

Transactivation of the Human Keratin 4 and Epstein-Barr Virus ED-L2 Promoters by Gut-enriched Krüppel-like Factor*

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The Krüppel-like family of transcription factors comprises genes that appear to have tissue-restricted functions. Expression of gut-enriched Krüppel-like factor (GKLF) may be important in the switch from proliferation to differentiation in the squamous epithelium. We sought to determine transcriptionally mediated effects of GKLF on two promoters active in the esophageal squamous epithelium, namely the Epstein-Barr virus ED-L2 and human keratin 4 promoters. Both promoters contain a CACCC-like motif previously shown to bind GKLF. To determine whether GKLF regulates genes containing this element, we first demonstrated expression and then cloned the full-length human GKLF from an esophageal squamous carcinoma cell line. In a transient transfection system, GKLF increased the activity of both promoters >25-fold, localized to regions containing the CACCC-like element. Recombinant GKLF specifically binds the CACCC-like motif in both promoters. GKLF epitope-tagged protein leads to the formation of two proteins of 65 and 34 kDa. The chromatographically purified 65-kDa protein binds the CACCC-like element from both Epstein-Barr virus ED-L2 and keratin 4 promoters, which is not attenuated by the 34-kDa protein. In summary, GKLF is expressed in esophageal squamous epithelial cells and transcriptionally activates two esophageal epithelial promoters important at the transition toward differentiation.

Zinc finger transcription factors bind DNA through motifs that contain a zinc atom bound to 4 amino acids, either cysteine or histidine (1, 2). While encompassing a broad and diverse group of proteins, zinc finger transcription factors also can be further subclassified based upon their homology to the *Drosophila* Krüppel protein (3). There is compelling evidence that a certain group of Krüppel-like transcription factors share structural and functional similarities and include erythroid Krüppel-like factor (EKLF)¹ (4), lung Krüppel-like factor

(LKLf) (3), gut-enriched Krüppel-like factor (GKLF) (5, 6) also referred to as epithelial zinc finger or EZF (7), basic transcription element binding-protein 2 (8), and basic Krüppel-like factor (9). These factors share zinc finger domains at the carboxyl terminus and transactivation domains at the amino terminus. Expression is relatively tissue-restricted based upon Northern blot analysis and RNA *in situ* hybridization studies. For example, EKLF is found predominantly in erythroid cells of the bone marrow and spleen (4); LKLf is found in the lung epithelium, hematopoietic organs, and testis (3); and GKLF is found in epithelial cells of the gastrointestinal tract (intestine and esophagus), lung, testis, and skin (5, 7). These factors have DNA binding domains that bind the cognate CACCC motif or CACCC-like variants, including CACACCC (3, 7, 10). EKLF binds the CCACACCCT site in the mouse and human β -globin promoters (10). A pSG5-EKLF expression vector transactivates a CACCC site-containing reporter in CV-1 cells 13-fold (10). Similarly, LKLf can transactivate a human β -globin promoter (3).

An emerging theme among the family of Krüppel-like transcription factors is their involvement in potentiating cell differentiation or quiescence. Targeted disruption of LKLf in mouse embryonic stem cells supports the notion that LKLf is critical in maintaining single positive T cells in a state of quiescence (11). Expression of GKLF is high in growth-arrested fibroblasts and nearly absent in cells in an exponential phase of proliferation (5). Constitutive expression of GKLF leads to inhibition of DNA synthesis (5). The role of GKLF in regulation of cellular growth is further supported by cellular localization studies which indicate that GKLF mRNA is abundant in the middle to upper crypt region of the colonic mucosa, an area in which proliferating cells make a commitment to early differentiation (5). Additionally, GKLF localizes to suprabasal cells in skin, tongue, and esophageal squamous epithelial cells (7). Proliferating basal cells in these epithelia lose this capacity and begin to differentiate in the suprabasal layer where GKLF is expressed. The localization of the GKLF gene on chromosome 9q22 has led to the speculation that dysregulation of GKLF gene expression may play a role in the pathogenesis of squamous epithelium-derived neoplasms which have been shown to have abnormalities in this chromosomal region (12–14).

While it has been demonstrated that EKLF and LKLf transactivate the β -globin promoter, gene targets for GKLF transactivation have yet to be identified. Given the localization of GKLF in the suprabasal esophageal squamous epithelium and the role of the Krüppel-like transcription factors in regulating cellular differentiation and quiescence, we postulated that

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¹ The abbreviations used are: EKLF, erythroid Krüppel-like factor; LKLf, lung Krüppel-like factor; GKLF, gut-enriched Krüppel-like fac-

tor; bp, base pair(s); kb, kilobase pair(s); PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; K4, keratin 4; EMSA, electrophoretic mobility shift assay.

genes expressed during the transition toward early differentiation may be transactivated by GKLF. Two such candidates are the Epstein-Barr virus ED-L2 (15, 16) and the keratin 4 promoters.

Previous work has shown that the ED-L2 promoter is active in esophageal squamous suprabasal cells of transgenic mice (17, 18). The ED-L2 promoter is basally regulated by a novel zinc-dependent nuclear protein that binds a CACCC-like motif (19). Phorbol ester leads to enhanced activation of the ED-L2 promoter through the binding of an E-box by upstream stimulatory factor by USF and a zinc-dependent factor that interacts specifically with CACACCC (20). Keratin 4 is also highly expressed in esophageal squamous suprabasal cells, and its expression is associated with the switch to differentiation that occurs as cells exit the proliferation zone (21, 22). Sequence analysis of the keratin 4 promoter (GenBank™ accession number X97566) reveals a GTGTGGG or inverted CACACCC motif in the proximal 5'-untranslated region. We investigated whether ED-L2 and K4, expressed in suprabasal squamous epithelial cells of the esophagus, are transactivated by GKLF, thereby providing a basis for GKLF's role *in vivo*.

EXPERIMENTAL PROCEDURES

Cloning and Analysis of the Human GKLF cDNA from the Human Esophageal Squamous Cancer Cell Line TE-11—Oligonucleotide primers were designed to amplify the zinc finger-encoding region of the transcription factor GKLF, yielding an 840-base pair (bp) polymerase chain reaction (PCR) product. The oligonucleotide primer sequences are as follows: GKLF-S, 5'-AGTTGGACCCAGTATACATTCCGCCACAGCAGCCT-3'; GKLF-AS, 5'-TTAAAAGTGCCTCTTCATGTGTAAGGCAAGGTGGT-3'. Total RNA was extracted from lysates of subconfluent 293, TE-11, and 3T3 cells with a denaturing solution consisting of 4 M guanidinium thiocyanate, 0.1 M β -mercaptoethanol, 0.5% sarcosyl, 25 mM sodium citrate (pH 7.0), and 10% volume of 2 M sodium acetate (pH 4.0). The cell lysate was mixed with a 5:1 ratio of water-saturated phenol and chloroform/isoamyl alcohol mixture (49:1), and incubated at 4 °C for 15 min. After centrifugation, the aqueous phase was precipitated with ethanol, and RNA was dissolved in denaturing solution. It was then reprecipitated with ethanol, washed with 80% ethanol, and redissolved in water treated with 0.1% diethyl pyrocarbonate. An aliquot of total RNA was poly(A)-selected (polyAT tract mRNA isolation system III; Promega Corp.).

Total and poly(A)-selected RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer), with a 3'-sequence-specific primer, random primers, and oligo(dT) primers (Perkin-Elmer). Following a single cycle of reverse transcription at 42 °C for 15 min, the sample was subjected to 30 cycles of polymerase chain reaction amplification (RT-PCR) using GKLF-specific primers. The 840-bp PCR product was ligated into a TA cloning vector (Novagen), and the DNA sequence was confirmed as the zinc finger coding domain of human GKLF. A ZAP express (Stratagene) cDNA library was prepared from TE-11 cell RNA using previously described methods (23, 24). *EcoRI* and *XhoI* sites were used for directional insertion of the cDNA prepared from poly(A)-selected, reverse transcribed RNA. The TE-11 library contained 3×10^9 plaque-forming units/ml. 1×10^6 plaques were screened in the initial round. Plaques were oriented and lifted on to Hybond N nylon membranes (Amersham Pharmacia Biotech). Filters were denatured, neutralized, and rinsed, followed by UV cross-linking (Stratalinker, Stratagene).

The 840-bp fragment was radiolabeled (Amersham Pharmacia Biotech megaprime labeling kit) with [α -³²P]dCTP (NEN Life Science Products). Filters were prehybridized for 2 h in a 2 \times PIPES, 40% formamide buffer containing herring sperm DNA (100 mg/liter) and 0.5% SDS, followed by hybridization in an identical solution containing the radiolabeled GKLF probe for 24 h at 42 °C. Filters were washed sequentially in 0.1 \times SSC and 0.1% SDS at 25 °C for 20 min, 0.1 \times SSC and 0.1% SDS at 55 °C for 15 min, and 1 \times SSC and 0.1% SDS at 65 °C for 15 min. Filters were exposed to Eastman Kodak Co. X-Omat AR film with an intensifying screen at -80 °C for 72 h. Two subsequent rounds of screening under identical conditions were performed, yielding several positive clones. *In vivo* excision of the pBK-CMV phagemids containing cDNAs from positive plaques was performed using previously described methods (23, 24). Purified plasmid was obtained from individual colonies using an alkaline lysis plasmid preparation method (Qiagen). 30

plasmids derived from separate positive colonies were sequenced from the 3'- and 5'-ends of the insert cDNA, and sequence analysis was performed using GenBank™. Approximately 50% of clones yielded sequences identical to the published sequence of human GKLF. One clone containing the apparent full-length sequence was selected and fully sequenced, confirming its identity with GKLF.

Construction of the GKLF Epitope-tagged Expression Vector—The pBK-CMV plasmid containing the full-length GKLF cDNA was digested with *Bam*HI to yield a 1.4-kb fragment containing the full-length open reading frame of GKLF. This fragment was ligated into pcDNA3.1/His B (Invitrogen) in frame and 3' to the HIS₆ and Anti-Xpress epitope tag coding sequence. The GKLF expression construct is represented in the schematic shown in Fig. 1A. The GKLF expression plasmid as well as pcDNA3.1/His B and pcDNA3.1/His-lacZ plasmids (Invitrogen) were purified by a modified alkaline lysis method (Qiagen).

Northern and Western Blot Analysis of GKLF Expression in the 293 Cell Line—GKLF-nonexpressing 293 cells were transiently transfected with the different pcDNA3.1/His plasmids, and total RNA was harvested 24 h after transfection, using methods previously described (20). RNA concentration was determined by spectrophotometry. 20 μ g of total RNA was resuspended in sample buffer consisting of 50% deionized formamide, 6.7% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA (pH 8.0) and heated at 60 °C for 10 min. Electrophoresis was performed at 20 V for 2 h followed by 40 V for 4 h with buffer consisting of 20 mM MOPS, 5 mM sodium acetate, and 1 mM EDTA (pH 8.0). The RNA was transferred onto a Hybond N nylon membrane (Amersham Pharmacia Biotech) followed by UV cross-linking (Stratalinker, Stratagene). The 840-bp probe used for GKLF cloning was used for Northern blot analysis and was labeled using a random primed labeling method (Amersham Pharmacia Biotech). To assess equivalent loading of RNA samples, equal amounts of 18 S and 28 S RNA were identified in each lane. Northern blot hybridization was carried out using Rapid-hyb buffer (Amersham Pharmacia Biotech) and the conditions described above.

For Western blot analysis, lysates from transfected 293 cells were prepared in a lysis buffer with protease inhibitors as described previously (25). 100 μ g of total protein from each sample was separated on a 10% SDS-polyacrylamide gel. Following electrophoresis, the protein was transferred to an Immobilon membrane (Millipore Corp.) at 10 V for 12 h at 4 °C. The membrane was treated with 5% milk, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20 for 1 h. The primary antibody (Anti-Xpress, Invitrogen) was used at a 1:4000 dilution, and the secondary antibody, horseradish peroxidase-conjugated goat anti-mouse (Amersham Pharmacia Biotech), was used at a 1:2500 dilution. Horseradish peroxidase activity was detected with a chemiluminescence system (ECL system, Amersham Pharmacia Biotech).

Tissue Culture Cell Lines and Transient Transfection Studies—The human esophageal squamous carcinoma TE-11, 3T3 fibroblast (ATCC), and human embryonic kidney 293 cell lines (ATCC) were cultured under standard conditions with Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin, 100 μ g/ml streptomycin (Sigma), and 2 mM L-glutamine (Sigma).

After PCR amplification with primers specific to the ED-L2 and K4 promoter sequences and confirmatory DNA sequencing of PCR products, fragments of the ED-L2 promoter and K4 promoter were agarose gel-purified and directionally ligated into the luciferase reporter gene promoterless vector pXP2 (26) to generate luciferase reporter constructs. The oligonucleotide sequences listed in Table I were used to generate promoter deletion constructs with PCR conditions previously described (20). The resulting luciferase reporter constructs (ED-L2-144, ED-L2-114, K4-340, K4-140) represent regions of the ED-L2 and K4 promoters that contain the CACCC-like motif. In addition, previously generated mutant reporter constructs of ED-L2-144 were used in transient transfections (20). Plasmid DNA constructs were checked by restriction digestion for correct length, verified by DNA sequencing, and purified by an alkaline lysis method (Qiagen).

Transient transfection of the ED-L2 and K4 constructs in 293 cells was carried out using a calcium phosphate precipitation technique (5 Prime \rightarrow 3 Prime, Inc.). 293 cells were plated at a density of 1×10^6 cells/35-mm well and transfected 24 h later with combinations of 2 μ g of the GKLF reporter construct, 2 μ g of the luciferase reporter plasmid, and 2 μ g of pGreen Lantern-1 (Life Technologies, Inc.), a reporter plasmid encoding a green fluorescent protein. The transfectant mixture consisted of a 250- μ l solution of 125 mM CaCl₂, 25 mM Hepes, pH 7.05, 0.75 mM Na₂HPO₄, 5 mM KCl, 140 mM NaCl, and 6 mM glucose. In a subset of the transfection experiments, cells were examined under

fluorescence microscopy (475-nm excitation peak, 490-nm emission peak) to assess green fluorescent protein production. The percentage of fluorescing cells was determined in each well and was found not to vary within a given transfection experiment, indicating that transfection efficiency was uniform.

Luciferase assays were performed as described previously (20). Incubations were performed in triplicate, and results were calculated as the mean \pm S.E. values for luciferase activity. Values were expressed as -fold increase compared with a base line established for each set of experiments and represented at least three independent transfection experiments.

Affinity and Bio-Gel P-60 Column Purification of Recombinant GKLF Fusion Protein—Following transient transfection of 293 cells with 2 μ g of the GKLF expression construct, total cellular protein was harvested after 24 h for purification of the GKLF histidine-tagged fusion protein. Cells were mechanically detached from the plates and resuspended in a total of 4 ml of native binding buffer (20 mM NaH_2PO_4 , 500 mM NaCl, pH 7.8) with aprotinin (5 μ g/ml) and phenylmethanesulfonyl fluoride (100 μ g/ml). Cells were lysed with two cycles of freezing-thawing, followed by passage through an 18-gauge needle four times.

ProBond affinity resin columns (Invitrogen) were equilibrated for purification of the histidine-tagged protein according to the manufacturer's specifications. 293 cell lysate was added to the equilibrated affinity column in a volume of 4 ml of native binding buffer. The column was sealed at both ends and incubated at 4 $^\circ\text{C}$ for 1 h with gentle agitation. The column was then packed, and the supernatant was removed. Next, the column was washed twice in wash buffer (20 mM sodium phosphate, 500 mM NaCl, pH 6.0) with aprotinin (5 mg/ml) and phenylmethanesulfonyl fluoride (100 mg/ml). The recombinant GKLF fusion protein was eluted by adding 4 ml of elution buffer (20 mM sodium phosphate, 500 mM NaCl, pH 4.0) with aprotinin (5 μ g/ml) and phenylmethanesulfonyl fluoride (100 μ g/ml) and incubating at 4 $^\circ\text{C}$ for 15 min with gentle agitation. The final elution volume of 4.0 ml was concentrated to 1.0 ml by vacuum centrifugation at 4 $^\circ\text{C}$.

Separation of the affinity column-purified 65- and 34-kDa GKLF fusion proteins was accomplished using gel filtration chromatography (27). Bio-Gel P-60 (Bio-Rad) was hydrated for 24 h in elution buffer, suspended in a 2-fold excess of buffer, and degassed for 15 min. A 50% slurry of gel was then allowed to settle in a 7.5-ml column. A 200- μ l solution of bovine serum albumin (66 kDa) and ovalbumin (40 kDa) in elution buffer (1 mg/ml) was passed through the column to calibrate and confirm the efficiency of size separation of these two proteins. After clearing the column with 5 ml of elution buffer, 200 μ l of recombinant GKLF solution was passed over the column and twenty 200- μ l fractions were collected. Purified protein concentration was determined by a colorimetric method (Bio-Rad protein assay). Qualitative purity of protein was assessed by a silver stain method (Bio-Rad) after 5 μ g of total protein from each fraction was separated on a 10% SDS-polyacrylamide gel (28).

Electrophoretic Mobility Shift Assays (EMSAs)—Purified recombinant GKLF protein was prepared as described above for use in EMSAs, except the buffers were supplemented with a mixture of 0.5 μ g/ml protease inhibitors aprotinin, chymostatin, and pepstatin (Boehringer Mannheim). 5 pmol of a double-stranded oligonucleotide, synthesized by the phosphoramidite procedure (Applied Biosystems) and purified by gel electrophoresis, was radiolabeled by the Klenow fill-in reaction in a buffer consisting of 10 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , 7.5 mM dithiothreitol, 33 μ M dATP, 33 μ M dGTP, 33 μ M dTTP, 0.33 μ M [α - ^{32}P]dCTP (NEN Life Science Products), and 1 unit of DNA polymerase I Klenow fragment (Amersham Pharmacia Biotech). The oligonucleotide (sense) sequences derived from the ED-L2 and K4 promoters are shown in Table I with the putative GKLF binding motif in boldface type. The 5'-end of the ED-L2 sense oligonucleotide corresponds to ED-L2 promoter position -135, and that of the K4 oligonucleotide corresponds to K4 promoter position -281. At the 5'-end of each oligonucleotide, a *Bam*HI restriction site was added to facilitate Klenow fill-in labeling.

EMSAs were carried out by incubating 10 μ g of recombinant GKLF fusion protein with 5 fmol of the α - ^{32}P -labeled oligonucleotide DNA probe in a 20- μ l binding reaction containing 10 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 1.0 μ g of poly(dA-dT) (Amersham Pharmacia Biotech). After incubation at room temperature for 15 min, the samples were loaded onto a 6% polyacrylamide, 0.25 \times Tris borate gel and electrophoresed at 10 V/cm for 2 h. The gels were dried and exposed to x-ray film (Kodak X-AR) at -80 $^\circ\text{C}$ for 12 h. For competition experiments, the recombinant protein was preincubated with 100-fold excess unlabeled double-stranded oligonucleotide before the addition of the α - ^{32}P -labeled oligonucleotide DNA probe.

RESULTS

GKLF Is Expressed in the TE-11 Human Esophageal Squamous Cancer Cell Line and Not in the 293 Human Embryonic Kidney Cell Line—Our previous studies suggest that tissue-restricted zinc-dependent nuclear factors are important in regulating gene expression in the esophageal squamous epithelium (19, 20). The Epstein-Barr virus ED-L2 promoter is highly active in cells derived from this tissue compartment, as a result of transactivation by a zinc-dependent factor designated keratinocyte-specific factor (19), and a phorbol ester-induced zinc-dependent factor (20). The recent description of GKLF led us to test the hypothesis that this factor may play a prominent role in the tissue-specific regulation of gene expression in the esophageal squamous epithelium.

Prior studies demonstrated that GKLF is expressed in the normal adult murine squamous epithelium (7). We initially determined whether TE-11 cells expressed GKLF using RT-PCR. In addition, we tested whether 293 cells and 3T3 fibroblasts express GKLF. As previous studies have shown, 3T3 fibroblasts express GKLF as determined by RT-PCR designed to yield an 840-base pair PCR product encompassing the zinc finger domain of GKLF (7). Concurrent RT-PCR reactions with RNA template from TE-11 and 293 cells yielded positive and negative results, respectively (data not shown). Based on these results, we chose to pursue a cloning strategy designed to obtain the full-length human cDNA of GKLF and any other Krüppel-like factor genes that might share homology in the zinc finger domain. Using the 840-bp human GKLF sequence as a probe under relatively low stringency conditions, a TE-11 cDNA library was screened. Approximately 30 individual positively hybridizing clones were identified, 50% of which had sequence identical to the GKLF published sequence. No other genes were identified that shared homology with GKLF, a result consistent with previous attempts to identify closely related Krüppel-like factor genes (11).

One full-length GKLF clone was selected for further analysis. A *Bam*HI fragment containing the entire open reading frame of GKLF was placed in frame into the expression vector pcDNA3.1/His B. This vector contains a HIS_6 and epitope tag 5' to the inserted GKLF cDNA (Fig. 1A). The GKLF fusion construct was transfected into 293 cells for determination of expression characteristics since these cells do not have endogenous GKLF. Separate aliquots of 293 cells were transfected with the native pcDNA3.1/His B vector and with a pcDNA vector containing the β -galactosidase cDNA (pcDNA3.1/His-lacZ). 293 cells were found to express high levels of a 3.5-kb transcript 24 h after transfection with the GKLF construct (Fig. 1B), whereas cells transfected with empty vector did not express GKLF. There was no smaller transcript identified, as reported previously in Northern blot analysis of mouse tissue (7).

Total protein lysates were harvested following transfection with the different plasmids. Cells transfected with the GKLF construct expressed a prominent 65-kDa band identified by the anti-Xpress epitope tag (Fig. 1C). Untransfected cells and cells transfected with empty vector did not express this protein. Cells transfected with the *lacZ*-containing construct expressed high levels of a protein of appropriate molecular mass for β -galactosidase (>120 kDa) (Fig. 1C). The 65-kDa protein corresponded to the approximate expected molecular mass of GKLF (58 kDa) with the addition of the histidine and epitope tags (7 kDa). A second minor band was identified on the Western blot with the anti-Xpress antibody at approximately 34 kDa (Fig. 1C). This band was not identified in lysates from cells

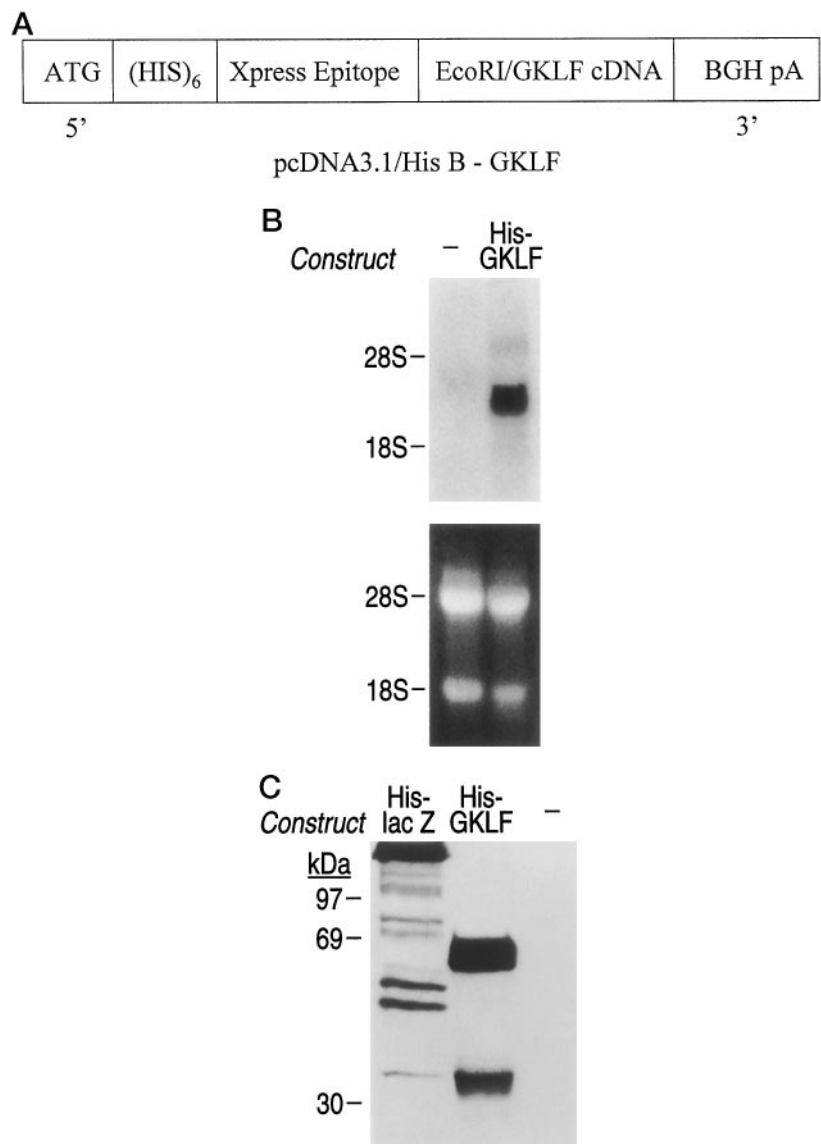


FIG. 1. GKLF histidine-tagged fusion protein expressed in 293 cells yields a single transcript and a major (65-kDa) and minor (34-kDa) fusion protein. 293 cells were transiently transfected with the GKLF expression vector depicted in *panel A*. This vector contains sequence encoding a polyhistidine tag and recognition epitope at the amino terminus of the expressed protein, the GKLF cDNA in an *EcoRI* fragment, and a bovine growth hormone polyadenylation signal. 24 h after transient transfection with the GKLF construct, 293 cells were harvested for analysis of RNA and protein. Northern blot analysis using a probe specific to the zinc finger coding domain of the transcription factor reveals a single 3.5-kb transcript, shown in *panel B*. Untransfected cells do not express GKLF RNA. Shown *below* the Northern hybridization is the relative abundance of 18 S and 28 S RNA to confirm equal loading of total RNA. Western blot analysis reveals the presence of bands immunoreactive with the anti-Xpress antibody specific to the epitope encoded by the pcDNA3.1/His B vector. In the pcDNA3.1/His-lacZ-transfected cells, a prominent >120-kDa band is present in addition to other nonspecific bands. There is no band present in the untransfected cells. The GKLF-transfected cells yield two bands with approximate molecular masses of 65 and 34 kDa. *A*, schematic of the GKLF expression construct; *B*, Northern blot analysis of 293 cells transfected with GKLF construct; *C*, Western blot analysis of 293 cells transfected with pcDNA3.1/His-lacZ and pcDNA3.1/His B-GKLF.

transfected with empty vector or pcDNA3.1/His-lacZ.

GKLF Transactivates the ED-L2 and K4 Promoters in the 293 Cell Line—To assess transcriptional activation by GKLF, we selected the 293 cell line which does not express GKLF as determined by RT-PCR. This cell line is highly transfectable, and we have determined previously that promoters that are generally active in keratinocyte-derived cell lines are active in 293 cells, although with reduced activity (19). Thus, our 293 cell transient transfection system took advantage of a low level of basal promoter activity coupled with the absence of endogenous GKLF.

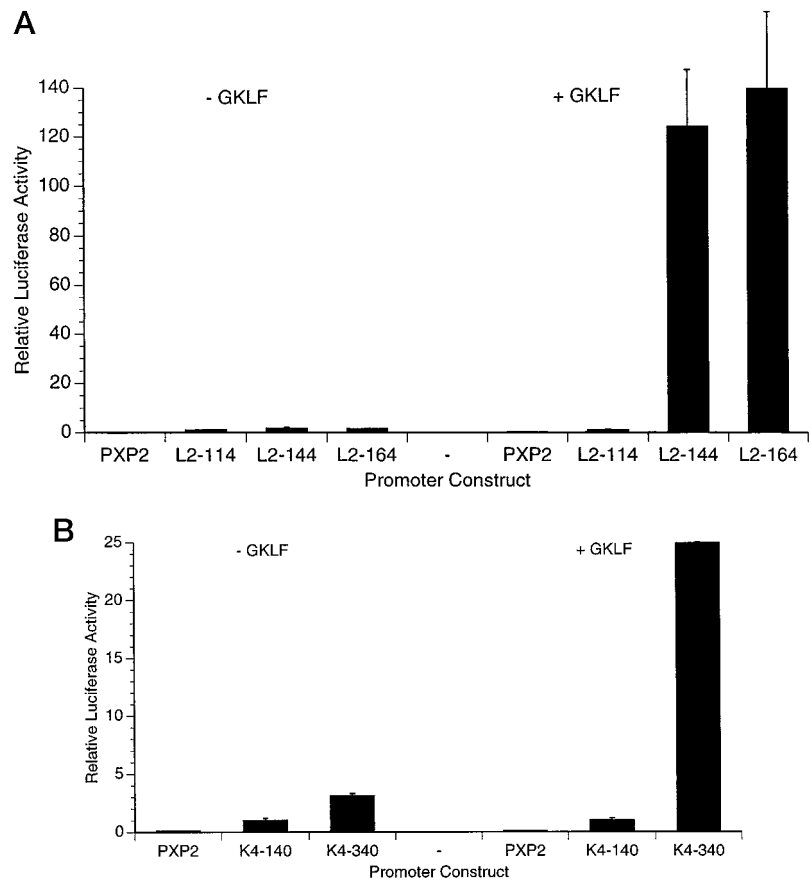
Different Epstein-Barr virus ED-L2 promoter-reporter constructs were used to test potential GKLF transactivation. These constructs had been previously shown by us to confer significant phorbol ester-induced promoter activity, attributable to the bp between -144 and -114 (20). These constructs were chosen for use in the GKLF experiments because the 5'-ends of the promoter deletions represent a DNA motif (CACACC) that is known to bind GKLF in EMSAs (7). In addition, it was previously demonstrated that mutation of this motif in the native ED-L2 promoter abolishes much of the phorbol ester-induced activity and that a zinc-dependent nuclear factor from TE-11 cells binds this motif in EMSAs (20).

The GKLF expression construct when co-transfected with

the ED-L2 promoter reporter constructs resulted in greater than 100-fold activation of promoter activity when the promoter construct spans the sequence including the CACACC motif (Fig. 2A). Transfection of the wild type pcDNA3.1/His B construct did not result in an increase in promoter activity. The increase in activity between the ED-L2-144 and ED-L2-114 constructs in the presence of GKLF is approximately 10-fold higher than that found with phorbol 12-myristate 13-acetate-treated TE-11 cells (20). Additional reporter constructs were used to test the specificity of GKLF transactivation. The promoterless pXP2 vector was not stimulated by co-transfection of the GKLF expression construct (Fig. 2A). The ED-L2-164 construct contains additional 5'-sequence not critical for phorbol ester-induced activation. There is also no CACCC-like element contained in the sequence between -164 and -144. When co-transfected with the GKLF construct, the ED-L2-164 reporter gene activity was not significantly higher than that of the ED-L2-144 construct (Fig. 2A). In addition, previously generated reporter gene constructs of ED-L2-144 (20) that contain mutations in the CACCC-like motif were not transactivated by GKLF (data not shown).

The GKLF expression construct was next tested with another promoter highly active in differentiated esophageal squa-

FIG. 2. GKLF transactivates the ED-L2 and K4 promoters, and the region of transactivation contains the CACCC-like motif. 293 cells were transiently transfected with the ED-L2 and K4 promoter deletion luciferase reporter constructs or empty pXP2 and co-transfected with pcDNA3.1/His B-GKLF (+GKLF) or pcDNA3.1/His B (-GKLF) constructs for 24 h followed by harvesting for the luciferase assay. Transfections of 293 cells with the ED-L2 constructs are shown in *panel A*, and K4 constructs are shown in *panel B*. Luciferase activity is expressed as -fold increase relative to ED-L2-114 or K4-140 (mean \pm S.E.) and is calculated from three independent transfections. Results have been normalized to green fluorescence protein production. *A*, ED-L2 co-transfection with pcDNA3.1/His B or pcDNA3.1/His B-GKLF; *B*, K4 co-transfection with pcDNA3.1/His B or pcDNA3.1/His B-GKLF.



mous epithelial cells, namely the human K4 promoter.² The 5'-untranslated region of K4 was recently submitted to GenBank™ (accession no. X97566), and PCR primers were used to amplify regions of the promoter from TE-11 genomic DNA. Two deletion luciferase reporter constructs were made, namely at -140 bp (K4-140) and -340 (K4-340). K4 promoter positions -340 and -140 also flank a palindromic CACCC-like motif (GTGTGGG), similar to the motif found in ED-L2. When transfected into 293 cells, the K4-340 promoter reporter construct containing the GTGTGGG motif was activated 25-fold relative to the K4-140 reporter in the presence of co-transfected GKLF (Fig. 2B). The pXP2 construct was not stimulated by GKLF, and wild type pcDNA3.1/His B construct did not transactivate the K4-140 or K4-340 constructs.

Histidine-tagged 65-kDa GKLF Fusion Protein Is Isolated and Purified from Transiently Transfected 293 Cells—To define the biochemical characteristics of the interaction of GKLF with the CACCC-like motif contained within the ED-L2 and K4 promoters, histidine-tagged GKLF was expressed and purified from 293 cells for use in electromobility shift assays. As indicated above, Western blot analysis of the expressed GKLF demonstrated that major (65-kDa) and minor (34-kDa) forms of the histidine-tagged recombinant GKLF were present in transiently transfected 293 cells. Previous analysis of the GKLF cDNA predicts that the open reading frame should yield a 58-kDa protein (5) in addition to the 7-kDa epitope and histidine tag. To investigate the DNA binding properties of GKLF, extracts were initially prepared that contained both 65- and 34-kDa forms of the fusion protein and that subsequently contained the 65-kDa protein size-fractionated from the 34-kDa protein using gel chromatography techniques.

The 293 cell lysate shown by Western blot to contain GKLF fusion protein contains several bands in addition to GKLF as demonstrated by silver staining (Fig. 3A). Using a nickel-based affinity column, the histidine-tagged proteins were purified from unrelated proteins until the final fraction contained predominantly 65-kDa GKLF with a lesser amount of the 34-kDa protein for use in EMSAs (Fig. 3A). Western blot analysis confirmed the identity of the histidine-tagged, purified proteins (data not shown).

Affinity column-purified GKLF was then fractionated over a gel filtration chromatography column to isolate the 65-kDa GKLF for use in a second set of EMSAs. This was accomplished using a 7.5-ml BioGel P-60 column to fractionate 200 μ l of affinity column-purified protein. After using size standards to calibrate the column, three early fractions were found to contain only the 65-kDa GKLF and not the smaller protein as detected by silver staining (Fig. 3B) which in this system is capable of detecting protein in excess of 10 ng. Thus, the inferred maximal amount of 34-kDa fusion protein in the final purified fraction was less than 1 ng/ μ l.

EMSAs of Recombinant Affinity Column-purified GKLF Demonstrate That the CACCC-like Motif Is Critical for Specific GKLF DNA Binding Activity—The affinity column-purified GKLF containing both 65- and 34-kDa GKLF fusion protein was used in EMSAs. Two series of radiolabeled probes and competitor oligonucleotides were used, which represented the wild type ED-L2 and K4 promoter sequences containing the CACACCC and GTGTGGG motifs, respectively. In addition, mutant double-stranded oligonucleotides were used to determine the specificity of binding of the recombinant GKLF.

The CACACCC motif in the ED-L2 promoter was found to bind the affinity-purified GKLF in the EMSA (Fig. 4A). This binding is specific as shown by competition studies using wild type and mutant unlabeled excess competitor oligonucleotides

² O. G. Opitz, T. D. Jenkins, and A. K. Rustgi, unpublished observations.

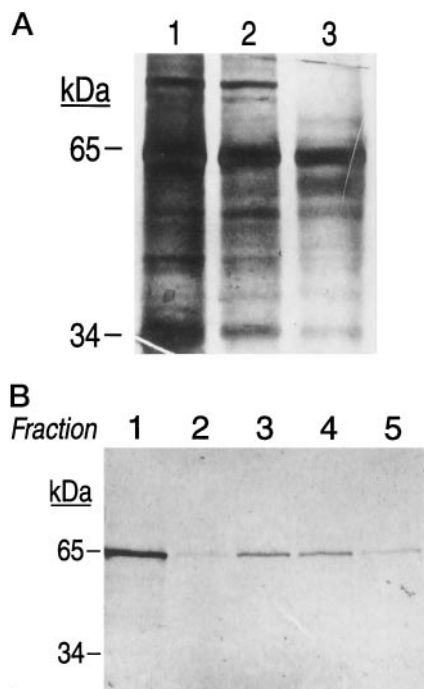


FIG. 3. Affinity column and gel filtration chromatography purification of GKLF. 293 cells were transfected with the GKLF expression construct, and protein was harvested after 24 h. Protein was purified over an affinity column with specificity for the polyhistidine-tagged GKLF, shown in *panel A*. Lane 1 shows the initial cell lysate, and lanes 2 and 3 show successive purification with the affinity column. Material shown in lane 3 was used in EMSAs. Size marker references are shown at the left in kDa. Affinity-purified protein was size-fractionated with gel filtration chromatography to isolate the 65-kDa fusion protein. Fraction 1 represents the starting material, and subsequent purification fractions are shown in *panel B*. Fraction 3 contains purified 65-kDa protein without detectable 34-kDa protein and was used in EMSAs. *A*, GKLF fusion affinity column purification; *B*, 65-kDa GKLF fusion gel chromatography size fractionation.

(Table I). Importantly, the CACACCC motif is critical for binding GKLF, since mutant competitor does not eliminate the GKLF-labeled probe complex. Radiolabeled mutant probe does not bind GKLF, and affinity-purified extracts from untransfected or empty vector-transfected cells do not reconstitute the complex demonstrated with GKLF (data not shown).

Similar results were found in EMSAs with the GTGTGGG sequence from the K4 promoter. Affinity column-purified GKLF also specifically binds the GTGTGGG as shown in competition studies (Fig. 4B). Comparisons of binding between equivalent amounts of labeled K4 and ED-L2 probe yielded a more intense signal with the K4-GKLF complex than with the ED-L2-GKLF complex (data not shown).

Purified 65-kDa GKLF Fusion Protein Is Sufficient to Reconstitute the EMSA Complex, and the 34-kDa Protein Has No Inhibitory Effect on Formation of the Specific Complex—Since the affinity column-purified GKLF contained detectable amounts of a histidine-tagged 34-kDa protein, we were interested in determining whether this protein had relevance in the DNA protein interaction between the 65-kDa predicted form of GKLF and its DNA target. The 65-kDa protein was separated from the 34-kDa protein and used in the EMSA. This result was compared with results from EMSAs utilizing both forms of the protein. Because of the small amount of 34-kDa protein relative to excess 65-kDa GKLF, it was not possible to completely isolate the 34-kDa protein using conventional chromatography techniques. Nonetheless, the addition of the 34-kDa protein did not affect the specific complex formed with the ED-L2 and K4 motifs. Competitor studies as above confirmed

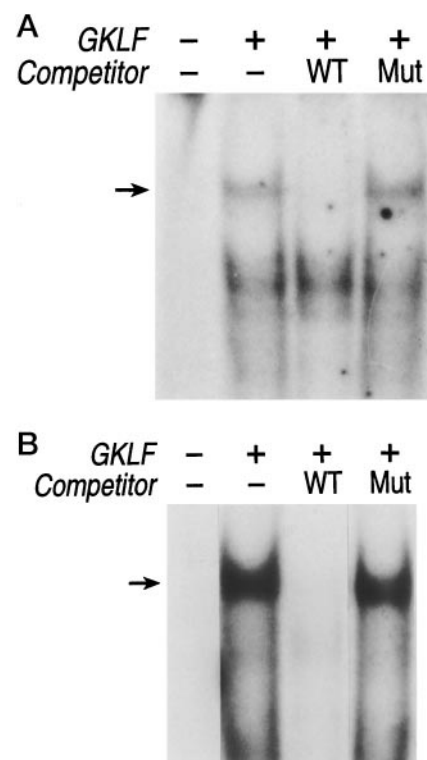


FIG. 4. Affinity-purified GKLF specifically binds elements of the ED-L2 and K4 promoters containing CACCC-like motifs in the EMSA. Double-stranded oligonucleotides corresponding to the CACCC-like element containing regions of ED-L2 and K4 were labeled with [α - 32 P]dCTP and used in EMSAs, shown in *panels A and B*, respectively. Total protein was extracted from 293 cells transiently transfected with GKLF and purified over an affinity column utilizing the histidine tag of the expressed GKLF (shown in Fig. 3A, lane 3). The arrow indicates the specific GKLF-DNA complex. Competition with a 100-fold excess of mutant unlabeled probe does not affect the GKLF binding of either the ED-L2 or K4 probes. Free, unbound radiolabeled probe runs below all complexes in each lane. *A*, EMSA with affinity-purified GKLF fusion and ED-L2 probe; *B*, EMSA with affinity-purified GKLF fusion and K4 probe.

TABLE I
Sequences of oligonucleotides from the Epstein-Barr virus ED-L2 and keratin 4 promoters used for PCR amplification and EMSAs

Oligonucleotide sequences used for PCR amplification of the ED-L2 and K4 promoter sequences for use in the reporter gene constructs are shown in the upper portion of the table. Sense sequences of the double-stranded oligonucleotide probes representing the CACCC-like elements from each promoter are shown in the lower portion of the table; each of these EMSA oligonucleotides has a 4-base pair *Bam*HI site added at the 5'-end to facilitate Klenow fill in radiolabeling.

Oligonucleotide	Sequence	Position
ED-L2-AS	5'-GAAGATCTCGAGAGTGAGGCACAGCTG-3'	0
ED-L2-144-S	5'-CGGGATCCTAATCCCTCTCTCACA-3'	-144
ED-L2-114-S	5'-CGGGATCCGCTAGCCCAAACCTCCA-3'	-114
K4-AS	5'-GAAGATCTGGCTGCAGAGAGCGAGCTG-3'	+59
K4-340-S	5'-GCGTCGACGTGATGGGGTCTCTTTTCT-3'	-340
K4-140-S	5'-GCGTCGACTAGTGACATGCTCAACGGGT-3'	-140
ED-L2/GKLF w/t	5'-TCTCACACCCAGA-3'	
ED-L2/GKLF mt	5'-TCTACGTAGAAGA-3'	
K4/GKLF w/t	5'-AGTGGGTGGGCA-3'	
K4/GKLF mt	5'-ATACTTATGCTCCA-3'	

that the complex required the CACCC-like element, and competition studies with affinity-purified GKLF indicated that these complexes were identical (data not shown). The 65-kDa GKLF fusion protein is sufficient to reconstitute the EMSA complex for both the CACACCC motif (Fig. 5A) and the GTGTGGG motif (Fig. 5B).

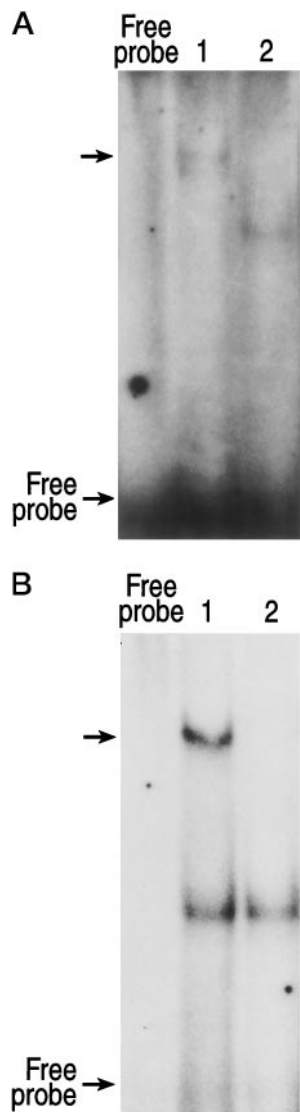


FIG. 5. The 65-kDa GKLF fusion protein is sufficient for reconstitution of the EMSA DNA-protein complex. Double-stranded oligonucleotides corresponding to the CACCC-like element containing regions of ED-L2, shown in *panel A*, and K4 shown in *panel B*, were labeled with [α - 32 P]dCTP and used in EMSAs. Gel filtration chromatography was used to purify the 65-kDa GKLF fusion protein for use in EMSAs (shown in Fig. 3B, fraction 3). *Lane 1* in each *panel* shows the 65-kDa protein incubated with radiolabeled oligonucleotide probe, and *lane 2* shows the addition of unlabeled 100-fold excess wild type competitor oligonucleotide. The *arrow* indicates the specific GKLF-DNA complex. The addition of the 34-kDa protein did not alter formation of the specific complex with either the ED-L2 or K4 probe (not shown). Free, unbound radiolabeled probe runs at the *end* of each *lane*. *A*, EMSA with ED-L2 probe, 65-kDa GKLF fusion, and ED-L2 competitor; *B*, EMSA with K4 probe, 65-kDa GKLF fusion, and K4 competitor.

DISCUSSION

Many epithelial cell types undergo defined programs of proliferation and differentiation. Such programs occur, for example, along the crypt-villus axis in the small and large intestine. In the squamous upper gastrointestinal tract, proliferating basal cells undergo a switch to become terminally differentiated superficial squamous cells in the stratified squamous epithelium. In this context, it is important to delineate both the genes responsible for this proliferation-differentiation gradient and transcription factors that orchestrate the pattern of gene expression in the switch from proliferation to differentiation. Other models of transcriptional regulation, particularly in the skin and hematopoietic systems, suggest that the process is

mediated by both ubiquitous (29, 30) and tissue-specific (31–33) genes and transcription factors.

The Krüppel-like family of transcription factors, comprising GKLF, EKLF, LKLF, and basic transcription element binding-protein 2, may be essential tissue-restricted factors influencing the proliferation-differentiation gradient. GKLF is localized to gut epithelial compartments at the transition between proliferating and early differentiated cells, and previous studies have implicated a role for GKLF in differentiation (5, 7). To investigate mechanisms of gene regulation in the squamous upper gastrointestinal tract, we have focused attention on potential gene targets of GKLF, particularly because TE-11 cDNA library screening yielded GKLF as the only Krüppel-like factor in cells derived from this tissue compartment. This finding supports those observations of other studies, which have to date found only four members of this restricted family. A recent screening of a murine embryonic cDNA library with a probe for the zinc finger domain of EKLF also identified the previously described basic transcription element binding-protein 2, LKLF, and GKLF (11).

Our previous studies have shown that the Epstein-Barr virus ED-L2 (19, 20) and human keratin 4 promoters² are active in suprabasal cells of the esophageal squamous epithelium. Both promoters contain multiple CACCC-like elements. ED-L2 contains a CACCC-like element between bp –218 and –187, which binds a tissue specific, zinc-dependent nuclear factor responsible for most of the unstimulated activity of this promoter in esophageal squamous epithelial cells (19). The CACCC-like element between –144 and –114 binds a phorbol 12-myristate 13-acetate-inducible, zinc-dependent factor whose molecular weight as estimated by UV cross-linking is in the range of several of the Krüppel-like factors (20). In addition, the human keratin 4 promoter contains a palindromic CACCC-like element at position –281. Given the potential importance of GKLF in tissue-specific gene regulation at the transition from proliferation to differentiation in the suprabasal layer of the esophageal squamous epithelium, the activity of these two representative promoters at this transition, and the presence of CACCC-like motifs in both promoters, we investigated whether GKLF is capable of specifically binding these sites in the two promoters and activating gene expression.

After generation of an expression construct of the full-length human GKLF cDNA fused to coding sequence for a polyhistidine and epitope tag, transient transfection into 293 cells revealed a single 3.5-kb transcript. This is in contrast to previous findings which identified a second 1.9-kb transcript postulated to result from alternative splicing (7). The smaller transcript was only identified in tissues with the highest GKLF expression, namely newborn mouse skin and lung. It is possible that the absence of this transcript in 293 cells reflects the absence of alternative splicing or instability of the smaller transcript.

A smaller 34-kDa protein in addition to the 65-kDa protein predicted to result from the GKLF open reading frame was detected by Western blot analysis using an antibody to the epitope tag at the amino terminus. Sequence analysis revealed that there was no stop codon in the region predicted to result in a smaller protein, and the Northern blot suggested that the smaller protein was not the result of alternative splicing at the 3'-end of the RNA. Ultimately, further analysis of the different size GKLF transcripts in newborn mouse skin and the proteins encoded by these transcripts is needed to determine biological relevance. It is conceivable that the smaller protein may have functional consequences for *in vivo* GKLF DNA binding. Alternatively, proteolytic cleavage in the 293 cell line at a site unrelated to *in vivo* post-translational processing of GKLF is

possible, although nonspecific proteolytic degradation of the 65-kDa GKLF appears unlikely, given a distinct and single 34-kDa band on Western blot analysis.

Our studies demonstrate that transiently expressed GKLF leads to transactivation of the ED-L2 promoter dependent upon the presence of sequence containing the CACACCC motif. Additionally, when the CACACCC motif is mutated within the endogenous ED-L2 promoter, GKLF does not transactivate the promoter, also suggesting that activation is mediated through the CACACCC *cis*-regulatory motif. A similar phenomenon was observed with the human keratin 4 promoter. Transient transfection of 293 cells with the K4 promoter reporter constructs and GKLF demonstrates that the region activated by GKLF resides between -340 and -140 of the promoter. There is a palindromic GTGTGGG motif at position -281 of the keratin 4 promoter. These experiments also suggest that a unique sense or antisense orientation of the CACCC-like element may not be essential for GKLF-mediated transactivation.

EMSA with affinity column and gel filtration chromatographically purified GKLF fusion protein confirm the DNA-GKLF interaction and the ability of GKLF to interact specifically with the CACCC-like motif as found in the ED-L2 and K4 promoters. Additionally, we found that the 65-kDa GKLF fusion protein is sufficient to reconstitute DNA binding in the EMSA. The mixture of the 34- and 65-kDa proteins had similar DNA binding characteristics with both the ED-L2 and K4 promoter elements. While it is tempting to speculate that these two proteins may have *in vivo* relevance in differential regulation of expression of different genes, the biochemical characteristics of the two proteins need to be established further.

The role of the Krüppel-like factors in the regulation of gene expression is underscored by emerging evidence suggesting that these factors may provide specific tissue compartments with a mechanism for governing tissue-restricted development and cell-specific differentiation. The recent demonstration that LKLF is required to maintain a quiescent state in single positive T cells and that absence of LKLF leads to a spontaneously activated phenotype is evidence in favor of this view (10). Additionally, LKLF was shown to play an important role in vascular development, specifically in the formation of the tunica media compartment and blood vessel stabilization during murine embryogenesis (34). Targeted disruption of EKLF leads to defective hematopoiesis in the fetal liver (35), as well as lethal β -thalassemia (36). Further study of the role GKLF has in governing proliferation and differentiation should include identification of additional gene targets in the squamous upper gastrointestinal tract apart from ED-L2 and K4 and the role that GKLF overexpression or absence has in control of these basic processes.

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