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# A modified version of a Fos-associated cluster in HBZ affects Jun transcriptional potency

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## ABSTRACT

**Like c-Fos, HBZ (HTLV-I bZIP factor) is able to interact with c-Jun but differs considerably from c-Fos in its ability to activate AP-1-responsive genes since HBZ rather inhibits transcriptional activity of c-Jun. To better understand the molecular mechanisms involved in this down-regulation of c-Jun activity, a large number of HBZ/c-Fos chimeras was constructed and analyzed for their ability to interact with c-Jun, to bind to the AP-1 motif and to stimulate expression of a reporter gene containing the collagenase promoter. By this approach, we demonstrate that the DNA-binding domain of HBZ is responsible for its inhibitory effect on the *trans*-activation potential of c-Jun. However, unexpectedly, we found that exchange of a cluster of six charged amino acids immediately adjacent to the DNA contact region altered significantly transcriptional activity of chimeras. This particular subdomain could be involved in efficient presentation of the AP-1 complex to the transcriptional machinery. To confirm this role, specific residues present in the cluster of HBZ were substituted for corresponding amino acids in c-Fos. Unlike the JunD-activating potential of wild-type HBZ, this mutant was no longer able to stimulate JunD activity, confirming the key role of this particular cluster in regulation of Jun transcriptional potency.**

## INTRODUCTION

Transcription initiation is regulated by the assembly of a large multiprotein complex on the promoter region of genes (1). In their simplest form, interactions regulating transcriptional activity involve direct protein–protein contacts between components of the general transcription machinery and transcriptional activators bound to the promoter. Heterodimerization of many transcriptional regulatory proteins provides mechanisms for combinatorial regulation of gene expression. Among these transcriptional factors, the Fos (c-Fos, FosB, Fra-1 and Fra-2) and Jun (c-Jun, JunB and JunD) proteins are proto-oncogene products that regulate a diverse array of genes in response to mitogenic signals (2). These proteins belong to the AP-1 family of transcription factors. Fos-Jun heterodimers and Jun homodimers bind DNA, whereas the Fos homodimer is unstable and does not bind DNA. The Fos-Jun heterodimer is also capable of binding cooperatively with members of other transcription factor families.

Fos, Jun and the other AP-1 proteins are DNA-binding proteins, which are members of the family of basic-leucine zipper (bZIP) factors. On the basis of their amino acid sequence, the zipper motif was first identified as a heptad repeat of leucines interspersed with other hydrophobic residues. These regions form long α-helices that dimerize by forming a parallel coiled-coil. The basic region, located N-terminal to the coiled-coil, mediates DNA contacts. The basic region becomes ordered upon binding to DNA, forming an α-helix that lies in the DNA major groove and contacts specific base and phosphate groups. Recently, we have demonstrated that the oncogenic retrovirus HTLV-I (human T-cell leukaemia virus type I) codes for a bZIP factor, which was appropriately named HBZ (HTLV-I bZIP factor) (3). This novel HTLV-I-encoded protein is a 31 kDa protein that resembles a prototypical bZIP transcriptional factor (3), with an N-terminal transcriptional activation domain, a central domain involved in nuclear localization, and a C-terminal bZIP domain (4). HBZ interacts with c-Jun (5,6), JunB (5) and JunD (7) but is unable to interact with c-Fos (6) and to form stable homodimers (5). However, although HBZ and c-Fos can interact with c-Jun, they differ greatly in their abilities to activate transcription of AP-1-regulated genes. Indeed, unlike c-Fos, the interaction of HBZ with c-Jun results in the decrease of c-Jun DNA-binding activity (5,6) and

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prevents this protein from activating transcription of AP-1-dependent promoters and the basal expression of the HTLV-I promoter (5).

This difference between HBZ and c-Fos function provides an opportunity to better characterize the molecular mechanisms involved in the HBZ-mediated down-regulation of c-Jun activity. In this study, we generated and analyzed a large number of HBZ/c-Fos chimeras and found that the HBZ-mediated repression of c-Jun transcriptional activity is due to several substitutions of important amino acid residues in its DNA-binding domain. However, unexpectedly, we also found that exchange of a cluster of six amino acids, containing basic and acidic residues immediately adjacent to the DNA contact region, affected the transcriptional potency of the chimeras. This new data suggest that HBZ contains a short modulatory domain that is involved in determining the strength of the transcriptional response. To explain this unexpected observation, we then speculated that this particular subdomain of HBZ could be involved in the presentation of the AP-1 complex to the transcriptional machinery. In order to test this hypothesis, specific residues present in the cluster sequence of HBZ were substituted for the corresponding c-Fos amino acids and the effect of the mutated HBZ was tested in the presence of JunD. Indeed, as already described (7), the JunD DNA-binding activity was not modified in the presence of the wild-type HBZ and its transcriptional activity was stimulated. On the other hand, the mutated HBZ was no longer able to stimulate the transcriptional activity of JunD although no decreasing of the JunD DNA-binding activity was observed. This result confirms the involvement of this particular cluster in the modulation of Jun transcriptional potency. In addition, taken together, our results demonstrate that HBZ can now be considered as a novel member of the AP-1 family.

## MATERIALS AND METHODS

### Plasmid constructs

The pcDNA-HBZ-Myc vector has already been described (7). pCMV-JunD, pcDNA-c-Jun, pcDNA-JunB and pcDNA-c-Fos constructs were obtained from M. Piechaczyk. To generate the HBZ209/Fos chimera, the c-Fos cDNA was PCR amplified from the pcDNA-c-Fos vector, digested with HindIII, and subcloned in frame into HindIII-linearized pcDNA-HBZ-Myc, to produce a Myc-tagged chimera. The pcDNA-HBZ122-Myc vector was constructed by digesting pBIND-HBZ122 (3) with BamHI and KpnI and by subcloning the resulting HBZ fragment into similarly digested pcDNA3.1(−)/Myc-His. The c-Fos c-DNA digested by KpnI was then introduced in frame into the KpnI cloning site of the linearized pcDNA-HBZ122-Myc to produce the Myc-tagged chimeras HBZ122/Fos and HBZ122/bZIPFos. Different fragments of HBZ DNA were amplified by PCR and subcloned into pcDNA3.1(−)/Myc-His generating pcDNA-HBZ130-Myc, pcDNA-HBZ140-Myc and pcDNA-HBZ163-Myc. The c-Fos cDNA was then cloned in frame in these latter constructs using the HindIII restriction site to obtain HBZ130/bZIPFos and HBZ163/ZIPFos. For the pcDNA-HBZ140/bZIPFos construct, amplified c-Fos cDNA was first cloned into BamHI/HindIII-digested pcDNA-HBZ140-Myc. The artificially introduced BamHI site was then deleted to eliminate the two amino acids due to the insert of the restriction site and to reintroduce accurate amino acid sequence. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene) to produce H3F, H6F, H9F, H12F, H14F, EEEERE, EEEERR, HBZ and c-Fos mutants. All of the constructs were sequenced to ensure that no unintended mutations were introduced during PCR amplification.

### Analysis of *in vivo* expression of the chimeras by western blot analyzes

293T cells were transfected with 3 μg of expression vector using the jetPEI™ transfection reagent (Qbiogene). A total of 200 μg of protein extracts from transfected 293T cells were electrophoresed on to SDS–PAGE and blotted to polyvinylidene difluoride (PVDF) membranes (Millipore). Membranes were then incubated 1 h at room temperature in a blocking solution (phosphate-buffered saline [PBS] containing 5% milk) prior to addition of antiserum. The chimeras tagged with the Myc epitope, fused to their C-terminal end, were detected with the mouse anti-Myc antibody 9E10 (Sigma). After 1 h, membranes were washed three times with PBS-0.5% Tween-20 and incubated for 1 h with goat anti-mouse immunoglobulin-peroxydase conjugate. After three washes, membranes were incubated with the enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). Membranes were then exposed to hyperfilms-ECL (Amersham Pharmacia Biotech).

### Immunofluorescence microscopy analysis

After transfection with 1 μg of expression vector as described above, COS cells were cultivated on the glass slides and then analyzed by fluorescent microscopy at 36 h after transfection. The chimeras tagged with the Myc epitope were detected with the mouse anti-Myc antibody 9E10 (Sigma) and secondary goat anti-mouse IgG antibody coupled to FITC (Pierce). The same approach was carried out with COS cells transfected with pcDNA-c-Fos labeled with mouse anti-c-Fos antibody from Santa Cruz Biotechnology.

### Cotransfections and luciferase assays

CEM cells were transiently cotransfected according to the previously published procedure (8). A total of 5 μg of pcDNA3.1-*lacZ* (β-galactosidase-containing reference plasmid) was added in each transfection for controlling of the transfection efficiency. The total amount of DNA in each transfection was kept constant through the addition of appropriate quantities of empty plasmids. Equal amounts of proteins from each cell extracts were then used for luciferase and β-galactosidase assays.

### Microwell colorimetric AP-1 assays

Nuclear extracts (15 μg) were incubated with 30 μl of binding buffer [10 mM HEPES (pH 7.5), 8 mM NaCl, 12% glycerol, 0.2 mM EDTA, 0.1% BSA] in microwells coated with probes containing the AP-1 site (Trans-AM™ AP-1 of Active Motif Europe, Belgium). After a 1 h incubation at room temperature, microwells were washed three times with PBS containing 0.1% Tween-20. The AP-1-bound complexes were detected

with a mouse anti-c-Jun antibody or a goat anti-JunD antibody followed by the addition of a secondary peroxydase-conjugated antibody. For colorimetric detection, tetramethylbenzidine was incubated at room temperature before addition of the stop solution. Optical density was read at 450 nm, using a 620 nm reference wavelength with a Tecan microplate reader.

### Two-hybrid assays in yeast

Interactions between the chimeras and c-Jun were analyzed by two-hybrid assay in *Saccharomyces cerevisiae* strain HF7c. Strain HF7c possesses the *Escherichia coli lacZ* gene driven by three copies of the *GAL4* consensus sequence. The region of the chimeras containing the bZIP domain (from 123 to 209 amino acid) was cloned in frame with the GAL4 DNA-binding domain of the pGBT9 vector. Chimera cDNA was PCR amplified from pcDNA-HBZ/c-Fos-Myc, digested by EcoRI and BamHI, and cloned into pGBT9. Yeasts were cotransformed with pGBT9 and pGAD-c-Jun or pGAD-JunD as already described (9). The β-galactosidase assay with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate was carried out on three independent colonies per transformation according to the manufacturer's protocol (Clontech). The β-galactosidase activity was calculated in Miller units (10).

## RESULTS

### Analysis of chimeric proteins between HBZ and c-Fos

We have previously reported that, unlike c-Fos, a typical heterodimer partner of c-Jun, HBZ inhibited the *trans*-activating potential of c-Jun (5). To analyze whether the distinct behavior of HBZ and c-Fos could be attributable to specific regions of the protein, chimeras were constructed by swapping fragments of c-Fos and HBZ using restriction sites artificially introduced within inactive regions located between their activation domains and their DNA-binding domain. On the other hand, for chimeric constructs directly targeting functionally important regions, such as the bZIP domain (11), a restriction site was strategically added thereby maintaining correct phasing between the basic region and leucine zipper. For constructs in which this strategy was not feasible, the restriction site was deleted to regenerate the accurate amino acid sequence. All chimeric DNAs were inserted into the mammalian expression vector pcDNA3.1(−)/Myc-His permitting Myc tagging of each chimera at the C-terminus. *In vivo* expression of each chimera was analyzed by western blotting and their ability to interact with c-Jun was tested by a two-hybrid assay in yeast. Finally, the transcriptional activity of the wild-type and chimeric proteins were tested by cotransfection of corresponding expression vectors into CEM cells with pcDNA-c-Jun and a luciferase reporter vector carrying the AP-1-driven collagenase promoter.

The c-Fos protein contains five activation modules: two in its N-terminal region (12) and three others at its C-terminal end (13,14) while HBZ only possesses a single activation domain located in its N-terminal end (3). We first produced two chimeric constructs, HBZ122/Fos and HBZ209/Fos, depicted in Figure 1. Both chimeras contain the N-terminal activation domain of HBZ and the three C-terminal activation modules of c-Fos but differ in amino acid sequence from residue 123 to 209, which includes their respective bZIP domain (from residue 140 to 200). While HBZ122/Fos possesses the c-Fos bZIP domain, HBZ209/Fos contains the reciprocal HBZ bZIP domain (Figure 1). We first confirmed that both chimeras were stably expressed in transfected cells (Figure 2A) and were capable of interacting with c-Jun (data not shown). Upon cotransfection of each expression vector with the collagenase promoter-luciferase construct in CEM cells, we found that, similar to HBZ, the HBZ209/Fos chimera inhibited c-Jun activity while HBZ122/Fos, like c-Fos, maintained its activating potential toward c-Jun-dependent transcriptional activation (Figures 1 and 2B). To determine if the c-Fos activation domains AD3, AD4 and AD5 might contribute to this difference in c-Jun activation between both chimeras, we produced a novel chimera derived from the first 209 amino acids and termed HBZ122/bZIPFos, possessing the N-terminal activation domain of HBZ fused to the c-Fos region (residues 123 to 209) that contains the c-Fos bZIP domain (Figure 1). In the presence of HBZ122/bZIPFos, c-Jun was able to activate AP-1-dependent transcription (Figures 1 and 2C). Taken together, these data suggest that the HBZ region located between 123 and 209 amino acids is responsible for the HBZ inhibitory effect on the *trans*-activation potential of c-Jun.

### The cluster of residues immediately adjacent to the HBZ bZIP domain can negatively modulate c-Jun-dependent transcription

To better define the domains of HBZ involved in its repression activity, HBZ/c-Fos chimeras targeting shorter regions were derived. As shown in Figures 1 and 2C, the HBZ163/ZIPFos chimera was unable to stimulate AP-1-transcription in the presence of c-Jun, confirming the involvement of the DNA-binding domain of HBZ in its inhibitory action on c-Jun activity, as already suggested (5,6). On the other hand, HBZ130/bZIPFos and HBZ140/bZIPFos, both containing the bZIP domain of c-Fos, retained their capacity to *trans*-activate AP-1-transcription in the presence of c-Jun. However, unexpectedly, whereas HBZ130/bZIPFos was found to stimulate c-Jun dependent expression of the luciferase reporter gene by about 195-fold, i.e. at comparable levels to HBZ122/bZIPFos, luciferase activity was only enhanced by 39-fold upon transfection of the HBZ140/bZIPFos expression vector (Figure 2C).

To identifiy amino acids responsible for the functional differences observed between HBZ130/bZIPFos and HBZ140/bZIPFos chimeras, amino acid sequences of HBZ and c-Fos were compared in the 130–140 amino acid cluster (Figure 3A). It is worth noting that c-Fos in this region contains a stretch of six charged residues (EEEEKR from residues 134 to 139) that are only partially conserved in the HBZ sequence (EQERRE). Mutational analysis was thus targeted towards these residues. To determine which of these amino acids were important, HBZ-specific residues were replaced in HBZ140/bZIPFos with the corresponding c-Fos amino acid. We first constructed a mutant for which EQERRE was changed to EEEERE in order to reintroduce two negatively charged residues. The effect of this double

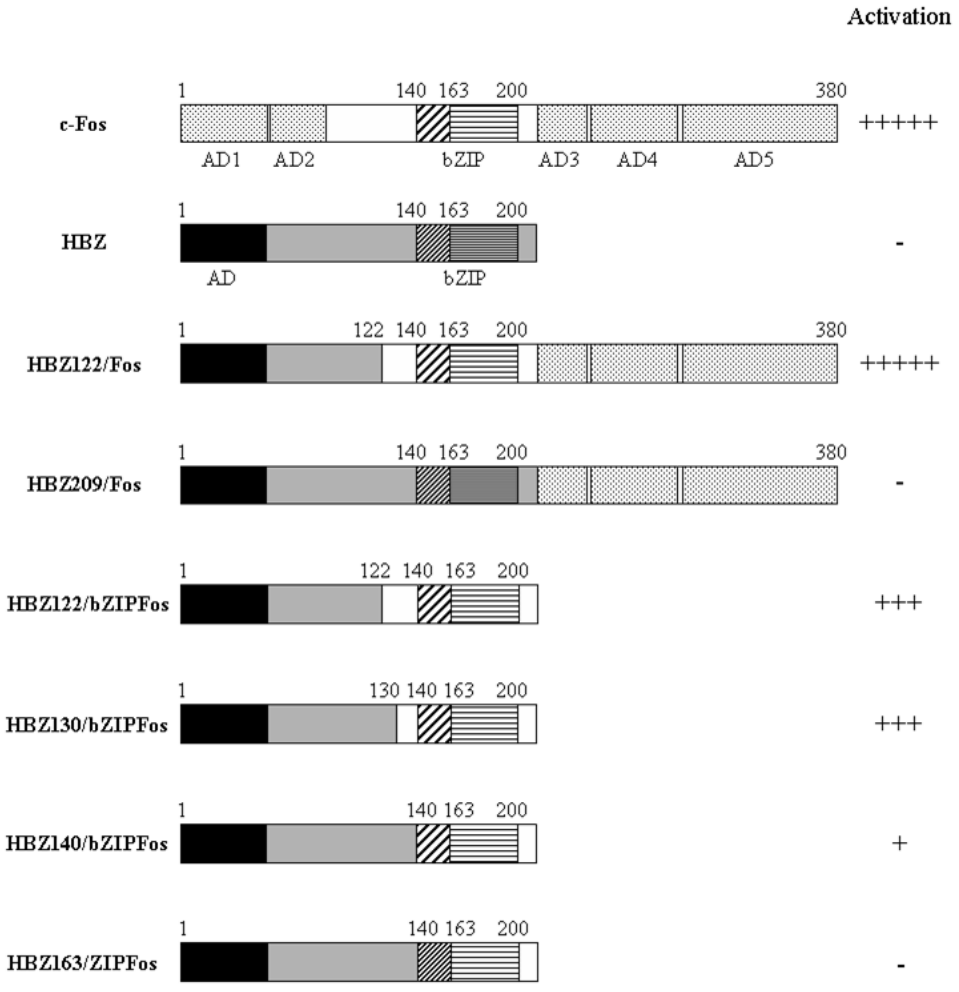


**Figure 1.** Structures of the chimeric proteins between c-Fos and HBZ and their effect on the *trans*-activation by c-Jun. Schematic representations of c-Fos, HBZ, and the various chimeras are shown; the chimeric proteins possess one or several activation domains (AD) and a bZIP structure including the DNA-binding domain (from residue 140 to 163) and the zipper (from 164 to 200). Expression plasmids of c-Fos, HBZ, and their chimeras were cotransfected with c-Jun expression plasmid together with a vector containing the luciferase gene driven by the collagenase promoter as described in the legend of Figure 2. The results are indicated on the right. Symbols: +++++, activation above 800-fold; +++, about 200-fold; +, about 40-fold; -, inhibition of c-Jun activity.

mutation was significant since the mutant activated expression of the luciferase reporter gene in the presence of c-Jun up to 125-fold (Figure 4A). A third modification ($E^{139}$ into R) was introduced in the cluster thereby generating the EEEERR amino acid stretch. This mutation resulted in 210-fold activation of the collagenase promoter, which was comparable to the activating potential of the HBZ130/bZIPFos chimera (Figure 4A). These data show that both the DNA-binding domain and the amino acid cluster adjacent to the bZIP domain can affect the *trans*-activating potency of the chimeras toward c-Jun-dependent transcription.

## The EQERRE motif of HBZ does not modify DNA-binding activity of the c-Fos bZIP domain

A possible explanation for our results is that the increased *trans*-activating potency of the chimeras results from an increase in their stability. This explanation was however ruled out by the results shown in the Figure 4B. HBZ140/bZIPFos and the two resulting mutants demonstrated protein levels comparable to HBZ130/bZIPFos in transfected cells. We also found that the chimeras were able to interact with c-Jun (Figure 4C). Another possible explanation for the observed difference in activating potential is that this cluster of six amino acids, particularly rich in charged residues and immediately adjacent to the DNA contact region, may interfere with the binding to the AP-1 motif. To test this possibility, we compared the DNA-binding activity of HBZ140/bZIPFos (EQERRE) with that of its mutated EEEERR form. To evaluate DNA-binding, we used the microwell colorimetric assay from Active Motif Europe (15) which we had previously used for the study of complex formation between c-Jun, HBZ and the AP-1 motif (5). Briefly, nuclear extracts of 293T cells transfected with c-Jun and either HBZ, c-Fos or HBZ/c-Fos chimera expression vectors were incubated in the presence of a double-stranded oligonucleotide containing the AP-1 site immobilized on a microwell plate. The DNA-binding activity was then measured by colorimetric assay using mouse anti-c-Jun antibodies. As shown in Figure 4D, there was no significant difference between the binding

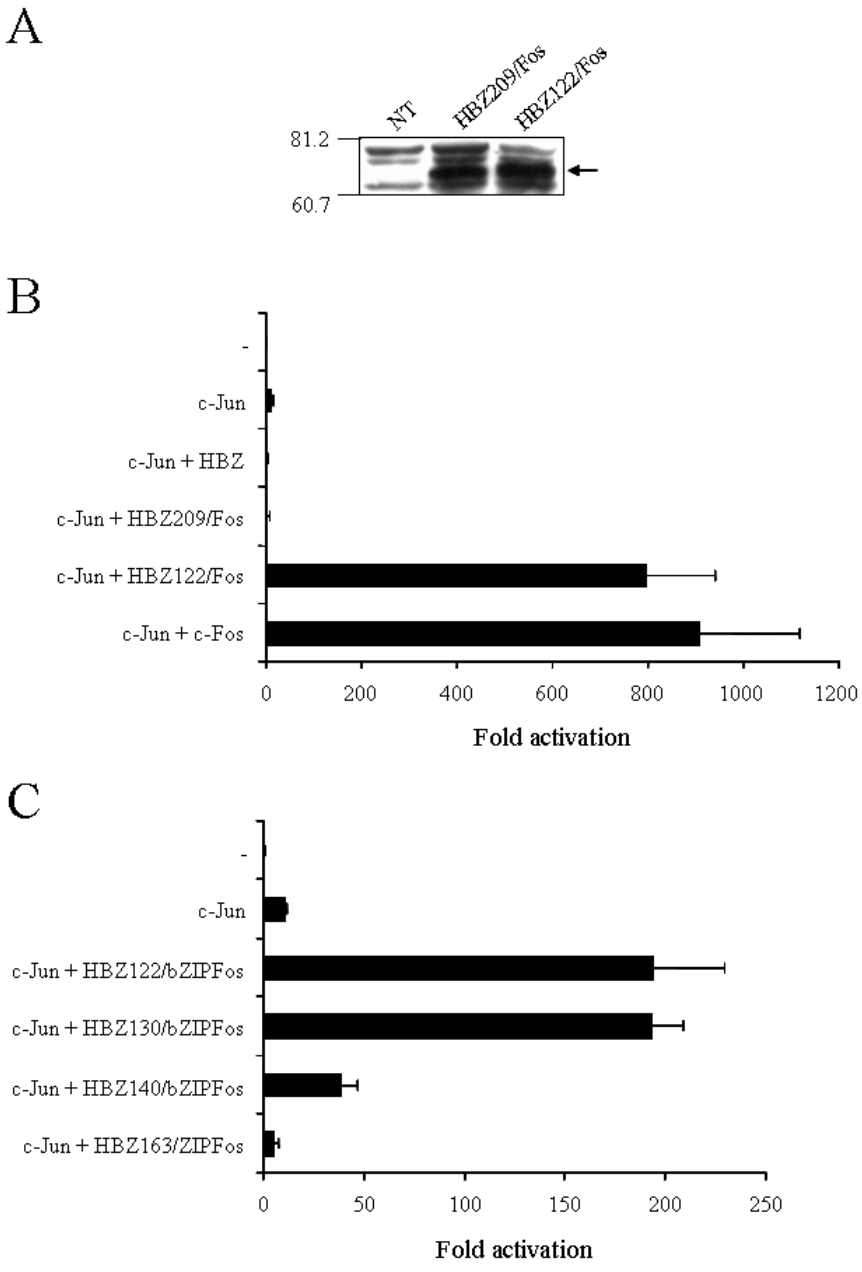


**Figure 2.** Characterization of the amino acid region involved in the differential activation of c-Jun by c-Fos and HBZ. (**A**) Expression of the chimeras HBZ122/Fos and HBZ209/Fos *in vivo*. Expression of the chimeric proteins in 293T cells was detected by western blotting using the mouse anti-Myc antibody (NT: not transfected). Molecular size markers (kDa) are shown on the left and migration of the chimeras is indicated by the arrow on the right. (**B** and **C**) Analysis of the *trans*-activation by the chimeras in the presence of c-Jun. CEM cells were cotransfected with 2 μg of a vector containing the luciferase reporter gene driven by the collagenase promoter, 5 μg of pcDNA3.1-*lacZ* (β-galactosidase-containing reference plasmid), 1 μg of pcDNA-c-Jun and 2 μg of the vector pcDNA3.1(−)/Myc-His expressing each of the tested chimera. Luciferase values are expressed as folf increases relative to values measured in cells transfected with empty pcDNA3.1(−)/Myc-His in the presence of the luciferase reporter vector. The total amount of DNA in each series of transfection was equal, the balance being made up with the empty plasmids. Luciferase values were normalized for β-galactosidase activity. Values represent the mean ± SD ($n = 3$).

activity of the tested chimeras while HBZ decreased c-Jun binding. This result was expected since the hexapeptide EEEEKR is not included in the amino acid sequence of the c-Fos bZIP domain known to be in contact with the AP-1 site in the presence of c-Jun (16). In conclusion, our data suggest that a stretch of six charged residues immediately adjacent to the bZIP domain of HBZ and c-Fos affects the transcriptional potency of the AP-1 complexes on targeted promoters.

### The DNA-binding domain of HBZ is involved in the down-regulation of c-Jun activity

We next studied the influence of association of the hexapeptide EEEERR with the DNA-binding domain of HBZ on c-Jun *trans*-activation. For these experiments, we decided to construct a mutant named H3F in which EQERRE was replaced in HBZ163/ZIPFos by the amino acid sequence EEEERR (Figure 3B). Unlike HBZ140/bZIPFos, this triple

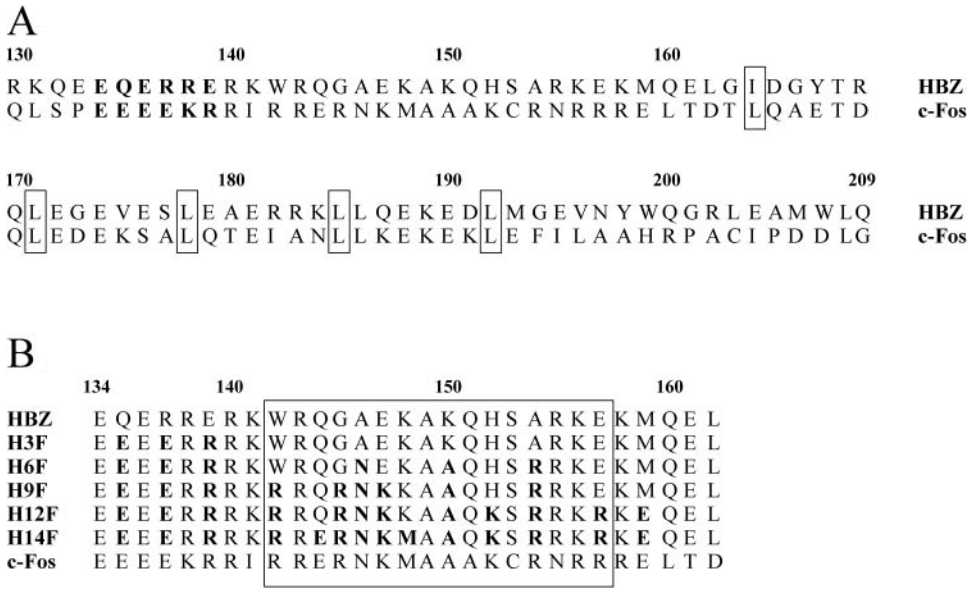


**Figure 3.** Comparison of the amino acid region adjacent to the bZIP domain between HBZ and c-Fos. The HBZ and c-Fos DNA-binding domains were aligned by using the basic motif and leucine zipper as a reference point. Numbering is relative to the +1 methionine of the chimeras shown in Figure 1. (**A**) The cluster of six charged residues studied in the paper (134–139 amino acids) is in bold and the leucine residues of the zipper are in boxes. (**B**) Comparison of the amino acid sequences adjacent to the leucine zipper of HBZ, c-Fos, and the different mutants produced from HBZ163/ZIPFos. The mutated residues are bold and the basic motifs of the DNA-binding domains (142–157 amino acids) are in a box. The conserved alanine and serine/cysteine residues of the basic motif correspond to position 149 and 153.

mutated HBZ163/ZIPFos chimera had no significant effect on c-Jun activity (Figure 5A). This difference can be explained by the presence of the particular DNA-binding domain of HBZ in the chimera HBZ163/ZIPFos. Indeed, the DNA-binding site in bZIP factors is composed of a stretch of predominantly basic amino acids lying immediately adjacent to the leucine zipper and containing an array of specific residues termed the basic motif corresponding to the consensus sequence bb-bN--AA-b(C/S)R-bb (17–19). The HBZ basic motif diverges from this consensus, lacking the conserved $N^{146}$, $A^{150}$ and $R^{154}$ and some basic residues ($R^{142}$, $R^{145}$, $K^{152}$ and $R^{157}$) present in the DNA-binding domain of c-Fos.

To precisely define residues that affect HBZ DNA-binding, specific amino acids in the HBZ basic motif were substituted for their equivalent in c-Fos (Figure 3). All mutants generated from this approach were derived from HBZ163/ZIPFos. Because $N^{146}$, $A^{150}$ and $R^{154}$ have been described to be highly conserved, these three residues were first reintroduced in the HBZ basic motif. This novel mutant, named H6F, did not however stimulate transcription in the presence of c-Jun (Figure 5A), although it was able to interact with c-Jun (Figure 5C). Three additional mutants were then produced: H9F, H12F and H14F, in which additional substitutions were generated in the HBZ basic motif (Figure 3). Only mutant H14F, for which 14 amino acid substitution had been introduced in the amino acid sequence encompassing residues 135 to 159 of HBZ, enhanced *trans*-activation in the presence of c-Jun (Figure 5A). All of the produced mutants had comparable stability (Figure 5B) and capacity to interact with c-Jun (Figure 5C).

To confirm that the *trans*-activity of H14F was effectively due to its modified basic region, we compared c-Jun DNA-binding activity in the presence of H14F and H3F, two chimeric proteins which only differ in their DNA-binding domain. Figure 5D shows that their ability to bind to the AP-1 motif was significantly different, with H14F/c-Jun heterodimers showing a higher affinity for AP-1 sequence than H3F/c-Jun heterodimers. Lastly, to rule out aberrant subcellular localization as an explanation for the absence of *trans*-activation for some chimeras, their localization was examined by immunofluorescence microscopy. As shown in Figure 6, all chimeras were found to localize to the nucleus.

In conclusion, our results demonstrate that HBZ down-regulates c-Jun activity mainly by forming a heterodimer with c-Jun that is severely reduced in its ability to recognize the AP-1 site.

## The EQERRE motif of HBZ positively modulates JunD transcriptional activity

To better understand the role played by the EQERRE motif in HBZ, this amino acid cluster was substituted by the amino acid sequence EEEERR, which is similar to the c-Fos motif (EEEEKR). Like the wild-type HBZ, this mutated protein, termed HBZ-mutMD (for mutated modulatory domain), could interact with Jun factors and was localized in the nucleus (data not shown). Moreover, HBZ-mutMD was stably expressed in transfected cells (Figure 7A). HBZ has been described to have different effects depending on its heterodimerization partner: while it represses c-Jun activity, HBZ augments the *trans*-activating potential of JunD (5,7). For this reason, the effect of the mutation on HBZ function was tested in the presence of either c-Jun, or JunD. We first analyzed the DNA-binding activity of c-Jun and JunD in the presence of HBZ-mutMD. As similarly described for the wild-type HBZ (5,7), HBZ-mutMD decreased DNA-binding activity of c-Jun (Figure 7B) but did not significantly alter

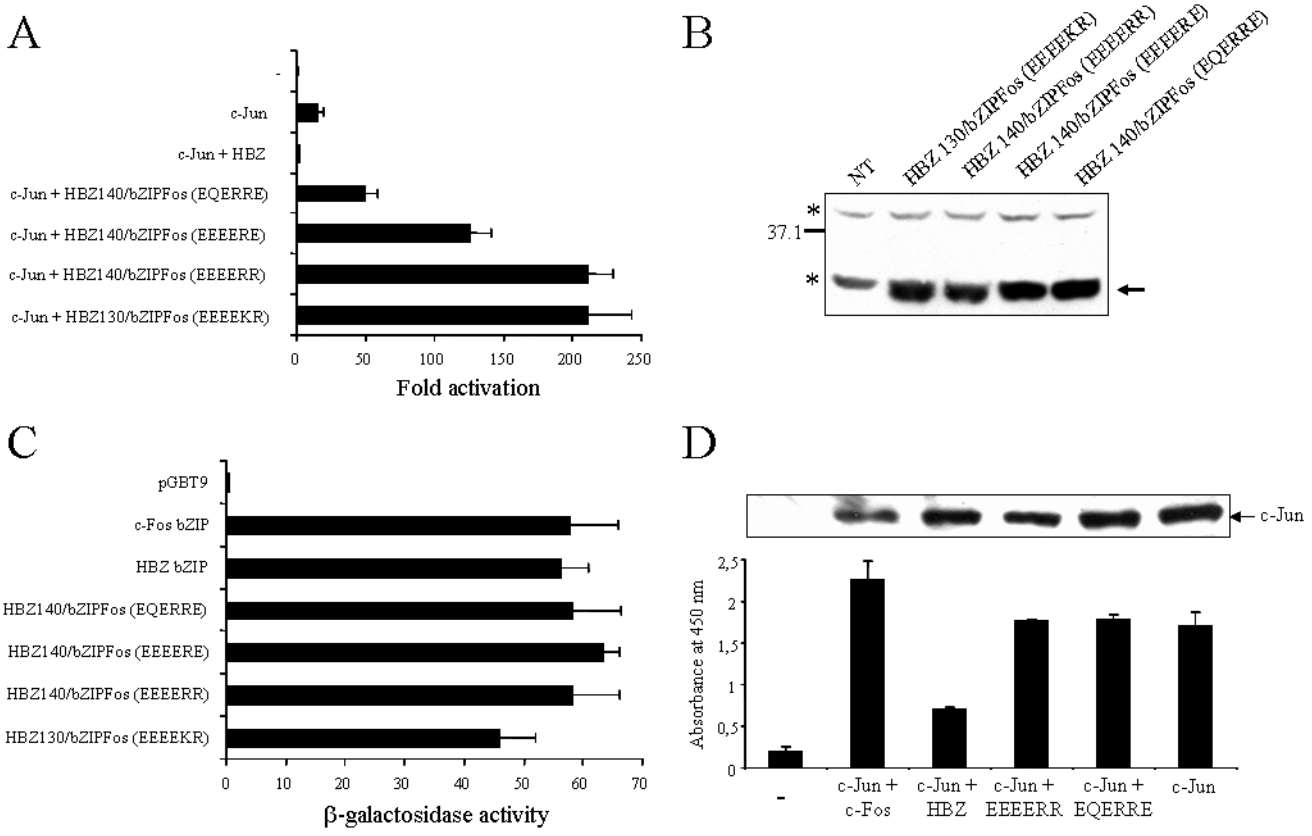


**Figure 4.** The EEEEKR sequence immediately adjacent to the bZIP domain of c-Fos confers a *trans*-activation potential to heterodimerized c-Jun. (**A**) The transcriptional activity of the mutated chimeras were analyzed as described in the legend of Figure 2. Bars labeled as EQERRE, EEEERE, EEEERR and EEEEKR correspond to cells tranfected by HBZ140/bZIPFos, the two mutated chimeras produced from HBZ140/bZIPFos, and HBZ130/bZIPFos, respectively. For comparison, luciferase activity was also measured from cells transfected with pcDNA-c-Jun alone or in the presence of the wild-type HBZ expression vector. (**B**) Expression of the chimeric proteins in 293T cells was detected by western blotting using the mouse anti-Myc antibody as already mentioned. Migration of the chimeras is shown by the arrow and the asterisks indicate non-specific bands. (**C**) Interaction study between the generated chimeras with c-Jun by yeast two-hybrid assay using a liquid culture β-galactosidase assay. Yeasts were transformed with the expression vector pGAD containing the entire coding sequence of c-Jun fused to the GAL4 activation domain along with pGBT9 expressing the GAL4 DNA-binding domain fused to the region encompassing residues 123 to 209 from c-Fos, HBZ, or the chimeras. The β-galactosidase was carried out on three independent colonies per transformation assay using ONPG as substrate. Mean values presented in the graph are expressed in Miller units. (**D**) DNA-binding activity of chimeras. Microwells containing the AP-1 binding probe were incubated with nuclear cell extracts of 293T cells cotransfected with 4 μg of pcDNA-c-Jun and 4 μg of the vector expressing c-Fos, HBZ, HBZ140/bZIPFos (EQERRE) or its mutated form (EEEERR). The negative and positive controls correspond to cells transfected by the pcDNA empty vector and pcDNA-c-Jun alone. The data represent the means of three values ± S.D. Above, immunoblotting of nuclear proteins from transfected 293T cells using anti-c-Jun antibodies.

JunD binding to the AP-1 site (Figure 7C). Transcriptional activities of both c-Jun and JunD were then studied in the presence of wild-type HBZ and its mutated form by cotransfection of their expression vectors into CEM cells. In comparison to wild-type, HBZ-mutMD could equally inhibit c-Jun-mediated transcriptional activity (Figure 7B). This result confirms that the repressing functional properties of HBZ on c-Jun activity are mainly due to its DNA-binding domain. On the other hand, the effect of HBZ-mutMD on JunD activity was found to be completely different from that of the wild-type since the mutant could no longer stimulate JunD transactivating potential (Figure 7C). We also tested the effect of HBZ-MutMD on JunB activity but, like c-Jun, no difference between the wild-type and the mutant was observed (data not shown). Moreover, during the preparation of this manuscript, it has been published that the HTLV-I genome can code for a new isoform of HBZ (20,21). We also mutated this isoform in the EQERRE motif and the same results were obtained (data not shown). Such an observation is not surprising since only the first four amino acids differ between both isoforms and our data have focussed on the modulatory role of the C-terminal region of the protein. In conclusion, our results demonstrate that the EQERRE cluster is involved in the activation by HBZ of JunD-mediated transcriptional activity.

In light of these results, it was interesting to determine whether an equivalent effect could be observed when the modulatory domain of HBZ was introduced in the context of the c-Fos amino acid sequence. Thus, the c-Fos EEEEKR motif was replaced by the EQERKE sequence, which is similar to the HBZ motif (EQERRE). As already shown in Figures 4 and 7, such a modification does not change the *in vivo* stability of the protein and its capacity for binding to the Jun factors and the AP-1 site. The activity of this new mutant (termed c-Fos-MutMD) was then analyzed in the presence of c-Jun, JunB and JunD. In the presence of c-Jun, whereas wild-type c-Fos stimulated expression of the luciferase reporter gene by 720-fold, luciferase activity was

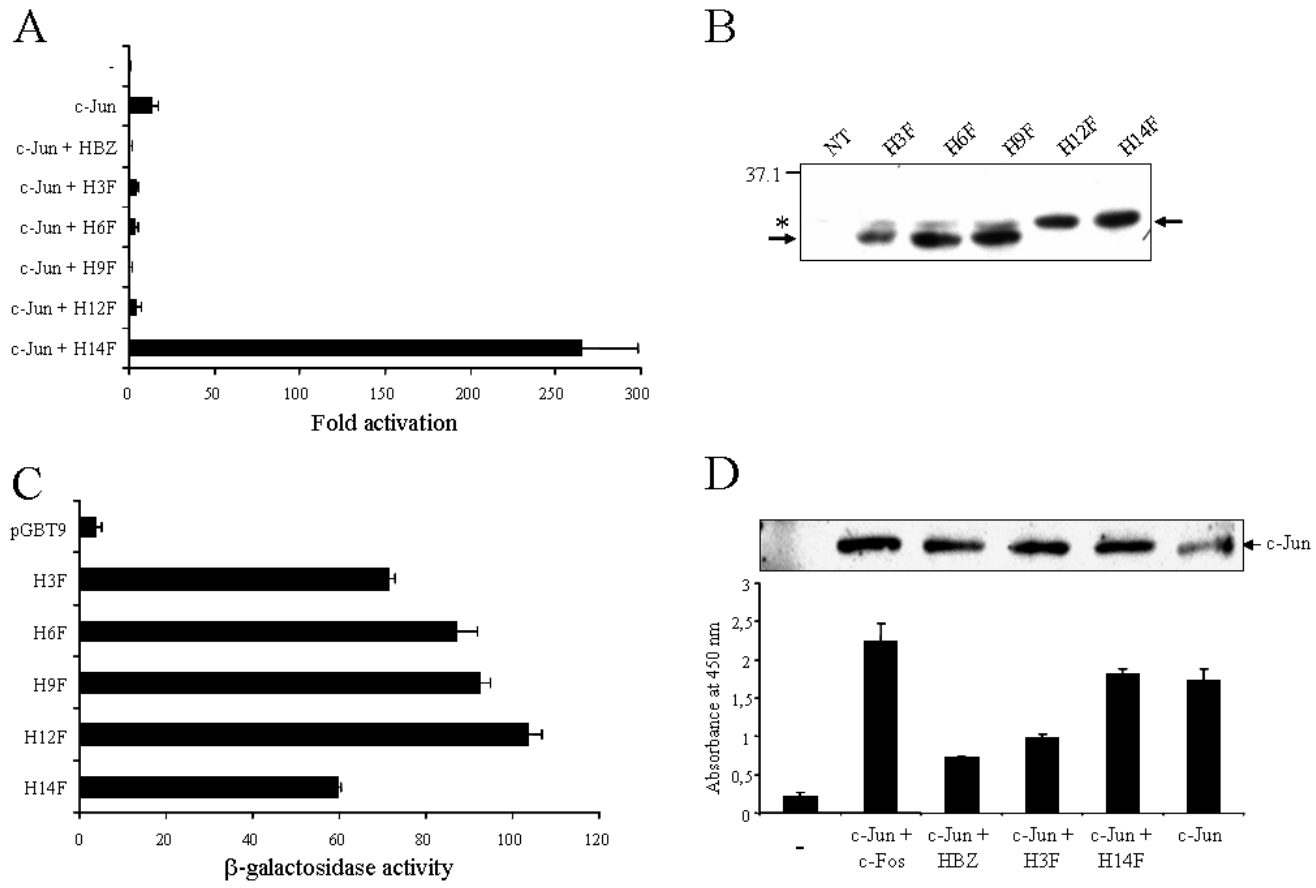


**Figure 5.** Activity analysis of c-Fos substitution mutants in the HBZ DNA-binding domain. (**A**) The transcriptional activity and (**B**) *in vivo* expression were analyzed as described in the legend of Figure 2. (**C**) The interaction with c-Jun and (**D**) the DNA-binding activity of H3F and H14F mutants were tested as described in the legend of Figure 4.

only enhanced by 240-fold upon transfection of the c-Fos-MutMD expression vector (Figure 8). This result shows that the modulatory domain of c-Fos affects the transcriptional potency of the c-Fos/c-Jun heterodimer, confirming our results with the HBZ/c-Fos chimeras. On the other hand, the effects were less drastic with the two other Jun factors, especially with JunB (Figure 8). In the presence of JunB, luciferase activity was stimulated by 400- and 320-fold by c-Fos and c-Fos-mutMD, respectively. Nonetheless, these data indicate that the modulatory domain is crucial for regulation of c-Jun transcriptional potency by c-Fos.

## DISCUSSION

An earlier study from our team had for the first time demonstrated the existence of a bZIP transcriptional factor encoded on the complementary strand of the HTLV-I RNA genome (3). Subsequent studies from our group and others have demonstrated that HBZ inhibits c-Jun activity by forming heterodimers deficient in their ability to form stable complexes on the AP-1 motif (5,6). Sequence comparison indicates that HBZ possesses a particular DNA-binding domain that lacks the consensus amino acid sequence bb-bN--AA-b(C/S)R-bb thought to be critical for DNA-binding (3,22). It was therefore anticipated that this defective DNA-binding domain would contribute importantly to the functional differences between HBZ and c-Fos. Indeed, in this study, the generation of the different chimeras through the swapping of various segments of c-Fos and HBZ indicates that this basic motif has an important impact on the transcriptional activity observed for both transcription factors in the presence of c-Jun. However, we also found that swapping of an amino acid cluster rich in charged residues (EEEEKRR in c-Fos substituted for EQERRER in HBZ) affects transcriptional potency of the chimeras. Thus, HBZ and c-Fos possess specific amino acid motifs immediately adjacent to their DNA-binding domain involved in determining the strength of their transcriptional response. This conclusion is not only based on our results but also on the study of the transforming properties of c-Fos. Indeed, analysis of mutant c-Fos proteins has shown that the presence of an intact EEEEKRR motif is a prerequisite for the induction of transformation (23). The presence of such a modulatory sequence is not the only common point between HBZ and the members of the Fos-protein family. Indeed, HBZ does not form stable homodimers (5) and is unable to interact with c-Fos (6). On the other hand, like c-Fos, HBZ interacts with c-Jun, JunB and JunD (5–7). Taken together, all these observations suggest that HBZ needs to be classified as a new member of the AP-1 family.

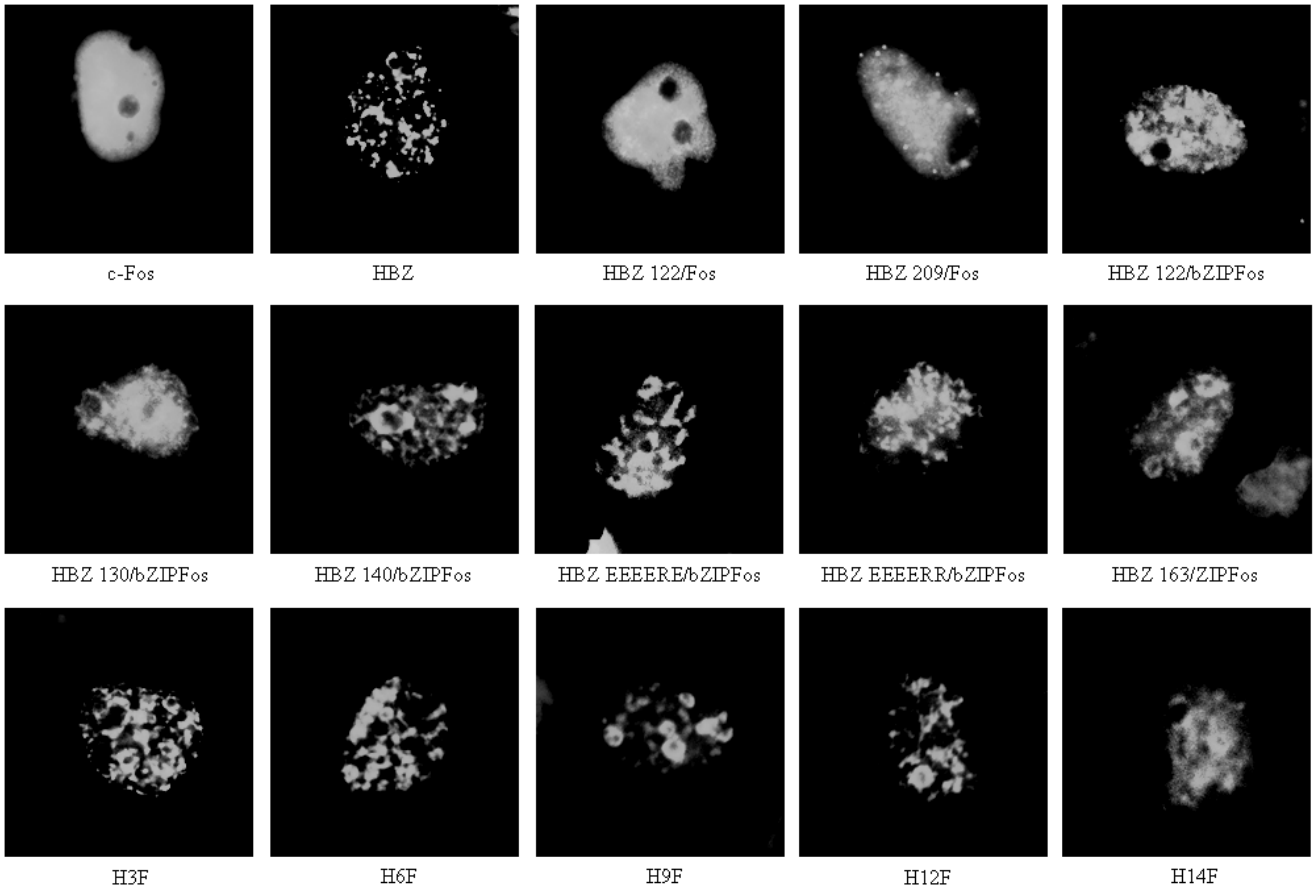


**Figure 6.** Subcellular localization of HBZ, c-Fos and the different chimeras in COS cells. Expression vectors for HBZ, c-Fos and the different chimeras were transiently transfected into COS cells. Cells were cultivated on glass sides, fixed, and stained with the Hoechst solution. The localization of the Myc-tagged chimeras was analyzed by immunofluorescence microscopy using the mouse anti-Myc antibody and goat anti-mouse IgG antibodies coupled to FITC. The blue fluorescence of the nuclei results from ultraviolet (UV) illumination of the fixed cells.

We also demonstrate that mutations in the c-Fos EEEEKRR motif do not alter the capacity of the protein to bind to the AP-1 site and to interact with Jun factors. These observations suggest that this sequence could play a role in the architecture of the protein complexes bound to the promoter. c-Fos/c-Jun heterodimers have been found to induce DNA bending (24,25) and substitution of two basic residues ($R^{139}$ and $R^{140}$ of the Figure 3) adjacent to the c-Fos bZIP domain by neutral and acidic residues resulted in a reduction in DNA bending (26). Thus, residues adjacent to the bZIP domain are likely to influence DNA bending (27,28). Moreover, it has also been demonstrated that DNA bending determines c-Fos/c-Jun heterodimer orientation and that the orientation of the DNA-bound complex strongly affects its transcriptional potency (29,30). Again it has been clearly shown that the basic residues KRR influence the orientation of heterodimer binding to the AP-1 site (30,31). All these observations could explain why mutation of the amino acid sequence EQERRER into EEEERRR was required to obtain full transcriptional activation in the presence of c-Jun. However, the chimeric mutant EEEERER was able to significantly stimulate AP-1-dependent transcription although it did not contain the KRR motif adjacent to its bZIP domain. These results suggest that the acidic domain adjacent to the basic residues could also be involved in the formation of a potent transcriptional complex. Moreover, a negatively charged region located at a more distant position from the bZIP domain of c-Jun has been suggested to induce DNA bending in the opposed direction from the bending caused by the binding of Jun homodimers and to counteract c-Fos-dependent DNA bending in the context of heterodimers (26). The transcription activation domains of c-Fos and c-Jun, which contain clusters of charged residues, also induce DNA bending (32). Altogether, these observations suggest that DNA bending by c-Fos and c-Jun is mediated at least in part by charge interactions. In conclusion, the cluster of acidic residues at the extreme N-terminal end of the c-Fos bZIP domain might be at a sufficiently close proximity to the DNA backbone to influence the protein architecture of the AP-1-bound complex. This issue would be of particular importance with respect to the spatial relationship of the activation domain with chromatin remodelling factors and proteins associated with RNA polymerase II.

It is worth noting that the presence of this particular cluster in HBZ modulates JunD activity. Unlike c-Jun, HBZ does not reduce the JunD ability to bind to the AP-1 site. Moreover, HBZ stimulates the JunD transcriptional activity (7). Interestingly, when the HBZ EQERRE motif was substituted by the c-Fos modulatory domain, the mutated HBZ was no longer able to stimulate the transcriptional activity of JunD although

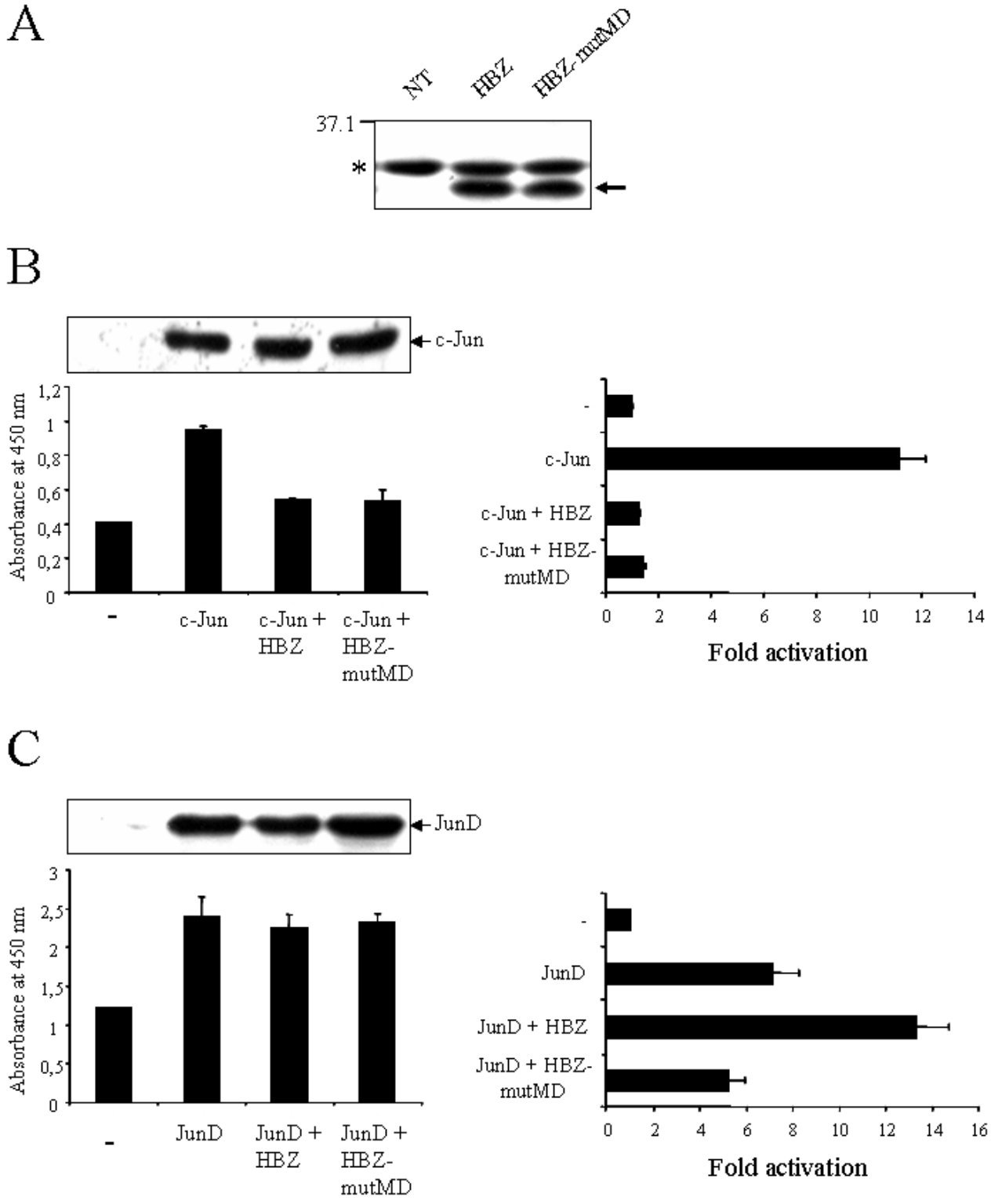


**Figure 7.** The EQERRE sequence immediately adjacent to the bZIP domain of HBZ confers a *trans*-activation potential to JunD. *In vivo* expression of the HBZ-mutMD protein (**A**) and its effect on c-Jun (**B**) or JunD (**C**) DNA binding (on the left) and transcriptional (on the right) activities were analyzed as described in the legends of the other figures.

no decreasing of the JunD DNA-binding activity was observed. At first glance, this result seems paradoxical even though the regulation of JunD activity, compared to c-Jun, is known to be different. Indeed, JunD might not normally exist in a free form, but only as a complex with menin (33). This factor is the product of the multiple endocrine neoplasia type 1 gene and is known to be a tumor suppressor. Menin specifically interacts with JunD (and not with c-Jun)

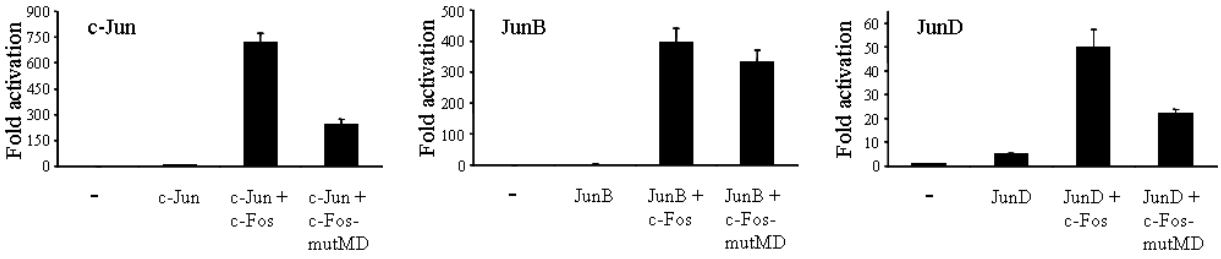


**Figure 8.** The modulatory domain of c-Fos affects Jun transcriptional potency. The transcriptional activity of c-Fos-mutMD was analyzed in the presence of c-Jun, JunB or JunD. For comparison, luciferase activity was also measured from cells transfected with the wild-type c-Fos expression vector. CEM cells were cotransfected with 2 μg of a vector containing the luciferase reporter gene driven by the collagenase promoter, 5 μg of pcDNA3.1-*lacZ*, 1 μg of Jun expression vector alone or with 1 μg of pcDNA-c-Fos or pcDNA-c-Fos-MutMD. Luciferase values are expressed as fold increases relative to values measured in cells transfected with the luciferase reporter vector alone. The total amount of DNA in each series of transfection was equal, the balance being made up with the empty plasmids. Luciferase values were normalized for β-galactosidase activity. Values represent the mean ± SD ($n = 3$).

through a N-terminal domain exclusively present in JunD (34,35). Menin represses JunD transcriptional activity by recruiting a histone deacetylase complex through association with mSin3A, a transcriptional corepressor (36). It is tempting to speculate that the presence of HBZ might influence the recruiting of HDACs by JunD. Experiments are underway to further evaluate further this possibility. Moreover, the results obtained with our HBZ mutant modified in the modulatory domain strengthen the idea that this particular cluster present in HBZ would influence the architecture of Jun proteins bound to the promoter, thus forming a complex with lesser accessibility to transcriptional regulators, i.e. activators for c-Jun and repressors for JunD.

In conclusion, we demonstrate that, in its C-terminal region, HBZ possesses two subdomains involved in the regulation of the *trans*-activation potential of Jun factors. The DNA-binding domain of HBZ seems preferentially involved in the down-regulation of c-Jun by forming heterodimers with a reduced stability on the AP-1 site when compared to c-Jun homodimers. This HBZ-specific negative effect on c-Jun transcriptional activating function could lead to the inhibition of viral transcription (5,37). On the other hand, the second domain corresponding to a cluster of six charged amino acids might rather be involved in the modulation of JunD activity. Moreover, the presence of the activation domain of HBZ has also been described to be necessary for *trans*-activation of JunD (7) but not to be an important modulator of viral transcription (3), suggesting that the effect of HBZ on JunD could have a stronger impact on the control of cellular promoters. In addition, taken together, our results demonstrate that HBZ can be considered as a novel member of the AP-1 family and confirm the potential role of HBZ in the control of the development of HTLV-I-associated pathologies including adult T-cell leukaemia (ATL) and inflammatory disorders.

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*Conflict of interest statement*. None declared.

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