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Gamma Interferon-Inducible Protein 10 Induces HeLa Cell Apoptosis through a p53-Dependent Pathway Initiated by Suppression of Human Papillomavirus Type 18 E6 and E7 Expression

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Gamma interferon-inducible protein 10 (IP10) is a member of the CXC family of chemokines. By differential mRNA display, we have demonstrated the upregulation of IP10 in coxsackievirus B3 (CVB3)-infected mouse hearts. Functional characterization of the IP10 gene in IP10-transfected Tet-On HeLa cells has found that IP10 induced cell apoptosis and inhibited viral replication. In the characterization of the IP10-induced apoptotic pathway, we found that overexpression of IP10 upregulated p53 and resulted in altered expression of p53-responsive genes such as the $p21^{Cip1}$, $p27^{kip1}$, NF-κB, Bax, and PUMA genes and the mitochondrial translocation of Bax. However, transduction of the IP10 cells with adenovirus expressing dominant negative p53 not only ablated p53-triggered gene expression but also abolished IP10-induced apoptosis and restored CVB3 replication to the control levels. These data suggest a novel mechanism by which IP10 inhibits viral replication through the induction of host cell death via a p53-mediated apoptotic pathway. We also found that constantly high-level expression of p53 in these tumor cells is attributed to the IP10-induced suppression of human papillomavirus E6 and E7 oncogene expression. Taken together, these data reveal not only a previously unrecognized link between chemokine IP10 and p53 in antiviral defense but also a mechanism by which IP10 inhibits tumor cell growth.

Gamma interferon-inducible protein 10 (IP10) is a CXC chemokine in the chemokine superfamily. It is a chemoattractant for T cells, monocytes (33, 58, 59), and NK cells (34). It has an antiproliferative effect on endothelial cells (35), as well as angiostatic and antitumor activity (1, 31, 34). Recently, it has been reported that coexpression of IP10 and its receptor CXCR3 plays an important role in human cardiac allograft rejection (37). On the other hand, IP10 upregulation was shown in a mouse model of hepatectomy to play a fundamental role in hepatic repair and regeneration (28). Among the current expanding list of cellular activities discovered for IP10, there has been no study on virus-induced IP10 upregulation in infectious heart diseases. Therefore, we focused our investigation on the role of IP10 in coxsackievirus B3 (CVB3) infection, particularly on the characterization of the IP10-induced apoptotic pathway.

CVB3 is the most common causative agent of viral myocarditis in humans (15). In addition, CVB3-induced acute myocarditis may develop into chronic infection leading to dilated cardiomyopathy, whose only treatment is heart transplantation (8). The pathogenesis of CVB3 infection has been studied for decades. However, it is only recently that the disease occurrence has been found to be determined by complex interactions among several variables, such as viral genome structure (6), host genetic background (6, 20, 68), and the age (30) and the immune status (17, 21) of the host. The molecular biology of CVB3 is well documented. However, the roles of host gene responses to CVB3 infection are poorly understood. Our previous studies using differential mRNA display identified 28 genes which were either up- or down-regulated in CVB3-infected mouse hearts, and five of these genes have been reported (68). In this paper, we report an additional upregulated gene, the IP10 gene, and focus on its functional analysis, which has demonstrated that this gene induces host cell apoptosis through a p53-dependent pathway.

p53 is a sequence-specific transcription factor and plays a pivotal role in cellular responses to a variety of genotoxic stresses, which result in cell cycle arrest or apoptosis (12, 16). While the p53-dependent cell cycle checkpoints are well characterized, the actual mechanism whereby p53 activates apoptosis is still not fully understood. p53 can induce the expression of several apoptotic genes, including genes those for the death receptors CD95/Fas and KILLER/DR5 and the proapoptotic Bcl-2 family members Bax and PUMA (39, 40, 67). However, their roles in apoptotic pathways remain to be defined. Moreover, the expression of these proapoptotic genes induced by p53 is variable depending on the experimental system used (48, 62, 63). Thus, investigation of the differential regulation of gene expression in the IP10-induced p53-mediated apoptotic pathway and further correlation of these gene responses to the IP10-mediated antiviral activity are critically important for understanding the host defense mechanism in CVB3 infection.

The p53 gene is a tumor suppressor gene, and its expression is down-regulated in certain tumor cell lines such as HeLa cells. This is because the cells harbor high-risk human papillomavirus type 18 (HPV-18) E6 and E7 oncogenes (75), which can mediate ubiquitin-dependent proteolytic degradation of

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tumor suppressors p53 and retinoblastoma protein (pRb), respectively (23, 46). Therefore, the objective of this study is to determine how p53 induces apoptosis in HeLa cells and what the function of IP10 is in this induction of apoptotic cell death.

To achieve this goal, we have performed experiments in this study to investigate the function of IP10 by establishing a Tet-On HeLa cell line, which expresses IP10 upon induction using doxycycline (Dox). We demonstrated that IP10 expression activated a p53-dependent apoptosis pathway. In the further characterization of the cell death pathway and exploration of the mechanism of IP10-mediated antiviral activity, we analyzed the expression of a number of p53-responsive genes and several other related proteins. We found that several proapoptotic genes downstream of p53 had an altered expression, resulting in an increased rate of cell death and a corresponding inhibition of viral capsid protein synthesis. We propose that IP10 upregulation may benefit the host by an early induction of cell apoptosis through a p53-dependent pathway, thereby limiting viral replication and spread of infection. Moreover, in an attempt to determine why p53 can keep consistently high levels of expression in HeLa cells expressing IP10, we finally found that p53 upregulation is due, at least in part, to the suppression of HPV E6 and E7 expression by IP10.

MATERIALS AND METHODS

Animals, cells, virus, and antibodies. Inbred, adolescent and young adult A/J (H-2a) mice were used for screening differentially expressed genes in CVB3-infected hearts. Stock CVB3 (CG strain) was propagated in HeLa cells (American Type Culture Collection, Manassas, VA) and stored at −80°C. Premade Tet-On HeLa cells (Clontech, Palo Alto, CA) were grown and maintained according to the manufacturer's instructions. Antibodies against p53, $p21^{Cip1}$ (p21), Bax, Bcl-2, $p27^{kip1}$ (p27), HPV-18 E7, and caspase-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against poly(ADP)ribose polymerase (PARP) was obtained from PharMingen (San Diego, CA). Antibody against PUMA (p53-upregulated modulator of apoptosis) was purchased from Oncogene (La Jolla, CA). Antibodies against IP10, VP1, and cytochrome *c* were obtained from BD Transduction Laboratories (Lexington, KY).

Differential mRNA display. Total RNAs were isolated from CVB3-infected and sham-infected (phosphate-buffered saline) 4- and 10-week-old mouse hearts at day 4 postinfection (pi). Differential mRNA display experiments were carried out using the RNAimage kit (Genhunter, Nashville, TN) as described previously (68).

RT-PCR. To further confirm the upregulation of IP10 gene expression in CVB3-infected hearts, total RNAs were isolated from the CVB3-infected and sham-infected mouse hearts and reverse transcription-PCR (RT-PCR) was conducted as described previously (64). The up- and downstream PCR primer sequences targeting murine IP10 are P1 (5′GGG TCT CTT ACT ACT TCA AT3′) and P2 (5′CAT CAA GTA TGT ATC AAT GGG3′), respectively. In addition, to determine whether IP10 expression inhibits transcription of the papillomavirus type 18 E6 gene (accession no. AY262282) in HeLa cells, RT-PCR was also performed using up- and downstream primers (upstream, 5′CTA CAA GCT ACC TGA TCT G3′; downstream, 5′TTC TCT GCG TCG TTG GAG T3′) targeting the open reading frame of the E6 gene.

Construction of pTRE/IP10 expression plasmid. A cDNA clone of mouse IP10 (pcDNA3/IP10; a generous gift from Tom Hamilton, Cleveland Clinic Foundation, Cleveland, OH) was digested with EcoRI to isolate the entire IP10 open reading frame and ligated into the plasmid vector pTRE (Clontech) prelinearized with EcoRI. The recombinant expression plasmid was checked for correct size and orientation of the IP10 gene by restriction enzyme digestion and agarose gel electrophoresis.

Establishment of the double-stable Tet-On/IP10 HeLa cell line. Tet-On HeLa cells (10^6) in six-well plates were transfected with 5 μg of pTRE/IP10 DNA mixed with 0.25 μg of pTK-Hyg plasmid containing the hygromycin B resistance gene and 8 μg of Lipofectamine (GIBCO, Grand Island, NY). After 8 h treatment of the cells with the mixture, the plasmids and Lipofectamine were replaced with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Tet system approved fetal bovine serum (Clontech) and incubation was continued for 2 days before initiating selection. The double-stable cells were screened with hygromycin B and G418 at concentrations of 200 μg/ml and 400 μg/ml, respectively, following the instructions described previously (72). Positive clones were further screened for IP10 protein expression in the presence of Dox at a final concentration of 1 μg/ml by Western blot analysis using an anti-IP10 antibody. Cells cotransfected with empty vectors pTRE and pTK-Hyg were also screened in parallel to be used as a negative control in later experiments.

Adenovirus vector and transduction. An adenovirus vector encoding dominant negative p53 protein (Ad.DN/p53) was constructed using the Adeno-X expression system and plasmid pCMV-p53mt135 (Clontech). pCMV-p53mt135 contains a G-to-A conversion at nucleotide 1017 of the p53 coding region and thus expresses a dominant negative p53 protein. When this DN/p53 is coexpressed with wild-type p53 in the cell, they form a mixed tetramer that is unable to interact with p53-binding sites; therefore, the downstream effects of p53 are blocked (61). The construction of this Ad.DN/p53 was achieved by following the manufacturer's instructions. Briefly, the p53mt135 fragment was excised by EcoRI and HindIII and blunted with T4 DNA polymerase. This DN/p53 fragment was then ligated into the blunt-ended XbaI site of the pShuttle vector. The resulting pShuttle-DN/p53 was digested with PI-SceI and I-CeuI, and the released DNA fragment was then inserted into the prelinearized pAdeno-X vector at the same site. After checking the orientation of the insert by restriction digestion and gel electrophoresis, the vector was linearized at a PacI site and packaged into infectious adenovirus particles by transfection of HEK-293 cells using Lipofectamine. The virus titer was determined using an Adeno-X rapid titer kit (Clontech) following the manufacturer's procedures. The empty adenovirus vector (Ad) was also grown and purified in parallel to serve as a control.

IP10 cells were grown in 60-mm plates overnight and then transduced with Ad.DN/p53 and Ad as described previously (47). Briefly, the IP10 cells at approximately 80% confluence were washed with serum-free DMEM and transduced with Ad.DN/p53 or Ad vector at a titer of 2×10^9 particles/ml for 90 min. The transduction medium was replaced by fresh DMEM containing 2% serum, and the incubation was continued overnight until the addition of Dox to induce IP10 expression.

Western blot analysis. Tet-On/IP10 cells and vector-transfected control HeLa cells were harvested at various time points following induction with Dox at a final concentration of 1 μg/ml. The sample preparation for Western blot analysis followed the method described previously (73). Briefly, for isolating total proteins, cells were resuspended in lysis buffer, incubated for 20 min on ice, and centrifuged at 12,000 × *g* for 5 min to collect the supernatant. For cytosolic extracts, cells were resuspended in S100 buffer and disrupted in a Dounce homogenizer by 20 strokes of a type B pestle. The supernatant was collected following centrifugation at 10,000 × *g* and further centrifuged at 100,000 × *g* for 1 h at 4°C. For the mitochondrial fraction, pellets were collected after centrifugation at 100,000 × *g* as described above and resuspended in the lysis buffer. This lysate was vortexed and freeze-thawed several times and centrifuged at 13,000 × *g* again. The resulting supernatants were used to perform Western blot analysis of specific proteins using corresponding antibodies.

ELISA. To measure the content of IP10 in the medium, culture media harvested after 2 days of Dox induction were used for measuring IP10 concentrations by enzyme-linked immunosorbent assay (ELISA). Sandwich ELISA was performed following the standard procedure (7) using a homemade kit containing IP10 antibody (ID Labs, London, ON, Canada), biotinylated IP10 antibody (ID Labs, London, ON, Canada), and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) substrate (Sigma).

Viral plaque assay. Tet-On/IP10 cells transduced with Ad.DN/p53 or Ad vector or not transduced with recombinant adenoviruses were inoculated in 60-mm plates at 10^6 cells per plate and induced with Dox. Two days later, cells were infected with CVB3 at a multiplicity of infection of 10, and incubation was continued for 24 h. The virus supernatant was obtained by freeze-thaw of the collected cells with medium three times and then centrifugation of the cell lysates to eliminate the cell debris. A viral plaque assay was performed on HeLa cell monolayers as described previously (72). The experiment was repeated three times.

Cell viability assay. To determine the effect of IP10 expression on cell viability, Tet-On/IP10 HeLa cells were cultured and induced with Dox as described above. Cell viability was determined using the MTS [3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tertrazolium salt] assay kit (Promega, Madison, WI) as described previously (72). All cells were treated with MTS at different time points and submitted for absorbency measurement at 490 nm. The percentages of cell viability of the induced Tet-On/IP10 cells and vector-transfected control cells were determined according to the absorbency corrected with the reading of the nontransfected HeLa cell culture.

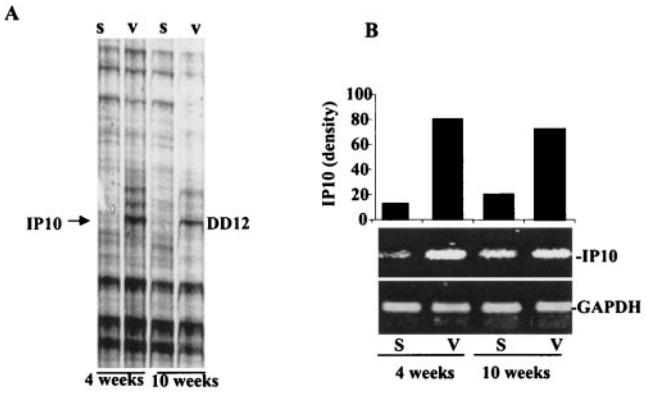

FIG. 1. IP10 gene transcription is upregulated in CVB3-infected mouse heart. (A) Differential mRNA display of IP10 expression in CVB3-infected (V) and sham-infected (S) 4- and 10-week-old A/J mouse hearts. The arrow (DD12) indicates the upregulation of the IP10 gene. (B) RT-PCR to further confirm the upregulation of IP10 expression. Quantitation of PCR product was conducted by densitometric scanning of the bands and normalized with that of a control that used water as template for RT-PCR (data not shown). These data are representative of two independent experiments. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene expression was used as a loading control.

siRNA and transfection. To selectively silence E6 or E7 oncogene expression, double-stranded small interfering RNAs (siRNAs) were synthesized. To minimize possible off-targeting effects, two different targets were selected for the E7 gene (E7a, 5′ GGCAACATTGCAAGACATT 3′; E7b, 5′ GCGACTCAGAGG AAGAAAA 3′) according to the manufacturer's (QIAGEN) recommendations and subjected to a BLAST search against the human genome sequence to ensure that targeting was only to the desired gene. The E6 siRNAs (5′ CUAACACU GGGUUAUACAATT 3′) were synthesized according to the published sequences, which have shown successful silencing of this gene expression (4). In addition, a siRNA with random sequence (5′ UUCUCCGAACGUGUCAC GUTT 3′) was synthesized after checking by BLAST search and was used as a negative control.

The transfection of siRNAs was performed following the procedures used previously by this laboratory (71) with minor modifications. Briefly, 2×10^5 Tet-On/IP10 HeLa cells were grown at 37°C overnight. When cells reached 50 to 60% confluence, they were transfected with Oligofectamide (Invitrogen)-siRNA complexes containing siRNA (300 nM) for 24 h. Cells were then induced with Dox as described above. After 48 h of induction, cell supernatants and cell lysates were harvested for analyses of gene silencing and alteration of p53 production.

Assessment of cell morphology. Morphological changes were evaluated by phase-contrast microscopy. To localize the apoptotic bodies in the culture, double staining with annexin V and propidium iodide was conducted. Briefly, Dox-induced cells were washed with 1× binding buffer and incubated in 5 μl of annexin V-fluorescein isothiocyanate (FITC) plus 10 μl of propidium iodide for 15 min at room temperature in the dark. The cells were then washed and fixed in 2% paraformaldehyde. Morphological observation of the apoptotic cells was carried out under a fluorescence microscope using a dual filter set for FITC and rhodamine.

Statistical analysis. All values are presented as means ± standard errors. Statistical significance was evaluated using the Student's *t* test for paired comparison, with a P value of <0.05 considered statistically significant.

RESULTS

Transcriptional determination of IP10 upregulation. To identify host genes that are responsible for cardiac susceptibility to CVB3 infection, differential mRNA display was conducted to screen altered gene expressions in CVB3-infected mouse hearts (68). In this study, we have chosen band DD12, which is an upregulated gene (Fig. 1A), and DNA sequence analysis revealed that it is identical to the murine IP10 gene. To further confirm the upregulation of IP10 expression observed in differential mRNA display gels, RT-PCR was performed using total RNAs isolated from CVB3-infected hearts harvested from mice of different ages. Figure 1B demonstrates that IP10 transcription was upregulated in both 4- and 10-week-old mice that were sacrificed 3 days pi. The changes in IP10 expression during CVB3 infection were further confirmed by our cDNA microarray analysis (60).

Overexpression of IP10 in Tet-On/IP10 HeLa cells inhibits CVB3 protein synthesis and virus replication. In establishing the inducible HeLa cell lines overexpressing IP10, a total of 13 clones were selected and confirmed by Western blot analysis. Figure 2A demonstrates the inducible overexpression of IP10 for one of the clones, which was named Tet-On/IP10 and used throughout this study. IP10 overexpression and secretion into the medium were further verified by ELISA measurement of IP10 concentration in the medium. Figure 2B indicates that IP10 concentration is 1.3 pg/ml in the supernatant, which is much higher than that in the medium of controls. To investigate the effect of IP10 expression on CVB3 infectivity, we performed Western blot to detect viral capsid protein VP1 production in the presence or absence of Dox induction following CVB3 infection. Figure 2C shows that VP1 expression was delayed until 9 h pi in the presence of Dox, while noninduced cells exhibited VP1 expression at 5 h pi. In addition, Dox-induced vector-transfected cells did not show inhibition of VP1 production, indicating that vector or Dox itself did not have a negative effect on VP1 expression (data not shown). These data were further confirmed by viral plaque assay, showing that samples from Dox-induced cultures contained much less viral particles than those from noninduced HeLa cells (Fig. 2D).

IP10 overexpression induces cytochrome *c* release from mitochondria, activation of caspases, and reduction of cell viability. To explore the mechanism by which IP10 expression inhibits viral replication, we tested whether IP10 expression can induce cell apoptosis, since viral replication relies on viable cells. We first performed an MTS assay to measure HeLa cell viability in response to IP10 expression and found that IP10 expression induced HeLa cell death and reduced overall cell viability (Fig. 3A). We then turned our attention to determining the redistribution or activation of several major apoptotic markers and found that IP10 expression induced cytochrome *c* release from mitochondria (Fig. 4) and activation of caspase-3 (Fig. 3B). The activation of caspase-3 was further verified by Western blot analysis to detect the cleavage of its substrate, PARP (Fig. 3B). Additional verification was performed by morphological assessment as shown in Fig. 3C, which demonstrates significant loss of cell adherence and appearance of apoptotic bodies 48 h postinduction as well as phosphatidylserine exposure on the outer membrane, as shown by annexin V staining.

IP10-induced cytochrome *c* release correlates to mitochondrial translocation of Bax. As cytochrome *c* release from mitochondria usually results from interactions between mitochondrial outer membrane proteins and the proapoptotic members of the Bcl-2 family, we first determined the expression levels of Bax in IP10-expressing cells. Figure 4A shows that Bax was slightly upregulated 48 h postinduction and reached much higher levels 72 h postinduction. Bax is primarily

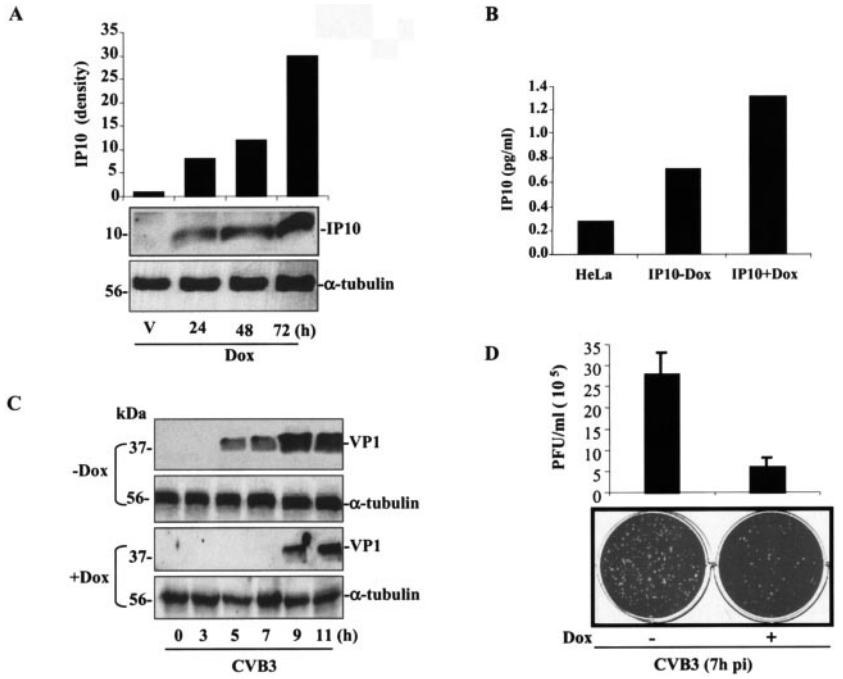

FIG. 2. IP10 expression in Tet-On/IP10 HeLa cells inhibits CVB3 replication. (A) IP10 was inducibly expressed in Tet-On/IP10 cells, and expression was detected by immunoblot with a rabbit anti-mouse IP10 antibody. Tet-On vector-transfected cell culture (V) was used as a negative control. Equal loading was assessed with an anti-α-tubulin antibody. The numbers indicated on the left are molecular masses in kDa. IP10 products were quantitated by densitometric scanning of the bands and normalized with the density of control. Values shown are averages of the data from two independent experiments. (B) IP10 contents in culture medium. Medium collected from the culture was used to measure IP10 concentration by ELISA. Media collected from HeLa cells and noninduced Tet-On HeLa cells were used as controls. (C) IP10 inhibits CVB3 VP1 protein expression. Dox-induced and noninduced IP10 cells were infected with CVB3 and harvested at the indicated time points pi for Western blot analysis of VP1 expression. Note that VP1 production in Dox-induced IP10 cells was delayed by 4 h compared to that in the noninduced cells, which exhibited VP1 production 5 h pi. (D) IP10 expression inhibits virus plaque formation. Supernatants collected from cell cultures described above for VP1 detection were used for viral plaque assays. The bars represent the values obtained from three separate experiments. Values shown are means ± standard errors; $P < 0.05$.

located in the cytosol and translocates to the mitochondrial outer membrane upon apoptotic stimulation. To test whether this occurred after IP10 expression, we performed Western blot to detect the redistribution of Bax and cytochrome *c* in both the cytosolic and mitochondrial fractions. Figure 4B demonstrates that in the cytosolic fraction the Bax protein is significantly decreased but cytochrome *c* is correspondingly increased 48 h postinduction. On the other hand, in the mitochondrial fraction, the Bax protein is dramatically increased, while cytochrome *c* is hardly detectable 48 h postinduction (Fig. 4C), indicating that the mitochondrial translocation of Bax protein correlates well to the cytochrome *c* release from mitochondria.

IP10 overexpression upregulates p53 and alters p53-responsive gene expression. Since p53 is an upstream inducer of Bax expression, we focused our study on the p53-mediated apoptotic pathway. We first determined the expression levels of p53 and then its responsive genes in IP10-expressing cells. As shown in Fig. 5, there was significant p53 upregulation at 24 h postinduction, and its downstream target genes, the p21 and p27 genes, were slightly upregulated at early (24 h) and late (72 h) time points, respectively. As mentioned above, Bax was significantly upregulated 48 h postinduction (Fig. 4). However, a strong but transient Bcl-2 expression signal was detected 24 h postinduction and then totally disappeared thereafter (Fig. 5). PUMA, a recently identified p53-upregulated modulator of the apoptosis gene, was found to be significantly upregulated 48 h postinduction in this system. However the slight NF-κB upregulation peaked at 24 h postinduction and returned to the control level 72 h postinduction.

Ad.DN/p53 transduction blocks IP10-induced expression of p53 and its responsive genes and prevents inhibition of CVB3 replication. To confirm that the IP10-induced antiviral effect occurs through a p53-mediated apoptosis pathway, we used Ad.DN/p53 viruses to transduce IP10 cells to detect the changes in gene expressions of IP10, p53, p53-responsive genes, and other apoptotic mediators. We then analyzed the end effects of these altered gene expressions on CVB3 replication. We found that Ad.DN/p53 transduction did not significantly affect the IP10 expression but dramatically reduced expression of p53 (Fig. 6A and B; the antibody used can only detect wild-type p53) and its target genes, including the PUMA, p21, and p27 genes (Fig. 6C). In addition, mitochondrial translocation of Bax, redistribution of cytochrome *c* (Fig. 7A and B), and activation of caspase-3 as well as cleavage of its substrate PARP (Fig. 6D) were all significantly blocked by Ad.DN/p53 transduction. Finally, we found by immunoassay for VP1 that Ad.DN/p53 transduction blocked IP10-induced death signal transduction and restored the expression of CVB3 VP1 protein to the earlier time point at 5 h pi in Dox-induced

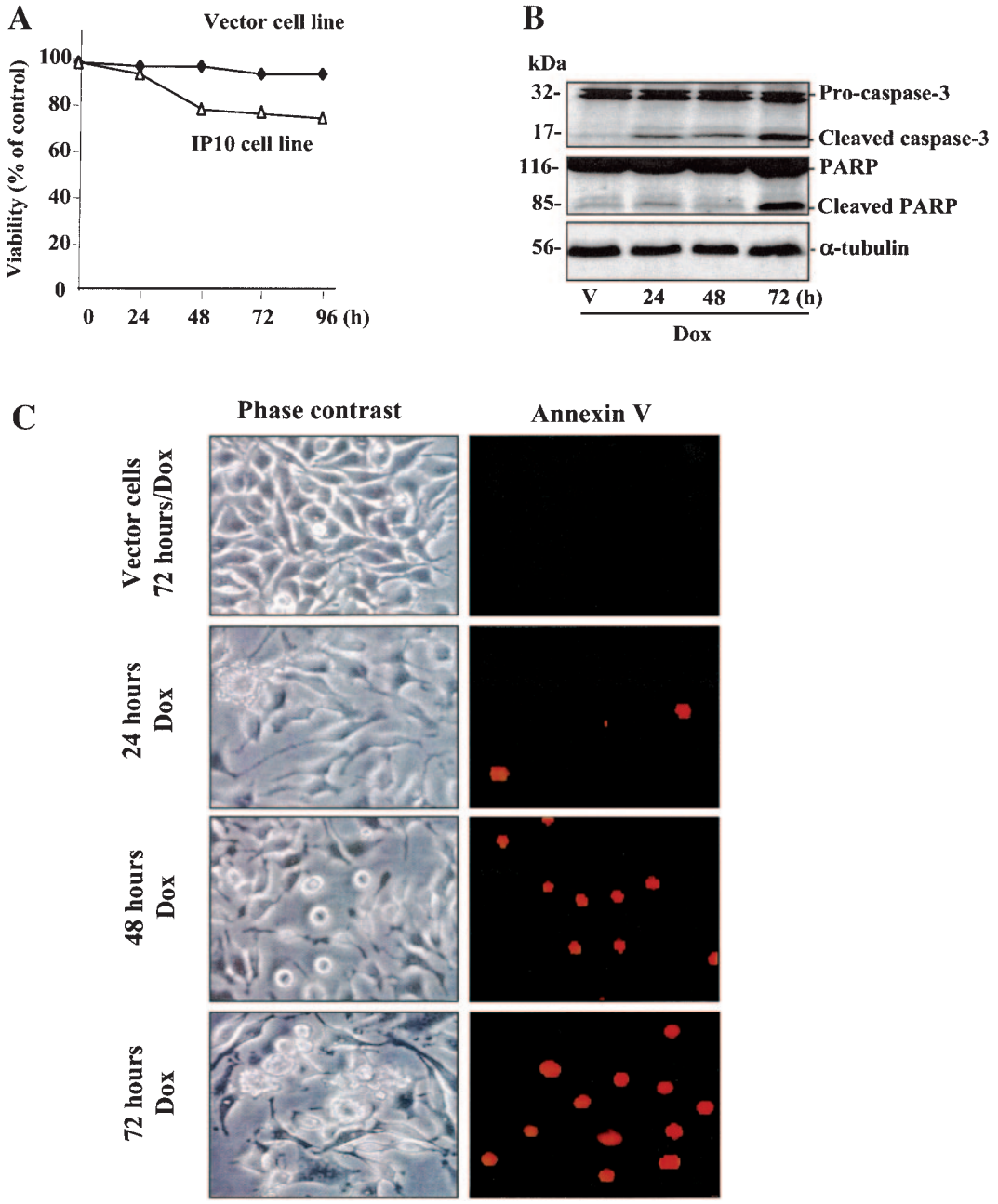

FIG. 3. IP10 expression induces caspase-3 activation, decreased cell viability, and apoptotic cell death. (A) MTS cell viability assay. The percentage of cell viability was determined by measuring the absorbency of the produced formazon after treatment with MTS. Note that, 72 h after Dox induction, approximately 95% and 75% of vector-transfected control cells and Tet-On/IP10 cells, respectively, are alive. (B) IP10 induces cleavages of pro-caspase-3 and caspase-3 substrate PARP. IP10 expression was induced, and the cells were harvested at the indicated time points. Western blot analyses show that activation of caspase-3 and cleavages of PARP were most noticeable 72 h postinduction. A vector-transfected cell line was used as a negative control as described for Fig. 2. (C) Apoptotic cell death. After induction and incubation, cells were washed with binding buffer and then double stained with annexin V and propidium iodide. Cells were then washed again and fixed in 2% paraformaldehyde. Morphological observation of the apoptotic cells was carried out under a fluorescence microscope using a dual filter set for FITC and rhodamine. A vector-transfected cell culture induced with Dox for 72 h was used as a control.

IP10 cells (Fig. 8A) compared to that in non-Ad.DN/p53-transduced but Dox-induced IP10 cells showing that VP1 expression started 7 h pi. These data suggest that Ad.DN/p53 transduction prevents IP10-induced inhibition of CVB3 replication, which was further substantiated by viral plaques assays showing that the PFU of CVB3 in the Ad.DN/p53-transduced sample are approximately fourfold higher than those in the non-Ad.DN/p53-transduced, Dox-induced sample (Fig. 8B).

IP10 expression suppresses HPV E6 and E7 expression. p53 expression is normally suppressed in HPV-positive cells. How-

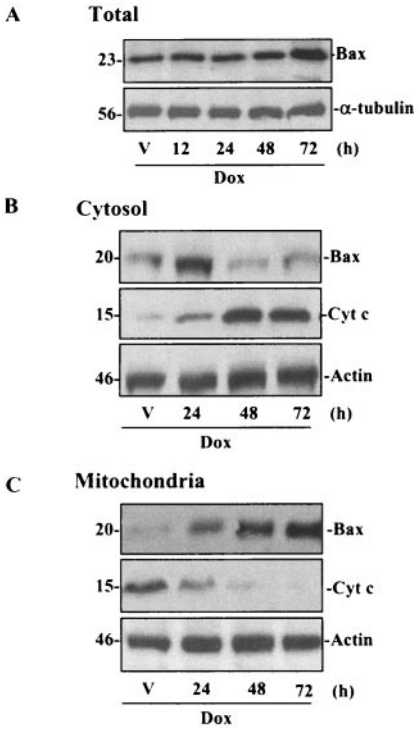

FIG. 4. IP10 expression induces upregulation and redistribution of Bax and cytochrome *c* release from mitochondria. Tet-On/IP10 cells and vector-transfected control cells were induced with Dox for IP10 expression and harvested at the indicated time points. Total cellular proteins as well as proteins in cytosolic and mitochondrial fractions were prepared. (A) Total cellular proteins were blotted onto nitrocellulose membranes and immunoanalyzed for Bax expression. (B) For cytosolic extract, the membrane was first detected for Bax and then stripped and reprobed for cytochrome *c* and finally for actin (equal loading control). Note that the amount of Bax and cytochrome *c* in the cytosolic fraction was decreased and increased, respectively, 24 h postinduction. (C) For the mitochondrial fraction, samples were analyzed as described above and the distribution of Bax and cytochrome *c* in this fraction was just opposite to that in the cytosolic fraction.

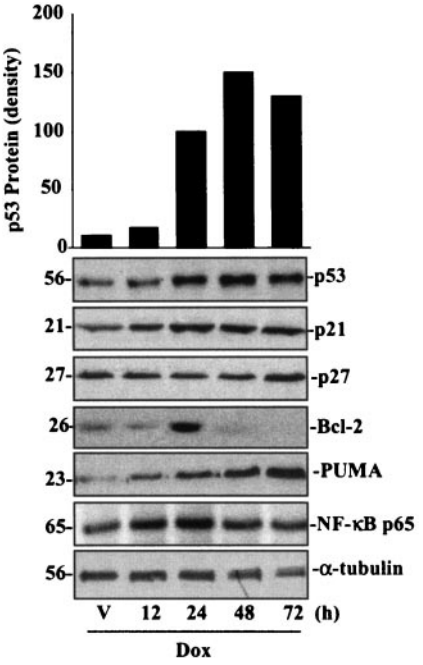

FIG. 5. IP10 upregulates p53 and alters p53-responsive gene expression. Tet-On/IP10 cells and vector-transfected control cells were induced with Dox and harvested at the indicated time points. Western blot analyses of whole-cell extracts to detect p53 and its target gene expression were performed using antibodies as indicated. The sample used for NF-κB analysis was prepared using the NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce). p53 upregulation was quantitated by densitometric scanning as described for Fig. 2A.

ever, in IP10-expressing HeLa cells, the p53 gene was not inhibited. On the contrary, it was steadily upregulated upon Dox induction. To determine the underlying mechanism, RT-PCR to detect HPV E6 expression was performed and demonstrated that E6 expression was strongly inhibited 24 h after Dox induction of IP10 expression. However, the vector-transfected control cells did not show notable change of E6 expression (Fig. 9A). We also detected E6 suppression by Western blot analysis but failed to obtain a clear band, which is largely due to the poor quality of E6 antibody (25). To check if E7 expression is altered by IP10 expression, we detected E7 protein using the same cell lysates by Western blot. Figure 9B shows that E7 protein production was strongly suppressed by IP10 expression.

Gene silencing of E6 or E7 with siRNAs increases p53 production. The role of E6 in ubiquitin-mediated degradation of p53 is well known; however the effect of E7 expression on p53 production is not very clear. To address this issue, we transfected the cells with E7 or E6 siRNAs and then induced or did not induce with Dox before detection of E7 expression and p53 accumulation. Figure 10 demonstrates that E7 and E6 siRNAs specifically inhibit their respective target genes' expression. Further detection of p53 expression by Western blot indicates that suppression of E6 or E7 can enhance p53 accumulation.

DISCUSSION

In this study, we conducted functional analyses of IP10, a chemokine identified by differential mRNA display to be upregulated in CVB3-infected mouse heart. We found that IP10 gene transcription was significantly increased during CVB3 infection. Further functional characterization was performed by establishing a cell line inducibly expressing IP10, which mimics the significant upregulation of IP10 in CVB3-infected cells. We found that IP10 possesses proapoptotic activity and its expression confers antiviral ability to the host cells.

Like some other chemokines, IP10 has been known to play a dual functional role in response to viral infection. This includes mediating development of inflammatory disease and contributing to viral clearance. The latter function largely depends on the recruitment and activation of innate immune responses, such as chemoattraction of natural killer cells and other immune cells. The presence of natural killer cells at the foci of infection mediates the cytopathic injury of infected cells through the enhancement of cytolytic activity. This indirect antiviral action of IP10 is obviously through the immune-mediated destruction of host cells. However, whether IP10 can initiate non-immune-mediated direct antiviral action is not known. Our in vitro finding on the IP10-induced apoptosis

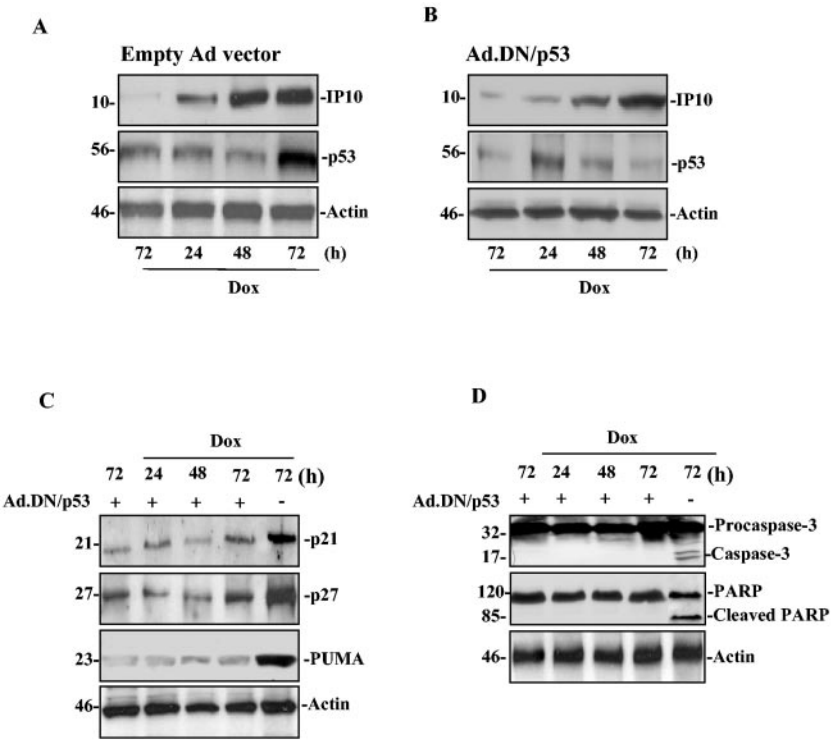

FIG. 6. Ad.DN/p53 transduction does not affect IP10 expression but suppresses expression of p53 and its responsive genes and inhibits cleavage of procaspase-3. The IP10 cells were transduced with either empty Ad viral vector (A) or Ad.DN/p53 (B) and then induced with Dox for IP10 expression. A cell culture transduced with Ad.DN/p53 or Ad vector but not induced with Dox was used as a negative control (first lane on the left). Whole-cell extracts were prepared at indicated time points postinduction and analyzed by Western blot to detect IP10 and p53 expression. These cell lysates were further used to detect expression of p53 responsive genes (C) and activation of caspase-3 as well as cleavage of PARP by activated caspase-3 (D). In addition to the negative control, cells that were not transduced with Ad.DN/p53 but induced with Dox for 72 h were used as a positive control (last lane on the right).

provides evidence to support our hypothesis that IP10 expression directly initiates the host cell death program and limits viral replication and subsequent spread of the infection.

Viral infections have been known to induce apoptosis through the activation of a number of proapoptotic genes (5, 18, 73). However, to our knowledge, IP10 has never been reported to be involved in such process. In this work, we clearly demonstrated that IP10 expression induced HeLa cell apoptosis, in the absence of CVB3 infection, through the activation of caspase-3. It is known that there are two major pathways that transduce death signals to the apoptotic machinery: the extrinsic death-receptor-ligated pathway and the intrinsic mitochondrion-mediated pathway (2, 44). To determine the mechanism of IP10-induced apoptosis, we performed a series of experiments to analyze the expression and activation of a number of potential mediators of apoptosis. The most informative data directing our experiment progress at the early stage of this work was the cytochrome *c* release from mitochondria and the upregulation of Bax in the mitochondrial fraction of the cell lysates. These data indicate that IP10-induced apoptosis occurs likely via an intrinsic mitochondrion-mediated pathway because the Bax protein normally resides in the cytosol and

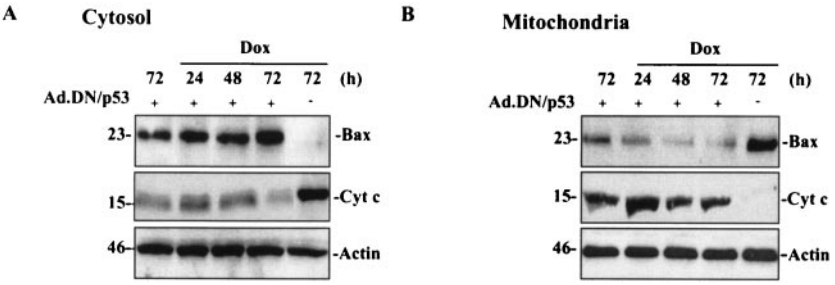

FIG. 7. Ad.DN/p53 transduction prevents IP10-induced Bax translocation and mitochondrial cytochrome *c* (Cyt c) release. IP10 cells were transduced with Ad.DN/p53 and then induced with Dox. The cytosolic and mitochondrial extracts were prepared and used to detect the redistribution of Bax and cytochrome *c* in cytosol (A) and mitochondria (B) by Western blot. The negative and positive controls are the same as those described for Fig. 6.

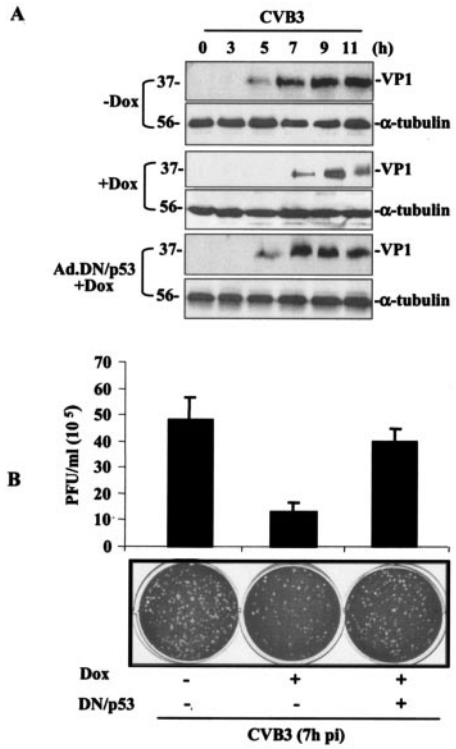

FIG. 8. Ad.DN/p53 transduction prevents IP-10-induced inhibition of CVB3 replication. (A) CVB3 VP1 detection. Three groups of IP10 cell lysates were prepared: (i) cells infected with CVB3 in the absence of Dox; (ii) cells induced with Dox for 48 h and then infected with CVB3; and (iii) cells transduced with Ad.DN/p53 overnight, then induced with Dox for 48 h, and finally infected with CVB3. All three groups of cells were harvested at the indicated time points. Total cellular proteins were prepared for Western blot analysis of CVB3 VP1 protein. (B) Viral plaque assay. IP10 cells were transduced or not transduced with Ad.DN/p53 and then induced or not induced with Dox for 48 h as indicated. All cells were infected with CVB3 for 24 h, and the supernatants were prepared and used for a plaque assay to determine viral titer.

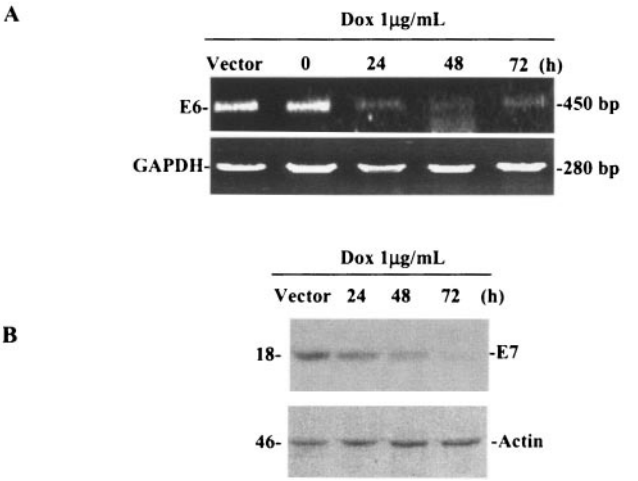

FIG. 9. IP10 inhibits human papillomavirus type 18 E6 and E7 gene expression in HeLa cells. Tet-On/IP10 cells and Tet-On/vector control cells were induced with Dox for IP10 expression and harvested at the indicated time points. (A) RT-PCR to detect E6 mRNA. Total RNAs were isolated, and RT-PCR was conducted to detect papillomavirus type 18 E6 gene expression using primers targeting the E6 coding region. Human GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was used as an equal loading control. (B) Western blot to detect E7 protein. Cell lysates were harvested at different time points postinduction, and E7 was detected by immunoblotting using a goat polyclonal anti-E7 antibody. Actin detection was used as an equal-loading control.

translocates to the mitochondrial outer membrane to interact with Bcl-2 family proteins upon apoptotic stimulation (10, 32). These preliminary data raised the question of what factor induces the translocation of Bax in our experimental system. To address this issue, Western blot analysis was performed to detect the redistribution of Bax protein and cytochrome *c* in fractions of cytosol and mitochondria using Dox-induced and noninduced Tet-On/IP10 HeLa cells; the analysis confirmed that IP10 overexpression played a role in inducing the translocation of Bax and stimulating cytochrome *c* release from mitochondria.

To further determine whether the upregulation and translocation of Bax occur directly by IP10 induction or indirectly through an IP10-induced mediator, we focused our attention to p53 expression, since this protein has been reported to be an upstream inducer of Bax in other experimental systems (27, 42). We performed Western blot to detect p53 expression in Dox-induced Tet-On/IP10 HeLa cells and found that IP10 expression induced upregulation of p53 as early as 24 h postinduction. This suggests that p53 serves as a mediator between IP10 expression and Bax upregulation. This finding was further verified by using Tet-On/IP10 cells transduced with Ad.DN/p53, in which the upregulation of Bax was blocked and the translocation from cytosol to mitochondria was abolished even at the presence of a Dox inducer.

As p53 is a tumor suppressor and also a transcription factor, its upregulation can trigger the altered expression of a number of responsive genes through its binding on the promoter of these genes. These genes include two major classes, one that controls the cell cycle and one that controls apoptosis (43, 52, 70). To further determine which genes are involved in the IP10-induced apoptosis pathway, we have analyzed several downstream p53-responsive genes including the PUMA and p21 genes and others. PUMA is a proapoptotic Bcl-2 family protein and predominantly locates in mitochondria (32, 40). Recent reports found that PUMA provides a connection between p53 and Bax and that its upregulation is parallel with p53-mediated apoptosis (32, 70). Our data have shown that the upregulation of PUMA in IP10-expressing cells correlates well with the increased expression or redistribution patterns of Bax and other downstream mediators of apoptosis such as cytochrome *c* and caspases. These data are further supported by the fact that Ad.DN/p53 transduction of the cells almost completely abolished the cascade from the upstream upregulation of both PUMA and Bax to the downstream redistribution of Bax and cytochrome *c*, and finally to the activation of caspase-3. As caspase-3 activation can also be induced by death receptor-mediated activation of caspase-8, our findings rule out the potential involvement of a death-receptor-mediated pathway because the transduction with Ad.DN/p53 could almost totally block the apoptotic pathways. However, another member of the Bcl-2 family, Bcl-2, shows an opposite expres-

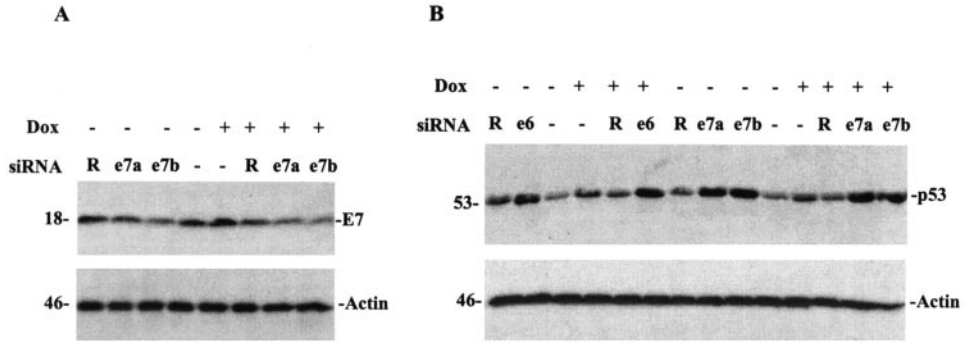

FIG. 10. Gene silencing of E7 or E6 with siRNA increases p53 accumulation. Cells were transfected with siRNAs and then induced or not induced with Dox. E7 (A) and p53 (B) production was detected by Western blot analysis. Nontransfected cells and random siRNA-transfected cells (R) were used as controls. Since the siRNA treatment is prior to Dox induction, siRNA seems to play an overwhelming role in altering E7 and p53 expression compared with Dox induction. Due to the poor quality of E6 antibody, the detection of E6 protein was not shown. Actin expression was used as a loading control.

sion pattern compared with PUMA, indicating that IP10 expression not only activates proapoptotic genes but also suppresses antiapoptotic genes.

p21 and p27 are cyclin-dependent kinase inhibitors, and their upregulation can cause cell cycle arrest at the G_1 or G_2 checkpoint (50, 52). The p21 gene is a p53-responsive gene; however, whether p27 expression is inducible by p53 is unclear. Recently, there was a report indicating that suppression of p21 expression by c-Myc, a transcription factor, may sensitize cells to p53-mediated apoptosis (50). However, in our experimental system, the expression of both p21 and p27 was not suppressed by IP10-induced p53 expression. Instead, a slight enhancement of p21 and p27 gene expression occurred upon Dox induction for IP10 expression. Since this slight upregulation was not sufficient to inhibit its target, cyclin-dependent kinase, it could not protect cells from apoptosis. Instead they may contribute to cell cycle arrest. On the other hand, p53 expression also caused a response from another downstream target gene, the NF-κB gene. NF-κB has been known to be a modulator in the p53-mediated apoptosis pathway, and its expression can protect or contribute to apoptosis depending on its expression levels (43, 51). In our experimental system, NF-κB was only slightly and transiently upregulated at the early time points and then downregulated 24 h postinduction, which correlated well with the time points when downregulation of Bcl-2 occurred. These data indicate that p53-induced downregulation of NF-κB may contribute to HeLa cell apoptosis.

The finding on IP10-induced upregulation of p53 is somewhat surprising because it has been reported previously that p53 can be upregulated only by alpha/beta interferon but not by gamma interferon in mouse embryonic fibroblasts (56). A possible explanation for this difference may be that the gamma interferon-induced p53 upregulation is cell type specific depending on the expression status of the gamma interferon receptor in the cell, since we have shown that gamma interferon treatment of HeLa cells induced elevated production of IP10 (data not shown), which implies that HeLa cell has a gamma interferon receptor on the surface of the cell membrane. Although we are not very clear on the mechanisms by which each type of interferon induces upregulation of p53, we have found that, at least for gamma interferon, a mediator such as IP10 is essential for the induction, which indicates again that IP10 is an important inducer of the p53-mediated apoptosis pathway.

Another interesting point is how IP10 can induce tumor suppressor p53 upregulation in HeLa cells, a cancer cell line harboring the HPV E6 and E7 oncogenes (69). It has been known that the high-risk HPV-18 E6 and E7 can interact with their associated proteins to initiate ubiquitination and degradation of p53 (46) and pRb (23), respectively. Therefore, in regular nontransfected HeLa cells, p53-mediated apoptosis may be suppressed by E6-mediated downregulation of p53 (19). However, in our IP10-transfected Tet-On cell line, p53 expression was not downregulated; instead it was significantly upregulated upon IP10 expression. These data imply that IP10 may possess the activity to suppress oncogene expression and thus protect p53 from degradation initiated by E6-mediated ubiquitination. This speculation has been confirmed by RT-PCR showing that E6 gene transcription is significantly downregulated by IP10 expression. We tried to authenticate these data by Western blot analysis; however we failed to obtain a satisfactory result. This is largely due to the low levels of E6 expression and poor reactivity of available E6 antibodies, which have been similarly reported by a number of publications (25, 29, 36).

For the mechanisms of IP10-induced suppression of E6 expression, although we are not clear at present, several potential mechanisms can be rationalized as follows. IP10 is an interferon (IFN)-inducible chemokine and IFN production is known to be induced by double-stranded RNA (dsRNA) (38, 57). During CVB3 infection, dsRNAs are intermediates of CVB3 replication and thus they can stimulate production of IFNs and in turn upregulate expression of a number of IFN-responsive genes such as the IP10 gene. These IFN-stimulated proteins can further activate downstream IFN-regulated genes such as the dsRNA-dependent protein kinase (PKR) (55), 2′5′-oligoadenylate synthetase (OAS), and RNase L (13, 74) genes. Activated PKR has been known to inhibit gene expression of host and virus at the translational level by phosphorylation of the α-subunit of the eukaryotic translation initiation factor 2 (eIF2α) (24, 53). OAS and RNase L have been reported to downregulate viral gene expression at the tran-

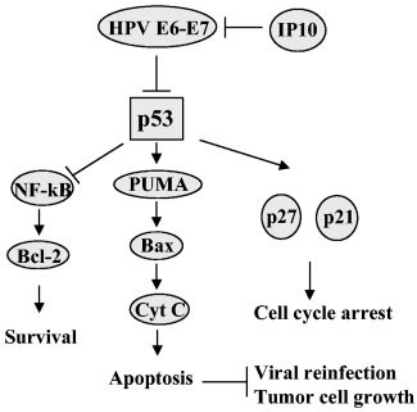

FIG. 11. A model of IP10 function in regulation of the p53-dependent apoptotic pathway in HeLa cells. IP10 expression (mimicking CVB3-induced upregulation of IP10) in HeLa cells suppresses HPV-18 E6 and E7 oncogene expression and initiates a p53-dependent apoptotic pathway through activation of its downstream responsive genes, including the PUMA and Bax genes, leading to release of cytochrome *c* from mitochondria. In addition, p53 can also downregulate NF-κB expression, leading to the inhibition of the transcription of the Bcl-2 gene, a prosurvival gene. On the other hand, IP10-activated p53 can upregulate p21 and p27 expression, resulting in cell cycle arrest. Apoptotic cell death or suppressed host cell survival will limit viral replication and reinfection or inhibit tumor cell growth.

scriptional level, as part of IFN-mediated antiviral artillery, by degradation of target mRNAs (13, 53). In addition to the IFN-regulated gene-mediated suppression, a question of whether IP10 can directly inhibit oncogene transcription is worth asking. This question is raised based on recent reports that introduction of a bovine or human papillomavirus E2 gene into HeLa cells could repress oncogene transcription and activate the p53 pathway (9, 14, 65). This E2 protein negatively regulates oncogene expression through direct interaction with their promoters, inhibiting transcription (41). Whether IP10 can function in a similar way as E2 needs to be elucidated. Regardless of whether all these speculated mechanisms are involved in IP10-induced suppression of E6 expression, IP10 is an ideal candidate for further investigation of its potential anticancer activity.

The role of E7 in oncogenesis has been established in several HPV-positive cell lines (11, 54); however the effects of E7 suppression on cell proliferation, senescence or apoptosis are controversial. There are reports indicating that E7 expression leads to an increase in the free E2F transcription factor, a condition that induces p53-mediated apoptotic cell death (45, 49, 65). However, several other reports indicated that E7 plays a role in cell immortalization (3, 26) and thus inhibition of E7 alone or both E6 and E7 could enhance the p53-mediated apoptosis pathway (9, 22, 66). This inconsistency may be attributed to the differences in cell types or experimental systems used. In our system, we found that IP10 induced downregulation of E7 and this suppression enhanced p53 production. These data have been further validated by E7 gene silencing with siRNAs. From this point of view, E7 may also be a target for anticancer gene therapy.

In summary, IP10 expression plays an important role in inducing apoptosis and in viral clearance. We have verified this in CVB infection by demonstrating the reduction of VP1 protein synthesis and inhibition of viral plaque formation. However, this antiviral activity could be blocked by the expression of a DN/p53 gene. Since p53 is a central player in the IP10-induced apoptotic pathway, this finding reveals a novel antiviral mechanism by which IP10 inhibits viral replication via nonimmune responses, which can be summarized as follows (Fig. 11). CVB3 infection triggers IP10 expression and further induces apoptosis by suppression of HPV E6 and E7 expression and upregulation of the p53 gene, followed by the death signal transduction from p53 to downstream targets PUMA and Bax, and finally to the mitochondrial outer membrane proteins. The induced premature cell death is beneficial to the host by limiting viral replication. This programmed process could reduce viral titer and inhibit its spread for reinfection of surrounding cells. From this consideration, the present study has revealed a previously unrecognized link between IP10 and p53 in antiviral defense. In addition, the suppression of E6 and E7 oncogene expression by IP10 implies the potential of IP10 as an anticancer agent for HPV infection.

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