Host cell factor (HCF) was initially discovered as a cellular co-factor required for the activation of herpes simplex virus immediate early gene expression by the virion associated transactivator VP16. HCF also participates in a variety of cellular processes, although the mechanism of its action is not known. VP16 binds to HCF through a 4-amino acid motif (EHAY), which closely resembles the HCF binding domain of two cellular basic leucine-zipper proteins, Luman and Zhangfei. Luman is a powerful transcription factor that, in transient expression assays, activates promoters containing cAMP or unfolded protein response elements (UPRE). In contrast, Zhangfei neither binds consensus recognition elements for basic leucine-zipper proteins nor does it activate promoters containing them. Here we show that Zhangfei suppresses the ability of Luman to activate transcription. HCF appeared to be required for efficient suppression. A mutant of Zhangfei, which was unable to bind HCF, was impaired in its ability to suppress Luman. Zhangfei did not suppress ATF6, a transcription factor closely related to Luman but that does not bind HCF, unless the HCF binding motif of Luman was grafted onto it. Zhangfei inhibited the HCF-dependent activation of a UPRE-containing promoter by a Gal4-Luman fusion protein but was unable to inhibit the HCF-independent activation by Gal4-Luman of a promoter that contained Gal4 binding motifs. Binding of HCF by Zhangfei was required for the co-localization of Luman and Zhangfei to nuclear domains, suggesting that HCF might target the proteins to a common location.
containing these sequences (12). When expressed in cells infected with HSV, Zhangfei appears to suppress the expression of Immediate Early and Late classes of genes (12). Recent theoretical (24) and experimental (25) studies that have examined the ability of various B-Zip domains to dimerize, suggest that, although Luman and Zhangfei are inefficient at forming heterodimers, Zhangfei can associate with XBP-1 and ATF4. These factors are known to mediate stress responses in the cell.

In this article we describe our studies of interactions between Zhangfei and Luman. We show that Zhangfei suppresses the ability of Luman to activate transcription. Efficient suppression of transcriptional activation by Zhangfei correlated with its ability to bind HCF. Suppression by Zhangfei was specific to HCF-dependent transcriptional activation by Luman. Zhangfei had no effect on the HCF-independent transcriptional activation of a Gal4 binding site-containing promoter by a Gal4-Luman fusion protein. In addition, Zhangfei did not affect the activity of ATF6, a transcription factor that is closely related to Luman, but that does not bind HCF, unless the HCF-binding domain of Luman was grafted on to it. Although Luman and Zhangfei did not appear to form heterodimers, even in the presence of HCF, binding of HCF by Zhangfei was required for the co-localization of Luman and Zhangfei with the promyelocytic leukemia protein, a component of nuclear domain 10, suggesting that HCF might target the proteins to a common location. The inhibitory effects of Zhangfei were underscored by the observation that, in a human epithelial cell line expressing Zhangfei, the transcripts for 89 of 1700 genes examined decreased while those of only one gene increased.

MATERIALS AND METHODS

Plasmids—The construction of the plasmids pcFL-Lu and pcLu (S2210p), which express full-length and truncated forms of Luman, and pcZF, which expresses Zhangfei, have been described (12, 17, 20). Plasmid CGNATF6(1–733), which expresses the constitutively active truncated form of ATF6, and pSXTF6G6L3, which contains five copies of the UPRE-containing oligonucleotide, CTCGAGACAGGTGCTAGCGTG- GCATT, were obtained from Ron Prywes, Columbia University, New York, NY (26). A plasmid expressing the functionally active, spliced form of the second HCF-encoding cDNA was obtained from R. Hirosumi, University of Tokyo, Japan (27). The plasmid G5EC, a CAT reporter plasmid with five copies of the yeast Gal4-UAS, and the pM series of plasmids for constructing Gal4 fusion proteins were obtained from I. Sadowski, University of British Columbia, Canada (28). The plasmid pS7L, which expresses the fused amino and carboxyl termini of HCF, was obtained from P. O’Hare, Marie Curie Institute, UK.

The coding sequences for ATF6 were transfected to the expression vector pCDNA3 (Invitrogen) by recovering the sequences by PCR using primers that added 5¢ terminal BamHI and 3¢ terminal Xhol sites. The PCR product, digested with BamHI and XhoI was then transferred to pCDNA3 cut with the same enzymes. The ATF6 sequences were similarly transferred to pGEX-KG generating pGEX-ATF6, a plasmid expressing a glutathione S-transferase (GST) ATF6 fusion protein. The CAT reporter plasmid pCAT3BATF6 was constructed by transferring the UPRE-containing repeats from pSXTF6G6L3 to pCAT3Basic (Promega). Plasmids expressing mutant proteins, pcZF (Y224A), pcLu (DHTY78AGTA), pcLu S2210p (DHTY78AGTA), pcLu (N160G), and pcLu S2210p (N160G) were constructed using the QuikChange site-directed mutagenesis kit (Stratagene) as modified by Wang and Malcolm (29). To construct pcLu-ATF6 (ELLA:EDK1), a plasmid that expresses a fusion protein comprising the amino-terminal 112 amino acids of Luman and amino acids 149–373 of ATF6, a unique Nhel site was added to pcLu (S2210p) by site-directed mutagenesis. Sequences coding for the carboxyl terminus of ATF6 were then recovered by PCR using primers that added 5¢ terminal Nhel and 3¢ terminal Xhol sites. The PCR product cut with Nhel and XhoI was then used to replace the Nhel-Xhol fragment from the modified pcLu (S2210p). The Luman-ATF6 sequences were transferred to pGEX-KG to make pGEXLu-ATF6. In the plasmid names the mutations are designated in parentheses with the original amino acid (or amino acid sequence) and its location in the protein followed by the altered amino acid. For plasmids expressing fusion proteins, the junction between the two sequences is indicated by a colon separating the amino acids from the two proteins.

Antibodies, Immunoblots, Immunofluorescence, and Immunoprecipitation—Antisera against Luman and Zhangfei were raised in rabbits or mice using recombinant GST fusion proteins purified from Escherichia coli (12, 18). Monoclonal antibodies against VP16 (LPI) as well as rabbit anti sera against PML and HCF were obtained from Peter O’Hare, Marie Curie Institute, UK. The procedure for immunoblotting and immunofluorescence using the antibodies has been described previously (18, 20). For co-immunoprecipitation experiments described in Fig. 4, proteins labeled with [35S]methionine were synthesized using the TNT kit (Promega) as suggested by the manufacturer. The TNT lysates were charged with pcLu, pcZF, and pS7L, either alone or in combination. Following synthesis, the lysates were diluted to 200 μl in radioimmune precipitation assay buffer (0.1 M Tris, pH 7.0, 0.15 M NaCl, 0.1% SDS, 1% Triton X-100, and 1% sodium deoxycholate) containing Complete mini protease inhibitor mixture (Roche Applied Science), sonicated, and centrifuged for 15 min in a Beckman Airfuge to remove particulate material. After removing an aliquot of the assay buffer, the cleared lysate was kept at 4 °C for 20 min. Incubation was continued after adding 50 μl of Protein A-Sepharose beads (Amersham Biosciences). The beads were then washed three times with radioimmune precipitation assay buffer and suspended in 50 μl of SDS-PAGE sample buffer. Samples were analyzed by electrophoresis, and radioactive bands were detected by autoradiography. Procedures for immunofluorescence microscopy have been described before (12).

Transfection and CAT Assays—Vero and Hep2 cells were transfected with plasmids, purified through CsCl gradients, using the calcium phosphate method as described previously (30, 31). 5 μg of DNA was used for 2.5 × 106 cells for immunofluorescence or 1 × 106 cells for chloramphenicol acetyl transferase (CAT) assays. For CAT assays, 250 ng of pCMVGal, a plasmid specifying β-galactosidase, were added to each transfection. Lysates were assayed for β-galactosidase activity (22) and for CAT using an enzyme-linked immunosorbent assay kit (Roche Applied Science). CAT values were adjusted for transfection efficiency using β-galactosidase values. In figures for CAT assays, each datum point is the average of duplicate transfections with the bar representing the range. The data are representative of several (at least two) independent experiments that gave the same results. For extracting RNA, 4 × 106 cells were cultured with 20 μg of plasmid DNA. Where indicated, transfected cells were treated with 5 μM MG132 for 24 h before harvesting for immunoblotting. MG132 was not used when cells were transfected for CAT assays.

Real-time PCR—RNA from transfected cells was purified using TRIzol (Invitrogen) as suggested by the manufacturer. To detect and quantitate Luman and other transcripts, we used a LUX (FAM) oligonucleotide primer set (supplied with the kit) for quantitating Luman transcripts a series of one-step real-time PCR protocols for quantifying other transcripts (12, 18). Monoclonal antibodies against VP16 (LP1) as well as rabbit anti sera against PML and HCF were obtained from Peter O’Hare, Marie Curie Institute, UK.

A 100-ng sample of total cellular RNA was incubated with 100 pmol of FAM-labeled human β-actin primer set (Invitrogen). Transcripts were amplified with Taq polymerase from the Platinum quantitative RT-PCR thermocycler one-step system (Invitrogen). To quantitate Luman transcripts a series of samples containing 4 pg to 0.4 μg of pcLu were included to derive a standard curve. To determine the relative amounts of other transcripts (Mx1, Mx2) we analyzed the data using the standard curve method. In each experiment the standard curves for the various transcripts were normalized to the amount of β-actin transcript. Maintenance media (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, 1% penicillin/streptomycin), were
added to the cells. 4 h later 6 ml of the same media was added to each well. Cells were harvested 24 h after infection.

To confirm expression of Zhangfei and Zhangfei (Y224A) in adenovirus-infected Hep2 cells, anti-Zhangfei polyclonal antibodies were used for immunoblotting and immunofluorescence analysis as described above. β-Galactosidase expression was assayed using 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) (34).

**RNA Isolation**—Total RNA was extracted from Hep2 cells treated with adenoviruses expressing Zhangfei, Zhangfei (Y224A), or β-galactosidase 24 h after adenovirus infection using 2 ml of TRIzol reagent (Invitrogen) per 100-mm diameter dish according to the manufacturer’s protocol. The quality and integrity of the RNA was evaluated using a 2100 Bioanalyzer (Agilent Technologies).

cDNA Microarrays—Microarrays (1700 human expressed sequence tag clones) were purchased from the Microarray Center (Ontario Cancer Institute, Canada). More information about the microarray layout can be found on the website www.microarrays.com.

For each array 10 μg of total RNA was converted into cDNA using SuperScript II (Invitrogen). The reverse transcription was performed using AccT primers (T20VN, Sigma Genosys), dATP, dGTP, dTTP (final concentration 0.5 mM), and dCTP (final concentration 0.05 mM). The mixture was heated at 70 °C for 10 min and cooled down to room temperature. SuperScript II (300 units), Cy3-dCTP or Cy5-dCTP (final concentration 0.025 mM), and 20 units of Superase-in (Ambion) was added, and the mixture was incubated at 45 °C for 1 h in a light protected incubator. Two hundred units of SuperScript II were added for the next hr. Reverse transcription was stopped with EDTA (final concentration, 4.5 mM) and RNA hydrolyzed with 1 N NaOH and heating at 70 °C for 10 min. The NaOH was neutralized with 1 x HCl. Cy3- and Cy5-labeled cDNA were mixed together followed by purification and hybridization using Montage PCR Centrifuge Filter Devices (Millipore) according to the manufacturer’s instruction.

Five microliters of cDNA probe was added to 60 μl of DIG Easy hybridization buffer (Roche Applied Science) to which was added 0.5 mg/ml COT-1 DNA (Invitrogen) and 0.5 mg/ml yeast tRNA (Invitrogen). The mixture was incubated for 2 min at 100 °C and cooled down at room temperature. The hybridization solution was pipetted onto a 24 × 50 mm coverslip, and a cDNA array slide was placed on the solution. Hybridization was performed at 37 °C for 14–16 h in a humid chamber. After incubation arrays were washed three times in 1 x SSC, 0.1% SDS at 50 °C for 15 min each time and rinsed 4 times in 1 x SSC at room temperature.

**Array Scanning and Data Analysis**—Arrays were scanned immediately after washing using a GenePix 4000B scanner. Images were analyzed using GenePix Pro 4.1 (Axon Instruments, Inc.) LOWESS normalization and statistical analysis was performed using Acuity 3.2 (Axon Instruments, Inc.) and the SAM (Significance Analysis of Microarrays) software package from Stanford University (35). Gene ontology analysis was performed using Onto-tools (available from vortex.cs.wayne.edu/projects.htm). Onto-Express was used to generate functional profiles of genes down-regulated by Zhangfei (36, 37).

**RESULTS**

**Zhangfei Suppresses the Ability of Luman to Activate Transcription**—Luman is a potent activator of the reporter gene, CAT, linked to a promoter containing multiple copies of the UPRE. 3 To determine the effect of Zhangfei on Luman, we transfected cells with the reporter plasmid pCAT3BATF6, which contains five copies of UPRE, a plasmid expressing Luman, and varying amounts of a plasmid expressing Zhangfei (pcZF).

For our experiments we used a truncated mutant of Luman that lacks its ER-anchoring trans-membrane domain and portions of the protein that lie distal to it (20). This mutant, Luman S221Op (serine at position 221 is replaced by an Opal termination codon), is constitutively active and does not require release from intracellular membranes for its activity. Luman S221Op is a powerful activator of transcription. However, in contrast to ER-associated full-length Luman, it is extremely unstable, and we are unable to detect Luman S221Op using anti-Luman serum unless cells expressing the protein are treated with a proteasomal inhibitor such as MG132. In MG132-treated cells Luman S221Op is detected as a 36,000 molecular weight protein. For convenience, for the remainder of the paper we will refer to Luman S221Op as Luman. Full-length, ER-anchored Luman will be referred to as FL-Luman.

In the absence of Zhangfei, Luman activated CAT almost 100-fold (Fig. 1A, 0 μg of pcZF) over cells transfected with the empty vector. Zhangfei inhibited activation by Luman in a dose-dependent manner (see also Figs. 2A, 2C, 3A, and 3D). To determine the relative levels of Luman and Zhangfei in transfected cells, we examined them by immunoblotting using antisera against either Luman or Zhangfei (Fig. 1B). Cells were either treated with MG132 (+) to allow for the detection of Luman or left untreated (−). As expected, the amount of detectable Zhangfei increased with increasing concentrations of pcZF and MG132 had relatively little effect on the amount of Zhangfei detected. Luman could only be detected in cells that had been treated with MG132. Zhangfei had a profound effect

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3 M. Sackville and V. Misra, unpublished data.
HCF Binding by Zhangfei Is Required for Its Ability to Suppress Luman—To determine whether HCF binding by Zhangfei was required for its ability to suppress Luman, we compared Zhangfei with a mutant (Zhangfei Y224A) in which a conserved tyrosine (Tyr) residue in its HBM had been replaced with alanine (Ala). The mutation has no effect on the stability of Zhangfei but completely abrogates its ability to bind HCF, as determined in vitro (12). In contrast to Zhangfei, the mutant was relatively inefficient at suppressing Luman (Fig. 1A). The mutant was also unable to decrease the amount of Luman in MG132-treated cells. The mutant may even have stabilized Luman as it could be detected even in the absence of MG132 in cells expressing ZF Y224A (Fig. 1B, arrowhead). These results suggested that HCF binding by Zhangfei was critical for its ability to both suppress the ability of Luman to activate transcription and to reduce levels of Luman in cells.

To rule out the possibility that Zhangfei suppressed Luman by competing with it for HCF, we used the following approaches: First we supplemented functional HCF in Zhangfei-expressing cells by cotransfecting them with a plasmid-expressing HCF. This plasmid (pSL7) can functionally compensate for mutations in endogenous HCF (3). Increasing the amount of HCF in cells did not have an effect on the ability of Zhangfei to suppress Luman (Fig. 2A). Next we compared the ability of Zhangfei to suppress Luman with that of other proteins with HBMs. Luman S221Op (N160G) is a mutant of Luman in which the conserved asparagine residue in its basic DNA binding domain has been replaced with a glycine. The mutant protein does not bind DNA but retains the ability to bind HCF. HSV-VP16 is the prototypical HCF-binding protein. In contrast to Luman none of these other HBM-containing proteins could activate the UPRE-containing promoter (Fig. 2B). Fig. 2C shows that, compared with Zhangfei, the other HBM-containing proteins were relatively inefficient at suppressing the transcriptional activity of Luman. VP16 did have some suppressive effect but only at concentrations more than 10 times higher than are optimal for it to activate transcription. In addition, VP16 Y364A, a mutant that does not bind HCF, inhibited Luman to the same extent as VP16. These results suggest that Zhangfei suppresses Luman by a mechanism that does not rely on it competing with Luman for functional HCF.

HCF Binding by the Target Is Also Important for Suppression by Zhangfei—Our results suggested that HCF binding by Zhangfei was important for its suppression of the activity of the HCF-binding transcription factor Luman. To determine if the effect was specific for transcription factors that bind HCF, we examined the effect of Zhangfei on ATF6. Like Luman, ATF6 is a basic leucine-zipper protein that is anchored in the ER. It is released by proteolysis in response to stimuli such as the act-
beads were analyzed by SDS-PAGE and autoradiography. DHTY78AGTA, a mutant impaired in its ability to bind HCF (without Zhangfei).

amounts of pcZF. CAT activity is expressed as a percentage of activity Luman, ATF6-(1–373) or Luman-ATF6-(1–373), and increasing amounts of glutathione-Sepharose beads used in experiment shown in Fig. 3B shows that like Luman, the Luman-ATF6 chimera was bound much more HCF than GST alone (compare lanes 1 and 2) while binding by the Luman DHTY78AGTA mutant, in which the three conserved amino acids in its HBM (D, H, and Y) were replaced (abbreviated as “Lu D78A” in Fig. 3B, C and D), was greatly reduced (lane 3). In contrast to ATF6, which bound very little HCF, binding by the Luman-ATF6 chimera was comparable to that of Luman (compare lanes 2, 4, and 5). Fig. 3D shows that like Luman, the Luman-ATF6 chimera was inhibited by Zhangfei in a dose-dependent manner, whereas ATF6 was not affected.

We next constructed a chimera that comprised the amino-terminal 112 amino acids of Luman, including its HBM, and amino acids 149–373 of ATF6. Fig. 3B illustrates the ability of various GST fusion proteins, attached to glutathione-Sepharose beads, to bind HCF, and Fig. 3C shows the relative amounts of GST-linked proteins used in the assay. GST-Luman bound much more HCF than GST alone (compare lanes 1 and 2) while binding by the Luman DHTY78AGTA mutant, in which the three conserved amino acids in its HBM (D, H, and Y) were replaced (abbreviated as “Lu D78A” in Fig. 3B, C and D), was greatly reduced (lane 3). In contrast to ATF6, which bound very little HCF, binding by the Luman-ATF6 chimera was comparable to that of Luman (compare lanes 2, 4, and 5). Fig. 3D shows that like Luman, the Luman-ATF6 chimera was inhibited by Zhangfei in a dose-dependent manner, whereas ATF6 was not affected.

Physical Association between Luman, Zhangfei, and HCF—To examine the possibility that Zhangfei might associate either directly with Luman or through HCF, we synthesized the three proteins, alone or in combination, in an in vitro transcription and translation-linked system. The proteins were labeled with [35S]methionine to facilitate detection. Proteins were then precipitated with either anti-Luman or anti-Zhangfei serum; or C, after immunoprecipitation with anti-Zhangfei serum. The positions of HCF (arrow), Luman (arrowhead), and Zhangfei (feathered arrow) are marked.

We next constructed a chimera that comprised the amino-terminal 112 amino acids of Luman, including its HBM, and amino acids 149–373 of ATF6. Fig. 3B illustrates the ability of various GST fusion proteins, attached to glutathione-Sepharose beads, to bind HCF, and Fig. 3C shows the relative amounts of GST-linked proteins used in the assay. GST-Luman bound much more HCF than GST alone (compare lanes 1 and 2) while binding by the Luman DHTY78AGTA mutant, in which the three conserved amino acids in its HBM (D, H, and Y) were replaced (abbreviated as “Lu D78A” in Fig. 3B, C and D), was greatly reduced (lane 3). In contrast to ATF6, which bound very little HCF, binding by the Luman-ATF6 chimera was comparable to that of Luman (compare lanes 2, 4, and 5). Fig. 3D shows that like Luman, the Luman-ATF6 chimera was inhibited by Zhangfei in a dose-dependent manner, whereas ATF6 was not affected.

cumulation of unfolded proteins in the ER (38). The basic domains of Luman and ATF6 are similar, and ATF6 also activates promoters containing UPRE. For our experiments we used the truncated, constitutively active mutant of ATF6 (ATF6-(1–373)), which does not possess the trans-membrane and ER luminal portions of ATF6 (the mutant is equivalent to Luman S221Op). Although Zhangfei completely suppressed the ability of Luman to activate the UPRE-containing promoter, it had no effect on the ability of ATF6 to do so (Fig. 3, A and D).
larly, anti-Zhangfei serum precipitated Zhangfei from all reaction mixtures that contained it (Fig. 4C, arrow, lanes 3, 4, 6, and 7), and HCF co-precipitated with Zhangfei (Fig. 4C, arrow, lane 6 and faint band in lane 7). However, at least in this assay, FL-Luman and Zhangfei did not associate with each other, even when HCF was present in the reaction mixture (Fig. 4C, lanes 4 and 7). When Luman, Zhangfei, and HCF were expressed together (lane 7) Zhangfei bound less HCF than Luman (compare lane 7 in Fig. 4, B and C). This suggests that Luman may have a greater affinity for HCF than Zhangfei does and supports our interpretation (Fig. 2) that Zhangfei does not deprive Luman of HCF when the two proteins are present in the same cell.

**Luman and Zhangfei Co-localize with PML in Nuclear Domains, and HCF Binding by Zhangfei Is Required For This Association**—Although both Luman and Zhangfei each bind HCF, our results suggested that HCF, Luman, and Zhangfei do not form a trimeric complex. We therefore investigated the possibility that HCF may act by targeting Luman and Zhangfei to a common cellular site where Zhangfei might affect the activity of Luman. Cells transfected to express Luman and Zhangfei were treated with MG132 and labeled with mouse anti-Luman and rabbit anti-Zhangfei serum. The bound antibodies were detected with Alexa546 (red)-labeled anti-mouse and Alexa488 (green)-labeled anti-rabbit antibodies. As expected, when Luman and Zhangfei were expressed together, very few cells showed both Luman and Zhangfei. However, in the few cells with both proteins, the proteins co-localized in punctate nuclear structures (Fig. 5A). Nuclear structures called nuclear domain 10 (ND10) have been implicated in the control of cell growth, cell-cycle regulation, transcription, apoptosis, nuclear proteasomes, the interferon response and the suppression of HSV and adenovirus replication (reviewed in Refs. 39–41). Several cellular and viral proteins that are also associated with these processes have been shown to co-localize with a component of nuclear domain 10 called the promyelocytic leukemia protein (PML). We therefore detected PML and Luman or Zhangfei in cells expressing these proteins. All cultures were treated with MG132 to stabilize the expression of activated Luman. Both Luman and Zhangfei co-localized with PML (Fig. 5B). Interestingly, Zhangfei Y224A, the mutant that does not bind HCF and is impaired in its ability to suppress Luman did not co-localize with PML (Fig. 5B). When Luman and Zhangfei were expressed on their own (not shown), the proteins also co-localized in nuclear dots with PML. For Zhangfei, this association was observed in the absence or presence of MG132.

**Depletion of Nuclear HCF by Retaining It In the ER with Full-length Luman**—Recently, Khurana and Kristie (9) developed an ingenious system for depleting cells of nuclear HCF by developing cell lines expressing actin containing the VP16 HBM. To retain HCF in the cytoplasm we used the natural ability of full-length Luman to bind and retain HCF in the ER (17). We used a mutant of full-length Luman (FL-Luman N160G), which does not bind UPRE. To control for the possibility that FL-Luman might retain critical factors, other than HCF, in the ER, we compared the effects of FL-Luman N160G with another mutant, FL-Luman DHTY78AGTA, which has an altered HBM. To retain HCF in the cytoplasm we used the natural ability of full-length Luman to bind and retain HCF in the ER (17). We used a mutant of full-length Luman (FL-Luman N160G), which does not bind UPRE. To control for the possibility that FL-Luman might retain critical factors, other than HCF, in the ER, we compared the effects of FL-Luman N160G with another mutant, FL-Luman DHTY78AGTA, which has an altered HBM. To retain HCF in the cytoplasm we used the natural ability of full-length Luman to bind and retain HCF in the ER (17). We used a mutant of full-length Luman (FL-Luman N160G), which does not bind UPRE. To control for the possibility that FL-Luman might retain critical factors, other than HCF, in the ER, we compared the effects of FL-Luman N160G with another mutant, FL-Luman DHTY78AGTA, which has an altered HBM. To retain HCF in the cytoplasm we used the natural ability of full-length Luman to bind and retain HCF in the ER (17).
scriptionally active forms of three factors that activate UPRE-containing promoters. Of the three factors only Luman possesses an HBM and requires HCF for efficient activity. ATF6 and XBP1 do not have HBMs. Fig. 6 shows that FL-Luman N160G drastically inhibited the ability of Luman to activate a UPRE-containing promoter, whereas FL-Luman DHTY78AGTA had no effect. This confirms our earlier observations that Luman relies on HCF for activity (10). In contrast to its effect on Luman, FL-Luman DHTY78AGTA had relatively little effect on ATF6 and XBP1. Similar amounts of the two FL-Luman mutants were expressed in these experiments (Fig. 6C).

Zhangfei Suppresses HCF-dependent Luman Activation of a UPRE-containing Promoter but Not HCF-independent Activation by a Luman Fusion Protein with a Heterologous DNA Binding Motif—To determine if the effect of Zhangfei on Luman was limited to its HCF-dependent activation of UPRE-containing promoters we constructed a chimera of Luman that contained the DNA binding domain of the yeast protein Gal4. The chimera, Gal4-Luman, can activate both UPRE and Gal4-UAS-containing promoters about 100-fold (not shown). Fig. 7A shows that, although the activation of a UPRE-containing promoter by both Luman and Gal4-Luman was inhibited by FL-Luman N160G, suggesting that it was dependent on HCF, activation of a Gal4-UAS-containing promoter by Gal4-Luman was not. This confirms our earlier observations that Gal4-Luman activation of a UAS-containing promoter is HCF-independent (10). As expected, Luman did not activate the Gal4-UAS promoter. Although the activation of the UPRE promoter by Luman or Gal4-Luman was inhibited by Zhangfei, activation of the Gal4-UAS promoter by Gal4-Luman was not (Fig. 7B). Interestingly, both Luman and Gal4-Luman were reduced in Zhangfei-expressing, MG132-treated cells (Fig. 7C). This suggested that, despite our inability to detect Gal4-Luman in immunoblots of Zhangfei and Gal4-Luman-coexpressing cells, these cells contained sufficient Gal4-Luman to activate transcription.

Suppression of Gene Expression in Zhangfei-expressing Cells—We next determined if Zhangfei had an effect on the expression of other cellular genes. To ensure the expression of Zhangfei in all cells in a culture, we cloned the coding sequences for Zhangfei in a human adenovirus 5-expression system. Preliminary experiments indicated that at a multiplicity of infection of 20 plaque-forming units per cell, which was not toxic to the infected cells, almost all cells in a culture expressed detectable levels of Zhangfei. Total RNA was extracted from cells infected with adenovirus vectors expressing Zhangfei,
Interactions of HCF-binding Proteins

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Interactions of HCF-binding Proteins

A

B

C

![Figure 7](image)

**Figure 7.** Zhangfei suppresses HCF-dependent promoter recognition by Luman. **A**, Luman requires HCF for the activation of a UPRE-containing promoter but not a Gal4-UAS promoter. Cells were transfected with a UPRE-containing reporter plasmid (pCAT3BATF6) or a Gal4-UAS-containing reporter plasmid (pG5EC) and plasmids expressing Luman (Lu) or a fusion protein of the Gal4 DNA binding domain and LuS221Op (Gal4-Lu). Transfection mixtures also contained empty vector (pcDNA3) or vector expressing full-length ER-associated Luman N160G (FL-Luman N160G) or FL-Luman DHTY78AGTA. **B**, Zhangfei suppresses Luman and Gal4-Luman on UPRE but not on Gal4-UAS promoters. Cells were transfected with UPRE or Gal4-UAS containing reporter plasmids and plasmids expressing Luman or Gal4-Luman alone or in conjunction with plasmid expressing Zhangfei. **C**, Zhangfei reduces both Luman and Gal4-Luman in cells. Transfected cells, similar to the ones in **B** were either treated with MG132 (+) or left untreated (−) and analyzed by immunoblotting using anti-Luman serum.

Zhangfei Y224A, or β-galactosidase. Mixtures of two cDNA samples (Zhangfei versus Luman Y224A or Zhangfei versus β-galactosidase and Zhangfei Y224A versus β-galactosidase), in which one was labeled with the dye Cy3 while the other was labeled with Cy5 were hybridized to microarrays of cDNAs representing 1700 human genes. To control for differences in labeling efficiency with the two fluorescent dyes each experiment contained microarrays in which the dyes used to label the cDNAs were reversed. In one experiment comparing Zhangfei Y224A and β-galactosidase we found no difference in the pattern of expression of any of the genes suggesting a “null” phenotype for the mutant. Each of the remaining pairs of experiments was repeated twice with fresh batches of RNA.

Analysis using the program Significance Analysis of Microarrays (SAM) showed (Fig. 8) that when RNA samples from cells expressing Zhangfei were compared with those from cells expressing Zhangfei Y224A or β-galactosidase, transcripts from 89 of the 1700 genes examined were suppressed. To validate these results we quantitated transcripts from 6 of the 89 genes by quantitative real-time PCR. The samples of RNA were used for the microarray analysis were also used for the quantitative real-time PCR procedure. Transcripts for three genes were consistently down-regulated, the transcripts of two other genes were suppressed less than 2-fold, whereas there was no significant difference for the sixth gene. In contrast to the 89 transcripts that were under-represented, RNA from only one gene was increased in Zhangfei-expressing cells.

The genes down-regulated by Zhangfei were analyzed by gene ontology-based OntoExpress software (vortex.cs.wayne.edu/projects.htm) on June 12, 2004. 65 genes of the 89 that were listed in the OntoExpress data base were distributed according to their participation in biological processes. Prominent among these were signal transduction (20) and cell growth and/or maintenance (19) and protein (16) and nucleotide and nucleic acid (13) metabolism.

**Discussion**

Host Cell Factor has been implicated in many cellular and viral processes, and at least some of these appear to be mediated through direct interaction between HCF and HBMs present in several transcription factors. For those factors such as VP16, Luman, Krox20, and E2F4, where the interaction has been examined in any detail, HCF binding appears to be required for the activity of the factors. Mutation of their HBMs or depletion of functional HCF in the cell by cytoplasmic retention or by elevated temperature in cell lines with temperature-sensitive lesions in HCF, leads to abrogation of the activities of the factors.

In this report, we show that Zhangfei, an HCF-binding protein, is a potent and specific suppressor of transcription induced by the HCF-binding transcription factor Luman. We believe that HCF appears to play a critical role in this interaction for the following reasons: 1) Zhangfei Y224A, a mutant that is unable to bind HCF in vitro was inefficient at inhibiting Luman (Fig. 1). We cannot, however, rule out the possibility that the mutation affects aspects of Zhangfei other than just its ability to bind HCF. Because Luman, the target of Zhangfei, also requires HCF for activity it would be difficult to confirm the reliance of Zhangfei on HCF by depleting the cell of functional HCF either by using a cell line with a temperature-sensitive lesion in HCF (4) or by sequestering HCF in the cytoplasm (9). 2) Zhangfei had no effect on the activity of ATF6, a protein very similar to Luman, unless its amino terminus was swapped with the HCF-binding amino terminus of Luman (Fig. 3). 3) Zhangfei inhibited the HCF-dependent transcriptional activation by Luman and Gal4-Luman of a UPRE-containing promoter, but had no effect on HCF-independent activation by Gal4-Luman of a Gal4-UAS-containing promoter (Fig. 7). These results are consistent with our previous observations (10) that suggested that Luman requires HCF for efficient recognition of its promoter but not for transcriptional activation.
There are several potential ways in which Zhangfei might suppress the activity of Luman as follows. If the concentration of Luman in the transcription assays is rate-limiting, Zhangfei might inhibit its ability to activate transcription by reducing levels of Luman in the cell. It could accomplish this by suppressing the transcription of its gene, suppressing translation of its mRNA, or by accelerating its degradation. Indeed, cells co-expressing Luman and Zhangfei had greatly reduced levels of Luman (Fig. 1B, although the interpretation of this observation is complicated by the lack of stability of the protein, which can only be detected in immunoblots if its proteasomal degradation is inhibited). The Y224A mutant, in addition to being impaired in its ability to suppress the transcriptional activity of Luman, was also less efficient at reducing Luman in cells (Fig. 1) suggesting that the two observations might be linked. However, despite our inability to detect Luman in cells by immunoblots the protein does not appear to be limiting. In the absence of Zhangfei and MG139, although no Luman was detected in immunoblots, it activated the UPR-containing promoter over 100-fold, and although Zhangfei reduced detectable Gal4-Luman as efficiently as it did Luman, it had no effect on the ability of Gal4-Luman to activate a UAS-containing promoter (Fig. 7). This suggests that, although Zhangfei may reduce amounts of Luman in cells, this is not sufficient to explain the inhibitory effect on the transcriptional activity of Luman.

Zhangfei might suppress Luman by binding and reducing the available HCF within a cell. Our results suggest otherwise. Synthesis of an excess of functional HCF in transfected cells had no effect on the ability of Zhangfei to suppress Luman and, in contrast to Zhangfei, other HCF-binding proteins had relatively little effect on Luman (Fig. 2).

Zhangfei might prevent Luman from recognizing its response element. This is a reasonable hypothesis. Zhangfei lacks a conserved asparagine residue in its basic domain thought to be critical for DNA binding (22, 23), and it is conceivable that Luman-Zhangfei heterodimers may not bind Luman response elements. The asparagine residue in question lies 18 amino acids upstream from the first leucine in the leucine zipper of almost all B-Zip proteins (Fig. 9, consensus). A survey of all human and viral B-Zip proteins shows that the only exceptions to this rule are Zhangfei, HBZ, K-bZip, and CHOP (Fig. 9). All these proteins are also efficient suppressors of closely related B-Zip transcription factors (42–50).

The mechanisms by which HBZ, K-bZip, and CHOP suppress other B-Zip proteins are not known. It is possible that HBZ, K-bZip, CHOP, and Zhangfei exert their effect on transcription by forming heterodimers with their B-Zip partners and preventing them from binding DNA. Indeed, K-bZip, HBZ, and CHOP are thought to interact with their targets through their B-Zip domains. However, a study by Vinson et al. (24) that examined the theoretical propensity of B-Zip proteins to form homo or heterodimers, and an analysis by Newman and Keating (25) using arrays of synthetic peptides representing all human B-Zip regions, suggest that Luman and Zhangfei have incompatible leucine-zippers and cannot form heterodimers. This is supported by our attempts to co-immunoprecipitate the proteins co-produced in vitro (Fig. 4). These observations argue against Zhangfei and Luman directly interacting through their leucine zippers to form heterodimers incapable of binding Luman response elements. In addition, our preliminary experiments indicate that Zhangfei also inhibits two other transcription factors that possess HCF binding domains, HSV-VP16 and Brn3a, in an HCF-dependent manner (not shown). Because neither HSV-VP16 nor Brn3a possess leucine zippers, it is unlikely that the effect of Zhangfei is mediated through leucine zipper interactions.

For Zhangfei to efficiently exert its suppressive effect, HCF appears to be required by both Zhangfei and its target. One might therefore expect HCF to be the bridging molecule. The co-immunoprecipitation experiment suggests that, at least for

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**Fig. 8. Identification of genes with significant change in expression in response to Zhangfei.** RNA from Hep2 cells infected with adenovirus vectors expressing Zhangfei, Zhangfei Y224A, or β-galactosidase was hybridized to cDNAs of 1700 representative human genes. A scatter plot of observed and expected relative difference in the expression of genes in cells expressing Zhangfei. The solid line indicates where the observed relative difference would be the same as the expected difference. The dotted lines are drawn at a distance (D) = 0.38 from the solid line. Eighty-nine genes (green dots) were down-regulated, and a single gene (red) was up-regulated. At a D setting of 0.38, 16 of 89 down-regulated genes could be expected to be false-positives.

**Fig. 9. The basic domains of inhibitory B-Zip proteins lack an asparagine residue conserved in B-Zip transcription activators.** The basic domains of Luman, Zhangfei, human T-cell leukemia virus HBZ, CHOP, and Kaposi sarcoma-associated herpes virus K-bZIP are shown below the consensus sequence for the basic region of basic leucine-zipper proteins. The single letter amino acid code is used. In the consensus, B = R or K. The first amino acid in the leucine-zipper is marked with an arrow. Amino acids that align with the consensus are in bold red, the N residue in the consensus and other sequences is boxed as are amino acids that replace it in ZF, HBZ, CHOP, and K-bZIP (blue). Conserved amino acids in the basic domains of Luman and Zhangfei are marked with double-headed arrows.
protein made in vitro, this is not the case. The results, however, do not rule out the possibility that in vivo other proteins may facilitate interactions between Luman, HCF, and Zhangfei.

In addition to acting as a bridging molecule, HCF might help to target Zhangfei to nuclear domains that harbor functionally active Luman. Our results show that if proteosomal degradation of Luman was suppressed by MG132, it accumulated at nuclear sites that contain PML. Zhangfei also accumulated at these sites (with or without MG132), whereas the mutant Zhangfei Y224A, which cannot bind HCF and cannot suppress these sites (with or without MG132), whereas the mutant nuclear sites that contain PML. Zhangfei also accumulated at

These results suggest that one way in which HCF might influence these processes is to target relevant cellular factors to appropriate nuclear compartments either to fulfill or interfere with their function.

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