MOLECULAR AND CELLULAR BIOLOGY, Aug. 2006, p. 5969–5982 Vol. 26, No. 16
0270-7306/06/$08.00+0 doi:10.1128/MCB.00696-06


# Differential Gene Regulation by Selective Association of Transcriptional Coactivators and bZIP DNA-Binding Domains†

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Received 22 April 2006/Returned for modification 12 May 2006/Accepted 2 June 2006

**bZIP DNA-binding domains are targets for viral and cellular proteins that function as transcriptional coactivators. Here, we show that MBF1 and the related Chameau and HBO1 histone acetylases interact with distinct subgroups of bZIP proteins, whereas pX does not discriminate. Selectivity of Chameau and MBF1 for bZIP proteins is mediated by residues in the basic region that lie on the opposite surface from residues that contact DNA. Chameau functions as a specific coactivator for the AP-1 class of bZIP proteins via two arginine residues. A conserved glutamic acid/glutamine in the linker region underlies MBF1 specificity for a subgroup of bZIP factors. Chameau and MBF1 cannot synergistically coactivate transcription due to competitive interactions with the basic region, but either protein can synergistically coactivate with pX. Analysis of Jun derivatives that selectively interact with these coactivators reveals that MBF1 is crucial for the response to oxidative stress, whereas Chameau is important for the response to chemical and osmotic stress. Thus, the bZIP domain mediates selective interactions with coactivators and hence differential regulation of gene expression.**

bZIP DNA-binding proteins represent one of the largest families of transcription factors in eukaryotic cells (15, 35). bZIP domains consist of two subdomains, the leucine zipper and the basic region, that are connected by a short fork (28). The C-terminal leucine zipper forms a coiled coil that mediates dimerization (37), and the connecting fork symmetrically positions a divergent pair of basic region α-helices along the major grooves of each DNA half-site (2, 42, 49). Upon binding to DNA, the previously unfolded basic region becomes α-helical (36, 38, 54) such that five conserved amino acid residues are positioned to contact specific base pairs in the target sites (12, 26). The bZIP family has been subdivided into classes of proteins based on DNA-binding specificity or heterodimerization properties. Examples of such bZIP subfamilies include AP-1, ATF/CREB, C/EBP, CNC, Maf, and Yap proteins, as well as those with divergent basic domains.

Although bZIP domains were initially characterized in terms of their DNA binding and dimerization properties, they possess other functional properties. The bZIP domains of several factors contain the information necessary and sufficient for nuclear translocation (32, 56). A cysteine residue lying in the basic DNA binding region of c-Jun, DJun, c-Fos, and EB1 is a sensor of oxidative stress (3, 24). The S186A mutation in EB1 abolishes its ability to initiate the viral lytic cascade, but does not impair its ability to activate transcription from an EB1 reporter (13). The R288P substitution in the basic domain of Maf mislocalizes the Maf/Sox transcriptional complex in the nucleus and causes cataracts (43).

bZIP domains can also interact with transcriptional coactivator proteins to affect DNA binding selectivity or affinity, modulate the interaction with the basal transcription machinery, or regulate the chromatin environment at bZIP DNA targets. The human T-cell leukemia virus Tax and the hepatitis B virus pX proteins target a broad range of bZIP-containing proteins and promote their dimerization and binding to DNA targets in vitro (5, 39, 40, 52). The multiple factor bridging protein 1, MBF1, promotes the interaction between bZIP proteins and the transcriptional machinery (25, 48), whereas the MYST histone acetyltransferase Chameau (Chm) interacts with AP-1 during *Drosophila* development to locally enhance H4 acetylation at the level of transcriptional targets (33). HBO1, the putative human homolog of Chm (17), interacts with androgen receptor (45) and replication factors ORC1 and MCM2 (7, 22), but has not yet been linked to bZIP proteins.

Interestingly, some of these coactivators selectively associate with a subgroup of bZIP domains. Drosophila MBF1 and human TAF1 interact with the basic region of Jun but not Fos in vitro (24, 29), whereas TORC strongly enhances the transcriptional activity of promoters responsive to CREB, but not AP-1 factors, in transient transfection assays (11). Tax can discriminate between CREB/ATF-1 and other bZIP factors in vivo despite extensive sequence similarities in a yeast two-hybrid assay (4), and it can also discriminate between CREB and ATF-1 for binding in vitro and in human cells (1, 50, 57). However, the various studies have identified different residues or structural features in the CREB basic region required for the interaction with Tax.

As the basic region of bZIP proteins contains a surface that directly contacts DNA, it is presumed that a distinct surface within the same region is involved in mediating the interaction with coactivators. Furthermore, as basic regions are the most highly conserved regions of the bZIP protein family, it seems likely that coactivator selectivity will be determined by subtle differences among family members. However, the structural

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† Supplemental material for this article may be found at http://mcb.asm.org/.

basis for bZIP/coactivator selectivity and the functional consequences in vivo of such selectivity are poorly understood.

More generally, the basis for coactivator specificity among individual members of transcription factor families is poorly understood. For instance, extensive dissection of the interaction of coactivators with nuclear hormone receptors has identified an LXXLL interaction motif (NR box) in coactivators (20). However, alanine-scanning mutagenesis has revealed that sequences C terminal (+1 to +9) to this LXXLL motif appear to have the greatest impact on the selectivity and affinity of binding (31, 34), whereas peptide competition experiments identified consensus residues in the N-terminal part (9, 19).

Here, we demonstrate that residues at specific positions of the basic region diversify bZIP/coactivator interactions in vitro and in vivo. We combine glutathione *S*-transferase (GST) pull-down and transient transfection assays to assess the function of pX, MBF1, and the related Chm and HBO1 coactivators on the transcriptional activity of a broad range of bZIP factors. We identify specific residues in the basic region that determine the selective interaction of these coactivators with subsets of bZIP domains. Furthermore, by using bZIP variants that are selectively defective in interactions with specific coactivators, we decipher unique functions of these coactivators under different cellular stress conditions. Therefore, although bZIP domains are well conserved, individual bZIP domains can selectively recruit a subset of coactivators to achieve a specific transcriptional response.

## MATERIALS AND METHODS

**DNAs, cell culture, and transient transfection assays.** Reporter constructs and protein expression plasmids are described in the supplemental material. HEK293, NIH 3T3, and HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum and penicillin/streptomycin antibiotics (Invitrogen-GibcoBRL). For transient transfection assays, cells were plated at a density of $2 \times 10^6$ per well in 10-cm tissue culture plates and transfected with the $Ca_3(PO4)_2$ precipitate technique. All transfections contained 1 μg of the cytomegalovirus (CMV)–β-galactosidase reporter as an internal standard, and the amount of DNA was scaled up to 10 μg with pBluescript DNA carrier. After transfection, cells were lysed in buffer (Tris-phosphate [pH 7.8], 25 mM; EDTA, 2 mM, dithiothreitol [DTT], 1 mM; glycerol, 10%; and Triton X-100, 1%) by three cycles of freeze-thawing and assayed for β-galactosidase activity and firefly luciferase activity. To test the impact of MBF1, Chm, and HBO1 on c-Jun and v-Jun activity under oxidative conditions (100 μM $H_2O_2$ for 1 h), each transfection point was run in duplicate, and luciferase expression was monitored by reverse transcription-PCRR (RT-PCR). Small interfering RNAs (siRNA) against MBF1 (Santa Cruz biotechnology), HBO1 (Ambion), and green fluorescent protein (GFP) (18) have been described previously. Statistical analyses were performed with the classical Student's *t* test, with significance at $P < 0.1$, $P < 0.01$, and $P < 0.001$, as shown on the figures.

**GST pull-down and in vivo immunoprecipitation assays.** His-$Chm^{Cter}$ and His-$Chm^{Nter}$ were expressed in BL21 cells and purified on $Ni^{2+}$-nitrilotriacetic acid (NTA) beads (QIAGEN). GST fusions of c-Jun and MBF1 were expressed in DH5α and purified on glutathione-agarose beads (Invitrogen). Radiolabeled proteins were produced with the TNT T7 Quick Coupled transcription/translation system (Promega). Preformed beads containing GST-MBF1, His-$Chm^{Nter}$, and His-$Chm^{Cter}$ were incubated with radiolabeled bZIP factors for 2 h at 4°C in binding buffer containing 50 mM $NaH_2PO_4$, 20 mM imidazole, and 150 (or 250) mM NaCl, and after extensive washing, the associated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Interaction of His-$Chm^{Cter}$ with GST derivatives of c-Jun was tested as described previously (33). All buffers are supplemented with ethidium bromide (1.2 μg/ml) to avoid bZIP-DNA association.

In vivo immunoprecipitation assays were performed with anti-HA–Sepharose or anti-Flag–Sepharose resins (Pharmacia) in binding buffer (150 mM KCl, 20 mM Tris-HCl [pH 7.5], 20% glycerol, 5 mM DTT). After electrophoresis, proteins of interest were detected with antibodies against Chm (kindly provided by J. Pradel), MBF1 (25), hemagglutinin (HA)-Tag and Flag-Tag (Sigma-Aldrich), and HBO1 (Santa Cruz Biotechnology).

**Chromatin immunoprecipitation.** Chromatin immunoprecipitation was performed as described previously (8) using HBO1 and c-Jun antibodies (Santa Cruz Biotechnology). Quantitative PCR analysis was performed in real time using the Applied Biosystem 7700 sequence detector based on SYBR green fluorescence. The primer pairs for PCR amplification were as follows: MMP-1 promoter region (GTGTGTCTCCTTCGCACACATCTTG and GAGTCCTTG CCCTTCCAGAAAGCC), thymidine kinase promoter region (GGATTCCTCC CACGAGGGGGCGGGCT and AGCCCCTGGTTCCCGCGCCGACCGCT), and histone H3 coding sequence (GGTATTGGCAGTTTTTCCATTTTC and CCAAATGCTGGCATTGTCC).

## RESULTS

**pX regulates the transcriptional activity of bZIP-containing factors.** In vitro, pX binds all bZIP domains tested and enhances DNA binding apparently without any requirement or preference in the amino acid sequence targeted (40). However, the effect of pX on transcriptional activation in vivo has only been described in the case of CREB (55). Here, we performed transient transfection experiments involving a nucleus-targeted pX factor (nuclear localization signal [NLS]-pX), a large number of bZIP proteins representing different families, and promoter constructs containing the appropriate binding sites (Fig. 1). In accord with its in vitro properties, NLS-pX significantly enhances transcription mediated by all bZIP proteins tested in a concentration-dependent manner. The bZIP proteins tested include AP-1 factors (DJun, DFos, and c-Jun) (Fig. 1D to G), the ATF/CREB factor ATF-1 (Fig. 1J and K), C/EBP factors (C/EBPα and C/EBPβ) (Fig. 1B and C), the Maf factor NRL (Fig. 1H), the Cap-N-Collar factor Nrf-2 (Fig. 1L and M), and the divergent viral bZIP factor EB1 (Fig. 1I). As expected, NLS-pX does not activate transcription in the absence of a bZIP protein (Fig. 1A), and a pX derivative that cannot enter the nucleus (SLN-pX) fails to enhance bZIP-dependent transcription (data not shown).

We also tested the impact of pX on transcription mediated by bZIP factors whose activity is regulated by appropriate protein kinases. Phosphorylation/activation of DJun by Jun N-terminal kinase (JNK) does not affect pX function (8.2-fold activation in the absence of JNK and JNKK versus 7.4-fold activation in their presence; Fig. 1E and F). In contrast, activation of ATF-1 by protein kinase A (PKA) reduces pX impact (2.0-fold activation in the presence of PKA versus 4.2-fold activation in its absence) (Fig. 1K and J), whereas JNK activation of Nrf-2 synergistically enhances pX coactivation function (4.7-fold activation in the presence of JNK and JNKK versus 2.7-fold activation in their absence) (Fig. 1M and L). Thus, pX directly regulates the transcriptional activity of a broad range of bZIP factors in vivo, although signal transduction pathways that lead to modification of the bZIP proteins can influence the magnitude of the pX-dependent effect.

**Chm/HBO1 and MBF1 regulate the transcriptional activity of a subgroup of bZIP factors.** In contrast to the properties of pX, the related Chm and HBO1 histone acetylases (Fig. 2) and MBF1 (Fig. 3) coactivators do not regulate the transcriptional activity of all of the bZIP factors tested. Chm was initially characterized as a transcriptional coactivator that enhances transcriptional activity of the DFos/DJun heterodimer and DFos homodimer only when the JNK pathway is stimulated (33). Here, we show that Chm enhances the transcriptional

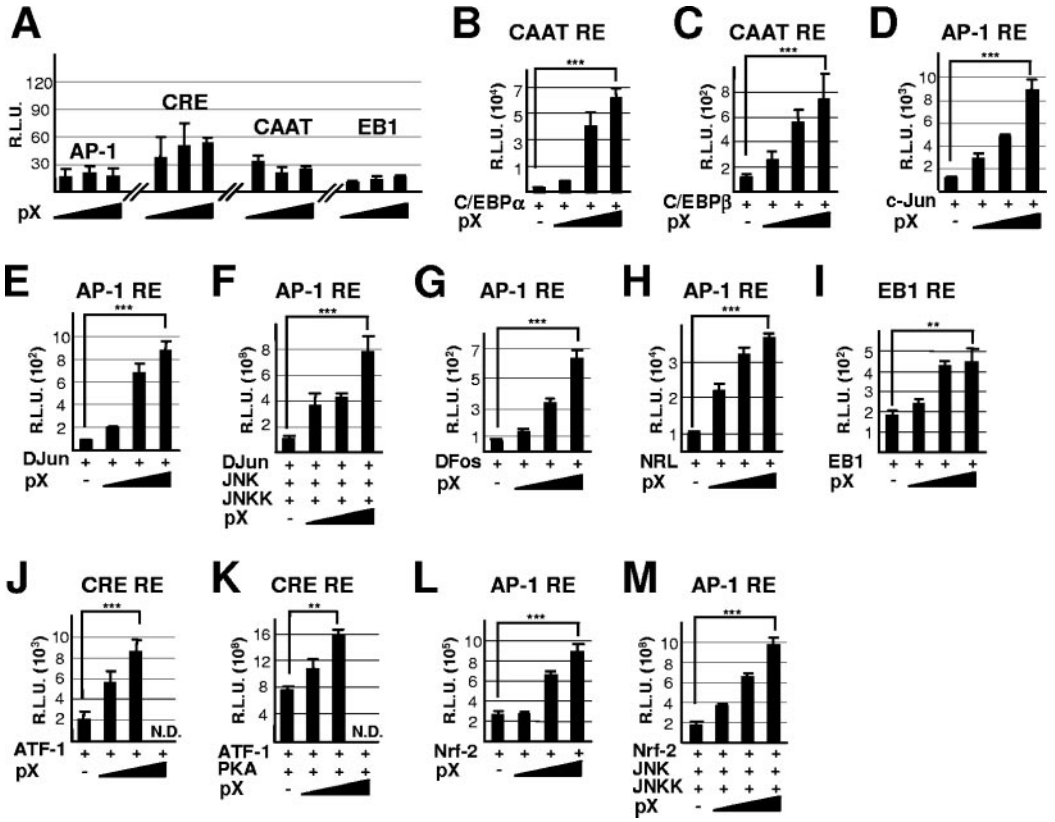



FIG. 1. pX enhances the transcriptional activity of bZIP-containing factors in HEK293 cells. (A) Control experiments showing the effect of pX concentration (from 100 to 300 ng) on the basal level of expression of the AP-1, CRE, CAAT, and EB1 reporters. R.L.U., relative light units. (B to M) Transcriptional activity mediated by the indicated proteins in response to pX (100 to 300 ng). Standard deviations from four independent experiments are indicated. N.D., data not determined due to extensive cell death. ***, $P < 0.001$; **, $P < 0.01$.

activity of DJun and c-Jun homodimer in response to JNK phosphorylation (Fig. 2A to C), but it fails to enhance the transcriptional activity of all other bZIP family factors tested, even under conditions where the appropriate kinase (PKA or JNK) is present (Fig. 2D to M; see Fig. S6 in the supplemental material). Furthermore, Chm does not enhance transcription mediated by the ATF-4 homodimer (Fig. 2L) or by the ATF-4/C/EBPβ heterodimer (Fig. 2N), but it does enhance the transcriptional activity of ATF-4/c-Jun heterodimer in a concentration-dependent manner (Fig. 2O). These observations indicate that Chm histone acetylase is a coactivator that is specific for the AP-1 class of bZIP proteins.

To gain further support in the physiological significance of this observation, we assessed the activity of two other MYST acetylases, hHBO1 and hTip60, on the transcriptional activity of different bZIP factors. HBO1, the putative human homolog of Chm (17), does not enhance the transcriptional activity of the divergent bZIP factors EB1 (Fig. 2P), ATF1 (Fig. 2Q) even in response to PKA (Fig. 2R), or C/EBPα (Fig. 2S) on an adequate transcriptional reporter but enhances in a concentration-dependent manner the activity of c-Jun (Fig. 2T), DFos (Fig. 2U), and DJun (Fig. 2V) in response to JNK activation. Therefore, HBO1, like Chm, specifically regulates the transcriptional activity of AP-1 bZIP factors. In contrast, hTip60 does not regulate transcription activated by AP-1, ATF-1, or EB1 (Fig. 2W and X; also data not shown), indicating that not all MYST acetylases regulate AP-1 and bZIP factors transcriptional activity. Furthermore, as HBO1 and Chm are both capable of regulating human and *Drosophila* AP-1, the residues involved in coactivator selectivity appear to be conserved through evolution.

MBF1 was initially identified as a direct target and coactivator of the bZIP factors Gcn4, c-Jun, and ATF-1 (25, 47). Here, we show that MBF1 also enhances the transcriptional activity of DJun, c-Jun, the ATF-4/c-Jun heterodimer, NRL, C/EBPα, Nrf-2, and ATF-1 (stimulated by PKA or not). However, MBF1 does not affect the transcriptional activity of either DFos (even in response to JNK stimulation), EB1, ATF-4, or the ATF4/C/EBPβ heterodimer (Fig. 3). Thus, MBF1 discriminates among bZIP proteins, although unlike the case for Chm/HBO1, this discrimination is not specific to a particular subfamily such as AP-1 proteins.

**Chm/HBO1 selectivity depends on two conserved arginine residues in the basic region of AP-1 factors.** To understand how Chm/HBO1 discriminates among bZIP proteins, we first asked whether Chm interacts in vitro with various bZIP proteins. The C-terminal portion of Chm ($Chm^{Cter}$) that includes the MYST domain (75% identical to $HBO1^{Cter}$) strongly binds the basic DNA-binding domain of DFos in vitro (33). Using GST fusion proteins containing various portions of c-Jun, we observe that $Chm^{Cter}$ interacts with the basic region of the bZIP domain of cJun (Fig. 4A). In contrast, and consistent

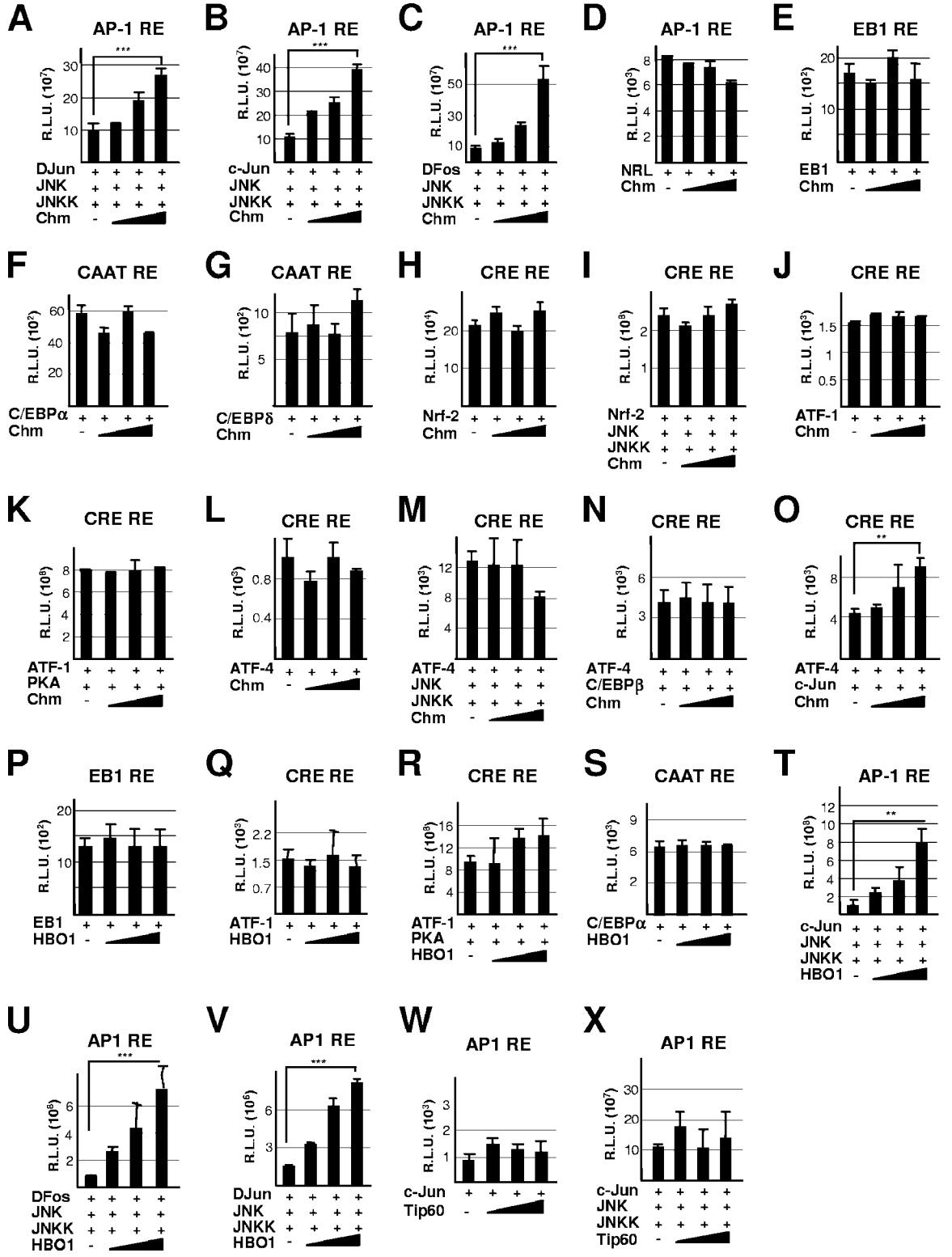



FIG. 2. Chm/HBO1 specifically regulates the transcriptional activity of AP-1 bZIP factors. The impact of Chm (50 to 250 ng), HBO1 (50 to 300 ng), and Tip60 (100 to 400 ng) in HEK293 cells on the transcriptional activity mediated by the indicated proteins ($n = 3$) is shown. R.L.U., relative light units. ***, $P < 0.001$; **, $P < 0.01$.

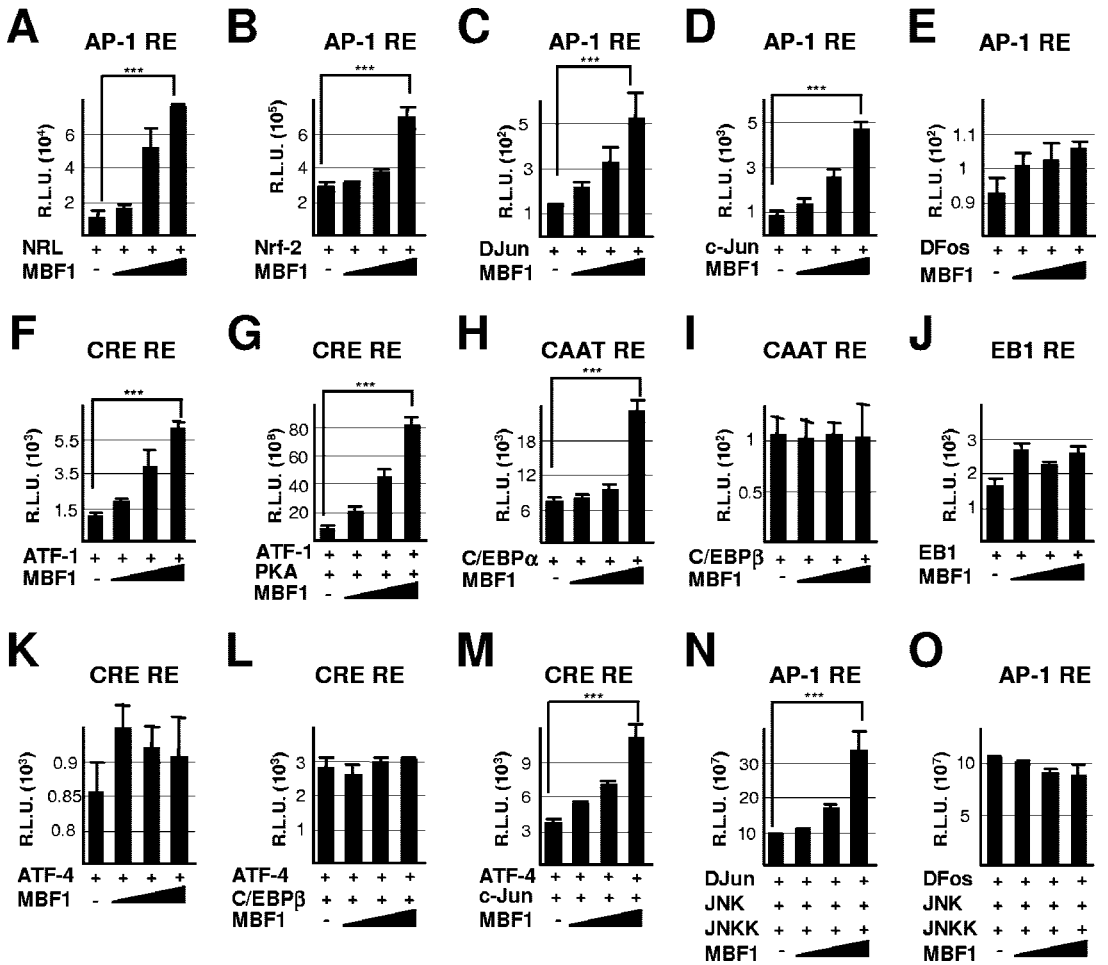



FIG. 3. MBF1 regulates the transcriptional activity of a subset of bZIP factors. The impact of MBF1 (from 200 to 400 ng) on the transcriptional activity mediated by the indicated bZIP proteins ($n = 4$) is shown. R.L.U., relative light units. ***, $P < 0.001$.

with the transcriptional analysis (Fig. 2), $Chm^{Cter}$ (and $Chm^{Nter}$; data not shown) does not interact with any in vitro-translated bZIP proteins tested that does not belong to the AP-1 subfamily (Fig. 4B; data not shown). In addition, Chm enhances AP-1 transcriptional activity in response to JNK activation at the collagenase and atrial natriuretic peptide promoters that contain canonical AP-1 binding sites (data not shown) and also at an artificial CRE target promoter (data not shown), suggesting that Chm function is not significantly affected by the architecture of the protein-DNA complex or sequences flanking the AP-1 site. Finally, whereas Chm does not regulate the transcriptional activity of EB1, it enhances the transcriptional activity of a chimeric EB1-$cJun^{Basic}$ protein in which the basic region of EB1 is replaced by the basic region of human c-Jun (Fig. 4C, lanes 1 to 8) and conversely does not regulate the transcriptional activity of the cJun-C/EBPβ fusion in which the bZIP domain of c-Jun is replaced by the bZIP domain of C/EBPβ (see Fig. S2 in the supplemental material). Thus, Chm functionally interacts with the basic domain of AP-1 proteins both in vitro and in vivo, and it appears to recognize some particular feature(s) in the basic region of AP-1 proteins.

To identify residues in AP-1 basic regions that are critical for interaction with Chm, we tested the impact of Chm on the transcriptional activity of two other chimeric factors, EB1-cJunA and EB1-cJunB, in which only half of basic regions are swapped. Both chimeric proteins activate the transcription from both AP-1 or EB1 target promoters, but EB1-cJunA is not responsive to Chm, whereas EB1-cJunB is only slightly responsive (twofold enhancement) at both promoters (Fig. 4C, lanes 9 to 16; see Fig. S3 in the supplemental material). This suggests that both parts of the c-Jun basic region might be required for a complete response to Chm. Sequence analysis (see Fig. S1 in the supplemental material) identifies two arginine residues that are specific to the basic region of AP-1 proteins (defined here as positions +8 and +19, respectively, which correspond to R221 and R232 in DJun) but are not located on the DNA-binding surface (15, 35). Derivatives of DJun containing R232K or R232A substitutions activate transcription in response to JNK activation, but are completely defective in the response to Chm (Fig. 4D). Furthermore, abolition of Chm function correlates with its loss of interaction with DJun as immunoprecipitation of HA-DJun from cotransfected cellular extracts pulls down Chm, whereas immunoprecipitation of Flag-$DJun^{R232K}$ does not (Fig. 4E).

In a converse experiment, we replaced the lysine residues at the

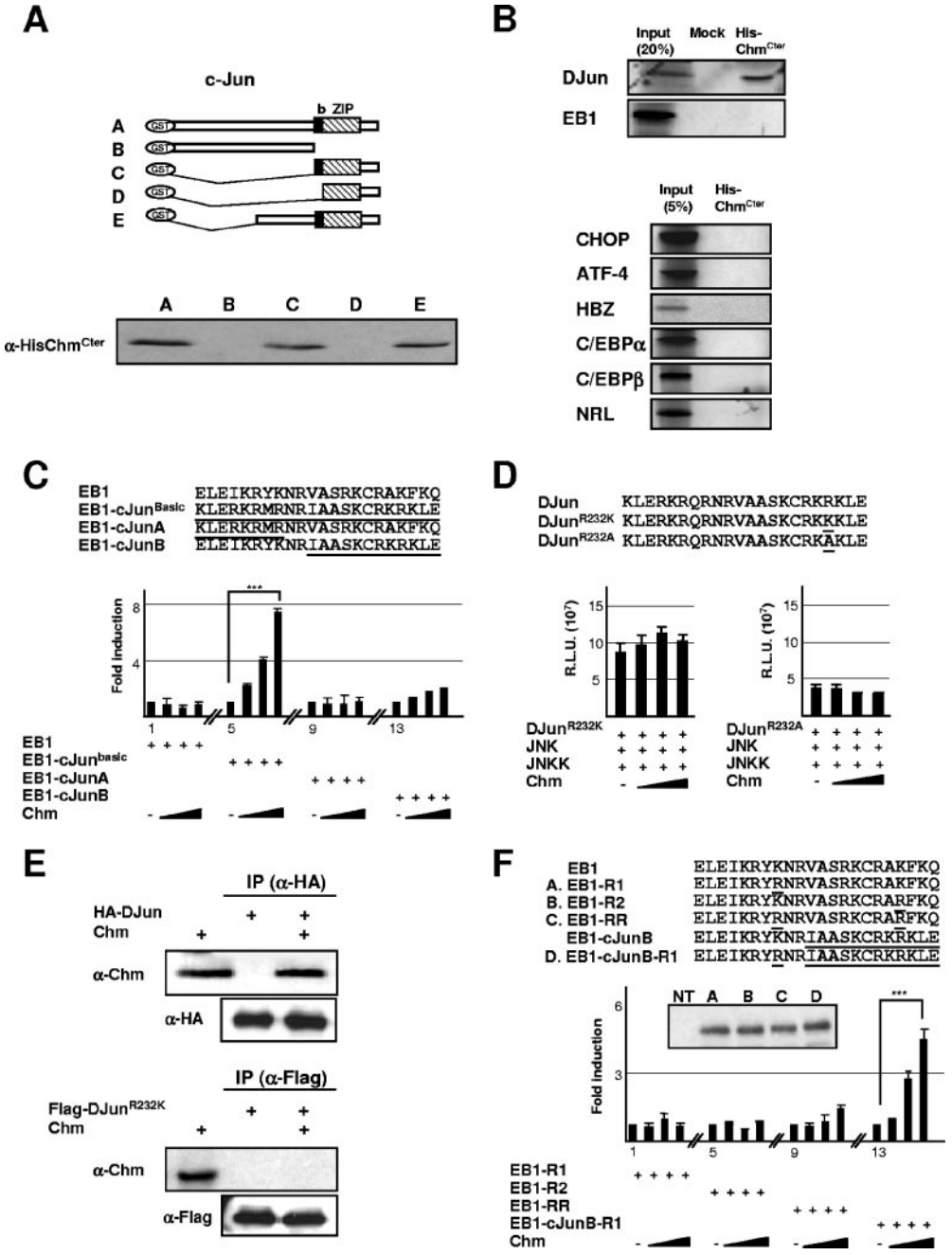



FIG. 4. Chm/AP-1 selectivity relies on two Arg residues in the AP-1 basic region. (A) Binding of Chm$^{Cter}$ to the indicated GST derivatives of c-Jun. α-HisChm$^{Cter}$, anti-His-Chm$^{Cter}$. (B) Interaction of His-Chm$^{Cter}$ beads with in vitro-transcribed/translated bZIP-containing factors. (C) Effect of Chm (from 50 to 250 ng) on the activity of the indicated EB1/c-Jun chimeric proteins on an AP-1 reporter ($n = 3$). A description of the basic DNA binding region of the different chimeric proteins is provided with c-Jun residues underlined. (D) Effect of Chm on the activity of DJun$^{R232K}$ and DJun$^{R232A}$ in response to JNK stimulation on an AP-1 reporter ($n = 3$). R.L.U., relative light units. (E) Immunoprecipitation of HA-DJun and Flag-DJun$^{R232K}$ from HEK239 cells overexpressing HA-DJun and Chm or Flag-DJun$^{R232K}$ and Chm. Chm was detected by Western blotting. α-Chm, anti-Chm; α-HA, anti-HA; α-Flag, anti-Flag. (F) Effect of Chm on the transcriptional activity of EB1 variants on an AP-1 reporter ($n = 5$). (Insert panel) Anti-HA immunodetection of the HA-EB1 variants in the nuclear pellet. ***, $P < 0.001$.

+8 and +19 positions in EB1 protein with arginine residues. All variants activate the transcription driven by an AP-1 or EB1 response element (RE) (see Fig. S3 in the supplemental material), but neither the single- nor double-arginine substitutions lead to enhancement by Chm. However, substitution of arginine for lysine at +8 in the context of the EB1-cJunB hybrid protein results in concentration-dependent coactivation by Chm (Fig. 4F; see Fig. S3 in the supplemental material). Taken together, these results suggest that Arg +8 and Arg +19 play a critical function in Chm selectivity, but additional residues in the B portion of the Jun basic region also contribute to Chm function.

Similar to Chm, HBO1 selectively regulates bZIP domains

containing arginine residues at +8 and +19. HBO1 enhances in a concentration-dependent manner the activity of EB1-cJun$^{Basic}$, but it does not enhance the activity of EB1 or the chimeric cJun-C/EBPβ protein (see Fig. S2 in the supplemental material). Furthermore, HBO1 also enhances the transcriptional activity of EB1 Chm-responsive variant, EB1-cJunB-R1 but not of Chm-unresponsive ones, EB1-cJunB, EB1-RR, and EB1-R1 (see Fig. S4 in the supplemental material). Finally, regulation of DJun activity by HBO1 is abrogated by mutation of R232 (see Fig. S4 in the supplemental material). Thus, the functional selectivity of HBO1/Chm for AP-1 bZIP factors and the mechanism of discrimination are conserved through evolution.

**MBF1 selectivity is strongly influenced by specific residues in the bZIP domain.** We analyzed the basis of MBF1 selectivity using chimeric proteins composed of c-Jun, whose activity is enhanced by MBF1, and EB1, whose activity is unaffected by MBF1 (Fig. 3). Unlike EB1 itself, transcription dependent on the EB1-cJun$^{Basic}$ hybrid protein is enhanced in a concentration-dependent manner (Fig. 5A), indicating that the basic region of c-Jun is important for the MBF1 response. As a control, MBF1 also enhances the transcriptional activity of EB1-cFos$^{Basic}$ where the basic domain of EB1 is replaced by a c-Fos one (data not shown). Furthermore MBF1 does not affect the transcriptional activity of the EB1-cJunA, EB-1-R1, EB-1-R2 nor EB1-cRR proteins, but it does enhance the transcriptional activity of EB1-cJunB and EB1-cJunB-R1 on an AP-1 or EB-1 reporter (Fig. 5A, lines 9 to 32; also data not shown). Finally, MBF1, which does not enhance C/EBPβ activity, does not enhance the transcriptional activity of the chimeric cJun-C/EBPβ protein as well (see Fig. S2 in the supplemental material). Thus, MBF1 appears to recognize some specific residues in the B portion of the bZIP domain that are conserved between c-Jun and c-Fos, but not present in EB1, C/EBPβ, and probably also in DFos, and ATF-4.

It has been suggested that arginine residues at positions +14, +15, +17, and +19 in the yeast bZIP protein Gcn4 are important for the binding and transcriptional function mediated by yeast MBF1 (48), and these residues are conserved in the basic regions of c-Fos and c-Jun (Fig. 5C). In accord with this suggestion, the R232A derivative of DJun (corresponds to the +19 arginine) abolishes MBF1 coactivation in the presence or absence of JNK stimulation (Fig. 5B). However, the R232K derivative of DJun is fully enhanced by MBF1 (Fig. 5B), indicating that a basic residue at this position is important for MBF1 coactivation. However, this residue cannot fully explain the specificity of MBF1, because several bZIP factors with an arginine (DFos) or lysine (EB1, ATF-4, and C/EBPβ) at this position are not targeted by MBF1.

Interestingly, the same pattern of positively charged residues occurs in nuclear hormone receptors in the domain targeted by MBF1 (Fig. 5C), and these residues are important for MBF1 binding of Ftz-F1 (48). By comparing this region of nuclear receptors and the basic region of bZIP factors targeted by MBF1, we identified a common glutamine or glutamate residue at position +22, which lies at the fork between the basic region and leucine zipper. This Gln/Glu residue is likely to be a determinant of MBF1 selectivity, as it is absent in bZIP domains not targeted by MBF1 (Fig. 5C). In accord with this suggestion, E235A or E235D derivatives of DJun abolish or drastically impair MBF1 function in the presence or absence of JNK stimulation (Fig. 5D), and HA-DJun coimmunoprecipitates endogenous MBF1, whereas HA-DFos and Flag-DJun$^{E235D}$ do not (Fig. 5E). Furthermore, GST-MBF1 interacts in vitro with DJun, NRL, and C/EBPα, but not with HBZ, CHOP, ATF-4, and C/EBPβ (Fig. 5F). Thus, a basic residue at +19 and a glutamine or glutamate residue at +22 are important for MBF1 association in vitro and coactivation in vivo.

Although important, the combination of a basic residue at +19 and a glutamine or glutamate at +22 is not sufficient for MBF1 function, because EB1 contains these residues yet does not respond to MBF1 in vivo (Fig. 5A). Interestingly, EB1 interacts weakly in vitro with MBF1 in the presence of 150 mM NaCl (Fig. 5F), but this interaction is abolished under more stringent conditions (250 mM NaCl) that do not affect the interaction of MBF1 with DJun (data not shown). Thus, residues in the basic region distinct from positions +19 and +22 are important for a strong MBF1 interaction in vitro, which in turn is required for transcriptional activation in vivo.

**Selective transcriptional synergy among coactivators.** Having characterized the specificity of pX, Chm/HBO1, and MBF1 for several bZIP proteins, we examined whether these coactivators could synergistically activate transcription mediated by DJun under conditions of JNK stimulation (Fig. 6A; data not shown for HBO1, which gives similar results to Chm). DJun activation under these conditions is further enhanced by pX (8-fold, lanes 2 and 3), Chm (3-fold, lanes 4 and 5), and MBF1 (3.5-fold, lanes 6 and 7). Cotransfection of pX with Chm or MBF1 results, respectively, in a 57-fold (lanes 8 and 9) or 36-fold (lanes 10 and 11) enhancement, indicating that pX synergistically activates transcription in combination with either Chm or MBF1 even though all three factors target the bZIP domain. On the contrary, cotransfection of MBF1 and increasing amount of Chm, or vice versa, only induces an approximately five- to ninefold enhancement in transcription (lanes 12 to 15), indicating that MBF1 and Chm cannot cooperate in transcriptional activation by DJun. As both coactivators require an arginine residue at +19 (Fig. 4 and 5), we tested whether Chm and MBF1 compete for the binding to the bZIP domain of DJun. Coimmunoprecipitation and GST pull-down experiments show that high levels of MBF1 inhibits the association between Chm and DJun (Fig. 6B), and conversely increasing Chm concentration in vivo displaces MBF1 from DJun (see Fig. S5 in the supplemental material). These observations suggest that the absence of transcriptional synergy between MBF1 and Chm is due to competition of these coactivators for binding the basic domain of DJun.

To demonstrate that cooperativity between coactivators reflects their direct interactions with the bZIP domains, we analyzed the transcriptional response at promoters activated by EB1 and the hybrid proteins EB1-JunB and EB1-JunB-R1. EB1 transcriptional activity is enhanced by pX but not by Chm or MBF1 (Fig. 6C, lanes 2 to 10), and Chm and MBF1 do not synergistically activate transcription in combination with pX (Fig. 6C, lanes 11 to 16). EB1-cJunB activity is enhanced by both pX and MBF1 but only marginally by Chm (Fig. 6D, lanes 2 to 10). In combination with pX, MBF1 synergistically enhances EB1-cJunB activity (10.6-fold enhancement compared to 1.3-fold enhancement for pX alone and 3.6-fold enhancement for MBF1 alone; Fig. 6D, lanes 14 to 16), whereas Chm

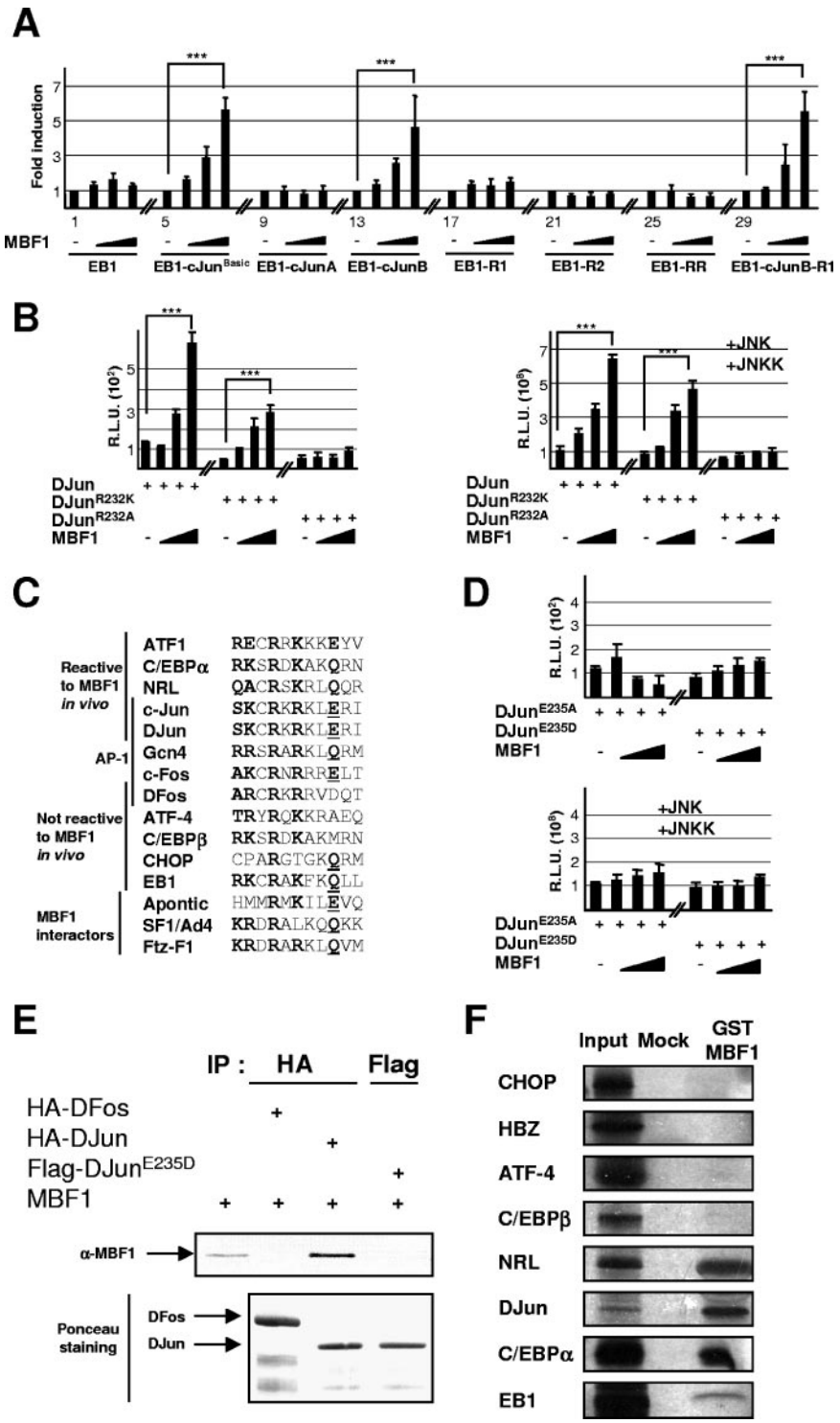



FIG. 5. MBF1 selectivity relies on the presence of a Glu/Gln residue in the linker region at position +22. (A) Impact of MBF1 (from 200 to 400 ng) on the activity of EB1 variants on an AP-1 reporter in HEK293 cells ($n = 5$). (B) Impact of MBF1 on the transcriptional activity of DJun variants at position +19 in absence (left panel) or presence (right panel) of JNK signalization in HEK293 cells ($n = 4$). R.L.U., relative light units. (C) Sequence alignment of the basic regions of AP-1 factors, bZIP factors regulated or not by MBF1, and the region targeted by MBF1 on hormonal nuclear receptor. Bold residues are conserved and are implicated in the interaction of yeast MBF11-MBP1 with yeast Gcn4 (47), and the Gln/Glu residue common to MBF1 targets is underlined. (D) Impact of MBF1 on the transcriptional activity of DJun$^{E235A}$ and DJun $^{E235D}$ in the absence (top panel) or presence of JNK stimulation (bottom panel) ($n = 4$). (E) Immunoprecipitation (IP) of HA-DFos, HA-DJun, and Flag-DJun$^{E235D}$ to access endogenous MBF1 binding. α-MBF1, anti-MBF1. (F) In vitro GST pull-down assays to test the interaction of MBF1 with in vitro-translated bZIP factors. ***, $P < 0.001$.

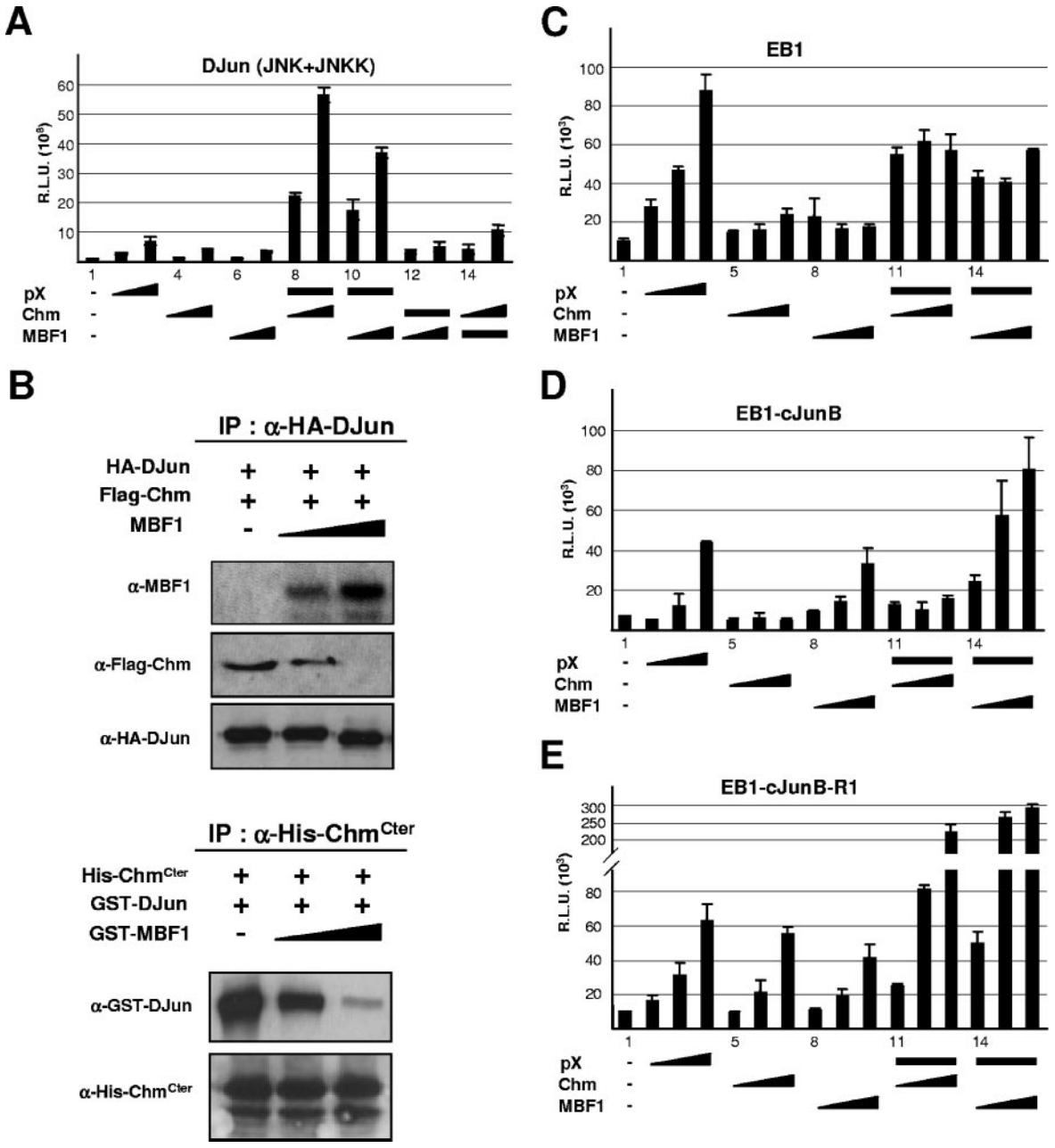
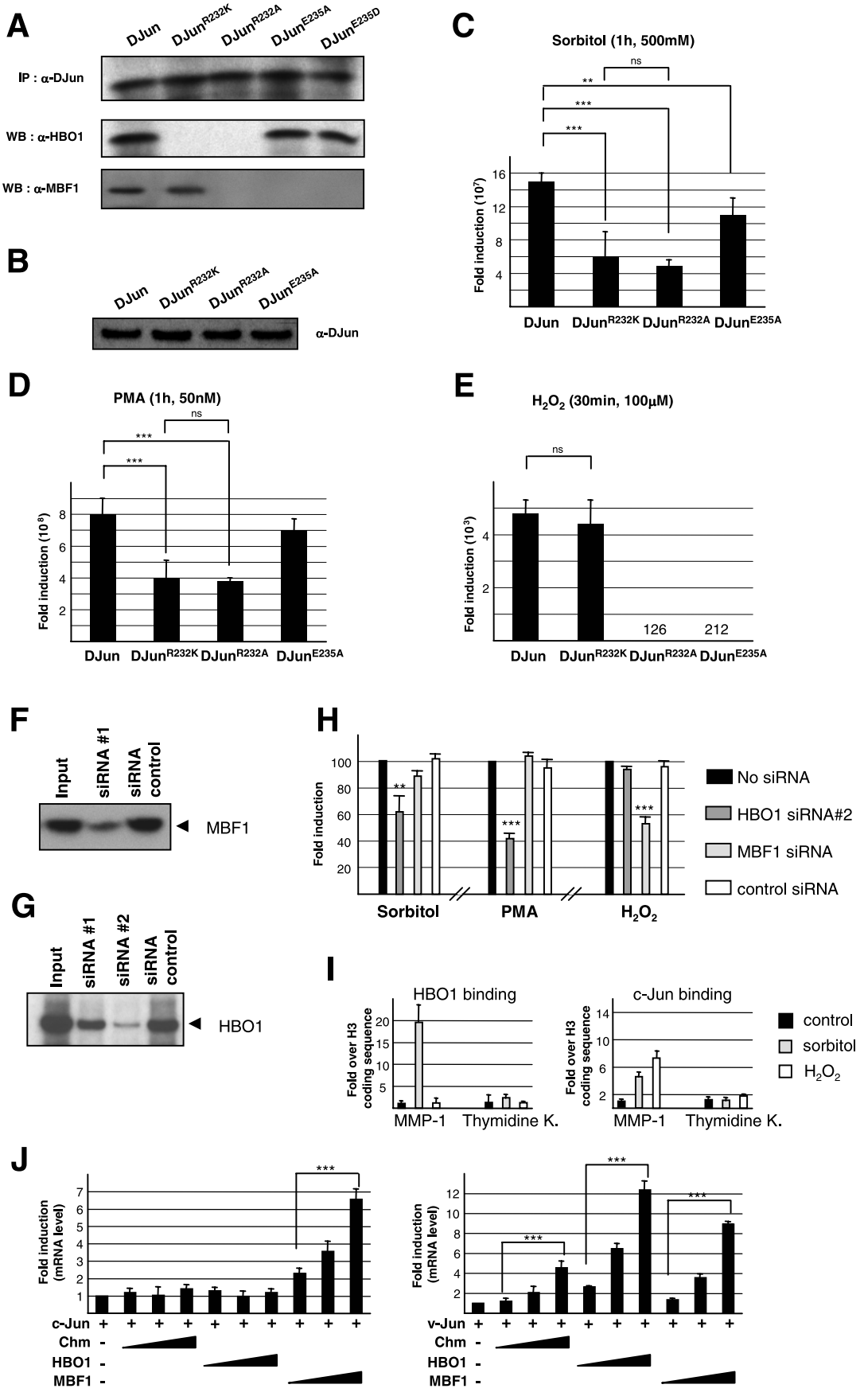



FIG. 6. Sequence of the basic region determines cofactor association and transcriptional synergy. (A) Impact of pX, MBF1, Chm, and the indicated on DJun transcriptional activity in response to JNK stimulation in HEK293 cells ($n = 5$). R.L.U., relative light units. (B) (Top) HEK293 cells were transfected with Chm (1 μg), DJun (2 μg), and increasing amount of MBF1, and MBF1 and Chm binding was assessed after immunoprecipitation (IP) of HA-DJun. α-MBF1, anti-MBf1; α-Flag-Chm, anti-Flag-Chm; α-HA-Djun, anti-HA-DJun; α-His-Chm$^{Cter}$, anti-His-Chm$^{Cter}$; α-GST-DJun, anti-GST-DJun. (Bottom) His-Chm$^{Cter}$/GST-DJun-preformed complexes on Ni-Nta$^{2+}$ beads were challenged with increasing amounts of GST-MBF1. (C to E) Transcriptional impact of MBF1, pX, and Chm combinations on EB1 (C), EB1-JunB (D), and EB1-JunB-R1 (E) transcriptional activity on a AP-1 reporter ($n = 4$).

does not (Fig. 6D, lanes 11 to 13). Finally, transcriptional activity of EB1-cJunB-R1 is individually enhanced by pX, Chm, and MBF1 and synergistically enhanced by all combinations except for Chm and MBF1 (Fig. 6E; also data not shown). Presumably, bZIP interactions with pX involve a different protein surface from interactions with MBF1 or Chm/HBO1, such that synergistic activation involves multiple (and perhaps simultaneous) interactions with the bZIP domain. In contrast, MBF1 and Chm/HBO1 interact with overlapping surfaces of bZIP domains and hence compete for binding and fail to cooperate in transcriptional activation.

**Coactivator specificity in the AP-1/JNK-dependent transcriptional response to environmental stresses.** To investigate the functional impact of coactivator selectivity, we tested DJun variants that selectively disturb Chm/HBO1 or MBF1 association for their ability to mediate the JNK-dependent response


A
DJun
DJunR232K
DJunR232A
DJunE235A
DJunE235D
IP : α-DJun
WB : α-HBO1
WB : α-MBF1
B
DJun
DJunR232K
DJunR232A
DJunE235A
α-DJun
C
Sorbitol (1h, 500mM)
ns
**
***
***
Fold induction (10^7)
16
12
8
4
DJun
DJunR232K
DJunR232A
DJunE235A
D
PMA (1h, 50nM)
ns
***
***
Fold induction (10^8)
8
6
4
2
DJun
DJunR232K
DJunR232A
DJunE235A
E
H2O2 (30min, 100μM)
ns
Fold induction (10^3)
4
2
126
212
DJun
DJunR232K
DJunR232A
DJunE235A
F
Input
siRNA #1
siRNA control
MBF1
G
Input
siRNA #1
siRNA #2
siRNA control
HBO1
H
Fold induction
100
80
60
40
20
**
***
***
Sorbitol
PMA
H2O2
No siRNA
HBO1 siRNA#2
MBF1 siRNA
control siRNA
I
HBO1 binding
c-Jun binding
Fold over H3 coding sequence
20
15
10
5
14
10
6
2
MMP-1
Thymidine K.
MMP-1
Thymidine K.
control
sorbitol
H2O2
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Fold induction (mRNA level)
***
7
6
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1
c-Jun + + + + + + + + + +
Chm -
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MBF1 -
Fold induction (mRNA level)
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12
10
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v-Jun + + + + + + + + + +
Chm -
HBO1 -
MBF1 -

of an AP-1-dependent promoter to chemical (phorbol myristate acetate [PMA]), osmotic (sorbitol), and oxidative ($H_2O_2$) stresses. Expression of DJun, DJun$^{R232K}$, DJun$^{E235A}$, DJun$^{E235D}$, or DJun$^{R232A}$ occurs at comparable levels (Fig. 7B), such that the observed differences in AP-1 transcriptional activity will reflect bZIP-coactivator association. DJun mediates strong transcriptional activation in response to sorbitol, PMA, and $H_2O_2$ (Fig. 7C, D, and E). DJun$^{R232A}$, which does not bind to HBO1 and MBF1 (Fig. 7A), is approximately twofold less active than DJun in the response to PMA and sorbitol, and it is essentially inactive in the response to $H_2O_2$. DJun$^{R232K}$, which selectively blocks the interaction with Chm/HBO1 (Fig. 7A), behaves similarly to DJun$^{R232A}$ in the response to PMA and sorbitol, but it strongly activates transcription in response to $H_2O_2$ at a level comparable to that of DJun (Fig. 7C, D, and E). These observations suggest that HBO1 association with AP-1 is dispensable for the response to $H_2O_2$, but important for the optimal response to chemical and osmotic stresses.

Conversely, DJun$^{E235A}$ and DJun$^{E235D}$, which selectively block the interaction with MBF1 (Fig. 7A), are unable to activate transcription in response to $H_2O_2$, but they are as active as DJun in the response to PMA and only marginally defective in the response to sorbitol (Fig. 7C, D, and E; data not shown). Therefore, MBF1 association with the bZIP domain of DJun is essential for the AP-1 transcriptional response to oxidative stress, but it plays little if any role in the response to osmotic or PMA stress. Furthermore, and consistent with the absence of interaction between MBF1 and DFos (Fig. 5E) (24), transfection of both DFos and DJun$^{E235A}$ (or DJun$^{E235D}$) is not able to activate the transcription under oxidative stress (data not shown). These observations are consistent with the fact that MBF1 protects DJun DNA-binding activity from oxidative modification in vitro and in *Drosophila* tissues (24).

To independently confirm the selective function of Chm/HBO1 and MBF1, we examined JNK/AP-1 activity in HeLa cells largely depleted for either coactivator by siRNA (Fig. 7F and G). Depletion of HBO1 affects AP-1 transcriptional activity on a reporter construct in response to sorbitol and PMA but not to $H_2O_2$ (Fig. 7H). Conversely MBF1 depletion abrogates the AP-1 response to $H_2O_2$ but has no effect on sorbitol and PMA response (Fig. 7H). We further confirmed the physiological relevance of this observation by monitoring HBO1 association with an AP-1 promoter (MMP-1 collagenase) by chromatin immunoprecipitation (Fig. 7I). In the absence of stress, HBO1 is not bound to the MMP-1 promoter, whereas sorbitol treatment induces strong recruitment of HBO1 along with c-Jun. In contrast, HBO1 is not recruited upon $H_2O_2$ treatment, even though these conditions result in strong binding of c-Jun.

We also confirmed the essential function of MBF1 in oxidative response by looking at the impact of HBO1 on c-Jun and v-Jun activity. v-Jun is the oncogenic counterpart of c-Jun but is insensitive to the oxidative state due to the C270S substitution in the basic region (3). In the presence of $H_2O_2$, increasing HBO1 or Chm concentration does not affect transcriptional activity mediated by c-Jun, but it significantly enhances the activity of v-Jun (Fig. 7J). Therefore, a single substitution in the basic region allows v-Jun to interact with HBO1 by bypassing the requirement in MBF1 interaction.

## DISCUSSION

**Specific residues in the basic region determine selective interactions between bZIP domains and coactivator proteins.** bZIP domains interact with a variety of coactivators, but the selectivity and transcriptional consequences of such interactions have not been previously described. Here, we show that Chm/HBO1 and MBF1 selectively regulate the activity of a subset of bZIP, whereas protein pX enhances the transcriptional activity of all the bZIP factors tested here. Furthermore, the discrimination among bZIP proteins by Chm/HBO1 and MBF1 is mediated by specific residues in the basic region that govern protein-protein interactions between the bZIP domain and the coactivators. Chm/HBO1 function and selectivity critically rely on the presence of two arginine residues at positions +8 and +19 of the basic region, whereas MBF1 selectivity is determined by the presence of a glutamine or glutamate residue at +22 and residues at positions +14, +15, +17, and +19.

X-ray crystal structures of bZIP/DNA complexes indicate that residues at +8, +19, and +22 do not contact DNA (12, 16, 41, 50). Residues at +8 and +19 extend outward from the major groove of DNA, but are interspersed with residues that directly contact DNA and hence represent a different surface of the recognition helix. The residue at +22 lies at the fork between the basic region and leucine zipper. In the context of DJun, mutations that selectively abolish the physical and functional interaction with either Chm/HBO1 or MBF1 nevertheless retain the ability to activate transcription from a promoter dependent on AP-1 sites. Thus, the mutations in the bZIP domain that abolish the interaction with coactivators do not affect DNA binding in vivo.

**Side chain length at key residues in the basic region is important for coactivator selectivity.** Chm/HBO1 is highly selective for the AP-1 subfamily of bZIP proteins, all of which

FIG. 7. Effect of coactivator selection in JNK/AP-1 transcriptional response to cellular stresses. (A) Binding selectivity of DJun variants as determined by immunoprecipitation (IP) assays. α-DJun, anti-DJun; α-HBO1, anti-HBO1; α-MBF1, anti-MBF1. (B) Expression level of DJun variants in HEK293 cells as determined by Western blotting (WB). (C to E) Transcriptional activity of DJun variants in response to sorbitol (C), PMA (D), and $H_2O_2$ (E). Relative luciferase activity is expressed as the luciferase activity ratio of DJun variants to control BlueScript ($n = 4$). (F to G) Western blot analysis of the depletion of MBF1 (F) and HBO1 (G) 48 h after transfection of HeLa cells with respective siRNA constructs. (H) Impact of the depletion of MBF1 or HBO1 on the AP-1 transcriptional response to sorbitol, PMA, and $H_2O_2$ treatment in HeLa cells ($n = 4$). (I) Chromatin immunoprecipitation analysis of HBO1 and c-Jun binding on the AP-1-dependent promoter MMP-1 and control thymidine kinase (Thymidine K.) promoter in HeLa cells under basal condition and in response to sorbitol and $H_2O_2$ treatments. (J) Effect of HBO1, Chm, and MBF1 on the transcriptional activity of c-Jun (left) and v-Jun (right) under oxidative condition ($n = 6$). Relative luciferase mRNA level is determined as the ratio of luciferase mRNA after $H_2O_2$ treatment to that with no treatment. ***, $P < 0.001$; **, $P < 0.01$; ns, no statistical difference.

contain arginine residues at +8 and +19, and our mutational analysis indicates that these arginine residues are important for interacting with Chm. Interestingly, reactivity of DJun to Chm is abolished by the R232K mutation, which does not alter the charge or hydrophobicity of the side chain, but rather its length. Although arginine and lysine residues are often functionally equivalent in proteins, they can differentially affect protein-protein interactions. For example, the K685R substitution of Stat3 impairs Stat3 dimerization (58), the K409R substitution of Smad3 enhances transcriptional activity and the interaction with coactivators (23), and the K630R substitution of androgen receptor reduces p300 binding and enhances corepressor binding (14).

Interaction of bZIP domains with MBF1 strongly depends on a glutamate (or glutamine) residue at position +22, and the MBF1 interaction with DJun is abolished by the E235D substitution. Although the E235D substitution does not alter the charge of the side chain, glutamate and aspartate residues are not always equivalent in mediating protein-protein interactions. For example, the D74E derivative of the rat β2-adrenergic hormonal receptor alters the affinity of the receptor for most of its antagonists (6), and the E64D substitution in the A subunit of the tumor suppressor protein phosphatase A2 abolishes subunit interaction in lung carcinoma (44). Conversely, MBF1 tolerates a glutamine residue at +22, whose side chain is of similar length as a glutamate, but lacks the negative charge. In a similar vein, the E180Q substitution in a glucoamylase does not disturb the association of the catalytic domain of the enzyme with its substrate (10), and the E233Q derivative (unlike the E233A derivative) of the mRNA cap-specific methyltransferase VP39 does not eliminate N7-methylguanosine binding (21). In both cases, it has been proposed that the carboxylate group that is present in glutamate and glutamine is important for the protein-protein interaction, suggesting the possibility that the interaction between bZIP domains and MBF1 might depend on the carboxylate group of Glu/Gln at position +22. Thus, for both MBF1 and Chm, it appears that selectivity of the bZIP domains for interaction with the coactivator is strongly influenced by the length of the side chain, but not the charge, of specific residues in the basic region.

These different modes of interaction with the bZIP domain might also underline the capacity of different cofactors to interact with a common bZIP target. Thus, we have demonstrated that Chm/HBO1 and pX or MBF1 and pX can transcriptionally cooperate at the level of DJun and EB1-JunB-R1 and on the contrary that Chm/HBO1 and MBF1 cannot. This might be due to the fact that pX is believed to enhance DNA binding and to be released from the DNA after binding (39), whereas MBF1 and Chm/HBO1 compete for association with a common surface that includes Arg +19 to sit on AP-1 binding sites, explaining their incapacity to form a tripartite bZIP/Chm/MBF1 complex.

**Selectivity of bZIP-coactivator interactions as a mechanism to diversify transcriptional responses.** Using DJun variants that selectively disturb Chm-HBO1 or Chm-MBF1 association, we observe that bZIP/coactivator specificity differentially affects the JNK-dependent response of an AP-1-dependent promoter to various stresses. Interaction of Chm/HBO1 with the DJun and/or DFos bZIP domain is important for the response to PMA and osmotic stress, but it plays little if any role in the response to oxidative stress. Conversely, the MBF1 interaction with the DJun bZIP domain is essential under oxidative stress, but of limited importance under other stresses tested, consistent with the phenotype of *Drosophila* mutants lacking Mbf1 (24). Interestingly, the *Drosophila* genome encodes only two AP-1 factors, DJun and DFos, and only DJun physically interacts with MBF1 (Fig. 5E and 7D) (24). Therefore, under oxidative stress, dMBF1 modifies the repertoire of AP-1 proteins, and presumably AP-1-dependent transcriptional output, by selectively inactivating DJun/DFos heterodimers, but not DJun homodimers. The differential interaction of ATF-1/4 or C/EBPα/β with MBF1 suggests that under specific environmental conditions, yet to be determined, one essential function of MBF1 could be to switch bZIP subunits.

Chm/HBO1 is a specific coactivator that only associates with the AP-1 class of bZIP proteins, and hence it is likely to preferentially regulate genes with AP-1 sites in their promoter/enhancer regions. In this regard, Chm/HBO1 resembles the TORC coactivator that specifically interacts with and enhances the transcriptional activity of CREB (11). Interestingly, TORC cooperatively regulates CREB activity in response to cyclic AMP (cAMP), whereas Chm/HBO1 enhances AP-1 response in response to JNK stimulation. As such, preferential expression of TORC and Chm/HBO1 may explain the ability of CREB and AP-1 to function as strong activators in specific developmental contexts. Chm is required for the JNK-dependent response in the proximal part of the wing imaginal disc during *Drosophila* metamorphosis, where its expression is progressively restricted (33). TORC2 is highly expressed in the liver and regulates CREB stimulation of glucogenesis and fatty acid oxidation (27). As another example of coactivator regulation, *Arabidopsis* MBF1 is rapidly induced by several stresses, and it predominantly localizes into the nucleolus, a stress sensor (30, 46). The selectivity of bZIP/coactivators mediated by the basic regions will greatly diversify bZIP-mediated transcriptional responses between different cell types and within the same cell under different conditions.

As subtle alterations in the basic region of bZIP domain can strongly affect the interaction of coactivators, bZIP-coactivator interactions might be affected by phosphorylation (or other modifications) in the basic region, and they might be altered in disease states. For example, the oncogenic and normal versions of Jun differ by three nonconservative amino acid substitutions, two of which are localized in the basic region. The S243F and C270S mutations play a role in allowing v-Jun to escape GSK-3 phosphorylation-dependent degradation (53) and oxidative sensitivity (3). Perhaps these mutations alter the interaction with a specific coactivator, as is the case for the differential behavior of c-Jun and v-Jun in response to oxidative stress.

The selectivity of bZIP-coactivator associations provides an additional mechanism for the specificity of transcriptional responses by bZIP proteins beyond that achieved by the heterodimerization properties of bZIP factors. Such bZIP/coactivator specificity might underlie alterations in transcriptional regulatory patterns in response to changes in physiological conditions or in disease states. For example, the K297R substitution in Maf, which is associated with cerulean cataract in human, lies at the +8 position of the basic region, which in Jun is required for Chm-HBO1 association and might therefore be

involved in Maf coactivator interaction (51). Transcriptional specificity is also provided by the selective ability of coactivators to synergistically affect transcription. For example, Chm and MBF1 cannot act synergistically, presumably because they recognize the same surface of the basic region, whereas either coactivator can function synergistically with pX. More generally, the expression patterns and responses to signal transduction pathways of individual bZIP proteins and individual bZIP coactivators and possibly corepressors should provide combinatorial input into transcriptional regulatory profiles and ultimately phenotype.

## ACKNOWLEDGMENTS

We are very grateful to A. Swaroop, J. Alam, A. Friedman, P. Johnson, J.-M. Mesnard, S. X. Hou, A. Sergeant, P. M. Lieberman, R. Prywes, S. Taylor, J. A. Goodrich, O. M. Andrisani, J. Auwerx, K. H. Klempnauer, P. Sassone-Corsi, and M. Safak for the different DNA plasmids they provide us. We also thank J. Pradel for the gift of the Chameau primary antibody and A. B. Lassar for the used of the Avoroskan Ascent F.L. luminometer.

This work was supported by grants to K.S. from the National Institutes of Health (GM30186).

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