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Regulation of Human Papillomavirus Transcription by the Differentiation-Dependent Epithelial Factor Epoc-1/skn-1a

KAZUNORI YUKAWA,[1]† KARIN BUTZ,[2] TERUHITO YASUI,[1]
HITOSHI KIKUTANI,[1] AND FELIX HOPPE-SEYLER[2]*

Institute for Molecular and Cellular Biology, Osaka University, 1-3, Yamadaoka, Suita, Osaka, Japan,[1] and Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, 69120 Heidelberg, Germany[2]

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Human papillomavirus (HPV) early gene expression is closely linked to the differentiation status of infected epithelial cells. Typically, HPV type 16 (HPV16) or HPV18 E6 and E7 transcripts are only barely detectable within the undifferentiated basal cell layer, but their levels increase concomitantly with higher degrees of epithelial cell differentiation in suprabasal cells. A similar differentiation-dependent distribution of expression has been reported for the recently cloned epithelial cell specific transcription factor Epoc-1/skn-1a. We therefore examined whether Epoc-1/skn-1a may be directly involved in the activation of HPV E6/E7 transcription. Transient transfection studies showed that Epoc-1/skn-1a specifically stimulated the HPV16 and HPV18 E6/E7 promoters. Moreover, ectopically expressed Epoc-1/skn-1a was sufficient to stimulate HPV transcription also in nonepithelial cells. By deletion analyses, the Epoc-1/skn-1a-responsive element was mapped to the promoter-proximal portion of the HPV18 transcriptional control region. Footprint analyses and gel retardation assays demonstrated direct binding of Epoc-1/skn-1a to a hitherto uncharacterized site within this region. Mutation of the Epoc-1/skn-1a recognition site within the context of the complete HPV18 upstream regulatory region inhibited Epoc-1/skn-1a-mediated transactivation. These results show that Epoc-1/skn-1a can directly activate the E6/E7 promoter by binding to the viral transcriptional control region. Thus, Epoc-1/skn-1a may be involved in the differentiation-dependent regulation of HPV transcription.

Human papillomaviruses (HPVs) are small DNA viruses with a strong epithelial tropism. They cause a number of benign proliferative lesions in humans, including skin warts and anogenital condylomas (31). Moreover, specific HPV types (in particular HPV type 16 [HPV16] and HPV18) are strongly associated with the development of anogenital cancers, most notably cervical carcinoma (43), in humans.

During natural HPV infection, the basal cells of the epithelium seem to represent the primary target cells for the virus (31). It is believed that HPVs gain access to these cells through epithelial microlesions. The transcriptional activity of the virus within the epithelium is closely linked to the cellular differentiation state. In situ hybridization analysis of HPV16- and HPV18-positive lesions indicate that infected basal cells contain low copy numbers of viral genomes with only very little transcriptional activity (13, 32). However, in parallel with the progression of epithelial cell differentiation, suprabasal cell layers exhibit increased levels of viral DNA replication and an activation of viral early gene transcription (13, 32). Only the terminally differentiated keratinocytes close to the epithelial surface are able to support transcription of the late genes and synthesis of the capsid proteins.

The regulatory mechanisms involved in the differentiation-dependent transcriptional control of HPV16 or HPV18 are still unknown, in large part because of the lack of an experimental system which mimics epidermal differentiation to the extent that it allows propagation of these viruses. Principally, the particular pattern of HPV16 and HPV18 gene expression could be explained by the presence of an activator of HPV transcription in the suprabasal epithelial cell layers, which is absent in undifferentiated basal cells.

For HPV16 and HPV18, several cellular transcription factors have been proposed to contribute to the epithelial cell specificity of the viral E6/E7 early gene promoter. These factors include JunB (36), the epithelial cell specific transcription factor KRF-1 (24), particular subsets of NF1 factors (2), and cell-type-specific coactivators associated with TEF-1 (22). With the exception of JunB, which is found predominantly in the well-differentiated upper layers of the epithelium (39), nothing is known about a possible differentiation-dependent distribution of these factors. JunB itself, however, is not sufficient for the transcriptional stimulation of the viral E6/E7 promoter (36).

Recently, a novel epidermal transcription factor, termed Epoc-1 (epidermal octamer-binding factor 1), was cloned from a murine cDNA library. Epoc-1 is a POU domain protein with homology to the octamer-binding transcription factors Oct-1 and Oct-2 (41). The rat counterpart of Epoc-1 has been cloned independently and was termed skn-1a (1). Epoc-1/skn-1a is expressed almost exclusively in the skin (1, 41), where transcription is linked to the state of epithelial cell differentiation. Epoc-1/skn-1a transcripts are detectable in differentiating suprabasal cells but undetectable in the undifferentiated basal cell layer (1, 40). Functionally, Epoc-1/skn-1 protein binds to octamer-like recognition sequences and can transcriptionally stimulate the cytokeratin 10 gene promoter (1, 41).

The similar, differentiation-dependent distributions of Epoc-1/

* Corresponding author. Mailing address: Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69120 Heidelberg, Germany. Phone: 6221-424872. Fax: 6221-424852.

† Present address: Center for Neurobiology and Behavior, Columbia University, New York, NY 10032.

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skn-1a expression and HPV early gene transcription within the epithelium raises the question of whether this factor may be directly involved in the regulation of HPV transcription. Using HPV18 as a model system, we investigated the effects of Epoc-1/skn-1a on the transcriptional activity of the viral E6/E7 promoter. We found that Epoc-1/skn-1a specifically binds to sequences within the promoter-proximal portion of the HPV18 transcriptional control region. Moreover, Epoc-1/skn-1a transcriptionally activated the HPV18 E6/E7 promoter, and ectopic expression of Epoc-1/skn-1a was sufficient to stimulate the activity of the viral E6/E7 promoter in nonepithelial cells. Mutational analyses identify the Epoc-1/skn-1a recognition site within the promoter-proximal region of the HPV18 upstream regulatory region (URR) as the key element for Epoc-1/skn-1a-mediated transactivation. These findings suggest that Epoc-1/skn-1a may be an important factor for the differentiation-dependent regulation of HPV transcription.

MATERIALS AND METHODS

Plasmids and oligonucleotides. Basic vector pBL, the HPV18 reporter construct p18URRL, which contains the complete HPV18 URR linked to the *Photinus pyralis* luciferase gene, and deletion constructs p363/18L and p232/18L have been described in detail before (6). p178/18L corresponds to p232/18L with a further deletion of 54 bp and contains HPV18 nucleotides 7768 to 88 (nucleotide numbering according to reference 11). Plasmid p230s/tk*L contains the 230-bp HPV18 enhancer upstream of a truncated herpes simplex virus thymidine kinase (HSV TK) promoter, while ptk*L represents the identical construct devoid of HPV18 enhancer sequences (19). Constructs pCMV-Luc and pAc-Luc contain the human cytomegalovirus promoter/enhancer and the human β-actin promoter cloned into pBL and have been described in detail before (21). Construct p18EpocMURRL, which contains the complete HPV18 URR with mutant Epoc-1/skn-1a recognition sequences, was generated by PCR-mediated site-directed mutagenesis and subcloning into p18URRL (6). HPV regulatory sequences within p18EpocMURRL were verified by DNA sequencing. Plasmid pCMV-Gal (21) contains the human cytomegalovirus enhancer/promoter region linked to the *Escherichia coli* β-galactosidase gene and served as an internal control. The Epoc-1/skn-1a expression vector pEpoc1S was constructed by inserting the complete murine cDNA sequence of Epoc-1 (41) into the *Xba*I site of the eukaryotic expression vector pEF-BOS (26) in sense orientation, using *Xba*I linkers. Control vector pEpoc-1AS contains the same Epoc-1 cDNA insert in inverse orientation.

For protein-DNA binding analyses, the following double-stranded oligonucleotides derived from the HPV18 URR were used (only sense strands are indicated; HPV18 sequences are underlined): A, 5′-GATCCAGGAGCTGTGCATACATAGTGCGG-3′; B, 5′-GATCCAGGATATGCAACCGAAATAGGCGG-3; C, 5′-GATCCAGGACATAGTGGCGG-3; D, 5′-GATCCAGGCTGTGCATGCGG-3′; G, 5′-GATCCAGGTGCATACATAGGCGG-3′; H, 5′-GATCCAGGTGCATAAACTAGCGG-3′; Fp (footprint) VIII, 5′-GATCCATAGTTTATGCAACCGAAATG-3′; EBS, 5′-CTGTGCATACATAGTTTATGCAA-3′; and EBSMUT, 5′-CTGTGgATcCATAGTTTcTagAA-3′ (mutations are indicated by lowercase letters). For competition assays, we used a 34-bp oligonucleotide containing an octamer consensus binding motif (5′-GATCCCAATTGATTTGCATATTCATGAGCACTCG-3′ [the octamer motif is underlined]). Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer.

Cell culture and DNA transfection. The spontaneously immortalized human keratinocyte cell line HaCaT (5), HPV18-positive HeLa cervical carcinoma cells, HepG2 hepatoma cells, and primary human fibroblasts from the uterine cervix were all maintained in Dulbecco's minimal essential medium (pH 7.2) supplemented with 10% fetal calf serum. Approximately 2×10^5 cells per 6-cm-diameter petri dish were transfected by calcium phosphate coprecipitation (9). Transfections mixtures usually contained 3 μg of the reporter construct, 0.2 μg of pCMVGal, and 0.5 μg of expression vector and were made up to 6.5 μg by the addition of Bluescript DNA. Luciferase and β-galactosidase assays were performed as described before (7). To account for variations in transfection efficiency, promoter activities were determined by calculating the luciferase/β-galactosidase ratios. Values represent the means of at least three independent experiments performed in triplicate and using at least two different plasmid preparations, each purified by two subsequent CsCl gradient ultracentrifugations. Typically, results from individual experiments varied by less than 20%.

DNase footprinting and gel retardation analyses. The glutathione *S*-transferase (GST)–Epoc-1/skn-1a fusion protein was generated by cloning the *Eco*RI fragment of the Epoc-1 cDNA (41) in frame with the GST cDNA into vector pGEX2TH. The fusion protein was produced in *E. coli* and purified by using glutathione-Sepharose (Pharmacia).

Single-end-labeled probes for footprint analyses were obtained after subcloning the *Acc*I-*Xba*I fragment of p18URRL (6), which contains the 178-bp 3′ region of the HPV18 URR, into pBluescript KSII (+) (Stratagene) and subsequent excision with *Apa*I and *Xba*I (for analyzing the plus strand) or excision with *Sac*I and *Xho*I (for analyzing the minus strand). Probes were radiolabeled by using DNA polymerase I (Klenow fragment). In the binding reaction, 100 ng of GST or GST–Epoc-1/skn-1a fusion protein was incubated with the labeled probe for 30 min at room temperature in a buffer containing 20 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 50 μg of bovine serum albumin per ml, and 67 μg of poly(dI-dC) per ml. Diluted DNase I was added at a final concentration of 2.0 μg/ml, and the reaction mixture was incubated for 1 min at room temperature. Subsequently, the DNA was extracted from the reaction mixture with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and resuspended for analysis on a 7% denaturing polyacrylamide gel. Chemical sequencing was performed as described by Maxam and Gilbert (25).

For gel retardation assays, the *Acc*I-*Xba*I fragment of p18URRL (see above) or double-stranded synthetic oligonucleotides were radiolabeled with DNA polymerase I (Klenow fragment). Then 100 ng of GST or GST–Epoc-1/skn-1a fusion protein was incubated with the radiolabeled probe for 20 min at room temperature in the binding buffer described above. For competition experiments, a 100-fold molar excess of an oligonucleotide containing an octamer consensus motif (41) was added to the binding reaction. The protein-DNA complex was separated on a native 4% polyacrylamide gel and subsequently visualized by autoradiography.

RESULTS

Transcriptional activation of the HPV18 and HPV16 E6/E7 promoter by Epoc-1/skn-1a. Transcription of the HPV18 E6 and E7 early genes is initiated at a common promoter, p105 (35), located at the 3′ terminus of the 825-bp viral transcriptional control region (URR). Thus far, p105 represents the only promoter identified within the HPV18 URR. The activity of p105 is modulated by a series of *cis*-regulatory elements contained within the HPV18 URR, which include binding sites for AP1 (14, 29), NF1 (16), KRF-1 (24), Oct-1 (6, 24), Sp1 (18), YY1 (4), and the glucocorticoid receptor (8). Functionally, the HPV18 URR can be subdivided into three different portions (15): (i) a 5′ region which encompasses almost half of the complete URR and only marginally contributes to the activation of the E6/E7 promoter (6, 34), (ii) a centrally located 230-bp constitutive enhancer domain, and (iii) a 3′ promoter-proximal region containing the E6/E7 promoter.

To investigate the influence of Epoc-1/skn-1a on the activity of the HPV18 E6/E7 promoter, Epoc-1/skn-1a expression vectors were cotransfected with reporter construct p18URRL, which measures the activity of the HPV18 E6/E7 promoter within the physiological context of the complete HPV18 URR (6). Plasmid pEpoc-1S expresses the complete Epoc-1 cDNA under control of the human elongation factor promoter. pEpoc-1AS, which contains the same cDNA in inverted orientation, served as a negative control.

As shown in Fig. 1A, cotransfection of pEpoc-1S led to an approximately four- to fivefold stimulation of the HPV18 URR in HeLa cells. In contrast, other viral and cellular transcriptional control regions, such as the human β-actin promoter (pAc-Luc), a truncated HSV TK promoter (ptk*-L), or the human cytomegalovirus enhancer/promoter region (pCMV-Luc), were not affected by Epoc-1/skn-1a. Likewise, the basic reporter construct pBL was not influenced by Epoc-1/skn-1a. Control vector pEpoc-1AS did not affect the activity of any reporter plasmid (Fig. 1A). These findings indicate that Epoc-1/skn-1a can act as a specific activator of HPV18 transcription.

We then addressed the question of whether Epoc-1/skn-1a can also modulate the transcriptional activity of HPV16. Cotransfection of reporter plasmid p16URRL, which measures the activity of the HPV16 E6/E7 promoter under control of the homologous URR (3), with pEpoc-1S led to an approximately 47-fold stimulation above the basal vector pBL (Fig. 1A).

To investigate, whether the observed activation of HPV transcription by Epoc-1/skn-1a in HeLa cells can also be de-

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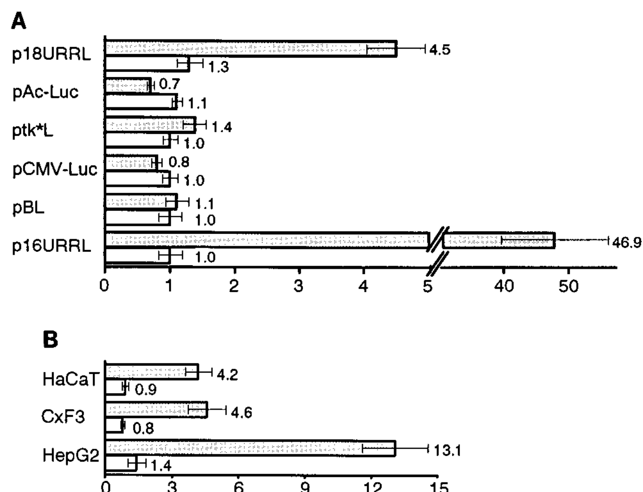


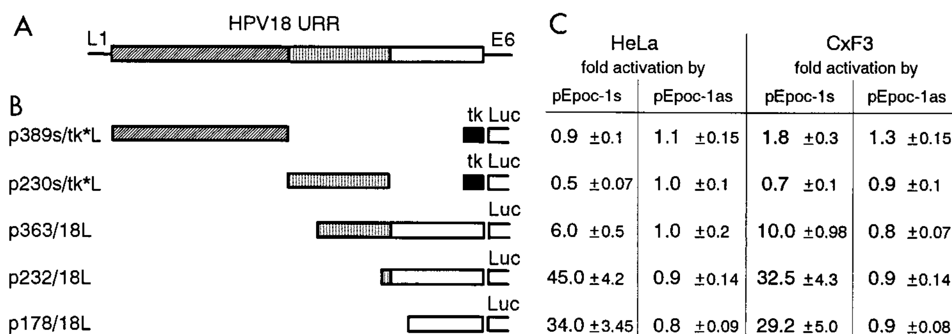
FIG. 1. (A). Epoc-1/skn-1a activates HPV18 transcription. Luciferase activities of reporter plasmids p18URRL, pAc-Luc, ptk*L, pCMV-Luc, pBL, and p16URRL after cotransfection with 0.5 μg of pEpoc-1S (shaded bars) or control vector pEpoc-1AS (open bars) into HeLa cells. Luciferase activities represent relative activities above the basal activities of the reporter plasmids in the absence of cotransfected Epoc-1/skn-1a expression vectors (arbitrarily set at 1.0). Standard deviations are indicated. (B) Epoc-1/skn-1a activates HPV transcription in epithelial and nonepithelial cells. Relative luciferase activities of p18URRL after cotransfection of 0.5 μg of pEpoc-1S (shaded bars) or 0.5 μg of pEpoc-1AS (open bars) in HaCaT keratinocytes, primary human fibroblasts from the cervix uteri (CxF3), or HepG2 hepatoma cells.

tected in other epithelial cells, cotransfection experiments were performed in the spontaneously immortalized human keratinocyte cell line HaCaT (5). Again, Epoc-1/skn-1a induced a stimulation of the HPV18 E6/E7 promoter in these cells (Fig. 1B). Moreover, pEpoc-1S activated the HPV18 URR in primary human fibroblasts (Fig. 1B), indicating that Epoc-1/skn-1a can stimulate HPV transcription also in nonepithelial cells. Furthermore, HPV transcription was activated by Epoc-1/skn-1a in HepG2 cells (Fig. 1B), a hepatoma-derived cell line in which the HPV18 URR otherwise is transcriptionally silent (18). These results show that Epoc-1/skn-1a is sufficient to stimulate HPV transcription in both epithelial and nonepithelial cells.

Mapping of the Epoc-1/skn-1a response element to the promoter-proximal region of the HPV18 URR. To gain insight into the molecular mechanisms underlying the transcriptional activation of the HPV18 E6/E7 promoter by Epoc-1/skn-1, we next attempted to map the *cis*-regulatory element(s) involved in this regulation. First, we tested the influence of pEpoc-1S on the HPV18 enhancer. This region within the center of the HPV18 URR contains at least two octamer-related sequence motifs, which are bound by Oct-1 with low affinity (6, 24). However, construct p230s/tk*L, which contains the 230-bp HPV18 constitutive enhancer region linked to a truncated HSV TK promoter, was not stimulated by pEpoc-1S (Fig. 2). Similarly, the 389-bp 5′ portion of the HPV18 URR was not stimulated by Epoc-1/skn-1a (Fig. 2). These findings suggested that Epoc-1/skn-1a-responsive elements may be located within the remaining 3′ promoter-proximal region of the HPV18 URR.

We therefore analyzed the effect of Epoc-1/skn-1a on 5′-deletion constructs of the HPV18 URR. Reporter plasmid p363/18L is a deletion mutant of p18URRL in which the complete 5′ region of the URR as well as the 5′ portion of the constitutive enhancer have been removed (Fig. 2). This construct contains the smallest fragment of the HPV18 URR, which is required for a detectable activation of the E6/E7 promoter (6). As shown in Fig. 2, Epoc-1/skn-1a activated p363/18L in HeLa cells sixfold. Further deletion of the complete HPV18 enhancer and 5′ portions of the promoter-proximal region resulted in constructs p232/18L and p178/18L. Cotransfection of pEpoc-1S again activated both constructs, resulting in HeLa cells in 45- and 34-fold stimulation, respectively. All cotransfection studies yielded qualitatively and quantitatively comparable results in nonepithelial cells, such as primary human fibroblasts (Fig. 2). Taken together, these experiments map an Epoc-1/skn-1a response element to the 3′ 178 bp of the HPV18 promoter-proximal region.

Binding of Epoc-1/skn-1a to the promoter-proximal region of the HPV18 URR. Gel retardation assays were performed to investigate whether Epoc-1/skn-1a directly binds to the promoter-proximal region of the HPV18 URR. As shown in Fig. 3A, Epoc-1/skn-1a protein bound to a DNA fragment containing the Epoc-1-responsive 178-bp 3′ portion of the HPV18 URR. Binding of Epoc-1/skn-1a was efficiently inhibited in competition experiments using increasing amounts of an octamer consensus sequence known to bind Epoc-1/skn-1a (Fig. 3A). These studies show that Epoc-1/skn-1a can bind to the promoter-proximal region of the HPV18 URR and suggest that Epoc-1-mediated transactivation stems from a direct interaction between Epoc-1/skn-1a and the viral transcriptional control region.



Construct	HeLa fold activation by pEpoc-1s	HeLa fold activation by pEpoc-1as	CxF3 fold activation by pEpoc-1s	CxF3 fold activation by pEpoc-1as
p389s/tk*L	0.9 ±0.1	1.1 ±0.15	1.8 ±0.3	1.3 ±0.15
p230s/tk*L	0.5 ±0.07	1.0 ±0.1	0.7 ±0.1	0.9 ±0.1
p363/18L	6.0 ±0.5	1.0 ±0.2	10.0 ±0.98	0.8 ±0.07
p232/18L	45.0 ±4.2	0.9 ±0.14	32.5 ±4.3	0.9 ±0.14
p178/18L	34.0 ±3.45	0.8 ±0.09	29.2 ±5.0	0.9 ±0.08

FIG. 2. An Epoc-1/skn-1a-responsive element is localized within the promoter-proximal region of the HPV18 URR. (A) Schematic representation of the HPV18 URR showing the 5′ region (hatched area), the constitutive enhancer (stippled area), and the promoter-proximal region (open area). (B) Reporter constructs p389s/tk*L and p230/tk*L, which contain the 5′ region and the constitutive enhancer portion of the HPV18 URR, respectively, linked to a truncated HSV TK promoter. p363/18L, p232/18L, and p178/18L contain successive 5′-deletion fragments of the wild-type HPV18 URR and carry the homologous HPV18 E6/E7 promoter. (C) Relative activities (see the legend to Fig. 1) of the constructs after cotransfection into HeLa cells and primary human cervical fibroblasts (CxF3) together with pEpoc-1S and pEpoc-1AS, as indicated. Standard deviations are indicated.

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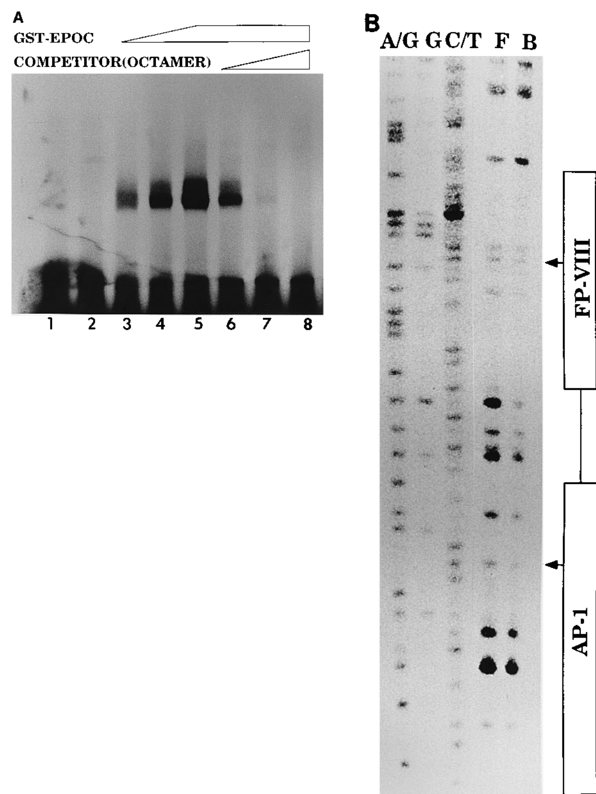

FIG. 3. Binding of Epoc-1/skn-1a to the promoter-proximal region of the HPV18 URR. (A) Gel retardation assay with increasing amounts of GST–Epoc-1/skn-1a fusion protein (lane 1, 0 ng; lane 2, 5 ng; lane 3, 20 ng; lane 4, 50 ng; lanes 5 to 8, 100 ng) and a radioactively labeled fragment containing the 178-bp 3′ region of the HPV18 URR. After maximal protein-DNA interaction was obtained (lane 5), increasing amounts of a 34-bp oligonucleotide containing an octamer consensus motif were included into the binding reaction as competitor DNA (5-fold [lane 6], 50-fold [lane 7], and 100-fold [lane 8] molar excesses). (B) DNase I footprint of the binding of Epoc-1/skn-1a to the promoter-proximal region of the HPV18 URR. The last two lanes (from the left) represent partial DNase I digestions of a radioactively labeled promoter-proximal region (minus strand) for 1 min, using 2.0 μg of DNase I per ml. Lanes F and B, absence and presence, respectively, of Epoc-1/skn-1a in the reaction mixture; lanes A/G, G, and C/T, sequencing reactions with dimethyl sulfate and hydrazine. Boxes to the right designate the Fp VIII region and the AP1 site (14). The region between the two arrows represents the DNase I-protected region in the presence of Epoc-1/skn-1a. DNase I protection by Epoc-1/skn-1a on the plus strand could not be detected in this assay.

To more precisely localize the site of Epoc-1/skn-1a interaction with the promoter-proximal fragment, DNase I footprint assays were performed (Fig. 3B). Incubation of a GST–Epoc-1/skn-1a fusion protein with a probe containing the 178-bp 3′ portion of the HPV18 URR resulted in the protection of a region located between and partially overlapping with the 3′ end of a previously described AP1 binding site (14) and the 5′ end of a yet uncharacterized footprint (footprint VIII in reference 14). DNA sequences around this protected area are shown in Fig. 4A.

To confirm and further map the binding of Epoc-1/skn-1a to this region, gel retardation assays were performed with synthetic oligonucleotides containing the HPV18 subregions depicted in Fig. 4A. As shown in Fig. 4B, Epoc-1/skn-1a specifically bound to region G, located between the Ap1 binding site and Fp VIII, and to region H, which resides 3′ from oligonucleotide G (Fig. 4A). In contrast, Epoc-1/skn-1a did not interact with oligonucleotides containing regions C and D, confirming that binding is sequence specific. In addition, purified Epoc-1/skn-1a weakly bound to oligonucleotide B (data not shown), which contains the sequence ATGCAACC, exhibiting only a two-nucleotide deviation from an octamer consensus motif (ATGCAAAT [23]).

These findings show that the promoter-proximal portion of the HPV18 URR contains at least two adjacent regions, G and H, with the capacity to bind Epoc-1/skn-1a in vitro. Inspection of these regions for similarities in DNA sequences reveal that they both contain the motif TGCATA(A/C)A, which is arranged in the form of a palindrome. Both regions do not contain an octamer consensus motif, indicating that Epoc-1/skn-1a can also bind to sequences exhibiting substantial deviations therefrom.

Mutation of the Epoc-1/skn-1a binding site abolishes Epoc-1/skn-1a-mediated transactivation of the E6/E7 promoter. To investigate whether the identified Epoc-1/skn-1a binding regions are involved in Epoc-1/skn-1a-mediated transactivation in vivo, the recognition sequences were mutated by PCR-mediated site-directed mutagenesis within the context of the complete HPV18 URR (p18EpocMURRL) or within the context of the 3′-deletion constructs, yielding p232EpocM/18L and p178EpocM/18L (Fig. 5). Gel retardation assays using oligonucleotides containing wild-type (EBS) or mutant (EBSMUT) Epoc-1/skn-1a recognition sites demonstrated that the introduced mutations inhibited Epoc-1/skn-1a binding in vitro. As shown in Fig. 6, binding of Epoc-1/skn-1a to only the wild-type element EBS, not to the mutant element EBSMUT, was detectable. Competition assays show that Epoc-1/skn-1a binding to EBS was inhibited by a 100-fold molar excess of unlabeled EBS but not by the same molar excess of EBSMUT, confirming specificity of binding (Fig. 6).

The effects of Epoc-1/skn-1a on the mutant reporter constructs were then analyzed by transient transfection assays in

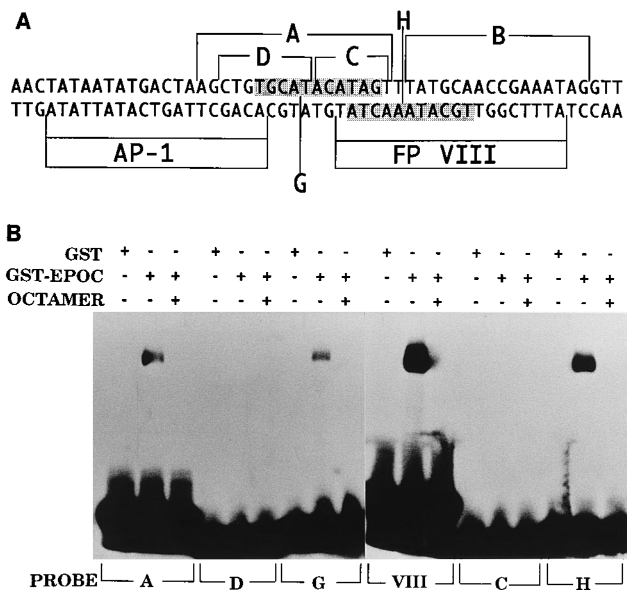

FIG. 4. Delineation of HPV18 sequences bound by Epoc-1/skn-1a. (A) Schematic representation of the DNA surrounding the DNase I-protected sequence within the promoter-proximal region of the HPV18 URR. (B) Gel retardation assay confirming the binding of Epoc-1/skn-1a to the DNase-protected regions (G and H in panel A) within the promoter-proximal region of the HPV18 URR. Radioactively labeled oligonucleotides (see Materials and Methods) containing sequences A, D, G, Fp VIII, C, and H were incubated with GST alone, GST–Epoc-1/skn-1a, or GST–Epoc-1/skn-1a together with an octamer consensus oligonucleotide as competitor DNA. Binding of Epoc-1/skn-1a is specifically detected to DNA sequences G and H.

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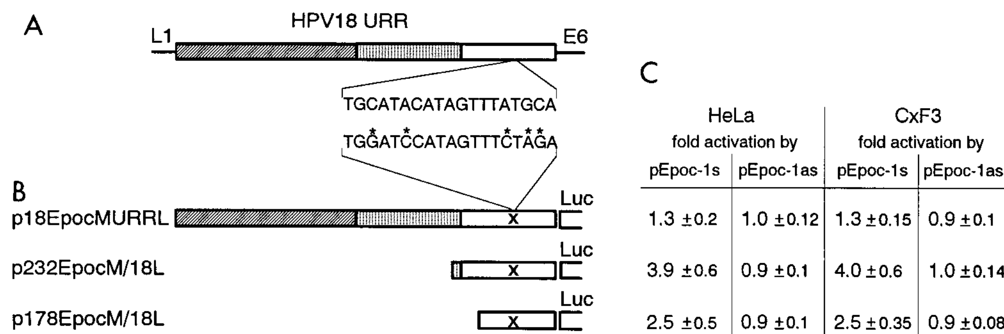

	HeLa fold activation by		CxF3 fold activation by	
	pEpoc-1s	pEpoc-1as	pEpoc-1s	pEpoc-1as
p18EpocMURRL	1.3 ±0.2	1.0 ±0.12	1.3 ±0.15	0.9 ±0.1
p232EpocM/18L	3.9 ±0.6	0.9 ±0.1	4.0 ±0.6	1.0 ±0.14
p178EpocM/18L	2.5 ±0.5	0.9 ±0.1	2.5 ±0.35	0.9 ±0.08

FIG. 5. Mutation of the Epoc-1/skn-1a binding region within the HPV18 promoter-proximal region inhibits Epoc-1/skn-1a-mediated transactivation. (A) Schematic representation of the HPV18 URR, highlighting the wild-type Epoc-1/skn-1a binding region within the promoter-proximal region. (B) Epoc-1/skn-1a binding sequences were mutated by site-directed mutagenesis in the context of the complete HPV18 URR (p18EpocMURRL) or in the context of 5′-deletion fragments of the URR (p232EpocM/18L and p178EpocM/18L). (C) Relative luciferase activities (see the legend to Fig. 1) of the constructs in HeLa cells and primary human fibroblasts (CxF3) after cotransfection with pEpoc-1S and pEpoc-1AS, as indicated. Standard deviations are indicated.

HeLa cells and primary human fibroblasts. Compared with the activities of the respective wild-type constructs (Fig. 2), which were tested in parallel, mutation of the Epoc-1/skn-1a-binding elements within the promoter-proximal region abolished Epoc-1/skn-1a-mediated transactivation almost completely in either cell type (Fig. 5). This was observed both within the context of the complete HPV18 URR and within the context of the deletion constructs. Titration analyses revealed that high concentrations of cotransfected pEpoc-1S resulted in a marginal stimulation of p18EpocMURRL (data not shown), raising the possibility that there are additional Epoc-1/skn-1a-responsive elements within the HPV18 URR. However, taken together, these findings clearly indicate that the Epoc-1/skn-1a binding region within the promoter-proximal region of the HPV18 URR can serve as a functional *cis*-activating element in vivo and represents the key element for transactivation of the E6/E7 promoter by Epoc-1/skn-1a.

DISCUSSION

This study demonstrates that the epithelial transcription factor Epoc-1/skn-1a activates the HPV18 E6/E7 promoter by direct and sequence-specific binding to a recognition site within the promoter-proximal region of the viral URR. Previous reports showed that both Epoc-1/skn-1a expression and efficient HPV E6/E7 transcription are restricted to the suprabasal, differentiated layers of the epithelium, while no Epoc-1/skn-1a expression and only very low levels of E6/E7 transcripts are detectable in the basal cells (1, 13, 32, 40). The observation that Epoc-1/skn-1a is a direct activator of the HPV16 and HPV18 E6/E7 promoters indicates an important role for this factor in the differentiation-dependent stimulation of HPV gene transcription.

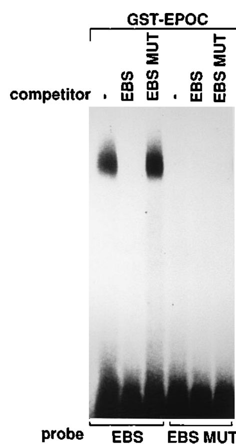

FIG. 6. Mutation of the promoter-proximal Epoc-1/skn-1a recognition site inhibits Epoc-1/skn-1a binding. Gel retardation assays were performed with purified GST–Epoc-1/skn-1a protein and either the wild-type (EBS) or mutant (EBSMUT) Epoc-1/skn-1a recognition element. Competition assays were performed with a 100-fold molar excess of unlabeled competitor oligonucleotides. The mutations within EBSMUT are identical to the mutations introduced into the reporter plasmids (Fig. 5).

Epoc-1/skn-1a belongs to the group of POU domain homeobox proteins (30). It exhibits substantial homology to the octamer-binding transcription factors Oct-1 and Oct-2 and has been demonstrated to bind to an octamer consensus element (1, 41). Interestingly, the recognition sequence for Epoc-1/skn-1a within the HPV18 URR does not contain a perfect octamer motif, indicating that DNA binding by this factor exhibits a somewhat relaxed sequence specificity. On the other hand, the functional data obtained in this study suggest that Epoc-1/skn-1a does not interact with every potential Oct-1 binding site. For example, we do not have evidence that the two sequence-aberrant and low-affinity Oct-1 binding sites within the HPV18 enhancer (6, 24) serve as critical Epoc-1/skn-1a-responsive elements. This is indicated by the observation that Epoc-1/skn-1a-mediated transactivation of the HPV18 URR was almost completely abolished after mutation of the promoter-proximal Epoc-1/skn-1a binding region, although the Oct-1 recognition sites within the viral enhancer were left intact. Furthermore, the HPV18 enhancer itself was unresponsive to Epoc-1/skn-1a even at high concentrations of pEpoc-1S. In this context, it is noteworthy that a recent study did not detect transactivation of an enhancer fragment from the HPV16 URR by Epoc-1/skn-1a (28). The observation in the present study that the complete HPV16 URR is clearly activated by Epoc-1/skn-1a may indicate that in the HPV16 URR, the critical Epoc-1/skn-1a-responsive elements are located outside the fragment tested in the former study.

The marginal stimulation of an HPV18 URR carrying a mutation in the Epoc-1/skn-1a binding site at high concentrations of pEpoc-1S and the observation that purified GST–Epoc-1/skn-1a protein retains some binding activity to the mutant promoter-proximal URR fragment (40) suggest that there may be additional binding sites for Epoc-1/skn-1a in the HPV18 URR. However, the severe reduction of Epoc-1-mediated transactivation after mutation of the promoter-proximal

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Epoc-1 recognition site strongly suggests that the element identified in this study represents the functional key element for the transcriptional activation of the HPV18 URR by Epoc-1/skn-1a.

Several previous studies addressed the question of which regulatory mechanisms contribute to the preferential transcriptional activity of the HPV16 or HPV18 URR in epithelial rather than nonepithelial cells. Comparative footprint analyses of the HPV16 and HPV18 URRs in epithelial and nonepithelial cultured cells did not detect marked cell-type-specific differences in the patterns of proteins binding to the viral URRs (summarized in references 20 and 37). It was therefore hypothesized that the differential activities of the HPV16 and HPV18 E6/E7 promoters in epithelial and nonepithelial cells may result from the activities of cell-specific members of the same multiprotein family, which bind to identical recognition sequences. This has been proposed for HPV18 AP1 elements, which in keratinocytes are preferentially bound by complexes containing JunB (36). Similarly, activation of the HPV16 enhancer has been reported to be dependent on specific subsets of NF1 proteins (2). Alternatively, cell-type-specific coactivators may functionally interact with proteins that directly bind to the URR, as postulated for a cell-type-specific coactivator interacting with TEF-1 (22). However, none of these candidate factors has been found to be sufficient for activation of the HPV E6/E7 promoter in nonepithelial human cells. In contrast, Epoc-1/skn-1a clearly activated the HPV18 E6/E7 promoter after introduction into human fibroblasts or into human hepatoma cells. Epoc-1/skn-1a thus represents the first epithelial cell-specific transcription factor which per se is sufficient to stimulate HPV transcription in nonepithelial cells.

HPV-associated carcinogenesis is closely linked to the expression of the viral E6 and E7 oncoproteins (38). The failure of cellular regulatory mechanisms normally involved in the control of HPV oncogene transcription and the concomitant increase in E6/E7 expression is believed to play a key role in the process of HPV-associated carcinogenesis (42). These deregulation phenomena in HPV-positive cervical carcinomas could be due principally to the loss of function of a transcriptional repressor, the gain of function of a transcriptional activator, or the combination of both events. It thus is of particular interest to define the factors involved in the transcriptional control of HPV E6/E7 expression. The definition in this work of Epoc-1/skn-1a as a transcriptional activator raises the question of whether this factor participates in the activation of viral oncogene transcription in HPV-transformed cells. Our data, however, suggest that this is not the case, since (i) the promoter-proximal fragment of the HPV18 URR, which is strongly activated by coexpression of Epoc-1/skn-1a, does not show any basal activity in HPV-positive cancer cell lines, and (ii) Epoc-1/skn-1a transcripts were undetectable among cDNAs prepared from HPV18-positive HeLa cervical carcinoma cells (40). This interpretation is in line with the notion that Epoc-1/skn-1a expression requires a certain degree of epithelial cell differentiation.

Overexpression of the ubiquitous transcription factor Oct-1 has been shown to inhibit the activity of the HPV18 E6/E7 promoter in a number of different cell systems (21, 27). Similar to the situation for the simian virus 40 enhancer, in which Oct-1 interferes with the binding of transcriptional activators to sph motifs (33), repression of the HPV18 URR may be partially due to the competitive binding of Oct-1 to overlapping recognition sites for transcriptional activators (20, 21, 24, 37). However, since Oct-1-mediated repression is observed in HeLa cells and fibroblasts (21, 27), in which neither Epoc-1/skn-1a expression nor Epoc-1/skn-1a-mediated transactivation was detected, it seems unlikely that Oct-1-mediated repression of HPV transcription in these cells relies on the competitive binding between Oct-1 and Epoc-1/skn-1a. This, however, does not exclude the possibility of a functional interplay between Oct-1 and Epoc-1/skn-1a in tissues in which both factors are expressed. As Oct-1 is considered to be ubiquitously expressed (17), such an interaction could take place in the differentiating layers of the epithelium.

It also should be noted that similar to the regulation of viral transcription, HPV replication appears to be strongly dependent on the differentiation status of epithelial cells, resulting in much higher viral copy numbers in the differentiated suprabasal cell layers than in undifferentiated basal cells (13, 32). The molecular basis for this observation is unknown. As cellular transcription factors can strongly influence the replication rate of viruses (12), it will be interesting in future studies to investigate the effect of Epoc-1/skn-1a on HPV replication in the available model systems (10).

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K.Y. and K.B. contributed equally to this work and should be considered equal first authors.

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