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Evidence for coiled-coil dimer formation by an Epstein–Barr virus transactivator that lacks a heptad repeat of leucine residues

(BZLF1 gene/protein crosslinking/coiled-coil dimerization/DNA binding)

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ABSTRACT **Two regions of the Epstein–Barr virus (EBV) BZLF1 gene product, ZEBRA, share sequence homology with c-Fos, one of which corresponds to the DNA binding domain of c-Fos. ZEBRA does not, however, contain the heptad repeat of leucines present in the dimerization domains of leucine zipper proteins. Here it is shown that ZEBRA binds its recognition sites as a homodimer and that the region adjacent to the basic DNA binding domain is essential for dimerization. This region contains a 4-3 repeat of predominantly hydrophobic residues, which is precisely in register with the hydrophobic heptad repeat present in the leucine zipper proteins with respect to the basic DNA binding domain. A mutational analysis of ZEBRA supports a model for dimerization involving a coiled-coil interaction. These results indicate that a heptad repeat of leucines is not a structural requirement for formation of coiled-coil dimers by transcription factors.**

Epstein–Barr virus is a lymphotropic human herpesvirus that predominantly establishes a latent infection in B lymphocytes. Latent viral infection results in growth transformation of the infected lymphocytes, yielding continuously proliferating lymphoblastoid cells that are immortalized but not oncogenically transformed. Disruption of viral latency is determined by an intricate cascade of events initiating at the plasma membrane. *In vitro*, the viral lytic cycle can be triggered by a variety of reagents, including anti-immunoglobulin, calcium ionophore, butyrate, and the phorbol ester phorbol 12-myristate-13-acetate (1–4).

Among the Epstein–Barr virus antigens known to be expressed during the onset of the viral lytic cycle, the BZLF1 gene product ZEBRA is unique in its ability to trigger the entire lytic cascade (5–7). ZEBRA shares two regions of homology with the cellular transactivator c-Fos, one of which is the DNA binding domain (8), and binds specifically to target sequences containing "AP-1 like" sites (8, 9, 11–13, *). However, the sequence similarity between ZEBRA and c-Fos does not extend into the leucine zipper dimerization domain of c-Fos adjacent to the basic DNA binding region (14–16). We have previously shown that mutations introduced into the basic region of ZEBRA abrogated DNA binding.* Furthermore, it has also been demonstrated that the region of ZEBRA adjacent to the basic domain is required for DNA binding (11, *), suggesting that it may be involved in dimer formation (12, 13).

MATERIALS AND METHODS

Mobility-Shift Assays. Plasmids were prepared and linearized by standard procedures (17). *In vitro* transcripts were generated by using either SP6 or T7 RNA polymerase, followed by phenol extraction and ethanol precipitation. Translations were carried out with a wheat germ extract (Promega) with [^{35}S]methionine according to the manufacturer's specifications. The [^{35}S]methionine-labeled translation products were analyzed by electrophoresis in a SDS/15% polyacrylamide gel. The gel was subsequently fixed in a solution containing 10% (vol/vol) acetic acid and 20% (vol/vol) methanol, followed by soaking in EN^{3}HANCE (NEN), dried, and subjected to fluorography.

The translation products were mixed with ^{32}P-labeled, double-stranded oligonucleotide containing either the ZIIIA or ZIIIB ZEBRA binding domains from the BZLF1 promoter (9) in 25 μl of 10 mM Tris·HCl, pH 7.9/6.7 mM Hepes, pH 7.9/33 mM KCl/0.5 mM EDTA/0.7 mM dithiothreitol/9.2% (vol/vol) glycerol/2% (wt/vol) polyvinylethanol/0.5 μg of poly(dI·dC). Binding reactions were carried out at room temperature for 20 min, followed by separation on a 5% polyacrylamide gel in 0.5× TBE (1× TBE = 90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3).

Glutaraldehyde Crosslinking. Site-directed mutations in the BZLF1 gene were introduced as described (9). The plasmids carrying the mutated BZLF1 genes were *in vitro* transcribed and translated as described in Fig. 1*A*. Equal amounts of each translation reaction were either mock treated or treated with 0.004% glutaraldehyde for 30 min at room temperature in 200 μl of 0.1 M NaCl. Samples were precipitated with 10% trichloroacetic acid, resolubilized, and separated by electrophoresis in SDS/10% polyacrylamide gels, followed by fixing and fluorography.

RESULTS AND DISCUSSION

To examine the issue of ZEBRA dimer formation, a truncated ZEBRA *in vitro* expression plasmid was constructed that generates a short form of ZEBRA containing residues 87–245. Both the full-length and truncated *in vitro* synthesized proteins were able to bind specifically to two oligonucleotides containing distinct ZEBRA target recognition sequences from the BZLF1 promoter (Fig. 1*A*). When the *in vitro* synthesized RNA templates for the full-length and truncated forms of ZEBRA were cotranslated, and the resulting products assayed for DNA binding, an intermediate shifted band was detected. This intermediate band corresponds to the anticipated shift in mobility by a heterodimer of full-length and truncated ZEBRA proteins. It is worth noting that only a small amount of the truncated ZEBRA protein homodimer was apparent in the presence of the full-length protein, indicating that the truncated protein preferentially dimerized with the full-length protein. This interpretation was supported by crosslinking studies, which showed that the truncated form of ZEBRA homodimerized less well than the full-length protein (data not shown). Analysis of the *in vitro*

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*Taylor, N., Flemington, E., Kolman, J. L., Baumann, R., Speck, S. H. & Miller G., 15th International Herpesvirus Workshop, August 2–8, 1990, Georgetown University, Washington, DC, abstr. 61.

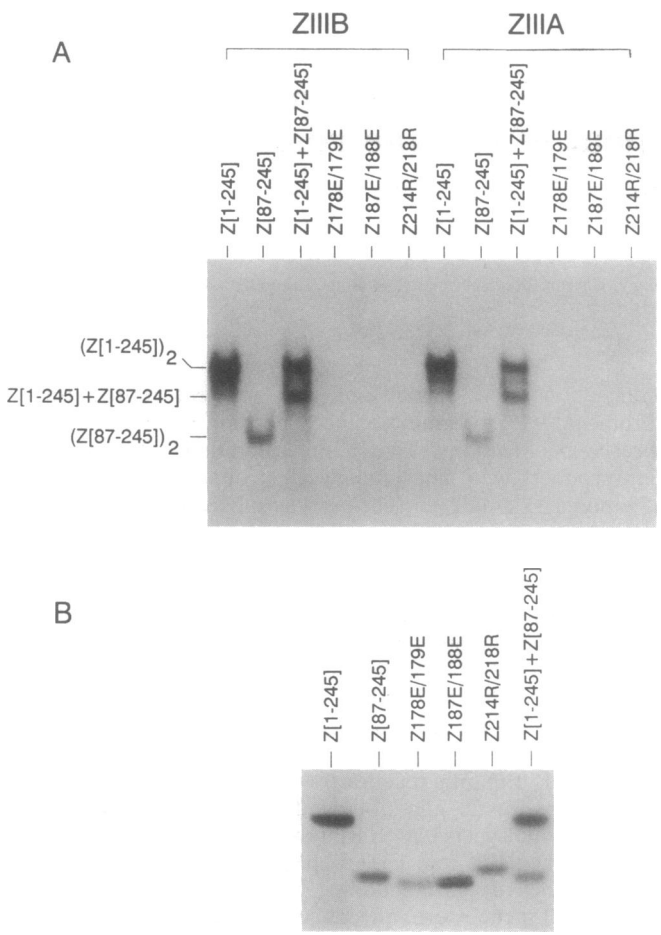

FIG. 1. (*A*) Mobility-shift analysis of full-length and truncated ZEBRA protein binding to ^{32}P-labeled oligonucleotides containing ZEBRA binding sites. (*B*) SDS/PAGE analysis of *in vitro* translation products. Z[1–245], full-length ZEBRA protein; Z[87–245], truncated ZEBRA protein containing residues 87–245; Z178E/179E, Z187E/188E, and Z214R/218R, full-length ZEBRA proteins containing mutations as indicated in Fig. 2*A*.

translated proteins by SDS/PAGE demonstrated that both the full-length and truncated ZEBRA proteins were present in the cotranslation reaction mixture (Fig. 1*B*). Dimerization of ZEBRA has also recently been demonstrated by two other groups (12, 13).

Characterization of truncated ZEBRA proteins has mapped the regions of the protein essential for DNA binding (11, *). In addition to the basic region, the only other region of the protein required is that immediately carboxyl terminal to the basic region. However, a minimal ZEBRA protein containing only the basic region and putative dimerization domain does not bind DNA, indicating that other regions of the protein, while not essential for dimerization and/or DNA binding, are involved in stabilizing these interactions.

Inspection of the region of ZEBRA adjacent to the DNA binding domain indicates that, while it lacks the heptad repeat of leucine residues, it does contain a 4-3 repeat of predominantly hydrophobic residues analogous to that found in the leucine zipper proteins. This heptad repeat in ZEBRA is in register with the heptad repeat in the leucine zipper proteins, with respect to the basic region (Fig. 2*A*). Indeed, it was recently shown that a c-Fos/ZEBRA chimeric protein in which the carboxyl terminus of ZEBRA from residues 196–245 (from the junction of between the basic and heptad repeat regions to the carboxyl terminus) was fused to the amino terminus of c-Fos to residue 161 (through the basic region, but lacking the dimerization domain) dimerized and bound DNA (13). The apparent conservation of a hydrophobic heptad repeat in ZEBRA, based on the characterization of GCN4, c-Jun, and c-Fos dimerization by Kim and coworkers (22, 23), suggests a model for ZEBRA dimer formation involving a coiled-coil interaction in which the predominantly hydrophobic residues at the a and d positions of the 4-3 heptad repeat are on one face of a helix. These residues are predicted to interact with the hydrophobic face of a helix on the dimerization partner in a parallel conformation (Fig. 2*B*).

To assess the validity of this model for ZEBRA dimerization, a number of site-directed mutants in this region of the protein were generated such that a charged residue(s) was introduced in place of a hydrophobic residue (or in some cases an uncharged residue) (Fig. 2*A*). These mutants were assayed for their ability to dimerize by crosslinking with glutaraldehyde (Fig. 3). In addition, as a control the ability of two basic region mutants to dimerize was determined. As expected, mutations in the basic region did not affect dimerization but, as previously shown,* they abrogated DNA binding (see mutants Z178E/179E and Z187E/188E; Figs. 1*A* and 3). Mutations introduced at positions containing hydrophobic residues predicted to be involved in the interaction between the two helices in the coiled-coil dimer generally interfered with the ability of ZEBRA to dimerize (see mutants Z197K/200S, Z200E, Z214D, Z214R/218R, Z214S/218S, Z225E, and Z200E/225E; Fig. 3). One of these mutants, Z214R/218R, was also assayed for DNA binding and, as expected, exhibited no ability to bind to either of two oligonucleotides containing ZEBRA recognition sequences (Fig. 1*A*).

Mutation of residue 204, predicted to be on the interacting face of the helix, from alanine to aspartic acid only slightly reduced the amount of dimer detectable (see Z204D; Fig. 3). An acidic residue at this position may be accommodated in a ZEBRA homodimer because, based on the coiled-coil model, it would be juxtaposed to lysine-207. The formation of a salt bridge may compensate for the introduction of a bulkier residue (see Fig. 2*B*). Indeed, mutation of alanine-204 to a histidine (Z204H) resulted in a greater reduction in dimer formation, although this mutation was not as effective as the Z200E, Z214D, or Z225E mutations. However, it is unlikely that all residues on the front face of the helix contribute equally to the binding energy.

Further support for the coiled-coil model was provided by mutations introduced in residues predicted to be on the back side of the helix. Two residues predicted to be in the f position at the back of the helix were independently mutated. Mutation of leucine-216 to glutamic acid (Z216E) had no apparent effect on dimerization, while mutation of serine-209 to arginine (Z209R) slightly reduced the amount of dimer detected. The latter result may reflect an unfavorable increase in the overall charge of the dimerization helix. Indeed, with the double mutant (Z209R/216E), in which both residues were mutated (resulting in no net change in the overall charge of the dimerization helix), no apparent decrease in dimerization was seen. Furthermore, mutation of the two alanine residues at positions 205 and 206, which are predicted to occupy b and c positions at the back half of the helix, to arginine and aspartic acid also had little effect on dimerization of ZEBRA (see Z205R/206D; Fig. 3).

A large decrease in the ability of ZEBRA to dimerize was obtained with the mutant Z216A/217Q, in which leucine-216 was converted to alanine and leucine-217 was converted to glutamine. This strong inhibition of dimerization is most likely due to mutation of residue 217, since independent mutation of residue 216 from leucine to arginine had no effect on dimerization. Leucine-217 is predicted to be positioned adjacent to the hydrophobic face of the helix, and the results obtained with this mutant suggest that the residues adjacent to the interacting faces of the helices are important for dimerization. In general, the leucine zipper proteins have a

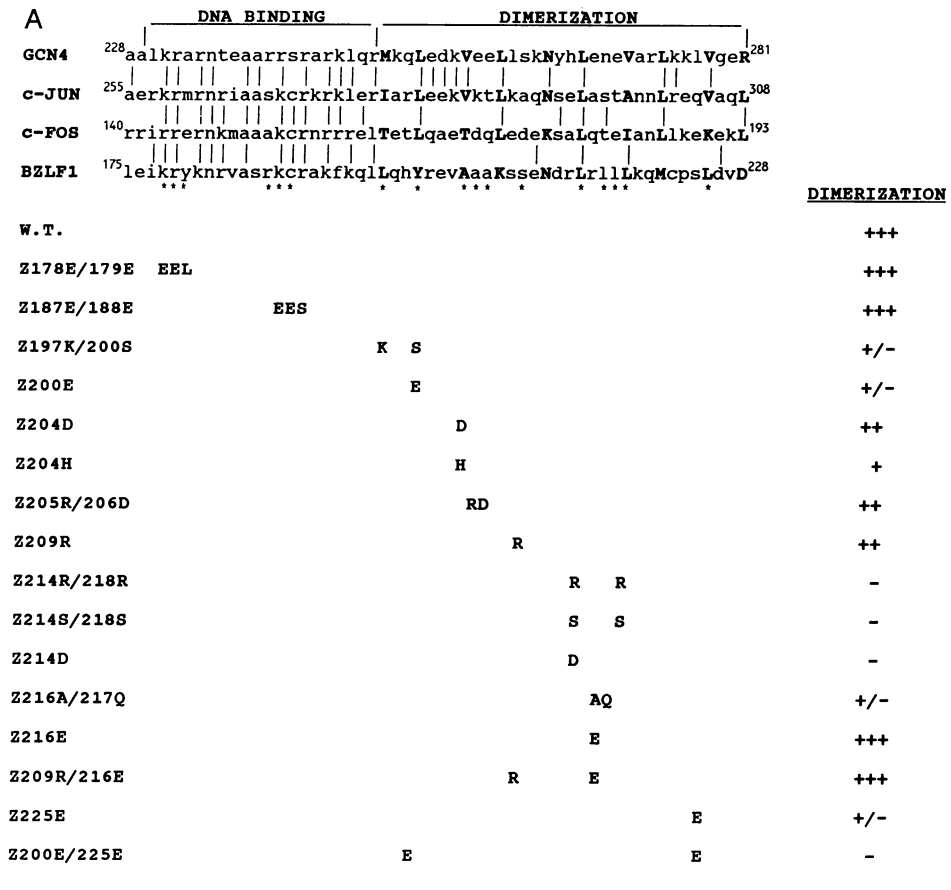

FIG. 2. (*A*) Sequence comparisons and summary of mutational analysis of ZEBRA dimerization domain. Sequences are denoted with the name of the protein and the number of the first and last amino acids shown (18–21). Sequence identities and conservative substitutions are indicated by vertical lines. Residues in ZEBRA that were subjected to mutation are marked with asterisks. The results of glutaraldehyde crosslinking experiments shown in Fig. 3 are summarized and the mutated residues are indicated. (*B*) Model of the ZEBRA coiled-coil homodimer. This represents a view down the helix axis from the amino terminus of the ZEBRA dimerization domain and illustrates the proposed interactions between the two helices as a coiled-coil.

high density of charged residues at the e and g positions of the helix, and these are thought to play an important role in stabilizing or destabilizing dimer formation (10, 22, 23). The predicted ZEBRA dimerization helix contains fewer charged residues at these positions, and two of the four residues in the g position are occupied by hydrophobic amino acids. Thus, in the ZEBRA homodimer there may be hydrophobic interactions as well as ionic interactions contributed by residues in the e and g positions. It should be noted that no electrostatic repulsion from the interactions of e and g residues in the ZEBRA homodimer should occur since there are two basic residues and no acidic residues in e positions, while there is a single acidic residue and no basic residues in g positions.

The presence of a proline residue in the f position of the fourth heptad repeat is expected to introduce a bend in the helix and might demarcate the end of the dimerization domain. To investigate whether this is the case, leucine-225 (predicted to be at the a position of a putative fifth heptad repeat) was mutated to glutamic acid (Z225E) alone and in conjunction with the Z220E mutation (Z200E/225E). In both cases, mutation of leucine-225 significantly diminished dimerization (Fig. 3), suggesting that this residue is also involved in dimer formation. It is possible, however, that the introduction of an acidic residue at this position leads to a destabilizing electrostatic effect due to the presence of an aspartic acid at residue 228 in the homodimer partner. If the dimerization helix extends past proline-223, then it undoubtedly ends with the proline at position 232, which is followed by another proline at position 235. Thus, the ZEBRA dimerization domain is composed of at least four complete heptad repeats and possibly a fifth.

The mutational analysis presented supports a coiled-coil model for ZEBRA dimerization. In addition, an independent mutational analysis carried out by Farrell and coworkers† also supports a coiled-coil model. However, it is also clear that there are a number of features of the ZEBRA dimerization domain that distinguish it from those found in the leucine zipper proteins, most notably the lack of a heptad repeat of leucines and the lower number of charged residues at e and g positions. Perhaps the most intriguing issue with regard to

†Packham, G., Sinclair, A., Rooney, C. & Farrell, P. (1990) 15th International Herpesvirus Workshop, August 2–8, 1990, Georgetown University, Washington, DC, abstr. 18.

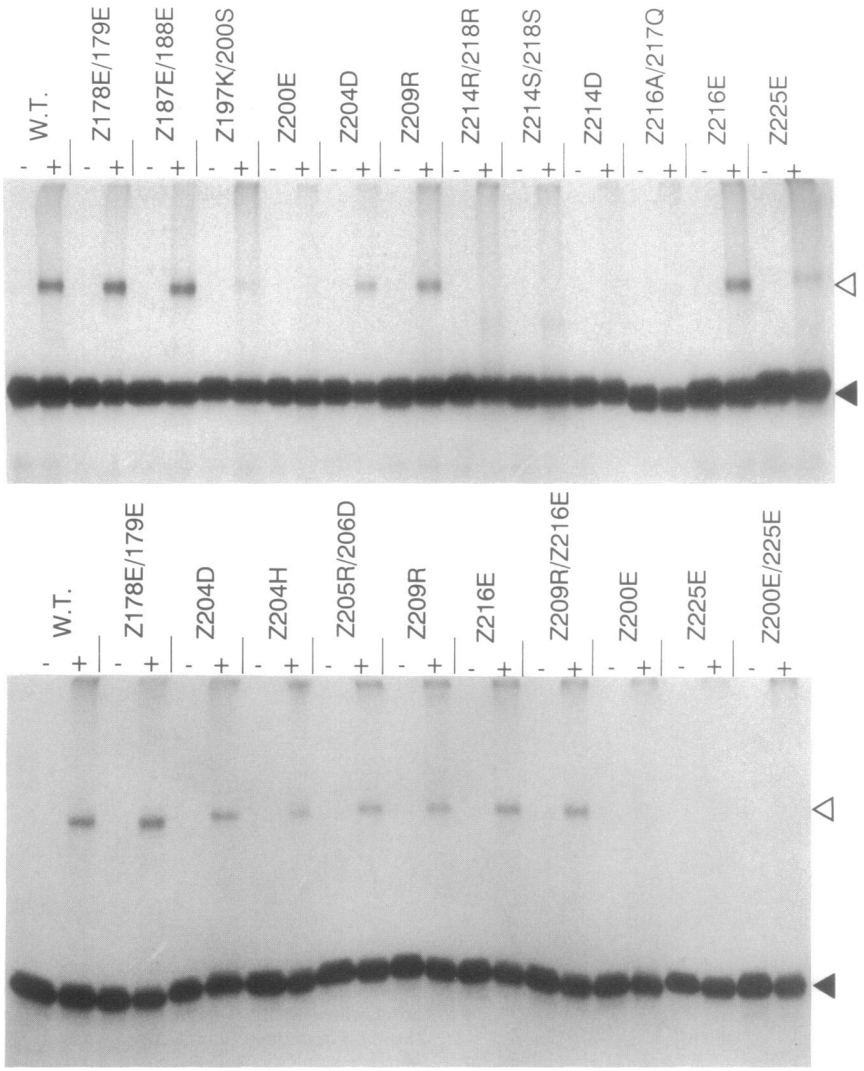

FIG. 3. Glutaraldehyde crosslinking of ZEBRA mutants. *In vitro* generated proteins were crosslinked, followed by fractionation on SDS/10% PAGE. Open arrowhead, migration of ZEBRA homodimer; solid arrowhead, migration of ZEBRA monomer.

the leucine zipper proteins is the presence of leucines virtually exclusively at the d position of the helix and not at the a position (14). In the coiled-coils of α-fibrous proteins leucine appears with equal frequency at both the a (32.2%) and d (34.7%) positions (10). In ZEBRA, there are two or possibly three leucines at the a position and a single leucine at the d position. Thus, in ZEBRA only the second heptad repeat of the dimerization domain lacks a leucine residue. If the coiled-coil model for ZEBRA dimerization is correct, then the significance of the heptad repeat of leucines in the leucine zipper proteins most likely is involved in specifying dimerization partners among these proteins rather than being a fundamental structural requirement.

Finally, the determination that ZEBRA binds DNA as a dimer raises the question of whether it functions exclusively as a homodimer or whether it forms heterodimers with other viral or cellular transcription factors. The fact that ZEBRA forms stable homodimers and that its dimerization domain has features that distinguish it from those of the leucine zipper proteins are consistent with ZEBRA existing only as a homodimer. Furthermore, preliminary studies indicate that ZEBRA does not dimerize with c-Jun, c-Fos, or CREB (ref. 13; data not shown). Alternatively, a class of transcription factors that form coiled-coil dimers with dimerization domains similar to that found in ZEBRA may exist.

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