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Influence of chromosomal integration on glucocorticoid-regulated transcription of growth-stimulating papillomavirus genes E6 and E7 in cervical carcinoma cells

(dexamethasone/cervical cancer/gene regulation/cell proliferation)

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ABSTRACT **In most cervical carcinoma cells the E6 and E7 genes of specific human papillomaviruses are transcribed from viral sequences integrated into host cell chromosomes. Glucocorticoids activate the promoter elements of various human papillomaviruses in transient-expression assays. We have analyzed the effect of dexamethasone on the transcription rate of human papillomavirus 18 E6 and E7 genes integrated at different chromosomal sites in four cervical cancer cell lines. Dexamethasone led to an increase in the transcription rate of the integrated E6–E7 sequences in C4-1 and C4-2 cells but led to a decrease in SW 756 cells and did not affect the transcription rate in HeLa cells. However, when the viral promoter elements derived from HeLa or SW 756 cells, in which dexamethasone does not activate transcription of the integrated E6–E7 sequences, were tested in transient-expression assays within the same cell lines, dexamethasone consistently activated the viral promoter. It thus appears that dominant regulatory mechanisms presumably depending on the chromosomal integration site are able to override the response of the viral promoter to steroid hormones. The growth rate of all dexamethasone-treated cell lines correlated consistently with the expression of the papillomavirus E6 and E7 genes, supporting their role in the maintenance of the proliferative phenotype of cervical carcinoma cells. Since human papillomaviruses are integrated into the host cell genome at variable, presumably randomly selected chromosomal loci, regulatory mechanisms that influence viral gene expression, and hence cell growth, may differ among cancers of independent clonal origin.**

Specific genotypes of human papillomaviruses (e.g., HPV 16, 18, 31, 33, 35, 39, 45, and 52) are involved in the etiology of premalignant and malignant lesions of the anogenital tract (1). In cervical carcinoma cells the DNA of these viruses is usually integrated into the host cell genome. No specific chromosomal integration locus is preferred, since viral DNA sequences have been mapped to different chromosomal loci in independent cervical cancer cell lines (2, 3). The viral genome, however, is often disrupted within its E1 or E2 open reading frame (orf), leading to functional inactivation of at least the E2 orf, which encodes trans-regulating functions for a viral promoter element located in the upstream regulatory region (URR) (4–8). The URR and the early genes E6 and E7 are preserved, while other viral genes may be deleted (7, 9–13). The E6 and E7 orfs are regularly transcribed into mRNA and the respective proteins have been identified in biopsy specimens or cell lines derived from cervical carcinomas (9, 11, 13–18). Transforming functions of the E6 and E7 genes have been demonstrated in various experimental systems (19–22). Specific inhibition of E6–E7 gene expression in cervical cancer cells by antisense RNA led to significantly reduced cell growth (23). Taken together, these data suggest that continuous expression of the viral E6 and E7 genes is involved in growth control of human cervical cancer cells and required for maintenance of the malignant state. Glucocorticoids enhance the transforming potential of the E6 and E7 genes (19, 21, 23), and glucocorticoid-responsive elements (GREs) were identified within the promoter element of several HPVs (24, 25). In transient-expression experiments, which serve to analyze the activity of isolated regulatory sequences, glucocorticoid hormones lead to significant activation of the viral promoter (24, 25).

Here we analyze the influence of dexamethasone on the expression rate of integrated E6–E7 genes of four HPV 18-positive cervical cancer cell lines. The cell lines used were C4-1 and C4-2 (26), which were derived from the same biopsy specimen of a squamous cell carcinoma and are therefore of identical clonal origin (27), HeLa, which was derived from an adenocarcinoma of the cervix (28, 29), and SW 756, more recently established from a squamous cell carcinoma (30). We found that depending on the origin of the cell line and the chromosomal integration site of the viral DNA, dexamethasone treatment modified the transcription rate of HPV genes differently. When the viral promoter was tested in transient chloramphenicol acetyltransferase (CAT) expression assays in the same cell lines, dexamethasone consistently activated the expression of the CAT reporter gene. Therefore, dominant cis-acting mechanisms mediated either by viral elements outside the URR, by viral–cellular junction fragments, or by flanking cellular sequences at the integration locus apparently are able to override the response of the viral promoter elements to glucocorticoid hormones. Analysis of the growth properties of these cell lines with and without dexamethasone treatment revealed that the growth capacities of these cells correlate consistently with the rate of viral E6–E7 gene expression. Regulatory features provided by the site of chromosomal integration may thus significantly influence viral gene expression and growth properties of cervical carcinoma cells.

MATERIALS AND METHODS

Cell Culture. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (40 units/ml), and streptomycin sulfate (50 μg/ml) at 37°C and 5% CO_2. Where indicated, dexamethasone (Sigma) was added in a final concentration of 1 μM.

RNA Analysis. Cytoplasmic RNA was extracted as described (31). For Northern blots, 10 μg was electrophoresed

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Abbreviations: HPV, human papillomavirus; URR, upstream regulatory region; orf, open reading frame; GRE, glucocorticoid responsive element; CAT, chloramphenicol acetyltransferase.

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in a nondenaturing 1% agarose gel in 20 mM Mops/5 mM NaOAc/1 mM EDTA and transferred onto GeneScreen filters (NEN). For RNA slot blots, the same amount was directly adsorbed onto nitrocellulose filters. The filters were hybridized with a ^{32}P-labeled (32) HPV 18 probe derived from the 7-23 cDNA clone (33). Parallel RNA slot blots were hybridized with a probe for ribosomal RNA (34), which served as internal standard for the amount of cytoplasmic RNA bound to the filter. Autoradiograms of RNA slot blot filters were evaluated by densitometry.

Nuclear Run-On Assays. Cells were cultured for 48 hr either with or without dexamethasone (1 μM). They were washed twice with ice-cold phosphate-buffered saline, harvested with a rubber policeman, and lysed in 10 mM Tris·HCl, pH 8.0/10 mM $MgCl_2$/0.5% Nonidet P-40/100 μM phenylmethylsulfonyl fluoride on ice. Nuclei were pelleted by centrifugation at 4000 × g for 5 min at 4°C, washed twice with storage buffer [50 mM Tris·HCl, pH 8.0/40% (vol/vol) glycerol/5 mM $MgCl_2$/0.1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride], and stored at a concentration of 2×10^7 nuclei per 0.1 ml of storage buffer in liquid nitrogen. The run-on experiment was performed as described (35) with minor modifications. In brief, nuclei were incubated in 5 mM Tris·HCl, pH 8.0/2.5 mM $MgCl_2$/0.15 mM KCl with 0.25 mM ATP, GTP, and CTP and 250 μCi of [α-^{32}P]UTP (10 mCi/ml; 400 Ci/mmol; Amersham; 1 Ci = 37 GBq) for 60 min at 28°C. Cellular DNA was digested with DNase I (20 μg/ml; (Boehringer Mannheim) for 5 min at room temperature. To terminate the reaction, 200 μl of 1% SDS/10 mM EDTA/20 mM Tris·HCl, pH 7.4, was added. Proteins were proteinase K-digested and extracted with phenol/chloroform. The radiolabeled RNA was passed through a Sephadex G-50 (fine) column and precipitated in ethanol. Labeled RNA synthesized by the respective nuclei was used at 10^6 cpm/ml as hybridization probe for nitrocellulose filters with 10 μg of the respective plasmid DNAs or 2 μg of cellular (HeLa) DNA serving as internal standard. Subsequently the filters were washed three times in 0.3 M NaCl/0.03 M sodium citrate, pH 7/0.1% SDS at 68°C and exposed to x-ray films.

CAT Assays. Cells grown in 25-cm^2 culture flasks (Greiner, Nürtingen, F.R.G.) were transfected by calcium phosphate precipitation (36) with 20 μg of pBLCAT3 (37) HPV 18 URR plasmids (see Fig. 3). Twenty-four hours after the addition of DNA, cells were split and plated in two 75-cm^2 culture dishes, of which one was incubated with 1 μM dexamethasone for 48 hr. CAT assays were performed as described (38). All experiments were repeated several times with the same result. Quantitation was achieved by liquid scintillation counting of chromatograms.

Cell Growth Assays. [*^{3}H*]*Thymidine incorporation.* The proliferation rate of cell lines cultured in either the presence or the absence of 1 μM dexamethasone was determined as described (23). Each value was determined 24-fold. The average incorporation rate and the standard deviation were calculated.

Growth in semisolid medium. Cells (10^3) were seeded in 6-cm dishes (Nunc) in 0.25% low-melting agarose (SeaKem, FMC) dissolved in culture medium either with or without dexamethasone (1 μM), layered on a 0.5% agarose medium basal layer, and incubated for 4 weeks in 5% CO_2 at 37°C.

RESULTS

Effect of Dexamethasone on the Steady-State Level of HPV 18 mRNA in Different Cervical Cancer Cell Lines. Cytoplasmic RNA was extracted from C4-1, C4-2, SW 756, and HeLa cells grown for 1 week in either the presence or the absence of dexamethasone (1 μM), and HPV 18 E6–E7 transcripts were analyzed by Northern and quantitative RNA slot blots (39) (Fig. 1). Dexamethasone treatment had no influence on

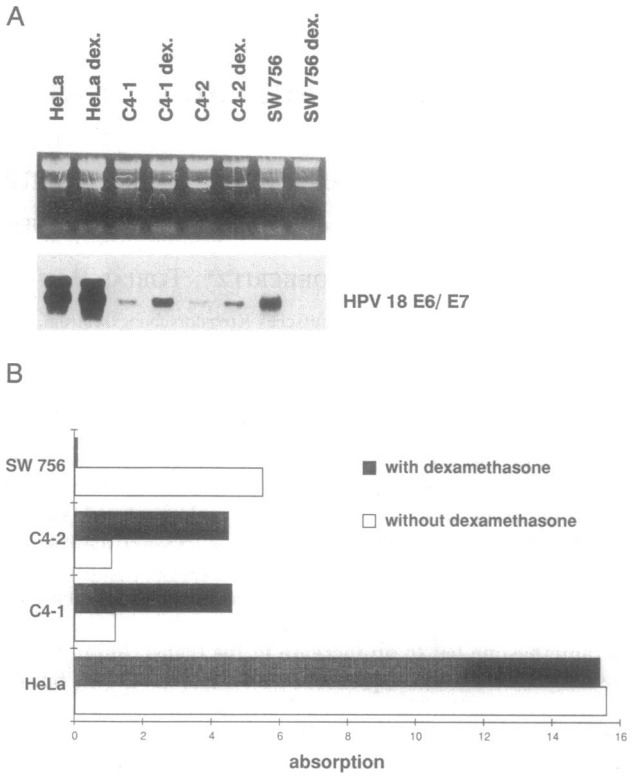

FIG. 1. Influence of dexamethasone on the steady-state level of the HPV 18 E6 and E7 mRNA in four HPV 18-positive cervical carcinoma cell lines. (*A*) Northern blots of cytoplasmic RNA of dexamethasone-treated (dex.) or untreated cells were hybridized with a ^{32}P-labeled HPV 18 E6–E7 probe (*Upper*, ethidium bromide-stained gel; *Lower*, autoradiogram). (*B*) In parallel, RNA preparations of untreated or hormone-treated cells were adsorbed onto nitrocellulose filters and hybridized with the same E6–E7 probe. The intensity of the signal (absorption) obtained on x-ray films was determined by densitometric scanning. As reference for the amount of RNA in each slot a parallel filter was hybridized with a ^{32}P-labeled probe for ribosomal RNA (34). Relative induction or reduction of HPV 18 E6–E7 mRNA is given as a histogram.

the steady-state level of HPV 18 E6–E7 transcripts in HeLa cells, but it resulted in a 4- to 5-fold increase of the steady-state levels of HPV 18 mRNA in C4-1 and a 3- to 4-fold increase in C4-2 cells. In contrast, dexamethasone treatment led to a significant reduction of the E6–E7 mRNA in SW 756 cells. Both the enhanced E6–E7 expression by dexamethasone in C4-1 and C4-2 cells and the reduced expression in SW 756 cells were dose-dependent.

Cellular glucocorticoid-controlled genes (e.g., those encoding class I major histocompatibility antigens or the epidermal growth factor receptor) are uniformly regulated by dexamethasone in these cervical cancer cell lines (ref. 40 and M.v.K.D., unpublished results). Thus, the differential response to dexamethasone is apparently restricted to papillomavirus genes integrated at different chromosomal sites.

Transcription of Integrated HPV 18 E6 and E7 Genes in Dexamethasone-Treated Cells. To test whether an altered transcription rate of the HPV 18 E6–E7 sequences was responsible for modified steady-state levels of the mRNAs in the cell lines, we performed nuclear run-on experiments. Cells were grown either with or without dexamethasone for 48 hr. Nuclei were isolated and *in vitro* synthesized ^{32}P-labeled RNA was hybridized to cloned HPV 18 E6–E7 sequences adsorbed onto nitrocellulose filters (Fig. 2). In C4-1 and C4-2 cells treated with dexamethasone, the transcription rate of the integrated HPV 18 E6–E7 sequences was increased, whereas in HeLa cells no quantitative difference

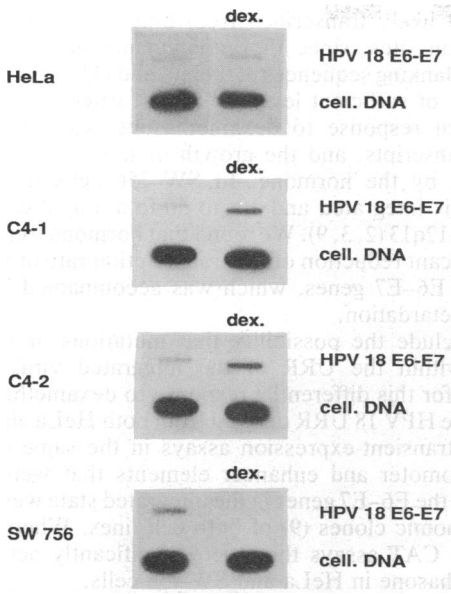

FIG. 2. Nuclear run-on assay. HeLa, C4-1, C4-2, and SW 756 cells were either treated or not for 48 hr with 1 μM dexamethasone (dex.). Nuclei were extracted and nuclear run-on assays were performed.

of radiolabeled HPV 18 E6–E7 transcripts was observed between hormone-treated and untreated cells. However, the transcription rate of the HPV 18 E6–E7 sequences in SW 756 cells was significantly reduced upon dexamethasone treatment. These results strongly suggest that hormone-induced alterations of the transcription rate are responsible for the effects of dexamethasone on the HPV 18 E6–E7 steady-state mRNA levels.

Influence of Dexamethasone on HPV 18 Promoter Activity in Transient-Transfection Experiments. The divergent effects of dexamethasone on transcriptional control of integrated HPV 18 E6–E7 genes in the respective cell lines might be due to loss or mutation of the GRE (25) in HPV 18 URR sequences in HeLa and SW 756 cells. However, DNA sequence analysis of the HPV 18 promoter elements derived from HeLa and SW 756 cells revealed a complete conservation of the GRE. Therefore, other cis- or trans-acting mechanisms mediate the differential response to dexamethasone.

To define which of the two is responsible here, the activity of the HPV 18 URR derived either from HeLa or SW 756 cells was tested in transient-expression assays in the same cell lines. Both promoter regions were cloned into a CAT expression vector (37) (Fig. 3) and the CAT constructions were transfected into HeLa and SW 756 cells. The CAT conversion rate was determined either with or without hormone treatment. CAT expression mediated by both plasmids was significantly enhanced by dexamethasone in HeLa and SW 756 cells (Fig. 4). Thus, in transient-expression assays, dexamethasone activates the HPV 18 URR derived from integrated viral genomes of HeLa or SW 756 cells. This suggests that in these cell lines, cis-acting elements at the site of chromosomal integration dominate over transcriptional control mediated by diffusible factors such as the hormone-activated glucocorticoid receptor, which binds to elements within the HPV 18 URR.

Growth Properties of Dexamethasone-Treated Cervical Cancer Cells with Divergent Rates of HPV E6–E7 Expression. To determine the proliferation rate of cells grown with or without dexamethasone treatment, we analyzed the [^{3}H]thymidine incorporation rate. Dexamethasone had no significant influence on the growth rate of HeLa cells (Fig. 5*A*). The proliferation rate of C4-1 and C4-2 cells was increased 3- to 5-fold by dexamethasone, as described previously for C4-1 (23). The proliferative capacity of SW 756 cells was significantly reduced under the same conditions. Thus, in all cell lines tested the influence of dexamethasone on cell growth reflected the alteration of viral early-gene expression.

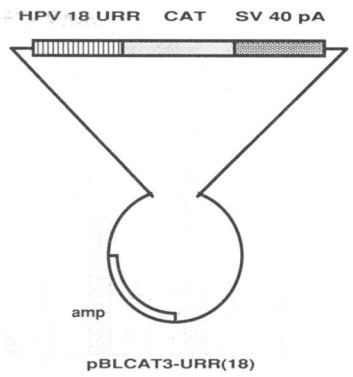

FIG. 3. Schematic diagram of CAT plasmids. HPV 18 promoter elements located in the URR were obtained from genomic clones derived from HeLa and SW 756 cells (9). The complete HPV 18 URR derived from integrated viral sequences of the SW 756 cells was isolated as a 1.1-kilobase *Bam*HI fragment from the genomic clone H1. The HPV 18 URR derived from the HeLa cells was isolated as a 1.05-kilobase *Bam*HI–*Nco* I fragment from the genomic clone H4. They were cloned 5′ to the CAT coding sequences in the pBLCAT3 vector (37). SV 40 pA, simian virus 40 polyadenylylation signal; amp, ampicillin-resistance gene.

Similar results were obtained when cells were cultured in semisolid medium (Fig. 5*B*). Growth properties of HeLa cells in soft agar were not influenced, whereas C4-1 cells had an increased cloning efficiency and grew faster in the presence of the hormone. The cloning efficiency and growth of SW 756 cells were significantly inhibited by dexamethasone in soft agar.

DISCUSSION

The papillomavirus genes E6 and E7 are expressed from viral sequences integrated at different sites into the chromosomal DNA of independent cervical carcinoma cells (2, 3). These

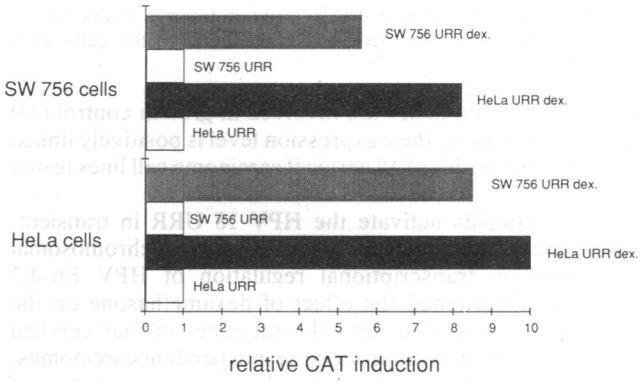

FIG. 4. Quantitative analysis of CAT assays by liquid scintillation counting of the chromatograms. HPV 18 URRs derived from HeLa and SW 756 cells were inserted into pBLCAT3 (see Fig. 3) and transfected into HeLa and SW 756 cells. Relative induction in dexamethasone-treated (dex.) HeLa and SW 756 cells is given. To control for sufficient levels of glucocorticoid receptors, cells were transfected in parallel with the glucocorticoid-responsive mouse mammary tumor virus (MMTV)–CAT construct (41), which was about 40-fold induced in both cell lines by dexamethasone (data not shown). Transfection of a negative control (pBLCAT3 DNA) gave no detectable CAT expression either in the absence or in the presence of dexamethasone.

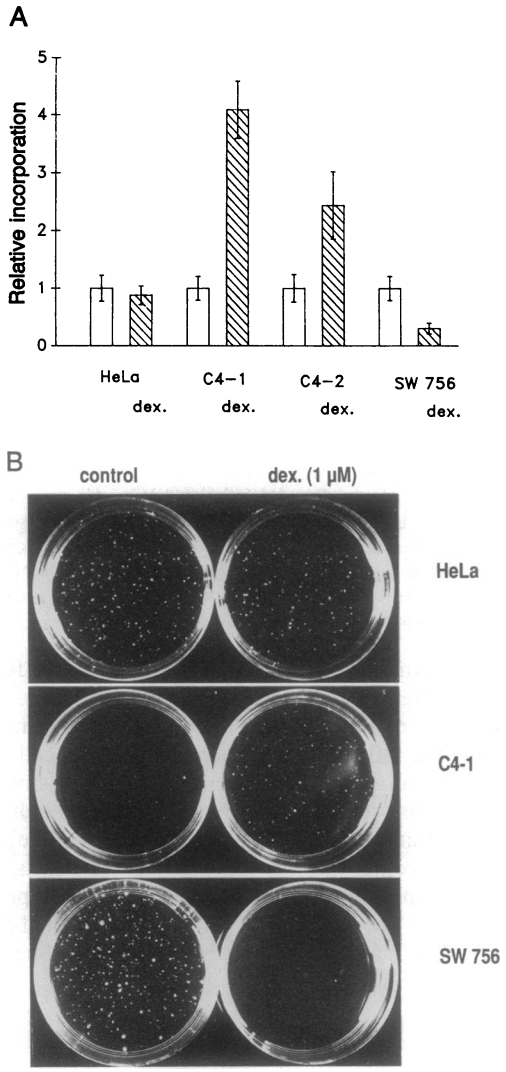

FIG. 5. (A) Relative [^{3}H]thymidine incorporation. Cells were cultured for 1 week with (hatched bars) or without (open bars) dexamethasone (dex.) treatment. Each value was determined 24-fold, with a standard deviation of 15–20%. (B) Growth of HeLa, C4-1, and SW 756 cells in soft agar with or without dexamethasone (dex.). Culture dishes were photographed 4 weeks after the cells were seeded.

transforming viral genes are involved in growth control (23) and, as shown here, their expression level is positively linked to growth properties of all cervical carcinoma cell lines tested so far.

Glucocorticoids activate the HPV 18 URR in transient-expression assays. To analyze the influence of chromosomal integration on transcriptional regulation of HPV E6–E7 genes, we determined the effect of dexamethasone on the transcription rate of integrated viral genes in four cervical cancer cell lines derived from three independent carcinomas. In cell lines of independent clonal origin, transcription of integrated HPV 18 E6–E7 genes was differently influenced by dexamethasone. In C4-1 and C4-2 cells, which were both derived from the same original tumor cell clone (27) and contain only one copy of the HPV 18 genome integrated on chromosome 8 (2), glucocorticoid treatment led to enhanced transcription of integrated viral genes leading to increases in E6–E7 mRNA that were associated with accelerated cell growth. HeLa cells contain 10–50 copies of the viral sequences, integrated preferentially on chromosome 8 at a different site than in C4-1 cells (2, 42) and additionally on chromosomes 5, 9, and 22 (43). The HeLa E6–E7 transcripts are most likely transcribed from only one of the multiple integration sites, since they contain identical stretches of cellular flanking sequences at their 3′ end (33, 44). Despite the presence of sufficient levels of glucocorticoid receptor, no significant response to dexamethasone was observed for these transcripts, and the growth of this cell line was not modified by the hormone. In SW 756 cells the HPV 18 genome is integrated and 10- to 50-fold amplified on chromosome 12q13 (2, 3, 9). We found that hormone treatment led to significant reduction of the transcription rate of integrated HPV 18 E6–E7 genes, which was accompanied by severe growth retardation.

To exclude the possibility that mutations or rearrangements within the URR of the integrated viral genomes account for this differential response to dexamethasone, we tested the HPV 18 URR derived from both HeLa and SW 756 cells in transient-expression assays in the same cell lines. URR promoter and enhancer elements that were directly linked to the E6–E7 genes in the integrated state were derived from genomic clones (9) of both cell lines. When tested in transient CAT assays they were significantly activated by dexamethasone in HeLa and SW 756 cells.

The differential effects of dexamethasone on the transcription rate of integrated HPV 18 E6–E7 sequences in independent cervical carcinoma cells are apparently due to cis-acting mechanisms in the respective tumor cells. They might be evoked by direct or indirect steroid hormone-sensitive regulatory elements within the adjacent sequences of the viral URR and presumably involve cellular elements at the site of chromosomal integration, or they might be due to the usage of cellular instead of viral promoters for E6–E7 transcription (44).

Our observations indicate that in independent cervical cancer cell clones, different control elements that dominate over viral control elements located in the URR may determine, at least in part, transcriptional regulation of integrated viral genes. This phenomenon presumably is not restricted to glucocorticoid regulation but may also involve negative regulatory factors acting on the viral promoter—e.g., the viral E2 gene product (4, 6–8). Integration of the viral sequences might not only inactivate the viral E2 gene but also, at least in some cases, render expression of the integrated viral E6–E7 genes independent of factors that inhibit the transcriptional activity of the viral URR. This might be of special relevance in those tumors where integrated as well as intact episomal forms of the viral DNA persist and where the integrated molecules appear to be transcribed preferentially (7, 11). Indirect evidence for this hypothesis is given by experiments in which the E2 orf was expressed in cervical cancer cell lines. Some of these (e.g., HeLa cells) were significantly growth-inhibited, most likely due to suppression of viral E6–E7 expression, whereas others (e.g., C4-1 cells) were only marginally inhibited in their growth capacities (M.v.K.D. and H.z.H., unpublished results). Similar mechanisms might also concern cellular factors acting on the papillomavirus URR. Integration of the viral genes into the host cell chromosomes might then represent, at least in some cases, a mechanism to escape cellular negative interfering factors that obviously control viral gene expression in undifferentiated human keratinocytes (45).

The significance of continuous expression of the viral E6–E7 genes for the maintenance of the proliferative phenotype of cervical cancer cells is further substantiated by the experiments described here. Modification of E6–E7 transcription in all cervical cancer cell lines tested was accompanied by concomitant alteration of growth capacities. Integration of HPV E6–E7 genes represents an important step in cervical carcinogenesis, since it contributes to deregulated expression of the viral growth-regulating genes. Since the chromosomal integration sites of the viral sequences differ in

cells of independent clonal origin, regulatory mechanisms that control expression of the integrated E6–E7 genes in individual cervical cancer cell clones are hard to predict and require specific elaboration for each cancer cell clone.

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