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Repression of the Human Papillomavirus Type 18 Enhancer by the Cellular Transcription Factor Oct-1

FELIX HOPPE-SEYLER,* KARIN BUTZ, AND HARALD ZUR HAUSEN

Projektgruppe Angewandte Tumorvirologie, Institut für Virusforschung, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 506, D-6900 Heidelberg, Germany

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The role of cellular factors involved in the transcriptional regulation of the cancer-associated human papillomavirus type 18 (HPV18) is yet poorly understood. The presence of an Oct-1-binding site within the HPV18 upstream regulatory region led us to investigate the influence of Oct-1 on viral transcription. Cotransfection of Oct-1 expression plasmids together with luciferase reporter constructs containing HPV18 regulatory sequences indicated that Oct-1 can transcriptionally repress the HPV18 upstream regulatory region. In contrast, heterologous control regions were not affected by Oct-1. HPV18 *cis* elements that can be repressed by Oct-1 mapped to a 135-bp subregion of the viral constitutive enhancer. Analysis of an Oct-1 mutant defective in DNA binding suggested that HPV18 down-modulation does not require direct binding of Oct-1 to DNA. These results make Oct-1 a candidate factor involved in the intracellular surveillance of HPV18 transcription and support the notion of a host cell mechanism that can specifically repress HPV E6-E7 transforming gene expression.

Papillomaviruses are epitheliotropic double-stranded circular DNA viruses responsible for a wide range of skin and mucocutaneous tumors in humans and other animals. The DNA of specific human papillomaviruses (HPVs) (in particular HPV type 16 [HPV16] and HPV18) is found almost regularly in cervical carcinomas, mostly integrated into the host cell genome (49). Viral early genes E6 and E7 are transcribed in tumor tissue and tumor-derived cell lines (39, 47). Both E6 and E7 possess transforming potential in vitro (2, 26, 31, 32), are required for the maintenance of the transformed phenotype of cervical cancer cells (44, 45), and form specific complexes with the putative tumor suppressor gene products p53 (E6) and Rb (E7) (19, 46). These findings strongly suggest that E6 and E7 play an important role in the process of malignant transformation.

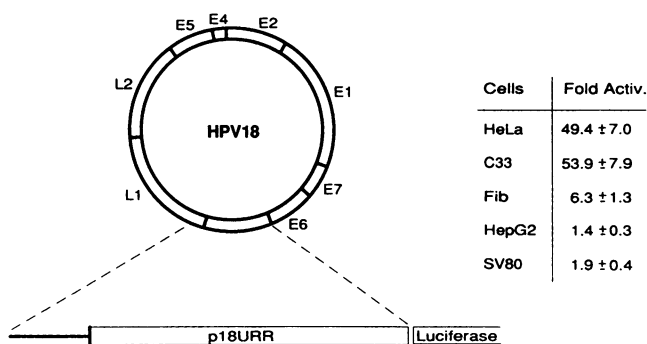
HPV18 E6-E7 transcription is controlled by *cis*-acting elements contained in the viral upstream regulatory region (URR), which separates the viral early (E) and late (L) genes in the viral genome (Fig. 1). The 825-bp viral URR has been functionally divided into three parts (see Fig. 4): a 5′-terminally located 389-bp E6-responsive enhancer, a central 230-bp constitutive enhancer, and a 206-bp 3′-terminally located E2-responsive region (22). However, although a number of studies indicated involvement of cellular factors in HPV gene regulation (12, 14, 15, 20, 23, 24), host control of viral transcription is still poorly understood.

In the course of experiments designed to characterize *cis* elements involved in HPV18 transcriptional regulation, we recently identified within the constitutive enhancer a binding site (AATTGCAT, nucleotide [nt] 7721 to 7728) for the ubiquitously expressed Oct-1 transcription factor (8). Owing to the lack of a proper transcriptional activation domain, Oct-1 itself cannot stimulate an mRNA minimal promoter containing a TATA box only (42). However, as shown for the herpes simplex virus VP16 transactivator (33), Oct-1 can stimulate transcription by forming multiprotein complexes with factors providing TATA box-type activation domains. Protein-protein interactions also seem to play an important role in the functional cooperativity between Oct-1 and steroid receptors, inducing the mouse mammary tumor virus promoter (7).

To study the influence of Oct-1 on transcription regulated by the HPV18 URR in vivo, we utilized transient expression assays employing the highly sensitive and readily testable luciferase reporter system (6, 17). Basically, this procedure consists of the introduction of an expression vector (encoding Oct-1 or mutant forms thereof) together with a plasmid containing HPV regulatory sequences linked to the *Photinus pyralis* luciferase gene. Transcriptional activation by the entire HPV18 URR was studied employing reporter plasmid p18URRL, in which the URR was cloned directly upstream of the *P. pyralis* luciferase-coding sequence (Fig. 1). To validate its use as a suitable reporter construct to measure HPV18 URR E6-E7 promoter activity, we determined p18URRL activity in cultured cells of different origins. As shown in Fig. 1, p18URRL luciferase activity was stimulated strongly in HeLa cells and C33 cells compared with the enhancer-promoterless pBL vector. In contrast, the HPV18 URR only weakly stimulated luciferase activity in primary human fibroblasts and was almost completely inactive in SV80 cells and HepG2 cells. These findings reflect the epithelial cell preference of the HPV18 regulatory region and are in good agreement with results obtained by others (3, 43) analyzing the HPV18 URR E6-E7 promoter activity using different reporter systems.

To analyze the effect of Oct-1 on the HPV18 URR, we cotransfected Oct-1 expression vectors together with reporter plasmid p18URRL. pCGOct-1 codes for a functional Oct-1 protein (42), pCGOct-1H$^-$ encodes a DNA binding-defective Oct-1 mutant (40–42) carrying a triple amino acid substitution in the homeodomain, while pCGOct-1AS (42) contains an inverted Oct-1-coding sequence and served as a negative control. Figure 2 shows the results of titration experiments performed in HeLa cells indicating that increasing amounts (0.01 to 1.0 μg) of cotransfected pCGOct-1 DNA led to a strong down-modulation of HPV18 URR-

* Corresponding author.



Cells	Fold Activ.
HeLa	49.4 ±7.0
C33	53.9 ±7.9
Fib	6.3 ±1.3
HepG2	1.4 ±0.3
SV80	1.9 ±0.4

FIG. 1. Transcriptional activity of the HPV18 URR. Reporter plasmids are based on basic vector pBL (37), which contains the firefly *P. pyralis* luciferase gene (17) fused to the SV40 poly(A) signal derived from pBLCAT2 (29) and cloned into the polylinker of Bluescript KSM13+ (Stratagene). p18URRL (indicated below) contains the complete HPV18 URR (nt 6929 to 88, numbering according to reference 12) with a 16-bp 3'-terminal deletion removing the E6 ATG to prevent a possible translational interference with the luciferase start codon. Luciferase assays were performed after transfection (11) of HeLa cells, C33 cells (a human cervical carcinoma cell line devoid of HPV sequences), Fib (early-passage human dermal fibroblasts derived from oral mucosa), SV80 cells (SV40-immortalized human fibroblasts), and HepG2 (human hepatocarcinoma cells). Each transfection contained 10 μg of p18URRL and 4 μg of pAc-Gal (4.3-kb *Eco*RI-*Alu*I fragment of the human β-actin promoter [28] linked to the *Escherichia coli* β-galactosidase gene) as an internal control and was adjusted to 20 μg by the addition of Bluescript DNA. Luciferase activities were quantitated as described previously (6), and β-galactosidase activities were measured (30) to account for variation in transfection efficiency. Promoter activities were determined by calculating the luciferase/β-galactosidase ratios. p18URRL luciferase activities (fold enhancement above activity of parental plasmid pBL) within different cells are indicated to the right.

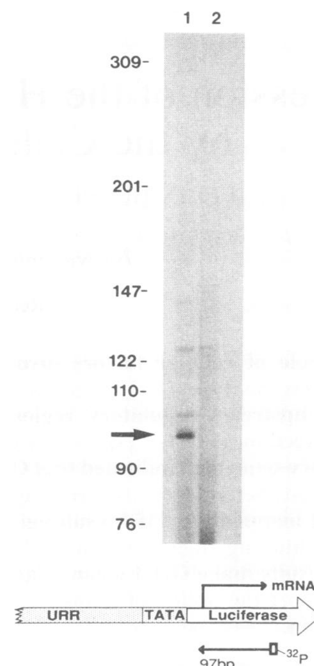


FIG. 3. Primer extension analysis of p18URRL transcripts in HeLa cells. Vector, radiolabeled primer, the 97-nt extended product, and luciferase mRNA are indicated schematically below. RNA (50 μg) treated with RNase-free DNase was hybridized to a molar excess of an end-labeled 20-nt synthetic oligonucleotide primer (5'-GCTCTCCAGCTTATCCATCC-3', complementary to *P. pyralis* luciferase gene [17] nt 105 to 124), and the assay was performed essentially as described previously (30). Products were analyzed on an 8% polyacrylamide sequencing gel, using *Hpa*II-digested pBR322 DNA as the size markers (numbers on left in nucleotides). Lane 1, extended product after transfection with 7 μg of p18URRL. The major transcriptional start site is indicated by an arrow. Lane 2, extended products after cotransfection of 7 μg of p18URRL with 0.5 μg of pCGOct-1.

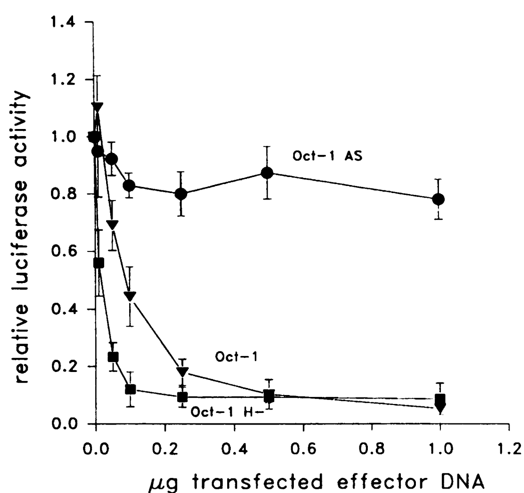


FIG. 2. Effect of Oct-1 wild-type and mutant proteins on the HPV18 URR in HeLa cells. p18URRL luciferase activity after cotransfection of increasing amounts of pCGOct-1 (▼), pCGOct-1H⁻ (■), and pCGOct-1AS (●) (described in reference 42) relative to p18URRL activity without cotransfected effector plasmid (normalized to 1.0). Values are the means of at least three independent determinations, and standard deviations are indicated.

regulated luciferase activity. Primer extension analysis (Fig. 3, lane 1) showed that the p18URRL major transcriptional start site mapped to the HPV18 E6-E7 promoter, initiating approximately 21 bp downstream of the HPV18 E6-E7 TATA box (43) and using a cap site approximately 23 bp upstream of the luciferase start codon in p18URRL (the authentic HPV18 E6 cap site is deleted in p18URRL [see the legend to Fig. 1]). Coexpression of Oct-1 strongly reduced the amount of correctly initiated transcripts (Fig. 3, lane 2), confirming that repression occurred at the transcriptional level.

Activity of p18URRL was also strongly down-modulated by pCGOct-1H⁻ (Fig. 2), indicating that Oct-1 binding to DNA is not required for this regulatory effect. In contrast, control vector pCGOct-1AS did not significantly affect p18URRL. As a further control, we cotransfected pCGOct-1, pCGOct-1H⁻, and pCGOct-1AS together with basic vector pBL and did not observe any effect on basal luciferase activity (Table 1).

We next examined whether the Oct-1-mediated repression is also observed for other transcriptional control regions of different viral and cellular origins. Cotransfection of a constant amount of pCGOct-1 or pCGOct-1H⁻ did not affect luciferase activity controlled by the truncated herpes sim-

TABLE 1. Specificity of Oct-1-mediated HPV18 URR repression

Effector plasmid	Relative luciferase activity[a] with the following reporter plasmid:						
	p18URRL	ptkL[b]	pCMV-Luc[b]	pAc-Luc[b]	pSV40-Luc[b]	pHMG-Luc[b]	pBL
None	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pCGOct-1	0.13 ± 0.05	1.29 ± 0.12	1.13 ± 0.11	1.29 ± 0.23	0.18 ± 0.05	0.94 ± 0.17	1.22 ± 0.12
pCGOct-1H⁻	0.09 ± 0.02	1.22 ± 0.29	1.16 ± 0.13	1.29 ± 0.27	0.82 ± 0.15	0.98 ± 0.05	1.11 ± 0.06
pCGOct-1AS	0.87 ± 0.09	1.05 ± 0.23	0.96 ± 0.11	0.99 ± 0.14	0.94 ± 0.18	0.84 ± 0.13	0.93 ± 0.16

[a] Luciferase activity relative to reporter plasmid activity without cotransfected effector plasmid. Values are the means of at least three independent experiments with DNA from at least two independent plasmid preparations. Standard deviations are indicated.

[b] Luciferase transcription is controlled by a truncated herpes simplex virus *tk* promoter (nt −105 to +19, derived from pBLCAT2 [29] in ptkL, by the human cytomegalovirus enhancer-promoter region (*Hin*cII-*Ava*II fragment [5]) in pCMV-Luc, by the human β-actin promoter (same fragment as pAc-Gal; see the legend to Fig. 1) in pAc-Luc, the SV40 early control region encompassing a single 72-bp repeat and the viral early promoter in pSV40-Luc (pBHE [1]), and by the hydroxymethylglutaryl-coenzyme A reductase promoter (1.4-kb *Not*-*Pvu*II fragment [21]) in pHMG-Luc.

plex virus *tk* gene promoter in ptkL, the human cytomegalovirus promoter in pCMV-Luc, the promoter of the cellular housekeeping gene hydroxymethylglutaryl-coenzyme A reductase in pHMG-Luc, and the human β-actin promoter in pAc-Luc (Table 1). In contrast, the HPV18 URR in p18URRL was down-regulated about 90% by both Oct-1 and Oct-1H⁻. At the concentrations used in these experiments, we did not observe nonspecific effects of the expression vectors on any of the examined regulatory regions, since cotransfection of control plasmid pCGOct-1AS did not affect the luciferase activities of the respective reporter constructs (Table 1).

The activity of the simian virus 40 (SV40) early control region in pSV40-Luc was repressed by Oct-1 (Table 1). However, in contrast to the repression observed for the HPV18 URR, down-modulation of the SV40 enhancer element required binding of Oct-1 to its cognate DNA motif since it was relieved by the homeobox mutation in Oct-1H⁻.

HeLa cells contain multiple copies of integrated HPV18 sequences and express viral transforming genes E6 and E7 (39). Both E6 and E7 possess transcriptional activation potential, and E6 was reported to transactivate the homologous HPV18 enhancer (22, 36). To investigate whether the Oct-1-mediated down-modulation of the HPV18 URR in HeLa cells is dependent on virus-specific gene products, we tested HPV-negative cell lines for Oct-1 repressor function. Both C33 cervical carcinoma cells and primary human dermal fibroblasts exhibited HPV18 URR *trans*-repression by Oct-1 that was qualitatively and quantitatively comparable to that of HeLa cells (Table 2), indicating that Oct-1 repressor function does not depend on HPV18 gene products. Since Oct-1 repressor function is also observed in human fibroblasts, the results further indicate that Oct-1 repressor function is not confined to epithelial cells (C33, HeLa).

TABLE 2. Oct-1-mediated repression of p18URRL in HPV-negative cells

Effector plasmid	Relative luciferase activity[a]	
	C33	Fib
None	1.0	1.0
pCGOct-1	0.18 ± 0.05	0.16 ± 0.05
pCGOct-1H⁻	0.09 ± 0.04	0.11 ± 0.05
pCGOct-1AS	0.92 ± 0.24	1.10 ± 0.19

[a] Luciferase activity relative to p18URRL activity without cotransfected effector plasmid. Values are means of four independent experiments with at least two independent plasmid preparations. Standard deviations are indicated.

To localize the HPV18 *cis* element(s) conferring repression by Oct-1, we tested different deletion mutants of p18URRL cotransfected with the Oct-1, Oct-1H⁻, and Oct-1AS expression vectors (Fig. 4). Deletion of HPV18 sequences 6929 to 7509 from p18URRL (Fig. 4) resulted in plasmid p436/18L, which thus contains the 230-bp *Rsa*I-*Rsa*I constitutive enhancer linked to the homologous promoter region. As indicated, removal of the putative E6-responsive enhancer decreased luciferase activity to about 80% of that of undeleted p18URRL. However, p436/18L could be efficiently repressed by Oct-1 and Oct-1H⁻. Deletion of nt 6929 to 7582 from p18URRL led to a further 71% decrease in HPV18 enhancer activity (p363/18L), but the residual activity could still be efficiently repressed by Oct-1 and Oct-1H⁻ (Fig. 4). Further deletion (nt 6929 to 7713) yielded plasmid p232/18L (Fig. 4), which was almost inactive (1.8-fold stimulation above that of pBL) and therefore could not be evaluated in Oct-1 repression assays. From these experiments, it can be concluded that Oct-1 repression is mediated by *cis* elements located in HPV18 sequences 7583 to 88.

To further map the Oct-1 target element(s), we investigated whether HPV18 enhancer elements could confer down-modulation by Oct-1 independent of their homologous promoter region. As shown in Fig. 4, the 230-bp *Rsa*I-*Rsa*I constitutive enhancer cloned upstream of the heterologous *tk* promoter (p230s/tkL) was efficiently repressed by both Oct-1 and Oct-1H⁻. Deletion of a 22-bp 3′-terminal *Afl*II-*Rsa*I fragment containing the Oct-1-binding site (nt 7721 to 7728) resulted in plasmid p208s/tkL. p208s/tkL luciferase activity was strongly inhibited by Oct-1 and Oct-1H⁻, which is consistent with the observation that Oct-1 binding to DNA is not required for repression. Deletion of a 73-bp 5′-terminal fragment from the 230-bp constitutive enhancer region resulted in p157s/tkL, which was efficiently repressed by both Oct-1 and Oct-1H⁻ (Fig. 4). The same 157-bp region also conferred Oct-1 repression when tested in the antisense configuration (p157as/tkL). Further deletion of a 22-bp 3′-terminal fragment from p157s/tkL resulted in p135s/tkL. p135s/tkL and the corresponding antisense construct p135as/tkL were both strongly down-modulated by Oct-1 and Oct-1H⁻ (Fig. 4). Although we cannot exclude the occurrence of additional Oct-1-responsive modules in the HPV18 URR, these results map an internal 135-bp *Bgl*I-*Afl*II enhancer fragment as sufficient to confer Oct-1 repressor function.

The results of this study showed that the ubiquitous Oct-1 homeodomain protein can transcriptionally repress the HPV18 enhancer. Interestingly, analysis of a DNA binding-defective Oct-1 mutant (40–42) indicated that this novel Oct-1 function does not require Oct-1 binding to DNA. To exclude the formal possibility that Oct-1H⁻ could bind to

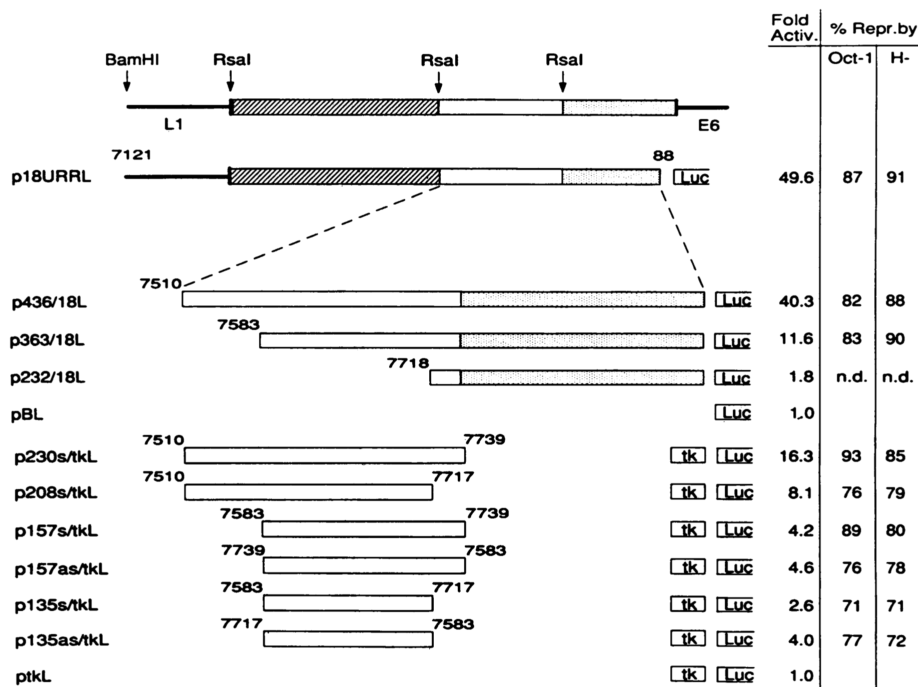


FIG. 4. Effect of Oct-1 wild-type and mutant proteins on HPV18 URR deletion mutants. Schematic representation of the HPV18 URR. E6-responsive enhancer (dashed area), constitutive enhancer (open area), and E2-responsive subregion (stippled area) (22) are indicated. Below: HPV 18URR fragments linked directly to the luciferase gene (Luc) or cloned in enhancer configuration upstream of the truncated herpes simplex virus *tk* promoter into ptkL (see Table 1). 5′ and 3′ boundaries of the HPV18 URR fragments (nucleotide numbering according to reference 13) are indicated. DNA manipulations followed standard cloning protocols (30), and constructs were confirmed by DNA sequencing. All experiments were performed in duplicate and repeated between 3 and 11 times. First column to the right, relative luciferase activities after transfection of 10 μg of reporter plasmid (fold activation above that of pBL or ptkL). Second and third columns, percent repression of luciferase activity after cotransfection with 0.5 μg of pCGOct-1 and pCGOct-1H$^-$, respectively. n.d., not determined.

degenerate octamer sequences within the repressible 135-bp enhancer region, we performed gel retardation assays using Oct-1H$^-$ protein (obtained by overexpression in HeLa cells) and the 135-bp fragment as probes. We did not detect binding of Oct-1H$^-$ to the HPV18 sequences (25), suggesting a more indirect mechanism of Oct-1-mediated repression which possibly involves protein-protein contacts. Consistent with this interpretation, Oct-1 has been shown before to possess an intrinsic ability to form protein-protein contacts with heterologous factors such as the herpes simplex virus transactivator VP16 (27, 40). Repression of the HPV18 enhancer could thus involve squelching (36) by Oct-1 complex formation with an essential HPV18 stimulatory factor, titrating out the number of activator molecules available for HPV transcriptional stimulation.

By deletion mapping, we identified a 135-bp fragment within the HPV18 constitutive enhancer as being sufficient to confer Oct-1 repressor function. This subregion contains several protein-binding sites protected in footprint experiments (12, 20). With the exception of a member of the AP1 family, however, the other factors binding to this subregion are not yet identified. We also observed Oct-1-mediated repression of the structurally related HPV16 constitutive enhancer (p7463-7752SVE-cat [15]), also involving a mechanism independent of Oct-1 binding to DNA (25).

Oct-1 repressor function exhibited specificity for HPV regulatory sequences, since the activity of other transcriptional control regions from other, both cellular (hydroxymethylglutaryl-coenzyme A reductase, actin) and viral (herpes simplex virus, cytomegalovirus), genes was not modulated by Oct-1. In this series of experiments, the only regulatory region also affected by Oct-1 was the SV40 early control region, containing a single 72-bp enhancer repeat and the SV40 early promoter. This finding confirms a previous observation reporting repression of the SV40 enhancer by Oct-1 through competitive binding to an octamer motif which overlaps with an activating Sph motif (42). This type of repression is steric, requires Oct-1 binding to DNA, and is therefore relieved by the homeobox mutation of Oct-1H$^-$. In contrast, DNA binding-defective Oct-1H$^-$ is a potent repressor of the HPV18 enhancer. These results strongly suggest a different mode of Oct-1-conferred repression of the HPV18 enhancer compared with that of the SV40 enhancer. From the results that Oct-1 can efficiently repress the HPV18 enhancer in HPV18-negative C33 cervical carcinoma cells as well as in primary human dermal fibroblasts, we conclude that Oct-1 repressor function does not involve interference with HPV-specific gene products but rather is related to cellular control functions.

A systematic analysis of Oct-1 transcription in different tissues has not yet been undertaken. A recent report described a differentiation-dependent regulation of Oct-1 levels in F9 cells (10). Since HPV gene expression largely depends on cellular differentiation processes (18), it will be interesting to examine the Oct-1 protein status at distinct stages of epithelial cell differentiation. To assess the potential role of Oct-1 for the process of HPV-associated malignant transformation, these investigations should include comparative

analysis of Oct-1 proteins in both normal and HPV-transformed epithelial cells. Furthermore, since maintenance of the transformed phenotype has been reported to require the continuous expression of viral E6-E7 transforming genes (16, 45), it will also be interesting to determine the biological effects of an inducible Oct-1 expression system stably introduced into HPV-positive cervical carcinoma cell lines.

The failure of an intracellular surveillance system controlling HPV transforming gene expression has been implicated to play a key role in HPV-associated carcinogenesis (4, 48, 49). Consistent with this view, recent reports related a deregulated E6-E7 expression to the development of HPV-associated malignancies (9, 14, 23, 34, 38, 44, 45). Although one has to be aware that cotransfection assays can involve an unphysiological overexpression of effector proteins, the results obtained in this study provide an experimental basis to investigate a cellular control mechanism that can specifically down-modulate HPV18 enhancer activity, which in turn affects the regulation of viral E6-E7 transforming gene expression. This mechanism could play an important role in the intracellular surveillance of viral gene expression and makes the human homeodomain Oct-1 protein a candidate protein involved in host cell control of HPV transcription.

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