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Definition of the transcription factors which bind the differentiation responsive element of the Epstein–Barr virus BZLF1 Z promoter in human epithelial cells

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Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus and an important human pathogen. Initiation of the EBV lytic cycle is dependent upon transcription of the EBV BZLF1 gene. Our previous studies of transcriptional regulation of the BZLF1 Z promoter (Zp) in human SCC12F epithelial cells identified a region within Zp that is responsive to epithelial cell differentiation. In the present study, we localize this differentiation responsive element to the CREB/AP-1-like binding site (TGACATCA) between −67 to −60 bp within Zp, previously designated ZII, and furthermore show that homodimers and heterodimers of CREB and ATF-1 specifically bind ZII. Consistent with a regulatory role for CREB and ATF-1 in differentiation dependent BZLF1 expression, ZII was able to bind approximately 3-fold more CREB and ATF-1 when incubated with nuclear extract obtained from populations of SCC12F cells enriched for the differentiated phenotype than when incubated with extract obtained from populations enriched for the undifferentiated phenotype. In addition, CREB and ATF-1 were found to increase in abundance during SCC12F differentiation. These results indicate a regulatory role for CREB and ATF-1 in differentiation-dependent expression of BZLF1 in human epithelial cells.

Introduction

Epstein–Barr virus (EBV) is a ubiquitous human herpesvirus, with at least 90% of the adult population worldwide being infected (Niederman *et al.*, 1970). Infection with EBV is usually an asymptomatic childhood disease; however, if primary infection is delayed until early adulthood it can cause infectious mononucleosis (Henle *et al.*, 1968). As with other members of the herpesvirus family, primary infection with EBV is followed by a lifelong persistence of the virus within the human host (Golden *et al.*, 1973). EBV can infect and replicate within certain epithelial cells (Sixbey *et al.*, 1984, 1986; Israele *et al.*, 1991) and B lymphocytes (Pattengale *et al.*, 1973), with oropharyngeal epithelium believed to be the major site of primary infection, replication and spread (Sixbey *et al.*, 1984) and B cells the major site of EBV persistent latency (Miyashita *et al.*, 1995). Furthermore, EBV is associated with malignancies of epithelial and lymphoid origin, including nasopharyngeal carcinoma, Burkitt's lymphoma, B cell lymphomas in immunosuppressed organ transplant and AIDS patients and T cell lymphomas (for review, see Rickinson & Kieff, 1996). The importance of EBV as a human pathogen has warranted the development of vaccines to prevent or modify EBV infection; however, the rational design of an effective EBV vaccine depends upon knowledge of the EBV life-cycle, which is still incompletely understood.

Much data have been generated regarding EBV gene expression in human B lymphocytes using latently infected cell lines. When primary human B lymphocytes are infected with EBV *in vitro* they form continuously proliferating lymphoblastoid cell lines (LCLs; Pope *et al.*, 1968). In most LCLs, the majority of the cells are latently infected with EBV, and EBV gene expression is limited to EBV encoded nuclear antigens (EBNAs) 1 to 6, latent membrane protein, terminal proteins 1 and 2, EBV encoded small nuclear RNAs (EBERs) 1 and 2 and *Bam*HI A transcripts (for review, see Kieff, 1996). Treatment of latently infected B cells with 12-*O*-tetradecanoylphorbol 13-acetate (TPA; zur Hausen *et al.*, 1978), butyrate (Luka *et al.*, 1979) or anti-immunoglobulin (Tovey *et al.*, 1978), results in B cell differentiation and induction of the EBV replicative cycle of infection (Crawford & Ando, 1986), during which 100 or more viral genes are expressed (Kieff, 1996). Studies have shown

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that the viral BZLF1 gene is one of the first genes to be expressed upon induction of the virus lytic cycle (Laux *et al.*, 1988; Takada & Ono, 1989) and that the product of the BZLF1 gene alone is sufficient to trigger the lytic cycle when transfected into latently infected B cells (Countryman & Miller, 1985; Chevallier-Greco *et al.*, 1986; Takada *et al.*, 1986). These results indicate that one of the initial events in the lytic cascade is activation of BZLF1 transcription.

Transcriptional regulation of the BZLF1 gene in B cells is complex. Two promoters can be used to transcribe the BZLF1 gene, Zp and Rp (Manet *et al.*, 1989). Several negative and positive regulatory elements have been defined within Zp (Flemington & Speck, 1990*a*, *b*; Montalvo *et al.*, 1991; Schwarzmann *et al.*, 1994). Negative regulatory elements include a distal 48 bp element (Montalvo *et al.*, 1991) to which the transcription factor YY1 has been shown to bind (Montalvo *et al.*, 1995) and five HI motifs (Schwarzmann *et al.*, 1994), while positive regulatory elements include four ZI elements, the ZII domain and two juxtaposed elements, ZIIIA and ZIIIB (Flemington & Speck, 1990*a*, *b*). The ZI domains are homologous and contain A+T-rich sequences (Flemington & Speck, 1990*a*). Several transcription factors have been shown to bind the ZI domains; MEF2D binds to the region of highest homology between the ZI domains, binding ZIA, ZIB and ZID (Liu *et al.*, 1997*b*). Sp1 and Sp3 bind ZIA, ZIC and ZID, the region of binding being slightly upstream to that of MEF2D (Liu *et al.*, 1997*a*). ZII contains a CREB/AP-1-like binding site (Flemington & Speck, 1990*a*), and recent studies have indicated that the transcription factor ATF-1 can bind ZII (Wang *et al.*, 1997). ZIIIA and ZIIIB are responsive to the BZLF1 gene product, Zta (Flemington & Speck, 1990*b*). ZIIIA contains an AP-1-like binding site while ZIIIB contains novel Zta-binding sequences.

In contrast, little is known regarding EBV gene expression in human epithelial cells, mainly because human epithelial cell lines latently infected with EBV are not available for such studies. However, there are indications that, as with B cells, EBV gene expression is dependent upon the differentiation status of epithelial cells. Studies in which normal human epithelial cell cultures were infected with EBV showed limited EBV gene expression; EBNA was expressed in the adherent, metabolically active cells while lytic cycle antigens were detected in the more differentiated cells shed into the culture medium (Sixbey *et al.*, 1983). Investigations where cultured human epithelial cells were sorted according to differentiation status, infected with EBV and EBV gene expression assessed, showed that EBNA was expressed by the small, largely undifferentiated cells while lytic cycle antigens were detected in the larger, more differentiated cells (Sixbey, 1989). Li *et al.* (1992) carried out studies in which human epithelial cells were stably transfected with the B cell EBV receptor, infected with EBV and EBV gene expression assessed before and after induced epithelial cell differentiation. EBV gene expression in the mainly undifferentiated cells was limited to the latent EBV genes EBNA1 and EBERs 1 and 2, while lytic cycle genes BZLF1, EA-R and VCA were expressed in cells induced to differentiate.

Transcriptional regulation of the BZLF1 gene has been little studied in human epithelial cells. Limited studies in HeLa cells identified two Zta responsive elements (Urier *et al.*, 1989) and a negative regulatory element (Montalvo *et al.*, 1991) within Zp. The HeLa cell line is, however, a fully transformed line that retains few of the characteristics of cultured normal keratinocytes and is known to contain human papillomavirus sequences (Schwarz *et al.*, 1985). The effects of epithelial cell differentiation upon transcriptional regulation of BZLF1 Zp were not investigated. Recently, we published data on differentiation responsive transcriptional regulation of the BZLF1 gene in the human epithelial cell line SCC12F (Karimi *et al.*, 1995), a line that retains many characteristics of cultured normal keratinocytes including responsiveness to terminal differentiation signals (Rheinwald & Beckett, 1980). We identified a region of −221Zp from −86 to +12 bp that is responsive to terminal differentiation signals of human epithelial SCC12F cells. In this report, we define the SCC12F differentiation responsive element within −86 to +12Zp, and identify the transcription factors that specifically bind this element during differentiation of SCC12F cells.

Methods

■ **Cell lines and culture.** SCC12F is an immortal, non-tumorigenic human epithelial cell line derived from a squamous cell carcinoma of the facial epidermis (Rheinwald & Beckett, 1980). SCC12F cells were cultured as previously described (Karimi *et al.*, 1995). Undifferentiated mouse embryo teratocarcinoma F9 (UF9) cells were cultured in Dulbecco's modified Eagle's medium plus 10% foetal calf serum on tissue culture plates coated with 0·1% porcine skin gelatin.

■ **Plasmids and transfection.** −221ZpCAT, BS-CAT, −221MIIZpCAT and PGL2CAT were kindly provided by S. H. Speck, Washington University School of Medicine, USA (Flemington & Speck, 1990*a*, *b*). $(CRE)_3$.TK.luciferase contains three copies of the fibronectin CRE upstream of the firefly luciferase gene and was the generous gift of N. C. Jones, Imperial Cancer Research Fund, UK (Benbrook & Jones, 1990). RSV-ATF-1 and RSV-CREB were constructed by inserting ATF-1 and CREB cDNAs into the RSV-pECE expression vector and were the kind gift of M. R. Green, University of Massachusetts Medical Center, USA (Liu *et al.*, 1993). pCMV*β*-gal was supplied by Stratagene. Constructs were transiently transfected into SCC12F and UF9 cells using Lipofectin Reagent (Life Technologies) as previously described (Karimi *et al.*, 1995). For UF9 cells, freshly confluent plates were split 1:10 5 h before transfection.

■ **Induction of terminal differentiation**

Suspension culture. SCC12F cells were cultured in suspension to induce epithelial cell terminal differentiation (Green, 1977; Karimi *et al.*, 1995). Aliquots of cells were taken before, and at various times during culture in suspension, for *β*-galactosidase and chloramphenicol acetyltransferase (CAT) assays, and for staining with anti-involucrin antibody.

Maintenance of cultures at confluence. Suspension culture of SCC12F cells is a good method for obtaining populations of cells enriched for the differentiated phenotype; however, this system generates small

numbers of cells. Large numbers of cells were required for nuclear extract preparation. SCC12F populations enriched for the differentiated phenotype were obtained from cultures maintained at confluency for 3 weeks. During this time the cells undergo extensive differentiation and after 3 weeks populations contain approximately 40% differentiated cells as assessed by involucrin positivity.

Subconfluent SCC12F cultures were used as a source of populations enriched for the undifferentiated phenotype, and contain up to 95% undifferentiated, involucrin-negative cells. Aliquots of cells from subconfluent and confluent SCC12F cultures were taken for staining with anti-involucrin antibody.

■ **β-Galactosidase assay.** Samples were assessed for β-galactosidase activity to allow standardization of transfection efficiencies and therefore enable a quantitative comparison within and between experiments. We have previously shown that β-galactosidase expression from pCMVβ-gal remains constant during differentiation of the SCC12F epithelial cell line (Karimi *et al.*, 1995). Cell extracts were prepared as previously described (Karimi *et al.*, 1995) and β-galactosidase activity determined using a Promega kit.

■ **CAT and luciferase assays.** Cell extracts containing similar β-galactosidase activities were assessed for CAT activity as previously described (Karimi *et al.*, 1995) or for luciferase activity using a Promega kit.

■ **Antibodies.** Rabbit antiserum to human involucrin (DH1) was the generous gift of F. M. Watt, Imperial Cancer Research Fund, UK (Dover & Watt, 1987). Rabbit antiserum to human CREB (KL2) was supplied by Serotec and recognizes all isoforms of CREB. Mouse monoclonal antibody to human ATF-1 (C41–5.1), rabbit antisera to human c-Jun (N), c-Fos (K-25) and OCT-1 (C-21) were provided by Santa Cruz; ATF-1 (C41–5.1) is non-cross-reactive with other ATF/CREB transcription factors, c-Jun (N) is non-cross-reactive with JunB or JunD while c-Fos (K-25) is broadly reactive with c-Fos, FosB, Fra-1 and Fra-2.

■ **Indirect immunofluorescence staining.** Cells were stained for involucrin using rabbit antiserum DH1 (Karimi *et al.*, 1995). Cells were photographed and at least 200 cells per sample counted to determine the percentage of involucrin-positive cells.

■ **Gel mobility shift and supershift assays.** The sequences of oligonucleotides used in gel mobility shift assays are shown below.

ZII 5′ AAACCA**TGACATCA**CAGAG 3′
3′ TTTGGTACTGTAGTGTCTC 5′
ZII(M) 5′ AAACGA**ATTCATCA**CAGAG 3′
3′ TTTGCTTAAGTAGTGTCTC 5′
CREB 5′ AGAGATTGCC**TGACGTCA**GAGAGCTAG 3′
3′ TCTCTAACGGACTGCAGTCTCTCGATC 5′
AP-1 5′ CGCTTGA**TGAGTCA**GCCGGAA 3′
3′ GCGAACTACTCAGTCGGCCTT 5′
OCT-1 5′ TGTCGA**ATGCAAAT**CACTAGAA 3′
3′ ACAGCTTACGTTTAGTGATCTT 5′

Oligonucleotides were end-labelled using T4 polynucleotide kinase and [γ-^{32}P]ATP. Nuclear extracts were prepared by the Dignam method (Dignam *et al.*, 1983). Nuclear extract (10 μg) was diluted with 5 × binding buffer [20% glycerol, 5 mM $MgCl_2$, 2·5 mM EDTA, 2·5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH 7·5), 1 mg/ml salmon sperm DNA] and incubated at room temperature for 10 min. End-labelled oligonucleotide was added to the mixture and incubated at room temperature for a further 20 min. DNA–protein complexes were resolved on a 4% non-denaturing polyacrylamide gel at 150 V for ∼ 1·5 h at room temperature.

For competition studies, 300-fold molar excess unlabelled oligonucleotide was added to the reaction mixture prior to the addition of radiolabelled probe. For supershift assays, 1 μg antibody was added to the reaction mixture before addition of end-labelled probe.

■ **Immunoblotting.** Immunoblotting was performed as previously described (Nicholson *et al.*, 1997). ATF-1-specific antibody C41–5.1 was used at a final concentration of 0·5 μg/ml, CREB antibody KL2 was used at a dilution of 1:400 and OCT-1 antibody C-21 was used at a concentration of 0·2 μg/ml.

Results

ZII domain involved in differentiation linked regulation of −221Zp

Previously we reported that the region of −221Zp from −86 to +12 bp contained sequences responsive to epithelial cell differentiation (Karimi *et al.*, 1995). This region has been shown to contain a CREB/AP-1-like binding site (TGACATCA) located between −67 to −60 bp that has been designated ZII (Flemington & Speck, 1990*a*). To determine whether ZII was involved in differentiation linked regulation of −221Zp, SCC12F cells were transfected with a CAT reporter plasmid, −221MIIZpCAT, containing a 3 bp mutation of ZII (TGACATCA to ATTCATCA). At 24 h post-transfection the cells were induced to differentiate in suspension culture and CAT activity was assessed before and at 72 h post-differentiation. Involucrin expression was used to assess the differentiation status of the SCC12F cells and was visualized by indirect immunofluorescence. Involucrin is the major protein precursor of the cornified envelope (Rice & Green, 1979) which is a structure formed beneath the plasma membrane of human keratinocytes at the end stage of differentiation. When human squamous epithelial cells are cultured on plastic all differentiating cells express involucrin, which as such serves as an ideal marker of epithelial cell differentiation. For comparison, results previously obtained using −221ZpCAT (Karimi *et al.*, 1995) and showing differentiation-linked regulation of −221Zp are represented in Fig. 1(A). Results shown in Fig. 1(B) are representative of three separate experiments. Involucrin expression was found to increase 5-fold from 4% involucrin-positive cells at time 0 to 20% involucrin-positive cells at 72 h post-differentiation, while CAT activity remained constant: 2·2% acetylation was observed at time 0 and 1·8% acetylation at 72 h post-differentiation. The promoterless PGL2CAT construct was included as a negative control. CAT activity was not detected with the PGL2CAT construct, either before or following induced terminal differentiation. Results indicate that mutation of ZII results in loss of −221Zp epithelial cell differentiation responsiveness.

Cellular factors specifically bind ZII

To determine whether ZII can specifically bind SCC12F nuclear factors and confirm the role of ZII in differentiation linked regulation of −221Zp, bandshift experiments were carried out using radiolabelled ZII oligonucleotide and nuclear

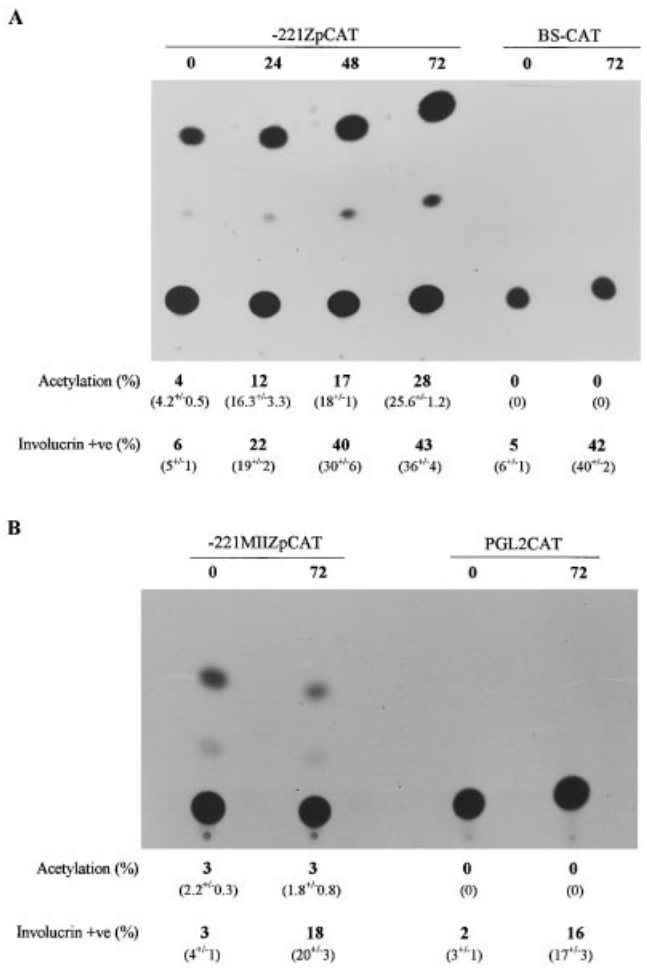

Fig. 1. −221ZpCAT and −221MIIZpCAT activity in SCC12F cells induced to undergo terminal differentiation in suspension culture. (A) SCC12F cells were transfected with 1·5 μg pCMV*β*-gal plus 10 μg −221ZpCAT or BS-CAT, and 24 h later were placed in suspension culture. CAT activity and involucrin expression were assessed after 0, 24, 48 and 72 h in suspension. (B) SCC12F cells were transfected with 1·5 μg pCMV*β*-gal plus 10 μg −221MIIZpCAT or PGL2CAT, and 24 h later were placed in suspension culture. CAT activity and involucrin expression were assessed after 0 and 72 h in suspension. *β*-Galactosidase expression was used to standardize transfection efficiencies and all samples assessed for CAT activity contained 0·8 milliunits *β*-galactosidase activity. Percentage acetylations were determined by liquid scintillation counting and involucrin expression was visualized by indirect immunofluorescence. Values in parentheses are averages of three separate experiments ± SEM. Panel (A) reproduced from Karimi *et al.* (1995).

extract from SCC12F populations enriched for undifferentiated (~ 5% involucrin-positive cells) and differentiated phenotypes (~ 40% involucrin-positive cells). Fig. 2 shows a representative result of three experiments carried out with different nuclear extract preparations. Results indicated the presence of four ZII binding complexes in SCC12F nuclear extracts, including three slower migrating complexes (bands a, b and c) and a faster migrating complex (band d). The mobilities of the four complexes were similar regardless of whether nuclear extract was obtained from populations enriched for the undifferentiated or the differentiated phenotype. The intensity of the bands was, however, higher when nuclear extract was obtained from cells enriched for the differentiated phenotype when compared with that obtained from cells enriched for the undifferentiated phenotype. In competition studies the slower migrating complexes (bands a, b, c) were specifically removed by inclusion of unlabelled ZII oligonucleotide but not by competition with mutated ZII [ZII(M)] or OCT-1 oligonucleotides. These results indicate that ZII can specifically bind SCC12F nuclear factors and that ZII is able to bind a greater quantity of factors upon differentiation of SCC12F cells.

Bandshift experiments were carried out to compare relative quantities of specific factors able to bind ZII in SCC12F nuclear extracts obtained from populations of cells enriched for the differentiated and undifferentiated phenotypes. A series of 5-fold dilutions of differentiated and undifferentiated nuclear extracts containing equal amounts of protein was prepared and incubated with radiolabelled ZII. Results showed that increasing dilutions of nuclear extracts resulted in a decrease in intensity of bands containing specific ZII binding factors (data not shown). The intensity of specific bands obtained when nuclear extract from populations enriched for the undifferentiated phenotype was diluted 1/5 was equivalent to the intensity of specific bands obtained when nuclear extract from populations enriched for the differentiated phenotype was diluted 1/15 (data not shown). These results indicate an approximate 3-fold increase in the quantity of specific factors able to bind ZII following differentiation of SCC12F cells.

Control bandshift experiments were carried out to demonstrate that the ability of ZII to bind more SCC12F proteins in response to epithelial cell differentiation was a specific response. Radiolabelled OCT-1 probe was incubated with nuclear extracts obtained from populations of SCC12F cells enriched for the undifferentiated and differentiated phenotypes. Results shown in Fig. 3 are representative of three experiments carried out using different nuclear extracts. Two OCT-1 binding complexes (bands a and b) were detected in extracts enriched for the undifferentiated and differentiated phenotypes. Competition experiments indicated that the slower migrating complex (band a) contained specific OCT-1 binding proteins. There was no apparent difference in the intensity of the bands obtained when OCT-1 was incubated with undifferentiated or differentiated nuclear extract, indicating that OCT-1 binds similar amounts of SCC12F proteins during epithelial cell differentiation. Consistent with this finding, immunoblot analysis indicated that similar amounts of OCT-1 protein were present in undifferentiated and differentiated SCC12F cells (see Fig. 7).

Identification of cellular factors that bind ZII

The ZII element (TGACATCA) resembles the binding motif for members of the CREB/ATF family of proteins (TGACGTCA) and the binding sequence for members of the

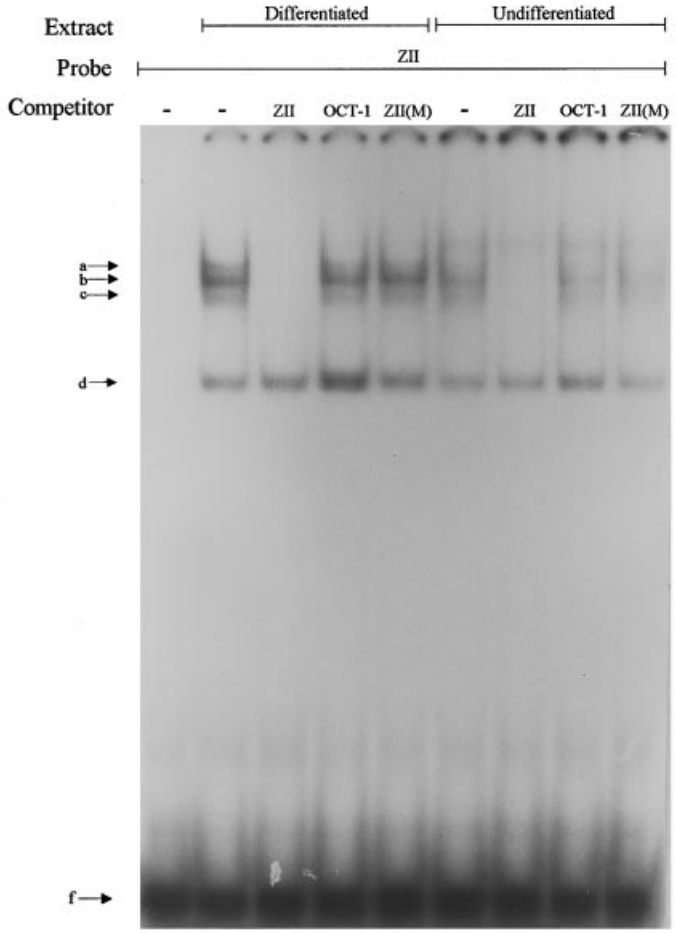

Fig. 2

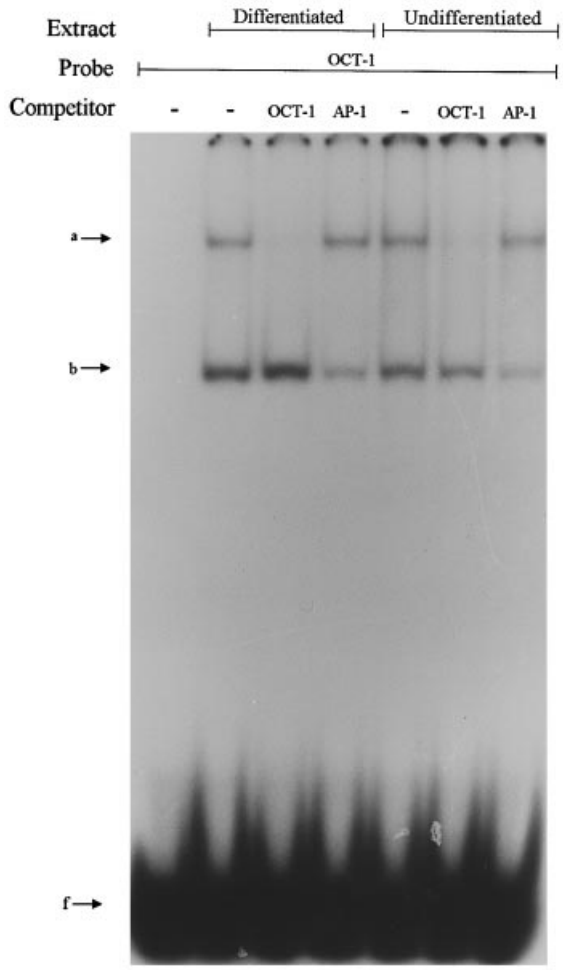

Fig. 3

Fig. 2. Specific binding of SCC12F nuclear factors to ZII. Nuclear extracts containing 10 μg protein from SCC12F cells enriched for the differentiated and undifferentiated phenotypes were incubated with radiolabelled ZII oligonucleotide and protein–DNA complexes separated from unbound probe (f) by electrophoresis through a 4% non-denaturing polyacrylamide gel. A control in which nuclear extract was replaced with water was included. Unlabelled competitor oligonucleotides were incubated with nuclear extract at 300-fold molar excess prior to the addition of probe. Protein–DNA complexes are indicated to the left of the gel.

Fig. 3. OCT-1 binding proteins in SCC12F cells. Nuclear extracts containing 10 μg protein from SCC12F cells enriched for the differentiated and undifferentiated phenotypes were incubated with radiolabelled OCT-1 oligonucleotide and protein–DNA complexes separated from unbound probe (f) by electrophoresis through a non-denaturing polyacrylamide gel. A control in which nuclear extract was replaced with water was included. Unlabelled competitor oligonucleotides were incubated with nuclear extract at 300-fold molar excess prior to the addition of probe. Protein–DNA complexes are indicated to the left of the gel.

Fos/Jun families of proteins (TGAGTCA). To determine whether consensus CREB or AP-1 sequences could compete effectively for SCC12F nuclear factors that bind ZII, bandshift experiments were carried out in which nuclear extract obtained from populations of SCC12F cells enriched for the differentiated phenotype was incubated with either radiolabelled ZII, CREB or AP-1 oligonucleotide. At least three experiments were carried out using different nuclear extract preparations; a representative result is shown in Fig. 4. When nuclear extract was incubated with radiolabelled ZII four protein–DNA complexes were formed (bands a, b, c and d) with the three slower migrating complexes (bands a, b and c) being specifically removed when excess unlabelled ZII was incubated with extract prior to the addition of radiolabelled ZII. Similarly, when unlabelled CREB oligonucleotide was used as competitor the three slower migrating complexes were specifically removed. In contrast, incubation of extract with unlabelled ZII (M) or OCT-1 oligonucleotide did not inhibit specific complex formation. A slight reduction in specific complex formation was observed when extract was incubated with unlabelled AP-

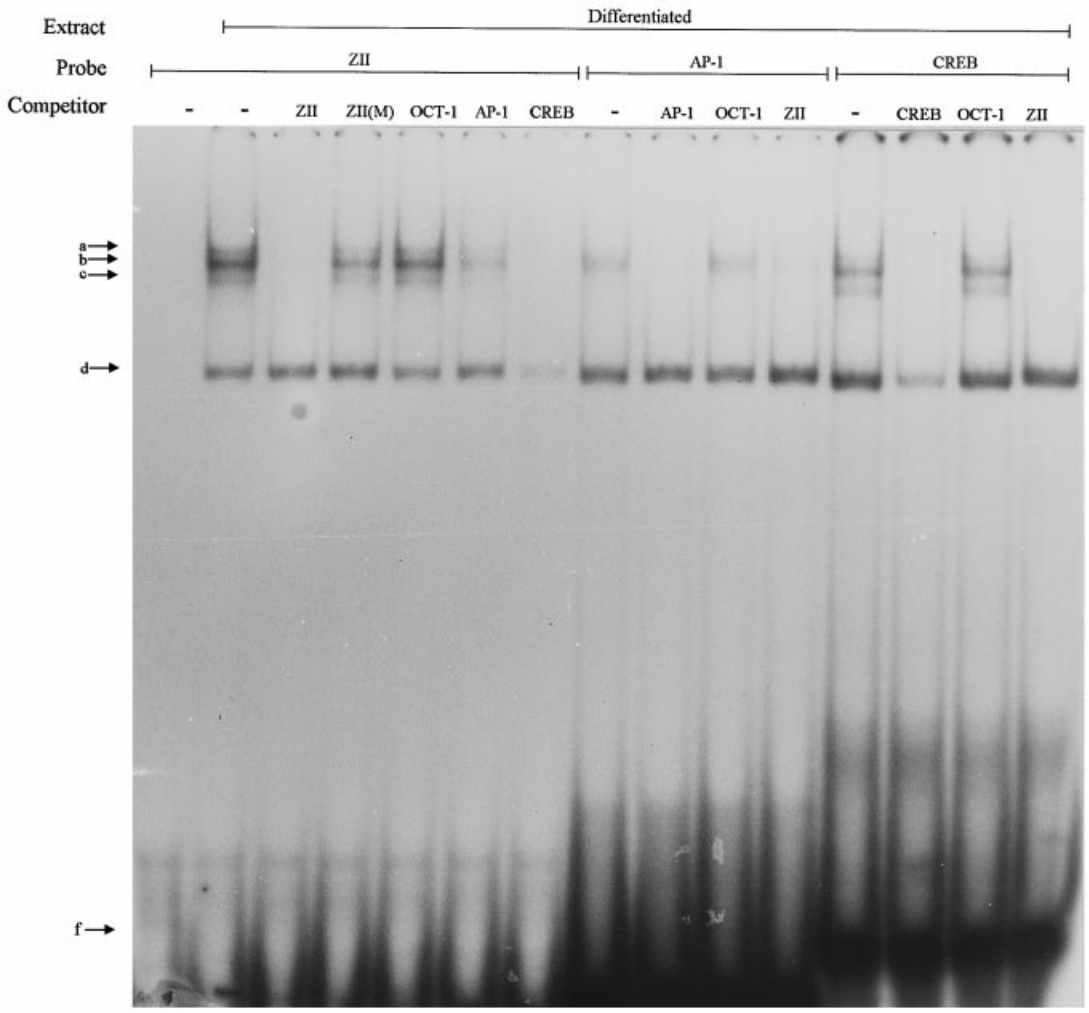

Fig. 4. CREB binding site competes with ZII for binding specific SCC12F nuclear factors. Nuclear extract containing 10 μg protein from SCC12F cells enriched for the differentiated phenotype was incubated with radiolabelled ZII, AP-1 or CREB oligonucleotide and protein–DNA complexes separated from unbound probe (f) by electrophoresis through a 4% non-denaturing polyacrylamide gel. A control in which nuclear extract was replaced with water was included. Unlabelled competitor oligonucleotides were incubated with nuclear extract at 300-fold molar excess prior to the addition of probe. Protein–DNA complexes are indicated to the left of the gel.

1 oligonucleotide. When SCC12F nuclear extract was incubated with radiolabelled CREB oligonucleotide, four protein–DNA complexes were formed (bands a, b, c and d) which had similar mobilities to the complexes formed when radiolabelled ZII was incubated with nuclear extract. The three slower migrating complexes (bands a, b and c) were specifically removed by incubation of extract with either unlabelled CREB or ZII oligonucleotide, while competition studies with OCT-1 oligonucleotide had no effect on complex formation. When SCC12F nuclear extract was incubated with radiolabelled AP-1 probe two protein–DNA complexes were formed; the faster migrating complex had a similar mobility to the non-specific complex formed when radiolabelled ZII or CREB was incubated with nuclear extract (band d), while the slower migrating complex had a slightly different mobility to the three specific complexes formed when nuclear extract was incubated with radiolabelled ZII or CREB. In competition studies, unlabelled AP-1 removed the slower migrating complex, while heterologous OCT-1 competitor had no effect on complex formation. A reduction in specific complex formation was observed when ZII competitor was incubated with nuclear extract. These results indicate that the CREB consensus sequence is an effective competitor for SCC12F factors that specifically bind ZII, that consensus AP-1 sequence competes ineffectively for specific ZII binding factors, and that ZII competes inefficiently for AP-1 binding factors.

Our data indicate that ZII and CREB sequences bind similar SCC12F nuclear factors. The consensus CREB binding sequence can bind members of the CREB/ATF family of transcription factors, which can form homodimeric and hetero-

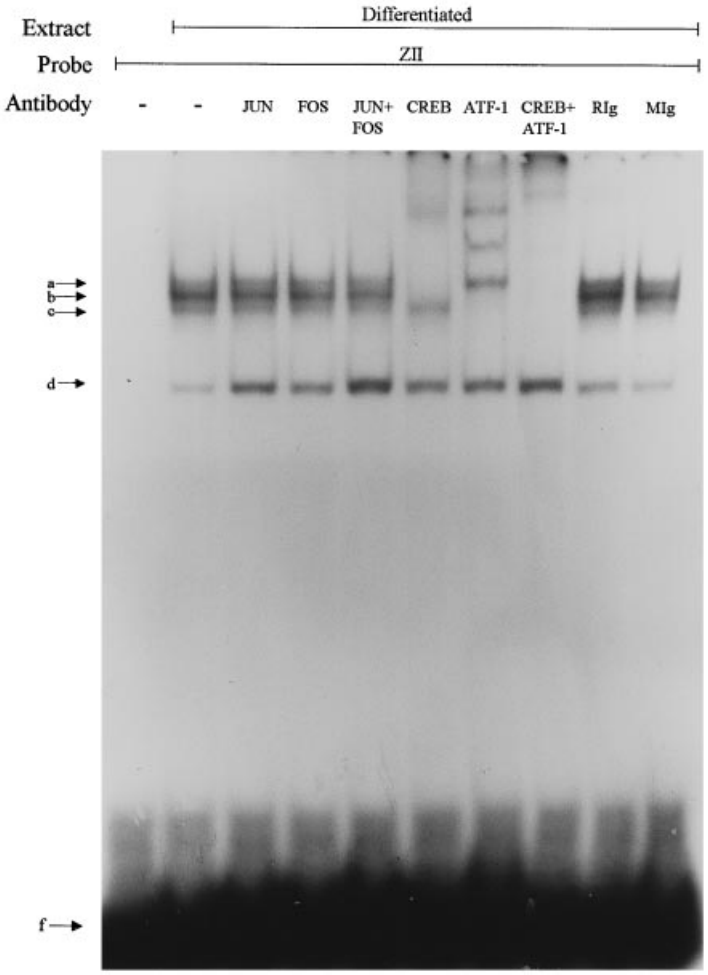

Fig. 5

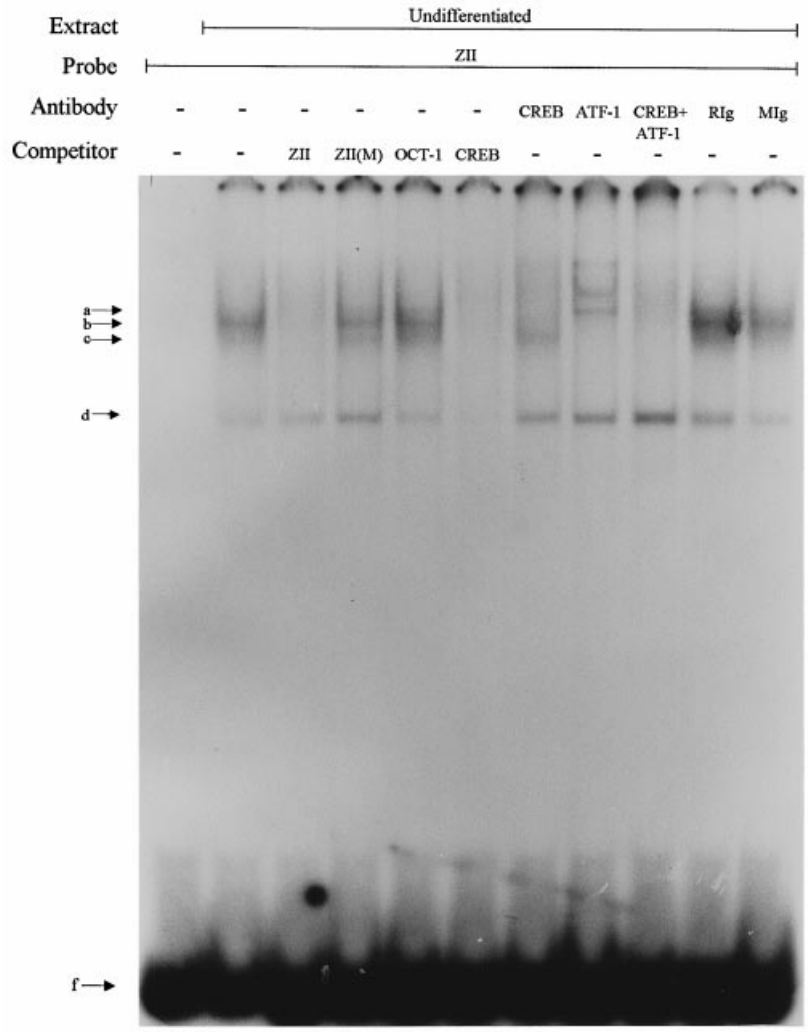

Fig. 6

Fig. 5. Specific binding of CREB and ATF-1 to ZII in SCC12F cells. Nuclear extract containing 10 μg protein from SCC12F cells enriched for the differentiated phenotype was incubated with radiolabelled ZII oligonucleotide and protein–DNA complexes separated from unbound probe (f) by electrophoresis through a 4% non-denaturing polyacrylamide gel. A control in which nuclear extract was replaced with water was included. Antibodies to Fos, Jun, CREB and ATF-1 were incubated with nuclear extract prior to the addition of probe. Control supershifts were carried out with rabbit anti-mouse immunoglobulins (RIg) and mouse anti-rabbit immunoglobulin (MIg). Protein–DNA complexes are indicated to the left of the gel.

Fig. 6. CREB and ATF-1 bind ZII in undifferentiated enriched populations of SCC12F cells. Nuclear extract containing 10 μg protein from SCC12F cells enriched for the undifferentiated phenotype was incubated with radiolabelled ZII oligonucleotide and protein–DNA complexes separated from unbound probe (f) by electrophoresis through a 4% non-denaturing polyacrylamide gel. A control in which nuclear extract was replaced with water was included. Unlabelled competitor oligonucleotides at 300-fold molar excess and antibodies to CREB and ATF-1 were incubated with nuclear extract prior to the addition of probe. Control supershifts were carried out with rabbit anti-mouse immunoglobulins (RIg) and mouse anti-rabbit immunoglobulin (MIg). Protein–DNA complexes are indicated to the left of the gel.

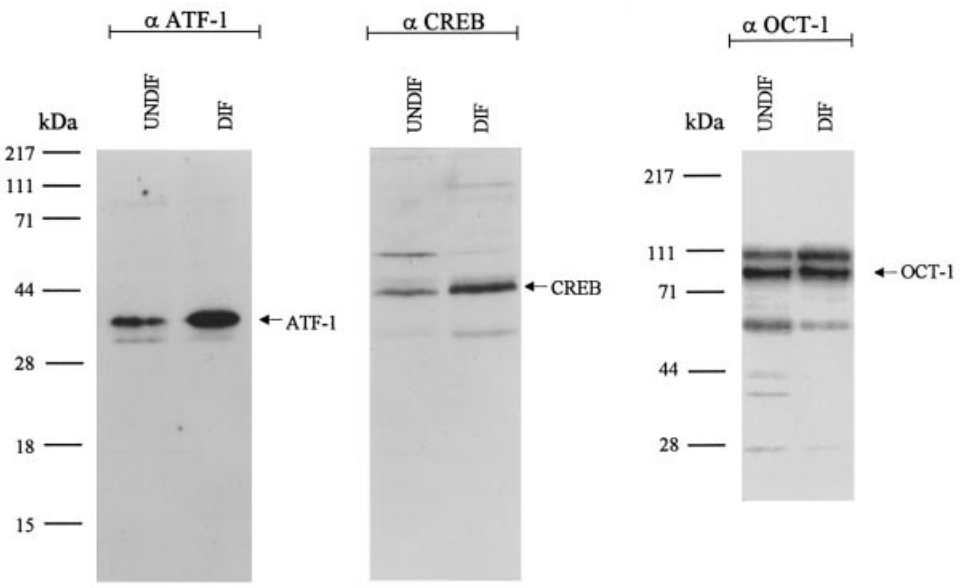

Fig. 7. CREB and ATF-1 increase in abundance while OCT-1 remains constant during differentiation of SCC12F cells. SCC12F nuclear extracts (40 μg) from populations enriched for the undifferentiated (UNDIF) and differentiated (DIF) phenotypes were electrophoresed through a 12% SDS–polyacrylamide gel, electrotransferred to PVDF and incubated with antibodies to ATF-1 (α ATF-1), CREB (α CREB) and OCT-1 (α OCT-1). Molecular size markers are indicated.

dimeric complexes, as well as dimeric complexes with various Fos and Jun family members (for review see Meyer & Habener, 1993). To identify the SCC12F nuclear factors that bind ZII, supershift experiments were carried out in which antibodies to CREB, ATF-1, Fos and c-Jun were incubated with SCC12F nuclear extract obtained from populations enriched for the differentiated phenotype prior to incubation with radiolabelled ZII. Control supershifts were carried out with rabbit anti-mouse and mouse anti-rabbit immunoglobulins. Three experiments were carried out using different nuclear extracts and representative results are shown in Fig. 5. As already demonstrated, three specific protein–DNA complexes were detected upon incubation of SCC12F nuclear extract with ZII probe (bands a, b, c). Incubation of CREB antiserum with SCC12F nuclear extract supershifted bands a and b, while addition of monoclonal antibody to ATF-1 supershifted bands b and c. Antibodies to Fos or c-Jun had no effect on the formation or mobility of specific DNA–protein complexes. These results identify homodimers and heterodimers of CREB and ATF-1 as the SCC12F nuclear proteins that bind ZII: band a contains CREB homodimers, band b CREB/ATF-1 heterodimer and band c ATF-1 homodimer. Fos and Jun family members do not appear to form part of the specific DNA–protein complexes produced following incubation of SCC12F nuclear extract with ZII.

Results from bandshift experiments indicated that similar factors within undifferentiated and differentiated SCC12F populations specifically bind ZII (Fig. 2). To confirm this, bandshift and supershift experiments were carried out using nuclear extracts obtained from SCC12F populations enriched for the undifferentiated phenotype. Fig. 6 is representative of three experiments using different nuclear extracts. Results indicated that three specific protein–DNA complexes (bands a, b and c) were formed following incubation of nuclear extract with radiolabelled ZII, and that unlabelled CREB oligonucleotide effectively competed with ZII for binding SCC12F nuclear factors. Incubation of CREB antibody with extract prior to addition of probe supershifted bands a and b while addition of ATF-1 antibody shifted bands b and c, indicating homo- and heterodimers of CREB and ATF-1 bind ZII in populations of SCC12F cells enriched for the undifferentiated phenotype.

Bandshift and supershift experiments indicated that homodimers and heterodimers of ATF-1 and CREB bind ZII in SCC12F cells and that amounts of these transcription factors able to bind ZII increased with epithelial cell differentiation. To determine whether the increased ability of ATF-1 and CREB to bind ZII with SCC12F differentiation was due, at least in part, to increased abundance of these factors, immunoblot analyses were carried out. Fig. 7 is representative of at least three experiments using separate nuclear extracts. Incubation of immunoblots with antibody to all isoforms of CREB detected a 43 kDa protein corresponding to CREB that increased in abundance with differentiation of SCC12F cells. Similar studies using antibody to ATF-1 detected a 35 kDa protein corresponding to ATF-1 that also increased in abundance with SCC12F epithelial cell differentiation. Immunoblot analysis using antibody to OCT-1 detected an 84 kDa protein corresponding to OCT-1 that remained constant with SCC12F differentiation. OCT-1 protein levels served as a loading control and in all immunoblot analyses similar amounts of

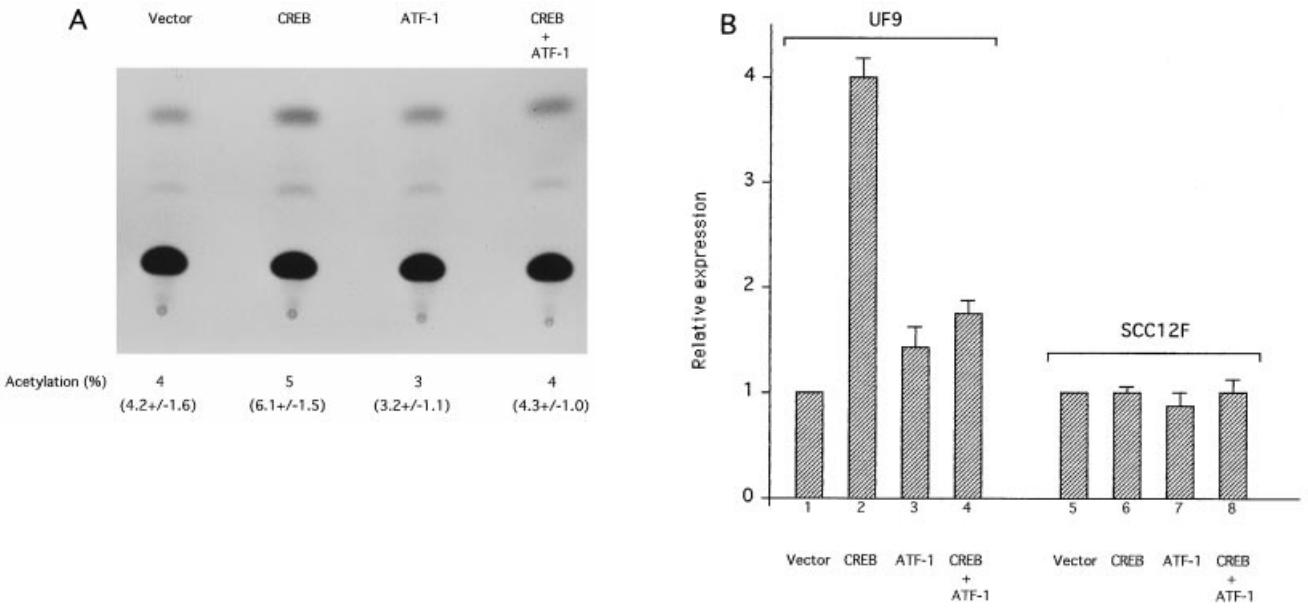

Fig. 8. Transcriptional activity of transfected CREB and ATF-1 cDNAs in undifferentiated SCC12F and F9 cells. (A) Undifferentiated SCC12F cells were transfected with 1·5 μg pCMVβ-gal and 5 μg −221ZpCAT. In addition, cells were transfected with 10 μg RSV-pECE, 5 μg RSV-CREB plus 5 μg RSV-pECE, 5 μg RSV-ATF-1 plus 5 μg RSV-pECE or 5 μg RSV-CREB plus 5 μg RSV-ATF-1. Cell extracts were prepared for β-galactosidase and CAT assays 40 h after transfection. β-Galactosidase expression was used to standardize transfection efficiencies and all samples assessed for CAT activity contained 0·8 milliunits β-galactosidase activity. Percentage acetylations were determined by liquid scintillation counting. Values in parentheses are averages of three separate experiments ± SEM. (B) Undifferentiated F9 and SCC12F cells were transfected with 1·5 μg pCMVβ-gal and 5 μg $(CRE)_3$.TK.luciferase. In addition, cells were transfected with 10 μg RSV-pECE (lanes 1 and 5), 5 μg RSV-CREB plus 5 μg RSV-pECE (lanes 2 and 6), 5 μg RSV-ATF-1 plus 5 μg RSV-pECE (lanes 3 and 7) or 5 μg RSV-CREB plus 5 μg RSV-ATF-1 (lanes 4 and 8). Cell extracts were prepared for β-galactosidase and luciferase assays 40 h after transfection. β-Galactosidase expression was used to standardize transfection efficiencies. Luciferase expression is presented relative to that seen for control expression vector RSV-pECE (equivalent to 1·0). Error bars show the variation observed in three separate experiments.

OCT-1 protein were present in extracts of undifferentiated and differentiated SCC12F cells.

Transcriptional activity of transfected CREB and ATF-1 cDNAs in undifferentiated SCC12F and F9 cells

Experiments were carried out to determine whether co-transfection of undifferentiated SCC12F cells with −221ZpCAT and CREB/ATF-1 expression vectors would give rise to similar levels of −221ZpCAT activity observed in differentiated cells. Results in Fig. 8(A) are representative of three experiments and indicate that neither CREB nor ATF-1 expression affected transcription of −221ZpCAT in undifferentiated cells. To confirm that the CREB/ATF-1 expression vectors were working, UF9 cells were co-transfected with CREB/ATF-1 expression vectors plus a synthetic promoter/reporter, $(CRE)_3$.TK.luciferase. Consistent with published work (Benbrook & Jones, 1990; Ellis *et al.*, 1995), CREB induced a 4-fold increase in luciferase activity while ATF-1 induced a slight increase when expressed alone and inhibited CREB induced activity when co-expressed with CREB (Fig. 8 B, lanes 1–4). When undifferentiated SCC12F cells were co-transfected with $(CRE)_3$.TK.luciferase plus CREB/ATF-1 expression vectors, CREB and ATF-1 had no detectable effect upon luciferase activity (Fig. 8 B, lanes 5–8). Results indicate that CREB and ATF-1 expressed in undifferentiated SCC12F cells are not functional, possibly due to the absence/presence of modifying enzymes in undifferentiated SCC12F cells.

Discussion

BZLF1 encodes an important activator of viral and cellular genes critical for induction of the EBV lytic cycle. Transcriptional regulation of BZLF1 has been little studied in human epithelial cells. Studies carried out in HeLa cells identified two Zta responsive elements (Urier *et al.*, 1989) and a negative regulatory element (Montalvo *et al.*, 1991) within Zp. More recently, we studied BZLF1 Zp transcriptional regulation in SCC12F cells that were induced to differentiate, and identified a region within Zp that is responsive to epithelial cell differentiation (Karimi *et al.*, 1995). In this report we used CAT assay analyses to identify a CREB/AP-1-like binding site (TGACATCA) located between −67 and −60 bp of Zp as the element responsive to SCC12F epithelial cell differentiation. This element has been previously designated ZII (Flemington & Speck, 1990*a*).

Data obtained from bandshift experiments indicated that CREB binding proteins bind ZII in SCC12F cells, and that

approximately 3-fold more CREB binding proteins were able to bind ZII upon differentiation of SCC12F cells. Furthermore, supershift experiments identified homodimers and heterodimers of CREB and ATF-1 as the factors that specifically bind ZII in SCC12F epithelial cells. These experiments are the first to indicate a regulatory role for CREB and ATF-1 in epithelial cell differentiation-dependent expression of EBV genes, and implicate these transcription factors in the regulation of cellular gene expression during normal human epithelial cell differentiation. Interestingly, the CREB binding sequence is found in the promoters of cellular genes known to be differentially expressed during epithelial cell differentiation, including c-*fos* and fibronectin (Sassone-Corsi *et al*., 1988; Dean *et al*., 1989; Bowlus *et al*., 1991; Nicholson & Watt, 1991; Gandarillas & Watt, 1995).

Transcriptional regulation of BZLF1 has been studied extensively in B cells. Regulation is complex involving the interaction of viral and cellular transcription factors with positive and negative regulatory elements within Zp. The ZII domain is believed to be involved in the initial activation of Zp, and has been shown to play a crucial role in responsiveness to TPA and anti-immunoglobulin (Flemington & Speck, 1990*a*; Daibata *et al*., 1994). Recent studies by Wang *et al*. (1997) have indicated that ATF-1 can bind ZII and is involved in transactivation of Zp in various B cell lines. In addition, CREB was implicated, although not identified, as an additional ZII binding protein. In Jurkat T cells, studies have indicated that phosphorylated CREB transactivates Zp at ZII in the presence of protein kinase A (Flamand & Menezes, 1996). These results suggest that ATF-1 and/or CREB may play a common role in the transactivation of Zp at ZII in epithelial cells and in B and T lymphocytes.

Immunoblot analyses indicated that ATF-1 and CREB increased in abundance during SCC12F differentiation. This may explain, at least in part, the increased binding of ATF-1 and CREB to ZII and their contribution to Zp transactivation following differentiation of SCC12F cells (Karimi *et al*., 1995). ATF-1 expression levels have similarly been reported to increase upon activation of normal human T lymphocytes (Hsueh & Lai, 1995). In contrast, bandshift studies carried out in T lymphocytes indicated equal binding of CREB to the ZII domain of Zp in mock-infected and human herpesvirus-6 (HHV-6)-infected Jurkat T cells (Flamand & Menezes, 1996). Differences between our results and those of other workers may reflect cell type-specific differences.

The transcriptional activity of both CREB and ATF-1 has been shown to be directly influenced by phosphorylation. Phosphorylation of CREB at serine-133 stimulates transcriptional activity (Gonzalez & Montminy, 1989; Lee *et al*., 1990; Sheng *et al*., 1991), while phosphorylation at serine-142 results in inhibition of transcriptional activity (Sun *et al*., 1994). Similarly, phosphorylation of ATF-1 has been shown to regulate transcriptional activity (Shimomura *et al*., 1996). The studies of Flamand & Menezes (1996) indicated that CREB was in a phosphorylated, transcriptionally active form in HHV-6-infected Jurkat cells. Data obtained from experiments in which undifferentiated SCC12F cells were co-transfected with −221ZpCAT and CREB/ATF-1 expression vectors indicated that CREB and ATF-1 were not transcriptionally active in undifferentiated SCC12F cells. We are currently investigating the phosphorylation and transcriptional activation status of CREB and ATF-1 in undifferentiated and differentiated SCC12F cells in order to gain an understanding of the mechanism by which these ZII binding transcription factors contribute to the transactivation of Zp in differentiating human epithelial cells.

The pathway by which CREB and ATF-1 regulate Zp in human epithelial cells is unclear, but one could speculate that Ca^{2+}-signalling pathways are involved as intracellular Ca^{2+} levels increase during epithelial cell differentiation. Indeed, it has recently been shown that expression of a Ca^{2+}/calmodulin-dependent protein kinase, CamKIV, activates −221Zp in B cells (Chatila *et al*., 1997). Activation of −221Zp was dependent upon functional ZII and ZI elements. The capacity of CamKIV to activate transcription through the ZII element is consistent with the ability of CamKIV to phosphorylate CREB at serine-133 (Sun *et al*., 1994).

Our data show that CREB and ATF-1 are present in nuclear extracts obtained from populations of SCC12F cells enriched for the undifferentiated phenotype. We do not know whether this is due to the presence of approximately 5% differentiated SCC12F cells or whether basal levels of CREB and ATF-1 are present in undifferentiated SCC12F cells – studies are in progress to resolve this issue.

In conclusion, we have identified ZII as the element within Zp that is responsive to terminal differentiation signals of human epithelial SCC12F cells, and have identified CREB and ATF-1 as the cellular transcription factors that specifically bind ZII, indicating a role for these factors in the differentiation dependent regulation of Zp in human epithelial cells. These results provide new information relevant to the life-cycle of EBV, and therefore of importance in the development of an effective EBV vaccine.

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References

Benbrook, D. M. & Jones, N. C. (1990). Heterodimer formation between CREB and JUN proteins. *Oncogene* **5**, 295–302.

Bowlus, C. L., McQuillan, J. J. & Dean, D. C. (1991). Characterization of three different elements in the 5′-flanking region of the fibronectin gene which mediate a transcriptional response to cAMP. *Journal of Biological Chemistry* **266**, 1122–1127.

Chatila, T., Ho, N., Liu, P., Liu, S., Mosialos, G., Kieff, E. & Speck, S. H. (1997). The Epstein–Barr virus-induced Ca^{2+}/calmodulin-dependent kinase typeIV/Gr promotes a Ca^{2+}-dependent switch from latency to viral replication. *Journal of Virology* **71**, 6560–6567.

Chevallier-Greco, A., Manet, E., Chavrier, P., Mosnier, C., Daillie, J. & Sergeant, A. (1986). Both Epstein–Barr virus (EBV)-encoded *trans*-acting factors, EB1 and EB2, are required to activate transcription from an EBV early promoter. *EMBO Journal* **5**, 3243–3249.

Countryman, J. & Miller, G. (1985). Activation of expression of latent Epstein–Barr herpesvirus after gene transfer with a small cloned subfragment of heterogeneous viral DNA. *Proceedings of the National Academy of Sciences, USA* **82**, 4085–4089.

Crawford, D. H. & Ando, I. (1986). EB virus induction is associated with B cell maturation. *Immunology* **59**, 405–409.

Daibata, M., Speck, S. H., Mulder, C. & Sairenji, T. (1994). Regulation of the BZLF1 promoter of Epstein–Barr virus by second messengers in anti-immunoglobulin-treated B cells. *Virology* **198**, 446–454.

Dean, D. C., Blakeley, M. S., Newby, R. F., Ghazal, P., Hennighausen, L. & Bourgeois, S. (1989). Forskolin inducibility and tissue-specific expression of the fibronectin promoter. *Molecular and Cellular Biology* **9**, 1498–1506.

Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Research* **11**, 1475–1489.

Dover, R. & Watt, F. M. (1987). Measurement of the rate of epidermal terminal differentiation: expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. *Journal of Investigative Dermatology* **89**, 349–352.

Ellis, M. J. C., Lindon, A. C., Flint, K. J., Jones, N. C. & Goodbourn, S. (1995). Activating transcription factor-1 is a specific antagonist of the cyclic adenosine 3′,5′-monophosphate (cAMP) response element-binding protein-1-mediated response to cAMP. *Molecular Endocrinology* **9**, 255–265.

Flamand, L. & Menezes, J. (1996). Cyclic AMP-responsive element-dependent activation of Epstein–Barr virus zebra promoter by human herpesvirus 6. *Journal of Virology* **70**, 1784–1791.

Flemington, E. & Speck, S. H. (1990*a*). Identification of phorbol ester response elements in the promoter of Epstein–Barr virus putative lytic switch gene BZLF1. *Journal of Virology* **64**, 1217–1226.

Flemington, E. & Speck, S. H. (1990*b*). Autoregulation of Epstein–Barr virus putative lytic switch gene BZLF1. *Journal of Virology* **64**, 1227–1232.

Gandarillas, A. & Watt, F. M. (1995). Changes in expression of the *fos* and *jun* families and *myc* network during terminal differentiation of human keratinocytes. *Oncogene* **11**, 1403–1407.

Golden, H. D., Chang, R. S., Prescott, W., Simpson, E. & Cooper, T. Y. (1973). Leukocyte-transforming agent: prolonged excretion by patients with mononucleosis and excretion by normal individuals. *Journal of Infectious Diseases* **127**, 471–473.

Gonzalez, G. A. & Montminy, M. R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* **59**, 675–680.

Green, H. (1977). Terminal differentiation of cultured human epidermal cells. *Cell* **11**, 405–416.

Henle, G., Henle, W. & Diehl, V. (1968). Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. *Proceedings of the National Academy of Sciences, USA* **59**, 94–101.

Hsueh, Y.-P. & Lai, M.-Z. (1995). Overexpression of activation transcriptional factor 1 in lymphomas and in activated lymphocytes. *Journal of Immunology* **154**, 5675–5683.

Israele, V., Shirley, P. & Sixbey, J. W. (1991). Excretion of the Epstein–Barr virus from the genital tract of men. *Journal of Infectious Diseases* **163**, 1341–1343.

Karimi, L., Crawford, D. H., Speck, S. & Nicholson, L. J. (1995). Identification of an epithelial cell differentiation responsive region within the BZLF1 promoter of the Epstein–Barr virus. *Journal of General Virology* **76**, 759–765.

Kieff, E. (1996). Epstein–Barr virus and its replication. In *Fields Virology*, 3rd edn, pp. 2343–2396. Edited by B. Fields, D. Knipe & P. Howley. Philadelphia: Lippincott–Raven.

Laux, G., Freese, U. K., Fischer, R., Polack, A., Kofler, E. & Bornkamm, G. W. (1988). TPA-inducible Epstein–Barr virus genes in Raji cells and their regulation. *Virology* **162**, 503–507.

Lee, C. Q., Yun, Y., Hoeffler, J. P. & Habener, J. F. (1990). Cyclic-AMP-responsive transcriptional activation of CREB-327 involves interdependent phosphorylated subdomains. *EMBO Journal* **9**, 4455–4465.

Li, Q. X., Young, L. S., Niedobitek, G., Dawson, C. W., Birkenbach, M., Wang, F. & Rickinson, A. B. (1992). Epstein–Barr virus infection and replication in a human epithelial cell system. *Nature* **356**, 347–350.

Liu, F., Thompson, M. A., Wagner, S., Greenberg, M. E. & Green, M. R. (1993). Activating transcription factor-1 can mediate Ca^{2+}- and cAMP-inducible transcriptional activation. *Journal of Biological Chemistry* **268**, 6714–6720.

Liu, S., Borras, A. M., Liu, P., Suske, G. & Speck, S. H. (1997*a*). Binding of the ubiquitous cellular transcription factors Sp1 and Sp3 to the ZI domains in the Epstein–Barr virus lytic switch BZLF1 gene promoter. *Virology* **228**, 11–18.

Liu, S., Liu, P., Borras, A., Chatila, T. & Speck, S. H. (1997*b*). Cyclosporin A-sensitive induction of the Epstein–Barr virus lytic switch is mediated via a novel pathway involving a MEF2 family member. *EMBO Journal* **16**, 143–153.

Luka, J., Kallin, B. & Klein, G. (1979). Induction of the Epstein–Barr virus (EBV) cycle in latently infected cells by *n*-butyrate. *Virology* **94**, 228–231.

Manet, E., Gruffat, H., Trescol-Biemont, M. C., Moreno, I., Chambard, P., Giot, J. F. & Sergeant, A. (1989). Epstein–Barr virus bicistronic mRNA's generated by facultative splicing code for two transcriptional trans-activators. *EMBO Journal* **8**, 1819–1826.

Meyer, T. E. & Habener, J. F. (1993). Cyclic adenosine 3′,5′-monophosphate response element binding protein (CREB) and related transcription-activating deoxyribonucleic acid-binding proteins. *Endocrine Reviews* **14**, 269–290.

Miyashita, E. M., Yang, B., Lam, K. M. C., Crawford, D. H. & Thorley-Lawson, D. A. (1995). A novel form of Epstein–Barr virus latency in normal B cells in vivo. *Cell* **80**, 593–601.

Montalvo, E. A., Shi, Y., Shenk, T. E. & Levine, A. J. (1991). Negative regulation of the BZLF1 promoter of Epstein–Barr virus. *Journal of Virology* **65**, 3647–3655.

Montalvo, E. A., Cottam, M., Hill, S. & Wang, Y.-C. J. (1995). YY1 binds to and regulates *cis*-acting negative elements in the Epstein–Barr virus BZLF1 promoter. *Journal of Virology* **69**, 4158–4165.

Nicholson, L. J. & Watt, F. M. (1991). Decreased expression of fibronectin and the $\alpha_5\beta_1$ integrin during terminal differentiation of human keratinocytes. *Journal of Cell Science* **98**, 225–232.

Nicholson, L. J., Hopwood, P., Johannessen, I., Salisbury, J. R., Codd, J., Thorley-Lawson, D. & Crawford, D. H. (1997). Epstein–Barr virus latent membrane protein does not inhibit differentiation and induces tumorigenicity of human epithelial cells. *Oncogene* **15**, 275–283.

Niederman, J. C., Evans, A. S., Subrahmanyan, L. & McCollum, R. W. (1970). Prevalence, incidence and persistence of EB virus antibody in young adults. *New England Journal of Medicine* **282**, 361–365.

Pattengale, P. K., Smith, R. W. & Gerber, P. (1973). Selective transformation of B lymphocytes by EB virus. *Lancet* **ii**, 93–94.

Pope, J. H., Horne, M. K. & Scott, W. (1968). Transformation of foetal human leucocytes *in vitro* by infiltrates of a human leukaemia cell line containing herpes-like virus. *International Journal of Cancer* **3**, 857–866.

Rheinwald, J. G. & Beckett, M. A. (1980). Defective terminal differentiation in culture as a consistent and selectable character of malignant human keratinocytes. *Cell* **22**, 629–632.

Rice, R. H. & Green, H. (1979). Presence in human epidermal cells of a soluble protein precursor of the cross-linked envelope: activation of the cross-linking by calcium ions. *Cell* **18**, 681–694.

Rickinson, A. B. & Kieff, E. (1996). Epstein–Barr virus. In *Fields Virology*, 3rd edn, pp. 2397–2446. Edited by B. Fields, D. Knipe & P. Howley. Philadelphia: Lippincott–Raven.

Sassone-Corsi, P., Visvader, J., Ferland, L., Mellon, P. L. & Verma, I. M. (1988). Induction of proto-oncogene fos transcription through the adenylate cyclase pathway: characterization of a cAMP-responsive element. *Genes & Development* **2**, 1529–1538.

Schwarz, E., Freese, U. K., Gissmann, L., Mayer, W., Roggenbuck, B., Stremlau, A. & zur Hausen, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314**, 111–114.

Schwarzmann, F., Prang, N., Reichelt, B., Rinkes, B., Haist, S., Marschall, M. & Wolf, H. (1994). Negatively cis-acting elements in the distal part of the promoter of Epstein–Barr virus trans-activator gene BZLF1. *Journal of General Virology* **75**, 1999–2006.

Sheng, M., Thompson, M. A. & Greenberg, M. E. (1991). CREB: a Ca^{2+}-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* **252**, 1427–1430.

Shimomura, A., Ogawa, Y., Kitani, T., Fujisawa, H. & Hagiwara, M. (1996). Calmodulin-dependent protein kinase II potentiates transcriptional activation through activating transcription factor 1 but not cAMP response element-binding protein. *Journal of Biological Chemistry* **271**, 17957–17960.

Sixbey, J. W. (1989). Epstein–Barr virus and epithelial cells. In *Advances in Viral Oncology*, pp. 187–202. Edited by G. Klein. New York: Raven Press.

Sixbey, J. W., Vesterinen, E. H., Nedrud, J. G., Raab-Traub, N., Walton, L. A. & Pagano, J. S. (1983). Replication of Epstein–Barr virus in human epithelial cells infected *in vitro*. *Nature* **306**, 480–483.

Sixbey, J. W., Nedrud, J. G., Raab-Traub, N., Hanes, R. A. & Pagano, J. S. (1984). Epstein–Barr virus replication in oropharyngeal epithelial cells. *New England Journal of Medicine* **310**, 1225–1230.

Sixbey, J. W., Lemon, S. M. & Pagano, J. S. (1986). A second site for Epstein–Barr virus shedding: the uterine cervix. *Lancet* ii, 1122–1124.

Sun, P., Enslen, H., Myung, P. S. & Maurer, R. A. (1994). Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. *Genes & Development* **8**, 2527–2539.

Takada, K. & Ono, Y. (1989). Synchronous and sequential activation of latently infected Epstein–Barr virus genomes. *Journal of Virology* **63**, 445–449.

Takada, K., Shimizu, N., Sakuma, S. & Ono, Y. (1986). *trans* Activation of the latent Epstein–Barr virus (EBV) genome after transfection of the EBV DNA fragment. *Journal of Virology* **57**, 1016–1022.

Tovey, M. G., Lenoir, G. & Begon-Lours, J. (1978). Activation of latent Epstein–Barr virus by antibody to human IgM. *Nature* **276**, 270–272.

Urier, G., Buisson, M., Chambard, P. & Sergeant, A. (1989). The Epstein–Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites. *EMBO Journal* **8**, 1447–1453.

Wang, Y.-C. J., Huang, J.-M. & Montalvo, E. A. (1997). Characterization of proteins binding to the ZII element in the Epstein–Barr virus BZLF1 promoter: transactivation by ATF1. *Virology* **227**, 323–330.

zur Hausen, H., O'Neill, F. J., Freese, U. K. & Hecker, E. (1978). Persisting oncogenic herpesvirus induced by the tumour promoter TPA. *Nature* **272**, 373–375.

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