1108–1116 *Nucleic Acids Research, 1997, Vol. 25, No. 6* *© 1997 Oxford University Press*

The ZiN/POZ domain of ZF5 is required for both transcriptional activation and repression

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Received January 3, 1997; Accepted January 23, 1997

ABSTRACT

ZF5 is a ubiquitously expressed protein originally identified by its ability to bind and repress the murine *c-myc* promoter. It contains five C-terminal zinc fingers and a conserved N-terminal ZiN/POZ domain. This motif, found in a growing number of zinc finger proteins, can inhibit DNA binding and mediate dimerization [Bardwell,V.J. and Treisman,R. (1994) *Genes Dev.,* 8, 1664–1677]. In the current study, a cyclic amplification and selection of targets (CAST) protocol detected preferred ZF5 binding sites which are highly GC-rich. Binding to these sites by ZF5 depended upon the zinc fingers and was enhanced when the ZiN/POZ domain was removed. Using transient cotransfection assays, ZF5 was shown to activate the HIV-1 LTR and repress the β-actin promoter. The ZiN/POZ domain was shown to mediate ZF5-dependent transcriptional activation and repression. From these data, we conclude that ZF5 can both activate and repress in the context of different natural promoters and that its ZiN/POZ domain can affect two functions; DNA binding and transcriptional modulation.

INTRODUCTION

ZF5 cDNA was cloned based on the ability of the ZF5 protein to bind a regulatory region of the murine *c-myc* promoter (2). In addition to five zinc finger domains at the C-terminus, ZF5 contains an N-terminal ZiN (Zinc finger N-terminus) (2), POZ (1) or BTB (3) domain. This motif identifies ZF5 as a member of a rapidly expanding family of zinc finger proteins which contain conserved ZiN/POZ domains (1,33).

ZF5 binds to two elements within the –290 to –240 bp region in the *c-myc* promoter (2). These sites flank a YY1 activator site (4,5) and overlap a Blimp-1 repressor site (6,7, Lin *et al.* submitted). ZF5 also binds to the –50 'Sp1' site of the herpes simplex virus 1 (HSV-1) thymidine kinase (tk) promoter (2). In these contexts and in a Gal4 fusion assay ZF5 is a transcriptional repressor. Additional target genes for ZF5 have not been identified.

ZF5 is ubiquitously expressed with highest levels found in brain and ovary tissues and fibroblast cell lines (2). Recently, ZF5 was independently cloned using a differential display technique as a gene specifically expressed in slow-growth phenotype female preimplantation embryos (James Crane, personal communication). This is consistent with a recent report demonstrating impaired growth of cell lines which overexpress ectopic ZF5 (8). Thus, ZF5 is expressed early in development and may be important for retarding cellular proliferation.

The number of known zinc finger proteins containing ZiN/POZ domains is growing rapidly; they have been found in species as diverse as *Drosophila melanogaster*, mice and humans. ZiN/POZ proteins usually either activate or repress transcription. Although the biological roles of mammalian ZiN/POZ proteins are poorly understood, two human ZiN/POZ proteins, BCL6 and PLZF, appear to play a role in leukemogenesis (9–14). In drosophila, approximately 40 ZiN/POZ family members have been detected (3) and several have been shown to regulate important developmental decisions (15–19).

The ZiN/POZ domain of the human ZID protein inhibits DNA binding by the ZID zinc fingers and is a dimerization domain with a high degree of specificity for dimerization partners (1). Consistent with its function as a dimerization domain, the ZiN/POZ domain of drosophila bric a brac has an alpha-helical structure with a highly hydrophobic face rich in leucine residues (18).

The studies reported here were undertaken to characterize the functional domains of ZF5 and to identify natural promoters which are subject to ZF5 regulation. Using a truncated form of ZF5 with enhanced DNA binding ability, a binding site selection protocol was used to define the preferred ZF5 binding sequence. This sequence is GC rich and shows similarity to Sp1 binding sites. Therefore, ZF5 was tested for its ability to regulate transcription of the HIV-1 LTR which depends upon three Sp1 sites. Interestingly, ZF5 activates this promoter. Activation of the HIV-1 LTR by ZF5 depends on ZF5 binding to DNA, requires intact Sp1 sites, is synergistic with the viral TAT protein and requires the ZF5 ZiN/POZ domain. The ZiN/POZ domain was also shown to be required for repression by ZF5 on a natural promoter and in a Gal4 fusion assay.

MATERIALS AND METHODS

Bacterial protein expression

pGEX-ZF5, pGEX-ΔZF or pALEX-ΔZiN bearing *Escherichia coli* cultures were grown to an OD_{600} of ~0.4–0.6 and induced with 1 mM IPTG for 2 h. The bacteria were centrifuged at 4000 *g* for 15 min and resuspended in GST binding buffer [50 mM Tris–HCl pH 8.0, 100 mM NaCl, 100 μM $ZnCl_2$, 1 mM DTT, 0.1% Triton X-100, 5% glycerol, 6.3 μg/ml aprotonin, leupeptin, pepstatin and benzamidine, 63 μg/ml *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) and *N*-tosyl-L-phenylalanine chloromethyl ketone

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(TPCK), 725 μM phenyl methane sulfonyl fluoride (PMSF)]. Cells were lysed by sonication, the bacterial debris centrifuged at 6000 *g* for 15 min at 4°C. The supernatant was saved for quantification and analysis. Because of the significant insolubility of bacterially expressed ZF5 proteins, the pellets were resolubilized by dialysis in GST binding buffer plus 725 μM PMSF, 500 mM NaCl and 6 M urea and sequentially renatured in 4 M, 2 M, 1 M, 500 mM and 0.0 M urea at 4°C. GST–ZF5 and GST–ΔZiN-ZF5 were purified by incubation with glutathione (GSH)–agarose beads (Sigma), washed three times with GST binding buffer and eluted with GST binding buffer plus 10 mM reduced glutathione but lacking Triton X-100. ZF5-ΔZiN was cleaved from the GSH–agarose beads/GST–ΔZiN-ZF5 complex by activated factor X (Boehringer Mannheim) in GST binding buffer at 4°C for 8 h. Proteins were analyzed by SDS–PAGE and quantified as described (20).

Cyclic amplification and selection of targets (CAST)

For each round of selection, 1.5 mg crude GST or GST–ΔZiN-ZF5 extract was incubated with a 50% slurry of GSH–agarose in a final volume of 100 μl in GST binding buffer. Binding of protein to the beads occurred for 2 h at room temperature followed by two washes in GST binding buffer. The bound beads were then equilibrated to ZF5 binding buffer (20 mM Tris–HCl pH 7.5, 50 mM NaCl, 5% glycerol, 1 mM DTT, 100 μM $ZnCl_2$) by two consecutive washes. Duplex degenerate oligonucleotide (10 μg), gift of Riccardo Dalla Favara (AGACGGATCCATTGCA[N_{20}]CT-GTAGGAATTCGGA) was added and binding to the immobilized protein occurred in a 50 μl reaction for 20 min at room temperature. The resulting complex was washed four times in ZF5 binding buffer and directly subjected to PCR amplification as described in the text. Following six rounds of selection and amplification, the resulting sequences were cloned into the pGEM-T vector (Promega). Nucleotide analyses were performed by the Consensus Program offered by the Genetics Computer Group (32).

Electrophoretic mobility shift assay (EMSA)

Probes for EMSA were produced by phosphorylation of oligonucleotides with [γ-^{32}P]ATP, PCR amplification and polyacrylamide gel purification. Unless otherwise noted, 20 ng of bacterially expressed, purified ZF5-derived proteins were used; binding reactions occurred in ZF5 binding buffer at room temperature for 20 min. Some EMSA experiments included 50 ng poly(dA-dT)(Pharmacia) as competitor for non-specific DNA binding activity. Competitors for specific DNA binding activity were preincubated with proteins in the binding reactions for 10 min before addition of 20 000–40 000 c.p.m. of labeled probe per reaction. Bound and free complexes were separated on a native 5% polyacrylamide gel in 0.25× TBE (22 mM Tris, 22 mM borate, 500 μM EDTA) at 4°C.

DNase I footprinting

End labeled probes were bound to the indicated proteins in 50 μl ZF5 binding buffer. DNase I (Worthington) was added at the indicated concentrations with a final $CaCl_2$ concentration of 2.5 mM for 1 min followed by addition of 100 μl stop buffer [1% SDS, 20 mM EDTA, 200 mM NaCl, 200 ng/μl glycogen (Boehringer Mannheim)]. Samples were phenol/chloroform extracted, ethanol precipitated and resolved on a 7 M urea/TBE, 8% polyacrylamide sequencing gel followed by autoradiography.

Transfections

NIH 3T3 cells growing in 10 ml Iscove's Modified Dulbecco's Medium (IMDM) + 10% newborn calf serum were split at a density of 5×10^5 cells/10 cm plate the day before transfection and the cells were again fed 3 h before transfection. For co-transfection experiments in NIH 3T3 cells, 1 μg of each reporter and the indicated amount of expression vectors were combined with pBluescript II SK(+) carrier DNA (Stratagene) to a total of 11 μg plasmid. These DNAs were added as a $CaPO_4$ precipitate (25 mM HEPES; 140 mM NaCl; 750 μM Na_2HPO_4; 125 mM $CaCl_2$) to the media of the cells to be transfected. The following day, 3T3 monolayers were shocked for 2 min (15% glycerol; 25 mM HEPES; 140 mM NaCl, 750 μM Na_2HPO_4) and incubated another 24 h in 10 ml medium. Cells were harvested and luciferase activity was assayed as described (2).

Western analysis

NIH 3T3 cells were transfected as described with 20 μg of each expression construct. The cells were harvested by scraping in PBS on ice, counted, centrifuged at 2500 *g* at 4°C, resuspended in Western substrate buffer (WSB) (50 mM Tris–HCl pH 6.8, 0.2% SDS, 1 mg/ml bromophenol blue, 0.1 M DTT, 10% glycerol), and boiled for 5 min. 1.6×10^5 cell equivalents (ce) were loaded into each lane of a 10% SDS–PAGE gel, followed by electroblot onto nitrocellulose. The blot was fixed for 1 min in isopropanol, rehydrated in water and blocked with 5% dry milk in PBS. For monitoring the expression of the Gal4 fusion proteins, anti-yeast Gal4 DNA binding domain antibody (Upstate Biotechnology Incorporated) was used at a 1:500 dilution in 2% dry milk in PBS; goat anti-rabbit IgG, peroxidase conjugated (Boehringer Mannheim), was used at a $1{:}10^4$ dilution in 2% dry milk/PBS. For the detection of FZF5LexA, FZF5-ΔZFLexA, FZF5ΔZiNLexA and FZF5 (Flu tagged), monoclonal anti-influenza hemaglutinin antibody 12CA5 (Boehringer Mannheim) was used at a 1×10^4 dilution and detected with rabbit anti-mouse IgG, peroxidase conjugated (Boehringer Mannheim). The bands were visualized by ECL Western Detection (Amersham) and exposure to X-ray film (Kodak).

Plasmid construction

To construct pGEX-ZF5, ZF5 cDNA was PCR amplified with a synthetic N-terminal *Bam*HI site engineered into the 5′ primer. A *Bam*HI–*Fsp*I fragment was blunt end cloned into the *Bam*HI site of pGEX-2T (Pharmacia). pGEX-ΔZF resulted from an internal deletion which removed the ZF5 zinc fingers from pGEX-ZF5 by partial *Bst*EII digestion, end filling and religation. To construct pALEX-ΔZiN, the same 5′ primer was used to amplify ZF5 cDNA and a *Hin*cII–*Sac*I (blunt) fragment was cloned into the *Sma*I–*Not*I (Blunt) sites of pALEX (33). To construct Gal4–ZF5, the *Bam*HI–*Fsp*I fragment was cloned into the *Bam*HI–*Ecl*136II sites of Gal4 1–147 (34). Gal4–ZF5 and Gal4(.76) were constructed as described (Numoto *et al.*). Gal4–ZiN and Gal4–ZiNAc contain ZF5 amino acids 1–90 and 1–209, respectively. The 5′-ends both use the synthetic *Bam*HI site, the 3′-ends use synthetic *Sal*I sites to clone into the *Bam*HI and *Sal*I sites of Gal4 1–147. Gal4 AcX has synthetic *Sma*I and *Xba*I sites bracketing the sequences encoding amino acids 159–283 and cloned into the same sites in Gal4 1–147. pFLexA was constructed by PCR amplifying the first 85 codons of LexA from pBTM116 (35) with primers which introduced *Sma*I

and *Bam*HI sites on the 5′- and 3′-ends, respectively. Also, on the 3′-end is an engineered stop codon after codon 85, this fragment was cloned into the *Sma*I and *Bam*HI sites of pGCN (36). To construct pFZF5LexA, ZF5 was PCR amplified from Gal4–ZF5 with a 5′ primer which bound to the Gal4 1–147 multiple cloning sequence and contained an engineered *Xba*I site. The 3′ primer bound to the last five codons of ZF5 before the first stop codon and contained an engineered *Sac*I site. This fragment was cloned into the pFLexA *Xba*I and *Sac*I sites (a partial *Sac*I digestion strategy was employed). For pFZF5, an *Xba*I stop-linker (New England Biolabs #1062) was cloned into the *Sma*I site between the ZF5 and LexA sequences, ensuring the expression of only a ZF5 protein. pFZF5-ΔZFLexA was constructed identically to pFZF5LexA except the 3′ primer bound to codons 274–278, just upstream from the zinc fingers. Construction of pFZF5-ΔZiNLexA was also identical to that of pFZF5LexA except the original Gal4 fusion construct contained a truncation in the 5′ ZF5 sequence at the *Acc*I site. LTR–Luc, –158ΔLTR–Luc, –93ΔLTR–Luc and pβactinLuc were as described (23,24). To construct pGL2wtLTR and pGL2mSp1LTR, the wild-type and mutant HIV-1 LTR sequences from –177 to 84 were PCR amplified from HIV–CAT and NSPALL (25) and blunt end cloned into the *Eco*RV site of pBluescript II SK+ (Stratagene). *Hin*cII/*Bam*HI fragments with the HIV sequences were cloned into the *Sma*I and *Bgl*II sites of pGL2Basic (Promega). RSV*tat* is as described (25).

RESULTS

The consensus binding sequence for ZF5 is GC-rich

In order to identify potential ZF5 binding sites in natural promoters, a CAST protocol (19) was employed to determine the consensus binding site for ZF5. Briefly, a pool of synthetic oligonucleotides was designed such that 20 bases of degeneracy were flanked by 15 base constant regions. Based on Bardwell and Treisman's data (1) showing that the ZiN/POZ domain of ZID inhibited its DNA binding ability, we used a truncated form of ZF5 lacking the ZiN/POZ domain in the CAST protocol. Bacterially expressed glutathione acetyl transferase (GST) and GST–ΔZiN-ZF5 (a GST fusion protein containing the C-terminal portion of ZF5 but lacking the ZF5 ZiN/POZ domain, Fig. 1A) were immobilized on glutathione (GSH)–agarose beads and incubated with the double-stranded oligonucleotide pool. The bound complexes were isolated and subjected to 10, 14 or 18 cycles of PCR amplification. By 10 cycles, a specific product from the GST–ΔZiN-ZF5 matrix could be detected by agarose gel electrophoresis. This product was selected by immobilized GST–ΔZiN-ZF5 in a second round of CAST. Six rounds of selection were performed followed by cloning and sequencing.

Twenty-nine sites were selected by CAST. In preliminary EMSAs 17 appeared to be bound by GST–ΔZiN-ZF5 with higher affinity than the remaining 12 (data not shown). Z44, a high affinity site, was then used as a probe in an EMSA with purified GST–ΔZiN-ZF5 and competitors corresponding to the Z11 site (high affinity), the Z5 site (low affinity) and a non-selected pool of degenerate sequences. The Z11 competitor competed efficiently for binding (Fig. 1B, lanes 3–5) while the Z5 competitor was much less efficient (Fig. 1B, lanes 6–8). The pool of unselected sequences, N_{20}, did not function as an efficient competitor for GST–ΔZiN-ZF5 binding activity (Fig. 1B, lanes 9–11). These data confirm that the CAST procedure successfully enriched for sequences with ZF5 binding specificity and that sequences bound with both high and low affinity were selected.

Several controls were performed to confirm the validity of the CAST experiment. To test if removal of the ZiN/POZ domain altered the specificity of DNA binding by ZF5, an EMSA was performed with full length GST–ZF5 using the same panel and concentration of competitors (Fig. 1C). As with GST–ZF5-ΔZiN, only the Z11 competitor could efficiently compete for GST–ZF5 binding (lanes 3–5) with no competition from the Z5 competitor (lanes 6–8) or the N_{20} pool (lanes 9–11). Since ZF5 proteins with or without the ZiN/POZ domain respond similarly to the same panel of competitors, we conclude that the ZiN/POZ domain of ZF5 does not affect its DNA binding specificity.

To determine if the GST moiety might have affected the DNA binding specificity of GST–ZF5-ΔZiN, an EMSA was conducted on a ZF5-ΔZiN protein (Fig. 1A) which was purified by binding GST–ZF5-ΔZiN to GSH–agarose beads and subsequently cleaved by Factor X (Fig. 1D). As observed with GST–ZF5-ΔZiN(Fig. 1B) only the Z11 competitor efficiently competed for ZF5-ΔZiN binding (lanes 3–5). The Z5 (lanes 6–8) and N_{20} pool (lanes 9–11) could not compete for complex formation. Since GST–ZF5-ΔZiN and ZF5-ΔZiN show similar DNA sequence specificity, we conclude that the presence of the GST moiety did not affect binding specificity in the CAST experiment.

We also wished to determine directly how the ZiN/POZ domain affected the binding affinity of ZF5 to DNA. We used the Z44 probe in an EMSA (Fig. 1E) with equivalent amounts of full length GST–ZF5 and GST–ZF5-ΔZiN proteins, as determined by Coomassie staining of the proteins on a SDS–PAGE gel (data not shown). Full length GST–ZF5 bound ~5-fold less efficiently than GST–ΔZiN-ZF5 (Fig. 1E, lanes 2 and 5). The complexes all represent specific protein–DNA interactions since excess Z11 competitor (lanes 3 and 6), but not the N_{20} Pool (lanes 4 and 7) competed for binding. Thus, although full-length ZF5 does bind DNA, the ZiN/POZ domain decreases its binding ability.

To demonstrate formally that the zinc fingers constitute the DNA binding domain of ZF5, an internal deletion, GST–ZF5-ΔZF (Fig. 1A), was constructed and used in an EMSA with Z44 (Fig. 1F). As compared to the complex formed with 50 ng of full length protein (lane 2), no complex could be detected with 50, 100, 150 or 200 ng of GST–ZF5-ΔZF (lanes 3–6). This experiment confirmed that the zinc fingers are in fact the DNA binding domain of ZF5. Protein concentrations were determined as described (20).

To identify the specific sequences which contribute to high affinity ZF5 binding, five high affinity sites (Z1, Z11, Z23, Z24 and Z44) were analyzed by DNase I protection. A typical footprint using the Z44 probe (Fig. 2A) shows a protection pattern which was dependent on the concentration of ZF5-ΔZiN protein. Based on the protection patterns of the footprinted sequences, all 17 high affinity sites were aligned. The preferred binding sequence consensus for ZF5 (GGGGRCGCGCW) is presented in Figure 2B at the bottom of the alignment. However, we note this is not a tight consensus. The degree of non-consensus bases in the high affinity sites varies from only 1/11 bases (Z11 and Z24) to as much as 5/11 bases (Z34 and Z42). The consensus sequence for Sp1 (21), which is also GC rich, is similar to the ZF5 sequence, being identical at 7 out of 10 bases. Previously identified ZF5 binding sites in the *c-myc* promoter and the –50 Sp1 site from the HSV-1 tk promoter (2) also match the ZF5 consensus.

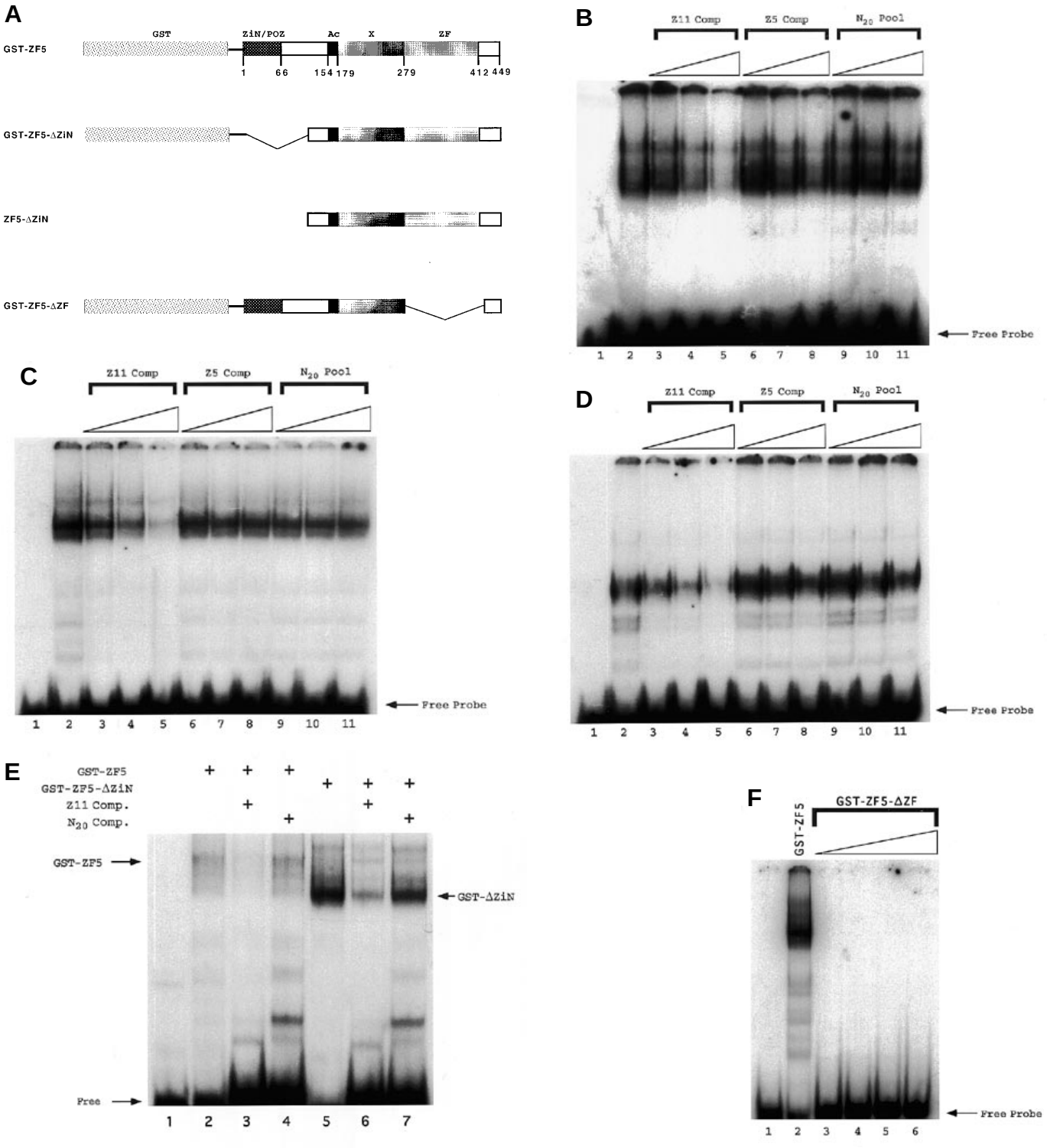

Figure 1. Analysis of the ZF5 binding sequences selected by CAST. **(A)** Schematic of the GST–ZF5 fusion proteins used to analyze the selected sequences. The GST, ZiN/POZ, acidic (Ac) and zinc finger (ZF) domains are indicated. Domain X is of unknown function and the amino acid numbers are indicated. **(B)** EMSA demonstrates that GST–ZF5-ΔZiN binds the Z44 probe specifically. Lane 1, no protein; lanes 2–11, 20 ng purified GST–ZF5. Competitors used were Z11 (high affinity), Z5 (low affinity) and N_{20} (non-specific) as indicated at the top and were used at 10-, 20- and 40-fold molar excess over the Z44 probe fragment. The free probe and specific protein–DNA complexes are indicated. **(C)** Demonstration that truncation of the ZiN/POZ domain does not affect the DNA binding specificity of ZF5. EMSA is similar to that in (B) except that 20 ng full length GST–ZF5 was used in lanes 2–11. **(D)** Demonstration that the GST moiety of the fusion proteins does not affect the binding specificity of the ZF5 moiety. EMSA is similar to (A) and (B) except that the ZF5-ΔZiN protein used in lanes 2–11 was cleaved from the GSH/agarose-immobilized GST moiety by activated Factor X. **(E)** EMSA analysis demonstrates the differential binding activities of GST–ZF5 (lanes 2–4) and GST–ZF5-ΔZiN (lanes 5–7). The Z44 probe was used with equivalent quantities of full length recombinant protein (~300 ng), as determined by Coomassie stained SDS–PAGE analysis (data not shown), in the binding reactions. The competitors used were Z11 (specific) and N_{20} (non-specific) at a 40-fold molar excess over the probe. **(F)** EMSA demonstrating that ZF5 binds DNA via its zinc fingers. Lane 1, no protein; lane 2, 50 ng GST–ZF5; lanes 3–6, 50, 100, 150 and 200 ng GST–ZF5-ΔZF.

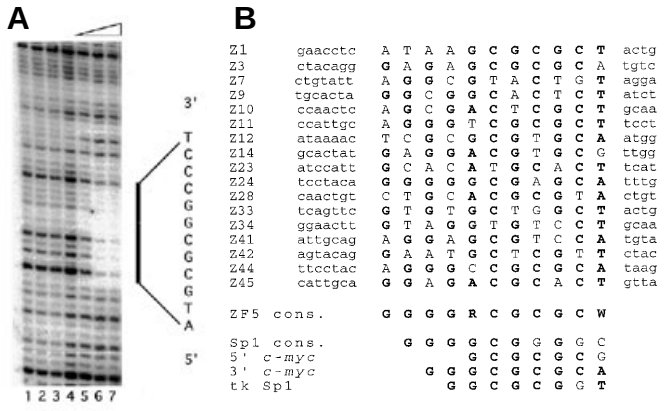

Figure 2. Identification of the ZF5 binding site consensus. **(A)** DNase I footprinting identifies a site specifically bound by GST–ΔZiN-ZF5 on a Z44 probe. Lanes 1–4, no protein and final DNase I concentrations of 1.25, 2, 3.3 and 10 μg/ml; lanes 5–7, final DNase I concentration of 2 μg/ml and 250, 500 and 750 ng purified ZF5-ΔZiN. The protected region is indicated by the bar, an arrow marks a DNase I hypersensitivity site, and the sequence was determined by a Maxam and Gilbert G ladder. **(B)** Alignment of the high affinity binding sequences. The 17 high affinity sequences were aligned and a ZF5 binding consensus was derived. For comparison, the Sp1 binding consensus and three previously defined ZF5 binding sites are provided. The center column represents the sequences that were used to generate the alignment, bold letters from each selected sequence match the consensus. Assignment of specific nucleotides to any position in the consensus required eight or more occurrences.

ZF5 activates the HIV-1 LTR

Based on the consensus binding sequence for ZF5, we hypothesized that ZF5 might regulate transcription from the HIV-1 LTR which contains a very GC-rich region and depends on three Sp1 sites. A co-transfection assay in NIH 3T3 fibroblasts was employed to test this possibility. A ZF5 expression plasmid, pFZF5, was engineered so that the influenza hemaglutinin (Flu) epitope tag was fused in frame to the N-terminus of ZF5 so that protein expression could be monitored. A similar construct, pFZF5-ΔZFLexA, has a truncation just upstream of the zinc fingers which are replaced by the LexA DNA binding domain. Expression of these fusion proteins in NIH 3T3 cells is shown in Figure 3A. pFLexA, a similar plasmid expressing the LexA DNA binding domain was used as a vector control in the co-transfection experiment shown in Figure 3B. Unexpectedly, co-transfection of pFZF5 resulted in a 12-fold activation of the pGL2wtLTR reporter plasmid. This was dependent upon the ability of ZF5 to bind DNA since the FZF5-ΔZFLexA protein could not activate the reporter (Fig. 3B).

The viral TAT protein synergizes with other activators to increase transcription from the HIV-1 LTR promoter (22). We wondered if ZF5 and TAT could cooperate to activate the LTR. Transient co-transfections were carried out using pFZF5 or a vector control; a TAT expression construct (RSV*tat*) or a vector control; and pGL2wtLTR. Figure 3C demonstrates the activities of both expression constructs, independently and together, relative to vector controls. Using this dual cotransfection system, the reporter was activated by ZF5 ~3.5-fold and by TAT ~25-fold. When these proteins were expressed together, the reporter was activated 68-fold (Fig. 3C). Thus, TAT can synergize with ZF5 to activate the HIV-1 3′ LTR.

In contrast, a reporter in which luciferase activity depended upon the human β-actin promoter (pβactinLuc) (23), included as a control, was strongly repressed by co-transfected pFZF5 (Fig. 3D). Subsequent computer analysis of the regulatory sequences of the β-actin promoter revealed at least two potentially strong ZF5 binding sites (data not shown). We have previously reported that ZF5 represses transcription dependent upon the *c-myc* and the HSV-1 TK promoters (2) and show here that it represses the β-actin promoter. Since we have shown that ZF5 activates the HIV-1 LTR, we conclude that in the context of natural promoters, ZF5 can function either as a repressor or an activator of transcription.

In order to locate the ZF5 response elements within the HIV-1 LTR, a deletion series was employed as depicted by the cartoons in Figure 4A (24). The largest HIV-1 reporter plasmid was activated 13.5-fold and two deletion plasmids, one of which contains only the Sp1 sites upstream of the TATA box, ~6.5-fold (Fig. 4A, –93ΔLTR–Luc).

Since the smallest reporter plasmid activated by ZF5 only contained the HIV-1 LTR Sp1 sites and TATA box, it seemed likely that ZF5 could bind this region of the promoter. To test this notion, ZF5 binding sites on the HIV-1 LTR were mapped by DNase I protection. ZF5 bound at the 5′ Sp1 site and at a region 3′ and partially overlapping the 3′ Sp1 site (Fig. 4B). This sequence is the best match to the consensus within the HIV-1 LTR as determined by computer analysis (data not shown). We note that, similar to high affinity sequences obtained by CAST, these binding sites vary in their similarity to the consensus sequence in Figure 2B. In addition, a region between –157 and –144 was protected by GST–ZF5-ΔZiN; however, transfections show that this region is not required for transactivation of the LTR promoter by ZF5 (Fig. 4A).

Mutations in the Sp1 sites of the LTR (25) were utilized to determine their requirement for ZF5-dependent transactivation of the LTR promoter (Fig. 4C). pGL2mSp1LTR contains site-specific mutations in all three Sp1 sites and was compared to the activity of the wild-type pGL2wtLTR luciferase reporter. The activity of the mutant promoter was severely reduced, but not ablated. Co-transfected pFZF5 caused a >7-fold activation of the wild-type promoter but no significant activation of the mutant promoter. Thus, ZF5-dependent activation of the HIV-1 LTR requires the 'Sp1' sites. Since the mutations do not extend into the –48 to –37 ZF5 binding region and do not affect the –157 to –144 bp site, the inability of ZF5 to activate the mSp1 LTR promoter shows that these ZF5 binding sites alone are unable to mediate ZF5-dependent activation.

The ZiN/POZ domain is necessary for transcriptional activation and transcriptional repression by ZF5

To determine the regions of ZF5 which are necessary for transcriptional repression, a previously described Gal4 fusion/co-transfection assay (2) was employed to execute a deletional analysis. A luciferase reporter driven by the HSV-1 tk promoter with or without five binding sites for the Gal4 protein was used in co-transfections with expression plasmids encoding ZF5 fused to the Gal4 DNA binding domain (Gal4 1–147, here abbreviated to G4 1–147). An expression plasmid, G4(.76), which encodes a fusion protein containing an N-terminal fragment of ZF5 lacking the zinc fingers, repressed transcription only in the presence of Gal4 sites, demonstrating that DNA binding was dependent upon the Gal4 1–147 portion of the fusion protein (2). Since this system eliminated ambiguity which might result from ZF5 binding to the HSV-1 tk promoter, G4(.76) and derivatives of it were used to monitor transcriptional repression by ZF5 fusion proteins.

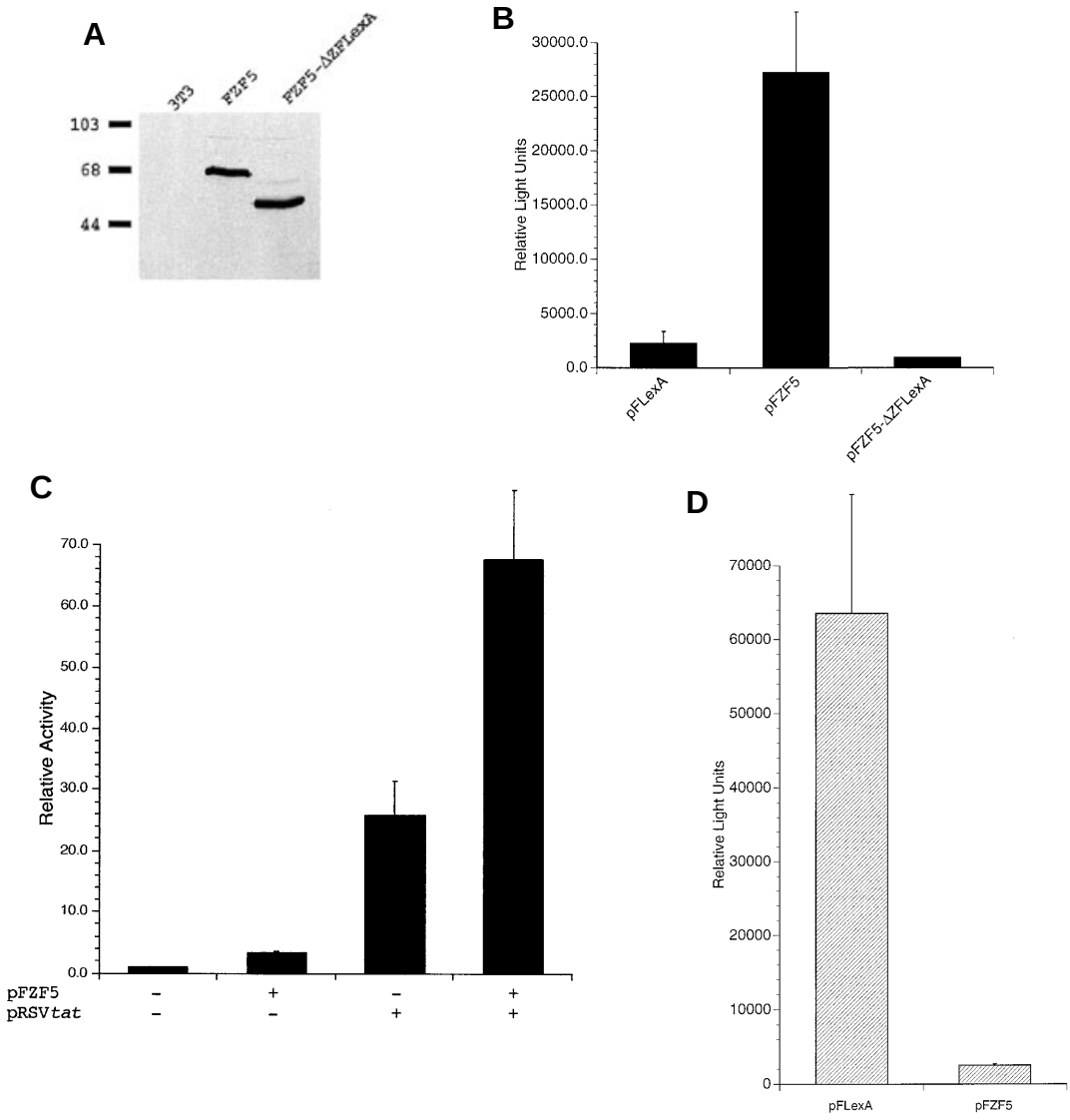

Figure 3. ZF5 is an activator of the HIV-1 LTR and repressor of the human β-actin promoter. **(A)** Western analysis of NIH 3T3 fibroblasts transiently transfected with 20 µg pFZF5 or pFZF5-ΔZFLexA demonstrates equivalent expression of these proteins. **(B)** ZF5 activates the HIV-1 LTR in NIH 3T3 fibroblasts and requires DNA binding. Co-transfection of 1 µg of the HIV-1 LTR reporter construct, pGL2wtLTR, with 1 µg of the indicated expression constructs demonstrates a requirement for the zinc fingers for activation. Triplicate values were averaged and presented as relative light units. **(C)** ZF5 activates the HIV-1 LTR synergistically with TAT. One µg pFZF5 and/or 1 µg RSV*tat* were co-transfected with 1 µg pGL2wtLTR. Triplicate values were averaged and presented relative to vector controls. **(D)** ZF5 efficiently represses the human β-actin promoter. Co-transfection of 1 µg pβactinLuc with 1 µg pFZF5 repressed reporter activity by ~97%. Each data point represents the average relative light units of triplicate transfections.

Repression activities were monitored by co-transfecting 1 µg G5–TK–Luc with 5 µg of each effector plasmid (Fig. 5A). A construct expressing only the ZiN/POZ and acidic domains, G4 ZiNAc, repressed transcription nearly as efficiently as G4(.76). G4 ZiN, containing only the ZiN/POZ domain, partially repressed transcription. However, G4 AcX, which is comparable to G4(.76) except for the deletion of the ZiN/POZ domain, was unable to repress transcription (Fig. 5A). Using antibodies to the Gal4 DNA binding domain, the expression levels of all the fusion proteins were monitored by western blot and found to be roughly equivalent (Fig. 5B). These data show that the ZF5 ZiN/POZ domain plays an important role in transcriptional repression. They also suggest that regions C-terminal to the ZiN/POZ domain participate in repression. This is consistent with the location of two repression domains in the BCL6 protein (26).

To test the importance of the ZiN/POZ domain for repression in the context of a natural promoter we used a reporter dependent on the human β-actin promoter. We found that an N-terminal truncated ZF5 protein, FZF5-ΔZiN, was not stably expressed in mammalian cells but that a similar form fused to the LexA DNA binding domain was. Therefore, FZF5-ΔZiNLexA was compared to the activity of the full length FZF5LexA fusion protein in a co-transfection assay. Protein expression was monitored by western analysis using anti-hemaglutinin antibody against the N-terminal epitope tag (Fig. 6A). To ensure there was no activity associated with the presence of the LexA region, the activity of FZF5LexA was compared to that of

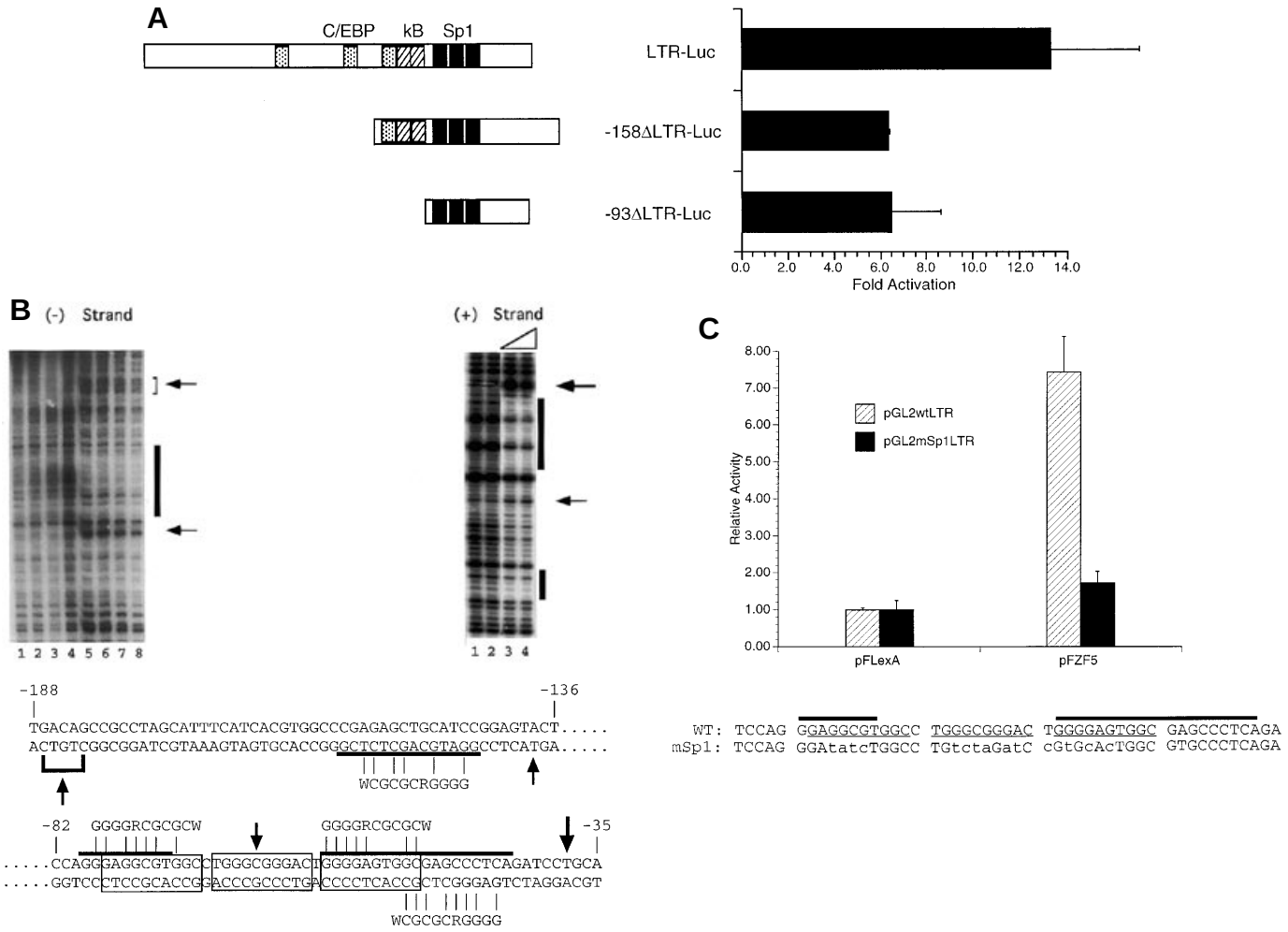

Figure 4. Identification of the ZF5-response elements in the HIV-1 LTR. **(A)** Deletion analysis of a LTR reporter identifies a functional element(s) within 93 bp upstream of the TATA box. Fold activation represents the value reported by co-transfection of 1 µg pFZF5 with 1 µg of the indicated reporter construct relative to the vector control, pFLexA. Each data point was repeated in triplicate, one standard deviation is indicated. **(B)** DNase I footprinting identifies ZF5 binding sites on the HIV-1 LTR. Footprints are indicated by bars, hypersensitive sites by arrows. Labeling of the (–) strand revealed the –157 to –144 footprint, the (+) strand revealed the –78 to –71 and –55 to –37 footprints. (–) Strand final DNase concentrations: lanes 1 and 5, 12.5 µg/ml; lanes 2 and 6, 25.0 µg/ml; lanes 3 and 7, 50.0 µg/ml; and lanes 4 and 7, 100 µg/ml. Lanes 1–4 have no protein and lanes 5–8 have 1 µg GST–ZF5-ΔZiN. (+) Strand final DNase concentrations: lane 1, 6.25 µg/ml and lanes 2–4, 13.0 µg/ml. 1 and 2 µg GST–ZF5-ΔZiN were used in lanes 3 and 4. **(C)** Site directed mutation of the HIV-1 LTR Sp1 sites ablates ZF5-mediated activation. One µg of the indicated reporter or effector plasmid was co-transfected into NIH 3T3 fibroblasts. Triplicate values were averaged and normalized to the effector plasmid control pFLexA. The mutations in the pGL2mSp1LTR reporter are indicated by lower case letters. The Sp1 sites are underlined and the ZF5 binding regions are indicated by bars.

FZF5 and no significant differences were observed (Fig. 6B). Consistent with the Gal4 studies, repression by the FZF5-ΔZiN-LexA fusion protein was decreased in comparison to the full-length protein. These data demonstrate that the ZiN/POZ domain is required for full repression by ZF5.

We also wished to determine if the ZiN/POZ domain is required for transcriptional activation of the HIV-1 LTR by ZF5. Co-transfection experiments similar to those described above were performed on pGL2wtLTR (Fig. 6C). The FZF5-ΔZiNLexA fusion protein was not able to activate the HIV-1 LTR reporter, demonstrating an absolute requirement of the ZiN/POZ domain for activation. Thus we conclude that the ZF5 ZiN/POZ domain is required for both transcriptional activation and transcriptional repression.

DISCUSSION

The studies reported here provide information about the mechanism of action of the ZF5 protein. We have demonstrated that the zinc fingers are required for ZF5 to bind DNA and that the ZiN/POZ domain reduces the affinity of the protein for DNA. Using a sensitive selection technique, a consensus high affinity binding site for ZF5 was determined and shown to be GC rich with similarity to Sp1 sites. This led us to examine its role in the transcriptional regulation of the HIV-1 LTR, a Sp1-dependent promoter. We have shown that the HIV-1 LTR is transactivated by ZF5 while the human β-actin promoter is repressed by ZF5. Finally, we have demonstrated that the ZF5 ZiN/POZ domain participates in transcriptional activation as well as repression.

ZF5 and Sp1 sites

Utilizing a binding site selection technique (CAST), high affinity binding sequences for ZF5 were enriched and from these a consensus sequence was derived. Consistent with previous findings, this sequence is rich in guanine and cytosine nucleotides and resembles the Sp1 consensus (2). Other zinc finger proteins

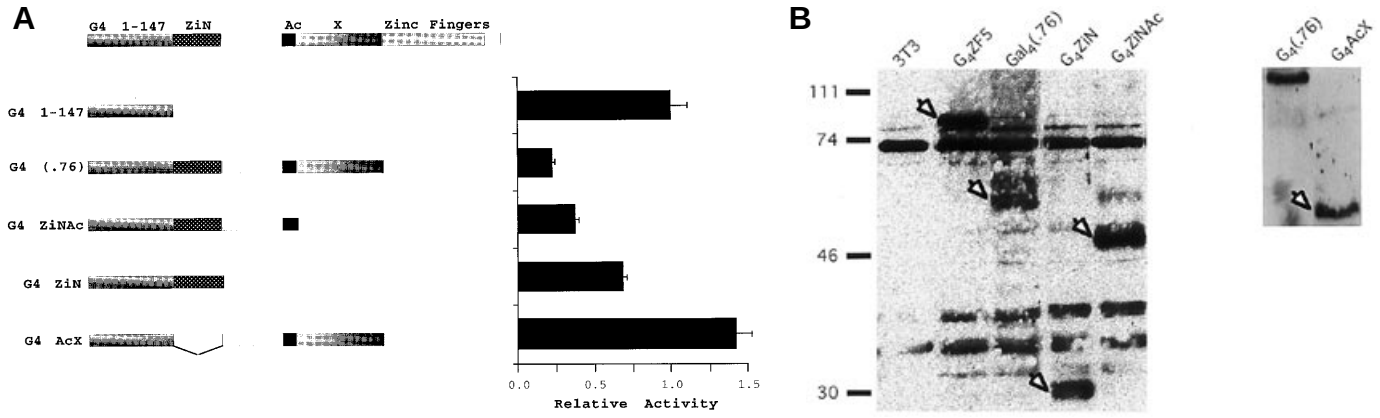

Figure 5. The ZF5 ZiN/POZ domain is required for efficient repression activity. **(A)** Co-transfection of Gal4 ZF5 fusion expression constructs with G5–TK–Luc. Five μg of each expression construct was co-transfected with 1μg G5–TK–Luc and correspond with the schematic of the indicated Gal4 ZF5 derived protein. The values represent an average of duplicate points and are all relative to the vector control. **(B)** Western analysis of NIH 3T3 fibroblast whole cell extracts transiently transfected with 20 μg of the Gal4–ZF5 fusion expression constructs. The molecular weight markers are indicated on the left side of each panel, the transfected expression construct on top. The arrows indicate specific Gal4 fusion proteins.

such as MAZ (27,28) have also been found to have overlapping binding specificity with Sp1 family proteins.

Binding studies on the HIV-1 LTR (Fig. 4B) and thymidine kinase (2) promoters show that ZF5 only binds a subset of Sp1 sites, consistent with the finding that the ZF5 consensus is slightly different from the Sp1 consensus (Fig. 2B). However, for the 'Sp1' sites which are recognized by ZF5, the two proteins may compete for binding *in vivo* and the regulation of some promoters previously attributed to Sp1 may in fact involve ZF5. It will be important in future studies to identify which known 'Sp1' sites are recognized by ZF5, to determine whether ZF5 activates or represses when bound to these sites and to determine whether it binds the sites *in vivo*. In addition, it may be that ZF5 and Sp1 act together or synergistically in some contexts such as the HIV-1 LTR. Our data show that ZF5 binds some but not all the Sp1 sites in this promoter and also binds sites not occupied by Sp1 (Fig. 4B). Our data are consistent with models in which ZF5 and Sp1 cooperate in binding or transcriptional activation, although additional studies will be necessary to test these possibilities.

The paradox of the ZF5 ZiN/POZ domain activities

The ZiN/POZ domain clearly confers on ZF5 a conformation which decreases binding affinity (Fig. 1E). Suppression of DNA binding by a region which is required for transcriptional modulation seems paradoxical since transcriptional regulation requires DNA binding. However our co-transfection data show that full-length ZF5 is indeed transcriptionally active, either activating or repressing transcription, depending on the gene context. In addition, it appears that the ZiN/POZ domains of other family members may have similar properties since the ZiN/POZ region of BCL6 was recently shown to be necessary for transcriptional repression (26).

To solve this paradox, it seems likely that *in vivo* there is a mechanism to regulate the ability of ZiN/POZ proteins to bind DNA and to modulate transcription. Indeed, Bardwell and Treisman noted that the ZID ZiN/POZ domain can direct assembly into subnuclear structures, presumably by dimerization, suggesting that ZiN/POZ-containing proteins may be unavailable for gene regulation unless they are modified (1). Phosphorylation or other post-translational modifications might alter the ability of the ZiN/POZ domain to suppress DNA binding. Alternatively, association with transcriptional or other regulatory proteins might stabilize ZiN/POZ proteins bound to DNA as part of a multiprotein complex or might induce a conformation with higher affinity for DNA.

Transcriptional activation and repression by ZF5

When ZF5 is tethered to an artificial promoter by the GAL4 DNA binding domain, transcriptional repression is observed. ZF5 also represses natural promoters including those of the murine *c-myc*, HSV-1 TK (2) and human β-actin genes. However, ZF5 strongly transactivates the HIV-1 LTR promoter. Thus, as with many activator/repressor proteins, an important question is what determines whether ZF5 will activate or repress a promoter.

There are several models which can provide an explanation for the ability of ZF5 to both activate and repress transcription. The binding site consensus for ZF5 is not palindromic and its orientation may affect ZF5 activity. Alternatively, ZF5 may function differently at high and low affinity binding sites, a possibility suggested by studies on the drosophila protein GAGA (29,30) and by our isolation of both high and low affinity sites by CAST. Binding of adjacent proteins may also affect ZF5 activity. It is interesting that there are multiple ZF5 binding sites interspersed with Sp1 sites on the HIV-1 LTR where ZF5 functions as an activator and, as suggested above, ZF5 and Sp1 may affect one another's activity. Adjacent proteins may affect the ability of ZF5 to associate with co-modulators, TAFs or the basal transcription machinery. YY1, another zinc finger protein which, like ZF5, can either activate or repress transcription, has recently been shown to require association with a co-repressor to repress transcription (31).

The growing list of ZiN/POZ proteins, their involvement in human tumors and their roles in drosophila development all underscore the importance of this class of zinc finger proteins. The experiments reported here provide the groundwork for addressing many intriguing questions regarding ZF5 and ZiN/POZ proteins in general. It will be important to study the paradox of the ZiN/POZ domain functions and to determine how DNA

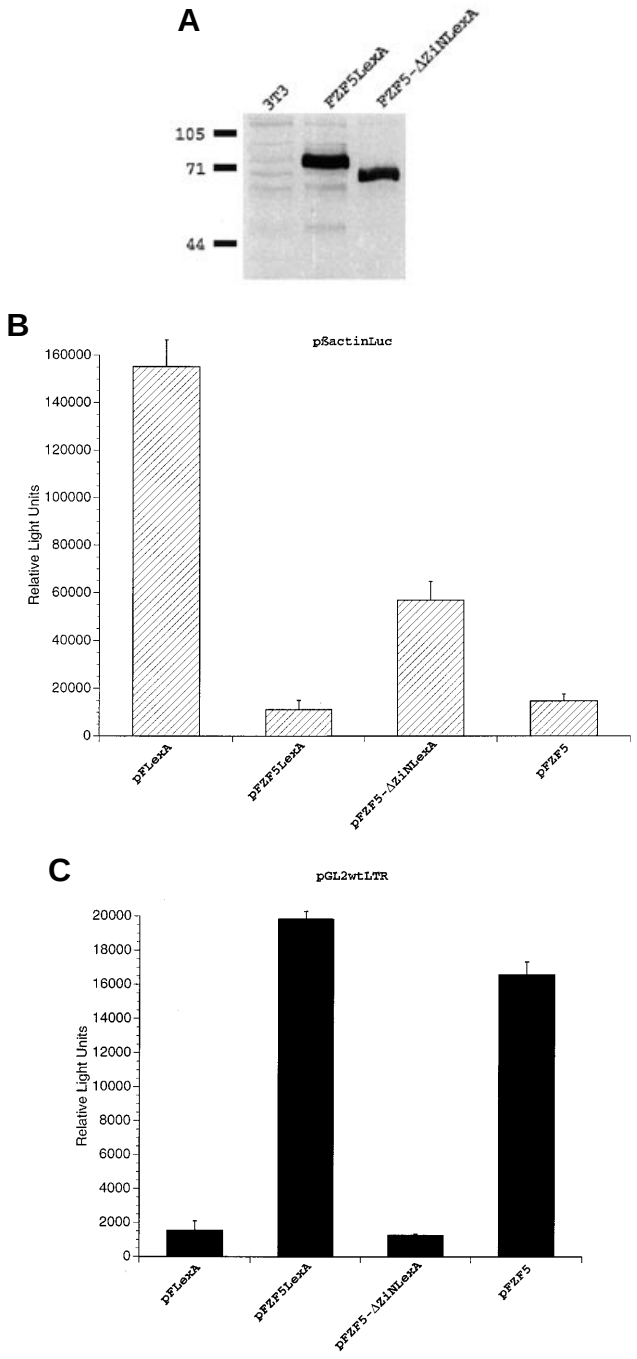

Figure 6. The ZiN/POZ domain is required for full repression and activation of natural promoters. **(A)** Western analysis of hemaglutinin-tagged FZF5LexA and FZF5-ΔZiNLexA fusion proteins transfected into NIH 3T3 fibroblasts. **(B)** The ZiN/POZ domain is required for full repression of the human β-actin promoter. One µg pβactinLuc was co-transfected with 1 µg of the indicated expression construct. Each bar represents the average relative light units of duplicate data points. **(C)** The ZiN/POZ domain is required for activation of the HIV-1 LTR. Co-transfections are as in (B) except the pGL2wtLTR reporter was used.

binding and transcriptional activity are regulated *in vivo.* It will also be important to determine the relationship between Sp1 and ZF5 binding in different genes and to identify additional target genes for ZF5. Finally, studies on the HIV-1 LTR and β-actin promoters should help us understand what determines whether ZF5 activates or represses transcription.

ACKNOWLEDGEMENTS

We would like to thank members of the Calame laboratory, Dr Andrew Henderson, Dr Christos Panagiotidis and Bob Soliman for their insightful discussions and generosity. We would also like to thank Dr Christian Schindler and Dr Gerald Siu for critically reading this manuscript. This work was supported by grants from the National Institutes of Health, the American Cancer Society and the Council for Tobacco Research to KC.

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