

The Keratinocyte-specific Epstein-Barr Virus ED-L2 Promoter Is Regulated by Phorbol 12-Myristate 13-Acetate through Two *cis*-Regulatory Elements Containing E-box and Krüppel-like Factor Motifs*

(Received for publication, April 30, 1997, and in revised form, June 12, 1997)

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We previously employed 782 base pairs of the Epstein-Barr virus ED-L2 early lytic cycle promoter in a transgenic mouse model to target cyclin D1 to the stratified squamous epithelium of the tongue and esophagus. This promoter is located 5' to the transcriptional start site of a short open reading frame BNLf-2A and is immediately 3' to the BNLf-1 (LMP-1 oncogene) open reading frame. We studied transcriptional regulation of the ED-L2 promoter by phorbol 12-myristate 13-acetate (PMA) as a means of understanding the tissue specificity of this promoter. The transcriptional activity of the ED-L2 promoter was stimulated 40-fold by PMA and could be blocked with the compound H7 through antagonism of protein kinase C. 5' deletion analysis of the 782-base pair promoter demonstrated that the sequences necessary for PMA-stimulated *trans*-activation were located in two separate *cis*-regulatory regions of the promoter: -187 to -164 and -144 to -114 base pairs from the transcription start site of BNLf-2A. Importantly, mutation of critical base pairs in each region was sufficient to abolish PMA-stimulated *trans*-activation in the native ED-L2 promoter. Region -187 to -164 contains a CACCTG (E-box) motif, and region -144 to -114 contains a CACACCC motif. Both of these motifs are necessary for *trans*-activation by PMA. These regions do not, however, demonstrate enhancer characteristics when tested in a heterologous minimal promoter system. Variations of the CACACCC motif are found in other keratinocyte-specific promoters, as well as in the DNA binding motifs of the Krüppel-like family of transcription factors. Electrophoretic mobility shift assays with specific competitors and factor-specific antibody supershift assays demonstrated that one complex binding the -187 to -164 region containing the CACCTG nucleotides has characteristics of the helix-loop-helix protein upstream stimu-

latory factor, whereas a factor binding the CACACCC motif may be a member of the Krüppel-like family. These experiments show how ubiquitous and tissue-specific transcription factors induced by PMA regulate the ED-L2 promoter in squamous epithelial cells.

The Epstein-Barr virus (EBV)¹ initially infects oropharyngeal esophageal epithelial cells, and a lytic state ensues (1). Oral hairy leukoplakia (2), nasopharyngeal carcinoma (2, 3), and esophageal squamous cell carcinoma (4) are among the epithelial neoplasms associated with EBV. One of the EBV-encoded gene products, latent membrane protein-1 (LMP-1), has been shown to have oncogenic properties mediated through induction of cellular proliferation, inhibition of terminal differentiation, and inhibition of apoptosis (5–12). It is likely that other EBV genes and gene products are important, if not necessary, in transformation of epithelial cells.

The EBV ED-L2 promoter (also referred to as the EDL2 promoter, BNLf-2 promoter, BNLf-2A promoter, and BNLf-2 5'-regulatory sequence) is an early lytic cycle promoter and is defined as containing the TATA-box found in an *EcoRI*-*Bam*HI fragment of the EBV genome, 30 base pairs 5' to a putative transcription start site for two short open reading frames (13, 14). These reading frames are designated BNLf-2A and -2B and encode 60 and 100 amino acids, respectively. The non-coding sequence containing the TATA element, designated the ED-L2 promoter, is located 3' to another open reading frame (BNLf-1) that encodes LMP-1. When a fragment of the EBV genome containing coding and flanking sequences for BNLf-1 and 2 was expressed under control of the BNLf-1 and ED-L2 promoters, 0.6-kilobase transcripts were expressed in tissues possessing a stratified squamous epithelium (tongue and esophagus), whereas the BNLf-1 transcript was expressed ubiquitously (15).

As a means of understanding oncogenesis in the stratified squamous epithelium of the aero-upper digestive tract, we previously generated transgenic mice utilizing the ED-L2 promoter (16). The transgene consisted of 782 base pairs of the ED-L2 promoter fused to the cyclin D1 oncogene. Mice harboring the transgene had high level cyclin D1 expression in the tongue, esophagus, and forestomach. Transgene expression was localized specifically to the basal and suprabasal layers of

* This work was supported by the American Digestive Health Foundation/American Gastroenterological Association Fiterman and Funderberg Awards (to A. K. R.), American Cancer Society Faculty Research Award JFRA-649 (to A. K. R.), National Institutes of Health Grant DK40561 (to A. K. R.), NIDDK Grant 5 T32 DK07191 D22 from the National Institutes of Health (to T. D. J.), a Glaxo Wellcome Institute for Digestive Health Basic Research Award (to T. D. J.), and Department of Energy DE-FG-2-91-ER61228 Award (to H. N. and A. K. R.). Oligonucleotides were synthesized through the CSIBD Molecular Biology Core National Institutes of Health Grant DK43351. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: EBV, Epstein-Barr virus; LMP-1, latent membrane protein-1; PMA, phorbol 12-myristate 13-acetate; PCR, polymerase chain reaction; PKC, protein kinase C; db-, dibutyl-; EMSAs, electrophoretic mobility shift assay(s); bp, base pair(s); KSF, keratinocyte-specific factor; RSV, Rous sarcoma virus; USF, upstream stimulatory factor.

the stratified squamous epithelia. These *in vivo* observations compelled us to postulate that the ED-L2 promoter is uniquely active in stratified squamous epithelial cells through cell type-specific interactions between *cis*-acting regulatory elements in the promoter and cellular *trans*-acting nuclear factors. In this context, we previously identified a CACCC-box like *cis*-regulatory element in the ED-L2 promoter between nucleotides -218 and -187 that interacts with several nuclear transcription factors, one of which is keratinocyte-specific (17). This factor has been designated as keratinocyte-specific factor and likely transcriptionally regulates other viral and eukaryotic promoters active in keratinocytes.

We further hypothesized that extracellular factors that govern the regulation of the ED-L2 promoter might be similar to those that govern other differentiation-critical promoters in keratinocytes. The most extensively studied keratinocyte system is the skin, and a key regulatory agent is phorbol ester. It has profound effects on the regulation of gene expression in keratinocytes, including expression of *c-fos* (18), *c-myc* (19), actin (20), vimentin (20), transforming growth factor- α (21), collagenase (22), stromelysin (23), and metallothionein (22). Phorbol ester mediates these effects through the protein kinase C (PKC) family, phospholipid-dependent enzymes which initiate a cascade of phosphorylation events resulting in many varied biological responses (24). Phorbol ester markedly changes keratinocyte differentiation and proliferation. Evidence supports the necessity of PKC activation for keratinocyte differentiation (25, 26). Ca^{2+} -induced expression of genes for late differentiation markers such as transglutaminase, loricrin, and profilaggrin is dependent upon PKC activation; conversely, Ca^{2+} -induced expression of the early markers keratins 1 and 10 is suppressed by PKC activation (27). The importance of PKC in keratinocyte differentiation is substantiated by the fact that PKC activators such as phorbol ester and synthetic diacylglycerols inhibit keratins 1 and 10 expression *in vitro* and *in vivo* (28, 29).

Study of the mechanisms by which phorbol ester produces its effects in keratinocytes has identified the transcription factor AP1 as central to its role (30). AP1 consensus binding motifs are found in the keratin promoters and are important determinants of the keratinocyte state of differentiation (31). For example, in the involucrin promoter, which is expressed specifically in the suprabasal layer, two of five AP1 sites in the 2500-base pair promoter are critical for tissue specificity (32). The effects are in turn mediated by transcription factors Fra-1, JunB, and JunD (32). Another critical keratinocyte differentiation gene, collagenase, has a phorbol ester-inducible enhancer in its promoter sequence (33, 34).

We postulated that phorbol ester regulates ED-L2 promoter activity for several reasons. First, because this promoter is specifically active in cells of keratinocyte origin, its regulation may be similar to the regulation of other keratinocyte genes, especially those involved in differentiation. Second, because the ED-L2 promoter shares motifs in common with other keratinocyte promoters under the influence of phorbol ester, it may also be governed by the same regulatory mechanisms. Third, a 0.6-kilobase transcript likely corresponding to BNL2A is induced in EBV-infected B lymphocytes upon phorbol ester treatment and was associated with latently infected lymphocytes entering the lytic cycle (14). Finally, because both the LMP-1 and ED-L2 promoters are early lytic promoters and in close physical proximity in the EBV genome, and since LMP-1 impairs differentiation, it is possible that the ED-L2 promoter may play a role in differentiation as well.

In this study, we demonstrate that phorbol ester activates the ED-L2 promoter. A series of deletion construct experiments

have led to the identification of two *cis*-regulatory elements in the ED-L2 promoter responsible for phorbol ester-induced transcriptional activity. These elements contain an E-box motif and a CACACCC motif, the latter recently shown to be important for binding of the Krüppel-like family of transcription factors, one of which is specifically expressed in epithelial cells of the squamous aero-upper digestive tract (35, 36). Electromobility shift assays demonstrate that specific factors, induced by treatment with PMA, bind these sequences. These experiments demonstrate how transcription factors regulated by phorbol ester through the protein kinase C pathway modulate an EBV promoter which is uniquely active in human stratified squamous epithelial cells.

EXPERIMENTAL PROCEDURES

Isolation of the ED-L2 782-Base Pair Promoter Sequence—A 782-base pair sequence between nucleotide positions 168,268 and 167,487 of the B95-8 Epstein-Barr virus genome contains the ED-L2 promoter (13). The 782-base pair fragment was isolated by the polymerase chain reaction (PCR) as described previously (17). PCR amplification conditions consisted of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min for 20 cycles. Identity of the ED-L2 PCR product was verified by DNA sequencing with the dideoxy-mediated chain termination method using the Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.).

Construction of the ED-L2 Promoter Reporter Genes, Deletion Constructs, Minimal Promoter, and Mutant ED-L2 Promoter Reporter Genes—After PCR amplification and confirmatory DNA sequencing, the reaction product was digested with *Bam*HI and *Xho*I, agarose gel-purified, and ligated into the luciferase reporter gene promoterless vector, pXP2 (37), to generate the ED-L2-782 plasmid containing 782 bp of the ED-L2 promoter. A subsequent series of ED-L2 promoter 5' deletion constructs (ED-L2-610, ED-L2-287, ED-L2-218, ED-L2-187, ED-L2-164, ED-L2-144, and ED-L2-114) was made in a similar fashion using ED-L2-782 as a template for PCR with sense primers designed at the different positions of the promoter and an antisense primer from +24 of the coding region of the luciferase reporter gene, as described previously (17). The ED-L2-435 plasmid was generated by digestion of ED-L2-782 with *Bam*HI and *Sma*I, deleting 347 bp of the promoter sequence, followed by ligation.

Minimal promoter DNA constructs containing wild-type or mutant nucleotides spanning promoter regions -188 to -159 and -144 to -115 of the ED-L2 promoter were generated by ligation of kinased double-stranded synthetic oligonucleotides (A_{35} and H_{35} from Table I) into the *Bam*HI site of the heterologous thymidine kinase promoter vector, pT81, with the luciferase reporter gene. Mutant promoter deletion constructs of regions -144 to 0 and -187 to 0 were made as follows. The ED-L2-782 plasmid was digested with *Bam*HI and *Nhe*I restriction enzymes (unique *Nhe*I site at position -114 of ED-L2 promoter), followed by subsequent directional ligation with a double-stranded oligonucleotide corresponding to the remaining promoter sequence. Mutations within the full-length sequence positionally correspond to block mutations shown in Table I for A_{MT1} and A_{MT2} and H_{MT1} and H_{MT2} . All plasmid DNA constructions were checked initially by restriction digestion for correct length and then verified by DNA sequencing by the dideoxy-mediated chain termination method using the Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.). Plasmids were purified by a modified alkaline lysis method (Primm Labs).

Tissue Culture Cell Lines and Transient Transfection Studies—The human esophageal squamous carcinoma cell line TE-11, pancreatic cancer cell line Panc-1 (ATCC Rockville, MD), cervical cancer cell line HeLa (ATCC), and lung cancer cell line LX-1 (ATCC) were cultured under standard conditions as follows: Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (BioWhittaker), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Sigma). A human B cell line, BJAB, was grown in RPMI 1640 medium (Sigma) with 10% serum and antibiotics. Human skin (SCC-13) and tongue (SCC-25) squamous cell carcinoma cell lines were grown in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (Sigma), supplemented with 0.4 μ g/ml hydrocortisone (Sigma), 10% serum, and antibiotics.

Transient transfection of the plasmid and minimal DNA constructions in cultured cells was carried out using the calcium phosphate precipitation technique (5 Prime \rightarrow 3 Prime, Inc.). For transient transfections, TE-11 cells were plated at a density of 1×10^6 cells per 35-mm well and transfected 24 h later with 2 μ g of the luciferase reporter

plasmid and 2 μg of pXGH5, a plasmid containing the mouse metallothionein-I promoter fused to the human growth hormone gene (Nichols Institute). The transfectant mixture consisted of a 250- μl solution of 125 mM CaCl_2 , 25 mM Hepes, pH 7.05, 0.75 mM Na_2HPO_4 , 5 mM KCl, 140 mM NaCl, and 6 mM glucose. After a 12-h incubation, cells were washed twice with phosphate-buffered saline, and 0.1% serum containing Dulbecco's modified Eagle's medium was added. In addition, phorbol 12-myristate 13-acetate (PMA) (Biomol, Plymouth Meeting, PA), forskolin (Sigma), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (Biomol), dibutyryl-cyclic AMP (db-cAMP) (Sigma), cyclosporin A (Sigma), or 10% serum were added at appropriate concentrations 12 h after transfections, and incubations were continued for a prescribed period.

Luciferase assays were performed using luciferin, ATP, and coenzyme A (Promega), with a Monolight Luminometer (Analytical Luminescence Laboratory). Cells were harvested 36 h post-transfection, washed twice with phosphate-buffered saline, lysed in 200 μl of 1 \times cell culture lysis reagent (Promega), and 40 μl of the lysate was mixed with 100 μl of luciferase assay reagent consisting of 20 mM Tricine, 1.07 mM MgCO_3 , 2.67 mM MgSO_4 , 0.1 mM EDTA, 33.3 mM dithiothreitol, 270 μM coenzyme A, 530 μM ATP, and 470 μM luciferin.

Incubations were performed in triplicate, and results were calculated as the mean \pm S.E. values for luciferase activity. Values were then expressed as fold increase or decrease compared with the control for each set of experiments. Activities were expressed as the mean of at least three independent transfection experiments. Expression of human growth hormone from the plasmid vector pXGH5, containing the human growth hormone gene under the control of the metallothionein-I promoter, was used as an internal control for a subset of the transfections to ensure consistency of transfection conditions. Empty pXP2, RSV luciferase, and the pT81 luciferase construct (37), in which the luciferase gene is driven by the enhancerless herpes simplex thymidine kinase promoter, served as additional controls.

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts from the different cell lines were prepared as described previously, except the buffers were supplemented with a mixture of 0.5 $\mu\text{g}/\text{ml}$ protease inhibitors aprotinin, chymostatin, and pepstatin (Boehringer Mannheim). The protein concentration was determined by a calorimetric method (Bio-Rad protein assay). α - ^{32}P -Labeled oligonucleotide DNA probes were constructed as follows. 5 pmol of a double-stranded oligonucleotide (shown in Table I), 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 μM dATP, 33 μM dGTP, 33 μM dTTP, 0.33 μM [α - ^{32}P]dCTP (NEN Life Science Products), 1 unit of DNA polymerase I Klenow fragment (Amersham Corp.), and then polyacrylamide gel-purified.

EMSAs were carried out by incubating 5 μg of nuclear extract with 5 fmol of the α - ^{32}P -labeled oligonucleotide DNA probe (20,000 cpm) in a 20- μl binding reaction containing 10 mM Tris-HCl, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, and 1.0 μg of poly(dA-dT) (Pharmacia Biotech Inc.). 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°C .

Competitor Oligonucleotides and Antibodies Used in EMSAs and Immune Supershift Reactions—For competition experiments, the nuclear extract was preincubated with 100-fold excess of unlabeled double-stranded oligonucleotides (Table I) prior to the addition of the α - ^{32}P -labeled oligonucleotide DNA probe. All oligonucleotides were synthesized by the phosphoramidite procedure (Applied Biosystems) and purified by gel electrophoresis. Immune supershift assays were performed using a polyclonal anti-Sp1 antibody (Santa Cruz) and a monoclonal anti-AP2 antibody (gift of T. Williams), a monoclonal anti-USF antibody, and a polyclonal anti-Egr antibody (gift of V. Sukhatme). The antibody was preincubated with the nuclear extract at room temperature for 15 min prior to the addition of the α - ^{32}P -labeled oligonucleotide DNA probe. Other conditions for the EMSAs are as described above.

Ultraviolet Light-induced Cross-linking—The A, A_{MT} , H, and H_{MT} single-stranded oligonucleotides corresponding to wild-type and mutant sequences of the ED-L2 promoter (Table I) were labeled by annealing corresponding 7-mers followed by the Klenow fill-in reaction as described above, except that 5-bromodeoxyuridine was substituted for dTTP. Binding reactions were performed identically to the EMSA reactions (38), except that the reaction was for 30 min at 4°C to inhibit protein degradation. Samples were then exposed to a medium wave (312 nm) UV transilluminator (UVP, Inc.) for 30 min on ice at a distance of 3 cm. Samples were then mixed with 2 \times sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer and boiled for 5 min,

and electrophoresis was carried out on 10% SDS-polyacrylamide gel electrophoresis. Gels were then dried and exposed to x-ray film (Kodak X-AR) at -80°C .

RESULTS

The Epstein-Barr Virus ED-L2 Promoter Is Responsive to Phorbol Ester in Human Esophageal Cancer Cell Line TE-11—To study the effect of different extracellular agents on ED-L2 promoter transcriptional activity, a luciferase reporter construct containing 782 base pairs of the ED-L2 promoter was used in a series of transient transfections of a cell line previously shown to exhibit high levels of promoter transcriptional activity, TE-11 (17). Additionally, we previously demonstrated that the ED-L2 promoter is active in a number of different cell lines of keratinocyte lineage, in the presence of medium with 10% serum (17). TE-11 cells were transiently transfected with 2.0 μg of ED-L2-782 reporter gene construct followed by administration of PMA in varying concentrations in 0.1% serum for 24 h. A dose-response analysis showed that transcriptional activation induced by PMA could be initially detected at 10^{-9} M, whereas maximal stimulation of the promoter occurred at 10^{-7} M (Fig. 1A). Stimulation decreased at higher concentrations, from a 40-fold maximal increase in promoter activity at 10^{-7} M to a 10-fold increase at 10^{-4} M. To demonstrate the transfectability of TE-11 cells, the RSV promoter-luciferase reporter gene construct was found to yield an 8-fold increase in luciferase activity. Time course studies of the maximally effective dose of PMA demonstrated that an effect was first detectable at 2 h (6-fold stimulation) with maximal stimulation occurring at 12–24 h (Fig. 1B). All subsequent transfection experiments examining the effects of PMA were carried out with PMA treatment for 24 h at 10^{-7} M. No PMA stimulation was observed with a heterologous enhancerless herpes simplex thymidine kinase promoter-luciferase reporter gene (pT81-luciferase) or a promoterless luciferase reporter gene, pXP2 (data not shown).

To examine further the relationship of the protein kinase C (PKC) pathway and ED-L2 promoter activity in TE-11 cells, down-regulation of PKC was achieved through prolonged treatment with PMA. In addition, antagonism of PKC was accomplished with the PKC inhibitor H7, a serine/threonine protein kinase inhibitor that inhibits all isoforms of protein kinase C. Transiently transfected cells were stimulated with the maximally effective PMA concentration (10^{-7} M) with or without H7 pretreatment for 1 h, as well as PMA (10^{-7} M) treatment for 24 h in the absence of H7. H7 pretreatment decreased the PMA response by 70% (Fig. 1C). Prolonged PMA pretreatment for 24 h followed by PMA treatment also abrogated the PMA response to a similar degree. To assess for nonspecific toxic effects of H7, cells were transfected and treated with H7 alone, in the absence of PMA. These conditions minimally affected basal ED-L2 promoter activity.

The Epstein-Barr Virus ED-L2 Promoter Is Not Primarily Regulated through Alternative Pathways—The effects of extracellular agents aside from PMA were also studied to establish the specificity of the PMA effect. Transfection of cells followed by administration of a fat-soluble derivative of cyclic adenosine monophosphate (db-cAMP) resulted in an approximately 35% decrease in basal promoter activity, tested at a concentration of 10^{-4} M (Fig. 2A). Other db-cAMP concentrations (10^{-3} M to 10^{-6} M) had a less pronounced effect (data not shown). Treatment of cells with varying concentrations of the phosphatase inhibitor cyclosporin A (10^{-5} M to 10^{-8} M, data shown for 10^{-6} M) led to no appreciable change in ED-L2 promoter activity (Fig. 2A). Forskolin, an activator of protein kinase A, increased the ED-L2 activity 1.7-fold at a concentration of 10^{-5} M and 10^{-6} M (data shown for 10^{-5} M) (Fig. 2A). Relative to the increase in promoter activity found with PMA stimulation,

FIG. 1. PMA stimulates ED-L2 promoter activity. TE-11 cells transiently transfected with the 782-bp EBV ED-L2 promoter were incubated in the presence of PMA in medium containing 0.1% serum. *A* shows the dose-response effect of different molar concentrations of PMA and *B* shows the time course of the PMA response. Additional transfections include ED-L2-782 without PMA (*Control* in *A-C*) as well as the RSV-luciferase (*RSV*) and pT81-luciferase (*pT81*) reporter genes. In the dose-response experiments, cells were treated with different concentrations of PMA and harvested after 24 h for luciferase assays. In the time course experiments, cells were treated with the maximal effective dose of PMA and harvested at different time points. *C*, shows the effects of the PKC inhibitor H7 (*H7 + PMA*) and prolonged PMA treatment (*PMA + PMA*). Promoter activity in the absence of PMA (*Control*), H7 treatment alone (*H7*), and PMA treatment alone (*PMA*) are also shown for comparison. Luciferase activity is expressed as fold increase relative to untreated control (mean \pm S.E. values) calculated from three independent transfections. *A*, PMA dose-response plot; *B*, PMA response time course; *C*, H7 inhibition and PKC depletion.

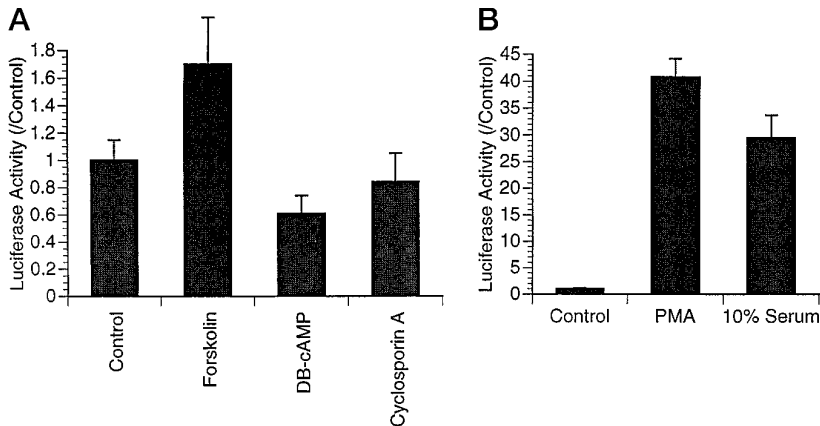
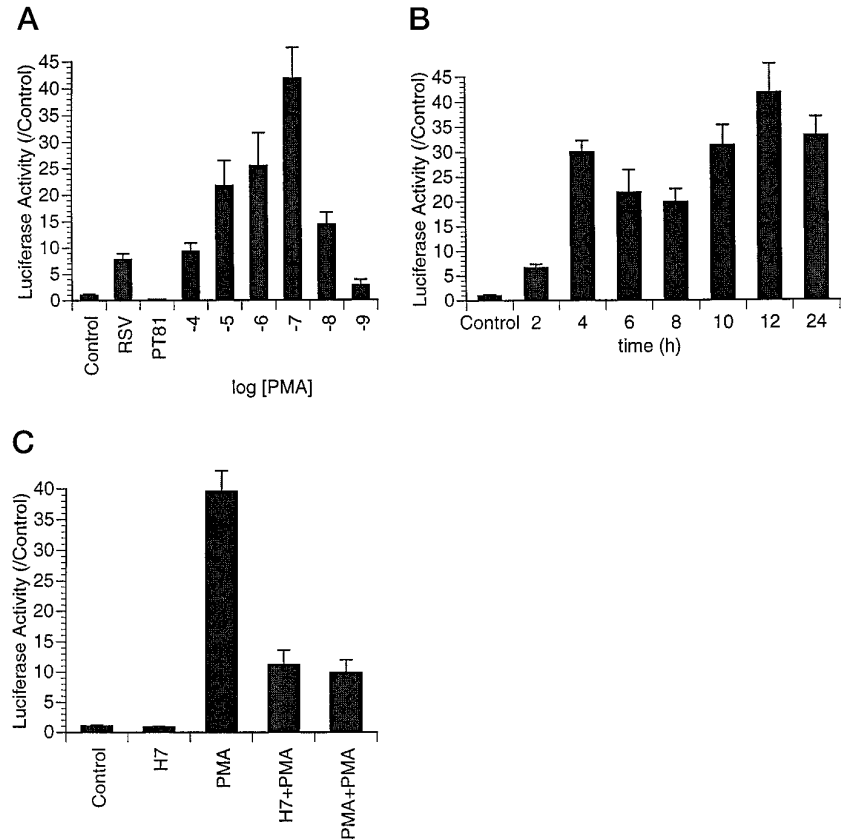


FIG. 2. Forskolin, db-cAMP, and cyclosporin A have minimal effects on ED-L2 promoter activity compared with PMA. TE-11 cells transiently transfected with the 782-bp EBV ED-L2 promoter were incubated in the presence of agents shown in *A* or medium containing 10% serum. Control transfections without PMA are also shown in *A* and *B*. Luciferase activity is expressed as fold increase relative to untreated control (mean \pm S.E. values) calculated from three independent transfections. *A*, ED-L2-782 response to other agents; *B*, ED-L2-782 serum response.

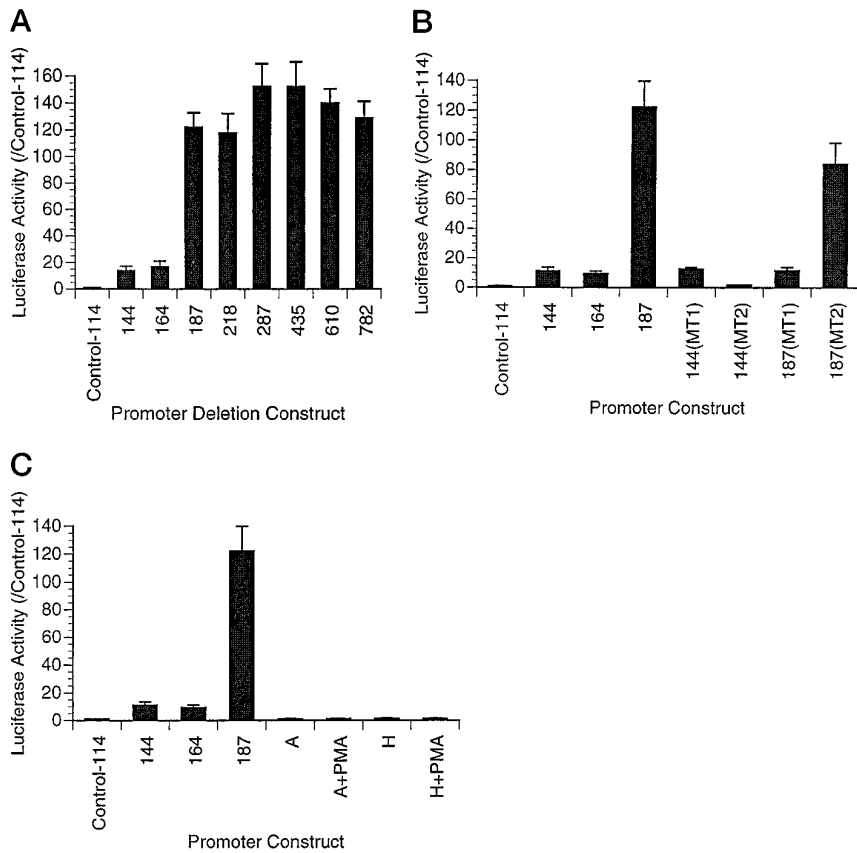
these results show that other extracellular agents that modulate non-PKC pathways have a minimal effect on the ED-L2 promoter.

The ED-L2 promoter activity in the presence of 10% serum without PMA was compared with the effect of PMA in 0.1% serum. We previously reported that the ED-L2 promoter is highly active in keratinocytes in the presence of 10% serum (17). Nucleotides -218 to -187 were shown to be responsible for the majority of this activity. TE-11 cells were transfected with the ED-L2-782 reporter construct, followed by 24 h treatment with PMA or 10% serum. Serum resulted in a 30-fold increase in promoter activity compared with the 40-fold stimulation found with the maximally effective PMA concentration (Fig. 2*B*), thereby indicating that PMA's effect is specific and not augmented by factors in the serum.

Deletion Analysis of the ED-L2 Promoter Sequences Demonstrates That Two cis-Regulatory Regions Are Necessary for the Response to Phorbol Ester—To identify the cis-regulatory elements mediating the transcriptional response of the ED-L2

promoter to PMA, deletion analysis of the promoter was performed. A series of 5' deletion constructs was made from the original ED-L2-782 plasmid, using PCR amplification and restriction enzyme digestion. Constructs containing 782, 610, 435, 287, 218, 187, 164, 144, and 114 of flanking DNA sequence 5' to the putative transcription start site of the BNL2A open reading frame were used in a series of transient transfections with and without PMA treatment (Fig. 3*A*). Transfection studies showed that two regions of the ED-L2 promoter were necessary for the PMA-induced transcriptional activity. A 10-fold decrease in promoter activity was observed when the -187 to -164 sequence was deleted. This region, designated as A, contains an E-box motif (CACGCG), common to many promoters. An additional 15-fold decrease in promoter activity was seen when the -144 to -114 sequence was deleted. This region, designated as H, contains a CACACCC motif to which transcription factors active in keratinocytes, among other factors, bind in electrophoretic mobility shift assays (17, 36). Deletion of the -218 to -187 region, however, had no effect on the

FIG. 3. Two cis-regulatory elements of the ED-L2 promoter are critical for PMA stimulation. TE-11 cells transiently transfected with the 782-bp EBV ED-L2 promoter, or deletion constructs as shown, were incubated with PMA for 24 h followed by harvesting for the luciferase assay. Control transfections of the ED-L2-114 construct in the presence of PMA (*Control-114*) are shown in A–C. Luciferase activity is expressed as fold increase relative to PMA-treated ED-L2-114 construct (mean \pm S.E. values) calculated from three independent transfections. A, 5' deletions of the ED-L2 promoter; B, mutant deletion ED-L2 constructs of regions A and H, compared with wild-type deletion ED-L2 constructs treated with PMA. Mutant construct 144(MT2) abolishes the CACACCC motif and mutant construct 187(MT1) eliminates the E-box motif; C, minimal (pT81) ED-L2 promoter constructs containing region A (–187 to –164) or region H (–144 to –114) PMA treated and untreated, compared with wild-type deletion ED-L2 constructs treated with PMA.



PMA-induced response. The 782-bp ED-L2 promoter under maximal PMA stimulation conditions had a nearly 200-fold greater activity than a construct containing only the first 114 base pairs of the ED-L2 promoter.

The E-box and CACACCC Motifs Are Critical for PMA Responsiveness of the ED-L2 Promoter—Mutational analysis of the deletion constructs in the pXP2 luciferase reporter system was carried out to determine the functional consequences of these motifs in the ED-L2 promoter. Double-stranded oligonucleotides containing mutation of different nucleotides within regions A and H were ligated into the ED-L2-114 construct 5' to the *NheI* restriction site at position –114, resulting in mutant ED-L2-144 and ED-L2-187 constructs. The wild-type and mutant sequences are identical to the sequences shown in Table I for EMSA probe oligonucleotides A₃₅, H₃₅, H_{MT1}, H_{MT2}, A_{MT1}, and A_{MT2}. To study region H, two mutants were constructed with one eliminating the CACACCC motif (144MT2). For region A, two mutants were also constructed, one of which abolished the E-box motif (187MT1). These constructs were used in transient transfection assays with TE-11 cells in the presence of PMA. Deletion of the CACACCC motif within region H reduced promoter activity of ED-L2-144 more than 10-fold (Fig. 3B). Mutation of a separate region resulted in no significant change in PMA-stimulated transcriptional activity. Within region A, disruption of the E-box motif had the strongest negative effect on promoter activity, reducing it by 85% (Fig. 3B). Mutation of an adjoining sequence had a significantly lesser effect on promoter activity, reducing it by 25%.

The –144 to –114 and –164 to –187 Regions of the ED-L2 Promoter Are Necessary but Not Sufficient to Permit Response to Phorbol Ester in TE-11 Cells—To further localize the cis-acting element necessary for PMA responsiveness of the ED-L2 promoter and to determine whether the critical regions identified in the deletion series transfections functioned as enhancer elements, additional luciferase reporter constructs were made

in a heterologous herpes simplex thymidine kinase promoter system. Minimal promoter DNA constructs containing wild-type or mutant nucleotides spanning promoter regions –188 to –159 (containing region A) and –144 to –115 (containing region H) of the ED-L2 promoter were generated by ligation of kinased double-stranded synthetic oligonucleotides (represented by A₃₅ and H₃₅ from Table I) into the *BamHI* site of the heterologous thymidine kinase promoter vector, pT81, with the luciferase reporter gene. Neither the H-pT81 nor the A-pT81 constructs had PMA-responsive transcriptional activity, compared with transfections with pXP2 deletion constructs depicted in Fig. 3C. In aggregate, the deletion analysis and minimal promoter construct data indicate regions A and H are necessary but not sufficient for PMA-induced promoter activity.

Distinct Nuclear Transcription Factors from the TE-11 Cell Line Bind the –144 to –114 and –187 to –164 Sequences and Are Induced by Phorbol Ester—To characterize the nuclear factors that bind regions A and H within the ED-L2 promoter and that mediate the PMA-induced response, electrophoretic mobility shift assays (EMSAs) were performed using nuclear extracts of PMA-treated and untreated TE-11 cells. These assays used as probes α -³²P-labeled, double-stranded oligonucleotides representing the sequences of regions A and H of the ED-L2 promoter (Table I). Two sets of EMSA probes were used. The first span the full-length of regions A and H and are designated A₃₅, H₃₅, H_{MT1}, H_{MT2}, A_{MT1}, and A_{MT2}, shown in Table I. Minimal-length DNA probes were also synthesized to test the DNA binding activity of the putative motifs contained within regions A and H. These short-length DNA probes are designated A, H, A_{MT}, and H_{MT}, also shown in Table I.

DNA probes A and H containing the E-box and CACACCC motifs bind several complexes that are induced by PMA (Fig. 4). Two complexes of similar mobility bind probe A (1 and 2) and three complexes (3, 4, and 5) bind probe H. Complexes 1–3

TABLE I
Sense sequences of double-stranded oligonucleotides of the EBV ED-L2 promoter used for EMSAs

Nucleotide positions in the EBV ED-L2 promoter are indicated in parentheses after each of the sequences. At the 5' end of each oligonucleotide, a *Bam*HI restriction site is added to facilitate Klenow fill-in labeling and cloning. Mutant sequences are shown in bold type.

Probe	Sequence	Position
A	5'-GAACACCTGTTG-3'	(-184 to -173)
A _{MT}	5'-GAAACGAGCTTG-3'	(-184 to -173)
A ₃₅	5'-GCAGGAACACCTGTTGTTGACACATTCTTT-3'	(-188 to -159)
A _{MT1}	5'-GCAGTCTACGAGCGAGTTGACACATTCTTT-3'	(-188 to -159)
A _{MT2}	5'-GCAGGAACACCTGTTG GACCATGCG ACTTT-3'	(-188 to -159)
H	5'-TCTCACACCCAGA-3'	(-135 to -123)
H _{MT}	5'-TCT ACGTAGA AGA-3'	(-135 to -123)
H ₃₅	5'-TAATCCCTCTCTCACACCCAGAACTAAGA-3'	(-144 to -115)
H _{MT1}	5'- GCTAAGAGG TCTCACACCCAGAACTAAGA-3'	(-144 to -115)
H _{MT2}	5'-TAATCCCTC GAAACGTAGAT GAACTAAGA-3'	(-144 to -115)

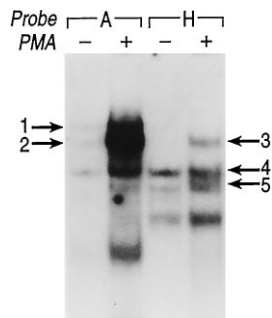


FIG. 4. PMA-induced nuclear transcription factors bind regions A and H within the ED-L2 promoter in EMSAs. The double-stranded DNA probes corresponding to regions A and H of ED-L2 (see Table I for sequence of probes A and H) were labeled with [α - 32 P]dCTP and used in EMSAs. Crude nuclear extracts were prepared from PMA-treated (10^{-7} M for 24 h) and untreated TE-11 cells. Numbered arrows indicate complexes induced by PMA treatment and referred to in subsequent EMSAs. Nonspecific induced bands below complexes 1–5 are not numbered in this and subsequent figures. Free, unbound radiolabeled probe (not shown) runs below all complexes in each lane in this and all subsequent figures.

and 5 are significantly induced by PMA, and complex 4 is also present to a lesser degree in unstimulated nuclear extracts. To investigate the possibility that these complexes also bind other motifs different from the motifs of interest, the full-length probes were tested in EMSAs. When the EMSA pattern from probes A and H was compared with that from probes A₃₅ and H₃₅, a similar EMSA pattern occurred (Figs. 5 and 6, upper panel). Mutation of the CACACCC within region A (probe A_{MT}) eliminated complexes 1 and 2, whereas mutation of the E-box motif within region H (probe H_{MT}) eliminated complexes 3 and 5 and partially eliminated complex 4 (Figs. 5 and 6, upper panel).

A series of competitor oligonucleotide experiments in EMSAs further strengthened the importance of these motifs within regions A and H. Competitor oligonucleotides were designed with mutations corresponding to mutations in the deletion constructs used in the transfection studies described above. For region A, competition with 100-fold molar excess unlabeled A₃₅ and a mutant competitor with an intact E-box motif (A_{MT2}) competed away complexes 1 and 2 (Fig. 5, middle panel). A different competitor (A_{MT1}) with mutation in the E-box did not compete for binding with wild-type A₃₅. Similarly, for region H, competition with 100-fold molar excess unlabeled H₃₅ and a mutant with an intact CACACCC motif (H_{MT1}) competed away complexes 3, 4, and 5 (Fig. 6, middle panel). EMSAs with probes A and H revealed a similar pattern of competition. Mutation of the E-box and CACACCC sequences (probes H_{MT} and A_{MT}) interfered with competition for binding to complexes 1–5 (Figs. 5 and 6, lower panel).

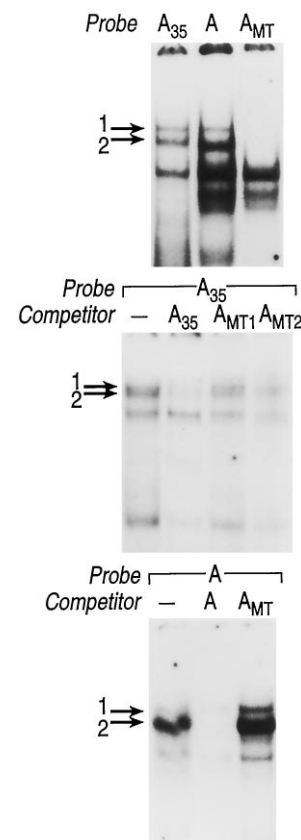


FIG. 5. EMSAs showing oligonucleotide competition studies for characterization of region A within the ED-L2 promoter. Double-stranded DNA probes corresponding to region A (see Table I for sequences of probes A, A₃₅, A_{MT}) were labeled with [α - 32 P]dCTP and used in the EMSAs. In the upper panel, the patterns of the DNA binding nuclear transcriptional factors with the three different radioactively labeled probes are compared. This reveals that the binding patterns to short-length DNA probe A and full-length DNA probe A₃₅ are similar for complexes 1 and 2 and that mutation of the E-box motif in probe A_{MT} eliminates complexes 1 and 2. The middle panel demonstrates competition assays using radioactively labeled probe A₃₅. 100-fold excess unlabeled competitor double-stranded oligonucleotides are incubated with radioactively labeled A₃₅. Complexes 1 and 2 are markedly attenuated by competitors A₃₅ and A_{MT2} but not by A_{MT1}. The lower panel demonstrates competition assays with radioactively labeled DNA probe A which contains the E-box motif. 100-fold excess unlabeled competitor A but not A_{MT} attenuates complexes 1 and 2.

Complexes 1 and 2 Are Found in Cell Lines of Different Lineage Whereas Complex 3 Is Unique to TE-11, among the Different Cell Lines Tested—To determine the tissue distribution of the complexes 1–5 binding regions A and H, EMSAs with nuclear extracts from different cell lines were performed. Region A contains an E-box motif common to many promoters

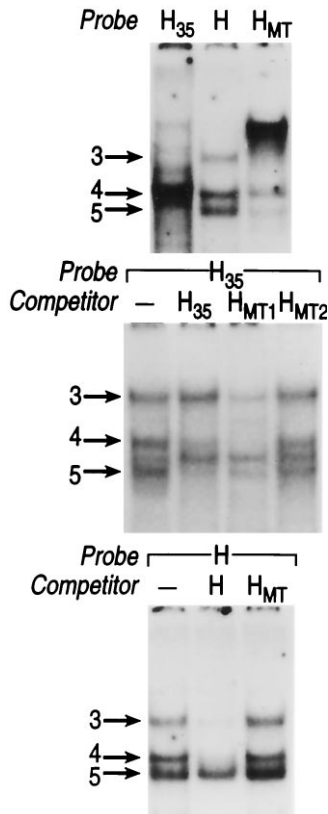


FIG. 6. EMSAs showing oligonucleotide competition studies for characterization of region H within the ED-L2 promoter. Double-stranded DNA probes corresponding to region H (see Table I for sequences of probes H, H₃₅, and H_{MT}) were labeled with [α -³²P]dCTP and used in the EMSAs. In the *upper panel*, the DNA binding patterns of the nuclear transcription factors with the three different radioactively labeled DNA probes are compared. This reveals that the binding patterns to short-length DNA probe H and full-length DNA probe H₃₅ are similar for complexes 4 and 5 and that mutation of the CACACCC motif in probe H_{MT} markedly attenuates complexes 3–5. The *middle panel* demonstrates competition assays using radioactively labeled DNA probe H₃₅. 100-fold excess unlabeled competitor double-stranded oligonucleotides are incubated with radioactively labeled H₃₅. Complexes 4 and 5 are markedly attenuated by competitors H₃₅ and H_{MT1} but not by H_{MT2}. Complex 3 is markedly attenuated by competitor H_{MT1} but not by H_{MT2}. The *lower panel* demonstrates competition assays with radioactively labeled DNA probe H which contains the CACACCC motif. 100-fold excess unlabeled competitor H but not H_{MT} eliminates complexes 3 and 4 and attenuates complex 5.

and active in many cell types. The *upper panel* of Fig. 7 shows that diverse cell lines contain factors, albeit with varying amounts, that have the same apparent mobility as complexes 1 and 2 in EMSA with PMA-treated TE-11 cell nuclear extract. Similar EMSAs were performed using a probe representing region H, shown in the *lower panel* of Fig. 7. Complex 3 was uniquely present in PMA-treated TE-11 cells but not in other cell lines tested. A complex with identical mobility to complex 4 was only present in minimal amounts in LX-1 and not in the other cell lines. Complex 5 was more ubiquitous, present in all cell lines but SCC-13.

Factor-specific Antibodies and Ultraviolet Light-induced Cross-linking Reveal Additional Characteristics of the DNA Binding Nuclear Proteins—Further information regarding the identity of the DNA binding nuclear proteins in EMSAs can be gained from immune supershift assays. EMSAs using antibodies specific for known transcription factors were used with probes A and H. The mobility of complexes 3–5 was not affected by antibodies to Sp1, USF, Egr, or AP2. Complex 1, however, was eliminated after preincubation with an antibody specific for USF (Fig. 8), a ubiquitous helix-loop-helix protein of molec-

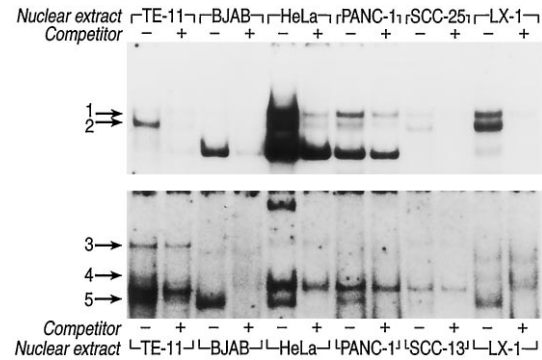


FIG. 7. Ubiquitous and cell type-specific nuclear transcription factors bind regions A and H within the ED-L2 promoter. EMSAs were performed using the [α -³²P]dCTP-labeled probes A (*upper panel*) and H (*lower panel*) with 5 μ g of crude nuclear extracts from different cell lines. 100-fold excess unlabeled competitor double-stranded oligonucleotide was incubated with extracts in the indicated lanes (unlabeled A for *upper panel* and unlabeled H for *lower panel*). In addition to TE-11 cells, other human cell lines included the following: *BJAB*, B-cell lymphoma; *HeLa*, cervical adenocarcinoma; *PANC-1*, pancreatic adenocarcinoma; *SCC-25* and *SCC-13*, squamous cell carcinomas of the tongue and skin, respectively; and *LX-1*, lung carcinoma. Arrows indicate complexes 1–5 referred to in previous figures.

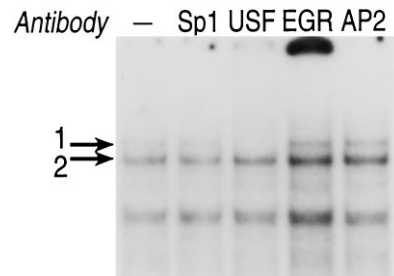


FIG. 8. Immune supershift assays indicate that complex 1 is USF. The double-stranded radioactively labeled DNA probe A was used in the EMSA along with antibodies to the transcription factors indicated. Antibodies were incubated with the EMSA reaction components prior to addition of the radioactively labeled DNA probe A. Arrows indicate complexes 1 and 2 binding DNA probe A. No effect on complex pattern is seen with antibodies to Sp1, Egr, or AP2. Complex 1 is eliminated when antibody to USF is used.

ular mass 43–44 kDa which binds the E-box motif (consensus CACGTG) (39). Additional EMSAs performed utilizing EGTA to identify factors which require Zn²⁺ for DNA binding showed that the binding activity of complex 3 but not complex 1 was attenuated relative to other complexes (data not shown). These data suggest that complex 3 may require zinc for binding the DNA sequences in the EMSA reaction, whereas complex 1 is zinc-independent.

To estimate the approximate molecular masses of complexes 1–5, ultraviolet light-induced cross-linking experiments were performed as described above (38). This technique takes advantage of the specificity of binding of the complexes to wild-type sequence, while not binding to the mutant sequence. EMSA reactions are performed in an identical fashion with ³²P-labeled wild-type and mutant probes, followed by UV-induced cross-linking and separation by SDS-polyacrylamide gel electrophoresis. This technique demonstrates the molecular masses in aggregate of all DNA-binding proteins comprising complexes 1–5. In this fashion, approximate molecular mass comparisons can be made between complexes 1 and 5 in the EMSA and known transcription factors. Probe A cross-links to specific proteins of molecular masses 75, 60, 43, and 25 kDa (Fig. 9). Of note, the USF proteins include members of molecular mass 43 and 44 kDa. Probe H cross-links to specific pro-

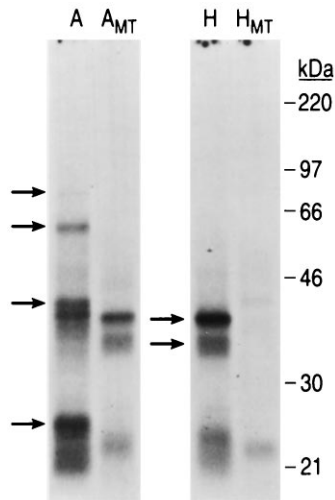


FIG. 9. UV light-induced cross-linking experiment with radioactively labeled probes corresponding to regions A and H within the ED-L2 promoter. DNA probes A, A_{MT}, H, and H_{MT} were labeled with [α -³²P]dCTP as described under "Experimental Procedures." EMSA reactions were the same except for the following modifications: DNA probes were Klenow filled-in by annealing corresponding 7-mers, 5-bromodeoxyuridine was substituted for dTTP, and reactions were for 30 min at 4 °C to inhibit protein degradation. Samples were exposed to a medium wave (312 nm) UV transilluminator for 30 min on ice at a distance of 3 cm and separated with 10% SDS-polyacrylamide gel electrophoresis. Arrows indicate the major protein complexes unique to the wild-type DNA probes A and H but not seen with the corresponding mutant DNA probes A_{MT} and H_{MT}. Molecular mass size markers are expressed in kilodaltons.

teins of molecular mass 33 and 38 kDa. The Krüppel-like binding factors include EKLF, GKLF, LKLF, and BTEB2 (Table II), two of which have molecular masses of 38 kDa.

DISCUSSION

This study demonstrates that the EBV ED-L2 promoter can be transcriptionally regulated by the phorbol ester PMA. We have previously shown that this promoter is selectively active in tissues with stratified squamous epithelia, namely the tongue, esophagus, and forestomach (16). This, in large measure, is attributable to a *cis*-regulatory element spanning -218 and -187 of the ED-L2 promoter and is mediated primarily through the binding of a novel transcriptional factor, designated keratinocyte-specific factor, to a CACACCT motif (17).

In the present study, a human esophageal cancer cell line in which the ED-L2 promoter has a high level of activity was used in a series of transient transfections to establish which *cis*-regulatory elements are important for regulation by phorbol ester. In this system, PMA stimulation of TE-11 cells led to 40-fold activation of the ED-L2 promoter, constituting greater activation than in the presence of 10% serum alone. The PMA response of the ED-L2 promoter could be blocked with pretreatment of TE-11 cells with H7, a nonspecific inhibitor of PKC, as well as prolonged treatment of TE-11 cells with PMA for 24 h. In contrast, other pathways such as those involving PKA and cAMP appear less important than the PKC pathway in regulation of this promoter.

Deletion analysis of the ED-L2 promoter to determine which *cis*-regulatory elements are necessary for the PMA responsiveness revealed that two regions are of critical importance, namely -144 to -114 and -187 to -164. Promoter region -114 to 0 has minimal activity in the presence of PMA when placed upstream of a luciferase reporter gene. Addition of nucleotides -144 to -114 yields a 15-fold increase in luciferase activity, whereas further addition of the sequence between -164 and -144 yields no additional luciferase activity. Inclu-

TABLE II
Proposed members of the Krüppel-like family of TFIIIA subclass of zinc finger transcription factors

Tissue-restricted members include basic transcription element binding factor 2 (BTEB2) (50) found in testis and placenta, erythroid Krüppel-like factor (EKLF) (46, 47), epithelial zinc finger (EZF) also designated gut-enriched Krüppel-like factor (GKLF) found in the squamous aero-upper digestive tract (35, 36), and lung Krüppel-like factor (LKLF) (45). While these members share a high degree of homology in the zinc finger domains, other related members of the TFIIIA subclass include Sp1, the Wilms tumor gene product WT-1, Egr-1, and Egr-2, BTEB, ZBP-89, and ht β (61), and zif268.

Nuclear transcription factor	cDNA size	Estimated mass	DNA-binding motif
	<i>kb</i>	<i>kDa</i>	
BTEB2	0.66	26.3	GGGGCGGGG
EKLF	1.07	38.0	CACACCC
EZF/GKLF	1.45	58.0	CACACCC
LKLF	1.06	37.7	CACCC

sion of the sequence between -187 and -164 gives an additional 10-fold increase in promoter activity compared with the -144 to 0 construct. The -187 to 0 construct is nearly 200-fold more active than the -114 to 0 construct.

Unlike the -218 to -187 element, which can function as an enhancer element in the pT81 heterologous promoter system (17), neither the -144 to -114 nor the -187 to -164 elements have enhancer characteristics. Thus, there are likely multiple PMA-induced nuclear transcription factors responsible for the ED-L2 promoter activity as further substantiated by mutational analysis of the native ED-L2 promoter and employed in transient transfection studies. This degree of complexity is similar to that found with other PMA-responsive elements. For example, the PMA-responsive element of the histidine decarboxylase promoter has enhancer characteristics, although the full promoter activity cannot be reconstituted in a heterologous promoter system with the minimal sequence required for the PMA response (40). In addition, there is no consensus 12-*O*-tetradecanoylphorbol-13-acetate response element (TGACTCA) in either the ED-L2 or HDC promoter regions necessary for the PMA response (40).

Functional mutational analysis of the ED-L2 promoter revealed motifs that, while not sufficient for full promoter activity, are clearly necessary for promoter activity. In a series of mutations within the native promoter sequence, mutation of the CACACCC motif within the -144 to -114 sequence at position -132 decreased promoter activity to that observed with the ED-L2-114 construct. Mutation of another region between -144 to -114 had no such effect. The -187 to -164 region appears more complicated. Mutation of the E-box motif in the region -187 to -164 at position -181 decreased promoter activity to that seen with the ED-L2-144 and ED-L2-164 constructs. However, mutation of a region adjoining the E-box also had an attenuating effect on promoter activity, although only by 20%. This region contains a motif (TGACACA) with similarity to the AP1 motif TGACTCA. It is conceivable there is cross-regulation between the E-box binding factors and the factors that bind this putative AP1 motif. In aggregate, these data are consistent with several different factors induced by PMA that subsequently bind the ED-L2 promoter in these two *cis*-regulatory regions, leading to its transcriptional activation.

The phorbol ester PMA, a known activator of protein kinase C, exerts its biological effect in squamous epithelial cells or keratinocytes by activating many diverse genes, including the proto-oncogenes *c-fos*, *c-myc*, and *c-sis* (21). Transcription factors known to be important for this response include AP1 (41) and Sp1 (42). Electromobility shift assays used in the current study are one means of gaining insight into the identity of

transcription factors induced by PMA. The region between -187 and -164 binds several nuclear proteins, two of which appear to bind specifically. The complex of slower mobility (complex 1) has characteristics of USF, a helix-loop-helix transcription factor, based on the immune supershift reactions. Elimination of the E-box motif CACCTG in EMSA experiments also eliminates this complex. The estimated molecular masses of complexes 1 and 2 include several proteins, one of which migrates with an apparent mobility of 43 kDa, the approximate molecular mass of USF1 (43 kDa) and USF2 (44 kDa) (43). DNA motifs recognized by the USFs include the CACGTG motif, a member of the canonical CANNTG recognition sequence of this family of helix-loop-helix transcription factors (43). As might be expected with the ubiquitous CANNTG motif, a similar pattern of binding appeared in EMSAs with nuclear extracts from different cell lines of non-keratinocyte lineage. Interestingly, the USF family of proteins was recently shown to have anti-proliferative properties, in that they inhibited transformation of fibroblasts by Ras and c-Myc (44).

The region between -144 and -114 also binds several complexes. Elimination of the CACACCC motif eliminates or attenuates complexes 3-5. The molecular masses of proteins binding this region include proteins clustering in the 35-40-kDa region (45-47). Supershift assays did not provide additional clues as to the identity of complexes 3-5. In examining the different cell lines, complex 3 was uniquely present in PMA-treated TE-11 cells and not in other cell lines tested, whereas complexes 4 and 5 were ubiquitous. Several previously described transcription factors would be candidates for binding to this motif in the ED-L2 promoter. The recently described Krüppel-like family of class TFIIIA transcription factors binds a consensus motif identical to the motif found at ED-L2 promoter position -132, namely CACACCC (45). The CACCC-box was originally identified as a *cis*-acting regulatory element in β -globin promoters that interacts with erythroid Krüppel-like factor (EKLF) which functions as a transactivator in a cell type-specific fashion (46-49). This family now includes several members in addition to EKLF (BTEB2 (50), GKLF/EZF (35, 36), and LKLF (45)) and have molecular masses ranging from 26 to 58 kDa (Table II). These transcription factors are relatively tissue-restricted: LKLF, for example, is found in lung epithelium. Recent studies of GKLF/EZF report expression in the suprabasal layer of esophageal squamous epithelium, precisely where the ED-L2 promoter is active (35). It is possible that this factor, or some related zinc finger transcription factor, contributes to the PMA responsiveness of the ED-L2 promoter. In addition, the GT1-box (CACCC) binding factors, related to the family of Sp1 genes (51-54), and the human papillomavirus E2 binding cellular transcription factors (55) would be candidates based on DNA binding motif homology.

PMA-induced activation of the ED-L2 promoter is complex; full activation requires at a minimum both a downstream CACACCC motif as well as an upstream E-box motif. The effects of phorbol ester are protean, but key among these is the ability to trigger the switch from proliferation to differentiation in the stratified squamous epithelium, mediated through the PKC pathway. There are certain observations that compel us to infer that the ED-L2 promoter is active in differentiating or differentiated cells. First, this promoter is highly active in differentiated cells in tissue culture (17). Second, in the ED-L2-cyclin D1 transgenic mice, the transgene is expressed in basal but mostly in the suprabasal cells. The latter compartment is where early differentiation is initiated (16). The data presented are consistent with the notion that phorbol ester activates the ED-L2 promoter and that two key *cis*-regulatory elements within the ED-L2 promoter interact with several ubiquitous

factors and a cell type-specific factor. Thus, a model emerging from this and other studies (56) is that along the proliferation-differentiation gradient of the stratified squamous epithelium there are viral and eukaryotic promoters that are variably active. These promoters are regulated by ubiquitous and cell type-specific transcription factors which may include the USFs, Krüppel-like factors, and keratinocyte-specific factor (17), as well as AP1, AP2, Sp1, Oct-6 (57), HOXC3 (58), basonuclein (59, 60) and ESE-1 (62). We speculate that these factors may act to regulate the switch from proliferation to early differentiation in keratinocytes.

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