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Recruitment of Nuclear Factor Y to the Inverted CCAAT Element (ICE) by c-Jun and E1A Stimulates Basal Transcription of the Bone Sialoprotein Gene in Osteosarcoma Cells*

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Bone sialoprotein (BSP), a major protein in the extracellular matrix of bone, is expressed almost exclusively by bone cells and by cancer cells that have a propensity to metastasize to bone. Previous studies have shown that v-*src* stimulates basal transcription of *bsp* in osteosarcoma (ROS 17/2.8) cells by targeting the inverted CCAAT element (ICE) in the proximal promoter. To identify possible downstream effectors of Src we studied the effects of the proto-oncogene c-*jun*, which functions downstream of Src, on basal transcription of *bsp* using transient transfection assays. Increased expression of endogenous c-Jun induced by the tumor promoter 12-*O*-tetradecanoyl-phorbol 13-acetate and ectopic expression of c-Jun increased basal transcription of chimeric reporter constructs encompassing the proximal promoter by 1.5–3-fold in ROS 17/2.8 osteosarcoma cells, with more modest effects in a normal bone cell line, RBMC-D8. The effects of c-Jun were abrogated by mutations in the ICE box and by co-expression of dominant negative nuclear factor Y, subunit A (NF-YA). The increase in *bsp* transcription did not require phosphorylation of c-Jun and was not altered by trichostatin treatment or by ectopic expression of p300/CREB-binding protein (CBP) or mutated forms lacking histone acetyltransferase (HAT) activity. Similarly, ectopic expression of p300/CBP-associated factor (P/CAF), which transduces p300/CBP effects, or of HAT-defective P/CAF did not influence the c-*jun* effects. Surprisingly, E1A, which competes with P/CAF binding to p300/CBP, also stimulated BSP transcription through NF-Y independently of c-*jun*, p300/CBP, and P/CAF. Collectively, these studies show that c-Jun and E1A regulate basal transcription of *bsp* in osteosarcoma cells by recruiting the NF-Y transcriptional complex to the ICE box in a mechanism that is independent of p300/CBP and P/CAF HAT activities.

Bone sialoprotein (BSP),[2] a prominent protein in the matrix of bone, is thought to regulate the formation and growth of mineral crystals (1). Although the expression of BSP is essentially restricted to mineralizing connective tissues, it is also expressed in various pathologies in which ectopic mineralization occurs. The involvement of BSP in physiological and pathological mineralization can be attributed to its ability to bind to collagen (2) and to nucleate hydroxyapatite formation through polyglutamate motifs (3). However, BSP is also expressed by cancer cells that have a propensity to metastasize to bone (4, 5). Although the mineral binding properties of BSP could also be involved in the formation of bone metastases, BSP has been shown to promote angiogenesis (6) and to confer protection against complement-mediated cell lysis through an RGD motif and a strong affinity for complement factor H (7). Recent studies have also reported that BSP can increase the invasive potential of metastatic cells by activating proMMP-2 (8).

Studies on the transcriptional regulation of *bsp* have identified a highly conserved proximal promoter region in which an inverted CCAAT element (ICE) and TATAA box are separated by 21 nucleotides (9). In addition to its critical role in basal transcription, the ICE is a target of *src* regulation through nuclear factor Y (NF-Y) (10). NF-Y comprises three subunits (A, B, and C), each having the DNA-binding domain required for CCAAT binding and transactivation. Subunits B and C form a stable dimer through interaction between complementary histone fold motifs. The dimer offers a complex surface for NF-YA association with co-activators. Subunits A and C also contain conserved Q-rich domains that have a transcriptional activation function (11–13). Recent studies of NF-Y-mediated transcription have shown that NF-YB binds p300/CBP. Because p300/CBP also binds P/CAF, which interacts with TFIID, a complex of these proteins has been suggested as transactivating NF-Y-mediated transcription in the proximal promoter of the human ferritin H gene (14).

The proto-oncogene c-*jun*, a transcription factor that functions downstream of Src, can regulate gene transcription by DNA-dependent and DNA-independent mechanisms. c-Jun is a basic region leucine zipper (b-ZIP) DNA-binding protein that heterodimerizes with Fos or other Jun family proteins to form activator protein 1 (AP-1), a transcription factor complex (15) that is a prototypical nuclear effector of the JNK signal transduction pathway. The AP-1 complex regulates gene transcription of specific target genes in a variety of cellular processes including proliferation, stress response, and tumorigenicity by binding to a specific nucleotide sequence known as the AP-1 element. However, c-*jun* can also regulate transcription by interactions with other transcription factors including Sp1 (16), PU.1 (17), and p300 (14) and by binding to the TATA-binding protein-associated factor TAF-7 (18).

In previous studies we have shown that 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-stimulated c-*jun* and c-*fos* expression in ROS 17/2.8

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[2] The abbreviations used are: BSP, bone sialoprotein; AP, activator protein; CBP, CREB-binding protein; C/EBP, CCAAT/enhancer-binding protein; ChIP, chromatin immunoprecipitation; CREB, cAMP-response element-binding protein; DN, dominant negative; HAT, histone acetyltransferase; HSP, heat shock protein; ICE, inverted CCAAT element; JNK, c-Jun NH_2-terminal kinase; JNKK, JNK kinase; Luc, luciferase; P/CAF, p300/CBP-associated factor; NF-Y, nuclear factor Y; PIC, pre-initiation complex; RICE, reverted ICE; rvt, reverted; siRNA, small interference RNA; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate; TSA, trichostatin A; wt ,wild-type.

cells suppresses transcription of long *bsp* reporter constructs in which the activity was located to an AP-1 site encompassed by a glucocorticoid response element (19). However, suppressed transcription was not observed in short constructs. Because c-*jun* functions downstream of c-*src*, we studied the effects of c-*jun* on basal transcription of *bsp* and show that c-*jun* stimulates BSP transcription by recruitment of NF-Y to the ICE box independent of DNA binding. In contrast to other studies (14, 20–22), neither the c-*jun* activity nor that of E1A, which also stimulates basal activity of *bsp* through NF-Y, requires the histone acetyltransferase (HAT) activity of the co-activators p300/CBP or P/CAF for transactivation.

MATERIALS AND METHODS

Cell Culture—Two bone cell lines, rat osteosarcoma cells (ROS 17/2.8) and rat stromal bone marrow cells (RBMC-D8), were used in this study. Both ROS 17/2.8 cells (provided by Dr. Gideon Rodan, Merck-Frost, Philadelphia, PA) and the clonal osteogenic rat bone marrow cell line (RBMC-D8) generated from spontaneously established rat bone marrow cells (provided by Dr. Sandu Pitaru, Tel Aviv University, Tel Aviv, Israel) were grown in α-minimal essential medium containing 10% fetal bovine serum and antibiotics (100 μg/ml penicillin G, 50 μg/ml gentamicin sulfate, and 300 μg/ml Fungizone).

Construction of Rat bsp Promoter Constructs—Constructs of different lengths of rat *bsp* promoter −116/+60, −84/+60, and −60/+60, were cloned as described previously (10). Briefly, rat *bsp* promoter inserts −116/+60, −84/+60, and −60/+60 were blunt end-ligated into the SmaI site of pGL3-Basic (Promega Life Science, Madison, WI). Constructs of single point-mutated CCAAT box (ptTTGG) and reverted (rvt) CCAAT box (prvtCCAAT) were cloned based on −60/+60 using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer sets used for cloning ptTTGG were 5′-GTGACCGTGTTTGGCTGCTGAG-3′ and 5′-GGCCGGTGCGACGTCAGGCTGT-3′, and those used for cloning prvtCCAAT were 5′-CCGGCCGTGACCGTGGCCAATTGCTGAGAGGAGAAGAAGGG-3′ and 5′-CCCTTCTTCTCCTCTCAGCAATTGGCCACGGTCACGGCCGG-3′ (the sequences of mutated and reverted CCAAT boxes are underlined). All constructs were confirmed by sequencing.

Cloning of Expression Vectors—The expression vectors for dominant negative analogs of NF-YA, NF-ΔYA13 m29 (23), and the wild-type NF-YA plasmid NF-YA 13 (24) were prepared as described previously. YA DN is a DNA-binding domain mutant that acts as the dominant repressor of NF-Y-DNA complex formation and NF-Y-dependent transcription (23). The c-Jun expression vector (human c-Jun cloned into pcDNA3.1+ at HindIII/XhoI) was kindly provided by Dr. S. Lye (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). The CBP expression vector pRc/Rsv mCBP-HA and the dominant negative version, pRc/Rsv mCBP-HA F(1541)A (25), were generously provided by Dr. T. Kouzarides (The Wellcome Trust/Cancer Research UK Institute, Cambridge, UK) with permission from Dr. R. H. Goodman (Vollum Institute, Oregon Health and Sciences University, Portland, OR). Expression vectors for p300, pCI p300-FLAG, P/CAF, pCI FLAG-P/CAF, and histone acetylase transferase domain-deleted P/CAF (pCI FLAG-P/CAFΔ579–608) (26), were generous gifts from Dr. Y. Nakatani (Dept. of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Fusion protein JNKK2-JNK1 (27), which alone is sufficient to stimulate c-Jun transcriptional activity, and JNKK2(K149M)-JNK1, in which lysine 149 is replaced by methionine and thus lacks the ability to phosphorylate c-Jun (27), were provided by Dr. A. Lin (Department of Pathology, University of Alabama, Birmingham, AL). Jun Ala, a c-Jun expression vector in which the serines and threonines targeted for phosphorylation were mutated to alanines (28), was kindly provided by Dr. D. Bohmann (Department of Biomedical Genetics, University of Rochester, Rochester, NY).

The E1A expression vector pCMX-Flag Ad5 13S was generously provided by Dr. J. S. Mymryk (London Regional Cancer Centre, University of Western Ontario, London, Ontario, Canada). Expression vector pCMV5-E1A was a gift from Dr. L. Penn (Dept. of Medical Biophysics, University of Toronto). The first Met was deleted in pCMV5-E1A; thus, the translation practically starts from amino acid 15, which is the first Met in the sequence. PCMV5-E1A is thus termed as E1A(−15) in this study. $E1A^+$ was constructed by PCR using pCMV5-E1A as template. The primers used were 5′-CGGGATCCGAAAATGAGACATATTATCTGCCACGGA-3′ (forward) and 5′-GGAATTCCTTGGCCTGGGGCGTTTACAGCTCAAGTC-3′ (reverse). The PCR product was digested with BamHI and EcoRI and then phosphorylated and inserted in-frame into pCMV5Tag4B at the BamHI/EcoRI site. $E1A^+$(−25), in which amino acids 4–25 were deleted, was cloned in the same way except that the forward primer used was 5′-CGGGATCCGAAAATGAGACATGAGGTACTGGCTGATAAT-3′. pE1A cDNA, in which the 116-nucleotide intron sequence between amino acids 185 and 186 in pCMV5-E1A is deleted, was cloned by the following steps. First, $E1A^+$ was digested with PpuMI. Then the 5-kb product was dephosphorylated and ligated with the phosphorylated double-stranded oligonucleotides 5′-GACCCAGATATTATGTGTTCGCTTTGCTATATGAGGACCTGTGGCATGTTTGTCTACA-3′ and 5′-GACTGTAGACAAACATGCCACAGGTCCTCATATAGCAAAGCGAACACATAATATCTGG-3′ (both from Qiagen Inc., Mississauga, Ontario, Canada). pE1A cDNA(−25) was constructed in the same way, except that the starting plasmid was $E1A^+$(−25).

Electrophoretic Mobility Shift Assay—Nuclear extracts used for electrophoretic mobility shift assays were prepared from ROS 17/2.8 cells as described (10). Protein concentrations of the nuclear extract were measured using the Bio-Rad assay. The double-stranded oligonucleotides used for electrophoretic mobility shift assay were synthesized by Invitrogen. The sequences of the probes (only the sense strand is shown) are: s-CCAAT (ICE), 5′-CCGTGACCGTGATTGGCTGCTGAG-3′; rvt-CCAAT (RICE), 5′-CCGTGACCGTGCCAATCTGCTGAGAG-3′; rvt-CCAAT+f2, 5′-CCGTGACCGAGCCAATCAGCTGAGAG-3′; rvt-CCAAT+f7, 5′-CCGTTCAGCAGCCAATCACGGTCGAG-3′; and rvt-CCAAT+f10, 5′-CCTCTCAGCAGCCAATC-ACGGTCACG-3′, where f2, f7, and f10 refer to 2, 7, and 10 flanking nucleotides, respectively. The double-stranded oligos were end-labeled with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Invitrogen). 2.5 μg of nuclear extracts were incubated with 50,000 cpm of labeled oligos at room temperature (21 °C). NF-Y binding was confirmed using antibodies to the NF-YA and NF-YB subunits (24). Nuclear extracts were incubated with or without antibody at room temperature for 10 min, and then the probe was added and incubated for an additional 20 min. The ability of the different reverted constructs to bind NF-Y was determined using radiolabeled probes and to inhibit NF-Y binding to the radiolabeled s-CCAAT probe by competition assays in which a 50-fold excess of the unlabeled probes was used.

Northern Hybridizations—Rat osteosarcoma cells (ROS 17/2.8) were grown to confluence in 60-mm culture dishes. The cells were treated with either 28 μg/ml cycloheximide or a combination of cycloheximide and 100 ng/ml TPA. Using the RNeasy mini kit (Qiagen), total RNA was extracted from triplicate dishes for each treatment at 0, 1, 3, 6, 9, 12, and 24 h following the addition of the reagents. Following purification, 20 μg of total RNA from each time point was used for Northern hybridization analysis as described (29). Hybridizations were carried out at 42 °C with

^{32}P-labeled probes to rat *fos* and human *jun* cDNA probes (generously provided by T. Curran of St Jude Children's Research Hospital, Memphis, TN and M. Breitman of Mount Sinai Hospital, Toronto, Ontario, Canada, respectively). Following hybridization, membranes were washed four times for 5 min each wash at 22 °C in 2× SSC (30 mM sodium citrate and 0.3 M NaCl, pH 7.0) containing 0.1% SDS. This was followed by two 20-min washes at 55 °C in 0.1× SSC and 0.1% SDS. The hybridized bands were photographed from radioautographs prepared by exposure on Kodak X-Omat film at −70 °C and quantitated by Quantify One (Bio-Rad).

Transcription Assays—ROS 17/2.8 and RBMC-D8 cells were plated on 24-well cell culture plates (0.4×10^5/well) 24 h prior to transfection. Cells (60% confluent) were then transfected using Lipofectamine 2000 (Invitrogen). A total of 1–1.5 μg DNA was used for transfection, normally 0.4 μg of *bsp* promoter construct (−116 *bsp*Luc, −84 *bsp*Luc, −60 *bsp*Luc, tTTGGC, or rvtCCAAT), 0.4 μg of expression vector (for c-Jun, P/CAF, JNK kinase, or E1A, etc.) and, as indicated, 0.4 μg of YA DN or YA wild-type (wt) expression vector. A *Renilla* luciferase expression vector, phRL-SV40 (Promega), was used as an internal control (one one-thousandth of total DNA). Some cells were treated with TPA or trichostatin (TSA) (using ethanol and Me_2SO as vehicle controls, respectively) 24 h after transfection. Cells were harvested either 24 h after transfection or 24 h after the addition of TPA or TSA. Cell lysates were prepared with the Dual Luciferase Reporter Assay System (Promega), and luciferase was analyzed by Berthold Lumat LB-9501.

siRNA—p300/CBP siRNAs were generated by cloning two pairs of siRNA oligos for p300 and two pairs of siRNA oligos for CBP (30), respectively, into pSilencer 3.1 H1-Hygro (Ambion, Austin, TX). A mixture of equal amounts (100 ng per siRNA per well) of the four siRNAs was used in transient transfections. The cells were harvested 24 h after transfection of the siRNAs, and the luciferase activity was analyzed.

Chromatin Immunoprecipitation (ChIP)—The ChIP procedure used has been described previously (31). Briefly, HeLa cells were transfected with −60 *bsp*Luc construct plus control vector or E1A-expression vector, pCMX-FLAG Ad5 13S. The cells were incubated for 24 h and fixed in 1% (v/v) formaldehyde. The chromatin was extracted, sonicated, and immunoprecipitated using antibodies that recognize NF-YB (24), FLAG tag (M2, Sigma-Aldrich), and c-Jun (H-79, Santa Cruz Biotechnology, Santa Cruz, CA). The chromatin immunoprecipitate was PCR-amplified using promoter-specific primers, resolved by agarose gel electrophoresis, and then visualized by UV fluorescence. The ChIP PCR primers 5′-GTGACCGTGATTGGCTGCTGAG-3′ (*bsp*; forward) and 5′-TTGCTCTCCAGCGGTTCCAT-3′ (Luc; reverse) generated a DNA fragment of 254 bp.

Statistical Analysis—Transcription assay results depicted in Figs. 1–3, 5–8, 10, and 11 are from one set of experiments in triplicate that were repeated at least twice. The significant effects of individual expression vectors are analyzed by paired Student's t test. The significance is indicated by asterisks where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

RESULTS

Basal Transcription of bsp Is Stimulated by c-jun—To determine whether an increase in endogenous c-*jun* expression would affect transcription of BSP, ROS 17/2.8 cells were stimulated with TPA. Following the addition of TPA, a rapid transient increase in c-*jun* and c-*fos* mRNAs were observed by Northern hybridization analysis in the presence of cycloheximide, which slows the rapid turnover of these mRNAs (Fig. 1*A*). In transient transfection assays, using short promoter constructs encompassing the basal promoter region of the *bsp* gene, treatment with TPA increased transcription 1.4–1.9-fold in ROS 17/2.8 cells with more modest effects observed in RBMC-D8, a normal bone cell line (Fig. 1*B*). Because the same increase was observed in the shortest construct, −60 *bsp*Luc, which encompasses only the ICE and TATA boxes, the effects of the TPA-stimulated expression appeared to be directed at the basal promoter.

To identify c-*jun* as the transcription factor directing the effects of the TPA, cells were co-transfected with a c-*jun* expression vector, and transcription was measured in transient transfection assays. Transcription by all three *bsp* promoter constructs, −116 *bsp*Luc, −84 *bsp*Luc, and −60 *bsp*Luc, was increased 1.7–2.8-fold in ROS 17/2.8 cells, with smaller changes observed in the RBMC-D8 cells (Fig. 2). These increases were similar to those observed with TPA and, as observed for TPA, the effects of c-*jun* appeared to target the basal promoter in both cell types. As the ICE and TATA boxes are the only recognizable elements in the basal promoter, the effects of c-*jun* appeared to be DNA binding-independent.

c-jun Targets the ICE Box—To demonstrate that c-Jun was targeting the ICE box, transcription analyses were performed in ROS 17/2.8 and RBMC-D8 cells using the −60 *bsp*Luc construct in which either a point mutation had been made in the ICE sequence (ATTGGC → tTTGGC) or the ICE was reverted (RICE) to a "CCAAT" (rvtCCAAT) sequence (Fig. 3). In both cases the constitutive and c-Jun-stimulated transcription was reduced significantly in both ROS 17/2.8 and RBMC-D8 cells. However, some stimulation by c-Jun was still evident, especially in the RICE construct.

c-jun Effects Are Mediated by NF-Y—Gel mobility shift assays were used to confirm that NF-Y is the major transcription factor binding to the ICE box (Fig. 4). A strong band containing the ICE sequence was super-shifted by specific antibodies to the NF-YB and NF-YA. Notably, the NF-Y binding was lost when the ICE sequence was reverted (RICE), and a new slower migrating band was observed. However, this unidentified band disappeared with the addition of reverted flanking sequences to the RICE, and NF-Y binding was progressively regained as an additional flanking sequence was reverted (Fig. 4). The transcriptional activity was also progressively regained with the flanking sequence, although with 10 flanking nucleotides reverted the transcription was consistently reduced, indicating possible adverse effects on transcription unrelated to NF-Y binding. That the reverted sequences were binding NF-Y was confirmed by competitive inhibition of NF-Y binding to s-CCAAT, which showed increasing efficacy for inhibition correlating with the increasing number of flanking nucleotides reverted (Fig. 4). In addition to the NF-Y-shifted band, a second faster migrating band was also observed that was partially competed with C/EBPα. To determine whether basal transcription might be regulated by C/EBPα, which can bind and regulate c-jun activity (32), ROS17/2.8 cells were transfected with a C/EBPα expression vector. However, ectopic expression of the C/EBPα had no effect on transcription through the reporter construct (results not shown).

That the NF-Y transcription factor is the target of c-Jun activity was determined by co-expressing a dominant negative expression vector for NF-YA (NF-YA DN) with c-Jun. In the presence of NF-YA DN the effects of c-Jun on −60 *bsp*Luc transcription were abrogated in both the ROS 17/2.8 and RBMC-D8 cells (Fig. 5). In comparison, co-expression of NF-YA with c-*jun* further increased transcription in the ROS 17/2.8 cells, but not in the RBMC-D8 cells.

c-Jun Effects Are Phosphorylation-independent—The transcriptional activity of c-*jun* in the AP-1 complex with c-Fos has been shown to be dependent upon the phosphorylation of specific serines in the Jun protein sequence by Jun kinase (28), and the phosphorylation of c-Jun

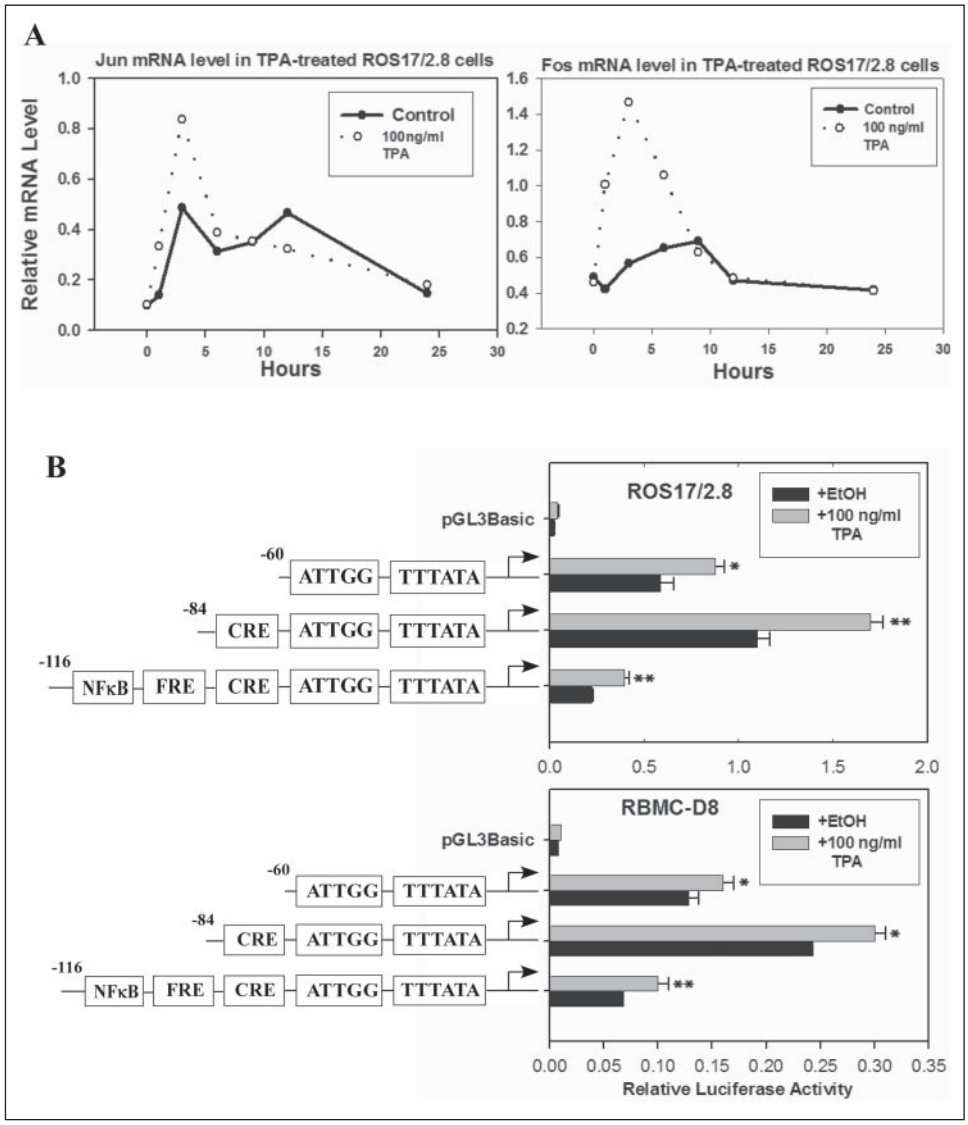

FIGURE 1. **Increased levels of c-Jun stimulate *bsp* promoter activity.** *A*, Northern hybridization analysis of TPA-treated ROS 17/2.8 cells. ROS 17/2.8 cells were treated with 100 ng/ml TPA for various time periods in the presence of 28 μg/ml cycloheximide. Total mRNA extracted from the cells was probed for c-*jun* and c-*fos* mRNA. *B*, transcription assays of TPA-treated ROS 17/2.8 and RBMC-D8 cells, which were transiently transfected with *bsp* promoter constructs of various lengths. Ethanol was used as a vehicle control for TPA. pGL3Basic was analyzed as an empty vector control for the *bsp* constructs. The significant increases by TPA are indicated by *asterisks*, where * indicates $p < 0.05$ and ** indicates $p < 0.01$. *CRE*, cAMP-response element; *FRE*, fibroblast growth factor-response element.

increases DNA binding-independent transcription of the H ferritin basal promoter (14). To determine whether the phosphorylation of c-Jun is required for the increased basal transcription of BSP, ROS 17/2.8 cells were co-transfected with c-Jun and expression vectors for the fusion protein JNKK2-JNK1, which alone is sufficient to stimulate c-Jun transcriptional activity, and for JNKK2(K149M)-JNK1, in which lysine 149 is replaced by methionine and thus lacks the ability to phosphorylate c-Jun. However, no significant effect of either the JNKK2-JNK1 or the JNKK2(K149M)-JNK1 was observed on c-Jun activity whether expressed alone on in combination with c-Jun (Fig. 6*A*). To confirm these results, we also transfected ROS 17/2.8 cells with a mutated form of c-Jun in which the serines targeted for phosphorylation had been changed to alanines (28). Although serines 63 and 73 have been shown to be phosphorylated by JNK in the activation of c-Jun, this mutated form of c-Jun was as effective as the wt construct in stimulating basal transcription of the −60 *bsp*Luc reporter construct (Fig. 6*B*). Thus, taken together with the lack of a JNKK effect, c-Jun stimulation of BSP basal transcription did not require JNK-directed phosphorylation.

c-Jun Stimulation Does Not Involve HAT—Because HAT activity has been associated with NF-Y-mediated transcription (14, 21, 22), we studied the effects of the histone deacetylase inhibitor TSA on c-jun-stimulated transcription. Although in the absence of ectopic c-Jun expression the transcriptional activity of the −60 *bsp*Luc reporter construct in the ROS 17/2.8 cells was increased by TSA, when cells were co-transfected with c-*jun* the increase was not significantly different (Fig. 7*A*). Moreover, in contrast to ROS 17/2.8 cells, TSA significantly decreased transcription of the −60 *bsp*Luc reporter construct in RBMC-D8 cells, both in the presence and absence of c-*jun* ectopic expression (Fig. 7*B*).

Role of p300 and P/CAF in c-Jun Stimulation—Because c-jun has been shown to bind the co-activator p300/CBP (14, 33), which also binds NF-Y and has HAT activity, the role of p300 in mediating the effects of c-Jun was analyzed. Co-transfection of expression constructs for wild-type and HAT-mutated CBP with c-*jun* had little effect on transcriptional activity (Fig. 8*A*). However, down-regulation of p300 mRNA, using an siRNA mixture that specifically targets p300 and CBP (30), significantly reduced c-Jun stimulated activity, indicating a requirement for p300 in transactivating the effects of c-Jun (Fig. 8*B*).

Previous studies have indicated that P/CAF binds to p300 and E1A/Twist in the pre-initiation complex (PIC) and thereby can transduce the HAT activity of p300 (34, 35). Thus, to determine the role of P/CAF in c-*jun* regulation, ROS 17/2.8 cells were co-transfected with expression vectors for P/CAF and P/CAFΔ in which the HAT domain had been partially deleted (26). Whereas P/CAFΔ lacking HAT activity had little effect on constitutive transcription, a consistent but not significant

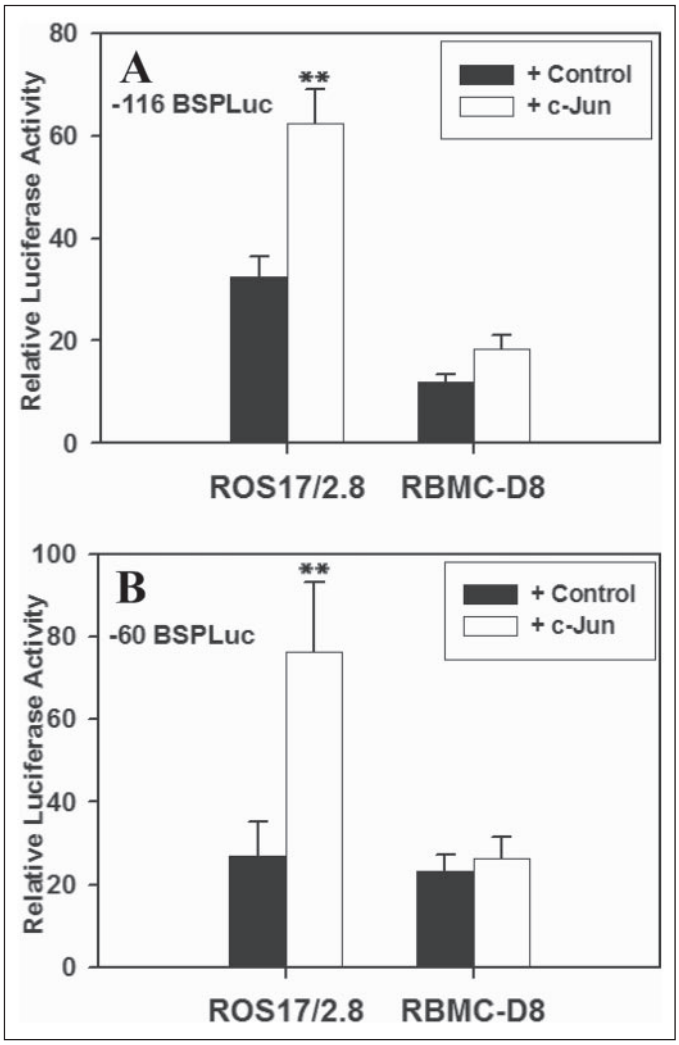

FIGURE 2. **Co-transfection of a c-Jun expression vector increases *bsp* promoter activity.** Transient transfection assays were used to show the effects of ectopically expressed c-*jun* on basal promoter activity of *bsp*. 300 ng of pcDNA3.1+ c-Jun or pcDNA3.1+ (empty vector control) was co-transfected with 300 ng of −116 *bsp*Luc (*A*) or −60 *bsp*Luc (*B*) in either ROS 17/2.8 or RBMC-D8 cells. Significant increases in response to c-Jun are indicated by *asterisks*, where ** indicates $p < 0.01$.

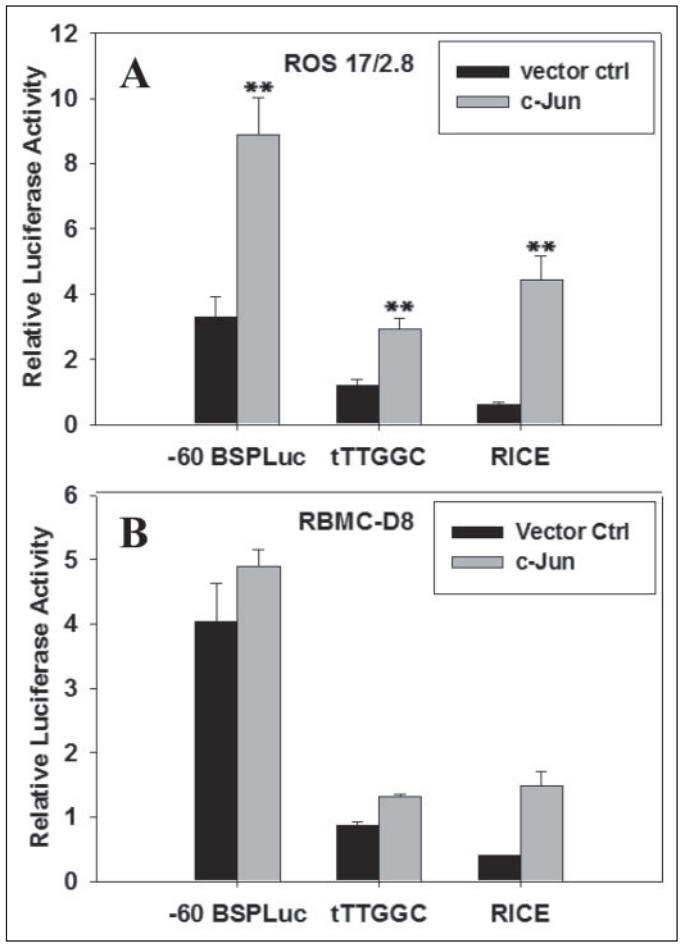

FIGURE 3. **c-Jun targets the BSP ICE box.** Mutations in the CCAAT element were used in transcription assays to show that the effects of c-Jun are mediated by the ICE box. The pcDNA3.1+ c-Jun was transiently transfected with −60 *bsp*Luc, tTTGGC (−60 *bsp*Luc with point-mutated ICE), and RICE (−60 *bsp*Luc with reverted ICE) in ROS 17/2.8 (*A*) and RBMC-D8 (*B*) cells. pcDNA3.1$^+$ was used as the vector control for pcDNA3.1$^+$ c-Jun. Significant increases in response to c-Jun in ROS17/2.8 are indicated by *asterisks*, where ** indicates $p < 0.01$.

decrease in transcription was observed with the constitutively active P/CAF (Fig. 9). Moreover, when the same constructs were co-transfected with c-*jun*, the increased transcription induced by c-Jun was suppressed by P/CAF, whereas P/CAFΔ increased transcription slightly (Fig. 9).

E1A Stimulates Basal Transcription of BSP—In an attempt to verify the results obtained with p300 and P/CAF, ROS 17/2.8 cells were transfected with a vector expressing E1A, which competes with P/CAF for p300 binding. Surprisingly, ectopic E1A expression increased transcription of the −60 bspLuc reporter to higher levels than c-*jun*, with no additional increase seen when both E1A and c-Jun were co-transfected (Fig 10*A*). Notably, the effects of p300 and P/CAF on E1A alone or when co-transfected with c-*jun* were similar to those observed with c-*jun* (not shown). To confirm the effects of E1A, the activity of three different E1A constructs (Ad5 13S, E1A$^+$, and E1A cDNA) were separately analyzed in the ROS 17/2.8 cells. Increased basal expression of the −60 *bsp*Luc was obtained with all constructs (Fig. 10*B*). To determine the importance of the p300 and P/CAF-binding region of the E1A (34), cells were transfected with various mutated E1A constructs in which the NH_2-terminal binding sites for p300 and E1A had been deleted. All three constructs showed stimulatory activity, indicating that the E1A effects did not involve binding to either p300 or P/CAF, which requires the NH_2-terminal region of E1A to be intact.

E1A Stimulates bsp Transcription through the ICE Box—To determine whether the E1A was acting through the ICE box, ROS 17/2.8 cells were co-transfected with wild-type and two ICE-mutated forms of the −60 *bsp*Luc construct, as described for the studies with c-*jun*. With both mutated constructs the E1A-stimulated expression was markedly suppressed, indicating that the E1A effects were mediated through the ICE box (Fig. 11, *A* and *B*). Furthermore, when transfected with NF-YA DN the E1A activity was suppressed whereas NF-YA had no significant effect, indicating that the E1A activity, similar to the c-*jun* activity, required NF-Y binding to the CCAAT box (Fig. 11*C*). We also examined whether the E1A effect occurred in response to an increase in c-*jun*, the expression of which has been reported to increase 50-fold in response to E1A in rat 3Y1 cells (36). However, we found no significant differences in c-*jun* transcription or protein expression when ROS 17/2.8 or HeLa cells were transfected with E1A as compared with cells transfected with the control vector (results not shown).

ChIP Analysis of NF-Y, c-Jun, and E1A—To determine whether c-jun and E1A formed transcriptional complexes with NF-Y on the *bsp* promoter, ChIP assays were performed. Transcription factors bound to the −60 BSPLuc transfected into HeLa cells were cross-linked and, follow-

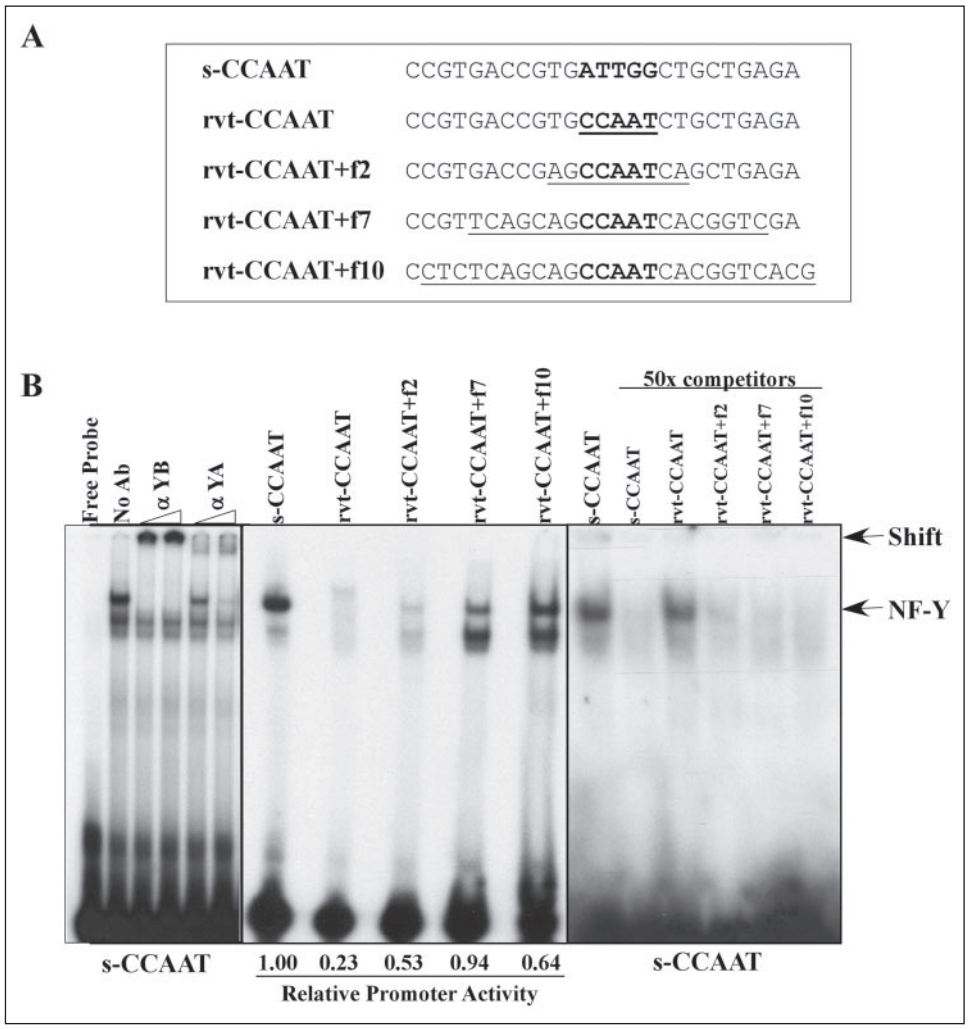

FIGURE 4. **Electrophoretic mobility shift assay using nuclear extracts from ROS 17/2.8 to show binding of NF-Y to the ICE box.** The sequences of the probes are shown in *panel A*. The *left section* of *panel B* shows that the band can be super-shifted by antibody against NF-YB (αYB) and NF-YA (αYA). The *middle section* of *panel B* shows the binding of NF-Y to the CCAAT box when the ICE is in its wt orientation, reverted (RICE) orientation, or reverted along with 2, 7, or 10 flanking nucleotides (*f*). The *right section* of *panel B* shows the competition for NF-Y binding by the cold probes in a 50-fold molar excess. *Ab*, antibody.

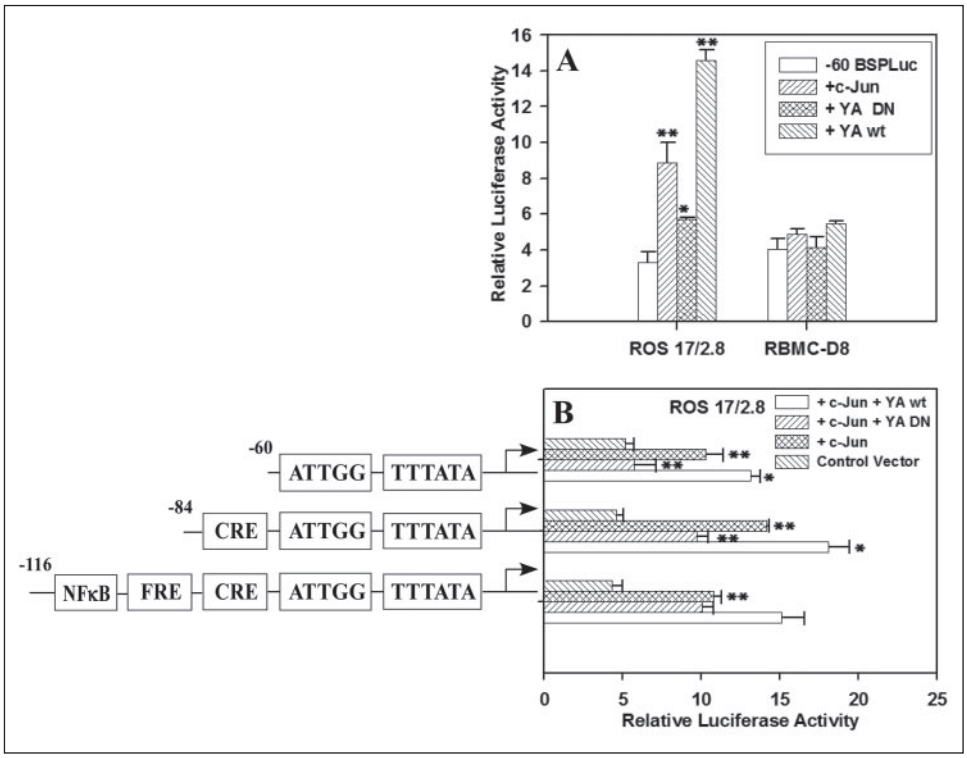

FIGURE 5. **Effect of NF-YA wt and NF-YA DN on c-Jun-stimulated *bsp* promoter activity.** The effects of NF-YA and NF-YA DN were studied using transient transfection assays. Expression vectors for c-Jun, YA wt, and YA DN were co-transfected with −60 *bsp*Luc into ROS 17/2.8 or RBMC-D8 cells (*A*). The effect of YA DN and YA wt on c-Jun stimulation was evaluated by co-transfecting c-Jun with YA wt or YA DN and different lengths of *bsp* promoter constructs (*B*). Significant increases by c-Jun, as indicated by *asterisks*, are compared with the activities of wt *bsp* promoter constructs (−60 *bsp*Luc, −84 *bsp*Luc, and −116 *bsp*Luc, respectively). The significances of the YA DN and YA wt effects are compared with that of the c-Jun effect and indicated by *asterisks*, where * indicates $p < 0.05$ and ** indicates $p < 0.01$. *CRE*, cAMP-response element; *FRE*, fibroblast growth factor-response element.

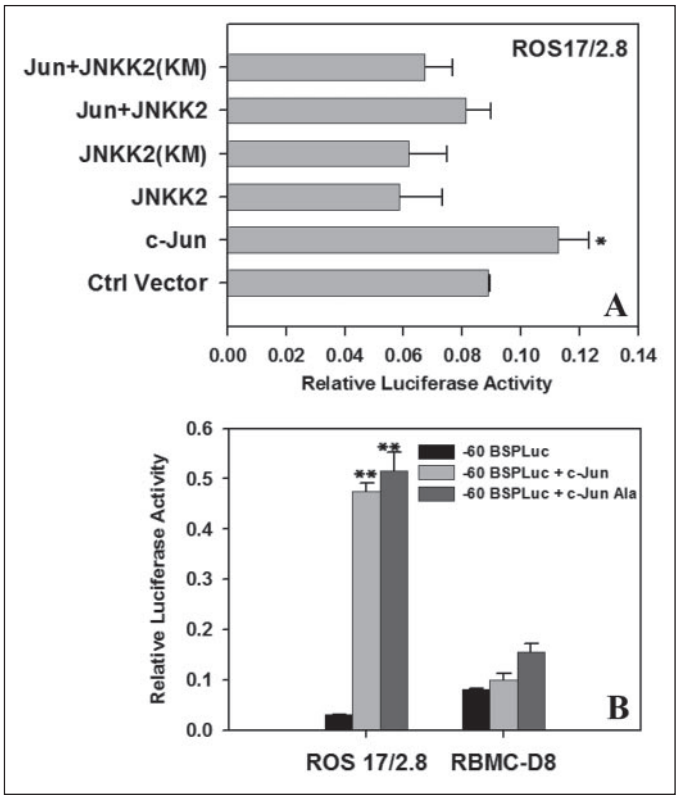

FIGURE 6. **Phosphorylation of c-Jun is not required to stimulate *bsp* promoter activity.** To determine the importance of c-Jun phosphorylation on transactivation of the *bsp* promoter, transcription assays were performed with ectopically expressed Jun kinase (JNKK2-JNK1; denoted *Jun+JNKK2* in *panel A*), a mutated Jun-kinase construct lacking phosphorylating activity (JNKK2(K149M)-JNK1; denoted *Jun+JNKK2(KM)* in *panel A*), and a mutated c-Jun construct lacking the phosphorylated serines (c-Jun Ala; denoted *c-Jun* in *panel A*). *A*, 300 ng of −60 *bsp*Luc was co-transfected with 300 ng of c-Jun and 300 ng of expression vectors for JNKK2-JNK1 or JNKK2(K149M)-JNK1 in ROS 17/2.8 cells. *Ctrl*, control. *B*, 300 ng of −60 *bsp*Luc was also co-transfected with 300 ng of either c-Jun or c-Jun Ala in ROS 17/2.8 or RBMC-D8 cells. The significant increases in response to c-Jun or c-Jun Ala are indicated by *asterisks*, where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

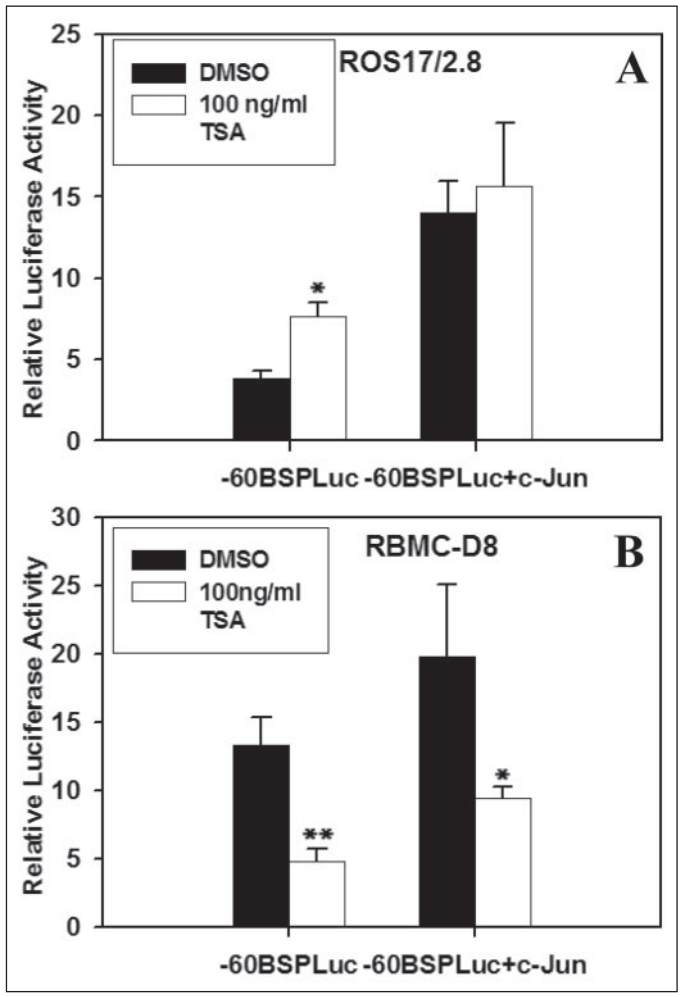

FIGURE 7. **TSA effect on basal and c-Jun-stimulated *bsp* promoter activity.** The involvement of histone acetylases in mediating c-Jun effects on *bsp* transcription was determined by analyzing the effects of the histone deacetylase inhibitor TSA. *A*, ROS 17/2.8 cells transfected with −60 *bsp*Luc with or without c-Jun, were treated with 100 ng/ml TSA for 24 h, and the transcription was measured by luciferase activity. *B*, the same experiment was performed in RBMC-D8 cells. The significance of TSA effects is indicated by *asterisks*, where * indicates $p < 0.05$ and ** indicates $p < 0.01$. *DMSO*, Me_2SO.

ing DNA fragmentation, were immunoprecipitated and subjected to PCR amplification using primers spanning the *bsp* promoter and luciferase genes. A significant increase in the amplification of a 254-bp fragment was observed in complexes immunoprecipitated with antibodies to NF-Y and c-Jun and with an anti-FLAG antibody for cells transfected with E1A (Fig. 12). Notably, when ectopic E1A was expressed, amplification for NF-Y was increased whereas c-*jun* amplification was suppressed, suggesting that E1A recruits NF-Y to the *bsp* promoter but competes with c-Jun for binding to the NF-Y complex.

DISCUSSION

These studies demonstrate that basal transcription of the *bsp* gene, which is activated in cancer cells that metastasize to bone, can be stimulated by increasing endogenous levels of c-Jun with TPA or by ectopic expression of c-Jun. In the absence of DNA binding and JNK-mediated phosphorylation, c-Jun increases BSP transcription by targeting an inverted CCAAT element, ICE, that is highly conserved in the *bsp* promoter. In ROS 17/2.8 osteosarcoma cells c-Jun recruits NF-YA to the ICE box, as indicated by the inhibitory effects of an ectopically expressed dominant negative form of NF-Y, NF-YA DN. In contrast to ROS 17/2.8 cells, basal transcription in normal RBMC-D8 cells is mediated by NF-Y and, as a consequence, c-Jun has more modest effects in these cells. The NF-Y-mediated transactivation in RBMC-D8 cells and ROS 17/2.8 cells does not involve the HAT activities of either p300 or P/CAF. Indeed, the stimulation of BSP transcription by E1A, and E1A lacking p300 and P/CAF binding, does not support the requirement of P/CAF in NF-Y-mediated basal transcription of the *bsp* gene. Interestingly, E1A stimulated *bsp* transcription in ROS 17/2.8 cells by recruiting NF-Y to the ICE box in a manner similar to, but independent of, c-Jun.

Previous studies of the mammalian *bsp* gene have demonstrated that the orientation, and location of the CCAAT and TATA boxes are highly conserved and that the inverted CCAAT is required for basal transcription (37, 38). Although the inverted TATA box in BSP is unique, the presence of a CCAAT element located, in either forward or reverse orientation, as a single copy element between nucleotides −60 and −100 of the transcription start site has been observed in 30% of eukaryotic promoters (39). Whereas CCAAT boxes are generally conserved within the same gene across species in terms of position, orientation and flanking sequences (39), inverted CCAAT boxes are critical for basal promoter activity, and regulation through this element is mediated by NF-Y (39). The importance of an inverted CCAAT box (located immediately upstream of the inverted TATA box) in the basal transcription of the *bsp* gene is indicated by the 10-fold increase in transcription of reporter constructs that include this element (38).

In previous studies we have shown that the oncogene v-*src* and serum up-regulate *bsp* transcription through NF-Y binding to the ICE box (10).

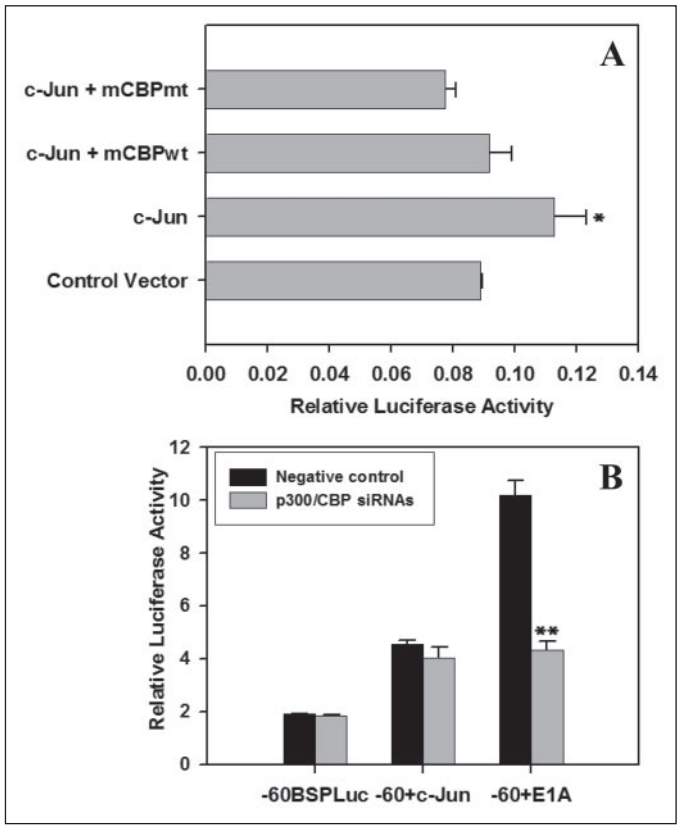

FIGURE 8. **Transcription assays showing the requirement of p300/CBP in c-Jun stimulation.** To determine whether the co-activator p300/CBP is involved in mediating c-Jun effects, endogenous levels of the co-activator were suppressed with siRNAs, and the effects on transcription were determined in transient transfection assays in ROS17/2.8 cells. *A*, a wt mouse CBP (*mCBPwt*) expression vector and a HAT domain mutated mouse CBP (*mCBPmt*) expression vector were used in co-transfection with c-Jun to examine the requirement of p300/CBP HAT activity. *B*, a mixture of p300/CBP siRNAs was co-transfected with −60 *bsp*Luc alone or with a combination of −60 *bsp*Luc and either c-Jun or E1A. The significant increase by c-Jun and the significant reduction by p300/CBP siRNAs on −60 *bsp*Luc with E1A are indicated by *asterisks*, where * indicates $p < 0.05$ and ** indicates $p < 0.01$.

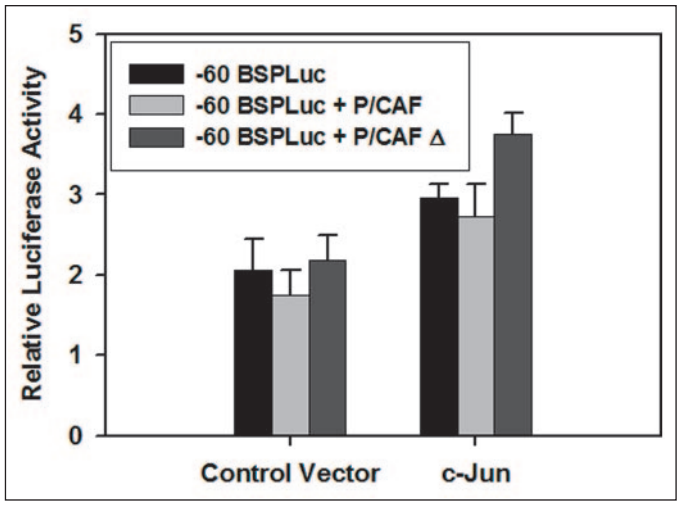

FIGURE 9. **Effect of P/CAF on basal and c-Jun stimulated *bsp* promoter activity.** −60 *bsp*Luc or the combination of −60 *bsp*Luc and c-Jun were co-transfected with P/CAF or P/CAFΔ579–608 in ROS 17/2.8 cells to determine the role of P/CAF in c-Jun stimulation of BSP transcription. No significant effects of either wt or mutated P/CAF on the luciferase activity of the *bsp* reporter construct were observed.

That expression of *bsp* is enhanced by serum through the ICE box (40) is consistent with the high levels of *bsp* expression early in bone development and at fracture sites (1), whereas the ability of v-*src* to increase

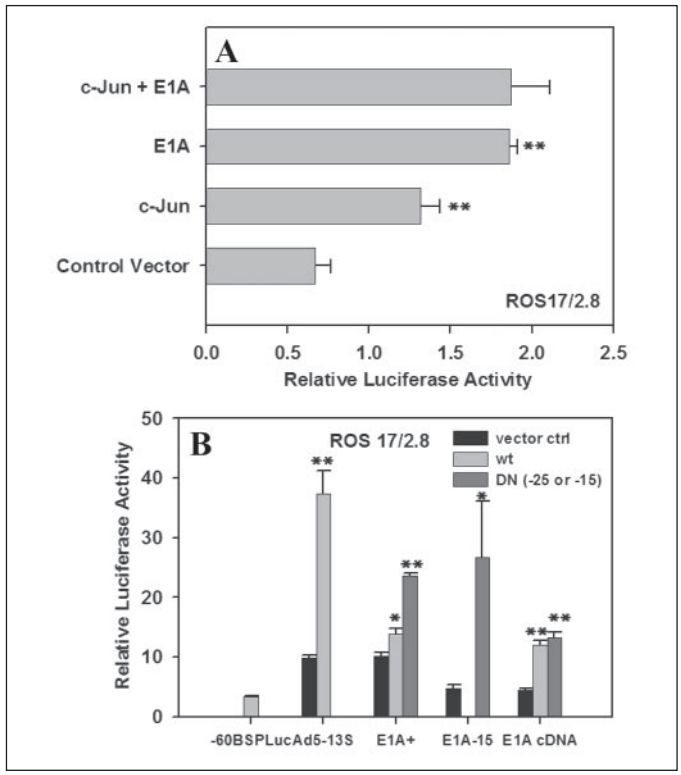

FIGURE 10. **Effect of E1A on basal and c-Jun-stimulated *bsp* promoter activity.** Several different wt and mutated E1A expression vectors were analyzed to determine the effects of E1A in BSP transcription in the presence and absence of ectopically expressed c-Jun. *A*, ROS 17/2.8 cells were co-transfected with −60 *bsp*Luc and E1A, c-Jun, or a combination of E1A and c-Jun, and the luciferase activity was measured in transient transfection assays. The significant increases by c-Jun or E1A are indicated by *asterisks*. *B*, three different E1A expression vectors, Ad5 13S, E1A$^+$, and E1A cDNA, and three NH_2-terminal deleted variants, E1A-15, E1A$^+$(−25), and E1A cDNA (−25), were co-transfection with −60 *bsp*Luc to confirm the E1A effects on BSP transcription. The significant effects of wt or DN E1A, as indicated by *asterisks* (where * indicates $p < 0.05$ and ** indicates $p < 0.01$), are compared with the respective vector controls (*vector ctrl*).

transcription of *bsp*, independent of serum (10), provides a mechanism by which transcription can become serum-independent as observed in retroviral promoters of the Rous sarcoma virus long terminal repeat (RSV LTR) (41) and Egr1/TIS8 (42) in which an inverted CCAAT box in the proximal promoter mediates both serum and *src* effects. In this study we show that the proto-oncogene c-*jun* and E1A also stimulate basal transcription of BSP by recruiting NF-Y to the ICE box.

The stimulation of *bsp* transcription by c-Jun occurs in the absence of DNA binding, because there are no binding sites for c-Jun in this region of the *bsp* promoter. Although initially characterized as a DNA binding basic region leucine zipper protein that is a target of the JNK signaling pathway, recent studies have demonstrated that c-Jun has co-activator functions that are independent of DNA binding. Thus, a functional interaction of c-Jun with the simian virus 40 promoter factor 1 (Sp1) mediates the regulation of p21 (WAF1/Cip1) (p21), a cell cycle inhibitor protein (16), the 12(*S*)-lipoxygenase gene (43), and the keratin 16 promoter (44). The region of c-Jun mediating interaction with Sp1 has been mapped within the basic region leucine zipper domain (16). c-Jun also binds to the Ets family member PU.1 and functions as a co-activator in the development of myeloid and lymphoid lineages (17, 45). Notably, the CCAAT-binding protein C/EBPα can interact with the DNA-binding domain of PU.1, displacing c-Jun and allowing granulocyte development. Although c-Jun and C/EBPα can also associate via their leucine zipper domains, preventing C/EBPα from DNA binding (46), this interaction does not appear to occur in basal *bsp* gene regulation because no

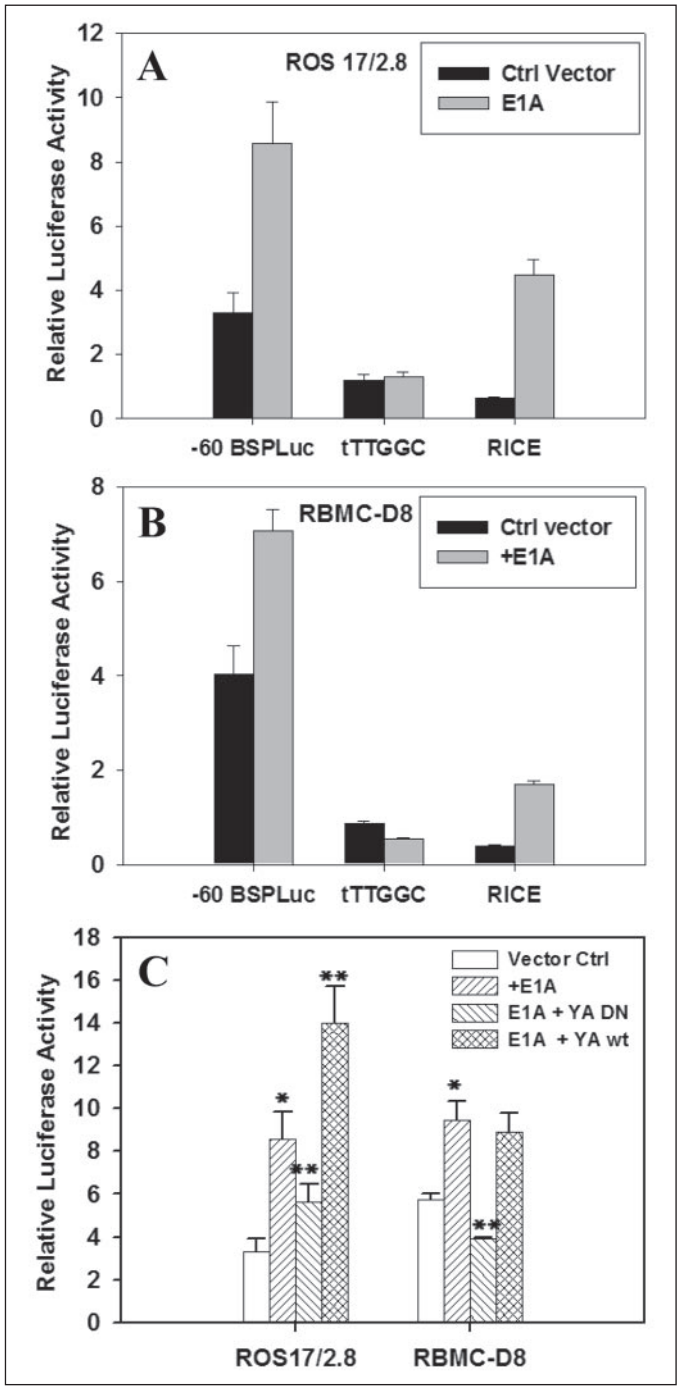

FIGURE 11. **Effect of mutated ICE and NF-YA DN on E1A stimulation.** Transient transfection assays were used to show that E1A exerted its effects on *bsp* gene transcription through NF-Y binding to the ICE box. *A*, Ad5 13S was transiently transfected with −60 BSPLuc, tTTGGC (−60 *bsp*Luc with point mutated ICE), and RICE (−60 *bsp*Luc with reverted ICE) in both ROS 17/2.8 and RBMC-D8 cells. pCMX was used as a vector control (*Ctrl Vector*). *B*, effects on luciferase activity when NF-YA wt or NF-YA DN were co-transfected with E1A and −60 *bsp*Luc in ROS 17/2.8. The significant stimulation by E1A is indicated by an *asterisk* (*, $p < 0.05$). The significant effects by YA DN or YA wt, as indicated by *two asterisks* (**, $p < 0.01$) are compared with the E1A effect.

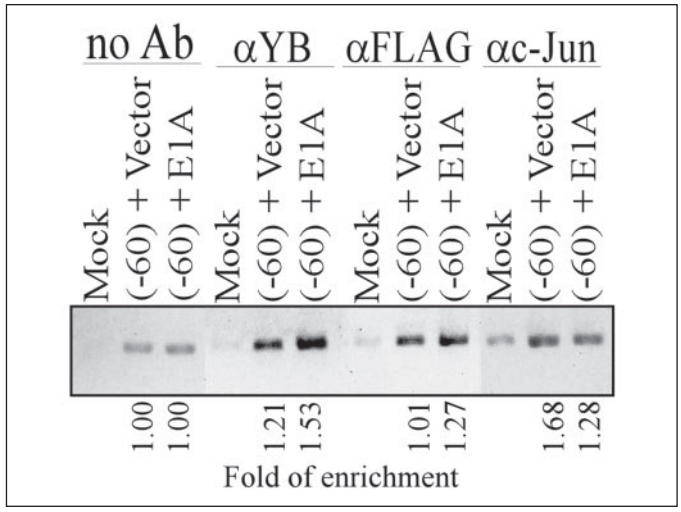

FIGURE 12. **ChIP analysis of transcription factor binding.** HeLa cells were mock-transfected or transient-transfected with −60 *bsp*Luc construct plus control vector or FLAG-tagged E1A expression vector. Chromatin immunoprecipitates of nuclear proteins pulled down by antibodies recognizing NF-YB, FLAG tag, or c-Jun were de-cross-linked and subjected to PCR amplification using primers encompassing the *bsp* promoter and luciferase gene. Gel electrophoresis of the PCR products is shown here with the fold of enrichment representing the average of two separate experiments. *Ab*, antibody.

affects were observed when C/EBPα was ectopically expressed alone or with c-Jun.

In the stimulation of H ferritin transcription, c-Jun has been reported to bind and recruit p300 (14, 47) to form a complex with P/CAF and NF-Y, which binds an ICE sequence in the proximal promoter. The involvement of p300 was deduced from an inhibitory effect of E1A, which competes with P/CAF binding to p300 (20). In our studies, E1A did not affect the c-Jun-stimulated increases in transcription. Although the proximal promoter of the H-ferritin gene is similar to the *bsp* proximal promoter, the mechanism of c-Jun-stimulated transactivation in the ferritin gene is clearly different, as the effects of c-Jun on the ferritin gene reporter construct were phosphorylation-dependent and involved HAT activity. The transcriptional activity of c-Jun is increased following phosphorylation on Ser-63 and Ser-73 by c-Jun NH_2-terminal kinase and JNK, (48, 49) and phosphorylation of serines 63 and 73 and threonines 91 and 93 in c-Jun has been considered as essential for signal-dependent AP-1 target gene activation (28). However, JNK phosphorylation-independent activation of c-Jun, as we have observed in our studies, has also been reported. Thus, phosphorylation of Ser-63 and Ser-73 on c-Jun by JNK does not alter the ability of c-Jun to enhance PU.1 transactivation (17). Similarly, c-Jun activation during DNA damage-induced neuronal apoptosis (50) and in response to Sgn2 overexpression (51) is also independent of JNK. However, the possibility that c-Jun activation may be mediated by an alternate CDK-regulated kinase has been indicated in DNA-induced apoptosis (50). Thus, although JNK-mediated phosphorylation of c-Jun does not appear to be a prerequisite for its co-activator functions in BSP, activation of c-Jun by an alternative phosphorylation mechanism that does not involve Ser-63 or Ser-73 cannot be discounted.

That p300 and P/CAF HAT activities are not involved in the c-*jun*-induced transactivation is supported by the lack of an inhibitory effect of E1A, which competes with P/CAF for p300 binding. Indeed *bsp* gene transcription was stimulated by several different E1A constructs and also by mutated constructs lacking the p300 and P/CAF binding (CR1) region. E1A strongly activates virus transcription by interacting with chromatin-remodeling enzymes but does not directly bind DNA (52). Although ChIP analyses have shown that E1A is present in the transcriptional complex formed on the ICE with NF-Y, it is not known how and to what the E1A is binding. The E1A protein contains several domains that regulate different aspects of the cell cycle, transcription activation, apoptosis, and stress responses in eukaryotic systems (44, 53). Thus, E1A binding to Rb family members through the CR2 region leads to the release of bound E2F transcription factors that repress tran-

scription (54). By binding the heat shock protein/CCAAT-binding protein HSP-CBP through the CR3 region (55), E1A also stimulates transcription of the heat shock protein HSP70 (56, 57). HSP-CBP was originally identified as a transcription factor that binds to the CCAAT box in the HSP70 promoter (58). However, recent studies have shown that HSP-CBP does not bind directly to the CCAAT box but acts as a co-activator of NF-Y (59).

Although c-Jun and E1A give similar increases in transcription, in conjunction they did not give higher effects. Therefore, they do not exhibit either additive or synergistic effects on transcription. Because they both are affected in a similar manner when cells are transfected with NF-Y and HAT vectors in different cell types, it is possible that they are both involved in the same regulatory pathway. Because we did not see any increase in c-Jun expression in response to E1A, it is unlikely that the E1A effects are mediated through c-Jun. ChIP assay also suggests that E1A in the transcription complex may compete with c-Jun binding (Fig. 12). However, the effects of c-*jun* and E1A could be mediated by p300, which binds both proteins. This is a potential mechanism that is currently being investigated in more detail, together with the possibility that E1A may function through an HSP-CBP co-activator or a related molecule such as the 110-kDa CCAAT box-binding factor, which transactivates E1A stimulation of the human *cdc* promoter (60).

In the absence of HAT activity it is unclear how the NF-Y, once recruited by c-Jun or E1A, promotes transcription. Given the proximal location of the CCAAT box in BSP and the demonstration that the promoter DNA in this region is wrapped around the PIC (61), a direct physical association of the NF-YB and NF-YC subunits with the PIC and/or PIC-bound regulators is possible. Indeed, the constitutive expression of NF-YB and NF-YC, as "house-keeping genes" and their ability to bind NC2 and TAF_{II}s through the histone motifs (62) suggest that these molecules are part of the PIC (63). Although expression of NF-YA is also held at a fairly constant level, it is biochemically separated from NF-YB and NF-YC (11), and protein levels can vary according to the cell cycle stage (64) and cellular differentiation (65, 66) and in response to serum starvation and senescence (65, 67, 68). Thus, NF-YA may be limiting in ROS 17/2.8 cells as indicated by the induction of NF-Y-mediated transcription when NF-YA is ectopically expressed. The possibility that c-Jun could transactivate NF-Y-mediated transcription through direct interactions with the PIC is suggested by studies showing that c-Jun can bind TAF-7 (18).

Because the histone-like subunits NF-YB and NF-YC have high intrinsic affinity for nucleosomal structures (41, 42), NF-Y binding to the CCAAT element can preset the chromatin configuration for co-activators to be recruited (12, 69). In these studies, however, the co-activators c-Jun and E1A stimulate basal promoter activity of *bsp* by recruiting NF-Y to the BSP ICE box in a mechanism that functions independently of p300/CBP and P/CAF HAT activity.

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