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The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription

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Summary

Post-translational histone modifications are crucial for the modulation of chromatin structure and regulation of transcription. Bromodomains present in many chromatin-associated proteins recognize acetylated lysines in the unstructured N-terminal regions of histones. Here, we report that the double bromodomain proteins Brd2 and Brd3 associate preferentially *in vivo* with hyper-acetylated chromatin along the entire lengths of transcribed genes. Brd2 and Brd3 associated chromatin is significantly enriched in H4K5, H4K12 and H3K14 acetylation and contains relatively little dimethylated H3K9. Both Brd2 and Brd3 allowed RNA polymerase II to transcribe through nucleosomes in a defined transcription system. Such activity depended on specific histone H4 modifications known to be recognized by the Brd proteins. We also demonstrate that Brd2 has intrinsic histone chaperone activity, and is required for transcription of the cyclin D1 gene *in vivo*. These data identify proteins that render nucleosomes marked by acetylation permissive to the passage of elongating RNA polymerase II.

Introduction

It has been appreciated for many years that eukaryotic DNA is packaged in structurally and functionally distinct forms of chromatin, termed heterochromatin and euchromatin (Peterson and Laniel, 2004). Important differences between these two types of chromatin include the number and nature of the posttranslational modifications present on the histones of their component nucleosomes. Specific residues in the unstructured, amino terminal segments of histones, which protrude from the nucleosome core (Luger et al., 1997), can undergo a variety of post-translational modifications, such as acetylation and methylation (Peterson and Laniel, 2004). It is well established that histone hyperacetylation is characteristic of regions of transcriptionally active chromatin (Hebbes et al., 1988). Histone methylation was shown initially to demark transcriptionally repressed heterochromatin (Lachner and Jenuwein, 2002), but more recent studies have also implicated this modification of specific histone amino acids in activation (Santos-Rosa et al., 2002). Recognition of the variety and combinatorial complexity of histone modifications led to the proposal that post-translational alterations of histone tail amino acids provide docking sites for effector proteins that induce changes in chromatin structure and hence regulate transcription (Jenuwein and Allis, 2001).

The observation that the chromodomain, a domain found in the HP1 family proteins that are components of transcriptionally silent heterochromatin (Elgin and Grewal, 2003), binds to

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histone H3 methylated at lysine 9 provides compelling support for a "histone code" (Jacobs and Khorasanizadeh, 2002). Furthermore, the HP1 homologue of *Drosophila melanogaster* is required for position effect variegation, the silencing phenomenon observed when a block of euchromatin is placed adjacent to a region of heterochromatin (Elgin and Grewal, 2003). Another specific methylation mark that is associated with regulation of transcription is trimethylation of lysine 4 of histone H3 (H3K4me3). This modification is characteristic of nucleosomes located near the sites of initiation of many transcribed genes (Bernstein et al., 2005; Santos-Rosa et al., 2002). Recently, the plant homeodomain finger (PHD) was identified as a motif that binds to H3K4me3, and proteins that contain this motif have been implicated in activation or repression of specific genes (Li et al., 2006; Pena et al., 2006; Shi et al., 2006; Wysocka et al., 2006).

Histones containing acetylated lysines are recognized by the bromodomain, which binds directly to these residues (Dhalluin et al., 1999; Jacobson et al., 2000). A single bromodomain is present in many histone acetyl transferases (HATs) and chromatin remodeling enzymes that regulate transcription, and mediates association of these proteins with acetylated nucleosomes (de la Cruz et al., 2005; Yang, 2004). Another group of bromodomain containing (Brd) proteins, the BET family, usually possess tandem bromodomains and an extraterminal domain (ET) of unknown function (Figure 1A) (Florence and Faller, 2001). The yeast Brd- related protein Bdf1 has been shown to interact with acetylated histones, to prevent heterochromatic spreading, and to regulate the expression of many genes (Ladurner et al., 2003). Several of the proteins specified by mammalian BRD genes have also been reported to bind to acetylated histones (Dey et al., 2003; Kanno et al., 2004; Peng et al., 2006; Pivot-Pajot et al., 2003). For example, Brd6, which is present only in the testis, exhibits acetylation-dependent binding to chromatin (Pivot-Pajot et al., 2003; Shang et al., 2004). Brd2, which like the closely related Brd3 and Brd4 proteins is present in nuclei in many tissues (Shang et al., 2004), has been shown to bind *in vivo* and *in vitro* to histone H4 via acetylated lysine 12 (Kanno et al., 2004). This lysine residue is a substrate of several histone acetyltransferase transcriptional coactivators, and its acetylation is associated with transcribed genes (Peterson and Laniel, 2004). The Brd2 protein, as well as Brd4, associate primarily with euchromatic regions of the genome, and are largely excluded from heterochromatin, suggesting that these proteins might regulate transcription (Crowley et al., 2002; Dey et al., 2003; Dey et al., 2000; Mattsson et al., 2002). Consistent with such a role, Brd2 and Brd4 can activate transcription from several promoters in transient expression assays (Denis et al., 2000; Jang et al., 2005). These properties suggest that Brd proteins might contribute to translation of the histone code. Here, we report the results of experiments that establish that such proteins recognize post-translational marks on chromatin, rendering it permissive to transcriptional elongation by RNA polymerase II *in vitro*.

Results

Brd2 and Brd3 bind actively transcribed, hyper-acetylated chromatin

For these studies, we chose to examine Brd2 and Brd3, which are produced in many cell types and the most closely related among the BET family proteins (Shang et al., 2004). Although it is well established that Brd2 binds to histones carrying acetylated-lysines and is associated with euchromatin (see Introduction), whether it is bound specifically to genes that are transcribed has never been examined. Furthermore, little is known about the properties of Brd3. To determine whether Brd2 and Brd3 bind to acetylated chromatin that is associated with transcribed genes, we performed chromatin immunoprecipitation experiments using FLAG-tagged Brd2 or Brd3 synthesized in 293 cells. It has been reported previously that expression of Brd2 from the human CMV enhancer-promoter used in our constructs does not lead to over-production of the exogenous protein (Kanno et al., 2004). Both Brd2 and Brd3 immunoprecipitated all four core histones, while no histones were detected in

immunoprecipitates obtained from cells not producing either FLAG-tagged protein (Figure 1B). These same immunoprecipitates were examined for histone H3 and H4 modifications by immunoblotting with antibodies that recognize particular modified residues. Neither Brd2 nor Brd3 associated strongly with chromatin acetylated on histone H4 lysine 16 (H4K16ac) or histone H3 lysine 9 (H3K9ac). In contrast, the Brd2- and Brd3- bound chromatin fractions were enriched in several acetylation marks associated with transcribed genes, including acetylated histone H4 lysine 5 (H4K5ac), H4 lysine 12 (H4K12ac), and histone H3 lysine 14 (H3K14ac) (Figure 1C). The histone H4 acetylations are those required for binding of Brd2 to this histone (Kanno et al., 2004). As Brd2 does not interact with histone H3 (Kanno et al., 2004), immunoprecipitation of histone H3 acetylated at lysine 14 must reflect the presence of such modified H3 in nucleosomes to which Brd2 binds directly via H4. Strikingly, the nucleosomes associated with Brd2 and Brd3 were not comparably enriched for dimethylated histone H3 lysine 9 ($H3K9me_2$) (Figure 1C), a mark that is generally considered to be characteristic of heterochromatin (Lachner et al., 2003).

To assess whether Brd2 and Brd3 were also associated preferentially with transcribed genes, chromatin isolated from human 293 cells synthesizing FLAG-Brd2, FLAG-Brd3 or no tagged protein was immunoprecipitated with anti-FLAG antibodies. The DNA was then purified and examined for the presence of various genes by limiting PCR. Representative results are shown in Figure 2. We observed that Brd2 and/or Brd3 were associated with the transcribed genes encoding geminin, β-actin, cyclin D1, RPL7 and RPS28 (Figure 2A). In contrast, neither protein was bound to chromatin containing the transcriptionally repressed β-globin and BMP4 genes, or a highly heterochromatic region of human chromosome 9 (Figure 2A). We also performed chromatin immunoprecipitations with antibodies recognizing all acetylated forms of histone H4 (K5, K8, K12 and K16) or acetylated histone H3 lysine 14 (H3K14). Both antibodies precipitated the transcribed genes encoding geminin, β-actin, cyclin D1, RPL7 and RPS28, but not the repressed β-globin, and BMP4 genes or the heterochromatic DNA (Figure 2B). These results indicate that both Brd2 and Brd3 bind to hyper-acetylated chromatin containing transcribed genes. Nevertheless, we observed differential binding of Brd2 and Brd3 to specific genes (Figure 2A). For example, the geminin and β-actin genes were associated with only Brd3, whereas both proteins bound to the RPL7, RPS28 and cyclin D1 genes (Figure 2A).

Brd2 and Brd3 are bound to hyper-acetylated chromatin along the cyclin D1 gene

As noted above, some proteins that bind to histones carrying specific modifications are associated primarily with promoter regions. To investigate whether the Brd2 and Brd3 proteins are similarly restricted, or present along the length of transcribed genes, we used ChIP and PCR to examine the interactions of these proteins with different regions of the ~ 16 kbp cyclin D1 gene. The chromatin from 293 cells that do not synthesize either FLAG-Brd2 or FLAG-Brd3 was again used as the negative control. As shown in Figure 2C, both Brd2 and Brd3 were bound along the entire length of this gene. The enrichment of the cyclin D1 gene sequences relative to the negative was substantial in both cases. However, sequences at and beyond the 3′ end of the gene were less abundant in Brd2-associated chromatin (Figure 2C). This same pattern was observed in a second, independent experiment, and sequences near or beyond the 3′ end of the RPS28 gene were also less abundant in Brd2- associated chromatin (supplemental data). We also attempted to examine the association of the Brd proteins with the cyclin D1 promoter (positions −197 to +44). Enrichment of this sequence in the Brd2- and Brd3- bound chromatin over the negative control was detected (data not shown). However, the immunoprecipitated chromatin fractions contained much less DNA from this region than from other regions of the gene precluding direct comparison of binding of the Brd proteins to the promoter and the other regions examined. Futhermore, only low signals were observed when the −197 +44 sequence was amplified from DNA recovered by ChIP using a pan-histone

antibody. These observations suggest that the promoter is largely nucleosome free and does not survive micrococcal nuclease digestion during the preparation of chromatin.

The chromatin associated with the different regions of the cyclin D1 gene was enriched in hyper-acetylated histone H4 and histone H3 acetylated at lysine 14, as demonstrated by immunoprecipitation with appropriate antibodies (Figure 2D). These acetylation marks are consistent with those found on chromatin immunoprecipitated with Brd2 and Brd3 (see Figure 1C). These data suggest that the Brd proteins coat hyper-acetylated chromatin that packages transcribed genes.

Brd2 is required for the transcription of cyclin D1 *in vivo*

To investigate the role of the Brd proteins in gene expression *in vivo*, we utilized short, interfering RNAs (siRNAs) that induce degradation of mRNAs to which they are targeted (RNAi). Our attempts to knockdown the expression of Brd3 resulted in cell death (data not shown), in agreement with a previous report (Ishii et al., 2005). In contrast, introduction into cells of siRNA specific for Brd2 was not lethal, but induced a large reduction in the concentration of the Brd2 protein, without altering the concentration of β-actin (Figure 3A). We therefore examined the effects of Brd2 knockdown on transcription of several genes by using RT-PCR with primers that detect only unprocessed, primary transcripts. The decrease in the concentration of the Brd2 protein resulted in a reduction in transcription of the cyclin D1 gene, whereas transcription of other genes that were bound by Brd2 was unaffected (Figure 3B). We also examined transcription of cyclin A and cyclin E, which has been reported to be regulated by Brd2 (Denis et al., 2000; Kanno et al., 2004). However, loss of Brd2 did not reduce the efficiency of transcription of either gene (data not shown).

Brd2 and Brd3 facilitate RNA polymerase II transcription through hyper-acetylated nucleosomes

The observations that the Brd2 protein is required for transcription of the cyclin D1 gene and is bound to nucleosomes associated with the entire length of this gene *in vivo* suggested that Brd proteins might function in transcriptional elongation through the acetylated nucleosomes characteristic of transcribed genes. No protein with such specificity has been identified in previous biochemical studies, despite the fact that transcription of nucleosomal templates in cell-free extracts is sensitive to acetylation (Loyola et al., 2001). To assess this possibility, we used a defined *in vitro* transcription system for RNA polymerase II transcription comprising highly purified components (Figure 4A) and nucleosomal templates that were assembled with either hypo- or hyper-acetylated histones purified from HeLa cells (Figure 4B). Comparison of the hypo- and hyper- acetylated histones by both acid-urea gel electrophoresis and direct examination of specific histone modifications established that the latter were indeed highly enriched in acetylated residues (Figure 4B). The chromatin templates were assembled enzymatically using RSF (remodeling and spacing factor), which assembles physiologically spaced arrays of nucleosomes (Figure 4C) (Loyola et al., 2001). The promoter used in these experiments contained 5 copies of the binding site for the yeast Gal4 transcriptional activator and chromatin assembly reactions contained a chimeric activator comprising the Gal4 DNA-binding domain and the human c-Myc activation domain to ensure that the promoter was nucleosome free (Loyola et al., 2001). Consequently, this chromatin template supports assembly of a preinitiation complex and initiation of transcription, but elongation of RNA polymerase II is blocked at the first nucleosome it encounters. A similar assay led to the identification of the FACT protein which facilitates the passage of RNA polymerase II through nucleosomes (Orphanides et al., 1998). As expected, only very short transcripts were detected in the absence of FACT, and the addition of this protein alleviated the nucleosomal block to elongation (Figure 4D). Consistent with previous reports (Loyola et al., 2001), transcription

of chromatin templates assembled with hypo- or hyper-acetylated histones was similar when reactions contained an optimal concentration of FACT (Figure 4E).

In order to assay proteins that might confer a preference for hyper-acetylated nucleosomal templates, we then utilized reactions that contained a concentration of FACT that was insufficient to support transcription elongation through nucleosomes (Figure 5B, lanes 3 and 8). When either purified FLAG-Brd2 or FLAG-Brd3 (Figure 5A) was added to such reactions, robust transcription was observed from the hyper-acetylated template (Figure 5B, lanes 4 and 9). Furthermore, in the absence of FACT, both Brd2 and Brd3 were capable of allowing transcription (Figure 5B, lanes 5 and 10), suggesting that they possess histone chaperone activity. This result is striking, because FACT and nucleolin are the only proteins ever reported to possess such activity (Orphanides et al., 1998; Rickards et al., 2007): of the many promising candidates, including Hmg-14, nucleophosmin, nucleoplasmin, TFIIS, Spt6 and human Swi/Snf, that have been tested in this type of assay, all failed to exhibit significant stimulation of transcription through nucleosomes (Orphanides et al., 1998; Orphanides et al., 1999).

In order to confirm that Brd2 and Brd3 are chromatin specific, we tested their activity in transcription reactions that contained naked DNA as a template. Neither protein altered the efficiency of transcription (Figure 5C). We next compared the abilities of Brd2 and Brd3 to stimulate transcription of hypo- and hyper- acetylated chromatin templates (Figure 5D). In stark contrast to FACT, both proteins displayed a striking preference for the hyper-acetylated templates (Figure 5D). As shown in Figure 5E, quantification of four independent experiments established that Brd2 and Brd3 exhibited an 11 and a 7-fold preference, respectively, for the hyper-acetylated compared to the hypo-acetylated chromatin template.

In one approach to investigate whether specific recognition of acetylated lysine residues is required for the Brd proteins to facilitate transcription, we deacetylated the histones prior to chromatin assembly with highly purified Hdac2 and Hdac3. Such treatment of hyper-acetylated histones greatly reduced the acetylation of H4K5, H4K12, H4K16, H3K9 and H3K14 (Figure 6A) and severely compromised the ability of both Brd2 and Brd3 to facilitate transcriptional elongation (Figure 6B, lanes 3,4,7 and 8). In addition, we tested the ability of histone H4 tail peptides, acetylated on specific residues, to inhibit Brd2 dependent transcription. None of the peptides altered transcription from naked DNA (Figure 6C), demonstrating that they do not inhibit transcription non-specifically. Furthermore, none inhibited FACT-dependent transcription of hyper-acetylated nucleosomal templates (Figure 6C). An unmodified peptide had no effect on Brd2 dependent transcription of a nucleosomal template (Figure 6C), consistent with results described above. In contrast, addition of a peptide specifically acetylated on K12 severely impaired transcription, whereas a peptide acetylated on K5 inhibited more modestly effect and a peptide containing acetylated K16 had little effect. These inhibitory effects are exactly as predicted from the previously reported binding specificities of Brd2 *in vivo* (Kanno et al., 2004). Brd3 dependent transcription of the hyper-acetylated nucleosomal template was also inhibited only by the K5Ac and K12Ac peptides, but to the same degree by both (data not shown).

We wished to determine whether the activity of Brd2 depended on the bromodomains previously reported to be required for binding to acetylated H4 (Kanno et al., 2004). We therefore examined the activity of an altered Brd2 protein, Bd(1+2)-Y/F, harboring a tyrosine to phenylalanine substitution in each of the two bromodomains, changes that inhibit severely interaction with acetylated H4 *in vivo* (Kanno et al., 2004). As shown in Figure 6D, the hyper-acetylated nucleosomal template was transcribed efficiently in the presence of Brd2 (lane 2), whereas the Bd(1+2)-Y/F protein failed to permit transcription of this template (lane 3). This observation demonstrates that functional bromodomains are required for Brd2 mediated transcription of acetylated nucleosomal templates.

Brd2 is a histone chaperone

In order to investigate whether the Brd proteins have histone chaperone activity, we assayed for the transfer of a histone octamer from oligonucleosome chains isolated from HeLa cells to a [^{32}P] labeled DNA fragment containing the 5S rDNA nucleosome positioning sequence. The nucleosome positioning sequence is AT-rich and exhibits a higher affinity for histone octamers than random DNA sequences (Hayes et al., 1991). Consequently, a histone chaperone should allow the transfer of histones from oligonucleosome chains or histones dissociated from these oligonucleosomes to a DNA molecule containing this 5S sequence. Formation of a nucleosome containing the [^{32}P] labeled 5S rDNA was observed only in reactions that contained Brd2 (Figure 7A compare lanes 5 and 6). We did not observe any nucleosomes formed on the 5S fragment in the absence or Brd2, nor did the Brd2 protein alone bind to the 5S fragment (Figure 7A). Furthermore, treatment of the oligonucleosomes with HDAC2/3 eliminated the ability of Brd2 to facilitate nucleosome assembly (Figure 7A lanes 7 and 8). The transfer reactions contained no exogenous ATP and addition of a non-hydrolyzable ATP analog did not inhibit Brd2-dependent nucleosome assembly (Figure 7B).

Discussion

It has been known for some time that mammalian Brd proteins bind to acetylated lysine residues in histones of euchromatic nucleosomes (see Introduction). However, no other biochemical activities had been ascribed to these proteins, nor had they been shown to associate with specific endogenous genes. Data presented here establish that the Brd2 and Brd3 proteins are associated *in vivo* with the highly acetylated chromatin of transcribed genes, but not with transcriptionally inactive chromatin (Fig 2). In particular, the Brd2 and Brd3 associated nucleosomes contained histone H4 acetylated on K5 and K12, but not on K16, and histone H3 acetylated on K14, but not on K9 (Fig 1C). Acetylation of K12 and K5 of histone H4 is found in chromatin that packages the entire length of transcribed genes in *Saccharomyces cerevisae* (Liu et al., 2005). Likewise, histone H4 carrying these modifications was found throughout the transcribed cyclin D1 and RPS28 genes in human cells, as were Brd2 and Brd3 (Fig 2). This property is in stark contrast to the restricted association of PHD domain-containing proteins that recognize histone H3 trimethylated on K4 near promoters (Li et al., 2006;Pena et al., 2006;Shi et al., 2006;Wysocka et al., 2006). The broad distribution of the Brd proteins along these genes might reflect a largely structural role for these proteins in establishing chromatin domains in which genes are accessible to the transcriptional machinery. However, Brd2 is required for transcription of the cyclin D1 gene (Fig 3), even thought this gene is also bound by Brd3 (Fig 2). This observation is difficult to reconcile with such a passive role, and rather suggests that Brd2 participates actively in transcription. Consistent with this view, Brd2 (and Brd3) allowed transcriptional elongation through acetylated nucleosomes *in vitro* (Fig 5). In the case of Brd2, such transcription required binding of the protein to the acetylated lysine residues with which it is known to associate *in vivo*, as well as the bromodomains that mediate this interaction (Fig 6). We therefore propose that Brd2 also facilitates movement of RNA polymerase II on nucleosomal templates during transcription of particular genes *in vivo*.

Previous studies have implicated several mammalian BET family proteins in the regulation of transcription (Denis et al., 2000; Kanno et al., 2004); Schweiger et al., 2006; (You et al., 2004) (Schweiger et al., 2006). For example, Brd4 can stimulate transcription from the human immunodeficiency virus type 1 LTR promoter by facilitating recruitment of the elongation factor pTEF-b (Jang et al., 2005; Yang et al., 2005). It was also observed that the binding of Brd4 to chromatin along the entire length of an integrated HIV-1 LTR-luciferase gene is required to establish a similar pattern of association of pTef-b (Jang et al., 2005). This finding suggests that the Brd4 protein might indirectly regulate elongation of transcription. The results of our *in vitro* experiments establish that Brd2 and Brd3 effectively remove nucleosomal

barriers to transcription elongation by RNA polymerase II, independently of pTef-b or any other elongation proteins (Fig 5). These data therefore identify a previously unknown function of these Brd proteins, one that depends on their ability to bind to acetylated histones (Fig 6). Furthermore, the inhibition of cyclin D1 transcription by RNAi-mediated knockdown of Brd2 (Fig 3B) provides the first example of transcriptional regulation of an endogenous gene in its natural genomic and chromatin context by a member of the Brd/BET protein family.

In the chromatin transcription assay, Brd2 and Brd3 were at least as effective in stimulating transcription through acetylated nucleosomes as FACT, which has been shown to bind to H2A and H2B, and to function as a histone chaperone (Orphanides et al., 1999). Brd2 interacts primarily with histone H4 acetylated on K5 and K12 (Kanno et al., 2004) and the former interaction is necessary for its function in transcription elongation (Fig 6C). In view of their different binding specificities, it is unlikely that Brd2 or Brd3 stimulate elongation through nucleosomes by the same mechanism of FACT. Nevertheless, their ability to replace FACT in the chromatin transcription assay (Fig 5) suggested that that these Brd proteins are also histone chaperones. Exactly how Brd proteins structurally alter and/or remove nucleosomes to allow passage of RNA polymerase remains to be established. Previous studies have shown that the ATP-dependent chromatin remodeling enzymes such as RSC can remove nucleosomal barriers to allow passage of elongating RNA polymerase II in an acetylation dependent manner. Moreover, SWI/SNF has been shown to displace acetylated nucleosomes and this activity requires its' bromodomain (Carey et al., 2006; Chandy et al., 2006). All our transcription reactions contain an ATP-dependent chromatin assembly and remodeling factor RSF. Consequently, it is possible that in this simplified system the Brd proteins altered nucleosomes in conjunction with RSF mimicking cooperation with any of the several chromatin remodeling enzymes present in cells. Alternatively, Brd proteins bound to acetylated nucleosomes might act in concert with a translocating RNA polymerase II molecule to drive nucleosome removal or restructuring.

It is likely that Brd proteins also contribute to the induction and maintenance of euchromatin (Mattsson et al., 2002). In contrast to other bromodomain proteins, Brd2 (and Brd4) remain bound to chromatin during mitosis, a property that suggests that they contribute to epigenetic inheritance (Dey et al., 2003; Kanno et al., 2004). Furthermore, extensive "ChIP on CHIP" experiments have shown that the yeast BET protein Bdf1 is bound to hyper-acetylated transcribed chromatin that is specifically non-acetylated at lysine 16 of histone H4 (H4K16) (Kurdistani et al., 2004). When acetylated, this residue provides a docking site for the heterochromatic protein Sir3 (Ladurner et al., 2003). It is therefore believed that the deacetylation of H4K16 in heterochromatin results in the replacement of Sir3 with Bdf1, and the subsequent induction of euchromatin (Kurdistani et al., 2004). In fact, the loss of Bdf1 leads to Sir3 mediated spreading of telomeric heterochromatin (Ladurner et al., 2003). The observation that the nucleosomes to which Brd2 and Brd3 are bound in human cells specifically lacked histone H4 containing K16Ac (Fig 1C) suggests that this distinguishing feature of euchromatin, and its recognition by Brd proteins, is likely to be conserved among eukaryotes.

In these studies, we examined the association of Brd2 and Brd3 with eight endogenous genes. Of these, Brd2 bound to three and its depletion by RNAi decreased the transcription of one (cyclin D1) (Figs. 2 and 3). However, Brd3 was also bound to all the genes associated with Brd2. These observations suggest some degree of redundancy in the functions of Brd2 and Brd3. This property underscores the need for systematic genome wide approaches, to catalog genes bound and regulated by Brd proteins. Such information will facilitate efforts to establish the physiological roles played by these proteins, and their mechanism(s) of action *in vivo*.

Experimental Procedures

Purification of transcription and chromatin assembly proteins

Purification of the general transcription factor proteins, Gal4-AD, FACT, Rsf and histones was adapted from previous work and described in Supplemental Data.

In vitro chromatin assembly and micrococcal nuclease digestion

Rsf mediated chromatin assembly has been optimized previously (Loyola et al., 2001; Loyola and Reinberg, 2003) and described in Supplemental Data.

In vitro chromatin transcription assay

The pG5MLP template for transcription by RNA polymerase II contained five binding sites for the yeast Gal4 protein fused to the adenovirus major late promoter and a 390 basepair G-less cassette. Reactions contained 200 ng of pG5MLP either naked or assembled into chromatin, 10 ng TBP, 10 ng TFIIB, 10 ng TFIIF, 50 ng TFIIE, 150 ng TFIIH and 100 ng RNA polymerase II, 3 mM each of ATP and CTP, 0.03 mM UTP, 12 units of RNAsin (Promega), and 5 μCi/reaction [α^{32}P]-UTP (800 Ci/mmol Perkin-Elmer) in 10 mM Hepes-KOH (pH 7.9), containing 50 mM KCl, 6 mM $MgCl_2$ and 1.25% PEG (w/v) (average mw 8000) and were incubated at 30°C for one hour (Flores et al., 1992; Orphanides et al., 1998). Reactions also contained varying concentrations of FACT, Brd2, Brd3, Brd2 Bd(1+2)-Y/F and 200 ng H4 tail peptides (Upstate 17–212), as indicated. Transcripts were digested with 50 units RNAse T1 (Roche) for 30 minutes at 30°C prior to addition of 20 mM EDTA, containing 200 mM NaCl, 1% SDS (w/v) and 25 μg glycogen (Kadonaga, 1990). They were then deproteinized and resolved by electrophoresis in 6% polyacrylamide gels, unless otherwise indicated, containing 8 M urea cast and run in 1X TBE (0.09 M Tris-borate pH 7.5, 2 mM EDTA). The gels were dried and subject to autoradiography at −80°C with intensifying screens. For quantification, transcripts synthesized in four independent experiments were quantified using Image J software.

Synthesis and purification of FLAG-Brd2, FLAG-Brd3 and FLAG-BD(1+2)-Y/P

Brd2 and Brd3 coding sequences were amplified by PCR from a human cDNA library. The mutant Brd2 cDNA, Bd(1+2)-Y/F, was a generous gift of Drs. T. Kanno and K. Ozato (Laboratory of Molecular Growth Regulation, NIH, Bethesda, MD). The cDNAs were cloned into the CβF expression vector that includes the hCMV enhancer-promoter and an amino terminal FLAG tag (Nikiforov et al., 2002). The vectors were introduced into 293 cells by calcium phosphate co-precipitation and the FLAG-tagged proteins isolated 24 hours later. Nuclei were isolated by hypotonic lysis in TMSD buffer (10 mM Tris-HCl pH 7.8, containing 1.5mM $MgCl_2$, 0.25 M sucrose, 1 mM DTT and 0.5 mM PMSF) (Conaway and Conaway, 1989). Nuclei were then lysed by dounce homogenization in 20 mM Tris-HCl, containing 0.5 M NaCl, 0.1 mM PMSF, 0.2 mM EDTA, and 10% glycerol (v/v) pH 7.5 at 4°C. The extract was clarified by centrifugation at 13000 rpm for 15 minutes. The FLAG-tagged proteins were purified by incubating the extracts with M2 FLAG beads (Sigma) overnight at 4°C with rotation. The beads were washed with buffer listed above and the proteins were eluted by incubation with FLAG peptide at 4°C for 12 hours. The proteins were analyzed by electrophoresis in SDS-PAGE gels, and stored at −80°C for subsequent usage.

Immunoprecipitation of chromatin

The vectors encoding FLAG-Brd2 and FLAG-Brd3 were introduced into 293 cells by calcium phosphate co-precipitation. 24 hours later, nuclei were prepared by hypotonic lysis in TMSD buffer on ice. The nuclei were resuspended in 10 mM Tris-HCl pH 7.5, containing 10 mM NaCl, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1 mM PMSF and 3 mM sodium butyrate, and chromatin

was released by digestion with micrococcal nuclease (Sigma) at 37°C for 10 minutes (Thorne et al., 2004). Digestion was optimized to produce primarily mono-nucleosomes. Digestion was stopped by addition of 20 mM EDTA. Nuclear debris was removed by centrifugation and the soluble chromatin was immunoprecipitated with M2 FLAG beads (Sigma) for the FLAG-Brd proteins by rotation at 4°C for 4-6 hours. The proteins were then eluted from the beads by competitive FLAG peptide elution at 4°C for 12 hours. Detailed descriptions of histone immunoblots are included in Supplemental Data. The DNA contained in each ChIP was purified by phenol extraction, precipitated with ethanol, and resuspended in H_2O. ChIPs to detect acetylated chromatin were performed by immunoprecipitation with an anti-tetra-acetyl-H4 antibody (Upstate, 06–866) or an anti-acetyl-H3K14 antibody (Serotec, AHP412) and preblocked A/G beads, or preblocked A/G beads alone. The immunoprecipitates were washed with PBS (containing 300 mM NaCl) and eluted with 1% SDS and 200 mM β-mercaptoethanol at 65°C for 15 minutes. The immunoprecipitated DNA was purified using QIAGEN PCR purification spin columns. PCR conditions were initially standardized utilizing the input DNA for the ChIPs. The number of PCR cycles and the concentration of DNAs were optimized for exponential amplification. For quantification PCR signals, the Brd2- and Brd3-immunoprecipitated chromatin were expressed relative to the corresponding signals from negative control chromatin immunoprecipitations (anti-FLAG immunoprecipitation of chromatin prepared in parallel from cells that do not synthesize either FLAG-Brd protein). Detailed descriptions of the PCR reactions are included in Supplemental Data.

Brd2 RNAi

siRNA specific for Brd2 (5′ GACAAAGGAGGAACUGGCUUUGGAG 3′) (IDT) or control Stealth RNAi Negative Control (Invitrogen) was introduced into 293 cells with TransFectin™ (BioRad) according to the manufacturer's protocols. At 24 hours post introduction, the medium was changed. Cells were harvested at 60 hours post siRNA introduction. Detailed procedures for protein and RNA preparation and detection are included in Supplemental Data.

Nucleosome transfer assay

The 190 basepair 5S rDNA sequence was isolated by digestion of the pG5MLP5S plasmid with EcoRI and purified by electrophoresis through a 1% agarose gel cast and run in 1X TBE followed by purification with the QIAquick gel extraction kit (Qiagen). 3 μg of the purified 5S fragment was end labeled with 100 μCi [γ-^{32}P]-ATP (3000 Ci/mmol) by using T4 polynucleotide kinase (New England Biolabs). 15 ng of the labeled fragment was incubated with hyper-acetylated oligonucleosomes or an equal quantity of sonicated salmon sperm DNA (Sigma) in reactions containing 10 mM Hepes-KOH (pH7.9), 150 mM KCl, 0.2 mM EDTA, 4 mM $MgCl_2$, 10% (v/v) glycerol, Brd2, and 2 and 4 mM AMP-PNP, as indicated, for 45 minutes at 37°C. The reaction products were resolved by electrophoresis in 5% polyacrylamide gels cast and run in 0.25X TBE (0.0225 M Tris-borate pH 7.5, 0.5 mM EDTA). The gels were dried and subject to autoradiography with intensifying screens. The mononucleosome reference marker was assembled by mixing 2.4 μg of hyper-acetylated histones and 1.5 μg of the [^{32}P] end labeled 5S DNA fragment in reactions containing 2M NaCl, 10 mM Hepes-KOH (pH7.9), 0.2 mM EDTA, 1 μg sonicated salmon sperm DNA (Sigma) and 10% (v/v) glycerol at 30°C for 30 minutes. The assembly reactions were subjected to sequential salt dilution with 10 mM Hepes-KOH (pH7.9), 0.2 mM EDTA and 10% (v/v) glycerol to a final salt concentration of 100 mM NaCl and incubated at 30°C for 15 minutes between dilutions.

Deacetylation of histones

Expression vectors for FLAG tagged Hdac2 and Hdac3 proteins were a kind gift from Thomas Shenk. These proteins were synthesized in 293 cells and purified as described for Brd2 and Brd3. Hyper-acetylated histones or oligonucleosomes were mixed with Hdac2 and Hdac3 and

incubated at 30°C overnight. The deacetylated histones were resolved by electrophoresis in 17% SDS-PAGE gels and immunoblotted with antibodies for specific lysine acetylations, or assembled into chromatin and used in the transcription assay as described in the "blotting of immunoprecipitated chromatin" section of Supplemental Data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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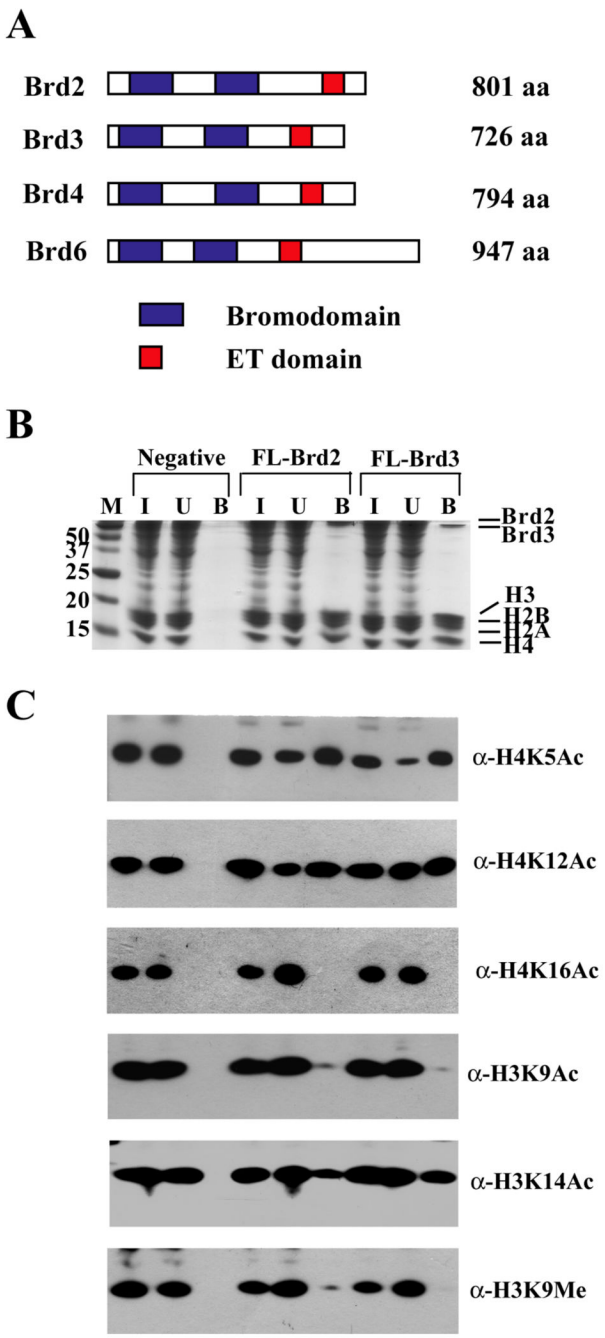

Figure 1. Brd2 and Brd3 associate with acetylated chromatin (A)

Alignment of the closely related Brd proteins that contain double bromodomains, shown to scale. Bromodomains in blue, ET domains in red. **(B)** The association of FLAG-Brd2 and FLAG-Brd3 with chromosomal histones was examined by immunoprecipitation. The proteins present in input chromatin (I), the immunoprecipitation unbound (U) and FLAG-peptide-eluted, bound (B) fractions were examined by SDS-PAGE and Coomassie Blue staining. **(C)** The proteins recovered from cells synthesizing FLAG-Brd2, FLAG-Brd3, or no tagged protein (Negative) by anti-FLAG immunoprecipitation were resolved by SDS-PAGE and examined by immunoblotting with antibodies specific for the histone modifications indicated.

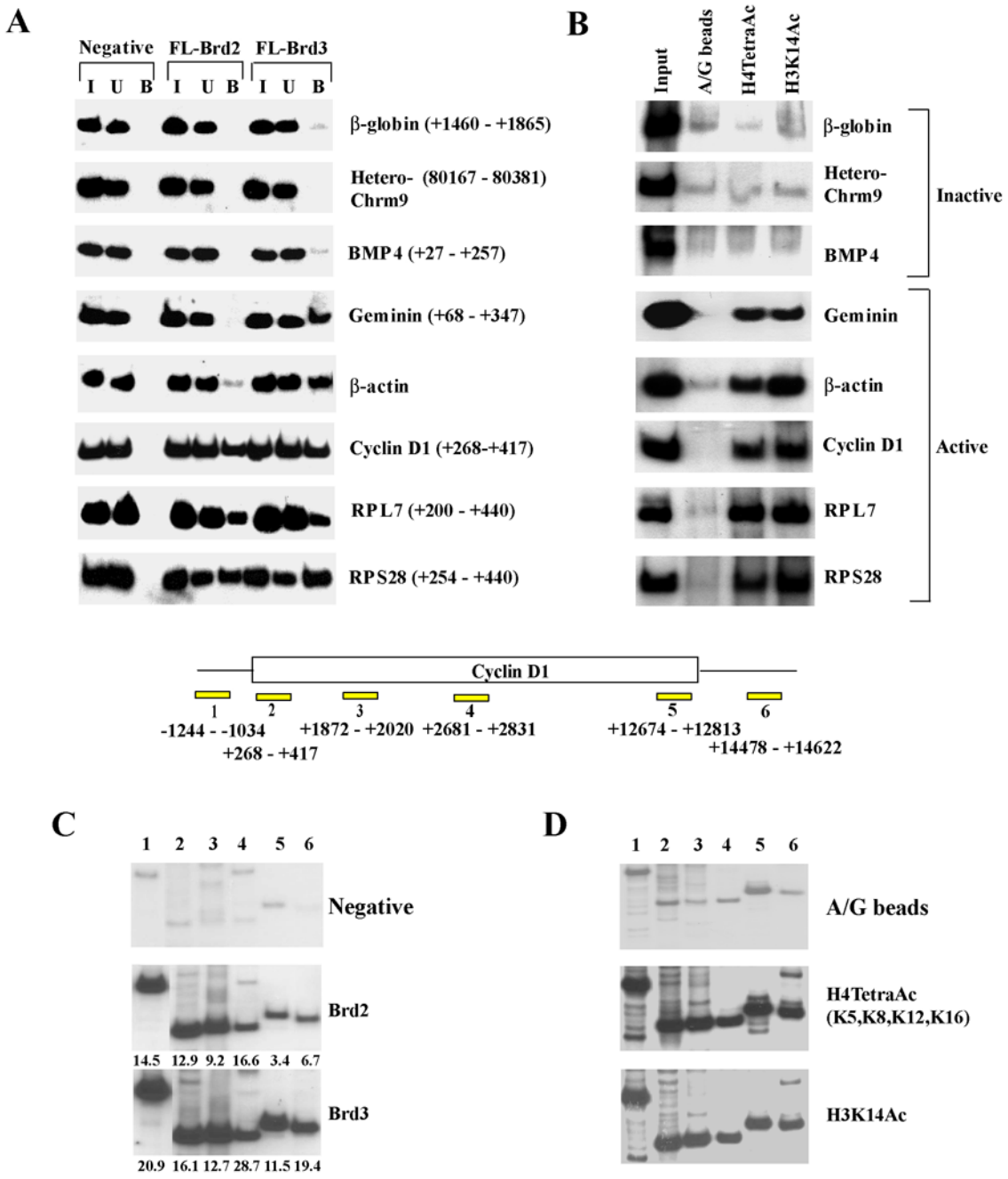

Figure 2. Brd2 and Brd3 associate with chromatin containing transcribed genes (A)

DNA from the immunoprecipitation reactions shown in Figure 1B was isolated and amplified by PCR for the regions of the genes indicated. All bound reactions contained immunoprecipitated DNA from the same number of cells. Parallel reactions contained input and unbound DNA from the same number of cells (1/10 that of bound reactions). **(B)** Chromatin-immunoprecipitation was performed with anti-acetyl-H4 (H4TetraAc) or anti-acetyl-H3 (H3K14Ac) antibody and or preblocked A/G beads alone (A/G beads). DNA from immunoprecipitation was then purified and amplified by PCR for the genes indicated. **(C)** The DNA samples used in the experiment shown in panel A were amplified by PCR for the regions of the cyclin D1 gene shown. The major PCR products observed are those indicated. The fold

enrichment of each sequence in the Brd2 and Brd3 associated ChIP relative to the negative control ChIP are listed under each lane. **(D)** The DNA samples used in the experiment shown in panel B were amplified by PCR for the regions of cyclin D1 gene shown.

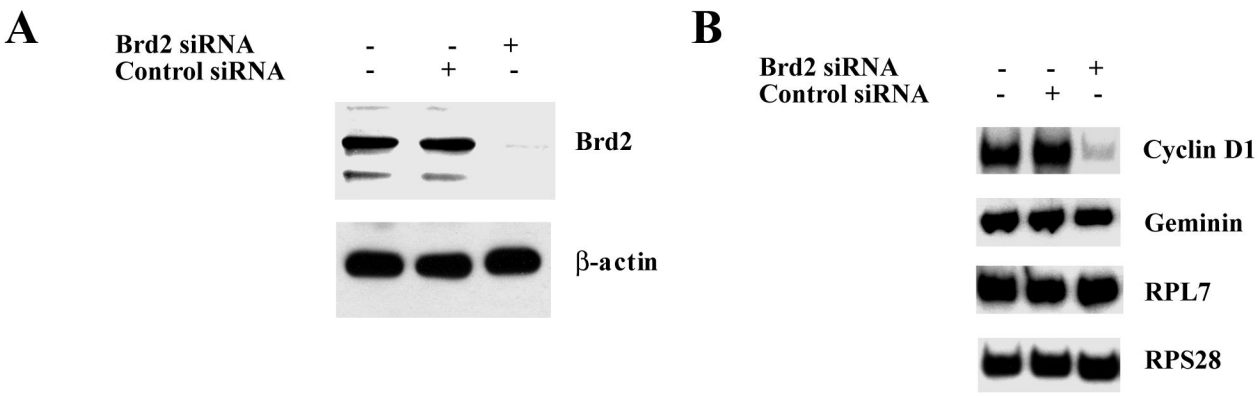

Figure 3. RNAi knockdown of Brd2 induces a reduction in cyclin D1 transcription (A) Immunoblotting of proteins isolated from cells treated with siRNA for Brd2, as indicated. **(B)** cDNA synthesized from the RNA isolated from cells treated with siRNA for Brd2 was amplified by PCR with primers for the genes indicated.

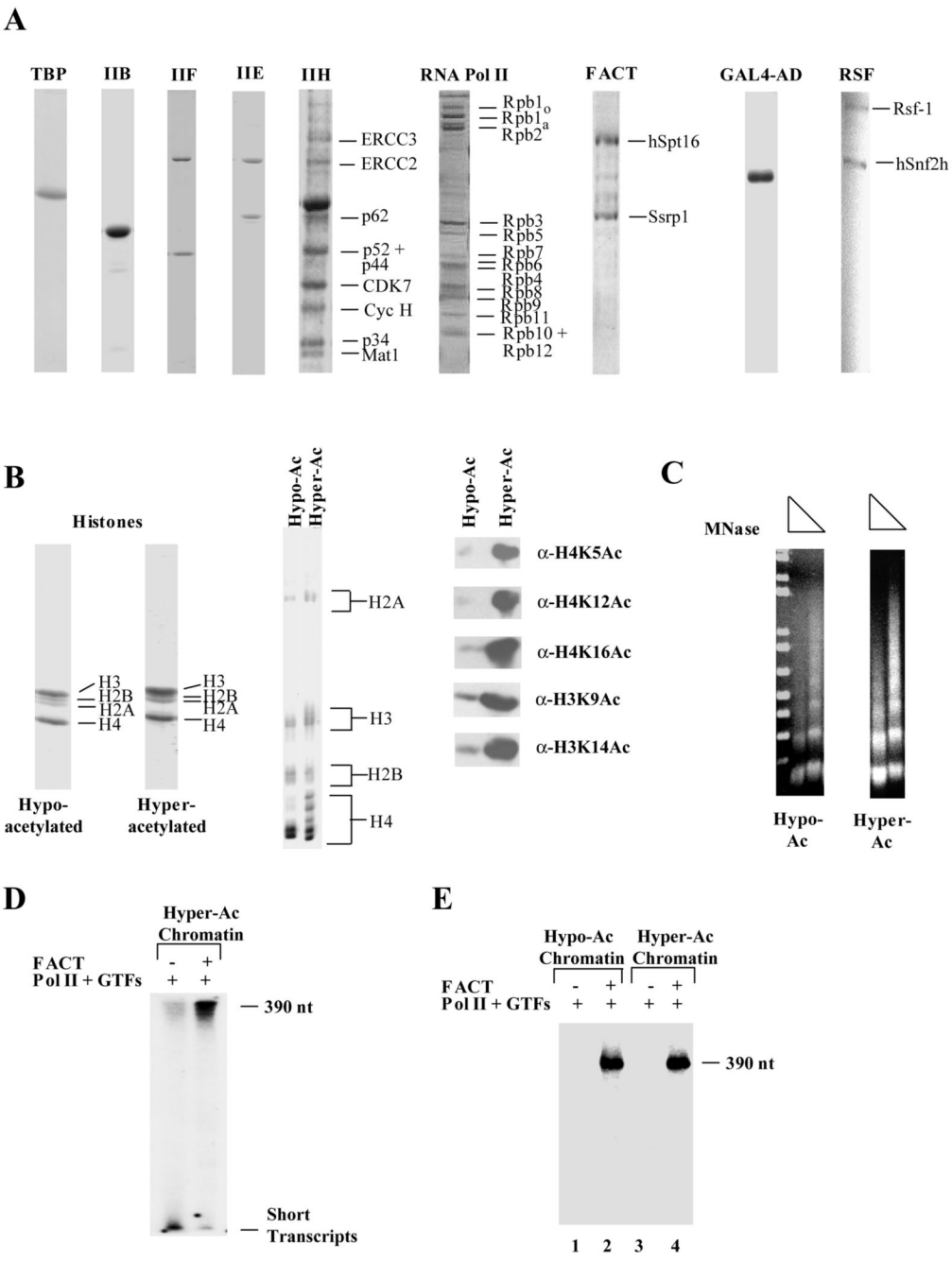

Figure 4. ***In vitro*** **chromatin transcription system (A)**

Purified RNA polymerase II, the general transcription factor proteins,, the chimeric Gal4 DNA binding domain-c-Myc activation domain (Gal4AD) activator and Rsf. Proteins were resolved by SDS-PAGE and stained with Coomassie Blue. **(B)** Hypo-acetylated and hyper-acetylated core histones were purified from HeLa cells, resolved by SDS-PAGE (left panel) or TAU-PAGE (middle) and stained with Coomassie Blue. Histones resolved on SDS-PAGE were subjected to immunoblotting with antibodies as indicated (right). **(C)** *In vitro* chromatin assembly system. Chromatin assembled with Rsf and either hypo-acetylated or hyper-acetylated histones onto the pG5MLP plasmid was digested with two concentrations of micrococcal nuclease, resolved by electrophoresis in a 1.3% agarose gel and visualized by

ethidium bromide staining. **(D)** Transcription reactions contained a hyper-acetylated chromatin pG5MLP template, TBP, TFIIB, TFIIF, TFIIE, TFIIH and RNA polymerase II. was added as indicated. RNA labeled *in vitro* was purified and visualized by electrophoresis in 10% polyacrylamide urea gels as described in the Experimental Procedures. **(E)** Transcription reactions contained either a hypo-acetylated or hyper-acetylated chromatin pG5MLP template and, as indicated.

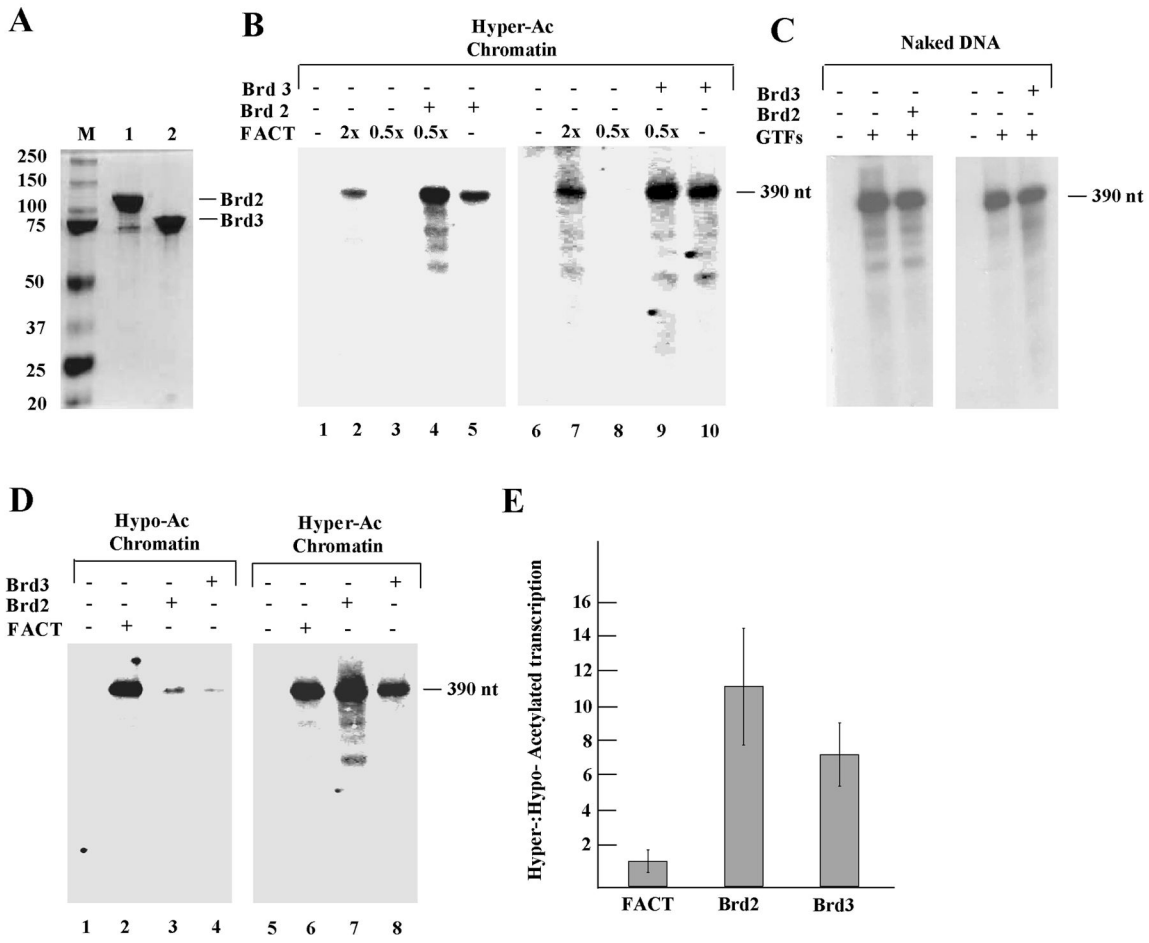

Figure 5. Brd2 and Brd3 preferentially allow transcription through hyper-acetylated chromatin

(A)

Affinity purified FLAG-Brd2 and FLAG-Brd3. Proteins were separated by SDS-PAGE and visualized by Coomassie Blue staining. **(B)** Hyper-acetylated chromatin templates were transcribed in the presence of and/or FLAG-Brd2 (left panel), or FLAG-Brd3 (right panel), as indicated. **(C)** The naked pG5MLP template was transcribed in the presence of the GTFs, FLAG-Brd2 and FLAG-Brd3, as indicated. **(D)** Comparison of transcription from hypo-acetylated (left panel) and hyper-acetylated (right panel) chromatin templates in the presence of, FLAG-Brd2 or FLAG-Brd3, as indicated. **(E)** Quantification of -, Brd2- and Brd3-dependent transcription from hypo-acetylated and hyper-acetylated chromatin templates. The values represent the mean of four independent experiments.

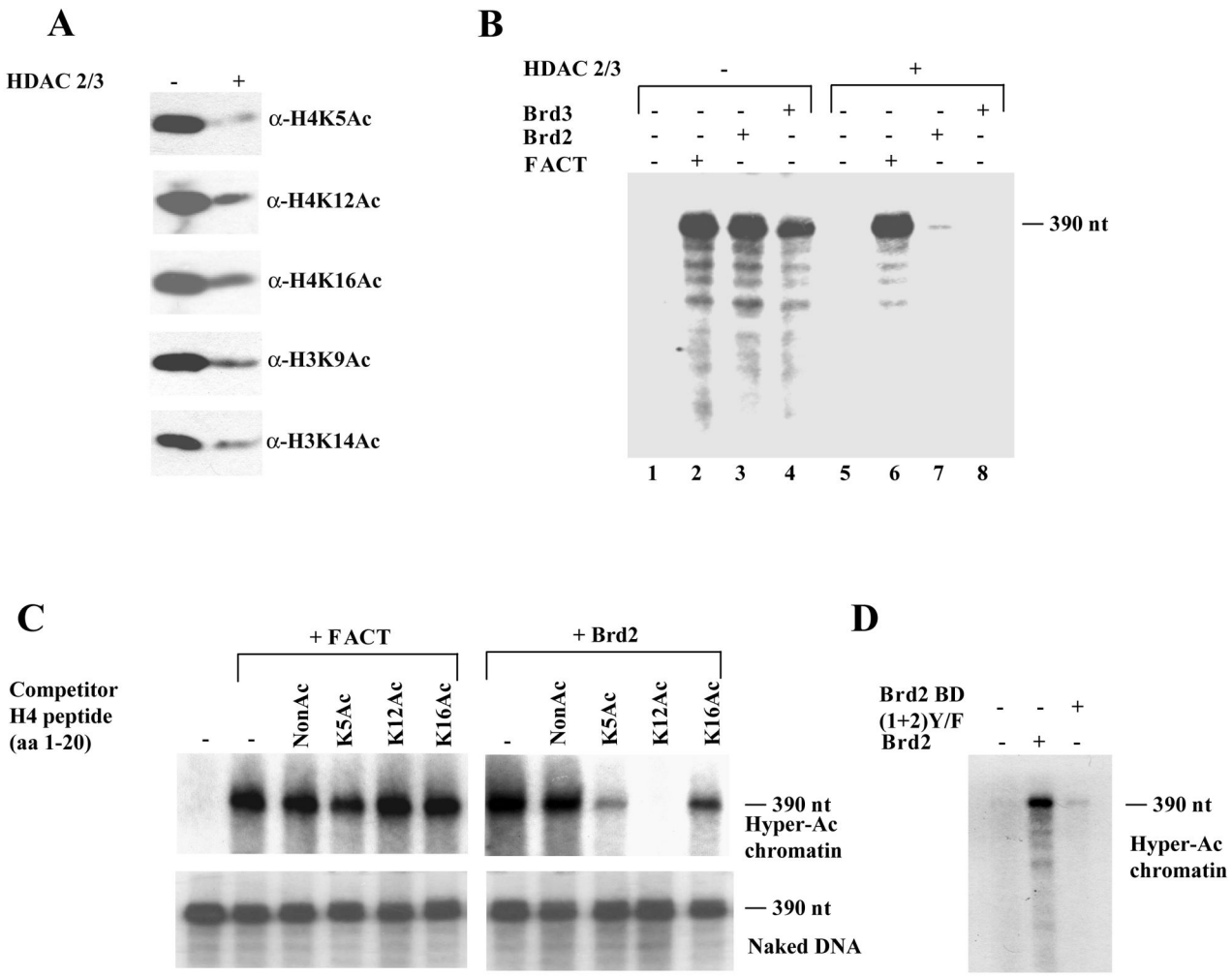

Figure 6. Brd2 and Brd3 require acetylation to facilitate transcription from chromatin templates

(A)
Immunoblotting of histones treated with Hdac2 and Hdac3 with the antibodies indicated. **(B)** Comparison of transcription from chromatin assembled with hyper-acetylated histones (lanes 1–4) or with hyper-acetylated histones treated with Hdac2 and Hdac3 (lanes 5–8) in the presence of, FLAG-Brd2, or FLAG-Brd3, as indicated. **(C)** Hyper-acetylated chromatin or naked DNA templates were transcribed in the presence of, FLAG-Brd2 and H4 tail peptides, as indicated. Peptides were at a ~20-fold molar excess over the concentration of histone H4. **(D)** Hyper-acetylated chromatin templates were transcribed in the presence of equal concentrations of FLAG-Brd2 or FLAG-Bd(1+2)-Y/F, as indicated.

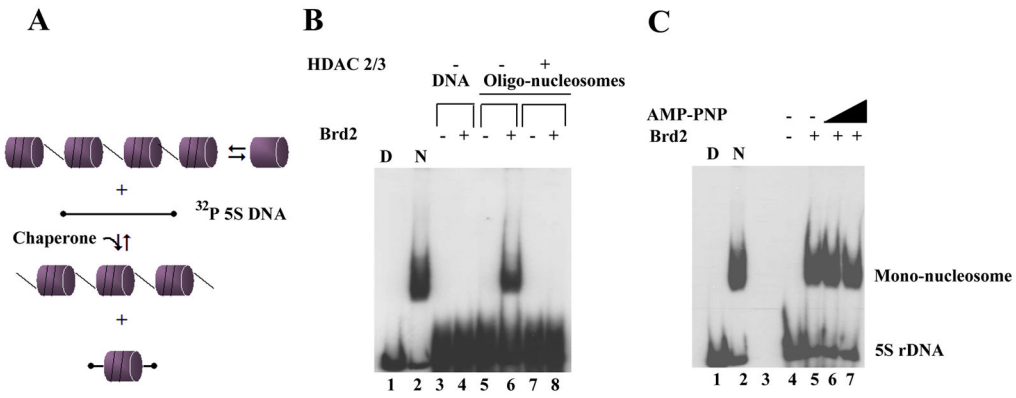

Figure 7. Brd2 functions as a histone chaperone (A)

Schematic diagram of the histone transfer assay. **(B)** The nucleosome transfer reactions contained sonicated salmon sperm naked DNA (lanes 3–4), hyper-acetylated oligonucleosomes (lanes 5–6) or hyper-acetylated oligonucleosomes treated with Hdac2 and Hdac3 (lanes 7–8) and FLAG-Brd2, as indicated. The first 2 lanes are markers, D: naked DNA, N: mono-nucleosome. **(C)** The reactions contained hyper-acetylated oligonucleosomes, FLAG-Brd2 and AMP-PNP, as indicated. Markers were as in panel B.