Bromodomain factor 1 (Bdf1) protein interacts with histones

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Abstract Using a yeast two-hybrid assay we detected an interaction between the N-terminal region of histone H4 (amino acids 1–59) and a fragment of the bromodomain factor 1 protein (Bdf1p) (amino acids 304–571) that includes one of the two bromodomains of this protein. No interaction was observed using fragments of histone H4 sequence smaller than the first 59 amino acids. Recombinant Bdf1p (rBdf1p) demonstrates binding affinity for histones H4 and H3 but not H2A and H2B in vitro. Moreover, rBdf1p is able to bind histones H3 and H4 having different degrees of acetylation. Finally, we have not detected histone acetyltransferase activity associated with Bdf1p. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Histone; Chromatin; Bromodomain factor 1 protein; Bromodomain; Histone acetyltransferase; Two-hybrid

1. Introduction

Activation of genes transcribed by RNA polymerase II requires the prior assembly of general transcription factors that form the pre-initiation complex. Recruitment of this complex is normally associated with a cascade of interactions between these general factors, DNA elements, and gene-specific *trans*-acting factors (i.e. transcriptional activators, mediators, and coactivators). Throughout the process of transcription initiation and subsequent activation, the chromatin at or near the promoter region is often modified or even disassembled (reviewed in [1,2]). Two main types of enzymatic activities can accomplish remodelling of chromatin structure: histone acetyltransferases (HATs) [3] and a class of ATPases able to alter nucleosome structure [4]. The reversible acetylation of lysines in N-terminal tails of the core histones has traditionally been correlated to the transcriptional activity of chromatin (reviewed in [1–3,5–7]). One model postulates that histone acetylation unfolds chromatin, thus resulting in a more accessible DNA interface. There are several lines of evidence implicating histones, and their modifications, in a more direct role for the activation of class-II genes. Thus, it has been proposed that transcription factors can be targeted to nucleosome-bound regions of DNA when the N-terminal tails of histones are acetylated in specific sites [3,8,9]. Furthermore, an increasing number of proteins related to transcriptional regulation have been shown to have HAT activity. In yeast there are seven different HAT activities described to date, six of them with physical and/or functional connections to transcriptional regulation: (1) the transcriptional adaptor Gcn5p [10]; (2) $Taf_{II}145$, a component of the basal transcription factor TFIID [11]; (3) Elp3p, a stimulator of elongation of RNA polymerase II [12]; (4) Esa1p, a protein required for cell cycle progression [13]; (5) Sas3p, a silencing-related protein [14]; (6) Nut1p, a subunit of mediator [15]. The seventh yeast protein with HAT activity is Hat1p, an enzyme originally found in the cytoplasm [16] but which also has been detected in the nucleus [17] and that has been recently implicated in telomeric silencing [18]. Histone N-termini can also be the target of non-HAT proteins such as Sir3p, Sir4p and Tup1/Ssn6, which act to repress gene expression in yeast [19]. Together, these data suggest that histones, and N-terminal histone modifications, may be specifically recognised by regulatory proteins.

Here we present the results obtained from a yeast two-hybrid screen designed for the purpose of identifying such proteins. We employed yeast genomic libraries in combination with a bait plasmid expressing a fragment of histone H4 (amino acids 1–59). Our results demonstrate that a portion of the bromodomain factor 1 protein (Bdf1p) interacts with this N-terminal fragment of histone H4. Furthermore, the region of Bdf1p shown to interact with H4 in vivo includes one of its two bromodomains. Additional in vitro analysis was also used to further characterise the interaction between Bdf1p and the core histones.

2. Materials and methods

2.1. Two-hybrid analysis

The yeast strain PJ69-4A (*MAT***a**, *trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*), vector plasmids (pGBD-C1, pGBD-C2 and pGBD-C3) and libraries (Y2HL-C1, Y2HL-C2 and Y2HL-C3) employed in this study were kindly supplied by Dr P. James and used as described [20].

To construct the bait plasmids, DNA fragments encoding amino acids 1–22, 1–37, 1–59 and 1–102 (the whole protein) of histone H4 were amplified from plasmid pUK421 (obtained from Dr M. Grunstein) using the sense primer 5′H4 (5′-CAATAAAGAATTCTCCGGTAGAGG-3′) and, respectively, the antisense primers 3′H4-22 (5′-ATGGGATCCTATAGAATCTTTCTGTGACGCTT-3′); 3′H4-37 (5′-TAGATCTCTGATAGCTGGCTTAG-3′); 3′H4-59 (5′-GGATTCCAAGATCTATTTCAAGAC-3′) and 3′H4-102 (5′-TTAACCATCGATTGTTTAACCACC-3′). The PCR products were cleaved and cloned into the appropriate digested vectors: fragment 1–22 into the *Eco*RI- and *Bam*HI-digested pGBD-C3 vector; the 1–37 into the pGBD-C3 digested with *Eco*RI and *Bgl*II; the 1–59 into the *Eco*RI- and *Bgl*II-digested pGBD-C2 vector and the 1–102 into the *Eco*RI- and *Cla*I-digested pGBD-C1 vector. The resulting plasmids: pGBD-H4(1–22), pGBD-H4(1–37), pGBD-H4(1–59) and pGBD-H4(1–102),

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Abbreviations: HAT, histone acetyltransferase; Gal4(AD), activation domain of Gal4; Gal4(DBD), DNA-binding domain of Gal4

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expected to contain in-frame fusions of each of the fragments of H4 with the DNA-binding domain (DBD) of Gal4, were confirmed by DNA sequencing.

The construct pGBD-H4(1–59) was used as bait plasmid in the screen of the yeast genomic libraries. The yeast reporter strain PJ69-4A was transformed with this bait plasmid by the LiAc method, and transformations of the library were made following the high efficiency method of Gietz and Schiestl [21] scaled up 10-fold. The two-hybrid transformants were plated on synthetic complete (SC) media lacking the appropriate amino acids. Interactions between proteins fused to bait and prey plasmids were expected to result in the activation of *HIS3*, *ADE2* and *lacZ* transcription. The positive clones, that arise on adenine and histidine omission plates, were screened by liquid β-galactosidase assays. In duplicate, approximately 80×10^6 cells were harvested and lysed by incubation at 30°C during 20 min with Zymolyase. Crude protein extracts, obtained after centrifugation at 12 000 rpm for 5 min, were assayed to measure β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside as substrate. The total proteins in the crude extracts were quantified by the method of Bradford [22]. One unit of β-galactosidase activity is defined as $A_{420}\times10^3$/min. Plasmid DNA was purified from yeast cells containing β-galactosidase activity and used to transform DH5α *Escherichia coli* cells by electroporation. DNA was analysed by agarose gel electrophoresis and pGAD plasmids were identified by their electrophoretical mobility. The presence of an open reading frame (ORF) fused in-frame to the Gal4 activation domain (AD) was confirmed by DNA sequencing using the forward primer pGAD5′ (5′-GGAATCACTACAGGGATG-3′) and the reverse primer pGAD3′ (5′-GGTGCACGATGCACAGTTGA-3′). The ORF sequences were identified on a database.

2.2. Preparation of 6×His-tagged Bdf1p

The complete ORF encoding the Bdf1p was amplified by PCR using yeast genomic DNA as template and the 5′-GTTAGGAGCTCGACCGATATCACACCCGTACAGAACGATGTG-3′ and 5′-GTCCTCGAATTCTCACTCTTCTTCACTTTCGCTGCTAACATC-3′ sense and antisense primers, respectively. The product was digested with *Sac*I and *Eco*RI and cloned into the cleaved pRSET T7 B expression vector (Invitrogen) and confirmed by sequencing. This construct was used to transform the *E. coli* strain BL21(DE3)pLysS. For expression of full-length 6×His-tagged Bdf1p, the transformed cells were grown in LB medium containing ampicillin (100 μg/ml) and cloramphenicol (25 μg/ml) until OD_{600} of 0.7, induced with IPTG at 1 mM and incubated for 4 h. Cells were harvested, washed once with disruption buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.05% Tween-20, 5 mM β-mercaptoethanol, 1 mM PMSF and a cocktail of protease inhibitors, Complete tablets from Roche) and resuspended in 20 ml of this buffer. Cells were disrupted by sonication. The cell extract was clarified by centrifugation and the supernatant was mixed with 50% Ni^{2+}-nitrilotriacetic (NTA) slurry (Qiagen, CA, USA) resin equilibrated with disruption buffer. The suspension was mixed gently for 1 h at 4°C and, after centrifugation, the resin was washed four times with 12 ml of wash buffer (50 mM sodium phosphate pH 7.4, 300 mM NaCl, 20 mM imidazole, 1% Tween-20, 10% glycerol and the same protease inhibitors indicated before). Bound proteins were eluted with the same buffer containing 250 mM of imidazole. Fractions were subjected to a 10% SDS–PAGE and the tagged protein was detected by Western blot analysis using the His probe H3 (SC-8036, Santa Cruz, CA, USA) followed by the phosphatase alkaline reaction. Fraction containing 6×His-Bdf1p was dialysed against buffer P (15 mM sodium phosphate pH 7.6, 150 mM NaCl, 20 mM imidazole, 0.05% Tween-20) and concentrated by ultrafiltration using a 30 kDa cut off filter device (Filtron Technology Corporation, MA, USA) before used to in vitro protein interaction assays.

2.3. Pull-down assays between Bdf1p and core histones

Chicken erythrocyte core histones and individual H3 and H4 histones were obtained as described elsewhere [23]. An aliquot of 15 μl of the recombinant 6×His-Bdf1p preparation was mixed with 20 μg of core histones or with 5 μg of each of the isolated H3 and H4 histones in a total volume of 50 μl. Samples were incubated during 1–2 h with gently agitation at room temperature. After adding 16 μl of 50% slurry Ni^{2+}-NTA agarose resin equilibrated in buffer P, the mixtures were incubated for 1–2 h more. The resin was washed five times with 500 μl of buffer P, resuspended in sample solution and subjected to SDS–PAGE analysis. AUT–PAGE [23] was used to assess the acetylation degree of retained histones. In this case, the pull-down assay was scaled up four times. Coomassie blue staining was used in both types of electrophoresis to detect proteins.

2.4. Search of HAT activity associated to Bdf1p

Liquid HAT activity assays were performed with the recombinant 6×His-Bdf1p and using chicken erythrocyte free histones and [1-^{14}C]acetyl-CoA (Amersham Pharmacia Biotech, 52 mCi/mmol) as substrates, exactly as described previously [24].

Preparation of cell extracts from yeast MCY3015 wild-type and MCY3017*bdf1* mutant strains (kindly supplied by M. Carlson), chromatographic fractionation of enzymes and analysis of HAT activities were carried out as described [24].

3. Results

3.1. Identification of potential histone H4-interacting proteins

To explore interactions between histones and proteins that could participate in the transcriptional process, a yeast two-hybrid screen was performed to identify proteins able to interact with a Gal4(DBD)-H4 fusion protein, including amino acids 1–59 of this histone. This H4 fragment contains the acetylatable Lys 5, 8, 12 and 16. Using the genomic library Y2HL-C3 fused to Gal4(AD), 5×10^6 transformants were

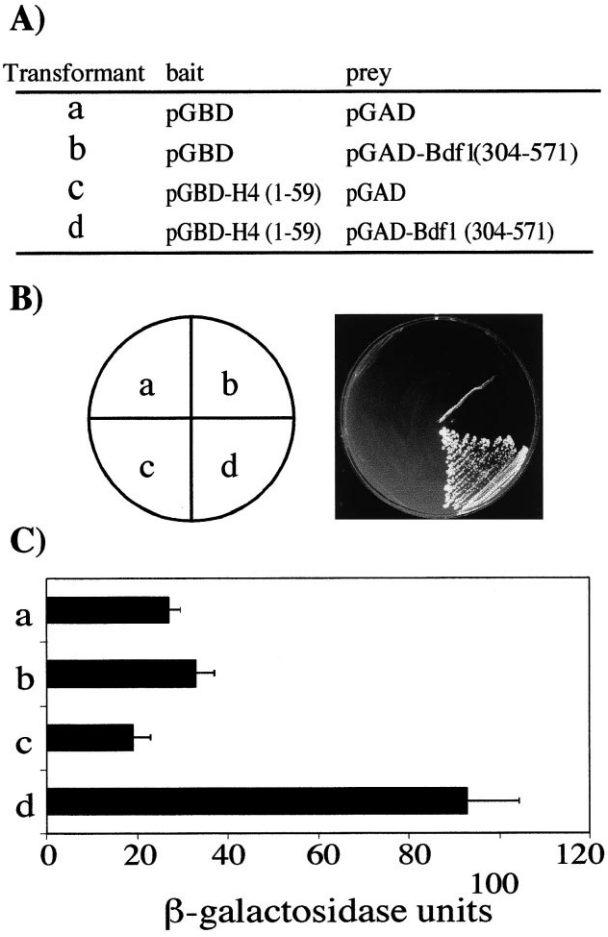

Fig. 1. Interaction of Gal4(DBD)-H4(1–59) with Gal4(AD)-Bdf1(304–571) fusion proteins in the two-hybrid system. (A) List of the plasmids used for double transformation. (B) Growth of the double transformants on SC-Trp,Leu,His,Ade medium. (C) β-Galactosidase activity of the indicated transformants; data represent the average values ± S.E.M. from at least three experiments in liquid SC-Trp,Leu medium.

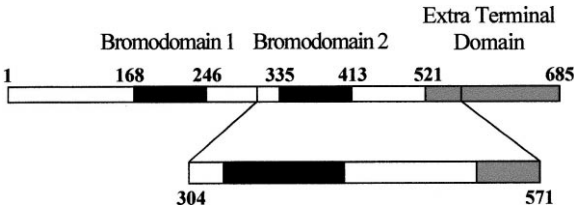

Fig. 2. Structure of the Bdf1p. The fragment isolated (amino acids 304–571) by two-hybrid screen using the N-terminal (amino acids 1–59) portion of H4 is represented below.

screened. Of these transformants, 25 grew on doubly selective media, and from these positive clones, only two exhibited significantly elevated levels of β-galactosidase activity (162 ± 33 U/mg of protein) relative to the reporter strain (30 ± 5 U/mg of protein). Plasmid purification and sequence analysis revealed that both clones (obtained from different screens) corresponded to the same fusion fragment (nucleotides 914–1715) of the yeast gene bromodomain factor 1 (*BDF1*; YLR399C locus). Confirmation of the H4–Bdf1p interaction was done by reconstruction. Yeast strain PJ69-4A was transformed with the bait and the prey plasmids (described in Fig. 1A), and plated on selective media. Cell growth was only observed in the case where both fusion proteins were expressed (Fig. 1B). Moreover, these transformants exhibit a 3–5-fold increase in β-galactosidase activity relative to the control strains (Fig. 1C). Fig. 2 illustrates the position of the isolated Bdf1p fragment (amino acids 304–571) with respect to the sequence of the entire Bdf1p [25]. This fragment contains the complete sequence of one of the two bromodomains, the bromodomain 2 (amino acids 335–413) and approximately one-third of the extra terminal domain.

3.2. Interaction between Bdf1p and different fragments of histone H4

In order to determine whether the isolated Bdf1p fragment (amino acids 304–571) could interact with N-terminal H4 fragments of various lengths, we assayed two-hybrid interactions with gene fusions expressing the regions indicated in Table 1. Yeast cells, coexpressing Gal4(AD)-Bdf1(304–571) and the various Gal4(DBD)-histone H4 fusions, were incubated on selective plates. Activation of reporter genes was not observed when Gal4(AD)-Bdf1 was coexpressed with any of the Gal4(DBD)-H4 fusions shorter than the first 59 amino acids. Interestingly, the construct Gal4(DBD)-H4(1–59) appears to activate transcription better than full-length H4 (102 amino acids), as colonies grew up within 2 days as opposed to 4 days in the case of the Gal4(DBD)-H4(1–102) construct. These results agree with those obtained when we measured the β-galactosidase activity of the respective transformants (results not shown). Together, these results suggest that in the context of the two-hybrid assay, the interaction between Bdf1p and histone H4 requires more than the first 37 amino acids of H4, and that this interaction is stronger in the case of the 1–59 N-terminal fragment than with the full-length histone.

Table 1
Relative growth on SC-Trp,Leu,His,Ade medium of transformants obtained on two-hybrid assays using the plasmid containing the fragment corresponding to amino acids 304–571 of Bdf1p fused to the AD of Gal4p and the different plasmids (pGBD-H4) that contain histone H4 fragments fused to the DBD of Gal4p

Transformant	Growth on plates
pGBD-H4(1–22)/pGAD-Bdf1	−
pGBD-H4(1–37)/pGAD-Bdf1	−
pGBD-H4(1–59)/pGAD-Bdf1	++++
pGBD-H4(1–102)/pGAD-Bdf1	++

3.3. Bdf1p interacts physically with histone H4 and histone H3 in vitro

To confirm the interaction between Bdf1p and histone H4 we performed in vitro pull-down assays using core and the recombinant full-length 6×His-tagged Bdf1p. We observed that histones H4 and H3 were retained by Bdf1p (Fig. 3A), while H2A and H2B were recovered in the non-retained fraction. The retention of H3 and H4 was not observed in the case where core histones were incubated with Ni^{2+}-NTA agarose resin alone (Fig. 3A), therefore it is a result of Bdf1p interaction. Further demonstration of the interaction specificity was provided by replacement of core histones with cytochrome *c* in the pull-down assay. Cytochrome *c* is similar to histones in that it is a small, basic protein. The data obtained indicate that there was no interaction between Bdf1p and cytochrome *c* (Fig. 3A). To eliminate the possibility that the interaction between H3 and Bdf1p was due to the fact that H3 and H4 can dimerise, pull-down assays were carried out on H3 and H4 individually. In these experiments, H3 and H4 were recovered in the respective retained fractions (Fig. 3A). Note that although our preparation of Bdf1p gives several

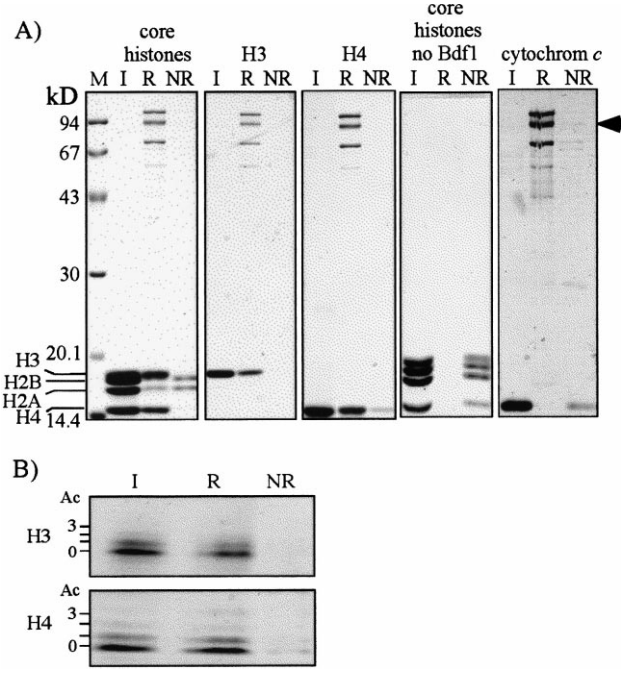

Fig. 3. In vitro interaction between recombinant Bdf1p and histones. Chicken core histones or individually histones H3 and H4 were mixed with recombinant 6×His-Bdf1p and, after incubation, pulled-down by adding Ni^{2+}-NTA agarose resin. As controls, core histones in the absence of 6×His-Bdf1p, and cytochrome *c* were incubated and processed identically. Input (I), retained (R) and non-retained (NR) fractions were analysed by SDS– (A) or AUT– (B) PAGE. Lane M corresponds to the markers of which molecular weights are indicated on the left of A. Arrow shows the position of recombinant full-length 6×His-Bdf1p. Ac denotes the acetylation degree of histones H3 and H4 resolved by AUT–PAGE.

bands in the SDS–PAGE gel, reported data [25] and Western analysis (results not shown) confirm that the band migrating as a 94 kDa protein corresponds to the full-length Bdf1p.

It has been suggested that bromodomains bind to N-terminal tails of histones with a strong preference for the acetylated forms (reviewed in [26]). In particular, a recent study suggests that Gcn5p bromodomain may discriminate between different acetylated lysine residues depending on the context in which they are displayed [27]. In order to examine the possible preference of Bdf1p for a particular acetylation state of histones H3 and H4, pull-down fractions were analysed by AUT–PAGE. As shown in Fig. 3B, we found that Bdf1p interacts to the same extent with non-acetylated H3 and H4 as with different acetylated isoforms. Thus, we do not detect preferential binding of Bdf1p to the acetylated forms of H3 or H4.

3.4. Does Bdf1p have HAT activity?

The fact that other bromodomain proteins have been reported to exhibit HAT activity [10,11,28] suggests that Bdf1p may also be able to acetylate histones, an idea that was further supported by its specific interaction with the N-terminal portions of H3 and H4. We employed two different experimental approaches to investigate this possibility. First, we assayed recombinant Bdf1p for HAT activity using core histones as substrate. HAT activity was not detected in these assays. Second, using yeast cell extracts from wild-type and *bdf1* mutant strains, the HAT activity profiles were compared following the strategy previously described in our laboratory [24]. There were no differences detected between the HAT profiles of these two strains (results not shown). In conclusion, in our experimental conditions, we are not able to detect HAT activity associated with Bdf1p.

4. Discussion

In recent years, modification of chromatin structure has been revealed to be an important step in the activation of gene expression. A relevant feature of certain proteins that modify or remodellate chromatin structure is to contain a conserved sequence called bromodomain, consisting of 60–110 amino acids [29,30]. In fact, many of the known HAT enzymes associated with transcriptional coactivators contain bromodomains. Examples of such proteins are yeast Gcn5p and P/CAF, a large complex in human cells with HAT activity [29,30]. Surprisingly, human $Taf_{II}250$ is also a HAT-containing bromodomain, while its yeast homologue (Taf145p) has HAT activity, but lacks the bromodomain [11]. This conserved module is also present in the ATPase subunit of several chromatin remodelling complexes such as RSC and SWI/SNF [29,30]. All these data suggest that bromodomain-containing proteins could interact in vivo with components of chromatin, like histones. Consistently with this idea, there are several studies showing that bromodomain-containing proteins, or the bromodomain fragment alone, can interact in vitro with the N-terminal region of histones H3 or H4 [8,9,27,31]. In this report, we show for the first time the existence of an in vivo interaction between a bromodomain-containing fragment of yeast Bdf1p and the N-terminal region of histone H4.

Yeast Bdf1p was originally identified as a transcriptional factor involved in the expression of several genes including snRNAs [32]. However, an additional study postulates that Bdf1p is a component of chromatin required for sporulation [25]. These authors explain the implication of Bdf1p in gene expression as a result of alterations in chromatin structure. Finally, using a yeast two-hybrid screen, it has recently been shown that Bdf1p interacts with Taf67p, a TBP-associated factor of yeast TFIID [33]. The existence of an in vivo interaction between Bdf1p and the N-terminal region of histone H4 is reinforced by all of these data. However, while it has been reported that other bromodomain proteins can interact with H4-derived peptides comprising of 1–12 [9], 1–34 [31], 1–36 [8] and 15–29 [27] in vitro, we have not been able to demonstrate any interaction between our Bdf1p fragment and H4 fragments smaller than the first 59 amino acids in vivo. This may simply be a consequence of different specific affinities, depending on the bromodomain sequence, or it may alternatively demonstrate that the in vivo assay requires a longer N-terminal fragment for detection. On the other hand, the weaker interaction shown by the full-length histone H4 with respect to the portion 1–59 on the two-hybrid assays could be a consequence of folding differences between both fusion proteins. On the other hand, the weaker interaction observed with the full-length histone H4 compared to the portion 1–59 in the two-hybrid assay could be due to folding differences.

Another variable to consider is the potential role of varying acetylation degree at the histone N-termini as a way of regulating their interactions with bromodomain proteins. It has been reported that P/CAF bromodomain [9], $Taf_{II}250$ dibromodomain [8] and Gcn5p bromodomain [27] preferentially bind the N-terminal peptides of H4 containing different acetylated lysine residues. In the case of Gcn5p, the bromodomain association is specific to histones H3 and H4, and can occur in the absence of acetylation [31]. A more recent study further demonstrated that the Gcn5 bromodomain can interact preferentially with a peptide corresponding to residues 15–29 of histone H4 acetylated at the lysine 16, but also shows that there are additional contacts between the peptide and the bromodomain at non-acetylated residues [27]. Thus, although we were unable to detect any preferential interaction between Bdf1p and acetylated forms of H3 and H4 (Fig. 3B), this does not discard the possibility of a binding dependence on the acetylation degree of histones. It has been proposed that bromodomains may participate in two discrete interactions with histone tails: a lower affinity interaction with the N-terminal region of the protein and a higher affinity interaction upon recognition of a specifically acetylated lysine residue [26]. Taken all together, in vitro and in vivo results suggest that the interaction between bromodomain proteins and histone H4 is not limited to the acetylatable 1–16 amino acids region, but that it can be exercised in a larger domain. This interaction could also be intensified by the acetylation of certain lysine residues.

Finally, unlike the reports of HAT activity for other bromodomain proteins such as yeast Gcn5p [10], human P/CAF [28] or human $Taf_{II}250$ [11], we have been unable to detect any HAT activity for recombinant Bdf1p. In this sense, based upon structural and functional comparisons, it has been proposed that Bdf1p corresponds to the carboxy-terminal region of higher eukaryotic $Taf_{II}250$ [33]. This protein exhibits acetyltransferase activity, has two bromodomains and an associated kinase activity, whereas its yeast homologue (Taf145p) has only the acetyltransferase activity. There are two bromodomains and kinase activity present in Bdf1p. Yeast Taf145p and Bdf1p combined could thus represent the actual homo-

logue of human $Taf_{II}250$ [33]. The fact that Bdf1p lacks HAT activity is in accordance with this hypothesis. Since Bdf1p interacts with Taf67p [33] and, as described here, with the N-terminal region of histone H4, this bromodomain protein could participate in the recruitment of the transcription factors needed to gene activation if acting in partnership with yeast Taf145p HAT.

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