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Interferon Kappa Inhibits Human Papillomavirus 31 Transcription by Inducing Sp100 Proteins

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ABSTRACT

High-risk human papillomaviruses (hr-HPV) establish persistent infections in keratinocytes, which can lead to cancer of the anogenital tract. Interferons (IFNs) are a family of secreted cytokines that induce IFN-stimulated genes (ISGs), many of which display antiviral activities. Transcriptome studies have indicated that established hr-HPV-positive cell lines display a reduced expression of ISGs, which correlates with decreased levels of interferon kappa (IFN-κ), a type I IFN constitutively expressed in keratinocytes. Prior studies have also suggested that IFN-β has anti-hr-HPV activity but the underlying mechanisms are not well understood. The downregulation of IFN-κ by hr-HPV raises the possibility that IFN-κ has anti-HPV activity. Using doxycycline-inducible IFN-κ expression in CIN612-9E cells, which maintain extrachromosomally replicating HPV31 genomes, we demonstrated that IFN-κ inhibits the growth of these cells and reduces viral transcription and replication. Interestingly, the initiation of viral early transcription was already inhibited at 4 to 6 h after IFN-κ expression. This was also observed with recombinant IFN-β, suggesting a common mechanism of IFNs. Transcriptome sequencing (RNA-seq) analysis identified 1,367 IFN-κ-regulated genes, of which 221 were modulated >2-fold. The majority of those (71%) matched known ISGs, confirming that IFN-κ acts as a *bona fide* type I IFN in hr-HPV-positive keratinocytes. RNA interference (RNAi) and cotransfection experiments indicated that the inhibition of viral transcription is mainly due to the induction of Sp100 proteins by IFN-κ. Consistent with published data showing that Sp100 acts as a restriction factor for HPV18 infection, our results suggest that hr-HPV target IFN-κ to prevent Sp100 expression and identify Sp100 as an ISG with anti-HPV activity.

IMPORTANCE

High-risk HPV can establish persistent infections which may progress to anogenital cancers. hr-HPV interfere with the expression of interferon (IFN)-stimulated genes (ISGs), which is due to reduced levels of IFN-κ, an IFN that is constitutively expressed in human keratinocytes. This study reveals that IFN-κ rapidly inhibits HPV transcription and that this is due to the induction of Sp100 proteins. Thus, Sp100 represents an ISG for hr-HPV.

Persistent infections with high-risk human papillomaviruses (hr-HPV) are a necessary risk factor for the development of anogenital and oropharyngeal cancers (1). HPV have circular double-stranded DNA (dsDNA) genomes of ~8,000 bp and infect keratinocytes of the basal layer of mucosal and cutaneous epithelium. In undifferentiated cells, HPV genomes replicate as extrachromosomal elements at a low copy number and express only early viral genes such as those for the oncoproteins E6 and E7 and the replication proteins E1, E2, and E8^E2C (2). hr-HPV E6 and E7 interact with critical regulators of the cell cycle such as p53 and retinoblastoma family members to ensure continuous proliferation of infected cells (3).

Transcriptome studies have shown that interferon (IFN)-stimulated genes (ISGs) are expressed at lower levels in hr-HPV-positive cell lines than in their uninfected counterparts (4–6). Type I IFNs such as IFN-α subtypes or IFN-β are induced upon viral infection and then secreted into the extracellular space (7). Secreted IFNs bind to the respective receptor on infected and neighboring cells, and kinases are activated, which results in the nuclear translocation of transcription factors such as STAT1 which then induce several hundred ISGs, many of which have direct antiviral activities (8).

The treatment of cell lines that maintain persistently replicating extrachromosomal HPV16 or -31 genomes with recombinant IFN-β resulted in growth retardation and the induction of apoptosis, which did not occur with HPV-negative keratinocytes (9, 10). A detailed analysis of the HPV16 copy number and transcription upon IFN-β treatment indicated that first the viral copy number is decreased, followed by a reduction of viral transcription (10). HPV31-positive CIN612-9E cells have reduced levels of STAT1, which is both an ISG and also a crucial transcription factor of the IFN signal transduction cascade. Reexpression of STAT1 resulted in a reduction of viral genomes (11). Taken together, these data strongly suggest that IFNs induce ISGs that inhibit the replication of HPV. In line with this, the ISG IFIT1 (or ISG56) directly interacts with the E1 helicases of HPV11 and -18, which results in the inhibition of the replication of the viral origin (12).

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The reduction of ISG expression in HPV-positive keratinocytes correlates with the inhibition of IFN-κ, which is an unusual member of the type I IFN family as it is constitutively expressed at high levels in normal keratinocytes and only weakly responds to inducers of IFN-αs and -β (13–16). The inhibition of IFN-κ expression is caused by the hr-HPV E6 oncoprotein and involves the induction of DNA methylation at the IFN-κ promoter (15, 16). This raises the question of whether HPV prevent IFN-κ expression because it displays anti-HPV activity by regulating ISGs in keratinocytes.

MATERIALS AND METHODS

Plasmids. Plasmid pENTR4-IFN-κ was constructed by subcloning a BglII fragment from pSG-IFN-κ (15) into the BamHI site of a modified pENTR4 plasmid (Invitrogen). The pInducer 20-IFN-κ plasmid was constructed by *in vitro* recombination of pInducer20 (17) with pENTR4-IFN-κ. Reporter plasmids pGL18 URR and pGL31 URR have been previously described (18). Expression plasmids for SP100 isoforms and the shSp100 expression construct and the respective control were kindly provided by S. Schreiner and have been previously described (19–21). The lentiviral packaging and envelope plasmids psPAX2 (Addgene plasmid 12260) and pMD2.G (Addgene plasmid 12259) were a gift from Didier Trono.

qPCR analysis. RNA was isolated using the RNeasy minikit (Qiagen). RNA (1 μg) was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen). Twenty-five nanograms of cDNA was analyzed in duplicate reaction mixtures by quantitative PCR (qPCR) using 0.3 μM gene-specific primers and 1× LightCycler 480 SYBR green I Master (Roche Applied Science) in a total volume of 20 μl as previously described (15). Relative expression levels were calculated using PGK1 as a reference gene as described previously (22). For the absolute quantification of Sp100 isoform expression levels, standard curves for PGK1 and Sp100 isoforms were generated using plasmid DNA and then used to determine copy numbers for Sp100 isoforms and PGK1 present in 25 ng of cDNA. RNA copy numbers were then corrected for the corresponding PGK1 value. The following gene-specific primers were designed with Primer 3 plus software (23): PGK1 sense (5′-CTGTGGGGGTATTTGAATGG-3′) and antisense (5′-CTTCCAGGAGCTCCAAACTG-3′), HPV31 E2 sense (5′-CTGTTGTGGAAGGGCAAGTT-3′) and antisense (5′-TCCCAGCAAAGGATATTTCG-3′), HPV31 E7 sense (5′-TAGGAGGAAGGTGGACAGGA-3′) and antisense (5′-GCTGTCGGGTAATTGCTCAT-3′), HPV16 E2 sense (5′-TGGAAGTGCAGTTTGATGGA-3′) and antisense (5′-CCGCATGAACTTCCCATACT-3′), HPV16 E7 sense (5′-GACAAGCAGAACCGGACAGA-3′) and antisense (5′-ACCGAAGCGTAGAGTCACAC-3′), BATF2 sense (5′-GACCCAGACAGACCCCAAG-3′) and antisense (5′-GTCTGCCTTGTCTGTGTGCT-3′), Sp100 sense (5′-TGAGAAGCAAGCATGGTGAG-3′) and antisense (5′-TTCAGGTTCTTGTGGCTGTG-3′), Sp100A sense (5′-GCATGGTGAGAAGGCTCCTA-3′) and antisense (5-CTAATCTTCTTTACCTGACCCTCT-3′), Sp100B sense (5′-TCACTCCCAGGGAATTTGAA-3′) and antisense (5′-TCACTTGATCATCACCTTTTTTCT-3′), Sp100C sense (5′-GTGAGGTGTGCAACAAATGG-3′) and antisense (5′-CTTTCTGGGCATCTTTCCTG-3′), and Sp100HMG sense (5′-GGACAAGGCCCATTATGAAA-3′) and antisense (5′-AGGCCAGGATGTTCTCCTTT-3′). The primer pair for IFIT1 (QT01852466) was commercially obtained (Qiagen).

To determine the HPV31 viral copy number, total genomic DNA from CIN612-9E/ind IFN-κ was isolated using the EZ1 instrument and EZ1 DNA tissue kit (Qiagen). Relative expression of viral copy numbers was determined using HPV31 E7 primers and PGK1 as a reference gene (22).

Cell culture. HPV16 wild-type-positive cell lines have been previously described (24, 25). The LKP1 and the CIN1 lesion-derived CIN612-9E cell lines contain replicating HPV31 genomes and have been described before (26, 27). HPV-positive keratinocyte cell lines were cocultured with mitomycin C-treated NIH 3T3 J2 fibroblasts in E medium supplemented with 5% fetal bovine serum as described previously (27).

For 2-, 4-, and 6-day measurements, 1×10^5 CIN612-9E/ind IFN-κ cells were seeded with mitomycin C-treated NIH 3T3 J2 fibroblasts in 6-well dishes 24 h before doxycycline (1 μg/ml) treatment. Fresh medium and doxycycline was added every other day, and cells were harvested 2, 4, or 6 days later. NIH J2-3T3 feeder cells were removed before harvesting keratinocytes. For growth curves, cells were trypsinized and counted in a Neubauer chamber. To extract protein, RNA, or DNA, cells were pelleted and lysed in the appropriate buffer.

For short-term measurements, 2.5×10^5 cells were seeded in 6-well dishes, and 24 h after seeding, cells were treated with doxycycline (1 μg/ml) or different concentrations of IFN-β (PBL Assay Science, 11415-1).

To obtain stable cell lines expressing pInducer/pInducer-IFN-κ or shControl/shSp100, amphotrophic lentiviruses were generated by transiently transfecting 293T cells with 1.5 μg each of the pInducer or short hairpin RNA (shRNA) lentiviral vector and the lentiviral packaging plasmids psPAX2 and pMD2.G using FuGENE HD reagent (Promega) and Opti-MEM (Life Technologies). CIN612-9E cells were infected and maintained on mitomycin C-treated NIH 3T3 J2-NHP fibroblasts in E medium. Cells were selected with G418 (300 μg/ml) or puromycin (0.6 μg/ml). For IFN-β treatment, 3×10^5 cells were seeded in 6-well dishes and treated 24 h later for 6 h.

RNA sequencing and data analysis. Sequencings were obtained from 1 μg total RNA using the TruSeq Stranded total RNA sample prep kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Each sequencing library was tagged with a 6-nucleotide (nt)-long barcode. After size selection, equimolar amounts of all six libraries were pooled and loaded onto two lanes of an Illumina single-read flow cell, and bound molecules were clonally amplified on a cBot instrument. Subsequently, the first 50 nt from each fragment were sequenced on an Illumina Genome Analyzer IIx, followed by a 7-nt sequencing run to decipher the barcode sequence in the adapter. In total, 4.49 Gb of sequence was generated. The raw base calls were simultaneously demultiplexed and transformed into fastq files, containing the sequence and an ASCII-coded PHRED quality score for each sequence read. The fastq files of each sample were merged and quality controlled using the fastqc quality control tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The sequences were first aligned against the *Homo sapiens* genome (hg19) using STAR with default parameters (28). Based on the alignment results, read counting was performed using htseq-count in the intersection-nonempty mode, and the resulting counts were summarized on the gene level (29). Additionally, all reads were aligned against the HPV31 genome (GenBank accession no. J04353.1) with tophat using default parameters (30). The statistical data analysis was performed with the Bioconductor R package "edgeR" (31). The raw count data were first normalized based on "counts per million mapped counts" (CPM), to account for differences in sequencing depth. Features with less than 10 reads in any sample were removed from the data set. A total of 13,022 out of 23,750 genes were included in the final analysis stage.

To account for cultivation effects, a design matrix was used to estimate the Cox-Reid common and tag-wise dispersion for the fitting of the negative binomial (NB) distribution, and then for each features an NB generalized linear model was fitted. The coefficients of these models were then used to assess differential expression using a likelihood ratio test. The resulting *P* values are adjusted for multiple testing as described by Benjamini and Hochberg (32), and a false-discovery rate (FDR) of <0.05 was chosen to extract differentially expressed genes. In addition, a gene ontology enrichment analysis was conducted using the Bioconductor R package "goseq" (33). The resulting *P* values have been adjusted for multiple testing, again using the FDR method described by Benjamini and Hochberg (32).

Reporter assays. CIN612-9E cells (3×10^4) were seeded into 24-well dishes at 24 h before transfection. Cells were cotransfected with 100 ng of pGL18 URR or pGL31 URR reporter plasmid and 100 ng of the vector control (pSG5) or Sp100 A, -B, -C, or -HMG expression vector using the FuGENE HD reagent (Promega) and Opti-MEM (Life Technologies). Furthermore, 0.5 ng of pCMV-Gluc plasmid (New England BioLabs) was

included as an internal control. *Gaussia* and firefly luciferase assays were carried out 48 h after transfection.

4sU labeling. CIN612-9E/ind IFN-κ cells (2×10^6) were seeded in six 100-mm dishes, and doxycycline (1 μg/ml) was added to three of the dishes 24 h later. Four hours later, 100 μM 4-thiouridine (4sU) (Sigma-Aldrich) was added to all six dishes. After 1 h, cells were lysed in RLT buffer (Qiagen), and RNA was isolated using the RNeasy minikit (Qiagen). Biotinylation and purification of 4sU-labeled RNA were carried out as previously described (34). Extracted RNA was reverse transcribed using the QuantiTect reverse transcription kit (Qiagen) and analyzed in a LightCycler 480 (Roche Applied Science).

Immunoblotting. Preparation of whole-cell extracts was done by lysing cell pellets in 20 to 30 μl of 4× SDS gel loading buffer (Carl Roth) and heating them to 95°C for 5 min. After brief sonication, equal amounts of extracts were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. After blocking with 5% milk powder in Tris-buffered saline–1% (vol/vol) Tween 20, the membranes were incubated overnight with diluted primary antibodies against HSP90 (Santa Cruz BT; sc-69703; 1:2,000), IFN-κ (Abnova; H00056832-M01; 1:500); IFIT1 (GeneTex; GTX103452; 1:1,500), p53 (Santa Cruz BT; sc-126; 1:1,000), Sp100 (Sigma; HPA016707; 1:200), or alpha-tubulin (Calbiochem; CP06; 1:1,000). Bound antibodies were detected with fluorescence-labeled secondary antibodies (Li-Cor Bioscience, IRDye 680RD goat anti-mouse IgG [925-68070] and IRDye 800CW goat anti-rabbit IgG [925-68071], dilution of 1:15,000) and an OdysseyFC infrared imaging system (Li-Cor Biosciences).

Southern blotting. Total cellular DNA from CIN612-9E cells was isolated and analyzed by Southern blotting as described previously (35).

siRNA transfection. CIN612-9E/ind IFN-κ cells (2×10^5) were transfected with control small interfering RNA (siRNA) (90 pmol; AllStars negative control [Qiagen, 1027281]), siBATF2 (90 or 360 pmol; Qiagen, 1027416) or siSp100 (90 pmol; Dharmacon, GE Healthcare, L-015307-00-0005) in 6-well plates using Lipofectamine RNAiMAX (Invitrogen). Doxycycline (1 μg/ml) was added at 48 h posttransfection, and 6 h later cells were lysed in RLT buffer (Qiagen) for qPCR analysis or in 4× SDS gel loading buffer (Carl Roth) to obtain whole-cell extracts for immunoblotting.

Flow cytometry. CIN612-9E/ind IFN-κ cells (1.5×10^5) were seeded in 6-well plates, and 1 μg/ml doxycycline was added the next day. Cells were harvested 2, 4, and 6 days later. Cell pellets were lysed in 1 ml of fluorochrome solution (36). Flow cytometry was carried out in a FACSCalibur (BD Biosciences) as previously described (36). The results were analyzed using the CellQuest Pro software (BD Biosciences).

Microarray data accession number. Transcriptome sequencing (RNA-seq) data have been deposited in the NCBI Gene Expression Omnibus (GEO) database under accession no. GSE74658.

RESULTS

The expression of IFN-κ in CIN612-9E cells reduces cell proliferation, viral genome copies, and viral transcription. In contrast to many other IFNs, biologically active IFN-κ is not commercially available. To analyze the effects of IFN-κ on HPV-positive cells, we therefore constructed a lentiviral vector from which IFN-κ can be expressed in a doxycycline-dependent manner (pInd20-IFN-κ). CIN612-9E cells, which harbor extrachromosomally replicating HPV31 genomes and have been previously shown to have an epigenetically silenced IFN-κ gene (15), were transduced with Ind20 as a control or Ind20-IFN-κ lentiviruses, and stable cell lines were obtained by drug selection. Southern blot analysis revealed that HPV31 genomes were maintained extrachromosomally at similar copy numbers in CIN612-9E/ind and CIN612-9E/ind-IFN-κ cells (Fig. 1A). The addition of doxycycline to CIN612-9E/ind-IFN-κ cells resulted in a robust induction of IFN-κ protein. The lower-migrating form detected by the IFN-κ antibody corresponds to a protein of 22 kDa, which matches the size of a processed form from which the predicted signal peptide has been cleaved off. Expression of the ISG IFIT1 was also upregulated, which indicated that the induced IFN-κ is biologically active (Fig. 1B). Next, the growth of doxycycline-treated or untreated CIN612-9E/ind and CIN612-9E/ind-IFN-κ cells was determined. This indicated that the addition of doxycycline had only very minor effects on the growth of CIN612-9E/ind cells, whereas the expression of IFN-κ inhibited the growth of CIN612-9E cells, which was most prominent at days 4 and 6 of IFN-κ expression (Fig. 2A and B). To analyze the effects on viral replication and transcription, total DNA, E2, and E7 transcripts were analyzed by qPCR. This revealed that viral gene expression was reduced to 50 to 60% at day 2 and slightly further repressed at days 4 and 6 (Fig. 2C). Interestingly, the viral copy number was only moderately changed at day 2 but decreased at days 4 and 6 (Fig. 2C). To address the consequences of reduced viral transcription and genome copies, the amount of the E6 target protein p53 and cell cycle profiles were analyzed in the absence and presence of doxycycline over 6 days. This showed that p53 protein levels were increased at day 2 (1.5-fold), day 4 (2-fold), and day 6 (2.1-fold) after IFN-κ induction (Fig. 3A). In addition, IFN-κ induction resulted in a gradual increase of cells in the G_1 phase and a decrease of cells in the G_2/M phase over time, but no changes in apoptosis (fraction of cells with a sub-G_1 DNA content) were observed (Fig. 3B). This experiment was repeated three times with similar outcomes. These findings are similar to the effects reported by the reduction of E6 and E7 transcript levels in cervical cancer cell lines by siRNA or expression of E2 or E8^E2C proteins (18, 37–40) and suggested that the decrease of viral genome copies and transcription levels resulted in lowered levels of the E6 and E7 oncoproteins, which causes a reduced proliferation.

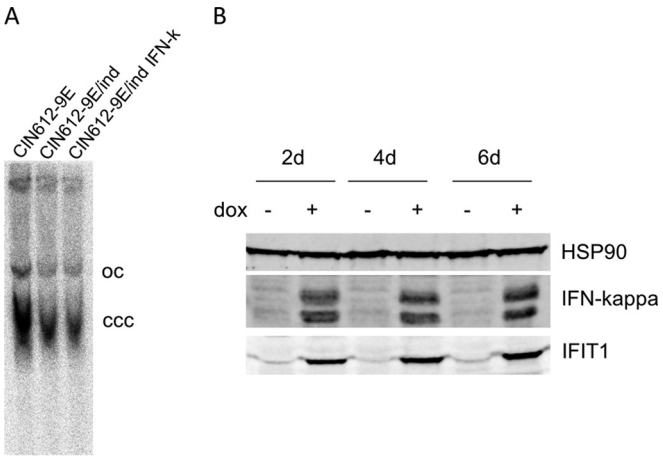


FIG 1 Characterization of CIN612-9E/ind and CIN612-9E/ind-IFN-κ cell lines. (A) Southern blot analysis of total cellular DNA from CIN612-9E (control), CIN612-9E/ind, and CIN612-9E/ind-IFN-κ cell lines. Open-circle (oc) and covalently closed circular (ccc) forms of HPV31 genomes are indicated. (B) Western blot analysis of CIN612-9E/ind-IFN-κ cells for IFN-κ and IFIT1 expression in the absence and presence of doxycycline (dox). Whole-cell lysates were prepared and analyzed by immunoblotting for the presence of IFN-κ and IFIT1 using HSP90 as a loading control.

IFN-κ represses viral transcription very rapidly. The data suggested that viral transcription is reduced before the viral genome copy number drops. To analyze this in more detail, total

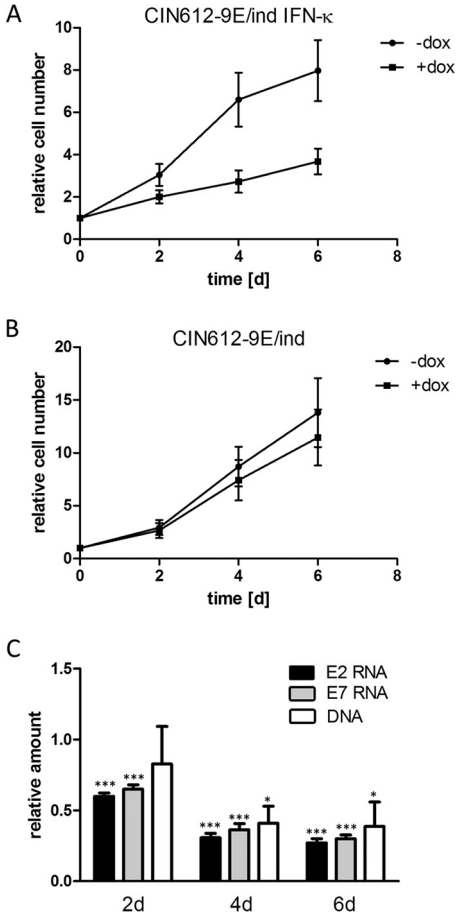
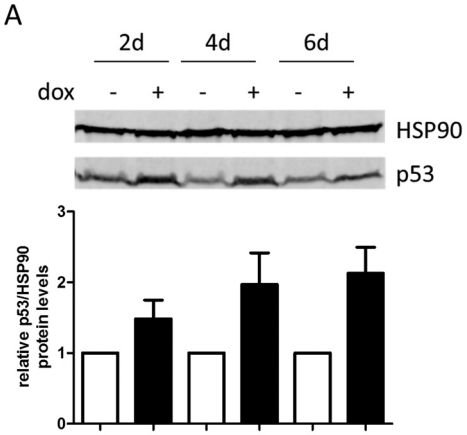


FIG 2 IFN-κ expression reduces cell proliferation, viral genome copies, and viral transcription. (A and B) CIN612-9E/ind-IFN-κ cells were seeded 1 day prior to doxycycline treatment. On the next day (day 0), cells were either treated with doxycycline (1 μg/ml) or left untreated. One dish from each treatment was harvested on days 2, 4, and 6, and viable cells were counted. Cell numbers are presented relative to day 0. The data represent the averages from three independent experiments, and error bars indicate the standard error of the mean (SEM). (C) RNA or total genomic DNA from doxycycline-treated CIN612-9E/ind-IFN-κ cells was analyzed by qPCR for expression of viral transcripts or viral copy number. Relative RNA expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. Relative DNA copy numbers were calculated using HPV31 E7 primers and PGK1 as a reference gene. The data represent the averages from three independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in untreated and treated cells was determined by one-way analysis of variance (ANOVA) and Dunnett's posttest (*, $P < 0.05$; ***. $P < 0.001$).



B

	2d		4d		6d	
	-dox	+dox	-dox	+dox	-dox	+dox
sub-G1	1.2	1.1	1.3	1.4	2.3	2.0
G1	72.8	75.6	72.5	78.5	64.4	76.2
S	4.0	3.5	2.2	1.9	2.6	2.2
G2/M	20.8	17.9	21.9	15.2	26.0	16.0

FIG 3 IFN-κ expression leads to p53 accumulation and changes in the cell cycle profile (A) Western blot analysis of p53 expression in CIN612-9E/ind-IFN-κ cells in the absence and presence of doxycycline (dox). Whole-cell lysates were prepared and analyzed by immunoblotting for the presence of p53 using HSP90 as a loading control. The quantification of four independent experiments is shown below the blots. Data are shown relative to each time point, and error bars indicate the SEM. (B) Flow cytometry analysis of the DNA content by propidium iodide staining of CIN612-9E/ind-IFN-κ cells. Sub-G_1 DNA content (<2n) indicates apoptotic cells, whereas G_1, S, and G_2/M represent cells in the different phases of the cell cycle. Shown are the data from one representative experiment out of three.

RNA was isolated at 2, 4, and 6 h after induction of IFN-κ expression. Interestingly, viral transcript levels were reduced to 50% already at 4 h and to 40% at 6 h compared to those in the untreated control (Fig. 4A). To confirm the effects of IFN-κ in a different cell line, we performed supernatant transfer experiments with the LKP-1 cell line, a human keratinocyte cell line that maintains episomal HPV31 genomes (27). CIN612-9E/ind-IFN-κ cells were incubated with or without doxycycline for 24 h. Cell-free supernatants were obtained by filtration, and immunoblots indicated that alpha-tubulin, a cytoskeleton protein, is present in very small amounts, whereas a robust IFN-κ signal can be detected only in the supernatant from doxycycline-treated cells (Fig. 4C). Equal volumes of supernatants were added to LKP-1 cells, and 6 h later RNA was isolated and analyzed by qPCR. Addition of IFN-κ-positive supernatants induced IFIT1 transcripts 63-fold and led to a significant reduction of HPV31 E2 and E7 transcript levels, comparable to those for induced CIN612-9E/ind-IFN-κ cells (Fig. 4B). This clearly suggested that the inhibition of viral transcription by IFN-κ is a very early event and precedes the reduction of viral genomes. A previous study by Chang and Laimins (4) observed similar growth-inhibitory effects and a long-term reduction of viral genomes by recombinant IFN-β on HPV31-positive cells, but viral transcription was not analyzed. We speculated that IFN-β may have the same effect as IFN-κ and treated CIN612-9E and LKP-1 cells with increasing amounts of recombinant IFN-β (50 to 1,000 U) for 6 h and analyzed viral transcription by qPCR (Fig. 4D and E). This revealed that viral transcription is reduced in a concentration-dependent manner to levels similar to those observed for IFN-κ in CIN612-9E and LKP-1 cells (Fig. 4A and B). This indicated that the rapid reduction of viral transcription is shared by IFN-κ and IFN-β. We also investigated the effects of IFN-β on HPV16 transcription. Three different stable human keratinocyte cell lines that maintain extrachromosomal genomes (24, 25) were treated with IFN-β (1,000 U/ml) for 6 h, and then E2 and E7 transcripts were analyzed by qPCR. Depending on the cell line, HPV16 transcripts were reduced to 60 to 80% (Fig. 4F).

To investigate whether initiation of transcription is influenced

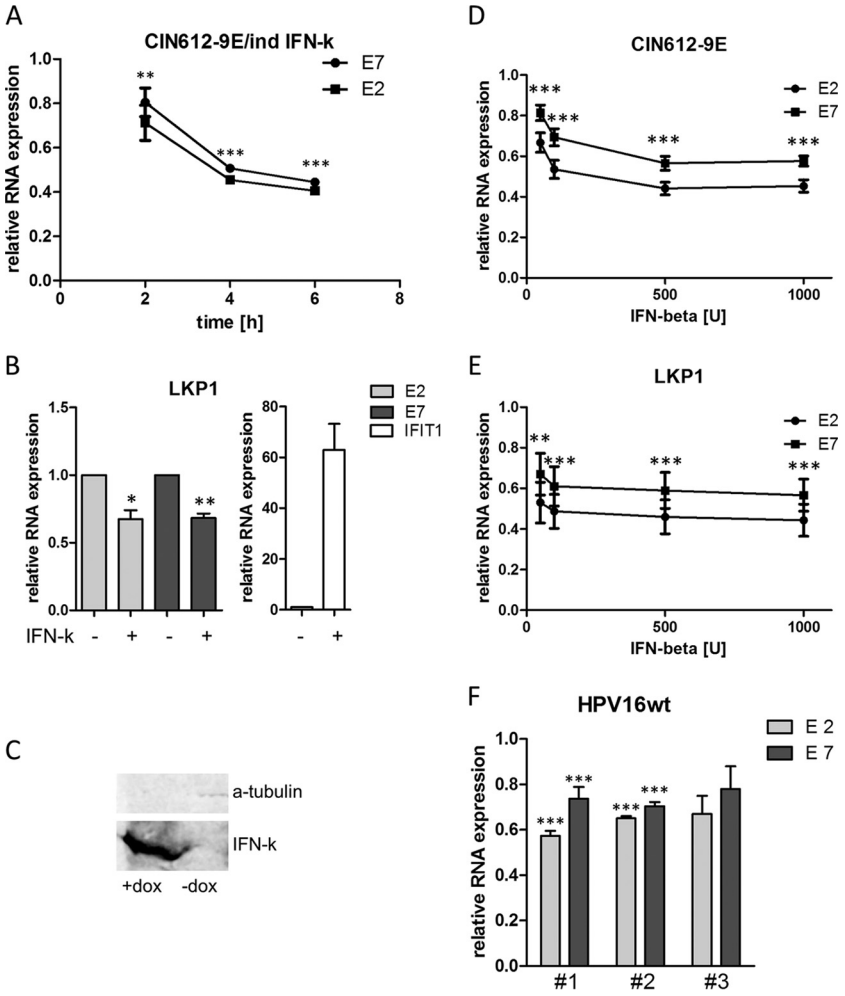


FIG 4 Rapid repression of viral transcription by IFN-κ and IFN-β. (A) CIN612-9E/ind-IFN-κ cells were incubated with doxycycline for 2, 4, and 6 h. RNA was analyzed by qPCR for expression of viral transcripts. Relative expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. The data represent the averages from four independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in untreated and treated cells was determined by one-way ANOVA and Dunnett's posttest (**, $P < 0.01$; ***. $P < 0.001$). Significance levels were identical for E2 and E7. (B) LKP-1 cells were incubated with cell-free supernatants from untreated (−) or doxycycline-treated (+) CIN612-9E/ind-IFN-κ cells, and HPV31E2, E7, and IFIT1 transcript levels were determined by qPCR as described for panel A. The data represent the averages from three independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in untreated and treated cells was determined by a paired Student *t* test (*, $P < 0.05$; **, $P < 0.01$). (C) Immunoblot analysis of α-tubulin and IFN-κ protein levels in cell-free supernatants from untreated (−dox) or doxycycline-treated (+dox) CIN612-9E/ind-IFN-κ cells. (D and E) LKP1 and CIN612-9E cells were treated with increasing amounts of IFN-β for 6 h. RNA was analyzed by qPCR for expression of viral transcripts. Relative expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. The data represent the averages from at least three independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in untreated and treated cells was determined by one-way ANOVA and Dunnett's posttest (**, $P < 0.01$; ***. p0.001). Significance levels were identical for E2 and E7. (F) Different HPV16wt cell lines (1 to 3) were treated with IFN-β (1,000 U/ml) for 6 h. RNA was analyzed by qPCR for expression of viral transcripts. Relative expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. The data represent the averages from three (lines 1 and 2) or two (line 3) independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in untreated and treated cells was determined by one-way ANOVA and Dunnett's posttest (***, $P < 0.001$).

by IFN-κ, RNA labeling experiments were carried out. IFN-κ expression was induced or not in CIN612-9E/ind-IFN-κ cells, and 100 μM 4-thiouridine was added to the culture medium 4 h later to label newly synthesized RNA. After 1 h of labeling, total RNA was isolated, *in vitro* biotinylated, affinity purified on streptavidin-coated magnetic beads, and then analyzed by qPCR. This revealed that the amounts of newly synthesized viral RNA were reduced compared to those in the uninduced control (Fig. 5), which strongly suggested that IFN-κ targets mainly the initiation of viral transcription.

Identification of IFN-κ-regulated genes in CIN612-9E cells. No global transcriptome analyses of IFN-κ-regulated genes in normal keratinocytes or HPV-positive keratinocytes are currently available. To identify IFN-κ-regulated genes that mediate the repression of viral transcription, RNA was isolated from CIN612-9E/ind-IFN-κ cells at 4 h after the addition of doxycycline or without doxycycline ($n = 3$) and analyzed by transcriptome sequencing (RNA-seq). Reads per sample ranged from 13.1 to 14.6×10^6, with a Spearman rank correlation of >0.975. A total of 1,362 significantly regulated cellular genes were identified. Of

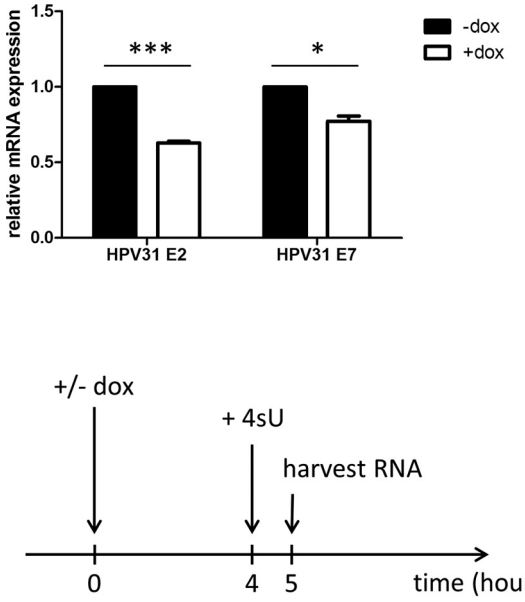


FIG 5 CIN612-9E/ind-IFN-κ cells were treated with doxycycline for 5 h. One h prior to harvesting, 4-thiouridine (4sU) was added to label newly synthesized RNA. After extraction, 4sU-labeled RNA was analyzed by qPCR for expression of viral transcripts. Relative expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. The data represent the averages from three independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in untreated and treated cells was determined using a two-tailed paired *t* test (*, $P < 0.05$; ***, $P < 0.001$).

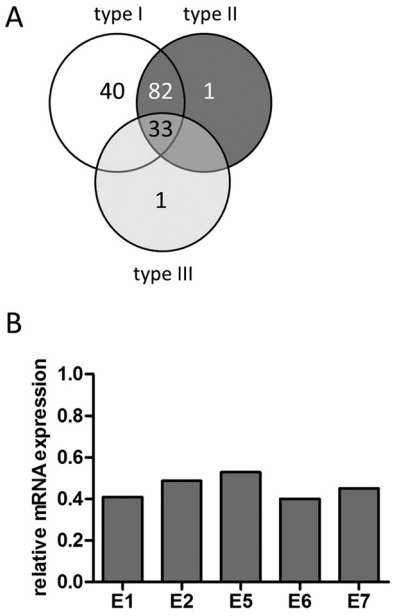


FIG 6 IFN-κ regulated genes. (A) Overlap between >2-fold IFN-κ-regulated genes, identified by RNA-seq in CIN612-9E/ind IFN-κ cells, and type I, II, and III IFN-regulated human genes extracted from the Interferome database. (B) Transcription of HPV31 early genes determined by RNA-seq in CIN612-9E/ind IFN-κ cells. Data are shown relative to untreated cells.

these, 193 genes were induced between 2- and 252-fold, and 28 genes were inhibited between 2- and 3.8-fold (see Table S1 in the supplemental material). Common targets between >2-fold IFN-κ-regulated genes with a corrected *P* value of ≤0.05 in CIN612-9E cells and human genes regulated by IFNs were analyzed using the Interferome database (v2.01) (41). This database contains experimentally and statistically (*P* value of ≤0.05 and fold change of ≥2) validated IFN-regulated genes. For humans, a total of 4,958 different genes are currently present in the database, of which 3,139 correspond to type I IFN, 2,864 to type II IFN, and 164 to type III IFN. Comparing the 221 differentially expressed genes to the genes in the database revealed that 157 (71.0%) of these have previously been identified as IFN regulated in humans. When using the 4-h and 6-h time points after IFN induction as the basis for comparison, a total of 105 (47.5%) out of the 221 could be identified in the database for the 4-h time point and 146 (66.0%) for the 6-h time point. A detailed analysis of the interferon types of the 157 IFN-regulated human genes showed that 33 are regulated by type I, II, and III IFNs, 82 by type I and II, 40 by type I, and 1 each by type II and III (Fig. 6A; see Table S1 in the supplemental material). When comparing IFN-κ-induced genes in CIN612-9E cells with HPV18-repressed genes in human keratinocytes, which have been shown to have reduced IFN-κ levels (5, 15), an overlap of 30/193 (15.5%) genes was evident. In summary, this indicated that IFN-κ induces a transcriptional program in CIN612-9E that is very similar to that for other IFNs in different experimental systems and also partially matches the effects of hr-HPV in human keratinocytes. A gene ontology enrichment analysis (FDR, <0.001) identified "immune system process" (GO:0002376; adjusted *P* value = 7.13E−31)," immune response" (GO:0006955; adjusted *P* value = 3.66E−26), "defense response" (GO:0006952; adjusted *P* value = 8.59E−25), and "response to type I interferon" (GO:0034340; adjusted *P* value = 8.59E−25) as the major categories, consistent with the idea that IFN-κ activates a type I IFN expression program in HPV-positive cells. Furthermore, the RNA-seq analysis revealed that the transcription of the HPV31 early genes E1, E2, E5, E6, and E7 was decreased to 40 to 53% by IFN-κ, confirming the qPCR data (Fig. 6B).

Knockdown of Sp100 increases HPV31 transcript levels. We then investigated the contribution of the IFN-κ-induced BATF2 and Sp100 genes to the inhibition of HPV31 transcription. BATF2 has been described as an IFN-β-induced inhibitor of AP1-dependent gene expression (42) and thus may act on the AP1-dependent enhancer of hr-HPV, which is crucial for viral early gene transcription (43). However, an efficient knockdown of IFN-κ-induced BATF2 by single or combinations of siRNAs had no influence on the IFN-κ-mediated inhibition of viral transcripts (data not shown). Sp100 is a component of ND10 bodies and has been described to inhibit the transcription of dsDNA viruses such as herpes simplex virus 1 (HSV-1), human cytomegalovirus (HCMV), and HPV18 during initial infection (44–47). CIN612-9E/ind-IFN-κ cells were transfected with an siRNA against Sp100 or a negative-control siRNA, and 48 h later IFN-κ expression was induced for 6 h. RNA and proteins were isolated and analyzed by qPCR or immunoblotting. IFN-κ induced Sp100 RNA 6.1-fold in siControl-transfected cells (Fig. 7A). In siSp100-transfected cells, the amount of Sp100 RNA in both uninduced and induced cells was reduced ~10-fold compared to the respective siControl (Fig. 7A). Immunoblots confirmed that Sp100 protein isoforms are induced by IFN-κ and that the amounts of all isoforms are reduced by siSp100 (Fig. 7A). As a control, the activation of IFIT1 transcripts was determined by qPCR. IFIT1 was induced 99-fold in

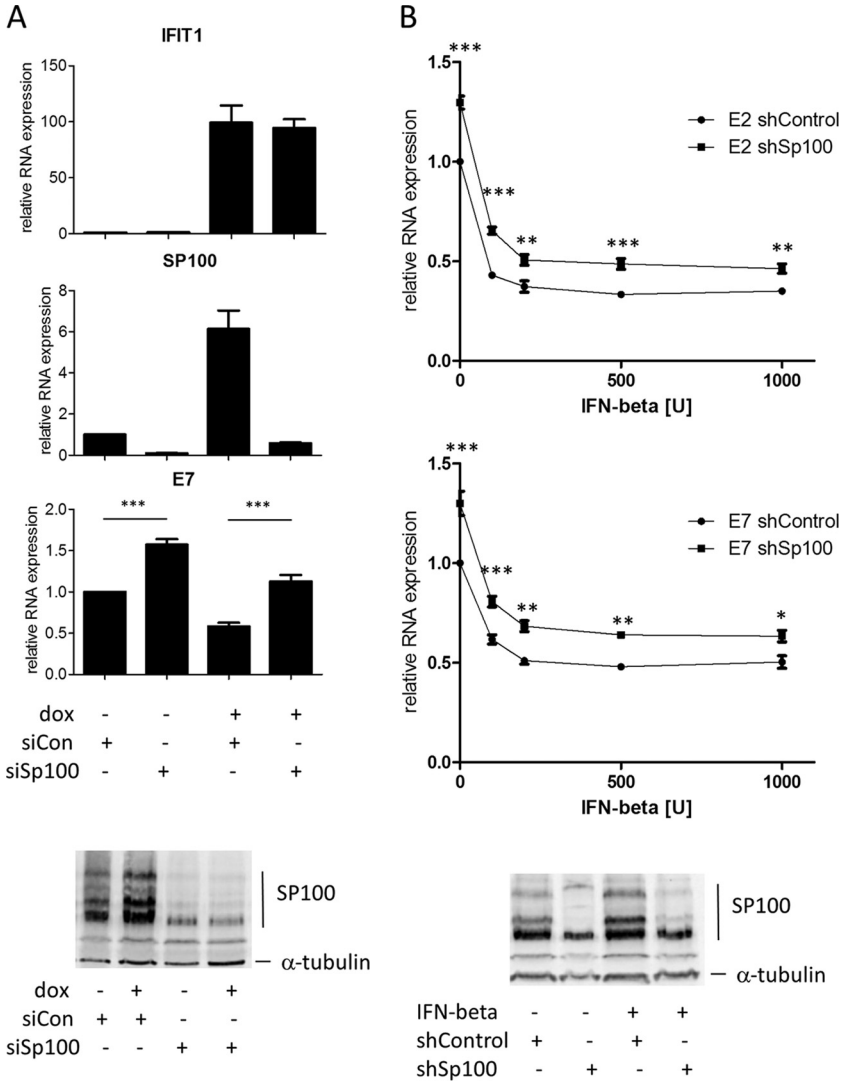


FIG 7 Influence of Sp100 knockdown on viral transcription in CIN612-9E cells. (A) CIN612-9E/ind-IFN-κ cells were transfected with a control siRNA or an siRNA against Sp100, and 48 h later IFN-κ expression was induced for 6 h. RNA was analyzed by qPCR for the expression of IFIT1, Sp100, and HPV31 E7. Relative expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. The data represent the averages from four independent experiments, and error bars indicate the SEM. Statistical significance between the E7 expression in control siRNA- or Sp100 siRNA-treated cells was determined using one-way ANOVA and Tukey's multiple-comparison test as a posttest (***, $P < 0.001$). Western blot analysis of CIN612-9E/ind-IFN-κ cells for Sp100 expression is also shown. Whole-cell lysates were prepared and analyzed by immunoblotting for the presence of Sp100 and α-tubulin as a loading control. (B) CIN612-9E cells stably transduced with either a lentiviral shControl or an shSp100 expression vector were treated with different concentrations of IFN-β for 6 h. RNA was analyzed by qPCR for expression of viral transcripts (E2 and E7). Relative expression values were calculated using PGK1 as a reference gene and are presented relative to untreated cells. The data represent the averages from three independent experiments, and error bars indicate the SEM. Statistical significance between expression levels in shControl- and shSp100-treated cells was determined using one-way ANOVA and Dunnett's posttest (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Western blot analysis of whole-cell lysates from CIN612-9E-shControl and -shSp100 cells for Sp100 and α-tubulin as a loading control is also shown.

siControl-transfected cells and 94-fold in siSp100-transfected cells by IFN-κ, indicating that siSP100 did not generally impede the ability of IFN-κ to induce ISG transcription. The reduction of Sp100 levels increased statistically significantly (1.6-fold) the amount of E7 RNA in the absence of IFN-κ and prevented the reduction by IFN-κ (0.58- versus 1.13-fold) (Fig. 7A). SiSp100 increased E7 transcript levels 1.6-fold in the absence of IFN-κ but 1.9-fold in its presence. Furthermore, Sp100 transcript levels were still elevated 5.5-fold by IFN-κ in the presence of siSp100. This most likely prevents E7 transcript levels from reaching the levels in siSp100-treated cells in the absence of doxycycline. In summary, this indicated that Sp100 proteins are involved in the reduction of viral transcription in the absence and presence of IFN-κ. To extend these findings to IFN-β, CIN612-9E cells that carry either a lentiviral shControl vector or a shSp100 expression vector were established (20). CIN612-9E/shControl or shSp100 cells were treated with different amounts of IFN-β for 6 h, and then the amounts of viral transcripts were determined by qPCR. This confirmed that the reduction of Sp100 by shRNA increased basal levels of viral transcription in the absence of IFN. Upon treatment with different amounts of IFN-β, viral transcription remained higher in shSp100 cells than in their control counterparts (Fig. 7b). This

suggested that Sp100 proteins also contribute to the inhibition of HPV31 transcription by IFN-β.

Regulation of Sp100 isoforms by IFN-κ and expression in stable HPV-positive cell lines. The Sp100 immunoblots suggested that in addition to the unmodified and SUMO-modified Sp100A isoform, which represent the two fastest-migrating species, other, slower-migrating isoforms are also present in CIN612-9E cells (Fig. 7). To get further insight into which isoforms are expressed and are regulated by IFN-κ, RNA from untreated or doxycycline-treated CIN612-9E/ind IFN-κ cells was isolated and analyzed by qPCR with specific primers for Sp100A, -B, -C, and -HMG and the cloned Sp100 isoforms as copy number standards (Fig. 8A). This revealed that Sp100HMG is the most abundant isoform but is not induced by IFN-κ. The second most abundant isoform is Sp100A, which is induced 3.7-fold by IFN-κ. Sp100B and -C are present at lower levels than Sp100A and are upregulated 2.7- and 8-fold, respectively. We next compared the relative levels of Sp100 isoforms in CIN612-9E, LKP1, and HPV16-positive keratinocytes to those in NHK cells (Fig. 8B). This revealed that in NHK cells Sp100HMG also is the most abundant form, followed by Sp100A, whereas the Sp100B and -C isoforms are present at lower levels. Interestingly, levels of Sp100A and -C were significantly reduced in HPV-positive cells, whereas Sp100HMG levels were similar in HPV-positive and NHK cells. Sp100B levels were also reduced in all HPV-positive cells, but this did not reach statistical significance. These data suggest that all Sp100 isoforms are present in HPV16- or HPV31-positive cells, but in different amounts. Interestingly, only Sp100 isoforms that are induced by IFN-κ in CIN612-9E cells display reduced levels in cell lines stably maintaining HPV16 or HPV31 genomes.

Sp100 isoforms B, C, and HMG inhibit HPV promoter activity in the absence of IFN. To gain insight into which Sp100 isoforms are responsible for the inhibition of viral transcription, expression vectors for Sp100A, -B, -C, and -HMG were cotransfected with HPV31 URR or HPV18 URR reporter plasmids into CIN612-9E cells. This revealed that the SP100B, -C, and -HMG inhibited both the HPV18 and the HPV31 URR plasmids to similar extents (Fig. 9). The HPV18 and the HPV31 URRs were repressed 8.0- and 6.2-fold by Sp100B, 8.0- and 5.2-fold by Sp100C, and 4.0- and 4.1-fold by Sp100HMG, respectively. The results for the Sp100A isoform were inconclusive, as the evaluation of the raw data indicated that Sp100A, in contrast to the other isoforms, activated the pCMV-gluc reporter plasmid used as a transfection control but had, in contrast to the other isoforms, no effect on the firefly luciferase activity from the HPV URR plasmids. Therefore, it is likely that Sp100A does not modulate HPV transcription, whereas Sp100B, -C, and/or -HMG inhibits HPV transcription.

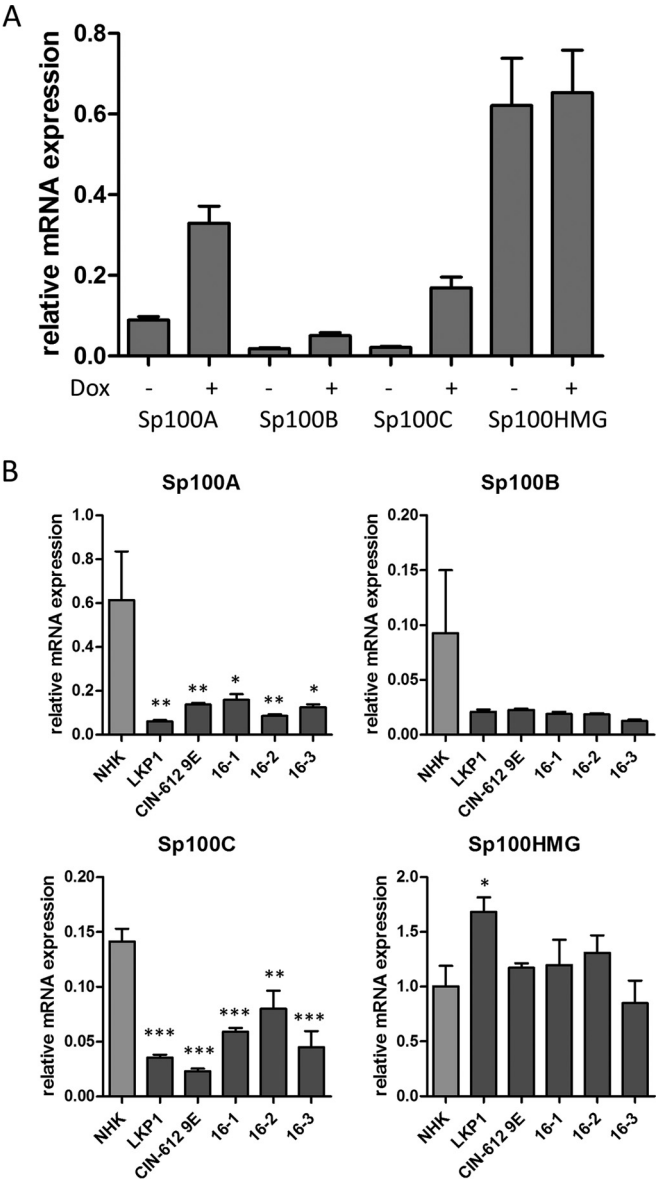


FIG 8 Expression of Sp100A, -B, -C, and -HMG isoforms. (A) CIN612-9E/ind-IFN-κ cells were incubated with doxycycline for 6 h, and RNA was analyzed by qPCR for the expression of Sp100A, -B, -C, and -HMG isoforms. Standard curves using plasmids for PGK1 and Sp100 isoforms were used to determine copy numbers. Data are presented as the ratio between Sp100 and PGK1 and represent the averages from four independent experiments; error bars indicate the SEM. (B) Comparison of Sp100 isoform expression in normal human keratinocytes (NHKs) and stable HPV-positive cell lines. Expression levels were obtained as described above. The data represent the averages for five different NHK donors and 2 to 4 replicates for HPV-positive cells. Error bars indicate the SEM. Statistical significance between expression levels was determined using one-way ANOVA and Dunnett's posttest (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

DISCUSSION

IFNs comprise a family of cytokines which induce ISGs in target cells and limit replication of diverse viruses. Classical type I IFNs such as IFN-αs and IFN-β, as well as type III IFNs, are strongly induced upon virus infection, which activates pattern recognition receptor pathways. In contrast, IFN-κ and IFN-ε are constitutively expressed in a cell type-specific manner and are only very moderately induced by pattern recognition receptor pathways, indicating a different way of regulation (13–16, 48). Recent studies demonstrated that IFN-κ expression is inhibited by hr-HPV both in tissue culture and *in vivo* (13, 15, 16). It had been previously shown that IFN-β inhibits the growth of cells lines that maintain replicating HPV16 or -31 genomes (4, 10). In the case of HPV16, kinetic analyses revealed that the number of replicating genomes decreased, which was followed by lowered viral transcript levels (10), strongly arguing that IFN-β has anti-HPV activity. Currently, only STAT1 and IFIT1 have been suggested as ISGs which

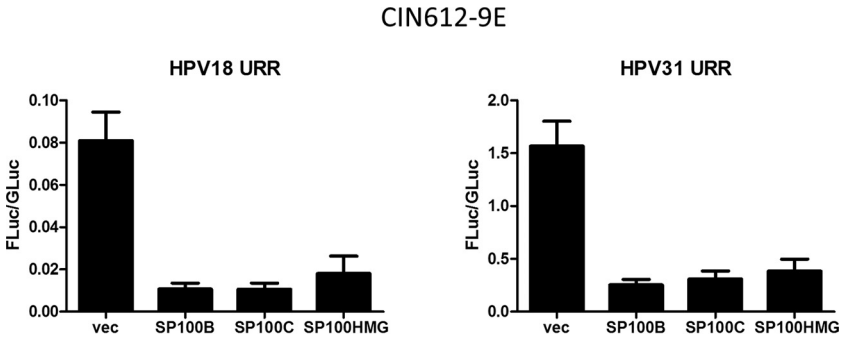


FIG 9 Sp100B, -C, and -HMG inhibit the HPV18 and HPV31 URR. CIN612-9E cells were transfected with 0.5 ng of pCMV-Gluc, 100 ng of Sp100B, -C, or -HMG or empty vector (vec) expression plasmid, and 100 ng of HPV18 URR or 31 URR reporter plasmid. Values are presented as the ratio between firefly luciferase (fluc) and *Gaussia* luciferase (gluc) activities. The data represent the averages from at least four independent experiments, and error bars indicate the SEM.

limit HPV replication (11, 12). STAT1 is a crucial transcription factor of the IFN signal transduction cascade, and its anti-HPV activity is most likely due to a reduced expression of a large number of ISGs (7, 8). IFIT1 has been reported to bind to and inhibit the HPV11 and -18 E1 replicative helicases (12), which may account for the loss of HPV16 genomes in W12 cells upon IFN-β treatment.

While IFN-κ has been reported to induce several standard ISGs such as IRF1, MX1 and STAT1 in different settings (14–16), it was unclear whether it would display anti-HPV activity. Using an HPV31-positive cell line that inducibly expresses IFN-κ, we show here for the first time that IFN-κ expression slows down cell growth, induces p53 protein expression, and changes the cell cycle profile. Consistent with these parameters being influenced by the expression of the hr-HPV E6 and E7 proteins, viral transcription and viral copy number declined over time when IFN-κ was expressed. Surprisingly, viral transcription was inhibited 2-fold already at 4 to 6 h after IFN-κ expression or addition of recombinant IFN-β. This suggested for the first time that IFNs very rapidly target HPV transcription. In order to get insight into IFN-κ-regulated genes in HPV-positive keratinocytes, we analyzed the transcriptome by RNA-seq. This indicated that more than 70% of the ISGs induced by IFN-κ more than 2-fold match to human ISGs in the Interferome database (V2.01) (41). IFN-κ induces ISGs that match mainly to type I IFN ISGs, further confirming the notion that IFN-κ signals through the IFNAR1/IFNAR2 receptor (14). Furthermore, IFN-κ-induced ISGs matched 15.5% of the previously reported genes repressed by HPV18 in human keratinocytes, which is consistent with the idea that mainly the repression of IFN-κ is responsible for the reduced ISG expression in such cell lines. In summary, IFN-κ behaves as a *bona fide* type I IFN in HPV-positive keratinocytes. Nevertheless, 63 IFN-κ-regulated genes have not been reported to be ISGs. This raises the question if these genes are keratinocyte-specific ISGs or if they have been missed in other experimental systems due to technical reasons. Future studies are required to address these questions.

RNA labeling experiments suggested that at early time points, mainly the initiation of viral transcription is targeted by IFN-κ. As it was unlikely that the reduction of viral transcript levels after 6 h was a consequence of the inhibition of viral replication by IFIT1, we focused on the IFN-κ target genes BATF2 and Sp100, as BATF2 has been described as an inhibitor of AP-1 activity and Sp100 proteins as repressors of transcription for different DNA viruses, including HPV18 (42, 47). We were unable to recover viral transcription despite an efficient knockdown of BATF2, suggesting that BATF2 is not involved in the control of viral transcription. In contrast, the knockdown of Sp100 by siRNA or shRNA increased viral transcript levels in the absence of IFNs and attenuated IFN-mediated repression. The expression of the isoform Sp100B, -C, or -HMG efficiently inhibited reporter activity driven by the major early promoter of HPV18 or -31, which is consistent with transcription initiation being targeted and Sp100 proteins being active in the absence of IFNs.

Sp100 was recently identified as a repressor of the early transcription of HPV18 (47). Interestingly, the effects of Sp100 were most pronounced during the establishment of the infection of human keratinocytes with HPV18, where a knockdown of Sp100 increased viral transcription 3- to 6-fold. In contrast, the knockdown of Sp100 in established cell lines with replicating HPV18 genomes increased viral transcription ~1.5-fold (47). Our data confirm and extend the finding that the knockdown of the basal levels of Sp100 by siRNA or shRNA increases HPV31 transcript levels in an established cell line. We now also show that IFN-κ is an activator of Sp100 in keratinocytes and that this is mainly responsible for the rapid, IFN-mediated inhibition of HPV transcription. Uninfected keratinocytes have high constitutive levels of IFN-κ, whereas established hr-HPV cell lines have reduced levels of IFN-κ and of several ISGs (5, 14, 15). Consistent with this, we now show that mainly IFN-κ-inducible transcripts for Sp100A and -C isoforms are present at reduced levels in cell lines stably maintaining HPV16 or HPV31 genomes compared to normal human keratinocytes (NHKs) (Fig. 8B). This may account for the different effects of the Sp100 knockdown on HPV18 transcription observed by Stepp et al. (47). In summary, we suggest that normal keratinocytes express IFN-κ to maintain high levels of Sp100, which limits viral expression and thus can prevent the establishment of hr-HPV. In the case of successful infections, E6 is expressed, which inhibits IFN-κ expression and thus restricts Sp100 levels to allow viral transcription and replication. Reexpression of IFN-κ or addition of exogenous IFN increases Sp100 levels and decreases viral transcription, which most likely contributes to the growth arrest of infected cells.

Sp100 and PML proteins are constituents of ND10 bodies (49). Previous studies have indicated that PV genomes are localized after infection to ND10 bodies (50–55). Experiments using bovine PV-1 and murine fibroblasts indicated a positive role of PML pro-

teins for viral transcription, and this was recently confirmed using HPV18 and human keratinocytes (47, 56). As pointed out above, Stepp et al. also observed in their study a negative role of Sp100 (47). This suggests that the interaction of ND10 bodies with PV is more complex than anticipated and requires further studies. Our studies reveal a negative role for IFN-induced Sp100 in established HPV-positive cell lines, which suggests that ND10 bodies may also regulate viral transcription in persistently infected cells.

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