JOURNAL OF VIROLOGY, Oct. 1990, p. 4767–4775
0022-538X/90/104767-09$02.00/0

Vol. 64, No. 10

Transforming Growth Factors Beta 1 and 2 Transcriptionally Regulate Human Papillomavirus (HPV) Type 16 Early Gene Expression in HPV-Immortalized Human Genital Epithelial Cells

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Received 11 May 1990/Accepted 29 June 1990

Human papillomavirus type 16 (HPV16) early proteins E6 and E7 have been implicated in maintenance of the malignant phenotype in cervical cancer. Transforming growth factors beta one and two (TGFβs 1 and 2), polypeptides that regulate cellular growth and differentiation, reversibly inhibited expression of the HPV16 E6 and E7 genes in several immortal genital epithelial cell lines. Loss of E6 and E7 protein expression followed a dramatic time- and dose-dependent decrease in E6 and E7 RNA levels and was accompanied by cessation of cell proliferation. TGFβs 1 and 2 inhibited HPV16 RNA expression at the transcriptional level; inhibition was dependent upon ongoing protein synthesis. TGFβs 1 and 2 also induced a six- to sevenfold increase in TGFβ 1 RNA. Cells became partially resistant to the inhibitory effects of TGFβ 1 on cell growth and HPV early gene expression after prolonged cultivation in vitro or after malignant transformation. Thus, TGFβ 1 may function as an autocrine regulator of HPV gene expression in infected genital epithelial cells.

Human papillomaviruses (HPVs) are a group of small DNA viruses that induce papillomas in mucosal and epidermal epithelia (35, 57). DNAs from several HPV types have been detected in intraepithelial neoplasias of the cervix, vulva, and penis, as well as in carcinomas (4, 6, 15, 29), implying that the virus may be a factor in the development of these tumors. Integration of the HPV genome usually occurs in cervical cancers and carcinoma cell lines in a specific manner that ensures that the E6 and E7 early genes are selectively retained and transcribed (43, 45, 46, 57). The E6 and E7 gene products regulate cell proliferation and gene expression, as transfection of primary human genital epithelial cells with recombinant E6 and E7 DNA confers immortality (19, 32) and resistance to terminal differentiation (23). The HPV type 16 (HPV16) E7 protein cooperates with an activated cellular Ha-*ras* gene to transform primary rodent cells (47), but continued expression of E7 is required to maintain the transformed phenotype (13). Normal genital epithelial cells possess an intracellular control mechanism directed against HPV gene transcription (56, 57); however, cellular functions downregulating HPV expression are absent in genital carcinoma cells (40), suggesting that this loss represents an important step in the development of cancer.

The beta transforming growth factors (TGFβs) are members of a family of polypeptides (TGFβs 1 to 5, inhibins, activins; reviewed in reference 39) that modulate cell proliferation and gene expression in diverse cells. The cellular response to TGFβs varies depending on the specific cells, growth conditions, and the presence of additional growth factors (2, 20, 21, 31, 33, 49). Generally, TGFβs inhibit proliferation of epithelial cells, but they can either inhibit (24) or stimulate (31, 33, 48) cellular differentiation. Alterations in the expression or responsiveness to TGFβs often occur in malignancy (7, 20–22, 44, 49). In cultured normal genital epithelial cells, TGFβ 1 inhibits proliferation (11, 44) and induces expression of its own RNA (2), suggesting that it might function as an autocrine regulator of growth and gene expression in normal genital epithelium. Limited information is available concerning the effect of TGFβs on expression of viral genes in infected cells (3).

In the present study, the effect of TGFβ on papillomavirus gene expression was determined in a series of immortal genital epithelial cell lines containing integrated and transcriptionally active HPV16 DNA (54, 55). TGFβs 1 and 2 dramatically down regulate expression of RNAs encoding the HPV16 E6 and E7 genes in several independently derived cell lines. Continuous serial passage in culture or malignant transformation by oncogenes results in partial resistance to the inhibitory effects of TGFβ 1 on cell growth and HPV early gene expression.

MATERIALS AND METHODS

Cell culture. Primary cultures of human genital epithelial cells were isolated from foreskin and cervical tissue as described before (54, 55). The derivation and characterization of cell lines immortalized by transfection with recombinant HPV16 DNA have also been described in detail (37, 54, 55). Normal foreskin or cervical cells and all HPV-immortalized cell lines were maintained in serum-free MCDB153-LB medium (37). In some experiments, cells were maintained in medium lacking growth factors (insulin, hydrocortisone, transferrin, triiodothyronine, epidermal growth factor, and bovine pituitary extract) to inhibit proliferation. Cervical carcinoma cell lines QGU, QGH (45), and SiHa (American Type Culture Collection, Rockville, Md.) were grown in a 1:1 mixture of Dulbecco modified Eagle medium (DMEM) and F12 medium supplemented with 5% fetal bovine serum. Early and late passages of HPV-immortalized cell lines were used. By definition, early passage refers to cells used within 60 population doublings after transfection with HPV DNA, and late passage refers to those maintained for more than 250 population doublings (each passage represents approximately four to five population doublings). Rapidly proliferating cultures at 60 to 80% confluence were

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used. In some experiments cellular protein synthesis was inhibited by treating cultures with cycloheximide (10 μg/ml of medium) (Sigma Chemical Co., St. Louis, Mo.), and the extent of inhibition of protein synthesis was monitored as described before (30). To measure RNA stability, dactinomycin (Fluka Chemical Corp., Ronkonkoma, N.Y.) was added to cultures at 10 μg/ml of medium.

Cloning experiments. Approximately 500 to 1,000 cells in 5.0 ml of medium were added to duplicate 60-mm tissue culture dishes and allowed to attach overnight. TGFβ 1 obtained from human platelets (Collaborative Research, Bedford, Mass.) or porcine TGFβ 2 (R and D Systems, Minneapolis, Minn.) was added in 5.0 ml of medium the next day, and cultures were incubated for 10 days. Cultures were fixed with 3% Formalin and stained with Giemsa, and colonies containing more than 20 cells were counted.

Immunoprecipitation and gel electrophoresis. After a 30-min incubation of subconfluent cell cultures in MCDB153 without methionine or cysteine, 4.0 ml of medium containing 100 μCi of [^{35}S]methionine and 100 μCi of [^{35}S]cysteine per ml was added. Cells were incubated at 37°C for an additional 4 h. Labeling was terminated by washing cells with cold phosphate-buffered saline. Cells were lysed in 1.0 ml per 10-cm plate of RIP buffer (1% Triton X-100, 1.0% sodium dodecyl sulfate, 0.5% deoxycholate, 0.1 NaCl, 0.1 M phenylmethylsulfonyl fluoride, and 20 μg of aprotinin per ml). Samples (200 μl) were incubated with rabbit polyclonal anti-HPV16 E6 (provided by J. Schiller), mouse monoclonal anti-HPV16 E7 (Triton Biosciences Inc., Alameda, Calif.), or rabbit polyclonal antiinvolucrin (Biomedical Technologies Inc., Stoughton, Mass.) for 2 h on ice before being precipitated with protein A-Sepharose. Immunoprecipitates were washed several times in RIP buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Parallel immunoprecipitations were performed with lysate samples with either equal total protein concentration (400 μg) or equal counts (75×10^6 cpm) to control for differences in amino acid uptake. Markers were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). Normal cervical cells were used as a negative control.

Recombinant plasmid DNAs. Plasmid DNAs included pD5DD1 (a 0.63-kilobase-pair [kbp] *Dde*I fragment containing HPV16 E6 and partial E7 open reading frames from nucleotides 25 to 653; provided by J. Doniger), a 0.7-kbp *Sac*I-*Pvu*II fragment of porcine TGFβ 1 cDNA (26), a 1.2-kbp *Hin*dIII fragment of simian TGFβ 2 cDNA (18), human c-*myc* gene (Oncor Incorp, Gaithersburg, Md.), a human β-actin gene fragment (28), a cDNA encoding the human laminin β1 chain (provided by Y. Yamada), and an 18S rRNA probe (provided by F. Mushinski). Plasmid DNAs were digested with the appropriate restriction endonucleases to cleave vector sequences, except for the 18S RNA probe, which was used as complete plasmid DNA. Insert DNAs were separated on agarose gels, and the purified fragments were used as probes for nick translation or for slot blots.

Northern (RNA blot) analysis. Total cellular RNA was purified from subconfluent cultures by lysis in guanidine thiocyanate (8), followed by centrifugation through cesium trifluoroacetate. RNA (10 to 15 μg) was separated by electrophoresis in 1.5% agarose gels containing formaldehyde, transferred to nylon membranes (Schleicher and Schuell, Keene, N.H.) by capillary blotting, baked at 80°C for 2 h, and hybridized to ^{32}P-labeled DNAs under stringent conditions (50% formamide, 10% dextran sulfate, 5× Denhardt solution (30), 1% sodium dodecyl sulfate) at 42°C for 16 to 24 h. Filters were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% sodium dodecyl sulfate for 30 min at room temperature and then with 0.1× SSC containing 0.1% sodium dodecyl sulfate at 50 to 65°C for 30 min. Filters were exposed to Kodak XAR-2 film with an enhancing screen at −72°C. RNA molecular weight standards were obtained from Bethesda Research Laboratories.

Runoff transcriptional analysis. Nuclei were isolated from subconfluent cell cultures as described before (42) and maintained at −70°C in freezing buffer (50% glycerol, 20 mM Tris hydrochloride, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM dithioerythritol, 100 U of RNase inhibitor per ml) for 2 to 3 weeks before use. Runoff transcription analysis was performed basically as described before (42). The in vitro elongation reactions were performed by the method of Gariglio et al. (16) in a mixture containing 100 mM Tris hydrochloride (pH 7.9), 200 mM NaCl, 0.4 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.3 M $(NH_4)_2SO_4$, 4.0 mM each $MgCl_2$ and $MnCl_2$, 1.2 μM dithioerythritol, 1.0 mM each GTP, ATP, and CTP, 250 μCi of ^{32}P-labeled UTP (3,000 Ci/mmol), 10 mM creatine phosphate, 20 U of placental RNase inhibitor per ml, 30% glycerol, and approximately 2×10^7 nuclei. α-Amanitin was added to some reaction mixes as a negative control. The reaction mixes were incubated for 30 min at 27°C. Labeled RNA was extracted from the reaction mixture and purified by gel filtration with Sephadex G-50 (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.), followed by precipitation with ammonium acetate. Nitrocellulose filters containing 2 μg of purified insert DNA from each recombinant plasmid were prehybridized for 24 to 48 h in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7)–0.75 M NaCl–50% formamide–0.5% sodium dodecyl sulfate–2.0 mM EDTA–10× Denhardt solution–200 μg of salmon sperm DNA per ml–10 μg of polyadenylic acid per ml. Equal amounts of labeled RNA (approximately 10^7 cpm/ml) were added to each filter, and hybridization was allowed to proceed at 42°C for 48 to 72 h. The intensity of hybridization signals was measured by scanning laser densitometry.

RESULTS

Inhibition of cell proliferation by TGFβ 1. TGFβ 1 at 3.0 ng/ml completely inhibited clonal growth in secondary cultures of normal genital epithelial cells derived from either foreskin (HK) or cervix (CX) (Table 1). Cells flattened and proliferation ceased approximately 24 to 48 h after treatment. Growth inhibition was partially reversible when foreskin-derived keratinocyte cultures that were exposed to TGFβ 1 for 48 h were washed and then refed with fresh medium lacking TGFβ 1 for an additional 8 days (data not shown); however, cervical epithelial cells were more susceptible and clonal growth was irreversibly inhibited by 3.0 ng of TGFβ 1 per ml by 48 h. Different cell lines derived by transfection and immortalization of normal cells with HPV16 DNA varied significantly in their response to TGFβ 1, with some being more resistant than normal cells. Cell lines of late passage (>250 population doublings) often exhibited a further increase in resistance.

Two immortalized cell lines that were malignantly transformed after transfection with the v-Ha-*ras* oncogene (14) or the herpes simplex virus type 2 (HSV-2) *Bgl*II N fragment (13a) were also examined. Both cell lines formed colonies in TGFβ 1-containing medium with a frequency greater than the parental lines. Three cervical carcinoma-derived tumor lines grew clonally in medium containing TGFβ 1, although

TABLE 1. Effects of TGFβ 1 on clonal growth and HPV16 E6/E7 RNA expression

Cell line	Cloning efficiency[a] (% of control)	HPV16 E6/E7 RNA[b] (% of control)
Normal cells		
Foreskin	0	
Cervical	0	
CX16-7		
Early passage	0	0
Late passage	20.6 ± 5.1	53
CX16-10		
Early passage	0	0
Late passage	5.5 ± 1.2	7
CX16-2		
Early passage	28.1 ± 7.6	12
Late passage	65.5 ± 11.6	13
ras transformed	74.8 ± 15.9	82
HK16-1		
Early passage	13 ± 11.8	19
Late passage	32.0 ± 10.1	12
HSV-2 transformed	72.7 ± 21.5	76
Carcinoma		
QGH	19.5 ± 7.8	20
QGU	74.6 ± 6.0	56
SiHa	114.8 ± 4.1	76

[a] Clonal growth was measured in the presence of TGFβ 1 (3.0 ng/ml) for 10 days. Data represent the mean of four dishes ± standard error. Untreated cultures served as the control.

[b] Northern blots were analyzed by scanning densitometry, and values are expressed as a percentage of the RNA in cultures grown in the absence of TGFβ 1 (control).

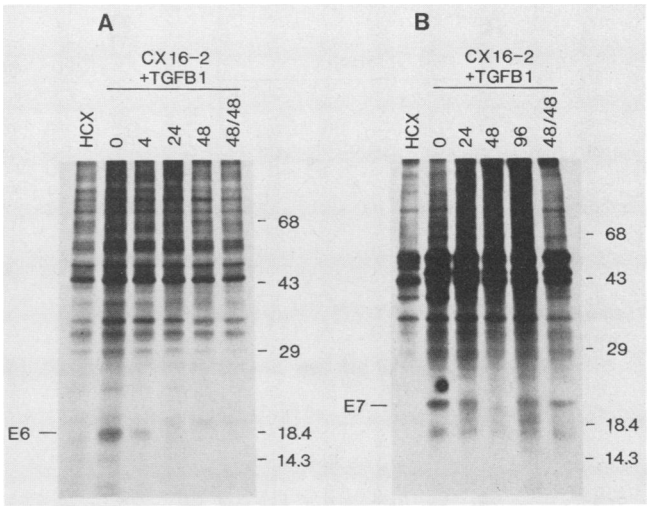

FIG. 1. TGFβ 1 effects on expression of HPV16 E6 and E7 proteins. CX16-2 cells were treated with TGFβ 1 (3.0 ng/ml) for various intervals (4, 24, 48, and 96 h), and expression of HPV E6 (A) and E7 (B) proteins was assessed after immunoprecipitation and polyacrylamide gel electrophoresis. Secondary cultures of normal cervical cells (HCX), which do not express HPV16 E6 or E7 proteins, were used as a negative control. The specificity of the E6 antiserum has been described previously (1). To determine whether TGFβ 1 effects on HPV expression were reversible, TGFβ 1-containing medium was replaced with fresh medium after 48 h (48/48). Numbers at the right of panels indicate molecular mass (in kilodaltons).

growth of one line (QGH) was reduced significantly with respect to untreated cultures. Another tumor line (SiHa) exhibited a slightly greater cloning efficiency in the presence of TGFβ 1.

Inhibition of HPV16 gene expression by TGFβ 1 and 2. The effect of TGFβ 1 on the expression of the HPV16 early genes E6 and E7 was examined in the CX16-2 HPV16-immortalized cervical cell line that was partially resistant to TGFβ 1 in clonal growth assays. All experiments were done with early-passage cultures (<60 population doublings) of subconfluent, proliferating cells. TGFβ 1 treatment for 24 h markedly decreased levels of E6 and E7 protein expression (Fig. 1). When cultures were maintained in the presence of TGFβ 1, E7 expression remained low to undetectable for 48 h, but expression was partially restored when cultures were switched after 48 h to fresh medium either with or without TGFβ 1 for another 48 h. In contrast, E6 expression remained undetectable even after TGFβ 1 was removed. DNA synthesis, measured by incorporation of tritiated thymidine, was decreased approximately 60% after treatment with TGFβ 1 for 24 or 48 h. TGFβ did not alter expression of involucrin (data not shown), a marker of squamous differentiation in cervical epithelium (52).

RNA analyses were performed to further define the level at which TGFβ 1 regulated papillomavirus gene expression. The CX16-2 cell line expressed three different RNAs that hybridized to the HPV16 E6 and E7 open reading frames (Fig. 2A). RNAs of 1.7 and 4.2 kilobases (kb) were expressed in each of seven HPV16-immortalized cell lines examined, and a 3.4-kb species was detected in four of seven lines (54). This implies that these transcripts begin and end within virus sequences and are not fusion RNAs containing both virus and cellular sequences. Exposure to TGFβ 1 resulted in time-dependent reductions in both cell proliferation and steady-state levels of E6 and E7 RNAs (Fig. 2A). The 1.7-kb RNA decreased within 6 to 8 h after treatment and remained low to undetectable by 24 to 48 h. In contrast, the 4.2- and 3.4-kb RNAs were reduced dramatically within 2 to 4 h, but later were reexpressed at reduced levels compared with the untreated cells. The pattern of inhibition of HPV RNA expression by TGFβ 1 was reproducible in two experiments with the CX16-2 cell line. These cells also expressed a 2.4-kb TGFβ 1 RNA (Fig. 2A). TGFβ 1 induced a time-dependent increase in TGFβ 1 RNA that was maximal (sevenfold) after 24 h. Rehybridization of the filter to a cDNA for glyceraldehyde phosphate dehydrogenase (GAPDH), a housekeeping gene, demonstrated that equal amounts of RNA were present in each lane.

To examine whether down regulation of virus gene expression by TGFβ 1 might occur secondarily to cessation of cell proliferation, cell growth was inhibited in replicate cultures by two different methods. CX16-2 cells were maintained for 2 or 4 days in basal medium lacking insulin, epidermal growth factor, transferrin, triiodothyronine, bovine pituitary extract, and hydrocortisone. This treatment induced cell flattening and inhibited cell division by approximately 72 to 83% in replicate experiments, as determined by direct cell counts. These cells could be stimulated to divide by replacing the basal medium with complete medium. Alternatively, cultures were grown to confluence, to inhibit proliferation by contact with adjacent cells. In both instances the CX16-2 cell line continued to express normal levels of all three virus RNAs (Fig. 2B). Maintaining cells in medium deficient in growth factors did not alter TGFβ 1 RNA levels but led to a slight decrease in expression of GAPDH RNA.

TGFβ 1 and 2 were equally effective in reducing HPV16 E6/E7 RNA levels after a 24-h treatment (Fig. 2C). Both TGFβ types inhibited virus RNA expression in a dose-dependent manner, and as little as 0.3 ng/ml was effective. In

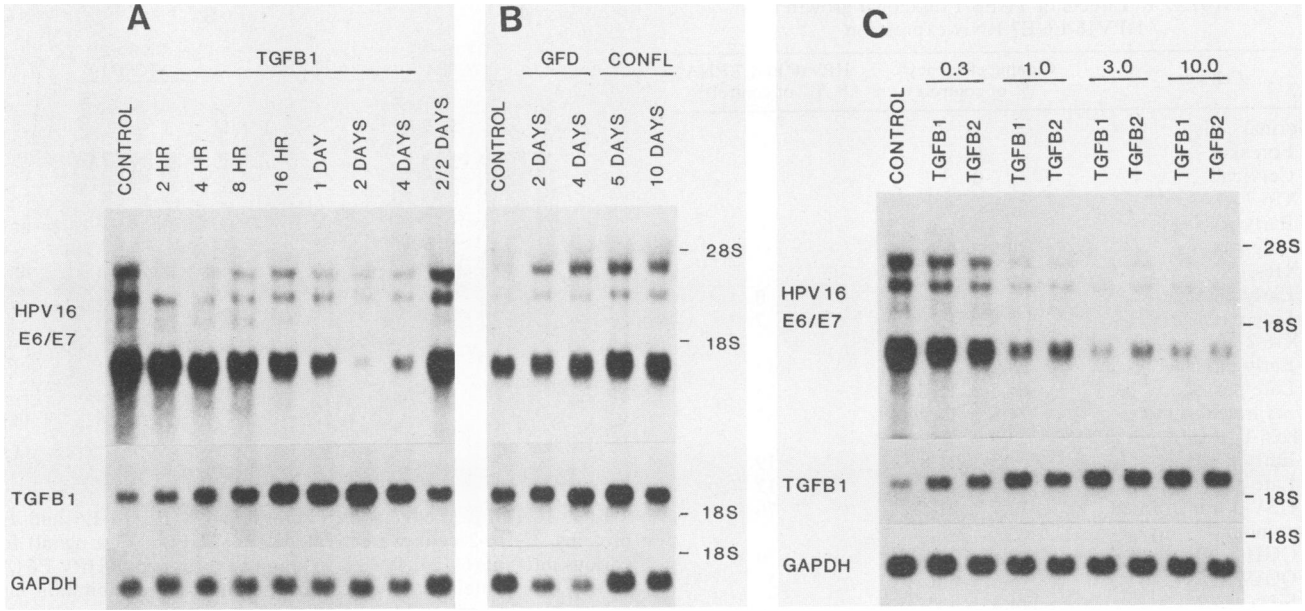

FIG. 2. Northern analyses of cells treated with TGFβs 1 and 2 and cells arrested in growth. (A) CX16-2 cells were treated with TGFβ 1 (3.0 ng/ml) for various intervals or for 2 days followed by removal of TGFβ 1 for 2 days (2/2 days). (B) Growth arrest was induced by maintaining CX16-2 cells in medium lacking growth factors (GFD) (see Materials and Methods) or by maintaining cultures for 5 to 10 days as confluent monolayers (CONFL). (C) CX16-2 cells were treated for 24 h with various concentrations (0.3, 1.0, 3.0, and 10.0 μg/ml) of TGFβ 1 and 2. Three RNAs of 4.2, 3.4, and 1.7 kb hybridized to the HPV16 E6/E7 probe. The probes used for hybridization were pD5DD1 (a 0.63-kbp *Dde*I fragment of HPV16 containing E6 and partial E7 open reading frames) and a portion of the TGFβ 1 cDNA (0.7-kbp *Sac*I-*Pvu*II fragment). To ascertain that each lane contained equal amounts of total RNA, blots were rehybridized to a cDNA for GAPDH. Numbers at the right of panels indicate the positions of the 18S and 28S rRNAs.

addition, both TGFβ 1 and 2 induced a six- to sevenfold increase in TGFβ 1 RNA. However, TGFβ 2 RNAs were not detected in this cell line even when the filter was exposed for as long as 1 week.

To determine whether inhibition of HPV16 RNA expression or induction of TGFβ 1 RNA by TGFβ 1 was dependent on continued protein synthesis, cultures of CX16-2 cells were treated with cycloheximide (10 μg/ml) in the presence or absence of TGFβ 1. This concentration of cycloheximide inhibited incorporation of [^{35}S]methionine into proteins in CX16-2 cells by approximately 80%. Cycloheximide treatment prevented the reduction in the 1.7-kb HPV RNA caused by TGFβ 1 (Fig. 3), but cycloheximide alone did not alter the level of this transcript. In contrast, cycloheximide treatment caused steady-state levels of the 4.2- and 3.4-kb RNAs to decline to undetectable levels by 8 h. The differential effects of cycloheximide on the HPV16 1.7- and 4.2-kb RNAs indicate that these RNAs are regulated differently.

The effect of cycloheximide on autoregulation of TGFβ 1 RNA was complex. Cycloheximide treatment for 8 h did not affect TGFβ 1 RNA levels but enhanced the autoinduction of TGFβ 1 RNA approximately fourfold. However, cycloheximide treatment for 24 h inhibited TGFβ 1 RNA expression.

Nuclear runoff experiments were performed to examine whether reductions in steady-state levels of HPV16 RNAs by TGFβs 1 and 2 were due to changes in transcription. Rapidly proliferating CX16-2 cell cultures were treated with TGFβ 1 or 2 for 24 h, and the elongation of transcripts for several genes was compared. Results were normalized to GAPDH expression, as previous experiments demonstrated that TGFβs 1 and 2 do not alter steady-state levels of this RNA. Transcription of HPV16 RNA was reduced dramatically in cells treated with either TGFβ 2 or 1 (Fig. 4; Table

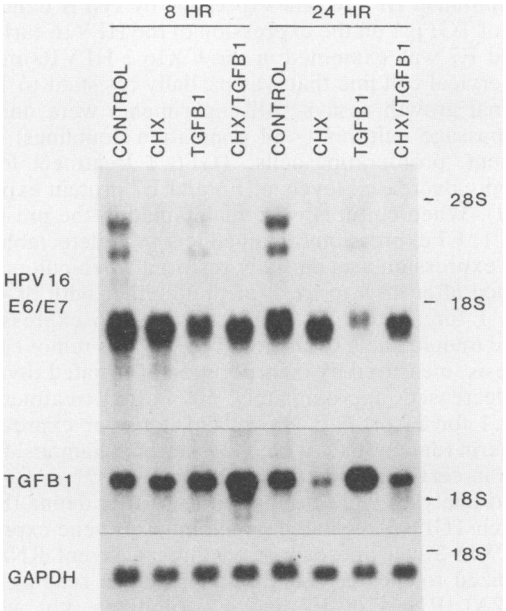

FIG. 3. Effect of cycloheximide on TGFβ 1-induced changes in gene expression. Proliferating CX16-2 cells were treated with 10 μg of cycloheximide (CHX) per ml, 3.0 ng of TGFβ 1 per ml, or both for either 8 or 42 h. Filters were hybridized to the probes described in the legend to Fig. 2.

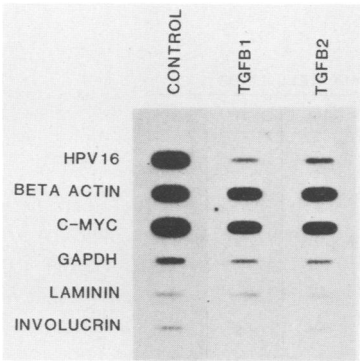

FIG. 4. Nuclear runoff transcription analysis of CX16-2 cells treated with TGFβ 1 or 2. Nuclei were isolated from proliferating cultures of CX16-2 cells that were treated with TGFβ 1 or 2 (3.0 ng/ml) for 24 h or from untreated cultures (control). Then, 2.5 μg of purified insert DNA from plasmids containing HPV16, β-actin, c-*myc*, GAPDH, laminin β1 chain, or involucrin DNA was immobilized on nitrocellulose filters and hybridized to an equal amount of radioactivity (10^7 cpm/ml) for 3 days at 42°C.

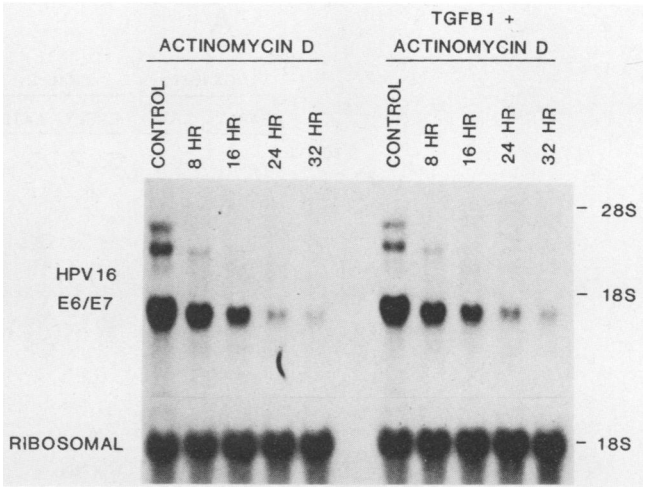

FIG. 5. Stability of HPV16 E6/E7 RNAs. Rapidly proliferating CX16-2 cells were treated with dactinomycin (10 μg/ml) or incubated for 2 h with TGFβ 1 (3.0 ng/ml) before addition of dactinomycin. Total cellular RNA was purified at various intervals and hybridized to the HPV16 E6/E7 probe described in the legend to Fig. 2. Filters were rehybridized to an 18S rRNA probe to ascertain that all lanes contained equal amounts of RNA.

2). In contrast, TGFβs 1 and 2 increased transcription of RNAs for the laminin β1 chain and β-actin. This increase was apparent after normalization of signal intensity to that of GAPDH (i.e., slightly more counts of nascent RNAs were present in the control lane). TGFβs 1 and 2 did not affect c-*myc* transcription in this immortalized cell line.

To determine whether the inhibition of virus gene expression by TGFβ 1 might be further regulated by posttranscriptional mechanisms, the stability of HPV16 early-gene transcripts was measured. Cultures were treated with dactinomycin (10 μg/ml) to inhibit RNA polymerase II activity, and the rates of decay of existing transcripts were compared in control cultures and those treated for 2 h with TGFβ 1. When the hybridization intensity of the 1.7-kb RNA was plotted against time, its half-life was interpolated as 14.5 h. Treatment with TGFβ 1 did not alter its stability (Fig. 5). In contrast, the half-life of the 4.2- and 3.4-kb RNAs was much shorter. When similar experiments were conducted over shorter intervals, the 4.2-kb RNA decreased within 1 to 2 h after treatment with dactinomycin, and TGFβ 1 did not alter this decay (data not shown).

Influence of TGFβ 1 on HPV16 gene expression in immortalized and tumorigenic cell lines. The biological significance of the ability of TGFβ 1 to downregulate papillomavirus early gene expression was examined with a series of cell lines derived from human genital epithelium. The primary objective was to determine whether inhibition of virus gene expression by TGFβ 1 occurred in a reproducible manner in different HPV16-immortalized cell lines. In addition, TGFβ 1 responsiveness was assessed in cell lines maintained for an extended interval in culture, in cells malignantly transformed by subsequent transfection with the v-Ha-*ras* oncogene or the HSV-2 *Bgl*II N fragment, and in carcinoma-derived cell lines.

TABLE 2. Changes in transcription induced by TGFβs 1 and 2[a]

Gene	Transcriptional activity (% of control)	
	TGFβ 1	TGFβ 2
HPV16	12	17
β-Actin	114	134
c-*myc*	91	98
GAPDH	100	100
Laminin β1 chain	353	237
Involucrin	46	70

[a] CX16-2 cells were treated for 24 h with TGFβ 1 or 2 at 3.0 ng/ml. Autoradiographs from transcriptional runoff analyses were analyzed by scanning densitometry, and values were normalized to levels of GAPDH transcription. Values are expressed as a percentage of transcriptional activity untreated cultures.

Treatment with TGFβ 1 for 24 h dramatically reduced steady-state levels of HPV16 RNA in early-passage cultures of all three immortal lines (CX16-2, CX16-7, and CX16-10) derived from cervical epithelium and one (HK16-1) from neonatal foreskin (Table 1; Fig. 6A). When the same cell lines were examined after extended maintenance in culture (>250 population doublings), down regulation of HPV gene expression by TGFβ 1 was often less pronounced but still detectable. One line (CX16-2) developed increased expression of HPV16 E6/E7 RNAs at late passage, whereas expression in another was decreased (data not shown). These cell lines originally were polyclonal but became monoclonal with increasing passage in culture, as reflected by their chromosomal constitution (38). Therefore, changes in steady-state levels of HPV16 RNA that occur in cells of late passage might have resulted from selection of a subpopulation which differs in the level of virus gene expression.

HPV16 E6/E7 RNA was decreased only minimally (56 to 76% of controls) by TGFβ 1 in the two cervical carcinoma cell lines QGU and SiHa (Fig. 6B); however, virus gene expression was down regulated significantly (80%) in another tumor line, QGH. Furthermore, virus RNA expression decreased only slightly after TGFβ 1 treatment of immortal cell lines that had been malignantly transformed with the HSV-2 *Bgl*II N fragment or the V-Ha-*ras* oncogene. Immortalized nontumorigenic and malignantly transformed cell lines varied widely in their ability to express RNA for involucrin, a structural protein that represents a marker of squamous differentiation in anogenital epithelium (52). TGFβ 1 treatment slightly enhanced expression of involucrin

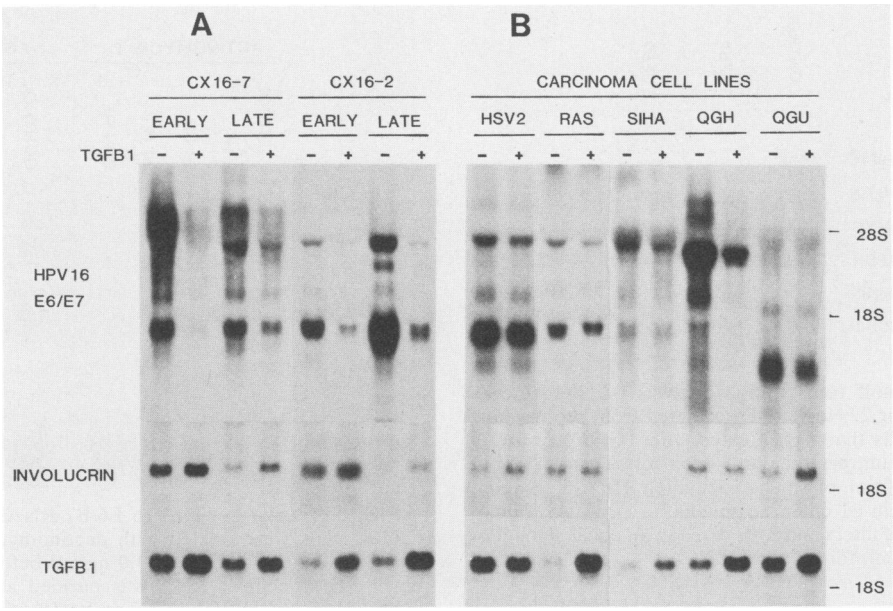

FIG. 6. Effect of cell passage or malignant transformation on TGFβ 1-mediated changes in gene expression. (A) Two HPV16-immortalized cell lines derived from cervical epithelium (CX16-7 and CX16-2) were tested at early (<60 population doublings) and late (>250 doublings) passage. Cells were either exposed to TGFβ (3.0 ng/ml) for 24 h (+) or left untreated (—). (B) In a replicate experiment, two immortal cell lines malignantly transformed by addition of a v-Ha-*ras* oncogene (RAS) or the HSV-2 *Bgl*II N fragment (HSV2) and three cervical carcinoma cell lines that express HPV16 RNA (SiHa, QGH, and QGU) were also tested. Filters were hybridized to probes for HPV16, involucrin, or TGFβ 1.

RNA in several lines. A direct correlation was observed between the basal level of involucrin expression in a particular cell line and the sensitivity of the line to inhibition of HPV16 RNA expression. Cell lines expressing higher levels of involucrin were more sensitive to TGFβ 1. TGFβ 1 also induced its own expression in most of the cell lines examined, but the level of TGFβ 1 autoinduction did not correlate with sensitivity to inhibition of HPV early gene expression. Furthermore, some cell lines (SiHa and RAS) were susceptible to autoinduction of TGFβ 1 RNA but were refractory to the effects of TGFβ 1 on growth and HPV16 gene expression. Therefore, the resistance of these lines to TGFβ 1 was not due solely to an absence of TGFβ 1 receptors at the cell surface.

DISCUSSION

The E6 and E7 proteins of the oncogenic HPVs are implicated in maintenance of the malignant phenotype in cervical cancer and cancer-derived cell lines (40, 43, 46, 51, 57). Transcription of the HPV16 E6 and E7 genes was dramatically inhibited by picomolar concentrations of TGFβs 1 and 2 in immortalized cell lines derived from human cervical and foreskin epithelium. Down regulation of virus gene expression was accompanied by inhibition of cell proliferation. In addition, TGFβ 1 induced the expression of its own RNA, thereby providing the potential to amplify and sustain inhibitory effects on HPV gene expression. Thus, these results suggest an autocrine function for TGFβ 1 in down regulating HPV gene expression in infected anogenital epithelium.

A host intracellular control mechanism that down regulates HPV gene expression in infected anogenital epithelial cells has been described previously (56, 57). Our observation that TGFβs 1 and 2 decrease HPV16 transcription in immortalized, nontumorigenic cervical cells has similarities to the report of Rosl et al. (40), who examined HPV gene expression in hybrids of normal keratinocytes and HeLa cervical carcinoma cells expressing HPV18. 5-Azacytidine, a demethylating agent, inhibited HPV18 gene transcription in nontumorigenic hybrid cells but not in the tumorigenic segregants that subsequently arose. Furthermore, inhibition of HPV gene expression was reversible and dependent on ongoing protein synthesis, suggesting that 5-azacytidine induced a cellular factor that controls virus gene expression. In contrast, 5-azacytidine did not inhibit HPV gene expression in cervical carcinoma cell lines or tumorigenic hybrids, implying that loss of responsiveness represents a step in malignant progression (40). The similarities in the mode of action of 5-azacytidine and TGFβ suggest that both may inhibit HPV gene expression by a similar mechanism, such as interaction with a transcription factor. Furthermore, autocrine regulation of HPV16 gene expression by TGFβ 1 could represent one component of an intracellular surveillance system directed against HPV transcription. In contrast to 5-azacytidine, the effects of TGFβ 1 on HPV expression varied significantly in different cell lines, and often the resistance that developed in tumorigenic cells was only partial.

A positive correlation was apparent between the magnitude of reductions in both HPV gene expression and cell growth in most cell lines (Table 1), suggesting that the two processes were related. To examine whether the effects of TGFβ on virus gene expression occurred secondarily to growth inhibition, cells were incubated under conditions that prevented cell replication prior to assessment of HPV RNA levels. Cultures maintained in growth factor-deficient medium or at confluent density expressed HPV RNAs at levels similar to rapidly proliferating cultures. Thus, HPV gene

expression is not necessarily inhibited by growth arrest. Furthermore, the results suggest but do not prove an alternative, that cell growth might depend on continued papillomavirus gene expression. Down regulation of the papillomavirus E6 and E7 gene products inhibits the growth rate of C4-1 cervical carcinoma cells (51), and continuous expression of the E7 gene is required for maintenance of the transformed phenotype in baby rat kidney cells transformed by EJ *ras* and HPV16 E7 (13). The current results are consistent with these observations.

TGFβ regulates expression of a wide variety of cellular genes (39), and regulation may occur at multiple levels (2, 11, 25, 26, 33, 34, 41, 50). Our results are the first report that TGFβs 1 and 2 inhibit transcription of viral genes. Papillomavirus transcription is regulated by several distinct enhancers located with a 5′ long control region (9, 12, 17, 27) which respond to glucorticoids (17), keratinocyte-specific factors (12), and autoregulation by products of the HPV E2 gene (5, 27). Sequence analysis of the long control region shows that the keratinocyte-specific enhancer contains binding sites for nuclear factor 1 and AP-1 (9), and mutational studies have shown that these sites are required for full enhancement of virus gene expression (9). TGFβ 1 stimulates transcription of the collagen type 1 promoter via nuclear factor 1 binding (41), and autoinduction of TGFβ 1 RNA is mediated by the AP-1 complex (25). Therefore, these factors might also be involved in the TGFβ 1-mediated downregulation of papillomavirus transcription.

Cells infected with papillomaviruses in vivo harbor virus DNA in episomal form, and transcriptional regulation is complex (10, 27). Analysis of transcription of the cancer-associated types HPV16 and HPV18 is hindered by the lack of an animal or cell culture model for studying productive infection. Much of the work on transcription of oncogenic HPV types has been done with cervical cancer cell lines (43, 45, 46). In carcinoma cell lines, the viral DNA characteristically integrates in a specific pattern, interrupting the E1/E2 genes and preserving the E6/E7 genes in a transcriptionally active form (43, 57). All of the HPV-immortalized cell lines used in this study contain one or more complete copies of the viral genome (54, 55); however, these copies are integrated within the host chromosome and therefore partially rearranged. Thus, these cells might resemble carcinoma-derived cell lines more than productively infected cells in their pattern of virus transcriptional regulation.

Alterations in the ability of cells to synthesize or respond to TGFβ occur during the development of neoplasia (7, 20–22, 44, 49), implying that these changes represent an important step in malignant progression. TGFβ 1 completely inhibited clonal growth of normal genital epithelial cells, but the resistance of HPV16-immortalized cell lines varied significantly. Some immortal cell lines were partially resistant to TGFβ 1, particularly when the cells were continually maintained in culture. In addition, the majority of the tumorigenic cell lines examined (four of five) also became resistant. Thus, loss of responsiveness to TGFβ 1 often precedes or accompanies malignant development in cultured genital epithelial cells. Different tumorigenic cell lines also varied significantly in their resistance to TGFβ, and one carcinoma-derived line, QGH, was partially sensitive. Therefore, while acquisition of resistance to TGFβ 1 might contribute to the carcinogenesis process, this study as well as others (20, 48, 49, 53) indicates that resistance to TGFβ is not a prerequisite.

The variability in responsiveness of different cell lines to TGFβ 1 was directly related to their ability to express involucrin RNA. Involucrin is a marker of squamous differentiation in normal cervical epithelium and is either absent or weakly expressed in poorly differentiated, dysplastic epithelium (52). Cells expressing high levels of involucrin were more susceptible to the effects of TGFβ 1 on growth and HPV16 gene expression than were cells that failed to express involucrin or expressed low levels of it. Thus, the degree of differentiation of genital epithelial cells influences their susceptibility to TGFβ 1. The current results are consistent with the report of Hooseien et al. (20), who demonstrated that resistance of colon carcinoma cells to growth inhibition by TGFβ 1 was related to the degree of cell differentiation. The mechanisms by which poorly differentiated cells become resistant is not known. However, TGFβ 1 maintained its ability to upregulate its own RNA in two cell lines (RAS and SiHa) that remained refractory to the effects of TGFβ 1 on growth and HPV RNA expression. Therefore, the primary defect in these resistant cells does not appear to be an absence of TGFβ 1 receptors, indicating that other changes in the signal transduction pathway must be involved.

ACKNOWLEDGMENTS

We thank Anita Roberts for helpful suggestions and critical review of the manuscript, John Schiller for providing antiserum to HPV16 E6, Frederic Mushinski for the 18S rRNA plasmid, and Yoshihiko Yamada for the laminin β1 chain plasmid.

ADDENDUM

During review of the manuscript, Pietenpol et al. (36) reported that human genital epithelial cells become resistant to growth inhibition by TGFβ 1 after transformation with papillomavirus or simian virus 40 DNA.

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