Acetylation-Dependent Binding Analysis of the Yeast Gcn5 Bromodomain Protein

Jesús Francisco Glaus Garzón*, Christopher Kupitz
Joshua Bailey, and Martin Thompson‡
Department of Chemistry
Michigan Technological University
1400 Townsend Drive, Houghton, Michigan 49931 USA

Received: February 9, 2008 Accepted: April 23, 2008

ABSTRACT

The 439 amino acid yeast Gcn5 protein contains a C-terminal bromodomain, which is required for SAGA (Spt-Ada-Gcn5-Acetyltransferase) mediated nucleosomal acetylation and transcriptional coactivation. Bromodomains are acetyl-lysine binding modules found in many chromatin binding proteins and histone acetyltransferases. Recently, both in vivo and in vitro studies indicate that bromodomains are able to discriminate the acetylation state of lysine side-chains within histone proteins. Here, the cloning, expression and bioactivity of a recombinant bromodomain from the yeast Gcn5 protein is described. The bromodomain from Gcn5 was cloned from yeast genomic DNA enabling effective one-step purification by affinity chromatography. Steady-state fluorescence anisotropy was used to quantify the interaction of Gcn5 with acetylated histone H3. The present cloning, expression, and purification procedure enabled the preparation of large quantity and high yields of biologically active recombinant Gcn5 bromodomain for in vitro structure and function studies.

I. INTRODUCTION

The yeast Gcn5 protein, a histone acetyltransferase (HAT), is the catalytic subunit of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) histone acetyltransferase complexes [1]. The 439 amino acid Gcn5 protein contains an amino-terminal N-acetyltransferase domain and a C-terminal bromodomain (BrD), which is required for SAGA-mediated nucleosomal acetylation. An Ada2p transcriptional coactivator domain, which creates a ternary structure (referred to as the SAGA subcomplex) is required to modulate the HAT activity of Gcn5 [2-4]. Taken together, the fact that BrDs bind acetylated histones [5], and acetyltransferases tag the ε-amine of lysine side-chains [6,7], the Gcn5 protein may serve an important role in transcriptional activation by targeting acetylhistone sites and modifying additional sites in the vicinity required for transactivation by SAGA [8,9]. Some HAT complexes, like SALSA (SAGA altered, Spt8 absent) and SLIK (SAGA-like) have unique subunits, such as the Gcn5 protein, that act as histone acetyl-transferases. The Gcn5 protein is an important part of such complexes and, depending on which complex it belongs to, it can determine the specific lysine to be acetylated [10].

BrDs, originally identified in the Drosophila (fruit fly) Brahma gene (hence the term bromo-domain), represent a family of evolutionarily conserved protein modules approximately 100 amino acids in length that are found in chromatin associated proteins [3,6,9]. A growing number of genetic studies indicate that biological function of bromodomains are dependent upon acetyl-lysine binding [11-13]. These new findings suggest an important posttranslational mechanism for regulating protein–protein interactions via lysine acetylation, and has broad implications for BrD directed assembly of multi-protein complexes at specific chromosomal sites, as observed for

‡ Corresponding author. E-mail: mthomps@mtu.edu

* Current address: Universidad de Sonora, Departamento de Ciencias Químico-Biológicas, Blvd. Luis Encinas y Rosales S/N. Col. Centro Hermosillo, Sonora, México

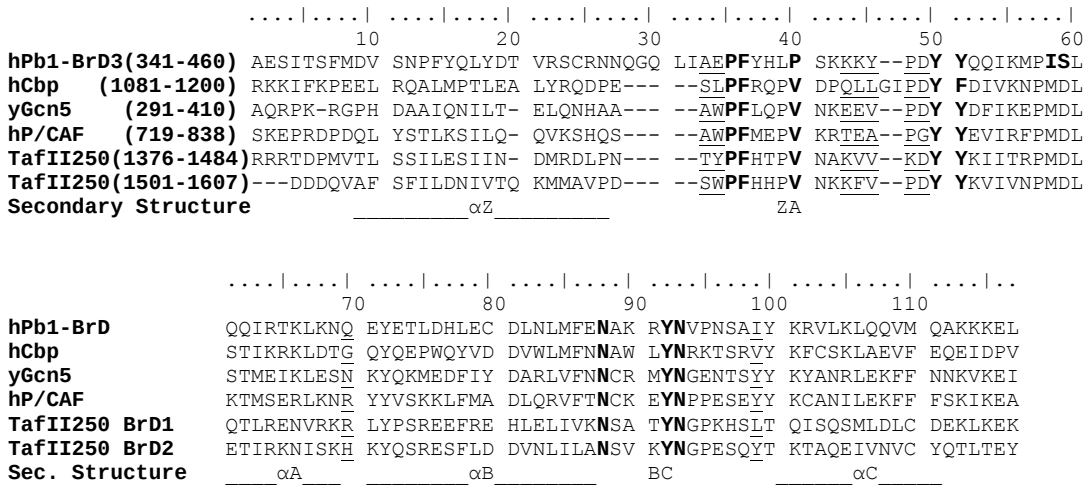

Figure 1. Sequence alignments of BrDs from representative BrD-containing proteins, including Gcn5. Amino acids making direct contact with acetyl-lysine (bold) or side chains flanking the acetyl-lysine of the histone tail (underlined) are indicated. ClustalW from within BioEdit was used to perform the alignment. Secondary structure (loop and helix) annotation is based on the structure of Gcn5 (PDB ID: 1E6I).

SAGA [14], SALSA [15], and RSC (Remodels Structure of Chromatin) [16-19]. *In vivo* and *in vitro* studies suggest that important biological role of Gcn5 proteins in human and yeast, no quantitative analysis of acetylation-dependent binding interactions has yet been performed. Here, bioactivity of the purified BrD from yeast Gcn5 is rigorously examined using fluorescence anisotropy to give solution-phase dynamics of the acetylation dependence of BrD-histone complex formation.

II. MATERIALS AND METHODS

a. Materials

All reagents used for standard molecular biology procedures were obtained from Fisher (Hanover Park, IL). Enzymes were purchased from New England BioLabs (Ipswich, MA). Ampicillin, chloramphenicol, and isopropyl-β-D-thiogalactopyranoside (IPTG) were ordered from Acros Organics (Hampton, NH). F-moc amino acids and peptide synthesis reagents were purchased from Advanced Chemtech (Louisville, KY).

b. Instrumentation

Polymerase chain reaction was performed using the Mastercycler Gradient isolated BrDs preferentially target only a subset of the many possible histone acetylation sites [20-22]. In spite of the Thermocycler from Eppendorf (Westbury, NY). Histone H3 peptides were synthesized on an Endeavor 90 peptide synthesizer (Aapptec, Louisville, KY), purified using high-performance liquid chromatography and characterized using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Fluorescence anisotropy measurements were performed on a Quantamaster-6/2003 Spectrofluorometer in T-format, with a 75W Xenon lamp and analyzed with Felix32 Fluorescence Analysis Software (PTI, Canada). An USB2000-UV-VIS Spectrometer from Ocean Optics (Dunedin, FL) was used to measure absorbance and a Gel Logic 200 imaging system from Kodak (New Haven, CT) was used to quantify DNA and protein gels.

c. Expression and Purification of Bromodomains

Cloning, expression and purification of the BrD from the GCN5 gene (GenBank Accession No. CAA97281), were performed using standard recombinant DNA techniques [23]. Sequence alignments were

used to determine the coding region expected to represent the fully functional BrD (Figure 1) and for the design of PCR primers. The gene region corresponding to the single BrD were PCR amplified using specific primers, which introduced the restriction enzyme sites Sap I (5'-GCTCTTC-3') and Pst I (5'-CTGCAG-3') on the sense and antisense primers, respectively, for subcloning into pTWIN1 (New England Biolabs). This construct places a hexa-histidine tag after the carboxy terminus of the BrD for effective one-step purification. Primers for the Gcn5 BrD 5' - GGTGGTGCTCTTCTAACGATGCGTTGGC ACAAC-3' and 5'- GGTGGTCTGCAGT TAATCAATAAGGTGAG-3' correspond to amino acids 321 through 439 in the native sequence. The PCR product was cloned into the parent vector and transformed into DH5α competent cells. The identities of the recombinant vectors were confirmed by PCR screening using universal T7 primers. Successful candidates were sent to the Biomedical Core Research Facilities at the University of Michigan for sequencing. Rosetta (DE3) cells (Novagen, San Diego, CA) were used for protein expression. Cells were grown in LB media supplemented with ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml) and induced with 1 mM IPTG. The high purity of each BrD was achieved by one-step chitin affinity chromatography and visualized by Coomassie stained SDS-PAGE analysis. Proteins were dialyzed overnight in a solution of 20 mM NaCl, 5 mM Tris, 0.33% β-mercaptoethanol, and 0.05% Tween20 at 4 ^{o} C. The concentration of purified BrD was determined by Bradford assay using a BSA standard.

d. Synthesis, purification and characterization of fluorescein labeled histone H3 peptides.

Fluorescein labeled histone H3 peptides with the sequence:
ARTKQTARKSTGGKAPRKQLASKAA
(locations of acetyl-lysine side chains are in bold) derived from amino acids 2-26 of yeast histone H3 (GenBank Accession No: P61830) were synthesized on PAL-PEG-PS resin by automated solid-phase peptide synthesis (Millipore 9050 peptide synthesizer) using F-moc chemistry. For the modified peptide, acetylated lysines (AcK) were incorporated at all lysine (K) positions and fluorescein was coupled to the α-amine of the N-terminal end using O-(7-azabenzotriazol-1-yl)-N, N, N9, N9, -tetramethyluronium hexafluorophosphate (HATU) activated coupling chemistry. The peptides were removed from the resin and deprotected by TFA cleavage methods for 4 h. Fluorescein is stable to standard deprotection and cleavage conditions. The peptides were purified by RP-HPLC on a Zorbax C8 column (9.4 mm X 25 cm) using a water (0.1 % TFA) to acetonitrile (0.1 % TFA) gradient. Identities and purities of the H3 peptides were confirmed by MALDI-TOF using α-cyano-hydroxycinnamic acid as matrix. The expected mass of the fully acetylated peptide is 3175.2 and multiple peaks centered at (m/z) = 3176.9 in each mass spectrum are observed for the singly charged species (data not shown). The expected mass of the unmodified peptide is 2969.2 Da and a single peak is observed at (m/z) = 2968.8 Da in the mass spectrum [23]. Stock solution concentrations of the fluorescein-labeled histone H3 peptides were determined spectrophotometrically based on the extinction coefficient of ε 491nm = 88,000 M-1 cm-1 for fluorescein [24].

e. Assay for bioactivity of BrD

All fluorescence anisotropy measurements were performed on a Quantamaster-6/2003 Spectrofluorometer in T-format, with a 75W xenon lamp and analyzed with Felix32 Fluorescence Analysis Software (PTI, Canada). Vertically polarized excitation at 488 nm was used with detection in the vertical and horizontal planes at 520 nm, corresponding to the spectral properties of fluorescein. Steady-state fluorescence anisotropy measurements were performed as forward titrations, as previously described [20]. In general, 10 - 50 nM of a given fluorescein-labeled histone peptide is titrated with 0 to 0.1 mM (final concentration) of the BrD until the maximum anisotropy was attained. Anisotropy values for each data point were calculated and plotted versus protein concentration using a two-state model of binding to attain the equilibrium dissociation constant (KD) for BrD binding to the unmodified or acetylated H3 peptide [20].

The data are the average of at least three independent measurements and fit using a nonlinear least-squared algorithm (SigmaPlot).

III. Results and Discussion

a. Expression and purification strategy for Gcn5 bromodomain

Sequence alignments using the Gcn5 sequence were used to determine the coding region expected to represent the fully functional BrD (Figure 1) and for the design of PCR primers. Gene regions corresponding to amino acids 321 to 439 in the native Gcn5 protein sequence were PCR amplified using specific primers, which introduced the restriction enzyme sites Sap I and Pst I for subcloning into pTWIN1. This system was developed to express the individual Gcn5 BrD fused to a cleavable chitin-affinity tag for effective, one-step purification (see Materials and Methods). The successfully screened and sequenced pTWIN1-Gcn5 BrD construct was transformed into the Rosetta (DE3) *E. coli* strain. Analysis of crude extracts and column purified proteins by SDS-PAGE indicate that 1 mM IPTG at OD600 of 0.4 results in the optimal expression of protein. Induction times of 8-12 hours proved to yield the most protein based on SDS-PAGE analysis. Under these conditions expression was 15-25 mg/L culture. Optimization of the lysis step for the native protocol to a final concentration of 3 mg/mL lysozyme for 30-60 minutes resulted in the highest amounts of crude protein. Protein was eluted from the column by addition of 50 mM dithiothreitol (DTT) to the elution buffer and adjusting the pH to 8.5.

SDS-PAGE analysis of each step of the purification process was examined for one liter of cell culture (typically yields 5 g of cell pellet). Total protein was determined for each BrD by quantifying the intensity of the indicated lane (Figure 2). The average total protein for the crude extract was approximately 60 mg, with about 30% of that being from the BrD. The final elution step yields roughly 15 mg of BrD, representing

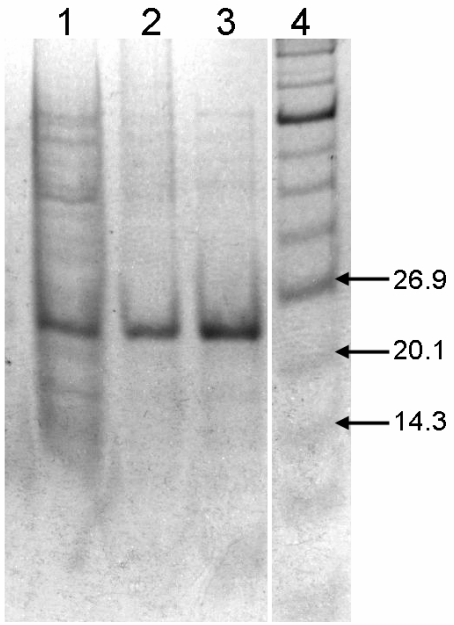

Figure 2. SDS–PAGE monitoring of Gcn5 BrD purification steps. Fractions of the BrD were collected and examined after successive purification steps. Lane 1: crude lysate, lane 2: lysate flow-through and wash, lane 3: eluted BrD protein, lane 4: broad range protein marker (New England Biolabs, Ipswitch, MA).

greater than 90% of the total protein. Overall, the average yield of the BrD is 10-15 mg per liter of culture. SDS-PAGE analysis was used to approximate the molecular weights of the expressed samples based on migration distance relative to a protein marker. The calculated molecular weight, based on amino acid sequence, for the BrD is 18.8 kDa. Based on SDS-PAGE analysis, the Gcn5 BrD has an approximate molecular weight of 21.2 kDa when compared with the migration distance relative to known protein standards (Figure 2). The observed difference between the calculated and observed molecular weight of approximately 10 % is within the margin of error for the method. The high purity of the Gcn5-BrD was visualized by Coomassie stained SDS-PAGE gel analysis (Figure 2). The difference between the calculated molecular weight and the size determined from migration distance was approximately 10% of the calculated molecular weight for

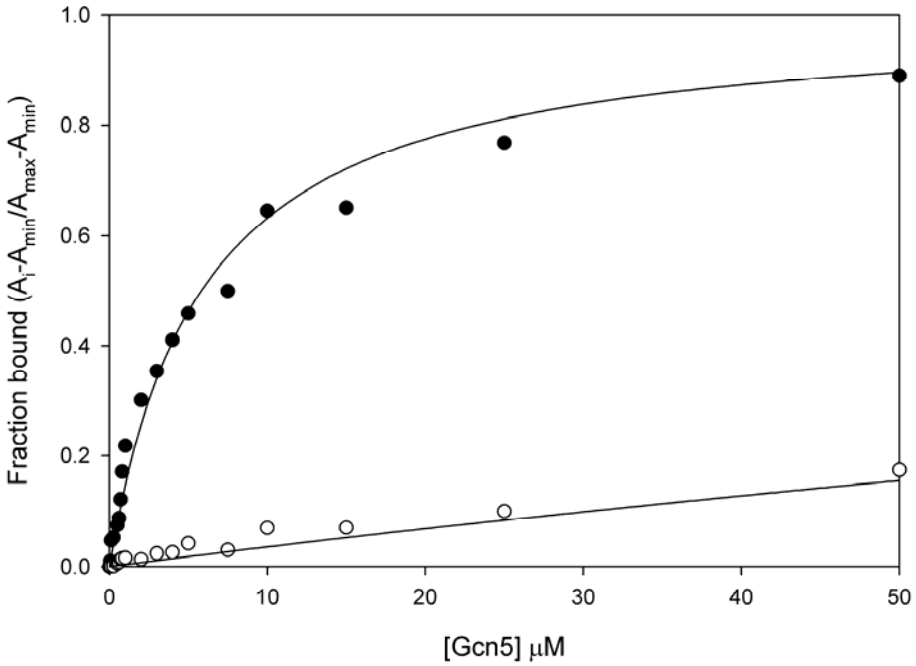

Figure 3. Binding curves for the Gcn5 BrD bound to acetylated or unmodified histone H3 peptides. 20 nM fluorescein-labeled H3 peptide containing modified (●) or unmodified (○) lysine side chains was titrated with Gcn5-BrD. Plots of the fluorescence anisotropy titrations are represented as fraction of the H3 peptide bound to the indicated BrD (A_i-A_{min}/A_{max}-A_{min}) by rescaling the raw anisotropy data along the y-axis and plotting the data as a function of the log of BrD concentration. All plots were fit to a two-state binding model.

different runs of the purified BrD sample. Dialyzed samples examined by SDS-PAGE show only one band confirming the purity of these preparations.

b. Characterization of Bioactivity

The specific binding affinity was examined for the Gcn5-BrD to either a fully acetylated histone H3 peptide or anunmodified histone H3 peptide to quantify the steady-state binding. In each case, this was accomplished by monitoring the change in fluorescence anisotropy of the indicated fluorescein labeled H3 peptide as a function of the concentration of the BrD. Binding studies were performed by titrating 20 nM H3 peptide with 0 to 100 μM of the BrD (Figure 3). Titrations were performed to attain an equilibrium constant for the histone-BrD interaction based on a two state binding model. Plots of the fluorescence anisotropy titrations are represented as fraction of the H3 peptide bound by the BrD (Ai-Amin/Amax-Amin). The raw anisotropy data are rescaled along the y-axis to represent the fraction of H3 peptide with bound BrD, simply by linearly rescaling the measured anisotropies from 0 (experimentally measured lower baseline) to 1 (measured upper baseline). The titration midpoint for individual determinations represents the equilibrium dissociation constant.

The binding of H3 peptide by Gcn5-BrDs shows an affinity in the micromolar range for the acetylated H3 peptide (Figure 3). Equilibrium dissociation constants (KD) determined from the binding assays indicate that the BrD has a KD value of 1.2 μM. This is consistent with prior studies of Gcn5 using NMR methods (25). For comparison, the binding of the Gcn5-BrD with unmodified H3 peptide indicate negligible binding affinity in

the range examined. The average KD value for the BrDs can only be estimated to be greater than 1 mM. In fact, the BrD does not form stable complexes with the unmodified histone peptide and only show modest binding at the highest concentrations examined. Control experiments substituting a BrD with different concentrations of BSA (1 μM, 10 μM, 100 μM and 1 mM) with the modified and unmodified histone H3 peptides shows no change in anisotropy (data not shown). This indicates the change in anisotropy is not due to solution conditions, but is attributable of the formation of BrD-histone peptide complex.

Bromodomains distinguish acetylated from unacetylated lysine side chains primarily by electrostatic effects. For example, the BrD is unable to bind to an unacetylated lysine, due to the strong electrostatic interaction between lysine and DNA. Even in the absence of DNA, as examined here, the charged lysine is not stabilized by the hydrophobic binding pocket of the BrD. However, upon lysine acetylation, the interaction between BrD and acetyllysine is stronger due to thermodynamic stabilization mainly attributed to the hydrophobic effect. Taken together, the electrostatic driving force that stabilizes histone-DNA interaction via lysine-phosphate contacts is lost upon acetylation, permitting the hydrophobic effect to drive the BrD-acetylhistone interaction. The structural and functional consequences of this event have shown that recognition between BrD-acetyllysine is a prerequisite for further transcriptional processes [21,24].

Based on results from these experiments, we were able to show that the single BrD from yeast Gcn5 protein preferentially interacts with acetylated histone H3 peptides than unacetylated H3 peptides. Equilibrium dissociation constant values attained here (KD ~ 1.2 μM) are comparable to those values previously (KD ~ 0.9 μM) attained for Gcn5 using NMR methods (25). The modest difference can be attributed to a variety of factors, including; solution conditions, experimental temperature and the different technique employed. To further our understanding of the molecular level details that drive such acetylation-dependent binding events by BrD proteins, mutagenesis, thermodynamic and kinetic studies will be performed. From a molecular perspective, rigorous examination of BrD-acetylhistone interactions will reveal fundamental information about acetylation-dependent protein interactions and general binding features for bromodomain proteins. From a macromolecular perspective, further studies will reveal important insights into the central role of these proteins in gene regulation and epigenetic phenomena.

ACKNOWLEDGEMENTS

We thank the Thompson lab for helpful discussions and contributions.

REFERENCES

1. Langer, M.R., Tanner, K.G., and Denu, J.M. (2001) Mutational analysis of conserved residues in the GCN5 family of histone acetyltransferases. J. Biol. Chem. 276, 31321-31331.
2. Eberharter, A., Sterner, D.E., Schieltz, D., Hassan, A., Yates, J.R., Berger, S.L., and Workman, J.L. (1999) The ADA complex is a distinct histone acetyltransferase complex in Saccharomyces cerevisiae. Mol. Cell. Biol. 19, 6621-6631.
3. Candau, R., Zhou, J.X., Allis, C.D., and Berger, S.L. (1997) Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo. EMBO J. 16, 555-565.
4. Sterner, D.E., Wang, X., Bloom, M.H., Simon, G.M., and Berger, S.L. (2002) The SANT domain of Ada2 is required for normal acetylation of histones by the yeast SAGA complex. J. Biol. Chem. 277, 8178-8186.
5. Zeng, L., and Zhou, M. (2002) Bromodomain: an acetyl-lysine binding domain. FEBS Lett. 513, 124-128.
6. Roth, S., Denu, J., and Allis, C. (2001) Histone acetyltransferases. Ann. Rev. Biochem. 70, 81-120.
7. Tanner, K., Langer, M., Kim, Y., and Denu, J. (2000) Kinetic mechanism of the histone acetyltransferase GCN5 from yeast. J. Biol. Chem. 275, 22048-22055.
8. Grant, P.A., Duggan, L., Cote, J., Roberts, S.M., Brownell, J.E., Candau, R., Ohba, R., OwenHughes, T., Allis, C.D., Winston, F., Berger, S.L., and

Workman, J.L. (1997) Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: Characterization of an Ada complex and the SAGA (Spt/Ada) complex. Genes Dev. 11, 1640-1650.
9. Wang, L.A., Mizzen, C., Ying, C., Candau, R., Barlev, N., Brownell, J., Allis, C.D., and Berger, S.L. (1997) Histone acetyltransferase activity is conserved between yeast and human GCN5 and is required for complementation of growth and transcriptional activation. Mol. Cell. Biol. 17, 519-527.
10. Jenuwein, T., and Allis, C.D. (2001) Translating the histone code. Science 293, 1074-1080.
11. Filetici, P., Ornaghi, P., and Ballario, P. (2001) The Bromodomain: A chromatin browser. Front. Biosci. 6, 866-876.
12. Horn, P., and Peterson, C. (2001) The Bromodomain: A regulator of ATP-dependent chromatin remodeling? Front. Biosci. 6, 1019-1023.
13. Jeanmougin, F., Wurtz, J., LeDouarin, B., Chambon, P., and Losson, R. (1997) The bromodomain revisited. Trends in Biochemical Science 22, 151-153.
14. Larschan, E., and Winston, F. (2005) The Saccharomyces cerevisiae Srb8-Srb11 complex functions with the SAGA complex during Gal4-activated transcription. Mol. Cell. Biol. 25, 114-125.
15. Sterner, D.E., Belotserkovskaya, R., and Berger, S.L. (2002) SALSA, a variant of yeast SAGA, contains truncated Spt7, which correlates with activated transcription. Proc. Nat. Acad. Sci. USA 99, 11622-11627.
16. Goodwin, G., and Nicolas, R. (2001) The BAH domain, polybromo and the RSC chromatin remodelling complex. Gene 268, 1-7.
17. Carey, M., Li, B., and Workman, J.L. (2006) RSC exploits histone acetylation to abrogate the nucleosomal block to RNA polymerase II elongation. Molecular Cell 24, 481-487.
18. Cairns, B., Lorch, Y., Li, Y., Zhang, M., Lacomis, L., Erdjument-Bromage, H., Tempst, P., Du, J., Laurent, B., and Kornberg, R. (1996) RSC, an Essential, Abundant Chromatin-Remodeling Complex. Cell 87, 1249-1260.
19. Cairns, B., Schlichter, A., Erdjument-Bromage, H., Tempst, P., Kornberg, R., and Winston, F. (1999) Two functionally distinct forms of the RSC nucleosome-remodeling complex, containing essential AT hook, BAH and bromodomains. Molecular Cell 4, 715-723.
20. Chandrasekaran, R., and Thompson, M. (2007) Polybromo-1 bromodomains bind histone H3 at specific acetyl-lysine positions. Biochem. Biophys. Res. Comm. 355, 661-666.
21. Hassig, C., Schreiber, S. (1997) Nuclear histone acetylases and deacetylases and transcriptional regulation: HATs off to HDACs. Current Opin Chem Biol 1, 300-308.
22. Kanno, T., Kanno, Y., Siegel, R., Jang, M., Lenardo, M., and Ozato, K. (2004) Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. Molecular Cell 13, 33-43.
23. Chandrasekaran, R., and Thompson, M. (2006) Expression, purification and characterization of individual bromodomains from human Polybromo-1. Prot. Expr. Purif. 50, 111-117.
24. Haugland, R.P. (1996) Handbook of Fluorescent Probes and Research Chemicals, (Spence, M. T. Z., Ed.) pp 144-156, Molecular Probes, Inc., Eugene, OR.
25. Hudson, B., M. Martinez-Yamout, H. Dyson, and Wright, P. (2000) Solution structure and acetyl-lysine binding activity of the GCN5 bromodomain. J. Mol. Biol. 304, 355-370.