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Transcriptional regulation of the human papillomavirus-16 E6–E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 *trans*-activator and repressor gene products: implications for cervical carcinogenesis

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The transcriptional promoter of the candidate E6–E7 transforming gene region of human papillomavirus (HPV)-16 (P97) was active in transiently transfected cervical carcinoma cells when linked to the HSV-1 *tk* or bacterial *cat* genes. Sequences 5′ to P97 contain a short enhancer element responding to cellular factor(s) in uninfected human foreskin keratinocytes and in cervical carcinoma cells, but not in human or animal fibroblasts. The E2 *trans*-activator products of HPV-16 or of the related bovine papillomavirus (BPV)-1 further elevated HPV-16-driven transcripts in co-transfections, and required the presence of E2-binding ACC(N)$_6$GGT cores in *cis*. A 'short E2' C-terminal repressor gene product (sE2) of HPV-16 or the BPV-1 sE2 repressor not only inhibited viral E2 *trans*-activation, but also suppressed enhancer response to keratinocytic factors. Suppression by the sE2 products was abolished by deletion of the E2-binding cores in *cis* or by a mutation in the sE2 DNA binding domain. The keratinocyte-dependent enhancer is likely to contribute to the epithelial cell tropism of HPV-16, and may direct persistent E6–E7 gene transcription in response to cellular factors in cervical carcinoma cells in which the viral E2 genes are inactive.

Key words: cat gene/cervical carcinoma/enhancer repression/HSV-1 *tk* gene/transient transfections

Introduction

Human papillomavirus (HPV)-16 is the most prevalent virus associated with cervical cancer and higher grade cervical intraepithelial neoplasia (Dürst *et al.*, 1983, 1985; Crum *et al.*, 1984). The circular DNA genomes of HPV-16 (Seedorf *et al.*, 1986) replicate as extrachromosomal plasmids in benign and premalignant cervical lesions (Crum *et al.*, 1984; Dürst *et al.*, 1985). In invasive carcinomas, however, DNA sequences of HPV-16 or the related HPV-18 are found integrated in the cellular genome. Viral integration observed in cervical carcinoma biopsies and derived cell lines shows a common pattern. The upstream early viral gene region is preserved, but the downstream early gene region is deleted, disrupted or inactivated (Schwarz *et al.*, 1985; Schneider-Gädicke and Schwarz, 1986; Baker *et al.*, 1987). The significance of HPV integration and of the viral gene rearrangements in cervical carcinogenesis is not well understood, since little is known about HPV functions.

Transfection with HPV-16 DNA extends the *in vitro* lifespan of human genital keratinocytes (Dürst *et al.*, 1987b; Pirisi *et al.*, 1987) and leads to neoplastic transformation of mouse fibroblasts (Tsunokawa *et al.*, 1986; Yasumoto *et al.*, 1986). The upstream gene region, comprising the E6–E7 open reading frames (ORFs; Seedorf *et al.*, 1985), may play a role in the maintenance of neoplastic growth. The E6 ORF of the related bovine papillomavirus (BPV)-1 encodes a *bona fide* transforming protein (Schiller *et al.*, 1984; Androphy *et al.*, 1985). The E6 and E7 viral proteins are expressed in cells transformed by HPV-16 DNA *in vitro* (Androphy *et al.*, 1987a). The HPV-16 E6–E7 gene region cooperates with the *ras* gene in tumorigenic transformation of primary rat fibroblasts (Matlashewski *et al.*, 1987). Moreover, the preserved HPV E6–E7 region is transcribed and translated into the predicted viral proteins in cervical carcinoma cells (Boshart *et al.*, 1984; Lehn *et al.*, 1985; Pater and Pater, 1985; Schwarz *et al.*, 1985; Yee *et al.*, 1985; Schneider-Gadicke and Schwarz, 1986; Smotkin and Wettstein, 1986, 1987; Androphy *et al.*, 1987a; Baker *et al.*, 1987).

The underlying causes of the apparent selection against the downstream early genes of HPV-16 in cervical carcinoma cells have not been explained so far. In the model BPV-1, the downstream E2 ORF encodes two *trans*-acting regulatory functions that can influence E6–E7 gene transcription. A full-length E2 gene product ('long E2'; further referred to as E2 here) *trans*-activates the E6–E7 promoter of BPV-1, P89 (Haugen *et al.*, 1987; Spalholz *et al.*, 1987). A C-terminal E2 gene product ('short E2', sE2 or 'E2-TR', Lambert *et al.*, 1987) inhibits E2 *trans*-activation, most likely by competitive binding to the same *cis* DNA target elements required for E2 *trans*-activator attachment. The shared C-terminal E2 peptide domain binds *in vitro* to viral DNA fragments that contain ACCG(N)$_4$CGGT cores (E2-responsive palindromes, *E2P*s; Androphy *et al.*, 1987b), and *E2P* cores upstream of the BPV-1 E6–E7 promoter are required for *trans*-activation by E2 (Haugen *et al.*, 1987; Spalholz *et al.*, 1987) and for its inhibition by the sE2 product (Lambert *et al.*, 1987).

HPV-16 shares some features of these regulatory circuits with BPV-1. Its upstream regulatory region (URR) 5′ to the E6–E7 ORFs contains potential *E2P* cores (Seedorf *et al.*, 1985), and the E2 ORF of HPV-16 encodes a *trans*-activator function (Phelps and Howley, 1987). In cervical carcinoma cells, disruption of the HPV E2 ORF could inactivate a putative sE2 repressor similar to that of BPV-1, but cannot account for the observed persistent E6–E7 gene expression in the absence of viral E2 *trans*-activation. We have therefore studied the activity of the authentic HPV-16 E6–E7 region promoter, P97 (Smotkin and Wettstein, 1986), in transient transfection assays to identify viral and cellular factors which can influence HPV-16 E6–E7 gene expression.

Our results provide a possible explanation for the apparent selection against viral E2 gene expression in cervical carcinomas. We have found that the level of transcripts initiated at the HPV-16 P97 promoter is driven by a keratinocyte-dependent upstream enhancer element in the absence of viral E2 products. The viral

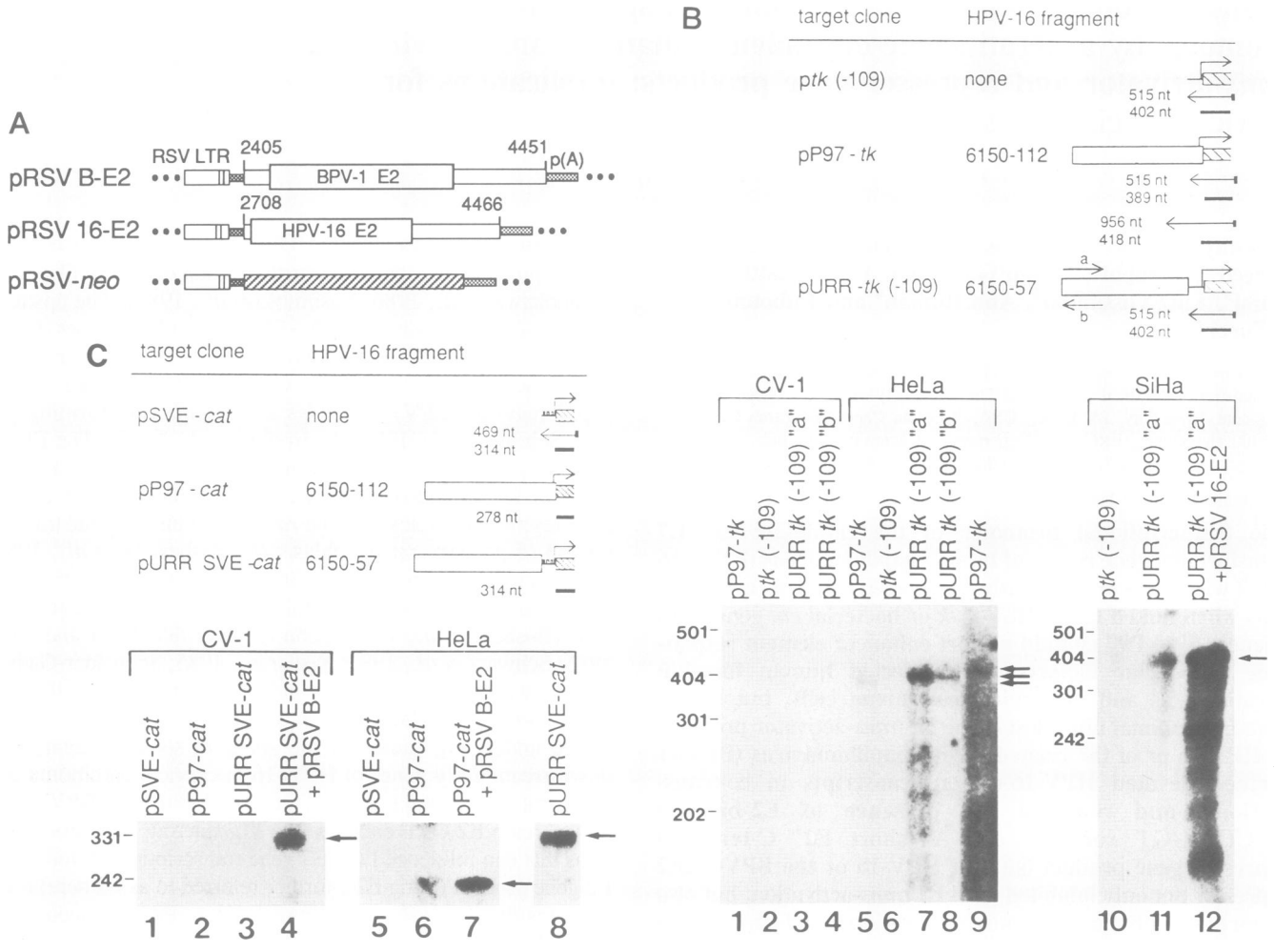


Fig. 1. RNase protection analysis of P97-initiated transcripts and HPV-16 URR enhancer activity in transient transfections. (**A**) RSV LTR-driven vectors expressing the E2 *trans*-activators of either BPV-1 or HPV-16, or the *neo* gene used as a carrier in co-transfections. (**B**) *tk* target clones and 5′ mRNA end detection. In pP97-*tk*, an HPV-16 fragment (open bar) with the P97 promoter was linked to the body of the *tk* gene at nucleotide +14. In pURR *tk*(−109)'a' and 'b', a fragment encompassing sequences 5′ to the P97 promoter was ligated upstream of the *tk* promoter at nt −109 in p*tk*(−109). These plasmids were co-transfected with pRSV-*neo* as a control or with E2 *trans*-activators. Predicted 5′ cap sites (→), SP6 probe (←), and protected fragment (solid bar) sizes are shown. The 515 nt SP6 probe complementary to p*tk*(−109) was used except in **lane 9**; the 956 nt probe used in this lane is complementary to the chimeric pP97-*tk* transcript. (**C**) *cat* target clones and 5′ mRNA end detection. All RNAs were hybridized to the 469 nt probe corresponding to the chimeric SVE-*cat* transcript.

E2 *trans*-activators of either HPV-16 or of the related BPV-1 further stimulate HPV-16-directed mRNA levels. C-terminal sE2 repressor gene products of the HPV-16 or the BPV-1 E2 ORFs not only inhibit the viral E2 *trans*-activation, but also repress the keratinocytic factor response. It is thus possible that inactivation of the viral E2 control mechanism leads to persistent E6−E7 gene expression in response to cellular factors, and is one of the critical events in the malignant progression of the infected cell.

Results

HPV-16 P97 promoter is differentially activated in cervical carcinoma cells

The major 5′ cap site of HPV-16 E6−E7 region transcripts has been mapped to nucleotide (nt) 97 in cervical carcinoma cells (Smotkin and Wettstein, 1986). To study the regulation of the P97 promoter by viral and cellular factors, we have linked HPV-16 sequences encompassing P97 upstream of a heterologous gene, HSV-1 thymidine kinase (*tk*), and tested the transcript levels of the chimeric promoter clone, pP97-*tk*, in transient transfections in different cells (Figure 1). Because the analogous E6−E7 promoter in BPV-1, P89, is *trans*-activated by a gene product of the BPV-1 E2 ORF (Haugen *et al.*, 1987), we have also investigated P97 activity in co-transfections with plasmids expressing high levels of BPV-1 E2 (pRSV B-E2; Haugen *et al.*, 1987) or HPV-16 E2 (pRSV 16-E2). pRSV-*neo*, which expresses the bacterial *neo* gene, served as a control carrier DNA (Figure 1A).

The pP97-*tk* clone was first transfected into monkey kidney CV-1 cells, and into two HPV-positive human cervical carcinoma cell lines, HeLa and SiHa. Total cellular RNA from transfected cultures was hybridized to a 515 nt-long SP6 riboprobe complementary to the 5′ end of the *tk* transcript (*tk* nt +1 to +402) and the *tk* upstream sequences (Figure 1B), treated with RNase A and RNase T1, and the resulting protected fragments were analyzed on polyacrylamide gels. Transcripts of pP97-*tk* were not detected in CV-1 cells (lane 1), but were readily observed in both transfected cervical carcinoma lines. Control CV-1 cultures, co-transfected with a *tk* plasmid containing the BPV-1 E2-responsive element (p*E2R-tk*; Haugen *et al.*, 1987) and the BPV-1 E2 *trans*-activator clone, pRSV B-E2, yielded the expected high *tk* transcript levels (data not shown). The absence of detectable *tk* transcripts in pP97-*tk*-transfected CV-1 cells was

Table I. Host range of the HPV-16 upstream *cis* enhancer[a]

	Keratinocyte-derived						Fibroblast-like			
	Human foreskin		Human cervical carcinoma				Human	Monkey kidney	Mouse	
cat plasmid	HFK	HPK-I	HeLa	CaSki	SiHa	C4-I	GM3498	CV-1	C127	3T3
pSVE-*cat*	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
pURR 5′-'a'	3.8	14.8	12.0	33.9	4.6	6.0	2.2	0.8	2.5	1.4
pURR 5′-'b'	12.8	20.1	16.1	18.1	2.6	7.2	1.9	0.9	1.0	1.2
pURR 3′-'a'	11.3	8.4	4.5	5.3	1.4	2.5	3.2	1.3	1.7	0.7
pURR 3′-'b'	2.3	19.2	11.2	9.9	1.9	3.8	2.3	0.6	1.4	1.5
pSV2-*cat*	3.7	40.5	29.6	48.1	9.1	16.8	ND	>7.6[b]	15.8	>5.2[b]
pRSV-*cat*	6.7	42.5	18.8	52.4	14.0	ND	8.7	>8.9[b]	9.8	>7.8[b]

[a]pURR SVE-*cat* clones containing the HPV-16 P97 enhancer fragment (nucleotides 6150–57) upstream (pURR 5′) or downstream (pURR 3′) of the SV40 early promoter-driven *cat* gene in the sense ('a') and anti-sense ('b') orientations, the enhancer-less SVE-*cat*, and the enhancer-positive pSV2-*cat* and pRSV-*cat* clones were transfected in duplicate into different recipient cells (see text), and CAT enzyme levels were determined 60 h later. CAT activity is expressed relative to the parent plasmid, pSVE-*cat*; expression of pSV2-*cat* and pRSV-*cat* served as positive controls of transfection efficiency.
[b]CAT enzyme levels in these cultures were above the linear range, resulting in an underestimate of relative CAT activity.

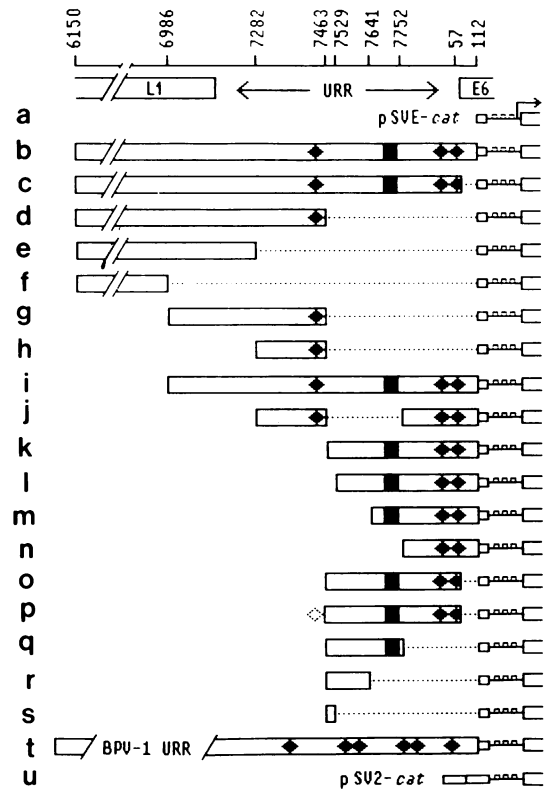
thus not due to poor transfection efficiency. In HeLa cells, transient transcripts of the clone pP97-*tk* protected the predicted shorter (~389 nt) fragment of this probe, corresponding to the specific *tk* gene sequence (lane 5). To detect whether the HPV-16 P97 promoter was used to initiate transcription, the pP97-*tk*-expressing RNA was also hybridized to a second, 956 nt-long riboprobe complementary to the chimeric pP97-*tk* clone (lane 9). This probe protected a 418 nt fragment consistent with correct initiation at the P97 promoter. A similar 5′ mRNA end was also observed in SiHa cells transfected with pP97-*tk* (data not shown). The P97 promoter was therefore preferentially expressed in cervical carcinoma cells.

The differential activity of the P97 promoter fragment in these clones suggested that its upstream sequences mediate its specificity. To test whether the viral 5′ sequences could activate other promoters, we linked an HPV-16 fragment (nt 6150–57), lacking the P97 transcription start site and TATAA box, upstream of the *tk* gene promoter in the transcriptional 'sense' ('a') or 'anti-sense' ('b') in the plasmids pURR *tk*(−109) 'a' and 'b' (Figure 1B). The enhancerless clone p*tk*(−109) served as a baseline control in transfections. While p*tk*(−109) transcripts were visible only after a long exposure (lane 2 in CV-1, 6 in HeLa, and 10 in SiHa cells), pURR *tk*(−109) 'a' and 'b' transcripts initiated at the *tk* promoter were readily detectable in HeLa and SiHa cells (~402 nt protected fragment in Figure 1B, lanes 7, 8, 11). In HeLa cells, the pURR *tk*(−109) 'a' and 'b' mRNA levels were 17-fold and 13-fold higher then p*tk*(−109) mRNA, as determined by densitometric scanning of a longer exposure. In CV-1 cells, the presence of the HPV-16 URR fragment did not increase *tk* transcript levels (Figure 1B, lanes 2–4; faintly visible on long exposure). Thus, the 5′ sequences of P97 functioned as an orientation-independent enhancer of transcripts correctly initiated at the heterologous *tk* promoter in HeLa and SiHa cells, but not in CV-1 cells. The level of *tk* transcripts from the clone pURR *tk*(−109) 'a' in SiHa cells was further increased by co-transfection with pRSV 16-E2 (lane 12) or pRSV B-E2 (not shown) in comparison with the pRSV-*neo* control.

To permit rapid dissection of the 5′ *cis* sequences, the HPV-16-directed promoter fragment was also linked to the bacterial chloramphenicol acetyl transferase (*cat*) gene (Figure 1C, clone pP97-*cat*). Again, HPV-16-initiated *cat* transcripts were easily detected in transfected HeLa cells (Figure 1C, lane 6), but not in CV-1 fibroblasts (Figure 1C, lane 2), despite high-level signal in parallel CV-1 cultures co-transfected with pRSV B-E2 and an SVE-*cat* plasmid containing the BPV-1 E2R element, pE2R-SVE-*cat* (data not shown). The upstream HPV-16 DNA fragment conferred this apparent cell specificity onto the enhancer-negative SV40 early (SVE) promoter in the clone pURR SVE-*cat* (Figure 1C, lanes 3 and 8, in comparison with pSVE-*cat*, lanes 1 and 5). Cotransfection with the E2 *trans*-activator clones of HPV-16 (not shown) and BPV-1 (pRSV B-E2) further increased HPV-16-initiated transcript levels in HeLa cells (lane 7). Since the SP6 *cat* probe used here did not extend into the HPV-16 URR, it is possible that the elevated transcripts were initiated at another promoter in the HPV-16 URR fragment. Both E2 products stimulated enhancer activity of the HPV-16 URR in CV-1 cells (lane 4) as well as in HeLa and SiHa cells (not shown), in agreement with the results of Phelps and Howley (1987). In the enzymatic CAT assay, however, the activity of the promoter construct, pP97-*cat*, was very low (data not shown), possibly due to an out-of-frame translation initiation at the upstream E6 AUG (nt 104). In contrast, the enhancer clone, pURR SVE-*cat*, showed a readily detectable CAT enzyme activity in both carcinoma lines, and was therefore used for further analysis in the enzymatic CAT test.

The HPV-16 enhancer is activated by cellular factors in uninfected human keratinocytes

The differential activity of the P97 promoter and the upstream enhancer element could be due to either viral or cellular factors present in the HPV-expressing cervical carcinoma cells. To distinguish between these possibilities, the host range of the HPV-16 P97 upstream enhancer-like element was determined in the enzymatic CAT assay (Gorman *et al.*, 1982) in different transfected cells. HPV-16 URR-*cat* constructs containing the viral DNA fragment in different orientations 5′ or 3′ to the SVE-*cat* chimera were transfected into primary human foreskin keratinocytes (HFK) devoid of detectable HPV sequences (Roman and Fife, 1986), a human keratinocyte line, HPK-I, established in culture after transfection with HPV-16 (Dürst *et al.*, 1987b), HPV-16 or 18-positive human cervical carcinoma cells, CaSki, SiHa, HeLa and C4-1, a primary human dermal fibroblast strain, GM3498, established mouse fibroblasts, NIH-3T3 and C127I, and the fibroblast-like monkey kidney cell line, CV-1 (Table I). The *cat* gene expression of these constructions was compared to that of the enhancer-negative plasmid, pSVE-*cat*, and the



	HFK	SiHa	HeLa	CV-1		
	+neo	+neo	+neo	+neo	+16-E2	+B-E2
a	1.0	1.0	1.0	1.0	1.6	4.0
b	8.2	3.6	29.8	1.4	13.3	42.7
c	3.8	9.8	29.9	1.9	14.8	50.9
d	0.9	ND	2.2	2.8	8.6	15.3
e	0.6	0.4	0.5	1.0	2.4	2.0
f	1.1	1.3	0.9	2.3	3.5	3.4
g	1.3	2.6	1.5	2.3	13.6	20.3
h	ND	1.5	1.8	1.4	12.7	22.1
i	ND	4.8	15.3	1.0	8.5	29.7
j	ND	0.5	1.3	1.6	15.0	35.7
k	3.1	2.4	14.0	1.2	2.9	7.0
l	3.3	3.5	23.8	1.1	4.8	12.7
m	2.4	4.6	8.6	0.9	5.7	7.8
n	1.3	1.0	1.4	0.9	6.1	12.2
o	6.1	24.1	24.1	1.2	5.0	8.5
p	7.9	22.1	35.6	1.1	16.9	49.6
q	6.8	27.6	31.6	0.7	2.3	4.6
r	1.0	3.2	2.8	1.3	2.2	5.0
s	1.1	0.8	1.4	0.8	3.0	4.0
t	5.4	9.0	3.9	5.5	40.0	>300
u	30.3	23.2	52.4	55.2	103.1	199.0

Fig. 2. Localization of HPV-16 *cis* regulatory elements upstream of the P97 promoter. In clone **b**, an ~1.8 kb *Bam*HI–*Ava*II fragment (nt 6150–112) containing the URR and P97 was cloned upstream of the SVE promoter in pSVE-*cat* (clone **a**). Clones **c**–**s** contain deletions of the HPV fragment. CAT activity is expressed relative to clone **a** in the absence of E2 (+*neo*), and represents the average from 2–5 experiments. Putative E2 binding cores, $ACCG(N)_4CGGT$ (< >), an inserted synthetic consensus E2 palindrome (< >), 5′-TCGACCGATATCGG(T)-3′, and homology with an HPV-18 enhancer (solid box) are shown. Clones containing the BPV-1 URR (BPV nt 6132–90, clone **t**) and the SV40 72-bp repeat enhancer (pSV2-*cat*, clone **u**) were used as controls for E2 *trans*-activation and transfection efficiency.

strong enhancer-positive clones pSV2-*cat* and pRSV-*cat* served as positive controls for transfection efficiency (Table I).

CAT enzyme activity was enhanced by the large HPV-16 URR fragment as much as 5- to 33-fold relative to the pSVE-*cat* levels, in some cases up to levels expressed by pSV2-*cat* or pRSV-*cat*, in all the human keratinocytic cells including uninfected foreskin keratinocytes (Table I). The URR-SVE-*cat* clones were as active as the pSV2-*cat* or pRSV-*cat* controls in some cells. The effect was seen to varying degrees with the HPV-16 URR inserted in either orientation upstream (5′-'a', 5′-'b') or downstream (3′-'a', 3′-'b') of the SVE-*cat* gene unit. The HPV-16 URR fragment thus fulfills the classical definition of a transcriptional enhancer. In contrast, relative activity of the HPV-16 enhancer clones varied between 0.7- and 2.5-fold in the murine or monkey fibroblast lines, and between 1.9- and 3.5-fold in the human skin fibroblast strain. These results indicate that the full activity of the cell-specific HPV-16 P97 enhancer element depends on factors present in human keratinocyte-derived cells, and does not require any viral factors.

The keratinocyte-dependent enhancer is distinct from the E2-responsive sequences

The above experiments demonstrate that the ~1.8-kb HPV-16 fragment upstream of the P97 promoter harbors an E2-independent, keratinocyte-dependent (*KD*) enhancer in addition to E2-dependent *cis* elements. To determine if both responses are mediated by identical or distinct *cis* sequences, the regulatory elements were identified by deletion mapping. Deleted HPV-16 URR fragments (Figure 2, clones 'b' through 's') were inserted into the enhancer-negative vector pSVE-*cat* (Figure 2, clone 'a'), and assayed in transiently transfected primary human foreskin keratinocytes (HFK), in cervical carcinoma cell lines, HeLa and SiHa, and in monkey CV-1 cells (Figure 2, columns '+*neo*'). pRSV-*neo* was included as a control in these cultures for comparison with E2 co-transfection experiments (see below). The enhancer-positive clone, pSV2-*cat*, was used as a transfection control.

In CV-1 cells, CAT activity of all target clones was within a 3-fold range of pSVE-*cat* ('a'). In the HeLa and SiHa lines and in the primary human foreskin keratinocytes, however, fragments extending to or beyond nt 7529 at the 5′ end retained *KD* enhancer activity (clones 'b', 'c', 'i', 'k', 'l'). In some cases, clones with the P97 TATAA box and transcription initiation site (HPV-16 nt 57–112) were less active than those without (e.g. 'k' versus 'o'). This could be due to transcripts initiated at the P97 promoter, and translated from the E6 AUG (nt 104) with the CAT protein out of frame. Upstream deletion to nt 7641 ('m') resulted in a three-fold reduced activity, and deletion to nt 7752 ('n') abolished *KD* activity. The downstream boundary was map-

ped by 3′ deletions in the 7463–112 fragment ('k') to nt 7752 ('q'). Further deletion to nt 7641 ('r') reduced CAT expression ~8-fold, and the fragment ending at nt 7529 ('s') did not exhibit any enhancer activity. The HPV-16 keratinocyte-dependent *cis* enhancer sequences are therefore located in a 224 nt fragment between nt 7529 and 7752 upstream of P97.

To define *cis* sequences required for E2 *trans*-activation, the different pURR SVE-*cat* target clones were also co-transfected with the BPV-1 and HPV-16 E2 expression vectors (Figure 1A) in CV-1 cells. E2 *trans*-activation of the BPV-1 P89 promoter is mediated by palindromic ACCG(N)$_4$CGGT cores (*E2Ps*) implicated in *in vitro* BPV-1 E2 protein binding (Androphy *et al.*, 1987b). The HPV-16 sequence 5′ to P97 contains three potential *E2P* cores (nt 7450, 35, 50; solid diamonds in Figure 2). It was thus of interest to see if these consensus sequences were required for either BPV-1 or HPV-16 E2 response. A large E2-responsive fragment of the BPV-1 URR served as a positive control for E2 *trans*-activation ('t').

All *cat* target clones, including the positive (pSV2-*cat*) and baseline (pSVE-*cat*) controls, were *trans*-activated by both E2 gene products to some degree (columns '+16-E2' and '+B-E2'). We ascribe this effect to the ability of the E2 gene products to *trans*-activate heterologous promoters in co-transfections (previously defined as E2 function B; Haugen *et al.*, 1987). While there was some variation in the relative response of individual target clones, only those HPV-16 fragments containing *E2P* palindromes responded to the E2 *trans*-activators to a greater extent than the controls. Deletion of the *KD* enhancer in clone 'j' (nt 7282 to 112 with a deletion of nt 7463–7752) inactivated its *KD* response, but did not compromise E2 *trans*-activation. The *KD* enhancer without *E2Ps* ('q'; nt 7463–7752) was no more responsive to E2 activation than the pSVE-*cat* control. The E2-responsive elements are therefore distinct from the *KD* enhancer.

Both *E2P*-containing elements were necessary for maximal effect since the lower E2 response of the deleted clone 'o' could be restored to full activity by the insertion of a synthetic *E2P* oligonucleotide, ACCGATATCGGT (open diamond) in clone 'p'. Full *trans*-activation by the viral E2 factors is therefore mediated by two *E2P*-containing *cis* elements flanking the *KD* enhancer. Since the HPV-16 E2 *trans*-activator requires identical *cis* elements as the BPV-1 E2 factor, it may also function by directly binding to the *E2P* cores.

Suppression by C-terminal 'short E2' gene products of BPV-1 and HPV-16

The E2 ORF of BPV-1 encodes a second protein (J. Schiller, E. Androphy and D. Lowy, personal communication), most likely from a co-linear mRNA initiated at an internal E2 promoter (P3080; Ahola *et al.*, 1987; Baker and Howley, 1987), and translated from an internal AUG at nt 3091 (Lambert *et al.*, 1987; T. Haugen and J. Schiller, in preparation). Molecular constructions expressing this 'short E2' protein (sE2; also denoted 'E2-TR') inhibit BPV-1 transformation and competitively inhibit E2 *trans*-activation (Lambert *et al.*, 1987; J. Schiller and T. Haugen, in preparation). We have thus sought to determine whether the HPV-16 E2 ORF has the potential to encode an analogous function, and whether the BPV-1 sE2 repressor or HPV-16 sE2 function could also suppress the keratinocyte-dependent enhancer activation.

We first tested the ability of the BPV-1 sE2 and the homologous C-terminal HPV-16 E2 product to repress the *KD* enhancer activity in SiHa cells, since these cells do not express endogenous E2 sequences (Baker *et al.*, 1987). The sE2 expression vector construction is given in Figure 3A. The *cat* target vectors with *KD* activity (Figure 3A) were co-transfected with increasing quantities of pRSV B-sE2 and pRSV 16-sE2, and tested for relative changes in CAT expression. pRSV-sE2 concentrations were adjusted with pRSV-*neo* as a carrier to prevent non-specific promoter competition. The *KD* enhancer activity of the large HPV-16 URR fragment (clone pBH SVE-*cat*, solid circles), and of the fragments in clones pSH SVE-*cat* (open circles) and pSP-SH SVE-*cat* (closed squares), was reduced by the sE2 products of both BPV-1 (Figure 3B, panel a) and HPV-16 (panel d). Co-transfection with a two-fold excess of the BPV-1 and HPV-16 sE2 clones over the *cis* target clones resulted in a 2- to 6-fold and a 1.5- to 3.5-fold reduction in CAT activity, close to enzyme levels obtained with the enhancer-negative control plasmid pSVE- *cat* (not shown). The reduction was sE2 dose-dependent. Since these HPV-16 *cis* fragments contain one or more *E2P* binding cores, we also tested the small *KD* enhancer fragment (clone pS-7752 SVE-*cat*, open squares) which lacks *E2P* elements. This fragment was not significantly influenced by either sE2. Therefore, the C-terminal portion of HPV-16 E2 can encode a function similar to the BPV-1 sE2 repressor, and both sE2 products require the presence of one or more *E2Ps* for their action.

As predicted from the shared *E2P* requirement of E2 and sE2 of both viruses, the sE2 products competitively inhibited *trans*-activation of pBH SVE-*cat* by pRSV 16-E2 in CV-1 fibroblasts (data not shown). Both sE2 products also interfered with the combined positive effect of keratinocytic factors and HPV-16 E2 *trans*-activation in SiHa cells (Figure 3, panels b and e). Again, the suppression was not apparent in the absence of the *E2P* core motifs in *cis* (clone pS-7752 SVE-*cat*; open squares). To determine whether the ability of the sE2 product to bind to *E2P* cores is critical for both inhibition of E2 *trans*-activation and suppression of the *KD* enhancer, we have used a BPV-1 sE2 amino acid insertion mutant (pRSV B-sE2 i; Figure 3A), which fails to bind *E2P* sequences *in vitro* (J. Schiller and T. Haugen, in preparation). This mutant did not repress either *KD* or E2-dependent activation in any of the *cat* plasmids tested in SiHa cells in the presence of HPV-16 E2 (Figure 3B, panel c).

In addition to competitive inhibition of E2 *trans*-activation, the sE2 repressor gene products of both HPV-16 and BPV-1 can therefore down-regulate activation of the upstream *KD* enhancer by cellular factors. It is surprising that the suppression is mediated by the *E2P* cores, which are distinct and remote from the *KD* enhancer. In contrast, we have found that co-transfection with molecular clones expressing the adenoviral E1a gene product(s) can suppress the minimal *KD* enhancer segment lacking *E2P* elements (data not shown).

Discussion

We have determined that the HPV-16 E6–E7 region promoter, P97 and its 5′ *cis* sequences, can be influenced by at least three diffusible regulatory factors in transient transfection experiments: by positive cellular factor(s) present in uninfected human genital keratinocytes and in cervical carcinoma cell lines, and by positive and negative viral factors encoded in the viral E2 ORF.

Keratinocyte-dependent P97 trans-activation

Our results represent the first indication that the gene expression of a human papillomavirus involves a transcriptional *cis* enhancer active in the natural host cells for viral infection, e.g. in uninfected human keratinocytes. The 224 nt long HPV-16 *KD* enhancer fragment is a part of the E6–E7 gene promoter, P97.

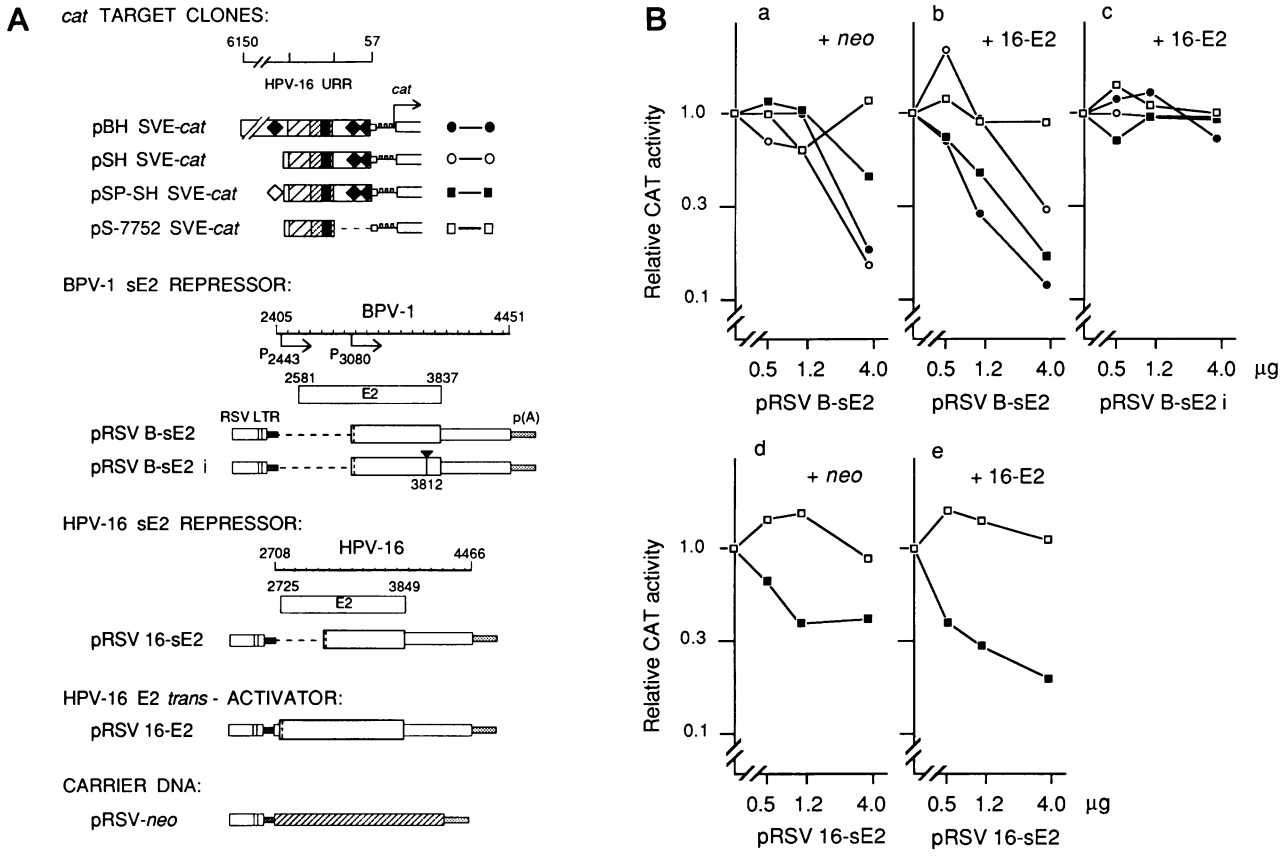


Fig. 3. Repression of HPV-16 keratinocyte-dependent enhancer activity and E2 *trans*-activation by sE2 products in SiHa cells. (**A**) *cat* repressor target clones, and vectors expressing sE2 repressors and the E2 *trans*-activator used in cotransfections (see text). The *cat* clones are the same as 'c', 'o', 'p' and 'q' in Figure 2; shaded regions are the two active domains of the *KD* enhancer. (**B**) Effect of co-transfections with sE2 repressor vectors. Duplicate cultures were co-transfected with a mixture of plasmids: 2 µg of a *cat* target clone, 0.5 µg carrier (pRSV-*neo*, panels **a** and **d**) or HPV-16 E2 *trans*-activator (pRSV 16-E2, panels **b**, **c**, and **e**), and from 0 to 4 µg of the BPV-1 or HPV-16 sE2 repressor vectors adjusted to 4 µg with pRSV-*neo* carrier DNA. Panels **a** and **b**: co-transfections with an increasing dose of the BPV-1 sE2 repressor (pRSV B-sE2); panel **c**: with a BPV-1 sE2 mutant (pRSV B-sE2 i); panels **d** and **e**: with the putative HPV-16 sE2 repressor (pRSV16-sE2). Curve symbols used for *cat* target clones are shown in (**A**). Relative CAT activity in each panel is given as fraction of CAT enzyme activity in triplicate control cultures transfected without sE2.

In BPV-1, E6–E7-encoded genes control plasmid copy numbers in replication (Lusky and Botchan, 1985; Berg *et al.*, 1986). In contrast to HPV-16, BPV-1 infection leads to fibroepithelial lesions *in vivo*, and the viral plasmid genomes replicate as unintegrated plasmids in both the dermal fibroblasts and in the warty epidermal cells (Lancaster, 1981). The BPV-1 E6–E7 region promoter, P89, is apparently expressed at a low constitutive level in susceptible fibroblasts due to the presence of a short upstream enhancer active in fibroblasts as well as in keratinocyte-derived cells (T.Haugen, T.Cripe and L.Turek, in preparation). A restriction of the HPV-16 E6–E7 gene expression to keratinocytes is likely to play a role in the strict epithelial tropism of HPV-16.

In addition, the HPV-16 E6–E7 promoter and its *KD* enhancer are active in cervical carcinoma cells (data presented here, and Gloss *et al.*, 1987). The HPV-18 upstream early promoter also functions in cervical carcinoma cells and in some tumor cell lines of epithelial origin (Thierry *et al.*, 1987a). This is due to an enhancer element in a similar location (Swift *et al.*, 1987; Thierry *et al.*, 1987b), showing partial sequence homology to the HPV-16 *KD* element (Swift *et al.*, 1987). Furthermore, a HPV-6vc URR fragment can act as an enhancer in cervical carcinoma cells (Rando *et al.*, 1986). It is possible that the upstream genes of other genital HPVs are regulated by the same cellular mechanism.

The keratinocyte-dependent HPV-16 enhancer contains the octamer TTTGGCTT (HPV-16, nt 7709–7716), which is found in a similar position upstream of the E6 ORF in all genital HPV genomes sequenced so far: HPV-6b (Schwarz *et al.*, 1983), HPV-11 (Dartmann *et al.*, 1986), HPV-18 (Thierry *et al.*, 1987a) and HPV-33 (Cole and Streek, 1986). The inverted consensus sequence, AANCCAAA, is present −410 to −65 nt upstream of several human and bovine cytokeratin genes and the human involucrin gene (Blessing *et al.*, 1987). It is possible that the viral *cis* element responds to the same cellular factors as these keratinocyte-specific genes. In addition, the HPV-16 enhancer fragment contains several short direct and inverted repeats, and a glucocorticoid receptor-binding element, TGTACATTGTGTCATA (nt 7640–7655; Gloss *et al.*, 1987). Which of these *cis* sequences interact with regulatory factors in the cell needs to be established in competition assays with specific HPV fragments and synthetic oligonucleotides, and in *in vitro* DNA binding experiments.

Trans-activation by viral E2 gene products

We have shown that in addition to cell-specific factors, the regulatory sequences of the P97 promoter are influenced by positive and negative regulatory factors encoded in the viral E2 ORF. *Trans*-activation by the E2 gene products of either HPV-16 (Phelps and Howley, 1987) or BPV-1 (Spalholz *et al.*, 1985) was required to detect HPV-16 URR-driven transcripts in CV-1 cells, and further increased transcript levels in cervical carcinoma cell lines. The E2 ORFs of other HPV types also encode positive

transcriptional regulatory factors (Hirochika *et al.*, 1987; F.Thierry and H.Pfister, personal communications; and our unpublished data), indicating that gene regulation by E2 products is conserved among papillomaviruses in general.

Genetic dissection of the HPV-16 *cis* sequences required for E2 action has identified two non-contiguous E2-responsive elements flanking the *KD* enhancer. Each element contains one or two consensus *E2P* cores, $ACC(N)_6GGT$, and at least one of these elements can be functionally replaced by the insertion of a synthetic *E2P* sequence. Consensus *E2P* cores are found in DNA sequences required for E2 *trans*-activation of the BPV-1 P89 promoter (Haugen *et al.*, 1987; Spalholz *et al.*, 1987), and for BPV-1 E2 protein binding *in vitro* (Androphy *et al.*, 1987a). Since the HPV-16 E2 gene product interacts with the same *cis* sequences as BPV-1 E2, it may also directly bind to the *E2P* cores.

Suppression of keratinocyte-dependent activation by the viral sE2 repressor

We have also demonstrated that the downstream part of the HPV-16 E2 ORF may encode a transcriptional repressor similar to the sE2 gene product of BPV-1 (Lambert *et al.*, 1987). The BPV-1 E2 *trans*-activator and sE2 repressor share the C-terminal E2 domain which has DNA binding activity *in vitro* (Androphy *et al.*, 1987b; Moskaluk and Bastia, 1987), but sE2 lacks the effector function mapped to the N-terminal E2 *trans*-activator domain (J.Schiller and T.Haugen, in preparation). Based on protein sequence homology and the results presented here, the HPV-16 E2 ORF has a similar organization. We do not know whether a C-terminal sE2 repressor product is made in HPV-16 infection since the transcriptional map of HPV-16 early genes is incomplete, and no HPV E2 proteins have thus far been identified. We have shown that the C-terminal part of the HPV-16 E2 ORF has the coding capacity for such a function.

Surprisingly, the sE2 repressor products of HPV-16 or BPV-1 not only inhibit E2 *trans*-activation, but also suppress the *KD* enhancer response to keratinocytic factors. We assume that this effect also involves sE2 binding to the target sequences because it requires *E2P* binding cores in *cis*, and is abolished in the BPV-1 sE2 binding mutant, pRSV B-sE2 i. The E2-binding *cis* elements appear to serve a dual role: they function either as a transcriptional 'enhancer' in the presence of the E2 *trans*-activators, or as a *cis* 'silencer' of *KD* enhancer response to cellular factors in the presence of the sE2 repressors. This is in contrast to suppression of the *KD* enhancer by adenoviral E1a products, also described for the HPV-18 enhancer (Swift *et al.*, 1987; Thierry *et al.*, 1987a). The E1a factors do not bind to DNA (Nevins, 1986), and they can repress the activation of the minimal HPV-16 *KD* enhancer fragment in the absence of *E2P* cores.

Inhibition of the E2 *trans*-activator function could be due to competitive binding of the sE2 repressor or of inactive E2/sE2 oligomers at the *E2P* sites (Lambert *et al.*, 1987), since the sE2 and E2 products interact with the same *cis* sequences. The apparent suppression of keratinocytic factor activation is more difficult to explain by steric hindrance or simple displacement of cellular factors at sites adjacent to the E2 binding elements. The *E2P* cores are located 79 and 277 nt from the boundaries of the minimal *KD* enhancer fragment, and the predicted sE2 products are not large enough to displace other proteins at such a distance. Alternatively, the attachment of sE2 could inhibit linear diffusion ('sliding') of cellular factors from their initial entry site to the promoter, or the cooperative binding of multiple cellular factors ('oozing') from the enhancer to the P97 promoter (see

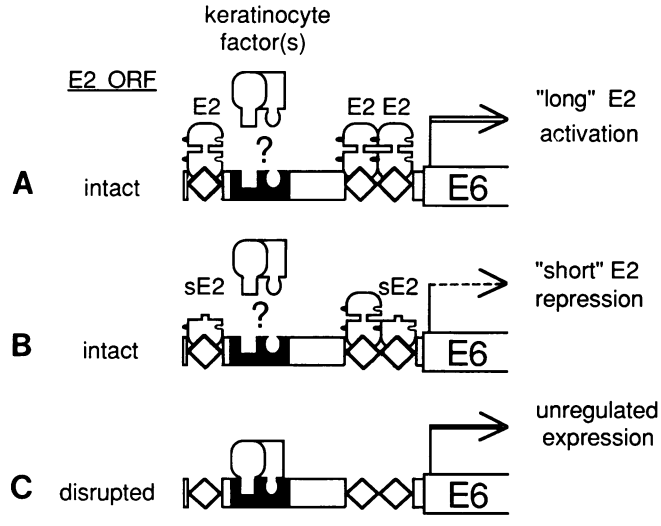


Fig. 4. Regulation of HPV-16 E6–E7 gene region promoter, P97: (**A**) in the presence of excess viral E2 *trans*-activator (E2); (**B**) in the presence of excess viral E2 C-terminal repressor (sE2); and (**C**) in the absence of viral E2 products. When the E2 genes are inactivated in integrated HPV DNA fragments, cellular factors interacting with the keratinocyte-dependent enhancer (bold segment) can sustain an unregulated expression of E6–E7 genes.

Ptashne, 1986, for discussion). We do not favor these possibilities since the *KD* element can function well at a distance from the promoter.

It is possible that the sE2 proteins share other functional domains with the E2 *trans*-activators, and that these play a role in the *KD* enhancer suppression. The predicted BPV-1 sE2 protein is ~100 amino acids larger than the minimal C-terminal DNA binding region of E2 (J.Schiller and T.Haugen, in preparation). The bound sE2 repressors could therefore interact with other cellular transcription factors attached at the promoter, but lack the ability to form an active transcription initiation complex. Experiments designed to determine whether the sE2 products suppress other strong enhancers in the presence of E2-binding cores in *cis* are in progress. Interaction of the sE2 repressors with the *E2P* cores and with cellular factors provides a model to study transcriptional repression of mammalian genes.

Viral gene regulation in HPV-16 infection and cancer

The results presented here suggest a possible explanation for the structure and expression of integrated HPV-16 DNA fragments in cervical carcinomas. We propose that transcription of the potential transforming gene(s) of the HPV-16 E6–E7 region (Matlashewski *et al.*, 1987) is tightly regulated in benign genital warts containing unintegrated, replicating HPV plasmids (Figure 4). This regulation, possibly tied into the cell cycle, is mediated by the E2 *trans*-activator (panel A) and sE2 repressor (B) proteins bound to the *E2P* cores. The E2 factor binding to the *E2P* cores may override the potential transcriptional activation of the promoter by cellular factors (Thierry *et al.*, 1987b) Furthermore, the bound viral E2 regulatory proteins may influence other viral promoters within the URR (Baker *et al.*, 1987). Cellular factors and other early viral functions are likely to further modulate E6–E7 transcription either directly or by controlling E2 and sE2 production. HPV-16 integration in the cellular genome could occur as a result of mutations in viral early genes needed for plasmid replication. In some random insertion events, disruption or inactivation of the viral E2 genes would be expected to lead to unregulated, persistent E6–E7 transcription under the control

of cellular factors (panel C), and result in an altered growth potential of the cell. In other cases, viral E6−E7 gene deregulation may be due to adjacent cellular *cis* elements near the site of integration, to deletions or mutations in the E2 attachment sites of the P97 promoter, or to duplications or mutations of the *KD cis* element that would result in its greater affinity for cellular factors. In addition, integration of the upstream HPV-16 *KD* enhancer has the potential to alter the expression of adjacent cellular genes (Dürst *et al.*, 1987a; Swift *et al.*, 1987).

If critical viral and/or cellular genes responsible for transformation maintenance were under the control of de-regulated viral upstream *cis* sequences in cells transformed by HPV-16 *in vivo* or *in vitro*, introduction of DNA clones expressing viral E2 factors would be expected to alter their neoplastic growth phenotype. This prediction can be tested experimentally using the molecular reagents described in this study.

Materials and methods

Plasmid constructions

Molecular cloning followed established protocols as described (Haugen *et al.*, 1987) and is illustrated in Figures. All nt numbers refer to the first nucleotide of the enzyme recognition site in the HPV-16 sequence (Seedorf *et al.*, 1985). HSV-1 *tk* constructs were generated from p*tk*(−109) by linking HPV-16 fragments to *tk* nt +14 in the promoter clone, pP97-*tk*, and to *tk* nt −109 in enhancer clones. pSVE-*cat* contains the *cat* gene (EC 2.7.1.28) linked to the enhancer-negative SV40 early promoter, and SV40 termination and splicing signals 3′ to the gene. The SVE promoter was deleted in pP97-*cat*. 5′, 3′ and internal deletion mutants of the HPV-16 URR DNA fragment were constructed using *Sph*I (nt 7463), *Dra*I (nt 6986, 7282, 7752) and *Rsa* I (nt 7529, 7641) restriction sites (Figure 2). Single-cut partial digests were obtained by DNA cleavage in the presence of ethidium bromide. DNA fragments were isolated in low melting point agarose gels and used directly for cloning.

The HPV-16 E2 *trans*-activator vector contains the entire E2 ORF (*Tth*111I−*Stu*I); the HPV-16 sE2 repressor vector has the 3′ domain of the E2 ORF (*Nde*I−*Bam*HI, nt 3126−4466); the BPV-1 sE2 clone corresponds to the co-linear mRNA starting at the P3080 (with only one upstream AUG at nt 3091; *Nco*I-*Bam*HI, nt 3089−4451); all are driven by the Rous sarcoma virus long terminal repeat (RSV LTR), and have the SV40 3′ signals as described for pRSV BPV E2 (Haugen *et al.*, 1987). The BPV-1 sE2 i mutant was made by insertion of a *Hpa*II to *Sal*I TAB linker (Pharmacia) resulting in an in-frame insertion of two amino acids at nt 3812 (Figure 3A). All plasmids were purified twice on CsCl gradients and visualized on EtBr agarose gels to exclude nicking and RNA contamination.

Cells and transfections

Transfection conditions were optimized for each cell strain with pRSV-*cat*. For RNA experiments, monkey kidney fibroblasts (CV-1) and human cervical carcinoma cells (HeLa, SiHa) plated at $3-4 \times 10^6$ cells/150 mm plate were transfected by the calcium phosphate procedure as described (Haugen *et al.*, 1987) with 35 μg *cat* or *tk* plasmid, 15 μg of pRSV-*neo*, pRSV B-E2, or pRSV 16-E2, and 40 μg of calf thymus DNA in 3 ml precipitate. Total cellular RNA was prepared at 60 h by the guanidinium thiocyanate procedure. RNase mapping was performed on 6 μg RNA hybridized to uniformly labeled SP6 riboprobes. HSV *tk* SP6 templates in Figure 1B contained the 5′ 402 nt *tk* sequences (from Nru I at +399) ligated to the SP6 promoter at the *Sma*I site of Gem II (PL Inc.) and linearized by *Hin*dIII cleavage (nt −109) upstream of the promoter. The chimeric P97-*tk* SP6 956 nt template had the HPV-16 *Sph*−*Ava*II (nt 7463−112) fragment ligated to *tk* nt +14, and was opened at an upstream *Hin*dIII site in Gem II (Figure 1B, lane 9). The *cat* DNA template in Figure 1C was a clone containing the 5′ 250 nt of *cat* coding sequences ligated to the SP6 promoter in the GEM II plasmid (Promega, Inc.) at the *cat Eco*RI site. The plasmid was linearized at an *Xba*I site upstream from the SV40 early promoter.

For CAT assays, cells were plated at $3-5 \times 10^5$ cells/35 mm well in duplicate, and transfected 24 h later by the calcium phosphate technique. 2.5 μg *cat* plasmid was adjusted to 6 μg with calf thymus DNA for each well. Co-transfections received 2 μg *cat* DNA and 1 μg of pRSV-based expression vector or pRSV-*neo* DNA per well. HFK cells were prepared by the procedure of Rheinwald and Green (1975), and transfected in T25 flasks by adding both 30 μg/ml Polybrene (Sigma Chemical Co., St. Louis) and 10 μg of *cat* plasmid DNA directly to the media (Farr *et al.*, 1987). All cells were treated 4−6 h later with either 30% DMSO (HFK, GM3498), 24% DMSO (CV-1, HPK-I), 20% DMSO (C127I), 20% glycerol (HeLa, CaSki, SiHa, C4-1) or 17% glycerol (NIH-3T3) in 1× Hepes-buffered saline (pH 7.1). CAT enzyme activity was determined from the acetylated Cm fraction using the linear portion of the standard curve as described (Haugen *et al.*, 1987).

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