Interferon treatment of human keratinocytes harboring extracomosomal, persistent HPV-16 plasmid genomes induces de novo viral integration

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Interferons (IFNs) have been used to treat epithelial lesions caused by human papillomavirus (HPV) persistence. Here, we exposed primary human keratinocytes (HFKs) immortalized by persistently replicating HPV-16 plasmid genomes to increasing levels of IFN-γ. While untreated HFKs retained replicating HPV-16 plasmids for up to 60–120 population doublings, IFN led to rapid HPV-16 plasmid loss. However, treated cultures eventually gave rise to outgrowth of clones harboring integrated HPV-16 genomes expressing viral E6 and E7 oncoproteins from chimeric virus-cell mRNAs similar to those in cervical and head and neck cancers. Surprisingly, every HPV-16 integrant that arose after IFN exposure stemmed from an independent integration event into a different cellular gene locus, even within parallel cultures started from small cell inocula and cultured separately for ≥25 doublings to permit the rise and expansion of spontaneous integrants. While IFN treatment conferred a growth advantage upon preexisting integrants added to mixed control cultures, our results indicate that IFN exposure directly or indirectly induces HPV-16 integration, rather than only selecting preexisting, spontaneous integrants that appear to be much less frequent. We estimate that IFN exposure increased integration rates by ≥100-fold. IFN-induced HPV-16 integration involved a wide range of chromosomal loci with less apparent selection for recurrent insertions near genes involved in cancer-related pathways. We conclude that IFNs and other potential treatments targeting high-risk HPV persistence that disrupt viral genome replication may promote increased high-risk HPV integration as a step in cancer progression. Therapies against high-risk HPV persistence thus need to be evaluated for their integration-inducing potential.

Introduction

High-risk (HR) human papillomavirus (HPV) types are associated with the majority of cervical and other anogenital carcinomas, 30% of head and neck cancers (HNC) overall and ≥50% of oropharyngeal carcinomas (1,2). Low-risk (LR) HPV types cause benign yet clinically intractable lesions such as recurrent respiratory papillomatosis. Vaccines against mucosal HPV prevent future infections yet are ineffective in eliminating virus persistence. HPV genomes have evolved complex mechanisms to evade and subvert molecular, cellular as well as immune responses during the establishment and maintenance of persistent viral infection (3,4). Currently, there are no effective therapies against HPV persistence (5) and the understanding of the underlying mechanisms has remained markedly incomplete.

Abbreviations: APOT, amplification of papillomavirus oncogene transcripts; CIN, cervical intraepithelial neoplasia; CI, caspase inhibitors; HCK, human cervical keratinocyte; HFKs, human keratinocytes; HR, high risk; HNC, head and neck cancers; HPV, human papillomavirus; IFNs, interferons; LR, Low risk; IRF, IFN-stimulated regulatory factor; MEP, major early promoter; IRE, IFN response element; RACE, rapid amplification of cDNA ends.

Interferons and the HPV life cycle

HPV infection and persistence in cervical and associated HNC lesions has been accompanied by altered cellular gene expression and immunogenic profiles (6). Interferons (IFNs) and other immune response signaling factors are differentially expressed in HPV-associated lesions and appear to play critical roles in modulating numerous cellular and viral processes through multiple phases of the HPV life cycle (3). Treatment of HPV-associated lesions with IFNs has historically resulted in refractory clinical outcomes (7). As one variable, we have shown that low-dose IFN exposure of keratinocytes immortalized by persistent plasmid HPV-16 genomes transiently stimulates early viral gene expression, augments initial plasmid amplification and increases viral plasmid copy numbers by activating IFN-stimulated regulatory factor (IRF)-1 (8). In turn, HPV oncoprotein products have been shown to modulate IFN response pathways (3,9). For example, the HPV E7 gene product directly targets the IRF-1 protein (10) while the E6 targets the IRF-3 protein (11) and inhibits activation of the signal transducers and activators of transcription-1 pathway (12). In contrast, high-dose IFN treatment of HPV plasmid-harboring keratinocytes inhibits viral gene expression (13) and decreases viral copy numbers in cell lines transformed with HPV-1 (14) and in immortalized human keratinocytes (HFKs) harboring HPV-16 (8,15) or HPV-31 (16). Further, IFN treatment has been shown to induce senescence of HPV-immortalized cutaneous keratinocytes (17) as well as increased accumulation of nucleotide substitutions in the HPV genomes (18).

HPV integration as a frequent event in HPV-associated cancers

Integration of HR HPV genomes leading to viral E6–E7 oncoprotein expression from chimeric virus-cell mRNAs has long been proposed to be an important, albeit not absolutely necessary, step in the development of cervical and anogenital cancers (19–21) and in HPV-associated malignancies of the head and neck (22). The integrated HR HPV fragments in high-grade precancerous cervical lesions, cervical carcinomas and derived cell lines, as well as HPV-associated HNCs express the viral oncoproteins, E6 and E7, from chimeric virus-cell mRNAs while the downstream early genes that are required for viral replication and regulated viral gene expression, E1, E2 and a spliced isomorph, E8*E2, are often disrupted or otherwise inactivated (23). Furthermore, chimeric HR HPV-cell mRNAs expressing E6 and E7 appear to be more stable than the full-length viral early mRNAs, which harbor a destabilization motif near their 3' end (24).

In addition, HPV integration in cancers as well as in culture often occurs within or near cellular gene loci involved in the control of cell division and migration with the potential of altering their function: c-myc, PTPN13, SGK1, HMG-AT hook (modulators of cell growth, signal transduction and chromatin modeling). In contrast, less is known about the viral integration process, the genetic structure of HPV-16 genomes and expression of their viral gene products in HNC (25).

HFK clones immortalized by the HPV genome have proven to be powerful tools in quantifying alterations to the viral genome as well as genetic and epigenetic changes within the host cell as the virus progresses through the establishment, persistence and productive phases of the HPV life cycle (26). In a series of large-scale assays utilizing primary HFK clones immortalized by unintegrated, plasmid HPV-16 genomes, we show that IFN causes rapid loss of HPV-16 genomes and promotes the outgrowth of cells with integrated HPV genomes expressing E6–E7 from chimeric HPV-cell mRNAs as seen in cancers. However, we demonstrate for the first time that IFN treatment dramatically increases the frequency of stochastic de novo HPV integration events.
Materials and methods

Cell culture

HPV-16 immortalized human foreskin keratinocyte (HFK) and human cervical keratinocyte (HCK) clones [HFK clone A (unintegrated), HFK-05 (integrated (21)) and HCK clone B (unintegrated (27)) were prepared and cultured as described (26). IFN inductions were performed in the presence of an irradiated J2 fibroblast feeder layer in E-media. Clonal HPV-positive primary keratinocyte clones were first starved for 24 h in charcoal-stripped, low serum medium (Dulbecco’s modified Eagle’s medium, 2% fetal bovine serum) prior to cytokine treatments. Cultures were then fed 10 ng/ml (‘low’) or 30 ng/ml (‘high’) human recombinant interferon gamma (IFN-γ) (Sigma, St. Louis, MO) in E-media (8) every 24 h with repeated visual monitoring via standard microscopy of the proliferating cell monolayers throughout the course of treatment before RNA or DNA harvest as described below. Lactacystin, MG132 and caspase inhibitors were incorporated with IFN treatment at optimal concentrations as indicated in the figure legends. IFN treatment periods were progressively extended in subsequent experiments to insure quantitative HPV plasmid loss.

Characterization of keratinocyte clones

For Southern blotting, 2 μg of whole cell DNAs of HPV-induced clonal cultures was resolved on 1% agarose gel, excised, purified (QIAquick PCR purification kit, Qiagen, Valencia, CA) and sequenced (University of Iowa DNA Core Laboratories). Chimeric HPV/cell sequences were then compared to available human genome data (GenBank) by BLASTN v.2.2 (National Institutes of Health).

Ethics statement

We confirm that all necessary ethical requirements and protocols related to tissue collection, where patient consent requirement was waived, and sample use are in conformity with the requirements of the Institutional Review Boards of the participating institutions and adhered to the principles outlined in the Declaration of Helsinki.

Results

IFN treatment reduces extrachromosomal HPV copy numbers in clonal keratinocytes

We recently isolated and characterized primary HFK clones immortalized by plasmid HPV-16 genomes that retain viral plasmid persistence up to 60–120 population doublings (PDs) without detectable integration. However, in some subcultures, they become overgrown by cells in which fragments or tandem arrays of HPV genomes are integrated and express the viral E6 and E7 oncogenes from chimeric HPV-cell mRNAs; these have the same types of structures as those found in cervical and HNC lesions (21). The defined clonal nature of the cells and the stability of plasmid HPV-16 genome persistence constitute a model for the study of HR-HPV integration in culture.

First, we wished to test HPV-16 plasmid persistence in response to IFN-γ. In a previous report where we focused on stimulation of HPV-16 and HPV-31 early gene transcription and replication by low-dose IFN-γ, we noted that higher levels of IFN-γ or IFN-beta rapidly reduce HR HPV plasmid genome levels (8). This initial finding was consistent with IFN effects on persistent HPV-31 plasmid genomes in mass cultures of HFK or in an HPV-16-positive cervical intraepithelial neoplasia (CIN) explant strain, W12 (16,29).

The exposure of clonal HPV-16-immortalized keratinocytes (clone A) to a high level (30 ng/ml) of IFN-γ resulted in a rapid, time-dependent loss of replicating extrachromosomal viral genomes 3–7 days post-induction (Figure 1A, lanes 5–9). We observed similar reductions in HPV copy numbers in explant-derived keratinocyte strains from CIN lesions which harbor extrachromosomal HPV-16 (W12-E (29,30)) or HPV-31 (CIN612 (16)) genomes in parallel (data not shown). IFNs are efficient inhibitors of cell growth yet the loss of viral plasmid genomes did not correlate with high cell mortality or a comparable decrease in apparent keratinocyte cell densities (via visual monitoring) during IFN treatment (data not shown), therefore the observed clearance of detectable HPV plasmids was not simply due to cell growth inhibition, cytopathic effects or arrested HPV-16 plasmid replication.

The plasmid HPV-16-immortalized keratinocytes (HFK clone A) were mixed at a ratio of 1:1 with a keratinocyte clone containing integrated HPV-16 genomes (HFK-05). After two PDs of the mixed starting culture had elapsed, Southern blotting revealed that the HFK-05 integrant outgrew the plasmid HFK clone A within 4–7 days in the absence of IFN; clone A disappeared from the culture by day 27 (Figure 1B; lanes 1–5). In the presence of IFN, the apparent decrease in plasmid levels in the unintegrated HPV clone was accompanied by increased selective outgrowth of the seeded HPV integrant (Figure 1C, lanes 5–8). These results confirm that even a slight decrease in PD times (i.e. an increased growth rate) in culture results in a selective growth advantage, as in our HR-HPV integrants and in the HPV-16 CIN strain W12 (30).

We previously demonstrated that expression of the viral E6 and E7 oncogenes from the major early promoter, P97, is dependent on an IFN response element, that is conserved in many mucosal HPV promoters. The P97 IRE binds constitutive levels of the IRF-1 and IRF-2 transactivators and is further induced by cytokine-stimulated levels of IRF-1 in response to low-level IFN (8,31). In this previous report, we treated clonal keratinocytes harboring HPV-16 plasmid genomes that contained a mutated IRE (HPV-16 IRE mut) with high levels of IFN, noting the same reduction in unintegrated viral copy numbers in both HPV-16 wt and IRE mut cultures (8). These previous results demonstrated that the mechanism of plasmid reduction is not dependent on the conserved HPV IRE. In subsequent experiments, we further noted that treatment with other extracellular agents, such as TNF-α and TGF-β1 (Figure 1F) or Cd340/40, also resulted in a similar dramatic decrease in HPV plasmid copy numbers in HPV-16 keratinocyte clone A. Some, but not all, of these agents are capable of modulating some of the same cytokine pathways induced by IFNs, resulting in transcriptional activation of the HPV major early promoter and IFN-responsive cellular promoters (data not shown).

We included proteolytic inhibitors in our IFN induction experiments to determine if a specific protein degradation pathway might be required for the observed IFN-dependent decrease in viral copy numbers (Figure 1D). Addition of Lactacystin—a broad-spectrum proteolytic inhibitor that targets, among other pathways, the catalytic proteosome subunit—did not inhibit IFN dependent plasmid loss and had no effect in the absence of IFN (Figure 1D, lanes 1–3). MG132, another broad-spectrum agent, also inhibits a variety of proteolytic pathways yet completely abrogated IFN-dependent plasmid loss in parallel (Figure 1D, lanes 8–11). Addition of caspase-8 or caspase-9 inhibitors (CI-8 and CI-9) similarly attenuated IFN-dependent plasmid loss (Figure 1E, lanes 12–18) while addition of other caspase inhibitors, CI-10 (Figure 1E, lanes 5–7) or CI-11 (data not shown) had no significant effect. These results indicate that IFN stimulation of caspase-8 and caspase-9-dependent proteolytic pathway(s) coincides with viral genome loss but the downstream targets responsible for this effect need to be identified in future studies.

The characterization of keratinocyte clones revealed that the HPV-16 plasmid genome was stably maintained in the absence of IFN but rapidly lost in the presence of IFN. This loss was not accompanied by a decrease in cell viability, suggesting that IFN was acting specifically on the viral genome. The addition of proteolytic inhibitors or caspase inhibitors completely abrogated IFN-dependent plasmid loss, indicating that proteolytic pathways were involved in this process. The results suggest that IFN stimulates a proteolytic pathway that results in the degradation of the viral genome, leading to its removal from the cells.
IFN-dependent induction of HPV integration

To characterize the process and the outcomes of HR HPV integration events that occur spontaneously in comparison to those associated with IFN treatment, we designed experiments to distinguish between the possibility that IFN treatment simply allows for the selective outgrowth of preexisting integrants that arose spontaneously before IFN exposure or, alternatively, that IFN may induce de novo integration as a consequence of disrupting HPV-16 plasmid genome persistence. In the first case, we would have predicted that integrants isolated from individual cultures would represent the progeny of one or more preexisting integration events with more than one integrant exhibiting the same, clonal integration pattern at the level of HPV DNA analysis and virus-cell mRNA sequence. Alternatively, if viral integrations were associated with IFN exposure, we would have expected most, if not all, outgrowing cells to stem from multiple independent, distinguishable integration events.

The design of the experiments and a summary of the results are illustrated in Figure 2. In experiment 1 (Figure 2A), we plated twelve 60 mm dishes with $3 \times 10^4$ cells of the unintegrated parent clone (HFK).
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clone A) and treated four with high and four with low dose IFN-γ. The remaining four dishes were left untreated as mock controls. The untreated control cultures kept on dividing; therefore, after an initial period of 14 days, they were passaged at a ratio of 1:3 every 3–4 days. After 60 days, we first isolated individual surviving colonies from a random selection of two cultures exposed to high-dose IFN treatment, expanded them and, again, tested them for integration by restriction analysis and 3′RACE (APOT). The representative colonies from the first high IFN-treated culture yielded four clones: one with HPV-16 in plasmid form and three with integrated HPV-16 DNA. The second high IFN treated culture gave rise to one clone harboring integrated and one clone with plasmid HPV-16. All integration patterns were clonally independent by both DNA integration pattern as well as by the 3′RACE (APOT) analysis (Table I). We then harvested total DNA from the control and remaining IFN-treated plates and analyzed HPV DNA physical status by restriction and Southern blotting. HPV plasmid genomes and integrated HPV sequences were readily distinguished from one another by characteristic patterns: in uncut DNA samples, the presence of fast-migrating supercoiled circular DNA as well as slow-migrating open circle genomes signified unintegrated HPV-16 plasmid genomes; the circles which converted to unit-length 7.9 kb DNA linear genome fragment upon restriction with a single-cut enzyme, Bam HI. The untreated cultures of HPV-16 clone A exhibited only this pattern and did not contain detectable integrants by 3′RACE (APOT). In contrast, both the low and high IFN-treated cultures contained predominantly integrated genomes comigrating with the cellular DNA.

In experiment 2, we started a series of 24 parallel cultures of the unintegrated parent (HFK clone A) from small inocula of 3000 cells per well of 12-well dishes (Figure 2B). Each separate culture thus had a low probability of harboring a preexisting integrant. These parallel cultures were passaged separately for ≥20 PDs (60 days) to allow time for spontaneous integration events to accumulate. At that point, each parallel culture was plated in triplicate at 3 × 10^4 cells per dish and exposed to low or high IFN-γ for 16 days. (C) Reconstitution experiment designed to compare selection of preexisting integrants versus de novo integration rates. 3 × 10^5 plasmid HPV-16 genome-containing clone A cells were either plated alone or seeded with 10 or 60 integrated HPV-16 cells (eight cultures each) and treated with high IFN-γ. Integration was analyzed by restriction enzymes followed by Southern blotting in all parallel cultures and by 3′RACE (APOT) analysis to identify specific virus-cell mRNAs in selected clones.

![Fig. 2.](image-url)
In experiment 3, we wanted to quantitatively define the emergence of de novo integration events and measure how effectively IFN treatment can select for a seeded integrant. A total of 24 parallel cultures containing $3 \times 10^4$ of the unintegrated HFK clone A were seeded alone or with either 10 or 60 cells of the integrated genome-containing clone HFK-05 (Figure 2C). These cultures were then treated with IFN-γ and surviving colonies isolated, expanded and analyzed for integration as in experiments 1 and 2. Seven of seven clones derived from the control cultures harbored unintegrated HPV-16 DNA. In plates seeded with integrant/plasmid mix of $1.3 \times 10^4$, we observed a 50% ratio of de novo integrants ($n = 4$) and colonies of the seeded, that is ‘preexisting’ integrated HFK-05 cells (also $n = 4$). Only integrated HFK-05 cell colonies were recovered from the $1.5 \times 10^3$ mix (Figure 2C). These results indicate that while IFN exposure can select preexisting

![Figure 3](image-url)

**Fig. 3.** HPV-16 mRNAs transcribed from plasmid viral genomes (A) and HPV-16-cell chimeric mRNAs expressed from (B) integrated HPV-16 genomes determined by 3'RACE (APOT) and listed in the tables. (C) Example of restriction mapping of HPV-16 DNA extracted from keratinocyte clones derived from high IFN-γ-treated HFK clone A cells in experiment 2.
HPV-16 integrants, it is IFN induction of de novo integration that is kinetically predominant.

Taken together, each of the 42 IFN treatment-associated HPV-16 integration events characterized in this study was individual, separate and unique, by restriction enzyme analysis and by direct sequencing of the chimeric HPV-16-cell mRNA by 3'-RACE (APOT) where we obtained the sequence of the HPV-16-cell mRNA junction. None of these integrations were detected in the starting cultures, demonstrating that IFN induced de novo integration events during treatment as opposed to solely selecting for preexisting integrants. All integrants that arose in the HPV-16 HFK clone A in 10 cultures in experiments 1–3 stemmed from de novo integration events. Therefore, we estimate that the frequency of preexisting integrants in the clone A is $3.3 \times 10^{-6}$ (<1 in 300,000). The frequency may be even lower as we do not have a quantitative assay that would detect low numbers of spontaneous integrants. However, in experiment 3, IFN treatment elevated the numbers of de novo integrants to the level of preexisting, seeded HPV-16 HFK-05, that is, $3.3 \times 10^{-6}$ (1:3000). Although these numbers are but rough estimates, the results indicate that IFN exposure increases the number of integrants by ≥100-fold.

We detected both type I (single copy) and type II (tandem repeat) DNA integration patterns within the clonal keratinocyte set (Figure 3; Tables I and II). The majority of integrants displayed the type II integration pattern. Both DNA integration structures are theoretically capable of expressing type ‘a’ (virus-to-cell splicing) or type ‘b’ (intragenomic viral splicing) mRNAs (Figure 3B). Consistent with this prediction and our previous studies (21), we also detected both type ‘a’ and type ‘b’ chimeric virus-cell mRNA structures with type ‘a’ representing the predominant detected structure (Tables I and II). In some samples, both mRNA types were simultaneously detected (Table II, clones B7(16-1) and C8(23-2)) as we would predict yet rarely recorded in previous studies.

The keratinocyte clones examined included integration events near loci with the potential to alter a range of host cell processes (Tables I and II). Integrations associated with DNA topoisomerases (Table I, clone 17C2), and pre-mRNA splicing factor 3B [Table II, clone C9(23-3)] and EGF [Table II, clone C7(23-1)], potentially target host cell replication, transcription and growth signaling, respectively. However, in contrast to recurrent integration loci previously noted in spontaneously arising integrations in culture, we noted no clear chromosomal loci preference and only one repeat integration in the RAP GNEF-4 locus (Table II, clones B7(16-1) and C8(23-2)). Furthermore, IFN-induced integrations included a wider array of cellular targets with a less pronounced preference for loci that would control cell growth, motility, metabolism or other potential changes in cell behavior.

**Discussion**

The results of our study show that IFN exposure directly or indirectly induces de novo HPV integration in clonal HFK immortalized by unintegrated, plasmid HPV-16 genomes. While IFN treatment rapidly eliminates extrachromosomal plasmid HPV-16 genomes from most cells in the cultures, it results in the outgrowth of cells expressing the

| Table II. De novo HPV-16 integration sites detected in IFN-exposed HPV-16 keratinocytes |
|-----------------|-----------------|-----------------|-----------------|
| Clone | HPV mRNA structure | Chromosome | Integration locus |
| A1 (1D4) | 880*3358-3794-chromosome 1 | 1 | Tissue factor isofrom 1 |
| A2 (1G1) | 880*3358-3664-chromosome 3 | 3 | CCR4-NOT |
| A4 (1G4) | 940*3456-chromosome 10 | 10 | Pancreatic lipase-related protein-3 |
| A5 (1G7) | 880*?? (no match) | Unknown | |
| B5 (2G3) | 940*chromosome 16 | 16 | TB53-target gene 3 protein |
| B6 (2G4) | (no signal) | | *
| B8 (2H2) | 880*chromosome 4 | 4 | MCGFR-2 |
| C5 (3H2) | 880*?? (no match) | Unknown | *
| A4 (3–4) | 880*chromosome 16 | 16 | Genetic suppressor element I |
| A5 (3–5) | 880*chromosome 2 | 2 | G protein-coupled receptor 39 |
| A7 (4–1) | 880-935-chromosome 9 | 9 | Lysine demethylase |
| A8 (4–2) | 880*3358–3838* | 14 | SPARC-related protein 1 |
| A10 (4–4) | 880*chromosome 14 | 8 | Protein odd-skipped-related 2 |
| B4 (11–4) | 880*chromosome 8 | 16 | Cadherin-13 preprotein |
| B8 (12–2) | 880*chromosome 16 | 16 | |
| B9 (12–3) | 880–927* | 16 | |
| B10 (12–4) | 880*3358–3762* | 16 | |
| B12 (12–6) | 880–948*3490–3764* | 16 | |
| C1 (20–1) | 880*3358-3520-7 | 16 | |
| C4 (20–4) | 880*chromosome 2 | 2 | Zinc finger RBD protein 3 |
| C6 (20–6) | 880*chromosome 2 | 2 | Regenerating islet-derived protein 3-g |
| C7 (19–1) | 880*chromosome 8 | 8 | Protein EFR3-A |
| C10 (19–5) | 880*chromosome 1 | 1 | LOC199870 |
| A12 (7–6) | 880*chromosome 14 | 14 | CHURC1 |
| B7 (16–1) | 880*3358-3492-chromosome 2 | 2 | Rap GNEF 4 |
| B8 (16–2) | 880*chromosome 7 | 7 | Mitochondrial inner membrane protease-2 |
| B10 (16–4) | 880*2709–3315* | 6 | AP-2 delta |
| B11 (16–5) | 880*chromosome 6 | 6 | |
| B14 (15–8) | 880*3358–3773* | 9 | Von Willebrand factor A; EGF |
| C7 (23–1) | 880*3358-3574-chromosome 9 | 2 | Rap GNEF 4 |
| C8 (23–2) | 880*3358-3494-chromosome 2 | 2 | Rap GNEF 4 |
| C9 (23–3) | 880*chromosome 1 | 1 | Pre-mRNA splicing factor 3B |
| C11 (24–7) | 880*3358-3541-chromosome 3 | 3 | Pseudoprophyl-3-trans isomerase A |
| C14 (24–10) | 880*chromosome 13 | 14 | Glypican-5 precursor |

*HPV integration status determined in all clones from experiment 3 (diagrammed in Figure 2C) and a replicate experiment without seeding by Southern blotting followed by 3' RACE(APOT) analysis of all integrants. Only sequences of chimeric HPV-cell mRNAs from de novo IFN-exposed HPV-16 integrants are shown.

*Cellular integration loci identified in GenBank databases by BLASTN search sequenced (where ‘a’ = splice junction and ‘*’ = incomplete 3’ RACE(APOT)).
IFN-dependent induction of HPV integration

HPV E6 and E7 oncogenes from integrated viral genomes in the form of chimeric HPV-cell mRNAs. All integrants we characterized in this study were clonal and stemmed from independent, de novo integration events. The impact of these findings is that prospective therapeutic approaches directed at eliminating high-risk HPV persistence will need to be tested for their integration-inducing potential.

Mechanisms of IFN-dependent extrachromosomal genome loss

Regulatory cis elements within HPV early promoters have been shown to bind cellular and viral trans-acting factors, resulting in tightly controlled early HPV gene expression, initial amplification and maintenance of viral plasmid genomes (32–34). We previously demonstrated that IRF-1 binding to a conserved IFN response element in the major early promoters of many mucosal HPV was required for IFN-dependent stimulation of viral transcription and replication (8). Disruption of the HPV-16 IRE, however, did not affect IFN-dependent loss of viral plasmid genomes in keratinocyte clones, indicating that an IRE-independent pathway is involved. These results, however, do not rule out the possibility that additional cis elements within the HPV genome may form alternative complexes with other IFN-stimulated factors at different points in the HPV life cycle, potentially inhibiting origin of replication function, plasmid maintenance, or late phase genome amplification (12).

HPV genomes are maintained as extrachromosomal plasmids by the full-length HPV E2 protein (E2-TA). E2-TA binds to E2 sites in the HPV genome through its DNA binding domain and to cellular chromatin through its multifarious N-terminal domain that associates with the cellular factor BRD4; this association further maintains E2-TA levels by preventing proteosome-mediated degradation (35). IFN treatment of HPV-16 keratinocytes harboring genomes with a disrupted PEST domain in the HPV-16 E2 hinge region, in an otherwise intact E2 cistron encoding a transcriptionally active and replication-competent E2 gene product, exhibited similar precipitous decreases in viral copy numbers upon IFN treatment (data not shown). These results suggest that IFN-dependent plasmid loss is not a function of proteosome degradation of the E2 protein under these conditions.

Limiting levels of the E1 replicase are required for HPV plasmid maintenance (33), potentially by interacting with cell factors (36). IFN has also recently been shown to inhibit E1-dependent replication via direct association between the HPV E1 and the IFN-inducible p56 protein (37). HPV has been shown to stimulate caspases-3, 7 and 9 in differentiating keratinocytes, resulting in HPV amplification in the absence of apoptosis via targeted proteolytic degradation of E1 which required a conserved caspase-3 and -7 target motif (DXXD) in HPV-11 and HPV-31 (38). The HPV-16 E1 protein potentially encodes a homologous putative caspase degradation motif. Caspases have emerged as mediators not only of apoptosis but of multiple functions in epithelial cells, including EGF signal transduction, IRF pathways (39) and cell migration (40). In this study, we demonstrated that IFN-dependent loss of HPV-16 plasmid genomes was abrogated by addition of protease inhibitors specific for caspase-8 and caspase-9 but not other caspase inhibitors, indicating that upstream components of the caspase pathway (i.e. caspase-9) are potential mediators of the maintenance of HPV plasmid genome persistence and potential targets in IFN-mediated plasmid loss.

Induction of dramatic plasmid loss by agents other than IFN, i.e. TNF-α, TGF-β1 or Cidofovir, indicated that the loss of plasmid copy numbers is not solely dependent on components of the IFN induction pathways. It remains to be determined whether treatment with these extracellular agents or other potential therapeutic interventions may also stimulate viral integration events. These results do not rule out the possibility that multiple mechanisms may contribute to viral genome loss at various points throughout the HPV life cycle.

HPV integration in response to IFN exposure

Spontaneous integration events could be the result of aborted replication that results in HPV DNA integration in the cell genome directly or due to subsequent spontaneous or induced events that occur in the course of further cell growth (41,42). Defined HPV-16-immortalized HFK cell clones mimic integration patterns observed in vivo (21). Apparent spontaneous integration rates differ among HR HPV types in culture (28). Furthermore, the distribution of cervical and other anogenital HPV lesions harboring integrated versus unintegrated HPV genomes varies by virus type (43).

Our results demonstrate that exposure to IFN induces de novo HPV-16 integration in primary human foreskin keratinocyte stably replating HPV-16 plasmid genomes since all 42 individual integrants isolated in this study stemmed from independent integration events into distinct chromosomal target sites. This is in contrast to a previous observation obtained in the HPV-16 CIN explant-derived W12 cell strain by Coleman, Stanley and coworkers (15). The W12 cells repeatedly yielded progeny outgrowth of the same clonal HPV-16 integrant apparently present in the W12 strain at passage 17 postexplantation. In our reconstitution experiment 3 (Figure 2C), in IFN-treated cultures that started with no known integrants, all integrations arose from independent events. In those cultures where the ratio of integrants to plasmid-containing cells was 1:3×10⁻⁴, we observed both de novo integration events as well as the outgrowth of the seeded integrant at a ratio of 1:1, and in those with a ratio of 2×10⁻⁴, that is, 2,100-fold. These results also are consistent with the results of one of the experiments performed in the W12 strain: when the authors treated W12 cells at passage 14 postexplantation, they identified both the progeny of the passage 17 integrant as well as integrants stemming from de novo integration events (15), thus resembling our mixed culture results.

In all HR-HPV-associated cervical and HNC lesions as well as in cell lines containing integrated HR-HPV DNA characterized so far (21,34), the 3’ ends of the integrated HPV genomes or genome fragments were found to be disrupted within the E2 or, less frequently, the E1 regions, expressing the viral E6 and E7 oncogenes from chimeric HR-HPV-cell mRNAs (illustrated in Figure 3). The same pattern of HPV-16 DNA integration was detected in all the 42 IFN-induced, de novo HPV-16 integrants characterized in this study, of which 34 also exhibited integrated mRNA expression patterns by 3’RACE (APOT). E1 encodes a replication helicase while E2 gives rise to a multifarious transcriptional activation/replication/chromatin attachment full-length product, E2-TA, and a shorter E8/E2 gene product, a potent inhibitor of viral transcription and replication. In addition, the chimeric mRNAs have lost a viral 3’ mRNA destabilizing sequence. Therefore, even under IFN exposure, there is a strong selection for the outgrowth of cells expressing HPV-16 E6 and E7 oncogenes from chimeric HPV-cell mRNAs (34).

In a previous study, we examined a range of viral integration loci associated with HNC tumors and clonal cell lines derived from primary human foreskin and tonsillar keratinocytes (21). We noted that approximately 35% of the cellular target loci encoded genes with the potential to influence cell cycle control, cell growth, motility and metabolism in cancer progression. In addition, a subset of these genes was recurrently associated with HPV integration in culture and in HPV-associated cancers (e.g. c-Myc and PTPN13). In IFN-associated HPV-16 integrants, we detected a more random array of integration loci and no recurrent integrations targeting potential cancer progression-associated genes. While we have no explanation for this phenomenon, it is consistent with the hypothesis that a strong disruptive genetic event promotes efficient integration. Alternatively, IFN exposure may further disrupt the structure and/or expression of as yet unidentified cellular genes so that targeted locus disruption becomes less necessary.

IFNs are potent pleiotropic modulators of gene expression, immune responses and cell proliferation. As such, IFN therapies (similar to other anti-viral regimens) can result in significant negative collateral
effects at the cellular level that are potentially manifested in the host system. Though viral integration is not a prerequisite for malignant progression, it may be a contributing factor as shown in a variety of HPV-associated carcinomas. This study demonstrates that, in addition to potentially stimulating early viral gene expression and initial genome amplification in HPV-immortalized keratinocytes, IFNs also induce de novo HR-HPV integration. Our results stress the necessity to understand the underlying biological processes. Above all, any therapies directed at eliminating HR-HPV persistence will need to be tested for the possibility that, like IFN exposure, they potentially may promote HPV-associated cancer progression.

Supplementary material

Supplementary Figure 1A, D, E and F can be found at http://carcin.oxfordjournals.org/

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References


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