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Different Sensitivities of Bromodomain Factors 1 and 2 to Histone H4 Acetylation

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Summary

The histone code hypothesis proposes that covalently modified histone tails are binding sites for specific proteins. In vitro evidence suggests that factors containing bromodomains read the code by binding acetylated histone tails. Bromodomain Factor 1 (Bdf1), a protein that associates with TFIID, binds histone H4 with preference for multiply acetylated forms. In contrast, the closely related protein Bdf2 shows no preference for acetylated forms. A deletion of *BDF1* but not *BDF2* is lethal when combined with a mutant allele of *ESA1* (a histone H4 acetyltransferase) or with nonacetylatable histone H4 variants. Bromodomain point mutations that block Bdf1 binding to histones disrupt transcription and reduce Bdf1 association with chromatin in vivo. Therefore, bromodomains with different specificity generate further complexity of the histone code.

Introduction

In eukaryotes, transcription occurs on a DNA template packaged into chromatin. DNA wraps around a histone octamer to form the basic subunit of chromatin: the nucleosome. Nucleosomes are further packaged into higher order structures, and these can be repressive to transcription and other processes (Workman and Kingston, 1998). Regulation of chromatin structure provides a means for controlling gene expression in vivo. Control is accomplished by at least two mechanisms: ATP-dependent chromatin remodeling and covalent histone modifications (Workman and Kingston, 1998). These activities are localized to promoters via interactions with transcriptional activators and are believed to modulate the accessibility of the transcription machinery to nucleosome-bound DNA sequences (Hassan et al., 2001). To function as gene regulatory mechanisms, these modifications need to be dynamic and coordinated with the transcription machinery.

Histones consist of core domains and N-terminal tails. The core domains mediate octamer formation and DNA wrapping. The tails are subjected to various covalent modifications such as acetylation, phosphorylation, and methylation (Jenuwein and Allis, 2001). There is a correlation between transcription activity and acetylation of specific lysine residues within the tails (Grunstein, 1997). The acetylation level of a locus is established by the competition between histone acetyltransferases (HATs) and deacetylases (HDACs) (Vogelauer et al., 2000). Acetylation may interfere with internucleosomal interaction and thereby open chromatin structure (Luger and Richmond, 1998). However, the "histone code" hypothesis predicts that histone tail modifications act as binding sites for proteins that may further modify chromatin or regulate chromatin accessibility (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Several HAT complexes (SAGA, P/CAF) and chromatin remodeling factors (Swi/Snf, RSC) contain one or more copies of a motif known as the bromodomain (Jeanmougin et al., 1997). An NMR study of the P/CAF bromodomain suggests that it binds to acetylated lysine of the histone H3 and H4 tails (Dhalluin et al., 1999). The bromodomain of the SAGA component Gcn5 has in vitro affinity for histones H3 and H4 tails, and affinity is increased when the tails are acetylated (Hudson et al., 2000; Ornaghi et al., 1999; Owen et al., 2000). However, evidence for an in vivo interaction between histones and bromodomains is still lacking.

Assembly of an RNA polymerase II preinitiation complex (PIC) on a promoter requires TFIID binding to core promoter elements. TFIID consists of TATA binding protein (TBP) and 10–14 TBP-associated factors (TAFs) (Pugh, 2000). Although TAFs are essential for viability in yeast, their functions are still not well understood. TAFs can interact with the initiator and downstream promoter elements of core promoters, can act as coactivators, and may possess kinase, acetyltransferase, and monoubiquitination activities (for reviews see Albright and Tjian, 2000; Burley and Roeder, 1996; Green, 2000; Verrijzer and Tjian, 1996). Several TAFs have histone-fold motifs that may form an octamer-like structure within TFIID (Gangloff et al., 2001; Selleck et al., 2001). Many of these histone-fold TAFs are also integral components of other complexes with HAT activity, such as the yeast SAGA complex (Grant et al., 1998a, 1998b). The histone-like TAFs may be structural components and are important for integrity of these complexes (Albright and Tjian, 2000; Durso et al., 2001; Green, 2000; Michel et al., 1998).

TAF1 of metazoan TFIID contains two copies of the bromodomain and these can preferentially bind acetylated histone H4 tail peptides in vitro (Jacobson et al., 2000). Although these bromodomains are not present in the homologous yeast TAF1, we found that Bromodomain Factor 1 (Bdf1) is associated with yeast TFIID and corresponds to the C-terminal domain of higher eukaryotic TAF1 (Matangkasombut et al., 2000). We also identified a homologous protein, Bdf2, that associates with TFIID when overexpressed or when Bdf1 is deleted (Matangkasombut et al., 2000; our unpublished data). The conserved presence of bromodomains in TFIID raises the possibility that acetylation of nucleosomes can directly recruit TFIID and thereby increase transcription. Here we present data that support a role for the Bdf bromodomains interacting with histone H4. Surprisingly, while Bdf1 preferentially binds acetylated histone H4

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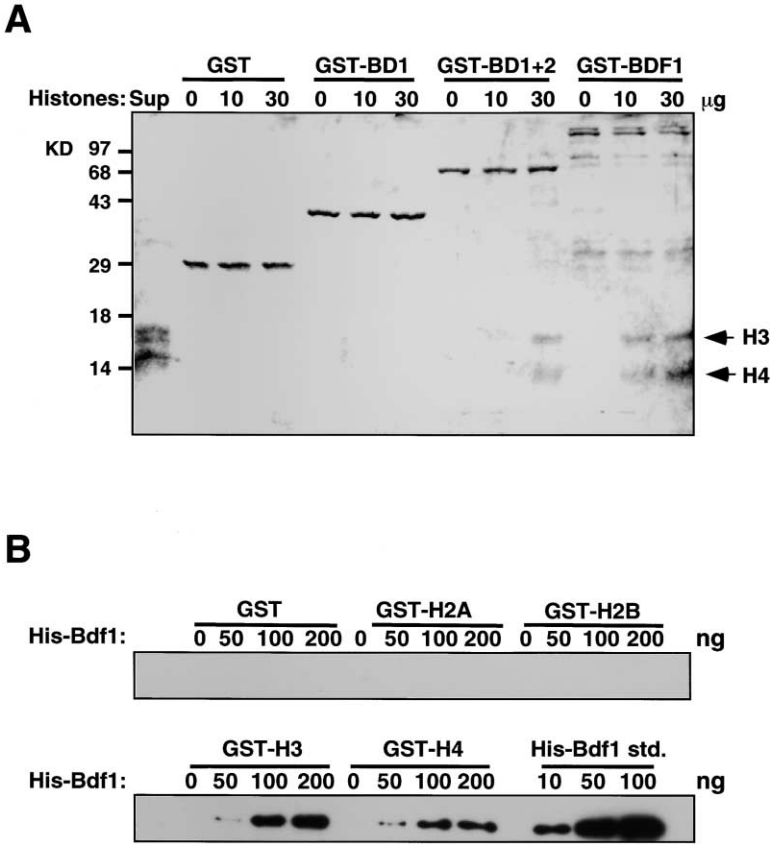

Figure 1. Bdf1 Interacts with the N Termini of Histones H3 and H4 In Vitro

(A) Recombinant GST or GST fused to the first bromodomain of Bdf1 (GST-BD1), both bromodomains of Bdf1 (GST-BD1+2), or full-length Bdf1 (GST-BDF1) was bound to glutathione-agarose beads and incubated with the indicated amount of calf thymus histones. Beads were washed extensively, and bound proteins were analyzed by SDS-PAGE and stained with Coomassie brilliant blue.

(B) Recombinant GST or GST fused to the N-terminal tails of histone H2A, H2B, H3, and H4 (Hecht et al., 1995) were bound to glutathione beads and incubated with the indicated amounts of His-tagged Bdf1 in GST binding buffer. After being washed extensively, the pellets were resolved by SDS-PAGE, blotted, and probed with α-His antibody. A His-Bdf1 standard (std.) was loaded for calibration of the immunoblot signal.

tails, Bdf2 shows no such preference. This suggests that different bromodomain specificities may confer a further level of complexity to the histone code.

Results

In Vitro Binding of Bdf1 to Histones H3 and H4

To examine the function of Bromodomain Factor 1, we performed in vitro experiments to determine whether Bdf1 interacts with histones. Glutathione S-transferase (GST) was fused to the first bromodomain, both bromodomains, and full-length Bdf1. Fusion proteins were bound to glutathione agarose beads and incubated with calf thymus histones. After extensive washing, proteins bound to the beads were analyzed by SDS-PAGE and Coomassie staining (Figure 1A). Binding to histones H3 and H4 was observed with full-length Bdf1 and the double bromodomain fusion proteins. If there was binding to the single bromodomain fusion protein, it was below the limit of detection in this assay. The ability of the double bromodomain to bind histones with higher affinity is consistent with previous findings that the two bromodomains can bind histones cooperatively (Dhalluin et al., 1999; Jacobson et al., 2000).

To determine whether Bdf1 binds directly to histone tails, GST fused to individual histone tails was tested for coprecipitation with full-length Bdf1. The GST-histone fusion proteins were bound to glutathione beads and incubated with recombinant, His-tagged Bdf1. The presence of His-tagged Bdf1 on the beads was detected by immunoblotting (Figure 1B). No binding to H2A or H2B tails was observed. In contrast, histone H3 and H4 tails could independently interact with Bdf1 in vitro. We note that a yeast two-hybrid screen using histone H4 tail as bait also revealed an interaction with Bdf1 (Pamblanco et al., 2001). That this interaction occurs in vivo is supported by data below, as well as by the finding that Bdf1 copurifies with a TAP-tagged histone H4 (N. Krogan and J. Greenblatt, personal communication).

Since the P/CAF, Gcn5, and TAF1 bromodomains show an in vitro preference for acetylated histone tails, we tested whether Bdf1 binds acetylated histone tails with higher affinity. To compare the binding of Bdf1 to different acetylated forms of histones, GST-Bdf1 was incubated with a mixture of HeLa histones. The presence of multiple acetylated forms of histones allows us to directly compare the binding affinity for different species in the same reaction. The bound and supernatant fractions were resolved on a Triton-acetic acid-urea (TAU) gel, which resolves the various modified forms of histones (Figure 2A). GST alone shows no binding to histones. Histones bound to Bdf1 match the distribution of the input histones, with the exception of histone H4. In the bound fraction, non-, mono-, and diacetylated forms of H4 are underrepresented while multiply acetylated forms are overrepresented relative to the input. The level of bound tetraacetylated form of H4 is approximately 4-fold higher than that of the nonacetylated form. Therefore, Bdf1 preferentially binds to multiply acetylated forms of histone H4.

Since Bdf2 is closely related to Bdf1, similar experi-

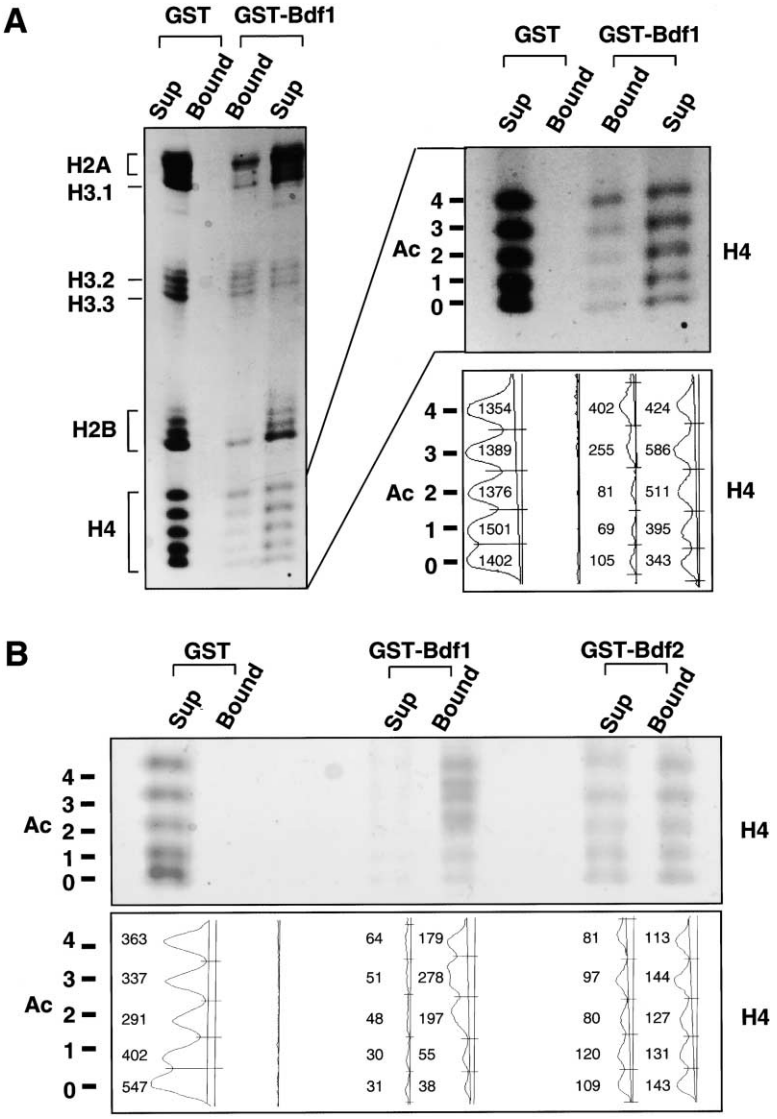

Figure 2. Bdf1 but Not Bdf2 Shows Preference for Interaction with Hyperacetylated Forms of Histone H4

(A) Recombinant GST or GST-Bdf1 (75 μg) immobilized on 5 μl of glutathione-agarose beads was incubated with 15 μg of hyperacetylated HeLa histones mixed with 5 μg of nonacetylated HeLa histones. The supernatants were precipitated with acetone and with the bound fractions resolved on a Triton-acetic acid-urea gel and stained with Coomassie brilliant blue. The position of each histone on the gel is marked. In contrast to Figure 1, some binding of GST-Bdf1 to all four histones in this preparation is observed. The area where histone H4 species run is shown at a higher magnification. The signal was quantified using NIH Image 1.62 program. A densitometric plot with quantified results is shown underneath.

(B) Recombinant GST, GST-Bdf1, and GST-Bdf2 were subject to GST pull-down assays and analyzed by Triton-Acid-Urea gels as performed in (A). The area of the gel containing histone H4 is shown. Densitometric plots with quantified results is shown in the lower panel.

ments were performed to compare binding of Bdf1 and Bdf2 to hyperacetylated HeLa histones. Strikingly, while Bdf1 prefers hyperacetylated forms of histone H4, Bdf2 binds equally well to all histone H4 species (Figure 2B). Therefore, different bromodomains can show distinct ligand specificity, and bromodomain binding to histones is not strictly acetylation dependent.

Genetic Interactions between Bdf1 and Histone Tails

To determine whether the interaction between Bdf1 and histone H3 and H4 tails was relevant in vivo, mutations in the histone tails and a deletion of *BDF1* were combined. The *BDF1* gene was deleted in a strain designed for plasmid shuffling of the histone H3 and H4 genes (Zhang et al., 1998). Both chromosomal copies of the histone H3 and H4 genes are deleted but viability is supported by histone H3 and H4 genes carried on a *URA3* marked plasmid. Histone mutant plasmids were shuffled in using 5-FOA selection. The phenotypes of the resulting strains are summarized in Table 1. With wild-type histones, a *BDF1* deletion strain is temperature sensitive and grows slightly slower than wild-type cells at 25°C. Under these conditions, *BDF2* supports viability but only partially compensates for the absence of *BDF1*. Although a *BDF2* deletion has no obvious phenotype, a *bdf1*Δ*bdf2*Δ strain is inviable, suggesting that the two genes are redundant for an essential function (Matangkasombut et al., 2000).

In the presence of wild-type *BDF1*, the tails of either H3 or H4 are nonessential. However, deletion of the H4 tail is synthetically lethal in combination with *bdf1*Δ. The H3 tail deletion is not lethal in combination with *bdf1*Δ, but the double mutant shows a synthetic slow growth phenotype. This suggests that both histone tails may play a role in the function of the Bdfs in vivo, with H4 being particularly important.

More compelling were interactions between the *bdf1*Δ and histone H4 alleles mutated at the lysines that are sites of acetylation. Acetylation of all four lysine residues of H4 is seen in actively transcribed euchromatin, while H4 is hypoacetylated in silenced domains (Grunstein, 1997). Although single lysine changes did not lead to

Table 1. Genetic Interactions between *bdf1*Δ and Histone N-Terminal Tail Mutations

Plasmids[b]	H3	H4	Growth on 5-FOA[a]	
			BDF1	*bdf1*Δ::KanMX
pWZ414-F13	WT	WT	+++	++
pWZ414-F14	Δ3-29	WT	++	±
pWZ414-F30	K9Q	WT	++	+
pWZ414-F53	K9R	WT	++	+
pWZ414-F31	K14Q	WT	+++	+
pWZ414-F36	K14G	WT	+++	+
pWZ414-F43	K14R	WT	+++	+
pWZ414-F15	WT	Δ4-19	++	−
pWZ414-F51	WT	K5,12Q	++	+
pWZ414-F52	WT	K5,12R	++	±
pWZ414-F23	WT	K5Q	++	+
pWZ414-F22	WT	K5R	++	+
pWZ414-F25	WT	K16Q	++	+
pWZ414-F26	WT	K16G	++	+
pWZ414-F24	WT	K16R	++	+
pWZ414-F47	WT	K8,16Q	++	+
pWZ414-F49	WT	K8,16R	++	−
pWZ414-F48	K14Q	K8,16Q	++	+
pWZ414-F50	K14R	K8,16R	++	−

[a] −, no growth; ±, very small colonies after 6 days; +, small colonies and slow growth; ++, intermediate colonies and slow growth (after 4 days); +++, normal growth (same as wild-type).
[b] Reference: Zhang et al., 1998.

synthetic phenotypes, alleles with two mutated lysines showed interactions with *bdf1*Δ. Strikingly, mutation of H4 lysines 8 and 16 to arginine (which mimics a constitutively nonacetylated lysine) is lethal in combination with *bdf1*Δ. Similarly, a double K5R/K12R mutant grew very slowly in the absence of Bdf1. These interactions were allele specific because no synthetic phenotypes were seen when the same residues were mutated to glutamine (which mimics a constitutively acetylated lysine). In contrast, mutations of lysine residues in H3, either to arginine or glutamine, did not show any synthetic phenotypes when combined with *bdf1*Δ. These results support the in vitro preference of Bdf1 for hyperacetylated H4 and suggest that Bdf1 function is sensitive to the acetylation state of transcriptionally relevant H4 lysines in vivo.

We previously found that *BDF1* and *BDF2* are partially redundant but that deletion of *BDF1* gives rise to more severe phenotypes than deletion of *BDF2* (Matangkasombut et al., 2000). Bdf2 shows distinct histone binding preference in vitro (Figure 2B). A *BDF2* deletion was combined with the mutations in histone H3 or H4. In contrast to *bdf1*Δ, *bdf2*Δ did not show genetic interactions with any of the histone mutants tested (data not shown). These results support the idea that Bdf1 and Bdf2 have different histone acetylation preferences.

Genetic Interaction between Bdf1 and HATs

The hypothesis that Bdf1 function depends upon the acetylation state of histones predicts that genetic interactions should be seen between *bdf1*Δ and mutants in enzymes that acetylate histones. In yeast, two major transcription-related HAT complexes are SAGA and NuA4 (Roth et al., 2001). The multisubunit SAGA complex primarily acetylates histone H3 and is not essential for viability (Grant et al., 1998a, 1998b). Gcn5 is the catalytic subunit, Spt20 and Ada1 are necessary for the structural integrity of the complex, and Spt3 and Spt8 mediate interactions with TBP. NuA4 also has multiple subunits and primarily acetylates histone H4. The essential gene *ESA1* encodes the catalytic subunit (Allard et al., 1999).

To test for genetic interactions between *BDF1* and HAT complex subunit genes, a *bdf1*Δ strain was crossed to strains carrying deletions of nonessential SAGA subunits or a temperature-sensitive allele of *ESA1*. The resulting diploids were sporulated, and tetrads were dissected. Approximately 20–40 tetrads were dissected and analyzed for each strain.

A temperature-sensitive *esa1* mutation exhibits synthetic lethality in combination with *bdf1*Δ at 30°C (Figure 3A). The double mutant is viable at room temperature, although growth is slow. The *esa1* mutation lies in the HAT domain and disrupts HAT activity at the nonpermissive temperature (Clarke et al., 1999). Since the major substrate of Esa1 is histone H4, this result further suggests that Bdf1 function is sensitive to H4 acetylation.

In contrast, deletion of *GCN5*, the HAT subunit of SAGA, has no synthetic phenotypes with *bdf1*Δ (Figure 3B). Diploids resulting from a cross of *gcn5*Δ and *bdf1*Δ had poor spore viability, so a *bdf1*Δ strain was transformed with a *URA3* marked plasmid carrying wild-type *BDF1* before crossing. Spores carrying both *gcn5*Δ and *bdf1*Δ alleles were recovered and tested for the ability to lose the plasmid on 5-FOA plates. As shown in Figure 3B, the strains with both genes deleted grew as well as strains with a single deletion. The *SPT3* deletion also shows no synthetic phenotypes in combination with *bdf1*Δ (data not shown). However, complete loss of the SAGA complex, caused by deletion of the Spt20 (Figure 3C) or Ada1 genes (data not shown), is lethal when combined with *bdf1*Δ. These results suggest that the SAGA complex but not its HAT activity affects Bdf1 function in vivo.

Correlation between Bdf1/Histone H4 Interaction and Transcription

The importance of the Bdf1 bromodomains in vivo was examined by analysis of deletion and site-directed mutants. Deletion mutants lacking either bromodomain fail to complement the temperature sensitivity of a *bdf1*Δ strain and cannot support viability in a *bdf1*Δ *bdf2*Δ strain (data not shown). However, these deletions may disrupt the overall structure of Bdf1 and interfere with other functions of the protein. Therefore, point mutants in the proposed acetyl-lysine binding pocket of the bromodomains were generated. The bromodomain consists of four α helices, and the putative binding pocket is made up of two loops between these helices (the ZA loop and the BC loop; see Figure 4A). We mutated conserved residues predicted to either contact acetyl-lysine directly (Y187, F229, N230 in bromodomain 1; Y354, F396, N397 in bromodomain 2) or to be required for proper folding of the binding pocket (P176, P194, M195 in bromodomain 1; P343, P361, M362 in bromodomain 2) (Dhalluin et al., 1999). The P194T/M195A and P361T/M362A double mutants were modeled on the P371T/M372A mutant of the Gcn5 bromodomain, which

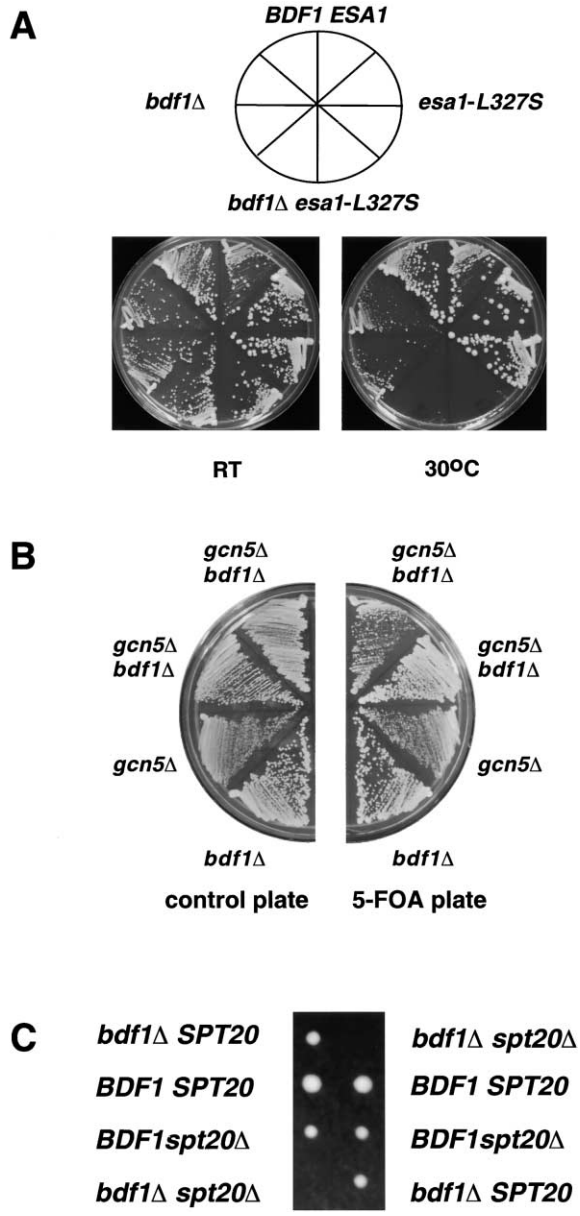

Figure 3. Genetic Interaction of *bdf1*Δ with HAT Components

(A) A diploid yeast strain (YSB791) was generated by crossing a *bdf1*Δ strain (YSB514) with a temperature-sensitive *esa1* mutant (*esa1-L327S*) strain (LPY3430). After sporulation, 20 tetrads were dissected and grown on YPD at room temperature. The *bdf1*Δ*esa1-L327S* spores were viable but grew more slowly than the single mutants. Upon restreaking, the *bdf1*Δ*esa1-L327S* strains failed to grow at 30°C.

(B) A *bdf1*Δ strain (YSB496) carrying the pRS316-*BDF1* plasmid was crossed to a *gcn5*Δ strain (FY1354). The diploids (YSB728) were sporulated and tetrads were dissected. A *bdf1*Δ *gcn5*Δ spore was selected and tested for loss of pRS316-*BDF1* with 5-FOA. The *bdf1*Δ*gcn5*Δ strain was able to grow without any synthetic phenotypes in the absence of the *BDF1* plasmid.

(C) A *bdf1*Δ strain (YSB514) was crossed to an *spt20*Δ strain (FY1076). The diploids (YSB 730) were selected by auxotrophic markers and sporulated. Twenty tetrads were dissected, and their genotypes were determined by auxotrophic markers. Two representative tetrads are shown with their genotypes specified.

causes defects in the ability of SAGA to recruit Swi/Snf to a promoter (Syntichaki et al., 2000). Residues were individually mutated in one or both of the Bdf1 bromodomains, and resulting mutant genes were tested for phenotypes (Figure 4A).

All *BDF1* alleles mutated in only one bromodomain were functional, as assayed by the ability to support viability in a *bdf1*Δ *bdf2*Δ strain. In contrast, most of the mutants changed in both bromodomains did not have full Bdf1 activity despite the fact that they were expressed at levels equal to wild-type protein (data not shown). This suggests some redundancy between the two bromodomains. The Y187A/Y354A and N230A/N397A mutants were unable to support viability in a *bdf1*Δ *bdf2*Δ strain, while the P194T/M195A/P361T/M362A quadruple mutant was slow growing and temperature sensitive. Surprisingly, even those Bdf1 mutants that could not support viability could suppress the temperature sensitivity associated with a *bdf1*Δ *BDF2* strain, suggesting there may be a second structural role for Bdf1 that is not dependent upon the bromodomains. These results show that the bromodomains of Bdf1 are important for viability, especially in the absence of Bdf2.

To determine if phenotypes caused by bromodomain mutations are due to defects in histone binding, histone binding activities of these mutants were tested. Recombinant GST-Bdf1 fusion proteins carrying the bromodomain mutations were incubated with HeLa histones. The supernatant and bound fractions were resolved on a TAU gel (Figure 4B). Histone binding activity correlated well with the in vivo phenotypes of the mutants. The P176A/P343A allele, which has no mutant phenotype, displays the same histone binding as wild-type protein, including a preference for the hyperacetylated forms of H4. The same result was observed with the F229A/F396A allele, which also has no mutant phenotype (data not shown). In contrast, Y187A/Y354A, which cannot support viability, shows significantly less H4 binding and no preference for the hyperacetylated form of histone H4, although it can still bind to other histones and nonacetylated H4. Based on the Gcn5 structure, this tyrosine is predicted to directly interact with the acetylated lysine of H4. The N230A/N397A mutant, which also fails to support viability, has little binding activity. The P194T/M195A/P361T/M362A mutant also has a defect in histone binding, consistent with its slow growth, flocculent, and temperature-sensitive phenotypes.

The conditional phenotype of the P194T/M195A/P361T/M362A mutant allowed us to determine the effect of Bdf1 bromodomain mutations on in vivo transcription. The wild-type and the P176A/P343A strains were used as controls. The strains were shifted to the nonpermissive temperature of 37°C. Nuclease protection assays were used to examine levels of specific transcripts. Levels of several genes rapidly and markedly decreased in the P194T/M195A/P361T/M362A mutant but not in the control strains (Figure 5). Interestingly, the genes that are most affected, such as ribosomal protein genes, HTA2, and TRP3, are genes that are considered TAF dependent (Kuras et al., 2000; Li et al., 2000). Furthermore, the ribosomal protein genes have also been identified as targets of the histone acetyltransferase Esa1 (Reid et al., 2000).

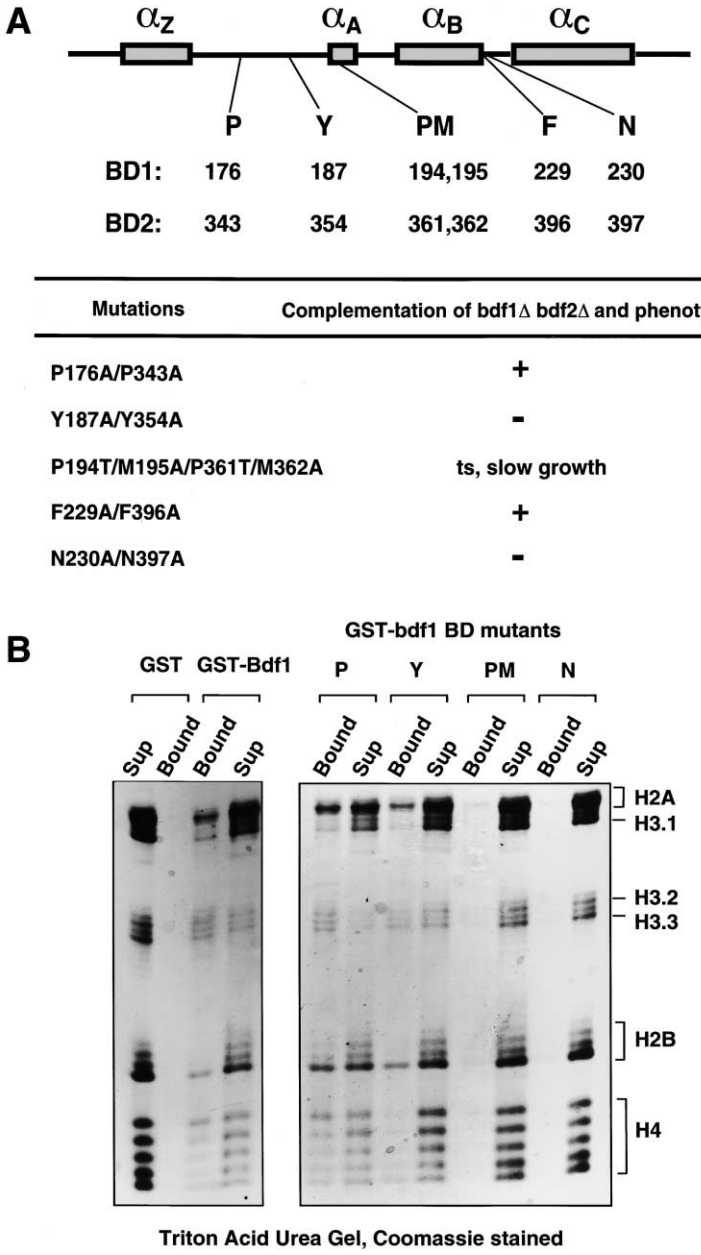

Figure 4. Bdf1 Bromodomain Mutants that Disrupt Binding to Acetylated Histone H4 Also Cannot Support Viability

(A) A schematic diagram of a bromodomain shows the positions of point mutations used in this experiment. The filled bars represent α helices as predicted from structural studies of other bromodomains. The ZA and the BC loops form the proposed binding pocket of the bromodomain. The position of each mutated residue in bromodomain 1 (BD1) and bromodomain 2 (BD2) is shown as a number underneath the diagram. The complementation and phenotypes of the mutants in a *bdf1Δbdf2Δ* strain (YSB529) are shown. These mutants were also tested in a *bdf1ΔBDF2* strain (YSB497) (data not shown).

(B) Recombinant GST and GST-Bdf1 mutants were bound to glutathione beads, incubated with hyperacetylated HeLa histones, and resolved with Triton-acetic acid-urea gel as in Figure 1C. P, P176A/P343A; Y, Y187A/Y354A; PM, P194T/M195A/P361T/M362A; N, N230A/N397A.

Association of Bdf1 with Chromatin

To determine if Bdf1 associates with chromatin in vivo, chromatin immunoprecipitation (ChIP) experiments were performed. Epitope-tagged wild-type and Y187A/Y354A Bdf1 were expressed in a *bdf1ΔBDF2* strain. An untagged *BDF1* strain was used as a negative control. Crosslinked chromatin was sheared and immunoprecipitated with α-HA (12CA5) antibody to precipitate HA3-tagged Bdf1 or an anti-TBP antibody. DNA coprecipitated with the indicated proteins was decrosslinked and analyzed by quantitative PCR (Figure 6A). Primer pairs specific for the promoters of ADH1, RPS5, RPL5, RPS8A, RPL9A, and RPS11B, and for a nontranscribed intergenic sequence on chromosome V were used to amplify input and coprecipitated DNA. HA-tagged Bdf1 is associated with all sequences tested, including the intergenic sequence on Chromosome V (Figure 6A), the coding sequences of ADH1 and PMA1 genes (data not shown), and the transcriptionally silent mating type locus and telomere VI (Figure 6B). This crosslinking is significant since α-HA antibody precipitates only a very small amount of DNA from the untagged strain. Furthermore, this association is disrupted by the *bdf1* Y187A/Y354A mutant. The changes in Bdf1 crosslinking is not due to differences in chromatin preparation because TBP crosslinks to promoters and at equivalent levels in all strains. PCR products from 10-fold dilutions of input chromatin show that input from the different strains is equivalent.

In summary, Bdf1 associates with chromatin in vivo, albeit without promoter localization as might have been predicted from the association with TFIID. Importantly,

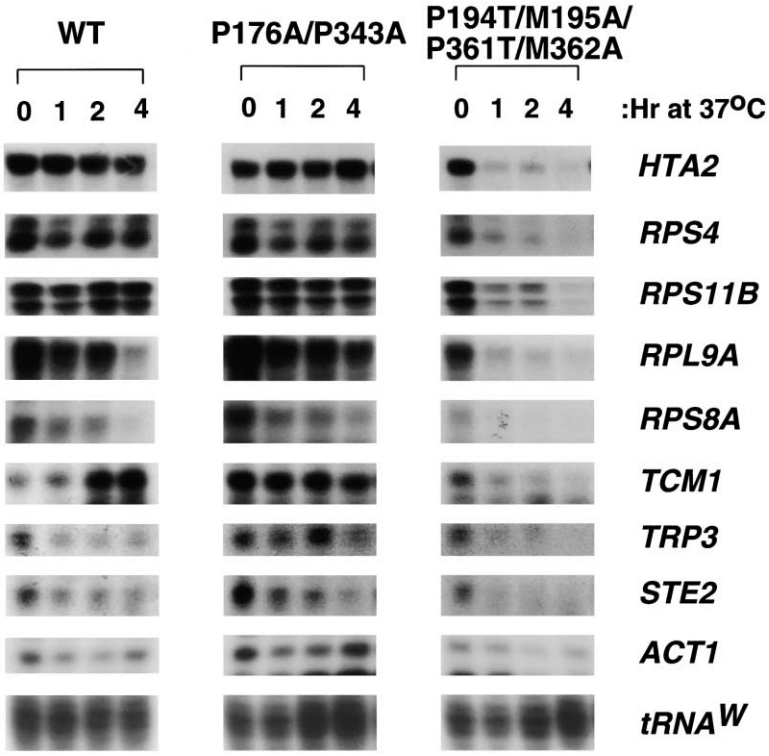

Figure 5. Effect of a Bdf1 Temperature-Sensitive Bromodomain Mutant on RNA Pol II Transcripts

A *bdf1*Δ*bdf2*Δ (YSB529) yeast strain carrying a plasmid copy of either a wild-type *BDF1*, P176A/P343A, or P194T/M195A/P361T/M362A mutants were grown in selective media (−Trp) at room temperature to early log phase. The cultures were then shifted to 37°C, and samples were collected at indicated time points. RNA was prepared by the acid phenol method. S1 nuclease protection assays were performed to determine the levels of specific transcripts as described (Michel et al., 1998; Reid et al., 2000).

this association is dependent on intact bromodomains. Since the *bdf1* Y187A/Y354A mutant is defective only in the interaction with hyperacetylated H4 (Figure 4B), it is likely that the interaction with hyperacetylated histone H4 is crucial for Bdf1-chromatin association.

Discussion

Using biochemical and genetic approaches, we show that Bdf1 preferentially interacts with acetylated histone H4 in vitro and in vivo. Genetic interactions are seen between *bdf1*Δ and H4 alleles mutated at some acetylation sites as well as between *bdf1*Δ and a mutation in the H4 HAT *ESA1*. Bromodomain point mutations that disrupt histone binding in vitro compromise Bdf1 function in vivo, leading to defects in transcription. Furthermore, the interaction between the bromodomains of Bdf1 and histone H4 is required for coimmunoprecipitation of Bdf1 with chromatin in vivo. There may also be some interactions between Bdf1 and histone H3. Although we observe direct binding in vitro, the in vivo data suggest that any role for H3 tails in Bdf1 function in vivo may be indirect.

Bdf1 and Bdf2 are closely related and genetically redundant in that one or the other is necessary and sufficient for viability (Matangkasombut et al., 2000). Surprisingly, Bdf2 differs from Bdf1 by binding equally well to all forms of histone H4 without preference for acetylation (Figure 2B). Furthermore, *bdf2*Δ does not exhibit the synthetic lethal interactions seen with *bdf1*Δ. It has been widely assumed that all bromodomains preferentially interact with acetylated histone tails. This feature of the histone code hypothesis must now be revised to include the possibility that any individual bromodomain may prefer specific acetylated lysines or histone tails that are not modified at all. It is possible that bromodomains could even be affected by other histone modifications such as phosphorylation or methylation. In support of the concept of different bromodomain specificities, we note a recent paper from Thanos and colleagues published while this paper was being reviewed. They found that in vitro recruitment of the bromodomain-containing complexes TFIID and Swi/Snf to a nucleosomal template specifically depend upon acetylation of histones H3 and H4, respectively (Agalioti et al., 2002).

The binding of Bdf1 to hyperacetylated histone H4 is approximately 4-fold higher than to the nonacetylated forms. Although it has been suggested that bromodomains bind only to acetylated histone tails (Dhalluin et al., 1999; Jacobson et al., 2000), we see significant binding of Bdf1 bromodomains to nonacetylated histone tails in vitro (Figures 1A, 1B, and 2A). The bromodomain of Gcn5 can also bind nonacetylated histones, and the critical tail residues for this interaction are arginines close to the acetylated lysine residues (Ornaghi et al., 1999). The cocrystal structure of Gcn5 bromodomain and histone H4 tail shows that the bromodomain forms secondary contacts with residues at +2 and +3 C-terminal to the acetylated lysine residue (Owen et al., 2000). Although an unacetylated lysine may be less favorable for bromodomain binding, secondary contacts might be sufficient for binding at higher concentrations of proteins. These secondary contacts may also play a role in determining ligand specificity of the bromodomains. Differences in the sequences of the bromodomains of Bdf1 and Bdf2 in the region that make these secondary contacts might lead to their distinct binding preferences.

The synthetic lethality between *bdf1*Δ and a histone

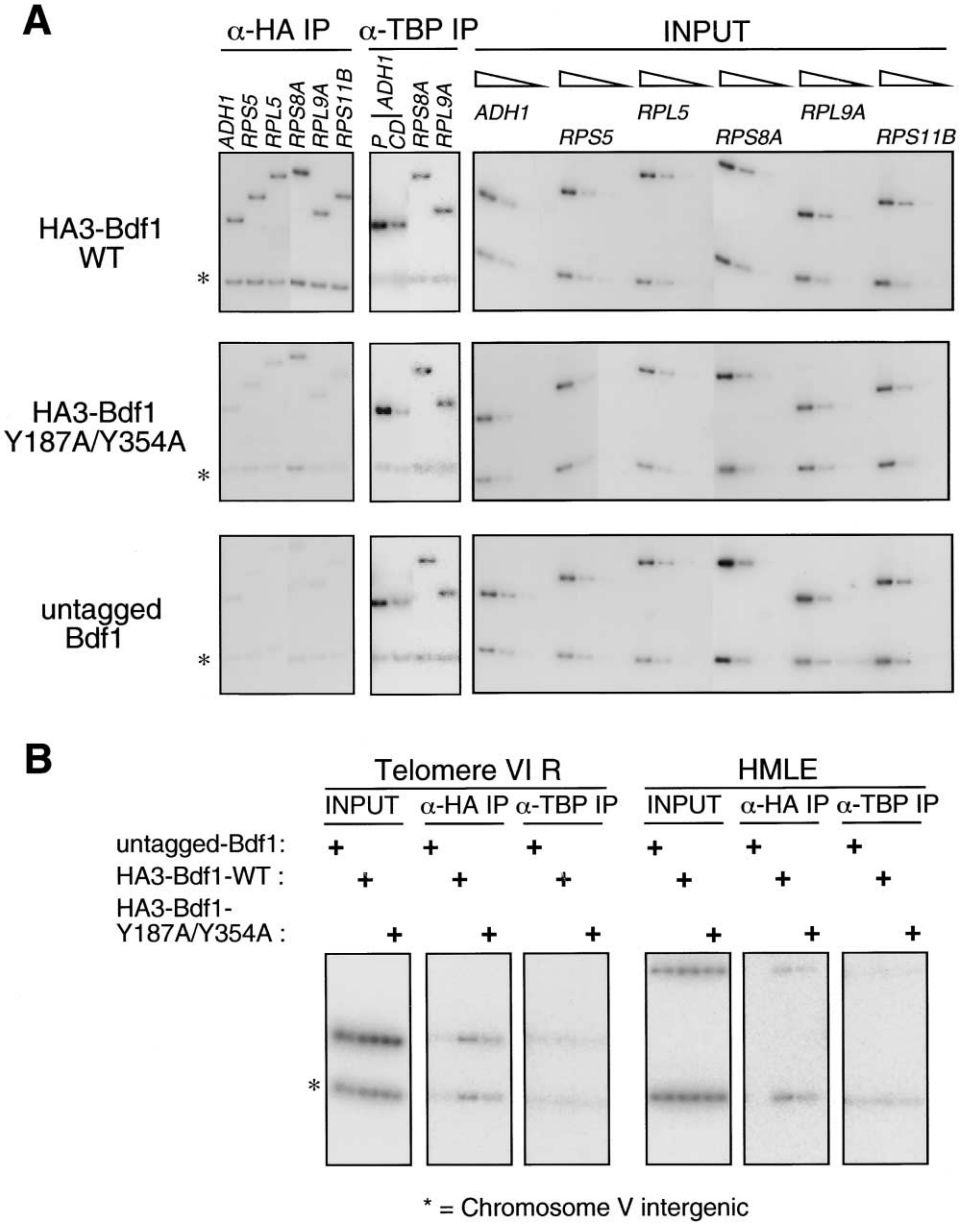

Figure 6. Bdf1 Association with Chromatin In Vivo Is Mediated by the Bromodomains

A *bdf1ΔBDF2* yeast strain (YSB497) with plasmids carrying either HA3-tagged wild-type *BDF1*, *bdf1*Y187A/Y354A (Y) mutant, or an untagged *BDF1* gene was grown in selective media (−Trp) to early log phase. Formaldehyde crosslinking was performed, and sheared chromatin was prepared. Input chromatin and chromatin immunoprecipitated with α-HA or α-TBP antibody was decrosslinked and analyzed by quantitative PCR with indicated primers (* indicates chromosome V intergenic sequence).

(A) All indicated primers were from the promoter of the indicated gene with the exception of the Adh1 coding sequence (ADH1 CD).

(B) Bdf1 also binds transcriptionally silent regions (telomere VI and silent mating type locus HMLE). TBP crosslinking is shown as a negative control.

H4 tail deletion or nonacetylatable mutants suggest that H4 acetylation is important for Bdf1 function. Particularly striking is the allele specificity seen for double substitutions at lysines 8 and 16. Changing both of these residues to glutamine, which may mimic an acetylated lysine, did not have an effect in the *bdf1*Δ background. However, changing these same residues to arginine, which preserves the charge of the unacetylated lysine, was lethal when combined with *bdf1*Δ. This finding echoes the observation that a histone H4 with all four acetylated lysines mutated to glutamine can support viability, while an allele with all four lysines changed to arginine is lethal (Megee et al., 1990).

Consistent with the histone interactions, a mutation in the H4 HAT Esa1 but not the H3 HAT Gcn5 also shows a synthetic lethal phenotype with *bdf1*Δ. These interactions are specific to *BDF1* as *bdf2*Δ shows no synthetic phenotype in combination with these muta-

tions (data not shown). It is intriguing that *bdf1*Δ is synthetically lethal with *spt20*Δ and with *ada1*Δ but not *gcn5*Δ or *spt3*Δ. Since *spt20*Δ or *ada1*Δ disrupts the entire SAGA complex, while *gcn5*Δ or *spt3*Δ disrupts only one of the two major functions of SAGA, the genetic interactions suggest that both functions of SAGA need to be disrupted for synthetic lethality with *bdf1*Δ. One function of SAGA is HAT activity and the other is TBP interaction. Both SAGA and Bdf1 may play a role in recruiting TBP to promoters with acetylated histones, so the combined deletion may not support viability.

The ChIP experiments presented here indicate that Bdf1 associates with chromatin in a bromodomain-dependent fashion. Bdf1 is not localized to promoters, contrary to our expectations based on the Bdf1/TFIID interaction. By immunofluorescence, Bdf1 localized to the chromosomes throughout the cell cycle but was excluded from the nucleolus (Chua and Roeder, 1995). A relatively high steady-state acetylation level is observed in bulk yeast histones, with an average of 13 acetylated lysines per nucleosome (Waterborg, 2000). Histone H3 and H4 display an average of two or more acetylated lysines per molecule. This may promote Bdf1 association at most loci. Global acetyltransferases that maintain the steady state of histone acetylation may also contribute to nonlocalized Bdf1-chromatin association (Vogelauer et al., 2000). Alternatively, the lower affinity of Bdf1 for unacetylated histone H4 and/or H3 tails may lead to binding throughout the genome.

We previously found that Bdf1 associates with TFIID and show here that Bdf1 binds specifically to histones H3 and H4. Therefore, Bdf1 may act as a bridge that mediates an interaction between TFIID and acetylated histones. The double bromodomain of higher eukaryotic TAF1 has been shown to interact specifically to acetylated histone H4 peptide, especially the diacetylated forms (Jacobson et al., 2000). It was proposed that the double bromodomain binds simultaneously to two acetylated lysines seven residues apart on the same tail. This distance corresponds to the frequently found patterns of H4 acetylation at K5/K12 and K8/K16. However, the Gcn5 bromodomain structure when superimposed onto TAF1 double bromodomain suggests the distance to be ten residues apart (Owen et al., 2000). It is possible that two double bromodomain modules bind to two histone tails to form a heterotetramer (Marmorstein and Berger, 2001). This could explain why only simultaneous point mutations in both bromodomains of Bdf1 lead to a defect in vivo. If only one bromodomain is mutated, the other one could still cooperate with another molecule of Bdf1 to maintain interaction with a multiply acetylated histone tail.

The finding that Bdf1 associates with regions in addition to promoters suggests that not all the Bdf1 in cells is associated with TFIID. Bdf1 may associate with TFIID in a regulated manner. One could speculate that binding to a particular pattern of acetylation induces a conformational change in Bdf1 that increases its affinity for TFIID. Thus, Bdf1 could associate with chromatin to monitor the patterns of acetylation and signal to TFIID when a specific pattern is present. Bdf1 is known to be phosphorylated (Matangkasombut et al., 2000), and its association with TFIID could also be regulated by its phosphorylation status. We are currently testing these possibilities.

Its widespread localization suggests that Bdf1 may also be part of other complexes or a general component of chromatin. These possibilities are supported by the fact that all eukaryotes contain several proteins (in addition to TAF1) with two bromodomains and an acidic C-terminal domain (Denis and Green, 1996; Dey et al., 2000; Jeanmougin et al., 1997; Lygerou et al., 1994; Maruyama et al., 2002). Although some of these proteins are implicated in gene expression, many also have roles in cell cycle progression. One interesting possibility is that this type of double bromodomain module is a general chromatin component, perhaps involved in sensing histone acetylation status. Although yeast TAF1 does not have bromodomains, TAF1 in higher eukaryotes may have evolved its current structure by a gene fusion with a Bdf1-like gene that incorporates the double bromodomain module covalently into TFIID. Other nuclear processes may respond to chromatin acetylation via interactions with other double bromodomain proteins. A recent paper found that DNA double-stranded break (DSB) repair was sensitive to histone H4 tail acetylation and Esa1 H4 HAT activity (Bird et al., 2002). Mutants in the H4 tails or Esa1 were sensitive to MMS, a DSB-inducing agent. Interestingly, strains lacking Bdf1 are also MMS sensitive (Chua and Roeder, 1995), and it will be important to determine whether Bdf1 has a chromatin-related role in DSB repair. Future biochemical and genetic studies, including the purification of Bdf1-containing complex(es) and characterization of Bdf1 phosphorylation sites, will help clarify its roles in the cell. It will also be of interest to determine whether Bdf2 has a specialized role in the cell or performs the functions of Bdf1 under growth conditions that lead to low levels of histone acetylation.

Experimental Procedures

Yeast Strains, Genetic Manipulations, and Media

Yeast strains used in this study are listed in Supplementary Table S1 at http://www.molecule.org/cgi/content/full/11/2/353/DC1. Yeast strains were transformed by the lithium acetate procedure (Gietz et al., 1992). Standard methods for media preparation, mating, sporulation, and tetrad analysis were used (Ausubel et al., 1991; Guthrie and Fink, 1991).

BDF1 was disrupted in strain FY24 to generate YSB778 by PCR-mediated gene disruption with KanMX cassette (Wach et al., 1994). Disruption cassette was amplified from pRS400 (Brachmann et al., 1998) using primers "BDF1-disruption-up" (ATGACCGATATCACAC CCGTACAGAACGATGTGGATGTCAAGATTGTACTGAGAGTGCAC) and "BDF1-disruption-down" (CTCTTCTTCACTTTCGCTGCTAA CAT CGTCATCTGAAGATCT GTGCGGTATTTCACACCG). The PCR product was transformed into FY24 and colonies were selected on YPD plates containing 500 mg/l of G418. Incorporation of the disruption cassettes was confirmed by PCR analysis of genomic DNA.

Site-Directed Mutagenesis and Plasmid Constructions

Single point-mutations of BDF1 were generated using PCR-mediated site-directed mutagenesis (Ho et al., 1989). A 5′ primer at BDF1-ATG (AACCATGGCCGATATCA CACCCGTACAGAAC) and the appropriate mutagenic primers were used to amplify the 5′ end of the BDF1 gene in pRS314-BDF1a. The resulting PCR product was gel purified and used as a 5′ megaprimer in a second PCR reaction with an BDF1-downstream (ACCATGGCAATGTATT ATAGTTTCTG CGTTG) 3′ primer for amplification of full-length BDF1 from the same template. The resulting ~2.5 kb amplified fragments were cloned

into pCRscript SK+ (Strategene). The incorporation of mutagenenic primers was verified by appropriate restriction digest. Mutant plasmids were digested with BamHI to release the Bdf1 open reading frames, which were then cloned into pRS314-BDF1a to replace the wild-type sequence. Plasmids with mutations in both bromodomains were generated by cloning ~1.5 kb MscI/SacI fragment carrying coding sequence of aa 330–686 from pRS314-bdf1 mutated in the second bromodomain into the ~7 kb MscI/SacI backbone of pRS314-bdf1 mutated in the first bromodomain. The BamHI fragments from double mutation plasmids were cloned into BamHI site of pGEX-1 for GST-fusion protein expression. The pRS314-bdf1 series with mutations in both bromodomains were used as templates to amplify a ~2.5 kb fragment carrying bdf1 ORF with oligos BDF1-ATG and Bdf1-downstream. The PCR product was digested with NcoI and cloned into the NcoI site of pRS314-TFA1pr-N-HA3.

Recombinant Protein Purification

Recombinant GST-fusion proteins were expressed in *E. coli* and were prepared as described (Matangkasombut et al., 2000). Recombinant His-tagged Bdf1 protein was expressed in *E. coli* BL21(DE3) and prepared similarly to GST-fusion protein with the following differences: lysis buffer is 20 mM Tris-HCl (pH 8.0), 100 mM potassium acetate, 20% glycerol, 1 mM PMSF, protease inhibitors; Ni-NTA agarose beads (Qiagen) were used for purification; wash buffer is lysis buffer supplemented with 30 mM imidazole; and elution buffer is lysis buffer supplemented with 250 mM imidazole. The fractions were pooled and dialyzed against GST binding buffer.

GST Pull-Down Assay

For GST-Bdf1 and calf thymus histone interaction, recombinant GST, GST-BD1, GST-BD1+2, or GST-Bdf1 proteins (500 ng) were immobilized on 10 μl bed volume of glutathione agarose beads. After washing with GST binding buffer, the beads were then incubated with the indicated amounts of calf thymus histones (Sigma) in 200 μl total volume at 4°C overnight on a rotator. The beads were washed five times with 200 μl of GST binding buffer, and bound proteins were resolved by SDS-PAGE. The bound proteins were detected by staining with Coomassie brilliant blue (Sigma).

For GST-histone tail and Bdf1 interaction, 10 μl bed volume of glutathione agarose beads prebound with recombinant GST or GST-histone tail proteins (500 ng) were incubated with the indicated amounts of recombinant His-tagged Bdf1 in 200 μl total volume at 4°C overnight on a rotator. The beads were processed similarly, but bound protein was detected by α-6His immunoblot (Clontech).

For the interaction of GST-Bdf1 and hyperacetylated HeLa histones, 10 μl bed volume of glutathione agarose beads were prebound with recombinant GST or GST-Bdf1 wild-type or mutants (50 μg). Beads were then incubated with a mixture of 15 μg of hyperacetylated HeLa histones and 5 μg of nonacetylated HeLa histones to allow an equal representation of all acetylated species in the reaction. After an overnight incubation at 4°C on rotator, the beads were washed with GST binding buffer containing either 100 mM or 600 mM potassium acetate. Supernatant fractions were precipitated with ice-cold 100% acetone for 3 hr or overnight at −20°C, washed with ice-cold 80% acetone, and dissolved in 5 μl acetic acid sample buffer (Ausubel et al., 1991). Washed beads were mixed with 5 μl acetic acid sample buffer and heated to 65°C for 5 min. All samples were loaded on Triton-acetic acid-urea (TAU) gel prepared as described (Ausubel et al., 1991). After electrophoresis, the TAU gels were stained with Coomassie brilliant blue.

RNA Analysis

Yeast cells were grown to early log phase at room temperature and shifted to 37°C by the addition of prewarmed media. At the indicated time points, cells were harvested and washed once with cold water. RNA was isolated by hot acid phenol extraction (Ausubel et al., 1991), and concentration was determined by measuring the A260. S1 nuclease protection assays were performed as described (Michel et al., 1998). The primers for RPL5A, RPS8A, RPL9A, and RPS11B are gifts from J. Reid and K. Struhl (Reid et al., 2000).

Chromatin Immunoprecipitations

Chromatin immunoprecipitations were performed essentially as described previously (Komarnitsky et al., 2000; Kuras and Struhl, 1999) with minor modifications. Two hundred milliliters of each yeast strain was grown to $OD_{600} \approx 0.6$ in synthetic complete medium supplemented as indicated. Formaldehyde was added to a final concentration of 1% for 20 min, and the reaction was quenched by the addition of glycine to 240 mM. Cells were collected by centrifugation, washed twice with TBS, and lysed with glass beads in FA lysis buffer (50 mM HEPES-KOH [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.01% Na-deoxycholate, 1 mM PMSF). Chromatin was sheared by sonication so that the average fragment size was between 200 and 700 bp and stored in aliquots at −80°C.

For immunoprecipitations, antibodies were preincubated for 1 hr at room temperature with protein A-Sepharose CL-4B beads (Amersham) as indicated and washed once with TE (pH 8.0). Chromatin was then added, and reactions were incubated overnight at 4°C. Immunoprecipitates were stringently washed, protease treated, and decrosslinked. Conditions for PCR and primers used in this study were as described previously (Komarnitsky et al., 2000; Reid et al., 2000). PCR products were quantified by a Fujix BSA 2040 PhosphoImager.

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