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# Nuclear localization of HTLV-I bZIP factor (HBZ) is mediated by three distinct motifs

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## Summary

The genome of the human T-cell leukemia virus type I (HTLV-I) codes for a basic leucine zipper protein, HBZ, capable of repressing JUN activity and viral transcription. Transient expression in mammalian cells showed that HBZ was targeted to the nucleus, where it accumulated in nuclear speckles. By using a complementary set of deletion mutants, we report here that the nuclear targeting of HBZ is mediated by three distinct nuclear localization signals and that at least two are necessary for the translocation of HBZ to the nucleus. Moreover, the resulting mutant proteins distribute throughout the nucleoplasm and/or into the nucleoli, whereas the wild-type HBZ exclusively accumulates in nuclear speckles, suggesting that the

integrity of the protein is required for its speckle localization. We also demonstrate that the HBZ-containing speckles do not correspond to Cajal bodies, splicing factor compartments, or promyelocytic leukemia oncoprotein bodies. Unexpectedly, by using immunogold electron microscopy, we found HBZ localized to heterochromatin. Until now, such characteristics had never been described for a transcription factor and could explain the inhibitory activity of HBZ.

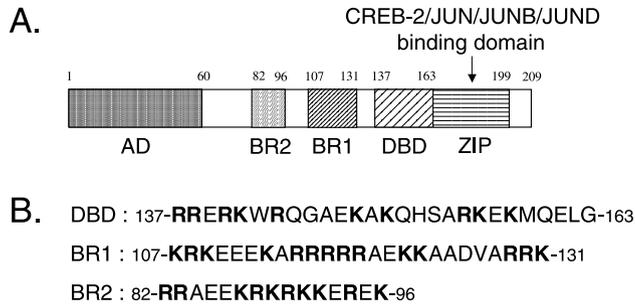
Key words: HTLV-I, HBZ, bZIP protein, Nuclear import, Heterochromatin

## Introduction

Basic leucine zipper (bZIP) factors have provided important insights into transcriptional regulation of cellular genes implicated in the regulation of processes relevant to energy metabolism, proliferation, differentiation, cell death and expression of cell-type-specific genes. The common structural feature of these regulatory proteins is the presence of a bZIP domain that consists of a basic region followed by heptad repeats of hydrophobic residues forming a leucine zipper. The segment containing these periodic arrays of leucine residues exists in an  $\alpha$ -helical conformation. The leucine side chains extending from one  $\alpha$ -helix interact with those from a similar  $\alpha$ -helix of a second polypeptide, facilitating dimerization. The ZIP motif thus allows the association of a single protein or potentially different proteins, explaining the heterotypic complex reported for some bZIP factors (Newman and Keating, 2003). Moreover, the 30 amino acid-long region, rich in basic residues, immediately preceding the ZIP is required for DNA binding and is also sufficient to ensure transfer of the protein to the nucleus. The bZIP pattern is present in many cellular transcription factors, such as the activating transcription factor/cAMP response element (CRE)-binding proteins (ATF/CREB), the activator protein-1 (AP-1) transcription factors, the CCATT-box/enhancer-binding proteins (C/EBP), the yeast general control protein 4 (GCN4), the *MYC*, *MYCL1* and *MYCN* oncogenes (previously *c-Myc*, *L-Myc* and *N-Myc*, respectively), the octamer-binding transcription factor (Oct-

2/OTF-2), the Maf transcription factors, and the Cap'n'Collar (CNC)-bZIP family. Moreover, the human  $\gamma$ -herpesviruses and human T-cell leukemia virus type I (HTLV-I) genomes encode proteins also related to the bZIP family and are involved in the regulation of viral and cellular transcription (Hivin et al., 2003; Sinclair, 2003).

HTLV-I is the etiological agent of the adult T-cell leukemia. This oncogenic retrovirus transforms T cells via its regulatory protein Tax, which dysregulates cell-cycle progression and causes genomic instability (Jeang et al., 2004; Mesnard and Devaux, 1999). In addition, Tax interferes with cell growth control pathways through activation of transcriptional factors including NF- $\kappa$ B (Sun and Ballard, 1999), E2F (Lemasson et al., 1998) and AP-1 (Iwai et al., 2001). Tax is also involved in viral transcription regulation by interacting physically with CREB (Adya and Giam, 1995; Goren et al., 1995; Yin et al., 1995), CREM (Suzuki et al., 1993), CREB-2 (Gachon et al., 2000), ICER (Newbound et al., 2000), or C/EBP $\beta$  (Hivin et al., 2004). The characterization of the interactions between Tax and different cellular bZIP proteins suggests that these factors play a key role in the HTLV-I cycle. Recently, we have confirmed this idea by demonstrating that HTLV-I itself codes for a bZIP factor, named by us HBZ (Gaudray et al., 2002). HBZ is a 209 amino acid-long protein with a C-terminal bZIP domain that interacts in HTLV-I-infected T-cell lines with CREB-2 (Gaudray et al., 2002), JUN (Basbous et al., 2003), JUNB (Basbous et al., 2003) and JUND (Thébault et al., 2004). Whereas Tax is able to activate viral transcription and the AP-



**Fig. 1.** Molecular structure of HBZ. (A) HBZ is composed of an N-terminal activation domain (AD), two basic regions (BR1 and BR2) and a DNA-binding domain (DBD) preceding its leucine zipper (ZIP). (B) Amino acid sequences of DBD, BR1 and BR2. The basic amino acids are indicated in bold.

1 pathway, HBZ inhibits Tax-dependent viral transcription (Gaudray et al., 2002) and JUN activity (Basbous et al., 2003). Indeed, by interacting with CREB-2 and JUN, HBZ forms heterodimers that are no longer able to bind to the viral promoter and to the AP-1 site (Basbous et al., 2003; Gaudray et al., 2002). Thus, HBZ could be a negative modulator of the Tax effect on the infected cell.

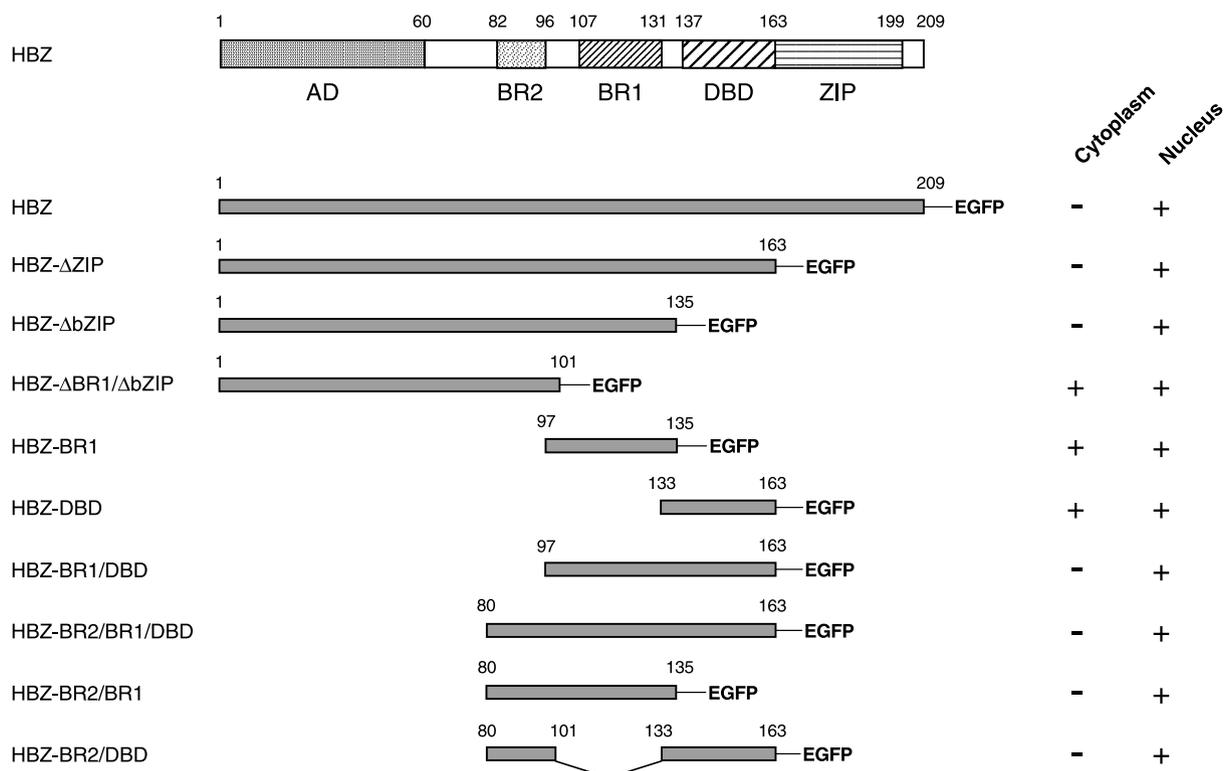
HBZ is targeted to the nucleus, where it exhibits a characteristic granular distribution (Gaudray et al., 2002). To define the protein domains required for HBZ translocation to the nucleus and subnuclear localization, mutants and fragments of HBZ fused to the enhanced green fluorescent protein (EGFP) were expressed in COS cells. This study has enabled

us to map three nuclear localization signals (NLSs) to a region in the central domain of HBZ that also contains the basic subdomain of the bZIP. Until now, the presence of three distinct NLSs had never been described for the other members of the bZIP protein family. Moreover, we found that the integrity of HBZ was necessary to observe its accumulation in nuclear speckles. We show furthermore that these nuclear sites containing HBZ correspond to heterochromatin. These studies support the hypothesis that HBZ could negatively regulate activity of some cellular bZIP factors by blocking them in transcriptionally inactive nuclear sites.

## Materials and Methods

**In vivo expression of HBZ polypeptides tagged with either EGFP or Myc epitope**

To express the different forms of HBZ protein with an EGFP tag, the coding sequences were subcloned into the plasmid pEGFP-N1. The HBZ cDNA fragments were generated by PCR amplification using Deep Vent DNA polymerase, pCI-HBZ as template (Gaudray et al., 2002), and specific sense (containing an *EcoRI* site) and antisense (containing a *BamHI* site) primers. The HBZ cDNA fragments were inserted in-frame into the *EcoRI/BamHI* cloning sites of the linearized pEGFP-N1 plasmid. Moreover, the cDNA fragment of two mutants, HBZ- $\Delta$ ZIP and HBZ-BR2/BR1/DBD, was subcloned in-frame into the *EcoRI/BamHI* cloning sites of the linearized pcDNA3.1(-)/Myc-His, to produce in vivo Myc-tagged HBZ proteins. All DNA constructs were verified by restriction analysis and by sequencing. The plasmid pcDNA3.1(-)/Myc-His-HBZ- $\Delta$ AD has been previously described (Thébault et al., 2004). COS cells were transfected by using the FuGENE 6 transfection reagent (Roche Applied Science) with 3  $\mu$ g of vector, and expression of the fusion



**Fig. 2.** A summary of subcellular locations of HBZ and its deletion mutants. The indicated HBZ-EGFP fusions were expressed in COS cells and their subcellular distribution was analyzed by fluorescence microscopy.  $\Delta$ , deletion.

proteins was analyzed by western blotting with either the mouse anti-EGFP antibody (Clontech) or the mouse anti-Myc antibody 9E10 (Sigma).

### Western blot assays

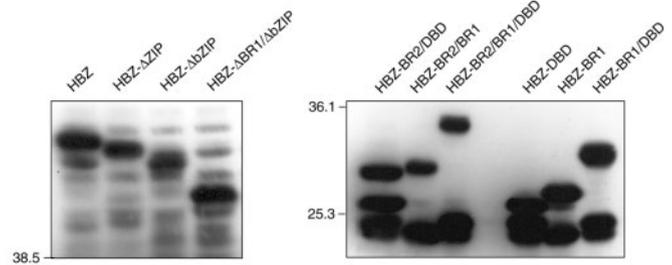
Protein extracts from transfected COS cells were electrophoresed onto sodium dodecyl sulfate-10% polyacrylamide gel (SDS-10% PAGE) and blotted to polyvinylidene difluoride (PVDF) membranes (Millipore). The blot was then incubated for 1 hour at room temperature with a blocking solution [phosphate-buffered saline (PBS) containing 5% milk] prior to addition of antiserum. After 1 hour, the blot was washed three times with PBS-0.5% Tween 20 and incubated for 1 hour with goat anti-mouse immunoglobulin-peroxydase conjugate. After three washes, the membrane was incubated with enhanced chemiluminescence (ECL) reagent (Amersham Pharmacia Biotech). The membrane was then exposed to hyperfilms-ECL (Amersham Pharmacia Biotech).

### Fluorescence microscopy analysis

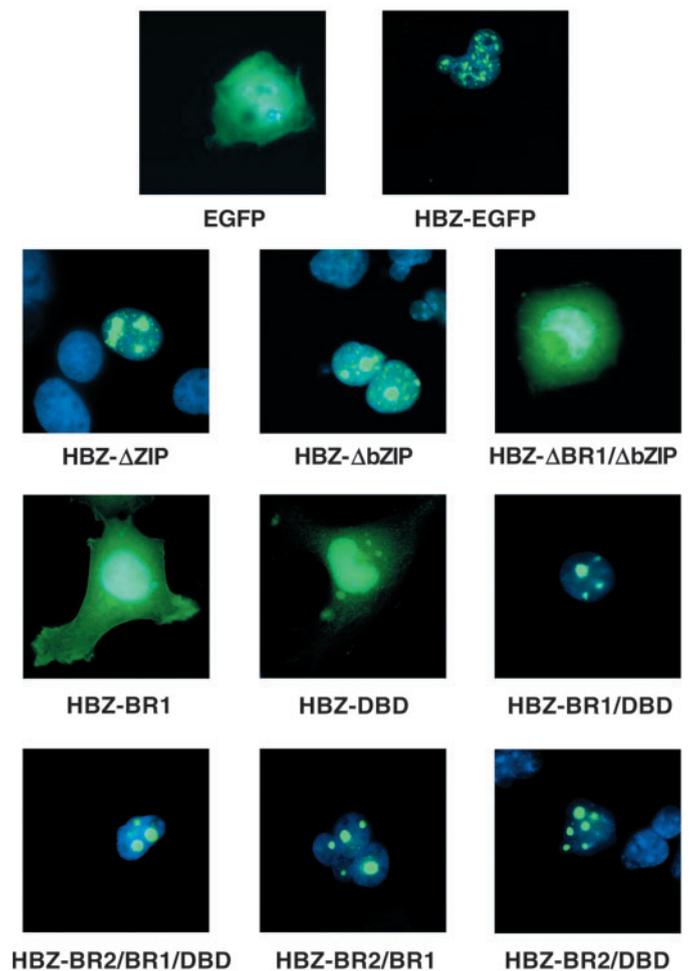
After transfection with 1  $\mu$ g of expression vector, COS cells were cultivated on glass slides and then analyzed by fluorescence 36 hours after transfection. HBZ tagged with the Myc epitope, fused to its C-terminal end, was detected using the mouse anti-Myc antibody 9E10 (Sigma) and secondary goat anti-mouse IgG antibody coupled to FITC (Pierce). For colocalization analyses, COS cells were first transfected with the mutants HBZ-BR1/DBD, HBZ-BR2/BR1 and HBZ-BR2/DBD fused to EGFP and cultivated on glass slides. Endogenous nucleolin was detected using the mouse anti-C23 antibody from Santa Cruz Biotechnology and goat anti-mouse IgG antibody coupled to TRITC (Pierce). Analysis of the green, red and merged fluorescence was performed by fluorescence microscopy. The same approach was carried out with COS cells cotransfected with HBZ-EGFP and PML protein or Tax expression vectors, and labeled with mouse anti-PML protein antibody or with culture supernatant of the anti-Tax 168A51-42 hybridoma, respectively. Anti-Tax was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). HTLV-I Tax hybridoma 168A51-42 (Tab176) was from B. Langton.

### Immunogold electron microscopy

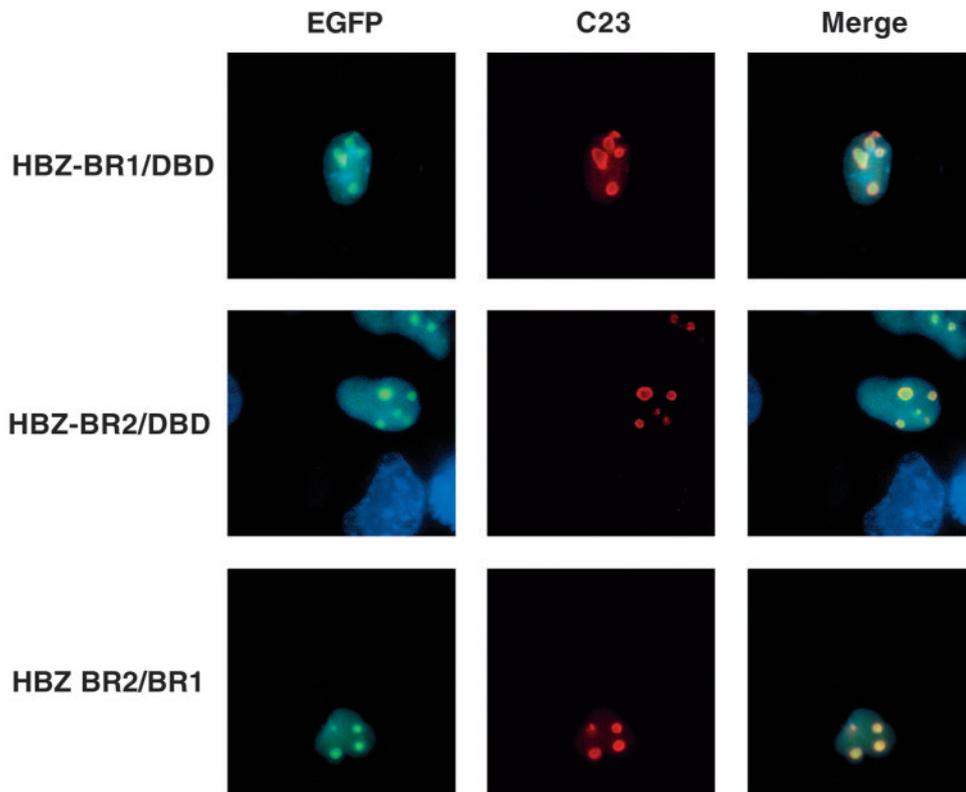
COS cells transfected with pcDNA3.1(-)/Myc-His-HBZ (Thébault et al., 2004) were fixed at 4°C in a 2.5% formaldehyde solution buffered with PBS for 90 minutes. They were thoroughly washed in PBS supplemented with 0.05 M NH<sub>4</sub>Cl to quench the free aldehyde groups for 2 hours at 4°C. A last wash without NH<sub>4</sub>Cl was performed for 1 hour. Next, the cells were peeling with a cell scraper and pelleted in a microtube at 120 g for 5 minutes. To dehydrate and embed the cells easily, the pellet was suspended in fibrinogen (5 mg/ml in PBS) and clothing of fibrin around the cell was induced by addition of thrombin (100  $\mu$ g/ml in 0.01% CaCl<sub>2</sub> in water). The specimens were dehydrated through progressive ethanol series and embedded in methacrylate resin (Lowicryl K4M, Chemische Werke Lowi) at -20°C to reduce extraction and damage of the antigens on the cells. They were photopolymerized by ultraviolet light (360 nm) at -20°C for two days. Ultrathin sections were cut with a Reichert OMU2 ultramicrotome, picked up with 300-mesh gold grids, and then processed for staining with the primary antibody, the mouse anti-Myc antibody, overnight at 4°C. Sections were washed, incubated with goat anti-mouse IgG antibody conjugated to 10 nm gold particles for 1 hour. After washing, the grids were stained with 2% uranyl acetate in water for 20 minutes and after a last washing the grids were air-dried and examined on a Hitachi H7100 electron microscope. Controls were done by omitting the first antibody and with COS cells transfected with the vector pcDNA3.1(-)/Myc-His.



**Fig. 3.** Western blot analysis of COS cells transfected with the different vectors expressing HBZ-EGFP fusion proteins. Expression of the different fusion proteins was analyzed by SDS-PAGE and western blotting with a mouse anti-EGFP antibody. Molecular size markers (kDa) are shown on the left.



**Fig. 4.** Subcellular localization of HBZ and its mutants in COS cells. HBZ and its mutants fused to EGFP were transiently transfected into COS cells. Cells were cultivated on glass slides, fixed and stained with Hoechst solution, and then green fluorescence was analyzed by fluorescence microscopy. The specificity of the fluorescent staining is indicated by the absence of signal in flanking untransfected cells. The blue fluorescence of the nuclei is visualized by UV illumination. Magnification, 1000 $\times$ .



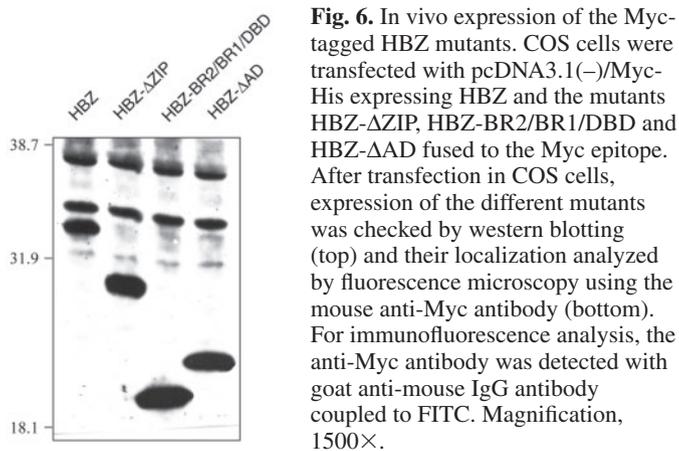
**Fig. 5.** Colocalization of HBZ mutants deleted of their N- and C-terminal domains with C23 in the nucleoli. COS cells transfected with the mutants HBZ-BR1/DBD, HBZ-BR2/DBD and HBZ-BR2/BR1 fused to EGFP (column EGFP) were labeled with a mouse anti-C23 antibody (column C23) and detected using goat anti-mouse IgG antibody coupled to TRITC. Analysis of the green, red and merged fluorescence was performed with a fluorescence microscope. Magnification, 1200 $\times$ .

## Results

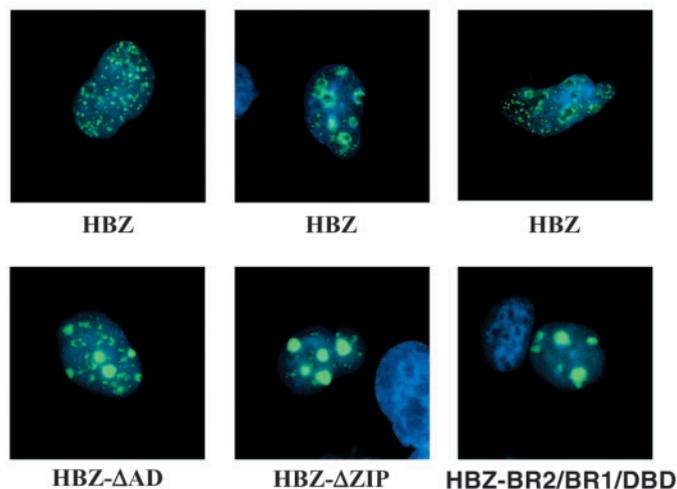
### Mapping of HBZ NLSs

The structure of HBZ resembles that of a prototypical bZIP transcription factor, with an N-terminal activation domain and a C-terminal bZIP domain (Fig. 1A). The NLS of several bZIP transcriptional factors such as CREB (Waeber and Habener, 1991), CREB-2 (Cibelli et al., 1999), JUN (Chida and Vogt, 1992), FOS (Tratner and Verma, 1991) and C/EBP proteins (Williams et al., 1997) has been identified. In all cases, the basic amino acids of their DNA-binding domain (DBD) are sufficient to ensure transfer to the nucleus. To test this possibility with HBZ, we made a series of EGFP-tagged C-terminal deletion mutants (Fig. 2) that were analyzed by western blotting (Fig. 3) and fluorescence microscopy (Fig. 4) after transfection in COS cells. As previously described (Gaudray et al., 2002), HBZ-EGFP exhibited a granular distribution exclusively localized in the nucleus, whereas EGFP showed a diffuse staining in the cytoplasm and the nucleus (Fig. 4). The subcellular distribution of the deletion mutants revealed that the mutant HBZ- $\Delta$ bZIP, although it does not possess a DBD, was exclusively nuclear. By contrast, the deletion of BR1, a basic region located upstream from the bZIP domain (Fig. 1B), affected the nuclear accumulation of HBZ (see HBZ- $\Delta$ BR1/ $\Delta$ bZIP in Fig. 4). These results suggest that BR1 is necessary for the translocation of HBZ to the nucleus. Moreover, compared with the wild type, the two mutants HBZ- $\Delta$ ZIP and HBZ- $\Delta$ bZIP showed a different pattern of staining since, in addition to the characteristic HBZ-speckled structures, intense spots resembling nucleoli were observed in the transfected COS cells (Fig. 4).

To confirm that BR1 was the NLS, BR1 was fused to EGFP (mutant HBZ-BR1) and its cellular distribution was analyzed. Unexpectedly, BR1-EGFP showed a diffuse staining in the cytoplasm and the nucleus like EGFP alone (Fig. 4), suggesting that BR1 is necessary but not sufficient. Effectively, the mutant HBZ-BR1/DBD, which contains BR1 and DBD, was exclusively nuclear, confirming that the two domains are involved in nuclear localization of HBZ. However, as already mentioned above, the deletion of DBD does not affect the



**Fig. 6.** In vivo expression of the Myc-tagged HBZ mutants. COS cells were transfected with pcDNA3.1(-)/Myc-His expressing HBZ and the mutants HBZ- $\Delta$ ZIP, HBZ-BR2/BR1/DBD and HBZ- $\Delta$ AD fused to the Myc epitope. After transfection in COS cells, expression of the different mutants was checked by western blotting (top) and their localization analyzed by fluorescence microscopy using the mouse anti-Myc antibody (bottom). For immunofluorescence analysis, the anti-Myc antibody was detected with goat anti-mouse IgG antibody coupled to FITC. Magnification, 1500 $\times$ .

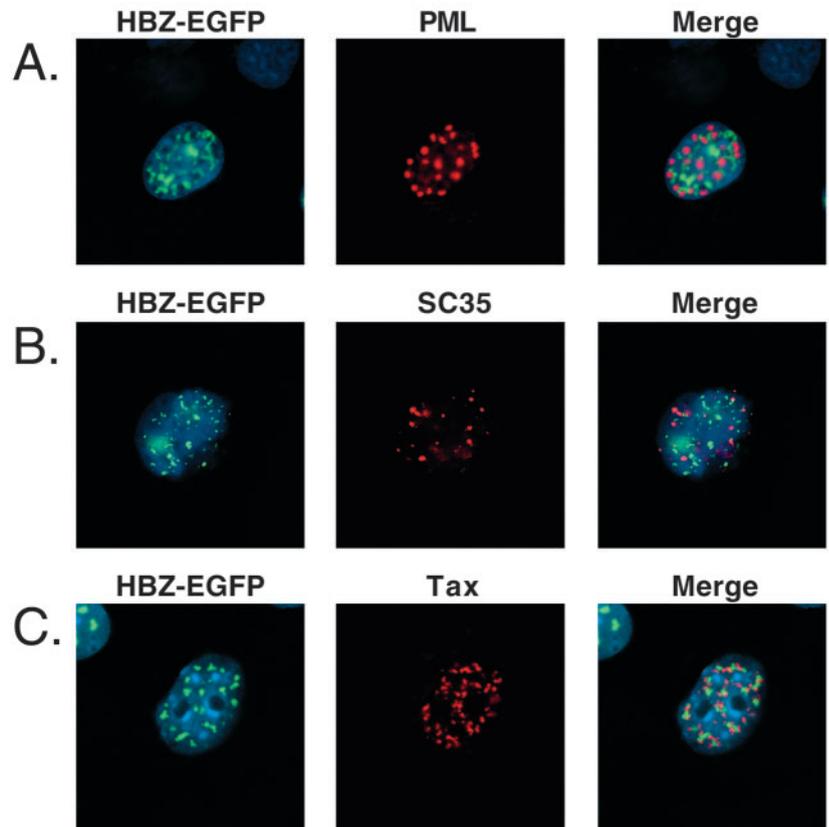


nuclear accumulation of HBZ (see the mutant HBZ- $\Delta$ bZIP), pointing to the existence of an additional NLS. Indeed, HBZ possesses another basic region, BR2 (Fig. 1B), located between the residues 82 and 96. For this reason, we produced a series of mutants corresponding to the different combinations of DBD, BR1 and BR2. Whereas fusions of at least two of these domains with EGFP were exclusively concentrated in the nucleus (Fig. 4), fusions of EGFP with sequences comprising either of these domains were present in both the cytoplasm and the nucleus (see the mutants HBZ-BR1, HBZ-DBD and HBZ- $\Delta$ BR1/ $\Delta$ bZIP). Collectively, the above data summarized in Fig. 2 indicate that HBZ contains three distinct NLSs, respectively located in DBD, BR1 and BR2. Moreover, at least two of these NLSs are necessary for the translocation of HBZ to the nucleus.

#### Integrity of HBZ amino acid sequence is required for its accumulation in nuclear speckles

Interestingly, some HBZ mutants, although located in the nucleus, showed a distinct staining pattern compared with that of the wild type. Thus, the four mutants truncated in their N-terminal and C-terminal domains (HBZ-BR1/DBD, HBZ-BR2/BR1, HBZ-BR2/DBD and HBZ-BR2/BR1/DBD) showed intense spots in the subnuclear structures resembling nucleolus organizing regions (Fig. 4). To confirm that these mutants were located in the nucleoli, colocalization experiments were carried out with a C23-specific antibody. C23, also designated nucleolin, is a major nucleolar phosphoprotein (Ginisty et al., 1998). As shown in Fig. 5, the analyzed HBZ mutants colocalize with endogenous C23 in the nucleoli. It is noteworthy that the localization of the mutants HBZ- $\Delta$ ZIP and HBZ- $\Delta$ bZIP, which do not possess the leucine zipper motif, is not limited to the HBZ-speckled structures but is also observed in the nucleoli (Fig. 4).

To be sure that the presence of the EGFP tag did not influence the subnuclear distribution of our HBZ mutants, a smaller tag, containing the Myc epitope, was fused to the C-terminal end of HBZ and of two mutants, HBZ- $\Delta$ ZIP and HBZ-BR2/BR1/DBD. Moreover, we also analyzed the cellular localization of a new mutant, HBZ- $\Delta$ AD, deleted of its 80 first amino acids (Fig. 1A). After transfection in COS cells, the *in vivo* expression of these Myc-tagged proteins was analyzed by western blotting and their subnuclear distribution was observed by immunofluorescence microscopy (Fig. 6). As already described for HBZ fused to EGFP, the wild-type HBZ showed a granular distribution in the nucleus and HBZ-BR2/BR1/DBD was exclusively located in the nucleoli. This result demonstrates that the presence of EGFP does not influence the localization of the different HBZ mutants. Moreover, the deletion of the activation domain and ZIP domain modifies the staining pattern of the wild-type HBZ (Fig. 6) since the nuclear distribution of both mutants was no longer limited to the HBZ-



**Fig. 7.** Analysis of HBZ subnuclear localization. Localization of the transiently expressed HBZ-EGFP was analyzed by immunofluorescence microscopy. (A) COS cells cotransfected with HBZ-EGFP and PML protein expression vector were labeled with a mouse anti-PML protein antibody and detected using goat anti-mouse IgG antibody coupled to TRITC. Analysis of the green, red and merged fluorescence was performed with a fluorescence microscope. (B) The same approach was performed with HBZ-EGFP-transfected COS cells to localize endogenous SC35 labeled with a mouse anti-SC35 antibody, and (C) with COS cells cotransfected with pEGFP-HBZ and pSG-Tax. Tax was labeled with a culture supernatant of the anti-Tax 168A51-42 hybridoma. Magnification, 1500 $\times$ .

speckled structures but was also nucleolar. Taken together, our results demonstrate that the integrity of the HBZ amino acid sequence is required to observe an exclusive accumulation of HBZ in nuclear speckles.

#### The nuclear sites containing HBZ correspond to heterochromatin

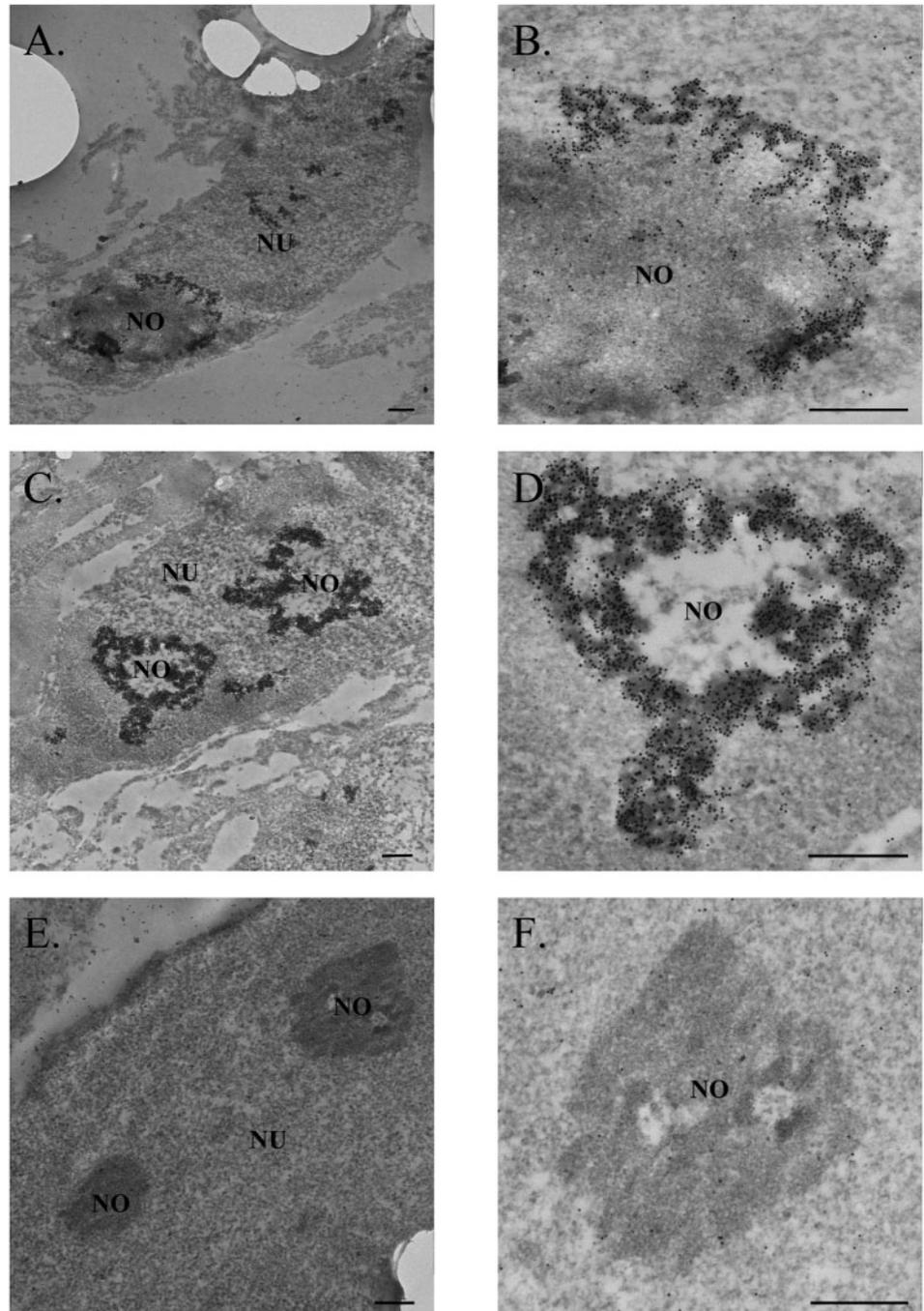
Different macromolecular structures in the nucleus, such as the nucleolus, the Cajal body (CB), the promyelocytic leukemia (PML) protein body, and the splicing factor compartments (SFCs), represent the compartments for nuclear functions (Dundr and Misteli, 2001). However, we first excluded the possibility that the HBZ-containing speckles could correspond to CBs because the number and size of CBs in COS cells are known to be small (Ogg and Lamond, 2002), unlike the HBZ-speckled structures that are large and numerous (Basbous et al., 2003; Gaudray et al., 2002). By contrast, the PML bodies, in which the activity of regulatory proteins is tightly controlled, have been shown to be a target of several viral proteins (Doucas, 2000). Hence, we examined whether HBZ and PML

are localized within the same subnuclear structures by cotransfecting COS cells with HBZ-EGFP and a PML protein expression vector. As shown in Fig. 7A, the two proteins do not colocalize.

SFCs correspond to perichromatin fibrils and interchromatin granule cluster, and are involved in the storage of splicing factors. To determine whether HBZ colocalizes with SFCs, we first checked the staining pattern of HBZ-EGFP with that seen in the same cell stained with anti-SC35 (Fig. 7B), an antibody that recognizes one component of an active spliceosome. Moreover, Tax has been described to colocalize with SC35 (Bex et al., 1997; Semmes and Jeang, 1996). For this reason, we next examined the subcellular localization of HBZ in COS cells transfected with HBZ-EGFP and a Tax expression vector, pSG-Tax (Gachon et al., 2000; Hivin et al., 2004). We found that HBZ neither colocalized with the endogenous SC35 nor with the transiently expressed Tax (Fig. 7B and C). Taken together, these results demonstrate that HBZ is not associated with the SFCs. Moreover, we show that HBZ and Tax do not interact in vivo as already suggested by in vitro approaches (Gaudray et al., 2002).

Given our results, we examined the nuclear localization pattern of HBZ more closely by using immunogold electron microscopy. Myc-tagged HBZ transiently expressed in COS cells was labeled with mouse anti-Myc antibody and detected using goat anti-mouse IgG antibody conjugated to 10 nm gold particles. Unexpectedly, we found HBZ specifically distributed throughout dense nuclear structures located in an irregular band around the nucleoli corresponding structurally to heterochromatin (Fig. 8A-D). When the same analysis was carried out with COS cells transfected with pcDNA3.1(-)/Myc-His, no labeling was detected (Fig. 8E-F). In conclusion, these results demonstrate that HBZ is targeted in the nucleus to heterochromatin.

The first structural components of heterochromatin to be identified were heterochromatin-associated proteins HP1 (James and Elgin, 1986). There are three HP1 protein family members in mammals, HP1 $\alpha$ , HP1 $\beta$  and HP1 $\gamma$ . Among the HP1 members, HP1 $\alpha$  is exclusively localized to the heterochromatic region



**Fig. 8.** HBZ localization in heterochromatin of transfected COS cells. COS cells transfected with pcDNA3.1(-)/Myc-His-HBZ (A-D) or pcDNA3.1(-)/Myc-His (E-F) for 36 hours were fixed with 2.5% formaldehyde and embedded in Lowicryl resin. Ultrathin sections were stained with the mouse anti-Myc antibody, then incubated with goat anti-mouse IgG antibody conjugated to 10 nm gold particles, and analyzed by electron microscopy. Bar, 0.5  $\mu$ m. NU, nucleus; NO, nucleolus.

whereas HP1 $\beta$  is associated with the heterochromatin and euchromatin, and HP1 $\gamma$  is predominantly distributed in euchromatic regions (Elgin and Grewal, 2003). We therefore examined whether HBZ was associated with endogenous HP1 $\alpha$  by visualizing their respective nuclear localization. In the presence of transfected HBZ, the two proteins colocalized within

the nucleoplasm of the majority of cells (Fig. 9A). However, we also observed a minority of cells where the two proteins did not colocalize (Fig. 9B). Taken together, our results show that HBZ can be targeted to heterochromatin but all HBZ is not associated with heterochromatin.

### Discussion

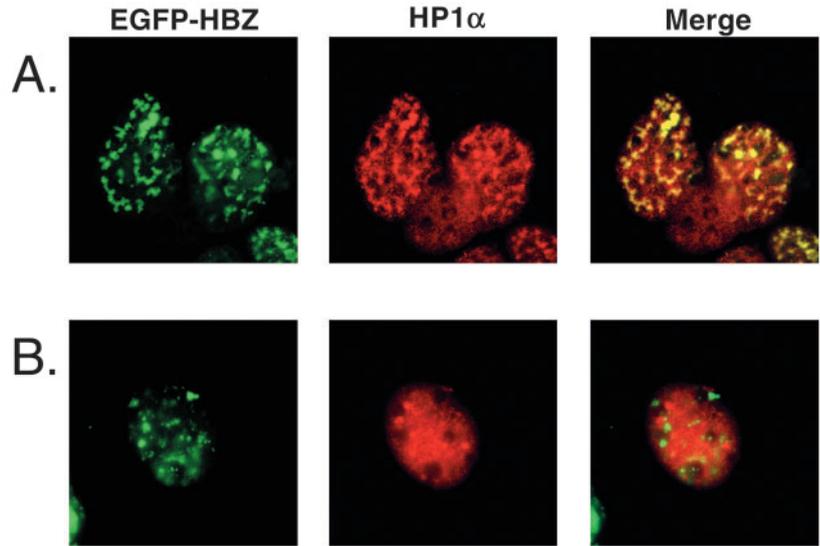
In this paper, we demonstrate that HBZ contains three distinct NLSs: NLS-1 and NLS-2, corresponding to two regions rich in basic amino acids, and NLS-3 corresponding to its DBD. At first glance, NLS-1 and NLS-2 contain the required stretch of 3-5 positively charged residues (KRKRKK from residues 87 to 92 and RRRRR from residues 116 to 120), known to be present in several NLSs of nuclear proteins. However, none of the NLS motifs alone is sufficient for HBZ to be retained exclusively in the nucleus and at least two NLS motifs are required to work together to promote nuclear translocation. It remains unknown why HBZ should have three NLSs for specifying its nuclear localization. Many nuclear proteins have been reported to possess two NLSs and it has been proposed that one signal might mediate nuclear import and the second might prefer to interact with DNA for nuclear retention of the protein. Indeed, the NLS and DNA- or RNA-binding domain in many proteins have been shown to overlap (LaCasse and Lefebvre, 1995). However, although HBZ interacts with CREB-2, JUN and JUNB in the nucleus, these complexes are unable to bind to DNA (Basbous et al., 2003; Gaudray et al., 2002). Thus, the DBD of HBZ is probably inefficient in allowing its nuclear retention, which can explain why HBZ possesses several NLS motifs. Moreover, it has also been suggested that the presence of multiple NLSs in a single protein can be associated with a faster nuclear uptake of the protein (Boulikas, 1993). In conclusion, our results confirm that HBZ exclusively acts at the nuclear level, as already suggested by our previous studies (Basbous et al., 2003; Gaudray et al., 2002).

In support of the notion that HBZ might function as a negative regulator of transcription, we found a localization of HBZ predominantly to heterochromatin. Indeed, heterochromatin is a condensed form of eukaryotic chromatin generally considered to be transcriptionally inactive. It is possible that this particular localization of HBZ is only representative of its overexpression in transfected COS cells. To confirm the relevance of the *in vivo* association of HBZ with heterochromatin, studies of localization in HTLV-I-infected cells should be carried out. Unfortunately, the anti-HBZ serum used in our previous studies (Gaudray et al., 2002; Thébault et al., 2004) does not work for immunofluorescence and immunohistochemistry. However, we demonstrate here that the HBZ-containing speckles do not correspond to CBs, SFCs, or PML oncoprotein bodies. Moreover, Ikaros, a cellular transcription factor, has also been found to be associated with heterochromatin in immature B lymphocytes (Klug et al., 1998). The *Ikaros* gene encodes multiple DNA-binding and non-binding Ikaros isoforms that act as positive or negative regulators of transcription. All these observations suggest that

the localization of HBZ to heterochromatin is probably specific and is not simply a result of its overexpression in transfected COS cells. From these data, a simple working model for the role of HBZ might be to regulate transcription activity negatively of some cellular bZIP factors such as JUN and CREB-2 (Basbous et al., 2003; Gaudray et al., 2002) by blocking them in transcriptionally inactive nuclear sites. Indeed, we have already observed that HBZ entails an intranuclear redistribution of JUN (Basbous et al., 2003) and CREB-2 (P.H., unpublished). However, HBZ is also capable of inhibiting JUN and CREB-2 activities by forming heterodimers that are no longer able to bind to AP-1 and CRE sites. Taken together, our data suggest that HBZ could act at different levels.

However, we also show that, in a minority of transfected cells, HBZ is not colocalized with HP1 $\alpha$ , suggesting that HBZ can be located away from heterochromatin. This observation is not unexpected since HBZ has been described as possessing an activation domain (Gaudray et al., 2002) involved in the stimulation of JUND transcriptional activity (Thébault et al., 2004). More unexpected is the fact that the pattern of HBZ association changes depending on the transfected cells. Does this modification correspond to a change of transcriptionally activated cells to inactivated cells? For the moment, we cannot answer this question and further studies are still necessary to reveal all the significance of our observations.

Another unexpected observation is the nucleolar translocation of HBZ when its activation domain and leucine zipper motif are truncated. However, the NLS-1 and NLS-2 motifs of HBZ are longer than most NLSs and are similar in sequence and in length to the consensus nucleolar localization signal (Thébault et al., 2000). The presence of the N- and C-terminal regions of HBZ seems to be necessary to prevent the transport of HBZ to the nucleoli probably by interacting with nucleoplasmic proteins. Moreover, it is worth noting that the



**Fig. 9.** HBZ colocalizes with HP1 $\alpha$ . (A,B) Endogenous HP1 $\alpha$  was localized in EGFP-HBZ-transfected COS cells with a mouse HP1 $\alpha$  antibody detected using goat anti-mouse IgG antibody coupled to TRITC. Analysis of the green, red and merged fluorescence was performed with a fluorescence microscope. Magnification, 2000 $\times$ .

nucleoli have been described as maintaining a tight association with the heterochromatin compartment, in which repressor proteins are highly concentrated. Interestingly, other proteins have been reported to localize both to heterochromatin and the nucleolus, including the *modulo* gene product, the Polycomb protein and pKi-67 (Carmo-Fonseca, 2002). Future studies on all these proteins will contribute to establishing new connections between chromatin silencing and nucleolar compartmentalization.

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