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Interferon-beta treatment increases human papillomavirus early gene transcription and viral plasmid genome replication by activating interferon regulatory factor (IRF)-1

Michael J.Lace[1,2,*], James R.Anson[1], Aloysius J.Klingelhutz[3], Hisashi Harada[4], Tadatsugu Taniguchi[5], Aaron D.Bossler[2], Thomas H.Haugen[1,2] and Lubomir P.Turek[1,2]

[1]Veterans Affairs Medical Center, 601 Highway 6 West, Iowa City, IA 52246, USA, [2]Department of Pathology and [3]Department of Microbiology, The Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA, [4]Department of Internal Medicine, Massey Cancer Center, Virginia Commonwealth University, Richmond, VA 23298, USA and [5]Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

[*]To whom correspondence should be addressed. Tel: +1 319 338 0581 ext. 7504; Fax: +1 319 339 7178;
Email: michael-lace@uiowa.edu

Interferons (IFNs) have been used to treat mucosal lesions caused by human papillomavirus (HPV) infection, such as intraepithelial precursor lesions to cancer of the uterine cervix, genital warts or recurrent respiratory papillomatosis, to potentially reduce or eliminate replicating HPV plasmid genomes. Mucosal HPVs have evolved mechanisms that impede IFN-β synthesis and downregulate genes induced by IFN. Here we show that these HPV types directly subvert a cellular transcriptional response to IFN-β as a potential boost in infection. Treatment with low levels of human IFN-β induced initial amplification of HPV-16 and HPV-11 plasmid genomes and increased HPV-16 or HPV-31 DNA copy numbers up to 6-fold in HPV-immortalized keratinocytes. IFN treatment also increased early gene transcription from the major early gene promoters in HPV-16, HPV-31 and HPV-11. Furthermore, mutagenesis of the viral genomes and ectopic interferon regulatory factor (IRF) expression in transfection experiments using IRF-1$^{-/-}$, IRF-2$^{-/-}$ and dual knockout cell lines determined that these responses are due to the activation of IRF-1 interaction with a conserved interferon response element demonstrated in several mucosal HPV early gene promoters. Our results provide a molecular explanation for the varying clinical outcomes of IFN therapy of papillomatoses and define an assay for the modulation of the HPV gene program by IFNs as well as other cytokines and signaling molecules in infection and therapy.

Introduction

High-risk oncogenic mucosal human papillomavirus (HPV) types are the major cause of most, if not all, carcinomas of the uterine cervix as well as many other anogenital tumors and are found in 20–30% of cancers of the head and neck (1). Interferons (IFNs) are potent modulators of cell growth and differentiation as well as host immune responses to viral infections (2,3). IFNs have historically been used in the treatment of laryngeal papillomas as well as other HPV-associated lesions, often with inconsistent clinical outcomes (4–6). Many viruses have evolved complex immunoevasive strategies and HPVs are no exception (7–10). HPV infection is associated with an altered immunogenic profile of keratinocytes *in vivo* (11,12) including altered cytokine expression (13,14). Cervical lesions undergoing malignant progression *in vivo*, as well as human keratinocytes harboring persistently replicating HPV plasmid genomes, exhibit compromised immunoreactive cellular pathways such as decreased expression of IFNs and IFN-dependent pathways (15,16). Decreases in IFN-inducible factors such as signal transducer and activator of transcription (Stat)-1 (17–19) and viral infection-induced interferon regulatory factor (IRF)-3 (20) have also been documented. A previous study described IRF-1 binding to the HPV-16 major early promoter (21) and E7 proteins from mucosal or cutaneous HPVs were shown to directly inhibit the function of IRF-9 (p48) (22) and IRF-1 (23–25).

IFNs induce the JAK–STAT signaling pathway to promote post-translational modifications of cellular factors, including Stats and IRFs, to facilitate activation or repression of cellular and viral transcription (26). Members of the IRF family of factors have been shown to be essential for cellular differentiation and proliferation as well as host response to viral infection (reviewed in ref. 27). Loss of IRF-1 (a putative tumor suppressor protein), for example, has been shown to be an important step for oncogene-induced transformation and apoptosis (28,29) and has also been detected in a variety of human leukemias and preleukemic myelodysplasias (30). IFNs have also been shown to modulate viral replication in non-HPV viruses (31,32). In HPV, recent studies have shown that high doses of IFN reduce viral plasmid load while selecting for latent integrated HPV genomes in explant-derived HPV-16-positive cervical keratinocytes (33,34).

In this study, we demonstrate that mucosal HPV early gene transcription and plasmid genome replication in keratinocytes are modulated by an IFN-induced, IRF-1-dependent mechanism as a conserved viral strategy to subvert cytokine-mediated host immune responses.

Abbreviations: HFK, human foreskin keratinocyte; HPV, human papillomavirus; IFN, interferon; IRE, interferon response element; IRF, interferon regulatory factor; PCR, polymerase chain reaction; SCC, squamous cell carcinoma; Stat, signal transducer and activator of transcription; wt, wild-type.

Materials and methods

Cell culture

Primary human foreskin keratinocyte (HFK) cultures were prepared from neonatal foreskins as described (35). Clonal HPV-16-immortalized HFK (36) and human cervical keratinocyte cultures (HCK-B) were similarly prepared (37). All keratinocyte cultures and HPV-negative human squamous cell carcinoma (SCC13) cells (38) were grown on irradiated J2 fibroblast feeder cells in E media, containing 0.5 μg/ml hydrocortisone, 0.1 nM cholera toxin, 5 μg/ml transferrin, 5 μg/ml insulin, 2 nM 3,3′-5-triodo-L-thyronine and 5 ng/ml epidermal growth factor (39). HPV-positive keratinocyte line-I cells were a gift from M.Dürst (40). HaCaT cells, a spontaneously immortalized human keratinocyte cell line that retains its capacity for *in vivo* differentiation, were a gift from Dr N.Fusenig (41) and HeLa, cervical carcinoma cells, were cultured as described (42,43). The IRF knockout fibroblast cell lines (29) and NIH 3T3 cells were cultured in 7% Dulbecco's modified Eagle's medium. The W12E cell line was a gift from Dr P.Lambert (44), whereas the CIN612 cell line (45) was a gift from Dr L.Laimins.

IFN inductions were performed in the absence of an irradiated J2 fibroblast feeder layer. Uninfected primary human keratinocyte or clonal HPV-positive keratinocyte cultures were first starved for 24 h in charcoal-stripped, low-serum medium (Dulbecco's modified Eagle's medium and 2% fetal bovine serum) prior to cytokine addition. Cultures were then fed an optimal range of 2–30 U/ml human recombinant IFN-β or 5–10 ng/ml IFN-γ (Sigma, St Louis, MO) in the same low-serum medium every 24 h for 2–5 days before DNA harvest or 15 min to 6 h post-induction for RNA or protein harvest as described below.

Plasmid constructions and transcription assays

Polymerase chain reaction (PCR)-generated probes were used in RNase protection assays as described (43). The HPV-16 P97-*cat* plasmids were constructed and tested in chloramphenicol acetyl transferase assays as described (42). The IRF-2-*cat* and IRF-1-*cat* reporter constructs (46) were tested in parallel. The AdE2-*cat* plasmid (47) was a gift from Dr M.Stinski. The HPV-16 E2 DNA-binding domain mutant genome was constructed by introducing a translation termination signal in the DNA-binding domain of the E2 open reading frame by PCR at AA 321 (36). Mutations introduced by PCR into

the interferon response elements (IREs) of HPV-16 and HPV-11 genomes are defined in Table I. The replication competent HPV-16 W12E plasmid (accession no. AF125673) was a gift from Dr P.Lambert (48). The HPV-31 plasmid (49) was a gift from A.Lorincz. The HPV-11 plasmid (50) was a gift from Dr H.zur Hausen. For RNase protection assays, total RNA was harvested from transfected HeLa cells using RNAqueous kits (Ambion, Austin, TX) for analysis as described (51).

Replication assays and Southern blotting

For initial HPV plasmid amplification assays, HPV-16-W12E and HPV-11 DNAs were first cleaved from pUC vector sequences with BamHI, whereas HPV-31 DNA was digested with EcoRI, and then religated at 5 μg/ml for 16 h. Ligated DNAs, reproducibly containing 30–50% of HPV sequences as single circles, were purified over plasmid purification columns (MaxiKit, Qiagen, Valencia, CA) and 3 μg transfected into SCC13 cells with Effectene (Qiagen). Beta-galactosidase staining of control cultures transfected with pCMV-*β-gal* indicated >50% transfection efficiencies. Cultures were split 1:2, 24 h post-transfection and cultured for up to 5 days before total DNA harvest (QIAamp DNA Blood Kit, Qiagen). DNA samples were then digested with DpnI and linearized with BamHI or EcoRI before Southern blotting. A total of 4 μg of each DNA sample was then digested with DpnI and linearized with BamHI and XbaI before Southern blotting. Digestion of DNA samples was confirmed by visualization of ethidium bromide-stained agarose gels following electrophoresis. DpnI-digested, whole-cell DNAs from transfected SCC13 cultures were resolved on 1.0% agarose gels, depurinated in 0.25 M HCl and blotted directly onto positively charged nylon membranes (Hybond-XL, Amersham Biosciences Corporation, Piscataway, NJ) by alkaline transfer with 0.4 N NaOH. Aliquots of linearized HPV DNA (1–30 pg) were included as positive Southern blotting controls and to normalize band intensities (quantified by scanning densitometry) between autoradiograms. Blots were hybridized at 65°C with probes (1.5×10^6 c.p.m./ml hybridization buffer) containing a representative fragment or equimolar cocktail of PCR-amplified segments of the HPV-16 W12E (nt 6226-3873/4471-6000), HPV-31 (nt 6161-872) or HPV-11 (nt 7080-225) genomes [α-^{32}P] deoxyadenosine triphosphate/deoxycytidine triphosphate labeled by random priming (HotPrime kit, GenHunter Corporation, Nashville, TN).

For stable replication assays, 2×10^6 per 100 mm dish of low-passage primary HFKs were transfected with 3 μg of recircularized HPV DNA and 1 μg of RSV-*neo*, G418-selected and clonal cultures isolated and expanded as described (36). Total cellular DNA (as described above) and total RNA (RNAqueous kits, Ambion) were harvested from clonal cultures to monitor HPV extrachromosomal replication and transcription, respectively.

Protein purification and mobility shift assays

Binding was performed in 25 μl at 0.1–0.3 nM (15 000 c.p.m.) of [γ-^{32}P] deoxyadenosine triphosphate end-labeled gel-purified oligonucleotides, 10 or 30 nM of unlabeled poly (dI-dC)(dI-dC) (Amersham–Pharmacia, Piscataway, NJ) and bovine papillomavirus E2#10 (51) as non-specific competitors and unlabeled specific competitor oligonucleotides at indicated concentrations. Protein–DNA complexes and unbound probe were resolved on non-denaturing 6% polyacrylamide gels and visualized by autoradiography. Dissociation constants (K_d) were determined by densitometric scanning from three or more competition experiments as described (51). Nuclear extracts were prepared by the ammonium sulfate protocol and fractionated over BioRex-70 before two cycles of adsorption and elution from a sequence-specific DNA affinity column as described (42,43). An IRF DNA affinity column was prepared by linking ~0.25 μg of an end-biotinylated oligonucleotide, corresponding to four tandem repeats of an IRF consensus hexamer 5′-AAGTGAAAGTGAAAGTGAAAGTGA-3′ (52) to 200 μl streptavidin-conjugated agarose (Sigma). *In vitro* translations of recombinant IRF-1 and IRF-2 were performed in a coupled transcription–translation system (TNT kit, Promega, Madison, WI) using IRF-1 or IRF-2 template plasmids, pCMIRS (52) and pIRF2-5 (53), respectively, and purified as described above. The supershifting of electrophoretic mobility shift assay complexes with polyclonal IRF antisera (Santa Cruz Biotechnology, Santa Cruz, CA) followed the manufacturer's protocols.

Chromatin immunoprecipitation assays

SCC13 cells (15×10^6) were transfected with 6 μg of religated HPV-16 wild-type (wt) 18 h prior to cross-linking with 1% formaldehyde and nuclear extract isolation as described above. Chromatin isolated from these extracts was precleared with 10 μl protein A sepharose then aliquoted and immunoprecipitated as described (54) with the appropriate antisera overnight at 4°C. Hemagglutenin antisera (Sigma) was used as a control while polyclonal Santa Cruz Biotech antisera was used to detect cellular factors, including histone 2A, in parallel. Immunoprecipitates were washed and the cross-linking reversed prior to total DNA extraction (BloodAmp kit, Qiagen). The immunoprecipitation of cross-linked protein–DNA complexes was measured by quantitative PCR using primers against the HPV-16 promoter, amplifying a 494 nucleotide fragment (nt 7665 to +155). A total of 3 fg of a truncated HPV-16 plasmid, which carries a deletion of nucleotide 7793-7902, was added to each sample as a competitor template that generates a PCR product with a greater mobility. The PCR products were resolved on an 1% agarose gel and the ratios of chromatin-dependent product to competitor were quantified by scanning densitometry to demonstrate immunoprecipitation enrichment of the protein-bound chromatin.

Table I. IRK-1 and -2 bind specifically to multiple mucosal HPV promoters

	Sequence[a]	Consensus match	Cell complex K_d (nM)[b]	rIRF-1 K_d (nM)[c]	rIRF-2 K_d (nM)[d]
a	HPV-16 (30-66) wt	11/13	2	2	2
b	HPV-16 IRE *mut* I		10	nd	nd
c	HPV-16 IRE *mut* II		10	nd	nd
d	HPV-6 (30-66)	10/13	10	10	10
e	HPV-6 (7851-7885)	12/13	3	1	1
f	HPV-11 (30-66) wt	10/13	10	10	10
g	HPV-11 (30-66) *mut*		30	nd	nd
h	HPV-11 (7874-7917) wt	11/13	3	1	1
i	HPV-11 (7874-7917) *mut*		10	nd	nd
j	HPV-13 (32-68)	9/13	10	10	10
k	HPV-31 (32-68)	10/13	3	1	1
l	HPV-33 (34-70)	10/13	3	1	3
m	HPV-35 (16-47)	9/13	3	nd	nd
n	HPV-52 (37-71)	11/13	3	10	10
o	HPV-56 (42-73)	9/13	10	nd	10
p	HPV-72 (24-57)	11/13	20	nd	20
q	IRF consensus	13/13	nd	nd	nd
r	ISRE (ISG15) wt	11/13	0.6	0.3	0.3
s	ISRE (ISG15) *mut*		10	nd	nd

nd, not determined.
[a]Mobility shift assays were performed with synthetic oligonucleotides (as shown in Figure 1A) and purified HaCaT nuclear extract and *in vitro* translated recombinant IRF-1 and IRF-2 proteins and derived from 10 mucosal HPV promoters that matched a 13 nt IRF consensus motif (IRE).
[b]Dissociation constants for keratinocyte extracts were determined from an average of two to four experiments as measured by scanning densitometry.
[c]Dissociation constants for rIRF-1 were determined from an average of two to four experiments as measured by scanning densitometry.
[d]Dissociation constants for rIRF-2 were determined from an average of two to four experiments as measured by scanning densitometry.

Results

IRF-1 and IRF-2 bind to a conserved IRE in the HPV E6–E7 promoters

Examination of the structure of numerous mucosal HPV E6–E7 promoters revealed a shared homology located 5′ of the E6–E7 start site which resembled the conserved sequences of IREs found in many cellular promoters (Figure 1A). Oligonucleotides spanning this putative IRE in the HPV-16 P97 promoter were constructed to detect the binding of IRFs in mobility shift assays (Figure 1B). Nucleotide substitutions designed to disrupt the binding of these factors were introduced into the homology shared by several mucosal HPV E6–E7 promoters, including HPV-31 (another high-risk HPV type) and HPV-11 (a 'low risk' type). A complex specifically formed between keratinocyte extracts or purified recombinant proteins and the putative HPV-16 IRE. The composite keratinocyte complex was identified as IRF-1 and IRF-2 by binding specificity, complex mobility and antigenicity (Figure 1B, lanes 5–8). In contrast to most other cell lineages, the ratio of constitutive binding of endogenous IRF-1 and IRF-2 to the P97 promoter of HPV-16 genomes was distinct in keratinocytes as demonstrated in transfected SCC13 cells using chromatin immunoprecipitations quantified by competitive PCR (Figure 1C, lanes 5 and 6). These results were consistent with *in vitro* mobility shift assays with keratinocyte extracts which also demonstrated a higher ratio of IRF-1 to IRF-2 binding (Figure 1B) in these cells even though rIRF-1 and rIRF-2 exhibited identical dissociation constants (K_d), ranging from 0.3 to 20 nM in parallel (Table I). We expanded this analysis to include multiple additional mucosal HPV types, demonstrating for the first time that IRF-1 and IRF-2 binding is a conserved feature among eight additional major early promoters in HPV types 6, 13, 31, 33, 35, 52, 56 and 72 (Table I).

IFN stimulates multiple cellular signaling pathways to modulate transcription in keratinocytes

IFNs modulate transcription by employing multiple signaling pathways to activate an array of protein complexes, which specifically bind to DNA elements to regulate the promoter activity of numerous cellular genes. We first monitored the formation of IFN-inducible complexes: Stat-1, IFN-stimulated gene factor-3 and IRF-1, using their cognate binding sequences (Figure 1A) and IFN-treated keratinocyte extracts in mobility shift assays (Figure 2A). All three

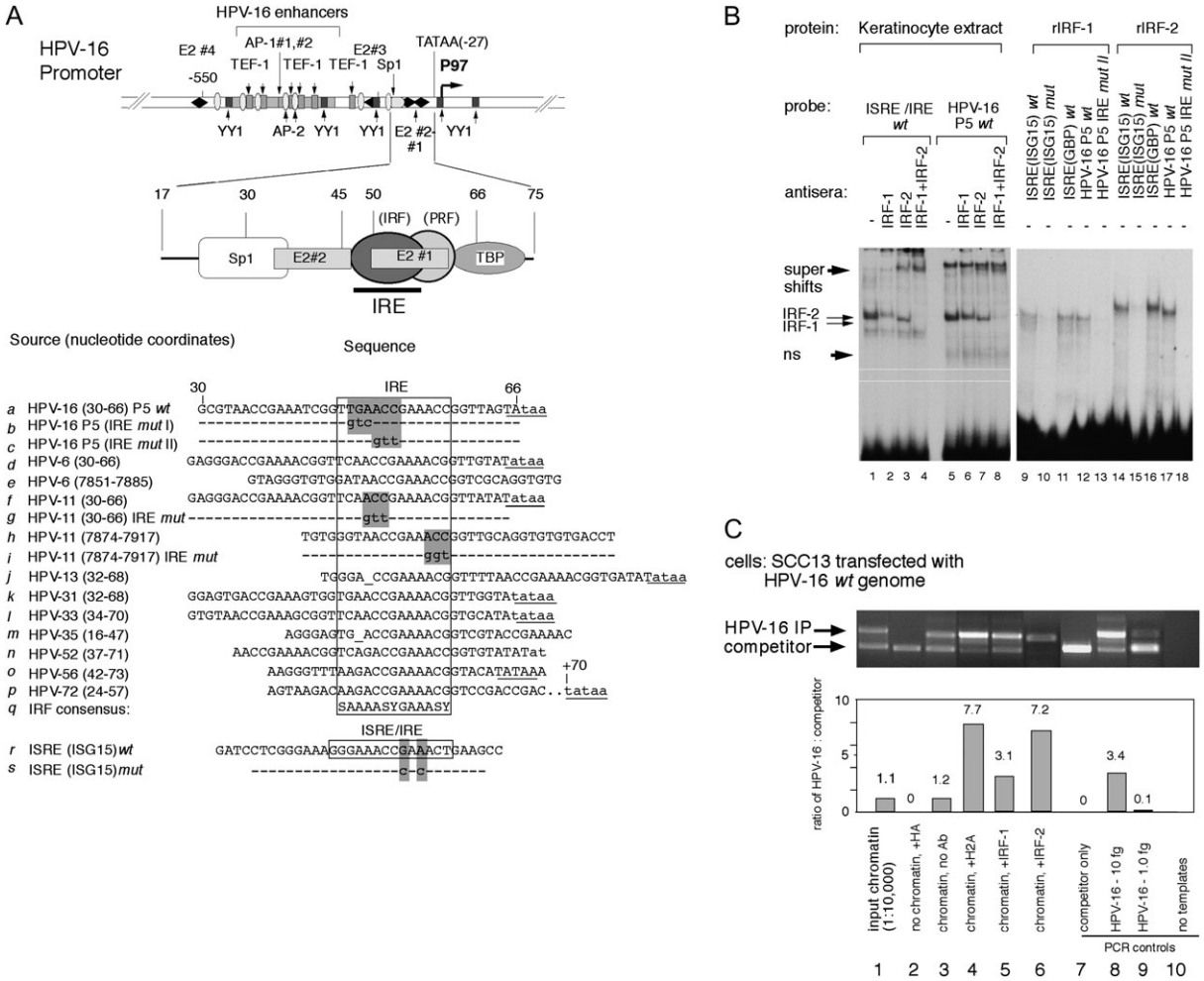

Fig. 1. IRF-1 and IRF-2 bind to a conserved IRE 5′ to the HPV-16 E6–E7 promoter. (**A**) Synthetic oligonucleotides (shown in upper case while adjacent sequences are shown as lower case) spanning a putative IRE in the HPV-16 P97 promoter and 10 other HPVs (boxed area). Nucleotide substitutions (shaded) disrupting IRF binding were introduced into a conserved IRE motif in HPV-16, HPV-11 and interferon-stimulated response element (ISRE) (ISG15 promoter) control sequence. Adjacent tataa boxes are underlined. (**B**) Specific cellular complexes formed with the HPV oligonucleotide probes in electrophoretic mobility shift assays. The composite cellular IRF complex consists of IRF-1 and IRF-2 while papillomavirus regulatory factor binding to HPV-16 had been described previously (55). Recombinant IRF proteins (rIRF-1 and rIRF-2) formed specific complexes with defined cellular ISRE, from the IFN-stimulated gene-15 (ISG15) and guanylate binding protein (GBP) promoters, as well as the HPV-16 wt IRE. Polyclonal antisera specific for each IRF protein was used to supershift these complexes. (**C**) Chromatin immunoprecipitations were performed with extracts derived from SCC13 cells transfected with HPV-16 wt plasmids. Antiserum, specific for histone 2A (αH2A), was included as a positive control in this composite of two independent experiments. HA refers to the hemagglutinin antisera control. Relative immunoprecipitation activity was expressed as a ratio of HPV-16 immunoprecipitation signal to the truncated competitor PCR product, measured by scanning densitometry.

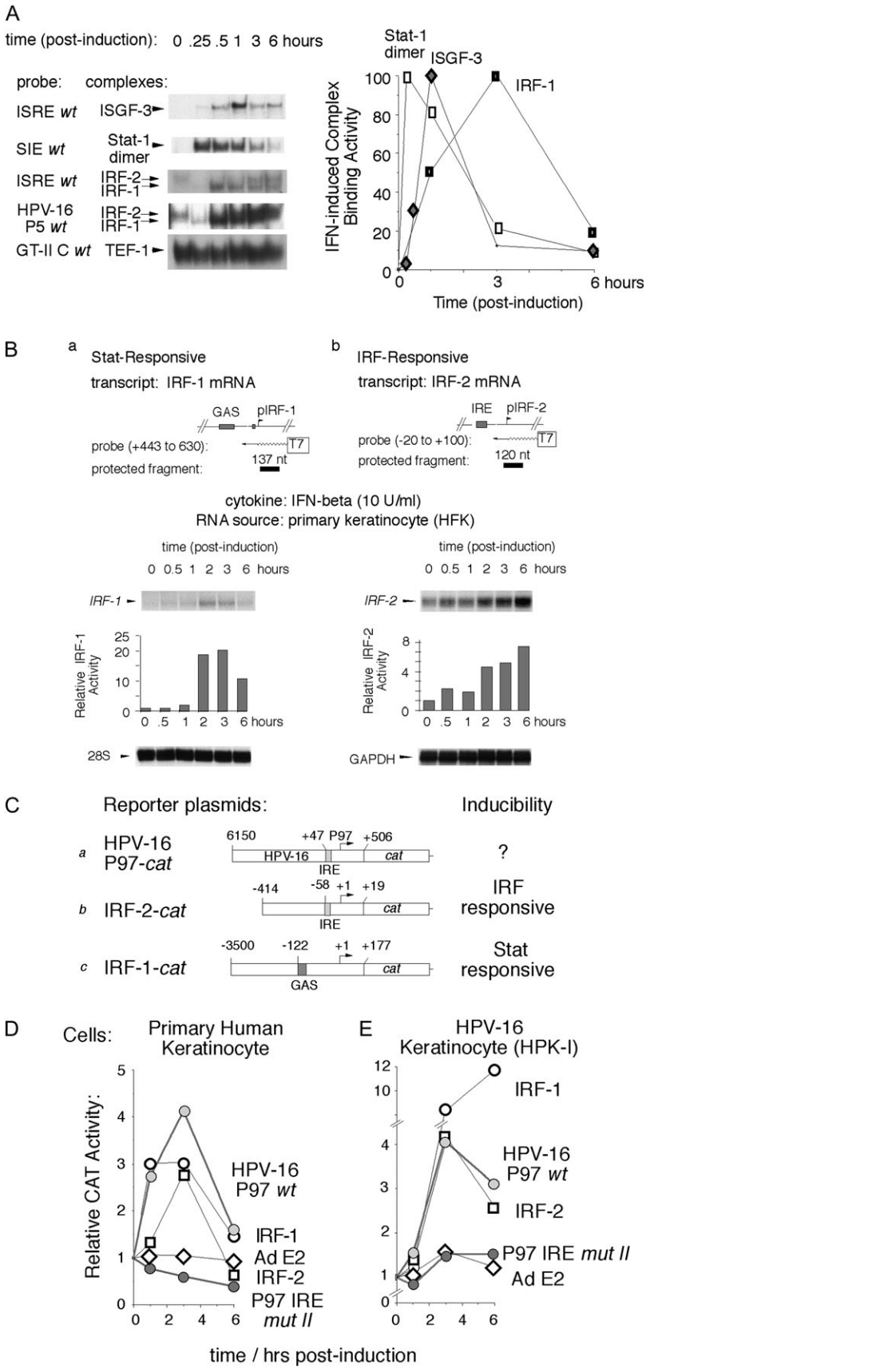

Fig. 2. IFN-mediated activation of the HPV-16 P97 promoter is dependent on the IRF pathway in keratinocytes. (**A**) Mobility shift assays were used to detect the binding of known IFN-inducible regulatory proteins in IFN-treated keratinocyte extracts to the HPV-16 IRE and control sequences *in vitro*. The ISRE probe (from the ISG15 promoter) detects the tripartite interferon-stimulated gene factor-3 complex and IRF protein binding while the consensus SIE probe

complexes formed with control sequences immediately following IFN treatment but only constitutive IRF-2 and IFN-induced IRF-1 binding to the HPV-16 IRE was detected. Rather than relying on sequence analysis to identify IFN-responsive *cis* elements in HPV-16, we performed mobility shift assays, as described above, using an array of overlapping synthetic oligonucleotides spanning the entire 1 kb upstream regulatory region. Interestingly, no Stat-1 or IFN-stimulated gene factor-3 binding was detected in the HPV-16 IRE or elsewhere in the major early promoter of HPV-16 (M.J.Lace, T.H.Haugen and L.P.Turek, unpublished data). We also monitored the transcription of Stat-responsive and IRF-responsive cellular promoters, pIRF-1 and pIRF-2, respectively, in primary HFK in RNase protection assays following IFN treatment (Figure 2B). We found that IFN-β activates the expression of both IRF-1- and Stat-1-responsive targets *in vivo*. Taken together, these results confirm that critical IFN-mediated IRF and Stat pathways are intact and functional in primary keratinocytes, the natural host cells for HPV infection.

IFN-mediated activation of the HPV-16 P97 promoter is dependent on the IRF-1 pathway in vivo

Having verified that keratinocytes contained functional IFN-mediated signaling pathways in our assays, we examined the effect of IFN on the papillomaviral machinery in these cells. We compared the activities of IRF- and Stat-responsive targets (Figure 2C) with the HPV-16 promoter in chloramphenicol acetyl transferase assays performed in both uninfected primary HFK and an HPV-positive keratinocyte line-I that harbors a single integrated HPV-16 genome (Figure 2D and E, respectively). The HPV-16 and IRF-responsive IRF-2 promoters were comparably induced in both cell types, whereas the IFN-unresponsive AdE2 promoter target was not, indicating that the IRF-2 and HPV-16 promoters could be induced by the same mechanism. Furthermore, the observed induction was unaffected by the expression of viral gene products, E6 and E7, from the integrated HPV-16 genome in the HPV-positive keratinocyte line-I cells. Mutation of the HPV-16 IRE that abolished IRF binding, abrogated IFN-mediated activation. We also tested the response of the HPV-16 and HPV-11 E6–E7 promoters to IFN in the context of the intact viral genomes in transfected HeLa cultures—a cell line that does not support HPV replication. Transcription from the HPV-16 (Figure 3A) and HPV-11 wt (Figure 3B) genomes was activated upon IFN treatment, whereas the corresponding IRE *mut* was unresponsive in each case, demonstrating that, in the context of the whole genome, IRF-1 binding to the promoter-proximal IRE was required for IFN-mediated activation of HPV transcription *in vivo*. Furthermore, the presence of endogenous levels of early viral gene products, including E2, failed to influence IFN-mediated induction of HPV transcription.

Interestingly, disruption of the IRE in HPV-16 and HPV-11 resulted in increased basal transcription from these templates in the absence of IFN (Figure 3A and B, respectively). Since mutation of the IRE also impinges on an overlapping E2-binding site, we disrupted the E2 DNA-binding domain alone or in combination with the IRE mutation in HPV-16 to delineate the contribution of E2 versus IRF-1 to this effect (Figure 3C). We found that the higher basal activity of the HPV-11 and HPV-16 IRE mutant constructs in these and subsequent experiments were solely due to the loss of E2 binding to a site overlapping the IRE.

Having demonstrated IFN-dependent transcriptional activation of transiently transfected HPV-16 and HPV-11 plasmids, we then examined the effect of similar doses of IFN on HPV transcription in clonal keratinocyte cultures that harbor stably replicating viral genomes. Similarly, HPV-16 P97 transcription in W12E cells (Figure 3D) and HPV-31 P99 transcription in CIN612 cells (Figure 3E) were transiently induced upon low-dose IFN-β or IFN-γ treatment.

IFN induction of HPV transcription kinetically correlated with the transient induction of IRF-1-dependent binding to the HPV-16 IRE *in vitro* (Figure 2A). However, Stat-1, IRF-1 and IRF-2 pathways are all activated upon IFN treatment of keratinocytes (Figure 2B). To determine which pathways were responsible for the observed activation of HPV-16 transcription *in vivo*, we examined the response of Stat- and IRF-dependent targets to IFN in fibroblast knockout cell lines in which the *IRF*-1, *IRF*-2 or both *IRF* genes have been disabled. The HPV-16 promoter as well as the IRF and Stat-responsive targets were activated in wild-type fibroblasts (Figure 4A). The IFN-unresponsive adenovirus promoter plasmid (AdE2-*cat*) was included as a negative control. In the IRF-1 knockout cells, however, the HPV-16 and IRF-dependent IRF-2 promoter constructs were unresponsive, whereas the Stat-dependent IRF-1 promoter was activated upon IFN treatment (Figure 4B). Loss of the IRF-2 gene alone had no effect (Figure 4C) and the simultaneous loss of both IRF-1 and IRF-2 (Figure 4D) confirmed that IFN-mediated activation of the HPV-16 P97 promoter was solely dependent on the IRF-1 pathway *in vivo*.

Treatment with low levels of IFN can increase HPV copy numbers in both transiently and stably transfected keratinocytes

Having demonstrated that IFN treatment can modulate HPV transcription via the IRF-1 pathway, we then examined its effects on HPV replication. Both wild-type and IRE-mutant HPV-16 and HPV-11 plasmids were transiently transfected into SCC13 cells and their replication monitored by Southern blotting following IFN treatment. Consistent with the transient induction of viral transcription, replication from both HPV-11 (Figure 5A) and HPV-16 (Figure 5B) wt genomes were also activated upon IFN treatment, whereas replication from the IRE *mut* genomes was not. We also noted an increased basal replication level with the HPV-11 and HPV-16 IRE *mut* genomes, consistent with the elevated baseline transcription levels (Figure 3C) as a result of collateral abrogation of E2 repressor binding to an overlapping motif. Titrations of linearized HPV-11 (Figure 5B, lane 9) and HPV-16 (Figure 5B, lanes 18 and 19) were included as positive Southern blotting controls.

We also examined the effect of IFN on the viral load of stably replicating HPV-transformed keratinocytes. Replication in both HPV-16-positive cervical (HCK) and foreskin (HFK) keratinocytes was stimulated upon IFN treatment (Figure 5B and C, respectively). Mutation of the HPV-16 IRE, however, abolished this stimulation; instead the viral load rapidly decreased upon IFN treatment over the same time course (Figure 5D). Subsequent experiments performed in our laboratory (M.J.Lace, J.R.Anson and L.P.Turek, unpublished data) have confirmed that the rapid loss of HPV plasmids upon IFN treatment of these keratinocyte cultures occurs via an IRE-independent mechanism. HPV replication in keratinocytes harboring HPV-31 wt plasmids was similarly stimulated by IFN

detects Stat dimer formation. The GTIIC wt probe (from SV40) detects IFN-unresponsive TEF-1 binding as a negative control. (**B**) Primary HFK cells were first starved in low-serum media prior to treatment with IFN-β, RNA harvest and analysis of IRF-1/IRF-2 messenger RNA by RNase protection. Scanning densitometry determined normalized promoter activities relative to the GAPDH internal control. The 28S message was monitored as an additional internal control. (**C**) The HPV-16 promoter, P97-cat, IRF-responsive (IRF-2 cat) and Stat-responsive (IRF-1 cat) constructs were used in these assays. (**D**) Primary keratinocytes were transiently transfected with the HPV-16 P97-cat, IRF and Stat-responsive targets prior to IFN-β treatment and extract preparation for chloramphenicol acetyl transferase assays. The adenovirus E2 promoter construct (AdE2-*cat*) served as an IFN-unresponsive negative control in these assays. Normalized activities represent an average of two to four independent experiments. (**E**) Transfections in HPK-I cells, which harbor integrated HPV-16, were similarly treated in parallel.

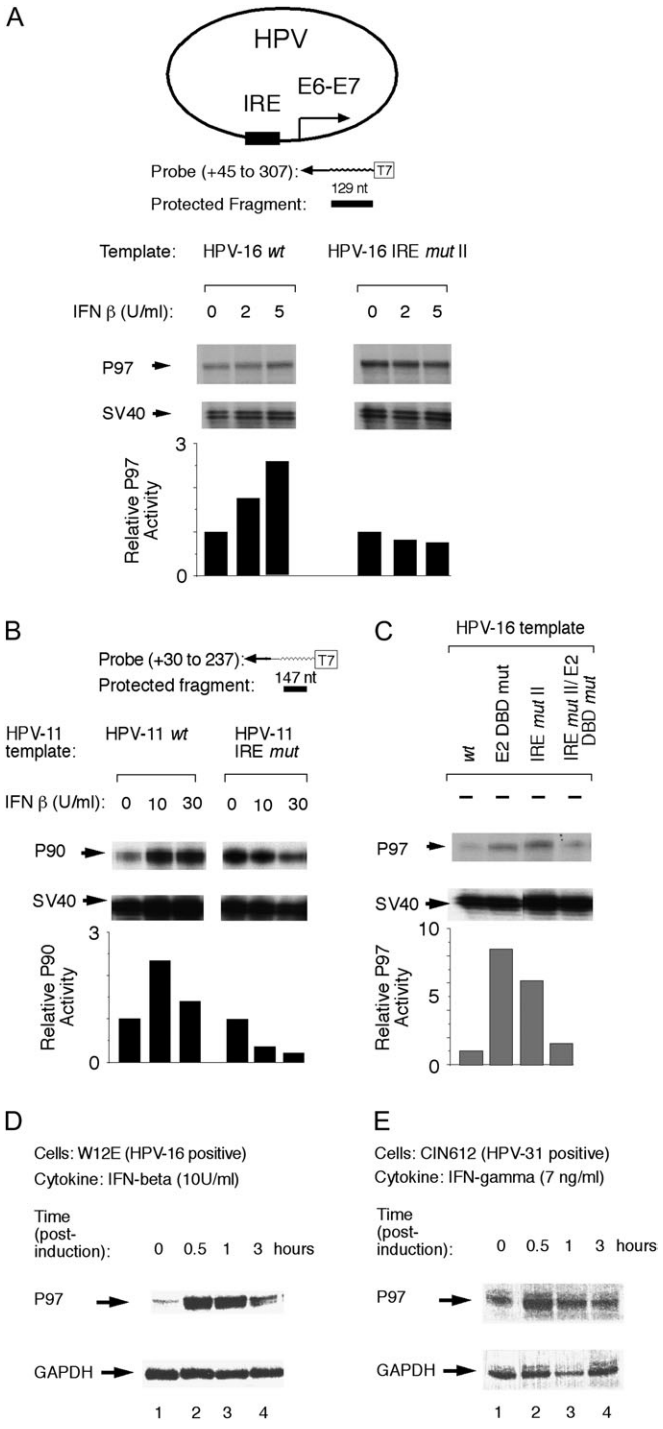

Fig. 3. IFNs activate HPV-16, HPV-11 and HPV-31 E6–E7 transcription from replicating viral genomes. Nucleotide substitutions in the HPV IRE that block IRF-1 binding were introduced into the (**A**) whole HPV-16 and (**B**) HPV-11 genomes, transiently transfected into HeLa cells and then treated with IFN-β prior to RNA harvest and analysis by RNase protection. (**C**) Baseline activities of constructs containing the indicated IRE and/or E2 DNA-binding domain (DBD) mutations were compared. Normalized, promoter activities (relative to the SV40 internal transfection control) were determined by scanning densitometry. (**D**) Clonal HPV-16 W12-E cells were treated with IFN-β prior to total RNA harvest and analysis by RNase protection assay. The normalized ratio of P97 transcription to GAPDH (as an internal control) was determined by scanning densitometry in a representative experiment. (**E**) Clonal CIN612 cells were treated with IFN-γ and similarly analyzed.

treatment (Figure 5E). These results demonstrate that treatment with low levels of IFN can augment initial HPV plasmid amplification and increase HPV plasmid copy numbers in clonal HPV-immortalized keratinocytes.

Discussion

Cytokines, such as IFNs, are pleiotropic mediators of HPV early gene expression and replication in keratinocytes—the natural host for infection. We have demonstrated that mucosal HPV types have evolved a conserved strategy to subvert a normal cellular response to viral infection in order to stimulate early viral gene expression, initial plasmid amplification and viral copy number. The IFN-dependent induction requires the binding of IRF-1 to a conserved IRE detected in major early promoters of HPVs associated with anogenital carcinomas and diseases of the head and neck. These viral promoters thus respond to activation of the IRF pathway in a manner similar to many IFN-inducible cellular genes.

We found that up to 90% of mucosal HPV types examined contained sequence matches within the E6–E7 promoter to the IRF binding consensus. While a previous study described IRF-1 binding to the HPV-16 promoter (21), we have demonstrated IRF-1 and IRF-2 binding to IREs in multiple mucosal HPV types at similar positions upstream of the transcription start site in the respective major early promoters. In contrast, only 22% of cutaneous HPV types examined revealed IRE sequence matches, suggesting a mechanism conserved among mucosal HPVs but possibly distinct from cutaneous HPVs. However, in contrast to previous studies that relied solely on purified proteins or cellular extracts forming complexes with chromatin-free DNA segments of the HPV-16 promoter *in vitro*, we utilized chromatin immunoprecipitation assays that quantitatively defined the binding of physiologic levels of IRF-1 to intact, replicating extrachromosomal HPV-16 genomes. We demonstrate for the first time in this study that IRF-2 also binds the HPV-16 IRE and that basal levels of DNA-binding competent IRF-1 exceed those of IRF-2 by >2-fold in keratinocytes. This pattern was not seen in extracts derived from other human and animal cell types where IRF-2 binding exceeds that of IRF-1 and this contrast did not correlate with differential steady state IRF messenger RNA levels in the absence of IFN treatment (M.J.Lace, T.H.Haugen and L.P.Turek, unpublished data). Interestingly, we detected no binding of IFN-inducible Stat-1 dimers or interferon-stimulated gene factor-3 complexes to the 1 kb HPV-16 early promoter *in vitro* using mobility shift or chromatin immunoprecipitation assays. However, we cannot rule out the possibility that other members of the Stat or IRF family of transcription factors may play a role in regulation of HPV transcription and replication at various stages of the viral life cycle.

Of particular interest was the demonstration that replication as well as early gene expression of HPV-11, a 'low-risk' HPV type frequently associated with benign proliferative lesions of the respiratory tract, was stimulated by low doses of IFN given the extensive use of IFNs in the treatment of recurrent respiratory papillomatosis (56). Previous studies had reported IFN-dependent reduction of HPV-31 copy numbers in keratinocyte cultures when high IFN doses were used (57). Similarly, IFN treatment of bovine papillomavirus type 1 transformed cultures resulted in the loss of bovine papillomavirus plasmids (58). In both studies, the IFN concentrations used were two orders of magnitude greater than the reproducibly effectual dose employed in our assays. The IFN-dependent induction of HPV transcription, initial plasmid amplification and plasmid copy numbers in keratinocytes was revealed by careful titration of the IFN dose coupled with stringent tissue culture conditions.

Low-dose IFN treatment stimulated HPV E6–E7 transcription in a kinetically transient manner consistent with the transitory transcriptional response of many IFN-inducible cellular genes. However, IFNs are pleiotropic mediators capable of activating multiple

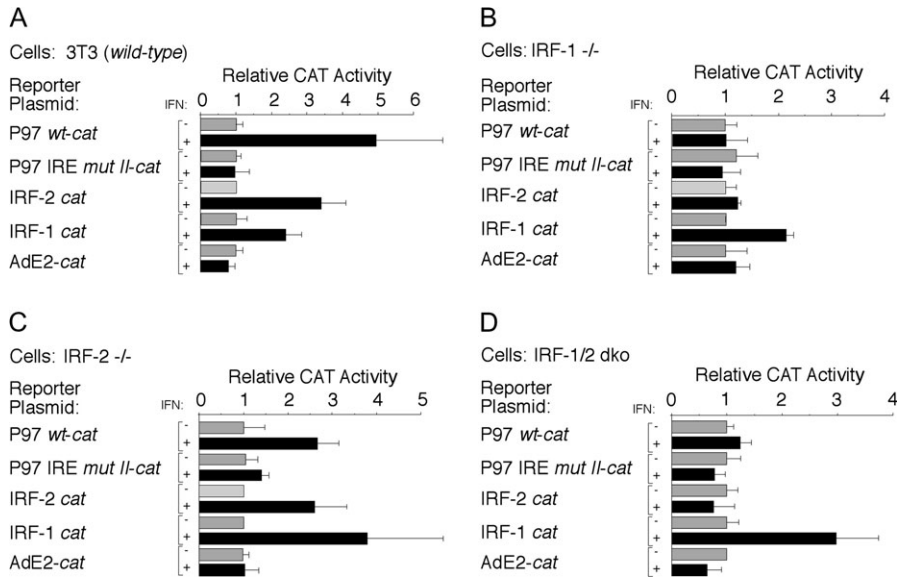

Fig. 4. IFN-mediated activation of the HPV-16 P97 promoter is dependent on the IRF-1 pathway *in vivo*. (**A**) IRF or Stat-responsive chloramphenicol acetyl transferase plasmids were transiently transfected into wt, 3T3, fibroblasts, expressing both IRF-1 and IRF-2, and then treated with IFN-β (10 U/ml) as described. Shaded bars indicate mock-induced target activities, whereas solid bars represent IFN-treated cultures. Normalized activities represent an average of three to six independent experiments. The IFN-unresponsive HPV-16 IRE *mut* II and AdE2-*cat* constructs were included as negative controls. (**B**) The IRF-1 knockout cells (IRF-$1^{-/-}$), (**C**) IRF-2 knockout cells (IRF-$2^{-/-}$) and (**D**) IRF-1 and IRF-2 double knockout cells (IRF-1/2 dko) were similarly transfected and treated in parallel.

cellular pathways *in vivo*. In contrast to previous studies, we utilized IRF knockout cell lines to resolve the complex interplay of IFN-dependent signaling pathways and define their effects on critical initial events influencing HPV persistence. These critical assays enabled us to identify the specific pathway responsible for the observed IFN-dependent HPV transcription phenotypes. The IFN-mediated stimulation of early HPV gene transcription was solely dependent on the induction of the IRF-1 pathway, presumably through initial phosphorylation of extant levels of the IRF-1 protein and supported by increased Stat-dependent IRF-1 expression. The transient nature of IRF-1-dependent effects following IFN treatment can be explained by limited IRF-1 messenger RNA and/or protein stability coupled with the eventual increase in IRF-2 expression that could competitively displace IRF-1 from its shared cognate binding motif to function as a DNA-binding repressor of IFN-responsive targets (53). The transient IFN-dependent induction of HPV early gene transcription and IRF expression observed in this study is consistent with the kinetics of this mechanism. Transient HPV induction is also consistent with a strategy of tightly regulated viral gene expression and genome amplification that would limit cellular responses to viral infection potentially triggered by unrestricted viral replication or elevated levels of viral gene products.

The IFN-dependent increase in transcription from the HPV-16 P97 and HPV-11 P90 promoters would result in increased expression of the viral E6 and E7 oncogenes that modulate cellular transformation. However, IFN treatment could also potentially drive the expression of other early viral gene products modulating HPV transcription and replication. An increase in normally limiting levels of E1 expression, for example, could be sufficient to drive the subsequent increase in HPV replication observed in these and parallel experiments (59).

Interestingly, in HPV-immortalized clonal keratinocyte cell lines harboring a mutated IRE, IFN treatment resulted in a dramatic decrease in HPV copy number, similar to previous reports that utilized much higher doses of IFN on cell lines harboring HPV-16 (W12E) (34) or HPV-31 (CIN612) plasmids (57), derived from explanted cervical lesions. As the IFN-mediated loss of plasmid copy number appears to be independent of the IRF pathway (this study), it probably competes with cytokine-dependent induction of plasmid replication *in vivo* to stabilize a homeostatic viral load in persistently infected keratinocytes. Therefore, when the IRE-dependent induction pathway is disabled, a significant decrease in overall plasmid replication and concomitant HPV copy number is revealed. At higher IFN doses, this alternate pathway appears to be kinetically dominant, resulting in a rapid loss of viral plasmids. This as yet undefined mechanism of HPV plasmid attenuation may account for partial clearance of HPV load following high therapeutic doses of IFN. However, following high-dose IFN treatment, any residual viral genomes could be restimulated after physiologic levels of IFN are restored, potentially resulting in a renewed cycle of HPV persistence and malignant progression.

IFN therapies have long been used to treat HPV-associated lesions with inconsistent outcomes; however, in order for similar interventional therapies to be reliably effective, they must incorporate an in-depth understanding of the underlying viral mechanisms that govern HPV persistence. This study demonstrates that low doses of IFN augment early viral functions through the cellular IRF-1 induction pathway. Since IRF-1 activity is influenced by a variety of cytokines, mucosal HPVs appear to have developed a potent mechanism to respond to multiple extracellular cues in the establishment of persistent viral infection, malignant progression and response to clinical treatment of HPV-associated lesions *in vivo*. This study defines sensitive assays capable of quantifying the effects of cytokines, growth factors, hormones and potential synthetic therapeutic agents influencing critical initial events in the HPV viral life cycle such as early gene expression and replication to identify potential mediators of the viral life cycle.

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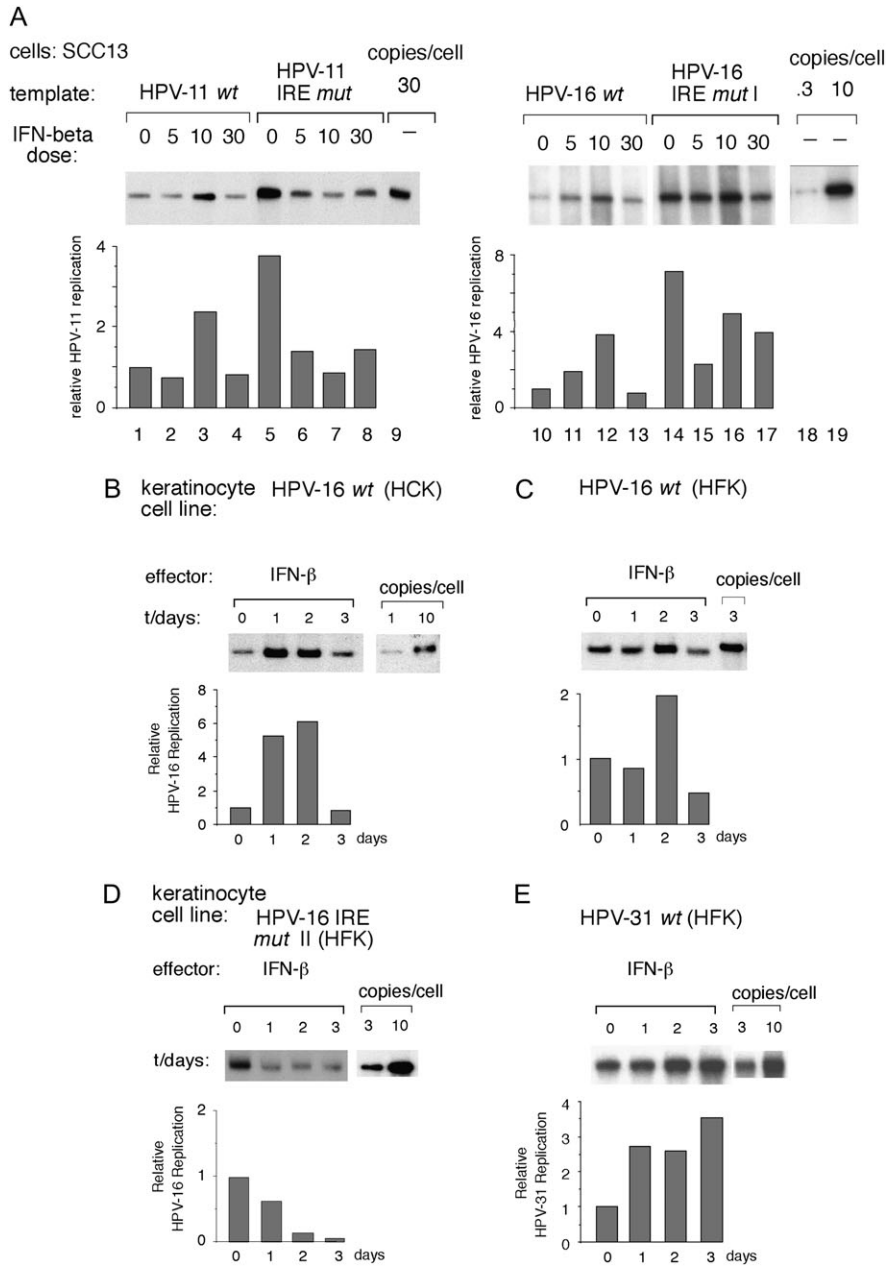

Fig. 5. Treatment with low levels of IFN-β can increase mucosal HPV copy numbers in transient transfections and stably replicating keratinocytes. (**A**) An increasing dose of IFN-β induced replication from HPV-16 and HPV-11 wt plasmids transiently transfected into SCC13 cells. Mutation of the conserved IRE (IRE *mut* II—as illustrated in Figure 1A), which disrupts IRF binding to these constructs, served as a negative control. (**B**) Stably replicating HPV-16 wt cervical keratinocyte cells (HCK) were treated with an optimal IFN-β dose (10 U/ml) in time-course inductions. (**C**) HPV-16 wt, (**D**) HPV-16 IRE *mut* and (**E**) HPV-31 wt cell lines derived from primary HFK were similarly treated. Changes in copy numbers were quantified by scanning densitometry.

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