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Progesterone and Glucocorticoid Response Elements Occur in the Long Control Regions of Several Human Papillomaviruses Involved in Anogenital Neoplasia

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We have previously identified in the long control region of the genome of human papillomavirus type 16 (HPV-16) a DNA segment which functions as a cell-type-specific enhancer as well as mediating glucocorticoid response. It contains multiple transcription-factor-binding sites, including several for nuclear factor I and one for the glucocorticoid receptor, which binds to the partially palindromic sequence TGTACANNNTGTCAT. We report here that this sequence element, when separated from the surrounding transcription-factor-binding sites and placed as an oligonucleotide into a test vector, retains its function as a glucocorticoid response element (GRE) in HeLa cells. In T47D cells, which express the progesterone receptor, the HPV-16 enhancer fragment mediates progesterone responsiveness. A point mutant in this fragment and the response of the oligonucleotide clone to both steroids prove the identity of the progesterone response element (PRE) with the GRE. The antiprogesterone and antiglucocorticoid RU486 interferes with both hormonal responses. In SiHa cells, the HPV-16 GRE mediates an increase in transcripts encoding E6 and E7 proteins, which are involved in transformation by HPV-16. Hormonal regulation is not restricted to HPV-16: DNA segments containing the cell-type-specific enhancers of HPV-11 and HPV-18 also mediate glucocorticoid and progesterone response. We identified sequence elements in the long control regions of HPV-11 and HPV-18 which function as GRE/PREs when tested as oligonucleotides. These findings suggest that GRE/PREs are an integral part of gene expression regulation in genital HPVs.

Infections with specific types of human papillomaviruses (HPVs) have emerged as a necessary factor in the etiology of anogenital neoplasia. Some HPV types, in particular HPV-6 and HPV-11, are associated with benign neoplasia, while numerous other virus types, in particular HPV-16 and HPV-18, are found in lesions frequently progressing toward malignancy (34, 46, 53). Transcription of the viral early genes, some of which code for gene products with transforming activity, appears to be largely dependent on regulatory elements in the long control region (LCR) of the HPV genome. In the case of HPV-16, the LCR encompasses 856 base pairs (bp) of the 7,904-bp-long viral genome (42) and contains the only promoter documented thus far in this virus, P97 (43, 44). From P97, transcription occurs toward open reading frames (ORFs) E6 and E7, which code for important transforming functions of HPV-16 (36, 49).

Immediately upstream of P97, footprint analysis identified two binding sites for nuclear proteins (17) which overlap with two binding sites for the papillomavirus E2 protein (10, 28, 35); therefore, steric hindrance of protein binding may modify the classical E2 regulatory pattern (24, 54). A further influence on P97 activity is exerted by a cell-type-specific enhancer which is centered around position 7650 about 250 bp upstream of P97. This enhancer is active in the cervical carcinoma cell line HeLa but inactive in the breast tumor cell line MCF7 (16) and may mediate transcriptional specificity for certain epithelial cells (10). A possibly homologous enhancer exists in HPV-6 (56), HPV-11 (24), and HPV-18 (52). DNase I protection analysis of the HPV-16 enhancer region with nuclear extracts from HeLa cells exhibited nine footprints, fp1e to fp9e (17). They include seven nonpalindromic nuclear factor I (NFI)-binding sites (18) and three potential AP-1 binding sites. Centrally in relation to the nine footprints, we have detected a binding site for purified glucocorticoid receptor. A 400-bp fragment including this binding site confers transcriptional regulation by glucocorticoids to a *cis*-linked heterologous promoter (16).

Transcriptional regulation by steroid hormones involves the specific complex formation between steroid and receptor protein. Subsequently, this complex recognizes specific binding sites on DNA (for reviews see references 13, 20, and 57). Independently, these binding sites were found to function as steroid response elements (25). Different DNA sequences are recognized by the various members of the steroid receptor family (23, 26, 27, 50). Glucocorticoid response elements (GREs) frequently involve the hexanucleotide 5′-TGTTCT-3′, which often occurs in sequences similar to the palindrome 5′-AGAACAN$_3$TGTTCT-3′ (26, 50). These elements can function as promoter elements (25, 51) or remote from the promoter, in cooperation with different transcription factors (3, 40, 51).

Interestingly, GREs from several genes can also mediate response to progesterone (6, 23, 50). In our research, we asked whether the GRE of HPV-16 can also function as a progesterone response element (PRE). Our data presented here confirm this inference, and beyond this, we found that several other HPV types infecting genital epithelia contain similar regulatory elements. We propose that progesterone responsiveness could play an important part in the HPV life cycle and the HPV-induced transformation process. This state could have evolved, as genital HPVs may benefit from the increase of progesterone concentrations during part of the ovulation cycle and during pregnancy. Our findings could give molecular explanations for how these physiological changes can lead to the much higher frequency of malignant HPV lesions in women than in men (34, 53), for the occa-

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sional onset of cervical cancer during pregnancy (53), and for the correlation observed in some studies between the use of oral contraceptives and the occurrence of cervical cancer (4, 48).

MATERIALS AND METHODS

Cell culture and transfection. The cell lines CaSki, SiHa (2), HeLa (39), and MCF7 (16) were grown in minimal essential medium supplemented with 10% or 5% (HeLa) fetal calf serum (HyClone, Logan, Utah); T47D cells (6) were grown in RPMI 1640 with 10% fetal calf serum. For RNA isolation, SiHa cells were treated with or without 10^{-7} M dexamethasone 6 h before harvesting. Transfections were done by electroporation using a Biorad Gene Pulser, applying 20 μg of uncut plasmid DNA, as described (51). The cells were split in two, and hormones were added at 10^{-7} M (dexamethasone) or 10^{-8} M (progesterone) 2 h after transfection. HeLa cells were harvested 20 h and T47D cells 40 h after transfection. Cell extracts were prepared by freeze-thawing, and chloramphenicol acetyltransferase (CAT) activity assays were performed using 50 μg of protein and 30 to 60 min of incubation at 37°C for HeLa extracts, or 125 to 250 μg of protein and 90 min of incubation for T47D cells (19, 51). The CAT activity was determined as percent conversion of chloramphenicol and is given in picomoles converted per minute per milligram of protein.

Synthetic oligonucleotides and plasmids. Oligonucleotides were synthesized on a Pharmacia gene assembler and were used directly for cloning or purified by gel electrophoresis for primer extension and synthesis of an S1 probe. To construct pHPV-16-GRE, the two complementary oligonucleotides 5′-CTAGTGTACATTGTGTCATT-3′ and 5′-CTA GAATGACACAATGTACA-3′ were annealed and ligated into the *Xba*I site of pBLCAT2 (29). Likewise, to construct pHPV-18-GRE and pHPV-11-GRE, oligonucleotides with the appropriate sequences were used, as given in Fig. 6. The resulting plasmid insertions were confirmed by sequence analysis. For the construction of pHPV-18-LCR1 and pHPV-18-LCR2, a 1,050-bp *Bam*HI fragment (positions 6929 to 119) from HPV-18 (8) was inserted into the *Bam*HI site of pBLCAT2 (29). The two resulting plasmids contain the fragment in the syn (pHPV-18-LCR1) and anti (pHPV-18-LCR2) orientation. Similarly, a 595-bp *Alu*I fragment from HPV-11 (positions 7219 to 7814) (11) was inserted into the *Hinc*II site of pUC18 and subsequently recloned into pBLCAT2 using *Hin*dIII and *Bam*HI sites, resulting in the two plasmids pHPV-11-LCR1 (syn orientation) and (pHPV-11-LCR2) (anti orientation). pHPV-16-LCR-MUT was obtained from a bisulfite mutation of the 400-bp HPV-16 enhancer fragment which was created in M13 and then reinserted into pBLCAT2 (16; B. Gloss, unpublished results); the mutated sequence is given in Results. The previously published plasmids pHPV-16-400.1 and pHPV-16-400.2 (16) have been designated pHPV-16-LCR1 and pHPV-16-LCR2, respectively. pHPV-16E67NDa contains a 377-bp, filled-in *Nde*I-*Dde*I fragment from positions 281 to 657 of the HPV-16 sequence (42) inserted into the *Sma*I site of bluescribe M13+ (Vector Cloning Systems, San Diego, Calif.). The plasmids were named pHPV-16E67NDa, in which the *Nde*I site was linked to the *Eco*RI-*Kpn*I region, and pHPV-16E67NDb, in which the *Nde*I site was linked to the *Hin*dIII-*Bam*HI region of the polylinker.

RNA isolation and primer extension. Total RNA was isolated from CaSki, SiHa, and MCF7 cells by using the guanidinium isothiocyanate method and a subsequent CsCl centrifugation step (50). Primer extension reactions were performed using a 20-nucleotide-long synthetic primer, complementary to the HPV-16 E6 gene from position 5′-169 to 3′-150, and 15 μg of RNA, as described (39). The hybridization was performed at 42°C, and for the primer extension reaction avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) was used. The reaction products, together with Sanger sequencing lanes and ^{32}P-labeled DNA marker fragments, were analyzed on an 8% sequencing gel and exposed to X-ray film.

S1 mapping. A 5′-labeled S1 probe covering the E6/E7 region of HPV-16 was created starting from a 40-nucleotide-long synthetic oligonucleotide spanning positions 5′-589 to 3′-550 of the HPV-16 genome (42), which was 5′-end labeled with ^{32}P (30). A 16-μg sample of pHPV-16E67NDa plasmid DNA was alkali denatured, neutralized, precipitated, and subsequently hybridized to 0.7 pmol of the labeled oligonucleotide in 50 mM Tris hydrochloride (pH 7.8)–10 mM $MgCl_2$–0.1 mM dithiothreitol–0.12 mM each of all four deoxynucleoside triphosphates. After addition of 10 U of Klenow DNA polymerase I (20 min of incubation at 37°C), 25 mM EDTA was added, followed by phenol-chloroform and ether extraction and ethanol precipitation. The extended probe was subsequently cut with *Eco*RI to yield a 323-nucleotide-long, 5′-labeled probe which spans the E7 N-terminal region from position 589 into the E6 gene (position 3′-281) plus a 14-nucleotide linker sequence at its 3′ end. The probe was purified using a preparative 6% polyacrylamide sequencing gel. Samples of the probe DNA (15,000 cpm) and total RNA (15 μg) were denatured at 95°C for 2 min in 80% formamide buffer, hybridized at 41°C for 15 h, and subsequently digested with S1 nuclease using 20 μg of calf thymus DNA per ml as a carrier, as described (30). The digested products were analyzed on a 8% sequencing gel together with Sanger sequencing lanes, for which the same primer was used as to produce the probe, and visualized by autoradiography.

RESULTS

The GRE in the LCR of HPV-16 can be functionally separated from the natural context of transcription factors. A 400-bp fragment from the LCR of HPV-16 (position 7454 to 7854) functions as a cell-type-specific enhancer in HeLa cells (16). It contains seven TTGGC motifs that bind pure NFI (17, 18). In the center of this 400-bp fragment lies fp4e (Fig. 1), a segment of about 40 bp in length with a complex overlap of binding sites for several factors, two of which are likely to be AP-1. fp4e also contains a 15-bp partial palindrome which binds purified glucocorticoid receptor (16). Proximity and overlap of the HPV-16 GRE with other motifs may lead in vivo to cooperation or competition between the glucocorticoid receptor and factors binding to fp4e (3, 40, 51).

To analyze the GRE separately from its natural sequence environment, we constructed pHPV-16-GRE, which contains the 15-bp oligonucleotide 5′-TGTACATTGTGTCAT-3′ inserted into the *Xba*I site of pBLCAT2, a vector that permits expression of the CAT gene by transcription from the herpes simplex virus thymidine kinase promoter (*tk*) (19, 29). pHPV-16-GRE was electroporated into HeLa cells, the cells were incubated with or without dexamethasone (a synthetic glucocorticoid) and harvested, and CAT enzyme activity was determined. As controls, we transfected pBLCAT2; pHPV-16-LCR1 and pHPV-16-LCR2 containing the whole HPV-16 enhancer segment; and pORFEXCAT, which contains the CAT gene under control of the cytomeg-

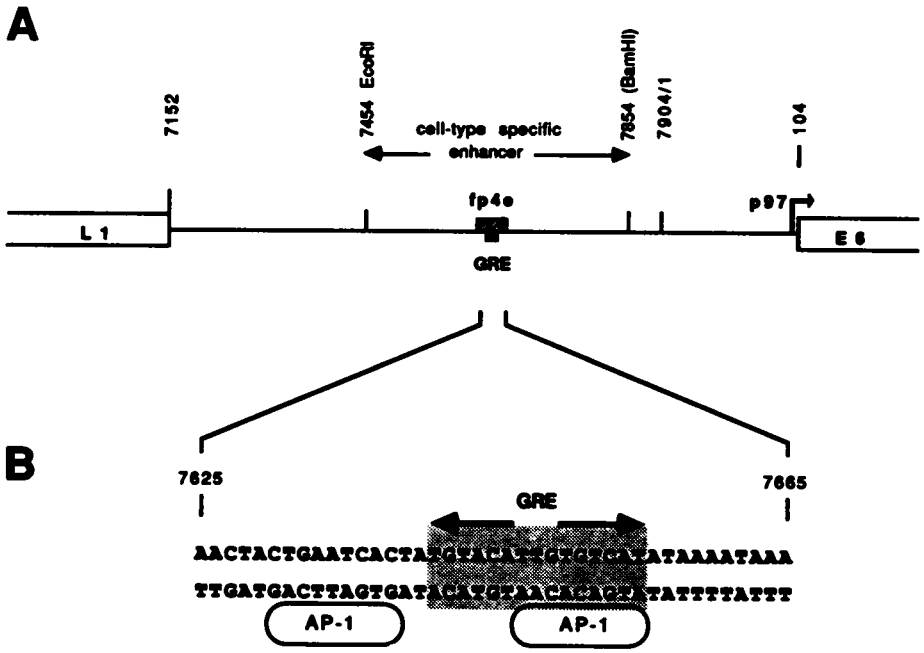

FIG. 1. Sequence elements surrounding the GRE in the LCR of HPV-16. (A) The LCR of HPV-16 with the cell-type-specific enhancer (10, 16) spans from ORF L1 to E6. fp4e is one of nine footprints identified with HeLa nuclear extracts (17). A binding site for the glucocorticoid receptor overlaps with fp4e. P97 is the only promoter identified so far, transcribing the early genes of HPV-16 (44). (B) DNA sequence of the fp4e region which includes the partial palindrome of the GRE of HPV-16 (arrows). Two AP-1 motifs are marked, which are protected by HeLa nuclear extracts (17). At the moment it is not known whether the glucocorticoid receptor and the proteins binding to the AP-1 motifs cooperate or compete for binding.

alovirus immediate early promoter (5) (Table 1). pHPV-16-GRE showed dexamethasone induction in the absence of constitutive enhancement. This demonstrates that the HPV-16 GRE is functional when separated from its surrounding transcription-factor-binding sites. CAT expression from pBLCAT2 is slightly down-regulated by glucocorticoids (16). pHPV-16-LCR1, pHPV-16-LCR2, and pORFEXCAT show strong constitutive CAT expression, while only in the HPV-16 constructs was CAT activity further induced by dexamethasone.

The HPV-16 GRE mediates response to progesterone. Several glucocorticoid-regulated promoters have been shown to be inducible by progesterone, and it is assumed that related DNA sequences mediate response to either hormone (6, 23, 50). A similar regulation could be of significant relevance for the etiology of HPV-16-induced lesions, first because the target cells of HPV-16 infection, the epithelia of the squamous-columnar junction of the cervix uteri, express progesterone receptors (38), and second, because progesterone is produced at high concentration during the luteal phase of the female ovulation cycle and during pregnancy. Since HeLa cells do not express substantial levels of the progesterone receptor protein, we decided to use T47D cells, a human breast carcinoma cell line (6, 50), as a test system for progesterone regulation. To test the possibility that the GRE of HPV-16 is also a PRE, we electroporated pHPV-16-LCR1, pHPV-16-LCR2, pHPV-16-GRE, and two control vectors into T47D cells, kept the cells with or without progesterone, and analyzed CAT activity. We observed a 3.1- to 10.2-fold hormonal induction both by the HPV-16 fragment and the HPV-16 oligonucleotide, but no induction of the control vectors (Table 1). Beyond this hormonal induction, constitutive enhancement of CAT expression could not be observed with the HPV-16 constructs, confirming the previously described cell type specificity of the HPV-16 enhancer (16).

TABLE 1. Glucocorticoid and progesterone induction with plasmids containing part of the HPV-16 LCR or GRE/PRE

Plasmid	Dexamethasone induction in HeLa cells[a]				Progesterone induction in T47D cells[a]			
	CAT activity (pmol/min per mg of protein)		Fold induction	Fold induction (corrected)[b]	CAT activity (pmol/min per mg of protein)		Fold induction	Fold induction (corrected)[b]
	− Dex	+ Dex			− Prog	+ Prog		
pBLCAT 2	29.7	21.0	0.7	1.0	8.4	7.2	0.86	1.0
pHPV-16-LCR1	410.8	1,845.0	4.5	6.4	16.0	140.4	8.8	10.2
pHPV-16-LCR2	364.8	1,058.8	2.9	4.1	8.2	22.1	2.7	3.1
pHPV-16-GRE	19.0	42.9	2.3	3.2	46.2	146.0	3.2	3.7
pORFEXCAT[c]	748.1	747.6	1.0	1.4	112.0	131.0	1.2	1.4

[a] Dexamethasone (Dex), a synthetic glucocorticoid, was applied at 10^{-7} M. Progesterone (Prog) was used at 10^{-8} M.
[b] Corrected values were obtained by correcting for down-regulation by the hormonal treatment of the CAT expression from the control plasmid pBLCAT2.
[c] pORFEXCAT is a control showing strong constitutive transcription without hormonal control by a cytomegalovirus promoter/enhancer.

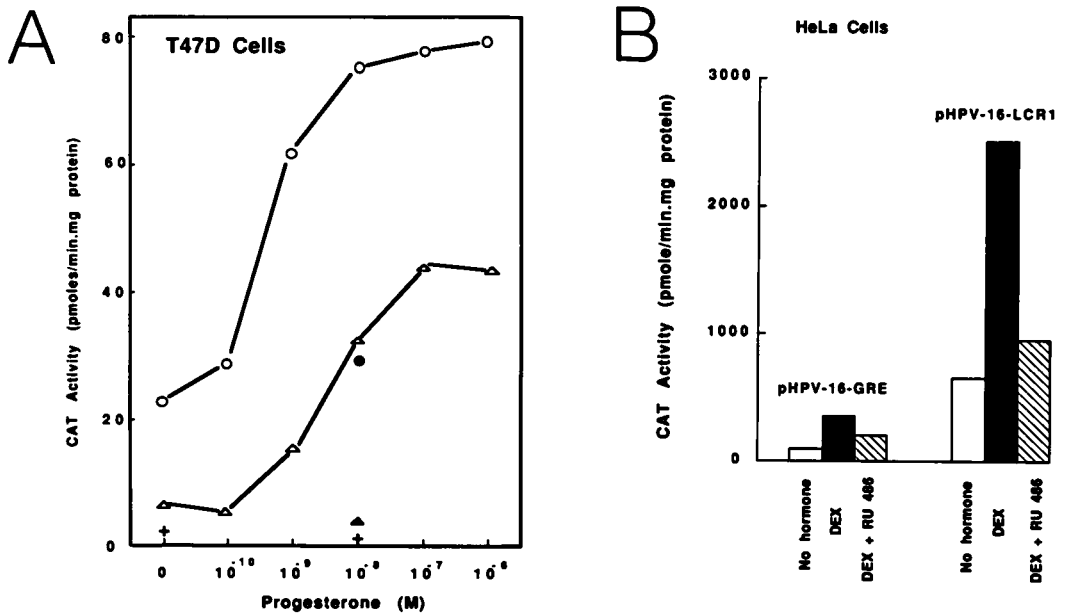

FIG. 2. Dependence of CAT expression from pHPV-16-LCR1 and pHPV-16-GRE on steroid concentrations. (A) Cultures of T47D cells transfected with either pHPV-16-LCR1 (△) or pHPV-16-GRE (○) were induced by various concentrations of progesterone. A 10-fold excess of RU486 reduced induction of CAT expression from both vectors (solid symbols). +, Controls with pBLCAT2. (B) Induction of CAT from pHPV-16-LCR1 and pHPV-16-GRE by dexamethasone (DEX) (10^{-7} M) is reduced by a 10-fold excess of RU486 (10^{-6} M).

In summary, we take these data as evidence that the same sequence element in the HPV-16 LCR mediates either glucocorticoid or progesterone control depending on the availability of the hormone receptor. This GRE/PRE can cooperate with a constitutive enhancer or can be uncoupled in vivo or through the design of the test vector from the interaction with neighboring transcription factors.

A point mutation in the GRE/PRE of HPV-16 reduces glucocorticoid and eliminates progesterone response. To address the question of whether the GRE/PRE we identified is an essential element for the hormone response of the HPV-16 enhancer, we have made use of a point mutant that resulted from a random bisulfite mutagenesis of the 400-bp enhancer fragment (Gloss, unpublished results). This mutation changed the GRE wild-type sequence TGTACATTGT GTCAT to TGTATATTGTGTCAT, eliminating the essential palindromicity of the TGT motif at positions 4-6/10-12 of the 15-mer. When pHPV-16-LCR1-MUT containing this mutant enhancer was tested in HeLa cells for response to dexamethasone, we observed in several transfection experiments a 2.9-fold rather than 6.4-fold induction with the wild-type plasmid. This suggests that the remaining binding site sequence permits a reduced recognition by the glucocorticoid receptor. The same mutant did not give any response to progesterone in T47D cells.

Concentration dependence of the HPV-16 progesterone response and effect of the antigestagen RU486. Figure 2 illustrates CAT expression from pHPV-16-LCR1 and pHPV-16-GRE under various progesterone concentrations. Half-maximal CAT expression from either plasmid occurred between progesterone concentrations of 5×10^{-10} and 5×10^{-9} M, a value typical for PREs tested in T47D cells (50). From both plasmids, progesterone-dependent CAT expression was strongly inhibited by the presence of a 10-fold excess of the antiprogesterone RU486. In a similar way, RU486, which is also known to function as an antiglucocorticoid (50), eliminated the dexamethasone response (Fig. 2).

Transcription of the transforming genes of HPV-16 is stimulated by glucocorticoids. ORFs E6 and E7 play an important role in the morphological transformation by HPV-16 (36, 49). It seems reasonable to assume a dosage dependence of this process, namely, an increased tendency of transformation of the host cell following an increase in expression of these genes. Such an increase of ORF E6 and E7 expression may occur under the control of the GRE/PRE of HPV-16. To test for this possibility, we analyzed E6/E7 gene transcription in SiHa cells, a cell line which contains a single-copy insertion of the HPV-16 genome (2). SiHa cells were grown in the absence or presence of dexamethasone, and RNA was isolated and subjected to primer extension analysis to monitor P97 activity (44) (Fig. 3). A cluster of bands identified P97 and documented an approximately threefold induction by dexamethasone, a hormone response similar to that observed in the transfection experiments (Table 1). This cluster of bands was absent from human MCF-7 cells, which do not contain HPV-16, but present in CaSki cells, which contain several hundred copies of HPV-16 (2).

To find out whether E7 mRNA is induced by dexamethasone, we extended this study by S1 analysis of these RNA preparations. Three bands (Fig. 4) occurred with RNAs from SiHa and CaSki cells but not with MCF-7 cells, as follows. Band A was derived from full-length transcripts encoding E6; its endpoint in this position was determined by the length of the probe fragment. Band B and the weak band C corresponded to the splice acceptor sites of the E6*1 and E6*2 mRNAs, two transcripts which may allow efficient E7 translation (39, 43). In SiHa cells, all three bands were induced by dexamethasone. It should be pointed out that we could analyze these gels downstream to position 554, 7 bp upstream of the ATG of ORF E7, without observing any promoter but P97. This makes it likely that E7 expression occurs via internal translation initiation on the hormone-inducible E6*/E7 mRNAs (43). Extensive transfection experiments involving GRE/PRE test vectors and progesterone

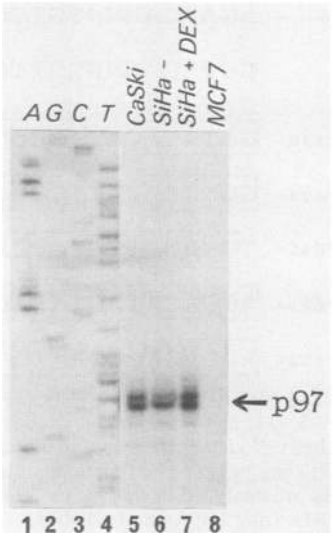

FIG. 3. Primer extension analysis of P97 transcripts in CaSki and SiHa cells. The primer extension reaction was performed using a 20-nucleotide-long primer complementary to the E6 mRNA, starting about 70 bp downstream of P97. RNAs from the following cell lines were used: lane 5, CaSki; lanes 6 and 7, SiHa; lane 8, MCF7 (a human breast carcinoma cell line, which served as a negative control). SiHa cells were grown in the absence (lane 6) or presence (lane 7) of 10^{-7} M dexamethasone, a synthetic glucocorticoid. The induction of P97 transcriptional activity by the hormone was estimated to be about threefold. A 15-μg sample of total RNA was used for each reaction, and the reaction products were separated on a 8% sequencing gel. Lanes 1 to 4 are sequencing lanes.

receptor expression clones showed that SiHa cells do not express significant levels of progesterone receptor, while CaSki cells expressed neither glucocorticoid nor progesterone receptor (our unpublished results).

GRE and PRE occur in the LCRs of HPV-11, HPV-18, and probably other genital HPVs. Papillomaviruses which infect the human genital tract, namely, HPV-6/11, HPV-16, HPV-18, and HPV-33, strongly diverge in sequence but show a similar general genomic organization (8, 9, 11, 41, 42). The diversity which is found in the organization of nuclear-factor-binding sites in the enhancers of HPV-11, -16, and -18 contrasts with the occurrence of a conserved cluster of NFI-binding sites associated with one of several AP-1 binding sites in all three viruses (17, 18). This suggests that in spite of organizational divergence, genital HPVs require a defined subset of transcription factors for peculiar features of their gene regulation. We decided to test whether glucocorticoid/progesterone response is another common property of the transcriptional regulation of genital HPVs. From the LCR of HPV-11, we cloned a 595-bp *Alu*I fragment into pBLCAT2 (pHPV-11-LCR1 and pHPV-11-LCR2), and from the LCR of HPV-18 we cloned a 1,050-bp *Bam*HI fragment into the same vector (pHPV-18-LCR1 and pHPV-18-LCR2). These four plasmids and plasmids pBLCAT2, pORFEX-CAT, pHPV-16-LCR1, and pHPV-16-LCR2 were transfected into HeLa cells and into T47D cells to analyze regulation by glucocorticoids and progesterone, respectively. All HPV-11 and HPV-18 constructs showed an up to 16.8-fold induction by dexamethasone or progesterone (Fig. 5A). No induction was observed with pBLCAT2 or pORFEXCAT. This demonstrates that GRE/PREs are contained in the LCR fragments of both viruses.

Sequence analysis showed that the LCRs of HPV-11 and

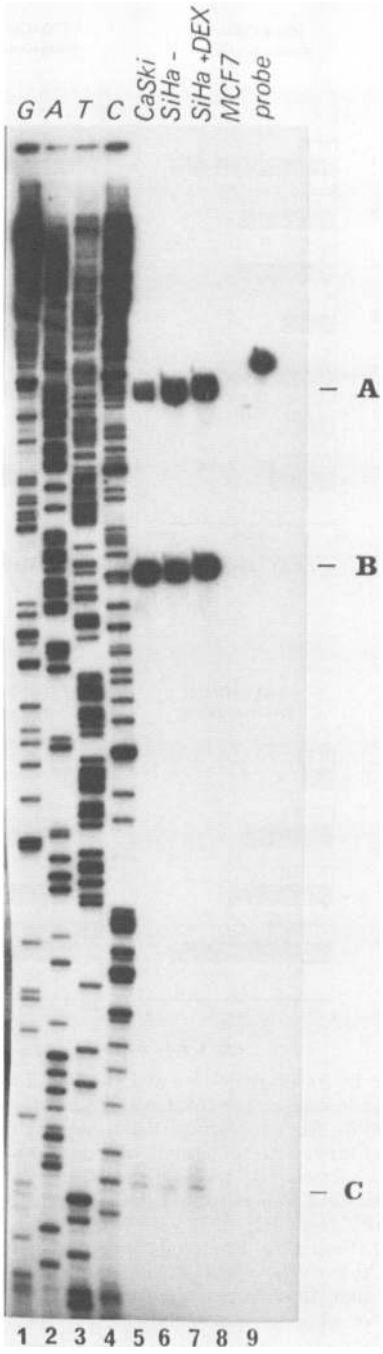

FIG. 4. S1 mapping of E6/E7 transcripts in CaSki and SiHa cells. Total RNA from different cell lines was hybridized to a 5'-labeled single-stranded DNA probe, complementary to E6/E7 mRNA of HPV-16, and subjected to S1 digestion. Lanes 1 to 4, Sequencing lanes for G, A, T, and C; lane 5 to 8, results from S1 analysis using RNAs from the following cell lines: lane 5, CaSki; lanes 6 and 7, SiHa; lane 8, MCF7 (negative control); lane 9, undigested probe. SiHa cells were kept without hormone (lane 6) or induced with 10^{-7} M dexamethasone (lane 7). The three protected fragments A, B, and C correspond to the following positions on the HPV-16 sequence: A identifies the end of the probe and unspliced E6 transcripts; B is the splice acceptor site at position 409 in E6 leading to E6*1; and C indicates the splice acceptor site at position 526 leading to E6*2 (43). The gel was overexposed to visualize band C; all three bands, A, B, and C, were inducible by dexamethasone.

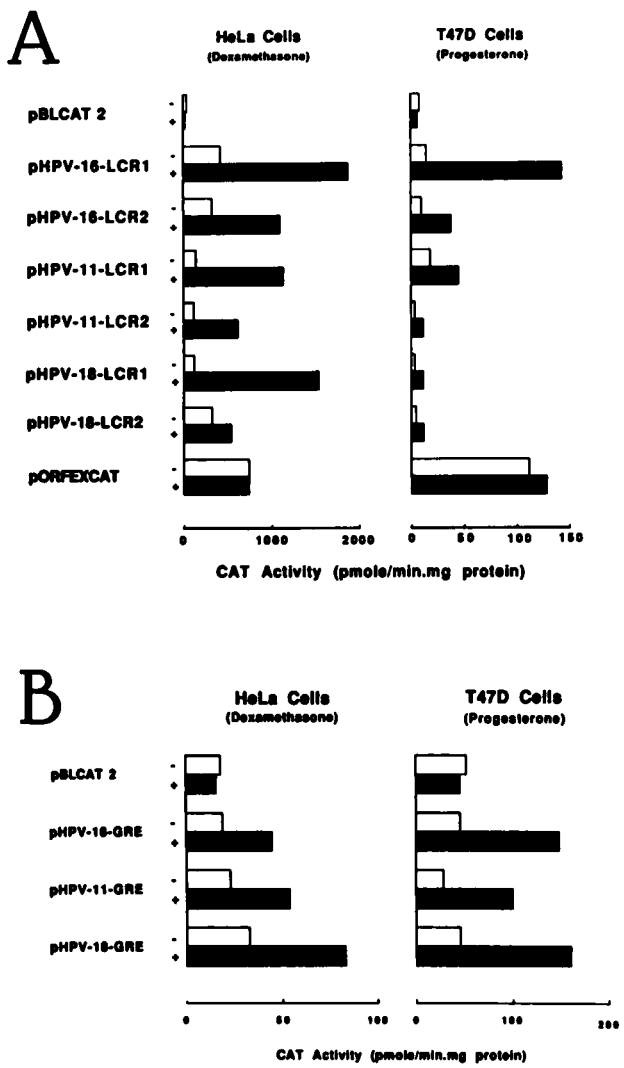

FIG. 5. The LCRs of HPV-11 and HPV-18 contain glucocorticoid and progesterone response elements. Both steroids induce *tk*-CAT expression from constructs that contain either a segment from the LCR of these viruses (A) or an oligonucleotide representing a GRE/PRE consensus (B) within these segments (Fig. 6). For comparison, the CAT values from Table 1 for pBLCAT2, pHPV-16-LCR1, pHPV-16-LCR2, pHPV-16-GRE, and pORFEXCAT were included in this figure. The basal expression (without hormone) of the HPV-LCR constructs was much lower in T47D than in HeLa cells, but the induction values were greater than twofold (plus progesterone) for all six LCR plasmids.

-18 each contain one segment homologous to the 15-bp partially palindromic GRE consensus sequence (26, 50) (Fig. 6). We cloned oligonucleotides representing these homologous sequences into the *Xba*I site of pBLCAT2, resulting in the plasmids pHPV-11-GRE and pHPV-18-GRE. These constructs were tested as described above. The inserted oligonucleotides led to a 3- to 4.4-fold induction of CAT expression by both steroids (Fig. 5B). We therefore believe that the homologies that we identified mediate the observed hormonal response. These data and the sequence analysis presented in Fig. 6 let us propose that progesterone and glucocorticoid regulation is a general phenomenon in gene regulation of genital HPVs.

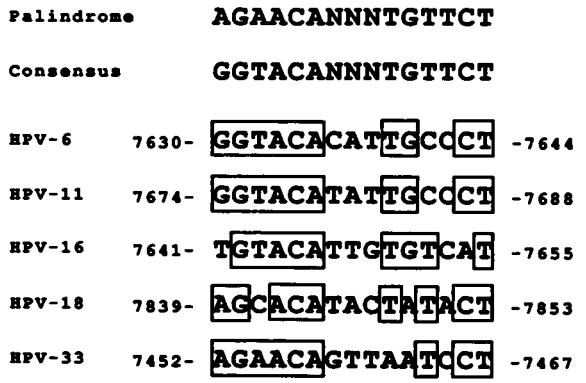

FIG. 6. GREs and PREs from genital HPVs. For reference, a consensus GRE/PRE (26) and a functionally analyzed palindrome (50) are listed. In the HPV sequences, bases identical to either of the reference GRE/PREs are boxed. The functions of the HPV-11, -16, and -18 GRE/PREs are analyzed in this paper. The figure includes two likely GRE/PREs from HPV-6 and HPV-33.

DISCUSSION

Research on the regulation of gene expression in papillomaviruses has concentrated for many years on bovine papillomavirus type 1 and, within this topic, largely on the unique properties of the E2 system, a papillomavirus-specific means for feedback regulation of transcription (21, 22, 28, 37, 45). At least six promoters are active in bovine papillomavirus type 1, each one specific for the expression of a subset of the viral ORFs (1, 12, 37, 47). Similar findings can be expected for HPVs, but so far in HPV-16 and HPV-18 only a single transcription start has been identified upstream of ORF E6 (39, 43, 44). While the understanding of these elements of HPV gene expression is still fragmentary, analysis of the interaction of cellular transcription factors with HPV genomes has advanced rapidly after the identification of an E2-independent enhancer in HPV-16 and HPV-18 (10, 16, 52). The enhancers of several HPV types seem to be identified by a cluster of NFI-binding sites associated with other transcription-factor-binding sites which vary between different HPV types (17, 18).

We have presented here evidence that in five genital HPVs one of these additional elements is mediating response to glucocorticoids and progesterone. We have analyzed in detail properties of the GRE/PRE in HPV-16, and we have pinpointed sequences which are likely to be corresponding elements in HPV-11 and HPV-18. These sequences diverge from established GREs only in 2 or 3 out of those 12 bp in the 15-mer palindrome which are considered to be relevant for receptor binding (26, 27, 50). Most of the differences have homologous base changes in proven GREs; e.g., in position 1, a T as in HPV-16 also occurs in the mouse sarcoma virus GRE, and in position 13 a C as in HPV-16 and HPV-11 also occurs in the GREs of at least three cellular genes (26).

Our results make it unlikely that the 400-bp HPV-16 fragment contains more than one GRE/PRE, since a point mutation in the GRE palindrome within the enhancer fragment strongly affects both hormone responses. To answer this question for the HPV-11 and HPV-18 LCR fragments is more difficult. One needs to compare the induction rates conferred by the enhancer fragments and by the oligonucleotides, and one may have to consider distance effects, contributions of other transcriptional elements to the hormone response, and cooperativity between two GREs (40, 51). Moreover, it cannot be ruled out completely that dex-

amethasone treatment in HeLa cells might lead to additional effects resulting from increased E6 and E7 gene expression of the integrated HPV-18 copies (39, 55). For both E6 and E7, transcriptional transactivating functions have been found (15, 36) that might contribute to the hormone stimulation mediated by the enhancer constructs, but most likely not by the GRE constructs.

GREs may occur generally in genital HPVs: HPV-6 exhibits complete sequence identity to HPV-11 in the position corresponding to the HPV-11 GRE, and HPV-33 has a perfect TGTTCT at position 7623 (lower strand). It is interesting that the elements of HPV-6, -11, -16, and -33 occur centrally in clusters of NFI sites shown by us to be characteristic of HPV enhancers (18). Diverging from this pattern, the HPV-18 GRE is located 120 bp outside of the NFI cluster and outside of a 230-bp *Rsa*I fragment shown to contain the constitutive HPV-18 enhancer (18, 52), but in close association with two other transcription-factor-binding sites (14). The occurrence of GREs may be a peculiarity of genital papillomaviruses, since we did not find sequence homologies in the genomes of HPVs infecting the epidermis (HPV-1 and HPV-8) or in bovine or cottontail rabbit papillomaviruses.

Recognizing GRE/PREs as elements of general occurrence among genital HPVs and as elements possibly restricted to this group among the papillomaviruses, one may ask questions about the function of these elements for viral biology. While we find it difficult to believe that a GRE/PRE is indispensable for HPV gene regulation, it is likely to confer some advantage to the virus, since it evolved and is maintained in many divergent HPV types. Genital HPVs frequently infect epithelial cells that bear progesterone receptors (38). In these cells, HPV gene expression would go through recurrent boosts during the female ovulation cycle and through extensive stimulation during pregnancy. These boosts of gene expression could result in an increase of the viral genome copy number or in increased multiplication of the virus-transformed cells. Both effects would stimulate the spread of the virus. For example, papillomavirus replication during pregnancy could increase the frequency of infection during birth, a possible pathway in the induction of juvenile laryngeal papillomata (32). At the moment, it is an open question whether virus release and subsequent infection of the newborn during parturition plays an important role in the viral epidemiology in addition to sexual transmission, which is believed to be the main infectious pathway of genital papillomaviruses (34, 53).

On the other side, hormonal induction of viral gene expression may constitute one of numerous events cooperating in the etiology of cervical carcinogenesis. The GRE/PREs of HPVs can modulate the transcription of the transforming genes E6 and E7 in established HPV-containing tumor cell lines. An increase in the concentration of the corresponding proteins should result in an increased propensity of the infected cell to assume a morphologically transformed state. Consequently, high concentrations of some steroid hormones should constitute a risk factor in cervical carcinogenesis (7). This hypothesis was also formulated to explain a glucocorticoid-dependent increase in proliferation of C4-1 cells, which contain the HPV-18 genome (55).

In addition to naturally circulating glucocorticoids and progesterone, steroids applied for medication, e.g., in contraceptives, may have to be considered as potential inducers of cervical cancer progression. On the other hand, the antihormone RU486, a potent inhibitor of both glucocorticoid and progesterone effects in human cells (50), affects the hormone response of the HPV-16 GRE/PRE. Antisense RNA expression of the E6/E7 region suppressed HPV-18 E6 and E7 gene expression and decreased cell growth of C4-1 cells (55), pointing to the importance of these ORFs for the growth properties of cervical carcinoma cells. These findings suggest that it may be possible to modulate the growth properties of HPV-carrying cervical malignant lesions by interfering with the expression of these viral genes. Whether antihormones can play such a role remains to be clarified. Among many other factors, the hormone responsiveness of viral gene expression may vary between individual HPV-containing lesions.

Several mechanisms may account for the transformation process by HPVs (36), one of them possibly involving cooperation with the *ras* tumor gene (31, 49). In fact, a stimulation of the transformation of newborn rat kidney cells by HPV-16 under the influence of glucocorticoid hormones has been reported (33). It will be rewarding to develop model systems that may help to define whether modulated levels of HPV transcripts directly change the growth or frequency of HPV-associated tumors.

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LITERATURE CITED

1. **Baker, C., and P. Howley.** 1987. Differential promoter utilization by the bovine papillomavirus in transformed cells and productively infected wart tissues. EMBO J. **6**:1027–1055.
2. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962–971.
3. **Becker, P., B. Gloss, W. Schmid, U. Straehle, and G. Schuetz.** 1986. In vivo protein/DNA-interactions within a glucocorticoid response element requires presence of the hormone. Nature (London) **324**:686–688.
4. **Beral, V., P. Hannaford, and C. Kay.** 1988. Oral contraceptive use and malignancies of the genital tract. Lancet **ii**:1331–1335.
5. **Bernard, H. U., T. Oltersdorf, and K. Seedorf.** 1987. Expression of the human papillomavirus type 18 E7 gene by a cassette-vector system for the transcription and translation of open reading frames in eukaryotic cell. EMBO J. **6**:133–138.
6. **Cato, A. C. B., R. Miksicek, G. Schuetz, J. Arnemann, and M. Beato.** 1986. The hormone regulatory element of mouse mammary tumour virus mediates progesterone induction. EMBO J. **5**:2237–2240.
7. **Chan, W. K., B. Gloss, and H. U. Bernard.** 1988. Human papillomavirus-16 and genital cancer: are tests for the viral gene expression in vitro indicators for risk factors in vivo? Ann. Acad. Singapore **17**:232–237.
8. **Cole, S. T., and O. Danos.** 1987. The nucleotide sequence and genome organization of human papillomavirus type 18. J. Mol. Biol. **193**:599–608.
9. **Cole, S. T., and R. E. Streeck.** 1986. Genome organization and nucleotide sequence of human papillomavirus type 33, which is associated with cervical cancer. J. Virol. **58**:991–995.
10. **Cripe, T. C., T. H. Haugen, J. P. Turk, F. Tabatabai, P. G. Schmid, M. Duerst, L. Gissmann, A. Roman, and L. Turek.** 1987. Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 transactivator and repressor gene products: implications for cervical carcinogenesis. EMBO J. **6**:3745–3753.
11. **Dartmann, K., E. Schwarz, L. Gissmann, and H. zur Hausen.** 1986. The nucleotide sequence and the genome organization of

human papillomavirus type 11. Virology **151**:124–130.
12. **Engel, L. W., C. A. Heilman, and P. M. Howley.** 1983. Transcriptional organization of the bovine papillomavirus type 1. J. Virol. **47**:516–529.
13. **Evans, R.** 1988. The steroid and thyroid hormone receptor superfamily. Science **240**:889–895.
14. **Garcia-Carranca, A., F. Thierry, and M. Yaniv.** 1988. Interplay of viral and cellular proteins along the long control region of human papillomavirus type 18. J. Virol. **62**:4321–4330.
15. **Gius, D., S. Grossman, M. A. Bedell, and L. Laimins.** 1988. Inducible and constitutive enhancer domains in the noncoding region of human papillomavirus type 18. J. Virol. **62**:665–672.
16. **Gloss, B., H. U. Bernard, K. Seedorf, and G. Klock.** 1987. The upstream regulatory region of the human papillomavirus-16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. EMBO J. **6**:3735–3743.
17. **Gloss, B., T. Chong, and H. U. Bernard.** 1989. Numerous nuclear proteins bind the long control region of human papillomavirus-16: a subset of 6 and 23 DNase I-protected segments coincides with the location of the cell-type-specific enhancer. J. Virol. **63**:1142–1152.
18. **Gloss, B., M. Yeo-Gloss, M. Meisterernst, L. Rogge, E. L. Winnacker, and H. U. Bernard.** 1989. Clusters of nuclear factor I binding sites identify enhancers of several papillomaviruses but alone are not sufficient for enhancer function. Nucleic Acids Res. **17**:3519–3533.
19. **Gorman, C. M., L. F. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
20. **Green, S., H. Gronemeyer, and P. Chambon.** 1987. Structure and function of steroid hormone receptors, p. 8–28. *In* M. Sluyser (ed.), Growth factors and oncogenes in breast cancer. Harwood, New York.
21. **Haugen, T. H., T. P. Cripe, G. D. Ginder, M. Karin, and L. P. Turek.** 1987. Transactivation of an upstream early gene promoter of bovine papillomavirus-1 by a product of the viral E2 gene. EMBO J. **6**:145–152.
22. **Hawley-Nelson, P., E. J. Androphy, D. R. Lowy, and J. T. Schiller.** 1988. The specific DNA recognition sequence of the bovine papillomavirus E2 protein is an E2-dependent enhancer. EMBO J. **7**:525–531.
23. **Hecht, A., A. Berkenstam, P. E. Stroemstedt, J. A. Gustafsson, and A. E. Sippel.** 1988. A progesterone responsive element maps to the far upstream steroid dependent DNase hypersensitive site of chicken lysozyme chromatin. EMBO J. **7**:2063–2073.
24. **Hirochika, H., T. R. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the trans-acting E2 proteins. Genes Dev. **2**:54–67.
25. **Hynes, N., A. J. J. van Ooyen, N. Kennedy, P. Herrlich, H. Ponta, and B. Groner.** 1983. Subfragments of the large terminal repeat cause glucocorticoid responsive expression of mouse mammary tumor virus and of an adjacent gene. Proc. Natl. Acad. Sci. USA **80**:3637–3641.
26. **Jantzen, M., U. Straehle, B. Gloss, F. Stewart, W. Schmid, M. Boshart, R. Miksicek, and G. Schuetz.** 1987. Cooperativity of glucocorticoid response elements located far upstream of the tyrosine aminotransferase gene. Cell **49**:29–38.
27. **Klock, G., U. Straehle, and G. Schuetz.** 1987. Oestrogen and glucocorticoid response elements are closely related but distinct. Nature (London) **329**:734–736.
28. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50**: 69–78.
29. **Luckow, B., and G. Schuetz.** 1987. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res. **15**:5490.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Matlashewski, G., J. Schneider, L. Banks, N. Jones, A. Murray, and L. Crawford.** 1987. Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. EMBO J. **6**:1741–1746.
32. **Mounts, P., and K. V. Shah.** 1984. Respiratory papillomatosis: etiological relation to genital tract papillomaviruses. Prog. Med. Virol. **29**:90–114.
33. **Pater, M. M., G. A. Hughes, D. E. Hyslop, H. Nakshatri, and A. Pater.** 1988. Glucocorticoid-dependent oncogenic transformation by type 16 but not type 11 human papillomavirus DNA. Nature (London) **335**:832–835.
34. **Peto, R., and H. zur Hausen.** 1986. Viral etiology of cervical cancer. Banbury Report 21. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional transactivation by the human papillomavirus type 16 E2 gene product. J. Virol. **61**:1630–1639.
36. **Phelps, W. C., C. L. Yee, K. Muenger, and P. M. Howley.** 1988. The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. Cell **53**:539–547.
37. **Prakash, S. S., B. H. Horwitz, T. Zibello, J. Settleman, and D. DiMaio.** 1988. Bovine papillomavirus E2 gene regulates expression of the viral E5 transforming gene. J. Virol. **62**:3608–3613.
38. **Sanborn, B. M., B. Held, and H. S. Kuo.** 1976. Hormonal action in human cervix. J. Steroid Biochem. **7**:665–674.
39. **Schneider-Gaedicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**: 2285–2292.
40. **Schuele, R., M. Muller, H. Otsuka-Murakami, and R. Renkawitz.** 1988. Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. Nature (London) **332**:87–90.
41. **Schwarz, E., M. Duerst, C. Demankowski, O. Latterman, R. Zech, E. Wolfsperger, S. Suhai, and H. zur Hausen.** 1983. DNA sequence and genome organization of genital human papillomavirus type 6b. EMBO J. **2**:2341–2348.
42. **Seedorf, K., G. Kraemmer, M. Duerst, S. Suhai, and W. G. Roewekamp.** 1985. Human papillomavirus type 16 DNA sequence. Virology **145**:181–185.
43. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63**:1441–1447.
44. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
45. **Spalholz, B. A., Y. C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papillomavirus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
46. **Steinberg, B. M., J. L. Brandsma, and L. N. Taichman.** 1987. Papillomaviruses. Cancer Cells (Cold Spring Harbor) **5**:275–300.
47. **Stenlund, A., G. L. Beam, and M. Botchan.** 1987. A promoter with an internal regulatory domain is part of the origin of replication in BPV-1. Science **236**:1666–1671.
48. **Stern, E., A. B. Forsythe, L. Yonkeles, and C. F. Coffelt.** 1977. Steroid contraceptive use and cervical dysplasia: increased risk of progression. Science **196**:1460–1464.
49. **Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford.** 1988. Comparison of the in vitro transforming activities of human papillomavirus types. EMBO J. **7**:1815–1820.
50. **Straehle, U., G. Klock, and G. Schuetz.** 1987. A DNA sequence of 15 basepairs is sufficient to mediate both glucocorticoid and progesterone induction of gene expression. Proc. Natl. Acad. Sci. USA **84**:7871–7875.
51. **Straehle, U., W. Schmid, and G. Schuetz.** 1988. Synergistic action of the glucocorticoid receptor with transcription factors. EMBO J. **7**:3389–3395.
52. **Swift, F. W., K. Bhat, H. B. Younghusband, and H. Hamada.** 1987. Characterization of a cell type specific enhancer found in the human papillomavirus type 18 genome. EMBO J. **6**:1339–1344.
53. **Syrjaenen, K. J., L. Gissmann, and L. G. Koss.** 1987. Papillo-

maviruses and human disease. Springer Verlag, Berlin.
54. **Thierry, F., and M. Yaniv.** 1987. The BPV-1-E2 transacting protein can be either an activator or a repressor of the HPV-18 regulatory region. EMBO J. **6**:3391–3397.
55. **von Knebel Doeberitz, M., T. Oltersdorf, E. Schwarz, and L. Gissmann.** 1988. Correlation of modified human papillomavirus early gene expression with altered growth properties in C4-1 cervical carcinoma cells. Cancer Res. **48**:3780–3786.
56. **Wu, T. C., and P. Mounts.** 1989. Transcriptional regulatory elements in the noncoding region of human papillomavirus type 6. J. Virol. **62**:4722–4729.
57. **Yamamoto, K. R.** 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**: 209–252.