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The DNA-Binding Domain of Two bZIP Transcription Factors, the Epstein-Barr Virus Switch Gene Product EB1 and Jun, Is a Bipartite Nuclear Targeting Sequence

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The Epstein-Barr virus BZLF1 gene product EB1 (also called ZEBRA and Zta), is a transcription factor belonging to the bZIP (basic domain leucine zipper) family of nuclear proteins. Translocation to the nucleus of EB1 (J. Becker, U. Leser, M. Marschall, A. Langford, W. Jilg, H. Gelderblom, P. Reichart, and H. Wolf, Proc. Natl. Acad. Sci. USA 88:8332–8336, 1991) and of two other bZIP proteins, c-Jun and c-Fos (P. Roux, J.-M. Blanchard, A. Fernandez, N. Lamb, P. Jeanteur, and M. Piechaczyk, Cell 63:341–351, 1990), has been shown to be subject to regulation. We show here that for both EB1 and Jun the nuclear targeting signals (NTS) in the proteins' primary sequences are two clusters of positively charged amino acids. These clusters, called BRA and BRB, are necessary and sufficient to direct β-galactosidase to the nuclear compartment and act as a bipartite NTS. They are conserved among all the bZIP proteins, and although they are not identical, they probably share the same function. Site-directed mutagenesis studies made on these basic clusters suggest that they also act as a bipartite NTS in the EB1 protein. Our results also demonstrate that in EB1 and Jun, these bipartite NTS are superimposed with bipartite DNA-binding domains, since BRA and BRB are required in vitro for direct and specific contact between these proteins and their DNA-binding sites.

Infection of B lymphocytes by Epstein-Barr virus (EBV) is generally followed by deregulation of cell proliferation (immortalization) and persistence of the virus in a latent state (see reference 32 and the references therein). The product of the EBV BZLF1 early gene, originally called EB1 (8) and later called ZEBRA (22) or Zta (12), is thought to be directly involved in the regulation of EBV latency. In B lymphocytes immortalized by EBV, the gene coding for this polymorphic 34- to 40-kDa protein is down regulated and this is thought to be the key for latency (10, 11, 41, 50). Indeed, upon induction of its expression by superinfection with the defective P3HR1 virus (3, 11, 22, 41), by tetradecanoyl phorbol acetate (8, 18) or by anti-immunoglobulin antibody (15, 53), EB1 activates its own synthesis (16, 56), transcription of the early genes (5, 6, 7, 8, 9, 11, 22, 23, 31, 39, 54, 56), and the lytic cycle (10, 48). EB1 may also play a critical role in *trans*-regulating the viral origins of replication which are active only during the lytic cycle (25).

EB1 is a DNA-binding protein related to the bZIP family of transcription factors (6, 14, 35). It activates transcription from different responsive elements, including AP-1 binding sites (14, 17, 36, 56), which are located at distances not greater than 100 to 200 bp from the TATA box (56). EB1 binds in vitro to its cognate DNA recognition sequences as a homodimer (6, 35). The dimerization region is probably a coiled-coil, but there is no heptad repeat of leucines characteristic of a leucine zipper (6, 19, 34). EB1 directly contacts the DNA through a region that consists of two clusters rich in basic amino acids, basic region A (BRA) and basic region B (BRB), separated by a spacer of alanines and conserved among the bZIP proteins (Fig. 1) (57). As proposed by Dingwall and Laskey (13), BRA and BRB could be a bipartite nuclear targeting signal (NTS).

Although EB1 is a nuclear protein, cytoplasmic localization of EB1 has been described in the basal epithelial cell layer at the lateral border and dorsum of the tongue in human immunodeficiency virus-infected oral hairy leukoplakia and -seronegative patients. However, EB1 was found to be nuclear in the differentiated cells of the stratum spinosum in patients with oral hairy leukoplakia (2). These results suggest that a potential site of latency for EBV could be controlled by a mechanism which is not at the transcription level but due to the sequestration of EB1 in the cytoplasmic compartment. Two other members of the bZIP family of proteins, c-Fos and c-Jun (49), are also regulated at the level of nuclear translocation, and this is also the case for other transcription factors (1, 4, 24, 27, 42, 44). It is therefore of importance to define carefully within the protein's primary sequence the signals regulating the subcellular localization of these transcription factors in general and more particularly of the bZIP proteins.

Numerous recent reviews have been published on specific signals (NTSs) required for the active transport of proteins to the nucleus (13, 20, 43, 51). Typically, NTSs are short sequences of 8 to 10 amino acids containing a high proportion of positively charged residues (lysine and arginine). The prototype is the simian virus 40 (SV40) T antigen NTS, PKKKRKV (30), and most NTSs identified in nuclear proteins have been found on the basis of homologies with the SV40 sequence. However, most of the basic clusters characterized, while necessary for nuclear accumulation of the protein, were inefficient in directing β-galactosidase or pyruvate kinase to the nucleus. The notion emerged that an NTS could be composed of several sequences rich in basic amino acids which act synergically, as in nucleoplasmin (46). Recently, Dingwall and Laskey proposed that, despite their

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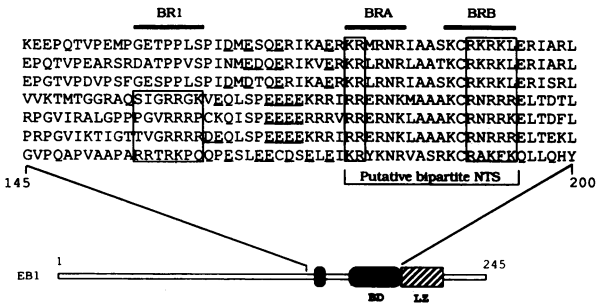

FIG. 1. Sequence comparison of the putative NTS contained in the DNA-binding domain of several bZIP proteins. The amino acid clusters BR1, BRA, and BRB, rich in positively charged residues, are indicated by thick bars over the sequence. Amino acids with a negative charge are underlined. The putative bipartite NTS suggested by Dingwall and Laskey (13) is delineated by thin boxes. In the schematic representation of EB1 at the bottom, BD represents binding domain and LZ represents leucine zipper. The amino acid numbers 145 to 200 are only valid for EB1.

diversity, many NTSs could be aligned as a consensus bipartite motif (13). This motif is present in the basic region of bZIP proteins (Fig. 1) and, as has already been shown for Fos, is probably part of the NTS (55).

Here we report that EB1 and c-Jun BRA and BRB, required for the specific contact between these proteins and their cognate DNA-binding sites (Fig. 1), are sufficient to direct β-galactosidase to the nucleus and act as a bipartite NTS. Domain-swapping experiments also demonstrate that c-Jun and EB1 basic clusters are interchangeable. A detailed site-directed mutagenesis in the EB1 cDNA demonstrates that BRA and BRB act as a bipartite NTS in the EB1 protein. However, another basic cluster, RRTRKP, located 15 amino acids upstream of BRA and called BR1, seems to influence the function of this bipartite NTS. Site-directed mutations in the BR1 sequence or in BRA, or in both sequences, progressively alter the nuclear localization of EB1. Similarly, site-directed mutagenesis in parts of BRB only alter the nuclear localization of EB1, but after complete mutation of BRB, EB1 is found almost exclusively in the cytoplasmic compartment. Our results strongly suggest that the EB1 and c-Jun NTSs are bipartite and coincide with the bipartite DNA-binding domain.

MATERIALS AND METHODS

Primers. The following primers have been used for the mutagenesis experiments described in the next paragraph: 1, 5′-GCAAGCTTCGGTAGTGCTGCAGCAG-3′; 2, 5′-GCG AATTCGGCACGACGCACACGG-3′; 3, 5′-GCGAATTCA CAACAGCCAGAATCGC-3′; 4, 5′-CCGGAATTCAAAGC GATACAAGAATCGG-3′; 5, 5′-GCGAATTCAGCAGACA TTGG-3′; 6, 5′-GCAAGCTTAGCTCCAGCGATTCTGG-3′; 7, 5′-CCGGAATTCAAAGGCAGAAAGAAAGCG-3′; 8, 5′-CCGGAATTCAAAGCGAATGAGGAATCGG-3′; 9, 5′-CCGGAATTCAGCTGCCTCAAAATGCCGG-3′; 10, 5′-GC GAATTCCAGAAAATGCCGGGCC-3′; 11, 5′-GCAAGCT TCGATTCTTCTTGTATCG-3′; 12, 5′-CATCTGCTTCAA CAGGAGGCG-3′; 13, 5′-TGTGCTGTGGCCGGTGCTGC C-3′; 14, 5′-GCAAGCTTCGGTAGTGCTGCAGCAG-3′; 15, 5′-GGCTGCCCCGGCAAATAACACAAACAACCCA CAACAGCCAG-3′; 16, 5′-GAACTAGAAATAGCGGCAT ACGCGAATGCGGTGGCTTCCAG-3′; and 17, 5′-CGGGT GGCTTCCGCAGCATGCGAGGCCGAGTTTGCGCAAC TGCTGCAGC-3′.

Plasmids and mutagenesis. (i) β-galactosidase fusion proteins. EB1–β-galactosidase fusions were constructed in a three-fragment ligation reaction. DNA fragments corresponding to different lengths of the EB1 or the c-Jun putative NTS were amplified by polymerase chain reaction (PCR), using pairs of primers generating double-stranded DNA fragments that could be further digested with *Eco*RI and *Hin*dIII. These *Eco*RI-*Hin*dIII fragments were amplified from a plasmid carrying an EB1 cDNA (pKSVZ41 [21]) or from plasmids carrying the c-Jun basic regions A and B (plasmid pZJ [21]), or the c-Jun basic region A fused to the EB1 basic region B (plasmid pZJA [21]), or the EB1 basic region A fused to the c-Jun basic region B (plasmid pZJB [21]). The following pairs of primers, 1 and 2, 1 and 3, 1 and 4, 1 and 10, 11 and 4, 5 and 6, and 6 and 2, were used to generate the PCR-amplified DNA fragments Z1, Z2, Z3, Z4, Z5, Z6, and Z7, respectively, with pKSVZ41 as template. Primers 1 and 3, 1 and 7, 1 and 8, and 1 and 9 were used to generate the PCR-amplified fragments ZJ, ZJ1, ZJ2, and ZJ3, respectively, with plasmid pZJ as template. Primers 1 and 3 and primers 1 and 3 were used to generate the PCR-amplified fragments ZBJA and ZAJB, respectively, with plasmids pZJA and pZJB as template.

These PCR-amplified DNA fragments were digested with *Eco*RI and *Hin*dIII and ligated with the *Eco*RI-*Sac*II and the *Hin*dIII-*Sac*II fragments generated from plasmid pβnls LacZ. Plasmid pβnlslacZ was constructed by ligating the *Sal*I-*Bam*HI fragment of pGemnlslacZ (30), carrying the β-galactosidase coding region, with the plasmid pAAC digested by *Bam*HI and *Hin*dIII.

Plasmid pAAC was constructed as follows. The blunt-ended *Eco*RI-*Hin*dIII fragment from plasmid p294 (a derivative of PHEBO vector [52]), containing the cytomegalovirus promoter, was ligated with plasmid pBluescript KSII$^+$ (Stratagene), digested with *Not*I, and made blunt ended. The resulting plasmid was subsequently digested with *Kpn*I and *Hin*cII and ligated with the *Rsa*I(blunt)-*Kpn*I fragment from pBLCat3 (37), containing the polyadenylation signal of SV40.

(ii) EB1 mutagenesis. The *Eco*RI fragment from pKSVZ41 (21) carrying the EB1 cDNA was inserted in the *Eco*RI site of pSG5 (Stratagene) to generate plasmid pZ. A PCR strategy requiring three universal primers selected from sequences in the plasmid pZ (primers 11, 12, and 14) and only one specific primer for each mutation was used to perform site-directed mutagenesis in EB1 (40). The specific primers 15, 16, and 17 were used to generate PCR-amplified DNA fragments that were purified, further digested with *Nhe*I and *Pst*I, and then ligated with plasmid pZ digested with *Nhe*I and *Pst*I to generate plasmids pZ1, pZA, and pZB. The construct expressing the double mutant pZ1A was made by using pZ1 as template for the PCR reaction, with the specific primer 16. The *Eco*RI fragments of the constructs expressing mutants 311, 310, and 306 (21) were ligated with pSG5 digested with *Eco*RI to generate plasmids pZ311, pZ310, and pZ306.

Cell culture and DNA transfection. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and seeded at 10^6 cells per 100-mm-diameter petri dish 4 h before transfection. The cells were transfected by the $CaPO_4$-DNA precipitate method (61). Typically, 15 μg of DNA was added to the medium which consisted of 1 μg of expression vector DNA and 14 μg of pUC18 DNA. At

12 h after transfection, cells were washed and fresh medium was added.

Detection of β-galactosidase in situ. β-galactosidase staining was done by the method of MacGregor et al. (38). Cells were plated in 100-mm-diameter petri dishes and transfected as described above. At 36 h after transfection, cells were washed in phosphate-buffered saline (PBS) and fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3). After three washes in detergent solution (2 mM $MgCl_2$, 0.01% sodium desoxycholate, and 0.02% Nonidet P-40 in 0.1 M phosphate buffer), cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside-potassium ferrocyanide-potassium ferricyanide.

Immunofluorescence staining. Indirect immunofluorescence staining was done as described previously (26). Cells were plated on sterile coverslips and transfected as described above. At 36 h after transfection, cells were washed with PBS and fixed for 15 min with 4% paraformaldehyde. The fixed cells were incubated twice for 10 min each in PBS–0.1 M glycine and once for 5 min in PBS–0.1% Triton X-100. The coverslips were then washed for 10 min in PBS–0.2% gelatin and incubated for 30 min at room temperature with a monoclonal antibody against EB1 (AZ125). After three washes in PBS, the coverslips were finally incubated with a fluorescein isothiocyanate-conjugated donkey anti-mouse immunoglobulin G (IgG) antibody (Jackson; 1:200 dilution), washed extensively with PBS, and mounted on microscope slides with Mowiol and 2.5% DABCO [1,4-diazabicyclo(2.2.2)octane] (Aldrich).

Immunoblots. About 5×10^6 HeLa cells were collected 36 h after the transfection and lysed with Nonidet P-40 (29). Nuclei were separated from the cytoplasmic fraction by centrifugation. The cytoplasmic extract was precipitated for 2 h at −20°C with 3 volumes of acetone. Each subcellular fraction was resuspended in sodium dodecyl sulfate (SDS) reducing buffer (0.05 M Tris-HCl [pH 6.8], 10% glycerol, 0.1% SDS, 0.14 M β-mercaptoethanol, and 0.05% bromophenol blue) and boiled for 2 min. One-third of each extract was then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose filter. Membranes were first incubated with an anti-EB1 monoclonal antibody (AZ125) and then incubated with a goat anti-mouse IgG (heavy and light chains) linked to alkaline-phosphatase (Bio-Rad).

RESULTS

Construction of EB1 mutants. We previously reported that EB1 has two short basic amino acid clusters required for nuclear translocation (21). One overlaps BRB and contains the sequence RKCRAKFK (amino acids 187 to 194), whereas the other, located between amino acids 140 and 165, encompasses the BR1 sequence RRTRKP (amino acids 157 to 162) (Fig. 1). These results were obtained by comparing the effects of deletions on the distribution of the mutated EB1 proteins between the nuclear and the cytoplasmic fractions, after transfection in HeLa cells, SDS-PAGE, and immunoblotting (21). Only BRB is part of the putative bipartite NTS proposed by Dingwall and Laskey (13). In order to refine our mutagenesis and to critically evaluate the nuclear targeting potential of EB1 and of c-Jun putative bipartite NTSs, several series of mutants were constructed.

Translational fusions consisting of different lengths of the EB1 protein placed at the N terminus of β-galactosidase were constructed. The c-Jun basic regions A and B and various deletion mutants were also fused to the β-galactosidase. The β-galactosidase constructions are depicted in Fig. 2. These hybrid proteins were expressed under the control of the cytomegalovirus promoter after transfection in HeLa cells, and their subcellular localization was vizualized by an in situ β-galactosidase activity assay. Expression of the β-galactosidase constructs in HeLa cells resulted in the synthesis of fusion proteins of the expected lengths and of identical stability, as vizualized in immunoblotting experiments with human serum containing anti-EB1 antibody (not shown).

Extensive site-directed mutagenesis was also performed in the EB1 region encompassing amino acids 157 to 195, and EB1-Jun domain-swapping mutants were also constructed (see Fig. 5). The subcellular localization of the mutants was evaluated either by indirect immunofluorescence or after cell fractionation, SDS-PAGE, and immunoblotting. In all cases, a monoclonal antibody recognizing an EB1 epitope localized between amino acids 59 to 93 was used.

In the series of transfections described here, 10 to 20% of the cells were positive either for β-galactosidase activity or by immunofluorescence. Therefore, although we only show selected areas on the photos, they are representative of a larger number of cells, with a very homogeneous subcellular distribution of the EB1 mutants.

EB1 basic region is sufficient to direct β-galactosidase to the nucleus. To further assess the nuclear targeting potential of the EB1 region encompassing amino acids 156 to 201, the constructs containing different lengths of the EB1 protein fused to the β-galactosidase were expressed in HeLa cells (Fig. 2A). As expected, when the construct expressing only the β-galactosidase (pB) was introduced in HeLa cells, β-galactosidase activity specifically localized to the cytoplasm (Fig. 3a). When the construct encoding amino acids 1 to 227 of EB1 fused to the β-galactosidase (mutant BZ) was introduced into HeLa cells, β-galactosidase localized exclusively to the nucleus (Fig. 3b). When amino acids 156 to 201 were fused to the β-galactosidase (mutant BZ1), the hybrid protein was also exclusively nuclear (Fig. 3c). Upon deletion of the motif BR1 (mutant BZ2), nuclear localization of the β-galactosidase was abrogated and the fusion protein was exclusively cytoplasmic (Fig. 3d). Surprisingly, upon deletion of the block of acidic residues between amino acids 163 to 178 (mutant BZ3), the EB1 amino acids 178 to 201 directed the β-galactosidase to the nucleus, with some residual activity still detected in the cytoplasm (Fig. 3e). Nuclear localization was no longer observed when BRB (mutant BZ4; Fig. 3f), BRA (mutant BZ5; Fig. 3g), or BR1 (mutants BZ6 and BZ7; Fig. 3h and 3i) was fused to β-galactosidase. Thus, the EB1 basic region appears to function as an efficient bipartite nuclear translocation signal when both regions A and B are linked to β-galactosidase.

c-Jun basic clusters A and B target β-galactosidase to the nucleus. We next asked whether the homologous c-Jun putative bipartite motif could target the β-galactosidase to the nucleus. In the fusion protein expressed from plasmid pBZ2 (Fig. 2A), EB1 amino acids 169 to 194 were replaced by the corresponding c-Jun peptide sequence (plasmid pBZJ; Fig. 2B). The corresponding fusion protein was targeted to the nucleus when expressed in HeLa cells (Fig. 4a). Progressive deletion of amino acids 163 to 178 (constructs pBZJ1 and pBZJ2) did not alter the nuclear localization of the β-galactosidase (Fig. 4b and c). However, deletion of c-Jun basic region A (construct pBZJ3) significantly altered the nuclear localization of the fusion protein (Fig. 4d). Finally, composite peptides (basic region A of c-Jun fused to basic region B of EB1 [mutant BZBJA] or basic region A of EB1

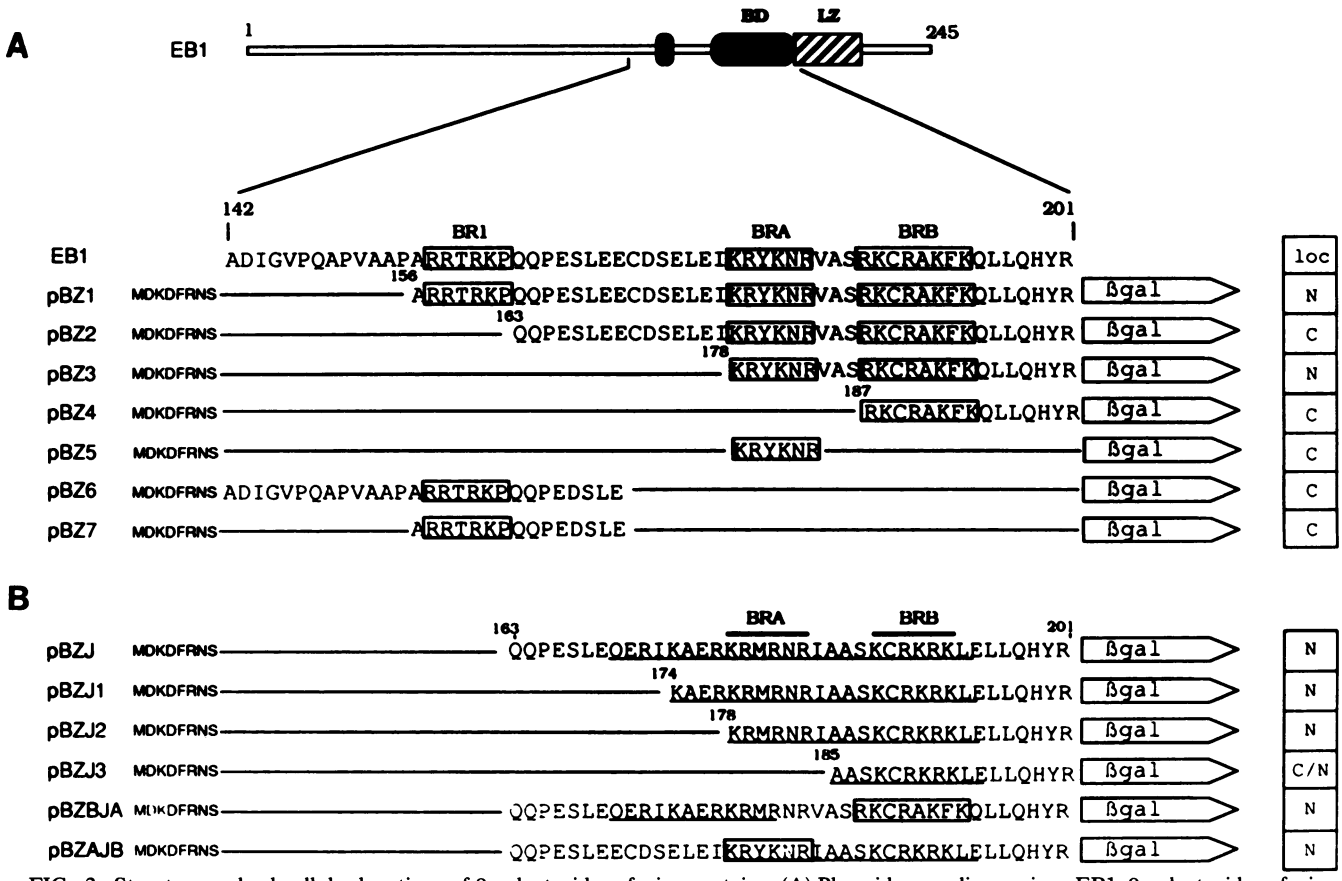

FIG. 2. Structure and subcellular locations of β-galactosidase fusion proteins. (A) Plasmids encoding various EB1–β-galactosidase fusion proteins. The basic clusters BR1, BRA, and BRB are boxed. (B) Plasmids encoding various Jun– or composite EB1–Jun–β-galactosidase fusion proteins. Jun sequences are underlined, and EB1 basic sequences BRA and BRB are boxed. The plasmids were transfected in HeLa cells. The subcellular location of the β-galactosidase was determined at 36 h after transfection. Cells expressing fusion proteins exhibited β-galactosidase activity solely in the nucleus (N) or solely in the cytoplasm (C) or equally in both (C/N).

fused to basic region B of c-Jun [mutant BZAJB]) could target β-galactosidase to the nuclear compartment when expressed in HeLa cells (Fig. 4e and f). Note that in mutants BZJ, BZAJB, and BZBJA, the block of amino acids rich in acidic residues had no effect on the nuclear localization of the fusion protein (Fig. 2B). These results suggest that c-Jun and EB1 BRA and BRB are interchangeable and cooperate to target β-galactosidase to HeLa cell nuclei.

Three clusters of positively charged amino acids participate in the nuclear localization of EB1. Having shown that EB1 basic regions BRA and BRB cooperate to direct the β-galactosidase to the HeLa cell nucleus, we next asked whether site-directed mutagenesis in these sequences, either alone or in combination, would affect the subcellular localization of the EB1 protein. We also mutated BR1, although it did not seem to be part of the NTS as discussed above. Because EB1 does not form heterodimers with any member of the bZIP family of proteins in vitro, it is unlikely that EB1 mutations could be compensated for by heterodimerization of EB1 with a wild-type heterologous bZIP protein. Several mutants were constructed (Fig. 5) and expressed in HeLa cells, and their subcellular localization was assayed by indirect immunofluorescence with a monoclonal antibody, AZ125, directed against an epitope localized within EB1 N-terminal amino acids 59 to 93 (Fig. 5).

Compared with HeLa cells transfected with the control

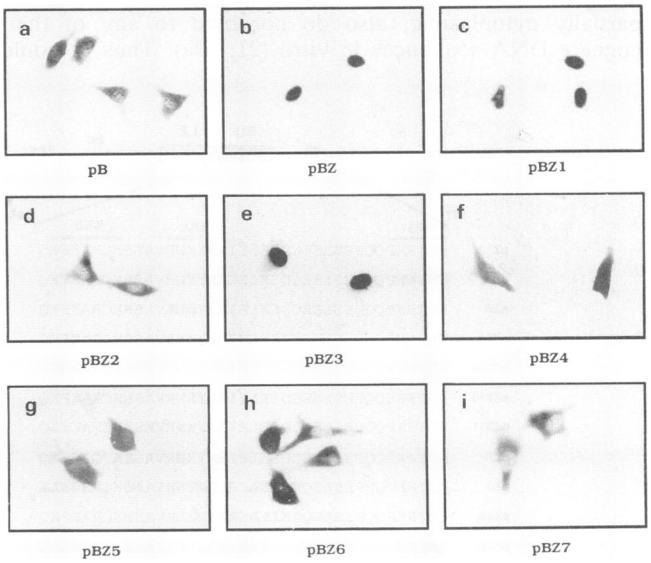

FIG. 3. Subcellular locations of EB1–β-galactosidase fusion proteins. HeLa cells were transfected with the plasmid DNAs encoding the EB1–β-galactosidase fusion proteins illustrated in Fig. 2A. Micrographs a to i represent β-galactosidase fusions, as visualized by in situ β-galactosidase activity assay 36 h after transfections.

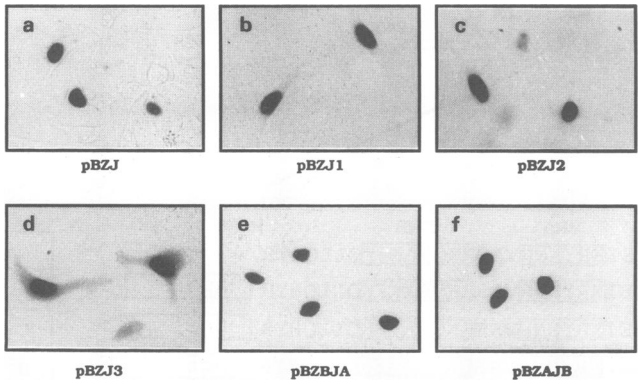

FIG. 4. Subcellular locations of Jun– and Jun–EB1–β-galactosidase fusion proteins. HeLa cells were transfected with plasmid DNAs encoding the fusion proteins illustrated in Fig. 2B. Micrographs represent in situ β-galactosidase assays as described in the legend to Fig. 3.

plasmid pSG5 (Fig. 6a), HeLa cells transfected with a plasmid expressing EB1 (pZ) showed an intense and even nuclear fluorescence (Fig. 6b). Although an intense nuclear fluorescence was also seen with EB1 proteins bearing nonconservative mutations in the sequence BR1 (mutant Z1) or in the sequence BRA (mutant ZA), significant fluorescence began to be observed in the cytoplasm, with mutant Z1 being less cytoplasmic (Fig. 6c) than mutant ZA (Fig. 6d). When both BR1 and BRA were mutated (mutant Z1A), the fluorescence was almost equally distributed between the nucleus and the cytoplasm (Fig. 6e).

In BRB, changing the sequence KFK to AAA (mutant Z306) resulted in an increase in the amount of mutated protein in the cytoplasm, with the nucleus significantly more stained than the cytoplasm (Fig. 6f). Changing the sequence KCR to RCK (conservative mutant Z310) resulted in accumulation of the mutated protein in the nucleus (Fig. 6g).

However, mutants Z1, ZA, Z1A, and Z306, which are partially cytoplasmic, also do not bind to any of their cognate DNA sequences in vitro (21, 39a). Thus, it could

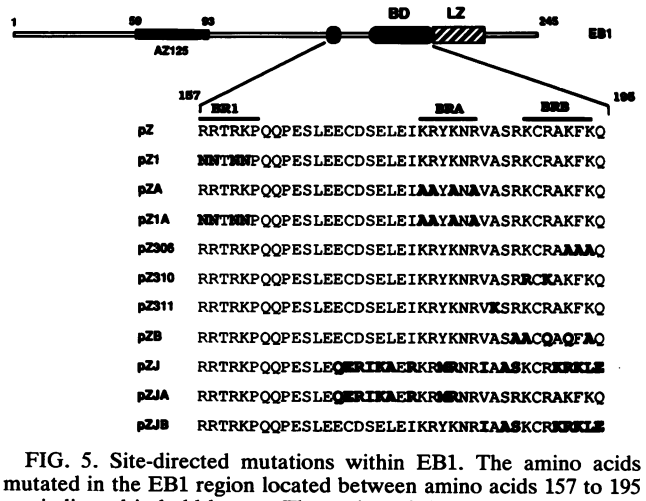

FIG. 5. Site-directed mutations within EB1. The amino acids mutated in the EB1 region located between amino acids 157 to 195 are indicated in bold letters. The region of the EB1 protein recognized by the monoclonal antibody AZ125 is located between amino acids 59 and 93.

then be that the loss of specific DNA-binding activity is the reason these proteins diffuse passively from the nucleus to the cytoplasm. However, this is unlikely since mutant Z311, in which the alanine in position 184 has been replaced by a lysine, does not bind specifically to DNA in vitro (21) but is exclusively localized in the nucleus (Fig. 6h). Finally, when a complete nonconservative mutation was done in BRB (mutant pZB), the protein was more cytoplasmic than nuclear (Fig. 6i).

EB1 and c-Jun BRA and BRB are interchangeable. We also examined the subcellular localization of EB1-Jun hybrid proteins pZJ, pZJA, and pZJB (Fig. 5). Replacing both EB1 basic regions BRA and BRB (mutant ZJ) or EB1 basic region BRA (mutant ZJA) or EB1 basic region BRB (mutant ZJB) with those of c-Jun did not alter the nuclear localization of the hybrid proteins (Fig. 6j to 6l) compared with that of EB1 (Fig. 6b). These results confirm that EB1 and c-Jun BRA and BRB are interchangeable.

Immunofluorescence data and subcellular fractionation data are inconsistent for some mutants. To test whether the immunofluorescences observed and shown in Fig. 6 were due to the recognition of bona fide intact proteins and to attempt to partially quantitate the results, the sizes and the subcellular localization of the EB1 mutated proteins were also determined by SDS-PAGE and immunoblotting after transfection in HeLa cells and separation of the nuclear fraction from the cytoplasmic fraction. As shown in Fig. 7, all the proteins had the expected sizes, with no obvious presence of degradation products, but the subcellular distribution of some mutants was not comparable to what was observed by immunofluorescence. Upon subcellular fractionation, the full-length EB1 protein expressed from construct pZ is recovered predominantly in the nuclear fraction (Fig. 7) and, as published elsewhere (21), the mutants in which various lengths of the protein between amino acids 2 and 140 were deleted were also in the majority found in the nuclear fraction. From these results, it appears that EB1 remains nuclear upon purification of the nuclei. For mutants Z1, ZA, Z1A, ZB, Z306, and Z311, the subcellular distribution following nuclear-cytoplasmic fractionation (Fig. 7) was clearly compatible with the immunofluorescence studies (Fig. 6). However, mutant Z310, which was detected almost exclusively in the nuclei by immunofluorescence (Fig. 6g), was found predominantly in the cytoplasmic fraction upon separation of the nuclei from the cytoplasm (Fig. 7). For the series of EB1-Jun hybrid proteins, the three mutants ZJ, ZJA, and ZJB were found exclusively in the nuclei by immunofluorescence (Fig. 6j to 6l). However, upon subcellular fractionation, mutants ZJ and ZJA were found predominantly in the nuclear fraction, whereas mutant ZJB was found predominantly in the cytoplasmic fraction (21).

DISCUSSION

EB1, a member of the bZIP family of proteins, can direct β-galactosidase, normally a cytoplasmic protein, to the nucleus of HeLa cells. Our deletion mutagenesis data suggest that the EB1 region targeting β-galactosidase to the nuclear compartment colocalizes with a domain rich in basic amino acids, thought to be required for specific and direct contact between this class of proteins and DNA. This observation is also true for the homologous domain of c-Jun. Since Fos BRA and BRB also target pyruvate kinase to the nucleus (55), three members of the bZIP family of proteins, c-Jun, EB1, and Fos, fall in the class of NTSs originally identified in the glucocorticoid receptor, the progesterone

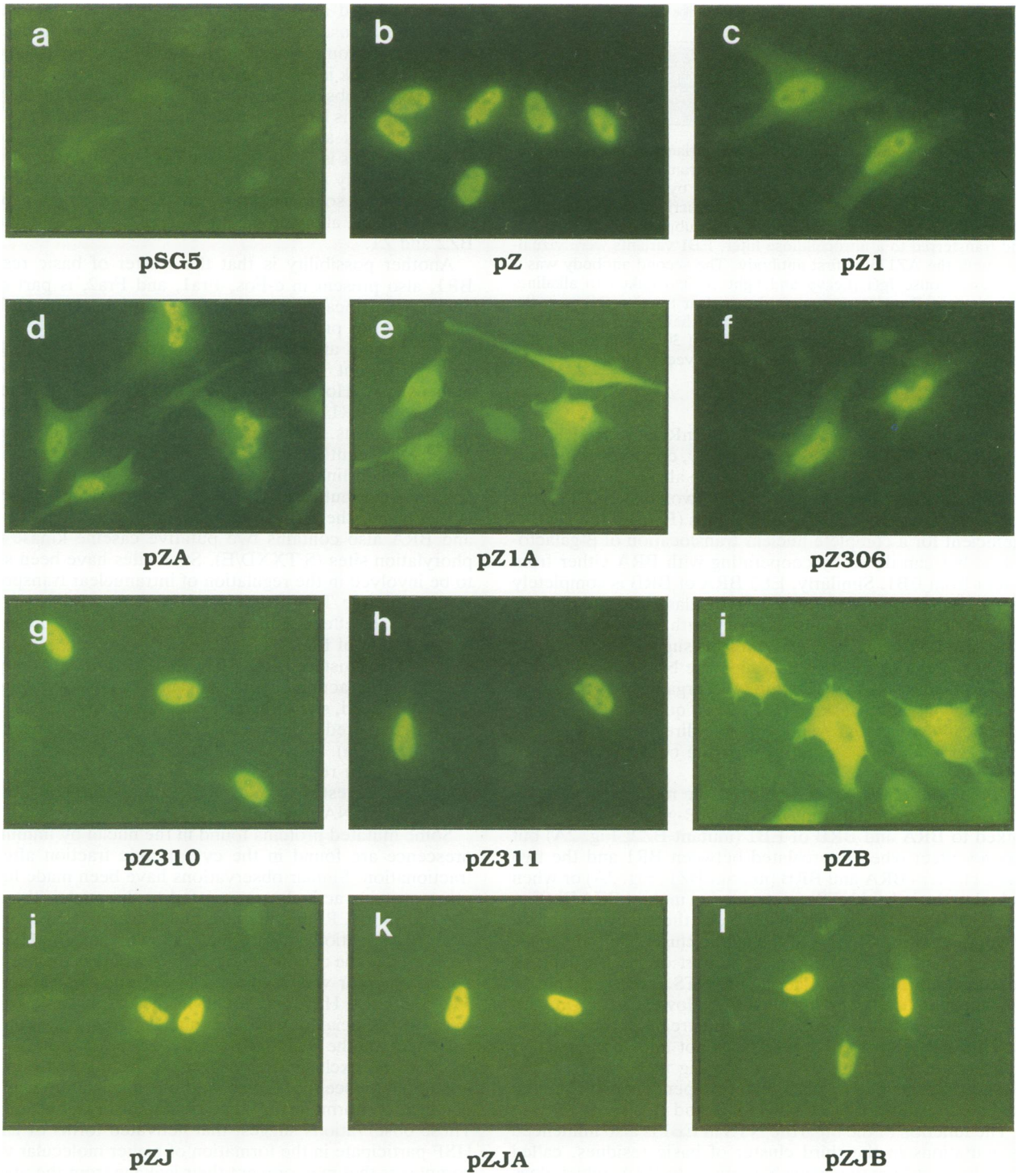

FIG. 6. Subcellular locations of the EB1 variants visualized by indirect immunofluorescence. HeLa cells were transfected with plasmid DNAs encoding the EB1 variants illustrated in Fig. 5. After transfection, cells were fixed and stained with the monoclonal antibody AZ125 as first antibody. The second antibody was a fluorescein isothiocyanate-conjugated donkey anti-mouse IgG antibody. Micrographs a to l show the subcellular distribution of EB1 variants as visualized by immunofluorescence microscopy.

receptor, p53, and nucleoplasmin 1, which many other karyophilic proteins have been proposed to carry (reference 13 and the references therein). As exemplified by nucleoplasmin (46) and VirD2 (28), these NTSs contain two arms rich in basic residues that are separated by a spacer of 10 to 12 amino acids. The two arms act cooperatively to target pyruvate kinase or β-glucuronidase to the nucleus, but neither arm alone can act as an efficient NTS. For Jun and

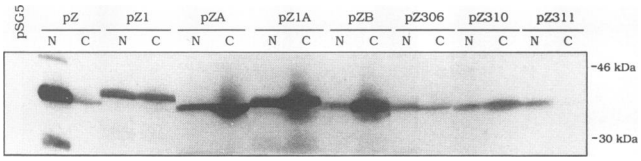

FIG. 7. Subcellular locations of EB1 variants visualized by cell fractionation and SDS-PAGE. HeLa cells transfected with plasmid DNAs encoding EB1 variants were lysed by Nonidet P-40, and nuclei were separated from cytoplasm as described in Materials and Methods. An aliquot of each fraction was subjected to SDS-PAGE and transferred to a nitrocellulose filter. EB1 variants were vizualized with the AZ125 as first antibody. The second antibody was a goat anti-mouse IgG (heavy and light chains) linked to alkaline-phosphatase. Proteins present in the nuclear fraction (N) or in the cytoplasmic fraction (C) are shown, and their relative molecular sizes are estimated from known proteins of 30 and 46 kDa. pSG5 represents HeLa cells transfected with the vector alone.

EB1, the amino acid sequences KRmRnRiaasKcRKRK and KRyKnRvasRKcRAKFK, respectively, contain putative bipartite NTSs (underlined), but there are also additional basic residues (capital letters), forming the two clusters BRA and BRB, separated by a spacer of alanines (Fig. 1). Jun BRB is inefficient for a complete nuclear translocation of β-galactosidase but can do so by cooperating with BRA either from Jun or from EB1. Similarly, EB1 BRA or BRB is completely inefficient for translocating β-galactosidase into the nucleus but can do so by acting cooperatively with each other or with BRA and BRB from Jun. These results suggest that EB1 and Jun basic regions function as a bipartite NTS when fused to β-galactosidase. Is this bipartite NTS organized as predicted by Dingwall and Laskey (13)? This question has to be specifically evaluated, since no site-directed mutagenesis has been made in the putative bipartite consensus depicted in Fig. 1.

A block of amino acids rich in acidic residues negatively influences the nuclear translocation of β-galactosidase when linked to BRA and BRB of EB1 (mutant BZ2; Fig. 2A) but has no effect when intercalated between BR1 and the two basic clusters BRA and BRB (mutant BZ1; Fig. 2A) or when linked to the hybrid NTS constructed in mutant BZAJB (Fig. 2B). How does this acidic cluster block the nuclear import of the mutant BZ2? Folding of the acidic cluster onto the basic cluster could impair the nuclear import of the β-galactosidase fusion protein by rendering the NTS unrecognizable by the nuclear transport machinery. However, in mutant BZAJB, the acidic domain present upstream of the composite EB1 BRA-JunA BRB NTS does not impair the nuclear transport of β-galactosidase, suggesting that the effect observed is not general and is probably dependent on the amino acid composition of basic clusters A and B.

The function of the bipartite NTS in EB1 is also influenced by mutations in the third cluster of basic residues, called BR1, located 15 amino acids N terminal to BRA, which does not seem to be required for the nuclear translocation of β-galactosidase. Site-directed mutagenesis in BR1 impairs slightly but significantly the nuclear import of EB1. As discussed above, BR1 could influence the folding of the region encompassing BR1, BRA, and BRB, such that the block of acidic residues located between BR1 and BRA would not mask the bipartite NTS. Mutation of BR1 will allow partial masking of BRA and BRB by the acidic block, influencing the nuclear transport of EB1. This putative masking effect is much more pronounced when the acidic block is placed at the N terminus of the β-galactosidase, probably because the structural constraints are reduced in this position compared with the intramolecular position of the acidic block in EB1. The above interpretation is compatible with the observation that the basic region of the bZIP protein GCN4 is largely unstructured in the absence of DNA and undergoes a coil-to-helix transition when it binds to DNA (58). This is likely to be the case for other members of the bZIP family of proteins, including EB1 and c-Jun, and could explain some flexibility in the conformation of the basic domains, allowing the masking of the NTSs in mutants BZ2 and Z1.

Another possibility is that the cluster of basic residues BR1, also present in c-Fos, Fra1, and Fra2, is part of the nuclear translocation signal of these proteins (Fig. 1). Interestingly, a Fos protein in which BRA, BRB, and the leucine zipper domain are deleted, but in which the basic cluster BR1 is present (Fig. 1), can still be translocated to the nucleus (55). However, neither the EB1 BR1 (this report) nor the Fos BR1 (55) can translocate a heterologous protein into the nucleus. However, it would be interesting to evaluate whether multimers of BR1 from EB1 or Fos have an NTS activity when linked to β-galactosidase, because multimerization of the mutated SV40 large T NTS restored its activity (47). Finally, the EB1 acidic domain located between BR1 and BRA also contains two putative caseine kinase phosphorylation sites (S/TXXD/E). Such sites have been shown to be involved in the regulation of intranuclear transport for the SV40 large T antigen (45). However, we have not yet evaluated the effect of mutations in these sites on the nuclear translocation of EB1.

The basic clusters BR1, BRA, and BRB are also required for stable interaction of EB1 with specific DNA-binding sites in vitro. Indeed, site-directed mutations in any of these three clusters impaired the capacity of EB1 to interact specifically with DNA (39a). However, although BR1 is outside the EB1 domain (bZIP) required for specific DNA binding, our results also suggest that BR1 might contact the DNA outside the specific DNA recognition sequence.

Some mutated proteins found in the nuclei by immunofluorescence are found in the cytoplasmic fraction after cell fractionation. Similar observations have been made for two other proteins actively transported to the nuclei, the estrogen receptor (ER) and the heat shock factor (HSF). Subcellular fractionation studies have shown that free ER was recovered in the cytoplasmic fraction, whereas the hormone-bound receptor was recovered in the nuclear fraction (33, 59). Similarly, HSF was found in the cytoplasmic fraction but was associated with the nuclear fraction upon heat treatment of the cells (60). However, both proteins were found to be exclusively localized in the nuclei by indirect immunofluorescence studies, and this localization was independent of hormone or heat treatments (33, 59 and 60). These observations suggest that activated forms of ER and HSF participate in the formation of higher molecular weight complexes that may prevent their leakage from the nuclei to the cytoplasmic fraction during the cell fractionation process. This could also be the case for EB1 mutants Z310 and ZJB, which were found to be nuclear by immunofluorescence but were cytoplasmic after cell fractionation.

In conclusion, our results strongly suggest that EB1 and c-Jun NTSs are composed of two motifs that are rich in basic amino acids, act cooperatively, and are related to the class of bipartite NTSs proposed by Dingwall and Laskey (13). The significance of such complicated NTSs has still to be addressed.

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