Transcriptional enhancer factor (TEF)-1 and its cell-specific co-activator activate human papillomavirus-16 *E6* and *E7* oncogene transcription in keratinocytes and cervical carcinoma cells

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The human papillomavirus (HPV)-16 oncogenes, *E6* and *E7*, are transcribed preferentially in keratinocytes and cervical carcinoma cells due to a 5' enhancer. An abundant peptide binding to a 37 nt enhancer element was purified from human keratinocytes by sequence-specific DNA chromatography. This protein was identified as transcriptional enhancer factor (TEF)-1 by complex mobility, binding to wild-type and mutant SV40 and HPV-16 enhancers and antigenic reactivity with two anti-TEF-1 antibodies. TEF-1 is cell-specific, but its transactivation also depends on a limiting, cell-specific TEF-1 'co-activator'. We show that both TEF-1 and the TEF-1 co-activator are active in human keratinocytes and essential for HPV-16 transcription. TEF-1 binding *in vivo* was necessary for HPV-16 P97 promoter activity. Excess TEF-1 and chimeric GAL4-TEF-1 specifically inhibited the P97 promoter by 'squenching', indicating that HPV-16 transcription also requires a limiting TEF-1 co-activator. TEF-1 and the TEF-1 co-activator functions mirrored HPV-16 transcription by their presence in keratinocytes and cervical carcinoma cells and their absence from lymphoid B-cells, but also functioned in liver cells where the HPV-16 promoter is inactive. TEF-1 and its associated co-activator are thus part of a complex mechanism which determines the restricted cell range of the HPV-16 *E6* and *E7* oncogene promoter.

Key words: cervical cancer/HPV-16 P97 promoter/ keratinocyte-specific transcription/NF-1/CTF/TEF-1

Introduction

Transcriptional regulation in ontogeny and differentiation is mediated by complex, cell-specific interactions between multiple transcription factors anchored to promoter and

enhancer elements. Although the cellular range of gene expression can be determined by factors present exclusively in the target tissue, such as MyoD in striated muscle (Weintraub *et al.*, 1991), or GHF-1/Pit-1 in the anterior pituitary (Bodner *et al.*, 1988; Nelson *et al.*, 1988), it is often the result of combinatorial interactions between factors of partial or low cell specificity (Fromental *et al.*, 1988; Ondek *et al.*, 1988; Dynan, 1989; Frankel and Kim, 1991). For example, the combined effect of multiple factors with different tissue distribution is responsible for cell-specific transcription in the liver (De Simone *et al.*, 1987; Lichsteiner *et al.*, 1987; Wuarin and Schibler, 1990) or in lymphoid cells (Gilmore, 1990; Ruvkun and Finney, 1991). Keratinocyte differentiation in stratified squamous epithelia and the epidermis is accompanied by specific activation of a series of genes coding for intermediate filaments and cell envelope proteins, keratins, filaggrins and involucrins (Fuchs, 1990; Steinert and Liem, 1990). As yet undetermined aspects of keratinocyte differentiation are also required for infection with human and animal papillomaviruses. The factors responsible for transcriptional activation of these cellular and viral genes in the epidermis or stratified squamous epithelia have not been identified.

Human papillomavirus (HPV)-16 infects and replicates in the form of unintegrated circular plasmids in keratinocytes of genital mucosa and perigenital skin. The major upstream early promoter of HPV-16, P97 (Smotkin and Wettstein, 1986; Smotkin *et al.*, 1989), initiates transcripts of the viral *E6* and *E7* oncogenes. HPV-16 or the related HPV type 18 DNAs are often present in cervical carcinomas as *E6*–*E7* fragments integrated within the cellular genome. In contrast, the downstream early viral *E2* gene region is disrupted or inactivated by integration (Schwarz *et al.*, 1985; Schneider-Gädick and Schwarz, 1986; Baker *et al.*, 1987; Wilczynski *et al.*, 1988; Cullen *et al.*, 1991). Since the *E2* gene product can downregulate the P97 promoter, *E2* disruption is likely to release *E6* and *E7* transcription from repression and may represent a critical step in progression to invasive carcinoma (Cripe *et al.*, 1987; Thierry and Yaniv, 1987; Romanczuk *et al.*, 1990; S.Parkkinen, T.Ishiji, Y.Yamakawa, M.Ushikai, M.Lace, T.Cripe, T.Haugen and L.Turek, in preparation). In the absence of *E2*, persistent transcription of the *E6* and *E7* oncogenes in cervical cancer is directed by cellular factors interacting with the HPV-16 P97 promoter.

We have shown that the sequences 5' to the HPV-16 P97 promoter in the upstream regulatory region (URR; also called the long control region, LCR) contain an enhancer that functions preferentially in the natural host cells, human keratinocytes of genital skin and mucosa (Cripe *et al.*, 1987). The restriction of HPV infection to squamous epithelial cells thus may be due in part to cell-specific transcription of viral genes. This upstream enhancer is also responsible for *E6* and *E7* transcription in cervical carcinoma cells (Cripe *et al.*, 1987; Gloss *et al.*, 1987). The *cis*-acting sequences in the

URR have a complex modular structure (Cripe *et al.*, 1990a; Chong *et al.*, 1991). The activity of a strong 88 nt enhancer fragment was reduced stepwise by deletions, with a 63 nt 'proximal enhancer' being the smallest fragment active as a single copy which retained the cell preference of the entire URR (Cripe *et al.*, 1990a). The nuclear proteins found to bind to the HPV-16 URR so far do not explain its cell specificity as they belong to ubiquitous factor families, including activator protein-1 (AP-1) (Chan *et al.*, 1990; Cripe *et al.*, 1990a), nuclear factor-1 (NF-1/CTF) (Gloss *et al.*, 1989b; Sibbet and Campo, 1990; Chong *et al.*, 1991) and the glucocorticoid or progesterone receptors (GR/PR) (Gloss *et al.*, 1987; Chan *et al.*, 1989).

The keratinocyte preference of HPV-16 transcription thus could be due to subtle variations in the concentration of ubiquitous factors or to the presence of related, but functionally distinct factors in different cells (Cripe *et al.*, 1990a; Chong *et al.*, 1991). Alternatively, the enhancer activity could be due to additional cellular factors, as suggested by complete DNase I protection of the enhancer at high nuclear extract concentrations (Cripe *et al.*, 1990a). We have therefore sought to identify the *cis*-acting elements and their cognate factors that contribute to its keratinocyte preference. In this paper, we have defined a 37 nt enhancer element active in transfected keratinocytes as a tetrameric repeat. An abundant nuclear protein binding to these sequences was enriched from keratinocytes by sequence-specific DNA affinity chromatography and identified as transcriptional enhancer factor (TEF)-1 (Davidson *et al.*, 1988; Xiao *et al.*, 1991). This is the first example of a promoter besides SV40 that is regulated by TEF-1. Activation of transcription by TEF-1 requires not only the TEF-1 protein itself, but also a cell-specific 'co-activator' protein that can be titrated by excess TEF-1 in intracellular competition or 'squenching' tests (Xiao *et al.*, 1991). We show here that both TEF-1 and TEF-1 co-activator function in human keratinocytes, are necessary for HPV-16 *E6* and *E7* gene transcription and determine in part its keratinocyte preference.

Results

Strategy for HPV-16 enhancer element detection

The 88 nt enhancer fragment in the HPV-16 URR comprises a 5' module with two AP-1 sites and an overlapping GRE/PRE and a complex, 63 nt 'proximal enhancer' with two half-palindromic NF-1/CTF sites (Cripe *et al.*, 1990a). The proximal enhancer sequence is shown in Figure 2A. The 88 nt or the 63 nt fragments increase correctly initiated mRNA levels in transfected cells (Cripe *et al.*, 1990a). To identify *cis*-acting elements in the proximal enhancer, we constructed reporter clones containing four head-to-tail tandem repeats of the previously characterized enhancer fragments next to the enhancer-negative SV40 promoter in pSVE-cat (Cripe *et al.*, 1990a). This approach has been used to define the functional motifs of the SV40 enhancer and their cognate factors (Ondek *et al.*, 1987, 1988; Schirm *et al.*, 1987; Davidson *et al.*, 1988; Fromental *et al.*, 1988). Activity of the reporter constructs was assayed in transfected cultures of an uninfected human keratinocyte cell line, HaCaT. The HaCaT cells retain the phenotype of epidermal basal keratinocytes in culture and undergo correct stratifica-

Fig. 1. The 37 nt HPV-16 enhancer fragment G is active as a tetramer. Fragments of the HPV-16 KDE region were cloned upstream of the enhancer-negative SV40 early promoter in pSVE-cat and transfected into cultures of the keratinocyte cell line, HaCaT. The CAT enzyme activity in transfected cell lysates is expressed relative to the parent plasmid, pSVE-cat. The results represent averages from duplicate cultures in three to six independent experiments.

tion and differentiation in transplants (Boukamp *et al.*, 1988).

The relative CAT activity of the reporter clones in the HaCaT keratinocytes is shown in Figure 1. In agreement with our previous results (Cripe *et al.*, 1990a), the 88 nt fragment ('O', clone a) and the 63 nt proximal enhancer ('RG', clone e) were the only constructs which exhibited detectable activity as single copies. Among the clones containing four tandem repeats, the only small enhancer element which gained activity was the 37 nt fragment G (clone g). The G element contains NF-1/CTF site #4 within the previously defined DNase I footprint 'E' (Cripe *et al.*, 1990a; also designated fp6e, Gloss *et al.*, 1989a). This site is part of a so-called 'cytokeratin (CK) element', TTTGGCTT, also found in other papillomaviruses and in keratin and involucrin promoters (Blessing *et al.*, 1987; Cripe *et al.*, 1987). Although this sequence does not bind any apparent cell-specific proteins (Cripe *et al.*, 1990a; Chong *et al.*, 1991), it is one of many NF-1/CTF binding sites in the URR (Gloss *et al.*, 1989a,b). However, reporter clones containing repeats of the NF-1 site #4 alone or in combination with different adjacent sequences were inactive (h, i and k), indicating that other factor(s) were required for G element function.

The 37 nt enhancer element G binds an abundant cell-specific protein, factor E

To identify factors binding to the G enhancer element, we synthesized oligonucleotides I and II to cover the fragment G sequence with a 14 nt overlap at the centre. In addition, oligo E covers the entire DNase I footprint E (fp6e) which partially overlaps fragment G (Figure 2A; Gloss *et al.*, 1989a; Cripe *et al.*, 1990a). Nuclear extracts from HaCaT keratinocytes were first partially enriched on the BioRex-70 resin and then fractionated on affinity columns prepared with multimerized oligos E or II. The eluted fractions were tested for DNA binding activity in mobility shift assays.

The starting BioRex material and the flow-through fractions were found to form multiple specific and non-specific complexes (Figure 2B, lanes 1 and 2; Figure 2C,

Fig. 2. The G element binds an abundant cellular protein. (A) The 88 nt HPV-16 enhancer sequence with previously identified footprints D (fp5e) and E (fp6e), factor binding sites (GRE, AP-1, NF-I/2 and CK, the 'cytokeratin element'), the boundaries of the 63 nt proximal enhancer (fragment RG) and the 25 nt AP-1–GRE module. Oligos I, II and E were used to detect factor binding to the G element. (B) Mobility shift with ³²P-labelled oligo II as probe and 3 µl of HaCaT nuclear extract fractions eluted from the oligo II affinity column by increasing KCl concentrations. Binding reactions contained 30 nM unlabelled unrelated competitor DNA corresponding to a BPV-1 E2 binding site with no sequence homology to the HPV-16 enhancer. F, free probe; E, factor E complex; ft, flow-through. (C) Mobility shift results with oligo E as a probe and fractions eluted from the oligo E affinity column with increasing KCl concentrations. X, a complex formed with the 3' part of oligo E outside the G element (see text). (D) DNA binding of factor E from an oligo E-purified fraction (from panel C, lane 10) or an oligo II fraction (from panel B, lane 12) to E and II probes. Reactions included 30 nM wild-type (wt) or mutant competitors. 'E mutant' is E-mut-12; 'II mutant' is II-mut-3 in Figure 4A.

Fig. 3. Factor E is found in keratinocytes and liver cells, but not in lymphoid B-cells. Nuclear extracts from human keratinocytes (HaCaT), liver cells (HepG2) and lymphoid B-cells (CA46) were fractionated on the oligo E column and tested for complex formation with ³²P-labelled oligo I probe Y, a ubiquitous complex (see text).

lane 1; and data not shown). A major binding activity we termed 'protein E' was eluted between 0.4 M and 1.0 M KCl from the oligo II column (labelled E in Figure 2B) and a similar activity was also eluted between 0.25 M and 0.4 M KCl from the oligo E affinity column (labelled E in Figure 2C). A second distinct activity was eluted between 0.1 M and 0.25 M KCl from the oligo E column (complex

X, Figure 2C). The X protein(s) did not bind to the shorter oligo I probe which matches the 3' end of the G element (Figure 3). Further, binding of the X protein(s) to scanner mutant probes was also different from that of factor E or NF-I/CTF (data not shown). The E complexes formed by the oligo E and II affinity column fractions were indistinguishable from each other by interchanging fractions and

Fig. 4. Factor E binds to two sites distinct from the NF-I/CTF sites. (A) Sequences of scanner (base pair substitution) mutations in oligos II, I and E. I-mut-8 and I-mut-9 have mutations identical to those in II-mut-8 and II-mut-9. I-mut-10–14 have the same mutations as those in E-mut-10–14. (B) Mobility shift results with 0.4 M oligo II affinity fractions from HaCaT cells (lanes 1–10) and HepG2 cells (lanes 11–20). Probes are oligo II wild-type (wt) and mutants from panel A. F, free probe; E, factor E complex. (C) Mobility shift results with a 0.4 M oligo E affinity fraction from HaCaT cells and oligo I wild-type and mutant probes from panel A. (D) Complex formation with E wild-type (wt) and mutant probes and oligo E affinity purified HaCaT fraction (lanes 1–11) or purified HeLa cell NF-I/CTF (lanes 12–22). NS, non-specific complex.

probes in mobility shift assays (Figure 2D) or in proteolytic clipping assays with proteinase K or trypsin (data not shown).

Factor E is found in keratinocytes and liver cells, but not in lymphoid B-cells

To study the cell distribution of factor E, we fractionated nuclear extracts from different cell lines on the oligo E affinity column. In addition to HaCaT cells, we also used two cell types that do not support HPV-16 transcription: the human liver carcinoma cells HepG2 and a human B-cell lymphoma line CA46 (Magrath *et al.*, 1980a,b). We included a B-cell line as a cell type that exhibited different DNA binding patterns in DNase I protections (A. Alderborn, P. Bergman and U. Pettersson, unpublished). The eluted fractions were tested in mobility shift assays using oligo I as a probe.

An activity binding to the oligo I probe (complex Y in Figure 3) was eluted from the column at low KCl concentrations (0.1 M and 0.25 M), indicating a weaker affinity for the DNA column E sequences. The protein(s) responsible for this activity was present in all extracts, including those of liver and lymphoid cells in which the HPV-16 enhancer fragment is inactive. The identification of this polypeptide is under study. In contrast, complex E was seen in the 0.25 M and 0.4 M KCl fractions from the keratinocyte and liver cell extracts, but not in the B-cell lymphoma CA46 cell extracts (Figure 3).

The lack of factor E DNA binding in the B-cell fractions

was not due to low extract concentration or proteolytic degradation since all three cell extracts formed indistinguishable AP-1 complexes with the control collagenase TRE probe (data not shown). Unlike factor E, AP-1 eluted in the flow-through fractions on the oligo E column which contains none of the three known HPV-16 AP-1 sites (Cripe *et al.*, 1990a). The oligo E affinity fractions were also tested for binding to the prototype *ras* NF-I site. All three cell lines contained NF-I/CTF-like factors that formed complexes with the *ras* NF-I probe and could be inhibited specifically by an anti-NF-I antiserum, but were clearly distinct from the factor E complexes (data not shown).

Factor E and NF-I/CTF bind to distinct but overlapping sites in the G element

To define factor E binding sites, we created a series of scanner mutant oligonucleotides in the G element (Figure 4A). These mutants were compared with the wild-type oligo E, I and II probes in mobility shift assays. Two distinct factor E binding sites were identified: one in oligo II and another in oligos I and E. These are referred to as sites II and I, respectively. For site II, mutants II-mut-2–5 did not bind factor E (Figure 4B, lanes 1–10). Factor E from HepG2 showed an identical binding profile (Figure 4B, lanes 11–20). Based on these results, the factor E recognition sequence in site II was ACATACCG. Site I sequence corresponds to ACATATTT, since mutants I-mut-9–12 and E-mut-10–12 did not form an E complex (Figures 4C and

Fig. 5. Factor E is identical to transcriptional enhancer factor (TEF)-1 by DNA binding and antigenicity. (A) Recognition sequences of factor E in the HPV-16 enhancer G element and of TEF-1 in the SV40 enhancer oligonucleotides. Homology between the HPV-16 factor E binding site II and the TEF-1 site within the SV40 GT-IIC oligonucleotide and between the HPV-16 factor E site I and the SV40 SphII TEF-1 motif are shown. The GT-I motif binds the unrelated factor TEF-2. (B) Factor E binds to the SV40 enhancer TEF-1 sites. Factor E from HaCaT cells was tested for binding to cognate sites in the SV40 enhancer (GT-IIC and Sph-I+II) and HPV-16 factor E binding sites in oligos II and E in the presence of 30 nM non-specific and specific competitors. GT-IIC contains a single motif, while SphI + SphII contains two motifs. *, supershifted complex with both TEF-1 sites occupied. 'E mutant' is E-mut-12 and 'II mutant' is II-mut-3 in Figure 4A. (C) Recombinant TEF-1 binds to the HPV-16 enhancer factor E sites. Recombinant human TEF-1 was tested for binding to HPV-16 and SV40 enhancer oligonucleotide probes as described for factor E affinity fractions in panel B. (D) Factor E binding affinity for HPV-16 and SV40 sites. The relative DNA binding affinity of HPV-16 and SV40 sites was determined from competitions with ^{32} P-labelled HPV-16 oligo E as a probe and factor E from lane 9 in Figure 2C. Lanes 1 and 10 contained the BPV-1 E2 competitor. (E) Factor E does not react with anti-NF-I/CTF antisera. Purified NF-I/CTF (lanes 3 and 4) and HaCaT factor E from panel D were preincubated with pre-immune serum (lanes 1 and 3) or immune anti-NF-I serum (lanes 2 and 4) and analysed in a mobility shift assay. (F) Factor E is recognized by two anti-TEF-1 antisera. Recombinant TEF-1 (lanes 1–4, lanes 7 and 8) or HaCaT factor E (lanes 5, 6, 9 and 10) were preincubated with antibodies raised against the C-terminus of TEF-1 (anti-C, lanes 2, 4 and 6) or against a synthetic N-terminal peptide, P2 (lanes 8 and 10) and tested for binding to TEF-1 sites of SV40, GT-IIC or HPV-16, oligo E in a mobility shift assay. Pre-immune sera were used as controls (lanes 1, 3, 5, 7 and 9). *, supershifted complex with anti-P2 antibody.

D, lanes 1–11). The oligo II sequence does not extend far enough 3' to include site I. In contrast, purified NF-I/CTF did not bind mutants E-mut-12–17 (Figure 4D, lanes 12–22). E binding site I thus partially overlaps with, but is distinct from and unrelated to the NF-I/CTF site #4 (the CK element, TTTGGCTT).

Factor E is identical to transcriptional enhancer factor (TEF)-1 by DNA binding and antigenicity

We noticed that the two factor E binding sequences shared 6 out of 8 nt with the binding sites for transcriptional enhancer factor (TEF)-1 in the SV40 enhancer, GT-IIC and SphII (Figure 5A). To determine the relationship between factor E and TEF-1, we compared their DNA binding properties (Figures 5B and C). Factor E from HaCaT cells bound to the SV40 GT-IIC probe and the factor E complex formation was competed by either the GT-IIC or E oligos.

The SV40 SphI + SphII oligo formed two complexes of different mobility due to the presence of two TEF-1 binding sites (Davidson *et al.*, 1988; Xiao *et al.*, 1991) and were both competed by oligo E. Vaccinia virus expressed, recombinant human TEF-1 protein (Xiao *et al.*, 1991) bound to the oligo E and II probes as well as to GT-IIC. Again, the SphI + SphII probe formed two different complexes. HPV-16 oligo E competed TEF-1 complex formation with all these SV40 motifs (Figure 5C, lanes 11 and 13).

Relative binding constants of factor E to HPV-16 and SV40 sites were estimated in competition experiments (Figure 5D). Titration of the DNA concentrations needed for 50% competition showed that the binding affinities of oligo E and oligo II were similar to one another and to SphI + SphII (lanes 2–9 and 15–18; 50% competition at 3×10^{-9} M), and 3- to 10-fold weaker than that of the GT-IIC enhancer (lanes 11–14). The SV40 GT-I enhancer

sequence containing a binding site for transcriptional enhancer factor (TEF) 2 did not form other complexes with the affinity E purified fractions, indicating that TEF-2 was not selectively enriched by binding to the HPV-16 E sequences. Furthermore, the GT-I oligonucleotide did not bind either TEF-1 or factor E (Figure 5B and C, lane 5) and did not compete with factor E binding (Figure 5D, lanes 19 and 20). Factor E and TEF-1 therefore were indistinguishable based on the mobilities of their respective complexes and DNA binding site preferences.

To confirm that the E protein was related to TEF-1, we

included antisera against TEF-1 or NF-I/CTF in the binding reactions. The anti-NF-I antiserum had no effect on factor E complex formation, but abolished the binding of NF-I/CTF (Figure 5E). Two different anti-TEF-1 antisera were tested (Xiao *et al.*, 1991). An antibody against the C-terminus of TEF-1 inhibited complex formation in mobility shift assays with either factor E or recombinant TEF-1. Antibody against the N-terminal P2 polypeptide of TEF-1 resulted in a super-shifted band for both TEF-1 and factor E (*; Figure 5F). Thus factor E and TEF-1 share at least two epitopes in different parts of the proteins. Based on these findings, we conclude that the HaCaT cell factor E is indistinguishable from and most likely identical to the HeLa cell-derived recombinant TEF-1 protein. This is further supported by the same binding properties of a factor E preparation from HeLa cell extracts (not shown).

TEF-1 binds to multiple sites in the HPV-16 regulatory region

To identify TEF-1 binding sites in the HPV-16 enhancer region, we performed DNase I protection experiments with vaccinia virus-expressed TEF-1 (Figure 6A). Both TEF-1 sites I and II within the 37 nt enhancer G element were protected from DNase I digestion. The TEF-1 site II lies between footprints D (fp5e) and E (fp6e) previously obtained with nuclear extracts; however, high nuclear extract concentrations were able to protect this segment fully (Gloss *et al.*, 1989a; Cripe *et al.*, 1990a). The footprint over the TEF-1 site I also protected the partially overlapping NF-I/CTF site #4 (the 'CK element') and was similar to nuclear extract footprint E (fp6e). Two additional TEF-1 footprints were located downstream of the G element in the URR. One matches nuclear extract footprint F (fp7e). This footprint overlaps an Oct motif adjacent to NF-I/CTF site #5 (Gloss *et al.*, 1989b; Chong *et al.*, 1991; and our data not shown). The Oct and NF-I/CTF site arrangement is similar in all genital HPV strains (Chong *et al.*, 1991) and the TEF-1 site position resembles the overlapping Oct and SphI + SphII enhancers in the SV40 enhancer. The other TEF-1 footprint corresponds to nuclear extract footprint fp9e (Gloss *et al.*, 1989a). Nucleotide sequences of the TEF-1 binding sites are shown in Figure 6B.

TEF-1 binding is necessary for HPV-16 enhancer activity

To determine if TEF-1 plays a role in HPV-16 transcription *in vivo*, first we introduced scanner mutations that blocked TEF-1 binding *in vitro* into the HPV-16 proximal enhancer (RG) by PCR. The mutant enhancer constructs were tested in transfected keratinocytes in comparison with the wild-type RG clone. The CAT enzyme activity was expressed relative to the enhancer-negative parental plasmid, pSVE-cat (Figure 7A). In agreement with our previous results (Cripe *et al.*, 1990a), deletion of 10 nt with NF-I site #4 and part of TEF-1 site I (in clone b) or of the 'R' segment with NF-I site #3 (in clone c) reduced CAT activity to that of the enhancer-negative pSVE-cat (clone l) or 10–15% of wild-type (clone a). Mutations in either TEF-1 site II (mut-2 in clone h) or site I (mut-9 and mut-12, in clones i and j) reduced relative CAT activity to 20–25% of the wild-type clone (a). Both TEF-1 sites were therefore necessary for enhancer function.

In contrast, the NF-I/CTF site #4 (the 'CK element') was not absolutely required for enhancer activity in this context.

Fig. 6. TEF-1 binds to multiple sites in the HPV-16 regulatory region. (A) DNase I protection assay. Fragments of the HPV-16 regulatory region were 5' end-labelled on the upper or lower strands and incubated with vaccinia-expressed recombinant TEF-1 protein. On the left panel, the probe extended from HPV-16 nucleotides 7645–7756 and was labelled on the upper strand at nucleotide 7645. In the right panel, the labelled probe was HPV-16 nucleotides 7477–7860 labelled on the lower strand at nucleotide 7860. Reactions in lanes 5, 6, 11 and 12 contained increasing concentrations of unlabelled GT-IIC oligonucleotide as competitor of TEF-1 binding. (B) TEF-1 binding motifs in HPV-16 and SV40.

This site partially overlaps TEF-1 site I (Figure 4). The sequence of mut-12 (clone j) spans both sites and mut-12 blocked binding to either factor *in vitro*. Therefore, we also tested mut-14 which abolished NF-I/CTF binding to NF-I site #4, but retained TEF-1 binding to TEF-1 site I. This mutant (clone k) retained 70% of wild-type CAT activity, arguing that the TEF-1 motif, not the overlapping NF-I/CK site, was mainly responsible for activation and was inactivated by the deletion in clone b. However, mutations in the upstream NF-I site #3 (clones e and f) abolished proximal enhancer activity. NF-I/CTF binding at site #3 (or possibly other factors anchored at nearby motifs) thus may be responsible for cooperation between the G element and the R segment of the proximal enhancer.

To confirm that TEF-1 is necessary for activation *in vivo*, we tested the activity of HPV-16 reporter clones in

cotransfections with competitor plasmids carrying multiple wild-type or mutant GTIIC enhancers of SV40 (Xiao *et al.*, 1991). The results are shown in Figure 7B. The prototype TEF-1-dependent enhancer clone 2GTIIC(R) (a) with four copies of two closely spaced GTIIC enhancers was active in HaCaT keratinocytes and excess wild-type GTIIC reduced its activity 3-fold. The HPV-16 G element multimer with TEF-1 sites I and II (Gx4; clone b) was reduced 4- to 5-fold. The HPV-16 P97 promoter clone (c) driven by the entire URR including the enhancer was reduced >2-fold. The mutant GTIIC enhancers which do not bind TEF-1 led to little or no reduction in activity, indicating that the wild-type GTIIC competitor specifically titrated active TEF-1 in the cells. In contrast, the TEF-1-independent major immediate-early enhancer/promoter (MIEP) of human cytomegalovirus (CMV; Stinski and Roehr, 1985) was not influenced by excess competing TEF-1 sites (clone d). Taken together, our mutagenesis and competition results demonstrate that the binding of active TEF-1 is required for HPV-16 P97 transcription *in vivo*.

HPV-16 P97 activation requires TEF-1 and its associated co-activator

TEF-1 requires a limiting, cell-specific co-activator protein for function. TEF-1 activity thus can be controlled by the availability of either TEF-1 or the TEF-1 'co-activator' proteins (Xiao *et al.*, 1991). Our results suggested that a functional co-activator was present in the HaCaT keratinocytes where the TEF-1-dependent 2GTIIC(R) and HPV-16 reporter clones were active (Figure 8B). To assay the function of the TEF-1 co-activator independent of TEF-1 binding sites, we also tested the response of a GAL4-dependent promoter in the UASG-*tk-cat* reporter clone to a chimeric GAL4-TEF-1 factor (Figure 8C).

The expression plasmids are illustrated in Figure 8A. The GAL4-TEF-1C chimeric construct combines the N-terminal GAL4 DNA binding domain (DBD) and the TEF-1 transcription activation domain (TAD). Low concentrations of the GAL4-TEF-1 chimera activated the UASG promoter, but increasing levels of the activator lowered the response by intracellular competition ('squenching'), in a fashion previously documented in HeLa cells (Xiao *et al.*, 1991). Increasing amounts of the control GAL4 DBD did not affect CAT activity.

Excess TEF-1 (or TEF-1 TAD in the GAL4-TEF-1 chimera) also squelched the activity of the prototype 2GTIIC(R) enhancer (Figure 8D), but not that of the composite CMV enhancer-promoter (Figure 8G). These results clearly show that TEF-1 and a specific co-activator are active in keratinocytes. Finally, the activities of the proximal enhancer (Figure 8E) and P97 promoter of HPV-16 (Figure 8F) were also titrated by increasing amounts of TEF-1, thus indicating that a limiting TEF-1 co-activator was necessary for their activity.

TEF-1 and its co-activator determine in part the cell preference of HPV-16 transcription

TEF-1 and its co-activator have been shown to be present and to function in HeLa but not in lymphoid cells (Xiao *et al.*, 1991). We compared the cell specificity of TEF-1 function with that of the HPV-16 P97 promoter and proximal enhancer in transient transfections (Figure 9). The HPV-16 reporter clones were active in keratinocytes (Figure 8) and in the cervical carcinoma cell lines, HeLa and SiHa, but not

Fig. 7. TEF-1 binding is necessary for HPV-16 activation *in vivo*. (A) Nucleotide substitution mutants deficient for TEF-1 binding to the TEF-1 sites or for NF-I/CTF binding to the NF-I motifs were introduced into the HPV-16 RG proximal enhancer sequence by PCR. The wild-type (wt) and mutant reporter clones were tested in transfected HaCaT cells. Relative CAT activities from duplicate cultures of two to three independent experiments are expressed as multiples of the enhancer-negative parent clone, pSVE-cat. (B) The reporter plasmids were cotransfected with a 2-fold molar excess of competitor plasmids containing eight copies of the wild-type (wt) or mutant TEF-1 enhancers, 2GTIIC(R)-MLP or 2GTIICm(R)-MLP. Relative CAT activities were confirmed from two to three independent experiments and are expressed relative to the 2GTIIC(R)-*tk-cat* clone.

Fig. 8. TEF-1 and TEF-1 co-activator are required for keratinocyte-dependent HPV-16 P97 activity. (A) TEF-1 expression vectors. (B) The prototype TEF-1-dependent enhancer of SV40 functions in keratinocytes. Duplicate cultures of HaCaT keratinocytes were transfected with reporter clones containing the prototype TEF-1 enhancer 2GTIC(R)-*tk-cat*, the HPV-16 P97 promoter or the HPV-16 RG enhancer. CMV-*cat* served as a positive control. Relative CAT activities from two to four experiments are expressed as multiples of those of the enhancer-negative parent clones pSVE-*cat* or *tk-cat*, respectively. (C) The GAL4-TEF-1 chimeric protein activates a GAL4-dependent promoter in keratinocytes. The UASG-*tk-cat* reporter clone was cotransfected with increasing amounts of the expression vectors GAL4(DBD), GAL4-TEF-1C or GAL4-VP16. CAT activities from duplicate cultures of two independent experiments are given as multiples of UASG-*tk-cat* alone. (D) TEF-1 activity in keratinocytes depends on a limiting 'co-activator'. The TEF-1-dependent GTIC-*tk-cat* reporter clone was cotransfected with increasing amounts of the expression vectors GAL4 (DBD), GAL-TEF-1C or TEF-1A to assay intracellular competition for limiting 'co-activator' factors ('squenching'). (E and F) HPV-16 P97 activity depends on the TEF-1 co-activator. HPV-16 P97-*cat* (panel E) or the HPV-16 RG-SVE-*cat* clone (panel F) were cotransfected with TEF-1 vectors and controls to detect squelching. (G) The complex, TEF-1-independent CMV MIEP used as a control is not inhibited by excess TEF-1.

in the lymphoid CA46 B-cells or the liver HepG2 cells. Similarly, the TEF-1 enhancer 2GTIC(R) was active in the cervical carcinoma cells and inactive in the lymphoid cells. However, this clone was also functional in the liver HepG2 cells where the HPV-16 elements functioned poorly. The presence of the titratable co-activator was tested by the activation of the UASG promoter in response to the chimeric GAL4-TEF-1 factor. Again, limiting amounts of the TEF-1 co-activator were found in all the epithelial and liver cell lines, but not the lymphoid B-cells (Figure 9). TEF-1 activity *in vivo* thus closely mirrored detectable TEF-1 DNA binding *in vitro*, while HPV-16 activation was more restricted. These results indicate that TEF-1 is only one of the factors that determine the keratinocyte preference of HPV-16 transcription.

Discussion

The P97 promoter directs the expression of HPV-16 *E6* and *E7* oncogenes in infected keratinocytes and in cervical carcinomas. Here we identify transcriptional enhancer factor (TEF)-1 as a critical activator protein required for HPV-16 P97 activity. TEF-1 requires a limiting cell-specific 'co-activator' for function (Xiao *et al.*, 1991) and our data show that both TEF-1 and its associated co-activator are active in keratinocytes and necessary for HPV-16 activation. In the SV40 enhancer, TEF-1 and its co-activator alternate

with a transcription factor specific for lymphoid cells, Oct-2, and thus extend the viral host range (Fromental *et al.*, 1988; Ondek *et al.*, 1988; Xiao *et al.*, 1991). In contrast, our results indicate that in HPV-16, TEF-1 and its co-activator are part of a complex mechanism that restricts the cell type preference of viral transcription.

Cell specificity of TEF-1 and its associated co-activator

TEF-1 activates the SV40 enhancer in the cervical carcinoma HeLa cell line by binding to the GTIC enhancer or 'core A' and the SphI and SphII enhancers in the 72 nt repeat (Davidson *et al.*, 1988; Fromental *et al.*, 1988; Ondek *et al.*, 1988). We have shown that there are two TEF-1 binding sites in element G of the HPV-16 enhancer. TEF-1 binding to these motifs is necessary for its function *in vivo*, since the HPV-16 P97 promoter or enhancer were specifically inhibited by competition with excess GTIC enhancers in cotransfections. Furthermore, mutations that abolished TEF-1 binding also interfered with enhancer function. Two additional TEF-1 binding sites were found in the 3' end of the HPV-16 URR upstream of the P97 promoter. The HPV-16 TEF-1 sites differ from the SV40 enhancers by two or more nucleotides, but have similar relative affinities for TEF-1 binding. A comparison of the HPV-16 and SV40 motifs in Figure 6B shows that only three of the 9 nt are preserved in each TEF-1 binding site. These findings confirm

Fig. 9. Cellular host range of HPV-16 and TEF-1. (A) Host range of 2GTIIC(R) and HPV-16 activation. The reporter constructs P97-*cat*, RG-SVE-*cat*, the prototype TEF-1-dependent 2GTIIC(R)-*tk-cat* and the positive control CMV-*cat* were transiently transfected into the cervical carcinoma cell lines HeLa and SiHa, liver carcinoma HepG2 cells or the lymphoid B-cell line, CA46. The results are from duplicate cultures of three to six independent experiments. (B) Host range of TEF-1 co-activator function. The UASG-*tk-cat* clone was cotransfected with increasing amounts of the expression vectors GAL4(DBD), GAL4-TEF1C or GAL4-VP16 into different cells as in panel A. CAT activities are relative to UASG-*tk-cat* alone in duplicate cultures in two independent experiments.

that TEF-1 can bind specifically to partially degenerate sites (Davidson *et al.*, 1988; Xiao *et al.*, 1991).

Many transcriptional activators, including the acidic transcription activation domains of GAL4 (Gill and Ptashne, 1988) and VP16 (Tasset *et al.*, 1990; Martin *et al.*, 1990), the glutamine-rich TAD of Sp1 (Pugh and Tjian, 1990) and both TADs of steroid receptors (Meyer *et al.*, 1989; Tasset *et al.*, 1990) function via secondary co-activators that can be titrated by intracellular competition or 'squelching' (for review see Ptashne and Gann, 1990). TEF-1 has been the first factor shown to require a cell-specific co-activator which

is present in HeLa cells, but not in the lymphoid MPC-11 cells (Xiao *et al.*, 1991). In this study, we have demonstrated that TEF-1 activity in human keratinocytes also requires a limiting cellular co-activator and that both TEF-1 and this co-activator are necessary for HPV-16 transcription. TEF-1 binds to specific sequences in the enhancer regions of other human and animal papillomaviruses (T.Ishiji and L.P.Turek, preliminary data). It will be interesting to determine if TEF-1 also plays a role in the activation of cellular genes during keratinocyte differentiation, such as cytokeratins, filaggrins or involucrins. These experiments will be facilitated by our expanded list of TEF-1 enhancer sequences.

Although TEF-1 is active as a transcriptional activator in keratinocytes and cervical carcinoma cells, it functions in a broader range of cell types. The lack of HPV-16 expression in lymphoid cells may be due to the absence of TEF-1 DNA binding activity and function (Xiao *et al.*, 1991; this study). In contrast, we have shown that TEF-1 is present and functional in human liver carcinoma cells (this study), in human and animal fibroblasts (T.Ishiji, M.J.Lace, R.D.Anderson and L.P.Turek, not shown) and mouse embryonal carcinoma F9 cells (Nomiyama *et al.*, 1987; Fromental *et al.*, 1988), even though the HPV-16 P97 promoter or its proximal enhancer function poorly in these cell types (Cripe *et al.*, 1990a). Interestingly, the TEF-1 co-activator appears to be extremely limiting in all cell types. The level of available co-activator in a given cell type thus may modulate TEF-1 function *in vivo*.

Determinants of the keratinocyte preference of HPV-16 transcription

It is apparent that the cell specificity of HPV-16 transcription is more restricted than that of TEF-1 and its associated co-activator. One possible mechanism of transcriptional restriction could involve alternative binding of a repressor (or a weaker activator) to other motifs overlapping the TEF-1 sites. In the SV40 72 nt repeat, TEF-1 competes for DNA binding with the Oct transcription factors at an octamer sequence overlapping the SphI + SphII enhancers (Fromental *et al.*, 1988; Ondek *et al.*, 1988). In HPV-16, a TEF-1 site in footprint F (fp7e) downstream of the proximal enhancer also overlaps an Oct element and the adjacent NF-I/CTF site #5 (Cripe *et al.*, 1990a; Chong *et al.*, 1991). A homologous motif cluster in the URR of the related HPV-18 (Royer *et al.*, 1991) was found to be repressed by excess Oct-1 in transient transfections (Hoppe-Seyler *et al.*, 1991). While these sequences are deleted in the 88 nt enhancer fragment, it is possible that alternative binding of TEF-1 and Oct factors modulates the activity of this Oct-TEF-1-NF-I site cluster in the context of the HPV-16 URR.

The proximal enhancer is also likely to comprise multiple overlapping motifs: for example, TEF-1 site I in the G element extends into NF-I/CTF site #4 (the 'cytokeratin element'). We previously interpreted the low enhancer activity of a deletion and a scanner mutant (Figure 7A, clones b and j) as evidence that NF-I/CTF interaction with site #4 is essential for activity (Cripe *et al.*, 1990a,b). However, data presented here clearly show that TEF-1 binding to the overlapping TEF-1 site I is mostly responsible for the function of this site cluster. It is possible that transcription depends on TEF-1 while NF-I/CTF is utilized primarily in viral DNA replication. Alternative binding of NF-I/CTF and

TEF-1 also could modulate transcription during the viral life cycle. However, there is no evidence so far that competitive inhibition of TEF-1 binding by NF-I/CTF or any other factor restricts the cell range of the proximal enhancer *in vivo*.

Alternatively, liver cells or fibroblasts may lack additional positive factor(s) that cooperate with TEF-1 in keratinocytes. In the SV40 enhancer, TEF-1 acts synergistically with other factors binding to adjacent, precisely spaced enhansons, for example, transcriptional enhancer factor (TEF)-2 at the GT-I enhanson or 'core C' (Davidson *et al.*, 1988; Fromental *et al.*, 1988; Ondek *et al.*, 1988). Chong *et al.* (1991) reported a candidate TEF-2 motif in the HPV-16 URR at a 23 nt sequence overlapping TEF-1 site I in the G element. The proposed consensus site has limited homology with active TEF-2 enhansons of SV40 and the β -globin promoter and contains altered nucleotides previously found to abolish TEF-2 binding and function (Xiao *et al.*, 1987; Davidson *et al.*, 1988; Fromental *et al.*, 1988). Furthermore, we have not observed any GT-I binding activity among proteins enriched on affinity columns containing these sequences (Figure 5). A more detailed analysis of the binding site and the candidate binding factor(s) will be necessary to resolve the role of TEF-2 in HPV transcription. In the related HPV-18 enhancer, Mack and Laimins (1991) have characterized another factor with a strikingly similar cell distribution to TEF-1, but there are no apparent homologies between its binding site and the HPV-16 enhancer. Other factor(s) interacting with TEF-1 at the HPV-16 G element remain to be identified.

The competitive inhibition and the cooperation models are not mutually exclusive since both mechanisms are likely to play a role in HPV-16 P97 regulation by the entire URR. For example, NF-I/CTF bound to the upstream NF-I site #3 (or possibly other proteins anchored to nearby motifs) cooperate with the G element factors to activate the proximal enhancer. At the next level of organization, the proximal enhancer factors clearly cooperate with the AP-1 module (Chan *et al.*, 1989, 1990; Cripe *et al.*, 1990a). Activator protein (AP)-2 has been implicated in the function of the human cytokeratin 14 promoter (Leask *et al.*, 1991). AP-2 binding to a proximal site (Cripe *et al.*, 1990a) may contribute to the keratinocyte dependence of the P97 promoter. To understand the interactions responsible for the keratinocyte preference of HPV-16 transcription, it will be necessary to define additional active enhansons within the HPV-16 enhancer and to identify their cognate binding proteins. The DNA binding and transfection experiments will be aided by the oligonucleotides and reporter clones representing the entire set of scanner mutations described in this study.

Materials and methods

Plasmid constructions

The nucleotide numbers refer to the corrected HPV-16 DNA sequence (Cripe *et al.*, 1990a). Reporter clones containing single copies of HPV-16 enhancer fragments of pSVE-*cat* have been described (Cripe *et al.*, 1990a). To obtain reporter clones with four tandem repeats, the HPV-16 enhancer fragments were excised as *Nde*I–*Bam*HI fragments from the starting pUC18 clones (Cripe *et al.*, 1990a) and repeatedly inserted into the corresponding pSVE-*cat* constructs opened with *Nde*I and *Bgl*III, giving head-to-tail duplications of the *Bgl*III–*Bam*HI enhancer segment. To generate reporter clones with enhancer scanner mutations in Figure 7A, oligonucleotides with 2–6 bp substitutions listed in Figure 4A were incorporated into the enhancer via a two stage polymerase chain reaction (PCR) method (Ho *et al.*, 1989). In the first set of two reactions, each single-stranded oligonucleotide carrying

the mutation was used together with a 5' or 3' flanking primer homologous to the RG sequence to amplify two enhancer segments with a central, mutated overlap. These two PCR products were then mixed and amplified with both flanking primers to generate a full-length enhancer fragment containing the mutation. These were restricted with *Bgl*III and *Xba*I and inserted into pSVE-*cat*. The HPV-16 P97–*cat* construct contains all 5' and 3' *cis* sequences necessary to initiate P97 mRNA correctly (S.Parkkinen *et al.*, in preparation). Other clones are illustrated in the Figures. All molecular constructions were verified by DNA sequencing on an Applied Biosystems, Inc. automated sequencer. The TEF-1 expression vectors in Figure 8A were described previously (Xiao *et al.*, 1991).

Cells and transfections

HaCaT, a spontaneously immortalized human keratinocyte cell line that retains its capacity for *in vivo* differentiation (Boukamp *et al.*, 1988), was a gift from Dr Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany). All cell cultures were grown in Dulbecco's MEM (DMEM) with 7% iron-supplemented newborn calf and 1% fetal calf serum. The B-cell lymphoma line CA46 (Magrath *et al.*, 1980a,b) was a kind gift from Dr I. Magrath (NCI-NIH) and was maintained in RPMI medium with 10% fetal calf serum. Except for HaCaT and CA46, all cells were transfected in duplicate by calcium phosphate coprecipitation, followed by glycerol or DMSO treatment 4–6 h later (Cripe *et al.*, 1987, 1990a). HepG2 cells were incubated in the presence of precipitated DNA for 24 h before refeeding. HaCaT cells and CA46 cells were transfected by lipofection (BRL, Gaithersburg, MD). The reporter cat plasmids were combined with the lipofectin reagent in water and added to cell cultures in a serum-free medium (OptiMEM; BRL). The culture medium was changed to DMEM with serum 22–24 h later. All cells were harvested 48–72 h after transfection. The enzymatic CAT assays were performed as described (Cripe *et al.*, 1987, 1990a; Haugen *et al.*, 1988).

Extract preparation and DNA affinity purification

Nuclear extracts from HaCaT, HepG2 and CA46 cells were prepared by the ammonium sulfate protocol as described (Cripe *et al.*, 1990a). Typical protein concentration of the extracts was 5–10 mg/ml; subsequent chromatography steps used volumes equal to column bed capacity at each step. Nuclear extracts were passed over a BioRex-70 ion exchange resin column followed by a stepwise KCl elution; the 0.4 M KCl fraction was the starting material for DNA affinity chromatography. The DNA–Sephrose columns were prepared as described by Kodonaga and Tjian (1986). Synthetic oligonucleotides E or II were phosphorylated and ligated twice with T4 DNA ligase, purified from free ATP by repeated Sephadex G25 chromatography and ethanol precipitation and covalently linked to cyanogen bromide-activated Sepharose CL2B. The DNA–Sephrose resins contained 100–170 μ g DNA per ml. Bovine serum albumin and HeLa cell nuclear extracts were passed over the column and washed off with 2 M KCl and 0.1 M KCl to reduce protein loss due to non-specific binding. The 0.4 M KCl BioRex-70 fractions were diluted 5-fold with buffer D and applied three times to the DNA affinity columns. Aliquots of the flow-through fractions were saved for analysis. Bound proteins were eluted two to three times with 0.1 M, 0.25 M, 0.4 M and 1.0 M KCl. All protein fractions were stored in a vapour-phase liquid nitrogen freezer.

Mobility shift assays

Oligonucleotides used as probes and competitors are shown in the figures; the *ras* NF-I, the collagenase TRE (AP-1) and the BPV-1 E2 site oligos have been described (Cripe *et al.*, 1990a). Binding was performed in a 25 μ l reaction volume as described (Haugen *et al.*, 1988; Cripe *et al.*, 1990a) with ~0.5–5 μ l of nuclear extract fractions, purified HeLa cell NF-I/CTF or with vaccinia virus-expressed recombinant TEF-1 protein and 15 000 c.p.m. (~4–8 fmol) of ³²P-labelled oligonucleotide probe in the presence of 30–100 ng of poly(dI·dC) (Pharmacia) at 30°C for 30 min. Protein–DNA complexes were resolved from the unbound probe on non-denaturing 6% acrylamide gels and visualized by autoradiography. Unlabelled oligonucleotides served as competitors of binding. Relative binding constants were determined from three or more independent competition experiments. Polyclonal antibodies against the C-terminus of NF-I (Santoro *et al.*, 1988) or against the C-terminus or the N-terminal P2 polypeptide of TEF-1 (Xiao *et al.*, 1991) were included in some binding reactions as indicated. Native HeLa cell NF-I/CTF and the anti-NF-I serum were kindly provided by Drs Naoko Tanese and Robert Tjian (UC Berkeley, CA).

DNase I footprinting

A 310 nt fragment which contained the HPV-16 regulatory region nucleotides 7645–7756 and SV40 early promoter sequences was 5' end-labelled using T4 polynukinase at nucleotide 7645 for the upper strand probe. For the lower strand probe, a 383 nt HPV-16 fragment (nucleotides 7477–7860) labelled

at nucleotide 7860 was used. Reaction mixtures containing vaccinia-expressed TEF-1 protein were preincubated at 30°C for 10 min in a final volume of 50 µl as described (Cripe *et al.*, 1990a). They were then incubated with 10 000 c.p.m. (3–10 fmol) of ³²P-labelled probes for 15 min. Digestion with DNase I (Pharmacia) was initiated by the addition of MgCl₂ to a final concentration of 5 mM. The digestion products were separated on a 6% polyacrylamide–urea denaturing gel and exposed to X-ray film with amplifying screens.

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