

Binding of the Ubiquitous Cellular Transcription Factors Sp1 and Sp3 to the ZI Domains in the Epstein–Barr Virus Lytic Switch BZLF1 Gene Promoter

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Induction of the Epstein–Barr virus lytic cycle in latently infected B cells requires the expression of the immediate-early lytic gene BZLF1. We have previously identified several *cis*-elements within the BZLF1 promoter that are required for induction by known inducers of the lytic cycle [E. Flemington and S. H. Speck (1990) *J. Virol.* 64, 1217–1226]. These include four elements termed the ZI domains (ZIA, ZIB, ZIC, and ZID) that share extensive homology and that have recently been shown to bind several cellular transcription factors [A. M. Borrás, J. L. Strominger, and S. H. Speck (1996) *J. Virol.* 70, 3894–3901]. Here Sp1 and Sp3 are identified as the cellular factors present in crude B cell nuclear extract preparations that bind to the ZIC domain. In addition, three of the four complexes observed in electrophoretic mobility shift analyses employing probes containing either the ZIA or the ZID domains also represent Sp1 or Sp3 binding. Binding of Sp1 and Sp3 to the ZI domains was shown to be significantly weaker than binding of these factors to a consensus Sp1 site. A heterologous promoter construct containing three repeats of a consensus Sp1 site, cloned upstream of a single copy of the ZII (CREB/AP1) element from the BZLF1 promoter linked to the β -globin TATA box, exhibited phorbol ester inducibility. The latter observation was consistent with the functional behavior exhibited by a heterologous promoter construct containing multiple copies of the ZIC domain linked to the ZII element. However, the basal activity of the heterologous promoter construct driven by the consensus Sp1 sites was ca. 10-fold higher than that of the heterologous reporter construct containing multimerized ZIC sites. Thus, the low affinity of Sp1 binding to the ZI domains may contribute to the low-level basal activity of the BZLF1 promoter. © 1997 Academic Press

INTRODUCTION

Epstein–Barr virus (EBV) is a human gammaherpesvirus that predominantly establishes a latent infection in B lymphocytes. Propagation of EBV from host to host is dependent upon the activation of an estimated 100 or more viral genes, culminating in the production of infectious virions (reviewed in Kieff and Liebowitz, 1989; Baer *et al.*, 1984; Bauer *et al.*, 1982; Hummel and Kieff, 1982; Cohen *et al.*, 1984). During latency, little or no viral replication takes place. Rather, immortalization of EBV-infected B cells is achieved through the expression of a small subset of viral genes which serve to establish and maintain cellular growth transformation (for a review see Kieff and Liebowitz, 1989). However, a switch in the genetic program leading to the expression of viral replication-associated genes can be accomplished *in vitro* by treatment of latently infected B cells with various activating agents, including phorbol esters, Ca²⁺ ionophores, and anti-immunoglobulin antibodies (Kallin *et al.*, 1979;

Luka *et al.*, 1979; Takada and Ono, 1989; Tovey *et al.*, 1978; zur Hausen *et al.*, 1978).

Activation of the viral lytic cascade by crosslinking surface immunoglobulin results in the initial expression of two viral genes, BZLF1 and BRLF1, which exhibit similar induction kinetics (Takada and Ono, 1989; Flemington *et al.*, 1991). The protein products of both the BZLF1 gene (referred to here as Zta, but also known as ZEBRA and EB1) and the BRLF1 gene (referred to as Rta) are transcriptional activators (Chevallier-Greco *et al.*, 1986; Countryman and Miller, 1985; Farrell *et al.*, 1989; Grogan *et al.*, 1987; Hardwick *et al.*, 1988; Miller *et al.*, 1984; Countryman *et al.*, 1987; Urier *et al.*, 1989; Lieberman and Berk, 1990; Chang *et al.*, 1990; Flemington and Speck, 1990b,c; Kouzarides *et al.*, 1991; Liberman *et al.*, 1990).

The BZLF1 promoter (Zp) exhibits very low basal activity, which is potently upregulated by inducers of the viral lytic cycle (Flemington and Speck, 1990a; Daibata *et al.*, 1994; Borrás *et al.*, 1996). The region from –221 to +12 bp of Zp harbors the necessary *cis*-elements for maintaining low basal activity and for transcriptional activation by lytic cycle-inducing agents (Flemington and Speck, 1990a; Borrás *et al.*, 1996). Within this sequence, three distinct types of response elements have been defined

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(see Fig. 1): (i) A + T-rich sequences termed ZI domains, four related copies of which are interspersed in the promoter (ZIA–D); (ii) a unique element, ZII, which shares homology with consensus CREB/AP1 binding sites (Flemington and Speck, 1990a; Borrás *et al.*, 1996; Ruf and Rawlins, 1995); and (iii) two adjacent sites, termed ZIIIA and ZIIIB, which bind the BZLF1 gene product Zta (Flemington and Speck, 1990b). ZIIIA, but not ZIIIB, is an AP1 response element (Flemington and Speck, 1990b). Induction of the BZLF1 gene appears to involve two steps: (i) initial activation of the promoter by inducers of the lytic cycle, mediated through the ZI and ZII domains, which results in low-level transcription of the BZLF1 gene; followed by (ii) autoactivation of Zp mediated through Zta binding to the ZIIIA and ZIIIB domains. We have previously shown that Zta autoactivation of Zp strongly synergizes with induction through the ZI and ZII domains (e.g., triggered by phorbol esters) (Flemington and Speck, 1990b; Borrás *et al.*, 1996). Thus, we have proposed that the duration and magnitude of the initial signal may determine whether enough Zta is generated in an appropriate time interval to trigger the entire lytic cascade.

We have recently reported a detailed analysis of the role of the ZIA and ZIC domains in TPA inducibility of Zp (Borrás *et al.*, 1996). Both domains exhibit phorbol ester inducibility when multimerized upstream of a minimal promoter. Phorbol ester inducibility was greatly augmented by inclusion of a single copy of the ZII domain, which on its own exhibited little activity. This suggested that TPA induction of Zp requires cooperation between cellular factors bound to the ZI and ZII domains. Analysis of cellular factor binding to the ZI sites revealed several complexes which could be resolved on nondenaturing polyacrylamide gels. Methylation interference analyses of these complexes demonstrated that the slowest migrating complex (complex 1) was centered over the region of sequence homology between the ZI domains (Fig. 1B). However, complexes 2, 3, and 4 were shifted slightly upstream of the homology region and exhibited identical patterns of sensitivity to methylation (Fig. 1B). In this paper we demonstrate that complex 2 reflects binding of Sp1, while complexes 3 and 4 reflect binding of the related transcription factor Sp3.

MATERIALS AND METHODS

Cell culture, transfections, and chloramphenicol acetyltransferase (CAT) assays

The EBV-negative Burkitt's lymphoma B cell line DG75 was grown at 37° in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% fetal calf serum, glutamine, and penicillin–streptomycin (Mediatech). DG75 cells were transfected using DEAE–dextran as described previously (Borrás *et al.*, 1996), with the following

modifications. Cells (10⁷ per transfection) were pelleted at 1000 *g*, washed once with sterile phosphate-buffered saline (PBS), and resuspended in 0.25 ml of PBS per transfection. Cells were added to sterile tubes containing 2 μ g of the appropriate vector in 0.25 ml of DEAE–dextran (1 mg/ml), incubated at room temperature for 20 min, and subjected to dimethyl sulfoxide shock (final concentration of 7%) for 2 min. Each tube was washed with 10 ml of PBS, resuspended in 10 ml of complete medium, and cultured at 37° in a 5% CO₂ incubator. For TPA induction, duplicate samples were transfected together in the same tube and following transfection split into two cultures resuspended in complete media \pm TPA. Final TPA concentration was 20 ng/ml.

Cells were harvested 72 hr posttransfection by centrifugation at 1000 *g*, washed once in PBS, and resuspended in 100 μ l of 0.25 *M* Tris–chloride, pH 7.5. The samples were lysed by three consecutive rounds of freeze-thawing, and the cellular debris was removed by centrifugation in a microcentrifuge at maximal speed. Eighty percent of the supernatant was used in CAT assays as previously described (Gorman *et al.*, 1982). The extent of acetylation was determined by exposure to a PhosphorImager plate (Molecular Dynamics).

Construction of vectors

The heterologous [3 \times ZI][ZII] constructs were cloned into BGCAT (Borrás *et al.*, 1996), which has the minimal β -globin promoter upstream of the CAT reporter gene in the pGL2 vector. This minimal β -globin promoter contains a 37-bp sequence with the TATA box and downstream sequences through the site of transcription initiation. Double-stranded oligonucleotides containing the three copies of a ZI element were then cloned into the *Aval/XbaI* restriction sites upstream of the β -globin TATA box. A single copy of the ZII element was cloned into the *XbaI/BamHI* sites. The sequences of the sense strand of the oligonucleotides used for cloning were as follows: 3 \times ZICwt, 5'-CCGGGACTGCCTCCTCTTTTAGAAACTAAGTGCCTCCTCCTCTTTTAGAACTAAGTGCCTCCTCCTCTTTTAGAACTAT-3'; 1 \times ZIIwt, 5'-CTAGACGTC-CCAAACCATGACATCACAGAGGAG-3'. The mutations introduced into the ZIC mutants are given in Fig. 4. Oligonucleotides with these base changes were synthesized and cloned into the appropriate restriction sites.

The [3 \times Sp1][ZII] β GCAT reporter construct was generated by cloning a double-stranded oligonucleotide containing three consensus Sp1 sites into the *Aval/XbaI* restriction endonuclease sites upstream of the ZII domain in the previously described [ZII] β GCAT reporter construct (Borrás *et al.*, 1996). The sequence of the sense strand of the oligonucleotide containing three copies of a consensus Sp1 site was as follows: 5'-CCGGGTCGA-TCGGGGCGGGGCGAGCTCGATCGGGGCGGGGCGA-GCTCGATCGGGGCGGGGCGAGCT-3'.

Electrophoretic mobility shift assays

DG75 crude nuclear extracts were prepared as described previously (Flemington and Speck, 1990a). A total reaction volume of 20 μ l containing 5 μ g of extract, 5 μ l of buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT), 4.5 μ g of bovine serum albumin, 0.5 μ l of 0.1 M DTT, and 2 μ g of salmon sperm DNA was incubated at room temperature for 5 min. If competitor oligonucleotides were used, they were added at this point. The relevant 32 P-labeled double-stranded oligonucleotide (0.4 ng per reaction, corresponding to 200,000 to 300,000 cpm) was added and incubated at room temperature for 30 min. Samples were loaded onto a running nondenaturing 4% acrylamide:0.1% bisacrylamide gel. The gel was run in 0.5 \times TBE (1 \times TBE is 90 mM Tris, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3). The 1 \times oligonucleotides used were designed to include the entire region protected from DNase I digestion (Flemington and Speck, 1990a). The sense strands of these oligonucleotides are as follows: ZIAwt, 5'-CCGGAAGTGGGCTGTCTATTTTGGACACCACT-3'; ZICwt, 5'-CTAGAACTGCCTCCTCCTCTTTTGAAGTATG-3'. The mutations introduced into the ZIC element are shown in Fig. 4. Oligonucleotides with these base changes were synthesized and used in the assay.

RESULTS AND DISCUSSION

Inspection of the region of the ZIC domain involved in binding complexes 2, 3, and 4 revealed homology to a CCT repeat motif present in the promoter for the epidermal growth factor (EGF) receptor gene (Johnson *et al.*, 1988). In the EGF receptor gene promoter two copies of this element are present, and it has been demonstrated that one of the factors that binds specifically to these elements is the ubiquitous cellular transcription factor Sp1 (Johnson *et al.*, 1988). A comparison of the sequences involved in binding Sp1 in the EGF receptor promoter to the Sp1 consensus binding site is shown in Fig. 1C (note that the sequences for ZIC and ZIA shown in Fig. 1C are the inverse complement of that shown in Fig. 1B).

Based on the homology between the ZIC domain and the EGF receptor promoter Sp1 binding sites, we assessed Sp1 binding to a double-stranded oligonucleotide probe containing the ZIC domain (Fig. 2). As shown in Fig. 2A, addition of an antibody directed against Sp1 to the EMSA assay resulted in the disappearance of complex 2 and the appearance of a weak supershift. Furthermore, when purified Sp1 was used a complex was observed with similar mobility to complex 2 (Fig. 2A). Addition of anti-Sp1 antibodies to the EMSA with purified Sp1 resulted in a supershifted band which migrated with similar mobility to the shifted complex observed with crude nuclear extract (Fig. 2A). Taken together these re-

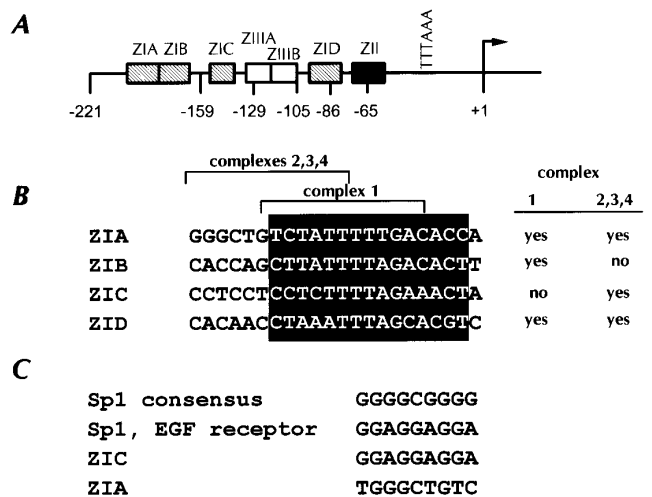


FIG. 1. (A) Organization of *cis*-elements in the BZLF1 promoter (Zp) as previously identified by DNase I footprinting in conjunction with functional analyses (Flemington and Speck, 1990a,b). The ZI domains were functionally grouped together on the basis of sequence similarity and binding competition studies (Flemington and Speck, 1990a). The ZII domain contains a consensus cyclic AMP response element-AP1 binding site, while the ZIIIA and ZIIIB domains are binding sites for the BZLF1 gene product, Zta, which serves to positively autoregulate Zp activity (Flemington and Speck, 1990b). (B) Sequence alignment of the ZI domains present in Zp. The core homology domain is highlighted in the shaded rectangle, while the regions identified as being involved in binding cellular transcription factors, as previously determined by methylation interference (Borras *et al.*, 1996), are indicated above. The ability to form complexes 1, 2, 3, or 4 for individual ZI domains is indicated to the right of the ZI sequences. (C) Sequence homology between the Sp1 consensus sequence and Sp1 binding sites present in the epidermal growth factor receptor (Johnson *et al.*, 1988) are shown aligned with the core sequences in the ZIA and ZIC domains involved in binding complexes 2, 3, and 4.

sults strongly argue that complex 2 represents binding of Sp1 to the ZIC domain.

Based on the similarities in binding of complexes 2, 3, and 4 to the ZIC element (Borras *et al.*, 1996), we considered the possibility that other Sp1 family members might also bind to this *cis*-element. Sp3 is a family member which has been shown to repress transcriptional activation by Sp1 (Hagen *et al.*, 1994). Thus, Sp3 binding to ZI domains might be involved in diminishing basal activity of Zp by blocking Sp1 binding. To address this possibility, antibodies which specifically recognize Sp3 (Hagen *et al.*, 1994) were added to the ZIC EMSA reaction. As shown in the righthand panel of Fig. 2A, anti-Sp3 antibodies specifically blocked the formation of complexes 3 and 4. It has previously been shown that this antibody reagent blocks Sp3 binding and does not give rise to a shifted complex(es) (Hagen *et al.*, 1994). In addition, the presence of two shifted complexes that react with the anti-Sp3 antibody has previously been observed (Hagen *et al.*, 1994). The faster migrating species (complex 4) may reflect binding of a smaller species of Sp3

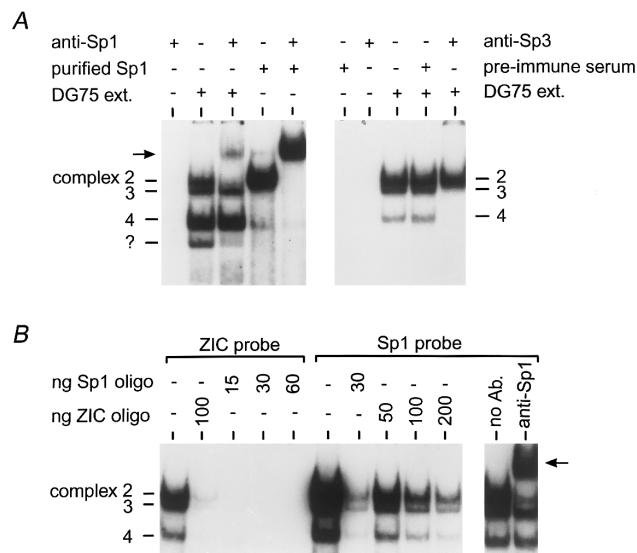


FIG. 2. (A) Sp1 and Sp3 bind to the ZIC domain. Electrophoretic mobility shift analyses (EMSAs) of the ZIC domain with crude nuclear extract prepared from the EBV-negative Burkitt's lymphoma cell line DG75 (prepared as previously described in Borrás *et al.*, 1996) or with purified Sp1 protein (Promega) were carried out under standard assay conditions (Borrás *et al.*, 1996). The ZIC probe employed has been previously described (Borrás *et al.*, 1996). The specific complexes are indicated to the left of the gel and correspond to those indicated in Fig. 1B. Antibodies specific for either Sp1 (Santa Cruz Biotechnologies) or Sp3 (Hagen *et al.*, 1994) were added as indicated. (B) Probes containing either the ZIC domain or a consensus Sp1 site exhibit the same pattern on EMSA gels which can be competed by either the ZIC or the Sp1 probes. EMSAs employed either the ZIC probe described in A or a probe containing a consensus Sp1 site (Promega), employing crude nuclear extract prepared from DG75 cells. The specific complexes formed are indicated to the left of the gel. Various amounts of unlabeled competitor oligonucleotide were added to the EMSA binding reaction as indicated. Supershift of the slowest migrating complex formed with the Sp1 probe with anti-Sp1 antibodies is also shown, carried out as described in A.

that arises from translation initiation at an internal AUG codon (G.S., unpublished data). The basis for the variation in the relative abundance of complexes 3 and 4 with different nuclear extract preparations is unclear (compare left and right panels in Fig. 2A).

To further characterize Sp1 and Sp3 binding to the ZIC element, competition with an unlabeled double-stranded oligonucleotide containing a consensus Sp1 binding site was carried out (Fig. 2B). This analysis clearly demonstrated that the Sp1 probe could readily compete for complexes 2, 3, and 4 (complete competition with only 15 ng of competitor). When the Sp1 probe was labeled and unlabeled ZIC double-stranded oligonucleotide was used as a competitor, ZIC was able to compete for binding but much less efficiently than the Sp1 oligonucleotide. This clearly indicates that the affinity of Sp1 and Sp3 for the ZIC domain is much lower than for a consensus Sp1 binding site. It should be noted that the pattern of cellular factor binding to the Sp1 probe was identical

to that observed with the ZIC probe, further substantiating the identification of Sp1 and Sp3 as the factors binding to ZIC. In addition, anti-Sp1 antibodies specifically supershifted the slowest migrating complex (complex 2; see Fig. 2B).

Based on our previous characterization of cellular factor binding to the other ZI domains, it is apparent that both the ZIA and the ZID domains also form complexes 2, 3, and 4 (Borrás *et al.*, 1996). That these complexes represent binding of the same cellular factors was established by binding competitions between the various ZI domains (Borrás *et al.*, 1996). To confirm that Sp1 does indeed bind to the ZIA domain, anti-Sp1 antibody was added to the ZIA EMSA reaction (Fig. 3). As expected, anti-Sp1 blocked binding of complex 2, but did not significantly affect the binding of complexes 1, 3, or 4 (note, no supershift of complex 2 was apparent under these assay conditions). Antibodies which specifically recognize the cellular transcription factors YY1 or c-myc were employed as negative controls and failed to alter the observed EMSA pattern. In addition, binding of cellular factors to the ZIB domain (which does not exhibit formation of complexes 2, 3, or 4) was unaffected by addition of anti-Sp1 antibodies (Fig. 3, righthand panel).

Our previous analysis of the nucleotides whose methylation blocked cellular factor binding to the ZIC domain indicated that the purine stretch on the antisense strand is involved in binding these factors (Borrás *et al.*, 1996). In addition, mutating blocks of three nucleotides across this region was shown to abrogate formation of complexes 2, 3, and 4 (Borrás *et al.*, 1996). To further investigate Sp1 and Sp3 binding to this region of ZIC, several additional mutants were generated (Fig. 4A). Binding to these mutants was assessed as shown in Fig. 4B, and

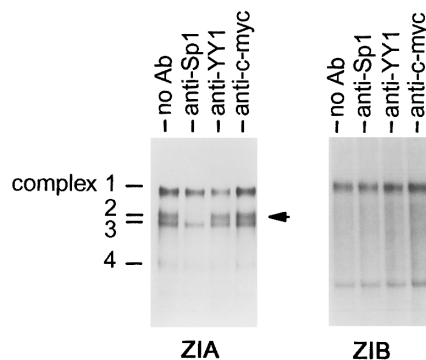


FIG. 3. Demonstration of Sp1 binding to the ZIA domain, but not the ZIB domain. EMSAs were carried out employing DG75 crude nuclear extract as described in Fig. 2. The specific complexes described in Fig. 1B are indicated to the left of the EMSA gels. The indicated specific antibodies were added to the EMSA binding reaction prior to addition of labeled probe as described in the legend to Fig. 2. Control supershifts were performed employing specific antibodies to the cellular transcription factors c-myc (Santa Cruz Biotechnologies) and YY1 (Santa Cruz Biotechnologies).

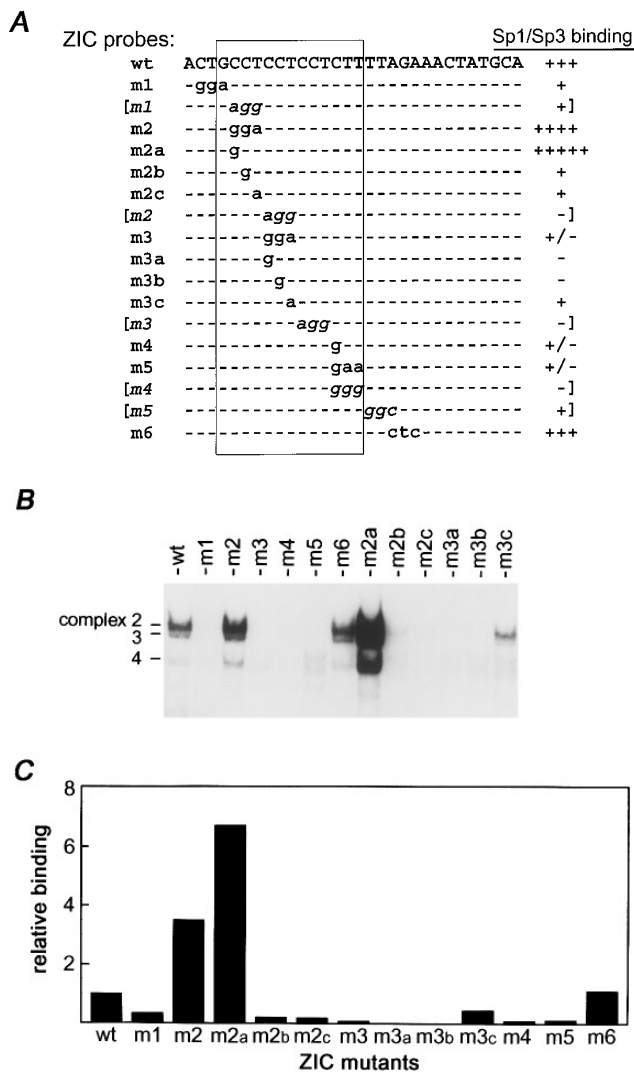


FIG. 4. Sequences of ZIC probes containing specific mutations and summary of Sp1 and Sp3 binding. Sequences within the open rectangle are within the region previously demonstrated by methylation interference to be involved in contacting the cellular transcription factors present in complexes 2, 3, and 4 (Borras *et al.*, 1996). Those ZIC mutants within the brackets were previously characterized for binding (Borras *et al.*, 1996) and are shown for comparison. (B) Analysis of specific mutations on Sp1 and Sp3 binding to the ZIC domain. EMSAs were carried out with DG75 crude nuclear extract as described in the legend to Fig. 2. The specific complexes are indicated to the left of the EMSA gel and are those depicted in Fig. 1B. (C) Quantitation of Sp1 and Sp3 binding to ZIC mutants. The EMSA analysis shown in B was quantitated employing a Phosphorimager (Molecular Dynamics). The level of binding for each mutant is shown relative to the binding observed with the unmutated ZIC probe (wt), which was assigned a value of 1.0.

the EMSA results were quantitated on a PhosphorImager (Molecular Dynamics). Mutation of the three nucleotides immediately upstream of the CCTCCTCCT motif decreased binding ca. threefold. It should be noted that the last nucleotide in the triplet was previously determined by methylation interference to be a contact residue for Sp1 and Sp3 binding (Borras *et al.*, 1996). As we pre-

viously showed, mutation of the first CCT repeat to AGG strongly diminished Sp1 and Sp3 binding (Borras *et al.*, 1996). However, surprisingly, mutation of the first CCT to GGA resulted in a strong induction of binding (see m2 mutant; Figs. 4B and 4C). Subsequent dissection of this mutation revealed that changing the first C to a G gave rise to a nearly sevenfold increase in the level of Sp1 and Sp3 binding, while mutation of the second C to a G or mutation of the T to an A strongly inhibited binding activity (see m2a, m2b, and m2c mutations; Figs. 4B and 4C). Notably, introduction of the identical mutation into the second CCT repeat resulted only in a strong inhibition of binding. This is consistent with our previous observation with another mutation introduced into this region (Borras *et al.*, 1996). An analogous dissection of this mutation failed to reveal any mutation which enhanced binding, although mutating the T to an A only modestly reduced binding of Sp1 and Sp3 (see mutation m3c; Figs. 4B and 4C). Thus based on this analysis, along with our previous mutagenesis, all three CCT repeats appear important for binding Sp1 and Sp3. Furthermore, as evidenced by mutation of the C immediately downstream of the CCT repeat to a G, nucleotides outside the CCT repeat also strongly affected binding (see mutations m4 and m5; Figs. 4B and 4C). These mutations lie within the region previously demonstrated by methylation interference to be involved in binding complexes 2, 3, and 4 (shown here to be Sp1 and Sp3) (Borras *et al.*, 1996). The only mutation that affects binding that lies outside the region identified by methylation interference is the m5 mutation we previously reported (Borras *et al.*, 1996), which mutates the region immediately downstream of the region protected from methylation. A slightly more distal mutation (m6), which also lies outside the region protected in the methylation interference assays, did not affect binding of Sp1 and Sp3 (Figs. 4B and 4C). Taken together, these results indicate that the affinity of Sp1 and Sp3 for the ZIC domain is strongly affected by critically placed G and C residues within the binding site. This notion is most strongly supported by the ca. sevenfold enhancement in binding observed with the m2a mutation, in which a single C was changed to a G.

To assess whether Sp1/Sp3 binding could account for the functional behavior displayed by heterologous reporter constructs containing ZIC sites, we generated a heterologous promoter containing three consensus Sp1 sites linked to a single copy of the ZII domain from Zp cloned upstream of a minimal β -globin promoter driving expression of the CAT reporter gene ($[3 \times \text{Sp1}][\text{ZII}]\beta\text{GCAT}$). This is analogous to reporter constructs that we previously characterized that contain three copies of individual ZI domains cloned upstream of the ZII domain in the β -globin promoter CAT vector (Borras *et al.*, 1996). TPA and ionomycin inducibility of the $[3 \times \text{ZIC}][\text{ZII}]\beta\text{GCAT}$ and $[3 \times \text{Sp1}][\text{ZII}]\beta\text{GCAT}$ reporter constructs

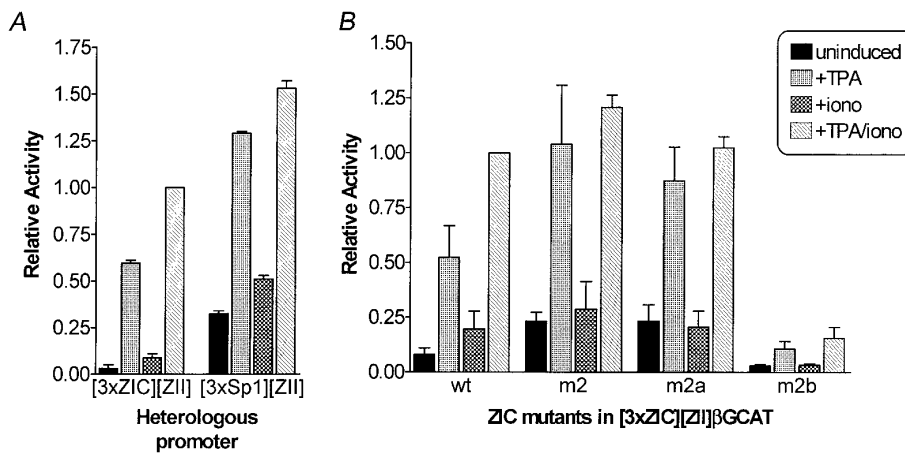


FIG. 5. Correlation between Sp1 binding and TPA inducibility of heterologous promoters. (A) A heterologous promoter containing multiple Sp1 sites exhibits phorbol ester inducibility. DG75 cells were transiently transfected with 2 μ g of the indicated reporter construct by the DEAE-dextran/DMSO shock protocol, as previously described (Borras *et al.*, 1996). Generation of the indicated heterologous promoter constructs is described under Materials and Methods. TPA (20 ng/ml final concentration) and ionomycin (1 μ M final concentration) were added immediately posttransfection as indicated. Transfected cells were harvested 72 hr posttransfection and analyzed for CAT activity as previously described (Borras *et al.*, 1996). Activities are expressed relative to the activity observed with the [3 \times ZIC][ZII] β GCAT reporter construct in the presence of TPA and ionomycin (defined as an activity of 1.0). Compiled data from three independent transfections is shown. (B) ZIC mutants with altered Sp1 binding exhibit altered basal and TPA-inducible activities. The mutations introduced into the ZIC domain are shown in Fig. 4A. Reporter constructs were transfected into DG75 cells, and CAT activity was assessed as described in A and under Materials and Methods.

was compared (Fig. 5A). As previously observed with the [3 \times ZIC][ZII] β GCAT reporter construct (Borras *et al.*, 1996), addition of TPA induced CAT activity. Ionomycin had little effect, and the combination of TPA and ionomycin enhanced activity less than twofold over that observed with TPA alone. Notably, the [3 \times Sp1][ZII] β GCAT reporter construct exhibited significantly higher basal activity than the [3 \times ZIC][ZII] β GCAT reporter construct (see Fig. 5A). However, as observed with the [3 \times ZIC][ZII] β GCAT reporter construct, the [3 \times Sp1][ZII] β GCAT reporter construct exhibited TPA inducibility which could be slightly augmented by the addition of ionomycin (Fig. 5A). It should be noted that we have previously demonstrated that heterologous promoter constructs containing only a single copy of the ZII domain cloned upstream of the minimal β -globin promoter exhibited little or no TPA inducibility (Borras *et al.*, 1996).

Overall, the functional characteristics of the [3 \times ZIC][ZII] β GCAT and [3 \times Sp1][ZII] β GCAT reporter constructs are similar. The higher basal activity exhibited by the [3 \times Sp1][ZII] β GCAT reporter construct correlates with the higher affinity binding of Sp1 and Sp3 for the consensus Sp1 site compared to the ZIC site (see Fig. 2B). As such, *in vivo* occupancy of the ZI sites by Sp1 (or Sp3) would be expected to be less than that of sites that more closely match the consensus Sp1 recognition sequence, thereby leading to lower constitutive activity. In addition, although there is no evidence from our binding analyses, it is possible that under physiological conditions the balance between Sp1 and Sp3 binding may be different between these sites. Since Sp3 has been

shown to interfere with Sp1-mediated transcriptional activation (Hagen *et al.*, 1994), this might also lead to lowering basal activity.

To further address the question of whether affinity of Sp1/Sp3 binding correlates with observed basal activity, we generated [3 \times ZIC][ZII] β GCAT reporter constructs in which the m2, m2a, or m2b mutations (see Fig. 4A) were introduced into all three copies of the ZIC element. As shown in Fig. 4B, the m2 and m2a mutations increased the affinity of Sp1 and Sp3 for the ZIC element, while the m2b mutation disrupted binding of Sp1 and Sp3 to the ZIC element. When these reporter constructs were assayed in the DG75 cell line, both the m2 and the m2a mutants exhibited increased basal and TPA-induced activities compared to that of the unmutated promoter (Fig. 5B). However, the m2b mutation significantly diminished both basal and induced activity (Fig. 5B). These results are consistent with the hypothesis that higher affinity Sp1/Sp3 binding leads to an increase in basal activity. Furthermore, the effect of the point mutation introduced in the ZICm2b reporter construct provides a strong correlation between decreased affinity of Sp1/Sp3 binding and loss of activity.

In the case of the ZIC domain, Sp1 and Sp3 are the only cellular factors we have detected binding to this site. However, for the ZIA and ZID domains, in addition to Sp1 and Sp3 binding, there is clearly another cellular factor involved in binding to these *cis*-elements (Borras *et al.*, 1996), which we have recently identified as MEF2D (a member of the myocyte enhancer factor 2 family, also known as related to serum response factor family) (Liu

et al., 1996). MEF2D binds to the region of highest homology between the ZI domains (see Fig. 1), while the region binding Sp1/Sp3 is shifted slightly upstream (Borras *et al.*, 1996; Liu *et al.*, 1996). Given the extensive overlap between these binding sites, it is very unlikely that both MEF2D and Sp1/Sp3 could bind simultaneously to a single ZI element. To rule out the possibility that low-affinity binding of MEF2D to the ZIC domain is functionally important, three linked copies of a truncated ZIC domain were cloned to generate a [3 × ZIC_{short}][1 × ZII]βGCAT reporter construct. The truncation of the ZIC domain retained the Sp1/Sp3 binding site, but partially deleted the region of ZIC which would be predicted to bind MEF2D based on the analysis of MEF2D binding to the ZIA and ZIB domains (Borras *et al.*, 1996). The ZIC_{short} reporter construct exhibited similar TPA inducibility to the artificial promoter containing the full-length ZIC element, although the basal activity was significantly higher (data not shown). Whether the latter reflects an alteration in the affinity of Sp1/Sp3 binding, perhaps due to the altered spacing between ZIC sites, remains to be determined.

At this point it is unclear whether binding of MEF2D, Sp1, and/or Sp3 at the ZIA and ZID sites is physiologically relevant. It should be noted, however, that MEF2D is the only cellular factor we have observed binding to the ZIB domain (summarized in Fig. 1) (Borras *et al.*, 1996; Liu *et al.*, 1996). Our previous studies demonstrated that the ZIB domain is functionally distinct from the other ZI domains in that it exhibits little TPA inducibility when multimerized and cloned upstream of the ZII domain (Borras *et al.*, 1996). Thus, it is unlikely that MEF2D binding to the ZIA and ZID domains can alone account for the TPA-inducible nature of these domains. Taken together these results argue for a role of Sp1 and Sp3 in phorbol ester inducibility of the BZLF1 gene promoter mediated through the ZIA, ZIC, and ZID domains.

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