N-terminal transcriptional activation domain of LZIP comprises two LxxLL motifs and the Host Cell Factor-1 binding motif

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Host Cell Factor-1 (HCF-1, C1) was first identified as a cellular target for the herpes simplex virus transcriptional activator VP16. Association between HCF and VP16 leads to the assembly of a multiprotein enhancer complex that stimulates viral immediate-early gene transcription. HCF-1 is expressed in all cells and is required for progression through G_1 phase of the cell cycle. In addition to VP16, HCF-1 associates with a cellular bZIP protein known as LZIP (or Luman). Both LZIP and VP16 contain a four-amino acid HCF-binding motif, recognized by the N-terminal β-propeller domain of HCF-1. Herein, we show that the N-terminal 92 amino acids of LZIP contain a potent transcriptional activation domain composed of three elements: the HCF-binding motif and two LxxLL motifs. LxxLL motifs are found in a number of transcriptional coactivators and mediate protein–protein interactions, notably recognition of the nuclear hormone receptors. LZIP is an example of a sequence-specific DNA-binding protein that uses LxxLL motifs within its activation domain to stimulate transcription. The LxxLL motifs are not required for association with the HCF-1 β-propeller and instead interact with other regions in HCF-1 or recruit additional cofactors.

Host Cell Factor (HCF-1) was first identified through association with the herpes simplex virus transactivator VP16 (Vmw65 or αTIF), an important regulator of herpes simplex virus lytic gene expression (1–4). Transcriptional activation of the viral immediate-early genes is initiated by VP16, which recruits two preexisting cellular proteins, HCF-1 and Oct-1. The resulting VP16-induced complex assembles on a VP16-responsive DNA sequence known as the TAATGARAT element, found in each immediate-early gene promoter. The relevance of HCF-1 to the viral regulatory machinery and, perhaps more importantly, its role in uninfected cells remain poorly understood.

HCF-1 is synthesized as a large precursor protein of more than 2000 amino acids and is subsequently cleaved at reiterated sites near the center of the polypeptide. Processing yields a family of N- and C-terminal fragments that remain stably bound together (5, 6). VP16 interacts with the first 380 residues of HCF (the HCF_{VIC} domain; VIC is the VP16-induced complex), which is composed of six kelch (HCF_{KEL}) repeats that fold into a β-propeller structure (7–10). The interaction between VP16 and HCF-1 retains VP16 in the nucleus and also stabilizes a conformation amenable to association with the Oct-1 POU domain and specific residues flanking the TAATGARAT element (2, 11).

HCF-1 is expressed in all cells but accumulates at highest levels in proliferating tissues and transformed cell lines, suggesting a role in cell proliferation (12). This hypothesis is supported by analysis of hamster tsBN67 cells, which contain a temperature-sensitive version of HCF-1 (13). When cultured at the nonpermissive temperature (39.5°C), tsBN67 cells undergo a G_1/G_0 arrest but remain viable, reentering the cell cycle when returned to the permissive temperature (33.5°C). The tsBN67 mutation, a proline-to-serine change at position 134 in the β-propeller domain, prevents transactivation by VP16 at the nonpermissive temperature and leads to a substantial reduction in viral yield (8, 13, 14).

In addition to VP16, the β-propeller domain of HCF-1 interacts with a broadly expressed basic leucine-zipper protein known as LZIP or Luman (15, 16). The basic region and leucine zipper of LZIP are highly homologous to other members of the ATF-6/CREB subfamily of bZIP proteins (15–17). Although the cellular target genes of LZIP are not known, LZIP binds to canonical cAMP-responsive elements (CREs) as a homodimer and can activate transcription from CRE-containing reporter genes (15, 17, 18). LZIP and VP16 recognize HCF-1 through a similar mechanism, involving a tetrapeptide sequence (D/EHXY, where X can be any residue) known as the HCF-binding motif or HBM (16, 18).

The functional relevance of the LZIP-HCF-1 interaction has yet to be determined. In this report, we show that the N terminus of LZIP (residues 1–92) contains a potent transcriptional activation domain composed of three functional elements: a pair of LxxLL motifs and the HBM. Optimal transcriptional activation by LZIP requires direct interaction with HCF-1 and is observed by using a heterologous DNA-binding domain demonstrating that HCF-1 does not act by modulating the DNA-binding properties of LZIP. In temperature-shifted tsBN67 cells, loss of LZIP-mediated transactivation occurs within a few hours. Interestingly, mutation of the LxxLL motifs significantly reduces transactivation by LZIP without affecting the interaction with the HCF-1 β-propeller, suggesting that the LxxLL motifs interact with other regions of HCF-1 or recruit additional cellular factors required for activation. HCF-1 may thus act as a molecular chaperone, promoting the association of DNA-binding transcription factors with other transcriptional coactivators.

Materials and Methods

Plasmid Constructs. Fragments encoding the N or C terminus of human LZIP were generated by PCR with an expressed sequence tag clone (GenBank accession no. R14706) as template and subcloned into the mammalian expression vector pCGN-Gal4(1–94) (ref. 19). Plasmids encoding Gal4-Sp1 and Gal4-c-jun were kind gifts of Naoko Tanese (New York University School of Medicine, New York; refs. 20 and 21). The plasmid encoding the Gal4-VP16 activation domain fusion (pCGNVP16AD) was generously provided by Bill Tansey and Winship Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The Gal4-responsive luciferase reporter plasmid used in this study was p5xGal4-E1b-luc (22).

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Abbreviations: HBM, HCF-binding motif; ER, endoplasmic reticulum.

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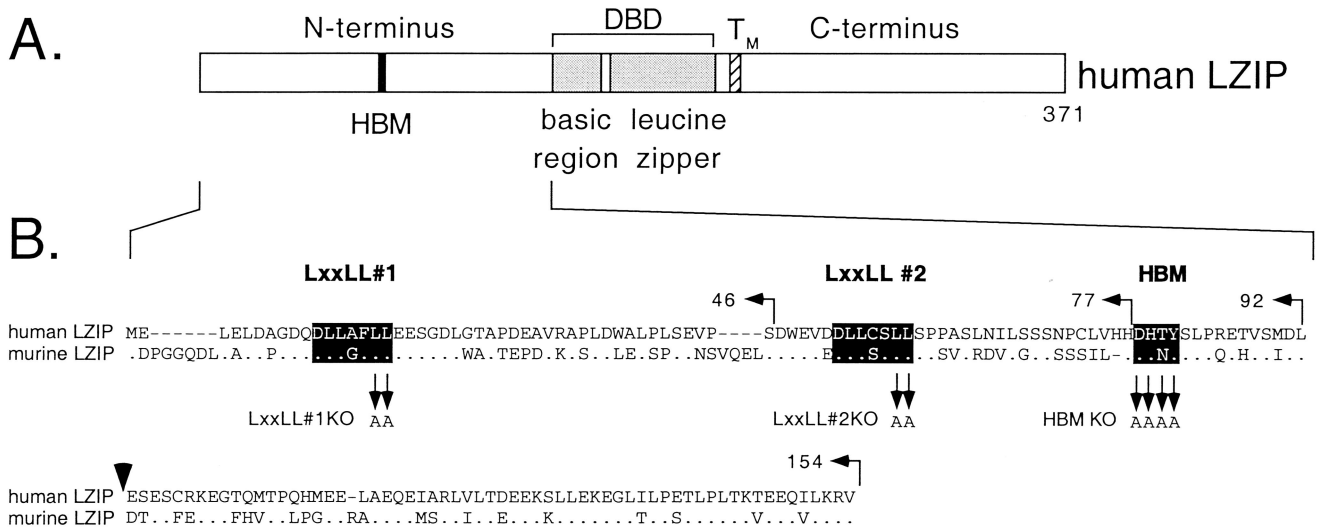

Fig. 1. (*A*) Structure of human LZIP. The bZIP DNA-binding and dimerization domain, composed of a basic region (residues 152–171) and adjacent leucine zipper (residues 178–220), lies near the center of the polypeptide (shaded) and is flanked by a putative transmembrane domain (T_M, residues 229–243; ref. 35). The HBM (filled box) is located in the N terminus (DHTY, residues 78–81). The N terminus is rich in bulky hydrophobic residues (especially leucine) and contains two clusters of acidic residues (residues 16–52, 30% acidic; residues 93–148, 27% acidic). The C terminus is also rich in bulky hydrophobic amino acids as well as prolines. (*B*) Alignment of the N-terminal sequences of human and mouse LZIP (15–17). The two LxxLL motifs and the HBM (highlighted) represent islands of greatest sequence conservation. The endpoints of truncations used in this study are indicated above the sequence, and residues targeted for alanine substitution are indicated below the alignment. A vertical arrowhead marks the insertion point of a mRNA-splice variant that adds an extra 15 amino acids (17).

The HBM and LxxLL motifs were disrupted by alanine-substitution mutagenesis with the Quick-change oligonucleotide-directed mutagenesis protocol (Stratagene). Diagnostic restriction sites were incorporated into each mutation. The oligonucleotides used in this study are as follows: Leu-16/Leu-17, 5′-CCAACCCCTGCCTTGTCCACCATgctgcagccgcc-TCCCTCCCACGGGAAACTGTCTC-3′ (*Pst*I$^+$) and its complement; Leu-57/Leu-58, 5′-GTAGATGATTTGCTGTGCT-CAgctgcgAGTCCCCCAGCGTCGTTGG-3′ (*Pvu*II$^+$) and its complement; and Asp-78 to Tyr-81, 5′-CCAACCCCTG-CCTTGTCCACCATgctgcagccgccTCCCTCCCACGGGA-AACTGTCTC-3′ (*Pst*I$^+$) and its complement. Lowercase letters indicate missense codons. Diagnostic restriction sites are underlined and identified in parentheses. PCR-generated templates and site-directed mutants were verified by DNA sequence analysis.

Cell Lines, Transfections, and Luciferase Assays. 293T, BHK21, and tsBN67 cells were transfected by electroporation (1×10^6 cells per assay) with a Bio-Rad Genepulser with Capacitance Extender set at 0.22 kV and 950 μF. For luciferase reporter assays, 300 μl of LCCLR buffer (Promega) was added to each 6-cm dish. For luciferase assays, 50 μl of cell extract and 300 μl of reaction buffer (25 mM glycine-glycine, pH 7.8/15 mM $MgSO_4$/1 mM ATP, pH 7.0/0.1 mg/ml BSA/1 mM DTT) were mixed, added to 1 mM D-luciferin substrate (Analytical Luminescence Laboratory, San Diego) and immediately assayed with a LB9507 luminometer (EG & G Berthold, Wellesley, MA). Values represent the means of three independent transfections, and error bars indicate standard deviation from the mean.

Yeast Two-Hybrid Analysis. Yeast expression plasmids pEG202-HCF_{N380} and pEG202-HCF_{N380}P134S contain the wild-type and tsBN67 mutant versions of the HCF-1 β-propeller domain (residues 1–380) fused to the LexA DNA-binding domain (kindly provided by Shahana Mahajan, New York University School of Medicine, New York). The *Xba*I–*Bam*HI fragment encoding the first 92 residues of LZIP was subcloned into a modified pUC119 polylinker, released by using *Eco*RI and *Bam*HI, and subcloned into the yeast expression vector pJG4-5 (23), creating B42 activation domain fusions. The $LexA_{DBD}$ and $B42_{AD}$ plasmids were cotransformed into the reporter strain EGY48 (trp1, his3, ura3, leu2), containing integrated LexA-responsive *LacZ* and *LEU2* reporter genes (pSH18.34), by using the lithium acetate method (24).

Results

LZIP Contains Two Potential Activation Domains. The primary structure of human LZIP is shown schematically in Fig. 1*A*. The protein can be divided into three general regions: the N terminus (residues 1–151), a basic-leucine zipper DNA-binding and dimerization domain (residues 152–220), and the C terminus (residues 221–371). The N terminus is enriched for acidic amino acids (20.5% Glu or Asp), whereas the C terminus is relatively proline-rich (11%). Bulky hydrophobic residues are interspersed throughout both terminal regions, reminiscent of many transcriptional activation domains (25–27). To map activation domains within LZIP, we fused the N-terminal (residues 1–154) or C-terminal (residues 244–371) regions to the yeast GAL4 DNA-binding domain (residues 1–94, $Gal4_{DBD}$) and measured activation of a Gal4-responsive reporter gene (5xGal4-E1B-luc) in transiently transfected 293T cells (Fig. 2*A*). Expression of both the N terminus (Gal4-$LZIP_{N154}$) and C terminus of LZIP (Gal4-$LZIP_{C244-371}$) gave rise to a substantial increase in reporter activity compared with the $Gal4_{DBD}$ alone. Activation by the N terminus was 2.7-fold greater than with the C terminus. Gal4-VP16, containing the C-terminal activation domain of herpes simplex virus VP16 (28), and Gal4-Sp1B, a glutamine-rich activator (29), served as positive controls. These results show that LZIP contains two potential activation domains, the strongest of which is located in the N terminus (residues 1–154).

The N-Terminal Activation Domain of LZIP Is Composed of Multiple Functional Elements. To define the boundaries of the N-terminal activation domain, we generated three truncations within the N terminus (shown in Fig. 1*B*). The first truncation deletes 63 residues from the C-terminal side of the HBM ($LZIP_{N92}$, residues 1–92) including a leucine-rich region; the second truncation

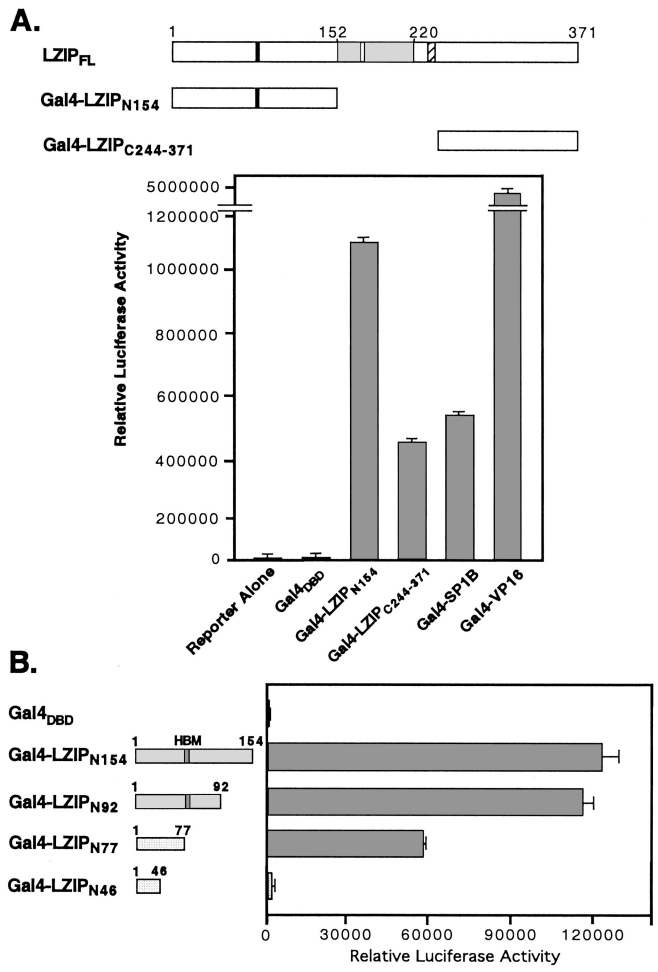

Fig. 2. The N terminus of LZIP contains a potent activation domain. (*A*) 293T cells were cotransfected with expression plasmids (25 ng) encoding Gal4$_{DBD}$, Gal4-LZIP$_{N154}$, Gal4-Sp1, and Gal4-VP16 together with the p5xGal-E1B-luc reporter gene (500 ng). Extracts were prepared after 36 h and assayed for luciferase activity. Values represent means and standard deviations of three independent transfections. (*B*) The boundaries of the activation domain were delineated with a series of C-terminal truncations. BHK21 cells were cotransfected with expression plasmids (250 ng) encoding Gal4$_{DBD}$, Gal4-LZIP$_{N154}$, Gal4-LZIP$_{N92}$, Gal4-LZIP$_{N77}$, and Gal4-LZIP$_{N46}$ together with the p5xGal-E1B-luc reporter gene (500 ng).

(LZIP$_{N77}$, residues 1–77) removes the HBM (residues 78–81) entirely; and the third (Gal4-LZIP$_{N46}$, residues 1–46) bisects the remaining N-terminal fragment. Each fragment was fused to the Gal4$_{DBD}$ and assayed for reporter gene activation in BHK21 cells (Fig. 2*B*). In these cells, expression of Gal4-LZIP$_{N154}$ increased reporter activity by 1,085-fold compared with the Gal4$_{DBD}$ alone. Deletion of residues 93–154 (Gal4-LZIP$_{N92}$) had a minimal effect on activation, whereas removal of an additional 16 residues including the HBM (Gal4-LZIP$_{N77}$) reduced activation by 55%. Further truncation to residue 46 (Gal4-LZIP$_{N46}$) reduced activation to only 9% of that of Gal4-LZIP$_{N154}$. These results show that the activation domain lies within the first 92 residues of LZIP and is composed of two or more functional elements.

HCF-1 Is Required for Activation by LZIP. The finding that deletion of the HBM reduced activation by half implies that HCF-1 is required for the function of the N-terminal activation domain. We addressed this in two ways: first using the conditional tsBN67 mutant HCF-1 allele to inactivate HCF-1 *in vivo* and second by mutating the HBM on LZIP itself.

The tsBN67 cell line is derived from BHK21 and contains a conditional version of HCF-1 because of a proline-to-serine substitution at position 134 (8, 13). In addition to causing a cell-cycle arrest, the mutation disrupts the LZIP–HCF interaction at the nonpermissive temperature (39.5°C; refs. 16 and 18). To address HCF-1's contribution to transactivation by LZIP, we transfected the Gal4-LZIP$_{N154}$ expression plasmid and p5xGal4-E1b-luciferase reporter gene into tsBN67 cells and assayed activation at both the permissive (33.5°C) and nonpermissive temperatures (39.5°C). The results of this assay are shown in Fig. 3*A*. At 33.5°C, Gal4-LZIP$_{N154}$ functions as a robust activator; however, this activity was reduced 9-fold in the parallel cultures incubated at the nonpermissive temperature. In contrast, Gal4-fusion proteins containing activation domains from c-jun and Sp1, which do not interact with HCF-1, were not significantly reduced at the higher temperature. Note that for ease of comparison, the activity of each fusion protein is expressed relative to its activity at 33.5°C. To ensure that Gal4-LZIP$_{N154}$ was not itself temperature-sensitive, we also measured transactivation at 39.5°C in a tsBN67-derivative that stably expresses wild-type recombinant HCF-1 (30). As shown in Fig. 3*B*, activation by Gal4-LZIP$_{N154}$ was similar to that observed in tsBN67 cells at 33.5°C. These results indicate that the differential response of Gal4-LZIP$_{N154}$ reflects a specific requirement for functional HCF-1 and is not caused by differences in temperature *per se*.

In the initial characterization of the tsBN67 arrest phenotype, Goto *et al.* (13) showed that there is a lag of around 36 h before the majority of cells within the population undergo a G_1/G_0 arrest. To determine whether loss of transactivation by Gal4-LZIP$_{N154}$ is immediate or shows a similar lag, we measured reporter activity at regular intervals after the shift from 33.5°C to 39.5°C (Fig. 3*C*). After transfection, cells were divided into aliquots and allowed to recover for 2 h at 33.5°C before half of the culture was transferred to 39.5°C. At 12 h after the temperature shift, there was a clear difference in the level of transactivation at 39.5°C compared with that at 33.5°C, with only a marginal increase in activity at 39.5°C over the remaining time points. In a similar analysis with Gal4-Sp1B, the reporter gene was more active at 39.5°C than 33.5°C for all time points (data not shown). Thus, loss of transactivation at the higher temperature is specific to Gal4-LZIP$_{N154}$ and occurs within a few hours of the temperature shift.

The HBM Is Important for Transactivation. We next asked whether LZIP requires direct interaction with HCF-1 by mutating the HBM (Fig. 3*D*). All four residues of the core tetrapeptide HBM were changed to alanine (Gal4-LZIP$_{N92}$HBMKO; Fig. 1*B*), a substitution known to abolish the LZIP-HCF-1 interaction. When assayed for transactivation in tsBN67 cells incubated at 33.5°C, activity of the mutant was reduced to 21% of that of wild-type LZIP$_{N92}$, demonstrating that the HBM in Gal4-LZIP$_{N154}$ is required to achieve robust stimulation of the Gal4-driven reporter gene. This result is consistent with the behavior of the temperature-sensitive tsBN67 allele of HCF-1, which specifically affects recognition of the HBM rather than HCF-1 stability (13) or subcellular localization (14).

LZIP Transactivation Requires the HCF Binding Motif and LxxLL Motifs. In addition to the HBM, the N terminus of human LZIP contains two leucine-rich motifs (11-DLLAFLL-17 and 52-DLLCSLL-58), indicated in Fig. 1*B*, that lie within two regions of highest sequence conservation between mouse and human LZIP. These leucine repeats strongly resemble LxxLL (where x is any residue) or nuclear receptor motifs, found in a variety of transcriptional coactivators, particularly those associated with nuclear hormone receptors (31, 32).

To address the role of the LxxLL motifs in transactivation, we

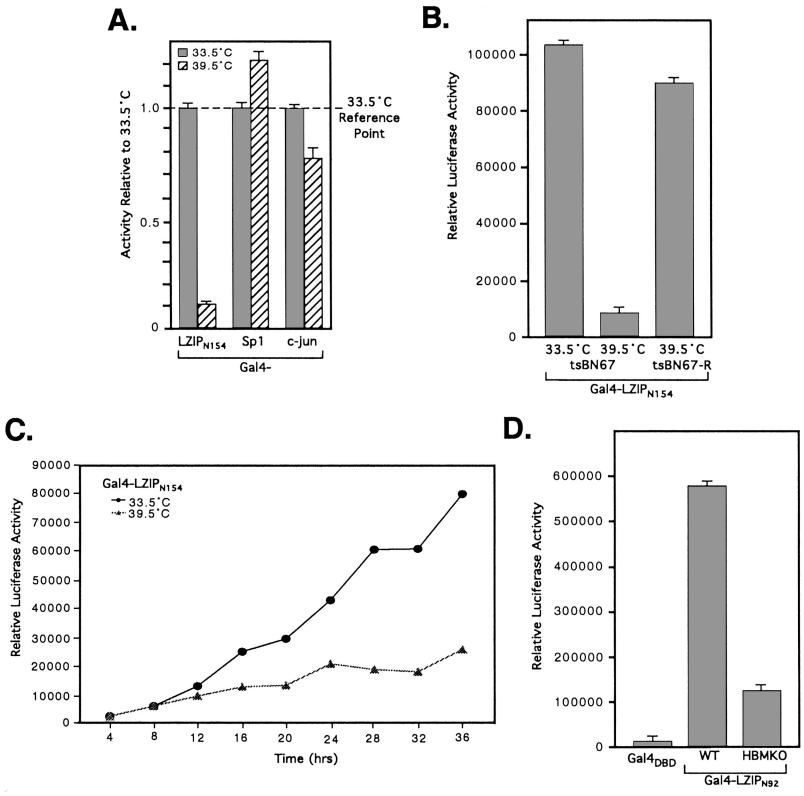

Fig. 3. Transactivation by Gal4-LZIP requires functional HCF-1. (*A*) Temperature-dependent transactivation by Gal4-LZIP$_{N154}$. Subconfluent tsBN67 cells were cotransfected with expression plasmids (250 ng) encoding Gal4-LZIP$_{N154}$, Gal4-Sp1, and Gal4-c-jun together with the p5xGal-E1B-luc reporter gene (500 ng) and incubated at 33.5°C or 39.5°C for 40 h. Because Gal4-Sp1 and Gal4-c-jun are much weaker activators than Gal4-LZIP$_{N154}$, activity at 39.5°C is plotted relative to the activity of the same activator at 33.5°C. (*B*) Transactivation by Gal4-LZIP$_{N154}$ is restored in tsBN67 cells stably expressing wild-type HCF-1 (tsBN67-R). (*C*) Time course analysis of tsBN67 cells. Transfected cells were maintained at 33.5°C for 2 h after electroporation with p5xGal-E1B-luc (500 ng) and Gal4-LZIP$_{N154}$ (250 ng), and then half of the culture was shifted to 39.5°C (time = 0). Aliquots of cells were then collected at 4-h intervals and assayed for luciferase activity. Values represent the means of three assays. (*D*) Activation by wild-type and HBMKO versions of Gal4-LZIP$_{N92}$ assayed in tsBN67 cells maintained at 33.5°C.

generated alanine substitutions within each of the two LZIP motifs. We targeted the second pair of leucine residues—positions +4 and +5 following standard nomenclature (33)—because alanine substitutions at these positions in the LxxLL motifs of human SRC-1 have been shown to abolish association with the estrogen receptor (31). The targeted residues correspond to Leu-16 and -17 within LxxLL motif 1 and the analogous Leu-57 and -58 within LxxLL motif 2 (see Fig. 1*B*). Each mutant (LxxLL 1KO and LxxLL 2KO) was expressed in BHK21 cells and assayed for transactivation (Fig. 4*A*). Disruption of either the first or second LxxLL motifs reduced activity to 42% or 40% of that of wild-type, respectively. Combining the two LxxLL mutations (LxxLL 1/2KO) resulted in a lower level of activation than either mutation alone, suggesting that each LxxLL motif performs a nonredundant role. Finally, disruption of both LxxLL motifs as well as the HBM (LLHKO) resulted in a further reduction in activation to only 13% that of wild-type Gal4-LZIP$_{N92}$. In summary, these results show that both LxxLL motifs are required for maximal transcriptional activation by the N-terminal activation domain of LZIP.

The LxxLL Motifs Are Not Required for Association with the HCF-1 β-Propeller. It is conceivable that the LxxLL mutations affect transactivation by compromising recruitment of HCF-1. To address this issue, we examined the association of the HCF-1 β-propeller with the LxxLL mutant with a yeast two-hybrid assay (Fig. 4*B*). The HCF-1 β-propeller domain (residues 1–380) and LZIP$_{N92}$ fragments were expressed as fusions to the LexA DNA-binding domain and B42 activation domain, respectively. Interaction was measured by activation of the LexA-responsive LacZ reporter gene. As expected, wild-type LZIP$_{N92}$ (B42-LZIP$_{N92}$) interacted strongly with the HCF-1 β-propeller (LexA-HCF$_{N380}$), resulting in a strong blue color on 5-bromo-4-chloro-3-indolyl β-D-galactoside-containing medium. Interestingly, each single LxxLL mutant (B42-LZIP$_{N92}$LxxLL 1KO and B42-LZIP$_{N92}$LxxLL 1KO) as well as the double LxxLL mutant (B42-LZIP$_{N92}$LxxLL1/2KO) also interacted strongly with HCF-1, demonstrating that the leucine motifs are not required for interaction with the HCF-1 β-propeller. In contrast, mutation of the HBM (B42-LZIP$_{N92}$HBMKO) abolished the interaction, resulting in white colonies. All of the B42-LZIP$_{N92}$ fragments failed to associate with LexA-HCF$_{N380}$ P134S, confirming that the interactions detected were specific.

Discussion

We report the characterization of a strong transcriptional activation domain located within the N-terminal 92 residues of human LZIP. By deletion analysis and site-directed mutagenesis, we show that the activation domain is composed of three functional elements: a pair of LxxLL motifs and the previously described HBM (16, 18). The importance of HCF-1 recruitment to activation domain function is demonstrated in two ways. First, point mutations within the LZIP HBM reduced the potency of the N-terminal activation domain to about half that of the wild-type version, and second, in transfected tsBN67, which carries a temperature-sensitive version of HCF-1, activation was reduced by 9-fold at the nonpermissive temperature. The fact that HCF-dependent transactivation is observed with fusions to

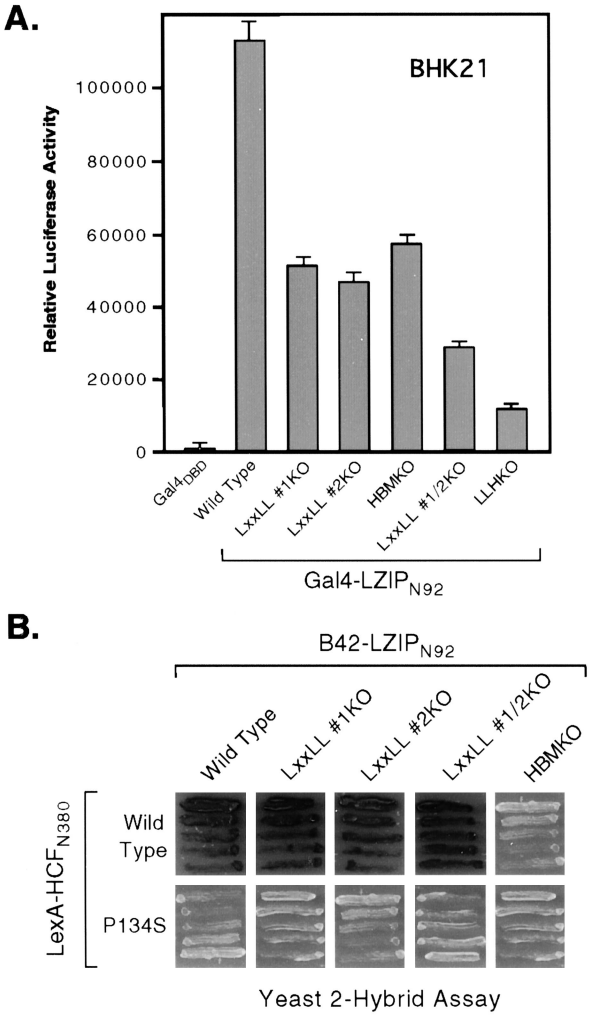

Fig. 4. The LxxLL motifs are critical for LZIP activation function. (*A*) Wild-type and mutant versions of Gal4-$LZIP_{N92}$ were cotransfected into BHK21 cells together with the p5xGal-E1B-luc reporter (500 ng), and luciferase activity was measured after 40 h. (*B*) Yeast two-hybrid interaction assay to monitor association between the β-propeller domain of HCF-1 and wild-type or mutant $LZIP_{N92}$ fragments. A yeast GAL1-LacZ reporter strain was transformed with expression plasmids encoding the wild-type or P134S mutant HCF-1 β-propeller domain (residues 1–380) fused to the LexA DNA-binding domain together with plasmids expressing wild-type, LxxLL 1KO, LxxLL 2KO, LxxLL1/2KO, and LxxLLHBMKO versions of $LZIP_{N92}$ fused to the B42 activation domain. Strains were grown on 5-bromo-4-chloro-3-indolyl β-D-galactoside indicator plates. For each combination, five independent transformants were patched.

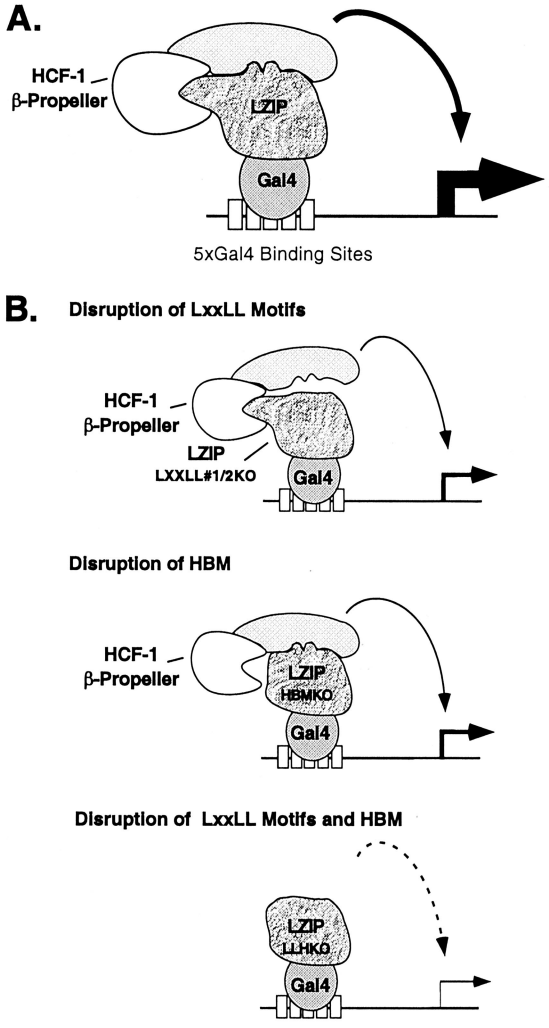

Fig. 5. Model for synergy between the HBM and LxxLL motifs. (*A*) The activation domain of LZIP recruits a coactivator complex including HCF-1. The two LxxLL motifs may interact with other regions in HCF-1 or possibly other cofactors. Recruitment of this complex results in strong transcriptional activation. (*B*) Mutations of the LxxLL motifs or HBM weaken the association by eliminating one or more points of contact and thus reduce activation.

the heterologous yeast $Gal4_{DBD}$ argues that HCF-1 is not functioning to promote recognition of specific promoter elements. Likewise, HCF-1 is unlikely to be required for nuclear localization of the fusion proteins, as described for VP16 (11), because the $Gal4_{DBD}$ contains a strong nuclear localization signal (34).

The recent finding that full-length LZIP is tethered to the endoplasmic reticulum (ER) brings into question the relevance of the C-terminal activation domain (35). ER association is mediated by a strongly hydrophobic sequence (LZIP residues 229 and 243) that resembles a single-pass transmembrane domain and separates the bZIP domain from the C terminus. An equivalent sequence is found in ATF-6, a closely related bZIP protein that is also associated with the ER (36). In response to stress, ATF-6 is cleaved at or close to the hydrophobic sequence, allowing the N terminus and bZIP domain to translocate to the nucleus. Whether LZIP is subject to similar regulation is unknown; however, the structural similarity to ATF-6 suggests that the C terminus may also remain within the lumen of the ER and thus does not participate in transcriptional activation. Full-length LZIP fused to the $Gal4_{DBD}$ is more than 100-fold less active than the N terminus, consistent with the majority of the overexpressed protein being retained at the ER (data not shown).

Targets of the LZIP LxxLL Motifs. LxxLL motifs (also referred to as NR boxes) have been identified in a number of non-DNA-binding transcriptional coactivators where they mediate recognition of other coactivators or DNA-binding proteins such as the nuclear hormone receptors (31). Cocrystallization studies of LxxLL-containing peptides complexed with the ligand-binding domains of estrogen receptor-α and PPARγ indicate that the LxxLL sequence forms an α-helix that fits into a groove on the surface of the nuclear receptor (37, 38). Consistent with this requirement, secondary structure modeling with the LZIP sequence predicts that both LxxLL motifs can be incorporated into

α-helices (data not shown). To our knowledge, LZIP provides the first demonstration of functional LxxLL motifs within the activation domain of a site-specific DNA-binding protein.

Detailed analysis of the interactions between nuclear hormone receptors and LxxLL-containing cofactors suggests that the rules underlying recognition of LxxLL motifs are complex. Specificity is thought to occur on several levels; studies of SRC-1/NCoA-1, for example, indicate that residues lying as much as eight amino acids C-terminal to the core leucine-rich motif play a critical role in cofactor selection (33, 39). The importance of flanking residues may explain why the sequences surrounding each of the LZIP leucine repeats are almost perfectly conserved between the mouse and human proteins and the spacing of the repeats may determine which cofactors can associate with LZIP. It is even possible that each LxxLL motif interacts with a different target protein, as suggested by the SRC-1/PPAR-γ receptor cocrystal, in which adjacent LxxLL motifs interact with separate subunits of the receptor dimer (38). Using a yeast interaction assay, we show that mutations of the LxxLL motifs do not prevent interaction with the HCF-1 β-propeller. This finding suggests that the LxxLL motifs interact with domains of HCF-1 lying outside of the β-propeller or that these motifs recruit additional cofactors, forming a coactivator complex (illustrated schematically in Fig. 5*A*). Our current data do not discriminate between these possibilities, and future studies will presumably require the cloning of cDNAs encoding additional proteins that interact with the N-terminal activation domain of LZIP.

While this study was in progress, Lu and colleagues (18) also mapped an activation domain located within the first 107 amino acids of LZIP. Using transfected tsBN67 cells, they showed that full-length LZIP requires HCF-1 for activation of a CRE-containing reporter gene, confirming the relevance of HCF-1 to LZIP function. In contrast to our results, however, these authors did not observe HCF-dependence when the N terminus of LZIP was fused to the $Gal4_{DBD}$, and this result led them to propose that HCF-1 facilitates transcription by stabilizing binding of LZIP to CRE elements or by inducing a specific "active conformation" required by the full-length protein. The basis for this discrepancy is not clear.

How Does HCF-1 Function as a Coactivator? The sequence of HCF-1 does not resemble other known coactivators and lacks functional modules such as a histone acetyltransferase domain (40, 41). We favor the idea that HCF-1 functions as a molecular chaperone, coordinating the assembly of multicomponent coactivator complexes. This role may be analogous to that of the corepressors Sin3 or Nurd/Mi-2, both of which lack intrinsic repressor function and instead coordinate the assembly of complexes between sequence-specific transcription factors and accessory proteins that include a histone deacetylase activity (42). Sin3 and Nurd/Mi-2 resemble HCF-1 in being large multidomain proteins and are known to provide a number of distinct interaction surfaces capable of gathering a variety of molecules into functional complexes. The identification of targets for the LxxLL motifs will allow us to address this model directly.

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