The Gut-enriched Krüppel-like Factor Suppresses the Activity of the CYP1A1 Promoter in an Sp1-dependent Fashion*

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The gut-enriched Krüppel-like factor (GKLF) is a newly identified zinc finger-containing transcription factor. Recent studies indicate that GKLF binds to a core DNA sequence of 5′-G(A)/G(A)GG(C/T)G(C/T)-3′, which is found in an endogenous cis element, the basic transcription element (BTE) of the cytochrome P-450IA1 (CYP1A1) promoter. The present study characterizes the ability of GKLF to regulate CYP1A1 expression. By electrophoretic mobility gel shift assay (EMSA) and methylation interference assay, GKLF was found to bind BTE in a manner similar to several other transcription factors known to interact with BTE including Sp1 and BTEB. Cotransfection studies in Chinese hamster ovary cells showed that GKLF inhibited the CYP1A1 promoter in a dose- and BTE-dependent manner. The same experiments also revealed that BTE was responsible for a significant portion of the CYP1A1 promoter activity. EMSA of nuclear extracts from Chinese hamster ovary cells showed that Sp1 and Sp3 were two major proteins that interacted with BTE. Additional cotransfection studies showed that GKLF inhibited Sp1-mediated activation of the CYP1A1 promoter. In contrast, GKLF enhanced Sp3-dependent suppression of the same promoter. Moreover, the ability of GKLF to inhibit Sp1-dependent transactivation was in part due to physical interaction of the two proteins. These findings indicate that GKLF is a negative regulator of the CYP1A1 promoter in a BTE-dependent fashion and that this inhibitory effect is in part mediated by physical interaction with Sp1.

Regulation of gene expression is dependent on the functions of sequence-specific DNA-binding proteins called transcription factors. Proteins with the zinc finger motif constitute a significant portion of the transcription factor family, primarily because of the stable nature of interaction between a zinc finger and its cognate DNA-binding sequence (1–5). Among the zinc finger proteins the Cys2-His2 (C2H2) type, initially identified in the Xenopus laevis transcription factor TFIIIA (6), represents the most common zinc finger motif for DNA binding (7). Additional homology in the amino acid sequence of C2H2 zinc fingers is found in a family of proteins closely related to the Drosophila segmentation gene product Krüppel (8). Examples of Krüppel-like proteins include Sp1 (9), zif268/Egr-1 (10), EKLF1 (11), and WT-1 (12), which collectively exhibit a diverse range of regulatory functions in the cell.

The gut-enriched Krüppel-like factor (GKLF) (also called epithelial zinc finger) is a newly identified Krüppel-type protein with three C2H2 zinc fingers located in the carboxyl terminus (13–15). The amino acid sequence in the zinc finger region of GKLF is closely related to several Krüppel-like proteins including LKLF (16), EKLF (11), and BTEB2 (17). However, we recently showed that the amino acid sequence required for the nuclear localization of this group of proteins is more conserved in GKLF, LKLF, and EKLF than in BTEB2 (18). These findings suggest that the former three transcription factors belong to a distinct subfamily of closely related Krüppel proteins (18).

The in vivo expression of GKLF is enriched in epithelial cells of the gastrointestinal tract (13, 14) and skin (14) and in vascular endothelial cells (15). In vitro, expression of GKLF is induced in conditions that promote growth arrest such as serum deprivation and contact inhibition (13). In addition, constitutive expression of GKLF inhibits DNA synthesis (13). Taken together, these observations suggest that GKLF may have an important function in regulating growth and proliferation of specific epithelial and endothelial tissues.

The best studied “first-degree” relative of GKLF is EKLF, which is crucial for expression of the β-globin gene (11, 19, 20). Recent studies utilizing the gene targeting technique also showed that EKLF is essential for erythropoiesis (21, 22). EKLF acts by binding to and activating the CACCC element in the promoter of the β-globin gene (19, 20). Because of the close homology in the zinc finger sequence between GKLF and EKLF, GKLF was also found to interact with the β-globin CACCC motif (14, 15). However, whether GKLF regulates endogenous β-globin gene expression is unclear from these studies. By using an empirical approach called the target detection assay, we recently obtained a consensus, minimal essential binding sequence of 5′-G(A)/G(A)GG(C/T)G(C/T)-3′ for GKLF (23). This sequence is similar but not identical to the CACCC element required for globin gene expression.

Another cis sequence that is similar to the empirically derived GKLF-binding sequence is the basic transcription element (BTE). This element is present in the promoter of a conserved family of genes encoding the cytochrome P-450 drug-
metabolizing enzymes including CYP1A1 (24, 25). GKLF has been shown to interact with high affinity with BTE (14, 23). These findings raised the interesting question whether GKLF is involved in the regulation of the CYP1A1 promoter through BTE. Previous studies have shown that BTE is essential for the basal CYP1A1 promoter activity (24) and that it is the focus of interaction for a multitude of transcription factors including Sp1, BTEB, and BTEB2 (17, 26). Of interest is that several BTE-containing cytochrome P-450 genes including CYP1A1 are expressed in the epithelial cells of the intestinal tract (27–29) in a similar distribution to that found for GKL F (13, 14). Our current study therefore represents a continuing attempt to decipher further the function of GKL F in regulating expression of endogenous genes, using CYP1A1 as a potential “target” gene.

EXPERIMENTAL PROCEDURES

Materials—Purified human Sp1 was purchased from Promega (Madison, WI). A rabbit polyclonal anti-GKLF serum was described before (13, 18, 23). Anti-Spa directed against human Sp1 and Sp5 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Oligonucleotides were synthesized at the Genetics Core Facility of the Johns Hopkins University School of Medicine. The sequences of the individual oligonucleotides in the sense orientation are as follows (shown in italics are linker sequences): BTE (17), 5′-gatcGAGAAGAGGAGGCTGC-CAACgact-3′; GKLF (23), 5′-atgcAGAGGAAAGGAGCCTAGTATGC-Taactg-3′; Sp1 (Promega), 5′-ATAGCTAGCCCGGCGCCGCGGCGT-3′; AP2 (Promega), 5′-GATGCAAGTACGCCGGCCGCGCCTG-3′.

DNA Constructs—Complementary DNA constructs containing various regions of the coding sequence of GKLF were generated in the mammalian expression vector PMT3 (30). Included were GKL F (1–483), containing the entire coding region, GKL F (350–483) containing the carboxyl-terminal nuclear localization signal and the three zinc fingers, and GKL F (1–401), containing the amino-terminal sequence including the zinc fingers but excluding the three zinc fingers (18, 23). Reporter constructs linking the chloramphenicol acetyltransferase (CAT) reporter to various regions of the CYP1A1 promoter have been described (24). Included were the following: pMC6.3k, containing 6.3 kilobase pairs of the rat CYP1A1 promoter; pMC6.3k(96/53) that has an internal deletion between nucleotides 96 and 53 of the promoter; pMC6.3k(96/44) that has an internal deletion between nucleotides 96 and 44 of the promoter; pSV/MC50, containing the SV40 enhancer linked to 44 bp of the CYP1A1 promoter; and pSV/MC44, containing the SV40 enhancer linked to 44 bp of the CYP1A1 promoter. Two of these constructs (pMC6.3k(96/44) and pSV/MC44) do not contain BTE, which is located between nucleotide positions 53 and 44 of the CYP1A1 promoter (24). CMV-Sp1 and CMV-Sp5, expression constructs containing Sp1 and Sp5, respectively, were generously provided by Dr. C. Suske (31). GST-Sp1ZnF and GST-Sp1Q1, fusion constructs of glutathione S-transferase (GST) to aa residues 620–778 (containing the three zinc fingers) and 1–262 (containing one of the transcription activation domains) of Sp1, respectively, were kindly provided by Dr. Y. Shi (32).

Production of Recombinant Protein—The production of bacterially expressed recombinant GKL F between as residues 350–483 was described previously (23). This protein contains the nuclear localization signal and all three zinc fingers of GKL F and will be referred to as “recombinant GKL F” throughout the text. Briefly, the prokaryotic expression plasmid pET-16b (Novagen (Madison, WI)) containing the SV40 enhancer linked to 44 bp of the CYP1A1 promoter activity was transformed into E. coli (BL21(DE3)) pLysS strain. Induction of recombinant protein production was achieved by the addition of 1 mM isopropyl-β-D-thiogalactosidase to logarithmically growing cells for 4 h. Cell lysates were prepared by treating pelleted bacteria with lysis buffer (20 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 6 mM urea, 5 mM imidazole, 1 mM g/ml leupeptin, 1 mM g/ml aprotinin, and 20 μM phenylmethylsulfonyl fluoride) on ice for 30 min, followed by sonication and purification by a Ni2+ column. The eluted protein was dialyzed against a solution of 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM ZnCl2, 10% glycerol, and gradually decreasing concentrations of urea from 6 M to nil. The purity of the protein was approximatively 90% as estimated by Coomassie Blue staining of the eluted protein resolved by denaturing polyacrylamide gel electrophoresis.

The production of GST-Sp1ZnF and GST-Sp1Q1 (32) protein was achieved using transformed DH5α strain of E. coli (Life Technologies, Inc.) after 2 h of induction with 1 mM isopropyl-β-D-thiogalactosidase. The bacterial pellet was collected by centrifugation and resuspended in a buffer containing 10 mM Tris-HCl, pH 7.8, 2 mM EDTA, 3% dimethyl sulfoxide, 1 mM leupeptin, 1 mM aprotinin, and 20 μM phenylmethylsulfonyl fluoride. The bacteria were sonicated and then centrifuged to remove debris. The soluble fusion proteins present in the supernatant were stored in aliquots at −80°C.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described previously (23). 0.1 pmol of 32P-end-labeled oligonucleotides and 100 ng of purified protein were used in experiments involving GST-Sp1ZnF or purified Sp1. In experiments with competitor DNA, a 10-fold molar excess unlabeled oligonucleotide over the labeled probe was included. Nuclear extracts from CHO cells were prepared by a modification of a published method (33). Briefly, 1 × 106 cells were washed twice with ice-cold PBS (phosphate-buffered saline), scraped in 5 ml of PBS, and collected by centrifugation at 400 × g for 5 min. The cell pellet was suspended in 4 packed cell volumes of buffer A containing 1 M phenylmethylsulfonyl fluoride). The bacteria were sonicated and then lysed by 10 strokes with a Dounce homogenizer. Nuclei were collected by centrifugation at 10,000 × g for 30 min. The supernatant was then dialyzed exhaustively against buffer C (20 mM Tris-HCl, pH 7.8, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol) and stored at −80°C. Ten μg of nuclear extracts were used for each EMSA reaction. In reactions that contained antibodies, 2 μg of affinity purified IgG were added to the mixture of nuclear extracts and DNA probe.

Methylation Interference Assay—Methylation interference assay was performed as previously described (34). The synthetic BTE oligonucleotide was first labeled with 32P-ATP using T4 polynucleotide kinase and annealed to the unlabeled complementary DNA strand. The single end-labeled double-stranded oligonucleotide was partially methylated with a solution of 0.5% dimethyl sulfate in 50 mM sodium cacodylate and 0.1 mM EDTA. Ten pmol of partially labeled probe and 2.5 μg of purified recombinant GKL F were used in each EMSA reaction as described above. Following electrophoresis in a 6% non-denaturing polyacrylamide gel, bands corresponding to the shifted GST-BTE protein complex and the free, non-shifted BTE probe were excised and the DNA within the bands eluted by electrophoresis. The eluted DNA was then cleaved with 0.1 μl piperidine at 95°C for 30 min. The cleaved products were resolved on a 12% denaturing polyacrylamide gel and visualized by autoradiography.

Transfection and Reporter Assays—Transient transfection of CHO cells by Lipofectin was described previously (18). The DNA contained a mixture of various amounts of effector and reporter constructs along with pCMV-SPORT-β-galactosidase (Life Technologies, Inc.) as an internal standard. The plasmid pBluescript (Stratagene (La Jolla, CA)) was added to bring the final DNA quantity up to 15 μg/10-cm dish. Cells were collected 24 h after transfection. CAT assays were performed as described before (35). Briefly, cell pellets were collected by centrifugation, resuspended in 100 mM Tris-HCl, pH 7.8, and lysed by repeated cycles of freezing and thawing. Extracts were incubated in 100 mM Tris-HCl, pH 7.8, 1 mM acetyl-CoA and 0.5 μCi of [14C]chloramphenicol (60 nCi/mmol (NEN Life Science Products)) at 37°C for 1 h. After extracting with ethyl acetate, the acetylated product and the substrate were resolved by thin layer chromatography using Silica Gel 60 (EM Science (Gibbstown, NJ)) in a solvent of 95% chloroform and 5% methanol. Following autoradiography of the developed gel plates, spots corresponding to the acetylated products and the substrate were excised and counted by liquid scintillation for quantification.

β-Galactosidase activity was determined by the chemiluminescent assay (36) using Lumi-Gal 530 (Lumigen Inc. (Southfield, MI)) as a substrate. In all measurements the amount of extract used for the CAT assay was first adjusted after standardizing to the β-galactosidase activity. The densitometric pull-down and pull-down experiments were performed as described previously (37). Five hundred μl of bacterial lysates containing GST-Sp1ZnF or GST-Sp1Q1 at an approximate concentration of 3 mg/ml were mixed with 250 μl of glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) equilibrated in PBS and rotated for 30 min at 25°C. After three successive washes with 1.5 ml of PBS, the beads were mixed with 50 μg of purified recombinant GKL F in a binding buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM MgCl2, 2 mM
The individual mutant oligonucleotides are shown. The bound proteins were eluted with a buffer containing 50 mM Tris-HCl, pH 8.0, and 10 mM reduced glutathione for 20 min at 25 °C. Two cases of protein from the flow-through and the eluant fractions were examined by Western blot analysis for the presence of recombinant GKLF. As shown in Fig. 1, therefore, established that GKLF binds to BTE and that such binding occurs at a sequence consistent with the empirically determined binding site for GKLF.

The Interaction between GKLF and BTE Is Similar to That between Sp1 and BTE—BTE is a cis element shown to interact with a number of transcription factors including Sp1, BTEB, and BTEB2 (17, 26). The nucleotides within BTE that are involved in contacting Sp1 are similar to those contacting BTEB (38). To determine whether GKLF also contacted these bases, methylation interference assay was performed using singly end-labeled double-stranded BTE oligonucleotides and recombinant GKLF. The results in Fig. 2 indicate that guanine residues in positions 9, 10, 12, 14, and 15 on the sense strand and those in positions 11, 16, 17, and 20 on the antisense strand were involved in contacting GKLF since their methylation resulted in an interference of binding. This pattern of methylation interference is very similar, if not identical, to that observed for Sp1 and BTEB (38), suggesting the three proteins bind to BTE in a similar fashion.

GKLF Suppresses the Activity of the CYP1A1 Promoter—To determine whether GKLF might influence the activity of the CYP1A1 promoter, cotransfection experiments were performed in CHO cells using a CYP1A1 promoter-linked reporter gene and an expression plasmid encoding either full-length or truncated forms of GKLF. The reporter pMC6.3k contains 6.3 kilobase pairs of the rat CYP1A1 promoter linked to CAT (24) and exhibited high basal activities in transfected cells (shown in the
The presence of a cotransfected expression plasmid containing full-length GKLF (PMT3-GKLF-(1–483)) caused a decrease in the reporter activity in a dose-dependent manner (Fig. 3). A similar suppressive effect was observed when cells were cotransfected with a plasmid containing a truncated form of GKLF that retained its zinc finger region (PMT3-GKLF-(350–483)) (Fig. 3B). In contrast, another truncated form of GKLF in which the three zinc fingers were deleted had no such suppressive effect on the CYP1A1 promoter (PMT3-GKLF-(1–401)) (Fig. 3C). This lack of effect was not due to failure of this zinc finger-minus form of GKLF to localize to the nucleus since the nuclear localization signal of GKLF is retained in this particular construct (18).

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To determine whether the suppressive effect of GKLF on the CYP1A1 promoter was mediated by BTE, additional cotransfection experiments were performed using reporters with various internal deletions in the CYP1A1 promoter sequence. Two such deletion constructs analyzed were pMC6.3kΔ-(96/53) and pMC6.3kΔ-(96/44), with the latter known to lack any BTE-mediated activity (24). As shown in Fig. 4, whereas full-length GKLF suppressed the activities of both pMC6.3kΔ-(96/53) and wild-type pMC6.3k reporters, it failed to suppress that of pMC6.3kΔ-(96/44). The truncated form of GKLF, PMT3-GKLF-(1–401), failed to suppress any reporters. These results indicate that the suppressive effect of GKLF on the CYP1A1 promoter is specifically mediated by BTE.

The BTE-specific nature of the effect of GKLF on the CYP1A1 promoter was substantiated by studying two additional promoter-reporter constructs, pSV/MC53 and pSV/MC44. pSV/MC53 contained 53 bp and pSV/MC44 contained 44 bp of the CYP1A1 promoter linked to the SV40 virus enhancer. They differed from each other by the presence and absence of BTE, respectively (24). As shown in Fig. 5, PMT3-GKLF-(1–483) inhibited the reporter activity of pSV/MC53 in a dose-dependent fashion (Fig. 5A) but not that of pSV/MC44 (Fig. 5B). When additional effector constructs were analyzed by similar transfection experiments, the inhibitory effect on the pSV/MC53 construct was observed for both full-length GKLF and GKLF that retained the zinc finger region (PMT3-GKLF-(350–483)) (Fig. 6). As in Fig. 3, GKLF lacking zinc fingers (PMT3-GKLF-(1–401)) had no effect on pSV/MC53. In no cases did any of the effector constructs exhibit any effect on pSV/MC44, which lacked BTE (Fig. 6).

Sp1 and Sp3 Are the Major BTE Binding Transcription Factors in CHO Cells—In addition to the findings that GKLF suppressed the activity of the CYP1A1 promoter, results of the preceding transfection experiments also indicated that BTE was responsible for a significant fraction of the CYP1A1 pro-
moter activity. This was demonstrated by the observation that deletion of BTE from the promoter resulted in approximately a 50% reduction in the promoter activity (Figs. 4 and 6). To determine which transcription factor(s) activated BTE in the transfected cells, EMSA was performed on BTE with nuclear extracts obtained from CHO cells. As shown in Fig. 7, multiple bands representing DNA-protein complexes were formed (lane 1). The addition of a 10-fold molar excess cognate BTE competitor oligonucleotide abolished many of the complexes (lane 2). Similarly, the presence of a competitor oligonucleotide representing the consensus Sp1-binding site decreased the formation of a number of complexes (lane 3). In contrast, the addition of a competitor oligonucleotide representing either an AP2-binding sequence or the minimal essential binding sequence for GKLF (23) failed to influence complex formation (lanes 4 and 5, respectively). These results suggest that the primary BTE-binding proteins in CHO cells include Sp1 and Sp1-related proteins.

GKLF Abrogates Sp1-dependent Activation and Enhances Sp3-dependent Suppression of the CYP1A1 Promoter—To determine whether Sp1 and Sp3 affected the CYP1A1 promoter and whether GKLF modulated Sp1- and Sp3-dependent activity of the same promoter, cotransfection experiments were conducted in CHO cells using the pMC6.3k reporter and various combinations of expression plasmids containing Sp1, Sp3, and GKL. Fig. 8 shows that Sp1 caused an increase in the CYP1A1 promoter activity in a dose-dependent fashion (lanes 1–6). The inductive effect of Sp1 was abrogated by the presence of increasing amounts of the expression construct containing full-length GKL (Fig. 8, lanes 7–12). In contrast to Sp1, Sp3...
caused an inhibition of the same promoter in a dose-dependent fashion (Fig. 9, lanes 1–6). The addition of GKL to the co-transfection further increased the inhibition of the CYP1A1 promoter (Fig. 9, lanes 7–12). These observations indicate that Sp1 and Sp3 modulate the CYP1A1 promoter in a reciprocal manner. They also show that GKL abrogates Sp1-dependent activation and enhances Sp3-dependent suppression of the CYP1A1 promoter.

Recombinant GKL and Sp1 Exhibit Similar Affinities for BTE and GKL Competes with Sp1 for Binding to BTE—We then compared the binding affinities of GKL and Sp1 to BTE using EMSA. Equal molar quantities of recombinant GKL or purified human Sp1 were incubated with increasing amounts of a labeled BTE oligonucleotide. The intensity of the shifted complex was quantified with a densitometer and plotted against the probe concentration (Fig. 10). By Scatchard analysis (not shown), it was estimated that the dissociation constant (K_d) was 0.7 nM between Sp1 and BTE and 1.6 nM between BTE and GKL. The estimated K_d of Sp1 to BTE is consistent with previous measurements of Sp1 with its binding site, which ranged between 0.5 and 3 nM (38–40).

To examine further the relationship among GKL, Sp1, and BTE, EMSA was performed using a mixture of recombinant GKL and purified human Sp1. As demonstrated in Fig. 11, the presence of GKL inhibited the binding of Sp1 to the BTE DNA in a dose-dependent fashion (lanes 11–15). As the purity of the two protein preparations was similar to each other, a unit quantity of recombinant GKL was approximately 4 times in molar quantity when compared with the same amount of Sp1. Lane 12 therefore contained a close to equal molar quantity of GKL and Sp1, which resulted in an approximately 50% decrease of binding of Sp1 to BTE due to the presence of GKL.

GKL and Sp1 Physically Interact with Each Other—Although the result in Fig. 10 indicated that the K_d of GKL to BTE was at least twice that of Sp1, GKL was nonetheless able to compete with Sp1 for binding to BTE at a similar molar ratio (Fig. 11). These findings suggested that there might be an additional mechanism by which GKL affected the binding of Sp1 to BTE. Protein-protein interaction is one such potential mechanism. To determine whether GKL and Sp1 could physically interact with each other, GST pull-down experiments were performed using recombinant GKL and two GST fusion proteins containing two different regions of Sp1. As can be seen in Fig. 12, GKL was retained by the glutathione-Sepharose 4B beads only in the presence of GST-Sp1ZnF, which contains the carboxyl-terminal 159 aa residues of Sp1 including its three zinc fingers (lane 7) (32). In contrast, GKL was recovered only in the flow-through fractions of beads that retained either GST alone (lane 3) or GST-Sp1Q1 (lane 5), a portion of Sp1 that contains a glutamine-rich transactivation domain (32). These results therefore provide strong evidence for a direct physical interaction between GKL and Sp1, which may potentially involve the zinc finger regions of the two proteins.

**DISCUSSION**

GKL was initially identified by low-stringency library screening with the zinc finger region of zif268/Egr-1 (13). Recent studies suggest that GKL is one of three members of a subfamily of closely related Krüppel proteins (18). The other two members are LKLF (16) and EKLF (11). Despite a significant degree of conservation in the zinc finger region (13) and in the nuclear localization signal (18) among these three proteins, their tissue distributions and presumed physiological functions are quite different. EKLF is expressed primarily in erythroid tissues including bone marrow and spleen (11). Gene targeting experiments indicated that EKLF-deficient mice were defective in erythropoiesis during fetal development (21, 22). LKLF, found primarily in lung and spleen (16), is necessary to maintain the quiescent state of single-positive T lymphocytes. T cells from mice deficient in LKLF exhibited a spontaneously activated phenotype and died in the peripheral lymphoid organs from Fas ligand-induced apoptosis (41). In addition, LKLF is required for the maintenance of vascular integrity during fetal development (42). The physiological function of GKL is less well defined although its epithelial (13, 14) and endothelial (15) nature of expression suggests that it may have a role in regulating tissue-specific gene expression. In addition, the growth arrest-associated nature of GKL expression points to a potential function in the regulation of cell proliferation.

Among the three aforementioned Krüppel-like proteins, only EKLF has a clearly established target gene. EKLF is a crucial transcription factor for β-globin gene expression and exerts its effect through a CACCC element present in the β-globin promoter (19, 20). Although both GKL (14, 15) and LKLF (16) have also been found to interact with the CACCC element, no definitive endogenous genes have been identified as the targets for either protein to date. It is within the context of identifying target genes that we conducted a study to empirically determine the binding sequence for GKL (23). That study not only established a minimal essential binding site of GKL, it also identified CACCC and BTE as two naturally occurring cis elements that form with the binding sequence of GKL. Our current effort is therefore focused on establishing the relationship between GKL- and BTE-mediated gene transcription.

BTE was selected for the present study because it is the focal point onto which multiple transcription factors converge to exert their effects. Along with the present study, BTE has been found to interact with no fewer than five Krüppel-like factors including Sp1 (26, 38), Sp3 (this study), BTEB (26, 38), BTEB2 (17), and GKL (14, this study). An additional significance of
BTE in transcriptional regulation is demonstrated by its presence in the promoter of a plethora of genes that belong to the cytochrome P-450 superfamily (43). Examples include, but are not limited to, CYP1A1 (24, 25), CYP1A2 (44, 45), CYP2B1 (25, 46), CYP2B2 (25, 46), CYP3A4 (47), CYP3A5 (48), CYP3A16 (49), and CYP11A (50). In many of these genes BTE was proven to be a crucial determinant of their promoter activity. Moreover, at least one BTE-binding protein, Sp1, has been shown to physically interact with transcription factors binding to other regions of the CYP1A1 promoter and to exert a cooperative influence on the drug-inducible expression of this gene (51).

These studies suggest that BTE is likely a crucial cis element involved in the coordinated expression of a large number of related genes. Of additional interest is that many cytochrome P-450 genes including CYP1A1, CYP1A2, and CYP2B2 are expressed in the epithelium of the gastrointestinal tract in a similar distribution to that of GKL (13, 14). These observations raise the distinct possibility that GKL is a major contributor to the intestinal epithelial expression of these physiologically important cytochrome P-450 genes.

By base-specific mutational analysis and methylation interference assay (Figs. 1 and 2, respectively), recombinant GKL is expressed in the gastrointestinal tract.
appears to interact with BTE in a very similar, if not identical, manner to Sp1 and BTEB (38). The dissociation constants of the three proteins in binding to DNA also appear to be reasonably similar to one another (Fig. 10; Ref. 38). These results suggest that GKLFL, Sp1, and BTEB exhibit similar affinities with respect to BTE binding. It is of interest to note that GKLFL binds poorly to a consensus Sp1-binding site (23). Conversely, Sp1 and Sp3 bind poorly to the empirically determined GKLFL-binding site (Fig. 7). To this end, BTE seems to represent a “composite” site to which all these proteins exhibit a high affinity. These features increase the attractiveness of BTE as a “model” element in studying the increasingly complex mechanism by which it mediates gene expression.

The results of our cotransfection experiments firmly established that GKLFL suppresses CYP1A1 promoter activity in a BTE-dependent fashion. Moreover, this inhibitory effect appears to be exerted at the expense of Sp1, which has an activating effect on the same promoter. This behavior of GKLFL is reminiscent of that observed for BTEB (26). In that study, BTEB exhibited a similar suppressive effect on the CYP1A1 promoter in a BTE-dependent manner. In addition, BTEB inhibited Sp1-mediated activation of the same promoter. However, this suppressive effect was dependent on the context of the DNA, BTEB was an activator when multiple copies of BTE were present in the promoter. Our laboratory has made a similar observation. When two tandem copies of either a consensus GKLFL-binding site (23) or BTE<sup>2</sup> were used to drive a reporter, GKLFL increased the reporter activity. The mechanism for this pleiotropic effect is unclear at this time although there are examples of other bifunctional transcription factors including Sp3 (31, 52, 53) and YY1 (54, 55) that exhibit a similar DNA-dependent positive and negative effect. GKLFL was also found to contain distinct domains that mediate repression and transactivation similar to Sp3 and YY1 (15, 56, 57). It is therefore possible that conformational changes resulting from protein-protein and/or protein-DNA interactions may be the basis for the pleiotropic effect of GKLFL.

Although a number of possible mechanisms may exist that are responsible for the suppression of the CYP1A1 promoter by GKLFL, the most likely explanation is that the suppression is based on a competitive mechanism. This is demonstrated by the inhibition of binding of Sp1 to BTE in the presence of recombinant GKLFL (Fig. 11). This passively accomplished repression may be augmented by GKLFL once it is bound to the promoter via an intrinsic repressive domain within GKLFL (15). The cooperative nature of suppression exerted by GKLFL and Sp3 on the CYP1A1 promoter (Fig. 9) further supports the latter hypothesis. A similar finding on the competitive binding to BTE by different proteins has previously been observed between Sp1 and BTEB (38). Taken together, GKLFL and BTEB appear to exhibit a very similar behavior with regard to their effects on a BTE-driven promoter. To the contrary, BTEB2, a more GKLFL-related Krüppel protein than BTEB (13, 18), is an activator of BTE-driven promoters (17). These findings further increase the complexity by which BTE is utilized as a basal promoter element to drive gene expression.

The competitive effect of GKLFL on the binding of Sp1 to BTE appears to be further augmented by a direct physical interaction between the two proteins (Fig. 12). Interestingly, in neither our study nor a previous report (38) was a stable ternary complex observed between GKLFL, Sp1, and BTE or between BTEB, Sp1, and BTE. In contrast, the interaction between the bifunctional Krüppel protein YY1, Sp1, and a YY1-binding sequence resulted in the formation of a ternary complex (32). The reason for the difference is unclear, although in the latter case the interaction of YY1 and Sp1 led to a cooperative induction of their target promoter. In a different system YY1 was shown to repress Sp1-mediated transcription and that such repression does not depend on physical interaction between YY1 and Sp1 (58). Additional studies are therefore necessary to delineate the exact effect of interaction between specific proteins on regulating promoter activity.

GKLFL is expressed in a tissue-selective manner (13, 14). Its interaction with a “housekeeping” transcription factor such as
Sp1 is reminiscent of other situations in which tissue-specific and ubiquitous transcription factors interact to control tissue-specific gene expression. Examples include the interaction between MyoD1 and E12/E47 during myogenesis (59) and between Oct-2 and Oct-1 during lymphogenesis (60). To this end, the expanding repertoire of proteins that interact with Sp1 increases its importance and versatility in regulating gene expression. Examples of other important cellular or viral proteins that are capable of physically interacting with Sp1 include YY1 (32), Sp3 (61), Rb (62), p53 (63), v-Rel (64), C/EBPβ (65), GATA-1 (66), AhR. Arnt (51), Tat (67), and Tax (68). As in the case of GKL, many of these proteins either have tissue-specific functions or are involved in crucial cellular functions such as growth and differentiation. The further examination of the involvement of Sp1 in the context of selective protein-protein interaction may further increase the understanding of the mechanisms regulating tissue-specific gene expression.

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Note Added in Proof—While this manuscript was under review, Jenkins et al. (69) demonstrated that GKL was able to transactivate the human keratin 4 and Epstein-Barr virus ED-1 promoters via a CACCC-like element.

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