

Feedback Regulation between Orphan Nuclear Receptor TR2 and Human Papilloma Virus Type 16

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The human TR2 orphan receptor (TR2), initially isolated from testis and prostate cDNA libraries, is a member of the steroid receptor superfamily. TR2 can regulate several target genes via binding to a consensus response element (AGGTCA) in direct repeat orientation (AGGTCA_nAGGTCA, n = 0–6). Here we show that TR2 is able to induce the expression of human papilloma virus type 16 (HPV-16) genes via binding to a DR4 response element in the long control region of HPV-16. Additionally, one of the HPV-16 gene products, the E6 oncogene, regulates TR2 gene expression. A likely mechanism for this regulation involves E6-mediated degradation of the tumor suppressor p53, a protein known to suppress TR2 expression. Together our data provide evidence for feedback regulation between TR2 and HPV-16, which represents a novel regulatory pathway involving a member of the steroid receptor superfamily and the HPV-16 DNA tumor virus.

Orphan receptors, or receptors without known ligands, make up the vast majority of members of the steroid receptor superfamily (1–3). Members of this family are characterized by a highly conserved DNA-binding domain (DBD)¹ and a carboxyl-terminal ligand-binding domain. The conserved amino acid sequence within the DBDs of these receptors is predicted to form two zinc finger motifs (1). Additionally, steroid receptors bind to specific DNA sequences known as hormone response elements (HREs) through which regulation of target gene expression may occur (1). The cDNA of the human TR2 orphan receptor (TR2) was isolated from the screening of human testis and prostate cDNA libraries for the androgen receptor. In the screening, an oligonucleotide probe homologous to the highly conserved DBD of the glucocorticoid receptor was used (2). Four TR2 cDNA clones (TR2-5, -7, -9, and -11) were isolated and found to share identical sequences in the N-terminal and DBD regions yet were found to differ in the length of the C-terminal putative ligand-binding domain (3).

Through further study of TR2, it was found that this orphan

receptor binds to a consensus HRE composed of a direct repeat (DR) with variable spacing (AGGTCA_nAGGTCA, n = 0–6) (4). Using the known TR2 HRE sequence as a guide, it was determined that TR2 has modulatory effects on several signaling pathways such as those involving retinoic acid (4), thyroid hormone (5), and ciliary neurotrophic factor (6). Moreover, expression of the erythropoietin gene (7), aldolase gene (8), histamine H1 receptor (9), and simian virus 40 (SV40) (10) is also regulated by TR2. To add to the list of TR2-responsive genes, we have found a TR2 DR4 consensus site within the long control region (LCR) of the human papilloma virus type 16 (HPV-16). In this report, we show that TR2 is able to bind the consensus response element (DR4RE) within the HPV-16 gene and modulate HPV-16 expression.

The human papilloma virus is a common sexually transmitted pathogen that is linked to increased risk for the development of cervical neoplasia, one of the most common cancers in women throughout the world. HPV is thought to contribute to the development of 10–15% of all human cancers (11). Papilloma viruses are epitheliotropic organisms and therefore result in chronic infection of the skin and mucous membranes. The most common site of infection of genital HPVs in women is the stratified squamous epithelium at the squamocolumnar junction of the cervix. Over 70 HPV types have been identified, and each is classified as either low risk (*e.g.* HPV-6 and HPV-11) based on association with benign lesions or high risk (*e.g.* HPV-16 and HPV-18) based on association with cervical carcinoma. Over 90% of cervical cancers show high risk HPV infection, and over 20% of genital HPV types are associated with cervical cancer; high risk HPV-16 is the predominant tumor-associated type (12).

A common element in pathways involving either TR2 or HPV is the p53 tumor suppressor. The p53 protein is a transcription factor that binds to a specific regulatory sequence and modulates the expression of various target genes such as p21^{waf1/cip1}, *bax*, *mdm2*, and cyclin G (for review, see Ref. 11). Through regulation of its target genes, p53 is able to suppress cell cycle progression in response to DNA damage. As a tumor suppressor, p53 is able to block DNA replication after damage has occurred allowing time for damage repair or the induction of the onset of apoptosis (13, 14). This process prevents mutations, induced via DNA-damaging agents, from being replicated and passed on, thus reducing the potential for cellular transformation. p53 expression has been shown to increase after insults such as γ -irradiation or actinomycin D treatments, which damage DNA directly. Along with p53 induction, cells demonstrate corresponding cell cycle arrest in phase G₁ (15). Relating this to TR2, it has been shown that ionizing radiation represses the expression of the orphan receptor at both transcriptional and translational levels and that this repression is mediated by p53 (16). The TR2 protein expression kinetics in cells after irradiation was opposite that of p53 expression; TR2 levels decreased dra-

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¹ The abbreviations used are: DBD, DNA-binding domain; TR2, human testicular orphan receptor-2; TR4, human testicular orphan receptor-4; HRE, hormone receptor response element; DR, direct repeat; HPV, human papilloma virus; HPV-16, human papilloma virus type 16; LCR, long control region; CAT, chloramphenicol acetyltransferase; TK, thymidine kinase; bp, base pair(s); ORF, open reading frame; Rb, retinoblastoma; AR, androgen receptor; RE, response element.

matically 1–2 h after treatment and were restored to pre-treatment levels over time, whereas p53 levels increased soon after treatment and decreased over time. The suggestion that p53 plays a role in the repression of TR2, which is induced by ionizing radiation, was further substantiated by the reduction in TR2 expression after p53 levels were increased in the absence of irradiation, as well as by the lack of TR2 repression when a mutant form of p53 was overexpressed (16). Having established a functional relationship between TR2 and p53, further study will be necessary to characterize the mechanism responsible for this phenomenon.

It is known that steroid hormone response elements exist within the HPV-16 viral regulatory region. Both progesterone and glucocorticoid have been shown to induce HPV-16 gene expression through receptor association with three glucocorticoid response elements within the HPV-16 LCR (17–20). We now report that TR2 is also able to modulate the expression of HPV-16 genes and that it does so through binding to a TR2 response element located in the HPV-16 LCR. We also demonstrate that E6 is able to reduce the repression of TR2 by p53, which is likely to occur through the binding of p53 by E6, an event that initiates p53 degradation. Cumulatively our data demonstrate a positive feedback regulatory pathway between TR2 and HPV-16, providing evidence for a novel relationship between a steroid receptor and the HPV-16 DNA tumor virus.

MATERIALS AND METHODS

Immunohistochemical Staining—Three 8–10-week-old female ICR mice (Taconic, Germantown, NY) were sacrificed, and the vagina, uterus, and cervix of each animal were removed. The tissue was fixed in Histochoice MB (Amresco, Solon, OH) at 4 °C overnight and embedded in paraffin. Sagittal sections through each of the three anatomic regions of interest were cut at a thickness of 7 μ m, dried at 37 °C overnight, and processed for modified hematoxylin/eosin (21) and immunohistochemical staining. For immunohistochemical staining, sections were stained with the mouse monoclonal anti-TR2 antibody G204 and a biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA). Staining was visualized upon development with the DAB peroxidase substrate kit (Vector Laboratories). Stained sections were analyzed via light microscopy (Nikon, Tokyo, Japan) and photographed.

Plasmids—The TR2pCAT reporter plasmid consists of the 2.7-kilobase 5'-flanking region of the TR2 orphan receptor (22) linked to the chloramphenicol acetyltransferase (CAT) gene. pC53-SN3 (a gift from B. Vogelstein, described in Ref. 23) was constructed by inserting wild-type p53 cDNA into the pCMV-Neo-Bam vector. p1321 and p1434 (gifts from P. M. Howley, described in Ref. 24) contain the E6 and E7 genes of HPV-16 driven by the human β -actin promoter. p1321 expresses both E6 and E7 genes, whereas p1434 contains a translation termination linker within E6. The plasmid pSG5-hTR2-11 (25) expresses full-length TR2, and the plasmid pCMX-TR4 (a gift from R. M. Evans, described in Ref. 26) expresses full-length TR4. For coupled *in vitro* transcription and translation, pSG5-hTR2-11 (25) was used. The HPV-16 LCR-CAT reporter plasmid was constructed using *EcoRV-HindIII*-digested pB-SCAT2 as a vector into which a 600-bp *SmaI-HindIII* fragment released from pGL2-LCR(wt) (gift from G. J. Sibbet, described in Ref. 27) was inserted. Dideoxy chain termination DNA sequencing (28) of HPV-16 LCR-CAT was conducted to confirm the integrity of the plasmid sequence. pSG5-TR2-AR-TR2 (2A2) was constructed by replacing the DNA-binding domain of TR2 with that of the androgen receptor (AR) in the context of the pSG5 expression vector using a method similar to that used to create the chimeric pSG5-AR-TR2-AR plasmid (25). Site-directed mutagenesis (25) was used to produce HPV-16 LCR-D27CAT, which lacks a 27-bp region of the HPV-16 LCR including the HPV-16 DR4RE. The AR2.3CAT plasmid was produced through insertion of 2.3 kilobases of the androgen receptor upstream promoter region into an expression plasmid containing a CAT reporter gene (29). The plasmids HPV-DR4RE(+)-TK-CAT and HPV-DR4RE(-)-TK-CAT were constructed by insertion of the 16-bp HPV-16 DR4RE oligonucleotide into the -32TK-CAT vector (a gift from R. C. Ralff) in either sense (+) or antisense (-) orientation. The pCMV β gal expression vector was co-transfected in all CAT assay experiments to enable normalization of transfection efficiency (25).

Cell Culture, Transfection, and CAT Assay—H1299 cells were cultured as described previously (16). Chinese hamster ovary cells were

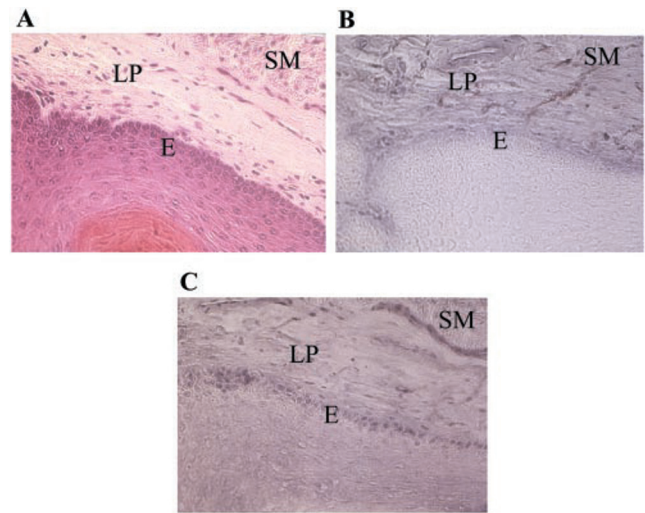


FIG. 1. TR2 protein is expressed at a potential site of HPV-16 infection. Sagittal sections of the vagina/cervix from adult female ICR mice (Taconic) were stained with hematoxylin/eosin (A) using phosphate-buffered saline in place of primary antibody (B) or with an anti-TR2 monoclonal antibody (C). Prominent TR2 staining is observed in the nuclei of cells in the basal layers of the stratified squamous epithelium (E) of the mouse vagina. LP, lamina propria; SM, smooth muscle.

cultured in Dulbecco's modified Eagle's F-12 medium supplemented with 5% fetal bovine serum, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin (Sigma). Twenty-four hours before transfection, cells were plated at a density of 10^6 cells/60-mm tissue culture plate. Cells were transfected with a total of 10.5 μ g of plasmid DNA/plate via the calcium phosphate method (30). Specific plasmids and doses used are described in the figures and associated figure legends. Twenty-four hours after transfection, the medium was changed. After another 24 h, cells were subjected to freeze-thaw lysis in 250 mM Tris-HCl (pH 7.8), and the resulting cell extracts were assayed for CAT activity. CAT activity was assessed via PhosphorImager scanning and quantitated using ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

In Vitro Transcription/Translation—The expression vector pSG5-hTR2-11 was used to produce *in vitro* transcribed and translated TR2 protein in the transcription/translation-coupled rabbit reticulocyte lysate system (Promega, Madison, WI).

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay analysis was performed as described previously (7). Briefly the HPV-16 DR4RE oligonucleotide was end-labeled with 32 P to serve as a probe to demonstrate the binding of *in vitro* expressed TR2 to the DR4 sequence within the LCR of HPV-16. For competition reactions, unlabeled HPV-16 DR4RE oligonucleotides were mixed with labeled probe prior to its addition to the reaction. HeLa cell nuclear extract was prepared as described previously (31). Antibody shift analysis involved the addition of 1 μ l of the monoclonal anti-TR2 orphan receptor antibody G204 to the reaction for 15 min at room temperature prior to gel loading.

RESULTS

TR2 Protein Is Expressed in Cell Types Commonly Infected by HPV-16—To establish physiological relevance for studying the interaction of regulatory pathways involving TR2 and HPV-16, staining of mouse vaginal and cervical tissue with a monoclonal antibody specific to TR2 (G204) was performed. Because HPV-16 is epitheliotropic and the genital form commonly infects the stratified squamous epithelial layers of the uterine cervix and vagina (12), we sought to determine whether TR2 was expressed in the same cell types within these regions. The anti-TR2 monoclonal antibody G204 was used in immunohistochemical staining of longitudinal sections of paraffin-embedded mouse cervix and vagina samples. After staining, it was clear that TR2 was present in the nuclei of stratified squamous epithelial cells within the regions known to be susceptible to HPV-16 infection (Fig. 1). These results supported our interest

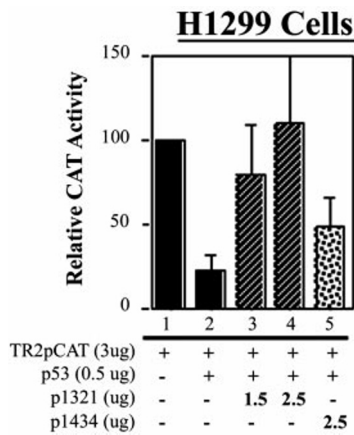


FIG. 2. The HPV-16 oncoprotein E6 antagonizes p53-mediated repression of TR2. Transient transfections were performed in the H1299 cell line using the TR2 promoter linked to the CAT gene (TR2pCAT) as a reporter. TR2pCAT was co-transfected with constructs expressing either wild-type p53, full-length E6 and E7 (p1321), or E6 and E7 with an early termination codon in the E6 open reading frame (p1434). In the histogram, CAT activity produced under each condition is displayed relative to that produced with the reporter alone (lanes 2–5 versus lane 1). Expression of p53 in the p53-null H1299 cell line resulted in suppression of TR2 promoter-linked reporter expression (lane 2). The addition of a construct expressing E6 and E7 eliminated the repression of TR2 mediated by p53 (lanes 3–4), whereas a truncated version of E6 was unable to reverse the suppressive effect of p53 (lane 5).

in further characterizing the potential regulatory relationship between TR2 and HPV-16.

HPV-16 Oncogene E6 Antagonizes p53-mediated Repression of TR2 Gene Expression—In an earlier study (16), we demonstrated that TR2 levels in MCF-7 cells were repressed by ionizing radiation and that the kinetics of the down-regulation of TR2 correlated with a concurrent increase in p53 expression. Additionally it was shown that upon co-transfection of the SV40 large T antigen, a viral oncoprotein known to associate with and inactivate p53 (32–35), TR2 reporter expression was not repressed after exposure of cells to ionizing radiation. Furthermore, using expression plasmids expressing either wild-type or mutant forms of p53, it was found that wild-type p53 was able to mediate suppression of TR2 expression. The mutant form of p53 was unable to affect expression of TR2. Having demonstrated repression of TR2 mediated by the p53 tumor suppressor (16) and recognizing that HPV-16 E6 binds and promotes degradation of p53 (36, 37), we sought to determine whether the suppressive effect of p53 on TR2 expression could be attenuated by E6. Using the lung-derived p53-null H1299 cell line, the effect of increasing levels of a plasmid expressing both the HPV-16 E6 and E7 proteins on TR2 promoter activity was tested (Fig. 2). To demonstrate the suppressive effect of p53 on TR2 reporter gene (TR2pCAT) (22) expression, TR2pCAT and an expression plasmid containing p53 (23) were co-transfected. This resulted in a 4-fold suppression of TR2-reporter gene expression (Fig. 2, lane 2) compared with reporter expression after transfection of the reporter alone (Fig. 2, lane 1). The suppressive effect was attenuated in a dose-dependent manner upon the addition of the p1321 expression plasmid (24), which expresses both the HPV-16 E6 and E7 proteins (Fig. 2, lanes 3 and 4). A plasmid containing a translation termination linker early in the E6 open reading frame (ORF) (p1434) (24) that expresses a truncated version of E6 along with full-length E7 was unable to efficiently attenuate the suppressive effect of p53 (Fig. 2, lane 5 versus lanes 3 and 4). These data suggest that the HPV-16 E6 oncogene, through its role in targeting p53 for ubiquitin-mediated degradation, is able to significantly reduce p53 suppression of TR2. Because

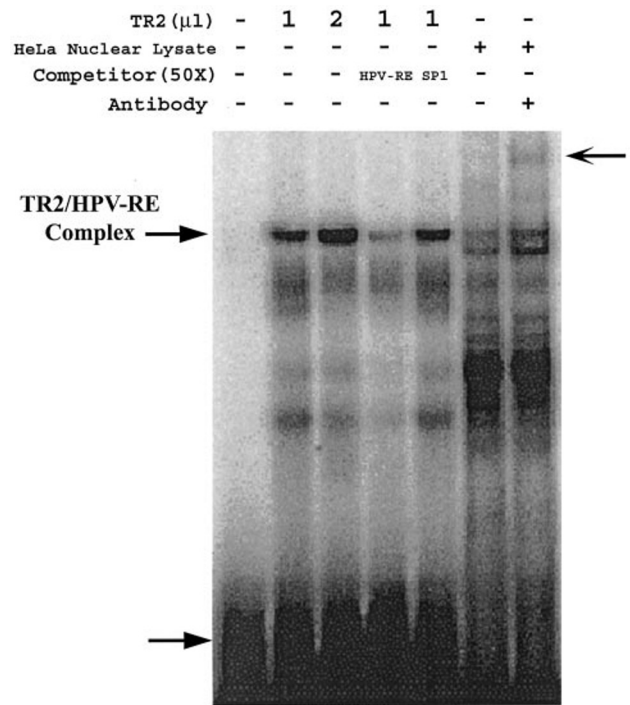


FIG. 3. Functional role of TR2 relative to HPV-16 LCR-directed gene expression. Gel shift analysis was performed using *in vitro* expressed TR2 protein and 32 P-labeled HPV-16 DR4RE. The binding reaction mixture containing the isotope-labeled probe was incubated with increasing amounts of TR2 alone (lanes 2 and 3) or in the presence of either excess unlabeled oligonucleotide (50X) (lane 4) or a nonspecific DNA competitor (SP1, lane 5). Lane 1 contains the 32 P-labeled probe alone. In lanes 6 and 7, endogenous TR2 from HeLa cell nuclear extract is shown to bind 32 P-labeled HPV-16 DR4RE, and in lane 7, addition of a monoclonal anti-TR2 antibody further demonstrates the specificity of TR2 in the formation of the observed complex. The slower migrating 32 P-labeled HPV-16 DR4RE-TR2-anti-TR2 antibody complex is indicated by the unlabeled arrow.

amino acids 106–115 of the C terminus of HPV-16 E6 are necessary for efficient association with p53 (38), it is unlikely that the 52-amino acid, truncated form of E6 expressed from plasmid p1434 (24) is able to bind p53. It has also been shown that the ability of E6 to bind p53 is necessary for p53 degradation (38), which may explain the loss of the attenuating effect of truncated E6 on p53-mediated suppression of TR2 promoter expression.

Identification of a Direct Repeat 4 Response Element in the HPV-16 LCR—Sequence analysis of the HPV-16 LCR resulted in the identification of a potential TR2 response element located 175 bp upstream of the TATA-box at base pair 65 of the E6 ORF. The site consists of two consensus A(G/T)(G/T)TCA half-sites with a spacing of four nucleotides (ATGTCACcctAGT-TCA). To determine whether TR2 was able to bind the HPV-16 DR4RE, gel shift analysis was performed. Fig. 3 demonstrates that *in vitro* transcribed and translated TR2 protein is able to form a complex with 32 P-labeled HPV-16 DR4RE oligonucleotides (Fig. 3, lanes 2 and 3) and that a 50-fold excess of unlabeled HPV-16 DR4RE is able to significantly reduce formation of the labeled complex (Fig. 3, lane 4). Furthermore, a nonspecific DNA competitor, SP1 (Fig. 3, lane 5), does not block complex formation, suggesting that the binding of TR2 to the sequence that makes up the DR4RE of HPV-16 is highly specific and can be competed away only by an unlabeled competitor of the same sequence (HPV-16 DR4RE).

To further confirm the binding specificity of TR2 for the HPV-16 DR4RE, a TR2-specific monoclonal antibody was added to a reaction containing HeLa cell nuclear extract (con-

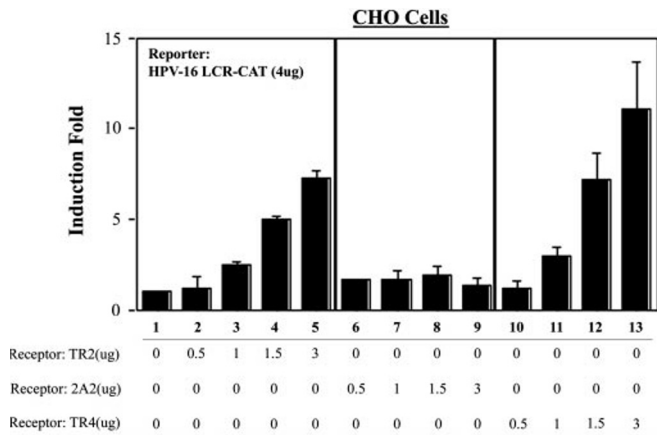


FIG. 4. TR2-mediated induction of HPV-16 gene expression. Transient transfection assays were performed in Chinese hamster ovary cells using an HPV-16 LCR-derived CAT reporter. Levels of CAT activity upon co-transfection of increasing doses of either pSG5-hTR2-11 (TR2) (lanes 2–5), pSG5-TR2-AR-TR2 (2A2) (lanes 6–9), or pCMX-TR4 (TR4) (lanes 10–13) are displayed relative to the CAT activity of the reporter alone (lane 1). The chimeric TR2 receptor, lacking the TR2-specific P-box, which is important for DNA binding, is unable to induce HPV-16 reporter gene expression.

taining endogenous TR2 protein) along with the 32 P-labeled HPV-16 DR4RE. In the absence of the antibody to TR2 (Fig. 3, lane 6), the TR2-HPV-RE complex is shown to form. Upon addition of the antibody to TR2 (Fig. 3, lane 7), slower migration and therefore an upward shift of the labeled complex is observed. These data provide evidence for the existence of a TR2 response element within the HPV-16 LCR.

Induction of HPV-16 LCR-directed Gene Expression by TR2—Having determined that E6 is able to affect TR2 expression (Fig. 2) presumably through induction of p53 degradation and after discovering a TR2 response element within the HPV-16 LCR (Fig. 3), we were interested in testing whether TR2 could regulate the expression of the E6 oncogene. An expression vector containing the region that controls E6 gene expression (pGL2-LCR containing the HPV-16 LCR) (27) was linked to a CAT reporter (HPV-16 LCR-CAT) and then co-transfected with a vector constructed to express TR2 (pSG5-hTR2-11) (25) in the Chinese hamster ovary cell line. As shown in Fig. 4, the HPV-16 LCR-driven CAT expression was significantly induced by TR2 in a dose-dependent manner (Fig. 4, lanes 1–5). A similar induction of HPV-16 LCR-CAT activity also occurred when we replaced the TR2-expressing plasmid with that which expresses the highly homologous TR4 orphan receptor (Fig. 4, lanes 10–13) (39). Both TR2 and TR4 recognize the same consensus HRE sequence and are able to modulate some of the same target genes (6, 7, 26, 40). Therefore, it is not surprising that both TR2 and TR4 are able to induce HPV-16 LCR-CAT activity. In contrast, when we replaced pSG5-hTR2-11 with pSG5-TR2-AR-TR2 (2A2) (25), which expresses a chimeric receptor in which the DBD of the androgen receptor is substituted for the TR2-DBD, the induction of HPV-16 LCR-CAT was lost (Fig. 4, lanes 6–9). This loss of reporter induction suggests that the DBD of TR2 is essential for recognition of the HRE located within the HPV-16 LCR.

Functional Analysis of the HPV-16 DR4RE—To determine whether the identified HPV-16 DR4RE is necessary for the regulatory effect of TR2 on E6 expression, we used a site-directed polymerase chain reaction mutagenesis strategy (25) to create a mutant of HPV-16 LCR-CAT. The mutant has an internal deletion of 27 bp including the HPV-16 DR4RE, the sequence recognized by TR2 (Fig. 5, A and B). The mutant (HPV-16 LCR-D27CAT) was co-transfected with expression

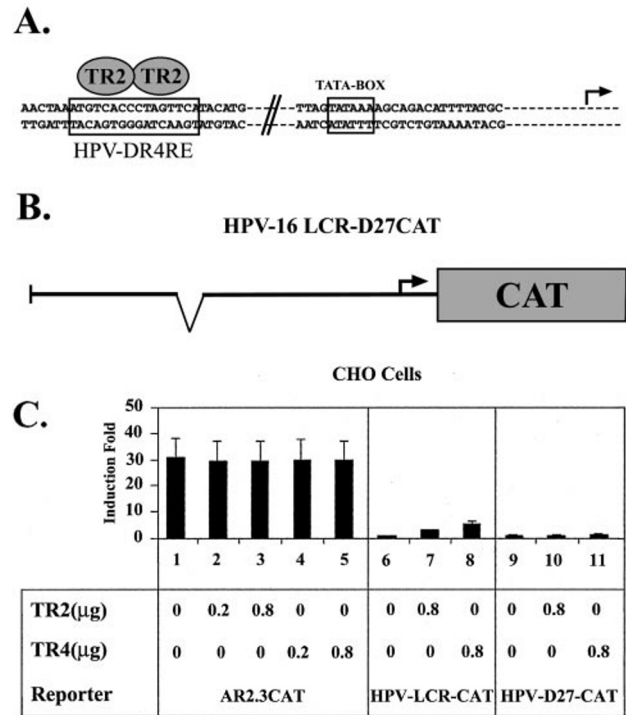


FIG. 5. Functional analysis of HPV-16 DR4RE. A, a portion of the HPV-16 LCR sequence containing the TR2-responsive HPV DR4RE is shown. The DR4RE contains two A(G/T)(G/T)TCA half-sites located 175 base pairs upstream of the TATA-box at the start of the E6 ORF. B, the HPV-16 LCR-D27CAT mutant plasmid was constructed via site-directed mutagenesis polymerase chain reaction. A 27-bp region containing the HPV-16 DR4RE was deleted from the HPV-16 LCR-CAT construct. C, the HPV-LCR-CAT reporter or the HPV-16 LCR-D27CAT mutant reporter was co-transfected with plasmids expressing either orphan receptor TR2 (pSG5-hTR2-11) or the highly homologous orphan receptor TR4 (pCMXTR4). Both TR2 and TR4 up-regulate expression of the wild-type reporter (lanes 6–8), whereas neither TR2 nor TR4 was able to induce expression of the mutant reporter (lanes 9–11) because the receptor binding site (DR4RE) is no longer present in the mutant construct. Additionally a non-TR2- or -TR4-regulated reporter AR2.3CAT was cotransfected with either TR2 or TR4. The lack of regulation of the AR2.3CAT reporter by TR2 or TR4 demonstrates the specificity of these orphan receptors for particular binding sequences and proves that they are not functioning as general regulators of transcription. CAT activities were represented as induction fold in comparison to the activity of the HPV-LCR-CAT reporter alone (lanes 6 and 9, set at 1-fold).

vectors that contain either TR2 or TR4 (25, 26) into Chinese hamster ovary cells. In contrast to HPV-16 LCR-CAT, which can be significantly induced by TR2 or TR4 (Figs. 4 and 5C, lanes 6–8), the CAT activity of HPV-16 LCR-D27CAT showed no significant increase with transfection of TR2 or TR4 (Fig. 5C, lanes 9–11) expression plasmids. Furthermore, neither TR2 nor TR4 were successful in modulating expression of a promoter/reporter gene that does not contain a response element recognized by these receptors (AR2.3CAT) (Fig. 5C, lanes 1–5). These data demonstrate that the HPV DR4RE, located 175 bp upstream of the TATA-box at bp 65 in the E6 ORF, is necessary for TR2-mediated induction of HPV-16 gene expression and that TR2 and TR4 are not general regulators of transcription but bind to specific sequences within particular target genes.

Identification of the HPV-16 DR4RE as an Enhancer for Induction of the Thymidine Kinase (TK) Promoter—To determine whether the TR2 response element sequence present in the LCR of HPV-16 is sufficient to allow TR2-mediated regulation of HPV-16 early gene expression, the response element sequence (HPV-16 DR4RE) was tested outside of the context of the HPV-16 LCR. The same unlabeled oligonucleotide (HPV-16

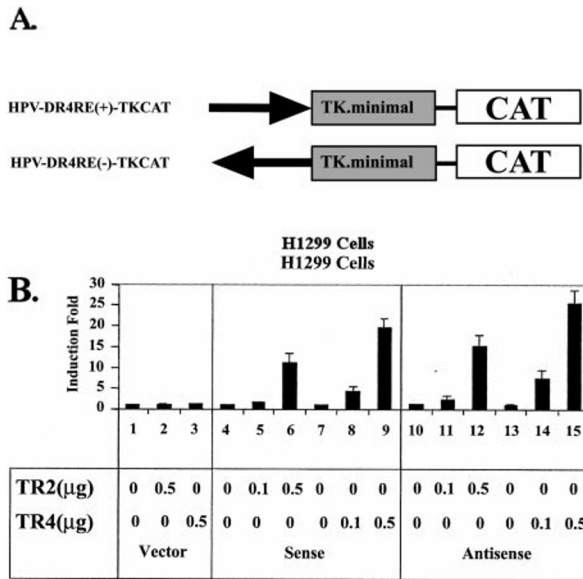


FIG. 6. HPV DR4RE up-regulates transcriptional activity of the TK minimal promoter in H1299 cells. A, the 16-bp HPV-16 DR4RE oligonucleotide (*bold arrow*) was inserted into the -32 TK-CAT vector in either sense (+) or antisense (-) orientation. B, the reporter gene expression of both constructs is significantly up-regulated upon addition of increasing amounts of either TR2 (pSG5-hTR2-11, lanes 4–6 and 10–12) or TR4 (pCMX-TR4, lanes 7–9 and 13–15). CAT activities were represented as induction fold in comparison to the activity of the -32 TK-CAT reporter alone (lane 1, set at 1-fold).

DR4RE) that was used in the gel shift assay (Fig. 3) was inserted into the -32 TK-CAT vector in either sense or antisense orientation (Fig. 6A). H1299 cells were then transfected with either parental vector, sense HPV-DR4RE(+)-TK-CAT, or antisense HPV-DR4RE(-)-TK-CAT along with increasing amounts of TR2 or TR4. Our data show no effect by vector (Fig. 6B, lanes 1–3) and induction of reporter activity by both TR2 (Fig. 6B, lanes 4–6 and 10–12) and TR4 (Fig. 6B, lanes 7–9 and 13–15) in a dose-dependent manner regardless of orientation. These data suggest that the HPV-16 DR4RE alone is sufficient to allow TR2- or TR4-mediated up-regulation of viral gene promoter expression as demonstrated by the induction of transcriptional activity of the TK minimal promoter by these receptors.

DISCUSSION

There are two molecular pathways that make up the feedback loop involving TR2, HPV-16, and p53. One of these pathways, which links HPV-16 and the p53 tumor suppressor, has been well characterized (36, 37, 41). The HPV-16 genome consists of an ~ 850 -bp regulatory LCR as well as early (E6, E7, E1, E2, E4, and E5) and late (L2 and L1) ORFs (42). Upstream of the HPV-16 E6-E7 gene region there exist E2-responsive core sequences as well as a short enhancer element that responds to E2-independent cellular factors in keratinocytes (43). The E2 ORF encodes two trans-acting factors that have the potential to bind upstream and modulate expression of the E6 and E7 genes. The long E2 gene product binds E2-responsive core sequences upstream of the E6-E7 promoter and has a transactivating effect. In contrast, there is a C-terminal E2 gene product that inhibits E2-independent transactivation as well as the keratinocytic cellular factor-dependent response (43). Viral integration in the infected host often occurs in the region of the E1-E2 ORFs, disrupting transcriptional regulation by E2. (24). Disruption of both the activation and suppression functions of the E2 gene products allows up-regulation of upstream enhancer-mediated, cellular factor-dependent transactivation of the

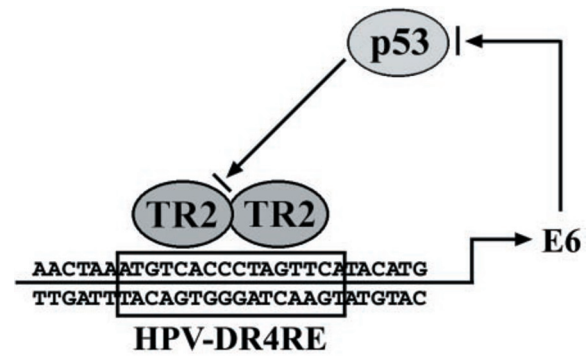


FIG. 7. Feedback regulation between TR2 and HPV-16. This figure is a schematic representation of the regulatory pathway involving both the TR2 orphan receptor and HPV-16 as suggested by our study. We hypothesize that through the binding of TR2 molecules at the DR4 response element sequence in the upstream long control region of HPV-16 TR2 is able to increase expression of the HPV-16 early gene product E6. From here, E6 is involved in promoting the ubiquitin-mediated degradation of tumor suppressor p53. Because p53 exhibits a repressive effect on TR2 expression, enhanced E6 expression and the resulting enhancement of p53 degradation may allow an increase in TR2 protein level.

E6 and E7 oncogenes (43). The HPV-16 protein E6 is capable of binding to the p53 tumor suppressor protein (36) and has been shown to stimulate ubiquitin-dependent degradation of p53 (37). Furthermore, on a functional level, HPV-16 E6 expression has been shown to disrupt the p53-mediated pathway, which is induced in response to DNA damage. This suggests that the E6 oncogene may, through its association with p53, disrupt a tumor-suppressive response and therefore contribute to the accumulation of genetic changes often associated with tumorigenesis (41).

The second molecular pathway that exists in the feedback loop involving TR2, HPV-16, and p53 is that linking TR2 with HPV-16. In a study examining the effect of both p53 and the retinoblastoma (Rb) protein on TR2 in the context of cryptorchidism, it was found that each tumor suppressor protein (p53 or Rb) had a repressive effect on TR2 expression (44). The known binding of proteins E6 and E7 to p53 and Rb, respectively, as well as the subsequent ubiquitin-mediated degradation of the tumor suppressor proteins known to result from such binding (36, 45–47) further implicated p53 and Rb as mediators of TR2 repression (44). The model proposed in the cryptorchidism study suggests that the down-regulation of TR2 by p53 involves a pathway in which p53 up-regulates one of its targets, p21. Because Rb can be regulated via phosphorylation by cyclin-dependent kinase and loses its ability to block cell cycle progression when hyperphosphorylated, the cyclin-dependent kinase inhibitor p21 prevents the inactivation of Rb. Rb is then able to bind transcription factor E2F and reduce expression of TR2 (44). Although the possibility exists that alternative pathways may be present that result in p53-mediated regulation of TR2, the above described p53 \rightarrow p21 \rightarrow cyclin-dependent kinase \rightarrow Rb \rightarrow E2F \rightarrow TR2 pathway may also occur in the context of HPV-16 infection and proliferation. Given the binding and degradation of p53 and Rb that occurs in the presence of the HPV-16 E6 and E7 proteins, it follows that upon HPV-16 infection and early gene expression less p53 protein would be present to induce p21 and inhibit cyclin-dependent kinase activity. Therefore, less active Rb would be present to bind E2F. Furthermore, the binding of Rb by E7 would directly inhibit the sequestration of E2F mediated by Rb-E2F binding. In both cases TR2 suppression would be attenuated, and TR2 would be free to bind its response element in the LCR of HPV-16, thus up-regulating viral gene expression.

TR2, a transcription factor that induces HPV-16 gene expres-

sion, is repressed by p53 and Rb and causes the potential for cell cycle disruption and for propagation of the viral infection. By inducing expression of the HPV-16 early gene E6, TR2 may lessen the regulatory effect exerted by p53 and on TR2 itself as well as on the cell cycle in general. Without the tumor-suppressive regulation afforded by p53, the human papilloma virus is able to infect growth-arrested cells and more readily induce proliferation of differentiated cells (11). Our studies reported here indicate that TR2 is likely to be expressed in the cell types prone to HPV-16 infection (the squamous epithelial cells of the vagina and cervix) and that a TR2 response element has been identified in the HPV-16 LCR. We also demonstrate the binding of TR2 to the HPV-16 DR4RE and the subsequent up-regulation of HPV-16 gene transcription. Together with our previous findings that the tumor suppressor p53 has a suppressive effect on the expression of TR2 (16), we hypothesize that there is a positive feedback regulatory mechanism involving TR2, HPV-16, and p53 (Fig. 7). In a more general sense to depict the involvement of HPV-16 in the context of papilloma virus infection, we suggest that TR2 induces HPV-16 E6, and E6 in turn reduces the available p53. In this way, p53-mediated cell cycle regulation as well as TR2 repression is attenuated. As a result, more TR2 will be available to continue to up-regulate E6 expression. As the cell cycle continues, more and more cells will undergo HPV infection, and loss of p53-mediated regulation of proliferation will occur. This process increases the potential for the accumulation of mutations that may eventually lead to malignancy. The positive feedback loop between TR2 and HPV-16 represents a relationship between a member of the nuclear hormone receptor family and HPV that may play a significant role in the propagation and maintenance of HPV infection as well as in the associated development and progression of cervical neoplasia.

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