP could rescue lethality. Flies carrying the

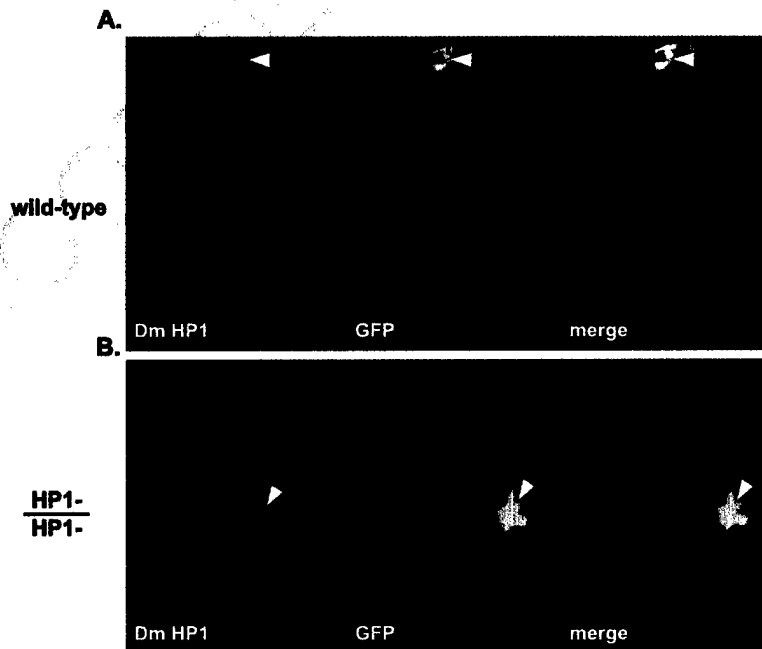


Fig. 4. (A) Polytene chromosomes from third instar larvae salivary glands from a stock carrying an $HP1^{Hs\alpha}$ -EGFP transgene. The chromosomes were fixed, squashed and stained with antibodies against Drosophila HP1 (DmHP1; red) and GFP (green). The DmHP1 antibody does not recognize $HP1^{Hs\alpha}$ by Western blot analysis (data not shown). Co-localization is observed in yellow. (B) Polytene chromosomes from HP1 mutants containing the $HP1^{Hs\alpha}$ -EGFP transgene stained with DmHP1 (red) and GFP (green). Arrowheads denote the chromocenter (formed by the fusion of all centromeres).

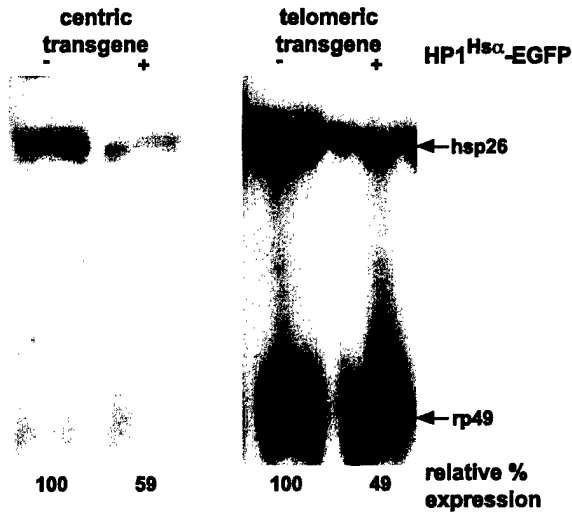


Fig. 5. Effects of $HP1^{Hs\alpha}$ -EGFP expression on gene silencing. Flies expressing the $HP1^{Hs\alpha}$ -EGFP transgene were crossed to flies that contain a reporter *hsp26* transgene inserted at a heterochromatic or telomeric location. RNA was isolated from the heatshocked progeny and analyzed by Northern analysis hybridized with radiolabeled sequences corresponding to the *hsp26* reporter gene and the *rp49* loading control.

485 $HP1^{Hs\alpha}$ transgene that were heterozygous for a mutant allele
 486 of the gene encoding HP1 [*Su(var)2-5⁰⁴*] balanced over a
 487 chromosome possessing a *Curly* wing mutation were crossed
 488 to flies heterozygous for a second mutant allele of the gene
 489 encoding HP1 [*Su(var)2-5⁰²*] over the *Curly* balancer chro-
 490 mosome. From this cross, *Curly* wing homozygotes, 25% of
 491 total progeny, die as early embryos. Flies heterozygous for
 492 the *Curly* wing balancer chromosome and a *Su(var)2-5*
 493 allele, 50% of the total progeny, were viable. The final class
 494 of progeny, heteroallelic for the *Su(var)2-5* mutant alleles,
 495 constituting 25% of the total progeny, would be lethal if no
 496 rescue is observed. Rescue of such individuals would give
 497 rise to straight wing adults. When $HP1^{Hs\alpha}$ -EGFP was
 498 expressed by daily heat shock treatment, 2% (4/218 adults)
 499 of the total progeny had straight wings, suggesting partial
 500 function of the $HP1^{Hs\alpha}$ -EGFP fusion protein. More convinc-
 501 ing, 14% (35/244 adults) of total progeny were rescued to
 502 adulthood by expressing $HP1^{Hs\alpha}$ without the EGFP tag.
 503 These data suggest that despite exhibiting the correct pattern
 504 of localization and gene silencing effects, EGFP was limiting
 505 the function of $HP1^{Hs\alpha}$. The ability of the untagged version
 506 to show appreciable rescue (14% vs. 25% for complete
 507 rescue) strongly suggests that $HP1^{Hs\alpha}$ is a functional homo-
 508 log of the *Drosophila* HP1 protein.

509 4. Discussion

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511 4.1. Conservation of gene structure

512 The *HP1* genomic structure is conserved from *Drosophi-*
 513 *la* to humans. *Drosophila Su(var)2-5*, mouse *mHP1 α* , *M31*,

and *M32*, and human $HP1^{Hs\alpha}$, $HP1^{Hs\beta}$, and $HP1^{Hs\gamma}$ are each
 comprised of five exons and four introns. Translation start
 begins in exon two in *Drosophila Su(var)2-5*, mouse
mHP1 α and *M31*, and human $HP1^{Hs\alpha}$, $HP1^{Hs\beta}$ and $HP1^{Hs\gamma}$.
 In contrast, the translation start of *M32* is within exon
 three. Due to the insertion of an intron within exon one,
M32 exon three corresponds to that of exon two in the other
HP1 genes (Jones et al., 2001). The CD of all of the
 mammalian *HP1* genes, except for *M32*, is contained in
 exons two and three (Jones et al., 2001). Exons three and
 four of *M32* have fused to become exon four. Therefore, the
 CD of *M32* is found within exons three and four. The CSD
 of all of the mammalian *HP1* genes is found in exons four
 and five (Jones et al., 2001). The amino acids at the splice
 junctions are conserved in all the mammalian *HP1* family
 members, except *M32*, but are distinctly different for *Dro-*
sophila Su(var)2-5. Therefore, the genomic structure, but
 not the intron/exon boundaries, are conserved from *Dro-*
sophila Su(var)2-5 to human *HP1* family members.

The *Su(var)2-5* gene, at cytological position 28F1-2,
 encodes a protein that is commonly referred to as *Drosophi-*
la HP1 and sometimes referred to as *HP1a* (Smothers and
 Henikoff, 2001). There are two additional *HP1*-like genes
 located at cytological positions 87C and 94C4, called *HP1b*
 and *HP1c*, respectively. The proteins encoded by these
 genes do not exhibit a chromosomal distribution pattern
 that significantly overlaps with *HP1* (Smothers and Henik-
 off, 2001). In addition, these two proteins have limited
 amino acid sequence identity with *HP1*; *HP1b* is 44%
 identical and *HP1c* is 31% identical to *HP1*. Furthermore,
HP1b and *HP1c* do not have a conserved genomic structure
<http://www.flybase.org/>) with the mammalian members of
 the *HP1* family, as does *Drosophila HP1*. Thus, *HP1* was
 exclusively used for comparisons in this study.

The three human genes encoding $HP1^{Hs\alpha}$, $HP1^{Hs\beta}$ and
 $HP1^{Hs\gamma}$ located at 12q13, 17q21 and 7q15, respectively,
 have several corresponding pseudogenes present in the
 human genome. In our screen of the human genomic library,
 we recovered one processed $HP1^{Hs\alpha}$ pseudogene (data not
 shown) corresponding to sequences on chromosome 3p11.1
<http://www.ncbi.nlm.nih.gov/>). Five pseudogenes have
 been previously published for $HP1^{Hs\beta}$, and four for $HP1^{Hs\gamma}$
 (Jones et al., 2001). The pseudogenes of $HP1^{Hs\beta}$, containing
 few, if any, introns are found on chromosomes 1q32, 3q26,
 14q24, Xp22, and Xq11 (<http://www.ncbi.nlm.nih.gov/>)
 suggesting they are processed pseudogenes. Eleven $HP1^{Hs\gamma}$
 pseudogenes are found on NCBI Genbank, four of which
 correspond to the previously published pseudogenes (<http://www.ncbi.nlm.nih.gov/>; Jones et al., 2001). The pseudo-
 genes of $HP1^{Hs\gamma}$, containing few, if any, introns are found
 on chromosomes 2q24, 3p22, 5q22, 6q22.2, 11p11, 11p14,
 11q14, 12p13, 12q23, 16p13, and 18p11 (<http://www.ncbi.nlm.nih.gov/>). Thus, each of the functional hu-
 man *HP1* family members is encoded by separate unlinked
 genes that have multiple pseudogenes scattered throughout
 the genome.

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571 4.2. *HP1^{Hsa}* regulation in breast cancer metastasis

572 *HP1^{Hsa}* is down-regulated in highly invasive/metastatic
 573 breast cancer cells compared to poorly invasive/non-metastatic
 574 cells (Kirschmann et al., 2000). This regulation likely
 575 occurs, at least in part, at the transcriptional level and does
 576 not involve differential DNA methylation. An analysis of
 577 the DNA sequences in the *HP1^{Hsa}* promoter region identified
 578 potential binding sites for transcriptional regulators that
 579 might be involved in differential regulation. Only three of
 580 the binding sites shown in Fig. 3, the two SP1 sites
 581 immediately upstream of *hnRNPA1*, the CAAT box (position
 582 -244) and the proximal USF/c-myc site (position
 583 -109), are conserved between mouse and humans; none
 584 of the elements can be identified upstream of the gene
 585 encoding *Drosophila* HP1.

586 Mutation of a δ EF1 binding site (at position -125),
 587 which associates with the AREB6 protein found to be up-
 588 regulated in highly invasive/metastatic breast cancer cells
 589 (Kirschmann et al., 1999), does not appear to be involved in
 590 the differential expression of *HP1^{Hsa}*. Mutation of a c-myc
 591 binding site at position +97 and a USF/c-myc site at
 592 position -109 does not appear to play a role in differential
 593 regulation either. In contrast, mutation of a USF/c-myc site
 594 at position -172 abolishes differential regulation. This
 595 USF/c-myc was protected from DNase I digestion, suggesting
 596 occupancy by protein(s) in HeLa cells (Biamonti et al.,
 597 1993). USF/c-myc sites, commonly called E-boxes, are
 598 frequently bound by a variety of proteins, including USF
 599 and Myc family members. USF proteins are involved in
 600 both gene silencing and activation, sometimes at the same
 601 site under different circumstances (Goueli and Janknecht,
 602 2003). Myc proteins are also involved in both gene activation
 603 and repression, depending on their dimerization partner
 604 (Queva et al., 1998). Therefore, the function of the distal
 605 USF/c-myc site in the differential regulation of *HP1^{Hsa}* is
 606 difficult to predict and warrants further investigation.

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608 4.3. Function of *HP1^{Hsa}*

609 Our data strongly suggest that *HP1^{Hsa}* is a functional
 610 homolog of the *Drosophila* HP1 protein. The results
 611 showing that *HP1^{Hsa}* can localize to the same sites on
 612 polytene chromosomes as *Drosophila* HP1 are consistent
 613 with previously published results (Ma et al., 2001). We
 614 extend these findings by demonstrating that *HP1^{Hsa}* exhibits
 615 the *Drosophila* HP1 pattern of localization even in the
 616 absence of *Drosophila* HP1. These results suggest a
 617 conserved mechanism for localization. Previously published
 618 results show that *HP1^{Hsa}* can enhance silencing
 619 induced by a transgene array in *Drosophila* (Ma et al.,
 620 2001). These arrays have similar, yet distinctly different,
 621 properties than heterochromatin (Prasad-Sinha et al.,
 622 2000). Our results clearly demonstrate that *HP1^{Hsa}* can
 623 participate in heterochromatin formation and silence eu-
 624 chromatic genes placed within heterochromatin. Thus,

HP1^{Hsa} has gene silencing functions similar to *Drosophila* HP1. 625
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Species specificity of protein function can be addressed
 by determining whether a protein from one organism can
 provide complete function of the homologous protein in
 another organism. *HP1^{Hsa}* can rescue the lethality of a
Drosophila HP1 homozygous mutant; therefore, *HP1^{Hsa}* is
 a functional homolog of *Drosophila* HP1. In contrast to our
 findings, the mouse M31 protein was unable to rescue
 mutant phenotypes associated with *S. pombe* Swi6 mutants
 (Wang et al., 2000). Rescue was obtained, however, when
 the Swi6 CSD was substituted for the M31 CSD (Wang et
 al., 2000). The overall amino acid sequence identity be-
 tween *S. pombe* Swi6 and mouse M31 is 37%. This is much
 less than the 44% overall amino acid sequence identity
 between *Drosophila* HP1 and human *HP1^{Hsa}*. In particular,
 the amino acid sequence identity between the *S. pombe*
 Swi6 CSD and the mouse M31 CSD is 39%, whereas the
 amino acid sequence identity between *Drosophila* HP1 CSD
 and the human *HP1^{Hsa}* CSD is 43%. Therefore, the CSD of
Drosophila HP1 and human *HP1^{Hsa}* is more conserved than
 the CSD of *S. pombe* Swi6 and mouse M31. The amino acid
 sequence differences between mouse and *S. pombe* might
 explain the species-specificity observed. 627
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4.4. Model for *HP1^{Hsa}* function in breast cancer metastasis 650

Given the conserved function of *HP1^{Hsa}* in gene regula-
 tion, one possible role for *HP1^{Hsa}* in breast cancer metas-
 tasis is gene silencing. Accordingly, the *HP1^{Hsa}* gene would
 be expressed in normal and primary breast cancer tumor
 cells where it produces protein that functions to silence
 genes required for invasion and metastasis. In highly in-
 vasive/metastatic breast cancer cells, *HP1^{Hsa}* expression is
 reduced and less *HP1^{Hsa}* protein is available to carry out
 gene silencing functions. Clearly in *Drosophila* and mice
 HP1 affects gene expression in a dosage-dependent manner
 (Weiler and Wakimoto, 1995; Festenstein et al., 1999).
 According to this model, loss of silencing would occur at
 genes encoding proteins that are required for invasion and
 metastasis. Therefore, the identification of *HP1^{Hsa}* regulated
 genes is a goal for future investigation. 651
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Heterochromatin Protein 1: Development of a Novel Breast Cancer Metastasis Marker

Laura E. Norwood¹, Lindsay Wright¹, Naira Margaryan², Diane E. Cryderman¹, Mary J.C. Hendrix², Dawn A. Kirschmann², Lori L. Wallrath¹

¹ Department of Biochemistry, University of Iowa

² Department of Anatomy and Cell Biology, University of Iowa

There was estimated to be 211,300 American women diagnosed with invasive breast cancer in 2003. The five-year survival rate for women with localized breast cancer is 97%, while the five-year survival rate for women with metastatic breast cancer is 23%. Currently, there is a need for molecular markers for metastasis. We have determined that the chromosomal protein, HP1^{Hs α} , is down-regulated in metastatic breast tissue compared to primary tumors. Similarly, HP1^{Hs α} is down-regulated in highly invasive/metastatic breast cancer cell lines compared to poorly invasive/non-metastatic breast cancer cell lines. We have determined that introduction of exogenous *HP1^{Hs α}* by adenoviral infection into a highly invasive/metastatic cell line reduces both the growth rate and the invasive potential. To determine the mechanism of *HP1^{Hs α}* down-regulation in highly invasive/metastatic cells, we undertook an analysis of the *HP1^{Hs α}* promoter region. Sequence analysis revealed no changes within the promoter region, splice junctions, or coding region of *HP1^{Hs α}* in the highly invasive MDA-MB-231 cells compared with the poorly invasive MCF-7 cells. In addition, sequence analysis revealed two CpG islands with the *HP1^{Hs α}* promoter region. Bisulfate sequencing detected little DNA methylation throughout these regions in both cell types. Since there were no differences in sequence or methylation status, we hypothesized that differential expression might arise through different interactions between transcription factors and *cis*-acting DNA elements of the *HP1^{Hs α}* promoter in the two cell types. We have determined that a USF/c-myc site within the *HP1^{Hs α}* promoter is responsible for the differential expression of *HP1^{Hs α}* observed between MDA-MB-231 and MCF-7 cell lines. Understanding the regulation and function of HP1^{Hs α} in breast cancer metastasis will aid in our general understanding of metastatic progression and may lead to new ways to diagnose and treat metastasis.

Structural and functional analysis of Heterochromatin Protein 1 in breast cancer cells

Timothy J. Moss^{1,2,3}, Laura E. Norwood^{1,3}, Daniel Ferraro^{1,2}, Sara L. Sloat¹, Michael J. Hitchler⁴, Mary J.C. Hendrix⁵, Dawn A. Kirschmann⁵ and Lori L. Wallrath^{1,3}

Department of Biochemistry¹, Medical Scientist Training Program²,
Genetics Program³, Biosciences Program⁴, Department of Anatomy and Cell Biology⁵

Heterochromatin protein 1 (HP1) is a conserved chromosomal protein enriched in heterochromatic regions of the genome. In humans there are three HP1 family members, HP1^{Hs α} , HP1^{Hs β} and HP1^{Hs γ} . HP1 proteins contain two conserved domains, an amino chromo domain that associates with methylated lysine 9 of histone H3, and a carboxy chromo shadow domain that interacts with a variety of nuclear proteins. These domains are thought to regulate protein-protein interactions that influence gene expression. HP1^{Hs α} is down-regulated in invasive/metastatic breast cancer cells compared with poorly invasive/non-metastatic breast cancer cells. Our working hypothesis is that HP1 plays a role in regulating invasion/metastasis through the regulation of gene expression. To address the *in vivo* function of HP1^{Hs α} in human breast cancer, RNAi knock-down experiments are underway. These experiments will determine the effects of reduced HP1^{Hs α} on invasion and gene expression. We have also taken a structural approach to understand the mechanism of HP1-mediated gene regulation. Full-length HP1 family members and mutants have been expressed in *E. coli* and purified for X-ray crystallography and biochemical analysis. These studies will allow mechanisms of silent chromatin formation and spreading to be better modeled. This information will be used to understand the role HP1^{Hs α} in breast cancer invasion/metastasis.