Distinct isoforms of the Drosophila Brd4 homologue are present at enhancers, promoters and insulator sites

Wendy A. Kellner1, Kevin Van Bortle1, Li Li1,2, Edward Ramos1, Naomi Takenaka1 and Victor G. Corces1,*

1Department of Biology, Emory University, Atlanta, GA 30322, USA and 2Department of Biostatistics and Bioinformatics, Emory University, Atlanta, GA 30322, USA

Received December 22, 2012; Revised July 22, 2013; Accepted July 24, 2013

ABSTRACT

Brd4 is a double bromodomain protein that has been shown to interact with acetylated histones to regulate transcription by recruiting Positive Transcription Elongation Factor b to the promoter region. Brd4 is also involved in gene bookmarking during mitosis and is a therapeutic target for the treatment of acute myeloid leukemia. The Drosophila melanogaster Brd4 homologue is called Fs(1)h and, like its vertebrate counterpart, encodes different isoforms. We have used ChIP-seq to examine the genome-wide distribution of Fs(1)h isoforms. We are able to distinguish the Fs(1)h-L and Fs(1)h-S binding profiles and discriminate between the genomic locations of the two isoforms. Fs(1)h-S is present at enhancers and promoters and its amount parallels transcription levels. Correlations between the distribution of Fs(1)h-S and various forms of acetylated histones H3 and H4 suggest a preference for binding to H3K9acS10ph. Surprisingly, Fs(1)h-L is located at sites in the genome where multiple insulator proteins are also present. The results suggest that Fs(1)h-S may be responsible for the classical role assigned to this protein, whereas Fs(1)h-L may have a new and unexpected role in chromatin architecture by working in conjunction with insulator proteins to mediate intra- or inter-chromosome interactions.

INTRODUCTION

The Bromo and Extra Terminal (BET) family of proteins has been reported to play a role in numerous functions including transcription regulation, chromatin boundary maintenance, genome structure, cell cycle regulation and viral replication and segregation (1,2). These proteins are characterized by two bromodomains capable of binding two acetylated lysines each, and an extra-terminus (ET) domain necessary for interactions with other proteins. Female sterile (1) homeotic [Fs(1)h] is the only BET family member in Drosophila melanogaster and is the closest orthologue to Brd4 in mammals (3–5). Because it is the only member of this protein family in Drosophila, Fs(1)h likely carries out many of the functions of BET proteins described in other organisms.

The fs(1)h gene encodes two isoforms, long [Fs(1)h-L] and short [Fs(1)h-S], both of which contain the tandem double bromodomains and the ET domain. The short isoform is identical to the short, but it contains an additional carboxy-terminal motif (CTM) that is essential for normal development of D.melanogaster (6). The best characterized binding partners of Brd4 via the bromodomains are histones, known to become hyperacetylated on transcriptional activation (7). Peptide binding assays have shown that the first bromodomain of Brd4 has a much higher affinity for acetylated H4, whereas the second seems to recognize acetylated residues in both the H3 and H4 tails with similar but weaker affinity (8,9). In mammals, once bound to the hyperacetylated histones at the promoter, Brd4 recruits Positive Transcription Elongation Factor b (P-TEFb) to the 5′ end of genes to release paused RNA Polymerase II (RNAPII) into productive elongation (10). Deletion mutants traced the interaction of Brd4 with P-TEFb to the CTM domain and additional regions, such as the second bromodomain, present in both the long and short isoforms (11). In addition, a recent study demonstrated that recruitment of Brd4 to the FOSL1 enhancer and promoter is required for P-TEFb recruitment on transcriptional activation. The recruitment of Brd4 to enhancers is also dependent on histone acetylation (12). For a large number of genes, release of RNAPII from the promoter-proximal pause appears to be the rate-limiting step for transcription. Brd4 has also been demonstrated to have a kinase activity capable of phosphorylating the carboxy-terminal domain of RNAPII, a step required for the release of
the polymerase into elongation (13). Therefore, determining where and when Brd4/Fs(1)h is recruited will help our understanding of this regulatory step.

In addition to transcription regulation, studies of Brd4 suggest a role for this protein in global chromatin structure. A recent study examined Brd4 depleted nuclei and reported disruption of chromatin architecture on a large-scale leading to nuclear disorganization and genome-wide micrococcal nuclease sensitivity. In particular, the Brd4 long isoform was demonstrated to be responsible for this phenomenon (14). In support of this, work in yeast with the BET family Bdf1 protein has shown this protein to be essential for maintaining heterochromatin-euchromatin boundaries. When Bdf1 is mutated, chromatin boundaries are blurred, and genes on either side of the boundary are misregulated, suggesting a role for this protein in the establishment or maintenance of chromatin domains (15). Taken together, it appears that BET proteins play a structural role in nuclear organization in addition to the classical function in transcription regulation.

Here, we explore the role of Fs(1)h, the Brd4 homologue and single BET family member found in *Drosophila*. We present evidence, suggesting that the two Fs(1)h isoforms may have distinct roles in chromatin architecture and transcription regulation. We analyze the genome-wide distribution of the Fs(1)h long and short isoforms. The results indicate that the two isoforms have different binding profiles, with the short isoform binding at promoters and enhancers. Surprisingly, the long isoform is present at chromatin insulators. The distribution of Fs(1)h-L provides an explanation for the chromatin architecture phenotypes previously observed, although it is possible that the two apparently distinct roles of each isoform are functionally related.

**MATERIALS AND METHODS**

**Antibodies and Co-IP experiments**

Fs(1)h-L&S rabbit polyclonal antibody was a kind gift from Drs D. Huang and I. Dawid (3). Antibodies to Fs(1)h-L were prepared by expression of a 220 amino acid fragment from the carboxy-terminal domain specific to this isoform (see Figure 6A) in *Escherichia coli*, followed by purification and immunization of rabbits and guinea pigs. For immunoprecipitation experiments, 2 × 10⁶ Kc167 cells were lysed in cell lysis buffer [5 mM PIPES (pH 8.0), 85 mM KCl, 1 mM Na₂EDTA, 1 mM EGTA, 0.5% TX-100], nuclei were spun down, incubated in 1 ml of RIPA buffer 20 mM Tris-PIPES (pH 8.0), 85 mM KCl, 0.5% TX-100, and guinea pigs. For immunoprecipitation experiments, 1 × 10⁶ Kc167 cells were lysed in 1 ml of RIPA buffer containing protease inhibitors (Roche 04 693 159 001) and briefly sonicated to ensure lysis. Lysates were treated with 5 µl of DNase I (Sigma D5319) plus 6 mM MgCl₂ at 25°C for 20 min to obtain single nucleosomes. Three microliters of the Glutathione S-transferase (GST)-Fs(1)h-L specific peptide were incubated in lystate at 4°C for 6 h and precipitated with 50 µl of glutathione-agarose beads (Novagen #70541). After four washes in RIPA buffer, bound proteins were eluted in 1x Laemmeli buffer and analyzed by western blots using antibodies to each of the insulator proteins.

**Cell culture and ChIP-seq experiments**

Kc167 cells were grown in CCM3 serum-free insect media (HyClone SH30065.01) at 25°C. ChIP experiments were performed using 2 × 10⁶ Kc167 cells cross-linked in 1% formaldehyde for 10 min as previously described (16–18). Nuclear lysates were sonicated to generate 200–1000 bp DNA fragments, incubated overnight with Fs(1)h antibodies (3), and washed and eluted following the Millipore protocol for ChIP preparation. To generate sequencing libraries, ChIP DNA was prepared for adaptor ligation by end repair (End-It DNA End Repair Kit, Epicenter Cat# ER0720) and addition of 'A' base to 3' ends (Klenow 3'-5' exo-, NEB Cat# M0212S). Illumina adaptors (Illumina Cat# PE-102–1001) were titrated according to prepared DNA ChIP sample concentration and ligated with T4 ligase (NEB Cat# M0202S). Ligated ChIP samples were PCR-amplified using Illumina primers and Phusion DNA polymerase (NEB Cat# F-530L) and size selected for 200–300 bp by gel extraction. ChIP libraries were sequenced at the HudsonAlpha Institute for Biotechnology, using an Illumina HiSeq 2000 sequencer.

**Data analysis**

Two biological replicates were used for ChIP-seq experiments, and only peaks found in both replicates were considered for further analyses. Sequences were aligned to the *Drosophila* dm3 genome using Bowtie v0.12.7. Peak files and wiggle files were generated using MACS v1.4.1 using a P-value cutoff of 10⁻¹⁰ with an equivalent number of reads from sonicated input sequences. The relationship between P-values and false discovery rate (FDR) is shown in Supplementary Figure S1. ChIP-chip data sets for H3K18ac, H3K9ac, H4K8ac, H4K16ac and GAGA Factor (GAF) were obtained from modEncode (modENCODE # 2996, 3007, 3289, 318 and 2568, respectively). ChIP-seq data sets for H3K4me3, H3K4me1, H3K27ac, H3K27acS28ph, H3K9acS10ph (GSE36374) and insulator proteins (GSE30740 and GSE36944) have been described previously (16–18). Negative values for ChIP-Chip data were set to zero color (white) due to the fact that only positive values are obtained for ChIP-seq data.
Promoter analysis was performed using transcription start sites (TSS) sorted by expression level from microarray data from *D. melanogaster* 60mer expression array (NimbleGen catalog no. A4351001-00-01) containing 15 634 genes (19). Heatmaps were generated by plotting the total number of reads (ChIP-seq) or log base 2 ratio enrichment (ChIP-chip) in 20bp bins covering 1 kb upstream and downstream from each site, followed by either clustering using Cluster 3.0 or sorting and viewed in Java Treeview. Histone acetylation data sets were sorted by the least to most correlated: H4K8ac, followed by H3K18ac, H4K16ac, H3K9ac and H3K27ac; the bottom cluster is not enriched for any of the acetylation marks tested. After sorting for the individual acetylation marks, the heatmaps for H3K9acS10ph and H3K27acS28ph were plotted using the anchors from the sort.

To determine the significance of the overlap in distributions between Fs(1)h-L, Fs(1)h-S, insulator sites and histone modifications, we followed the approach of Negre et al. (20). Briefly, we first generated permutations of insulator and histone modification sites by sampling N sites per chromosome from a random distribution of sites over the length of the chromosomes. We then mapped these simulated sites relative to those of Fs(1)h isoforms, and this process was repeated 10^4 times for each combination. We calculated P-values by determining the fraction of simulations that produced a number of mapped features as extreme as that detected in the actual data. Using this approach, we find that pairwise interactions between Fs(1)h-L and insulator proteins dCTCF, Su(Hw), BEAF-32, CP190 and Mod(mdg4) have P-values of 10^-4. As a control, we find that the interaction of Fs(1)h-S with Mod(mdg4) is not significant.

**RESULTS**

Fs(1)h is found at promoters, enhancers and other sites of unknown function. Brd4 has been found to play a critical role in transcription by releasing RNAPII from the promoter proximal pause. Current results suggest that this function is carried out by recognition of acetylated lysines in histones H3 and H4 tails via the tandem double bromodomains. To analyze the role of the *Drosophila* Brd4 homologue Fs(1)h in nuclear biology, we determined its genome-wide distribution by ChIP-seq using an antibody that recognizes both protein isoforms, Fs(1)h-L and Fs(1)h-L (3); we will use the term Fs(1)h-L&S to indicate situations in which the two isoforms cannot be distinguished experimentally. We predicted we would find enrichment of Fs(1)h-L&S at active enhancers and promoters in light of previous reports describing the presence of Brd4 at the FOSL1 enhancer and promoter in mammalian cells (12). To test this possibility, we compared the distribution of Fs(1)h-L&S with that of H3K4me3 and H3K4me1 obtained by ChIP-seq (16). Peaks of Fs(1)h-L&S identified by MACS in two biological replicates (n = 8423) were used as anchors to generate heatmaps of H3K4me3, which is correlated with active promoters, and H3K4me1, which is correlated with enhancers (16). Although we cannot be sure that all H3K4me1 enriched regions act as enhancers, this histone modification best defines this class of regulatory elements. The heatmaps were then K-means clustered using Cluster 3.0, and Fs(1)h-L&S was found at clusters enriched with H3K4me3 and H3K4me1 (Figure 1). In addition, Fs(1)h-L&S occurs in two clusters of unknown identity that are not correlated with enrichment of either H3K4me3 or H3K4me1. The cluster with the strongest Fs(1)h-L&S signal falls into this category, suggesting that one or both Fs(1)h isoforms might have a role different from that previously studied in transcription activation (Figure 1).

**Fs(1)h-S is present at enhancers and promoters**

Studies of *fs(1)h* mutants in *Drosophila* have suggested different functions for the long and short Fs(1)h isoforms based on the different phenotypes observed in mutants that affect each isoform specifically (4,21). To further explore whether Fs(1)h-L and Fs(1)h-S perform distinct functions, we determined differences in their genomic distribution by generating an antibody specific to the long isoform (Figure 2A). It is not possible to obtain antibodies specific to the short isoform because the entire length of Fs(1)h-S is identical to the first 1110 amino acids of Fs(1)h-L. ChIP-seq was then performed with this Fs(1)h-L specific antibody, peaks were identified using MACS, and those found in both biological replicates were analyzed (n = 4867). Peaks present in the Fs(1)h-L&S data set that are not present in the Fs(1)h-L ChIP-seq data set should be specific for the Fs(1)h-L isoform (n = 4015). This number is close to but higher than the expected 3556 peaks if both antibodies were completely efficient and specific. The 11.4% discrepancy between expected and observed suggests that the Fs(1)h-L antibody recognizes some sites that are not recognized by the Fs(1)h-L&S antibody. It is possible that this selection approach results in loss of Fs(1)h-S sites that also
contain the Fs(1)h-L isoform. Nevertheless, although not complete, the Fs(1)h-S data set will contain sites where this isoform is present without Fs(1)h-L. Cluster analysis shows that the Fs(1)h-S peaks are found in regions containing H3K4me3 but not H3K4me1. At these sites, the levels of Fs(1)h-L are close to background (Figure 2B). Scanning of these sites in the genome browser confirms differences in binding between the two data sets. Fs(1)h-S (sites at which Fs(1)h-L&S is present but Fs(1)h-L is absent) is found enriched at promoters marked by H3K4me3, and intergenic regions marked by H3K4me1 and H3K27ac likely to be enhancers (Figure 2C). From these data, it appears that the two isoforms have non-redundant distributions and therefore functions, at least at most sites in the genome.

The presence of Fs(1)h-S at enhancers and promoters correlates with active transcription

To further investigate the role of Fs(1)h-S present in regions enriched in H3K4me3 presumed to be promoters, TSSs from a Kc cell expression array (18,19) were sorted by expression level and given strand-specific orientation. The enrichment of Fs(1)h-L&S follows the same pattern as H3K4me3, a well-characterized histone modification correlated with transcription levels (Figure 3A). As

Figure 2. The Fs(1)h-S and Fs(1)h-L isoforms are present in different genomic locations. (A) Western blot analysis of Kc167 cell lysates using Fs(1)h antibodies recognizing both the long and short isoforms (left panel) and long specific isoform (right panel). (B) Peak summits present in the Fs(1)h-L&S data set but not found in the Fs(1)h-L data set were used as anchors to generate heatmaps showing co-localization with H3K4me3 and H3K4me1. The number of reads is plotted for each ChIP-seq data set in 20 bp bins covering 1 kb upstream and downstream from each unique Fs(1)h-S peak summit. The heatmap was subjected to K-means clustering using Cluster 3.0 and viewed in Java Treeview. (C) ChIP-seq wiggle files for Fs(1)h-L&S and Fs(1)h-L were visualized using the SignalMap software program (NimbleGen) along with a track of all Drosophila annotated genes from the UCSC Genome Bioinformatics website. The panel represents a region spanning coordinates 815 000–855 000 of chromosome 3L (genes CG13900, CG42553, CG42554, CG13901, CG13887, CG13902, Gr61a, CG13889, CG13890, dpr20) and shows Fs(1)h-L&S binding at active promoters marked by H3K4me3 enrichment, and active enhancers marked by H3K4me1 and H3K27ac enrichment. Fs(1)h-L is absent from promoters and enhancers. Y-axis represents number of reads.

Figure 3. Sites of Fs(1)h-S correlate with gene expression and show a different binding profile than Fs(1)h-L. (A) TSSs were sorted by expression level from highest to lowest and given up/downstream orientation according to strand. The total number of reads is plotted for each ChIP-seq data set in 20 bp bins covering 1 kb upstream and downstream from each TSS and viewed in Java Treeview. (B) Peaks unique to Fs(1)h-h-S and Fs(1)h-h-L data sets present around TSSs were plotted in 20 bp bins, and the average profile was plotted covering 200 bp upstream and downstream from the TSS and compared with the average profile of H3K4me3. Read counts within the window were normalized to the total reads within the window and plotted as a fold enrichment over the baseline calculated, as the average number of reads across the window to compare between data sets. (C) Example of a gene with an Fs(1)h-L&S peak shows that the Fs(1)h-L summit is found slightly upstream of the TSS, and the Fs(1)h-S summit is found slightly downstream of the TSS but still has enrichment upstream from the Fs(1)h-L cross-reactivity. (D) Enhancers identified by H3K4me1 enrichment were used as anchors around which the total number of reads is plotted for each ChIP-seq data set in 20 bp bins covering 1 kb upstream and downstream. The enrichments were then sorted by Fs(1)h-L&S intensity and viewed in Java Treeview.
expected, the binding profile of Fs(1)h-L is different from that of Fs(1)h-L&S. The heatmap in Figure 3A indicates that some sites of Fs(1)h-L are present at TSSs, but when present close to promoters, the long Fs(1)h isoform appears to bind at a slightly different location than Fs(1)h-S and does not correlate as well with transcription levels (Figure 3A and B). Fs(1)h-L binds on average at ~20 bp with respect to the TSS, in what is characterized as the histone-free region of the promoter. Conversely, Fs(1)h-S is present at +140 bp with respect to the TSS at the same location as the peak of H3K4me3, a histone modifications known to occur at promoters along with hyperacetylated histone H3 and H4 to which Fs(1)h is reported to bind during transcription activation (Figure 3B). The location of the summit of Fs(1)h-L is slightly upstream from the TSS, whereas the Fs(1)h-S summit is slightly downstream at promoters having both isoforms (Figure 3C), supporting the hypothesis that the two isoforms perform different functions. The results also suggest that, if the bromodomains of the two isoforms are involved in their targeting to chromatin, they may do so by recognizing acetylated residues in different proteins.

The initial clustering presented in Figure 1 suggests that there are significant numbers of Fs(1)h-L&S peaks at regions enriched for H3K4me1 and lacking H3K4me3, a pattern of histone modifications characteristic of enhancers. To further explore the presence of Fs(1)h at these sequences, a previously published set of enhancers (16) was used to generate enrichment heatmaps of Fs(1)h-L&S, Fs(1)h-L and H3K27ac, a histone modification found at active enhancers. Sorting by Fs(1)h-L&S intensity shows a correlation with H3K27ac and absence of Fs(1)h-L, suggesting that the enrichment from the Fs(1)h-L&S data set is contributed specifically by the Fs(1)h-S isoform (Figure 3D). Although all regions with H3K4me1 enrichment may be enhancers, the correlation of Fs(1)h-L&S binding with the H3K27ac modification, carried out by the histone acetyltransferase CBP, supports a role in active transcription (22).

The Fs(1)h-S isoform is present at sites where histone H3 is phosphoacetylated

Current results suggest that Brd4 function is carried out by recognition of acetylated lysines in histones H3 and H4 tails via the tandem double bromodomodomains to recruit P-TEFb. This correlates well with the presence of hyperacetylated histone tails described at active enhancer and promoter regions, whose role could be recruitment of Brd4 to elicit productive elongation by tethering P-TEFb to the promoter. However, it is not clear which acetylated lysine residues on the histones H3 and H4 tails are bound by the Brd4 bromodomodomains in vivo. In vitro peptide binding assays of each of the bromodomodomains have resulted in conflicting data, and a recent comprehensive analysis has shown that binding of Brd4 in vitro is not specific for any particular lysine residues. Instead, it appears that any combination of multiple acetylated lysines will result in Brd4 binding (8). To test whether this is also the case in vivo, Fs(1)h-L and Fs(1)-S specific peaks were used as anchors to cluster genome-wide ChIP data sets for H4K8ac, H4K16ac, H3K9ac, H3K18ac, H327ac, H3K9acS10ph and H3K27acS28ph. Sorting of acetylation marks from the least abundant (H4K8ac) to the most abundant (H3K27ac) demonstrates that Fs(1)h-S sites (Figure 4A) are more enriched for acetylated histones than Fs(1)h-L (Figure 4B). Most Fs(1)h-L sites show no enrichment for any of the histone acetylation marks investigated. A quantitative analysis of the relationship between the genomic locations of Fs(1)h isoforms and these histone modifications is presented in the scatter plots shown in Supplementary Figures S2 and S3.

Fs(1)h-S sites do not appear to co-localize extensively with regions of enrichment for H4K8ac (Figure 4A), contrary to results showing strong in vitro binding of mammalian Brd4 to peptides containing this modification (8,23). Similarly, H3K18ac, a mark reported to be carried out by CBP at enhancers and promoters along with H3K27ac (24,25), showed enrichment at only a small fraction of Fs(1)h-S sites. H4K16ac, deposited by MOF, is also a requirement for Brd4 recruitment at the FOSL1 enhancer (12). Although about half of all Fs(1)h-S peaks show H4K16ac enrichment, this modification is not present in the other half. These genomic sites of Fs(1)h-S must therefore require some combination of acetylated residues other than H4K8ac and H4K16ac for recruitment. H3K27ac, a modification associated with active enhancers as well as promoters, is enriched at most Fs(1)h-S sites but has not been shown to bind Brd4 bromodomodomains in vitro (8). Fs(1)h-S overlaps extensively with H3K9ac and H3K27ac, which likely occur simultaneously on neighboring residues of the same histone tail. Interestingly, phosphoacetylated histone H3 at H3K9acS10ph is enriched at most Fs(1)h-S binding sites (Figure 4A), and it shows the highest correlation in scatter plots (Supplementary Figure S2). This agrees with previous results, suggesting that H3K9acS10ph is necessary for Brd4 recruitment at the FOSL1 enhancer and promoter on gene activation (12). Taken together, it appears that no one individual acetylated histone H3 or H4 residue investigated here is essential for Fs(1)h-S binding, and it is likely that recruitment of this protein to chromatin is carried out by a combination of histone acetylation marks that may vary depending on the gene. On the other hand, H3K9acS10ph and H3K27acS28ph are the only modifications investigated here that are present at most Fs(1)h-S sites. As the phosphorylation of these two serine residues is carried out by JIL-1 in Drosophila (16), these results support a role for this kinase in recruitment of Fs(1)h to release paused RNAPII and elicit productive elongation. Interestingly, and in agreement with a different role for Fs(1)h-L, this isoform does not appear to be associated with acetylated histones H3 and H4 at most sites in the genome (Figure 4B and Supplementary Figure S2).

One clear exception is H3K9acS10ph, which co-localizes with both Fs(1)h-S and Fs(1)h-L. The functional significance of the presence of H3K9acS10ph at Fs(1)h-L sites is unclear at the moment and will require further analysis in view of the results presented later in the text.
Fs(1)h-L binds insulator sites with multiple insulator proteins

To gain insights into the possible nature of the sequences where Fs(1)h is present in the unknown cluster shown in Figure 1, we first analyzed whether Fs(1)h-L or Fs(1)h-S is present at these sites (Supplementary Figure S4). Motif analysis was performed on the Fs(1)h-L&S peaks from these clusters to try to identify DNA-binding proteins with which Fs(1)h might associate at these genomic locations. In Drosophila, there are five characterized insulator DNA-binding proteins, Drosophila CCCTC-binding factor (dCTCF), Suppressor of Hairy-wing [Su(Hw)], GAGA Factor (GAF), Boundary element-associated factor of 32 kDa (BEAF-32) and Zeste-white 5 (Zw5) (17,18,26). Surprisingly, Multiple EM for Motif Elicitation (MEME-Chip) (27) identified binding motifs for several of these insulator proteins, including BEAF-32, dCTCF, GAF and Su(Hw), suggesting a possible association of Fs(1)h with insulators that has not been previously reported.

Enrichment profiles viewed in a genome browser show Fs(1)h-L enriched at insulator sites, particularly in locations where multiple insulator proteins are present. Interestingly, the overlap of Fs(1)h-L at peaks of individual insulator binding proteins is less pronounced (Figure 5A and Supplementary Figure S5). Overall, Fs(1)h-L overlaps with 2373 peaks of CTCF, 3329 peaks of Su(Hw), 2650 peaks of CP190 and 2433 peaks of Mod(mdg4). To explore the relationship between the genomic locations of Fs(1)h isoforms and insulator proteins, we performed K-means clustering with ChIP-seq data sets for these proteins using Fs(1)h-S sites as anchor points. The heatmaps shown in Figure 5B indicate that Fs(1)h-S specific sites (top) are depleted of insulator proteins (see also Supplementary Figure S6), whereas Fs(1)h-L sites are enriched for all insulator DNA-binding proteins with the exception of GAF. We have recently shown that insulators containing binding sites for multiple insulator proteins constitute a special subset, named ‘aligned insulators’, which are enriched at the borders between topologically associating domains defined by Hi-C (17). Therefore, sites where Fs(1)h-L is present appear to correspond to sites of aligned insulators (Figure 5B). We have previously shown that some insulator sites are located close to promoters, whereas results in Figure 2A–C show that some Fs(1)h-L sites are present in the nucleosome-free region upstream of TSSs. To further explore this issue, we carried out K-means clustering of ChIP-seq data sets for Fs(1)h-L and insulator proteins using TSSs as anchors and sorted by expression levels of the corresponding genes. The results show an overlap between Fs(1)h-L and DNA-binding insulator proteins, with the exception of GAF, at this region (Figure 5C). Mod(mdg4) and CP190 are non-DNA-binding insulator proteins that help mediate interactions between the different insulator DNA-binding proteins (18,28,29). To examine the relationship of the location of these two proteins to that of Fs(1)h-L, we performed K-means clustering of insulator protein ChIP-seq data sets, including CP190 and Mod(mdg4), using Fs(1)h-L peaks as anchors. A subset of Fs(1)h-L sites colocalize with aligned insulators where BEAF-32, dCTCF, Su(Hw), CP190 and Mod(mdg4) are present (Figure 5D). In addition, the results show that the pattern of Fs(1)h-L distribution most closely resembles that of Mod(mdg4) (Figure 5D and E).

The co-localization of Fs(1)h-L with insulator proteins does not necessarily indicate a direct interaction. To determine whether Fs(1)h-L physically interacts with any of these insulator proteins in vivo, co-immunoprecipitation experiments were carried out with antibodies to the Fs(1)h-L isoform. Supporting the ChIP-seq data, interactions were detected between the Fs(1)h-L isoform and GAF, BEAF-32, Mod(mdg4) and CP190 (Figure 6A). The reciprocal
Figure 5. The Fs(1)h-L isoform is found at insulator sites. (A) ChIP-seq wiggle files for Fs(1)h isoforms, and insulator proteins were visualized using the SignalMap software program (NimbleGen) along with a track of all Drosophila annotated genes from the UCSC Genome Bioinformatics website in the region covering 3L: 486 000–552 000. The panel shows Fs(1)h-L present at sites containing multiple insulator proteins but not at sites where only one protein is found. (B) The total number of reads is plotted for each ChIP-seq data set indicated above in 20 bp bins covering 1 kb upstream and downstream from each Fs(1)h peak present in Fs(1)h-L&S but not in Fs(1)h-L (top), each Fs(1)h peak present in both Fs(1)h-L&S and Fs(1)h-L (middle) and peaks found in Fs(1)h-L but not Fs(1)h-L&S (bottom) (C) The total number of reads is plotted for each ChIP-seq data set indicated above in 20 bp bins covering 1 kb upstream and downstream from all annotated TSS sorted by expression level and viewed in Java Treeview. (D) Data sets for each of the indicated proteins were plotted in 20 bp bins covering 1 kb upstream and downstream from each Fs(1)h-L peak summit. The heatmap generated was subjected to K-means clustering using Cluster 3.0 and viewed in Java Treeview. (E) The Fs(1)h-L and Mod(mdg4) data sets from D were sorted by Fs(1)h-L intensity to show the correlation between the location and abundance of the two proteins.

Figure 6. Fs(1)h-L interacts with insulator proteins. (A) Co-IP experiments with a protein lysate from Kc167 cells (Input) were performed using either IgG (control) or antibody against Fs(1)h-L (guinea pig). Western analysis was performed on all samples using antibodies designated on the right side of the panel at 1:10 volume of input to IP. (B) Co-IP experiments were performed as described earlier in the text with only one immunoprecipitation from the lysate using antibodies against each of the indicated insulator proteins followed by western blotting with Fs(1)h antibodies that recognize both isoforms. (C) Organization of the Fs(1)h locus. Amino acids 1169 through 1389 were placed in an expression vector with a GST tag at the N-terminus and His6 tag at the C-terminus for expression in E. coli followed by purification to 1.3 mg/ml using the His6 and GST tags. (D) The Fs(1)h-L specific peptide was incubated with a Kc cell protein lysate, precipitated with glutathione-agarose beads and analyzed by western blot to test for interaction with each of the insulator proteins. Results suggest that GAF, Su(Hw), CP190 and Mod(mdg4) interact, directly or indirectly, with the CTM domain of Fs(1)h-L.
co-IP experiment, using antibodies to various insulator proteins followed by western analysis using antibodies that recognize both Fs(1)h isoforms, confirms the previous observation, suggesting that GAF, Su(Hw), CP190 and Mod(mdg4) interact with Fs(1)h-L (Figure 6B). One apparently contradictory result from these experiments is the observation of only a partial overlap between GAF and Fs(1)h-L in ChIP-seq experiments (Figure 5B and D), whereas the co-IP experiments suggests a strong interaction between GAF and Fs(1)h-L (Figure 6A and B). One possible explanation is that the interaction between these two proteins is indirect, through looping between sites containing GAF and sites containing Fs(1)h-L with other insulator proteins. Although the proteins are not located at the same genomic locations, they come down together in co-IP experiments. A co-IP experiment to test possible interactions between insulator proteins and Fs(1)h-S could not be done, as the Fs(1)h-L&S antibodies recognize both isoforms. However, results in Figure 6B suggest that antibodies to insulator proteins fail to pull down Fs(1)h-S, supporting the conclusion that this isoform does not interact with insulators. To further test the idea of an interaction between Fs(1)h-L and insulator proteins, a 220 amino acid GST-tagged Fs(1)h-L specific peptide containing the CTM domain of this isoform (Figure 6C) was incubated with a Kc cell protein lysate, precipitated with GSH-agarose beads and analyzed by western blot using antibodies to various insulator proteins. Results suggest that GAF, Su(Hw), CP190 and Mod(mdg4) interact, directly or indirectly, with the CTM domain of Fs(1)h-L (Figure 6D). Therefore, despite the fact that Fs(1)h-S and Fs(1)h-L have identical sequences except for the extra carboxy-terminal domain of Fs(1)h-L, these data suggest a specific function for the later at insulator sites, and this function is carried out by the extra 932 amino acids found on the Fs(1)h-L isoform.

**DISCUSSION**

Regulation of transcription by restricting the release of promoter-proximal paused RNAPII appears to be the rate limiting step for the regulation of many genes. Release from this promoter-proximal pause requires recruitment of P-TEFb, which is carried out by Brd4 in mammals. Brd4 has recently been identified as a therapeutic target for acute myeloid leukemia and other cancers (30,31), but the mechanisms by which it affects gene expression are not well understood. Brd4 reminds bound to chromatin in mitotic chromosomes, and the presence of the two bromodomains suggests that it interacts with acetylated histones (7,32,33). Recent results show a preference of Brd4 for binding to polyacetylated histone H3 and H4 peptides in vitro, including K5, K8, K12 and K16 in histone H4 and K14 in histone H3 (8). The affinity of Brd4 for acetylated histone peptides increases when neighboring S and T residues are phosphorylated. Once tethered at the promoter by interactions with acetylated histones, Brd4 recruits P-TEFb, which in turns phosphorylates the Ser2 residue of RNAPII (10). Brd4 is also an atypical kinase that can interact with the carboxy-terminal domain of RNAPII and phosphorylate Ser2 directly (13).

Brd4 encodes two different isoforms but, despite its importance as a regulator of transcription and its significance as a target for cancer therapeutics, the possible functional differences between the two isoforms have not been explored. Here, we show that Fs(1)h, the *Drosophila* Brd4 orthologue, is present at active promoters and likely plays a general role in transcription regulation as is the case in mammals. Importantly, we find that the two Fs(1)h isoforms display different genomic localization patterns, suggesting that they may play distinct roles in nuclear biology. We find that the Fs(1)h-S isoform is present at enhancers and promoters of active genes. The summits of Fs(1)h-S peaks at promoters are located at +140 bp from the TSSs and coincide with the summits of H3K4/me3 peaks, suggesting that Fs(1)h-S may interact with histones. This is confirmed by the strong clustering of Fs(1)h-S peaks and those of histones H3 and H4 acetylated in various residues. The results suggest a non-uniform distribution of acetylated histones at different genomic sites of Fs(1)h-S, suggesting that perhaps Fs(1)h-S can be recruited to different genes by different acetylated residues. This would allow different histone acetyltransferase complexes with different specificities to participate in transcriptional regulation of different genes with the same mechanistic outcome. Interestingly, we find that Fs(1)h-S correlates best with the presence of the phosphoacetylated marks H3K9acS10ph and H3K27acS28ph. Phosphorylation of S10 and S28 in the tail of histone H3 is carried out by the JIL-1 kinase, and these two modifications are found at all active promoters and enhancers in *Drosophila* (16). JIL-1 is in turn necessary for recruitment of P-TEFb and release of the paused RNA polymerase (34,35). The finding of Fs(1)h-S at sites of phosphoacetylated H3 supports the proposed role of JIL-1 in the transcription process and offers additional mechanistic insights for how JIL-1 contributes to the release of RNAPII from the promoter-proximal pause.

It is possible that recruitment of Brd4 requires acetylation events that do not involve histone tails but may act combinatorially to tether this protein to the promoter. We have previously demonstrated that CBP, the histone acetyltransferase that carries out H3K27 acetylation at both enhancers and promoters, is required for phosphorylation of serine 2 of the C-terminus tail of RNA polymerase II (16). Studies of P-TEFb in mammalian cells have shown that p300 acetylates Cyclin T of the P-TEFb complex to release it from inactivating factors (36). The acetylated lysine residues on Cyclin T can then bind the bromodomain of Brd4 (37). This suggests that Brd4 may serve as a bridge between acetylated histone tails and other acetylated residues in Cyclin T to tether P-TEFb to the promoter. Interestingly, a subset of Fs(1)h-S sites show low enrichment of H3K27ac (Figure 4A), suggesting that either recruitment of Fs(1)h-S requires simultaneous phosphorylation of the adjacent S28 residue or that acetylation of other residues take place at other genes. The possibility of different combinations of acetylated histone residues being able to recruit Fs(1)h-S in *Drosophila* and Brd4 in mammals would explain the discrepancies between
different studies, demonstrating different histone acetylation requirements for Brd4 recruitment to chromatin (12,38).

Although the recruitment of Brd4 to the FOSL1 enhancer has been previously shown to be required for recruitment of P-TEFb and productive transcription elongation (12), the widespread presence of Brd4 at enhancers has not been reported before as a general mechanism of transcriptional activation. The data presented here demonstrate binding of Fs(1)h-S at the majority of active enhancers in the Drosophila genome. Other events that take place at the promoter and are required for recruitment of P-TEFb and release of RNAPII, such as recruitment of JIL-1, 14-3-3 and histone acetyltransferases, also occur at enhancers genome wide (16). This raises the possibility that binding is seeded at the enhancer and, on contact of the enhancer with the promoter, Fs(1)h-S can bring P-TEFb to the promoter to release the polymerase. Alternatively, Fs(1)h-S and P-TEFb may have alternative functions at enhancers, such as transcription of eRNAs required for enhancer function (39–41). A third speculative but interesting possibility is that various signaling pathways in the cell contribute to building a code of epigenetic signatures at enhancers and promoters in the form of acetylation of various residues, which may then cooperate to recruit Brd4/Fs(1)h. The requirement for acetylation of histones at enhancers and promoters to recruit Brd4 would ensure that several different signaling events have taken place before recruitment of PTEF-b by Brd4 can release RNAPII into productive elongation.

Contrary to Fs(1)h-S, Fs(1)-L is present in the histone-free region overlapping the binding sites of insulator proteins, suggesting that this isoform may not be recruited to chromatin by interactions between the bromodomains and acetylated histones. Rather, it is more likely that Fs(1)-L recognizes acetylated residues in other proteins, as has been shown for the interaction between Brd4 and Cyclin T. Given the strong correlation between the genome sites of insulator proteins and Fs(1)-L, particularly between Mod(mdg4) and Fs(1)-L, it is possible that this isoform is recruited to insulator sites by acetylated residues in insulator components. The role of Fs(1)-L in the function of chromatin insulators is not clear at this time, but such a possibility is supported by previous work in yeast, where Bdf1, a BET family protein, has been shown to be involved in the function of chromatin boundaries (15). In addition, depletion of Brd4 in mammalian cells results in large-scale chromatin unfolding. This effect requires the carboxyterminal domain of Brd4, suggesting the long isoform is involved in this process (14).

Although Fs(1)/Brd4 has been regularly studied in the context of its role in transcription elongation, our genome-wide interrogation of Fs(1)h suggests that the poorly characterized long isoform of this protein localizes to sites bound by insulator proteins throughout the genome. This observation may suggest a role for Fs(1)/Brd4 in the establishment or maintenance of chromosome architecture.

ACCESSION NUMBERS

ChIP-seq data has been deposited in NCBI’s Gene Expression Omnibus (GEO) under accession GSE42086.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGMENTS

The authors thank Dr Margaret Rohrbaugh, Keith Jones and Brian Jones for help with some of the ChIP experiments, Xiya Zhu for help during the preparation of Fs(1)-L antibodies, and Drs Igor Dawid and Der-Hwa Huang for the gift of Fs(1)-L&S antibody. They also thank The Genomic Services Lab at the HudsonAlpha Institute for Biotechnology for their help in performing Illumina sequencing of ChIP-Seq samples.

FUNDING

National Institute of General Medical Sciences of the National Institutes of Health under award [R01GM035463]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Funding for open access charge: NIH [R01GM035463].

Conflict of interest statement. None declared.

REFERENCES


