Purification and Functional Analysis of a Novel Leucine-Zipper/Nucleotide-Fold Protein, BZAP45, Stimulating Cell Cycle Regulated Histone H4 Gene Transcription†

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ABSTRACT: Regulation of histone gene transcription at the G1/S phase transition via the Site II cell cycle control element is distinct from E2F-dependent mechanisms operative at the growth factor-related restriction point. E2F-independent activation of histone H4 gene expression combines contributions of several promoter factors, including HiNF-M/IRF2 and the HiNF-D/CDP-cut complex which contains pRB, CDK1, and cyclin A as non-DNA binding subunits. Mutational analyses suggest additional rate-limiting factors for Site II function. Using sequence-specific Site II DNA affinity chromatography, we identified a 45 kDa protein (KIAA0005 or BZAP45) that is embryonically expressed and phylogenetically conserved. Based on amino acid sequence analysis, BZAP45 contains a unique decapeptide that is part of a putative leucine-zipper protein with a nucleotide (ATP or GTP) binding fold. Bacterial expression of a full-length cDNA produces a 45 kDa protein. Binding studies reveal that highly purified BZAP45 does not interact with Site II, suggesting that BZAP45 function may require partner proteins. Forced expression of BZAP45 strongly stimulates H4 promoter (nt −215 to −1)/CAT reporter gene activity. Deletion analyses and point mutations indicate that BZAP45 enhances H4 gene transcription through Site II. Thus, BZAP45 is a novel regulatory factor that contributes to transcriptional control at the G1/S phase transition.

The onset of S phase marks a key cell cycle transition when cells have integrated growth factor dependent signaling mechanisms and are competent to initiate replication of the genome. Because the length of the human haploid genome is approximately 5 orders of magnitude larger than the diameter of the nucleus, newly replicated DNA must be rapidly compacted to dimensions that fit within the constraints of the nucleus. Furthermore, because transcriptional regulation of gene expression requires the recruitment of appropriate chromatin modifying enzymes, correct organization of nucleosomes and higher order chromatin structure of newly synthesized DNA is important for fidelity of gene expression. Therefore, achieving stringent control of histone gene expression and tight functional coupling with DNA synthesis represents one of the most critical regulatory challenges for cells progressing through S phase (*1*−*3*).

The human histone H4 gene promoter has been used extensively as a paradigm to define the key regulatory factors that control transcription and ultimately the stoichiometric biosynthesis of the four classes of histone proteins (H4, H3, H2B, and H2A) that together form nucleosomes [for example, (*4*−*14*)]. Our previous studies indicate that the highly conserved histone H4 genes are transcriptionally regulated by an E2F-independent mechanism (*15*−*17*). In contrast to other genes expressed during the late G1/S phase transition, the histone H4 genes lack E2F responsive regulatory elements. Our laboratory has identified at least three functionally distinct classes of factors that (i) activate histone gene transcription in proliferating cells, (ii) enhance histone mRNA synthesis at the G1/S phase transition, or (iii) suppress histone transcription (*1, 2*). For example, we have shown that the histone H4 gene contains two sites of in vivo genomic protein/DNA interactions, designated Sites I and II (*18*). Site I interacts with a series of regulatory factors (i.e., SP1/HiNF-C, YY1/HiNF-E, HMG-I/HiNF-A, and ATF factors) that together stimulate histone H4 gene transcription (*13, 19*−*21*). Site II has been shown to interact with several DNA binding proteins (i.e., IRF2/HiNF-M, CDP-cut/HiNF-D, and HiNF-P) (*15, 16, 22*−*26*). Point mutational analyses have revealed that each of these proteins contributes to control of histone H4 gene transcription (*27, 28*). Furthermore, co-regulatory proteins that interact with histone gene transcription factors may control expression of histone genes (*29*). Recent evidence suggests that the cdk2-cyclin E dependent protein NPAT is involved in global regulation of histone gene expression (*5, 6*). Apart from histone H4 genes, we note that the majority of DNA replication dependent genes (i.e., H1, H2A, H2B, H3, and H4) are not E2F dependent due to the absence of canonical E2F binding sites, indicating that the regulatory mechanism involving CDK2/cyclin E and NPAT is at least in part E2F-independent.

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It is necessary to account for regulatory mechanisms that are operative at the G1/S phase transition by E2F-dependent and -independent transcriptional control. Our mutational analyses of the histone H4 promoter suggest that additional factors may regulate histone H4 gene transcription. We performed DNA affinity chromatography using conserved histone H4 gene-specific regulatory sequences located in the distal segment of Site II. We purified a novel basic leucine-zipper protein with a putative nucleotide binding fold we designated BZAP45. BZAP45 stimulates Site II-dependent transcription, but does not appear to bind DNA directly.

MATERIALS AND METHODS

Cell Culture. HeLa S3 cells were grown at 37 °C and maintained at (3−6) × 10^5/mL in Joklik modified minimum essential medium (Gibco/BRL) supplemented with 5% fetal calf serum, 2% horse serum, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and L-glutamine (2 mM).

Plasmid Construction and Mutagenesis. The human histone H4 promoter−chloramphenicol acetyltransferase (CAT) gene fusion construct, −215/CAT, was described previously (*4*). Construct PSP/CAT contains a three nucleotide point-mutation in a conserved pentanucleotide within Site II (5′ GGTCC to 5′ tcTaC; mutations in lowercase letters). Construct MPSP contains an additional two nucleotide mutation in the HiNF-M/IRF-2 core binding sequence (5′ TTTCGGTTTT to 5′ TTcaGGTTTT). Constructs PSP/CAT and MPSP/CAT were derived from plasmids H4/CAT and MSP16/CAT (*28*), respectively, by PCR-based site-directed mutagenesis as described (*30*) using the forward primer 5′ CAATCTTCTACGATACTC and the reverse primer 5′ GAGTATCGTAGAAGATTG. Mutations were confirmed by automated DNA sequencing. Other constructs used in this study are described elsewhere (*4*, *19*, *28*). The DHFR/CAT construct was generously provided by Dr. Jane Azizkhan-Clifford (Roswell Park Cancer Institute, Buffalo, NY).

Biochemical Purification of BZAP. Nuclear extract was prepared from a 20 L HeLa S3 culture of exponentially growing cells as described (*23*). The nuclear preparation was made with KN-400 buffer (20 mM HEPES, pH 7.5, 20% glycerol, 400 mM KCl, and 0.01% NP-40) and diluted with 3 volumes of the same buffer without KCl (KN-0). The diluted extract was loaded onto a phosphocellulose column, washed with KN-100 (as KN-400 but with 100 mM KCl), and eluted with KN-200. The eluate was then loaded on a Fast Q-Sepharose column (Pharmacia) and washed with KN-300, KN-400, and KN-500. The KN-400 fraction, which has DNA binding activity, was mixed with 10 μg of salmon sperm DNA, 1 mM DTT, 20 mM $MgCl_2$, 10 mM $ZnCl_2$. The mixture was passed through a nonspecific DNA column and loaded onto a Site II specific oligonucleotide affinity column. The affinity column was washed with KN-100, KN-300, and KN-1000. The KN-300 fraction, which has Site II DNA binding activity, was concentrated on Fast Flow Q, and subjected to 11% SDS−PAGE, followed by copper staining and electroblotting to a nylon membrane. A prominent 45 kDa band was analyzed by Matrix-Assisted-Laser-Desorption-Ionization Time-of-Flight (MALDITOF)/mass spectrometry and peptide microsequencing by the UMass Protein Microsequencing Core Facility. Peptide microsequencing revealed that the 45 kDa protein contains the amino acids QQKPTLSGQR.

Cloning and Overexpression of BZAP45. The peptide sequence QQKPTLSGQR was used to search the GenBank DNA database. We identified a cDNA KIAA0005 (Kazusa B,cDNA-DataBase, GenBank Accession No. D136300). The full-length cDNA contains a 2.9 kb insert and a 1260 bp open reading frame, encoding a protein with a predicted size of 419 amino acids. The complete cDNA was assembled by subcloning two distinct cDNA segments (kindly provided by Dr. Takahiro Nagase, Kasuza DNA Research Institute). The cDNA segment designated HA00071 spans nt 298−2998 and was initially cloned in the *Eco*RI−*Xho*I sites of the pNTT1104 vector which contains a *Bam*HI site immediately upstream of the *Eco*RI site. We transferred the HA00071 fragment into the *Bam*HI−*Xho*I site of pGEX5X1 (Pharmacia) to yield pHA71. The cDNA fragment designated a263 spans nt 1−477 and was initially cloned in the *Sma*I site of M13mp18. We amplified the a263 fragment using the polymerase chain reaction with the high-fidelity Vent DNA plymerase (New England Biolabs, Beverly, MA) with two primers (5′ TCAGGGATCCGTAATAATCAAAAG-CAGCAAAAG and 5′ CTTTCTCCAGGTATTTGTAGC) and cloned the PCR product (approximately 380 bp) into the pCR-blunt vector (Invitrogen, CA). The insert of the resulting clone was released with *Eco*RI, gel-purified, and then digested with *Bam*HI−*Bsm*I. This fragment was cloned into the *Bam*HI−*Bsm*I sites of pHA71 to yield the full-length 2.9 kb cDNA encoding BZAP45 (pGEX5X1/BZAP45). For forced expression of BZAP45 in eukaryotic cells, we cloned the 2.9 kb *Bam*HI−*Xho*I fragment into pCDNA3.1/A (Invitrogen, CA) which contains a cytomegalovirus-derived promoter (pCMV/BZAP45).

To produce a glutathione-*S*-transferase (GST)−BZAP45 fusion protein, we transformed exponentially growing BL21 bacteria with pGEX5X1/BZAP45 and induced recombinant protein with 0.1 mM IPTG for 2 h at 37 °C. Cells were harvested and resuspended in Buffer A (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 1 M NaCl, 15% glycerol, 4 mM PMSF, 2 mM DTT) followed by incubation for 1 h with prewashed glutathione beads. The beads were washed 3 times with 1×PBS. One part of the protein preparation was eluted using glutathione to yield the GST−BZAP45 fusion protein. The remainder was digested with factor Xa overnight at 4 °C and centrifuged at 3000 rpm for 10 min to collect the supernatant containing the released BZAP45 protein. Samples were analyzed by 10% SDS−PAGE and Coomassie blue staining.

In Vitro Transcription and Translation. Plasmid DNA (1.2 μg) was incubated in 25 μL of rabbit reticulocyte lysate, 30 units of RNasin, 1 μL of T7 RNA polymerase (Promega), and 1 μL of 1 mM L-methionine or 15 μCi of [^{35}S]methionine (1000 Ci/mmol; New England Nuclear) in a reaction mixture of 60 μL. After incubation for 2 h at 30 °C, the synthesis was stopped by adding 20 μL of 80% glycerol, and an aliquot was analyzed by10% SDS−PAGE.

Western and Northern Blot Analyses. Proteins from reticulocyte lysate and HeLa whole cell extracts were analyzed by 10% SDS−PAGE and transferred to a nylon membrane. The membrane was blocked by 5% non fat milk for 1 h at room temperature to reduce nonspecific protein binding, and then incubated overnight at 4 °C with primary antibody diluted in 1% non fat milk. The blot was washed 4 times with PBS/Tween-20 (0.05%) and incubated with a

secondary antibody at room temperature for 1.5 h. After washing 6 times with PBS/Tween-20, immunoreactive bands on the blot were visualized by chemiluminescence detection (ECL Kit, Amersham). Northern blot analyses were performed with RNA samples from HeLa and HL60 cells using established procedures (*30*).

Transient Transfection. HeLa cells were plated at a density of $(2-2.5) \times 10^5$ cells per 10 cm plate and incubated overnight at 37 °C. Plasmid DNA (20 μg) was transfected using the calcium phosphate method as described (*31*). Cells were harvested 48 h following transfection and washed 2 times with cold phosphate-buffered saline. To each plate was added 0.5 mL of 1× lysis buffer (Promega), and samples were incubated at room temperature for 10 min. Extracts were collected and centrifuged at 10000*g* for 10 min at 4 °C. Supernatants were analyzed for CAT activity (*32*) and luciferase activity. CAT activity was normalized using luciferase as an internal control and is presented as percent conversion or fold activity. Statistical significance of the values was determined by Student's *t*-test.

RESULTS

To identify histone gene transcription factors that act through Site II, we performed biochemical purification of proteins interacting with Site II. We prepared nuclear extracts from exponentially growing HeLa cells (>5×10^{10} cells) and fractionated these using cationic and anionic resins followed by DNA affinity chromatography with wild-type and mutant Site II oligonucleotides (Figure 1A). Samples from the eluates were separated by SDS−PAGE, and copper staining revealed one prominent 45 kDa protein which was most enriched in the final fraction (Figure 1B). Amino acid sequence analysis of this protein showed the presence of one peptide (QQKPTLSGQR) with complete identity to an open reading frame (ORF) encoded by a cDNA clone designated KIAA0005. The KIAA0005 cDNA was initially characterized by the Kazusa DNA Research Institute as the conceptually assembled product of two separate cDNA segments (*33*). We constructed a composite full-length cDNA with an ORF of 419 amino acids encoding a protein designated BZAP45 with a predicted molecular mass of 45 kDa. BZAP45 contains a basic N-terminus fused to a leucine-zipper domain and a C-terminal region displaying strong identity with a nucleotide (ATP or GTP) binding fold (Figure 1C). Consistent with conceptual translation, bacterial expression of the BZAP45 cDNA fused to glutathione-*S*-transferase (GST) coding sequences yields a 71 kDa GST fusion protein (Figure 2A). The GST fusion protein is converted into the predicted 45 kDa protein upon cleavage by factor Xa (Figure 2B). A protein of the same size is observed following coupled in vitro transcription and translation (Figure 2C) or upon expression in mammalian cells (Figure 2D).

BZAP45 mRNA is endogenously expressed in a broad spectrum of cell types, tissues, and tumor cells and is detected in day 3 embryos based on EST analysis. BZAP45 expression in tumor cells and during early embryogenesis suggests that it may be coupled to cell growth, S phase progression, and/or histone H4 gene expression. Therefore, we used TPA-induced differentiation of HL60 cells to assess the coupling between cell proliferation, histone gene expression, and the levels of BZAP45 mRNA. Our previous studies have shown

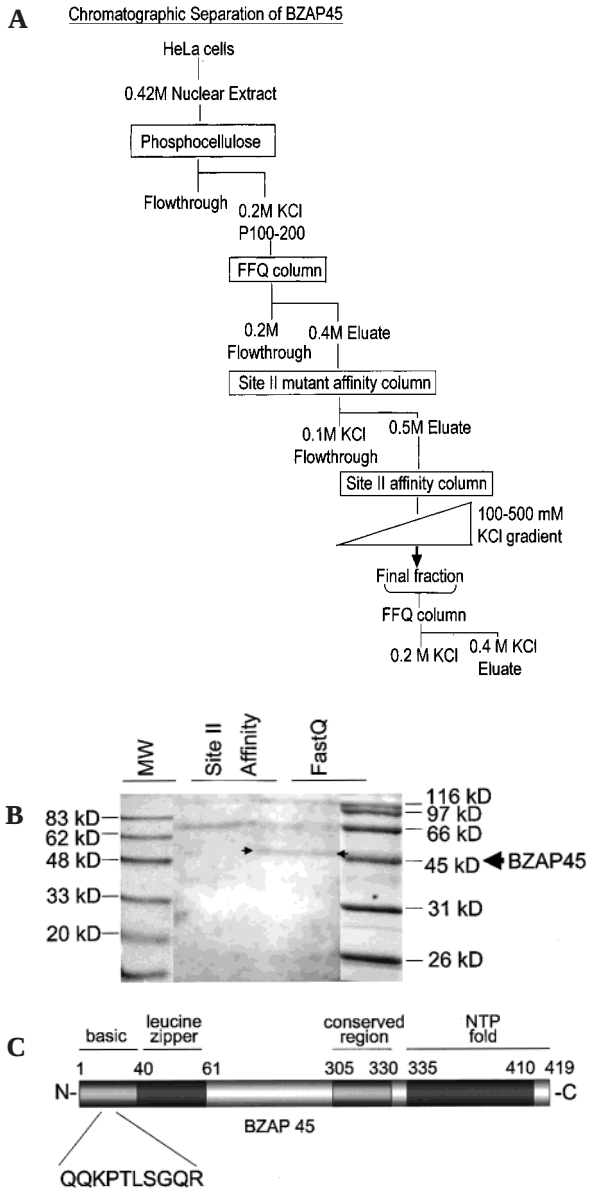

FIGURE 1: (Panel A) Biochemical purification of BZAP45. Diagram of the chromatographic separation procedure used in our studies. Nuclear extracts in 0.42 M KCl from HeLa S3 cells were adjusted to 0.1 M KCl and applied to a phosphocellulose column. Fractions containing Site II binding activity were eluted with 0.2 M KCl and bound to a Fast Flow Q (Pharmacia) column. The 0.4 M eluate of this column was diluted and applied to a mutant Site II DNA affinity column that retains nonspecific DNA binding proteins, and the flow-through was collected. This flow-through sample was further purified using a specific Site II DNA affinity column, and the final fractions were eluted using a salt gradient from 100 to 500 mM KCl. Fractions containing Site II binding activity were pooled and concentrated by passage through a Fast Flow Q column. (Panel B) Fractions from the Site II DNA affinity column and the Fast Flow Q column were analyzed by 10% SDS−PAGE. Protein bands were visualized by copper staining of the gel and photographed against a dark background. The major factor in the final fraction is a 45 kDa protein (designated BZAP45) indicated by the arrowhead. (Panel C) Schematic representation of the BZAP45 protein structure based on conceptual translation of the BZAP45 cDNA. BZAP45 contains several conserved domains (different shades of gray) including an N-terminal basic leucine-zipper domain and a C-terminal nucleotide ATP or GTP fold. The peptide identified by MALDI-TOF analysis and amino acid sequencing is shown below the diagram.

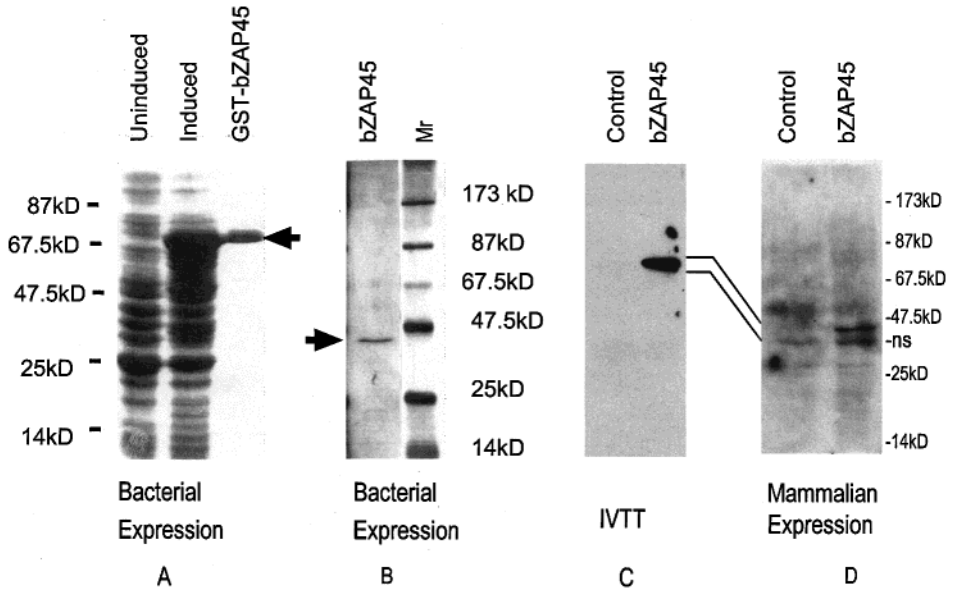

FIGURE 2: Analysis of recombinant BZAP45 protein synthesized in bacteria (panels A and B), by coupled in vitro transcription/translation (IVTT, panel C), or in mammalian cells (panel D) using prokaryotic and eukaryotic expression vectors containing the BZAP45 cDNA. (Panel A) Control lysates (uninduced) or lysates from bacteria induced to produce a GST/ BZAP45 fusion protein were analyzed by 10% SDS−PAGE followed by Coomassie Blue staining. The fusion protein (approximately 71 kDa) was purified by glutathione-affinity chromatography (right lane) and eluted with buffer containing glutathione. (Panel B) Glutathione-bound fusion proteins similar to those described in panel A were subject to enzymatic digestion with factor Xa protease. The cleaved proteins were analyzed by 10% SDS−PAGE and Coomassie blue staining. The cleavage occurs at the junction of the GST fusion protein and releases the recombinant 45 kDa BZAP45 protein into the eluate. (Panel C) Western blot analysis of a control reticulocyte lysate (left lane) or a lysate programmed with a vector containing BZAP45 protein coding sequences fused to an N-terminal epitope tag (Xpress). Recombinant protein was visualized using an Xpress antibody (Invitrogen). (Panel D) Western blot analysis of Xpress-tagged BZAP45 protein expressed in HeLa S3 cells transfected with the empty CMV-driven expression vector (left lane) or a vector containing the BZAP45 cDNA (right lane).

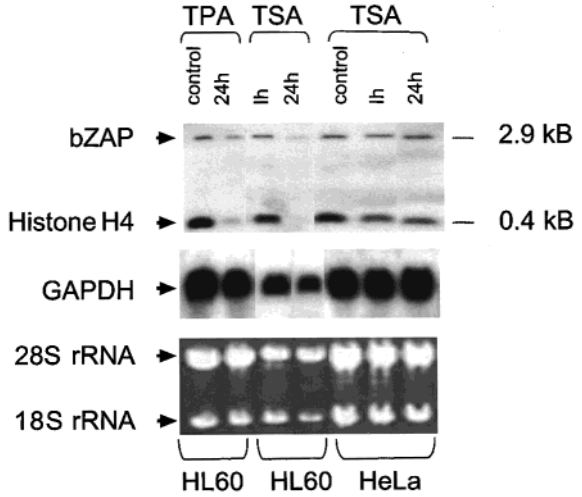

FIGURE 3: BZAP45 expression and histone H4 gene expression are independently regulated. BZAP45 and histone H4 mRNA levels were monitored during TPA (tumor-promoting agent, phorbol 12-myristate 13-acetate) induced myeloid differentiation of HL60 cells, as well as following Trichostatin (TSA) treatment of both HL60 and HeLa cells. Northern blot analysis was performed with gene-specific probes (upper panel), and blots were rehybridized with a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalization (middle panel). Ethidium bromide staining of the gels was used to assess the integrity of RNA samples and to account for differences in RNA quantitation (lower panel).

that histone H4 gene expression is down-regulated following TPA-induced HL60 differentiation (*34*). Northern blot analysis with mRNA samples isolated from proliferating and TPA-treated HL60 cells (Figure 3) shows that histone H4 gene expression is down-regulated during HL60 differentiation (Figure 3), while BZAP45 mRNA levels are not significantly affected (Figure 3). Similarly, histone H4 mRNA levels are down-regulated in HL60 cells following treatment with the histone deacetylase inhibitor Trichostatin A (TSA), and while there appears to be a concomitant decrease in the levels of BZAP45 mRNA, BZAP45 mRNA is still detected at low levels in TSA-treated cells. Treatment of HeLa cells with TSA did not affect BZAP45 mRNA levels. Thus, BZAP45 is expressed in both proliferating and nonproliferating cells.

We directly assessed the functional involvement of BZAP45 in regulating H4 gene transcription by analyzing the effect of cotransfecting cells with CAT reporter gene constructs and a CMV-driven BZAP45 expression vector. Expression of BZAP45 enhances H4 promoter activity 3−4-fold based on results of the wild-type −215/CAT construct (Figure 4A). For comparison, BZAP45 does not increase the promoter activity of the DHFR gene, which is another gene up-regulated at the G1/S phase transition (Figure 4A). We conclude that BZAP45 is a gene-selective activator of H4 gene transcription that discriminates between two S phase regulated genes.

To address whether BZAP45 regulates H4 gene transcription through specific elements in the H4 promoter, we examined a series of H4 promoter deletion mutants fused to the CAT reporter gene (Figure 4B). Deletion of H4 promoter Site I (−97/CAT), which interacts with ATF-1, SP-1, YY1, and HiNF-A (*1*), reduces the basal transcription as expected by approximately 3-fold, but this deletion does not influence the responsiveness to BZAP45 expression. However, upon deletion of the distal segment of Site II (−46/CAT) which removes binding sites for all known Site II interacting proteins (i.e., IRF-2/HiNF-M, CDP-cut/HiNF-D, and HiNF-P), transcriptional enhancement of H4 promoter activity by

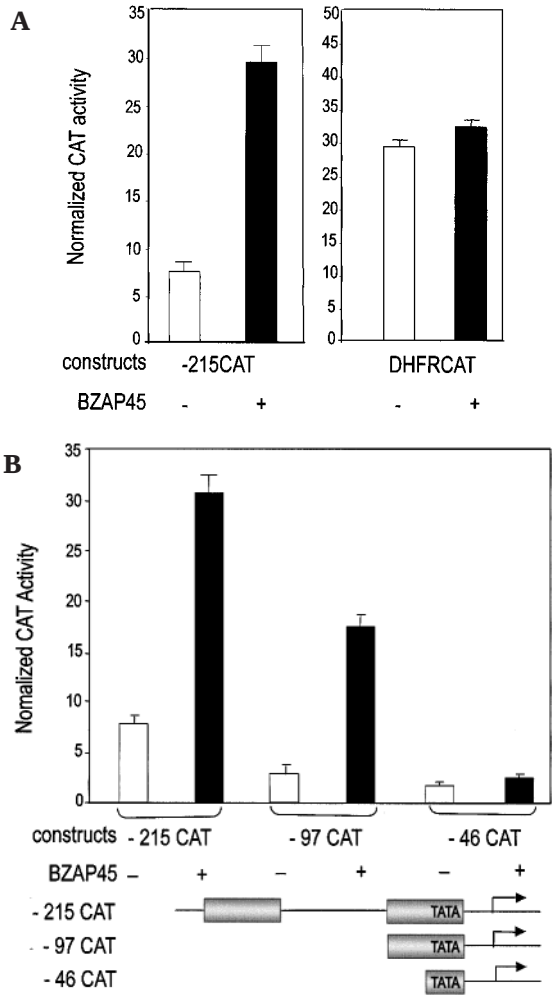

FIGURE 4: Selective activation of histone H4 gene promoter activity by BZAP45 through Site II. (Panel A) HeLa cells were transiently transfected with CAT-reporter gene constructs containing the histone H4 or DHFR promoter in the presence of an empty expression vector (−) or a construct expressing BZAP45 (+). The graph shows the relative CAT activity for each promoter/reporter gene construct in the absence or presence of BZAP45. Values were normalized for the activity of a promoterless Renilla luciferase construct to account for variations in transfection efficiency. (Panel B) HeLa cells were transiently transfected with histone H4 gene/CAT reporter gene constructs with different H4 promoter deletions (−215/CAT, −97/CAT and −46/CAT) and either empty vector (−) or an expression vector driving synthesis of BZAP45 (+). The graph shows the relative CAT activity for each H4 promoter construct in the absence or presence of BZAP45 and was normalized for activity obtained with a promoterless Renilla luciferase construct.

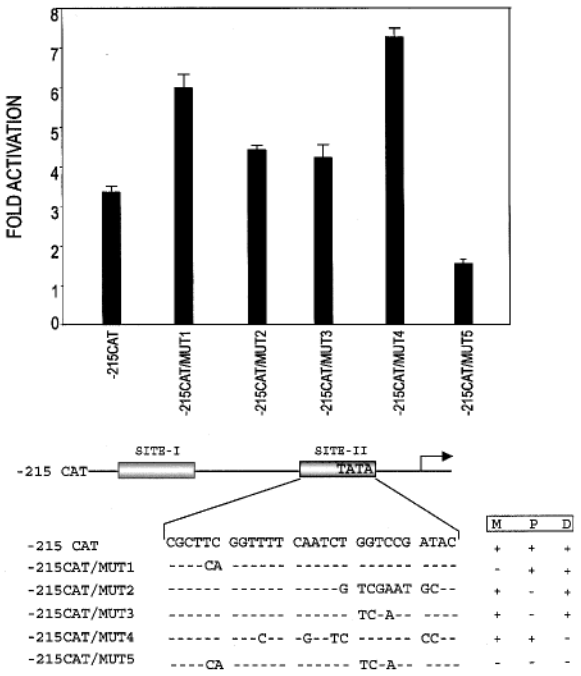

FIGURE 5: Point mutational analysis of BZAP45 function on distal Site II sequences. Transcriptional enhancement of histone H4 gene promoter activity by BZAP45 was assessed with promoters containing mutations in the recognition motifs for the Site II binding proteins HiNF-M/IRF2 (Mut1), HiNF-P (Mut2 and Mut3), and HiNF-D/CDP-cut (Mut4). The Mut5 construct contains mutations that abolish binding of all three factors. HeLa cells were transiently transfected with this panel of histone H4 promoter/CAT reporter gene and either empty vector or an expression vector driving synthesis of BZAP45. The graph shows the fold activation for each H4 promoter construct in the presence or absence of BZAP45. Values were corrected for differences in transfection efficiency using data obtained with a promoterless Renilla luciferase construct.

BZAP45 is no longer observed. Thus, BZAP45 enhances H4 gene transcription through the distal segment of Site II, the conserved gene regulatory element used for DNA affinity purification.

To understand the mechanism by which BZAP45 influences H4 gene transcription, we performed DNA binding assays with purified recombinant BZAP45 proteins (see Figure 2). Using a variety of DNA binding conditions which permit detection of other Site II binding proteins, we have been unable to demonstrate electrophoretically stable protein−DNA complexes in gel shift assays (data not shown). These results suggest that BZAP45 does not bind Site II directly and may enhance transcription through other Site II factors. We investigated whether BZAP45 may function through HiNF-M, HiNF-D, or HiNF-P by performing transient transfection assays with H4 promoter/CAT constructs containing factor-specific point mutations that selectively abrogate binding of HiNF-M, HiNF-D, or HiNF-P. The results show that abolishing the interaction of any one of these three proteins with Site II does not affect BZAP45 enhancement of H4 promoter activity (Figure 5). Strikingly, when all three known HiNF binding sites are mutated, BZAP45 is no longer capable of enhancing H4 gene transcription. Hence, Site II binding proteins appear to be functionally redundant in mediating the stimulatory effect of BZAP45 on H4 gene transcription.

DISCUSSION

In this study, we have purified to homogeneity a novel regulatory factor, BZAP45, a 45 kDa protein containing an N-terminal basic leucine-zipper domain and a C-terminal nucleotide (ATP or GTP) binding fold. We demonstrate that BZAP45 strongly activates histone H4 gene transcription through sequences required for cell cycle dependent regulation (Site II), but the protein does not appear to contact Site II directly. We propose that BZAP45 may regulate histone H4 gene transcription through its interactions with other Site II proteins (e.g., HiNF-M, HiNF-P, and/or HiNF-D), but formal demonstration of this proposal requires additional experimentation. The data presented here suggest that BZAP45 is an important coregulator of a subset of genes that are controlled at the G1/S phase transition during the cell cycle.

The N-terminal basic leucine-zipper domain of BZAP45 classifies this factor as a member of the superfamily of bZIP transcription factors, which includes the FOS/JUN (AP-1), ATF/CREB, C/EBP, and MAF proteins (*35*−*47*). One distinguishing feature of BZAP45 is the N-terminal location of the bZIP domain, because these domains are usually located in the C-terminal half of bZIP transcription factors. We postulate that the bZIP domain may function as a protein/protein interaction domain with other regulatory factors.

The second distinguishing feature of BZAP45 is its C-terminal nucleotide binding fold. This region exhibits a high degree of similarity with domains present in eukaryotic translation initiation factors (eIFs) that are highly conserved between yeast and human (*48*−*50*). The nucleotide binding fold of BZAP45 is most similar to that of eIF-5 and the Epsilon subunit of eIF-2B (*48*−*52*) which function as GDP−GTP exchange factors. The presence of the bZIP domain and nucleotide binding fold suggests that BZAP45 is a natural chimera of domains derived from two distinct classes of proteins.

The functional significance of the nucleotide binding fold in BZAP45 remains to be established. However, it is of interest to note that many processes involving transcription factors require energy input through nucleoside triphosphate hydrolysis, including SWI/SNF-dependent chromatin remodeling and covalent modifications of nucleosomal proteins (i.e., phosphorylation, acetylation, and ubiquitination) (*53*, *54*). Furthermore, nuclear import and correct protein folding of transcription factors are energy-dependent processes (*55*−*58*). Thus, the nucleotide binding fold of BZAP45 may represent a molecular adaptation to transport or hydrolyze nucleoside triphosphates to support a function in transcriptional activation.

The most striking result of our study is that BZAP45 activates histone H4 gene transcription but that the protein functions independent of DNA binding. Interestingly, the histone gene regulator NPAT (*5*, *6*) also does not bind directly to DNA. Histone H4 gene transcription is regulated by multiple factors via E2F-independent mechanisms. Deletion analyses of the histone H4 gene promoter revealed that the cell cycle regulatory domain (Site II) is required and sufficient for BZAP45-dependent transactivation. However, point-mutations affecting the interactions of each of the three known Site II binding proteins (i.e., HiNF-M/IRF2, HiNF-D/CDP-cut, or HiNF-P) (*1*, *2*) separately do not abrogate activation by BZAP45. Thus, these three Site II proteins may functionally compensate for each other to mediate the coregulatory properties of BZAP45. For example, BZAP45 may influence basal components of the transcriptional initiation machinery that bind to the TATA-box contained within Site II, and occupancy of the TATA-box may be supported by any of the three sequence-specific Site II binding proteins. Interestingly, our data show that the activity of the dihydrofolate reductase (DHFR) promoter is not enhanced by BZAP45, and this gene is regulated in part by E2F-dependent mechanisms and lacks a TATA-box (*59*). These molecular distinctions may represent the basis for differential gene regulation by BZAP45.

In summary, we have biochemically purified and functionally characterized a novel coregulatory factor of H4 gene transcription. Additional studies aimed at understanding the functional mechanisms by which BZAP45 selectively upregulates the histone H4 gene and does not affect transcription of other cell cycle related genes (i.e., DHFR) will provide further insight into gene regulatory mechanisms operative during the onset of S phase.

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